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Experimental evidence that workers recognize reproductives through cuticular hydrocarbons in the ant *Odontomachus brunneus*

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Abstract Eusociality is characterized by a reproductive division of labor, wherein workers respond to the presence of reproductive individuals by refraining from reproduction themselves and restricting the reproductive efforts of others. Our understanding of how eusociality is maintained therefore depends on characterizing the mechanism by which workers detect the presence of a reproductive. Variations in cuticular hydrocarbons correspond to changes in reproductive ability in ants, and experimental studies are beginning to reveal the function of hydrocarbons as signals. In this study, we compare the cuticular hydrocarbon profiles of dominant and reproductive workers and queens of the ant *Odontomachus brunneus* with profiles of non-reproductive workers. Using split/reunification tests we document the existence of worker policing in both queenless and queen-right colonies; supernumerary reproductives were treated aggressively by nestmates. Finally, we induce aggression and replicate queen-like submissive nestmate responses by supplementing the hydrocarbon profile of workers with (*Z*)-9-nonacosene, a compound that was significantly more abundant on the cuticles of reproductives. In three bioassays, we compare this manipulation to various control manipulations of the hydrocarbon profile and demonstrate that workers gauge the reproductive activity of nestmates through changes in their cuticular hydrocarbon profiles.

Keywords Cuticular hydrocarbon · Fertility signal · Pheromone · Policing · Dominance

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Introduction

Reproductive division of labor is a defining characteristic of eusocial species (Wilson 1971). However, successfully partitioning the task of reproduction to one or a few individuals requires that nestmates can assess the reproductive ability of those individuals. In the eusocial Hymenoptera, whereas non-reproductive "workers" can be completely sterile in some species, more often they are physiologically capable of reproduction under appropriate conditions (Bourke 1988). When reproduction by workers is possible, they usually show self-restraint, and even physically restrain, or police, the reproductive efforts of other nestmates while in the presence of an established reproductive (Monnin and Ratnieks 2001; Ratnieks et al. 2006). In the absence of a reproductive, workers may lose their reproductive restraint and develop their ovaries or reform social hierarchies to replace the reproductive (Bourke 1988). The production and detection of signals that communicate the presence of reproductives is therefore crucial to maintaining a successful society.

Among social insects, the queen or an established reproductive may honestly communicate her reproductive ability to nestmates which then respond by refraining from reproduction (Seeley 1985; Keller and Nonacs 1993; Le Conte and Hefetz 2008; Heinze and d'Ettorre 2009; van Zweden

2010; Kocher and Grozinger 2011). Cuticular hydrocarbons apparently are the source of those signals in ants, wasps, and some bees (Liebig 2010). Cuticular hydrocarbons vary in chain length, branching patterns, and number and positions of double bonds, and complex blends serve to prevent desiccation (Lockey 1988). In numerous insect taxa, cuticular hydrocarbons have been co-opted for intraspecific signals (Howard and Blomquist 2005; Martin and Drijfhout 2009a). In social insects, differences in hydrocarbon profiles among individuals may communicate information about nestmate status, within-nest tasks, and reproductive status (Blomquist and Bagnères 2010).

Changes in reproductive capability have been correlated with changes in cuticular hydrocarbon profiles in both the queen and worker caste of several ant species (reviewed in Monnin 2006; Liebig 2010), and recent research suggests that hydrocarbons signal reproductive status. For example, workers of the ant *Myrmecia gulosa* inspect (antennate) the solvent-extracted hydrocarbons of fertile queens for longer periods than they do hydrocarbons of infertile workers (Dietemann et al. 2003). Antennae of *Pachycondyla inversa* responsed strongly to 3,11-dimethylheptacosane, a compound correlated with reproductive ability (D'Ettorre et al. 2004). Workers of the ant *Camponotus floridanus* usually police (eat) viable worker-produced eggs in the presence of a reproductive queen but do not eat worker-produced eggs that are coated with hydrocarbons from queen-produced eggs. Furthermore, the presence of queen-produced eggs seems to inhibit ovarian development in *C. floridanus* workers (Endler et al. 2004). Changes in the abundance of the cuticular hydrocarbon, pentacosane, are correlated with reproductive activity in *Aphaenogaster cockerelli*, and non-reproductive workers that are artificially treated with the compound are policed by their nestmates (Smith et al. 2009, 2011). Finally, the cuticular hydrocarbon 3-methylhentriacontane is correlated with queen fecundity in the ant *Lasius niger*, and has a primer effect on workers, inhibiting ovarian development (Holman et al. 2010). The latter two instances represent the only direct evidence available to date that cuticular hydrocarbons are used to both gauge and regulate reproductive activity in ants.

Dominance and reproductive activity are often linked in ants of the subfamily Ponerinae (Oliveira and Hölldobler 1990; Medeiros et al. 1992; Heinze et al. 1996; Peeters et al. 1999; Cuvillier-Hot et al. 2002). Dominance hierarchies are usually established before reproductive signals develop (Peeters et al. 1999; Cuvillier-Hot et al. 2002; D'Ettorre and Heinze 2005). After dominance is established, high-ranking individuals begin oogenesis and develop hydrocarbon fertility signals (Peeters et al. 1999; Liebig et al. 2000; Cuvillier-Hot et al. 2002, 2004, 2005). Comparative studies in both wasps and ants demonstrate that cuticular hydrocarbon profiles are better correlated with ovarian development than with hierarchy rank (Heinze et al. 2002; Izzo et al. 2010). Thus, although dominance and fertility can be correlated, cuticular hydrocarbon profiles are better described as indicators of fertility rather than solely indicators of dominance or rank.

In this study, we investigate the role of cuticular hydrocarbons as fertility signals in the ponerine ant *Odontomachus brunneus*. A previous study of *O. brunneus* identified frequent dominance behaviors between workers, consisting of stereotypical aggression in the form of rapid antennation, and postural indications of dominance and submission (Powell and Tschinkel 1999). Workers display submissive reactions of crouching and retraction of antennae, combined with retreat, when in proximity to a reproductive queen. We hypothesize that workers recognize queens and reproductive workers based on cuticular hydrocarbons. Thus, we characterized the cuticular hydrocarbon profiles of dominant and reproductively active queens and workers and compare their profiles to those of non-reproductive workers. Using split/reunification tests, we document nestmate aggression towards supernumerary reproductive individuals. Finally, using a series of bioassays in which we manipulate the hydrocarbon profiles of workers, we replicate both the high levels of aggression seen towards reproductives in split/reunification tests and the submissive reactions that nestmate workers display in proximity to reproductive individuals. Our results support the hypothesis that cuticular hydrocarbons serve as reproductive signals in *O. brunneus* and suggest that an increase in the relative amount of a single compound, (*Z*)-9-nonacosene, comprises a major component of the reproductive signal in *O. brunneus*.

Methods

Study species

Nine nests of *O. brunneus* were excavated from the Apalachicola National Forest, southeast of Tallahassee, Florida. Colonies are potentially polydomous, spreading across as many as three nesting sites (Hart and Tschinkel 2012). For this study, the most active nesting sites were excavated, resulting in two queenright colonies and seven queenless colonies, ranging in size from approximately 25 to 80 workers. Evidence suggests that colonies of this species are monogynous, usually containing a single queen (Powell and Tschinkel 1999; Hart and Tschinkel 2012).

In the laboratory, colonies were housed in two interconnected 60×15 mm Petri dishes with plaster-lined bottoms that were kept moist. Colonies received a constant supply of water and sugar water and were fed 5 days a week on live termites and freeze-killed crickets. All colonies were

kept under a 12-h light–dark cycle at an average temperature of 27 °C.

Split/reunification

Twenty-four workers from the largest queenright (two colonies) and queenless (three) colonies were separated from their colonies and each housed in a single Petri-dish nest. All workers were given a distinguishing color mark of Testors® paint (Rockford, IL, USA) on their head, thorax, abdomen, or a combination thereof. After eggs had appeared in the isolated worker group and an individual could be clearly identified as dominant (see "Results" section), the group was reunited with their original nest. After multiple workers were observed simultaneously biting and pulling on the newly established dominant, she was removed from the colony and dissected to gauge ovarian development. Additionally, five reunited workers that received comparatively minimal amounts of aggression (only occasionally receiving bouts of rapid antennation) were also removed from the colony and dissected.

Chemical analysis

Cuticular hydrocarbons of reproductively active queens, dominant and reproductive workers, and non-reproductive nest workers were sampled from the nine colonies collected from Tallahassee, Florida. Live ants were sampled using solid-phase microextraction (SPME). A SPME fiber (100 μm polydimethylsiloxane; Supelco Inc., Bellefonte, PA, USA) was lightly rubbed on the gaster of the ant for 5 min. The compounds on the fiber were then thermally desorbed for 5 min in the injection port of a Hewlett-Packard 6890 series gas chromatograph (H-P now Agilent Technologies: Santa Clara, CA), equipped with a nonpolar capillary column (DB-5MS, 30 m × 0.25 mm × 0.25 μm film; J&W Scientific, Folsom, CA), connected to an H-P 5973 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, then increased to 220 °C at 40 °C/min, and then to 315 °C at 4 °C/min. Helium was used as a carrier gas at 1 ml/min, and samples were injected in splitless mode with a purge time of 2 min. Electron impact ionization mass spectra were measured at 70 eV, with a source temperature of 230 °C.

Compounds that appeared in at least two-thirds of the ants sampled in each category were included in the analysis. For each compound, its relative abundance within the overall profile was calculated. Straight-chain compounds were identified from their mass spectra, including the molecular ion when visible, and by matching retention times with authentic standards. Methyl-branched compounds were identified by a combination of their enhanced ions from fragmentation on either side of methyl branch points, and their retention indices relative to straight-chain hydrocarbon standards. Alkenes and dienes were identified from their retention indices (slightly smaller than the corresponding alkanes on the DB-5 column), their molecular ions, and their mass spectral fragmentation patterns. Identifications were confirmed by comparisons of retention times and mass spectra to those of authentic standards when available. To determine the positions and geometries of double bonds in alkenes, individual ants were freeze-killed and extracted in 500 μl of hexane for 5 min. The crude extracts were epoxidized by treating an aliquot of an extract with a few drops of *m*-chloroperbenzoic acid in CH_2Cl_2 (25 μl of a 2 mg/ml solution). After 2 h at room temperature, the mixture was extracted with 1 M aqueous NaOH, and the hexane layer was dried over anhydrous Na_2SO_4 and analyzed by GC-MS as described above.

Hydrocarbon bioassays

To determine whether observed differences in relative abundance of cuticular hydrocarbon compounds (see "Results" section) were biologically relevant, we added synthetic hydrocarbons to the surface of ants and measured the responses of their nestmate workers. Three bioassays were performed, all comparing the effect of adding (*Z*)-9-nonacosene (*Z*9:C_{29}) to worker cuticles against various control groups. *Z*9:C_{29} was chosen as the focal treatment because the relative amounts of that compound showed the greatest increase in abundance in the cuticular profile of reproductive individuals compared to non-reproductives (see "Results" section). Bioassay I compared nestmate responses to workers treated with *Z*9:C_{29}, pentacosane (C_{25}), and sham (hexane)-treated workers. Bioassay II compared nestmate responses to workers treated with *Z*9:C_{29}, 3-methylheptacosane (3Me-C_{27}), and untreated non-nestmate workers. Bioassay III compared nestmate responses to workers treated with *Z*9:C_{29}, (*Z*)-9-heptacosene (*Z*9:C_{27}) and untreated non-nestmate workers. Control compounds were chosen because their relative abundances were significantly different between reproductive and non-reproductive individuals (C_{25} and *Z*9:C_{27} are more abundant on non-reproductives, 3Me-C_{27} is more abundant on reproductives; see "Results" section).

*Z*9:C_{29}, *Z*9:C_{27}, and 3Me-C_{27} were synthesized using previously published methods (Ginzel et al. 2006; Millar 2010). Solutions with a ratio of 1 mg of hydrocarbon per 7 ml of hexane were used. Aliquots (1 ml) of working solutions were used for all of the test compounds. The concentrations of hydrocarbons in these aliquots were verified by GC-MS analysis and comparison of peak abundances. The same 1 ml aliquots were used throughout the experiments. For hydrocarbon treatments of live ants,

25 μl of the hydrocarbon working solutions (3.6 μg of hydrocarbon) were dropped onto the surface of deionized water in a 10 ml glass beaker. The hexane was allowed to evaporate, leaving a thin hydrocarbon film on the surface of the water. Before treatment, the ants were given a unique paint mark, and were temporarily immobilized by 30 s exposures to freezing temperatures. Before the ants reanimated, they were dropped onto the surface of the water with the hydrocarbon films and swirled, thereby transferring the hydrocarbons onto the surface of their cuticle (Smith et al. 2011, 2012). Treatments resulted in ~16 % increase in the relative abundance of the test compounds on the cuticle, as revealed by solid-phase microextraction sampling of treated individuals. All treatments resulted in an increase in the abundance of test compounds that was slightly beyond the normal reported range (see Table 1). However, because all of the control treatments resulted in this exaggeration, any aberrant nestmate response results should be controlled for in our analysis.

Two queenright colonies and five queenless colonies of those collected from Tallahassee, FL were used for these bioassays. On the same day, three workers were removed from each colony, paint-marked, treated, and consecutively reintroduced to their colonies. The ants were reintroduced in random order, so that each colony only responded to one treated ant at a time. Following reintroduction, the treated ants were video-recorded for 10 min. The resulting videos were assigned a randomly coded title and analyzed blindly for the number of rapid antennations that the treated ants received and the number of submissive responses (crouching and retraction of antennae, combined with retreat) displayed by workers encountering the treated ants.

The resulting raw data from each bioassay were analyzed for differences using a non-parametric Friedmann's ANOVA, followed by post-hoc analysis between groups using Wilcoxon signed-ranks tests, using the software package STATISTICA 7. Bonferroni-corrected *P* values were not used because they resulted in type-II error: false support for the null hypothesis of no differences between treatments. Type-II error was attributed to obvious differences (non-overlapping data ranges) between treatments and statistically significant overall ANOVA tests.

Results

Split/reunification

Prior to reunification, a single individual could be clearly identified as dominant in both the newly formed group and the original colony from which that group was formed. If a queen was present in the colony, she was the dominant individual, but individual workers also displayed queen-like dominance in queenless colonies. Dominant individuals were identified by their elevated body posture and by observing the reactions of their nestmates during close encounters, behaviors previously described for this species (Powell and Tschinkel 1999). Specifically, in proximity (1–2 cm) to dominants, nestmates adopt a submissive posture, crouching, retracting antennae, and retreating.

Upon reintroduction, newly established dominants elicited aggressive reactions from workers (Video 1). Multiple workers rapidly antennated the dominant worker and in all (5/5) colonies, queenright and queenless, the dominant was bitten, held, and pulled by multiple nestmate workers (Video 1). The newly established reproductive individuals were eventually separated from their attackers for analysis; therefore, the fate of these reunited workers in their colonies was not determined. Reintroduced non-dominant workers received low levels of rapid antennation from nestmate workers. No non-dominant reintroduced workers were observed being bitten, held or pulled by multiple workers. Aggression towards the original established reproductive was not observed.

All (5/5) dominant workers that elicited aggression had ovaries that were more fully developed than those of the five non-aggressed reintroduced workers sampled per colony (2–8 versus 0–1 developing oocytes, respectively; Mann–Whitney $U=0$, two-sided $P<0.001$).

Chemical analysis

The cuticular hydrocarbon profile of *O. brunneus* consisted of alkanes, alkenes, dienes, and methyl- and dimethyl-branched alkanes ranging in chain length from 23 to 35 carbons (Table 1). There were no qualitative differences in compound presence/absence between queens and reproductive workers; however, reproductive individuals (queens and workers) were qualitatively and quantitatively different in their cuticular hydrocarbon profiles than non-reproductive workers (Table 1). The most pronounced individual compound differences between reproductives and non-reproductives were in the relative abundances of hydrocarbons that were shared between them, rather than presence/absence differences (Fig. 1; Table 1).

Hydrocarbon bioassays

In bioassay I, workers treated with *Z*9:C_{29} elicited significantly more aggression (rapid antennation) than workers treated with the hydrocarbon control, C_{25}, and the solvent control treatments (Fig. 2a; Video 2; Friedman's ANOVA: $P=0.003$; Wilcoxon signed-ranks tests: *Z*9:C_{29} versus C_{25}: $T=0$, $P=0.018$; *Z*9:C_{29} versus hexane: $T=0$, $P=0.018$; C_{25} versus hexane: $T=7$, $P=0.24$). The number of rapid antennations received per individual ranged from 0 to 32

Table 1 Identification of cuticular hydrocarbons of *Odontomachus brunneus*, Kovat's retention indices, and relative percent concentration: means (minimums, maximums); *N*=9

Peak number	Identification	Rentention index	Reproductive	Non-reproductive
1	3-Methyltricosane	2371	0.07 (0, 0.26)	0.18 (0.11, 0.28)*
2	2-Methyltetracosane	2461	0.24 (0.11, 0.51)	0.3 (0, 0.53)
3	Pentacosane	2500	2.86 (2.17, 3.8)	8.27 (6.27, 10.1)*
4	11-;13-Methylpentacosane	2531	0.16 (0, 0.26)	0.37 (0.26, 0.52)*
5	5-Methylpentacosane	2547	0.58 (0.41, 0.96)	1.52 (1.3, 1.76)*
6	4-Methylpentacosane	2562	0.08 (0, 0.10)	0.06 (0, 0.13)
7	3-Methylpentacosane	2573	2.7 (1.76, 3.2)	5.18 (4.23, 6.25)*
8	5,15-Dimethylpentacosane	2581	0.11 (0, 0.43)	0.65 (0.36, 1.14)*
9	Hexacosane	2600	0.43 (0.28, 0.61)	0.88 (0.8, 1.03)*
10	4-Methylhexacosane	2655	0.06 (0, 0.11)	0*
11	2-Methylhexacosane	2661	0.14 (0.10, 0.21)*	0.04 (0, 0.16)
12	(*Z*)-9-Heptacosene	2677	6.73 (4.91, 8.19)	14.34 (11.52, 16.41)*
13	4,8-Dimethylhexacosane	2686	0.44 (0, 0.71)	0.3 (0, 0.51)
14	Heptacosane	2700	4.34 (2.99, 6.22)*	2.48 (1.96, 3.36)
15	13-Methylheptacosane	2729	0.65 (0.44, 0.99)	1.56 (1.35, 1.75)*
16	7-Methylheptacosane	2738	0.23 (0.18, 0.32)	0.24 (0.2, 0.29)
17	5-Methylheptacosane	2745	0.61 (0.3, 1.07)	0.6 (0.5, 0.72)
18	Unknown	2760	0.17 (0, 0.3)	0.33 (0.28, 0.4)*
19	3-Methylheptacosane	2778	11.4 (10.19, 12.91)*	9.5 (8.74, 10.84)
20	Octacosane	2800	0.73 (0.4, 1.14)	0.62 (0.5, 0.7)
21	3,9-; 3,11-; 3,13-Dimethylheptacosane	2804	0.13 (0, 0.64)	0.36 (0, 2.06)
22	3,7-Dimethylheptacosane	2806	0.4 (0.14, 1.17)	0.21 (0, 0.29)
23	10-; 11-; 12-; 13-Methyloctacosane	2830	0.18 (0, 0.33)*	0
24	*x*, *y*-Nonacosadiene	2849	3.16 (2.56, 3.88)*	1.23 (0.93, 1.54)
25	*x*, *y*-Nonacosadiene	2856	1.67 (1.45, 1.97)	1.88 (1.56, 2.3)
26	*x*, *y*-Nonacosadiene	2865	3.66 (3.1, 4.02)	5.72 (5.01, 6.91)*
27	(*Z*)-9-Nonacosene	2886	36.75 (31.75, 40.83)*	30.59 (28.87, 33.81)
28	Nonacosane	2900	1.11 (0, 1.98)*	0.25 (0.17, 0.39)
29	Unknown—possible conjugated nonacosatriene	2922	0.46 (0, 0.83)*	0
30	11-;13-Methylnonacosane	2928	0.96 (0.68, 1.28)	1.43 (0.25, 1.85)*
31	Unknown—possible molecular ion of 390	2938	0.66 (0.40, 1.09)*	0.43 (0, 1.88)
32	Unkown	2948	0.97 (0.35, 1.24)*	0.09 (0, 0.35)
33	Uknown	2952	0.45 (0, 0.74)	0.57 (0.41, 0.68)
34	x-Triacontene	2975	0.86 (0.38, 2.98)	0.49 (0.38, 0.63)
35	Triacontane	3000	0.27 (0.17, 0.44)*	0
36	12-Methyltricontane	3028	0.32 (0.21, 0.44)	0.44 (0.24, 0.59)*
37	*x*, *y*-Hentriacontadiene	3046	0.94 (0.59, 1.32)*	0
38	*x*, *y*-Hentriacontadiene	3053	2.7 (1.99, 3.57)*	1.77 (1.18, 2.45)
39	*x*, *y*-Hentriacontadiene	3061	3.55 (2.89, 4.31)	3.87 (2.83, 4.55)
40	(*Z*)-9-Hentriacontene	3074	2.29 (1.21, 3.43)*	0
41	x-Hentriacontene	3079	1.37 (0.82, 2.58)*	0.45 (0.27, 0.68)
42	11-;13-Hentriacontane	3129	0.41 (0.33, 0.51)	0.76 (0.39, 1.01)*
43	Unknown	3139	0.25 (0.10, 0.62)*	0
44	Unknown	3149	0.16 (0.01, 0.41)	0.14 (0, 2.83)
45	Unknown	3155	0.12 (0, 0.29)	0.08 (0, 0.22)
46	Unknown	3230	0.11 (0, 0.22)*	0
47	*x*, *y*-Tritriacontadiene	3247	0.39 (0, 0.56)*	0
48	*x*, *y*-Tritriacontadiene	3254	2.18 (0.43, 3.89)*	1.41 (0.88, 2)

Table 1 (continued)

Peak number	Identification	Rentention index	Reproductive	Non-reproductive
49	*x*-Tritriacontene	3271	0.28 (0, 0.54)*	0.01 (0, 0.1)
50	11-;13-Methyltritiacosane	3327	0.17 (0.07, 0.32)	0.24 (0, 0.41)
51	Unknown	3338	0.12 (0, 0.4)	0.09 (0, 0.21)
52	*x*, *y*-Pentatriacontadiene	3447	0.2 (0, 0.8)*	0

*Indicates compounds that are significantly more abundant (Mann–Whitney *U* test, two-sided $P<0.05$). Reproductive cuticular hydrocarbon profiles consist of seven workers and two queens

X, *Y* indicates unknown bond position

throughout the experiment. Workers encountering nestmates treated with *Z*9:C_{29} adopted a submissive posture significantly more often than when encountering nestmates treated with C_{25} or the solvent control treatments (Fig. 2a; Video 2; Friedman's ANOVA: $P=0.005$; Wilcoxon signed-ranks tests: *Z*9:C_{29} versus C_{25}: $T=0$, $P=0.018$; *Z*9:C_{29} versus hexane: $T=0$, $P=0.018$; C_{25} versus hexane: $T=2.5$, $P=0.36$). The number of submissive reactions observed per treatment ranged from 0 to 14 throughout the experiment.

In bioassay II, workers treated with *Z*9:C_{29} elicited significantly more aggression (rapid antennation) than workers treated with 3Me-C_{27}, the hydrocarbon control, and the same amount of aggression that non-nestmate workers received (Fig. 2b; Friedman's ANOVA: $P=0.023$; Wilcoxon signed-ranks tests: *Z*9:C_{29} versus 3Me-C_{27}: $T=0$, $P=0.028$; *Z*9:C_{29} versus non-nestmate: $T=9$, $P=0.4$; 3Me-C_{27} versus non-nestmate: $T=11$, $P=0.61$). The number of rapid antennations observed per individual ranged from 2 to 100 throughout the experiment. Nestmate workers encountering workers treated with *Z*9:C_{29} adopted a submissive posture significantly more often than both the 3Me-C_{27} treated workers, and non-nestmates (Fig. 2b; Friedman's ANOVA:

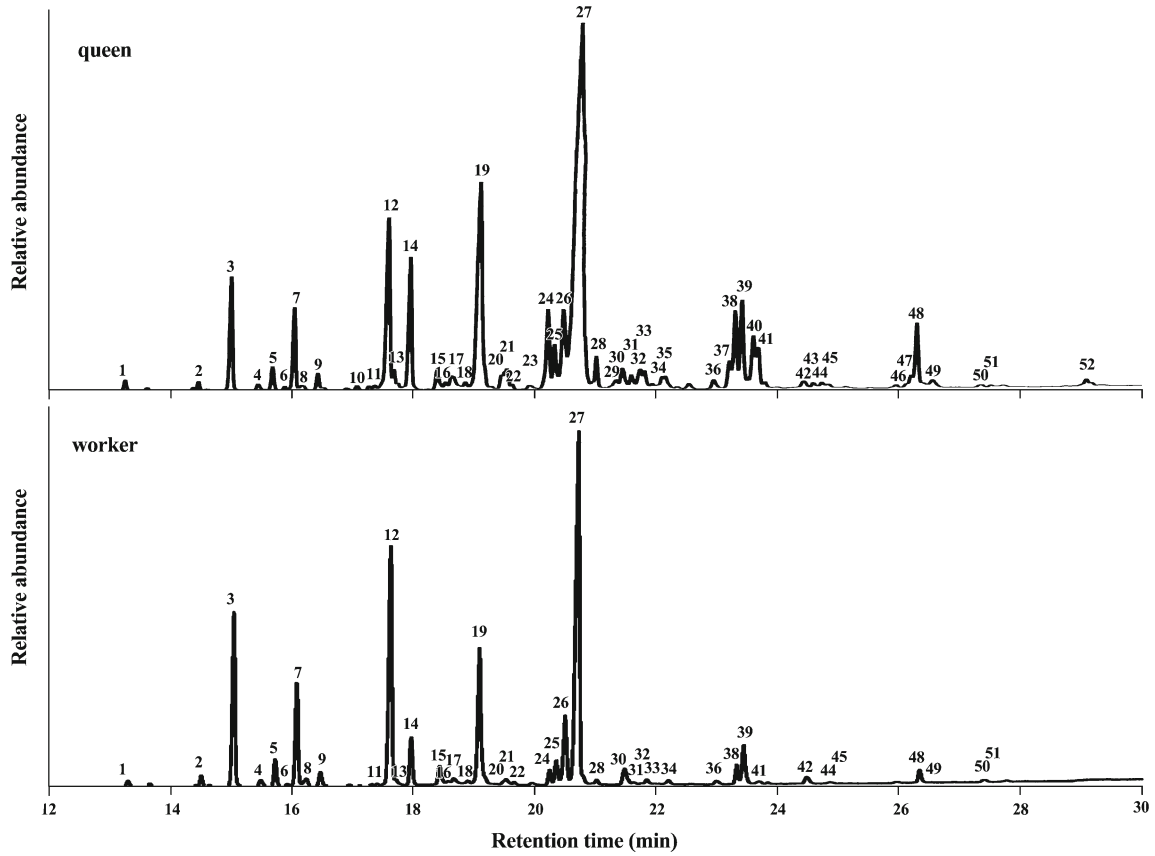

Fig. 1 The cuticular hydrocarbon profile of *Odontomachus brunneus*. Representative chromatograms are from solid-phase microextraction samples of a queen cuticle (*top*) and a non-reproductive worker (*bottom*). The *numbers* above the peaks correspond to the data presented in Table 1

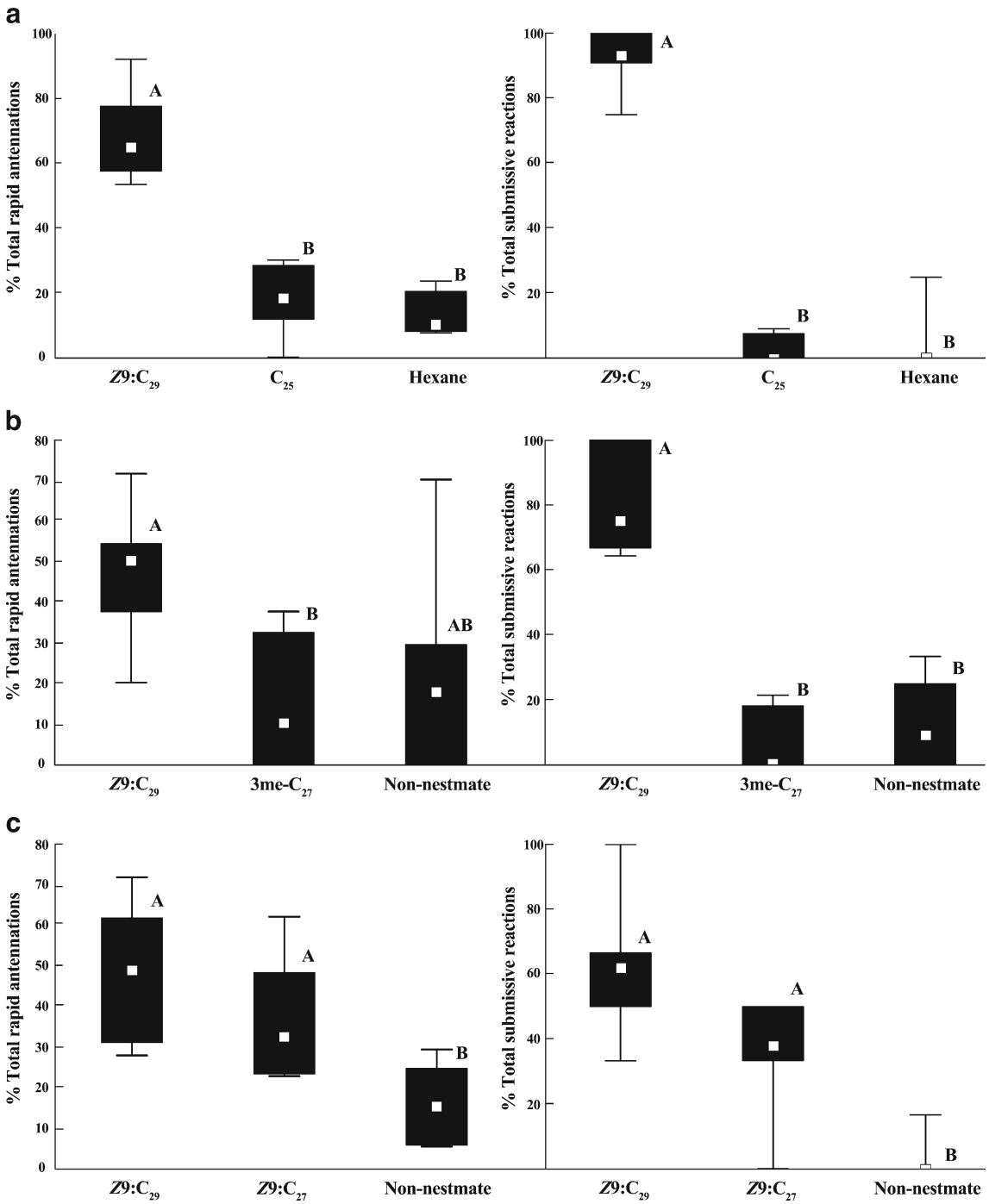

Fig. 2 Nestmate responses to hydrocarbon-treated and non-nestmate workers of *Odontomachus brunneus* in bioassays I (*top*), II (*middle*), and III (*bottom*). Medians, 25–75 %, minimums and maximums, and $N=7$ for all groups in all graphs. *Letters* indicate statistically significant differences between groups. All statistical analyses were performed on raw data rather than the data transformed into percent of total observations per colony

$P=0.003$; Wilcoxon signed-ranks tests: $Z9{:}C_{29}$ versus 3Me-C_{27}: $T=0$, $P=0.018$; $Z9{:}C_{29}$ versus non-nestmate: $T=0$, $P=0.018$; 3Me-C_{27} versus non-nestmate: $T=3$, $P=0.47$). The number of submissive reactions observed per treatment ranged from 0 to 9 throughout the experiment.

In bioassay III, workers treated with $Z9{:}C_{29}$ and $Z9{:}C_{27}$ elicited significantly more aggression (rapid antennation) than non-nestmate workers (Fig. 2c; Friedman's ANOVA: $P=0.004$; Wilcoxon signed-ranks tests: $Z9{:}C_{29}$ versus $Z9{:}C_{27}$: $T=8$, $P=0.31$; $Z9{:}C_{29}$ versus non-nestmate: $T=0$,

P=0.018; $Z9$:C_{27} versus non-nestmate: T=0, P=0.018). The number of rapid antennations observed per individual ranged from 3 to 69 throughout the experiment. Nestmate workers encountering workers treated with $Z9$:C_{29} and $Z9$:C_{27} adopted a submissive posture significantly more often than non-nestmates (Fig. 2c; Friedman's ANOVA: P=0.004; Wilcoxon signed-ranks tests: $Z9$:C_{29} versus $Z9$:C_{27}: T=1.5, P=0.06; $Z9$:C_{29} versus non-nestmate: T=0, P=0.018; $Z9$:C_{27} versus non-nestmate: T=0, P=0.03). The number of submissive reactions observed per treatment ranged from 0 to 13 throughout the experiment.

Discussion

Our split/reunification experiment demonstrated that aggression may be used to limit the number of reproductives in both queenright and queenless colonies of *O. brunneus*. Aggression, in the form of nestmate policing and dominance, is common both broadly throughout social insects and within the Ponerinae (Heinze et al. 1994; Monnin and Ratnieks 2001; Ratnieks et al. 2006). Dominance interactions between workers in queenless colonies serve to limit the number of reproductives in several ant genera (Oliveira and Hölldobler 1990; Heinze et al. 1997; van Walsum et al. 1998; Heinze and Oberstadt 1999; Gobin and Ito 2003; Denis et al. 2008). Because dominance interactions are common among workers in queenright colonies of *O. brunneus* (Powell and Tschinkel 1999), it is not surprising that these dominance interactions are also exhibited in queenless colonies. Our observation of only a single behaviorally dominant individual with developed ovaries per nest suggests that those individuals are the sole reproductive, as supported by high levels of aggression towards supernumerary reproductives. However, closer study is needed to determine whether or not a reproductive monopoly is maintained.

Our bioassays demonstrated that increasing the abundance of a single component of the cuticular hydrocarbon profile, $Z9$:C_{29}, on the cuticle of *O. brunneus* workers resulted in their nestmates treating them like reproductive individuals. Workers treated with $Z9$:C_{29} received substantial aggression from their nestmates (Fig. 2), similar to reactions triggered by introduction of a dominant worker to a queenright colony during the split/reunification tests. They also evoked submissive responses from nestmates (Fig. 2) that were similar both to our observations on identifying dominant and reproductive individuals and to observations made by Powell and Tschinkel (1999). Aggression, expressed as rapid antennation, comprised the majority of nestmate responses to $Z9$:C_{29}-treated workers in all bioassays (results above). Although submissive displays were evoked by treatment with $Z9$:C_{29}, those displays often were followed or preceded by aggression in the form of rapid antennation (Video 2). This response was consistent with our split/reunification tests wherein aggression was the most common response of nestmates towards newly established dominant workers. Our bioassay results reflect natural changes in the hydrocarbon profile of reproductive individuals, especially the greater relative abundance of $Z9$:C_{29} when compared to non-reproductives (Table 1). The next largest increase in compound abundance was the homologous compound (*Z*)-9-hentriacontene ($Z9$:C_{31}) (Table 1). We found this to be true both for queens and workers of this species because the profiles of both reflect similar compound increases. Similarity in reproductive worker and queen profile has also been reported in other species of ants (Heinze et al. 2002; Dietemann et al. 2003; Smith et al. 2008, 2011).

The control groups used in our bioassays further support our conclusion that workers associate higher proportions of $Z9$:C_{29} with reproductive status. Bioassays I and II (Fig. 2) demonstrated that the observed differences in nestmate reactions were not due to simply altering the cuticular hydrocarbon profile of workers. Workers treated with C_{25} and the 3Me-C_{27}-treated workers received significantly less aggression and evoked significantly fewer submissive reactions than workers treated with $Z9$:C_{29}. Although 3Me-C_{27} was surprising because this compound was significantly more abundant on reproductive individuals, application of that compound to workers did not result in reproductive-specific nestmate responses. Similarly, some compounds that are correlated with reproduction in the ant *L. niger* do not evoke reproductive-specific responses by workers (Holman et al. 2010). In bioassays II and III (Fig. 2), non-nestmate workers did not evoke submissive reactions from workers, suggesting that the submissive responses elicited by $Z9$:C_{29}-treated workers were not due to altered nestmate signals that triggered a foreign worker response, but rather were a specific response to a particular signal molecule.

In bioassay III (Fig. 2), workers treated with $Z9$:C_{27} and $Z9$:C_{29} received similar amounts of aggression, and both treatments evoked submissive reactions from nestmates. This result is somewhat puzzling in that $Z9$:C_{27} was more abundant in the profiles of non-reproductive workers (Table 1; Fig. 2c). A possible explanation for this reaction is that workers associate increases in homologous compounds with reproductive ability. Recent work on ant nestmate recognition systems suggests that homologous hydrocarbon compounds, because of likely shared biosynthetic pathways, convey similar information and workers may generalize their responses to homologous changes accordingly (Martin and Drijfhout 2009b, c; van Wilgenburg et al. 2010). Indeed, associative learning experiments with the ant *Camponotus aethiops* suggest that individuals generalize learned hydrocarbon compounds with shorter chained homologs (Bos et al. 2012). In *O. brunneus*, an increase in $Z9$:C_{29} was associated with a change in

reproductive ability but was not correlated with an increase in $Z9{:}C_{27}$. Both compounds are almost certainly products of the same general biosynthetic pathway, suggesting that the chain shortening or lengthening steps, at least for this homologous pair, are under tight control. The decrease in relative proportion of $Z9{:}C_{27}$ is most likely the result of the upregulation of the formation of $Z9{:}C_{29}$ and other fertility-associated compounds rather than a decrease of the amount of that compound on the cuticle of reproductives, because the total amounts of $Z9{:}C_{27}$ in the profiles of non-reproductive and reproductive individuals were equivalent (Mann–Whitney $U=22.0$, two-sided $P=0.11$). Because increases in another homologous compound, $Z9{:}C_{31}$, also appear to be correlated with reproductive status (Table 1), it is possible that workers generalize increases in *Z*9-alkenes as signals communicating reproductive ability. Systematically testing nestmate responses to *Z*9-alkenes of different chain lengths might improve our understanding of how information is conveyed and interpreted through the cuticular hydrocarbon profile.

Alkenes and specifically $Z9{:}C_{29}$ have been linked to changes in fertility in other social insect species. $Z9{:}C_{29}$ is significantly more abundant on dominant reproductives compared to nestmates in the wasp *Polistes dominulus* (Sledge et al. 2001). Increased amounts of a two-carbon homolog, 9-hentriacontene, are believed to be solely responsible for distinguishing dominant reproductives from nestmates in the queenless ant *Dinoponera quadriceps* (Peeters et al. 1999). Additionally, in the ponerine ants *Harpegnathos saltator*, *Streblognathus peetersi*, and *Hypoponera opacior*, changes in alkene abundance correlate with reproductive ability (Liebig et al. 2000; Cuvillier-Hot et al. 2004; Foitzik et al. 2011). A higher abundance of alkenes with chain lengths of 27, 29, and 31 carbons distinguishes physogastric queens from all other individuals in the stingless bee *Frieseomelitta varia* (Nunes et al. 2009). However, in all of these studies, associations between hydrocarbon profiles and insect behavior are correlative, and bioassays confirming the use of these potential signals have not been reported.

In summary, this study presents direct evidence that $Z9{:}C_{29}$ is a major component of the fertility signal of *O. brunneus*, adding to the small but growing number of experimental studies that link cuticular hydrocarbons to the perceptual basis of reproductive ability in ants. Because discriminating reproductive from non-reproductive individuals is a fundamental process in insect societies, identifying the basis of these signals is a crucial step in understanding how eusociality is maintained. Future work aimed at identifying the regulatory pathways that control the biosynthesis of these signals and how they relate to reproductive condition in both solitary and social species also may add to our understanding of the evolution of eusociality.

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References

Blomquist GC, Bagnères A-G (2010) Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge

Bos N, Dreier S, Jorgensen CG, Nielsen J, Guerrieri FJ, d'Ettorre P (2012) Learning and perceptual similarity among cuticular hydrocarbons in ants. J Insect Physiol 58:138–146

Bourke AFG (1988) Worker reproduction in the higher eusocial Hymenoptera. Q Rev Biol 63:291–311

Cuvillier-Hot V, Gadagkar R, Peeters C, Cobb M (2002) Regulation of reproduction in a queenless ant: aggression, pheromones and reduction in conflict. Proc R Soc Lond B 269:1295–1300

Cuvillier-Hot V, Lenoir A, Crewe R, Malosse C, Peeters C (2004) Fertility signalling and reproductive skew in queenless ants. Anim Behav 68:1209–1219

Cuvillier-Hot V, Renault V, Peeters C (2005) Rapid modification in the olfactory signal of ants following a change in reproductive status. Naturwissenschaften 92:73–77

Denis D, Chameron S, Costille L, Pocheville A, Chaline N, Fresneau D (2008) Workers agonistic interactions in queenright and queenless nests of a polydomous ant society. Anim Behav 75:791–800

D'Ettorre P, Heinze J (2005) Individual recognition in ant queens. Curr Biol 15:2170–2174

D'Ettorre P, Heinze J, Schulz C, Francke W, Ayasse M (2004) Does she smell like a queen? Chemoreception of a cuticular hydrocarbon signal in the ant *Pachycondyla inversa*. J Exp Biol 207:1085–1091

Dietemann V, Peeters C, Liebig J, Thivet V, Hölldobler B (2003) Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proc Natl Acad Sci USA 100:10341–10346

Endler A, Liebig J, Schmitt T, Parker JE, Jones GR, Schreier P, Hölldobler B (2004) Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. Proc Natl Acad Sci USA 101:2945–2950

Foitzik A, Fröba J, Rüger MH, Witte V (2011) Competition over workers: fertility signalling in wingless queens of *Hypoponera opacior*. Insect Soc 58:271–278

Ginzel MD, Moreira JA, Ray AM, Millar JG, Hanks LM (2006) (*Z*)-9-nonacosene-major component of the contact sex pheromone of the beetle *Megacyllene caryae*. J Chem Ecol 32:435–451

Gobin B, Ito F (2003) Sumo wrestling in ants: major workers fight over male production in *Acanthomyrmex ferox*. Naturwissenschaften 90:318–321

Hart LM, Tschinkel WR (2012) A seasonal natural history of the ant, *Odontomachus brunneus*. Insect Soc 59:45–54

Heinze J, d'Ettorre P (2009) Honest and dishonest communication in social Hymenoptera. J Exp Biol 212:1775–1779

Heinze J, Oberstadt B (1999) Worker age, size and social status in queenless colonies of the ant *Leptothorax gredleri*. Anim Behav 58:751–759

Heinze J, Hölldobler B, Peeters C (1994) Conflict and cooperation in ant societies. Naturwissenschaften 81:489–497

Heinze J, Trunzer B, Oliveira PS, Hölldobler B (1996) Regulation of reproduction in the neotropical ponerine ant, *Pachycondyla villosa*. J Insect Behav 9:441–450

Heinze J, Puchinger W, Hölldobler B (1997) Worker reproduction and social hierarchies in *Leptothorax* ants. Anim Behav 54:849–864

Heinze J, Stengl B, Sledge MF (2002) Worker rank, reproductive status and cuticular hydrocarbon signature in the ant, *Pachycondyla* cf. *inversa*. Behav Ecol Sociobiol 52:59–65

Holman L, Jorgensen CG, Nielsen J, d'Ettorre P (2010) Identification of an ant queen pheromone regulating worker sterility. Proc R Soc B 277:3793–3800

Howard RW, Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annu Rev Entomol 50:371–393

Izzo A, Wells M, Huang Z, Tibbetts E (2010) Cuticular hydrocarbons correlate with fertility, not dominance, in a paper wasp, *Polistes dominulus*. Behav Ecol Sociobiol 64:857–864

Keller L, Nonacs P (1993) The role of queen pheromones in social insects—queen control or queen signal. Anim Behav 45:787–794

Kocher SD, Grozinger CM (2011) Cooperation, conflict, and the evolution of queen pheromones. J Chem Ecol 37:1263–1275

Le Conte Y, Hefetz A (2008) Primer pheromones in social Hymenoptera. Annu Rev Entomol 53:523–542

Liebig J (2010) Hydrocarbon profiles indicate fertility and dominance status in ant, bee, and wasp colonies. In: Blomquist GJ, Bagnères A-G (eds) Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge, pp 254–281

Liebig J, Peeters C, Oldham NJ, Markstadter C, Hölldobler B (2000) Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? Proc Natl Acad Sci USA 97:4124–4131

Lockey KH (1988) Lipids of the insect cuticle—origin, composition and function. Comp Biochem Physiol B Biochem Mol Biol 89:595–645

Martin S, Drijfhout F (2009a) A review of ant cuticular hydrocarbons. J Chem Ecol 35:1151–1161

Martin SJ, Drijfhout FP (2009b) How reliable is the analysis of complex cuticular hydrocarbon profiles by multivariate statistical methods? J Chem Ecol 35:375–382

Martin SJ, Drijfhout FP (2009c) Nestmate and task cues are influenced and encoded differently within ant cuticular hydrocarbon profiles. J Chem Ecol 35:368–374

Medeiros FLN, Lopes NP, Moutinho PRS, Oliveira PS, Hölldobler B (1992) Functional polygyny, agonistic interactions and reproductive dominance in the neotropical ant *Odontomachus chelifer* (Hymenoptera, Formicidae, Ponerinae). Ethology 91:134–146

Millar JG (2010) Chemical synthesis of insect cuticular hydrocarbons. In: Blomquist GJ, Bagnères A-G (eds) Insect hydrocarbons: biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge, pp 163–186

Monnin T (2006) Chemical recognition of reproductive status in social insects. Ann Zool Fenn 43:515–530

Monnin T, Ratnieks FLW (2001) Policing in queenless ponerine ants. Behav Ecol Sociobiol 50:97–108

Nunes TM, Turatti ICC, Lopes NP, Zucchi R (2009) Chemical signals in the stingless bee, *Frieseomelitta varia*, indicate caste, gender, age, and reproductive status. J Chem Ecol 35:1172–1180

Oliveira PS, Hölldobler B (1990) Dominance orders in the ponerine ant *Pachycondyla apicalis* (Hymenoptera, Formicidae). Behav Ecol Sociobiol 27:385–393

Peeters C, Monnin T, Malosse C (1999) Cuticular hydrocarbons correlated with reproductive status in a queenless ant. Proc R Soc Lond B 266:1323–1327

Powell S, Tschinkel WR (1999) Ritualized conflict in *Odontomachus brunneus* and the generation of interaction-based task allocation: a new organizational mechanism in ants. Anim Behav 58:965–972

Ratnieks FLW, Foster KR, Wenseleers T (2006) Conflict resolution in insect societies. Annu Rev Entomol 51:581–608

Seeley TD (1985) Honeybee ecology: a study of adaptation in social life. Princeton University Press, Princeton, New Jersey

Sledge MF, Boscaro F, Turillazzi S (2001) Cuticular hydrocarbons and reproductive status in the social wasp *Polistes dominulus*. Behav Ecol Sociobiol 49:401–409

Smith AA, Hölldobler B, Liebig J (2008) Hydrocarbon signals explain the pattern of worker and egg policing in the ant *Aphaenogaster cockerelli*. J Chem Ecol 34:1275–1282

Smith AA, Hölldobler B, Liebig J (2009) Cuticular hydrocarbons reliably identify cheaters and allow enforcement of altruism in a social insect. Curr Biol 19:78–81

Smith AA, Hölldobler B, Liebig J (2011) Reclaiming the crown: queen to worker conflict over reproduction in *Aphaenogaster cockerelli*. Naturwissenschaften 98:237–240

Smith AA, Hölldobler B, Liebig J (2012) Queen-specific signals and worker punishment in the ant *Aphaenogaster cockerelli*: the role of the Dufour's gland. Anim Behav 83:587–593

van Walsum E, Gobin B, Ito F, Billen J (1998) Worker reproduction in the ponerine ant *Odontomachus simillimus* (Hymenoptera: Formicidae). Sociobiology 32:427–440

van Wilgenburg E, Sulc R, Shea KJ, Tsutsui ND (2010) Deciphering the chemical basis of nestmate recognition. J Chem Ecol 36:751–758

van Zweden JS (2010) The evolution of honest queen pheromones in insect societies. Commun Integr Biol 3:50–52

Wilson EO (1971) The Insect Societies. Belknap, Cambridge, MA