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RESEARCH PAPER

Conserved male-specific cuticular hydrocarbon patterns in the trap-jaw ant *Odontomachus brunneus*

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Abstract Cuticular hydrocarbons have been identified as the source of sex-recognition signals for many insects, but for social insects, specifically ants, cuticular hydrocarbon profiles of males are often ignored. This study reports male-specific cuticular hydrocarbon patterns for the trap-jaw ant *Odontomachus brunneus*. Analysis of samples from four Florida populations demonstrated that male-specific overabundance of four hydrocarbons is conserved across populations despite population-level divergence of the remainder of the profile. In addition, hydrocarbon patterns unique to adult males were not present on the cuticle of final instar male larvae, indicating that male-specific profiles arise late in development. The pattern of an abundant subset of conserved cuticular hydrocarbons characteristic of males across divergent populations was compared to earlier findings of the conservation of fertility signals of females across these same populations.

Keywords Pheromone · Sex pheromone · Male signal · Phenotypic variation

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Introduction

Insect species of many orders use cuticular hydrocarbons as sex-specific mate recognition signals (e.g., beetles: Ginzel 2010; flies: Ferveur and Cobb 2010; crickets: Tregenza and Wedell 1997). Most studies of cuticular hydrocarbons consider only male perception of female-specific profiles (e.g., Ginzel 2010; Tregenza and Wedell 1997). The perception of males through male-specific hydrocarbon profiles has been demonstrated in only a few species, such as fruit flies (Lacaille et al. 2007) and thrips (Olaniran et al. 2013). However, the utility of perceiving different sexes through chemical signals is not limited to mating. In social insects such as ants, where males are present in the colony for some time before leaving to disperse and mate, recognition of males by workers is undoubtedly important for colony spatial distribution and task allocation as well as potential conflicts over male production and sex ratios.

Whereas the cuticular hydrocarbon profiles of female worker ants have been described for over 80 species (Martin and Drijfhout 2009), hydrocarbons of males have been identified for only seven species (Chernenko et al. 2012). Profiles of male ants have been found to be qualitatively similar to those of females (gynes or workers) in most species studied to date. Differences in these profiles mostly consist of small quantitative differences in the relative abundances of individual compounds (Cremer et al. 2002, 2008; Antonialli et al. 2007; Beibl et al. 2007; Hojo et al. 2009; Chernenko

et al. 2012; Johnson and Sundstrom 2012). The most pronounced sexual differences in hydrocarbon profiles have been reported in the ponerine ant *Diacamma ceylonense*, with tenfold greater abundance of certain components in one sex compared to the other (Cuvillier-Hot et al. 2001).

Surprisingly, there is not yet any direct experimental evidence linking male-specific hydrocarbon patterns to recognition of males, for any ant species. Presumably this is because males do not participate in most colony activities and, in most species, have one mating bout outside of the nest that is difficult to replicate under laboratory conditions. However, more generally, there is abundant experimental evidence for several signaling roles of the hydrocarbon profile in ants, including distinguishing nestmates from non-nestmates, encoding task-specific signals, and signaling the fertility status of both queens and workers (Greene 2010; Liebig 2010; van Zweden and d'Ettorre 2010). These signals consist of individual compounds or a subset of compounds from the hydrocarbon profile. For example, we found that changes in abundance of a single compound, out of a profile of more than 50 hydrocarbons, are primarily responsible for signaling fertility status in the trap-jaw ant *Odontomachus brunneus* (Smith et al. 2012, 2013). This signal was conserved across populations, consistent with its being under stabilizing selection, whereas the remainder of the hydrocarbon profile was highly divergent (Smith et al. 2013).

In the present article, we characterize the cuticular hydrocarbon profiles of male *O. brunneus* across populations to test the hypothesis that male-specific sex recognition signals are conserved. We also demonstrate that the signals arise within the hydrocarbon profile of males post larval development.

Methods

Sample collection

Colonies of *O. brunneus* were collected by manual excavation in October of 2011 and August of 2012 from four locations in Florida: the Apalachicola National Forest southeast of Tallahassee, Leon County (henceforth referred to as "Tallahassee"), the MacArthur Agro-Ecology Research Center near Lake Placid, Highlands County ("Archbold"), the Chuluota Wilderness Area in Chuluota, Seminole County ("Chuluota"), and the Pine Jog Environmental Education Center in West Palm Beach, Palm Beach County ("West Palm Beach"). Colonies collected within each location were separated by no more than 1 km. Species identifications were confirmed according to the morphological traits of the workers and adult males (Deyrup and Cover 2004). The colonies were housed under laboratory conditions as described by Smith et al. (2012), and individuals were sampled from laboratory colonies after at least a month under these conditions.

Adult males were obtained from haploid, worker-laid eggs from queenless laboratory colonies ($n = 23$). When an adult male was removed from a colony for chemical analysis, an adult female worker also was removed for analysis ($n = 23$). Alate gynes that were present in some colonies when collected ($n = 10$) were also sampled.

In addition, we sampled ultimate (fourth) instar larvae, defined by having a head width of 0.59–0.65 mm and lacking "door-knob" tubercles (A.A. Smith, unpublished data). Only last instar larvae were sampled, for several reasons. First, we did not sample eggs because the hydrocarbons present on eggs are produced and deposited on the eggs by the ovipositing female (Endler et al. 2006), and in *O. brunneus*, egg-laying workers and queens are indistinguishable from one another in hydrocarbon profile (Smith et al. 2012). Second, there is no reason to suspect that differences in male–female larval profiles would arise in an early instar and be lost by later instars. Finally, pupae were not sampled because they are enclosed in a pupal case during metamorphosis, which is synthesized by late fourth instar larvae. Pupal cases likely occlude transmission and perception of contact chemical signals present on the cuticle of the developing pupa, or the relevant chemical signals present on the pupal case may instead be larval-synthesized hydrocarbons. Male larvae were sampled from queenless colonies ($n = 8$). Female larvae, presumably destined to be workers, were taken from queenright colonies ($n = 10$; with a fertile queen that produced only female workers in the laboratory). Sample sizes for each group represent independent samples from different colonies.

Chemical analysis

Cuticular hydrocarbons were extracted from individual ants by submerging them in 150 µl of hexane for 5 min. The extracts were concentrated using a stream of nitrogen to 10 µl from which 1 µl was injected into 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), interfaced 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, increased to 220 °C at 40 °C/min, and then to 315 °C at 4 °C/min. Helium was used as 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. A control sample of hexane was run through the GC–MS every day

before samples were analyzed, to confirm that the column was clean.

Alkanes and monoalkenes were identified as described in Smith et al. (2012, 2013). Dienes were identified by the presence of diagnostic ions in the mass spectra of their dimethyldisulfide derivatives (Buser et al. 1983). Thus, a cuticular lipid extract was fractionated on a Discovery™ Ag-ION solid phase extraction cartridge (750 mg; Supelco, Bellefonte PA) to isolate a diene fraction from the remainder of the cuticular lipids. The cartridge was conditioned with acetone (4 ml) and hexane (4 ml). A hexane extract of 7 males was prepared by soaking in 1 ml hexane for ~5 min, and the extract was concentrated to ~100 µl under a stream of nitrogen. The concentrated extract was loaded onto the conditioned cartridge, rinsing on with a few drops of hexane. The cartridge was then eluted sequentially with hexane (4 ml), hexane/acetone 96:4 (2 × 3 ml), hexane/acetone 90:10 (2 × 2 ml), hexane/acetone 50:50 (2 × 2 ml), and acetone (4 ml). The dienes eluted in the second 90:10 fraction and the first 50:50 fraction, and the two fractions were combined and concentrated to ~100 µl. An aliquot (25 µl) was combined with 10 µl of a 5 % solution of iodine in ether and 5 µl of dimethyldisulfide in a screw-cap vial with a Teflon lid liner, and the mixture was heated overnight at 50 °C. After cooling, the solution was diluted with 0.5 ml pentane and 100 µl of 0.5 M aqueous sodium thiosulphate, then vortexed until the pink color of iodine had completely disappeared. The pentane layer was removed, dried over anhydrous Na_2SO_4, concentrated to ~10 µl, and analyzed by coupled gas chromatography–mass spectrometry, using a Hewlett-Packard 6890 GC interfaced to a 5973 mass selective detector. The GC was fitted with a 30 m × 0.25 mm i.d. DB-17 column (J&W Scientific, Folsom CA), programmed from 100 °C/1 min, 10 °C/min to 300 °C, hold for 30 min. Injector and transfer line temperatures were set to 300 °C. A 25-µl aliquot of a 1 mg/ml solution of (6*Z*,9*Z*)-6,9-heneicosadiene and (6*Z*,9*Z*)-6,9-tricosadiene standards (available from previous work), was derivatized in similar fashion.

Compounds were included in the analysis if they occurred in ≥70 % of the sampled individuals within at least one of the classes per population (adult male, female worker, gyne, male larva, worker larva). Raw measures of compound abundances were transformed into relative compound abundances within the overall cuticular profile. Relative compound abundances were compared using Kruskal–Wallis ANOVAs across all groups followed by multiple comparisons between all groups (Siegel and Castellan 1988). Statistics were performed using the software package STATISTICA 7 (StatSoft, Inc., Tulsa, OK). Significant differences between groups were defined as having a Bonferroni-corrected p value < 0.05. The diagnostic power (DP) for each compound was calculated according to the equation given in van Zweden and d'Ettorre (2010), where DP is the standard deviation of the standardized peak area of all sampled individuals divided by the pooled standard deviation within the grouping of interest. Groupings of interest were population and caste (male, worker, gyne, worker larvae, and male larvae); therefore, two measures of DP for each compound were calculated. Compounds with the highest DP are the most consistent within group while being variable between groups; therefore, they are compounds most likely to be "diagnostic" of the grouping.

We performed non-metric multidimensional scaling to analyze the similarity of profiles between sampled groups across populations (Primer 6, PRIMER-E Ltd., Ivybridge, UK). Chord distances (normalized versions of Euclidean distances) were used to calculate the distance matrices. Stress values, representing how well the data are represented in three dimensions, were also calculated.

Results

Two compounds, (*Z*)-9-pentacosene and 6,9-pentacosadiene, made up ~45 % of the cuticular hydrocarbon profile of *O. brunneus* males across all populations (Fig. 1; Table 1). In contrast, these compounds accounted for less than 1 % of the profiles of the female workers and gynes, and were not detected in larvae of either sex (Fig. 1; Table 1). Only two other compounds were significantly more abundant on males than on other individuals: the chain-length homolog (*Z*)-9-tricosene, and tricosane (Table 1). The remaining compounds were identical to those previously identified from workers and queens across these populations (Smith et al. 2012, 2013; Supplemental Fig. 1, Supplemental Table 1).

The four compounds that were significantly more abundant on males also ranked among the five compounds that had the largest diagnostic power according to the caste grouping (Table 1; Supplemental Table 1). These same compounds also ranked among the compounds with the lowest diagnostic power according to grouping with population of origin (Table 1; Supplemental Table 1).

The compounds in the profile of males that were not significantly different in abundance than the corresponding compounds in the profiles of female workers or gynes varied across populations in parallel with the worker profile (Fig. 1). When compared by a multidimensional scaling approach, males, while distinct from workers and gynes, clustered with other males sampled from the same population (Fig. 2a). The stress value of 0.1 for this three-dimensional configuration (Fig. 2a) indicates a good graphical fit of the data. The female workers and male fourth instar larvae lacked the dominant male compounds and so clustered according to their source population

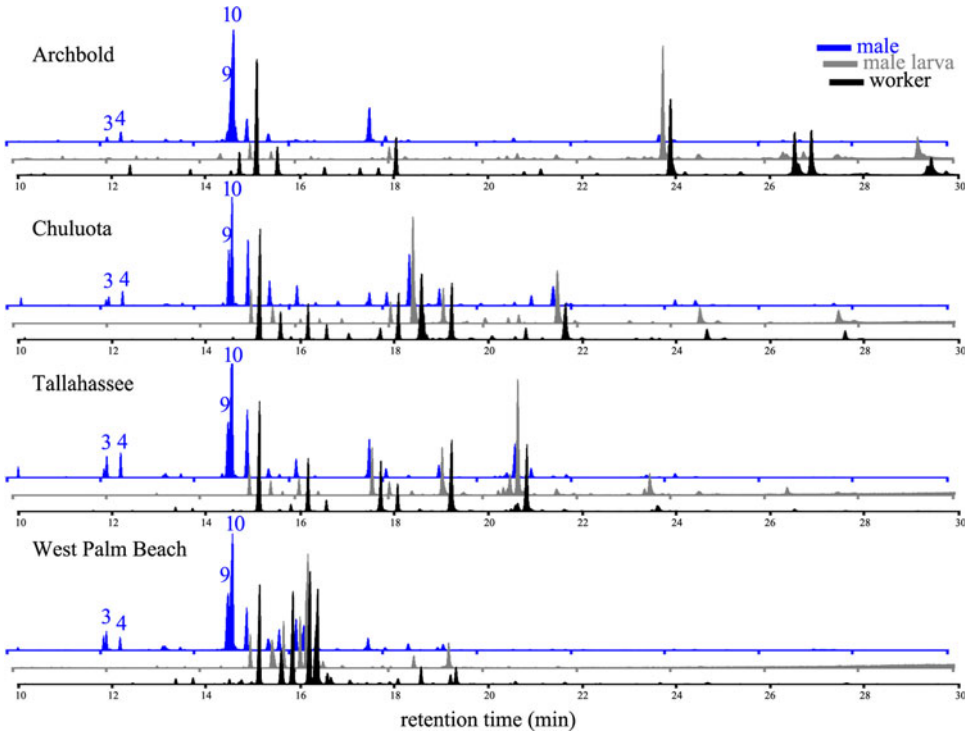

Fig. 1 Representative chromatograms of the cuticular hydrocarbon profiles of adult male *Odontomachus brunneus*, fourth instar male larvae, and female workers from four sampled populations. Numbers above individual peaks correspond to the compound numbers in Table 1 and Supplementary Table 1. The identities of the remainder of the compounds are listed in Supplementary Table 1

Table 1 Identification and mean percentage relative abundances (minimum, maximum) and diagnostic power (DP) of compounds that were significantly more abundant in the cuticular hydrocarbon profiles of male *Odontomachus brunneus* as compared to female workers, gynes, or last instar larvae

Compound number	Kovat's index	Compound	Males ($n = 23$)	Workers ($n = 23$)	Gynes ($n = 10$)	Male fourth instar larvae ($n = 8$)	Worker fourth instar larvae ($n = 10$)	Caste DP (rank)	Population DP (rank)
3	2275	(*Z*)-9-Tricosene	1.5 (0, 3.7)[a]	0 (0, 0)[b]	0 (0, 0)[b]	0 (0, 0)[b]	0 (0, 0)[b]	1.48 (5)	1.01 (34)
4	2300	Tricosane	2.6 (0.1, 6.6)[a]	0.3 (0, 2.8)[b]	0.2 (0, 0.7)[b]	0 (0, 0)[b]	0 (0, 0)[b]	1.53 (4)	1.04 (28)
9	2472	6,9-Pentacosadiene	12.4 (0, 40.1)[a]	0.2 (0, 0.6)[b]	0.1 (0, 0.2)[b]	0 (0, 0)[b]	0 (0, 0)[b]	1.56 (3)	1.00 (37)
10	2479	(*Z*)-9-Pentacosene	23.5 (0.6, 54.7)[a]	0.3 (0, 3.7)[b]	0 (0, 0.1)[b]	0 (0, 0)[b]	0 (0, 0)[b]	1.91 (1)	0.99 (38)

Means were significantly different for all compounds (Kruskal–Wallis ANOVA $P < 0.001$)

[a, b] Bonferroni-adjusted values for multiple comparisons between groups indicate significant differences ($P < 0.001$). Compound numbers refer to Fig. 1. Analyses were carried out on a DB-5 column, and Kovat's indices were calculated relative to straight-chain alkanes. For a more comprehensive identification of cuticular compounds on males and male larvae, see supplemental material

(Figs. 1, 2b). We also found no evidence of male-specific larval hydrocarbon profiles as compared to worker larvae (Fig. 2b). The stress value of 0.07 for this three-dimensional configuration (Fig. 2b) again indicates a good fit of the data.

Discussion

Our results demonstrate that adult male *O. brunneus* have a unique hydrocarbon profile relative to conspecific females, primarily characterized by a high relative abundance of (*Z*)-9-pentacosene and 6,9-pentacosadiene and lesser amounts of tricosane and (*Z*)-9-tricosene. Males could be differentiated from female workers and gynes in all four populations through the significantly increased abundance of these four compounds, with relative amounts in males being an order of magnitude greater than in females, as was found for another ponerine ant, *D. ceylonense* (Cuvillier-Hot et al. 2001). The four compounds significantly more abundant on males were diagnostic of caste and not diagnostic of population. In support of our hypothesis, the remainder of the profile of males diverged between populations, as did the profiles of female workers, gynes, and larvae. These findings are analogous to the conservation of fertility signaling compounds in this species that we reported in an earlier study (Smith et al. 2013). Together, these results support the hypothesis that specific

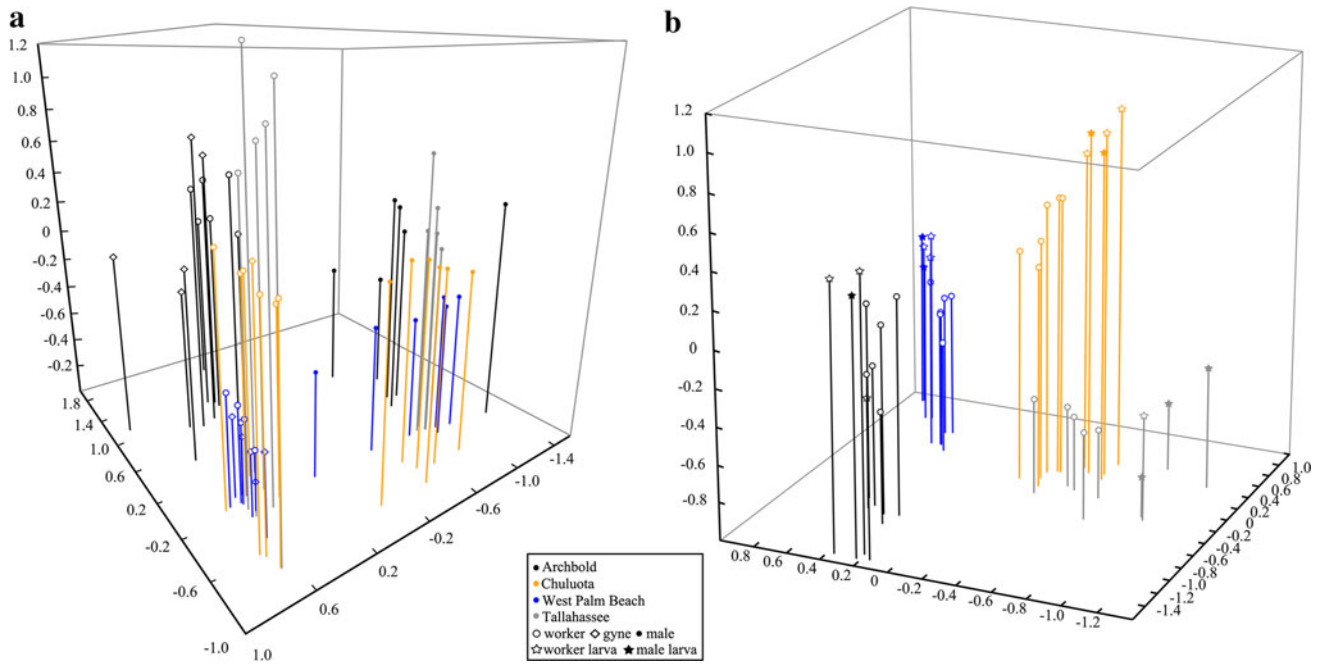

Fig. 2 Three-dimensional configuration of non-metric multidimensional scaling of cuticular hydrocarbon profiles for different sexes and castes of *Odontomachus brunneus*: **a** adult males, workers, and gynes across populations, **b** male-destined and female worker-destined fourth instar larvae across populations. Key applies to both Figures **a** and **b**

compounds in the cuticular hydrocarbon profile that constitute sex-recognition signals are under stabilizing selection, whereas the remainder of the hydrocarbon profile, portions of which may be used for nestmate discrimination (Smith et al. 2013), diverges. Similarly, Holman et al. (2013) recently found that hydrocarbon-based queen pheromones are conserved among several ant species in the genus *Lasius*.

In our laboratory colonies, males did not participate in any colony tasks and usually remained sedentary on the walls or ceilings of the nests, with minimal contact with workers. Thus, we were not able to identify a consistent behavior that workers displayed towards males that could be used as the basis for a bioassay of male-specific recognition. When males were placed outside the nest (anesthetized to prevent them from flying away) they were retrieved by workers, indicating that they were indeed recognized as colony members, and anesthetized nest workers also were retrieved at a similar rate.

Final (fourth) instar larvae destined to become males did not have the four compounds associated with adult males on their cuticles. In fact, larvae destined to become either workers or males did not have obvious differences in their cuticular hydrocarbon profiles. The destruction of worker-produced brood (usually eggs) in queenright colonies is a widespread means of enforcing a reductive division of labor in social insect societies (Ratnieks et al. 2006). For ants, discrimination of worker-produced from queen-laid brood is mediated by hydrocarbons on the egg surface (Endler et al. 2004). Whether worker-derived brood is destroyed in queenright *O. brunneus* colonies is unknown. However, our analytical data suggest that there may not be a chemical basis for brood discrimination because sex-specific hydrocarbon patterns only appear to be present in the adult stage. Therefore, the resolution of potential intracolony conflicts over male parentage or sex ratio biasing is not likely to be through selective discrimination and destruction of brood in this species.

(*Z*)-9-Pentacosene, the dominant compound in male hydrocarbon profiles males, is a chain-length homolog of the fertility signal of this species, (*Z*)-9-nonacosene (Smith et al. 2012, 2013). Sex recognition through cuticular hydrocarbon differences is widespread throughout the Insecta (e.g., Ginzel 2010; Ferveur and Cobb 2010; Tregenza and Wedell 1997), and likely to be a trait ancestral to ants. This suggests that intracolony fertility signals in insect societies may have evolved from an already established sex-recognition system. Studies of the chemical ecology of males and reproductive females of related species will be required to further test this hypothesis.

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