PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2008) **275**, 1271–1278
doi:10.1098/rspb.2007.1708
Published online 4 March 2008

Chemical basis of nest-mate discrimination in the ant *Formica exsecta*

Stephen J. Martin[1,*], Emma Vitikainen[2], Heikki Helanterä[1] and Falko P. Drijfhout[3]

[1]*Laboratory of Apiculture and Social Insects, Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK*
[2]*Department of Biological and Environmental Sciences, PO Box 65, University of Helsinki, Helsinki 00014, Finland*
[3]*Chemical Ecology Group, Lennard-Jones Laboratory, School of Physical and Geographical Sciences, Keele University, Keele, Staffordshire ST5 5BG, UK*

Distinguishing nest-mates from non-nest-mates underlies key animal behaviours, such as territoriality, altruism and the evolution of sociality. Despite its importance, there is very little empirical support for such a mechanism in nature. Here we provide data that the nest-mate recognition mechanism in an ant is based on a colony-specific Z9-alkene signature, proving that surface chemicals are indeed used in ant nest-mate recognition as was suggested 100 years ago. We investigated the cuticular hydrocarbon profiles of 10 *Formica exsecta* colonies that are composed almost entirely of a Z9-alkene and alkane component. Then we showed that worker aggression is only elicited by the Z9-alkene part. This was confirmed using synthetic Z9-alkene and alkane blends matched to the individual colony profiles of the two most different chemical colonies. In both colonies, only glass beads with 'nest-mate' alkene profiles received reduced aggression. Finally, changing the abundance of a single Z9-alkene on live ants was shown to significantly increase the aggression they received from nest-mates in all five colonies tested. Our data suggest that nest-mate discrimination in the social insects has evolved to rely upon highly sensitive responses to relatively few compounds.

Keywords: recognition; cuticular hydrocarbons; *Formica*; ants

1. INTRODUCTION

The ability of organisms to communicate with each other is common to all biological systems. Communication is most highly developed in societies where complex social decisions are made. This is exemplified by the social insects (ants, termites, wasps and bees) since their colonies can contain thousands or even millions of workers. The primary mode of communication in most insects, and many other animals, is chemical (Wyatt 2003). Insect sex pheromone systems are reasonably well understood, but almost nothing is known about the 'recognition language' of insect societies (Boomsma & Franks 2006). Recognition plays a critical role in the evolution of cooperative behaviour (Hamilton 1964), since the ability to discriminate between related and non-related individuals ensures that aggressive and altruistic behaviours are directed towards the correct individuals. Although nest-mate recognition is widespread among eusocial insects and has been found in a solitary bee (*Manuelia postica*; Flores-Prado *et al.* 2008), theoretical studies (Rousset & Roze 2007) show that kin recognition may not exist. However, the underlying mechanisms of both nest-mate and kin recognition remain poorly understood.

Ants are among the most dominant animals in the world and employ complex forms of chemical communication (Hölldobler & Wilson 1990). Over 100 exocrine glands have been described in social insects with more than half of these found in ants (Billen 2004). These glands are believed to produce a vast array of signals that encode information about an individual's species, sex, age, caste, status and relatedness, in addition to alarm and trail pheromones (Howard & Blomquist 2005). It has long been believed (Fielde 1901) that nest-mate discrimination signals are encoded in cuticular lipids, particularly hydrocarbons that coat all insects (Singer 1998). Biochemical investigations have shown that insect cuticular hydrocarbons (CHCs) are synthesized internally in oenocytes (Blomquist & Dillwith 1985) and are under strong genetic influence in flies (Ferveur & Jallon 1996; Savarit *et al.* 1999; Dallerac *et al.* 2000) and *Formica* ants (Beye *et al.* 1998, 2004). Therefore, the CHC profile is a reflection of an insect's genotype (Lockey 1991). The CHCs consist of homologous series of long straight-chain saturated alkanes, which can be modified by the addition of methyl groups attached to the chain or the introduction of one of more double bonds (Lockey 1988; Jackson & Morgan 1993). These simple modifications have allowed very rich and complex CHC profiles to evolve within insects. For example, from only seven species of *Cataglyphis* ants, 241 different CHCs were detected (Dahbi *et al.* 1996). Within the CHC profile many different recognition signals are believed to be encoded, e.g. species, nest-mate identity, fertility and task. Until recently (Akino *et al.* 2004; Ozaki *et al.* 2005), the evidence linking CHCs to nest-mate discrimination was at best circumstantial (Breed 1998) based largely on correlative studies. Most species have complex CHC profiles and comparing them requires very sensitive multivariate statistical methods (principal component and discriminate analysis) that

* Author for correspondence (s.j.martin@sheffield.ac.uk).

Received 13 December 2007
Accepted 19 February 2008

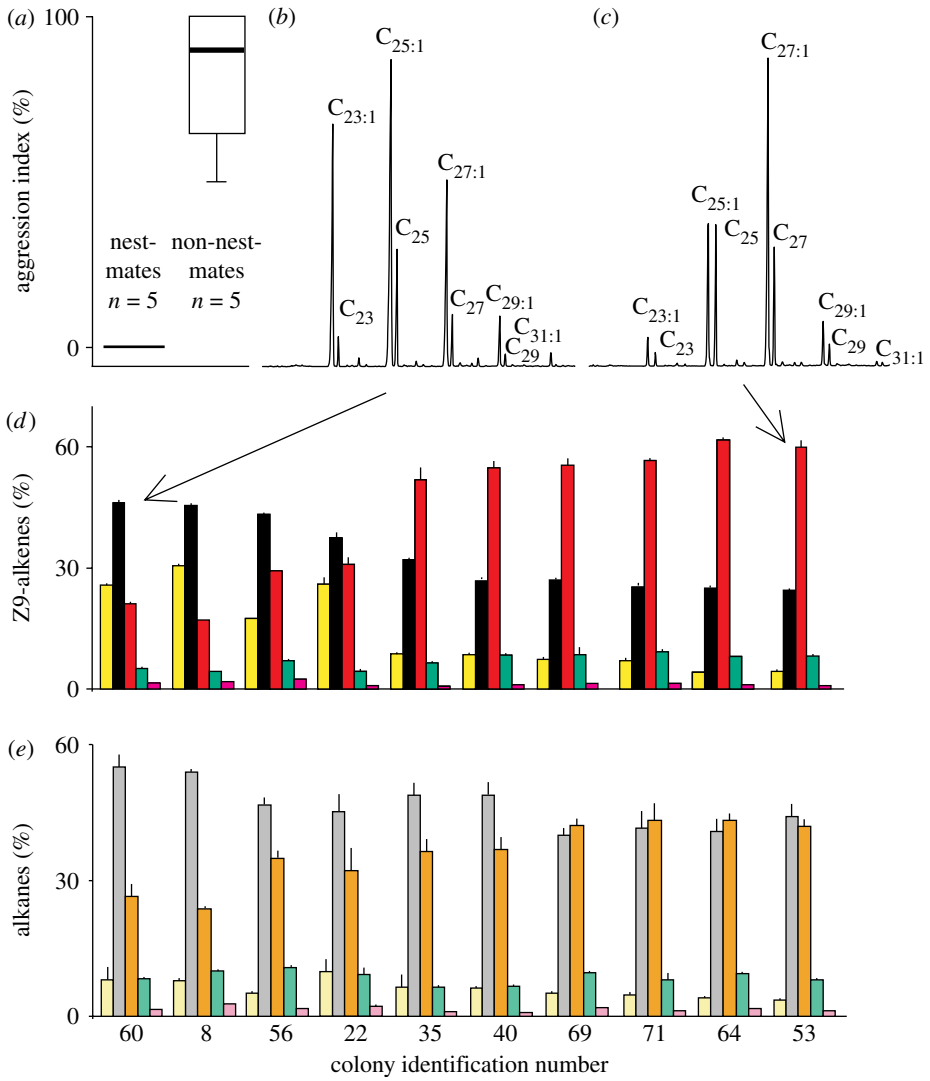

Figure 1. (*a*) Intra-colony and inter-colony aggression levels based on 500 interactions from 10 colonies. Chromatograms of a *F. exsecta* worker from (*b*) colony 60 and (*c*) 53 showing the dominance of C_{23}–C_{31} alkanes and Z9-alkenes. The average (*d*) Z9-alkene (yellow bars, $C_{23:1}$; black bars, $C_{25:1}$; red bars, $C_{27:1}$; green bars, $C_{29:1}$; pink bars, $C_{31:1}$) and (*e*) alkane (pale yellow bars, C_{23}; grey bars, C_{25}; orange bars, C_{27}; light green bars, C_{29}; pale pink bars, C_{31}) profiles (+s.d.) for each study colony in 2007 is presented separately to highlight inter-colony variation. The data have been normalized to obtain 100% for each class and colonies ordered in decreasing amounts of $C_{25:1}$.

often provide limited insights, since these methods combine rather than separate out various signals. Therefore, it is crucial that future studies attempt to tease out the different components of the profile and test their effects experimentally (Akino *et al.* 2004; Greene & Gordon 2007). This is especially true when temporal and environmentally induced variation in the CHC profiles might mask the more stable nest-mate recognition cues (Liu *et al.* 2001).

Formica is a keystone ant genus, comprising over 160 species that include the well-known mound-building wood ant species. Analysis of the CHC profiles of 13 *Formica* species found the evolution of two distinct chemical groups characterized by the presence of either dimethylalkanes or Z9-alkenes (Martin *et al.* in press). The latter group, which includes *Formica exsecta*, has the simplest profiles and is dominated (greater than 95%) by C_{23}–C_{29} alkanes and Z9-alkenes (figure 1), that is hydrocarbons with 23, 25, 27 and 29 carbons that are either saturated (C_n) or have a double bond in the ninth *cis* position (Z9-$C_{n:1}$). We concluded that decoding potential nest-mate discrimination cues would be easiest in this group of ants, due to the simple CHC profile and the ants' highly aggressive nature towards intruders placed on their nest mound. The aim of this study is to combine for the first time the genetic and chemical analysis of 10 colonies from a single population, and to reveal the compounds involved in the nest-mate recognition system of *F. exsecta* by mimicking and altering different parts of the CHC profile using synthetic compounds. Furthermore, the relationships of the CHC compounds have been investigated in a new way.

2. MATERIAL AND METHODS

(a) *Study site and species*

All chemical analyses (April 2006 and April 2007) and behavioural bioassays (April 2007) were conducted using 10 *F. exsecta* colonies that are part of the well-studied monogynous *F. exsecta* Joskär island population at the Tvärminne zoological station in Hanko, Finland (Sundström *et al.* 2003).

(b) *Genetic methods*

To determine the genetic structure of the colonies, 16 workers from each colony were genotyped at 10 highly variable microsatellite loci, Fe11, Fe13, Fe17, Fe37, Fe38, Fe42,

Fe49 (Gyllenstrand *et al.* 2002), Fl21 (Chapuisat 1996), P22 (Trontti *et al.* 2003) and Fy3 (Hasegawa & Imai 2004). DNA was extracted following the Chelex protocol and fluorescent-labelled F-primers were used for sizing the PCR products. Single PCR amplifications were carried out for Fe13, Fe17, Fl21, Fe37 and P22 in 10 µl of reaction solution of 10 mM Finnzyme Taq Buffer, 0.8 mM dNTPs, 0.3 µM of each primer, 0.25 unit of DyNAzyme and 1 µl of template DNA. A multiplex PCR amplification was carried out for Fe42 and Fe49 in 10 µl of reaction solution of 15 mM Finnzyme Taq Buffer, 1 mM dNTPs, 0.15 µM of Fe42 primers, 0.45 µM of Fe49 primers, 0.25 unit of DyNAzyme and 1 µl of template DNA. Fe11, Fy3 and Fe38 were amplified in 10 µl multiplex containing 15 mM Finnzyme Taq Buffer, 1 mM dNTPs, 0.2 µM of each primer, 0.6 units of DyNAzyme and 1 µl of template DNA. PCR products were analysed using a MegaBACE 1000 sequencer with ET400-R size standard. The genetic structure of the colonies was inferred as the minimum number of parents needed to explain the observed worker multilocus genotypes.

(c) *Aggression bioassays*

Preliminary trials indicated that when *F. exsecta* non-nest-mate workers were placed in a neutral arena such as a Petri dish, they just avoided each other. However, fights were initiated rapidly when a worker was placed on the surface of a foreign nest. Therefore, all aggression bioassays were carried out using a colony fragment that contained 200–300 workers along with part of the nest material from their mound. Colony fragments were transported into the laboratory and allowed to settle before the bioassays were conducted. Treated or untreated workers, or glass beads, were then introduced into the colony fragment and the level of ant aggression was recorded using a five-point scale: 1, ignore; 2, antennation; 3, mandible gaping; 4, attack; 5, fighting, sustained biting, etc. Introduced workers were carefully followed without marking them, which avoids the problems of marking techniques influencing interactions. This was possible since irrespective of treatment all workers received intense bouts of antennation from nest-mates. The outcomes of the first five interactions between the test ants and colony ants or first 10 interactions between the glass bead and colony ants were recorded. Fewer interactions were used with the test ants as the resulting behaviour was much less variable. To ensure all behavioural observations were unbiased, the observer was always unaware of the source of the test subject (bead or ant). All introduced test subjects were removed immediately after the interaction data had been collected. When ants from the same colony were used in different bioassays, a new colony fragment was used for each experiment.

(d) *CHC profile identification and synthesis of compounds*

During April 2006, the CHC profile of each study colony was determined by analysing five workers using GC–MS (see below). The two colonies with the most different Z9-alkene profiles (60 and 53) were chosen for the manipulation experiments. The main Z9-alkenes ($C_{23:1}$, $C_{25:1}$, $C_{27:1}$ and $C_{29:1}$) were synthesized at Keele University by Dr G. R. Jones and had a purity above 99%, while the synthetic alkanes (C_{23}, C_{25}, C_{27} and C_{29}) were purchased from Sigma-Aldrich Ltd. (Dorset, UK). During 2007, the 10 colonies were re-analysed to confirm that their profiles had remained relatively stable, which was the case.

(e) *Single compound bioassays*

We altered the profile of individual ants from five colonies by either adding a single synthesized alkane (C_{25} or C_{27}) or Z9-alkene ($C_{25:1}$ or $C_{27:1}$) to its cuticle and observed the behavioural responses of its nest-mates when returned to its own colony. Thirty ants were removed from each colony fragment and cooled in a fridge until they became torpid. Of these 10 were then treated with approximately 0.5 µl of HPLC grade hexane containing 10 ng of the alkane (C_{25} or C_{27}), 10 with Z9-alkene ($C_{25:1}$ or $C_{27:1}$) and the remaining 10 with only hexane that acted as the control. Treatment was applied via a 10 µl Hamilton syringe to the gaster of the ant, since application of hexane to the ant's head or thorax resulted in its immediate death through asphyxiation via hexane entering the spiracles. Treated ants were then individually reintroduced to their colony fragment in a random order and an aggression bioassay conducted. Five treated ants from each class were killed by freezing and their CHC profile analysed to confirm that their CHC had indeed being altered. The bioassay was repeated using fragments from five different colonies.

(f) *Glass bead bioassays*

Based on the 2006 data, we used eight synthesized hydrocarbons to recreate synthetic profiles similar to colonies 60 and 53, which were confirmed by GC–MS analysis. This profile or parts of the profile were applied to groups of 10 oval-shaped glass beads (5×2 mm) in a hexane solution. Glass beads contained the Z9-alkene plus alkane profiles, only the Z9-alkene, only the alkane part or the Z9-alkene plus a 'generic' alkane profile (figures 2 and 3). The generic profile consisted of 25% by weight of each of the four synthesized alkanes. The beads were introduced individually into a colony fragment of either colony 60 or 53 and a blind aggression bioassay conducted as described above.

(g) *Chemical analysis*

Individual *Formica* ants or glass beads were placed into glass vials with 50 µl of HPLC grade hexane. After 10 min, the ants or beads were removed and the hexane evaporated. Vials were then sealed and stored at 5°C. Just prior to analysis, 30 µl of hexane was added to the vials and the sample analysed on a HP 6890 GC (equipped with a HP-5MS column; length, 30 m; ID, 0.25 mm; film thickness, 0.25 µm) connected to a HP5973 MSD (quadrupole mass spectrometer with 70 eV electron impact ionization). Samples were injected in the splitless mode and the oven was programmed from 70 to 200°C at 40°C min^{-1} and then from 200 to 320°C at 25°C min^{-1} and held for 2 min at 320°C. Helium was used as carrier gas at a constant flow rate of 1.0 ml min^{-1}. The CHCs were characterized by the use of standard MS databases, diagnostic ions and their Kovats indices. We had previously determined by DMDS derivatization that the double bond was at the ninth position in *F. exsecta* alkenes (Martin *et al.* in press).

(h) *Statistical analyses*

Using the frequency distribution of aggression score levels, an aggressivity index was calculated by

$$\text{aggressivity index} = \frac{\text{total number of interactions scoring 3, 4 or 5}}{\text{total number of interactions}}.$$

Table 1. Mean correlation values ($r^2 \pm$s.d.) across the 10 study colonies between successive major (most abundant) Z9-alkenes ($C_{*:1}$) and alkanes (C_*) within a homologous series, or between compounds of the same chain length but from different homologous series. (For example, in each of the 10 colonies, the ratio (relative abundance) of any two successive Z9-alkenes and hence the overall shape of the Z9-alkene distribution was constant among workers ($n=10$) from the same colony. Although the shape of the Z9-alkene distribution varied between colonies (figure 1), the Z9-alkene distribution shape always remained stable within the colony because the compounds within the series were so highly correlated, shown in italics.)

homologous series	comparisons	$r^2 \pm$s.d.
Z9-alkenes versus Z9-alkenes	$C_{23:1}$ versus $C_{25:1}$	1 ± 0.01
	$C_{25:1}$ versus $C_{27:1}$	*0.99* ± 0.01
	$C_{27:1}$ versus $C_{29:1}$	*0.97* ± 0.03
alkanes versus alkanes	C_{23} versus C_{25}	0.56 ± 0.22
	C_{25} versus C_{27}	0.87 ± 0.13
	C_{27} versus C_{29}	0.88 ± 0.16
Z9-alkenes versus alkanes	C_{23} versus $C_{23:1}$	0.77 ± 0.18
	C_{25} versus $C_{25:1}$	0.25 ± 0.24
	C_{27} versus $C_{27:1}$	0.23 ± 0.26

Aggressivity indices were compared using the Kruskal–Wallis test, and for significant differences pairwise analyses using Mann–Whitney *U* tests were used. The Bonferroni correction was used to adjust the significance level where multiple comparisons were made. All tests were carried out by SPSS v. 14 and *p* values are based on the asymptotic significance (two tailed).

3. RESULTS

(a) *Genetic structure of the study colonies*

Microsatellite analyses using 10 highly variable loci showed that all colonies were monogynous with one reproducing queen producing all the workers. In all colonies, the queen was mated by one male and so all workers were full sisters. This minimizes the expected amount of variation in individual CHC profiles due to the low within-colony levels of genetic variation.

(b) *Intra-colony CHC profiles*

The CHC profiles of the 10 study colonies were composed almost entirely (greater than 95%) of a C_{23}–C_{29} alkane and a Z9-alkene homologous series. Within each colony, the ratios (relative amounts) of any two successive compounds within a homologous series were always correlated (table 1). Successive Z9-alkenes were very highly correlated ($r^2 > 0.9$, table 1), whereas the correlation of successive alkanes were significantly lower ($t=5.3$, d.f.$=58$, $p<0.001$) and more variable (table 1). However, poor correlation was found between the two homologous series, i.e. a Z9-alkene and alkane with the same chain length (table 1). This is because the total alkane : alkene ratio of individuals within a single colony can vary enormously (from 1 : 0.3 to 1 : 3), even in colonies where workers are highly related. This high level of intra-colony variation in the total alkane : alkene ratio rules it out as a reliable nest-mate signal. Therefore, in *F. exsecta* the CHC colony profile consists of a single fixed distribution of Z9-alkenes and a more variable distribution of alkanes. The low intra-colony variation *within* the alkenes and alkanes makes their distributions potential nest-mate signals.

(c) *Inter-colony CHC profiles*

Each of the 10 *F. exsecta* colonies possessed an individual and distinctive CHC profile in 2007 (figure 1*d*,*e*) and 2006 (data not shown). Despite small changes occurring in the precise proportions of Z9-alkenes between 2006 and 2007, the colony-specific profiles based on the Z9-alkenes remained similar. Aggression between colonies of monogynous *F. exsecta* colonies was always high with significantly higher ($U=15$, $p<0.0001$) levels of aggression between non-nest-mates than nest-mates (figure 1*a*). Even colonies with very similar CHC profiles, e.g. colonies 53 versus 69 or 8 versus 60 (figure 1*d*,*e*), exhibited high aggression levels. The CHC analysis showed that the amount of intra-colony variation was significantly lower (Wilcoxon signed-rank test, $z=-5.488$, $p<0.0001$, $r=-0.78$) among the $C_{23:1}$–$C_{29:1}$ Z9-alkenes than in the corresponding C_{23}–C_{29} alkanes, i.e. pooled s.d.$=1.8\%$ for $C_{27:1}$ versus 3.1% for C_{27}. By contrast, the inter-colony variation of $C_{23:1}$–$C_{29:1}$ Z9-alkenes significantly exceeded (Wilcoxon signed-rank test, $z=-2.023$, $p<0.05$ $r=-0.9$) that of the corresponding C_{23}–C_{29} alkanes, i.e. pooled s.d.$=15\%$ for $C_{27:1}$ versus 6% for C_{27} (figure 1*d*,*e*).

(d) *Mimicking the nest-mate profile using synthetic compounds*

Focusing on the two colonies with the most different Z9-alkene profiles, one dominated by $C_{25:1}$ and the other by $C_{27:1}$ (figure 1), we used synthetic Z9-alkenes and alkanes to mimic the profiles of colonies 60 and 53. These synthesized profiles, or parts of them, were placed onto glass beads and the behaviour of nest-mate and 'non-nest-mate' workers recorded using an aggression bioassay. Coated beads with a nest-mate profile (Z9-alkenes plus alkanes) received significantly less aggression (col-60: $U=20$, $p=0.02$, $n=20$; col-53: $U=16$, $p=0.009$, $n=20$) from 'nest-mates' than 'non-nest-mates' (figure 2). Control (uncoated) glass beads received a similar level of aggression as beads with non-matching profiles in col-53 ($U=22$, $p=0.032$, $n=20$; due to Bonferroni correction critical, $p=0.025$) or were more aggressed in col-60 ($U=10.5$, $p=0.003$, $n=20$). Furthermore, in both colonies, beads coated with the colony's synthetic Z9-alkene profile received significantly less aggression from nest-mates than those with the synthetic alkane part of a colony's profile (col-60: $U=11$, $p=0.003$, $n=20$; col-53: $U=10$, $p=0.002$, $n=20$). In fact, in both colonies there was no significant difference in the response of nest-mates to uncoated beads and those coated in alkanes (col-60: $U=41$, $p=0.5$, $n=20$; col-53: $U=32$, $p=0.2$, $n=20$; figure 3). This indicates that the Z9-alkene component of the profile is eliciting the response.

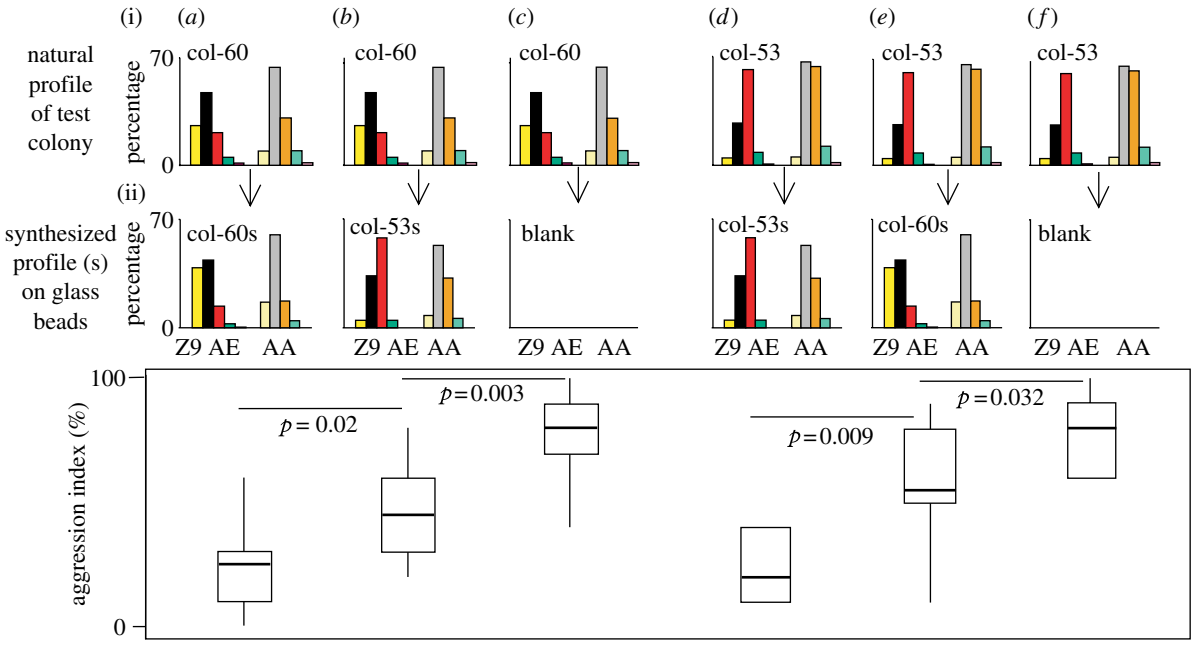

Figure 2. Response of ants from colonies 60 and 53 to beads coated with a synthetic profile based on their (*a*,*d*) nest-mates, (*b*,*e*) non-nest-mates or (*c*,*f*) blank glass beads. The CHC profiles (Z9-AE, alkenes; AA, alkanes) of (i) the test colony and (ii) on the glass bead are given. The box plots indicate the range, interquartile range and median scores of the aggression response shown by the 100 ant–bead interactions (10 beads per treatment group).

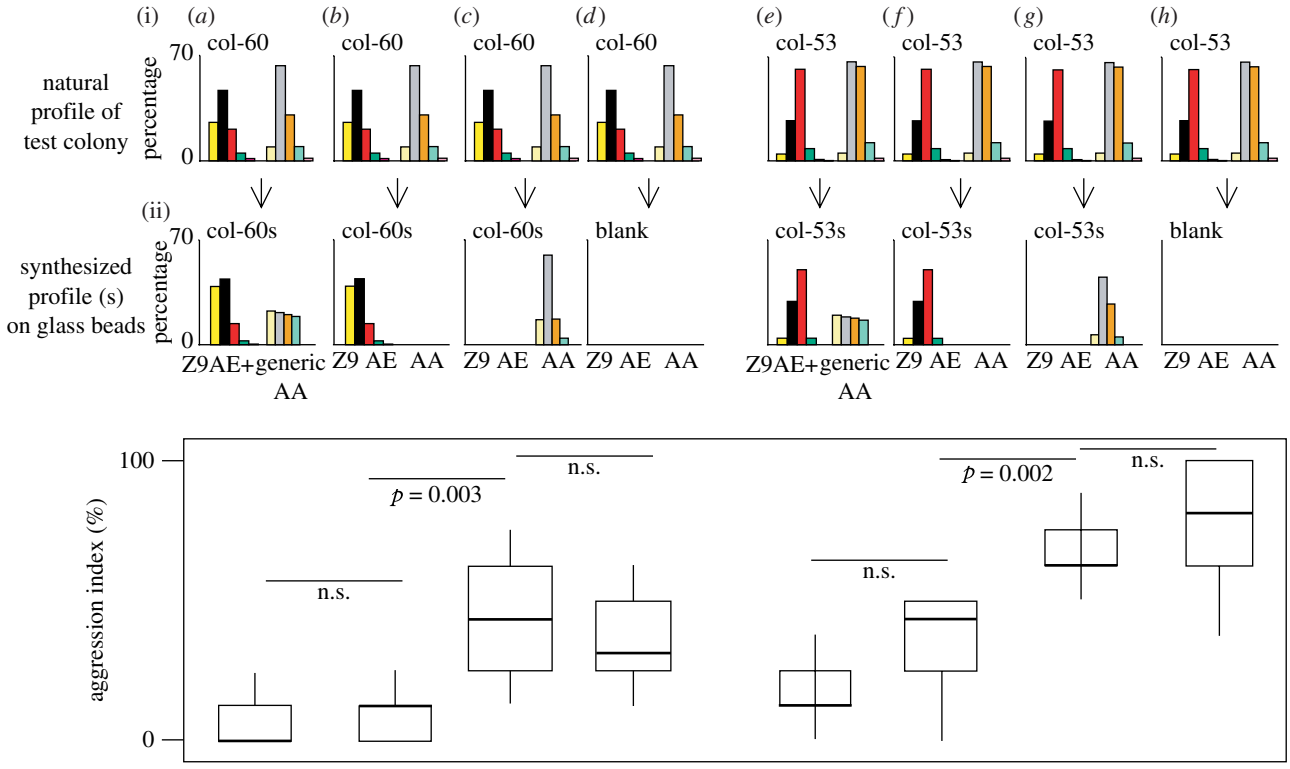

Figure 3. Response of ants from colonies 60 and 53 to beads coated with different parts of a synthetic profile based on their nest-mates. These were (*a*,*e*) a Z9-alkene + a generic alkane, (*b*,*f*) Z9-alkene only, (*c*,*g*) alkane only or (*d*,*h*) blank profiles. The CHC profiles of (i) the test colony and (ii) on the glass bead are given. The box plots indicate the range, interquartile range and median scores of the aggression response shown in the 100 ant–bead interactions (10 beads per treatment group).

(e) *Altering* in vivo *the nest-mate profile using synthetic compounds*

In both experiments and in a previous study (Akino *et al.* 2004), the beads that elicited the overall lowest level of aggression contained the nest-mate Z9-alkene and alkane profiles together. However, the effect of the alkanes occurs irrespective of their profile (figure 3) and they may simply make the beads appear more 'ant like', thus enhancing the effects of the Z9-alkenes. To solve this potential problem, we directly altered the CHC profiles of live individual nest-mates that were then allowed to freely interact with their nest-mates. We achieved this by the application of a single synthesized alkane or Z9-alkene via a hexane solvent onto their cuticle (figure 4*c*,*d*). A total of 150 ants were treated (5 colonies × 10 ants × 3 treatments). The five colonies chosen (col-8, 40, 53, 60 and 71)

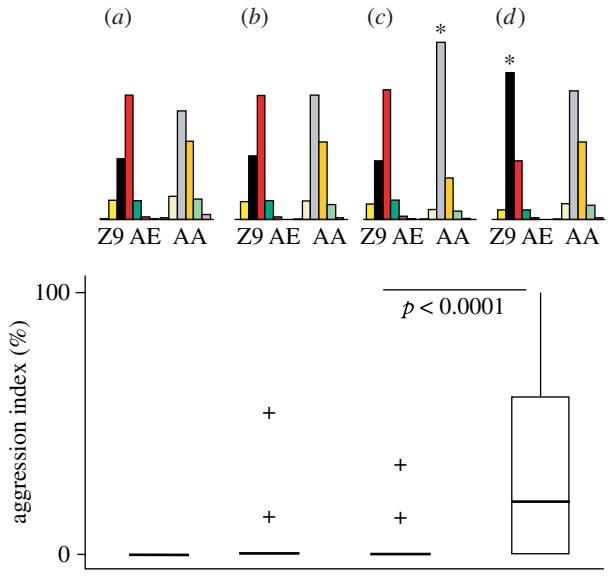

Figure 4. Profile of (*a*) an untreated *F. exsecta* ant and the effect on the profile of treatment with (*b*) hexane, (*c*) hexane containing a synthetic alkane (C_{25}) or (*d*) hexane containing a synthetic alkene Z9-$C_{25:1}$. The altered peaks are indicated by the asterisk. All profiles are of ants from colony 40 (figure 1) but aggression indices (box plots) are based on pooled data from five colonies (col-35, 40, 53, 60 and 71; $n=150$ ants (30 ants$\times$5 colonies)), with crosses indicating outliers.

represented the full range of Z9-alkene profiles (figure 1). In controls, the profile of hexane-treated ants remained unchanged (figure 4*b*). After being returned to their natal colonies, all treated ants were subjected to immediate attention from their nest-mates, which often involved bouts of intense antennation between both parties. There was no significant difference among the five study colonies in their response to the treated ants (Kruskal–Wallis, $H=7.6$, d.f.$=4$, $p=0.1$), but there was a highly significant difference between treatments ($H=56.3$, d.f.$=2$, $p<0.0001$). *Post hoc* tests indicated that the Z9-alkene-treated nest-mates elicited a significantly higher level of aggression than alkane-treated ones ($U=494$, $p<0.0001$). Whereas, alkane-treated nest-mates produced an aggression level not significantly different ($U=1226$, $p=0.8$) from the hexane-treated control nest-mates (figure 4). The high variability in the Z9-alkene data may be due to the difficulties in consistently applying small amounts of solvents to the ants. This was confirmed by the post-bioassay chemical analysis of 45 treated ants that revealed in two Z9-alkene and three alkane-treated ants, their profiles had not being changed. Both of these Z9-alkene 'treated' ants were not aggressed. However, of the 150 encounters with the highest level of aggression (attack or fighting), 86% involved nest-mates treated with a synthesized Z9-alkene. Again this strongly indicates that ants are responding to changes in the Z9-alkene but not the alkane part of the profile.

4. DISCUSSION

The initial chemical investigations and subsequent experiments demonstrate that in *F. exsecta* only the Z9-alkene part of the CHC profile is being used for nest-mate discrimination, which explains the precise and unique colony-specific distribution of Z9-alkenes found across the 10 study colonies in both 2007 (figure 1*d*) and 2006. The anti-desiccation role of long-chained (greater than C_{19}) alkanes is well known (Toolson & Kuper-Simbrón 1989), since their presence can reduce the insect's permeability to water by up to 1300% (Edney 1977). However, their function as discrimination cues in social insects has recently been questioned, since alkane extracts elicit a lower level of behavioural response than corresponding alkene extracts (Châline *et al.* 2005; Dani *et al.* 2005, but see Greene & Gordon 2007). Our results confirm this pattern. The Z9-alkenes: alkanes ratio in the *F. exsecta* study population could range widely within individuals from a single colony making it unsuitable for nest-mate discrimination. As all study colonies were monogynous and monandrous, the differences between individuals are not due to sampling different matri- or patrilines, but may reflect sampling workers that have been involved in different tasks that can be indicated by different alkane levels (Greene & Gordon 2003). This study demonstrates clearly that in *F. exsecta* alkanes have no role in nest-mate discrimination, despite their abundance on the cuticle. By contrast, alkenes are known to be involved in sexual communication (Howard & Blomquist 1982) especially in Diptera (Wicker-Thomas 2007), where Z9-$C_{23:1}$ is the sex pheromone of the housefly (*Musca domestica*; Carlson *et al.* 1971) and Z7-$C_{23:1}$ is an inhibitor of male–male courtship in *Drosophila melanogaster* (Tillman *et al.* 1999). The role of alkenes in nest-mate discrimination explains why the ratios of successive Z9-alkenes are so highly correlated ($r^2>0.9$) since there will be a strong evolutionary force to maintain unique colony alkene distributions found in each colony. Comparison of the two most similar pairs of alkene profiles (col-49 and 69) showed that there was still a significant difference in the proportion of at least one alkene ($C_{23:1}$, $p=0.002$) within the profile. However, bioassay data are needed to confirm whether these sometimes small but significant differences are biologically relevant. Therefore, bioassays are now required to establish the discriminative abilities of these ants with respect to changes in the alkene ratios. In this study, we used profiles from very different colonies to maximize the chances of finding differences, since we were unsure how fine the discrimination abilities of *F. exsecta* were. The low levels of intra-colony alkene variation suggest that these differences may be very small. Therefore, in future the natural and synthetic colony alkene profiles will need to be very closely matched.

Contrary to previous studies with ants (Akino *et al.* 2004; Greene & Gordon 2007), the blank glass beads we placed on the colony mound fragment were attacked rather than ignored. When disturbed, workers of species such as *F. japonica* (Akino *et al.* 2004) tend to run away. *Formica exsecta*, however, is a particularly aggressive species, with workers continuously patrolling the nest mound and readily attacking unfamiliar objects placed on the mound. During preliminary experiments, it was observed that when *F. exsecta* non-nest-mates were placed in a neutral arena they avoided each other, but would fight if placed on a nest-mound, indicating that their behavioural response is context dependent. This may help explain the overall reduced level of aggression observed in col-60 during the second experiment (figure 3), as the colony underwent a migration during the study, and the colony fragment used in the study was composed of both mound and migrating workers.

This study has, for the first time, combined data collected using well-established methods, with a new way of analysing CHC profiles. Our new approach has shown that Z9-alkenes are the nest-mate discrimination cues used by *F. exsecta* and this widens the role of alkenes in insect communication. It has long been suggested that nest-mate recognition information emanates from a social insect's CHC profile, but it has always been thought that discrimination was based on complex relationships between all the CHC compounds, making the CHC profile resembles a blurred barcode (Boomsma & Franks 2006). However, our identification of the nest-mate discrimination mechanism in *F. exsecta* has brought the blurred CHC barcode into sharp focus by showing that this species has evolved a highly sensitive nest-mate discrimination system based on relatively few compounds.

Many thanks go to Liselotte Sundström of Helsinki University for allowing access to the *F. exsecta* study population, Edward Jenner for help with the bioassays, Duncan Jackson and Kevin Foster for detailed comments. Funding for S.J.M. and F.P.D. was provided by NERC (NE/C512310/1) and to H.H. and L. Sundström by Academy of Finland grants (213821 and 206505), respectively.

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