Nest volatiles as modulators of nestmate recognition in the ant
Camponotus fellah

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Abstract

When ants from alien colonies encounter each other the stereotypic reaction is usually one of aggressive behavior. It has been shown that factors such as queen-derived cues or nest-odors modulate this reaction. Here we examined whether nest volatiles affect nestmate recognition by observing the reaction of nestmates towards individual workers under one of four regimes: completely isolated; isolated but receiving a constant airflow from the mother colony; as previous but with the passage of nest volatiles towards the isolated ants blocked by adsorption on a SuperQ column; or reversed airflow direction—from the isolated ants to the nest interior.

Ants that had been completely isolated for three weeks were subjected to aggressive behavior, but not those that had continued to receive airflow from the mother colony. Adsorbing the nest volatiles from the airflow by SuperQ abolished this difference, with these ants now also being subjected to aggression, indicating that nest volatiles can modulate nestmate recognition. Reverse airflow also reduced the level of aggression but to a lesser extent than airflow directed from the mother colony. In queenless colonies the overall aggression was reduced under all regimes, and there was no effect of flow, suggesting that the volatiles involved are queen-borne. The SuperQ adsorbed volatiles originated from Dufour’s gland secretions of both workers and queen, implicating them in the process. Cuticular hydrocarbon profiles were not affected by exposure to nest volatiles, suggesting that the latter either constitute part of the recognition cues or affect worker behavior via a different, as yet elusive mechanism.

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1. Introduction

Nestmate recognition constitutes a key element in insect social organization, with its manifestation and magnitude being structured by nest architecture, colony size, and colony demography. Recognition involves matching of a chemical label, present throughout the body surface, to a neural template (Lacy and Sherman, 1983); a mismatch usually results in aggression and exclusion of the intruders. It is generally accepted that recognition cues are mostly composed of cuticle-associated lipids (of which hydrocarbons (HCs) are the dominant constituents), but external substances may also be adsorbed onto the cuticular surface and thus incorporated into the colony label (reviewed by Vander Meer and Morel, 1998; Lenoir et al., 1999; Hefetz, 2007). The queen also plays a role in nestmate recognition, either by contributing the recognition cues to the workers (Carlin and Hölldobler, 1983, 1986, 1987; Provost et al., 1994) or by affecting worker motivation to keep their colony insulated from con- or heterospecific ants (Vander Meer and Alonso, 2002; Boulay et al., 2003).

Although alien ants may also engage in aggressive attacks away from the nest, nestmate recognition is manifested with the strongest response in the vicinity of the nest (Velasquez et al., 2006). The sense of “home” is probably conveyed through the overall nest atmosphere, including compounds that comprise nest odors, which should be fairly volatile and able to penetrate the whole nest environment. Studies in ants, honeybees, and wasps support the hypothesis that volatile substances are involved in the recognition process (Jaffe and Marcuse, 1983 for...
ants; Mann and Breed, 1997 for honeybees; Steinmetz and Schmolz, 2005 for wasps), but the origin and mode of action of these compounds are not clear. The in-nest environment modulating the ants’ aggressive or amicable responses may include volatiles emanating from nest substrate, food, feces, brood, workers, and the queen.

In previous studies with Camponotus fellah we were able to isolate several olfactory as well as social factors that affect nestmate recognition. By keeping ants in complete isolation we demonstrated that a shift in their pattern of cuticular HCs was accompanied by a concomitant alienation of the isolated ants by their nestmates (Boulay et al., 2000; Boulay and Lenoir, 2001). By enabling limited access to various nest odors, we further demonstrated that both volatile and non-volatile substances affect nestmate recognition. Isolated ants confined within their nest in a single mesh cage were treated as nestmates, whereas nestmates isolated outside of the colony were treated as alien after only two weeks of isolation. The reaction towards ants isolated within their nest in a double-mesh cage was intermediate. After two weeks of isolation they were still treated as nestmates, as opposed to completely isolated ants, but after two months of isolation they were treated as alien ants (Katzav-Gozansky et al., 2004). Differences in chemical profiles were uncorrelated in the case of single or double mesh ants. These findings suggest that keeping the ants within the nest environment affected nestmate recognition in additional ways than merely altering their HC profile thus modulating aggressive behavior. These findings, however, may be confounded by the fact that complete isolation may affect the ants in more ways than just preventing them from conforming to the colony odor.

The aim of this study was to evaluate whether workers can perceive the social environment through volatile compounds present in the nest and whether such perception affects nestmate recognition. We tested this hypothesis by exposing individually isolated ants to their mother-nest odors by directing airflow from the mother nest towards them. To ascertain that nest volatiles were responsible for any changes found in the ants’ behavior, we repeated the experiment but adsorbed all nest volatiles onto a column filled with SuperQ before they could reach the isolated ants. In an additional experiment we reversed the airflow, directing it from the isolated ants to the nest, to assess whether worker-born odors under stressed conditions also affect nestmate recognition. Finally, we performed complementary chemical analyses to verify whether nest volatiles affect these profiles in any way.

2. Materials and methods

2.1. Animals

Colonies of C. fellah were established from mated queens collected in Tel Aviv (Israel) after nuptial flights. In the lab, the colonies were reared under constant temperature of 27 ± 2 °C and supplied with an identical diet of dead insects twice a week and 60% w/v sugar water ad libitum. During the experiment the ants were fed with sugar water only. Twenty-four colonies, each containing at least 500 workers as well as brood, were selected for the experiments (see below for details).

2.2. Experimental set-up

To study the effect of nest odors on isolated ants, individual ants were placed in glass tubes (1.5 cm diameter, 10 cm long), closed at either end with a 50 mesh cloth. The isolated ants were regularly fed by removing one cloth and supplying a drop of sugar water (60%). All the ant-containing tubes were placed in a larger box through which air was passed. The airflow was maintained at approximately 11 ml/min throughout the experiment (tested daily). Fig. 1 depicts the experimental set-up. Three airflow regimes were conducted: direct flow from the mother colony to the isolated ants; direct flow as before but with a SuperQ column (SQ, 80/100 mesh, Alltech Associates, Inc.) inserted between the mother colony and the isolated ants, and reverse flow, i.e. flow directed from the isolated ants to the mother colony. Two types of mother colonies were

![Fig. 1. Scheme of the experimental set-up: workers were isolated from the colony in glass cages exposed to air-flow from the mother nest (direct flow). Isolated ants were kept in cages outside the nest preventing transfer of both contact and volatile cues (isolated ants). Additionally, reversed flow was directed from the isolated ants to the nest (reverse flow). A further experiment blocked the air-flow by using SuperQ column placed between the colony and the isolated workers. Mother colonies were either QR or QL.](image-url)
used: queenright (QR) and queenless (QL), both of which also contained brood. The QL colonies were kept as such for several months prior to the experiment. The boxes containing both the mother colony and the isolated ants were completely sealed; the only air flowing through the system was that of one of the three regimes as described above. A control regime comprised isolated ants kept as described above but in a separate box (isolated ants). Each experiment lasted 21 days after which the isolated ants were confronted with nestmates from their mother colony in group-encounters (see below).

2.3. Group encounter bioassays

Bioassays to measure aggression were performed in a circular arena (9 × 7 cm) containing 50 workers from the mother colony (resident ants) into which a marked ant (a dot of paint on the thorax, Unipaint, Mitsubishi pencil Co., Ltd., Japan) was introduced (introduced ant). The resident workers were placed in the arena for 30–60 min before each test to allow acclimation. During each session, the number of resident ants and their behavior towards the introduced ant was recorded 20 times at 10 s intervals. The frequencies of recorded behavior among QR or QL workers was strongly reduced, but still significantly higher when the mother colony was QL, aggression towards the isolated ants that had not been exposed to such nestmate recognition in QR and QL colonies, respectively. For the QR colonies, the type of regime had a significant effect on the aggression between ants of the various groups (ANOVA followed by LSD post hoc test p = 0.0001, albeit still higher than that expressed towards non-isolated nestmates (p = 0.006). However, absorbing the mother-nest volatiles by placing a SuperQ column between the mother colony and the isolated ants increased the level of aggression, which was significantly higher towards the isolated ants under this latter regime than towards isolated ants that had been freely exposed to volatiles from the mother nest, or to non-isolated ants (ANOVA followed by LSD post hoc test p = 0.02 and p < 0.0001, respectively) and as high as towards completely isolated ants (p = 0.9).

The effect of exposing the mother colony to volatiles emanating from the isolated ants (reverse flow) was more ambiguous. Although aggression of the resident ants towards the isolated ants was found to be reduced to the level of that of the direct flow regime (p = 0.3), it was still not statistically different from that towards the isolated ants that were not subjected to an air flow or the SuperQ blocked flow regime (p = 0.4 and 0.3, respectively). When the mother colony was QL, aggression towards the isolated workers was strongly reduced, but still significantly higher than that between non-isolated nestmates (Fig. 2b, ANOVA, df = 2, F = 7.4, p < 0.003). In these QL colonies, there was no effect of nest volatiles on the level of aggression towards the isolated ants (ANOVA, followed by LSD post hoc test p = 0.85).

3. Results

3.1. The effect of nest volatiles and the queen on nestmate recognition

Figs. 2a and b depict the effect of nest volatiles on nestmate recognition in QR and QL colonies, respectively. The effect of exposing the mother colony to nest volatiles from the mother nest, or to non-isolated ants (ANOVA followed by LSD post hoc test p = 0.0001) was significantly lower compared to that exhibited towards isolated ants that had not been exposed to such volatiles (ANOVA followed by LSD post hoc test p < 0.0001, respectively). Absorbing the mother-nest volatiles by placing a SuperQ column between the mother colony and the isolated ants increased the level of aggression, which was significantly higher towards the isolated ants under this latter regime than towards isolated ants that had been freely exposed to volatiles from the mother nest, or to non-isolated ants (ANOVA followed by LSD post hoc test p = 0.02 and p < 0.0001, respectively) and as high as towards completely isolated ants (p = 0.9).

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3.2. The effect of isolation and nest volatiles on HC profiles

Completely isolated ants experience a shift in cuticular and PPG gland HCs, due to the lack of exchange with nestmates (Boulay et al., 2000). Here we examined whether such shifts were affected by the various regimes using discriminant function analyses of the PPG HCs (since these are congruent with the cuticular HCs but represent greater amounts of material) in two colonies (Fig. 3). As expected, the profiles of volatiles of the two colonies were clearly distinct. In both colonies, the isolated ants clearly separated from the non-isolated ants, whereas there was a complete overlap between the two isolation regimes (with or without flow) for each of the two colonies.

Fig. 2. The effect of nest volatiles on a number of aggressive interactions (mean ± se) exhibited by nestmates toward introduced ants that were reared under various social conditions: (A) queenright, (B) queenless). Different letters denote statistical differences at α = 0.05 according to LSD post hoc test. n = number of replicates (introduced ants).

Fig. 3. Discriminant function analysis of major hydrocarbon peaks (see Materials and methods for the list of peaks) in the PPG of isolated workers with or without direct flow and non-isolated workers from two different colonies. The ellipses represent the range of distribution under 95% confidence.
Thus, the regime (exposure of the ants to their mother-nest volatiles) did not affect the course of HC profile changes of these ants.

3.3. Chemical composition of Dufour’s gland secretions and volatiles adsorbed by SuperQ column

Analysis of worker and queen Dufour’s gland secretion is presented in Fig. 4. Both secretions are mostly composed of a series of alkanes ranging from C\textsubscript{10} to C\textsubscript{25}, of which undecane is the major constituent. There are, however, some differences in the minor constituents, especially the non-HCs. Chemical analysis of the nest volatiles adsorbed on the SuperQ column revealed a similar series of saturated alkanes also dominated by undecane. This suggests that the alkanes adsorbed on SuperQ originated from the Dufour’s gland. In addition, the eluents from SuperQ contained small amounts of dimethylbenzaldehyde, dimethylaceto-phenone, and short chain alkenes, all of which may be contaminants from the adsorbents.

4. Discussion

Cuticular HCs are considered the primary mediators of nestmate recognition in social insects. Every colony possesses a unique blend that is shared by all nest members (for review, see Singer, 1998; Vander Meer and Morel, 1998; Lenoir et al., 1999). The recognition label, however, is not rigidly determined but shows flexibility and is affected by environmental factors such as diet (Liang and Silverman, 2000; Richard et al., 2004), nest substrate (Heinze et al., 1996) and the demographic composition of the colony (Hefetz et al., 1992). Moreover, the response to these cues is not always stereotypic: a mismatch causes mutual aggression while a match induces amicable reaction, but the response can also be context-dependent (Errard et al., 2006; Vander Meer and Alonso, 2002b; Boulay et al., 2003; Katzerke et al., 2006). In a previous study, we have shown that in the carpenter ant C. fallah, ants that were isolated outside of their mother nest for more than three weeks and then reintroduced into it were attacked by their nestmates due to changes having taken place in their cuticular profiles. However, if the ants were individually isolated within their nest no such aggression occurred (Katzav-Gozansky et al., 2004). In the present study we further demonstrate the effect of nest volatiles on nestmate recognition in this species.

The results clearly show an effect of nest volatiles on the ants’ nestmate recognition behavior. As expected (Boulay et al., 2000), isolated ants that were encountered with their non-isolated nestmates were subjected to aggression. However, when exposed to a constant flow of mother-nest volatiles during their isolation period they were significantly less aggressed by their nestmates, albeit not to the level of encounters between non-isolated nestmates. This clearly demonstrates that exposure of the isolated ants to nest volatiles mitigates aggression towards the isolated ants. This exchange of volatiles seems to be bi-directional, since exposing the resident ants in the mother nest to flow from the isolated ants also reduced the level of aggression directed towards the isolated ants. However, this latter effect was more variable, and consequently, aggression was also not significantly different from that towards isolated ants that did not receive any flow regime. Our ability to abolish the effect of such volatiles by adsorbing them on SuperQ before reaching the isolated ants supports our hypothesis that nest volatiles are involved. Although several studies have demonstrated that volatiles other than cuticular HCs may play a role in nestmate recognition, this is the first demonstration in ants that nest volatiles modify nestmate recognition. A similar effect of nest odors on nestmate recognition was shown in the paper wasp Vespula vulgaris, where exposing wasps to volatiles emanating from an alien nest made them more acceptable to the resident wasps of that nest (Steinmetz and Schmolz, 2005). In the case of C. fallah the effect is most probably derived from odors that are associated with the colony rather than nest substrate, because all the experimental colonies were kept in identical standard nests and received a uniform diet.

The origin and nature of such odors remain unclear. Analysis of the eluents from the SuperQ column clearly showed that volatiles from Dufour’s glands of both queens and workers are released into the nest environment. The experiments with the QL colonies shed more light on the effect of nest odors. As expected, QL colonies were less aggressive than QR colonies, but nonetheless still showed aggression towards the isolated compared to non-isolated nestmates. The interesting finding in these experiments was that the direct flow from the QL colony to the isolated ants did not reduce the level of aggression towards the latter. This indicates that the queen’s volatiles may play an important role in inducing aggression towards seemingly alien ants. However, these compounds may have been released in small quantities, and although adsorbed by the SuperQ, we were not able to recover them by eluting the adsorbed compounds. It is unclear whether the queen-borne odors affect nestmate recognition directly through the recognition cues or by indicating to the workers that a queen is present in the colony.
In many formicine species, Dufour’s gland is composed of mostly short chain HCs and is species specific (Blum and Hermann, 1978). Moreover, undecane and tridecane were found in several studied Camponotus species (Hefetz and Orion, 1982). Generally, the function of Dufour’s gland secretion in formicine ants is to act as a wetting agent for formic acid, the poison gland constituent, as well as to act as an alarm pheromone (review in Blum and Hermann, 1978). It was further demonstrated in several Camponotus species that the alarm responses to the gland constituents vary with the species and correlate to the ants’ foraging behavior (Hefetz and Orion, 1982). In Formica lugubris, Dufour’s gland secretion was shown to possess queen-specific HCs, of which undecane was found to be the sex pheromone (Walter et al., 1993).

The mechanism underlying nest odor effect on nestmate recognition remains unknown. The volatiles do not appear to affect the presumed label because we could not find differences in the composition of PPG HCs between isolated ants exposed to nest odors and those that were not. We presume that nest odors, in particular those originating from the queen, affect the motivational state of the workers. It is also possible that when the resident ants encountered the isolated ants the latter emitted their own volatiles from their Dufour’s gland and perhaps also from additional glands. This may have given the resident ants a sense of familiarity and thereby reduced their aggression.

It was previously suggested that ants use primarily chemical cues for discrimination between nestmates and alien ants and that the response is mostly stereotypic. Here we have further demonstrated that the level of aggression shown towards alien ants is not only driven by differential recognition cues but is also context-dependent and is affected by the colony requirements. Moreover, we suggest here a new function of Dufour’s gland secretion in *Camponotus fellah*, as a component in nestmate recognition.

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