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RESEARCH ARTICLE

A conserved fertility signal despite population variation in the cuticular chemical profile of the trap-jaw ant *Odontomachus brunneus*

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SUMMARY

Contact pheromones in the form of cuticular hydrocarbons are widespread among insects. Eusocial insects present a special challenge for understanding the evolution of the cuticular hydrocarbon profile because this blend is responsible for multiple distinct roles such as nestmate recognition and signalling fertility status. This study investigates these two signalling roles of the hydrocarbon profile in the trap-jaw ant *Odontomachus brunneus*. We demonstrate that the cuticular hydrocarbon profile is highly variable across populations and provide evidence that these differences are used for nestmate discrimination. Through manipulative experiments we also show that (*Z*)-9-nonacosene (*Z*9:C_{29}) is used as a fertility signal and its role is conserved across populations. Our data demonstrate that both fertility and nestmate signalling influence the cuticular hydrocarbon profile and specifically the relative abundance of *Z*9:C_{29} on the cuticle of *O. brunneus*. Our study suggests that natural selection works on the cuticular chemical profile through multiple regulatory pathways, diversifying nestmate signals while conserving fertility signals.

Key words: cuticular hydrocarbons, fertility signal, pheromone, phenotypic variation, nestmate recognition.

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INTRODUCTION

Chemical signals are a primary means of communication for insects. Contact pheromones, in the form of cuticular hydrocarbon components, are widespread throughout the Insecta (Blomquist and Bagnères, 2010). For example, contact sex pheromones have been identified for a number of species of flies and beetles (Carlson et al., 1971; Ferveur, 2005; Ginzel, 2010). In some *Drosophila* species, sexual dimorphism in hydrocarbon profiles provides a means for mate choice and reproductive isolation between species (Savarit et al., 1999; Higgie et al., 2000). Whereas mate and species recognition are the primary signalling functions of cuticular hydrocarbon profiles of these solitary species, hydrocarbon profiles in eusocial insects serve several other signalling roles (Howard and Blomquist, 2005). How natural selection maintains multifunctional roles of the individual components and blends that constitute the cuticular hydrocarbon profiles of eusocial species remains largely unexplored.

The cuticular hydrocarbon profiles of social insects, and ants specifically, typically consist of up to 50–60 compounds of 23–35 carbons in length, varying in the number and position of methyl branches and double bonds (Martin and Drijfhout, 2009; but see Menzel et al., 2008). To date, species-specific hydrocarbon profiles of over 80 species of ants have been described. These complex blends of hydrocarbons serve to prevent desiccation (Lockey, 1988); however, they also provide a means of intraspecific communication. They are used for distinguishing nestmates from non-nestmates, encoding task-specific cues, and signalling the fertility status of both queens and workers (Liebig, 2010; van Zweden and d'Ettorre, 2010).

Recent experimental work on cuticular hydrocarbons and nestmate recognition has shown that changes in specific subsets of the overall chemical profile are primarily responsible for distinguishing non-nestmates from nestmates (Greene and Gordon, 2007; Martin et al., 2008a; Martin et al., 2008c; Brandt et al., 2009; Guerrieri et al., 2009; van Wilgenburg et al., 2010). Aggression seems to be triggered by recognizing cuticular profiles as being unfamiliar, and the degree of difference from the colony's template correlates with aggression (Suarez et al., 2002; Ozaki et al., 2005; Guerrieri et al., 2009; Martin et al., 2012; Sturgis and Gordon, 2012). Within species, populations may have divergent hydrocarbon profiles (Nowbahari et al., 1990; Dahbi et al., 1996; Akino et al., 2002; Evison et al., 2012) or they may be relatively invariable across large geographic areas (Martin et al., 2008b). Between species, however, hydrocarbon profiles are consistently different, and divergent profiles have been successfully used to delineate closely related species (Lucas et al., 2002; Evison et al., 2012).

Fertility-associated differences in cuticular hydrocarbon profiles of ants have been well documented (Monnin, 2006; Liebig, 2010). Direct experimental evidence linking specific hydrocarbons with worker perceptions of nestmate reproductive ability has been provided for three ant species. In *Aphaenogaster cockerelli*, changes in worker reproductive ability correlated with changes in the relative abundance of pentacosane in the hydrocarbon profile (Smith et al., 2008). Non-reproductive workers that have this compound experimentally added to their cuticle are policed, and treated as reproductive, by their nestmates (Smith et al., 2009). In *Lasius niger*, 3-methylhentriacontane is correlated with queen fecundity and inhibits worker ovarian development (Holman et al., 2010). Finally, in the trap-jaw ant *Odontomachus brunneus* (Patton 1894), we recently demonstrated that (*Z*)-9-nonacosene (*Z*9:C_{29}) is correlated

with reproduction, and showed that artificial application of this compound to workers resulted in their being treated as reproductives by their nestmates (Smith et al., 2012). Nestmates responded to treated workers with rapid antennation, an aggressive dominance behaviour described by Powell and Tschinkel (Powell and Tschinkel, 1999) performed towards reproductive workers in the nest (Smith et al., 2012). Nestmates also occasionally adopted a stereotypical submissive pose when near the treated workers, also indicating perception of reproductive individuals (Powell and Tschinkel, 1999; Smith et al., 2012).

Very few studies of the cuticular hydrocarbons of social insects have considered both the fertility and nestmate recognition roles of the hydrocarbon profile simultaneously. Dapporto et al. (Dapporto et al., 2004) reported that hydrocarbon profiles of the paper wasp *Polistes dominulus* were population specific and that chemical differences associated with fertility were also variable across those populations, with fertility being associated with a proportional increase in higher molecular weight compounds (Dapporto et al., 2004). Denis et al. (Denis et al., 2006) suggested that compounds within the hydrocarbon profile of the ant *Pachycondyla goeldii* could be responsible for both nestmate and fertility signalling. Evison et al. (Evison et al., 2012) reported that both the overall cuticular profile in the ant *Pachycondyla verenae* and the compounds associated with fertility diverged across populations within species and between two morphospecies. However, the compounds that correlated with fertility across populations and morphospecies in that study were conserved, consisting of three structurally similar alkenes (Evison et al., 2012). It is important to note that in the above studies the focal compounds were only correlated with fertility, and direct experimental evidence linking those compounds to worker perception of fertility was lacking. Recently, Holman et al. (Holman et al., 2013) addressed this limitation by identifying a methylalkane that functions as a queen pheromone in two *Lasius* species. The authors also found that methylalkanes in general are correlated with the reproductive queen caste within several more related *Lasius* species. Within this group overall hydrocarbon profiles are divergent; however, the putative queen-specific signals appeared to be conserved.

In the present study we document variability in the cuticular hydrocarbon profile of *O. brunneus* by sampling three Florida populations, and demonstrate population-specific hydrocarbon profiles that differ from a previously sampled population (Smith et al., 2012). We conducted aggression assays between and within populations to correlate hydrocarbon profile differences with levels of aggression towards non-nestmates. In our previous study of *O. brunneus*, we demonstrated that changing the relative abundance of certain components of the cuticular hydrocarbon profile resulted in manipulated nestmates receiving non-nestmate levels of aggression, indicating that cuticular hydrocarbons are used as nestmate signals in this species (Smith et al., 2012). This previous study also tested multiple components of the hydrocarbon profile for their potential function as a fertility signal and found that treatment of non-reproductive individuals with *Z*9:C_{29} could replicate the nestmate responses elicited by fertile individuals. Therefore, the present study tests whether *Z*9:C_{29} is associated with fertility in different populations by comparing the hydrocarbon profiles of reproductive queens with those of workers from those populations. Finally, with these three populations, we repeated a series of bioassays that confirmed the role of *Z*9:C_{29} as a fertility signal (Smith et al., 2012). We conclude by discussing the implications of our main finding: that whereas the overall cuticular hydrocarbon profile of *O. brunneus* is highly divergent across Florida populations, the chemical signal that is responsible for signalling fertility has been conserved across populations.

MATERIALS AND METHODS

Collections and laboratory conditions

Queenright colonies of *O. brunneus* were collected by manual excavation in August 2011 and 2012 from three locations in Florida. Colonies referred to in this study as from 'Archbold' were collected at the MacArthur Agro-Ecology Research Center, near Lake Placid, Florida. Colonies from 'Chuluota' were collected at the Chuluota Wilderness Area in Chuluota, Florida. Colonies from 'West Palm' were collected at the Pine Jog Environmental Education Center in West Palm Beach, Florida. Additionally, colonies previously collected (Smith et al., 2012) from the Apalachicola National Forest in Tallahassee, FL, were used in our nestmate discrimination assay. Colonies from each location were collected within approximately 1 km of one another. Species identifications were confirmed according to morphological traits described by Deyrup and Cover (Deyrup and Cover, 2004).

In the laboratory, individual 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 20% sugar water solution and were fed 3 days a week on live termites and freeze-killed crickets. All colonies were kept under a 12 h:12 h light:dark cycle at an average temperature of 27°C. Colonies were sampled for behavioural and chemical analysis after being held in laboratory conditions for at least 1 month.

Chemical analysis

Cuticular hydrocarbons of live, reproductively active queens and non-reproductive workers were sampled from 18 colonies collected across three Florida locations. A queen and a worker were sampled from six colonies per location. The non-reproductive status of workers was verified through ovary dissections conducted after chemical sampling, whereas the reproductive activity of queens was assessed by observing stereotypical reproductive dominance behaviours (Powell and Tschinkel, 1999; Smith et al., 2012) and the presence of an egg pile. All ants were sampled after being held for at least 1 month under laboratory conditions. Cuticular hydrocarbons were sampled from individual ants by solid-phase microextraction (SPME). An SPME fibre (100 μm polydimethylsiloxane; Supelco, Bellefonte, PA, USA) was lightly rubbed on the abdomen of the ant for 5 min and compounds then were thermally desorbed for 5 min in the injection port of a Hewlett-Packard 6890 series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a nonpolar capillary column (DB-5MS, 30 m×0.25 mm×0.25 μm film; J&W Scientific, Folsom, CA, USA), connected to an HP 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^{-1}, and then to 315°C at 4°C min^{-1}. Helium was used as a carrier gas at 1 ml min^{-1}, 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.

Straight-chain compounds were identified from their mass spectra, including the parent ion if present, 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 (Carlson et al., 1998). Alkenes and non-conjugated

dienes were identified from their retention indices (slightly smaller than the corresponding alkanes on the DB-5 column), their parent 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^{-1} solution). After 2 h at room temperature, the mixture was evaporated just to dryness, a few drops of 1 mol l^{-1} aqueous NaOH and ~200 µl of hexane were added, and the mixture was vortexed, the hexane layer was removed and dried over anhydrous Na_2SO_4, and analyzed by GC-MS as described above.

Compounds were included in the graphical analysis if they occurred in ≥70% of the sampled individuals within at least one of the classes (queen, worker, population). Compounds are presented in terms of relative abundance within the entire profile (individual compound amount/sum of all compound amounts). We performed non-metric multi-dimensional scaling to analyze the similarity of profiles within and between populations (Primer 6, PRIMER-E, Ivybridge, UK). Chord distances were used to calculate the distance matrices. Stress values, representing how well the data are represented in two and three dimensions, were also calculated.

Nestmate discrimination assay

Six colonies from each of the Archbold, Chuluota, Tallahassee and West Palm populations were used in this bioassay. At least 24 h before testing, a subset of workers from each colony was marked with a single spot of Testors model paint (Rockford, IL, USA). Responses of workers to non-nestmates were tested by placing two individuals in a 55×15 mm glass Petri dish. Workers from each colony were exposed to non-nestmates from each of the four populations and to a nestmate worker, resulting in three treatments: nestmate pairing (control; N=24), non-nestmate pairing from the same population (N=24), and non-nestmate pairing from different populations (N=36). Each pairing of workers was unique and individuals were used only once. Immediately after two workers were added to the Petri dish, their interactions were video recorded for 5 min or until aggression escalated to the highest level.

The resulting videos were assigned a randomly coded title and analyzed, without knowledge of treatment, for level of aggression: (1) no aggression (workers only antennated one another); (2) rapid antennation [workers performed rapid antennation, an aggressive dominance behaviour (Powell and Tschinkel, 1999; Smith et al., 2012)]; and (3) mandible strike (workers performed mandible snap directed at the other ant). Mandible strikes have previously been reported to occur both in prey capture and in agonistic interactions with conspecific non-nestmates (Spagna et al., 2009). Although biting, holding and stinging did occur in some of our trials, the three behaviours used in the analysis represent the majority of those observed in this experiment.

Bioassay of cuticular hydrocarbons

In a previous study linking *Z*9:C_{29} with perception of nestmate fertility, we performed a bioassay wherein we treated workers with hexane or hexane solutions of either *Z*9:C_{29} or pentacosane (C_{25}), and reintroduced them to their nestmate workers. Workers treated with *Z*9:C_{29} elicited significantly more aggression from nestmates, in the form of rapid antennation, than did the other treatments. Rapid antennation is part of the aggressive policing response that workers perform towards supernumerary reproductives in their colony (Smith et al., 2012). Also, at a much lower frequency, nestmate workers responded to workers treated with *Z*9:C_{29} by adopting a submissive pose, crouching and retracting their antennae while retreating, significantly more often as compared with the control treatments (Smith et al., 2012). In proximity to reproductive workers and queens, non-reproductive workers have been reported to adopt a stereotypical submissive pose (Powell and Tschinkel, 1999; Smith et al., 2012).

For this study, we repeated this bioassay across three populations. *Z*9:C_{29} was synthesized using previously published methods (Ginzel et al., 2006; Millar, 2010). Solutions with a concentration of ~1 mg of hydrocarbon per 7 ml of hexane were used. One millilitre aliquots of working solutions were used for all of the test compounds throughout the experiments. The concentrations of hydrocarbons in these aliquots were verified by GC-MS analysis and comparison of peak areas. For hydrocarbon treatments of live ants, 15 µl of the hydrocarbon working solutions (2.1 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., 2012). Treatments resulted in an ~16% increase in the relative abundance of the test compounds, which was slightly beyond normal measures of relative compound abundances (Smith et al., 2012).

Six queenright colonies from each of the three populations were used in these bioassays (N=6 for all treatments per population). On the same day, three workers were removed from each colony, paint-marked, treated and consecutively reintroduced to their colonies. The ants were reintroduced to their colonies in random order, and each colony was subjected to one treated ant at a time. Following reintroduction, the treated ants were video-recorded for 5 min. The resulting videos were assigned a randomly coded title and analyzed, without knowledge of treatment, 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, each population separately, for differences between treatment means 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 (StatSoft, Tulsa, OK, USA).

RESULTS

Chemical analysis

The cuticular hydrocarbon profiles of *O. brunneus* were variable across all sampled Florida populations (Figs 1, 2, Table 1). Workers could be differentiated from queens in all populations, in that *Z*9:C_{29} (compound 20; Table 1) was significantly more abundant on the cuticle of queens as compared with workers, as noted previously for a single population (Smith et al., 2012). In fact, increased abundance of *Z*9:C_{29} marked the largest single compound increase in a queen profile (Table 1) across all of the populations sampled. Workers and queens within a population otherwise shared similar profiles (Fig. 2, Table 1). Stress values of 0.1 (Fig. 2A) and 0.03 (Fig. 2B) indicate a good graphical representation of the differences in cuticular hydrocarbon profiles between and within populations.

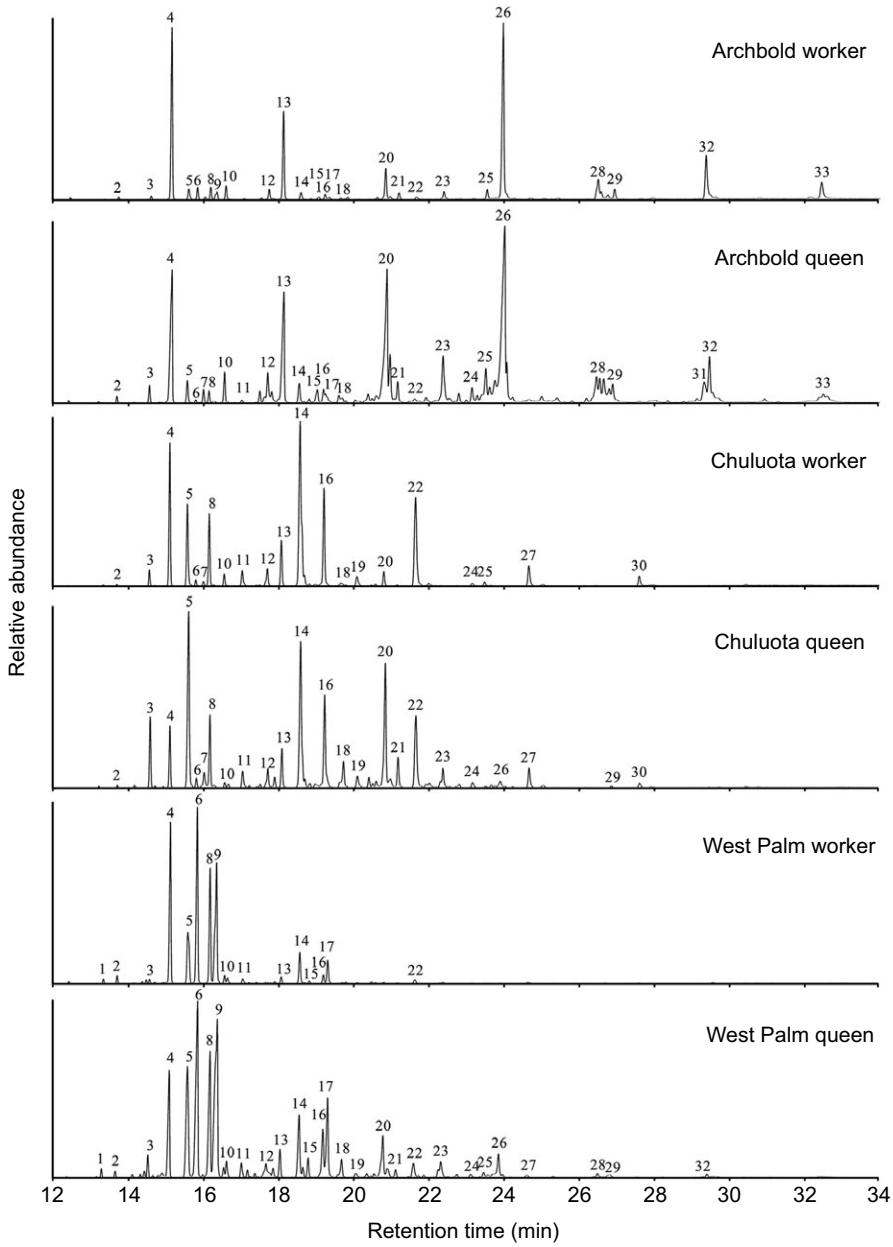

Fig. 1. Representative chromatograms of *Odontomachus brunneus* queens and workers across populations. Numbers above peaks correspond to the compounds presented in Table 1.

Cuticular hydrocarbon variation within populations was much less pronounced than differences between populations (Figs 1, 2, Table 1).

Nestmate discrimination assay

The type of pairing of individuals (nestmates, within-population non-nestmates and between-population non-nestmates) had a significant effect on level of aggression (χ^2=79.6, P<0.001; Table 2). No aggression was most commonly observed in pairings of nestmate workers. Aggression in the form of rapid antennation was most common in pairings of non-nestmates from the same population. Mandible snaps were only observed in pairings of non-nestmates from different populations.

Hydrocarbon bioassay

In all three populations, hydrocarbon treatments had a statistically significant effect on eliciting nestmate rapid antennations [Friedman's ANOVA: $\chi^2(2)>8.4$, $P<0.015$]. Workers that were treated with $Z9$:C_{29} received significantly more aggression, in the form of rapid antennation, than did workers treated with C_{25} or hexane (Wilcoxon signed-ranks test; Archbold, $Z9$:C_{29} *versus* C_{25} and $Z9$:C_{29} *versus* hexane: t=0, P<0.044; C_{25} *versus* hexane: t=6.5, P=0.79; Chuluota, $Z9$:C_{29} *versus* C_{25} and $Z9$:C_{29} *versus* hexane, t=0, P<0.044; C_{25} *versus* hexane: t=0, P=0.068; West Palm, $Z9$:C_{29} *versus* C_{25} and $Z9$:C_{29} *versus* hexane, t=0, P=0.028; C_{25} *versus* hexane: t=4.5, P=0.86; Table 3).

In all three populations, hydrocarbon treatments had a statistically significant effect on nestmate submissive reactions (Friedman's ANOVA; Chuluota and West Palm, $\chi^2(2)$=6, P=0.049; Archbold, $\chi^2(2)$=7.5, P=0.023), as previously reported for the same bioassay performed on colonies from Tallahassee (Smith et al., 2012). However, nestmate submissive reactions were observed less frequently in all three populations as compared with Tallahassee colonies. Direct comparisons of treatment groups within populations, though trending towards that reported in Smith et al. (Smith et al., 2012), did not indicate statistically significant differences in all three

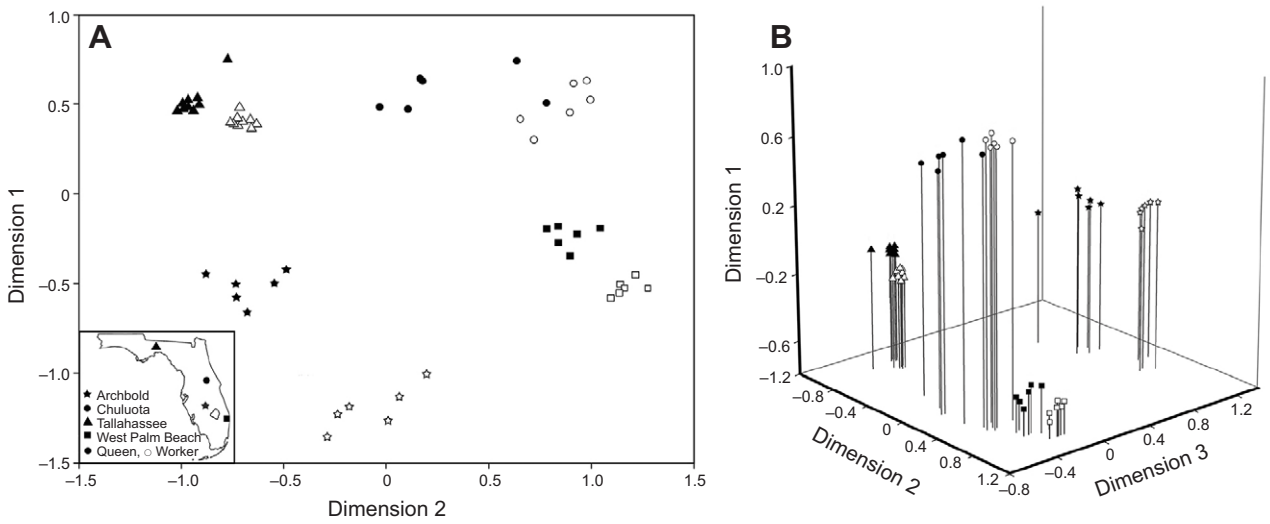

Fig. 2. Two-dimensional (A) and three-dimensional (B) configuration of non-metric, multidimensional scaling of differences in *O. brunneus* cuticular hydrocarbon profiles across populations. Profiles from Tallahassee are from Smith et al. (Smith et al., 2012). In that population only, the nine 'queen' data points are two queens and seven reproductive workers.

populations (Wilcoxon signed-ranks tests: all comparisons, $t<1.8$, $P>0.068$; Table 3).

DISCUSSION

Our results demonstrated that the cuticular hydrocarbon profile of the ant *O. brunneus* is highly variable across populations in Florida (Figs 1, 2, Table 1). The major factor discriminating queens from non-reproductive workers was the relative abundance of *Z*9:C_{29}, for all three populations sampled (Table 1). This is in accordance with what had been previously reported for *O. brunneus* from Tallahassee, where both queens and reproductive workers were distinguished by an analogous increase in the relative abundance of *Z*9:C_{29} (Smith et al., 2012). Our bioassays demonstrated that *Z*9:C_{29} is used by workers in the three sampled populations to assess the reproductive ability of their nestmates (Table 3) because workers treated with *Z*9:C_{29} received more aggression from nestmates in the form of rapid antennation than those treated with C_{25} or hexane, across all populations. They also elicited submissive responses from nestmates (Table 3). Whereas the omnibus statistical test of the effect of hydrocarbon treatment on eliciting nestmate submissive responses indicated a significant treatment effect, the low frequency of submissive responses resulted in non-significant differences between treatment groups. Submissive reactions were also observed at a much lower frequency than rapid antennations in our previous study (Smith et al., 2012). Submissive reactions may have been relatively more common among ants from the Tallahassee population because they had much larger amounts of *Z*9:C_{29} already on their cuticle. In fact, *Z*9:C_{29} was the most abundant single compound on the cuticle of non-reproductive workers from Tallahassee (Smith et al., 2012). It is possible that adding *Z*9:C_{29} to a cuticular hydrocarbon layer already rich in that compound, as with Tallahassee ants, may be more effective in reaching the threshold level necessary for eliciting submissive reactions than is the case when adding it to hydrocarbon blends with lower relative amounts of *Z*9:C_{29}, as with ants from the other populations.

Previous studies have documented variation in cuticular hydrocarbon profiles across populations of conspecific ants (Nowbahari et al., 1990; Dahbi et al., 1996; Akino et al., 2002; Evison et al., 2012). In accordance with these previous studies, we found substantial diversification of hydrocarbon profiles among populations, and our nestmate recognition assays correlated this diversification with worker perception of non-nestmates and corresponding behaviours (Table 2). This correlation, along with our previous manipulative work that showed changing the abundance of non-fertility signalling compounds resulted in aggression that was equivalent to non-nestmate aggression (Smith et al., 2012), suggests that, like other ants, nestmate status in *O. brunneus* is encoded in the cuticular hydrocarbon profile.

Most studies of cuticular hydrocarbons tackle only a single function of the hydrocarbons, and many of these studies show that one group or a single compound is primarily responsible for a specific function. However, that does not mean that this compound does not have signalling value in another context. The compound that we identify as the fertility signal, *Z*9:C_{29}, is present in the profile of non-reproductive workers in different levels of abundance across our sampled populations. *Z*9:C_{29} was the most abundant cuticular hydrocarbon on workers from the Tallahassee population (Smith et al., 2012), but it was present in much lower relative abundance in workers from Archbold and Chuluota populations, and nearly absent in workers of the West Palm population (Fig. 1, Table 1). Non-reproductive worker profiles do not display a fertility signal, yet in one of our populations *Z*9:C_{29} is highly abundant on the profile on non-reproductive workers. This suggests that *Z*9:C_{29} also influences perception of nestmate status; however, this hypothesis has yet to be specifically tested.

Contact chemical signals that serve intra-colony functions, such as those signalling reproductive status or caste differences, should be much more constant among populations because the organization of the colony is crucially dependent on the correct transmission and interpretation of such signals. Thus, in contrast to nestmate recognition signals, which diversify across populations in this species, it would be expected that selection should favour conservation of reproductive signals among populations. Deviation from this signal could result in a breakdown of colony organization by failure to recognize the presence of a reproductive queen or not being able to suppress or police worker reproduction. Our data suggest that this is indeed the case, because workers from all four populations associated an increase in the relative abundance of a

Table 1. Cuticular hydrocarbons of queens and workers from three populations of the ant *Odontomachus brunneus*, Kovat's retention indices and relative percent abundance

Peak number	Identification	Kovat's index	Relative percent abundance					
			Archbold queen	Archbold worker	Chuluota queen	Chuluota worker	West Palm queen	West Palm worker
1	3-Methyltricosane	2371	0.04 (0, 0.13)	0	0.14 (0, 0.59)	0.2 (0, 0.34)	0.52 (0.13, 1.12)	0.39 (0.19, 0.6)
2	Tetracosane	2400	0.27 (0.17, 0.34)	0.53 (0, 1.68)	0.17 (0, 0.57)	0.17 (0, 0.3)	0.38 (0.19, 0.87)	0.59 (0.38, 0.74)
3	4-Methyltetracosane	2462	1.59 (0.4, 2.9)*	0.58 (0, 1.01)	2.65 (0.09, 4.53)*	0.91 (0.38, 1.35)	0.64 (0.30, 1.11)*	0.27 (0.18, 0.42)
4	Pentacosane	2500	9.38 (7.56, 12.2)	26.3 (20.5, 31.9)	5.77 (3.5, 11.7)	14.8 (10.7, 21)	9.55 (7.06, 12.3)	17.4 (13.2, 20.7)
5	11- and 13-Methylpentacosane	2534	4 (0.61, 8.73)	1.79 (0.58, 2.68)	11.3 (6.76, 15.4)	9.36 (6.5, 11.7)	9.44 (8.08, 10. 6)	9.18 (8.27, 9.8)
6	5-Methylpentacosane	2548	0.20 (0, 0.53)	1.1 (0, 3.01)	0.54 (0.25, 0.74)	0.56 (0, 0.85)	14.1 (12.2, 14.9)	20 (16.7, 22)
7	4-Methylpentacosane	2562	0.74 (0.41, 1.54)*	0.24 (0, 0.64)	1.01 (0.14, 1.42)	0.43 (0, 1.11)	0.1 (0, 0.24)	0
8	3-Methylpentacosane	2573	0.97 (0.34, 1.73)	1.26 (0.27, 2.76)	5.91 (4.62, 7.77)	7.62 (5.72, 8.73)	9.41 (8.06, 11.3)	14.3 (13, 15.3)
9	5,8-Dimethylpentacosane	2588	0.08 (0, 0.37)	1.03 (0, 2.61)	0.23 (0, 0.45)	0.05 (0, 0.28)	15.63 (13.85, 18.08)	21.47 (18.89, 25.65)
10	Hexacosane	2600	0.93 (0.51, 1.5)	1.43 (1.16, 1.88)	0.62 (0.27, 1.41)	1.17 (0.71, 1.36)	0.66 (0.38, 0.96)	0.91 (0.85, 0.96)
11	14-Methylhexacosane	2631	0.23 (0, 0.62)*	0	1.81 (1.44, 2.52)	1.77 (1.13, 2.18)	0.73 (0.46, 1.01)	0.72 (0.56, 0.95)
12	(*Z*)-9-Heptacosene	2675	2.61 (1.84, 4.03)*	1.36 (1.07, 1.94)	2.67 (1.13, 4.17)	3.31 (1.81, 6.31)	1.11 (0, 1.58)	0
13	Heptacosane	2700	6.09 (5.03, 8.19)	9.58 (7.81, 11)	2.54 (1.08, 3.25)	3.99 (2.06, 6.61)	1.65 (1.36, 2.37)	0.86 (0.71, 1.05)
14	11- and 13-Methylheptacosane	2732	1.15 (0.43, 2.24)	0.79 (0, 1.99)	16.6 (11.4, 23.8)	24.1 (21.1, 27)	4.46 (4.14, 4.6)	4.45 (4, 4.95)
15	4-Methylheptacosane	2764	0.24 (0.14, 0.38)	0.09 (0, 0.52)	0.19 (0, 0.48)	0	1.09 (0.78, 1.24)*	0.29 (0.24, 0.31)
16	3-Methylheptacosane	2774	0.34 (0, 0.71)	0.5 (0, 1.26)	8.88 (7.45, 10.61)	9.13 (7.07, 10.76)	4.05 (2.77, 5.19)*	1.18 (1.05, 1.41)
17	5,19-Dimethylheptacosane	2782	0.92 (0.45, 1.79)*	0.26 (0, 0.76)	0	0	5.14 (3.89, 5.98)*	3.41 (2.96, 4.88)
18	3,7-Dimethylheptacosane	2806	0.11 (0, 0.26)	0.04 (0, 0.24)	2.54 (0.66, 5.44)*	0.82 (0.43, 2.26)	0.7 (0, 1.68)	0.27 (0, 0.82)
19	11-Methyloctacosane	2831	0.14 (0, 0.25)	0	1.36 (1.19, 1.87)	1.21 (0.84, 1.43)	0.2 (0, 0.4)	0.09 (0, 0.18)
20	(*Z*)-9-Nonacosene	2878	14.9 (12.6, 22.6)*	3.48 (2.2, 4.45)	10.3 (2.53, 14.7)*	2.12 (0.57, 4.46)	4.38 (2.22, 7.53)*	0.2 (0, 0.33)
21	Nonacosane	2900	0.68 (0.41, 1.18)	0.68 (0.49, 0.86)	1.5 (0.3, 2.63)*	0.03 (0, 0.18)	0.65 (0.38, 1.04)*	0.04 (0, 0.21)
22	11- and 13-Methylnonacosane	2931	0.38 (0.23, 0.51)	0.31 (0, 0.6)	7.83 (5.15, 10.66)	12.3 (11.2, 14.1)	1.37 (0.96, 1.69)*	0.63 (0.56, 0.72)
23	Unknown	2980	4.16 (2.97, 5.92)*	1.01 (0.58, 1.34)	0.99 (0, 1.74)*	0	1.43 (0.93, 2.22)*	0.16 (0, 0.3)
24	12-Methyltricontane	3028	0.64 (0.19, 0.94)*	0	0.57 (0.43, 0.76)*	0.18 (0, 0.45)	0.15 (0, 0.29)	0
25	x,y-Hentriacontadiene	3054	2.22 (1.6, 2.54)*	0.76 (0, 1.4)	0.21 (0, 0.5)	0.68 (0.43, 1.13)	0.22 (0, 0.5)	0
26	(*Z*)-9-Hentriacontene	3076	20.3 (17.7, 24.3)	25 (18.5, 36.8)	0.39 (0, 0.82)	0	0.6 (0, 1.99)*	0
27	13-Methylhentriacontane	3126	0.21 (0, 0.36)	0.12 (0, 0.4)	1.76 (1.44, 2.25)	2.29 (1.83, 2.78)	0.29 (0, 0.51)	0.18 (0, 0.3)
28	x,y-Tritriacontadiene	3251	3.21 (1.59, 7.65)	4.24 (0.69, 11.47)	0.03 (0, 0.19)	0	0.52 (0.2, 1.13)*	0
29	x-Tritriacontene	3272	1.63 (0.97, 2.86)	3.27 (0.88, 9.04)	0.12 (0, 0.45)	0	0.8 (0.28, 1.41)*	0.03 (0, 0.15)
30	13-Methyltritriacontane	3323	0.02 (0, 0.14)	0	0.5 (0.44, 0.6)	1.12 (0.85, 1.56)	0	0.04 (0, 0.25)
31	x,y-Pentatriacontadiene	3446	0.14 (0, 0.27)	0	0	0	0.05 (0, 0.31)	0
32	x,y-Pentatriacontadiene	3457	1.23 (0.33, 1.88)	5.99 (2.71, 8.41)	0	0	0.09 (0, 0.54)	0
33	x,y-Heptatriacontadiene	3655	0.08 (0, 0.29)	2.09 (0, 3.92)	0	0	0.03 (0, 0.19)	0

Data are means (minimums, maximums); N=6 for all groups. Asterisks indicate compounds that are significantly more abundant on queens than workers within populations (Mann–Whitney U-test, two-sided, P<0.05). x,y indicates unknown double bond position. Details of compound identifications were described in Smith et al. (Smith et al., 2012).

Table 2. Responses of workers of the ant *Odontomachus brunneus* when encountering nestmates *versus* non-nestmates

	No aggression	Rapid antennation	Mandible snap
Nestmates (*N*=24)	15	9	0
Within population (*N*=24)	5	19	0
Between populations (*N*=36)	1	4	31

The maximum level of aggression is recorded per pairing of two nestmates or two non-nestmates from within the same population or between populations.

Table 3. Responses of nestmate workers to hydrocarbon-treated workers

	Rapid antennations			Submissive reactions		
Population	*Z*9:C_{29}	C_{25}	Hexane	*Z*9:C_{29}	C_{25}	Hexane
Archbold	16.3 (7, 28)*	6.2 (0, 10)	5.8 (0, 12)	1.5 (0, 5)	0.17 (0, 1)	0
Chuluota	9.7 (1, 18)*	4.5 (1, 12)	2.3 (0, 6)	0.83 (0, 2)	0	0
West Palm	6.7 (2, 13)*	3.2 (1,9)	2.5 (1, 4)	1 (0, 3)	0	0

Mean (minimum, maximum) numbers of observations per trial, *N*=6 for all groups. Asterisks indicate significant differences between treatment groups within a population.

single compound, *Z*9:C_{29}, with nestmate reproductive status (Smith et al., 2012) (Table 3). Queen fertility signals were recently reported to be conserved across a group of closely related *Lasius* ant species (Holman et al., 2013). This suggests that fertility signals in *Odontomachus* might also be conserved among congeneric species.

Our data clearly indicate that the cuticular hydrocarbon profile of *O. brunneus* varies across populations, with variation among components of the profile providing the means for discrimination of nestmates from non-nestmates, and conservation of some components providing a conserved fertility signal recognized in all populations. Given the complexity of the overall hydrocarbon profiles, it is clear that they must be the products of numerous genetic regulatory processes. This in turn provides a plethora of opportunities for natural selection to influence the basic hydrocarbon template in divergent (nestmate profiles) and conservative (fertility signal) ways. Thus, cuticular profiles may be regulated by expression of genes coding for endogenous nestmate profiles, whereas different regulatory elements or processes (e.g. ovarian activity or hormone levels) are likely to be responsible for production of the fertility signal *Z*9:C_{29} (Holman, 2012).

The idea that components of cuticular profiles might serve multiple signalling functions in ants has been previously noted (Peeters and Liebig, 2009). For example, Le Conte and Hefetz (Le Conte and Hefetz, 2008) proposed a hierarchical model based on worker perception thresholds that assumed that non-nestmate recognition had the lowest discrimination threshold and that subsequent perception of fertility signals necessitated prior acceptance of nestmate status. There is experimental evidence both for (Le Conte and Hefetz, 2008; Cournault and de Biseau, 2009) and against (Moore and Liebig, 2010) this model. Regardless of whether this perception model holds true for most social insects, our data confirm the idea that Denis et al. (Denis et al., 2006) and Le Conte and Hefetz (Le Conte and Hefetz, 2008) put forth that individual cuticular compounds can serve multiple functions as signals in different contexts.

The genetic and physiological underpinnings of cuticular hydrocarbon profiles have been documented for only a few species of solitary insects. In *Drosophila melanogaster* and *Musca domestica*, for example, alkenes similar to *Z*9:C_{29} act as contact sex pheromones (Ferveur and Cobb, 2010). Precursors of these alkenes are biosynthesized by desaturase enzymes that are coded for by genes that have been identified in both systems (Wicker-Thomas and Chertemps, 2010). Analogous investigations into the enzymatic and genetic underpinnings of hydrocarbon production by eusocial insects may shed light on the evolution of the complex chemical blends that they exhibit, and ultimately the maintenance of eusociality.

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AUTHOR CONTRIBUTIONS

A.A.S., J.G.M., L.M.H. and A.V.S. conceived the study and drafted the manuscript. J.G.M. synthesized and identified chemicals in this study. A.A.S. designed the experiments and acquired and analyzed the data. All authors read and approved the final manuscript.

COMPETING INTERESTS

No competing interest declared.

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