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RESEARCH PAPER

Alteration of cuticular hydrocarbon composition affects heterospecific nestmate recognition in the carpenter ant *Camponotus fellah*

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Abstract Nestmate recognition is a ubiquitous phenomenon in social insects as a means to prevent entry of undesired individuals aiming at exploiting the rich nest resources. The recognition cues in ants were shown in a few cases to be cuticular hydrocarbons, although there are a quite number of correlated associations. In the present study we modified the cuticular profiles of workers *Camponotus fellah* hydrocarbons with cuticular washes from a closely related, yet undescribed species, *Camponotus sp*. Although these sympatric species are morphologically indistinguishable, cuticular washes of *C. sp*. contain 9,13-dimethylpentacosane and 11,15-dimethylheptacosane that are either absent or occur as traces in *C. fellah*. In addition, *C. sp*. contains significantly greater amounts of 3-methylpentacosane than *C. fellah* workers. The cuticle modification was done solventless in a manner that minimized disruption to the cuticular structure of the ant being modified. Judging from the 3 focal compounds, such treatment added between 20 and 30% of the original amounts present in *C. sp*. to the treated *C. fellah* workers. This addition changed consistently the cuticular hydrocarbon profile of the treated ant. Dyadic assays between *C. fellah* and their nestmates treated with *C. sp*. cuticular rinses revealed a significantly higher level of aggression compared to non-treated nestmates. There was no aggression between nestmates of *C. sp*. These results demonstrate that in heterospecific interactions between the two *Camponotus* species there is a correlation between cuticular hydrocarbons and a nestmate recognition response, albeit not as high as the response of *C. fellah* to of *C. sp*. workers. This is consistent with the hypothesis that cuticular hydrocarbons may play a role in nestmate recognition.

Keywords Cuticular hydrocarbons · Nestmate recognition · *Camponotus fellah*

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Introduction

Colony insularity is a widespread characteristic of ant societies as means for protecting the rich colony resources from exploitation by variety of organisms. It is generally mediated through a sophisticated recognition system whereby individuals are classified, through a specific chemical label they carry, either as nestmates or alien individuals (Hefetz 2007; Lenoir et al. 1999; Vander Meer and Morel 1998). There are numerous reports that correlate cuticular hydrocarbons with ant nestmate recognition (Bonavita-Cougourdan et al. 1987; Clément et al. 1987; Henderson et al. 1990; Lahav et al. 2001; Lavine et al. 2003; Provost et al. 1993; Tentschert et al. 2002). More direct evidence for cuticular hydrocarbon involvement in nestmate recognition was obtained from experiments using isolated cuticular hydrocarbons on various types of carriers (Akino et al. 2004; Lahav et al. 1999; Ozaki et al. 2005; Thomas et al. 1999; Wagner et al. 2000).

Nestmate recognition in *Camponotus fellah* has been studied extensively in the past years. Social isolation for over 2 weeks results in a shift in recognition cues that was large enough so that these ants were treated as non-nestmates when returned to their mother nest.

Correspondingly, there was marked shift of the isolated worker's cuticular hydrocarbon profiles as compared to non-isolated nestmates (Boulay et al. 2004; Boulay and Lenoir 2001). If isolated workers are introduced before the time required for rejection, their reintegration into their mother colony initiates high levels of trophallaxis (Boulay et al. 2000; Boulay et al. 2004). Trophallaxis is the major route by which colony members share their recognition cues to create a uniform colony odor (Lenoir et al. 2001). Furthermore, there is evidence that for this species nest volatiles also affect nestmate recognition. While 2-week isolated workers are attacked by their nestmates upon return to their mother colony, if these workers are exposed to nest volatiles during their isolation period they are accepted without aggression (Katzav-Gozansky et al. 2008), indicating that these volatile compounds also act as nestmate recognition cues. Finally, although the queen does not contribute to the composition of the recognition cues, her presence is crucial for proper worker recognition, presumably through maintaining worker motivation to being territorial (Boulay et al. 2003; Vander Meer and Alonso 2002). Similar phenomenon was shown also for the fire ant *Solenopsis invicta* (Vander Meer and Alonso 2002). This is presumably mediated by octopamine, since QL workers that are tolerant to alien conspecific ants to the point that the alien colonies fuse (Boulay et al. 2003) also have lower octopamine levels in the brain (Vander Meer et al. 2008).

Here we demonstrate that altering the cuticle composition of workers *C. fellah* with heterospecific cuticular wash in a way that does not disrupt the integrity of the epicuticular lipid layer (Torres et al. 2007) alters the recognition cue composition, such that nestmates are recognized as alien and attacked.

Materials and methods

Ants

Colonies of *C. fellah* and *C. sp*. were established in the laboratory from newly mated queens collected in Tel Aviv (Israel) after nuptial flights, and reared in artificial nests in a controlled temperature room (28 ± 2°C) and a photoperiod of 14:10LD. Ants were provided with an identical diet of sugar water and crickets twice a week. Neither the queens nor workers of these two sympatric species are morphologically distinguishable, but have marked differences in cuticular hydrocarbon composition (see below). For the purpose of this study all colonies were typed using the cuticular hydrocarbon composition and classified as either *C. fellah* or *C. sp*.

Chemical analyses and extract preparations

Chemical analyses of *C. fellah*, *C. sp*., and *C. fellah* treated with *C. sp*. cuticular hydrocarbons (see below) were performed by GC/MS (VGM250Q) using a DB5MS fused silica capillary column that was temperature programmed from 150 to 300°C at 5°C/min. The eluting compounds were identified by their mass fragmentation pattern, and further verified by comparing the retention times to those of synthetic *n*-alkanes. Routine quantifications of cuticular hydrocarbons of untreated and treated workers were performed by GC (Varian 3800, FID, N_2 as carrier gas in splitless mode), using the above column and running conditions.

Cuticular compounds from *C. sp*. used for transference to *C. fellah* workers were obtained by immersing pools of 20 thoraces (including legs) and the corresponding dissected postpharyngeal glands of workers *C. sp*. in 2 ml of pentane for 1 h, after which the thoraces were removed and the extract was kept at −20°C until further use. The amount of hydrocarbons in the extract was quantified by transferring an aliquot into pentane containing 250 ng tetracosane as internal standard. Quantifications of the hydrocarbons present in the extract were done both before and after inoculations of *C. fellah* (see below).

C. fellah workers were inoculated with the *C. sp*. or *C. fellah* extracts as follows. The extracts described above were slowly evaporated to dryness at room temperature while swirling the test tube so that the solutes were slowly deposited on the test tube walls. For inoculation each *C. fellah* worker was anesthetized by chilling on ice and then vortexed in the hydrocarbon-laden test tube for 1 min. Vortex level was adjusted at moderate speed. This procedure did not harm the workers (Torres et al. 2007). Inoculated workers were then either immediately extracted for chemical analysis to assess the amount of *C. sp*. transferred from the test tube wall onto the worker's cuticle, or within 20 min for behavioral assays. Quantifications of hydrocarbons transferred after inoculation were performed on total body washes of the inoculated workers, achieved by immersing whole ants for 10 min in pentane containing the internal standard.

Behavioral assays

Behavioral assays constituted dyadic encounters. The assayed workers were placed in a 3 cm Petri dish that had a partition that separated the two workers. The floor of the dish was covered with filter paper that was replaced after each test. After an acclimation period of 5 min the partition was removed and all interactions between the two workers have registered for an additional 5 min, starting from the first contact between the ants, using a digital event recorder

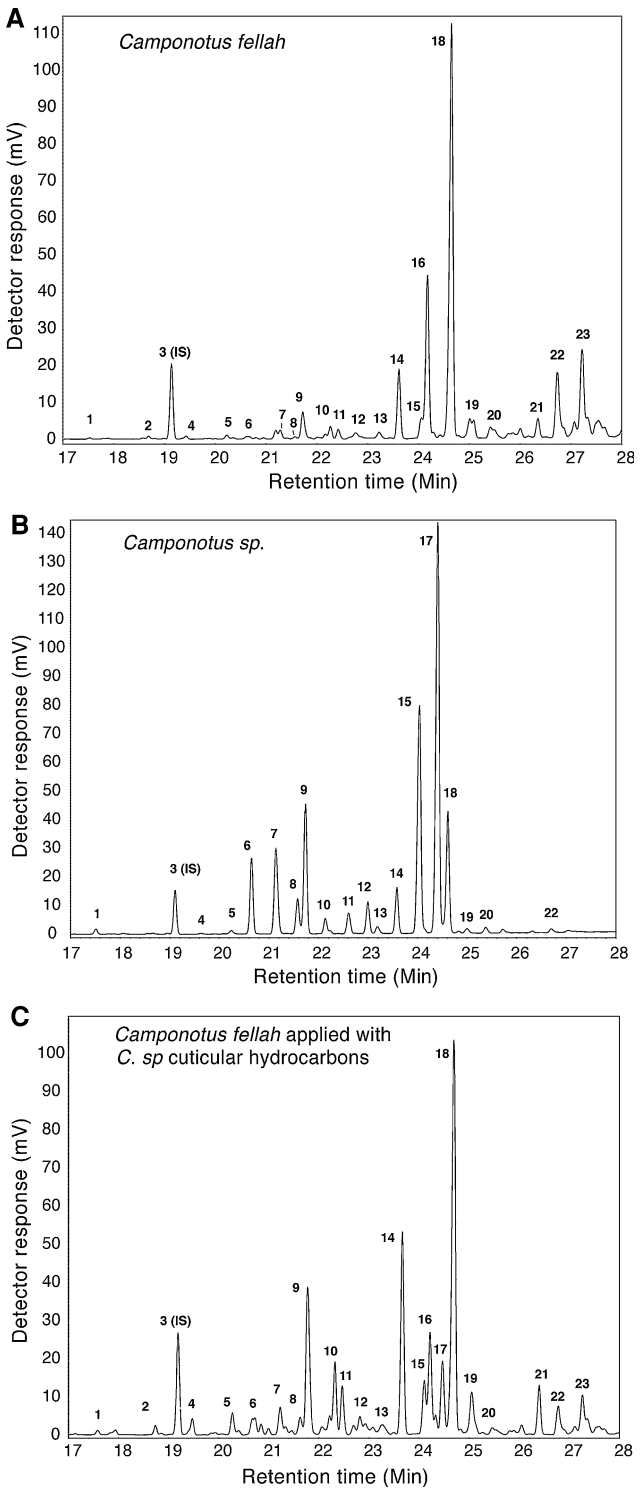

Fig. 1 Gas chromatogram of cuticular washes of *Camponotus fellah* (**a**), *Camponotus sp*. (**b**), and *C. fellah* that were applied with cuticular hydrocarbons of *C. sp*. Peak identity is as follows: *1* tricosane, *2* 3-methyltricosane, *3* tetracosane (internal standard), *4* 12-methyltetracosane, *5* 2-methyltetracosane, *6* pentacosane, *7* 11- +13-methylpentacosane, *8* 9,13-dimethylpentacosane, *9* 3-methylpentacosane, *10* hexacosane, *11* 12- +14-methylhexacosane, *12* 10,12-dimethylhexacosane, *13* 2-methylhexacosane, *14* heptacosane, *15* 11- +13-methylheptacosane, *16* 7-methylheptacosane, *17* 11,15-dimethylheptacosane, *18* 3-methylheptacosane, *19* octacosane, *20* 12-methyloctacosane, *21* nonacosane, *22* 13- +15-methylnonacosane, *23* 3-methylnonacosane. See "Materials and methods" for analyses conditions

(Jwatcher version 1.0—http://www.jwatcher.ucla.edu/). The following behaviors were registered and scored according to a ladder of escalating aggression: antennation (0), mandibular opening (1), biting (2), and abdomen curling while ejecting formic acid (3). The frequency and duration of each behavioral component were registered and the overall aggression exhibited in each encounter was calculated as follows:

$$\sum_{i=1}^{n} \frac{AI_{\mathrm{i}} \cdot t_i}{T}$$

where *AI* is the aggression index, *t* the duration of each act, and *T* the total time of interactions during a session (Lahav et al. 1999).

Statistical analyses

Cuticular hydrocarbon profile comparisons were done by principle component analysis using the hydrocarbons presented in Fig. 1. Comparisons of hydrocarbon quantities between groups (non-treated *C. fellah* and *C. sp*. and *C. fellah* painted with *C. sp*. cuticular hydrocarbons), as well as the results of the dyadic aggression tests were achieved by ANOVA followed by Tukey post hoc test. All data are presented as means ± SE.

Results

Comparative chemical analysis of cuticular washes of *C. fellah* and *C. sp*. revealed significant differences between these two species (Fig. 1). Notably *C. sp*. possesses large amounts of 11,15-dimethylheptacosane (peak 17; the major compound) that was present only in trace amounts in *C. fellah*. Likewise, 9,13-dimethylpentacosane (peak 8) was present, albeit in moderate amounts, in *C. sp*., but was almost completely absent in *C. fellah*. On the other hand, 7-methylheptacosane (peak 16) is a prominent component in *C. fellah* but was present at most in trace amounts in *C. sp*. There were also differences in the relative abundance of common components, e.g. 3-methylpentacosane (peak 9) was at a much higher concentration in *C. sp*. than in *C. fellah*, and vice versa for 3-methyl heptacosane (peak 18; the major component in *C. fellah*). In neither *C. fellah* nor *C. sp*., nor *C. fellah* inoculated with *C. sp*. cuticular washes could we detect components other than hydrocarbons. The trace compounds showing in the respective chromatograms were identified as hydrocarbons by their mass fragmentation, but correct structure assignment was impossible because of their small amounts.

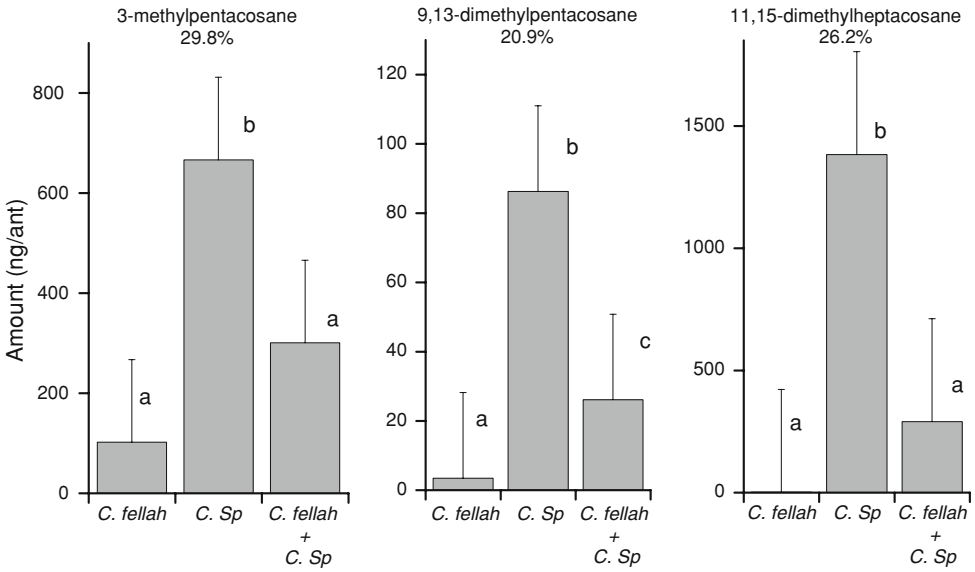

Fig. 2 The amounts per ant of 3-methylpentacosane, 9,13-dimethylpentacosane, and 11,15-dimethylheptacosane extracted from workers *Camponotus fellah* ($n = 10$), *C. sp*. ($n = 10$) and *C. fellah* after 1 min vortex in a test tube laden with *Camponotus sp*. cuticular hydrocarbon extract ($n = 30$). Note that the scale for each compound is different. Data are presented as means ± SE

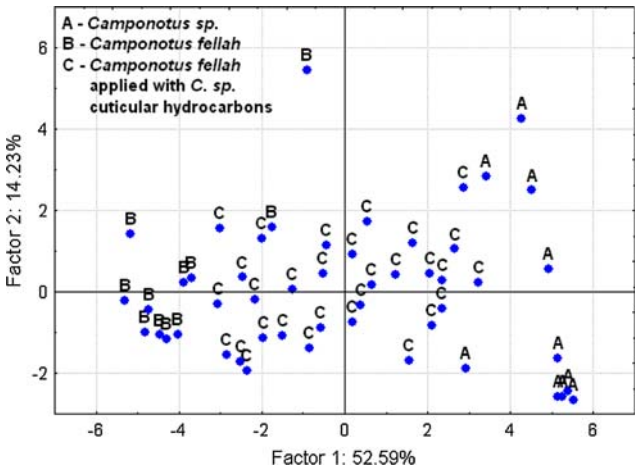

Fig. 3 Principle component analysis based on cuticular hydrocarbon profiles of *Camponotus fellah*, *Camponotus sp*., and *C. fellah* that were applied with cuticular hydrocarbons of *C. sp*., demonstrating a clear separation between the three profiles

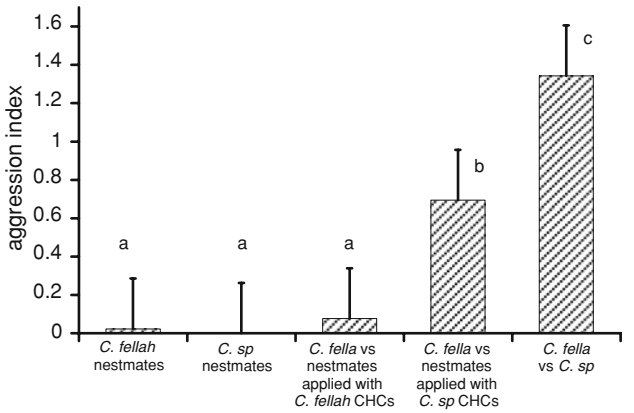

Fig. 4 The aggressive response of workers *Camponotus fellah* in dyadic assays (see "Materials and methods" for details) towards non-treated nestmate *C. fellah* ($n = 26$), or *Camponotus sp*. ($n = 40$), or *C. fellah* that were applied with cuticular hydrocarbons of *C. sp*. ($n = 35$), or *C. fellah* that were applied with cuticular hydrocarbons of *C. fellah* ($n = 32$). Also depicted is the response of *Camponotus sp*. towards its non-treated nestmates ($n = 9$). Data are presented as means ± SE

The transfer of *C. sp*. cuticular hydrocarbons to *C. fellah* workers was successful as seen in Fig. 1. There was a significant effect of the treatment on the amount of each of the components (one way ANOVA, $F_{2,49} = 13.48$ $p < 0.001$; $F_{2,49} = 42.3$ $p < 0.001$; $F_{2,49} = 30.8$ $p < 0.001$, for 3-methylpentacosane, 11,15-dimethylheptacosane, and 9,13-dimethylpentacosane, peaks 9, 17, and 8 respectively). Judging from the occurrence of the three compounds presented in Fig. 2 the degree of transfer was between 20 and 30% of the average amount present in *C. sp*. This changed the profile of the modified ants, which separated them from either species and positioned them between the two species clusters in the PCA (Fig. 3).

The application of heterospecific extracts on workers of *C. fellah* also had a significant effect on the outcome of the behavioral encounters (Fig. 4, one way ANOVA $F_{4,141} = 52.06$ $p < 0.001$). Encounters between non-treated nestmates, whether of *C. fellah* or *C. sp*. were at most antennal contact and not significantly different from each other (Tukey's post hoc test $p = 0.99$). On the other hand, aggression between non-treated workers of *C. fellah* and *C. sp*. were highly aggressive, significantly higher than either of the encounters involving nestmates (Tukey post hoc test $p < 0.001$ for both), often culminating in injury to the ants. Aggression between non-treated and *C. sp*.-inoculated nestmates *C. fellah* was significantly higher compared to that between two non-treated nestmate *C. fellah*, or between non-treated *C. fellah* and *C. fellah*-inoculated nestmates

(treatment control; Tukey post hoc test $p < 0.001$, for both). Aggression between non-treated *C. fellah* and *C. fellah*-inoculated nestmates was as low as that between two non-treated nestmate *C. fellah* (Tukey post hoc test $p = 0.99$). However, these encounters were still significantly less aggressive than those between heterospecific workers (Tukey post hoc test $p = 0.003$). The reverse experiment, i.e., testing aggression of *C. sp*. workers toward nestmates *inoculated* with *C. fellah* cuticular hydrocarbons was not possible because of shortage in *C. sp*. colonies.

Discussion

Cuticular hydrocarbons fulfill all premises of a nestmate recognition signal. Their composition varies qualitatively between species and quantitatively between colonies within a species. They are relatively non-volatile and are spread throughout the cuticular surface in line with the observations that recognition generally requires antennal contact, irrespective of the body-part encountered. While cuticular hydrocarbon specificity, both at the species and colonial levels has been demonstrated for many ant species (Hefetz 2007), direct causative proof of their role in nestmate recognition exists for only a handful of species (Akino et al. 2004; Lahav et al. 1999; Ozaki et al. 2005; Thomas et al. 1999; Torres et al. 2007; Wagner et al. 2000).

Here we examined whether alteration of cuticular hydrocarbons of workers of *C. fellah* by applying a heterospecific cuticular wash results in their alienation by nestmates. Alteration was achieved by solventless coating of such workers with the cuticular hydrocarbons of a closely related, yet unnamed, *Camponotus* species. This undescribed species is sympatric with *C. fellah* (dealated queens of both species were collected at the same site), and although morphologically indistinguishable these species show a consistent difference in the composition of their cuticular hydrocarbons. We used these chemical differences to alter the cuticular hydrocarbon composition of workers *C. fellah*, in order to test whether this affect nestmate recognition. Application of *C. fellah* workers with the heterospecific hydrocarbons in a way that minimized cuticle structural disruption is very important in obtaining meaningful results (Torres et al. 2007). Gas chromatography of the inoculated ants revealed that their natural hydrocarbon composition was not disrupted, but only supplemented with the donor's hydrocarbons. Furthermore, observations of the ants behavior did not reveal any post treatment traumatic symptoms. A Principle component analysis, based on the cuticular hydrocarbon profiles, further showed that the treated ants were distinguishable from both conspecific and heterospecific workers. The behavioral bioassays support the hypothesis that profile alteration renders the treated ants as alien to their nestmates. They were significantly more aggressively attacked than non-treated nestmate, albeit with still lower intensity as compared to the ants' reaction towards non-treated heterospecific ants. Similar assays using workers *C. fellah* applied similarly with cuticular hydrocarbons of conspecific did not elicit aggression, indicating that the treatment indeed did not disrupt the ants except for the addition of the applied hydrocarbons onto the cuticular surface.

It is also interesting that the major cuticular hydrocarbon differences between these two sympatric species are almost exclusively in the branched alkanes. Whether branched alkanes, as well as alkenes have greater information content than linear hydrocarbons (Dani et al. 2005; Dani et al. 2001) is still unclear. Recent studies with *Linepithema humile* and *Aphenogaster cockerelli* have modified our understanding of how profile differences are perceived by the ants (Greene and Gordon 2007). It was shown that among the three structural classes of hydrocarbons generally present in ant epicuticle (alkanes, alkenes and methylalkanes), at least two are needed for eliciting aggression, but the particular composition of the hydrocarbon in each class is less crucial. It is possible that in the present experiment the substantial alteration in the branched alkanes was the prime elicitor of aggression in *C. fellah*, although we can not completely exclude the involvement of compounds other than hydrocarbon in the process. We presume that such changes in cuticular hydrocarbon composition in these closely related species was shaped by the fact that they are sympatric, and at least *C. fellah* and presumably also *C. sp*. are highly polydomus (Hefetz, personal observations). Such chemical character displacement was also reported for sympatric *Cataglyphis* species (Dahbi et al. 1996).

These results support in the part the earlier, indirect evidence of the role of cuticular hydrocarbons as nestmate recognition pheromones in *C. fellah*, that showed an extensive hydrocarbon (and likely other classes compounds) exchange between nestmates mostly by trophallaxis (Lenoir et al. 2001) and that trophallaxis is essential for reintegration of isolated *C. fellah* workers into their colony (Boulay et al. 2000).

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