
Direct Behavioral Evidence for Hydrocarbons as Ant Recognition Discriminators

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Nestmate recognition in social insects involves matching chemical cues on the surface of one individual with the experience-derived neural template of nestmate discriminators of another individual. We report here the first direct experimental evidence that hydrocarbons are responsible for nestmate recognition in the ant Cataglyphis niger. Isolated hydrocarbons, but not other lipids, applied to one of a pair of live nestmates or alien ants significantly modified aggressive behavior in the direction expected if hydrocarbons were nestmate recognition discriminators. The behavior of treated ants did not change, supporting the view that the existing template was not altered by the treatment. Elevation in aggressive behavior by artificially exposing an ant to alien odor was more pronounced than its reduction when the ant was exposed to nestmate odor. We hypothesize that during the recognition process the ants rely more on differences between the template and label than on the similarities between the two.

Ants have a highly developed recognition mechanism enabling them to accept nestmates and reject alien conspecifics. Despite this well-defined behavior, the mechanisms involved in this process are still elusive, and subject to many theories and speculations. Among these is the nature of the chemical signal on the cuticle of the ant (label), by which they reveal their colonial identity (Fletcher and Michener 1987; Vander Meer and Morel 1998). Although two encountering ants may recognize each other from a short distance (1-2 cm), actual physical contact anywhere on the partner's body is generally needed for full expression of the behavior. This indicates the involvement of compounds that are widespread on the cuticle and have low volatility. Hydrocarbons constitute a major group of heritable cuticular lipids that exhibit high species and colony specificity. These compounds have been implicated as recognition cues in various social insects such as polistine wasps, honeybees, termites, and ants (Howard and Blomquist 1982; Lockey 1988). However, the evidence linking hydrocarbons to nestmate recognition is not yet substantial (Breed 1998), being based on either correlation studies or bioassays using removal and replacement of cuticular compounds by solvent extraction. The critical link between hydrocarbons and nestmate recognition was not made. In the present study we use a behavioral bioassay and isolated hydrocarbons to directly assess the role of these compounds as recognition cues.

There is a close link between cuticular chemistry and the chemistry of the postpharyngeal gland (PPG) of ants (Bagneres and Morgan 1991; Do Nas-
cimento et al. 1993; Soroker et al. 1995b). Recent studies have demonstrated that newly synthesized hydrocarbons are exchanged not only between the epicuticle and the postpharyngeal gland of individual ants by self-grooming but also between members of the colony through trophallaxis and allogrooming. Thus the PPG (a "Gestalt organ"; Soroker et al. 1994) is a site at which individual ants can acquire the cuticular chemical profile of their nestmates and admix them with their own to create a uniform colony odor (Lahav et al. 1998, Soroker et al. 1995b; Soroker et al. 1998). Direct application of PPG contents or its pentane extract on the cuticle of ants from different nests modifies the aggressive behavior of the encountering ants, thus supporting the hypothesis that it contains nestmate recognition signals (Soroker et al. 1994; Hefetz et al. 1996). In this study we used PPG content to test the hypothesis that the hydrocarbon constituents, but not the other lipid classes, are responsible for modifying the aggressive behavior of the ants and thus form a part of their nestmate recognition system.

To test this hypothesis we used two distant queenright colonies of Cataclyphis niger (A and B) collected from two localities in the coastal plains of Israel, about 30 km apart. In the laboratory they were kept in separate rooms and received the same diet of dead insects 3 days a week and sugar water ad libitum. Fifty postpharyngeal glands were extracted in chloroform:methanol:aqueous 2 N NaCl (1:2:0.8 v:v) to yield a total lipid extract (Bligh and Dyer 1959). The total lipid extract was separated into hydrocarbon and other nonpolar and polar lipids on a silica gel column (Machery and Nagel, 70–230 mesh), 6.5 cm long, and 0.6 cm wide by stepwise elution with hexane (14 ml), hexane: chloroform (1:1) (4 ml), chloroform (6 ml), and methanol (6 ml). The hexane fraction contained only hydrocarbons as evidenced by thin-layer chromatography and gas chromatography–mass spectroscopy (Mclafferty and Stauffer 1989). All other fractions were pooled, dried under N2, and redissolved in chloroform:methanol (1:2) to give the non-hydrocarbon lipid fraction. Another total lipid extract of PPGs was used to test the effect of the total PPG content. Solvent volumes were adjusted to give a concentration of 0.7–0.8 PPG equivalents per microliter.

Dyadic encounters between nestmates or alien ants were used to determine the ability of the various fractions to change the individual nestmate recognition label. This method simulates natural encounters more closely than previous behavioral assays that tested the reaction of ants to treated dummies or dead ants. To overcome the variation in individual responses of the ants, paired tests were designed in which the same untreated ant was encountered once with a solvent-treated ant (control) and once with an extract-treated ant (test). These were presented to the untreated ant randomly. Treated ants, nestmates or alien, had 1 μl of the appropriate extract or solvent applied to it from head to tip of the abdomen with a micropipet. The tests conducted are outlined in Table 1. Encounters were conducted in a neutral arena (9 cm in diameter lined with a filter paper that was changed after each test) with size matched worker pairs. Before each test the ants were confined to the arena in glass tubes for 2 min for acclimation. The test started when the glass tubes were removed and lasted 2 min. All tests were videotaped. The behavior of one of the ants was registered continuously using a computerized event recorder, whereas the behavior of the second ant was recorded in the same way from video replay. Aggression was scored by the following index: 0, antennation; 1, mandibular threat; 2, short biting with jumping; 3, biting; 4, spraying of formic acid. Scoring of aggressive behavior was calculated using the following formula:

$$\frac{n \sum A_i t_i}{T}$$

where $A_i$ and $t_i$ are the aggression index and duration of each act respectively, and $T$ is the total interaction time. This calculation excludes the times in which the ants were not in actual contact.

Aggression among alien workers in C. niger is usually moderate. Often competitive encounters between rivals (at least under laboratory condi-

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**Table 1. The test groups used for assessing the role of PPG extract or its fractionation product in nestmate recognition in the ant C. niger**

<table>
<thead>
<tr>
<th>Encounter</th>
<th>Treatment of A or B</th>
</tr>
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<tbody>
<tr>
<td>A* vs. A</td>
<td>Control (untreated)</td>
</tr>
<tr>
<td>A* vs. B</td>
<td>Control (untreated)</td>
</tr>
<tr>
<td>A* vs. A</td>
<td>Total ppg extract of B applied on A, chloroform (control)</td>
</tr>
<tr>
<td>A* vs. B</td>
<td>Total ppg extract of A applied on B, chloroform (control)</td>
</tr>
<tr>
<td>A* vs. A</td>
<td>Hc fraction extract of B applied on A, hexane (control)</td>
</tr>
<tr>
<td>A* vs. B</td>
<td>Hc fraction extract of A applied on B, hexane (control)</td>
</tr>
<tr>
<td>A* vs. A</td>
<td>NHL fraction of B applied on A, C.M. (1:2) (control)</td>
</tr>
<tr>
<td>A* vs. B</td>
<td>NHL fraction of A applied on B, C.M. (1:2) (control)</td>
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</tbody>
</table>

Untreated ants from nest A (asterisk) were encountered either with alien ants (nest B) or nestmates that were treated with glandular extracts or their fractions (approximately 0.8–0.7 ant eq/test except for the test with NHL where 1.4 eq/ant were tested, see text for details). In each paired test ant A* was the same individual, tested once with an ant applied with the solvent and once with an ant applied with the treatment solution, in a random manner. Hc, Hydrocarbons fraction derived from PPG secretion; NHL, non-hydrocarbon lipids; C.M, chloroform:methanol (1:2).
tions are settled by ritualized aggression. Nonetheless, aggression towards alien ants was significantly higher than between nestmates (Figs. 1a, d; $P<0.0006$ for both reciprocal observations). Figure 1b, c presents the behavioral response of the untreated ant towards the treated ant, while Fig. 1e, f presents the behavioral response of the treated ant in the corresponding tests. Although overall aggression in tests using PPG extracts was lower than for the untreated cases, aggression towards nestmates applied with alien PPG extracts was significantly higher than towards nestmates applied with solvent alone (Fig. 1b, PPG; $P=0.01$). Although obtained in different tests, comparing the results presented in Fig. 1b vs. 1c reveals that the aggression exhibited toward a nestmate treated with alien PPG extract was as high as that exhibited towards an alien ant treated with solvent. Correspondingly, treating an alien ant with nestmate PPG extract reduced the aggression towards it to lower level than toward nestmates treated with solvent. Modification of aggressive behavior was even more pronounced when the purified hydrocarbon fraction was utilized (Fig. 1b, c, Hc). However, the fraction containing the nonhydrocarbon lipids (NHL), failed to alter the ants' behavior. At first, 0.7 gland eq/test was used, but since there was no demonstrable effect, we doubled the concentration (1.4 gland eq/test) to ensure that the lack of activity is real. Even at that high concentrations the NHL fraction did not alter the ants' behavior (Fig. 1b, c, NHL; data shown for the high concentration only). These results clearly demonstrate that the activity of the PPG extracts in C. niger nestmate recognition is attributable to its hydrocarbon constituents, and by inference that colonial identity is expressed in the cuticular hydrocarbons and not in the other lipid constituents.

The results of tests with the purified hydrocarbon fraction provide insight to the mechanism of perception and behavioral response. It is commonly accepted that such a mechanism in ants involves the comparison of a perceived signal with a neural reference imprint called a template. Application of alien hydrocarbons elicited aggression comparable to that exhibited in the encounters between untreated alien ants (Fig. 1b vs. 1a, Hc). In contrast, application of nestmate hydrocarbons on an alien ant significantly reduced aggression but not to the level displayed when two nestmates meet (Fig. 1c vs. 1a, Hc). It should be noted that because of individual variability the level of aggression exhibited by the ants depended on the respective test, which made statistical comparisons between tests impossible. Worker responses to nestmates treated with alien discriminators (test vs. control in Fig. 1b, Hc; $P<0.01$) appeared to have a greater effect than when alien workers were treated with nestmate discriminators (test vs. control in Fig. 1c $P<0.03$). This implies that the ants are more sensitive to label/template differences than to similarities. These results may also reflect the limits of our experimental protocol, where it is not possible homogeneously to apply 1 µl of treatment or control solution to the test ant. It is easier to create a label difference in nestmates then it is to totally mask the label of nonnestmates. The advantage of assays using live ants lies in simulating a natural situation; however, the behaviors manifested by the encountering ants may not be independent, for example, one ant's reaction could provoke a reaction in the other ant. We tested this possibility using Kendall's correlation analyses, which revealed that the response of each of the two interacting ants is independent of the behavior of their opponent ($r=0.13$). The tendency of specific ants to be aggressive was also examined, revealing a relatively high correlation ($r=0.58$, i.e., at least in 50% of the tests the ants that were either aggressive or amicable in
the first encounter showed the same tendency in the second encounter). Thus ants exhibited different response thresholds. We accounted for individual variability in aggressive behavior by comparing the reaction of the same ant to the appropriate signal (stimulus) and control. The fact that application of hydrocarbons changed the ants' behavior in the direction expected from recognition cues, despite this inherent variability, supports their role as signals for nestmate recognition in this species.

Observations on the behavior of the treated ants indicate that in all but one case they were not significantly affected by the treatment (Fig. 1e,f), as previously reported (Soroker et al. 1994). The ants treated with alien extract did not appear to change their template. Ants treated with alien PPG or fractionated extracts were uniformly amicable towards their original nestmates and aggressive to alien ants, although these carried (naturally) the same odor as the one applied (artificially) on the treated ants. The only case in which aggression of the treated ant toward an alien was significantly lower than expected was when an ant was treated with total PPG extract from nestmates of the encountering ant (Fig. 1f). Our results, however, may be confounded by incomplete coverage with the treatment and/or lack of adequate time for a self-based template change. A similar case has been reported for Lasio1glossum zepbyrum in which individual bees do not sense their own odor but rely on the odor of nestmates for constructing their template (Buckley and Greenberg 1981). The PPG hydrocarbons of C. niger consist of a complex mixture of normal, mono, dimethyl and trimethyl branched alkanes (Soroker et al. 1995a). The individual compounds are not available synthetically, nor can they be effectively isolated from natural sources. Thus, testing individual compounds and selected mixtures has not yet been possible. Correlative evidence for some ant and wasp species suggest that branched hydrocarbons account for most of the between-colony variability found when all hydrocarbons are used in the analyses (ants: Provost et al. 1993; Dahbi et al. 1996, wasps: Bonavita-Courgordan et al. 1991; Espe 1994). Fatty acids have also been implicated as recognition cues in the ant Leptothorax (Francks et al. 1990), while in the honeybee,Apis mellifera,n-alkanes and fatty acids have been found to be more significant than n-alkanes (Breed 1998). While these correlative studies are interesting, we have provided the first direct behavioral evidence that isolated hydrocarbons influence nestmate recognition in C. niger. It also lends credence for the use PPG hydrocarbons as a model system for demonstrating the role of the PPG as a “gestalt organ.” The generality of this phenomenon awaits similar experimentation with other ant species.

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Bagnères AG, Morgan ED (1991) The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. Experientia 47:106-111
Buckley GR, Greenberg L (1981) Nestmate recognition in a sweat bee (Lasio1glossum zephyrum): does an individual recognize its own odor or only odours of its nestmates? Anim Behav 29:802-809