# The Regulation of Worker Reproduction

# in the Ant *Aphaenogaster cockerelli*

by

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

Approved October 2011 by the
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ARIZONA STATE UNIVERSITY

December 2011

# ABSTRACT

The repression of reproductive competition and the enforcement of altruism are key components to the success of animal societies. Eusocial insects are defined by having a reproductive division of labor, in which reproduction is relegated to one or few individuals while the rest of the group members maintain the colony and help raise offspring. However, workers have retained the ability to reproduce in most insect societies. In the social Hymenoptera, due to haplodiploidy, workers can lay unfertilized male destined eggs without mating. Potential conflict between workers and queens can arise over male production, and policing behaviors performed by nestmate workers and queens are a means of repressing worker reproduction.

This work describes the means and results of the regulation of worker reproduction in the ant species *Aphaenogaster cockerelli*. Through manipulative laboratory studies on mature colonies, the lack of egg policing and the presence of physical policing by both workers and queens of this species are described. Through chemical analysis and artificial chemical treatments, the role of cuticular hydrocarbons as indicators of fertility status and the informational basis of policing in this species is demonstrated. An additional queen-specific chemical signal in the Dufour's gland is discovered to be used to direct nestmate aggression towards reproductive competitors. Finally, the level of actual worker-derived males in field colonies is measured. Together, these studies demonstrate the effectiveness of policing behaviors on the suppression of worker reproduction in a social insect species, and provide an example of how punishment and the threat of punishment is a powerful force in maintaining cooperative societies.

# ACKNOWLEDGMENTS

Many thanks go to my committee members, who have provided support, encouragement, and mentorship throughout my development as a biologist.

Kevin Haight has been a mentor to me from the first day I set foot in a biology lab, I am thankful for his continual encouragement, advice, and friendship. I also thank Walter R. Tschinkel for his mentorship early in my academic life, and the phone call he made that got me into graduate school. Joshua R. King, Jon N. Seal, and Chris R. Smith were also influential in my early academic development.

I am grateful to Bethany Cutts for her unending support. I thank my parents and grandparents for encouraging and being proud of my work. I thank Adam and Kelly Dolezal for being my friends. I thank Clint Penick for being my bud. I thank Heidi Fisher and Prasad Boradkar for having me in their classrooms. I thank Tate Holbrook, Joshua Gibson, Dani Moore, Rick Overson, Brenda Rascón, Takao Sasaki, Zach Shaffer, Adam Siegal, and the rest of my colleagues for their company and encouragement.

The School of Life Science, the Graduate & Professional Student Association, Bert Hölldobler, and The Design School at Arizona State University all provided financial support for me during my time in Arizona; I am thankful for their support.

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# Chapter 1

# INTRODUCTION

In the evolution of cooperation and the formation of societies, repressing intra-group competition stands beside such factors as kin selection and reciprocal altruism as a key component of successful societies. Individuals that cheat group members of resources for personal gain, at costs for the group, threaten all social groups. Punishment of cheaters promotes cooperation in human societies (Fehr and Gachter 2002; Hauert et al. 2007), and models of the effect of punishment on group cohesion can be extended throughout other animal societies (Frank 1995, 2003). Key factors determining the competitive success of a group are the presence of a means by which within-group cheaters can be suppressed and the level at which those cheaters are kept (Brandvain and Wade 2007; Van Dyken, Linksvayer, and Wade 2011). While the role of cheater suppression in promoting group cohesion has been established, understanding the methods and mechanisms of suppression utilized within a society would provide fundamental insights into how cooperation is sustained.

Cheaters occur in a diverse array of group living organisms, from quorum-sensing pathogenic bacteria (Sandoz, Mitzimberg, and Schuster 2007) and cellular slime moulds (Strassmann, Zhu, and Queller 2000), to the eusocial insects (Ratnieks, Foster, and Wenseleers 2006). In the eusocial insects, cheating takes the form of non-established reproductive individuals exploiting group resources to gain personal reproductive benefits at a cost to the group. Even though eusocial insects are defined by having a reproductive division of labor, in most genera, subordinate workers retain functional ovaries and the ability to, at least, lay unfertilized male-destined eggs (Bourke 1988; Choe 1988; Wilson 1971). Haplodiploidy and colony kin structure favor worker reproduction when a colony has a single, singly mated queen, as workers are more related to their sons ($r = 0.5$) and to males from sister-workers ($r = 0.375$) than males produced by their mother ($r = 0.25$). However, restraint of worker reproduction is favored when worker reproduction has negative effects on colony efficiency and sex allocation (Cole 1986; Ratnieks 1988; Ratnieks, Foster, and Wenseleers 2006). Under queenright conditions, reproductive restraint is a result of workers either behaviorally inhibiting (policing) the reproductive efforts of other workers or individual workers exhibiting reproductive self-restraint (Ratnieks 1988; Wenseleers et al. 2004).

## Policing and Fertility Signals in Insect Societies

The two forms of policing behaviors found in insect societies are physical policing of potential egg layers and egg policing (Monnin and Ratnieks 2001; Ratnieks, Foster, and Wenseleers 2006). The reproductive efforts of individuals that are physically policed are inhibited when nestmates attack the policed individual. Egg policing occurs when newly worker-laid eggs are destroyed by nestmates. Queens of many species are also known to police the reproductive efforts of their offspring through both physical and egg policing (Ratnieks, Foster, and Wenseleers 2006).

In order for either one of these policing behaviors to be carried out effectively, the policing individuals must have reliable information indicating which workers should be policed and which eggs should be destroyed. Hydrocarbon blends present on the cuticle of workers and queens, and on the surface of eggs are thought to contain these signals (Liebig 2010; Peeters and Liebig 2009). Evidence from the eusocial hymenopteran groups (ants, bees, and wasps) links changes in hydrocarbon patterns to changes in reproductive status (reviewed in Liebig 2010). However, direct casual evidence is missing, linking hydrocarbon differences to indicators of fertility and policing behaviors.

Hydrocarbons present on the cuticle and the surface of eggs provide desiccation resistance (Lockey 1988). Oenocytes within the fat body synthesize hydrocarbons that are transported through the hemolymph to target tissues, including the cuticle and the ovaries (Schal et al. 1998). In ants, a diverse blend of hydrocarbons, varying in chain-length, bond number and location, and branching patterns, are present both on the cuticle and the surface of the egg (Peeters and Liebig 2009; Smith et al. 2008 [Appendix II]), making them prime candidates for facilitating chemical communication. Indeed, cuticular hydrocarbons serve as the basis of nestmate recognition in ants (Howard and Blomquist 2005).

Hormonal changes associated with changes in reproductive ability have been correlated to changes in cuticular hydrocarbon profiles in a queenless ant species, *Streblognathus peetersi* (Brent et al. 2006; Cuvillier-Hot et al. 2004). In many non-social insect species, hormonal changes linked with changes in reproductive development result in changes in sex-specific cuticular hydrocarbon signals: German cockroach *Blattella germanica* (Schal et al. 1991; Schal et al. 1994), housefly *Musca domestica* (Adams, Dillwith, and Blomquist 1984; Dillwith, Adams, and Blomquist 1983), the fly *Calliphoria vomitoria* (Trabalon et al. 1994), and a burying beetle *Nicrophorus vespilloides* (Steiger et al. 2007). Hormonal linkages that connect changes in reproductive status with changes in hydrocarbon profile have received comparatively little attention in ants, although hydrocarbon profile differences that correlate with reproductive differences have been described in 21 different species of ants (Liebig 2010).

For queens, conveying reliable information about personal reproductive status and ability is thought to be crucial in maintaining colony organization (Keller and Nonacs 1993). Rather than emitting queen-pheromones aimed at controlling or directly suppressing worker reproduction, queen-produced fertility signals (in the form of surface hydrocarbons) are thought to accurately reflect physiological changes correlated with reproductive ability. Indeed, in a number of ant species, cuticular hydrocarbon profiles of egg-layers (queens or mated workers) change according to changes in egg-laying rate and ability (Cuvillier-Hot et al. 2001; Endler, Liebig, and Hölldobler 2006; Hannonen et al. 2002). Because cuticular and egg-surface hydrocarbons are thought to accurately signal the presence and status of an established egg layer, and to reveal ovarian activation in potential reproductive cheaters, they are thought to serve as the informational basis of both worker reproductive self-restraint and nestmate policing.

## The Ant *Aphaenogaster cockerelli*

The research herein focuses on fertility signaling and the policing of worker reproduction in the ant *Aphaenogaster cockerelli* (formerly *Novomessor cockerelli*) (Formicidae: Myrmicinae). *A. cockerelli* occurs throughout the southwestern region of North America, inhabiting open intermountain plains throughout most of its range and rocky hillsides in the westernmost area of its distribution (Johnson 2000). This species is active above-ground during the evening, night, and early morning, and co-occur with the army ant *Neivamyrmex nigrescens* from which they regularly experience predatory raids (Mirenda et al. 1980). In response to these raids *A. cockerelli* rapidly evacuate their nests carrying out their brood and queen. This nest-evacuation response to army ant invasions was used to develop a methodology for easily extracting mature colonies of *A. cockerelli* from their nests (Smith and Haight 2008 [Appendix I]). This collection method allowed us to study the regulation of worker reproduction in mature colonies of *A. cockerelli*; colonies that had developed to the point sexual-alate production.

Mature colonies of *A. cockerelli* can exceed a worker population of 8,000, while being spread across multiple (2-5) nesting sites, one of which contains a single queen (Chapter 3). Nest workers do not regurgitate and share liquid food via trophalaxis between colony members, instead nest workers produce non-viable trophic eggs that are feed to brood or eaten by fellow nestmates (Hölldobler and Carlin 1989).

## Policing and Fertility Signals in *Aphaenogaster cockerelli*

An earlier study on the regulation of worker reproduction in *A. cockerelli* described the occurrence of worker policing in this species (Hölldobler and Carlin 1989). When workers were isolated from their queen, for periods as short as two weeks, they began to produce viable male-destined eggs. Reuniting this separated group of workers with the queenright portion of their nest resulted in a few of the isolated workers being physically attacked by their nestmates. The ovarian status of those attacked workers compared to non-attacked nestmates revealed that only reproductive workers were attacked, indicating that physical policing is used as a means of regulating worker reproduction (Hölldobler and Carlin 1989).

To build on this original study, other means of regulating worker reproduction that might be used by *A. cockerelli* were investigated. Egg policing, or the selective destruction of viable worker-produced eggs, is studied in Smith, Hölldobler, and Liebig (2008 [Appendix II]). Queen policing, or aggression from the queen towards reproductive workers, is described in Smith, Hölldobler, and Liebig (2011 [Appendix IV]). The information basis of aggression towards reproductive workers is also studied in Smith, Hölldobler, and Liebig (2008, 2009, 2011 [Appendices II, III, IV]). Causal evidence for hydrocarbons being fertility signals that are the basis for policing behaviors is also, for the first time, given in these studies (Smith, Hölldobler, and Liebig 2009, 2011 [Appendices III, IV]).

Upon discovering that queens will physically police the reproductive efforts of their daughter workers, an additional queen-specific signal that is used for directing worker aggression towards reproductive workers was discovered (Chapter 2). The source and the function of the signal which, although it is queen-specific, is not used as a fertility signal, is described.

Finally in Chapter 3 the actual level of successful worker reproduction in field colonies of *A. cockerelli* is measured. Samples from mature colonies that spread across multiple nesting sites were taken to see if nesting outside of the presence of the queen effects worker reproduction.

Chapter 2

# QUEEN SPECIFIC SIGNALS, WORKER PUNISHMENT, AND THE MULTIPLE RECEIVER HYPOTHESIS IN THE ANT APHAENOGASTER COCKERELLI

## Abstract

Chemical communication between reproductives and subordinates within social insects is fundamental to maintaining colony organization. Cuticular hydrocarbons are thought to be the dominant source of fertility signals among ants, however differences found within the Dufour's glands could also serve as fertility signals. The multiple receiver hypothesis, generated to explain multiple male ornaments in birds, presents a means for explaining the function of these seemingly similar signals: they have distinctly different receivers.

The function of the queen Dufour's gland in *Aphaenogaster cockerelli*, an ant species in which cuticular hydrocarbon profiles serve as fertility signals, is investigated. The queen's Dufour's gland contents distinguish her from all other members of the colony. When she encounters a competing reproductive worker she uses her gland to mark the worker, inducing punishment from nestmates. It is shown that only the queen's Dufour's gland can induce the observed amount of aggression.

The Dufour's gland and the cuticular hydrocarbon profile of *A. cockerelli* queens are prime examples of signal functions explained by the

multiple receiver hypothesis. This study also points out striking similarities in the use of the gland that span several subfamilies and forms of colony organization in ants, leading to a task separation of queen specific signals.

## Introduction

In most insect societies, chemical communication plays a central role in colony organization. Chemical signals involved in communication can originate from many glands, are typically composed of multiple components, and may be used for very specific purposes (Hölldobler and Wilson 2009). Determining the purpose or function of a specific signal that has similar properties to other potential signals, such as a queen that produces two queen-specific chemical blends, presents a major challenge to our understanding of chemical communication in these societies.

The problem of distinguishing the functions of multiple signals that convey seemingly similar information is found in other, non-insect and non-chemically based studies of animal communication (Andersson 1994; Møller and Pomiankowski 1993). In birds, multiple male signals of quality may convey multiple or complementary messages to a single receiver (Andersson 1994; Candolin 2003; Møller and Pomiankowski 1993). However, theoretical predictions indicate that mate choice based on multiple costly signals is evolutionarily unstable (Iwasa and Pomiankowski 1994; Johnstone 1996; Schluter and Price 1993).

An alternative approach to considering multiple male traits indicating quality is using the multiple receiver hypothesis, which suggests that males may display multiple ornaments because they are messages intended for different receivers (Andersson et al. 2002). In the red collared widowbird the carotenoid colored collar is a result of selection through male competition over territories (Pryke, Lawes, and Andersson 2001), while the elongated tail feathers are a result of selection for female choice preference (Pryke and Andersson 2005). Male competition and female preference is also thought to have led to multiple male plumage traits in peacocks (Loyau, Jalme, and Sorci 2005), red-backed fairy-wren (Karubian et al. 2009), and the yellow-browed leaf warbler (Marchetti 1998).

In social insect colonies, chemical signals are used to distinguish queens and established reproductive individuals from non-reproductive nestmates (Liebig 2010; Peeters and Liebig 2009). These signals are generated by the reproductive individuals and act as indicators of reproductive ability (D'Ettorre and Moore 2008; Keller and Nonacs 1993). Subordinate individuals respond to queen produced fertility signals in two ways: refraining from personal reproduction or self-policing, and restraining (policing) the reproductive efforts of their fellow nestmates (Ratnieks 1988). Fertility signals therefore have multiple receivers: workers deciding whether or not to reproduce and workers punishing reproductive subordinates. Although there is some evidence that the workers responsible for restraining the reproductive efforts of their nestmates are the same workers that are potential reproductives (Stroeymeyt, Brunner, and Heinze 2007), there is also evidence against this being the case in other species (van Zweden et al. 2007).

Substantial data indicate that cuticular hydrocarbons are the source of fertility signals in ants, bees, and wasps (Liebig 2010). However, potentially any chemical signal that accurately distinguishes reproductive individuals or queens from the non-reproductive members of the colony could serve as a fertility signal. For instance in honeybees, worker reproduction does not occur in the presence of queen mandibular pheromone, which, in colonies with a reproductive queen, is a substance produced only by the queen and may act as fertility signal (Hoover et al. 2003; Kocher et al. 2009). However, egg-laying honeybee queens also possess unique chemical blends in their Dufour's gland (Katzav-Gozansky et al. 1997). Workers who become reproductive when outside of the presence of the queen also develop both queen-like mandibular gland substances and queen-like esters in their Dufour's gland (Katzav-Gozansky et al. 2004, 2006; Malka, Katzav-Gozansky, and Hefetz 2009; Malka et al. 2008). Work attempting to disentangle the effects of the honeybee Dufour's gland from that of the queen mandibular pheromone suggests that Dufour's gland acts as a true fertility signal while the mandibular gland substance acts as a signal of reproductive dominance (Dor, Katzav-Gozansky, and Hefetz 2005; Malka et al. 2008).

In ants, cuticular hydrocarbons convey information about reproductive ability however, like honeybees, there is also evidence that the Dufour's gland contains reproductive-specific compounds. This has been well documented in one species, the queenless ant *Dinoponera quadriceps*. Reproductive alpha-individuals are mainly distinguished from their nestmates through the relative amount of 9-hentriacontene present on their cuticle (Monnin, Malosse, and Peeters 1998; Peeters, Monnin, and Malosse 1999). Changes of the relative proportion of this component of their cuticular hydrocarbon profile correlate with changes in reproductive status (Peeters, Monnin, and Malosse 1999). Reproductive alphas can also be distinguished from subordinate individuals through the contents of their Dufour's gland (Monnin et al. 2002). The Dufour's gland is one of two exocrine glands that empty at the base of the sting apparatus (Hölldobler and Wilson 1990). However, alphas only dispel the contents of their gland when challenged by a beta worker, sting-smearing the beta with the contents of the Dufour's gland. The attacked and sting-smeared beta is then immediately immobilized and punished by other nestmate workers (Monnin et al. 2002). Although the alpha worker is distinguishable from her nestmates through both her cuticular hydrocarbon profile and the contents of her Dufour's gland, it is clear that these chemical signals differ in their intended receivers.

The multiple receiver hypothesis is thus useful when explaining the function of multiple queen-specific substances in ants. In this study organism, the ant *Aphaenogaster cockerelli*, cuticular hydrocarbons correlate with reproductive ability in both the queen and worker caste and are used by policing workers for assessing the reproductive activity of their nestmate workers (Smith, Hölldobler, and Liebig 2011; Smith, Hölldobler, and Liebig 2008; Smith, Hölldobler, and Liebig 2009). This study reports that the Dufour's gland is being used during intracolony conflict over reproduction between queens and workers. It has been previously shown that queens respond aggressively towards reproductive daughter workers, biting them on the dorsal portion of their thorax or petiole (first abdominal segment), while thrusting the tip of their gaster towards the attacked individual (Smith, Hölldobler, and Liebig 2011). This report is furthered through time-lapse observations revealing that nestmate workers respond aggressively towards both the queen and the queen-aggressed worker. Evidence is provided that queens discharge the contents of their Dufour's gland while attacking nestmate workers and that the contents of the queen's Dufour's gland are distinct from the contents found in the Dufour's glands of all other females in the nest. It is demonstrated that placing contents of the queen Dufour's gland on the cuticle of workers triggers nestmate aggression towards treated workers on a level equal to that seen in unmanipulated observations of queen aggression. Finally, by compiling the reports of the Dufour's gland being used in intra-colony conflict in ants, it is suggested that directing intracolony conflict is a previously unrecognized major function of the gland. Differences in Dufour's gland contents between reproductive and non-reproductive individuals are not used as fertility signal like the cuticular hydrocarbons, but are addressed to individuals who actively police potential contenders of the established queen or reproductive alpha workers.

## Methods

### *Study Species*

Mature colonies of *A. cockerelli* were collected from the Chihuahuan desert between Portal, Arizona and Rodeo, New Mexico. Colonies were collected using army ants to trigger nest evacuation (Smith and Haight 2008). Mature colonies are polydomous, therefore only the colony section which contained the queen was used. Previous work has shown that queens are singly mated based on 31 colonies and 487 workers genotyped for two microsatellite loci (Mösl & Gadau, unpublished data).

In the laboratory, the ants were housed in a dental-plaster nest with molded chambers darkened by red acetate over glass. The nests were attached to a foraging arena in which they received a constant supply of water, sugar-water, and pieces of cricket (*Acheta domestica*) and beetle larvae (*Zophobas morio*). For this study, temperature was maintained at 26°C and the foraging arenas were kept in constant light.

### *Worker Response to Queen Aggression*

Five groups of 50 workers were separated from their queenright colony and allowed to reproduce. Once eggs were present in the isolated worker group, the mother-queen was introduced into the group of workers. The colonies were recorded using time lapse video software (Studio Surveillance v. 8.1) set at maximum frame rate capture (4 - 5 frames per second) for 20 hours. The data collected from the resulting video included nestmate-worker aggressive responses (holding of queen and holding of queen-aggressed worker), number of queen aggressive acts (biting, biting and holding or pulling), and any resulting mortality.

### *Dufour's Gland Contents*

In order to verify that queens were discharging compounds from their Dufour's glands during instances of aggression towards reproductive nestmate workers, the compounds present on the tip of the gaster of two queens were sampled, directly before and after aggression. The sampling was performed via Solid Phase Microextraction (Arthur and Pawliszyn 1990). A fiber (SUPELCO, coated with a 30µm polydimethylsiloxane film) was directly rubbed on the surface of the gaster for 5 min. The fiber was directly inserted into the injection port of a GC/MS, as mentioned below.

Dufour's glands of reproductively active queens (n = 9), reproductive workers (n = 10), virgin alate females (n = 9), workers with trophic egg producing ovaries (referred to below as nest workers; n = 9), and workers with minimal ovary development (referred to below as foragers; n = 9) were dissected from the ants for content analysis. To determine which workers were reproductive, queen aggressive behavior was used. From a previous study it is known that queens are only aggressive towards reproductively active workers (Smith, Hölldobler, and Liebig 2011). Therefore, a queen was introduced to a group of workers and as soon as any rapid antennation or biting occurred, the queen was separated and collected the rapidly-antennated or bitten reproductive worker.

Each dissected gland was placed directly into a 250µl glass vial. The gland was then broken open and extracted in 80µl of hexane solvent. The solvent and the extract were then evaporated and suspended in 10µl of hexane from which 1µl was injected into the injection port of an Agilent 6980N series gas chromatograph (GC), equipped with DB-1MS (J&W Scientific) nonpolar capillary column (30m X 0.25mm X 0.25µm), connected to an Agilent 5975 series mass selective detector. The GC injection port was set to 250 °C and the transfer line to 300 °C . The column temperature was held isothermal at 60°C for 2 min before rising to 320°C at 7°C min$^{-1}$. Helium was used as a carrier gas at 1 ml min$^{-1}$, and samples were injected in the splitless mode with a splitless time of 2 min. Electron impact mass spectra were measured at 70 eV, with a source temperature of 230°C.

Resulting peak areas were used to determine the number of compounds present in the glands. Verification of compound identifications was done by comparison of Kovats retention indices to published references. Only compounds that appeared in at least 70% of the glands sampled in at least one of the female types were used to compare gland contents. Statistical comparisons of gland contents were performed using STATISTICA 7.0 Software (StatSoft, Inc, Tulsa, OK, USA).

### *Dufour's Gland Treatments*

To test for the effect of Dufour's gland on worker aggressive responses, non-reproductive workers were treated with the contents of various nestmate glands. Three treatment groups were used, consisting of nestmate Dufour's glands from queens, reproductive (queen-aggressed) workers, and foragers. From previously dissected and extracted glands (see above), 45% of the gland contents suspended in hexane solvent were added to the cuticle of queenright workers. 10µl of Dufour's gland in hexane solvent was dropped into a 5ml beaker filled with de-ionized water which leads to a compound film on the water surface after solvent evaporation. After evaporation of the hexane a worker was dipped and lightly swirled on the surface of the water in the beaker, a technique similar to other water-based hydrocarbon application methods (Roux et al. 2009). Each treated worker was then allowed to air-dry, and was marked with a single dot of white Testors® enamel paint on the thorax, gaster, or head before being reintroduced to nestmate workers.

Test groups of 30 nestmate workers from a queenright colony were placed in an isolated nest for 24 hours before the treated workers were introduced to them. The treated workers, 30 isolated workers, and the Dufour's glands used for the treatment all originated from the same nest. 30 minutes after treatment and marking, the three treated workers were simultaneously introduced to the nest of 30 workers and video recorded for 20 hours (see above). The first two hours, wherein all observed aggression occurred, were analyzed, blindly, for the number and effect of any aggressive acts towards the treated workers.

## Results

### *Worker Response to Queen Aggression*

All transferred queens ($N = 5$) were accepted into the nest of their daughter workers as reported in Smith, Hölldobler, and Liebig (2011). Upon introduction into the foraging arena of the worker nests, queens either found their way into the interior of the nest or were carried by outside workers into the nest. Queens always initiated aggression towards reproductive workers. Aggression included rapid antennation following immediately by biting. Queens then held the workers while they flexed their gaster under their bodies, pointing towards the worker. In all cases, the aggressed worker and the queen were separated by nestmate workers who bit, held onto, and pulled at both the queen and the aggressed worker. Subsequently, the aggressed worker (in all cases) and the queen (in 4 of 5 cases) were held by other nestmate workers (queens were held for median: 68 min, range: 0 – 1,098 min; aggressed workers were held for median: 6 min, range: 4 – 347 min). As a result of this aggression, in the five recorded trials, one queen and two workers were killed by their nestmate workers who pulled off the gaster, head, or other appendages. Three out of five of the queens committed multiple acts of aggression (10, 13 and 24 acts were recorded for the respective queens). In the 20 hours of video observation for each of the five colonies used, queens spent a substantial percentage of their time in aggressive conflicts with workers (median percent of time spent in aggressive interactions: 6.75%, range: 1.9 - 72.7%).

### *Dufour's Gland Contents*

After observing the aggression that both queens and queen-aggressed workers received from nestmates, a hypothesis was made that nestmate aggression was being elicited through a chemical signal. Due to the aggressive queen's behavior of flexing her gaster towards the worker she is aggressing, the queen was hypothesized to be the generative source of the chemical. All of the additional compounds that appeared on the tip of the queen's gaster after aggression could be accounted for in the contents of the queen Dufour's gland (Fig. 1).

The contents of the queen's Dufour's gland differed significantly from all other females (Fig. 1 – 2, Table 1). Queens have more compounds in their glands, compared to other workers and gynes (Fig. 1 - 2). Qualitative differences are evident in that all the queen Dufour's glands appear filled with a yellow and oily substance, while the gland contents of all other females appear clear.

The majority of the compounds found in the Dufour's gland were long-chained hydrocarbons ranging from 13 to 33 carbons long. The compounds were identified, when possible, according to the class of compound. Queens have a higher proportion of methyl-branched hydrocarbons and a lower proportion of alkenes in their glands compared to all other females (Table 1).

*Dufour's Gland Treatments*

Workers treated with the Dufour's gland of their queen received significantly higher levels of aggression from their fellow nestmates than workers treated with reproductive nestmate-worker Dufour's glands and non-reproductive nestmate-worker Dufour's glands (Fig. 3). One of the workers treated with a queen Dufour's gland was killed (dismembered as described above) by her nestmates. No workers were killed in the other treatment groups. Workers treated with worker Dufour's glands only received brief instances of biting from their nestmates, while workers treated with queen Dufour's glands were bitten, held, and pulled by their nestmates.

## Discussion

*A. cockerelli* queens use the contents of their Dufour's gland to direct aggression towards reproductive workers. The contents of the queen's Dufour's gland distinguish her from all other members of the colony. In addition, the cuticular hydrocarbon profiles of queens also distinguish them from the non-reproductive workers and are used as signals of fertility status (Smith, Hölldobler, and Liebig 2011; Smith, Hölldobler, and Liebig 2008; Smith, Hölldobler, and Liebig 2009). Thus, two different signals are unique to queens, but these signals have different functions and are addressed to multiple receivers: workers responding to the presence of a fertile queen by practicing reproductive self-restraint, and workers who are restraining the reproductive efforts of others.

The use of the Dufour's gland by an established reproductive to direct aggression towards a nestmate has unique features in *A. cockerelli*, but is also very similar to the case of the queenless ant species *Dinoponera quadriceps* (Monnin et al. 2002). Both *A. cockerelli* queens and *D. quadriceps* reproductives can be distinguished from nestmates through their cuticular hydrocarbon profiles as well as the contents of their Dufour's gland (Fig. 1-2) (Monnin et al. 2002; Peeters, Monnin, and Malosse 1999; Smith, Hölldobler, and Liebig 2011). Both *A. cockerelli* queens and *D. quadriceps* reproductives use their Dufour's gland to direct aggression towards reproductive nestmates while their cuticular hydrocarbon profiles are used to signal reproductive ability (Fig. 3) (Monnin et al. 2002; Peeters, Monnin, and Malosse 1999; Smith, Hölldobler, and Liebig 2011; Smith, Hölldobler, and Liebig 2008; Smith, Hölldobler, and Liebig 2009). However, the colony characteristics and the reproductive potential of colony members differ strongly between the two species. *D. quadriceps* has small colonies (mean size of 81 individuals), and there is no reproductive dimorphism among all colony members, *A. cockerelli* differs from *D. quadriceps* in having both large colonies (mature colonies contain 2,000 – 9,000 workers spread across multiple nest sites) and a high degree of worker-queen reproductive dimorphism (workers average five ovarioles while queens average 33 and are the only individuals that can fertilize eggs with sperm stored in their spermatheca) (Hölldobler and Carlin 1989). These characteristics make queen aggression in *A. cockerelli* an exceptional case because direct queen to worker physical conflict is not predicted to occur in these types of colonies (Beekman and Ratnieks 2003; Bourke 1999; Hölldobler and Wilson 2009; Keller and Nonacs 1993).

*A. cockerelli* is a clear exception to this prediction as queens not only display a stereotypical aggressive behavior towards reproductive workers (Smith, Hölldobler, and Liebig 2011), they also have a chemical marker that is used during reproductive conflict to punish reproductive workers. One explanation for why this behavior and chemical marker are used in *A. cockerelli* is because reproductive workers have the ability to produce cuticular hydrocarbon profiles qualitatively similar to those of a reproductive queen (Smith, Hölldobler, and Liebig 2011; Smith, Hölldobler, and Liebig 2008). It is possible that when a reproductive queen is introduced into a group with reproductive workers some of those reproductive workers are indistinguishable from a queen. Therefore, this chemical similarity would necessitate queens to have a queen-specific marker that enables workers to distinguish who the reproductive cheater is. This suggests that the primary cause for the evolution of such a punishment system is not the lack of reproductive dimorphism as in the case of *D. quadriceps* but rather the high reproductive potential of workers and the associated signaling system.

A surprising observation in this study was of a queen being killed by her nestmates after attacking a reproductive nestmate worker. Although both worker and queen Dufour's gland secretions elicit nestmate aggression, only the contents of the queen's gland elicit a sometimes lethal amount of aggression equal to that observed in the unmanipulated instances (Fig. 3). A hypothesis as to why queens receive aggression from nestmates is that queens have difficulty avoiding self-contamination when they attempt to expel their Dufour's gland on reproductive workers. The Dufour's gland empties through the sting and in *A. cockerelli* the sting is greatly reduced and is nonfunctional as a weapon (Hölldobler, Stanton, and Markl 1978). Indeed, in this study Dufour's gland compounds were retrieved from the tip of a queen's gaster directly after she had been aggressive towards a reproductive worker (Fig. 1), indicating that queens do not entirely avoid self-contamination. If the observed death of a queen that was recorded in the study was due to self-contamination it is strong supporting evidence that queen-produced Dufour's gland compounds do not serve as a fertility signal.

While it may be possible that the self-contamination observed in this study is an experimental artifact caused by the artificial nest conditions in which this study was done, this queen behavior seems to have high potential costs and raises the question of why queens might risk damage to themselves. One hypothesis is that this behavior is not only used in queen/worker conflict but potentially also in all-or-none situations where queens must compete for securing the position of a reproductive. This may occur if queens invade other colonies with established reproductive queens. *A. cockerelli* colonies are victim to army ant raids during witch the entire colony including the queen evacuate their nests (Smith and Haight 2008). During these incidents it may be possible that colonies merge and queens encounter one another. Queens treat reproductive workers as reproductive competitors and in laboratory observations when two queens encounter one another they are aggressive in a similar way (personal observation). Therefore it is possible that this behavior may be used during queen to queen conflict, however this potential aspect of *A. cockerelli*'s biology has yet to be explored.

More evidence for the use of Dufour's gland content in intracolonial conflicts comes from two other ant species, *Leptothorax gredleri* and *Streblognathus peetersi* (Table 2). *S. peetersi* is a queenless ant where sting smearing between competing alpha workers has indicated that they may be dispelling the contents of the Dufour's gland while being aggressive towards one another. The involvement of the Dufour's gland, however, has not been directly shown in this case. Similarly, competing *L. gredleri* queens sting smear each other and induce nestmate aggression towards marked individuals.

Besides their use in intracolonial conflict, Dufour's gland contents are also used by several social parasites to modify aggression (Table 2). In two of these cases (*Harpagoxenus sublaevis* and *Protomognathus americanus*), if parasitic queens (*H. sublaevis*) or workers (*P. americanus*) directly apply their Dufour's gland contents to the cuticle of workers from the parasitized colony, then the affected workers may receive deadly amounts of aggression from their nestmates. In the remaining reports of the usage of the Dufour's gland in parasitic species the effects are either enhancing alarm (*Formica subintegra*) or minimizing aggression towards the parasite (*Rossomyrmex minuchae*, *Polyergus samurai*, *Polyergus rufescens*). In all cases, modifying the host workers aggressive responses is the reported function of the Dufour's gland. In total, these reports from both dulotic and non-dulotic span several subfamilies of ants that have vastly different social structures, suggesting that a major function of the Dufour's gland is eliciting nestmate aggression during intra-colony conflict or social parasitism.

With distinct differences in the Dufour's gland contents and the cuticular hydrocarbon profile between queens and workers of *A. cockerelli* and at least one other documented ant (*D. quadriceps*), two potential sources of fertility signals are present in ants. However, as this study shows, the presence of signal differences that correlate with reproductive ability does not always indicate that the signal functions as an indicator of fertility. The multiple receiver hypothesis presents an alternative view of the function of multiple signals of quality (Andersson et al. 2002). The application of this hypothesis to social insect research could reformulate how signals of quality are interpreted.

Several examples of multiple signals of quality in regards to queen-worker differences are present in social insects. The Dufour's gland also contains a queen-specific chemical blend in bees (Amsalem et al. 2009; Katzav-Gozansky et al. 1997) and wasps (Bhadra et al. 2010; Mitra and Gadagkar 2011). The function of this unique chemical blend is often initially assumed to be a fertility signal, informing self-policing or whether or not workers decide to become reproductive. However, tests of the effect of the Dufour's gland alone on worker behavior and physiology both in honeybees and wasps does not account for all of the effects expected if workers were self-policing (Bhadra et al. 2010; Katzav-Gozansky et al. 2004). Considering alternative functions for the gland contents could prove to be beneficial to our understanding of chemical communication in insects.

### *Conclusion*

Up to now the multiple receiver hypothesis has mostly been attributed to explaining the existing of multiple male ornaments and visual signals in birds. Chemical signaling, rather than visual signaling, is the main mode of communication used by social insects. This study shows that approaching investigations into the function of queen-specific chemical signals in ants through the framework of the multiple receiver hypothesis can provide a great service in understanding the complexities of communication within insect societies.

The Dufours's gland and the cuticular hydrocarbon profile of queens in at least two species of ants (*A. cockerelli* and *D. quadriceps*) are prime examples of how the multiple receiver hypothesis can explain the existence of seemingly similar signals. While both signals distinguish reproductives from non-reproductives the Dufour's gland functions as a chemical marker used to punish reproductive subordinates rather than signaling fertility to nestmates. From compiling the published accounts of the Dufour's gland being used during conflict in ants, directing the aggressive responses of nestmates seems to be a common major function of this gland across ants.

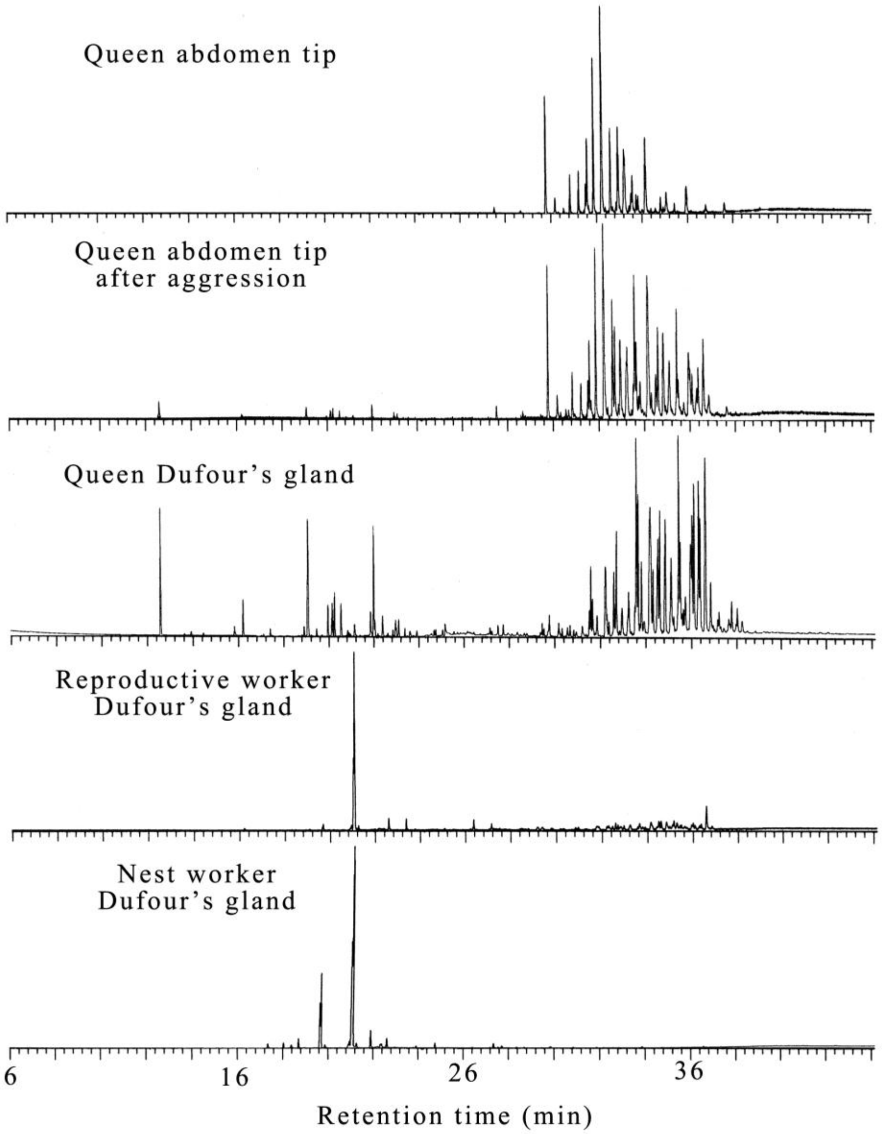

Figure 2.1. Representative chromatograms showing the Dufour's glands contents of queens and workers. The top three chromatograms are from the same individual queen. The first was generated from sampling the tip of the queen's gaster, the following is sampling the same area immediately after the queen had aggressed a reproductive worker. The reproductive worker Dufour's gland is from a worker that received aggression from a queen.

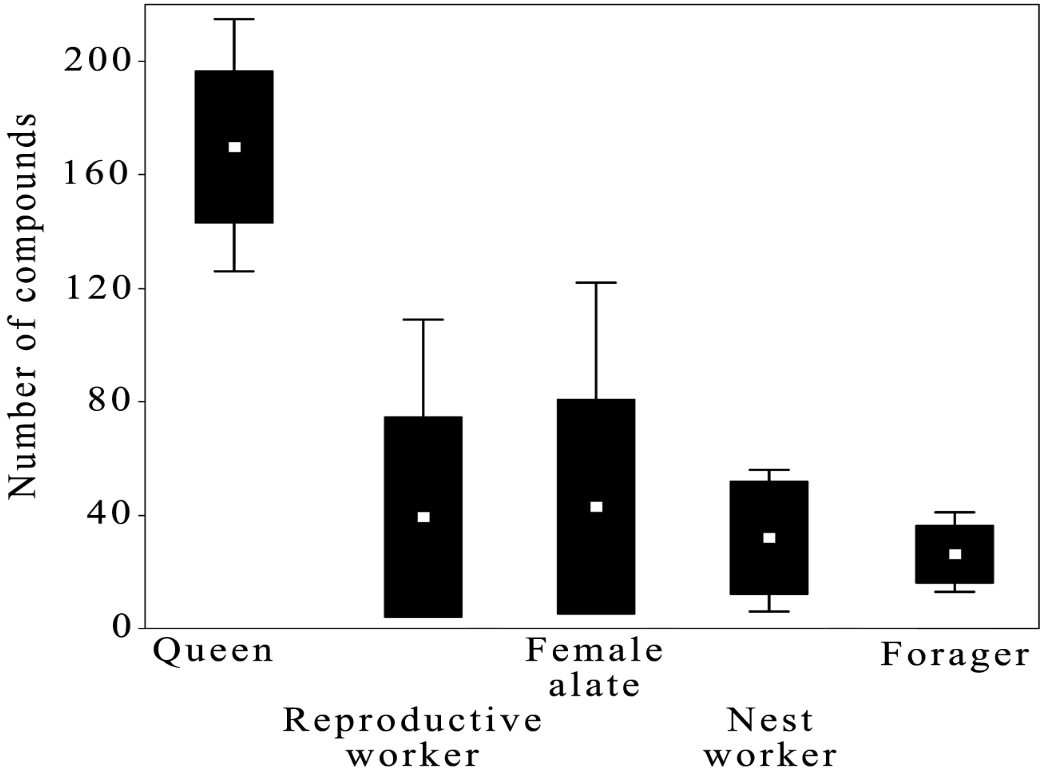

Figure 2.2. Number of compounds found in the Dufour's gland. *N* = 9 for all groups, except Reproductive worker *N* = 10. Number of compounds presented in means, +/- standard deviation, maximums and minimums. Reproductive workers are workers that have elicited aggressive responses from queens. Levene's test: $P = 0.066$. ANOVA: $F_{1,4} = 42.05$, $P < 0.001$. Post hoc analysis, Tukey HSD: Queen vs. all other groups $P < 0.001$, all other comparisons not significant.

Table 2.1. Median Relative Proportions of Classes of Compounds Found in the Dufour's Gland of *A. cockerelli* Females.

| Compound Type | Queens | Reproductive Workers | Female Alates | Nest Workers | Foragers |
|---|---|---|---|---|---|
| Alkanes | 0.14 | 0.19 | 0.33 | 0.15 | 0.17 |
| Methyl-alkanes | 0.36* | 0.14* | 0.19 | 0.05* | 0* |
| Dimethyl-alkanes | 0.19 | 0.11 | 0.08 | 0.17 | 0.16 |
| Diene-aldehydes | 0.03 | 0.01 | 0.03 | 0.05 | 0.06 |
| Alkenes | 0.16* | 0.25 | 0.21 | 0.21 | 0.31* |
| Unidentified | 0.11 | 0.21 | 0.13 | 0.3 | 0.33 |

*Indicates significant differences between queens and workers (Kruskal-Wallis ANOVA: $P < 0.01$, non-parametric multiple comparison 2 tailed $P < 0.05$). All other differences are not statistically significant. Comparisons between relative proportions of unidentified compounds were not made. $N = 9$ all groups except reproductive workers were sample size is 10.

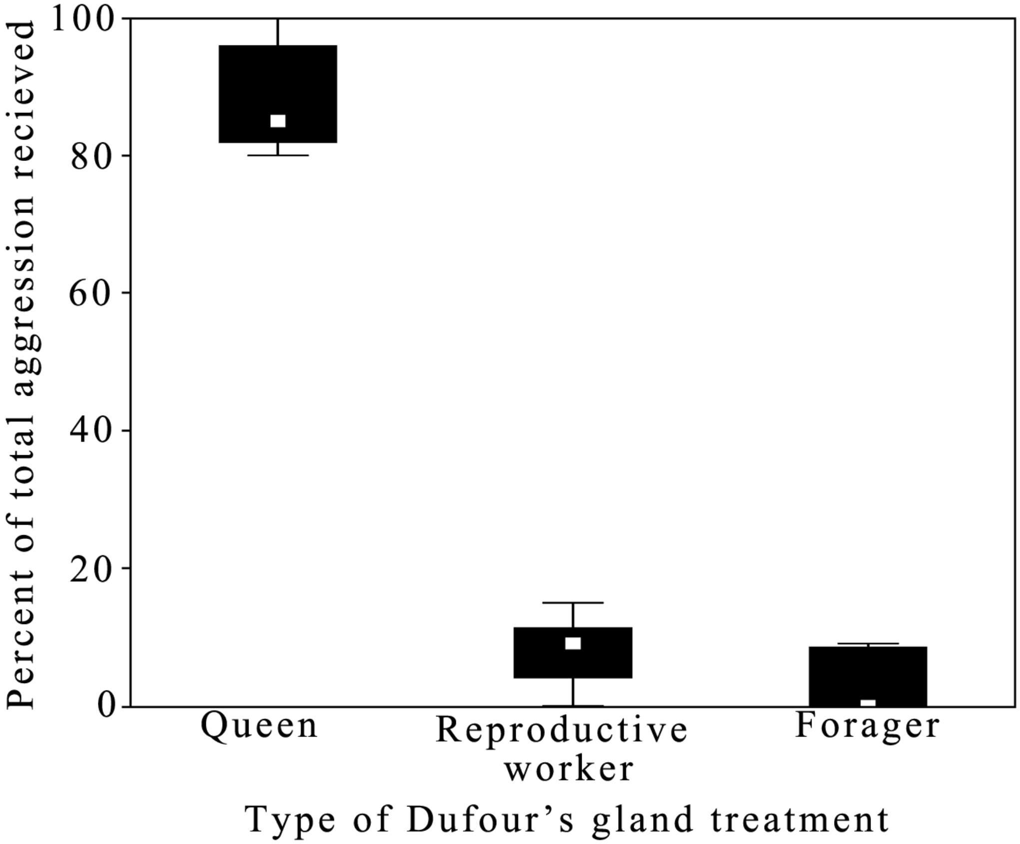

Figure 2.3. Effect of Dufour's gland treatment. *N* = 5 all groups, medians, 25 – 75%, maximums and minimums. One of the workers represented here in the Queen Dufour's gland treatment was killed by her nestmates. No workers were killed in either of the other treatment groups. Friedman's ANOVA: *P* = 0.009. Wilcoxon matched pair test: Queen vs. Reproductive worker and Forager *Z* = 2.02, *P* = 0.043; Reproductive worker vs. Forager *Z* = 1.60, *P* = 0.108.

Table 2.2. Reported Usages of the Dufour's Gland in Conflict Among Ants.

| Species | Response to Dufour's gland | Reference |
|---|---|---|
| *Dinoponera quadriceps* | Induces directed nestmate aggression towards queen-marked individual | (Monnin et al. 2002) |
| *Leptothorax gredleri* | Competing queens are marked and receive aggression from nestmate workers | (Heinze, Lipski, and Holldobler 1992; Heinze et al. 1998) |
| *Streblognathus peetersi* | Competing alphas are sting-smeared and immobilized by nestmates | (Cuvillier-Hot, Renault, and Peeters 2005) |
| Dulotic species | | |
| *Harpagoxenus sublaevis* | Induces deadly fights among marked nestmate workers of the parasitized colony | (Allies, Bourke, and Franks 1986; Buschinger 1974; Foitzik, Fischer, and Heinze 2003) |
| *Protomognathus americanus* | Induces strong aggressive responses among nestmates towards contaminated workers of the raided colony | (Brandt et al. 2006) |
| *Formica subintegra* | Induces panic and dispersion among workers of parasitized colony | (Regnier and Wilson 1971) |
| *Polyergus rufescens* | Lowers aggression towards the social parasite either through appeasement or repulsion | (D'Ettorre et al. 2000; Mori, Grasso et al. 2000; Mori, Visicchio et al. 2000; Topoff et al. 1988) |
| *Polyergus samurai* | Repels host workers from the invading social parasite queen | (Tsuneoka and Akino 2009) |
| *Rossomyrmex minuchae* | Repels host workers from the invading social parasite queen | (Ruano et al. 2005) |

Chapter 3

# THE REGULATION OF WORKER REPRODUCTION IN THE POLYDOMOUS ANT SPECIES *APHAENOGASTER COCKERELLI*

## Abstract

A hallmark of eusociality is a reproductive division of labor between subordinates and established reproductives. In most groups, however, workers retain some reproductive capabilities. Measures of successful worker reproduction within the presence of a queen across insect societies, with few exceptions, indicate that worker reproduction, if it occurs at all, is kept at very low levels. Certain colony-level characteristics such as queen number, queen mating frequency, and physical presence of a queen in species with multiple nesting sites may influence the degree to which worker-queen reproductive conflict is swayed to promote worker reproduction.

In this study, the level of worker reproduction in field colonies of the ant species *Aphaenogaster cockerelli* is measured. *A. cockerelli* is a monogynous and polydomous species, so worker reproduction across nesting sites is investigated. None of the 297 males sampled provided any evidence of worker reproduction. Worker reproduction was detectable at and above a level of 1.5% of the total male population. An effective mating frequency for queens of this species was found to be 1.03. Although *A. cockerelli* colonies have many colony-level factors potentially promoting

worker reproduction (workers with trophic egg-laying active ovaries, a single singly-mated queen, workers who are physically separated from the queen) it is evident that worker reproduction is highly regulated. Synthesizing the extensive amounts of policing and fertility signaling data previously reported on this species, *A. cockerelli* is presented as case study for how worker reproduction is repressed and cooperation is maintained in insect societies.

## Introduction

In most social insect species, workers have retained functional ovaries, capable of producing unfertilized male-destined eggs (Bourke 1988). Worker reproduction in the presence of the queen has been documented within several social insect genera (Barron, Oldroyd, and Ratnieks 2001; Bourke 1988; Brunner et al. 2005; Choe 1988; Hammond and Keller 2004; Tsuchida et al. 2003). Relatedness alone predicts that worker reproduction in the presence of a queen is promoted when the colony contains a single queen who has mated only once, as workers are more related to their son's and nephews rather than their brothers, and general patterns seem to support this prediction (Wenseleers and Ratnieks 2006). Relatedness asymmetries are, however, just a portion of the factors that might promote or discourage worker reproduction. Conflict over sex allocation and losses in colony performance or efficiency might lead to the restraint of worker reproduction (Ratnieks 1988; Ratnieks, Foster, and

Wenseleers 2006). Indeed, among the ants, worker reproductive restraint has been found is several species wherein a lack of genetic conflict would predict worker reproduction (Endler, Liebig, and Hölldobler 2006; Hartmann et al. 2003; Helanterä and Sundström 2007b; Iwanishi, Hasegawa, and Ohkawara 2003; Kikuta and Tsuji 1999). The question of whether or not workers are reproducing in their colony and why they might be prevented from doing so remains a central motivator behind the study of social insects.

The restraint of worker reproduction takes the form of workers either behaviorally inhibiting (policing) the reproductive efforts of other workers or individual workers exhibiting reproductive self-restraint (Ratnieks 1988; Wenseleers et al. 2004). Two dominant modes of policing behaviors in ant societies are physical policing of potential egg layers and egg policing (Ratnieks, Foster, and Wenseleers 2006). The reproductive efforts of individuals that are physically policed are inhibited when nestmates attack the policed individual (Dietemann et al. 2003; Gobin, Billen, and Peeters 1999; Hartmann et al. 2003; Hölldobler and Carlin 1989; Iwanishi, Hasegawa, and Ohkawara 2003; Liebig, Peeters, and Holldobler 1999; Monnin and Peeters 1999; van Zweden et al. 2007). Egg policing occurs when newly worker-laid eggs are destroyed by nestmates (D'Ettorre, Heinze, and Ratnieks 2004; Endler et al. 2004; Helanterä and Sundström 2005; Kikuta and Tsuji 1999).

In order for either one of these policing behaviors to be carried out effectively, the policing individuals must have reliable information indicating which workers should be policed and which eggs should be destroyed. Hydrocarbon blends present on the cuticle of workers and queens, and on the surface of eggs are thought to contain these signals (reviewed in: Liebig 2010; Peeters and Liebig 2009). Reproductive workers can be policed when they are differentiated from non-reproductive workers and queens by their cuticular profile; while worker-produced eggs can be selectively destroyed when they are distinguishable from the established egg profile produced by the queen.

The queen-produced egg surface hydrocarbon profile is also an important means of advertising queen presence and fertility status to workers who otherwise might not exhibit reproductive self-restraint (Endler et al. 2004). In the ant *Camponotus floridanus*, workers isolated from their queen but in the presence of queen produced eggs refrain from personal reproduction (Endler et al. 2004). It has been hypothesized that in monogynous ant species that nest across multiple non-connected sites (polydomous species), eggs are transported across nesting sites to advertise the presence of a fertile queen and promote worker reproductive restraint.

The effects of polydomy in monogynous ant species on worker-queen reproductive conflict have only received attention in a handful of species. In four monogynous and polydomous species workers seem to bias the worker-sexual brood ratio in their favor by sexualizing diploid brood in queenless satellite nests (Banschbach and Herbers 1996; Cerda, Dahbi, and Retana 2002; Denis, Pezon, and Fresneau 2007; Ito, Higashi, and Maeta 1988; Snyder and Herbers 1991). This is modeled to be a result of queen-worker conflict over investment in colony growth vs. reproduction (Herbers, DeHeer, and Foitzik 2001; Pamilo 1991).

In colonies with singly mated queens, solely based on relatedness, there is also potential queen-worker conflict over male production. In one of the species reported to bias sex-ratios in queenless nests, *Myrmica punctiventris*, worker male production has been reported, however a correlation between queen location and successful worker reproduction was not made (Herbers and Mouser 1998). In another monogynous and polydomous species, *Pachycondyla goeldii*, workers in queenless satellite nests have an intermediate level of ovarian development compared to workers in orphaned and queenright nests, and distinctly shaped worker-produced eggs were found in queenless satellite nests (Denis, Pezon, and Fresneau 2007). However, in the former study, the presence of realized worker-derived males in field colonies was not determined.

In this study, genetic data is used to search for the presence of worker-derived males in the monogynous and polydomous desert ant species *Aphaenogaster cockerelli*. To determine the effective mating frequency of queens, two microsatellite loci are used. The question of whether or not any of the males present in the queenright or queenless nesting sites are worker-derived is also answered. Basic colony demographic data is provided, such as worker and male distribution across nesting sites. Finally, the lack of evidence of any worker reproduction is explained by reviewing what is known about how worker reproduction is regulated in *A. cockerelli* colonies.

## Methods

### *Sample Collections*

Mature colonies of *A. cockerelli* were collected and sampled in the Chihuahuan desert between Portal, Arizona and Rodeo, New Mexico, in early June of 2009 and 2010 before the monsoonal rain-triggered mating flights of this species occurred. Mature colonies consist of a queenright nest and 1 – 4 non-connected satellite nests containing workers and brood (Hölldobler and Carlin 1989), therefore worker and male samples were taken from each nest present. For the seven colonies used in this study, entire colonies were collected by triggering nest evacuations using the army ant *Neivamyrmex nigrescens* (Smith and Haight 2008). The number of males (adults and pupae) and workers (adults) captured from each nest of five of these colonies were hand counted to make measures of mature colony sizes (Table 1). (Undoubtedly some workers escaped collection and foragers were not accounted for, so the measures of colony size are conservative.) The remaining two colonies were not counted. For this study, the nest identified as queenright was the nest from which the queen exited during nest evacuation.

*Worker Reproduction*

After collection, samples from each colony were stored in 100% ethanol at -80°C. DNA was extracted by first drying the sample and removing the gaster. Then the sample was crushed in 200µl of 10% Chelex 100 and 1µl Proteinase K. This mixture was then incubated at 57°C for 1 hour, which was followed by heating to 95°C for 5 minutes. Finally, the mixture was centrifuged at 14,000 rpm for 10 minutes and the supernate, containing the DNA, was extracted.

Males and workers were genotyped using two microsatellite loci, E19 (J. Gadau unpublished data) and LxAGT-1 (Bourke, Green, and Bruford 1997). A subset of the total workers and males from seven colonies collected were genotyped (Table 1). The queen genotype and mating frequency were inferred by the worker genotypes using Matesoft (Moilanen, Sundstrom, and Pedersen 2004).

Polymerase chain reaction (PCR) amplifications were performed in 12µl reaction volumes at heating rates of 5 °C/s. The reaction mix contained 1 µl of a 1:20 dilution of extracted supernate (DNA), 2.5µl 5X Colorless GoTaq® Reaction Buffer (Promega), 6.35 µl of de-ionized water, 0.5 µl of dNTP and $MgCl_2$, 0.125 µl Taq DNA polymerase (5 U/µL, MBI Fermentas), and 0.5 µl of each primer. All loci were amplified using the following parameters: 37 cycles of 30 s at 94 °C, 30  at the annealing temperature (E19: 51.3 °C; LxAGT-1: 57 °C), and 30 s at 72 °C; an initial denaturation step of 5 min at 94 °C and a final elongation step of 5 min at 72 °C was added.

*Power to Detect Worker Reproduction*

Independent assortment of the two loci used for this study is tested for following the protocol of Smith et al. (2007). Both haploid males and diploid workers were used to test for linkage disequilibrium in colonies where the queen genotype was heterozygous at both loci (four of the seven colonies). The observed frequencies of allele combinations for both loci were tested for independence using a chi-square goodness of fit test. The null hypothesis was that the alleles assort independently, and the test was performed separately for each colony.

Worker-derived males were able to be detected only if the queen genotype and her mates had different alleles and the males had inherited the paternal allele of the worker (informative alleles). The following equation taken from Foster, Ratnieks, and Raybould (2000) was used to calculate the total number of assignable males ($N_a$) in this study.

$$N_a = \sum_{j}^{n} \left( 1 - \prod_{i}^{l_j} \left( 1 - 0.5 p_{ij} \right) \right) N_j$$

Where $l_j$ denotes the number of loci and $N_j$ the number of males analyzed for the $j$th of $n$ nests and $p_{ij}$ is the proportion of informative worker genotypes at the $i$th loci of the $j$th nest. This equation was used instead of the more widely used and generalized equation presented in Foster and Ratnieks (2001), because workers with more than one locus containing both informative and non-informative alleles were not encountered.

Male non-sampling error was also calculated by following Foster, Ratnieks, and Raybould (2000) by using the equation $(1-x)^{Na}$ that calculates the probability of not sampling any worker-derived males, if workers are producing a proportion $x$ of the total males.

## Results

### *Mating Frequency and Colony Demographics*

All of the mature colonies collected for this study contained only a single queen. A total of 135 workers, 18 – 20 per colony were successfully genotyped (Table 1). Any worker genotypes that introduced a new allele that was not seen in any other workers or males sampled from its colony were excluded from the data; there was one worker that fit this description and was excluded. It was excluded from the analysis becuase it was impossible to determine if that individual genotype was due to a sampling or a collection error. Genetic evidence verified the presence of only one reproducing queen per colony, a single queen genotype was deducible in all cases. The effective mating frequency as calculated by Matesoft from the worker genotypes was 1.03. Only genotypes from two workers from one of the seven sampled colonies indicated the presence of more than one male genotype.

Mature colony sizes were previously estimated to range between 1,000 – 3,000 workers (Johnson 2000). These data reveal that colony sizes are much greater (Table 1; average total worker number/colony: 6,294; min, max: 4,961, 8,375). Number of workers per nest was not a reliable indication of queen location, as queenless satellite nests contained more workers in 3/5 cases (worker number in queenright vs. mean worker number in queenless-satellite nest; Wilcoxon Matched Pairs Test: $Z = 0.94$, $P = 0.35$). The nests of all colonies, with the exception of one, also contained female alates. All nests contained larvae. Eggs were found in exactly half of queenless satellite nests of the colonies that were fully counted. This report of brood in queenless nests verifies earlier observations of above ground brood transfer happening between nests in this species (Hölldobler and Carlin 1989).

### *Worker Reproduction and Detection Power*

A total of 297 males from the seven colonies were genotyped, 20 – 60 males per colony were sampled for this study (Table 1). Neither the sampled males nor workers from any colony indicated that the two loci used did not independently assort (for all tests: $\chi^2 < 1.8$, $P > 0.6$).

Therefore, both loci could be used to calculate worker reproduction detection power.

All male haplotypes from all colonies and nests were consistent with being queens' sons; no evidence of worker reproduction was found. The number of assignable males, $N_a$, was estimated to be 206.5. The calculated detection power is 0.95 for worker reproduction greater than 1.5% (Fig. 1).

## Discussion

No indication of worker reproduction in either queenright or queenless satellite nests of *A. cockerelli* was found. These data, along with unpublished data gathered from 31 colonies and 487 workers genotyped for two microsatellites (Mösl and Gadau), suggest that *A. cockerelli* queens are singly mated (effective mating frequency of 1.03). Relatedness alone would predict that workers should favor raising their own son's and nephews over brothers. However, this study along with previous reports, finds that this prediction does not hold (Hammond and Keller 2004). Several other factors besides within nest relatedness led towards predict finding some degree of worker reproduction in *A. cockerelli*.

Nest workers have active ovaries that are used to produce trophic eggs for distributing nutrients inside the nest, as this species does not perform liquid food sharing through trophalaxis (Hölldobler and Carlin 1989). Workers are known to switch to laying viable male eggs when, in the lab, they are separated from their queen for as short of a time as two weeks (Hölldobler and Carlin 1989; Smith, Hölldobler, and Liebig 2008). In the field, mature colonies have been reported to consist of up to five independent nests, not physically connected by any excavated chambers or tunnels (Hölldobler and Carlin 1989; Smith, Hölldobler, and Liebig 2008). This physical separation from the queen has been correlated in other species with increasing worker influence over colony reproduction and even increasing the degree of worker reproductive activity (Banschbach and Herbers 1996; Cerda, Dahbi, and Retana 2002; Denis, Pezon, and Fresneau 2007; Ito, Higashi, and Maeta 1988; Snyder and Herbers 1991). It is therefore somewhat surprising that with these potential factors influencing worker reproduction, no evidence of it in this study population was found.

A proximate mechanism for preventing workers who are physically separated from the queen from reproducing might be nestmate policing. In the presence of a reproducing queen, workers are under selection (whether from relatedness, colony efficiency, or sex ratio pressures) to police the reproductive efforts of their nestmates (Ratnieks 1988; Ratnieks, Foster, and Wenseleers 2006). In fact, the presence of an effective policing system is thought to promote worker reproductive self-restraint (Ratnieks and Reeve 1992; Wenseleers et al. 2004). One mechanism through which queens advertise their presence and reproductive activity is through their eggs (Endler et al. 2004). In this study eggs were found in queenless satellite nests, and in a previous study above ground brood transport between nests had been documented (Hölldobler and Carlin 1989). It is therefore likely that workers in satellite nests act as if they are in the presence of a reproductive queen and police the reproductive efforts of their nestmates.

Policing in *A. cockerelli* has been well documented. Workers do not police (destroy) the viable eggs produced by their nestmates (Smith, Hölldobler, and Liebig 2008). They do, however, physically police (aggress) nestmates who become reproductively active (Hölldobler and Carlin 1989; Smith, Hölldobler, and Liebig 2008; Smith, Hölldobler, and Liebig 2009). Additionally, if workers allow their nestmates to develop into egg laying workers (i.e. in prolonged episodes of isolation from the queen), queens will physically police those reproductive workers themselves (Smith, Hölldobler, and Liebig 2011) (Fig. 2).

Causal evidence that suggests *A. cockerelli* workers judge nestmate fertility and police their nestmates according to fertility associated changes in the cuticular hydrocarbon profiles (Smith, Hölldobler, and Liebig 2009). Further correlative evidence linking policing behaviors and hydrocarbon signals also supports the fertility signaling role of surface hydrocarbons: 1) *A. cockerelli* workers do not police eggs and worker-produced viable eggs have a surface hydrocarbon profile qualitatively indistinguishable from that of queen eggs (Smith, Hölldobler, and Liebig 2008), 2) workers who are allowed to establish themselves as reproductive individuals are capable of developing a queen-like hydrocarbon profile, and when their queen encounters them she physically polices them as she would a reproductive competitor (Smith, Hölldobler, and Liebig 2011). Synthesizing our knowledge of the policing behaviors found in this species and the reproductive signals involved in informing the behavior, reveals that worker reproduction in this species is highly regulated (Fig. 2).

As workers activate their ovaries for viable egg production this is accompanied by a shift in their hydrocarbon profile, placing them between the profile of a non-reproductive worker and a queen. If they escape worker policing and further develop their viable egg laying activity the presence of fertility-associated hydrocarbons on their cuticle increases, making them more queenlike in appearance and more likely to be policed by the queen herself. This gradual development of a cuticular hydrocarbon fertility signal has been documented in other ant species (Cuvillier-Hot et al. 2001; Endler, Liebig, and Hölldobler 2006; Hannonen et al. 2002), and this linkage of reproductive development with the development of fertility signal ensures that *A. cockerelli* workers will face policing from two different parties if they attempt to successfully reproduce (Fig. 2).

Although worker reproduction was not detected at any level, detection power was very low for finding low levels (<1.5%) of worker-derived males (Fig. 1). Worker reproduction at frequencies lower than 1.5% has been documented in honey bees (Page and Erickson 1988; Ratnieks 1993; Visscher 1989). In honey bees, workers actively police worker laid eggs and greatly reduce the proportion of worker-laid eggs that survive to maturity (Ratnieks 1993). Modeling the effects of an effective policing system on the level of reproductive cheaters within a colony predicts that cheaters will be rare even in colonies where relatedness favors a relatively higher level of worker-reproduction (Wenseleers et al. 2004). Furthermore, competition between groups or colonies is thought to favor colonies that effectively police reproductive cheaters and maintain a low level of reproductive cheaters over evolutionary time (Brandvain and Wade 2007; Van Dyken, Linksvayer, and Wade 2011).

Evidence of effective policing systems is found in many studies of policing behaviors in social insects, as is the case with *A. cockerelli*. However, genetic data describing the pattern of male parentage across colony and nesting sites are needed to reveal the actual degree to which worker reproduction regulated. These results verify that policing behaviors are an effective means through which worker reproduction can be regulated, even in social insect systems where within-colony relatedness and colony structure might promote worker reproduction.

Table 3.1. Colony Sizes and Sample Sizes, Per Colony and Per Nest, of Individuals Successfully Genotyped for at least one of the Microsatellite Loci.

| | | Colony | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Fh2 | Fh3 | Dw8 | Dw9 | Dw10 | 901 | 97 |
| Queen nest | # workers; # genotyped | 4612; 10 | 3121; 10 | 1824; 9 | 1605; 5 | 3047; 7 | x; 7 | x; 7 |
| | # males; # genotyped | 119; 15 | 413; 10 | 227; 18 | 50; 18 | 103; 18 | x; 18 | x; 18 |
| Satellite nest 1 | # workers; # genotyped | 766; 8 | 1840; 10 | 5244; 9 | 2157; 5 | 3127; 7 | x; 6 | x; 7 |
| | # males; # genotyped | 19; 18 | 210; 10 | 143; 18 | 85; 18 | 223; 18 | x; 18 | x; 18 |
| Satellite nest 2 | # workers; # genotyped | | | | 1271; 5 | 2201; 6 | x; 6 | x; 6 |
| | # males; # genotyped | | | | 36; 18 | 10; 10 | x; 18 | x; 12 |
| Satellite nest 3 | # workers; # genotyped | | | | 658; 5 | | | |
| | # males; # genotyped | | | | 6; 6 | | | |

*x denotes samples that were not counted

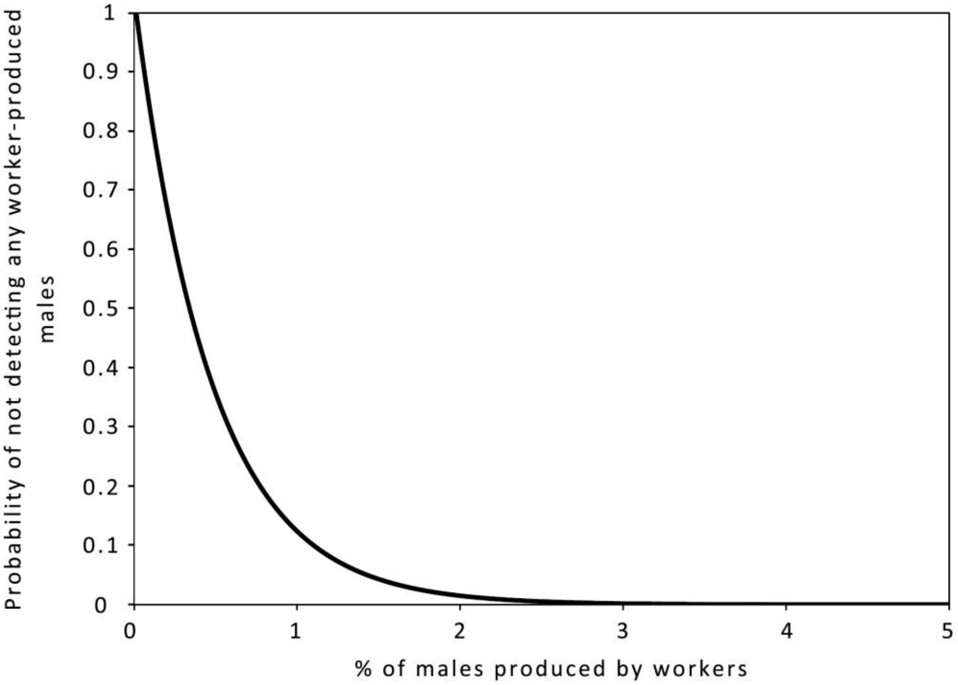

Figure 3.1. Power of detection a worker-derived male at various levels of worker reproduction within colonies. From the 206.5 assignable male genotypes, $N_a$, worker reproduction, if at all present, accounts for fewer than 1.5% of the total males present in a colony.

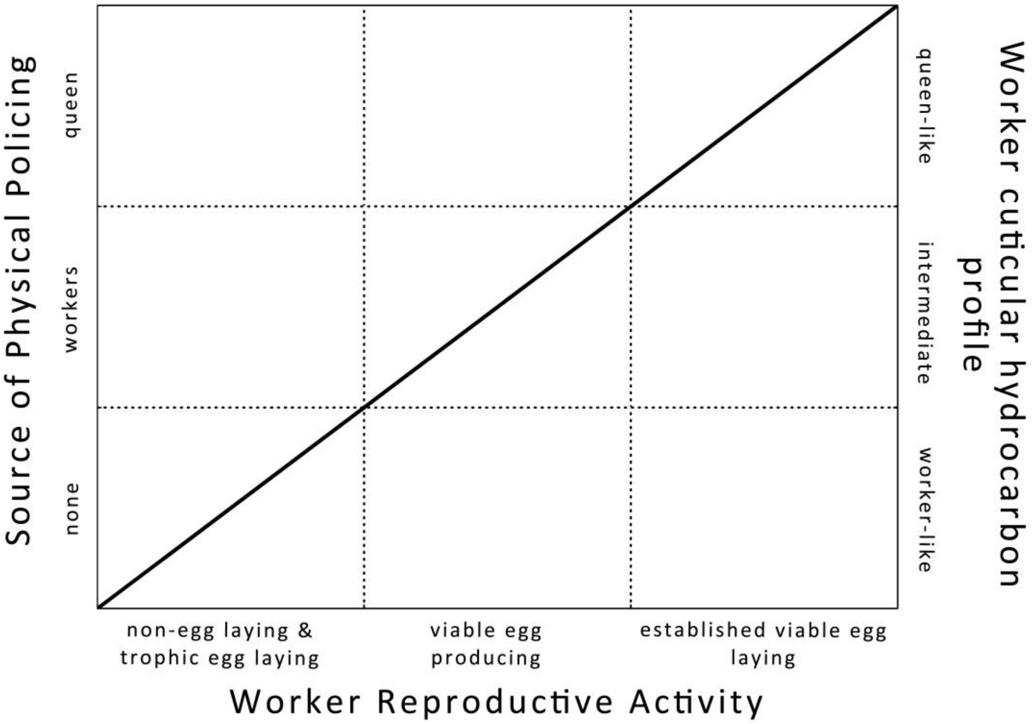

Figure 3.2. The means of restraining worker reproduction in *A. cockerelli*. The known policing behaviors (left y-axis) and the chemical signals (right y-axis) that are used to inform are seen in relation to the level of worker reproductive activity (x axis). They are synthesized here from previously published accounts (Hölldobler and Carlin 1989; Smith, Hölldobler, and Liebig 2011; Smith, Hölldobler, and Liebig 2008; Smith, Hölldobler, and Liebig 2009).

## Chapter 4

## CONCLUSION

### Synthesis and Impact of Research

Collectively, this work describes the means by which worker reproduction is regulated in *A. cockerelli*. This work could be performed after developing a means for collecting entire mature colonies of *A. cockerelli* from the field (Smith and Haight 2008 [Appendix I]). Herein, the repertoire of policing behaviors performed by this species of ant is described (Chapter 2; Smith, Hölldobler, and Liebig 2008, 2011 [Appendices II, IV]). The chemical signals that are the informational basis for policing worker reproduction are described (Chapter 2; Smith, Hölldobler, and Liebig 2008, 2009, 2011 [Appendices II, III, IV]). The first case of causal evidence demonstrating the role of cuticular hydrocarbon fertility signals in policing is provided, filling a long-standing gap in the understanding of fertility signals in social insects (Smith, Hölldobler, and Liebig 2009 [Appendix III]). Genetic data that determines the degree to which worker reproduction is suppressed in field colonies of *A. cockerelli* is presented (Chapter 3). The discussion section of chapter three is a summary of how the modes of policing and the fertility signals used in *A. cockerelli* work in concert in the process of regulating worker reproduction.

Within the field of social insect research, this body of work is significant in that it describes the system of reproductive regulation in an ant species with derived colony characteristics rather than one with more primitive traits (i.e. small colony size and low level of reproductive dimorphism). The majority of studies on policing behaviors in ants have focused on species with primitive colony characteristics (Monnin and Ratnieks 2001); however, there are a few exceptions (e.g. Endler, Holldobler, and Liebig 2007; Helanterä and Sundström 2007a; Moore and Liebig 2010). Physical policing, especially from the queen, is predicted to occur only in social insect colonies with low levels of queen-worker reproductive dimorphism and small colony sizes (Bourke 1999; Hölldobler and Wilson 2009), however these behaviors are found in *A. cockerelli*. One possibility as to why *A. cockerelli* relies on physical policing to maintain worker reproductive restraint is that nest workers have constantly active ovaries for producing trophic eggs that can switch to producing viable eggs in a short period of time and workers have the ability to produce fertility signals equivalent to that of queens (Smith, Hölldobler, and Liebig 2011 [Appendix IV]). The ability of *A. cockerelli* workers to produce cuticular hydrocarbon profiles and egg profiles equivalent to those produced by the queen makes them an interesting system for further studies of fertility signaling and its effects on reproductive restraint in ants. If worker-produced eggs have an effect on worker reproduction similar to any self-restraint effect caused by queen eggs, this would be evidence of queen-produced hydrocarbons not serving a queen signal but rather a generalized fertility signal. Since workers can produce cuticular hydrocarbon profiles that are equivalent to that of a queen, this attribute combined with the high degree of anatomical dimorphism between queens and workers, makes *A. cockerelli* an ideal system to test if fertility signals are solely responsible for making a fertile queen seem like a queen to her nestmates. In other words, can a queen hydrocarbon-mimicking worker evoke similar nestmate responses to those evoked by a queen. This experiment again would highlight any potential differences between the concept of queen signals and general fertility signals.

This body of work is a case study in how cooperation is maintained in a social organism. The importance of policing and punishment has been theoretically demonstrated in many social species and is thought to be a key attribute of successful societies (Frank 2003). Although no evidence of successful reproductive cheaters was found, and no evidence suggesting that workers are attempting to cheat in natural colonies was found, what was found was an extensive repertoire of behaviors and adaptations for suppressing potential reproductive cheaters. It is predicted that the presence of an effective policing system alone will sufficiently lower the benefits of reproductive cheating so that reproductive self-restraint is favored (Ratnieks and Reeve 1992; Wenseleers et al. 2004). Policing behaviors are thought to occur more commonly in societies where within colony relatedness is low due to multiple mating of the queen, rather than in societies where the queen is singly mated and workers are highly related to one another (Foster and Ratnieks 2000; Wenseleers and Ratnieks

2006). This body of works demonstrates that *A. cockerelli* uses multiple forms of policing behaviors even though workers are highly related due to a singly mated queen (Chapter 3). This suggests that relatedness is, alone, an insufficient predictor of both the degree of worker reproduction and the presence of presence of policing behaviors within insect societies. Instead, other aspects of a species' biology such as degree of worker ovarian development, and polydomy might necessitate the need of a well developed system for regulating reproduction (Chapter 3).

This work demonstrates the degree to which and the various means through which reproductive regulation is relied upon in maintaining successful insect societies. It is hoped that this work moves the field forward towards the discovery of other unknown mechanisms promoting social cohesion and cooperation.

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# APPENDIX A

# ARMY ANTS AS RESEARCH AND COLLECTION TOOLS

# Army ants as research and collection tools

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## Abstract

Ants that fall prey to the raids of army ants commonly respond by evacuating their nests. This documented behavior has been underexploited by researchers as an efficient research tool. This study focuses on the evacuation response of the southwestern desert ant *Aphaenogaster cockerelli* André (Hymenoptera: Formicidae) to the army ant *Neivamyrmex nigrescens* Cresson. It is shown that army ants can be used to collect mature colonies of ants. The applicability of this tool to ecologically meaningful areas of research is discussed.

**Keywords:** *Neivamyrmex nigrescens, Aphaenogster cockerelli*, nest evacuation, absconding
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**Received:** 27 September 2007 | **Accepted:** 24 January 2008 | **Published:** 14 November 2008

**ISSN:** 1536-2442 | Volume 8, Number 71
**Note:** Bob Jeanne was editor of this paper

**Cite this paper as:**
Smith AA, Haight KL. 2008. Army ants as research and collection tools. 5pp. *Journal of Insect Science* 8:71, available online: insectscience.org/8.71

## Introduction

A common defense response of ants to army-ant raids is nest evacuation. The rapid fleeing from the nest of workers with brood, sometimes in the company of the queen, is a well documented response of preyed-upon ant species throughout the New World (Schneirla 1971). The army ant *Neivamyrmex nigrescens* Cresson (Hymenoptera: Formicidae), which is distributed throughout the southwestern United States, is a particularly well studied species in which nest evacuation is a common response of their prey. Several species of ants across multiple genera are raided by this ant. The reported list of preyed-upon genera which are known to evacuate workers and brood from their nests in response to *N. nigrescens* includes: *Pheidole* (Droual 1983, 1984), *Trachymyrmex* (Topoff et al. 1980), *Camponotus* (LaMon and Topoff 1981) and *Aphaenogaster* (McDonald and Topoff 1986). Species in which the full colony (workers with brood and queen) evacuates in response to *N. nigrescens* include *Camponotus festinatus* (LaMon and Topoff 1981), *Aphaenogaster (=Novomessor) albisetosa* and *Aphaenogaster cockerelli* (McDonald and Topoff 1986).

Researchers documenting nest evacuation responses have successfully induced evacuations by introducing a relatively small number (100–150) of army ants to the targeted colony (McDonald and Topoff 1986; LaMon and Topoff 1981; Droual 1983, 1984). Although triggering an evacuation has been proven to be simple, this behavior has been poorly exploited in other areas of myrmecological research. A study by Helms and Rissing (1990) utilizes the evacuation response of *Pheidole* species to census alate production and is, to our knowledge, the lone example of utilizing army ants as a research tool. It is surprising that in the twenty years since nest evacuation behavior has been thoroughly documented that this method of extracting ants from the ground has not been more generally applied as a field research tool. In this methodological paper the evacuation response of *A. cockerelli* to *N. nigrescens* is demonstrated. The benefits of exploiting this behavior as a means for collecting mature colonies and its research applications are discussed.

## Methods and Results

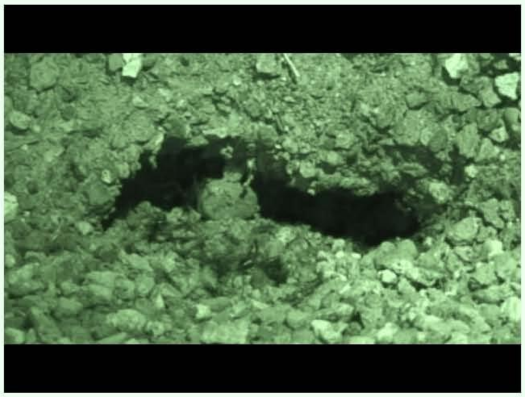
*Aphaenogaster cockerelli* occurs in intermountain plains throughout the southwestern United States and into Mexico (Johnson 2000). Colonies are monogynous and mature colonies are polydomous, with 2–5 distinct nests (Hölldobler and Carlin 1989). For this study colonies were collected primarily from the Chihuahuan desert between Portal, Arizona and Rodeo, New Mexico from the end of August through the beginning of October, 2006 and in August, 2007 (N= 30). Collections were also made 12 km east of Apache Junction, Arizona in August, 2006 (N = 2). Three different collection methods were attempted: physical excavation, flood-triggered nest evacuation, and army-ant triggered nest evacuation.

Physical excavation was attempted on *A. cockerelli* colonies with small (~15 cm diameter) nest mounds. Five days (45 hours) were spent on the excavation of 14 colonies; resulting in the collection of a total of four queenright colonies of a few hundred workers. The majority (n=10) of excavations yielded only workers and brood. Excavations frequently went one meter deep, and the queens that were collected were probably caught because they happened to be located near the surface. Single nest excavations, dug using picks and shovels, took approximately two to three hours.

A total of six colonies were flooded. The evacuation of brood was triggered after approximately 1.9 liters of water was poured into the nest entrance. Incrementally streaming water into the nest entrance gradually induced workers with brood to evacuate to a neighboring satellite nest but never resulted in the evacuation of the queen. After two to three hours of incremental flooding, the number of workers exiting the nest carrying brood diminished to zero.

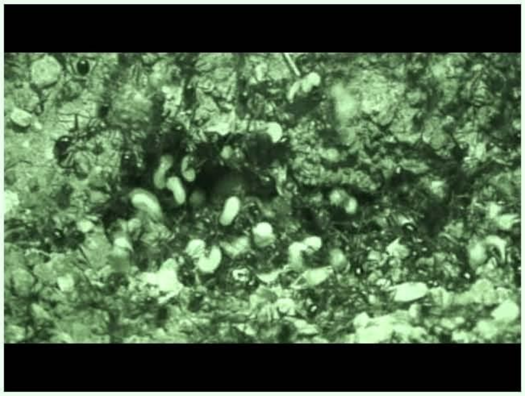
The third method used workers of the army ant, *N. nigrescens*, to induce evacuation of *A. cockerelli* nests. Due to both *N. nigrescens* and *A. cockerelli* being crepuscular/nocturnal ants the collections of both species were done between 1800 hours and 0100 hours. *N. nigrescens* workers were collected from raiding columns. In a total of four collection trips, raiding columns of *N. nigrescens* were encountered by randomly walking and scanning the ground for raiding columns. To collect *A. cockerelli*, groups of 100–150 army ants where aspirated into 50 ml centrifuge tubes, shaken into ball-like masses, and poured directly into the entrances of *A. cockerelli* nests. Evacuation response was nearly instantaneous. Within fifteen seconds a steady stream of workers were running out of the nest (Video 1). Within thirty seconds to one minute workers were exiting carrying brood. Additional army ants were not introduced unless the rate of brood exiting the nest diminished significantly or workers reentered the nest with brood. Queens usually exited, or were carried, out of the nests after the first 150–300 army ants were introduced (Video 2). On some occasions when large nests (~5,000–6,000 workers) were evacuated the queen appeared only after more than 300 army ants had been added. On average three colonies were collected per hour of work. A total of 28 queenright colonies with an estimated average size of two to three thousand workers were collected from two field sites using this method.

Queenright colonies of *A. cockerelli* could be collected much more effectively using army ants (Figure 1). Physical excavation yielded a single queenright colony per 11.25 hours of work, while a single queenright colony



**Video 1.** Worker nest evacuation response of *Aphaenogaster cockerelli* triggered by placing 150 *Neivamyrmex nigrescens* workers at the pictured nest entrance.

**View this video online at** http://www.insectscience.org/8.71/ref/video1.html



**Video 2.** *Aphaenogaster cockerelli* workers evacuating carrying brood in response to the introduction of *Neivamyrmex nigrescens* workers, followed by the evacuation and collection of the queen. Video slowed to half-speed.

**View this video online at** http://www.insectscience.org/8.71/ref/video2.html

could be caught in just twenty minutes of work using army ants.

## Discussion

Utilizing the nest-evacuation response of *A. cockerelli* when collecting mature colonies proves to be the most efficient means of colony collection. Not only is the method much

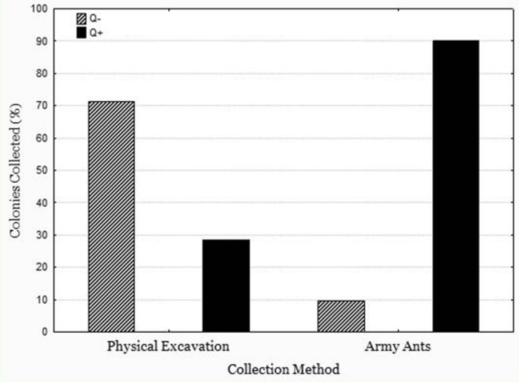


**Figure 1.** Collection of *Aphaenogaster cockerelli* colonies by physical excavation (n=14) and utilizing *Neivamyrmex nigrescens* ants (n=31). Using army ants proves to be a more effective method than physical excavation for capturing queenright (Q+) colonies (2-tailed Fisher exact test, p<0.0001). Queenless (Q-) colony collection represents both nests without a queen and failed collections where the queen (if present) could not be captured.

faster than all others tried, but it also allowed the collection of large mature nests. Excavating small nests of a few hundred workers required two to three hours of work, whereas when using *N. nigrescens* as a collection tool collection of a queenright colony with 4,000 workers took approximately twenty minutes.

Knowledge of the natural behavioral patterns of these two ant species proves to be essential when undertaking field collections. The army ant *N. nigrescens* forages at night when *A. cockerelli* is most active and has been observed to preferentially raid *A. cockerelli* nests in late summer and early fall when conditions are dryer and other prey species (*Pheidole*) become less active (Mirenda et al. 1980). The collections described here were done under these conditions. The same species of army ant is known to prey on several other co-occurring ant species, as listed above, and elicit a full colony evacuation (with queen) in at least two other species (Lamon and Topoff 1981; McDonald and Topoff 1986). This common evacuation response of several ant genera to this species of army ant suggests that this approach to collection could be an effective method for the collection of other ant species that are preyed upon by army ants.

Beyond collecting mature colonies from the field, the evacuation responses of preyed-upon species can be exploited in other areas of research. After evacuation, species from several genera have been documented to reenter the original nest (*Phiedole*: Mirenda et al. 1980; Droual 1984. *Trachymyrmex*: LaPolla et al. 2002. *Aphaenogaster*: personal observations). By triggering evacuations and collecting the evacuating workers and brood, sociometric data can be easily gathered. Since the colony will reenter the nest colonies can be non-destructively assessed multiple times throughout development, providing valuable descriptive life history data (Tschinkel 19991). The question of the impact of such repeated evacuations on the colony remains to be investigated.

Promising studies of queen turnover or replacement are possible in systems where queens evacuate with their colony and nests are re-inhabited. Three monogynous species that are known to evacuate with the queen in response to *N. nigrescens* include *Camponotus festinatus, Aphaenogaster albisetosa* and *A. cockerelli*. Queen replacement in orphaned monogynous species has been shown to occur both by adoption of foreign queens and daughter queens from within the nest (Sanetra and Crozier 2002; Tschinkel 1996; DeHeer and Tschinkel 1998). Studies of genetic colony kin structure predict queen replacement in monogynous species to be an important occurrence in the life cycle of a colony (Heinze and Keller 2000). Using army ants to remove the queen without damaging the nest structure or the worker population is quite possible and would be a worthwhile method to study the behavior and reproductive efforts of orphaned colonies in their natural environment.

Finally, studies of interspecific competition often involve the removal of one of the competing species (King and Tschinkel 2006; LeBrun et al. 2007). Removing a targeted species in an ecologically non-destructive and responsible way is of great importance to these studies and has taken considerable effort (Tschinkel and King 2007). Using army ants to non-destructively remove a competitive species is an as-of-yet unused but promising method. For example, ***A. cockerelli*** is a species that co-occurs with other seed harvesting ants such as ***Pogonomyrmex barbatus*** and ***Pogonomyrmex rugosus*** (Johnson RA 2000; Hölldobler et al. 1978), and is likely to compete with these species along with other smaller omnivorous ant species such as ***Solenopsis xyloni and Monomorium minimum*** (Hölldobler et al. 1978). ***A. cockerelli*** is therefore a prime example of a competitive species that can easily be removed from an ecosystem without harming the habitat or co-occurring fauna in the process.

Utilizing army ants to induce nest evacuations in prey species is a promising, and often overlooked tool. The applicability of this method seems to be far reaching if the faunal system including *N. nigrescens* and it's preyed-upon species is any indication of the potential for using other army ant predator-prey interactions.

## Acknowledgments

We thank Jürgen Liebig for comments on this manuscript, and Dani Moore, Clint Penick, and Jürgen Liebig for their help in the field.

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# APPENDIX B

# HYDROCARBON SIGNALS EXPLAIN THE PATTERN OF WORKER AND EGG POLICING IN THE ANT APHAENOGASTER COCKERELLI

J Chem Ecol (2008) 34:1275–1282
DOI 10.1007/s10886-008-9529-9

# Hydrocarbon Signals Explain the Pattern of Worker and Egg Policing in the Ant *Aphaenogaster cockerelli*

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Received: 29 April 2008 / Revised: 4 June 2008 / Accepted: 30 July 2008 / Published online: 16 August 2008

**Abstract** In ant societies, worker reproduction is regulated through policing behaviors, such as physical aggression or egg eating. The information used by policing individuals is thought to be in blends of hydrocarbons present on the cuticle and the surface of eggs. These fertility signals have been studied in numerous genera. However, signaling patterns that emerge across distinct subfamilies of ants have yet to be explained. We investigated policing behavior and the chemical signaling upon which policing behaviors are informed in the ant *Aphaenogaster cockerelli*. We found that worker-produced eggs are not policed, and we showed that there is a lack of chemical signaling for effective egg policing to occur in this species. Furthermore, we identified the available signals that demarcate workers to be policed physically. We showed that in *A. cockerelli*, a species with derived social organization, workers produce fertility signals identical to the queen. This queen-like signaling may be due to workers maintaining a high level of ovarian activity, linked to trophic egg production, in the presence of the queen.

**Keywords** Physical policing · Egg policing · Cuticular hydrocarbons · Worker reproduction · Fertility signal

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## Introduction

Conflict resolution often requires suitable information. In ant societies, reproduction by specific colony members may reduce the fitness of other nestmates. Defending fitness interests can be achieved by reliable identification of fitness-reducing individuals and their offspring. It is assumed that hydrocarbon profiles that reveal the reproductive status of workers and the identity of their eggs are used for this purpose in ant societies. These profiles and the abilities of individuals to produce them are variable throughout ant genera. Factors that might influence patterns of reproductive signaling have not been identified.

In ant societies, a single or a few individuals within the society specialize in reproduction while other individuals make up the workforce, caring for the offspring and maintaining the colony (Hölldobler and Wilson 1990). In most species, these workers have retained functional ovaries, capable of producing unfertilized male-destined eggs (Bourke 1988). In some species, workers have active ovaries in the presence of a queen, producing non-viable trophic eggs, morphologically distinct from viable male-destined eggs (e.g., Brian and Rigby 1978; Hölldobler and Carlin 1989). However, worker production of male offspring is typical only after the loss of the primary reproductive caste or queen (Bourke 1988; Choe 1988).

When workers forgo reproduction, the lack of direct fitness is balanced by fitness gains received through raising related offspring and maintaining an efficient and productive colony (Hammond and Keller 2004). Haplo-diploidy and colony kin structure theory predicts worker reproduction to be favored when a colony has a single, singly mated queen, as workers in these colonies are more related to their sons ($r=0.5$) and to males from sister-workers ($r=0.375$)

than males produced by their mother ($r=0.25$). Although worker reproduction may be favored due to relatedness, other factors such as maintaining colony efficiency and conflict over sex allocation favor reproductively restrained workers (Cole 1986; Ratnieks 1988). Under queenright conditions, workers usually either behaviorally inhibit (police) the reproductive efforts of other workers or are selected to exhibit reproductive self-restraint (Ratnieks 1988; Wenseleers et al. 2004).

The two most common policing behaviors in ant societies are physical policing of potential egg layers and egg policing (Monnin and Ratnieks 2001; Ratnieks et al. 2006). The reproductive efforts of individuals that are physically policed are inhibited by nestmates attacking the policed individual (Hölldobler and Carlin 1989; Dietemann et al. 2003; Hartmann et al. 2003). Egg policing occurs when worker-laid eggs are destroyed by nestmates (Kikuta and Tsuji 1999; D'Ettorre et al. 2004; Endler et al. 2004). In order for either one of these policing behaviors to be carried out effectively the policing individuals must have reliable information that indicates which workers should be policed or which eggs should be destroyed. Hydrocarbon blends present on the cuticle of workers and queens, and on the surface of eggs, are thought to contain these signals.

Cuticular hydrocarbons provide resistance to desiccation (Lockey 1988) and have the potential for holding information through recognizable quantitative and qualitative blend differences (Monnin 2006; Le Conte and Hefetz 2008). Changes in cuticular hydrocarbons in numerous ant species reliably correlate with changes in reproductive ability (e.g., Liebig et al. 2000; Dietemann et al. 2003, 2005; Cuvillier-Hot et al. 2004; Lommelen et al. 2006). This potentially allows the effective policing of individuals that change their hydrocarbon blends while becoming reproductively active. However, experimental data to prove a causal relationship has not yet been presented. Similar correlative evidence exists for egg policing. Policing occurs where eggs can be identified according to the reproductive caste of the egg-laying worker (Monnin and Peeters 1997; D'Ettorre et al. 2004; Endler et al. 2004). A causal relationship between surface hydrocarbons and egg policing has been shown in the ant *Camponotus floridanus* (Endler et al. 2004). In this species, workers destroy significantly fewer worker-produced eggs when the eggs are given a queen-derived profile. This indicates that egg surface hydrocarbons are the signal that provides the information necessary for effective egg policing.

In the current study, we examined behavioral regulation of reproduction and fertility signaling within the worker caste of the desert-ant *Aphaenogaster cockerelli*. The derived status of this species is indicated by a distinct worker–queen dimorphism and large-size colonies with a single queen. Workers of this species have highly active ovaries that produce trophic eggs in the presence of a queen and viable male eggs in as few as 2 weeks after the removal of the queen (Hölldobler and Carlin 1989). In the presence of a queen, workers have been shown to police reproductive nestmates physically (Hölldobler and Carlin 1989), thus limiting workers to trophic egg production in queenright colonies. However, egg policing has not yet been examined in this species. Furthermore, the hydrocarbon signals that potentially underlie both of these behaviors have yet to be identified. Herein, we study egg policing and identify the hydrocarbon signals available for both egg and physical policing behaviors.

Studies of fertility signaling and policing in ants have mostly focused on more primitive species with low to non-existent worker–queen reproductive dimorphism. Our findings on the signaling and policing behavior of *A. cockerelli* provide new insights into the evolution of fertility signals. We conclude that the policing behaviors performed in this species are a direct result of the signals available to the policing workers. In comparison to other ant species, our results provide the first insights into the physiological adaptations that may influence patterns of reproductive signaling.

## Methods and Materials

*Study Species* Mature colonies of *A. cockerelli* were collected from the Chihuahuan desert between Portal, Arizona, and Rodeo, New Mexico, from the end of August through the beginning of October 2006 and in August 2007. Colonies were collected by triggering nest evacuation using army ants (*Neivamyrmex nigrescens*; Smith and Haight 2008). Collected colonies averaged 1,500 workers; all had a single queen. Mature colonies consist of a nest with a single queen and one to four distinct satellite nests containing workers and brood (Hölldobler and Carlin 1989). The colonies used for this experiment consisted primarily of workers collected from the nest containing the queen. Previous work based on 31 colonies and 487 workers genotyped for two microsatellites has shown that queens are singly mated (Mösl and Gadau, unpublished data).

*Laboratory Conditions* In the laboratory, ants were housed in a dental-plaster nest with molded chambers, darkened inside by red acetate over glass. The nests were attached to a foraging arena in which they received a constant supply of water, sugar–water, and pieces of cricket (*Acheta *domestica*) and beetle larvae (*Zophobas morio*). For this study, temperature was maintained at 25°C, and the foraging arenas were kept in constant light.

*Egg Policing* To determine whether or not workers selectively destroy viable eggs produced by sister workers, we measured egg acceptance by using discriminator worker groups (a method used to show egg policing; Endler et al. 2004, 2006). These worker groups consisted of 50 randomly chosen nest workers. Each group was placed into a nest connected to a foraging arena similar in construction to the queenright colonies from which they originated. These workers were given food (as mentioned above), water, sugar–water, and 24 h to acclimate to their new nests. There were three discriminator worker groups from each colony ($N$=9) in this experiment. Each group simultaneously received one of three egg treatments, introduced into the nest area. One group received 20 mother-queen-produced eggs, another received 20 sister-worker produced eggs, and the final group received a mixture of ten mother-queen eggs and ten sister-worker eggs. Sister-worker-produced eggs were taken from isolated worker groups that had become reproductive, exhibiting an egg-carrying behavior indicative of tending viable eggs rather than trophic eggs (Hölldobler and Carlin 1989). This setup allowed us to test if workers would police worker-produced eggs in the presence and absence of queen-produced eggs. *A. cockerelli* workers are often separated from the queen due to polydomous colony nest structure (mentioned above). Therefore, the short-term absence of a queen present in these discriminator groups should not have affected the outcome of our egg-policing tests.

The number of eggs remaining after 24 h was recorded in all treatments. This period of time has proven to be suitable for egg policing to occur (Endler et al. 2004; Dietemann et al. 2005; Helanterä and Sundström 2007). Egg survival in the experimental groups was analyzed by using a non-parametric Friedman's ANOVA.

*Hydrocarbon Profiles* Queen ($N$=16), non-reproductive worker ($N$=14), and reproductive worker ($N$=10) cuticular hydrocarbon profiles were collected from our laboratory colonies. Note that the sample size (above) represents ants from independent colonies, except the reproductive worker group in which two workers from the same colony were sampled, thus making colony sample size actually $N$=9. Queen profiles were taken from live queens that were reproductively active, as indicated by the presence of eggs. Non-reproductive workers consisted of non-callow workers that were randomly picked off of the brood pile of a queenright colony; therefore, these ants were likely to be trophic-egg producing, brood-tending workers. Reproductive workers were taken from reproductive groups of workers (100–200 individuals) that had been isolated from their queen and brood for an extended time span (3 weeks to 6 months). Workers were classified only as reproductive (1) when they had been observed laying an egg that was then deposited onto the pre-existing egg pile (to ensure that the worker was producing viable eggs, not trophic eggs, as the latter are immediately fed to brood or eaten by other workers; Hölldobler and Carlin 1989), and (2) when subsequent dissection of these workers confirmed ovarian activity and the condition or type of yolky oocytes indicated the development of viable eggs.

Eggs used for gathering egg profiles were taken directly from the egg piles of both queenright colonies and isolated worker groups (mentioned above) that had started to reproduce.

*Chemical Analysis* Cuticular hydrocarbon profiles were gathered from live queens and workers by using solid-phase microextraction (Arthur and Pawliszyn 1990). A fiber (Supelco Inc., coated with a 30-μm polydimethylsiloxane film) was rubbed on the gaster surface for 5 min (Monnin et al. 1998). The fiber was inserted into the injection port of an Agilent 6980N series gas chromatograph (GC) equipped with a DB-1MS (J&W Scientific) non-polar capillary column (30 m×0.25 mm×0.25 μm), connected to an Agilent 5975 series mass selective detector. The GC injection port was set to 260°C and the transfer line to 300°C. The column temperature was held at 60°C for 2 min before increasing to 200°C at 40°C min$^{-1}$, and then to 320°C at 5°C min$^{-1}$. Helium was used as carrier gas at 1 ml min$^{-1}$, and samples were injected in the splitless mode. Electron impact mass spectra were measured at 70 eV with a source temperature of 230°C. Identification of the compounds was tentatively based on a comparison of their mass spectra with published spectra, synthetic alkane standards, and retention indices.

Egg-surface hydrocarbon profiles were gathered by extracting groups of 20 eggs in 20 μl of hexane for 5 min. The extract was evaporated and suspended in 2 μl of hexane from which 1 μl was injected into the GC.

Compounds were included in the statistical analysis if they occurred in ≥70% of the sampled individuals within at least one of the classes (queen, reproductive worker, nest worker, worker-produced egg, and queen-produced egg). We performed non-parametric multi-dimensional scaling to analyze the similarity of cuticular and egg profiles by using Primer 6. Chord distances were used to calculate the distance matrices.

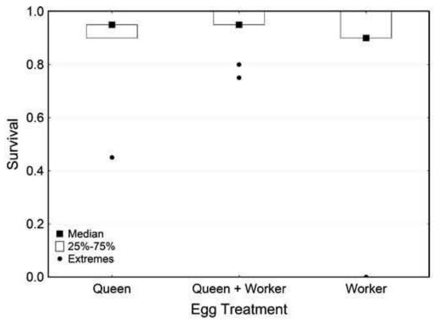

**Fig. 1** Bioassay of egg survival over 24 h. Survival of 20 queen eggs is compared to ten queen eggs mixed with ten worker eggs and to 20 worker eggs. There was no statistical difference among treatments, Friedman's ANOVA, $n_{\mathrm{groups}}=9$, $P=0.875$

## Results

*Egg Policing* There was no significant difference in the survival of eggs among the different treatments (Fig. 1). Median survival of queen eggs and a mix of worker and queen eggs was 0.95, while median survival of worker eggs was 0.9. If workers effectively police worker-produced eggs, survival of the queen and worker mixed egg group would have been 0.5, while worker egg survival would have been zero.

*Egg Surface Hydrocarbon Profiles* The two classes of eggs (worker-produced and queen-produced) were not qualitatively different in their hydrocarbon profiles. Only small relative abundance differences were distinguishable (Fig. 2, Table 1), which was confirmed by the lack of a clear separation of the two classes of eggs in the non-parametric multi-dimensional scaling (Fig. 3). The stress value of 0.09 indicates a good graphical representation of the data structure.

*Cuticular Hydrocarbon Profiles* Quantitative and qualitative differences separated the classes of individuals (queen, nest worker, and reproductive worker; Fig. 2, Table 1). For instance, compounds 1 9 (Fig. 2) were unique to reproductive individuals. There were only three of these compounds that occurred in all reproductive workers sampled (peaks 3, 7, and 8; Table 1). In two of these three compounds, quantitative differences separated reproductive workers and queens; e.g., median relative abundance of pentacosane (peak 3; Table 1), queen = 25.2 and reproductive worker = 6.8.

Non-parametric multi-dimensional scaling separated queens and non-reproductive workers well, with a large gap between the two groups (Fig. 4). The reproductive workers were between the two groups as expected, including profiles that were relatively similar to those of queens and those of non-reproductive workers. The stress value of 0.07 indicates a good graphical representation of the data structure.

## Discussion

The crucial requirement for effective reproductive policing is the ability to identify which workers or which eggs should be policed. Our findings suggest that the policing behaviors of *A. cockerelli* can be explained by discriminatory information present in hydrocarbon blends on the worker cuticle and the surface of eggs. Workers of *A. cockerelli* do not police worker-produced eggs; viable worker egg survival was not different from that of queen eggs in the egg policing experiment. Correspondingly, workers produce eggs with a surface hydrocarbon blend nearly identical to that of queen-produced eggs (Table 1, Fig. 2). In contrast, the cuticular profiles of non-reproductive workers showed strong qualitative differences from queens. Reproductive egg-laying workers fall between the two groups. This difference provides potential to identify reproductive workers, while eggs cannot be distinguished.

*A. cockerelli* is a species with workers that have active ovaries used for trophic egg production within a queenright nest. There are striking parallels between *A. cockerelli* and another trophic egg-producing ant, *Myrmecia gulosa*, in which policing and hydrocarbon signaling has been studied. Like *A. cockerelli*, *M. gulosa* do not police worker-produced viable eggs. Instead, reproductive workers are singled out and policed. Correspondingly, reproductive workers develop a distinct cuticular hydrocarbon profile, and their eggs are covered with a suite of hydrocarbons similar to queen eggs (Dietemann et al. 2003, 2005). In both phylogenetically distant systems (*Aphaenogaster*, Myrmicinae; *Myrmecia*, Myrmeciinae), workers can distinguish only reproductive castes and not their eggs. Thus, policing is limited to physical attacks and immobilization of reproductive workers.

In contrast to the mode of policing seen in *M. gulosa* and *A. cockerelli* is that of the ant *Camponotus floridanus* (subfamily Formicinae). When workers become reproductive, their cuticular hydrocarbon profile does not change.

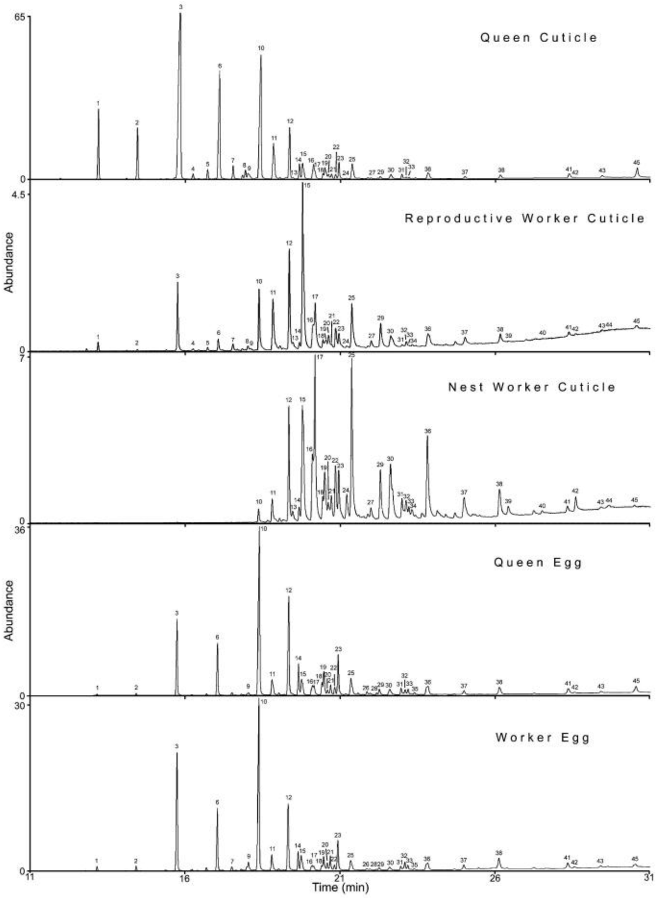

**Fig. 2** Representative chromatograms showing hydrocarbon profiles among sampled classes of individuals and eggs. *Numbered peaks* correspond to compounds in Table 1 and are the compounds included in the statistical analyses

Their eggs receive hydrocarbon blends that are similar to those occurring on the cuticle of workers. This makes worker eggs distinguishable from queen eggs, which contain hydrocarbons that vary with fertility and indicators of fertility such as colony size (Endler et al. 2006). Correspondingly, workers in this system are not physically policed, but their eggs are (Endler et al. 2004, 2006, 2007). In contrast to *M. gulosa* and *A. cockerelli*, *C. floridanus* workers are not trophic egg producers and do not have active ovaries under normal queenright conditions.

The ability to produce queen-like hydrocarbon blends may be associated with workers retaining active ovaries. It has been suggested recently (Endler et al. 2007) that workers of more derived ant genera, with low reproductive potential (inactive ovaries, e.g., *C. floridanus*), may have lost the ability to produce a fertility signal due to their

**Table 1** Tentative identification of chemical compounds found on *Aphaenogaster cockerelli,* along with retention indices and relative concentration

| Peak number | Compound | Retention index | Group | | | | |
|---|---|---|---|---|---|---|---|
| | | | QC | RWC | NWC | QE | WE |
| 1 | Tricosane | 2,300 | 3.3 (1.5, 14.9)* | 0.8 (0, 5.5)* | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) |
| 2 | Tetracosane | 2,400 | 1.9 (0.7, 6.8)** | 0.1 (0, 2.3)** | 0 (0, 0) | 0.2 (0, 0.4) | 0.4 (0, 0.6) |
| 3 | Pentacosane | 2,500 | 25.2 (11.8, 36.8)** | 6.8 (0.6, 24.5)** | 0 (0, 0) | 8.1 (5.8, 13.4) | 10.9 (5.8, 18.1) |
| 4 | 9-Methylpentacosane | 2,530 | 0.4 (0, 1.3)*** | 0 (0, 0.7)*** | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) |
| 5 | 3-Methylpentacosane | 2,567 | 0.4 (0, 1.0) | 0.3 (0, 1.7) | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) |
| 6 | Hexacosane | 2,600 | 12.5 (10.0, 4.3)** | 1.2 (0.4, 6.0)** | 0 (0, 0) | 6.0 (3.4, 7.0) | 4.7 (3.1, 7.9) |
| 7 | 10-;14-Methylhexacosane | 2,630 | 0.9 (0, 2.7) | 0.7 (0.2, 1.6) | 0 (0, 0) | 0.4 (0, 0.6) | 0 (0, 0.6) |
| 8 | x,y-Heptacosadiene | 2,658 | 0.9 (0.2, 3.7)* | 0 (0, 1.3)* | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) |
| 9 | x-Heptacosene | 2,665 | 1.0 (0, 3.5)** | 0 (0, 0.5)** | 0 (0, 0) | 1.5 (0, 2.9) | 0.8 (0, 2.1) |
| 10 | Heptacosane | 2,700 | 20.4 (11.6, 32.1) | 8.0 (3.0, 19.3) | 1.0 (0.6, 3.5) | 24.6 (18.0, 36.8) | 24.2 (15.5, 30.3) |
| 11 | 9-;11-;13-Methylheptacosane | 2,730 | 3.5 (0.5, 13.8) | 3.5 (0, 9.6) | 1.5 (0, 3.8) | 3.4 (1.2, 4.5) | 1.7 (0.9, 3.0) |
| 12 | 3-Methylheptacosane | 2,775 | 6.1 (3.7, 8.7) | 7.1 (0.6, 16.1) | 6.2 (3.4, 12.1) | 12.1 (10.1, 15.5) | 12.7 (9.5, 16.3) |
| 13 | unknown | 2,782 | 0 (0, 0.5) | 0 (0, 1.6) | 0.7 (0, 1.2) | 0 (0, 0) | 0 (0, 0) |
| 14 | Octacosane | 2,800 | 2.2 (1.2, 4.0) | 2.4 (0, 16.2) | 1.0 (0.4, 2.0) | 3.9 (3.0, 4.4) | 3.5 (2.5, 5.3) |
| 15 | 3,7-;3,11-Dimethylheptacosane | 2,808 | 2.3 (0.6, 6.0) | 5.6 (0, 31.0) | 11.1 (4.0, 14.8) | 2.5 (1.4, 4.1) | 3.2 (2.0, 5.0) |
| 16 | 10-;12-;14-Methyoctacosane | 2,835 | 1.1 (0, 4.5) | 5.8 (0.7, 11.4) | 12.6 (1.1, 20.2) | 1.5 (1.1, 2.2) | 1 (0.9, 1.5) |
| 17 | 8,12-Dimethyloctacosane | 2,839 | 0.6 (0, 2.4) | 0 (0, 7.7) | 0 (0, 4.8) | 1.6 (0.5, 2.4) | 1.3 (0.6, 1.9) |
| 18 | unknown | 2,859 | 0.5 (0, 1.1) | 1.3 (0, 5.0) | 1.1 (0, 1.9) | 1.4 (0, 2.4) | 1.2 (0.6, 1.8) |
| 19 | 2- or 4-Methyloctacosane | 2,864 | 1.6 (1.0, 2.2) | 2.5 (0, 4.3) | 4.2 (3.3, 8.7) | 3.1 (2.1, 4.3) | 4.2 (1.2, 6.5) |
| 20 | x-Nonacosene | 2,872 | 0 (0, 1.5) | 1.5 (0, 2.8) | 1.4 (0, 1.9) | 0.8 (0, 1.0) | 1.2 (0, 2.3) |
| 21 | x-Nonacosene | 2,880 | 0 (0, 0.9) | 0.9 (0, 2.9) | 1.8 (0, 3.9) | 1.4 (0.8, 2.0) | 2.1 (0.6, 2.6) |
| 22 | 4,12-Dimethyloctacosane | 2,890 | 0.2 (0, 1.2) | 2.6 (0.4, 30.2) | 3.5 (1.1, 4.5) | 1.1 (0, 1.4) | 1.1 (0.6, 1.6) |
| 23 | Nonacosane | 2,900 | 3.0 (1.5, 5.9) | 0.3 (0, 5.4) | 3.7 (2.3, 9.7) | 5.4 (4.4, 8.6) | 6.2 (4.4, 9.9) |
| 24 | unknown | 2,918 | 0 (0, 0.3) | 0 (0, 0.9) | 1.3 (0, 2.6) | 0 (0, 0) | 0 (0, 0) |
| 25 | 9-;11-;13-;15-Methylnonacosane | 2,933 | 1.3 (0, 5.9) | 8.0 (3.4, 12.5) | 11.1 (8.9, 18.7) | 3.4 (2.7, 5.2) | 3.3 (2.2, 6.1) |
| 26 | 3-Methylnonacosane | 2,975 | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) | 0.4 (0, 1.0) | 0.5 (0, 0.8) |
| 27 | 5,11-;5,13-;5,15-Dimethylnonacosane | 2,983 | 0 (0, 0.3) | 0.8 (0, 1.5) | 1.1 (0, 1.6) | 0 (0, 0) | 0 (0, 0) |
| 28 | Triacontane | 3,000 | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) | 0.3 (0, 0.9) | 0 (0, 0.8) |
| 29 | 3,9-;3,11-;3,13-Dimethylnonacosane | 3,007 | 0 (0, 1.0) | 3.2 (0.7, 4.6) | 4.4 (1.5, 8.0) | 0.9 (0, 1.4) | 1.2 (0, 1.5) |
| 30 | 10-;12-;14-Methyltriacontane | 3,035 | 0 (0, 0.3) | 0.8 (0, 1.5) | 1.1 (0, 1.6) | 1.5 (0, 2.4) | 1.5 (0.6, 2.5) |
| 31 | 2- or 4-Methyltriacontane | 3,064 | 0.4 (0, 0.6) | 1.2 (0, 3.2) | 1.5 (0.9, 3.1) | 0.8 (0, 2.7) | 1.5 (0.5, 2.7) |
| 32 | x-Hentriacontene | 3,075 | 0 (0, 0.5) | 0.7 (0, 2.4) | 1.4 (1.0, 3.2) | 0.8 (0, 1.4) | 1.4 (0.7, 3.7) |
| 33 | x-Hentriacontene | 3,083 | 0 (0, 0.2) | 0 (0, 1.1) | 0.9 (0, 2.4) | 0.9 (0, 1.3) | 1.0 (0, 1.4) |
| 34 | unknown | 3,092 | 0 (0, 0) | 0.2 (0, 3.4) | 0.6 (0, 1.7) | 0 (0, 0) | 0 (0, 0) |
| 35 | Hentriacontane | 3,100 | 0 (0, 0) | 0 (0, 0) | 0 (0, 0) | 0.4 (0, 1.7) | 0 (0, 1.9) |
| 36 | 9-;11-;13-;15-Methylhentriacontane | 3,136 | 0.8 (0, 2.6) | 3.5 (1.1, 8.7) | 6.5 (5.1, 12.5) | 2.2 (0.9, 4.8) | 2.7 (1.1, 5.4) |
| 37 | 10-;12-;14-Methyldotriacontane | 3,235 | 0.1 (0, 0.9) | 1.7 (0.8, 3.2) | 2.2 (1.7, 3.7) | 1.1 (0, 1.6) | 1.1 (0.8, 1.7) |
| 38 | 9-;11-;13-;15-Methyltritriacontane | 3,335 | 0.4 (0, 1.3) | 1.9 (1.1, 4.4) | 3.4 (1.8, 4.9) | 2.4 (1.1, 3.7) | 2.3 (1.6, 3.0) |
| 39 | 13,17-;15,17-Dimethyltritriacontane | 3,357 | 0 (0, 0) | 0 (0, 1.2)*** | 1.0 (0, 2.1)*** | 0 (0, 0) | 0 (0, 0) |
| 40 | unknown | 3,358 | 0 (0, 0) | 0 (0, 0.5)*** | 0.3 (0, 0.4)*** | 0 (0, 0) | 0 (0, 0) |

**Table 1** (continued)

| Peak number | Compound | Retention index | Group | | | | |
|---|---|---|---|---|---|---|---|
| | | | QC | RWC | NWC | QE | WE |
| 41 | 9-;11-;13-Methylpentatriacontane | 3,535 | 1.0 (0.3, 2.4) | 0.6 (0, 1.9) | 2.0 (0.8, 3.1) | 1.7 (0.7, 2.6) | 1.3 (0.7, 1.6) |
| 42 | 13,19-;15,19-Dimethylpentatriacontane | 3,555 | 0 (0, 0.3) | 0.5 (0, 1.7) | 2.0 (0.8, 3.1) | 0.3 (0, 0.5) | 0 (0, 0.8) |
| 43 | 10-;12-Methylhexatriacontane | 3,636 | 0.4 (0, 1.2) | 0.1 (0, 1.2) | 0.4 (0, 1.0) | 0.6 (0, 0.9) | 0 (0, 0.8) |
| 44 | unknown | 3,680 | 0 (0, 0) | 0 (0, 2.6)*** | 0.5 (0, 2.2)*** | 0 (0, 0) | 0 (0, 0) |
| 45 | 9-;11-;13-Methylheptatriacontane | 3,736 | 2.5 (0.5, 6.2) | 0.5 (0, 2.4) | 0 (0, 1.0) | 2.4 (0.8, 3.5) | 1.4 (0.8, 2.0) |

Compounds were tentatively identified on the basis of mass spectrum and retention index; assignment of *n*-alkanes confirmed by comparison with authentic compounds. X, Y: position of double bond unknown. 10-;14-Methylhexacosane=a mixture of 10- and 14-methyl isomers. Mann–Whitney *U* test, $n_{\mathrm{QC}}=16$, $n_{\mathrm{RWC}}=10$, and $n_{\mathrm{NWC}}=14$) in relative compound concentrations. Concentrations are provided as mean (min, max). Group: *QC* queen cuticle, *RWC* reproductive worker cuticle, *NWC* non-reproductive worker cuticle, *QE* queen egg, *WE* worker egg

**P*<0.01, two-sided (significant difference)

***P*<0.001, two-sided (significant difference)

****P*<0.05, two-sided (significant difference)

inability to replace the primary reproductive. However, the social organization of *A. cockerelli* is highly derived with distinct worker–queen dimorphism, large colony size, and a single queen, similar to that of *C. floridanus*. The most obvious difference between the two species is the ovarian activity level of workers in the presence of the queen. *A. cockerelli* is, to our knowledge, one of the most derived species of ant in which workers can produce the reproductive signals utilized by the primary reproductive or queen. This is commonly found in more primitive ant societies, in which worker–queen dimorphism is low or non-existent (Liebig et al. 2000; Cuvillier-Hot et al. 2004; Lommelen et al. 2006). In these systems, nestmates have a reproductive potential that rivals that of the queen. The high level of ovarian activity for trophic egg production of *A. cockerelli* workers may be the underlying physiological condition that has led to workers and queen sharing the same reproductive signaling capabilities. Further fertility signaling studies in other trophic egg-producing derived species of ants are needed to verify this hypothesis.

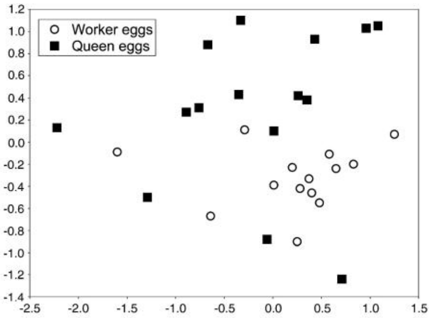

**Fig. 3** Two-dimensional configuration of non-metric, multi-dimensional scaling of surface hydrocarbon blend differences between queen-produced eggs and worker-produced eggs

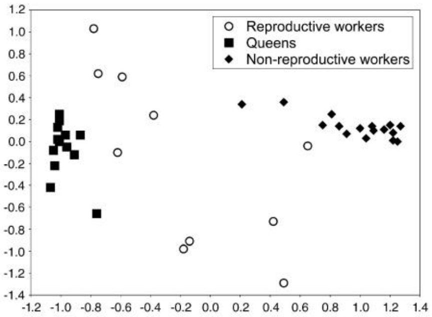

**Fig. 4** Two-dimensional configuration of non-metric, multi-dimensional scaling of cuticular hydrocarbon blend differences among queens, reproductive workers, and non-reproductive workers

**Acknowledgment** We thank Kevin Haight, Dani Moore, Clint Penick, and Dorit Eliyahu for assistance throughout the time this research was performed. C. T. Holbrook provided helpful comments on the manuscript.

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# APPENDIX C

# CUTICULAR HYDROCARBONS RELIABLY IDENTIFY CHEATERS AND ALLOW ENFORCEMENT OF ALTRUISM IN A SOCIAL INSECT

Current Biology *19*, 78–81, January 13, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2008.11.059

**Report**

# Cuticular Hydrocarbons Reliably Identify Cheaters and Allow Enforcement of Altruism in a Social Insect

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## Summary

**Cheaters are a threat to every society and therefore societies have established rules to punish these individuals in order to stabilize their social system [1–3]. Recent models and observations suggest that enforcement of reproductive altruism (policing) in hymenopteran insect societies is a major force in maintaining high levels of cooperation [4–6]. In order to be able to enforce altruism, reproductive cheaters need to be reliably identified. Strong correlational evidence indicates that cuticular hydrocarbons are the means of identifying cheaters [7–14], but direct proof is still missing. In the ant *Aphaenogaster cockerelli*, we mimicked reproductive cheaters by applying a synthetic compound typical of fertile individuals on nonreproductive workers. This treatment induced nestmate aggression in colonies where a queen was present. As expected, it failed to do so in colonies without a queen where workers had begun to reproduce. This provides the first direct evidence that cuticular hydrocarbons are the informational basis of policing behaviors, serving a major function in the regulation of reproduction in social insects. We suggest that even though cheaters would gain from suppressing these profiles, they are prevented from doing so through the mechanisms of hydrocarbon biosynthesis and its relation to reproductive physiology. Cheaters are identified through information that is inherently reliable.**

## Results and Discussion

### Induction of Worker Policing in Queenright Colonies by Application of a Synthetic Compound Indicating Fertility

In the ant *Aphaenogaster cockerelli*, we find a strong correlation between reproduction, cuticular profiles, and policing. On the cuticle of reproductive queens, there are straight alkanes (tri-, tetra-, penta-, and hexacosane) in high quantities that do not appear in the profiles of nonreproductive workers [15] (Figure 1). However, workers that are separated from the queen and allowed to reproduce develop profiles with these compounds [15] (Figure 1). Previous experiments showed that such workers with active ovaries receive aggression upon reintroduction to their colony [16]. In these experiments, when groups of workers are experimentally separated and reunited with their nestmates and queen, individuals capable of producing viable eggs are immediately singled out and attacked. This observation in *A. cockerelli* is strongly indicative of policing, because studies in other ant species have shown that such aggression will lead to inhibition of any ovarian activity [7, 17]. The correlation of worker policing with fertility-related changes in the cuticular hydrocarbon profile of *A. cockerelli* workers suggests the involvement of reproductive-specific alkanes in cheater identification. If such alkanes are indeed responsible for worker policing, a transfer of such compounds on nestmates in similar concentrations should trigger worker aggression toward the manipulated workers.

We transferred the most pronounced reproductive-specific alkane, pentacosane, onto the gaster of nonreproductive workers in a concentration matching that of queenless workers that had fully developed ovaries, capable of producing viable eggs (Figure 1). (Pentacosane on workers after addition treatment [median relative abundance: 5.3%, range: 3.3%, 15.1%, n = 11] was not different than pentacosane abundance on workers with reproductively active ovaries [median relative abundance: 6.3%, range: 0.4%, 30.5%, n = 10; Mann-Whitney U-test: $z = -0.493$, 2-sided $p = 0.654$].) After reintroduction of the treated ants, we recorded directed aggression of nestmate workers that encountered the manipulated individual. For each trial, we used a different colony to exclude any colony-level effects (n = 11). To exclude any manipulation effect, we also transferred similar amounts of nonacosane, an alkane that occurs on the cuticle of all workers and queens, on a second group of workers in addition to a solvent control (Figure 1). (The increase in relative abundance of nonacosane on treated workers [median increase: 5.9%, range: 3.3%, 17.5%, n = 12] was not different from the relative increase of pentacosane on pentacosane-treated workers [median relative abundance: 5.3%, range: 3.3%, 15.1%, n = 11; Mann-Whitney U-test: $z = 0.246$, 2-sided $p = 0.833$].)

If simply manipulating the cuticular hydrocarbon profile induces aggression, we should find the same level of biting in this control group. However, aggression was significantly more frequent toward the pentacosane group than in the nonacosane group (Figure 2A, Movies S1–S4 available online). In addition, antennal inspection, indicative of treatment perception, was significantly raised in the pentacosane-treated group in comparison to the hexane control. Nonacosane-treated workers received intermediate levels of antennation (Figure 2B). By applying the major hydrocarbon compound of fertile workers, we were able to induce aggression toward these manipulated workers. This strongly suggests that cuticular hydrocarbons reveal reproductive status and are used to identify reproductive cheaters.

### Lack of Worker Policing toward Pentacosane-Treated but Nonreproductive Workers in Queenless Colonies

When *A. cockerelli* workers are kept in queenless groups, some of them will activate their ovaries and eventually lay eggs [15]. This change in ovarian activity is accompanied by a shift in the cuticular hydrocarbon profile in *A. cockerelli* as

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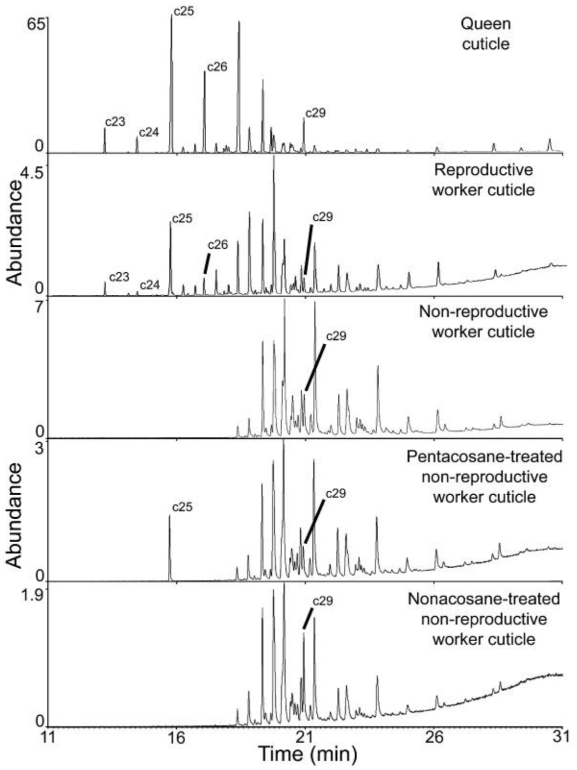

**Figure 1. Chromatograms Showing Differences in Cuticular Hydrocarbon Profiles**

Natural profiles of a queen, reproductive worker, and nonreproductive worker are shown. The profiles of pentacosane- (c25) and nonacosane- (c29) treated nonreproductive workers are provided for comparison. Straight chained alkanes tricosane (c23), tetracosane (c24), pentacosane (c25), hexacosane (c26), and nonacosane (c29) are labeled in the profiles where they are present.

well as in other ant species [11, 14]. Although these workers were policed in queenright colonies upon reintroduction, we did not observe aggression directed to them when they became reproductively active in queenless worker groups. We predict that nonreproductive workers treated with pentacosane should not be policed in queenless worker groups in contrast to the outcome in queenright colonies, because such workers are already present in the queenless group.

We repeated the same experiment in queenless worker groups already containing egg-laying workers. As predicted, pentacosane-treated workers were not policed but received the same low level of aggression as the nonacosane group and the controls (treated only with hexane), whereas foreign workers were attacked at significantly higher rates (Figure 3A). Workers in the queenless groups antennated pentacosane-treated workers more frequently than the hexane control group, indicating that workers still detected the profile manipulation (Figure 3B). The acceptance of the pentacosane-treated nonreproductive workers in queenless worker groups indicates that they were accepted as fertile workers even though they would have been policed in queenright colonies. This again is in line with our hypothesis that pentacosane indicates the reproductive status of a worker. It also confirms that pentacosane-treated workers are still considered as nestmates and that the aggression received in queenright colonies is not a consequence of mistaking them as foreign workers.

### The Mechanism of Hydrocarbon Synthesis and Distribution and Its Unique Advantages as a Reliable Information System of Reproductive Status

It is counterintuitive that workers express changes in their cuticular hydrocarbon profile that consistently reveal themselves as reproductive cheaters. Such cheating in ant colonies is represented by egg-laying in the presence of the queen to gain direct fitness benefits [18, 19]. However, the linkage of cuticular hydrocarbon profiles with the surface profiles of their eggs may prevent a successful cheater strategy. Reproductive cheaters would need to escape physical policing as an adult and ensure that their eggs escape policing. In several species, nestmate workers are able to recognize worker-laid eggs with a surface hydrocarbon profile differing from the established queen's eggs, leading to the destruction of worker-laid eggs [9, 20–24]. In fact, in the egg-policing species *Camponotus floridanus*, the policing of viable worker eggs is strongly decreased when these eggs are treated with an artificial queen egg hydrocarbon profile [9]. Egg profiles, though not identical, are correlated to the cuticular hydrocarbon profile of the egg layer resulting from the mechanism of their production and distribution. Oenocytes within the fat body synthesize new hydrocarbons, which are transported through the hemolymph to target tissues, including the cuticle and the ovaries [25]. *A. cockerelli* workers produce viable eggs that are indistinct in surface hydrocarbon profile to queen-laid eggs and are not policed by workers [15]. Although egg policing has not been demonstrated in *A. cockerelli*, we would expect to see policing of eggs with a hydrocarbon profile that does not have the reproductive-specific compounds. This, however, cannot be tested, because all our analyzed viable eggs showed the reproductive profile. Because the presence of the reproductive-specific pentacosane on the cuticle of workers identifies these individuals as

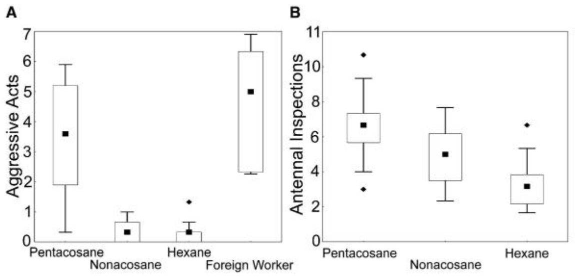

Figure 2. Response to the Introduction of Treated Workers into Queenright Colonies

Medians, 25%–75%, nonoutlier range, and outlying data points. Sample size (n = 11 all groups) represents number of independent colonies.

(A) Acts of aggression were more frequently directed toward pentacosane-treated workers and foreign workers. Friedman's ANOVA, $p < 0.001$; Wilcoxon-Wilcox multiple comparisons, $p < 0.001$ (foreign worker versus nonacosane and hexane), $0.01 > p > 0.005$ (pentacosane versus nonacosane and hexane), and $p > 0.05$ (pentacosane versus foreign worker and hexane versus nonacosane).

(B) Antennal inspections toward treated individuals. Friedman's ANOVA, $p = 0.003$; Wilcoxon-Wilcox multiple comparisons, $0.005 > p > 0.001$ (pentacosane versus hexane), $p > 0.05$ (nonacosane versus pentacosane and hexane).

reproductive cheaters [15], a successful cheater strategy of reproductive *A. cockerelli* workers would require two conditions: (1) to completely suppress the reproductive profile on the cuticle and (2) to continue to express it on the eggs. Only with these two requirements fulfilled would they be capable of gaining direct fitness benefits. However, this would require physiological changes leading to a complete uncoupling of the profile expression on eggs and the cuticle in workers, which has never been observed. Furthermore, this is opposed by strong selection on maintaining this physiological connection in queens that benefit from indicating their reproductive status on the cuticle and on the surface of their eggs to their workers [26, 27].

Hydrocarbon synthesis, transport, and uptake in target tissues are most likely under the regulation of epistatically interacting genes, so that single mutations in any involved genes are unlikely to remove the reproductive compounds on the cuticle and leave them on the eggs. On the other hand, a stepwise uncoupling of these profiles would lead to a transitionary phase that is prevented by efficient policing. In addition, pleiotropic constraints may be involved, as indicated by the fact that changes in one profile (cuticular or egg surface) result in changes in the profile expression of the other. For a general understanding of the absence of cheating, it is important to uncover the specific genetics and physiology of these underlying mechanisms. The inability to cheat may not be based on costs associated with producing or not producing a fertility signal, but rather on the conservation of basic biochemical mechanisms that are difficult to change. Epistatic and pleiotropic gene interactions are likely to play an important role in reliable cheater identification [29].

## Conclusions

Correlational evidence has indicated that hydrocarbons contain sufficient information necessary to identify cheating egg-layers and their eggs [30–33]. Herein we provide the first direct evidence that reproductive cheaters are identified by nestmates through changes in their cuticular compounds. Cuticular hydrocarbons represent the proximate mechanism for the regulation of reproduction in insect societies for which only indirect evidence has been presented so far [10, 11, 34]. Cheater resistance in this system is crucial in ensuring social stability. The mechanism of hydrocarbon synthesis and transport provides a system that is uniquely resistant to cheating, which may explain its widespread occurrence among social insects [28, 30, 31, 33]. Similar to means of cheater prevention in slime molds [29], pleiotropic and epistatic effects may be constraining cheating. Such communicative mechanisms should be expected in other cooperative groups.

## Experimental Procedures

Colonies of *A. cockerelli* were collected and cultured as described [15]. Solutions of 1 μg hydrocarbon per 40 μl of hexane were made for pentacosane and nonacosane (Sigma Aldrich). 0.5 μl of the solution was applied to the dorsal anterior portion of the gaster. The treated individual was marked with a single dot of flat blue Testors enamel paint on the dorsal section of the thorax and introduced into the appropriate colony 20 min after treatment. Hexane controls were treated with 0.5 μl of hexane and also marked with

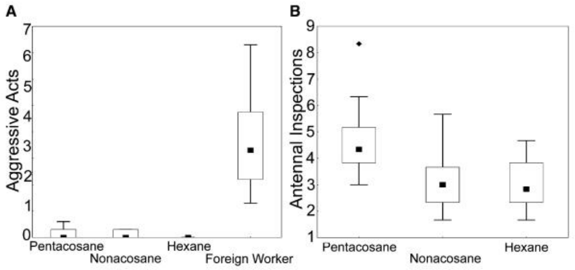

Figure 3. Response to the Introduction of Treated Workers into Groups of Queenless Reproductive Workers

Medians, 25%–75%, nonoutlier range, and outlying data points. Sample size (n = 12 all groups) reflects number of independent colonies.

(A) Acts of aggression were more frequently directed toward foreign workers. Friedman's ANOVA, $p < 0.001$; Wilcoxon-Wilcox multiple comparisons, $p < 0.001$ (foreign worker versus hexane), $0.005 > p > 0.001$ (foreign worker versus nonacose and pentacosane), and $p > 0.05$ (pentacosane versus nonacosane and hexane).

(B) Antennal inspections of treated individuals. Friedman's ANOVA, $p = 0.002$; Wilcoxon-Wilcox multiple comparisons, $0.005 > p > 0.001$ (pentacosane versus hexane), $p > 0.05$ (nonacosane versus pentacosane and hexane).

a color dot. To verify the effect of treatment, in-between each replicate colony, cuticular profiles were gathered [15] from pentacosane- and nonacosane-treated workers before and after treatment. After introduction into either queenless reproductive-nestmate groups or queenright colonies, any interactions that involved prolonged antennal inspection of the treated area were recorded. Aggressive actions (open mandible contact, clearly distinguishable from allogrooming, holding, and pulling) were recorded only when they followed antennal contact with the treated area. Ants introduced into queenright colonies (300–1000 workers) were observed for 5 min directly after reintroduction, whereas, because of smaller colony size, individuals placed in queenless colonies (50–150 workers) were observed for 10 min. A paint-marked foreign worker was introduced into each colony (queenright and queenless), and aggressive acts were recorded. Each data point for antennal inspection and aggression is the average of three trials with separate treated workers and foreign workers. Reactions to all four groups of individuals were tested on the same day, under the same laboratory conditions. A single queenright colony that did not respond aggressively to foreign workers in all three trials was excluded from analysis.

## Supplemental Data

Supplemental Data include four movies and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01619-9.

## Acknowledgments

We thank C. Peeters and K.R. Foster for their comments and K. Haight, D. Moore, C. Penick, and D. Eliyahu for their assistance.

Received: October 24, 2008
Revised: November 25, 2008
Accepted: November 26, 2008

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# APPENDIX D

# RECLAIMING THE CROWN: QUEEN TO WORKER CONFLICT OVER REPRODUCTION IN *APHAENOGASTER COCKERELLI*

Naturwissenschaften (2011) 98:237–240
DOI 10.1007/s00114-011-0761-8

SHORT COMMUNICATION

# Reclaiming the crown: queen to worker conflict over reproduction in *Aphaenogaster cockerelli*

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Received: 29 November 2010 / Revised: 24 December 2010 / Accepted: 5 January 2011 / Published online: 21 January 2011

**Abstract** In many social taxa, reproductively dominant individuals sometimes use aggression to secure and maintain reproductive status. In the social insects, queen aggression towards subordinate individuals or workers has been documented and is predicted to occur only in species with a small colony size and a low level of queen–worker dimorphism. We report queen aggression towards reproductive workers in the ant species *Aphaenogaster cockerelli*, a species with a relatively large colony size and a high level of reproductive dimorphism. Through analysis of cuticular hydrocarbon profiles, we show that queens are aggressive only to reproductively active workers. Non-reproductive workers treated with a hydrocarbon typical for reproductives are attacked by workers but not by queens, which suggests different ways of recognition. We provide possible explanations of why queen aggression is observed in this species.

**Keywords** Queen policing · Cuticular hydrocarbons · Policing · Aggression · Reproductive regulation

## Introduction

The threat of breeder replacement in some animal societies drives dominant reproductive individuals to physically aggress and suppress potential reproductive competitors. In vertebrate societies, these potential reproductive competitors often have the same, or nearly the same, reproductive capacity as the established reproductive (Reeve 1992; Young et al. 2006). Similar low levels of reproductive dimorphism are also found in some eusocial insects. Accordingly, it is thought that queen control of nestmate reproduction through physical aggression only occurs in insect societies where queen–worker dimorphism is low and group size is small (Bourke 1999; Hölldobler and Wilson 2009).

In ant societies, physical policing of workers by queens has only been reported in species that have an exceptionally low level of worker–queen dimorphism (Bourke 1988; Heinze and Smith 1990; Heinze et al. 1999; Peeters and Hölldobler 1995), or have colonies with mated workers, capable of a reproductive output equivalent to that of the queen (Sommer and Hölldobler 1992; Nakata and Tsuji 1996; Gobin et al. 2001). Similarly, in ant species that lack a true queen caste, the established reproductives are known to physically police reproductive subordinates (Peeters and Higashi 1989; Oliveira and Hölldobler 1991; Monnin and Peeters 1999). Queen aggression directed towards reproductives has yet to be documented in a species where workers or subordinates are not potentially able to take the place of the established queen.

We investigated policing by the queen in the ant *Aphaenogaster cockerelli* André, formerly *Novomessor*. Mature colonies are usually monogynous and polydomous, consisting of one to six physically separate nests. A mature colony is populated by 2,000–9,000 workers across multiple nest sites (personal observation; Hölldobler and Carlin 1989). Queens are morphologically and reproductively distinct from workers. Only queens can fertilize eggs with sperm stored in their spermatheca. Their number of ovarioles

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averages 33 compared to five in workers (Hölldobler and Carlin 1989). In this study, we report direct aggression from queens to reproductive workers. We induced this aggression by experimentally introducing queens to groups of daughter workers that have been separated from the queen and allowed to reproduce. Using cuticular hydrocarbon profiles and ovary dissections, we show that queens are aggressive only towards reproductively active workers. Finally, we attempt to trigger queen aggression by artificial hydrocarbon treatments of non-reproductive workers.

## Materials and methods

### Study species

All colonies of *A. cockerelli* used for this study were mature when collected from the Chihuahuan desert between Portal, Arizona and Rodeo, New Mexico.

The ants were housed in a dental-plaster nest with molded chambers darkened by red acetate over glass at a temperature of 26°C. The nests were attached to a constantly illuminated foraging arena in which they received a constant supply of water, sugar water, and pieces of cricket (*Acheta domestica*) and beetle larvae (*Zophobas morio*).

### Queen aggressed workers

Groups of 50 workers (35 nest workers and 15 foragers) were separated from their queenright colony and allowed to reproduce. Once workers started laying fertile eggs, the parental queen was introduced into the worker groups. As soon as queen aggression had been observed, the queen and the aggressed worker were removed from the nest. The aggressed worker and five workers that the queen encountered but did not attack were sampled for their cuticular hydrocarbons. For this procedure, workers were sedated using carbon dioxide gas and placed in 80 μl of hexane for 5 min. Identical GC/MS protocols used in previous cuticular hydrocarbon studies of this species were used for the chemical analysis (Smith et al. 2008, 2009). This was repeated in ten colonies. Ovaries of aggressed and non-aggressed workers from seven of those colonies were imaged for analysis of ovarian development.

### Eliciting queen aggression with artificial hydrocarbon treatments

Pentacosane, a compound that is dominant in the cuticular hydrocarbon profile of reproductive individuals (Smith et al. 2008, 2009), was added to the cuticle of non-reproductive workers to test for whether or not it elicits queen aggression. Nonacosane, a compound present on cuticles of all female castes, was used as a hydrocarbon treatment control. As an additional control, we used ants treated with the hexane solvent.

Solutions of 1 μg hydrocarbon per 30 μl of hexane were made for pentacosane and nonacosane (Sigma Aldrich). Ten microliters of the solution was dropped on the surface of a 5 ml beaker filled with deionized water. After the hexane solvent had evaporated, a worker was dipped and lightly swirled on the surface of the water. The treated individuals were allowed to air dry and were sampled using solid phase microextraction to verify the treatment. Each treated worker was then marked with a single dot of white Testors® enamel paint and introduced into the appropriate colony 30 min after treatment. Hexane controls were treated with 10 μl of hexane and also marked with a color dot.

In order to verify that the compounds used represented a "reproductive," "non-specific," and "control" treatment, we first presented queenright workers from six colonies with the treated ants. Thorax-marked treated workers were introduced into the foraging arena of their queenright nest and filmed for 5 min. This was repeated with three workers for every treatment within a colony, for a total of nine workers recorded per colony. The three treated workers were introduced one at a time. The order of the introductions was random, and the three treatments were introduced consecutively. The batches of introduced workers were separated by 1–3 h. The video was analyzed, blindly, for number of aggressive acts (biting, holding, and pulling) directed towards the treated individual.

Finally, the response of queens from five of the corresponding colonies to hydrocarbon treatments was tested. Workers were treated as described above. The workers were paint marked on the dorsal portion of their head, thorax, or abdomen. Three daughter workers of each treatment type (total of nine workers) were simultaneously placed into a nest that was identical to the nests used for the previous observations of queen policing. The queen was then introduced into the group of nine workers and video recorded for 10 min. Queen aggression towards naturally reproductive individuals had been observed in similar laboratory conditions; therefore, we were confident that the setup chosen for the experiment would not affect the behavior of the queen involved. The video was later analyzed in the same way as in the worker treatment.

## Results

### Queen aggressed workers

All queens transferred into the isolated worker groups were accepted, and occasionally carried, into the nest of their daughter workers. Ten of the ten queens always initiated aggression towards workers. Aggression included rapid antennation followed immediately by biting and holding (Fig. 1).

All seven sampled workers (from seven colonies) that were attacked by the queen had ovaries that indicated viable egg production. In contrast, non-aggressed worker ovaries were highly variable, from showing no ovarian development to indicating trophic egg laying activity. Thirty-four non-aggressed workers (out of a total of 35 from seven colonies) were classified as having ovaries that were not producing viable eggs. One of these workers had a level of ovary development that was not absolutely distinguishable as either trophic or viable. We conservatively counted it as having viable egg-producing ovaries. The difference in ovary condition between aggressed and non-aggressed workers is statistically significant (Fisher's exact test, two-tailed $p<0.0001$).

Queens and reproductive workers show nine cuticular compounds that are unique to reproductive individuals (Smith et al 2008). The relative proportion of these compounds present on the cuticle of aggressed workers was significantly higher than the relative proportion of the same compounds on non-aggressed workers (Fig. 2). The cuticular hydrocarbon profile of reproductive workers that are attacked by queens statistically differ in proportion to a reproductive queen's hydrocarbon profile as presented in Smith et al. (2008; Fig. 2).

### Eliciting queen aggression with artificial hydrocarbon treatments

Similar to the results from Smith et al. (2009), pentacosane elicited the most aggression from queenright nestmate workers (Fig. 3), indicating our treatment elicited a worker policing response in six of six colonies. However, queens ($n=5$) did not respond aggressively to any of the treated workers.

## Discussion

In *A. cockerelli*, queens are aggressive to reproductive workers that occur in experimentally orphaned groups of workers (Figs. 1 and 2). The highly directed queen attacks strongly suggest a chemical recognition mechanism. Cuticular hydrocarbons are potential candidates, since they play a major role in signaling reproductive status in ants (reviewed in Liebig 2010). In fact, *A. cockerelli* workers respond to changes in cuticular hydrocarbon profiles and prevent nestmate workers from attempting to reproduce under queenright conditions (Smith et al. 2009). In this study, hydrocarbon treatments elicited aggression from queenright nestmates according to what was previously

**Fig. 1** Queen aggression towards a reproductive daughter worker. Upon antennal contact with a reproductive worker, queens respond by biting and holding the worker, while flexing their abdomens towards the worker

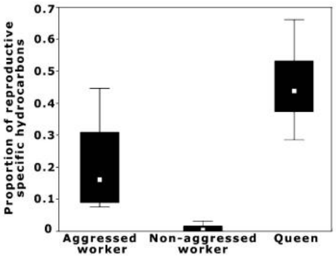

**Fig. 2** Proportion of cuticular hydrocarbon compounds specific to reproductive status in the hydrocarbon profiles of queen aggressed workers, non-aggressed workers, and queens. Each non-aggressed worker sample is an average of five non-aggressed workers from the same colony. The proportion of hydrocarbon compounds specific to reproductive status in aggressed workers is significantly different from that of non-aggressed workers (paired data; Wilcoxon signed rank test $T=0.00$, $p=0.005$). The proportion of hydrocarbon compounds specific to reproductive status in aggressed workers is also significantly different from that of queens (independent samples; Mann–Whitney $U$ Test, $Z=-3.32$, two-sided $p<0.001$). Queen hydrocarbon data is taken from Smith et al. (2008). $n=10$ both worker groups, $n=16$ queens, medians, 25–75%, maxima and minima

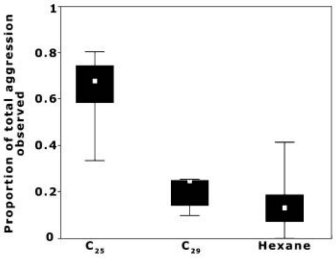

**Fig. 3** Proportion of worker aggression towards workers treated with hydrocarbons or solvent. Only worker aggression is shown, since queens ($n=5$) were not aggressive to any of the treated workers. Friedman's ANOVA $p=0.042$; Wilcoxon signed rank tests: $C_{25}$ vs. $C_{29}$ $Z=2.20$, $p=0.027$; $C_{25}$ vs. hexane $Z=1.99$, $p=0.046$; $C_{29}$ vs. hexane $Z=0.94$, $p=0.345$. $n=6$ for all groups (pentacosane=$C_{25}$, nonacosane=$C_{29}$, hexane), medians, 25–75%, maxima and minima

found by Smith et al. (2009), indicating again that pentacosane ($C_{25}$) is used by workers as a sign of reproductive activity (Fig. 3). However, queen aggression could not be induced by the same treatment.

The different queen response to pentacosane-treated workers suggests that queens do not use exactly the same information as workers to discriminate reproductively active workers. In fact, for workers the presence of a single compound out of nine identified chemicals that are predominantly expressed on the cuticle of reproductive workers and queens is sufficient for discrimination (Smith et al. 2008, 2009). Queens may either use information provided by several or all of these additional components or they may rely on additional yet to be identified compounds that indicate reproductive status.

Queen aggression towards reproductive workers in a species with relatively large colony sizes and a high degree of worker–queen dimorphism is a surprising observation. We hypothesize that this may be associated with a certain chance of workers activating their ovaries in a queenright colony. This may occur after hibernation when queen ovarian activity and worker policing may be low. In the functionally monogynous ant species, *Leptothorax* sp. A, queens fight out their hierarchy after hibernation each spring before they activate their ovaries (Ortius and Heinze 1999). A similar situation may occur in *A. cockerelli*. Alternatively, queen aggression may be associated with the polydomous colony structure of the species. If this separation from the queen or queen-produced eggs allows for workers to escape the influence of the queen and begin reproducing on their own, queens may encounter a situation similar to what has been documented in this study. Ongoing research is focused on genetic studies searching of evidence of worker-produced males and the effect of hibernation and polydomy on worker reproduction.

**Acknowledgments** We thank Tanja Bloss, Adam Dolezal, James Garcia, Kevin Haight, Dani Moore, Navdeep Mutti, and Clint Penick for their assistance and three anonymous referees for helpful comments.

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# APPENDIX E

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