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Ants recognize foes and not friends

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Discriminating among individuals and rejecting non-group members is essential for the evolution and stability of animal societies. Ants are good models for studying recognition mechanisms, because they are typically very efficient in discriminating 'friends' (nest-mates) from 'foes' (non-nest-mates). Recognition in ants involves multicomponent cues encoded in cuticular hydrocarbon profiles. Here, we tested whether workers of the carpenter ant *Camponotus herculeanus* use the presence and/or absence of cuticular hydrocarbons to discriminate between nest-mates and non-nest-mates. We supplemented the cuticular profile with synthetic hydrocarbons mixed to liquid food and then assessed behavioural responses using two different bioassays. Our results show that (i) the presence, but not the absence, of an additional hydrocarbon elicited aggression and that (ii) among the three classes of hydrocarbons tested (unbranched, mono-methylated and dimethylated alkanes; for mono-methylated alkanes, we present a new synthetic pathway), only the dimethylated alkane was effective in eliciting aggression. Our results suggest that carpenter ants use a fundamentally different mechanism for nest-mate recognition than previously thought. They do not specifically recognize nest-mates, but rather recognize and reject non-nest-mates bearing odour cues that are novel to their own colony cuticular hydrocarbon profile. This begs for a reappraisal of the mechanisms underlying recognition systems in social insects.

Keywords: recognition systems; nest-mate recognition; cuticular hydrocarbons; ants

1. INTRODUCTION

The organization of individual organisms into social groups is one of the major transitions in evolution (Maynard Smith & Szathmáry 1997). The stability of many social groups requires that individuals are able to discriminate group members from non-group members in order to direct potentially costly helping behaviour only to the former and to reject the latter that can be expected to steal the society resources. Therefore, one of the conditions favouring the evolution and maintenance of sociality is the ability to discriminate friends and foes (Hamilton 1987). Social animals have evolved multifaceted recognition systems (Starks 2004), the study of which can contribute to an integrated understanding of the ultimate evolutionary forces shaping social life. Social insects, particularly ants, represent one of the pinnacles of social evolution and are tremendously successful (Hölldobler & Wilson 1990; Boomsma & Franks 2006). The ecological dominance of social insects probably depends on the remarkable organization of their societies, where individuals are highly efficient in recognizing friends and foes (nest-mates versus non-nest-mates).

Insects live in a world of odours. Ants have been described as 'walking chemical factories' (Hölldobler & Wilson 1990), so it is not surprising that their recognition system is based on chemical cues (Hölldobler 1995). Ant bodies are covered by a layer of cuticular hydrocarbons that probably evolved to prevent desiccation and has since been co-opted to function in recognition (Howard & Blomquist 2005). Cuticular hydrocarbon cues are perceived by other individuals by direct antennal contact or at a short distance (Cuvillier-Hot *et al.* 2005; Brandstaetter *et al.* 2008). The pattern of cuticular hydrocarbons can be complex and dynamic, with many compounds varying both qualitatively (different species typically have different compounds) and quantitatively (different colonies of the same species have different relative proportions of the same hydrocarbons) (Lenoir *et al.* 1999, 2001). A typical ant cuticular hydrocarbon profile is composed of linear and methyl-branched molecules (alkanes) and sometimes unsaturated molecules (alkenes), with a chain length ranging generally from 20 to 40 carbon atoms. Molecules differing in their structure may carry different information, and probably not all cuticular substances are relevant for recognition (d'Ettorre & Moore 2008). For instance, in honeybees (*Apis mellifera*), unsaturated molecules (alkenes) seem to be more important than linear alkanes in nest-mate recognition (Châline *et al.* 2005; Dani *et al.* 2005). In paper wasps (*Polistes dominulus*),

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the topical supplementation of alkenes and methyl-branched alkanes interferes with nest-mate recognition, while linear alkanes do not have any significant effect (Dani *et al.* 2001). This may be a consequence of the fact that more configurations per chain length are possible for methyl-branched alkanes and alkenes than for linear alkanes (Châline *et al.* 2005). Also, recent studies on ants have suggested that unsaturated or branched hydrocarbons contain more information than linear ones and are more often used in nest-mate recognition (Akino *et al.* 2004; Martin *et al.* 2008), although in some species also linear alkanes appear to play a role (e.g. Greene & Gordon 2007). However, despite the growing body of evidence identifying recognition cues in social insects, the perceptual, neural and cognitive mechanisms underlying nest-mate recognition remain substantially unknown.

It is often implicitly or explicitly assumed that recognition occurs when the 'label' of the encountered individual (e.g. a specific pattern of cuticular hydrocarbons) matches a 'template' consisting of the neural representation of the typical colony label (colony odour) in the discriminating individual (Crozier 1987; Breed 1998; Lenoir *et al.* 1999; Starks 2004). This model requires storage of the template into long-term memory, and different forms of either simple or more complex learning have been proposed. Recognition is thus inferred to occur via an assessment of the overall similarity between colony odour and alien odour, as the bouquet of individuals living in the same colony generates a 'Gestalt colony odour' (Crozier & Dix 1979; Lenoir *et al.* 1999). In a more general approach, Sherman *et al.* (1997) proposed the 'D-present/U-absent' model that consists of two mechanisms for template matching, and has been recently tested in stingless bees (*Friesomelitta varia*; Couvillon & Ratnieks 2008). According to this recognition system, an ant guard at the nest entrance would (i) accept newly arriving individuals when they possess desirable cues (D-present: these cues are present mostly on nest-mates, but rarely on non-nest-mates) or (ii) accept incomers when they do not possess undesirable cues (U-absent: these undesirable cues are absent on nest-mates, but present on most non-nest-mates). Thus, this model assumes the recognition and acceptance of *nest-mates* by evaluating either the presence or the absence of the cues they carry, and the rejection of all other individuals.

Here, we present an alternative model suggesting that ants specifically recognize *non-nest-mates*, which are rejected, leading to the acceptance of all other individuals. We hypothesized that ants use a simple 'U-present' rule only: individuals bearing an undesirable cue, i.e. a cue that is present on the cuticle of undesirable individuals and *not* present on the cuticle of desirable nest-mates, would be rejected. Our model gives the same observable discrimination behaviour as other models (acceptance of nest-mates/rejection of non-nest-mates), but it is based on a different mechanism of perception. We tested the U-present hypothesis by supplementing the cuticle of *Camponotus herculeanus* ants with synthetic hydrocarbons that were not originally present in their cuticular profile. We used a natural supplementation procedure (via food manipulation), which is novel and we recorded the discrimination behaviour of ants in two different kinds of bioassays, one using free-walking ants and the other using harnessed ants.

2. MATERIAL AND METHODS

(a) *Origin of hydrocarbons and supplementation of the cuticular profile*

Four colonies of the carpenter ant *C. herculeanus* were collected in Denmark during summer 2006 and 2007, brought to the laboratory and kept under standardized laboratory conditions (24°C; 12 L: 12 D). From each laboratory colony, we created pairs of subcolonies consisting of 35 workers each. These were housed in plastic boxes with a plaster floor (18×10×6 cm). For supplementation, we coated the central surface (5 mm diameter circle) of a microscope cover slip (18×18 mm) with a hydrocarbon solution (2 µg pure hydrocarbon per ant present in the subcolony). After the solvent had evaporated, we added 20 µl of honey-water solution (1:1) and stirred to mix the hydrocarbon with the honey. One subcolony of each pair received honey supplemented with a pure hydrocarbon, the other was sham treated (with only pentane, the solvent). We used three different hydrocarbons: a dimethylated alkane, 3,11-dimethylheptacosane (thereafter coded as d); a monomethylated alkane, 11-methylhexacosane (m); and an unbranched, linear alkane, eicosane (l). Thus, we had three different pairs of subcolonies: d⁺ (d supplemented) and d⁻ (d deficient); m⁺ and m⁻; and l⁺ and the l⁻. In all groups, the ants immediately consumed the honey solution and exchanged it via trophallaxis. None of the three hydrocarbons was originally present on the ant cuticle (see figure S3 in the electronic supplementary material), but all of them are representative of those compounds commonly found on the cuticle of carpenter ants (Bonavita-Cougourdan *et al.* 1987 for *Camponotus vagus*). Eicosane (n-C₂₀) was purchased from Sigma-Aldrich (99% purity). The dimethyl alkane, 3,11-dimeC₂₇ (3,11-dimethylheptacosane), was synthesized by the laboratory of Prof Wittko Francke (University of Hamburg, Germany), synthetic pathway in d'Ettoire *et al.* (2004). The monomethyl alkane, 11-meC₂₆ (11-methylhexacosane), was synthesized for the present study (figure 1; see the electronic supplementary material).

Bioassays were done 24 hours after supplementation. To verify that the hydrocarbons had been incorporated into the ant cuticular profile, a sample of the treated workers for each pair of subcolonies was analysed by Solid Phase Micro Extraction, rubbing the ant body with a 7 mm polymethylsiloxane fibre (Supelco, Bellefonte, PA, USA) for 5 min. The fibre was injected into an Agilent Technologies 6890N gas chromatograph (capillary column: HP5MS 30 m×250 µm×0.25 µm; injector: *split-splitless*; carrying gas: helium at 1 ml min⁻¹). The temperature programme ranged from 70 to 200°C at 30°C min⁻¹, and from 200 to 300°C at 3°C min⁻¹. Compounds were identified on the basis of their mass spectra, produced by an Agilent Technologies 5975 inert mass selective detector (70 eV electron impact ionization) coupled with the gas chromatography.

(b) *Aggression test*

Each aggression test consisted of an encounter between three 'resident' ants and one 'alien' ant, with alien and residents coming from the same subcolony pair. We had six possible combinations: d⁺ versus d⁻; m⁺ versus m⁻; l⁺ versus l⁻ and their reciprocal combinations: d⁻ versus d⁺; m⁻ versus m⁺; l⁻ versus l⁺. Each ant was used in one test only. Each subcolony consisted of 35 ants, of these 8 were used as alien and 24 as resident for a total of 16 aggression tests per each pair of subcolonies. Aggression tests were performed in a circular

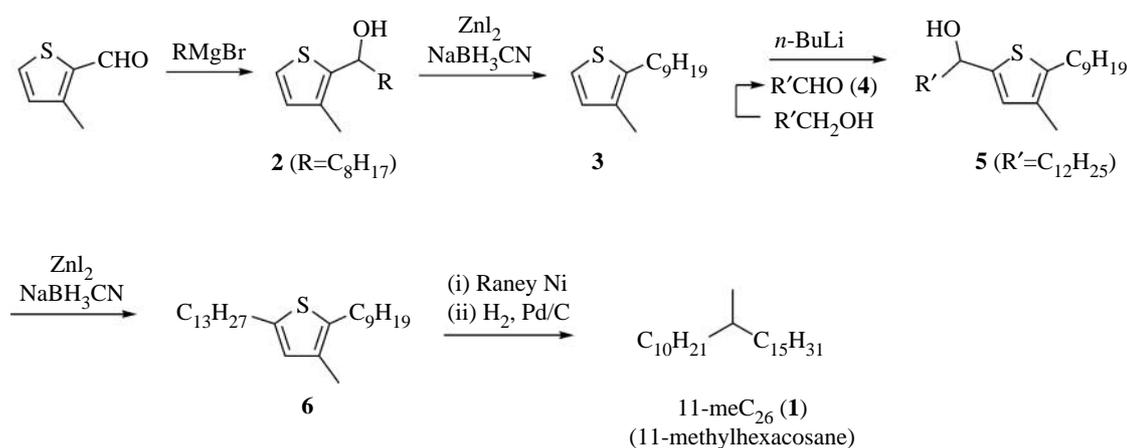


Figure 1. Synthesis of 11-meC₂₆ (11-methylhexacosane).

arena (diameter 5 cm) lined with filter paper, which previously had been in the residents' nest-box for at least 2 hours to acquire the residents' colony odour. Thus, the arena was not a neutral environment but was probably perceived as home territory by the residents and foreign territory by the alien. To ensure acclimatization, we introduced the ants into the arena 5 min prior to the start of the experiment; the alien ant was separated from the residents by a plastic cylinder. We started the experiment by removing the cylinder and recorded the behaviour of the residents towards the alien during 3 min. The experiment was replicated four times for each hydrocarbon treatment (total sample size in figure 2). All aggression tests were performed by the same experimenter who was not aware of the origin of the tested ants (blind trials).

We quantified the duration of the following behaviours (received by the alien) using the software ETHOLOG v. 2.2 (Otoni 2000): antennal contact; mandible opening (representing a threat, the first aggressive display in ants); biting; and gaster flexing. At any time, the strongest aggression display was recorded, e.g. if the alien received mandible opening and biting at the same time by two different residents, the duration of biting was recorded. Behaviours were ranked from minimum to maximum aggression level (a): antennal contact ($a=0$), mandible opening ($a=1$), biting ($a=2$) and gaster flexing ($a=3$). For each aggression test, an overall aggression index (AI) was computed according to the formula (cf. d'Ettorre *et al.* 2000)

$$\text{AI} = \frac{\sum_{i=1}^n a_i \cdot t_i}{T},$$

where a_i and t_i are the aggression level and total duration of each action, respectively, and T is the total interaction time. Only encounters during which the residents interacted with the alien ant were used for data analysis (approx. 90% of the encounters).

(c) Mandible opening response

This test consists in recording the aggressive response (mandible opening) of harnessed ants to a series of chemical stimuli (Guerrieri & d'Ettorre 2008). Ants were supplemented with hydrocarbons in the same paired design as above, except that subcolonies consisted of 15 workers. Test individuals from the six subcolonies (d^+ , d^- , m^+ , m^- , l^+ and l^-) were cooled on ice until they stopped moving, and harnessed in an ant holder only allowing them to move their antennae

and mouth parts (Guerrieri & d'Ettorre 2008). The ants were left undisturbed for 2 hours to habituate to the harness. After resting, those individuals that could actively move their antennae and mandibles (more than 90%) were used for testing.

The chemical stimuli were: (i) solvent, (ii) cuticular hydrocarbon extract of ants from the same subcolony as the test ant, (iii) the synthetic hydrocarbon used for supplementation, (iv) cuticular hydrocarbon extract of ants from the other subcolony of the pair, and (v) cuticular hydrocarbon extract of ants from an additional colony (control). Ten microlitres of each ant extract (one ant equivalent) were poured on the tip of a Pasteur pipette (hereafter stimulation pipette) using a Hamilton syringe. For pure hydrocarbons, 10 μl of solution in pentane (0.01 mg ml^{-1}) were poured on the tip of the stimulation pipette, similarly to cuticular extracts. Thus, 100 ng of hydrocarbon were deposited on the stimulation pipette. The pipette was held with its tip downwards to keep the extract around the outer part, up to 3 mm from the tip, until pentane completely evaporated.

Each test was composed of seven trials (two blank stimulations followed by the five different stimuli mentioned above). Each trial lasted 1 min and involved presenting one stimulus at a time to each test ant. One individual was placed under a stereomicroscope, after 25 s the antennae were gently touched for 5 s with the tip of one of the stimulation pipettes, after another 25–30 s the individual was returned to its resting place. The inter-trial interval was 10 min to avoid adaptation of the antennal receptors. Then, the ant was set under the stereomicroscope again to be presented with the next stimulus. The procedure was performed twice using a clean pipette (blank stimulation) to reduce spontaneous aggression as a reaction to tactile stimulation. Then, the five stimuli were presented in a randomized order. Mandible opening response (MOR) was recorded as a categorical variable: when an ant opened its mandibles wide, i.e. displacing them from their resting position, as the antenna was touched with the stimulation pipette, the response was noted aggressive; else it was noted non-aggressive (Guerrieri & d'Ettorre 2008).

3. RESULTS

(a) Synthesis of methylated hydrocarbon and cuticle supplementation

We developed a new pathway for the synthesis of mono-methyl alkanes: 11-meC₂₆ (11-methylhexacosane, 1, figure 1) was synthesized using a 3-methylthiophene structure as a 4-carbon synthon installing the methyl

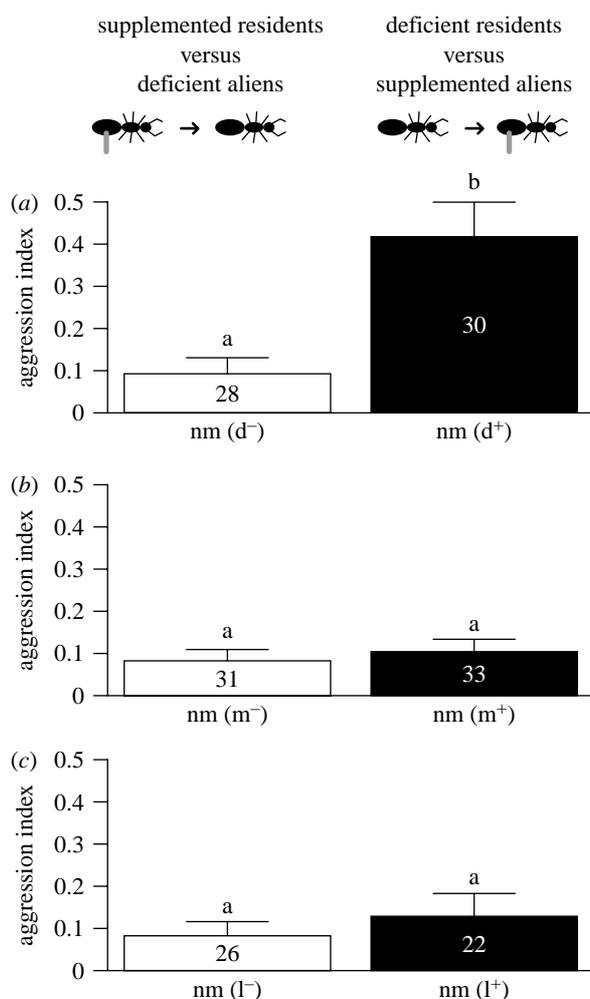


Figure 2. Aggression level of resident ants (either supplemented with a hydrocarbon or deficient of it) towards alien ants (either deficient or supplemented, respectively). White bars show the aggression level of ‘supplemented resident’ towards ‘deficient alien’ ants; black bars show the aggression level of ‘deficient resident’ towards ‘supplemented alien’ ants. (a) ‘nm (d⁺)’ are alien ants supplemented with 3,11-dimeC₂₇, ‘nm (d⁻)’ the respective deficient aliens, lacking the extra hydrocarbon. (b) ‘m’ stands for 11-meC₂₆ and (c) ‘l’ for *n*-C₂₀. Aggression differed significantly among all six assayed resident–alien combinations ($F_{5,164}=7.71$, $p<0.0001$). Bars depict mean and s.e.m.; different letters indicate significantly different aggression indices (Scheffe *post hoc* comparisons between all six combinations $p<0.01$); numbers inside bars refer to sample sizes.

group in the 11-position of the final product. Previously, thiophenes have been used successfully within other research areas as 4-carbon synthons (Krishna *et al.* 2000; Mohr *et al.* 2005). Also, Pomonis *et al.* (1989) prepared related hydrocarbons from thiophenes, although with a different set of subreactions than presented here. In our synthesis strategy (figure 1), the initial steps involved a Grignard reaction of 3-methylthiophene-2-carbaldehyde followed by reductive removal of the hydroxyl group in compound 2. Compound 3 was then lithiated selectively in the 5-position and treated with aldehyde (4). The resulting hydroxyl compound 5 was subsequently reduced to thiophene compound 6. Using commercial Raney nickel, the conditions for reducing thiophenes in general (Mohr *et al.* 2005) were optimized

by reducing structure 2 to 3-methyltridecane. To ensure full reduction of double bonds, it was necessary to introduce a hydrogenation step after removal of sulphur with Raney nickel. These conditions were successfully used in the isolation of 11-meC₂₆ (see the electronic supplementary material for details).

We used a new method to supplement the ant cuticle with synthetic hydrocarbons: we added each chemical to food (diluted honey). Previously, supplementation has been achieved by direct topical application of chemicals onto the cuticle (e.g. in *Apis mellifera*: Dani *et al.* 2001, 2005, Breed *et al.* 2004a; in *C. vagus*: Meskali *et al.* 1995; in *Camponotus floridanus*: Leonhardt *et al.* 2007). Using that approach, the change in cuticular complements is immediate, and the animal needs time to habituate to its own, changed odour. Instead, we succeeded in incorporating the new chemical into the insect cuticle by mixing the hydrocarbon with liquid food (diluted honey). The added hydrocarbons were relocated onto the animals’ cuticles within 24 hours, as shown by chemical analysis (figure S3 in the electronic supplementary material).

(b) Aggression tests

We used freely moving ants (residents) to test their aggressive behaviour towards individuals (aliens) with less or with more hydrocarbons on the cuticle compared with residents. Across tests, we found highly significant differences in aggression levels (overall ANOVA $F_{5,164}=7.71$; $p<0.0001$, figure 2). Specifically, we tested the role of three factors on the aggression level of residents towards aliens: (i) whether the alien ant was supplemented or sham treated, i.e. whether the alien’s cuticle contained one hydrocarbon more (alien-supplemented) or one hydrocarbon less (alien-deficient) than the residents’ cuticle, (ii) whether the residents were supplemented or deficient, and (iii) what type of hydrocarbon was used for supplementation (i.e. dimethylated, mono-methylated or linear molecule). In pairwise comparisons, only aliens supplemented with 3,11-dimeC₂₇ (d⁺) received significantly more aggression by resident-deficient ants (d⁻) than vice versa (figure 2a; Scheffe *post hoc* tests, $p<0.01$), showing that presence, but not absence, of this substance elicits aggression. By contrast, as shown in figure 2b,c, there was no significant difference between the aggression received by alien-supplemented and alien-deficient ants when the extra substance was 11-meC₂₆ (m⁺ versus m⁻) or *n*-C₂₀ (l⁺ versus l⁻), regardless of whether the residents were deficient or supplemented, showing that not all substances are evaluated in the same way with respect to nest-mate recognition.

(c) Mandible opening response tests

When using freely moving ants, it is only possible to test a single pair of treatments. We therefore further investigated aggressive behaviour in harnessed ants using the procedure described in Guerrieri & d’Ettorre (2008), where each ant can be sequentially challenged with a battery of stimuli (figure 3). The results of MOR were consistent with those observed with freely moving ants. There was a clear difference in MOR levels of each category of resident ants when presented with the five different stimuli (ANOVA, $p<0.01$, in all cases; figure 3a–f). In particular, d⁺ resident ants (supplemented with 3,11-dimeC₂₇) reacted with equally low aggression levels

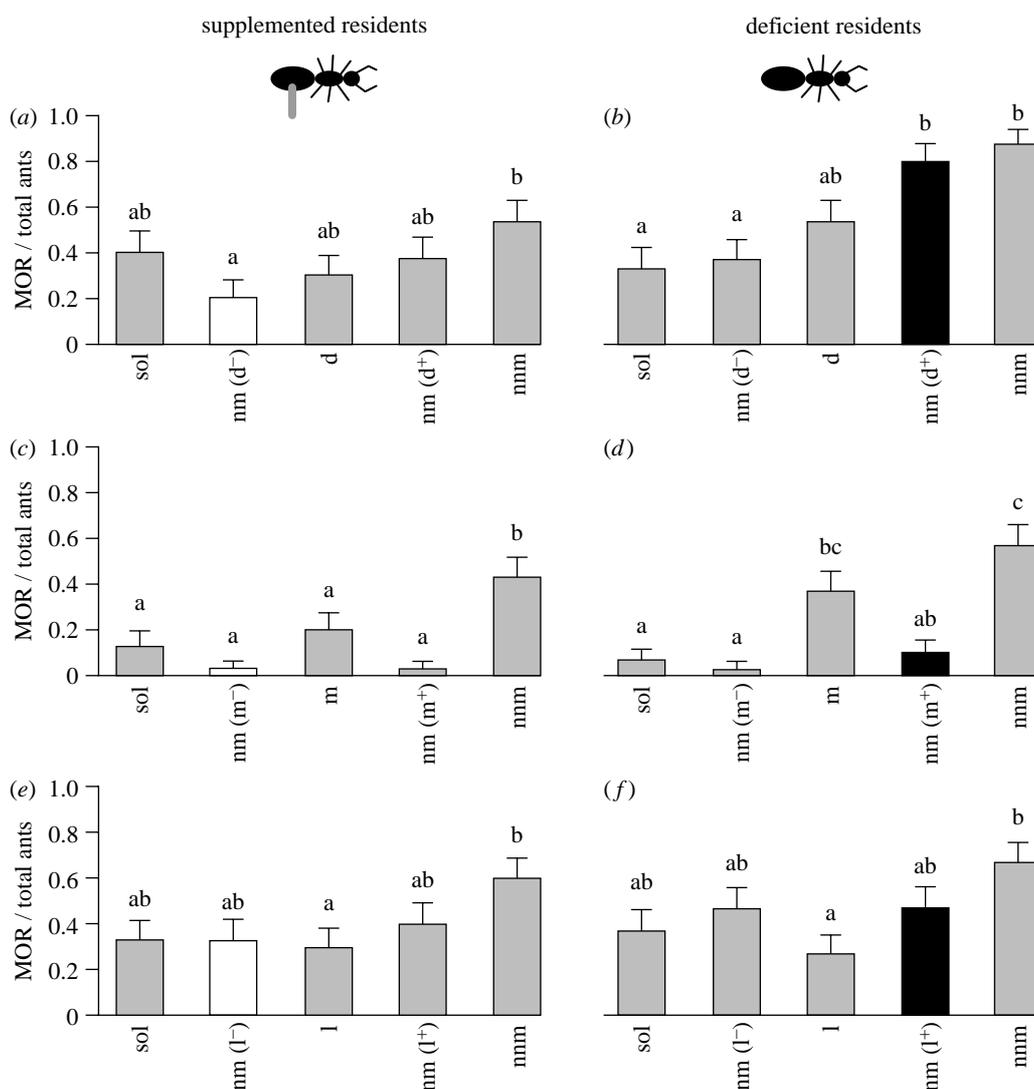


Figure 3. MORs displayed by six groups of test ants ('residents') to five different test stimuli. (a,c,e) MOR level of resident-supplemented ants; (b,d,f) MOR level of resident-deficient ants. The hydrocarbons used for supplementation were (a,b) 3,11-dimeC₂₇ (group d), (c,d) 11-meC₂₆ (group m) and (e,f) *n*-C₂₀ (group l). The stimuli were solvent (sol); extracts of alien-deficient ants (nm(d⁻), nm(m⁻) and nm(l⁻)) and extract of the respective alien-supplemented ants (nm(d⁺), nm(m⁺) and nm(l⁺)); pure hydrocarbons (groups d, m and l); extracts of control non-nest-mate ants (nmm) coming from another colony than the test ants. Each of the residents ($n=30$ per subset) was stimulated with each of the five stimuli. Bars show mean and s.e.m.; different letters indicate significant differences (Scheffe *post hoc* tests; $p < 0.05$, in all cases). White and black bars indicate those tests that correspond, in their logic, to the situation in figure 2.

to extracts of supplemented (d⁺) and deficient (d⁻) ants (figure 3a; Scheffe *post hoc* test, $p > 0.05$). This was not due to a reduced level of activity following harnessing, as shown by the control: when presented with a non-nest-mate (nmm) stimulus, aggressive behaviour was strong (figure 3a). By contrast, d⁻ resident ants (deficient of 3,11-dimeC₂₇) were as highly aggressive towards extract of d⁺ ants as towards those of a control non-nest-mate colony (nmm) (figure 3b; Scheffe *post hoc* test, $p > 0.05$). Thus, it is the presence, and not the absence, of a compound that is eliciting aggression. The aggressive behaviour is stronger when the added substance (d) is given in the cuticular bouquet, than when it is given as a pure synthetic substance.

In the case of 11-meC₂₆ or *n*-C₂₀ (figure 3c-f), stimulation with extracts of ants from a control non-nest-mate colony (nmm) always induced a higher MOR response compared to stimulation with extracts from the same or the paired subcolony, regardless whether residents were supplemented (m⁺, l⁺) or deficient (m⁻, l⁻). Thus,

these substances were not effective in modifying nest-mate recognition. In contrast to *n*-C₂₀ (l), pure 11-meC₂₆ (m) elicited more aggressive behaviour than when presented in a blend with the cuticular hydrocarbons (figure 3d). This also shows that evaluation of pure substances might be different from evaluation of complex blends.

4. DISCUSSION

Our results clearly demonstrate that discriminating ants reject (attack) an individual if it displays additional chemicals on its cuticle (alien-supplemented), but not if it displays less molecules (alien-deficient) than the discriminator. Furthermore, we show that this effect does not apply to every molecule. Specifically, in our experiments, a dimethylated hydrocarbon (3,11-dimeC₂₇) was effective, while a mono-methylated (11-meC₂₆) and a linear (*n*-C₂₀) hydrocarbon were not effective in eliciting aggression, either when they were added to the cuticular

bouquet or when they were missing. This agrees with previous work showing that linear hydrocarbons might have little role in nest-mate recognition (Dani *et al.* 2001, 2005; Martin *et al.* 2008; but see Greene & Gordon 2007). We also show that 24 hours are sufficient to change the cuticular composition by food supply. This technique provides a new tool for investigating the mechanisms of chemical recognition, in addition to the often used direct topical application of substances (Dani *et al.* 2001, 2005; Breed *et al.* 2004a; Leonhardt *et al.* 2007). Our technique is likely to mimic a more natural situation, because the new substance is incorporated through the metabolic pathway of the animal. Most importantly, when cuticular composition was modified by supplementing the diet, we did not observe any aggression within the supplemented group. This suggests that a gradual change over several hours is possible, and that the reference system (the so called *template*) can be modified accordingly, as also indicated by other experiments (Leonhardt *et al.* 2007). Thus, the semiochemical composition of the ants' cuticles may slowly change without deleterious effects on the colony aggression levels in natural conditions (e.g. when foragers collect from a new food source). Furthermore, in *Camponotus* ants, trophallaxis (exchange of liquid food between nest-mates) is common within a nest, which causes all individuals to have a similar diet and hydrocarbon composition. Our finding that supplemental hydrocarbons in the diet end up on the ants' cuticle supports the role of trophallaxis in the homogenization of the colony odour (Soroker *et al.* 1994; Lenoir *et al.* 1999).

Nest-mate recognition in social insects needs to be strict enough to allow the rejection of individuals if they come from a different colony, while at the same time being flexible enough to accept variations in the colony odour composition without rejecting nest-mates (Reeve 1989). One proposed mechanism for nest-mate recognition is based on a stimulus identification/generalization task, often referred to as template-label matching (Lenoir *et al.* 1999; Starks 2004). This consists in a stimulus-similarity evaluation, in which case the cuticular bouquet is interpreted as a colony odour *Gestalt* (Crozier & Dix 1979; Crozier 1987). It has been suggested that matching can be achieved by evaluating the presence of desirable cues (D-present) or the absence of undesirable cues (U-absent) (Sherman *et al.* 1997). On the basis of our results, we propose an alternative mechanism; namely that only added, but not missing, components elicit aggression. This U-present model accounts for recognition of non-nest-mates (which bear undesirable cues), but not for recognition of nest-mates (which bear the same label as the discriminator).

Our results raise the question of what physiological mechanisms could elicit aggression based on the occurrence, but not the lack of a cuticular hydrocarbon. The effect could be entirely peripheral, i.e. that olfactory receptor responses to biologically relevant cuticular substances are downregulated (Ozaki *et al.* 2005), which could be realized by sensory adaptation (desensitization). In this case, antennal receptors of the discriminator would not respond to substances that are around all the time, e.g. the cuticular pattern of nest-mates (shared by the discriminator). However, experiments have shown that slower effects and simple memory may be also involved in nest-mate recognition (Leonhardt *et al.* 2007). In the data

presented here, there are two aspects that indicates that sensory adaptation is not likely to play the main role in this system: first, adaptation should be equally effective for all substances, but we find that some substances are effective (3,11-dimeC₂₇), while others are not (11-meC₂₆ and *n*-C₂₀). Second, sensory systems rarely adapt to a level of no response, but rather to a level of activity that allows for coding both an increase and a decrease in stimulus concentration (Stengl *et al.* 1992). We expect, therefore, that central plasticity plays a major role, i.e. that ants learn about their nest-mates' odours. The most parsimonious mechanism involves habituation, a non-associative form of elemental learning. In this case, the response to the supplemented substances would be reduced by synaptic changes in the brain, most likely in the antennal lobes, the first brain area that processes olfactory information—analogue to the olfactory bulbs in mammals (Galizia & Szyszka 2008). This would represent an efficient and economic way of updating the reference system (template) without involving the formation of long-term memory.

Odours are coded by activity in sensitive receptor neurons. In most instances, an odour will activate more than one family of olfactory receptors, and elicit a specific pattern of activity across glomeruli, which are the processing units in the antennal lobe (Galizia *et al.* 1999; Galizia & Szyszka 2008). Our data suggest that the combinatorial pattern of glomeruli elicited by the cuticular odour of a nest-mate does not cause aggression, but also that a pattern consisting of fewer active glomeruli (the deficient case) does not elicit aggression. To reject an alien intruder, additional activity in some glomeruli is needed. As an implication, we propose that for nest-mate recognition, odours are not judged by the similarity of the glomerular pattern that they elicit, but rather by an inclusion criterion: any odour that elicits a subpattern of the nest-mates' odour will be acceptable. This is evidenced by comparing d⁺ ants (figures 2 and 3), which did not display aggressive behaviour towards d⁻ ants, with d⁻ ants which did show aggressive behaviour towards d⁺ ants, even though the odour similarity of d⁺ to d⁻ is logically equal to the odour similarity of d⁻ to d⁺. Indeed, ant social parasites that invade colonies of other ant species have a very weak complement of cuticular hydrocarbons (the 'chemical insignificance' hypothesis, cf. Lenoir *et al.* 2001). The same applies to young workers that are readily accepted in non-nest-mates colonies (Breed *et al.* 2004b). The fact that social parasites can successfully break the recognition code of ants by being chemically insignificant cannot be explained by the D-present model (Sherman *et al.* 1997).

A recent study in stingless bees has showed that guards reject nest-mates that had acquired odour cues from non-nest-mates, but do not accept non-nest-mates that had acquired equivalent amounts of odour cues from the guard's nest-mates. These results have been interpreted as supporting the U-absent system in recognition (Couvillon & Ratnieks 2008). However, they can be easily explained by our U-present model, focusing on the observation that additional odours cause rejection. Experimental symmetrical swapping of wax combs between two honeybee hives resulted in decreased reciprocal rejection, since the odour of bees of the two hives became more similar to each other due to the acquisition of cues present on the wax comb (d'Ettore *et al.* 2006). However, when only a receiver

hive (A) was provided with the combs of a donor hive (B), rejection of B-bees by A-guards decreased, but not the other way around (Couvillon *et al.* 2007). These results are also consistent with our U-present model, since after the experimental manipulation the odour of A-guards contained B-cues, but this was not the case for B-guards, which reacted to the presence of undesirable A-cues, even if A-bees carried also some B-cues.

Our finding that some chemicals are effective, while others are not, can be explained in two ways. First, ants may lack olfactory receptors for the ineffective substances (e.g. 11-meC₂₆). Alternatively, and in our view more likely, the data suggest that not all glomeruli are equal. Thus, it is conceivable that there is a subset of glomeruli specifically devoted to nest-mate recognition. In fact, when compared to approximately 160 glomeruli in bees, 60 in moths and 50 in fruitflies (Galizia & Menzel 2001; Galizia & Szyszka 2008), ants have very high numbers of glomeruli: *C. floridanus* has approximately 460, and these glomeruli are arranged in morphologically distinct groups (Zube *et al.* 2008). Separate subgroups of glomeruli devoted to processing of sexual pheromones are well known in many insect species, and recently an example of a non-sexual pheromone system has been reported in leaf-cutting ants (Kleineidam *et al.* 2005). The restriction of cuticular recognition cues to a limited set of molecules might make nest-mate recognition robust against other environmental odours.

In summary, we show that an added substance to the cuticle of an ant will elicit aggressive behaviour while a missing one will not, indicating that a comparison is made based on inclusiveness and not on odour similarity. This principle may also work for differences in relative proportions of the same substances, which is the common situation in an ant's environment of competition among colonies of the same species. An alien ant bearing a higher relative concentration of some hydrocarbons present also on the cuticle of the discriminator, and a lower relative concentration of others, will be efficiently rejected on the basis of the former only. Conversely, in its own colony, the alien will be the 'discriminator', and it will use the second group of substances, which will then be higher in the intruder. This remains to be tested. Further work may also elucidate how widespread the U-present model might be in social insects.

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