

Nestmate recognition in ants is possible without tactile interaction

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Abstract Ants of the genus *Camponotus* are able to discriminate recognition cues of colony members (nestmates) from recognition cues of workers of a different colony (non-nestmates) from a distance of 1 cm. Free moving, individual *Camponotus floridanus* workers encountered differently treated dummies on a T-bar and their behavior was recorded. Aggressive behavior was scored as mandibular threat towards dummies. Dummies were treated with hexane extracts of postpharyngeal glands (PPGs) from nestmates or non-nestmates which contain long-chain hydrocarbons in ratios comparable to what is found on the cuticle. The cuticular hydrocarbon profile bears cues which are essential for nestmate recognition. Although workers were prevented from antennating the dummies, they showed significantly less aggressive behavior towards dummies treated with nestmate PPG extracts than towards dummies treated with non-nestmate PPG extracts. In an additional experiment, we show that *cis*-9-tricosene, an alkene naturally not found in *C. floridanus*' cuticular profile, is behaviorally active and can interfere with nestmate recognition when presented together with a nestmate PPG extract. Our study demonstrates for the first time that the complex multi-component recognition cues can be perceived and discriminated by ants at close range. We conclude that contact chemosensilla are not crucial for nestmate recognition since tactile interaction is not necessary.

Keywords Communication · Social insects ·
Cuticular hydrocarbons · Close range detection ·
Camponotus floridanus · Ants

Introduction

Ants recognize colony members (nestmates) or members of other colonies (non-nestmates) by means of chemical cues (Hölldobler and Wilson 1990). Nestmate recognition is important for colony fitness, since it allows workers to restrict altruistic behavior to nestmates and to avoid or attack rivals. The major nestmate recognition cues in ants, honeybees, and wasps have been found in lipids of the insect cuticle (Howard and Blomquist 2005). These lipids originally served as protection against desiccation (Lockey 1988; Buckner 1993). In ants, the lipids essential for nestmate recognition are long-chain hydrocarbons (Singer 1998; Lahav et al. 1999; Thomas et al. 1999; Wagner et al. 2000; Akino et al. 2004; Howard and Blomquist 2005). Across colonies of the same species the cuticular hydrocarbon profile consists of the same components but varies in its ratios (Howard 1993; Vander Meer and Morel 1998), and thus is colony specific (colony profile).

The carpenter ant *Camponotus floridanus* has become a model system to study the nestmate recognition system of ants. *C. floridanus* is an evolutionary-derived eusocial species and its distinct nestmate recognition behavior was described in great detail already in the 1980s (Carlin and Hölldobler 1986, 1987; Carlin et al. 1987). Recently, the cuticular hydrocarbon profiles of *C. floridanus* workers, queens, and eggs were analyzed in detail: they consist of different chemical classes (methyl-branched alkanes, *n*-alkanes, and *n*-alkenes), and the hydrocarbons with chain-

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length ranging from C29 to C34 are important for nestmate recognition (Endler et al. 2004, 2006). As *C. floridanus* colonies are both monogynous and monoandrous (Gadau et al. 1996), workers show a great amount of genetic homogeneity, and heritable components of the colony profile are probably important for nestmate recognition in this species (Carlin and Hölldobler 1986). In addition, the cuticular hydrocarbon profiles of individuals within a colony are not stable, but are influenced by environmental factors and vary with age, reproductive status, and/or caste membership of their bearers. Thus, the colony profile changes over time in the course of weeks and months (Morel et al. 1988; Wagner et al. 1998; Nielsen et al. 1999; Heinze et al. 2002; Dietemann et al. 2003; D'Ettorre et al. 2004; Endler et al. 2004; Buczkowski et al. 2005; Katzerke et al. 2006). The cuticular colony profile shows a strong concordance with the content of the postpharyngeal glands (PPG; Bagnères and Morgan 1991; Soroker et al. 1995; Akino et al. 2004), and it is thought that PPG content is exchanged between colony members through tactile interaction and trophallaxis (Lahav et al. 1999; Boulay et al. 2004). Hydrocarbon exchange via the PPGs might further reduce slight hydrocarbon profile variability between individuals and provides a possibility of integrating environmental influences into the colony profile (Hefetz 2007).

Long-chain hydrocarbons have low volatility. Therefore, it has been assumed that contact chemoreception by tactile interaction is a precondition for detection and discrimination of nestmate recognition cues (Wilson 1971; Howard 1993; Meskali et al. 1995). It is not known, whether ants can receive nestmate recognition cues as odors. Due to the enormous sensitivity of the olfactory system in ants, several authors have speculated that nestmate recognition at close range might be possible (Jutsum 1979; Hölldobler and Michner 1980; Vander Meer and Morel 1998; Lahav et al. 1999; Akino et al. 2004).

In this study we investigated whether workers of *C. floridanus* can discriminate nestmate recognition cues from non-nestmate recognition cues without tactile interaction. We treated dummies with PPG extracts of nestmates and non-nestmates and observed the behavior of single workers without allowing physical contact. To investigate whether long-chain hydrocarbons in general can be received, we studied whether a long-chain alkene (C23) is volatile enough to be detected by the ants at close range and can interfere with nestmate recognition cues.

Materials and methods

Animals The very common carpenter ant *C. floridanus* reaches colony sizes of over 10,000 individuals. Colonies

have only one single-mated queen (Gadau et al. 1996). Queens of *C. floridanus* were collected in Florida (USA) at Orchid Island and the Florida Keys after the mating flight in August 2001 and July 2003, respectively. For our experiments, one colony with a founding queen from Orchid Islands (collected by A. Endler and C. Strehl; colony C90) and one colony with the founding queen from Florida Keys (collected by A. Endler and S. Diederling; colony C316) were kept in the laboratory in artificial plaster nests at a constant temperature of 25°C and 50% humidity (12 h/12 h photoperiod). Both colonies were provided with the same artificial diet (Bhatkar and Whitcomb 1970), honey–water, and dead cockroaches (*Nauphoeta cinerea*) twice a week and water ad libitum. At the time of the experiments, colony size of colony C316 was approximately 3,000 workers, colony C90 constituted about 8,000 workers. Both colonies were classified as mature colonies in a similar colony life cycle (reproductive stage), and thus no difference in the nestmate recognition system due to size disparity is expected (Pricer 1908; Hölldobler and Wilson 1990).

Small workers (head width <3 mm) from both colonies were used for the preparation of PPGs, while behavioral experiments were conducted only with workers of colony C316. Thus, nestmate PPG extracts were obtained from workers of colony C316, while workers of colony C90 were used for non-nestmate PPG extracts. We selected these two colonies after pilot experiments with freely interacting workers, assuring that members of both colonies can discriminate nestmates from non-nestmates. Due to our selection of two colonies and our behavioral studies on workers of a single colony, we can investigate whether nestmate recognition based on odor cues in principle is possible. Our experiments were not designed to investigate to what extent odor cues can be used for nestmate recognition across colonies within the population of *C. floridanus*.

PPG extraction The colony profiles were obtained from PPG extracts of nestmate and non-nestmate workers. For this purpose, a small worker (head width <3 mm) was immobilized with carbon dioxide (Tyczka Industrie-Gase GmbH, Mannheim, Germany). The gaster was removed and the thorax was pinned upside down onto a silicone elastomer (Sylgard 182, Dow Corning, MI, USA) in a Petri dish. The head was covered with distilled water, the maxillo-labial apparatus was removed and the PPGs were taken out by pulling out the pharynx. In order to compensate for differences in content quantity between glands, three PPGs were collected in 500 µl of distilled hexane and the glandular content was extracted for at least 2 h. Prior to experiments, hexane was evaporated under a constant stream of pure N₂ (Tyczka Industrie-Gase GmbH,

Mannheim, Germany) to a volume of 75 μl . Using a hexane-rinsed Hamilton syringe (Hamilton Company, Bonaduz, Switzerland), 25 μl of this extract (one PPG equivalent) was applied onto each dummy, and the hexane was allowed to evaporate at room temperature for at least 2 min before experiments were conducted. Using PPG extracts instead of commonly used cuticular surface extracts is advantageous for our approach since the latter extracts often contain short-chain exocrine gland products, regurgatory and/or excretory products in addition to the long-chain cuticular lipids (Fig. 1; Vander Meer and Morel 1998).

As we used whole worker PPG extracts we did not correct for possible fertility signals contained in the PPG extracts of the two different colonies. However, no fertility



Fig. 2 Picture taken from a high speed video of a *Camponotus floridanus* worker orienting aggressively towards a dummy treated with non nestmate PPG extract. Workers were allowed to individually climb a T bar located close to the nesting area. A dummy (magnetic stir bar) was presented at a distance of 1.5 cm from the T bar so that the workers could not reach the dummy with their antennae. Dummies were treated with different extracts and the workers' behavior towards the dummy was observed. Mandibular threat towards the dummies was defined as aggressive behavior

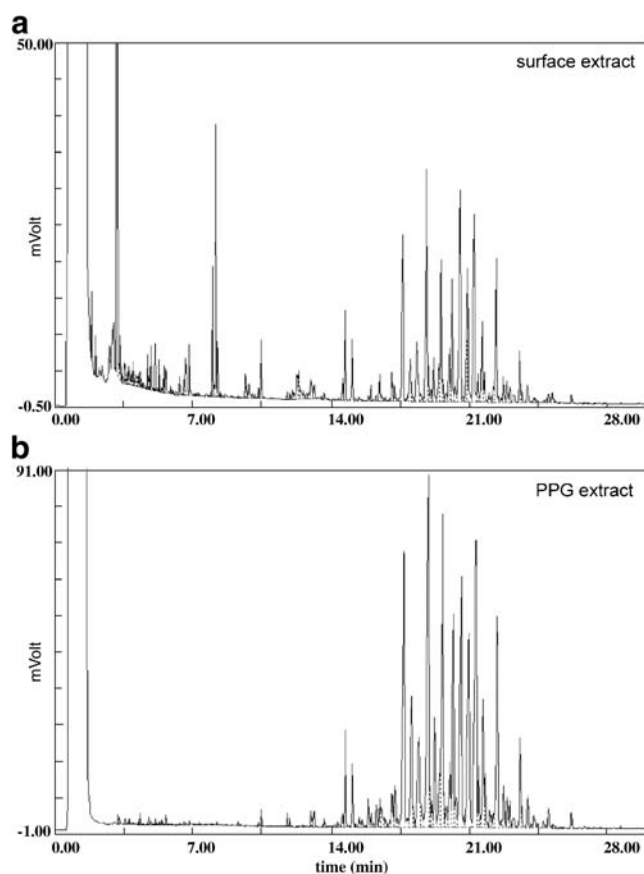


Fig. 1 Two examples showing chromatograms of a surface extract (a) and a PPG extract (b). a Five workers of colony C316 were anesthetized with CO_2 and the cuticular hydrocarbons extracted in distilled hexane for 15 min. b Five PPGs of workers of colony C316 were dissected and the hydrocarbons extracted in distilled hexane for at least 2 h. In both chromatograms, an initial solvent (hexane) peak is followed by the distinct colony profile beginning after approximately 14 min and ending at about 25 min (for comparison and procedure see: Endler et al. 2004, 2006). In the surface extract (a) additional peaks are visible between the hexane peak and the colony profile, representing hydrocarbons of shorter chain length with probably higher volatility than the hydrocarbons constituting the colony profile

signal comparable to the known fertility signal of queens (Endler et al. 2004, 2006) could be detected in our worker PPG extracts (Fig. 1b).

Behavioral experiments Differently treated dummies (magnetic stir bars; 14×4 mm) were presented to individual workers in two separate experiments with identical experimental design. An aluminum rod with a bore on one end containing a small piece of steel mounted on a micromanipulator was used as a holder for a single dummy. The dummy was positioned at a distance of 1.5 cm inclined above the end of a T-bar positioned close to the nest area (and kept there also between experiments). The inter-space between T-bar and dummy was wide enough so that the workers could not reach the dummy with their antennae, and the closest distance between the head of a worker and the dummy was about 1 cm (Fig. 2). Workers were allowed to individually climb the T-bar. During the first 30 s, the workers were allowed to calm down and orientate themselves on the T-bar. The animals' most aggressive behavior towards the dummy was recorded during the following 150 s according to a behavioral index shown in Table 1 (modified from Carlin and Hölldobler 1986). Experiments were performed under red light conditions to reduce visual cues, while a plastic tube was used by the observer for exhaling to prevent air current disturbance. In order to avoid repeated measurements of individuals, workers were branched off onto a bridge after testing and removed from the colony.

In experiment 1, the behavioral response of workers to one of four differently treated dummies was recorded. Control dummies were treated with distilled hexane (control dummies) and three test dummies were treated each with an extract equivalent to one PPG dissolved in distilled hexane: (a) nestmate (NM dummies), (b) non-nestmate (n-NM dummies), and (c) nestmate plus 1 μg *cis*-

Table 1 Six category behavioral index

Category	Description
0	No antennal scanning
1	Antennal scanning not directed towards dummy
2	Antennal scanning directed towards dummy
3	Weak mandibular threat (mandibles slightly opened)
4	Strong mandibular threat (mandibles widely opened)
5	Body jerking (repeated, rapid forward and back jerking with open mandibles)

9-tricosene (NM + C9T dummies). The mixture of nestmate extract plus *cis*-9-tricosene was achieved by adding 3 μ g *cis*-9-tricosene to three PPGs in hexane prior to evaporation and application to the dummies.

In experiment 2, the behavioral response to dummies treated with nestmate PPG extract plus 1 μ g *cis*-9-tricosene (NM + C9T dummies) was compared to the response towards dummies treated solely with 1 μ g *cis*-9-tricosene (C9T dummies).

Allocation of different PPG extracts was balanced in the majority of all conducted experiments and dummies were used only once. The observer was blind to the dummy treatment in all experiments. For statistical analysis, the recorded behavior was classified as non-aggressive behavior (levels 0–2) or aggressive behavior (levels 3 and more) towards the dummy. In order to test for differences in the number of workers showing aggressive behavior towards the differently treated dummies, a χ^2 test was performed for each of both experiments. In order to compare the behavioral response to NM dummies with that to all other dummies in experiment 1, a one-tailed Fisher's exact test was used to evaluate whether no tactile interaction is needed for nestmate recognition (a priori, one-sided hypothesis). Significance levels in this case were corrected for multiple tests ($n=3$; NM vs. n-NM dummies, NM vs. NM + C9T dummies, NM vs. control dummies) via sequential Bonferroni procedure (Holm 1979; Rice 1989), setting α_1 to 0.017, α_2 to 0.025, and α_3 to 0.05. For single tests, significance level was set to 0.05. Statistical analysis was performed with Statistica 7.1 (StatSoft, Tulsa, OK, USA).

Results

After climbing onto the T-bar, the workers started to run from one end to the other (described as level 0 behavior), usually accompanied by intensive antennal scanning behavior (level 1) and rarely by opening of the mandibles anywhere on the T-bar. While being quite agitated during the first 30 s of the experiment, the workers usually continued running up and down the T-bar and scanning with their antennae more calmly during the rest of the

experiment. In many cases, workers briefly stayed at the end of the T-bar where the dummy was presented, trying to reach the dummy with their antennae (level 2). In these cases, some workers opened their mandibles (levels 3 and 4), which was counted as threat towards the dummy. Some workers also opened their mandibles during antennal scanning near the dummy, occasionally jumping towards the dummy with open mandibles from up to 1 cm near the end of the T-bar (levels 3 and 4). In rare cases, workers showed body jerking behavior near the dummy (level 5). This fine scale behavioral index was established to best possibly describe the workers' behavior. For statistical analysis, however, it appeared most appropriate to split the behavioral index into aggressive and non-aggressive behavior. Mandibular threat towards and near the dummy (levels 3 and 4) and body jerking near the dummy (level 5) were considered as aggressive behavior towards the dummy, while other behavior was classified as non-aggressive behavior.

Experiment 1

More workers showed aggressive behavior towards n-NM dummies (24 of 52) and NM + C9T dummies (30 of 60) than towards NM dummies (15 of 58) and control dummies (14 of 60). A χ^2 test revealed significant differences in the number of workers showing aggressive behavior towards the differently treated dummies (Fig. 3; $\chi^2=14.2$; $df=3$; $p=0.003$). A one-tailed Fisher's exact test showed signifi-

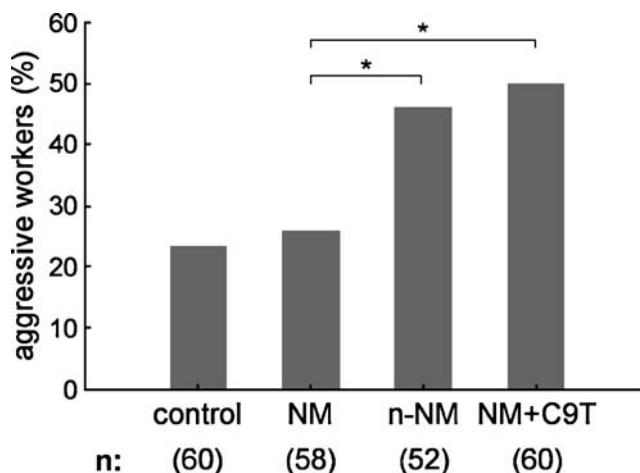


Fig. 3 Number of workers showing aggressive behavior towards dummies treated with different PPG extracts or the solvent hexane (experiment 1). Dummies treated with nestmate PPG extracts (NM) elicited aggressive behavior significantly less often than dummies treated with non nestmate PPG extract (n NM) or dummies treated with nestmate PPG extract plus *cis* 9 tricosene (NM + C9T). No significant difference was found in the number of aggressive workers encountering dummies treated with distilled hexane (control) and nestmate PPG extract. Significance levels were corrected for multiple tests via sequential Bonferroni procedure and an *asterisk* denotes a significant difference. *n* Total number of ants tested

cant differences in the number of aggressive workers towards NM dummies vs. NM + C9T dummies ($\chi^2=7.28$; $df=1$; $p=0.006$) and towards NM dummies vs. n-NM dummies ($\chi^2=4.93$; $df=1$; $p=0.022$). No significant difference in the number of aggressive workers was found between control and NM dummies ($\chi^2=0.1$; $df=1$; $p=0.458$).

Experiment 2

The larger number of aggressive workers encountering NM + C9T dummies in experiment 1 could have been due to two different reasons: (a) *cis-9*-tricosene may interfere with the assessment of nestmate recognition when added to nestmate PPG extract, rendering it non-nestmate; (b) *cis-9*-tricosene alone may elicit aggressive behavior. However, both possibilities do not exclude each other. In order to clarify whether *cis-9*-tricosene alone is a behaviorally active component, we tested workers' responses towards C9T dummies and NM + C9T dummies. Aggressive behavior was elicited even more often by C9T dummies (44 of 50) than by NM + C9T dummies (34 of 49). A χ^2 test revealed a significant difference in the number of aggressive workers found in the two groups (Fig. 4; $\chi^2=5.13$; $df=1$; $p=0.024$).

Discussion

Our study provides experimental evidence that ants can discriminate multi-component nestmate recognition cues without tactile interaction from a distance of 1 cm.

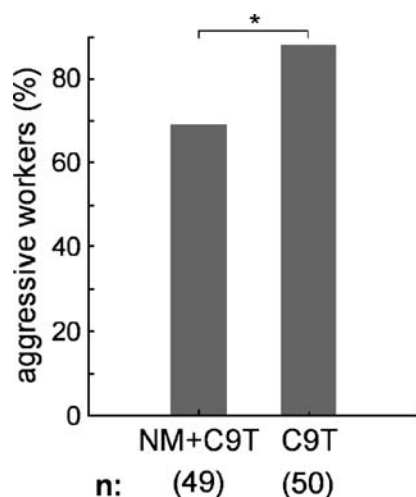


Fig. 4 Number of workers showing aggressive behavior towards dummies treated with nestmate PPG extract plus *cis-9* tricosene and *cis-9* tricosene alone (experiment 2). Dummies treated with nestmate PPG extract plus *cis-9* tricosene (NM + C9T) elicited aggressive behavior significantly less often than dummies treated solely with *cis-9* tricosene (C9T). An asterisk denotes a significant difference. *n* Total number of ants tested

Nestmate PPG extracts elicited aggressive behavior in significantly less workers than non-nestmate PPG extracts. Therefore, we conclude that contact chemosensilla are not necessary for nestmate recognition. Airborne cues received via olfactory sensilla are sufficient. Our behavioral observations suggest that workers may even recognize nestmates and non-nestmates from larger distances than 1 cm.

The PPG extracts of workers from two different colonies contained long-chain hydrocarbons in a pattern typical for *C. floridanus* colony profiles, ranging from C29 to C34 (see: Endler et al. 2004, 2006), and hydrocarbons of shorter chain-length in far less quantity than cuticular surface extracts (Fig. 1). Our results indicate that at close range the same long-chain hydrocarbons are used for discrimination as during contact. However, the airborne components may differ in their ratios compared to the ratios found on the cuticle, and possibly nestmate recognition based on contact chemosensation is more reliable than nestmate recognition based on odor cues. Our finding demonstrates once again the powerful olfactory recognition system of ants, and neuroanatomical data suggest adaptations of the olfactory system to social life (Kleineidam and Rössler 2008).

The hypothesis that long-chain cuticular hydrocarbons can be detected at close range has been supported recently by several investigations. Alkanes present on the cuticle of honeybees were also found in headspace up to a chain length of C29 (Schmitt et al. 2007), prompting new considerations about long-chain hydrocarbon volatility and supporting earlier studies on the importance of airborne cues in the nestmate recognition system of honeybees (Mann and Breed 1997). Several studies indicate that long-chain cuticular hydrocarbons mediate discrimination between reproductive and non-reproductive ants (Liebig et al. 2000; Dietemann et al. 2003). Ants of the genus *Pachycondyla inversa* use a C27 hydrocarbon to communicate fertility, and this fertility signal can be detected with olfactory sensilla (D'Etorre et al. 2004). Similarly, gamergates (mated and reproductive workers) of *Streblognathus peetersi* are able to detect other gamergates from a distance of up to 0.35 cm on the first and up to 2.67 cm on the second encounter, and long-chain hydrocarbons with low volatility are supposed to act as a fertility signal in this case (Cuivillier-Hot et al. 2005).

We used a C23 alkene (*cis-9*-tricosene) to show that long-chain hydrocarbons can be detected by workers at close range and it interferes with nestmate recognition when added to nestmate PPG extracts (NM + C9T dummies). In experiment 1, about the same number of workers responded aggressively towards NM + C9T dummies and n-NM dummies. However, in experiment 2 more workers responded aggressively towards NM + C9T dummies than in experiment 1. Thus, the colony (its workers' response) changed over time between the two experiments, which

were conducted consecutively at different times of the year. This shows that the workers' response depends on the nature of the presented stimuli and also is influenced by changing conditions within the colony. Our finding illustrates the importance of a balanced and parallel presentation (on the same day) of differently treated dummy types, as we did in both experiments, in order to compensate for varying colony conditions over time.

In *C. vagus*, *cis*-9-tricosene (applied onto the cuticle of a worker) elicits aggressive behavior when workers freely interact (Meskali et al. 1995). We found that *cis*-9-tricosene alone is behaviorally active since even more workers showed aggressive behavior towards C9T dummies than towards NM + C9T dummies. In the natural environment, *cis*-9-tricosene may act as a cue to detect the presence of other insects, e.g., heterospecific ants (army ants; Bagnères et al. 1991). The strong effect of *cis*-9-tricosene on the behavior of workers seems to superpose the response to nestmate recognition cues. Interestingly, fewer workers responded aggressively towards the mixture of nestmate PPG extract and *cis*-9-tricosene. Apparently, some workers perceived the nestmate recognition cues and therefore did not respond to the *cis*-9-tricosene.

Discrimination between nestmates and non-nestmates is achieved by template matching, where a detected label (the nestmate recognition cues) is compared to an intrinsic template (Vander Meer and Morel 1998). It is still not clear where in the nervous system this matching process takes place. Recently, Ozaki et al. (2005) suggested that template matching may take place at the sensory periphery (receptor neurons), and the authors described a contact chemosensillum with receptor neurons responding only to non-nestmate colony profile but not to the nestmate colony profile. Thus, sensory adaptation to the nestmate colony profile might be sufficient for non-nestmate recognition. Although the simplicity of the idea is tempting, it fails to explain how workers can discriminate between foragers, scouts, nest-maintenance workers, and nurses, which show subtle differences in their cuticular hydrocarbon profile (Wagner et al. 1998, 2001; Kaib et al. 2000; Greene and Gordon 2003; Howard and Blomquist 2005). In addition, as the colony profile is highly variable, changing over time in the course of weeks and months, template acquisition and reformation might include higher forms of learning comparable to associative learning and probably is localized in the central nervous system (Leonhardt et al. 2007). In order to understand the complex processes underlying nestmate recognition, it is important to access its neuronal basis. Our finding that nestmate recognition cues can be received by olfactory sensilla and that ants can discriminate nestmate recognition cues at close range opens new approaches in researching the recognition process. We now can adopt

techniques already widely used in olfaction research (e.g., calcium imaging, intra- and extra-cellular recording) in order to investigate how and where nestmate recognition cues are processed in the ants' brain.

What is the biological significance of our findings for the social organization of ant colonies? Nestmate recognition with odor cues considerably expands the range of information propagation in ant colonies. It enables ants to assess the presence and colony identity of other ants at close range and may help workers to control the integrity of their nest and foraging trails against the presence of rivals without the need to touch every passing ant individually. In addition, volatile nestmate recognition cues might be used to assess worker density which is a measure probably important for nest building behavior. Bonabeau et al. (1998) report that queens in termites (*Macrotermes bellicosus*) emit a pheromone which serves as a template for the construction of the royal chamber built directly around the queen at a distance of 2 cm. The colony profile could serve a comparable purpose in ants of the species *Leptothorax tuberointerruptus*, where nests are built according to actual colony size (Franks and Deneubourg 1997). Whereas recognition on contact allows only successive assessment of worker density in direct proximity, the overall concentration of volatile nestmate recognition cues in headspace possibly allows an ant to assess the number of workers in close vicinity. Finally, the possibility to recognize non-nestmates without tactile interaction considerably increases an ant's chances of survival when encountering rivals by allowing early behavioral reaction to avoid or prepare for aggressive interaction. Odor-guided nestmate recognition enables workers to further assist the queen in reproduction, and thus increases each individual ant's inclusive fitness.

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