Chem. Senses 30: 477–489, 2005

doi:10.1093/chemse/bji040
Advance Access publication May 25, 2005

Nestmate Recognition Cues in the Honey Bee: Differential Importance of Cuticular Alkanes and Alkenes

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Abstract

In social insects, recognition of nestmates from aliens is based on olfactory cues, and many studies have demonstrated that such cues are contained within the lipid layer covering the insect cuticle. These lipids are usually a complex mixture of tens of compounds in which aliphatic hydrocarbons are generally the major components. The experiments described here tested whether artificial changes in the cuticular profile through supplementation of naturally occurring alkanes and alkenes in honeybees affect the behaviour of nestmate guards. Compounds were applied to live foragers in microgram quantities and the bees returned to their hive entrance where the behaviour of the guard bees was observed. In this fashion we compared the effect of single alkenes with that of single alkanes; the effect of mixtures of alkenes versus that of mixtures of alkanes and the whole alkane fraction separated from the cuticular lipids versus the alkene fraction. With only one exception (the comparison between *n*-C_{19} and (*Z*)9-C_{19}), in all the experiments bees treated with alkenes were attacked more intensively than bees treated with alkanes. This leads us to conclude that modification of the natural chemical profile with the two different classes of compounds has a different effect on acceptance and suggests that this may correspond to a differential importance in the recognition signature.

Key words: cuticular hydrocarbons, cuticular lipids, honeybees, nestmate recognition

Introduction

The recognition and discrimination of nestmates from alien conspecifics is a well-documented feature of social hymenopterans. Exclusion of aliens from the colony allows it to (i) maintain genetic colonial integrity against conspecific reproductive females attempting to take over the nest [as in the case of intraspecific parasitism (Cervo and Dani, 1996)] or covertly entering the nest to lay eggs; (ii) preserve colony reserves against plundering of colony food by conspecifics (Winston, 1987; Yamaguchi, 1995, Jeral *et al.*, 1997); (iii) protect colony immature brood from conspecifics preying on (Driessen *et al.*, 1984) or abducting the brood (Le Moli *et al.*, 1993); and (iv) maintain control over a territory around the nest (Pfeiffer and Linsenmair, 2001).

Studies on the proximate factors at the basis of insect nestmate recognition have shown that the recognition cues involved are olfactory and are located on the surface of the insect body. Such cues may be both self-produced and exogenous (see Gamboa, 1996; Breed *et al.*, 1998). Self-produced cues may have both a genetic and an environmental component, for example depending on the type of food (Lian and Silverman, 2000), while exogenous cues may also have a genetic component (as, for example, the odours acquired from other nestmates or from the nest material produced by nestmates) as well as an environmental one. In some species, e.g. the honeybee, heritable self-produced cues appear to be of little importance in nestmate recognition, at least in a natural context, and guard bees appear to be unable to discriminate between related and unrelated conspecifics if these have lived within their hive during adulthood (Downs and Ratnieks, 1999). Among the environmental components, nest material (Breed *et al.*, 1998a) rather than food source (Downs *et al.*, 2001) and odours acquired from flowers (Bowden *et al.*, 1998; Downs *et al.*, 2000), appear to be the most important source of recognition cues.

In social insects, cuticular lipids have been reported as recognition pheromones involved in nestmate recognition (see

Lorenzi *et al.*, 1996; Breed, 1998a; Singer, 1998; Singer *et al.*, 1998) but also as cues involved in forms of intracolony recognition, such as task (Greene and Gordon, 2003), fertility (Peeters *et al.*, 1999; Liebig *et al.*, 2000; Sledge *et al.*, 2001a; Cuvillier-Hot *et al.*, 2002; Dietemann *et al.* 2003) and sex recognition (Cremer *et al.* 2002). Evidence of the importance of cuticular lipids as nestmate recognition pheromones derives from the common observation that their composition is less variable among nestmates than among individuals belonging to different colonies (see Breed, 1998a), and also from bioassays in which such lipids have been removed, reapplied or changed in their composition (see Breed, 1998a; Dani *et al.*, 2001; Ruther *et al.*, 2002; Breed *et al.*, 2004a,b). Additional indirect evidence derives from the study of cuticular lipids in social parasites; in fact, the social parasites studied so far either change their cuticular hydrocarbon profile to match that of the host when entering host colonies (Lenoir *et al.*, 2001; Sledge *et al.*, 2001b) or show a reduction in the quantity of their own cuticular hydrocarbons before invasion of the host colonies (Lenoir *et al.*, 2001; Lorenzi and Bagnères, 2002). Both these strategies are thought to improve the parasites' chances in the host colonies (Lenoir *et al.*, 2001).

Insect cuticular lipids are complex mixtures, composed mainly of long-chain hydrocarbons (Howard and Blomquist, 2005), though oxygenated compounds have been reported for several species (see Buckner, 1993). Although there is a large number of studies dealing with the chemical basis of recognition in social insects, and the many species for which cuticular lipids have been chemically characterized, very little is known on how insects perceive and process the information contained in the cuticular lipid mixture. This was highlighted in a review by Howard (1993), but since then only limited progress has been made. In fact, studying how such a complex stimulus, composed sometimes of tens of components, is perceived by insects and used in recognition contexts is a very challenging goal, which requires both reliable bioassays and the availability of the compounds to be tested, which may be difficult to isolate or to synthesize, e.g. branched hydrocarbons.

Attempts in this direction have been made for honeybees (Breed and Stiller, 1992; Breed *et al.*, 1992, 2004a; Breed, 1998a,b), for two social wasps (Dani *et al.*, 2001; Ruther *et al.*, 2002) and for an ant (Meskali *et al.*, 1995) through what Breed (1998a) has named 'chemical supplementation studies'. Many aliphatic compounds, belonging to different chemical classes (fatty acid esters, free fatty acids, alkanes and alkenes) have been tested on honeybees in recognition bioassays, in which both the bees applied with the compound (or compounds) to be tested and the bees to which the treated individuals were presented to (the discriminant bees) were isolated from the hive. Of these, many fatty acid esters, several free fatty acids and some hydrocarbons have been found to affect nestmate recognition significantly, but the picture emerging from these results is complex and it is difficult to extrapolate whether specific molecular features affect recognition more than others. In a recent paper, Breed *et al.* (2004a) performed supplementation experiments, with single or mixtures of free fatty acids, aimed at understanding the degree of deviation from the familiar recognition cues tolerated by guards. The authors found that although changes in concentration are generally tolerated, the total absence of one component often leads to non-acceptance by guards.

Floral oils, compounds of environmental origin, have also been tested for their effect on nestmate recognition in bioassays both on laboratory hive-isolated bees (Bowden *et al.*, 1998) and in the field (Bowden *et al.*, 1998; Downs *et al.*, 2000). While the application of floral oils to hive-isolated newly emerged bees affected their acceptance by nestmates (Bowden *et al.*, 1998), both the studies conducted in the field (Bowden *et al.*, 1998; Downs *et al.*, 2000) reached the same conclusion that floral oils do not affect nestmate recognition.

Only aliphatic hydrocarbons have been tested on social wasps (Dani *et al.*, 2001; Ruther *et al.*, 2002), although it has been demonstrated that other compounds, even those not naturally present in the cuticular lipid mixture, may affect nestmate recognition (Pickett *et al.*, 2000). In the social wasp *Polistes dominulus*, we recently investigated the effect on acceptance by nestmates of the supplementation in the epicuticlar lipid mixture of the three classes of aliphatic hydrocarbons present: linear alkanes, methyl branched alkanes and alkenes (Dani *et al.*, 2001). Using this approach, we found that linear alkanes never modified acceptance of treated wasps by their nestmates, while both alkenes and methyl branched alkanes did. However, in a later report on *Vespa crabro*, Ruther *et al.* (2002) found that application of *n*-C_{21} did modify acceptance by nestmates. A somewhat intermediate result has been obtained by Lorenzi *et al.* (2004), who found that linear alkanes modified acceptance by nestmates if applied to newly emerged *P. dominulus* females, but not if applied to older individuals.

In this work we have conducted bioassays, similar to those already performed on *P. dominulus*, on honeybees, focusing our interest on the hydrocarbon fraction of the cuticular lipids. The bioassays consisted of the manipulation of the cuticular hydrocarbon profile through supplementation of the concentration of alkanes or alkenes naturally present on the cuticle. The compounds used were either synthetic hydrocarbons or the whole alkane or alkene fraction obtained from the extraction of honeybees. Bioassays were conducted in the field, in the natural context of hives, by presenting live, non-anaesthetized treated foragers to their nestmate guards. Similar bioassays have been used by Downs and Ratnieks (1999, 2000), Downs *et al.* (2000, 2001) and Ratnieks *et al.* (2001).

Material and methods

Experimental apiaries

Experiments were performed on two different apiaries of *Apis mellifera ligustica*. Apiary A, consisting of 10 hives, was

located on the pre-Apennines at ~600 m above sea level in the Council of Vernio (North of Tuscany). Apiary B, consisting of 30 hives was located at 350 m above sea level in the Council of Pelago (35 km NE from Florence). All the colonies used for the experiments were queenright and housed in Dadant-Blatt hives with one box for honey storage on the top.

Bioassays

General description

The day before each bioassay, 70–140 2 ml glass vials were numbered. Pentane or pentane solutions (10 μl) of hydrocarbons were inserted randomly into the vials, the solvent was allowed to evaporate completely and the vials were capped. In each experiment 3–5 different treatments were performed. Each vial number and the compound or compounds it contained was recorded. The following day, 10 forager bees leaving the hive were collected from the front of their hive with a net and marked individually on the back of the thorax with small dots of water-based paint. Each single bee was then inserted into one of the vials prepared the day before and left there for 1 h. Gas chromatographic analysis of pentane extracts of bees treated in this fashion had been previously performed to verify that the treatment actually affected the cuticular hydrocarbon profile (see below). After this time the 10 vials containing the bees were brought back to the hive in a polystyrene box, which was slightly cooled in very hot weather. Each bee was individually returned to the landing board of its own hive. In order to minimize manipulation of the treated bee and disturbance of the hive, the open extremity of the vial was inserted in a hole of similar diameter drilled in the vertical boards contiguous to the landing board (experiments A and B); alternatively the open vial was gently laid on the landing board (experiment C). The introduced bees were not lethargic after having been in the polystyrene box, the temperature within the box being only slightly lower than the outside temperature. The intention of cooling was to avoid the bees overheating rather than to prevent them from flying away. In the majority of our experiments we presented nestmates, which tend not to fly away from the hives (F.R. Dani and S. Corsi, personal observation), unlike alien bees, which do and for which chilling is necessary [as in the experiments by Downs and Ratnieks (1999, 2000) and Downs *et al.* (2000, 2001)].

The behaviour of the nestmate bees toward each presented bee was observed for five min starting from the first interaction. If the presented bee left the hive or was chased away from it or entered inside it, before the end of the 5 min, recording lasted for a shorter time. If bees returned to the landing board within the 5 min, the recording of the behaviours resumed and continued until a total of 5 min was reached. Experiments were considered null if the bee left the hive without interacting and did not returned within 5 min. Moreover, since we wanted to be sure that hostile behaviours or acceptance occurred after close investigation of the introduced bees (and therefore that cuticular cues could have been perceived by the guards) and were not based on visual stimuli such as rapid exit from the vial, experiments were also considered null if the introduced bee was immediately chased away; if it immediately entered the hive without interacting; or if the number of interactions was <2. Deciding which experiments were null was made immediately while the experiment was performed. Although 50 replicates were planned for each single treatment, the null experiments sometimes lowered the final number of replicates considered in the data analyses. The number of replicates for each treatment is reported in Figures 1, 3 and 4. Experiments A and B were performed by two people, and the person observing the behaviour was unaware of the number of the vial in which the presented bee had been kept. Experiment C was performed by one single experimenter, who recorded the observed behaviours on a tape-recorder. In both experiments the researchers were not aware of the compound(s) contained in the vial of the presented bee. Bees subjected to different treatments were presented in a random order and never simultaneously, so that the behaviour of the guards towards each bee could be carefully observed. A few minutes (2–5) were allowed between the end of one experiment and the beginning of the next one. The treatment of each bee and the behaviour elicited from the guards were matched either at the end of the day or when all the experiments of a given series were completed.

Aliens versus nestmates presentation experiment

The same method was also used to test the behaviour of guard bees towards untreated nestmate and alien foragers. In this case foragers were collected from the hive on which we intended to perform the presentations and from a different hive of the same apiary. Bees were inserted individually into untreated vials, kept there for 1 h and then presented to the nest. In this experiment, the polystyrene box in which the vials were kept was at a lower temperature than in all the other experiments to prevent alien bees from flying away immediately from the hive to which they were presented. This experiment was performed a few days before the start of experiment A.

Effect of the treatment on the cuticle lipid profile

The effect of the enclosure in the vial treated with hydrocarbons was verified by comparing the gas chromatograms of the pentane extracts of bees kept for 1 h in vials containing 200 μg (deposited by evaporation of 10 μl of a pentane solution) of the compound (if a mixture of compounds was used, 200 μg was the total quantity) to be tested with the pentane extracts of a control bee (kept in a vial from which 10 μl of pentane only had been evaporated). After the 1 h enclosure, each bee was removed from the vial and frozen at −20°C. Bees were then extracted with 1 ml of pentane in

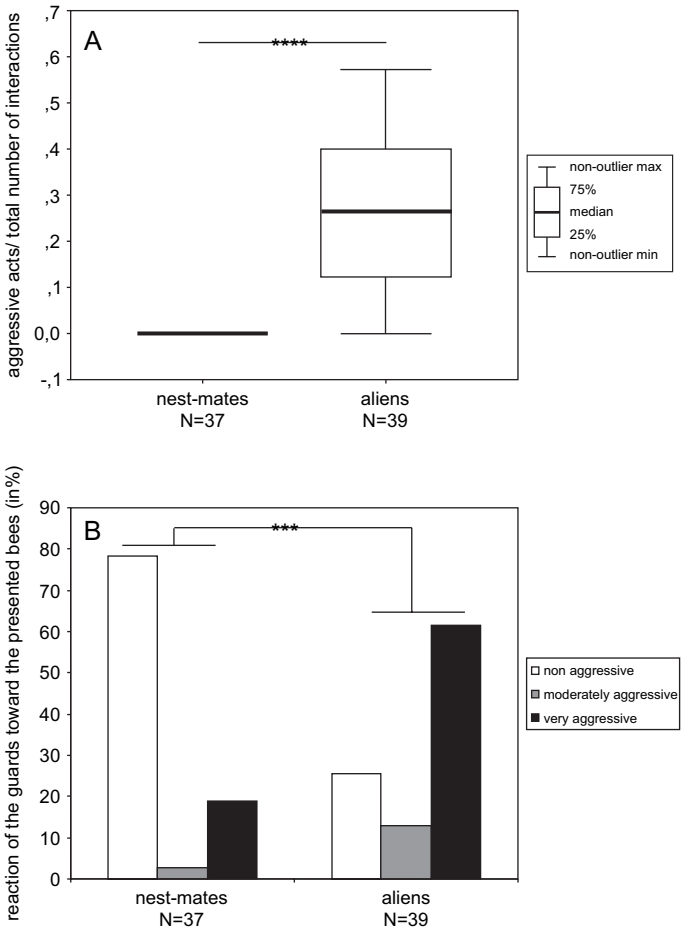

Figure 1 **(A)** Boxplot of the ratio between the number of aggressive acts (see text) and the total number of acts each bee, either nestmate or alien, received by the guard bees (Mann–Whitney test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$); **(B)** percentage of replicates where the presented bees, either nestmates or alien, were treated non-aggressively, moderately aggressively or very aggressively by guard bees, *G*-test: $***P < 0.001$; $****P < 0.0001$.

an ultrasonic bath for 10 min, solvent was then evaporated under a nitrogen stream and extracts were re-suspended in 100 µl of heptane. A 1 µl aliquot of the extracts was then injected in a gas chromatograph coupled to a quadrupole mass detector, with electronic impact ionization. The equipment used was the same as described in Sledge *et al.* (2001b). The oven temperature was increased from 70°C to 150°C at a rate of 30°C/min. This temperature was maintained for 5 min, before being raised to 300°C at a rate of 5°C/min.

Efforts were made to quantify the amounts of compound transferred to the bees; however, this proved difficult due to the variability both among treated bees and among untreated bees. It may be possible to re-extract compounds from the vials; however, this would require the use of a relatively large amount of solvent, which would have to be evaporated, and we suspect that the losses involved in the extraction and evaporation process are probably greater than the amounts transferred to the bees.

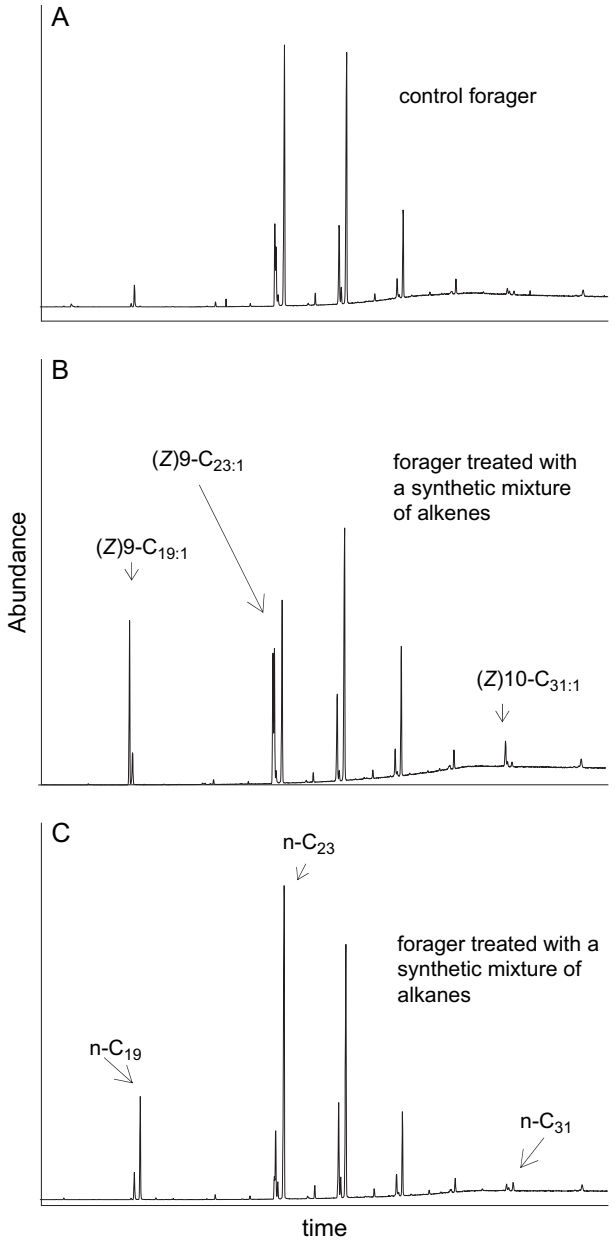

Figure 2 GC traces of the pentane extracts of three nestmate foragers, **(A)** the first untreated (bee kept for 1 h in a 2 ml vial previously treated with 10 µl of pentane and the pentane allowed to evaporate); **(B)** the second treated with a mixture of synthetic alkenes [bee kept for 1 h in a 2 ml vial previously treated with 10 µl of pentane containing 66.6 µg of (*Z*)-9-$C_{19:1}$, (*Z*)-9-$C_{23:1}$, (*Z*)-10-$C_{31:1}$ and the pentane allowed to evaporate]; and **(C)** the third treated with a mixture of synthetic alkanes (bee kept for 1 h in a 2 ml vial previously treated with 10 µl of pentane containing 66.6 µg of *n*-C_{19}, *n*-C_{23}, *n*-C_{31} and the pentane allowed to evaporate).

Experiment A

Choice of compounds to be tested in the bioassays

During spring 2000, 20 bees originating from two different colonies were collected in apiary A for the chemical analysis of their cuticular lipids. All these bees were collected from

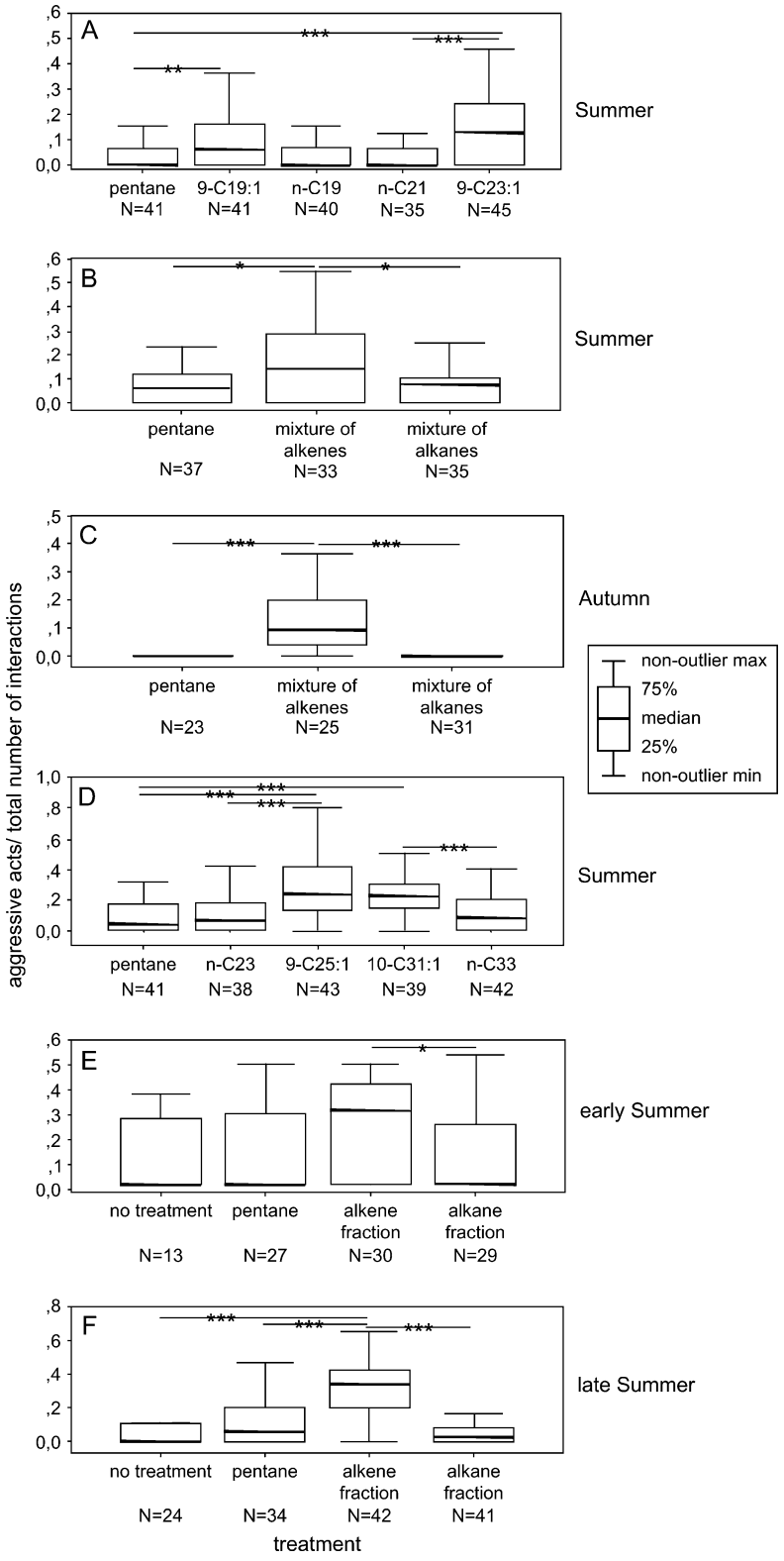

Figure 3 Boxplot of the ratio between the number of aggressive acts (see text) and the total number of acts each bee received by its nestmates **(A)** when treated with single compounds; **(B)** when treated with synthetic mixtures in summer 2001; **(C)** when treated with synthetic mixtures in autumn 2001; **(D)** when treated with single compounds selected in the stepwise discriminant analysis between colonies (see text); **(E)** when treated with the alkane and the alkene fractions of the cuticular hydrocarbons in summer 2002; and **(F)** when treated with the alkane and the alkene fractions of the cuticular hydrocarbons in autumn 2002. Mann–Whitney test with the Dunn–Šidàk correction: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

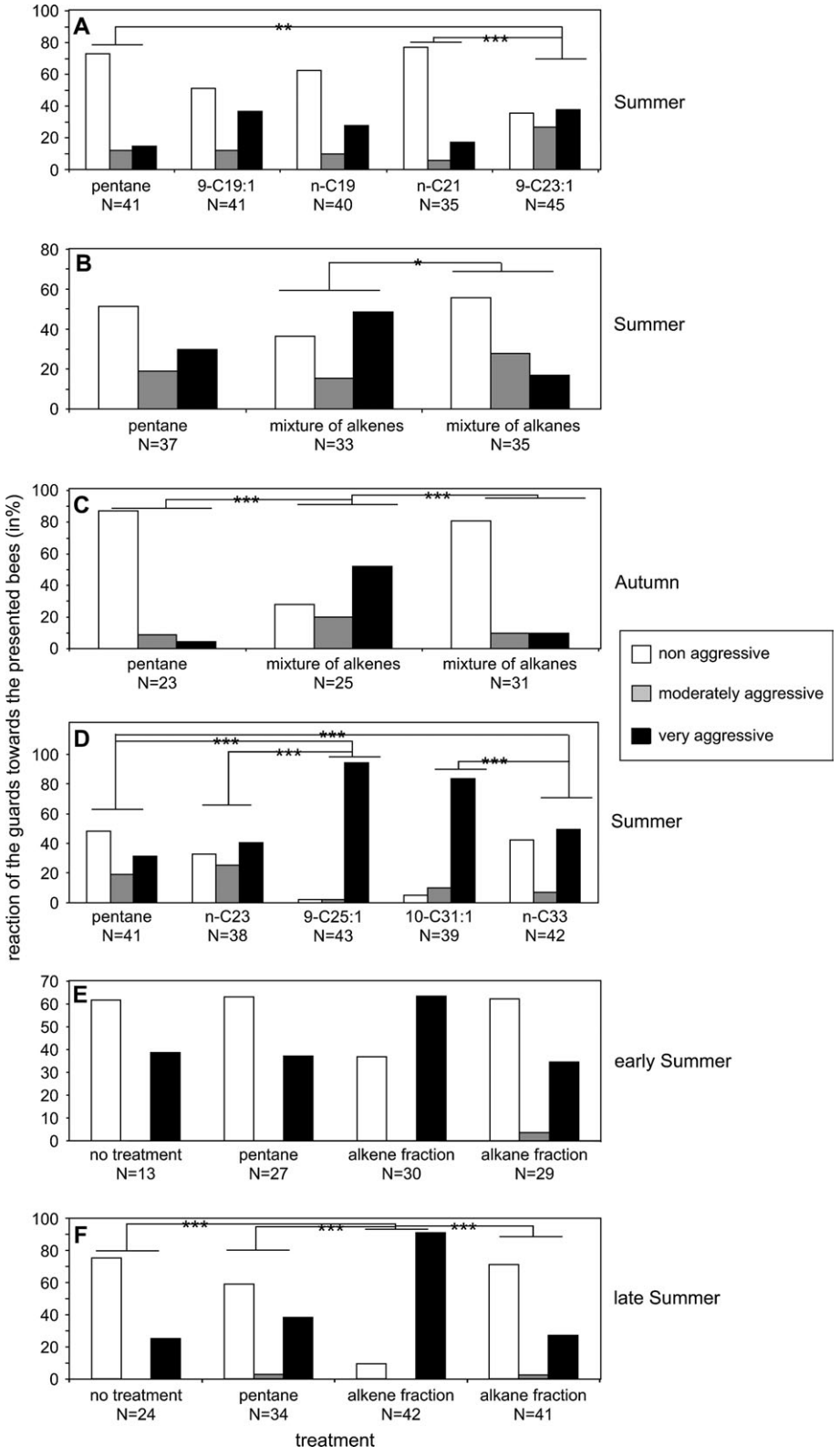

Figure 4 Percentage of replicates in which bees were treated non-aggressively, moderately aggressively or very aggressively (see text), **(A)** when treated with single compounds; **(B)** when treated with synthetic mixtures in summer 2001; **(C)** when treated with synthetic mixtures in autumn 2001; **(D)** when treated with single compounds selected in the stepwise discriminant analysis between colonies (see text); **(E)** when treated with the alkane and the alkene fractions of the cuticular hydrocarbons in summer 2002; and **(F)** when treated with the alkane and the alkene fractions of the cuticular hydrocarbons in autumn 2002. *G*-test with the Dunn–Šidàk correction: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

inside the hives. Results regarding the composition of the cuticular lipids of these specimens are reported elsewhere (Dani *et al.*, 2004). The compounds selected for testing were based on these results. We chose to test for each alkene an alkane with a similar concentration and a similar or equal chain length. By using these criteria, the following compounds were selected: (*Z*)-9-nonadecene [(*Z*)-9-$C_{19:1}$]; *n*-nonadecane (*n*-C_{19}), *n*-heneicosane (*n*-C_{21}), (*Z*)-9-tricosene [(*Z*-9-$C_{23:1}$], (*Z*)-10-hentriacontene [(*Z*)-10-$C_{31:1}$] and hentriacontane (*n*-C_{31}). The average percentages and standard errors of the chromatographic peaks of these compounds over the area of the 24 compounds considered in the chromatogram integration (see Dani *et al.*, 2004) were respectively 0.29 ± 0.07, 0.19 ± 0.04, 0.44 ± 0.06, 0.49 ± 0.13, 3.82 ± 0.43 and 7.93 ± 0.96.

Bioassays

(*Z*)-9-$C_{19:1}$, *n*-C_{19}, *n*-C_{21} and (*Z*)-9-$C_{23:1}$ were tested individually. A solution in pentane (20 μg/μl) was prepared and 10 μl of it was placed in the vials in which bees were due to be confined, while 10 μl of pentane only were used for the control experiments. Experiments were carried out on July 3–18, 2001 on a single hive, on which the alien versus nestmate experiments had previously been performed.

A pentane solution containing 6.66 μg/μl of each of the following alkenes (*Z*)-9-$C_{19:1}$, (*Z*)-9-$C_{23:1}$, (*Z*)-10-$C_{31:1}$ and a mixture containing 6.66 μg/μl of each of the following alkanes *n*-C_{19}, *n*-C_{21}, *n*-C_{31} were also prepared. 10 μl of solution were inserted in the vials. Experiments testing the effect of these solutions were carried out from July 30 to August 1, 2001 on a single hive and then repeated on October 15–26, 2001 on two different hives, one of which had been used in the summer. For each type of treatment, in the October experiments, the data of the two hives regarding the number of introduced bees eliciting reaction of different intensity by the nestmates (see Analysis of the bioassay data) were verified using a G test to not be significantly different (Sokal and Rohlf, 1995), and not to differ at the Mann-Whitney (Siegel and Castellan, 1989) for the ratio between the aggressive interactions and the total number of interactions received (see Analysis of the bioassay data), after which the data were cumulated.

Experiment B

Identification of the most relevant compounds in the discriminant analysis between colonies

Since the choice of the compounds tested in the previous experiment was not based on a presumed relevance they may actually have in nestmate recognition, we decided to perform a further experiment to test the effect of alkanes and alkenes whose relative abundance was important in separating different colonies, and therefore could, at least potentially, be relevant in nestmate recognition. To acquire this information, we collected, during the summer 2001, a total of 133 bees from four different hives of apiary A and analysed their cuticular lipids. All these bees were caught by placing a net in front of the hives and were therefore probably foragers. The identified compounds and their relative average abundance have been reported elsewhere (Dani *et al.*, 2004), while here (in the result section) we report the results of a stepwise discriminant analysis (using the SPSS 11.0 program) aimed to verify which compounds are more important in separating the foragers of the four colonies.

Bioassays

Among the compounds selected in the stepwise discriminant analysis we decided to use in the bioassays, 10-$C_{31:1}$, 9-$C_{25:1}$ and *n*-C_{23}. The compound *n*-C_{33} was tested as control for 10-$C_{31:1}$. These compounds were tested singly, by treating the vials with 200 μg of substance (diluted in 10 μl of pentane, which was evaporated), as in the previous experiments. Experiments were performed on July 12–23, 2002.

Synthetic hydrocarbons

The linear alkanes used in the bioassays were purchased either from Fluka or Aldrich. (*Z*)-9-$C_{23:1}$, (*Z*)-9-$C_{19:1}$ and (*Z*)-9-$C_{25:1}$ were all synthesized according to Dani *et al.* (2001). Synthesis of (*Z*)-10-$C_{31:1}$ was not possible using the Wittig method previously employed due to the lack of available starting materials. Therefore synthesis was achieved by stereoselective reduction of the corresponding 10-C_{31} alkyne. The alkyne was synthesized in 67% yield through coupling of 1-decyne with 1-bromoeicosane according to Savoia *et al.* (1981). Following the procedure of Chan *et al.* (1976) the alkyne was selectively reduced using Lindlar catalyst to the *Z*-alkene (yield 100%, determined by GC-MS analysis).

Experiment C

Fractionation of alkanes and alkenes from the cuticle lipid extracts

About 6000 bees were collected from different hives of apiary B during late spring 2002 and immediately frozen at −20°C. Groups of ~500 bees were placed in a beaker, pentane was added and the beaker was inserted in a ultrasonic bath for 10 min. Extract were then passed through a silica column and eluted with hexane to eliminate any polar compounds and the solvent evaporated.

A Flash 40 Biotage chromatographic cartridge was conditioned with CH_3CN, after which a solution of 10 g of $AgNO_3$ in 10 ml of CH_3CN was injected onto the column. The column was successively washed with ether (100 ml) and then hexane (300 ml). The bee extract was then applied to the column in 1 ml of hexane and the column eluted with hexane and 10 ml fractions were collected. Each fraction was spotted onto a silica TLC plate and visualized with $KMnO_4$ in order to locate

which fractions contained alkenes. Each fraction was then analysed by GC-MS. Fractions containing alkanes were combined the solvent evaporated on a rotary evaporator, and the material weighed and then redissolved in pentane. Those fractions containing the alkenes were treated similarly.

Bioassays

In these experiments the whole alkane and alkene fraction separated from the bee extracts were tested, using the same procedure as described for experiments A and B. A 10 µl aliquot of a pentane solution containing 20 µg/µl of the alkene or alkane mixture was placed in the vials in which bees were due to be inserted. As a control, untreated vials or vials treated with pentane only were used. Experiments were performed on July 25–29, 2002 and repeated on September 10–18, 2002. Experiments were performed on a single colony.

Analysis of the bioassay data

The reaction of the nestmates towards each treated bee was evaluated in two different ways. First we considered the number of aggressive acts towards the treated bee over the total number of acts registered (a measure of the intensity of the aggression). The following behaviours were considered aggressive: attacks (darting at the treated bee), biting, pulling (legs/antennae/wings) and fighting (when pulling and biting were very prolonged and when stinging attempts occurred). Statistical differences between the results obtained with different treatments were tested through the Mann–Whitney test. When the results of one treatment were compared more than once with the results of other treatments, significance thresholds were corrected with the Dunn–Šidàk correction (Sokal and Rohlf, 1995; $\alpha_1 = 1 - (1 - \alpha)^{1/K}$, where $\alpha = 0.05$, 0.01 or 0.001 and K is the number of times the same treatment is compared.

Secondly, using an analogous method to that described in the study on nestmate recognition in paper wasps by Dani *et al.* (1996), we scored the general behaviour of the colony in different categories of aggressiveness. The output of each single experiment was scored as (i) non-aggressive if aggressive behaviours had not occurred or if only one single attack had occurred; (ii) moderately aggressive if only attacks and bites had occurred and the total number of these acts was ≤4; and (iii) very aggressive if fights or pulling had occurred, or if the number of bites and attacks was >4. Statistical differences between the distributions in the three different categories were tested with the *G*-test with the Williams' correction (as recommended for small sample sizes, Sokal and Rohlf, 1995) and in case the results obtained for a specific treatment were compared with more than one other treatment, the level of significance was corrected using the Dunn–Šidàk correction (see above).

In several presentations guards managed to eject the presented bee from the nest, in some cases the guards flew away from the nest while holding the presented bee by its legs or wings, letting it drop several meters from the hive. Therefore we also considered for each treatment (i) the number of experiments in which the treated bee was ejected from the colony over the total number of experiments; and (ii) the number of bees entering the nests over the total number of experiments. Differences between treatments were tested with the *G*-test. These results are reported in the Results section only if statistically significant differences between treatments were observed.

Results

Aliens versus nestmate presentation experiment

The fraction of aggressive acts over the total number of interactions was found to be much higher for presented alien bees than for nestmates (Figure 1A). The distribution into the three classes of response was also significantly different, with more very aggressive reactions found for the alien bees (Figure 1B). A significant difference was also found between the number of aliens and nestmates entering and being ejected from the nest (18/37 nestmates entering the nest versus 9/39 alien bees, G with Williams' correction = 5.37, df = 1, $P < 0.05$; 3/37 nestmates ejected from the nest versus 10/39 alien bees, G with Williams' correction = 4.15, df = 1 $P < 0.05$).

Effect of the treatment on the cuticular lipid profile

The analyses of the chromatograms of the extracts of the bees inserted in the vials treated with the alkanes or alkenes and of those inserted in vials treated with solvent only showed that the former treatment was effective in modifying the cuticular hydrocarbon profile (Figure 2). It is of note that, as already highlighted in Dani *et al.* (2004), the relative concentrations of the different compounds (Figure 2A) differ from those reported elsewhere in the literature (Francis *et al.*, 1989) for the higher concentrations of low-molecular-weight hydrocarbons versus high-molecular-weight ones, in particular C_{31} and C_{33} alkenes. While the higher concentration of low-molecular-weight components may be due to contamination from the hemolymph (where they tend to be present in higher concentrations; see Francis *et al.*, 1989), caused by a harsher extraction procedure compared with that used by previous authors, the low concentration of C_{31} and C_{33} hydrocarbons is difficult to explain.

Experiment A

Figure 3A shows for each treatment the boxplot for the number of aggressive acts over the total number of acts recorded in the experiments with *n*-C_{19}, (*Z*)-9-$C_{19:1}$, *n*-C_{21}, (*Z*)-9-$C_{23:1}$ and pentane only. A similar distribution was obtained for pentane and the two alkanes, while higher median values were obtained for the two alkenes. The results obtained for both the alkenes were significantly different from those

obtained for the pentane. The results obtained for 9-$C_{23:1}$ were significantly different from those of *n*-C_{21}, while the results of 9-$C_{19:1}$ were not significantly different from those of *n*-C_{19}. Figure 4A reports for the same experiments the number of bees towards which the nestmate guards reacted non-aggressively, moderately aggressively and very aggressively (see above). The distribution into the three classes for bees treated with $C_{23:1}$ was significantly different from that obtained for pentane and for *n*-C_{21}, the $C_{23:1}$ treatment eliciting more moderate and very aggressive reactions. No statistical differences were found between the distributions obtained for *n*-C_{19} and 9-$C_{19:1}$ and between 9-$C_{19:1}$ and pentane.

Figures 3B, 4B, 3C and 4C show the results of the bioassays in which we applied a mixture of alkanes (*n*-C_{19}, *n*-C_{21}, *n*-C_{31}) or of alkenes [(*Z*)-9-$C_{19:1}$, (*Z*)-9-$C_{23:1}$, (*Z*)-10-$C_{31:1}$]; Figures 3B and 4B refer to the experiments performed in summer 2001, Figures 3C and 4C to the experiments performed in autumn 2001.

In both series of experiments, bees treated with the alkene mixture received a significantly higher number of aggressive acts (over the total number of interactions) than bees treated with the alkane mixture or with pentane. No differences were found between the pentane and alkane treated bees (Figure 3B,C). In the summer bioassays, the distributions in the three response categories differed between bees applied with the alkane mixture and those applied with the alkene mixture, but not between the latter and bees applied with pentane (Figure 4B). We first thought that the relatively high fraction of very aggressive reactions toward the pentane treated bees could be due to contamination of the pentane used, but GC-MS analysis showed that it was not contaminated.

In the autumn experiments, a strong difference was found in the distribution of the three behavioural categories between the pentane and alkene treated bees and between the alkane and alkene treated bees (Figure 4C).

Experiment B

Identification of the most relevant compounds in the discriminant analysis between colonies

The concentration of 24 compounds was considered in the discriminant analysis. Of them, 22 were hydrocarbons (10 monoenes, 11 linear alkanes, one methyl-branched alkane) and two were primary alcohols, (*Z*)-11-eicosen-1-ol and a shorter chain primary alcohol, possibly 1-hexadecanol. The discriminant functions obtained correctly assigned 77.4% of the bees to their colony and the variables entered in the analysis were the percentages of the following compounds: 10-$C_{31:1}$, *n*-C_{23}, 11-eicosen-1-ol, 9-$C_{25:1}$, 7-$C_{25:1}$, 8-$C_{31:1}$, 10-$C_{33:1}$, *n*-C_{24}, 9-$C_{23:1}$. The three discriminant functions accounted for the 100% of the variance, with the first one accounting for 59.3%, the second for 32.7% and the third for 8.0%. Among the selected compounds, we tested in the bioassays 9-$C_{25:1}$, *n*-C_{23} and 10-$C_{31:1}$. Considering the standardized canonical discriminant functions, 9-$C_{25:1}$ and *n*-C_{23} had similar coefficient values at least in the first function (respectively −0.422 and −0.328), and therefore contributed in a comparable manner to this function. *n*-C_{33} was used as control for 10-$C_{31:1}$.

Bioassays

Figures 3D and 4D show the results obtained in the experiments. Each alkene was found to differ significantly from its alkane-control and from the pentane both in the higher number of aggressive acts over the total number of interactions (Figure 3D) and in the higher frequency of moderately and very aggressive reactions (Figure 4D). In this set of bioassays, we also observed a strong difference between some treatments in the number of treated bees ejected from the nest and in the number of bees entering the nest. In fact, a significantly higher number of 9-$C_{25:1}$ treated bees (26/43) were ejected in comparison with pentane- and *n*-C_{23}-treated bees (5/41 and 7/38 respectively; *G* with Williams' correction = 22.06, df = 1, $P < 0.001$ for the 9-$C_{25:1}$ versus pentane comparison; *G* with Williams' correction = 15.8, df = 1, $P < 0.001$ for the $C_{25:1}$ versus *n*-C_{23} comparison). Conversely a significantly higher number of pentane and *n*-C_{23} treated bees (respectively 16/41 and 17/39) entered the nest compared with the $C_{25:1}$ treated bees (6/43; *G* with Williams' correction = 15.24, df = 1, $P < 0.001$ for the pentane versus $C_{25:1}$ comparison; *G* with Williams' correction = 6.53, df = 1, $P < 0.05$ for the *n*-C_{23} versus $C_{25:1}$ comparison).

Experiment C

Bioassays

Figures 3E,F and 4E,F report the results for experiments in which we presented bees applied with either the alkane or the alkene fraction of the bee extracts. In the July experiments, bees treated with the alkene fraction elicited both a higher number of aggressive acts over the total number of interactions (Figure 3E) and a higher proportion of very aggressive responses (Figure 4E) than all the other treatments; however, the only statistically significant difference was found between the alkene and the alkane fraction for the number of aggressive acts (Figure 3E). The results for all the other treatments (non-applied, pentane-applied and alkane fraction-applied bees) were very similar. A significant difference was also found between the alkene and the alkane fraction for the number of treated bees ejected from the nest (8/30 alkene-treated bees were ejected versus 1/29 alkane-treated bees, G with Williams' correction = 6.53, df = 1 $P < 0.05$).

Figures 3F and 4F show the results for the experiments performed in September 2002. Similarly to what had already been observed for the experiment using synthetic hydrocarbon mixtures (experiment A), the difference between the alkene fraction treatment and the other treatments were

clearer in the experiments performed in September than in those performed during July.

A highly significant difference was found between the bees treated with the alkene fraction and all the other treatments for the ratio between the number of aggressive acts and the total number of interactions (Figure 3F), and between the distribution in the three response categories (Figure 4F). A significant difference was found for the number of bees entering the nests between bees to which alkene fraction had been applied (5/42) and all the other treatments (9/24 non-treated bees, 12/34 pentane-treated bees, 16/41 alkane fraction-treated bees; G with Williams' correction = 5.56, df = 1, $P = 0.05$ for the alkene versus non-treated bees; G with Williams' correction = 5.80, df = 1, $P < 0.05$ for the alkene versus pentane comparison; G with Williams' correction = 8.17, df = 1, $P < 0.05$ for the alkene versus alkane comparison). A significant difference was also found in the number of ejected bees between the bees treated with the alkene fraction (14/42) and the pentane-treated bees (3/34; G with Williams' correction = 6.81 df = 1 $P < 0.05$), but not with the alkane-treated bees (5/41 ejected).

Discussion

These experiments demonstrate that by supplementing one individual alkene on a bee, the cuticular hydrocarbon profile is sufficiently changed for discriminant nestmate bees to act aggressively towards that individual or to eject it from the colony. Most importantly the level of supplementation was tested using GC-MS and found to have a moderate effect on the hydrocarbon profile, although it was not possible to fully quantify this data. One suggestion is that changes in the acceptance by guards may be caused by a masking or dilution effect of the alkenes over the 'true' recognition cues, which hinders the guards' perception. However, as there is no effect on acceptance observed when the corresponding alkanes were tested at the same concentration, a more probable explanation is that the alkenes are part of the profile of hydrocarbons which are the recognition cues.

This result holds for all but one of the experiments in which the cuticular hydrocarbon profile was modified through application of individual synthetic compounds, synthetic mixtures and fractions separated from cuticle extracts, the exception being the comparison between the (*Z*)9-$C_{19:1}$ and the *n*-C_{19} treated bees. Even in this case, the alkene-treated bees received more intense attacks than the alkane-treated bees, but the difference was not statistically significant. Moreover, in the analysis of the intensity of the attacks we found no significant difference between the alkane-treated bees and the untreated bees (confined in vials from which solvent alone had been evaporated or in untreated vials). In contrast, a difference between the alkene-treated and untreated bees was found in all but one experiment (the summer series of experiments C, in which the alkene and alkane fractions were tested).

Similar, but sometimes less definitive, results were found when we considered the proportion of treated bees suffering attacks ranked in different intensity categories (very aggressive, moderately aggressive or non-aggressive). The number of treated bees ejected from the hive was found to be significantly higher for the treatment with 9-$C_{25:1}$ than for that with *n*-C_{23}, and in the experiments with the separated fractions we found a higher number of ejected bees among the alkene-treated bees than among the alkane-treated bees.

A strong difference between alkenes and alkanes on the acceptance by guard nestmates has also been found in the experiments testing some of the hydrocarbons selected in the stepwise discriminant analysis between colonies. Therefore the differential effect appears to be general, with the only exception being the comparison between 9-$C_{19:1}$ and *n*-C_{19}. Breed and Stiller (1992; but see also Breed, 1998a,b) have reported that two medium-length chain alkanes, *n*-C16 and *n*-C18, affect the response of nestmates in the honeybee, while other alkanes of a similar chain length had no effect. In our experiments, however, the lack of a difference between 9-$C_{19:1}$ and *n*-C_{19} seems to be due to the facts that bees treated with *n*-C_{19} elicited more aggressive responses by guards compared with control bees (though this difference was not statistically significant) and that 9-$C_{19:1}$-treated bees were treated less aggressively than those treated with the other alkene tested simultaneously [(*Z*)9-$C_{23:1}$].

A difference was noted when the same experiment was performed in early summer compared with the repeats in late summer or at the beginning of the autumn, when a clearer response was obtained. In the early summer, because of the high temperatures, a large number of bees were often stationary on the landing board during the experiments. Although a majority of bees appeared completely inactive, this situation may have somehow disturbed the guard bees and resulted in less effective recognition. Downs and Ratnieks (2000) have reported that a shift in the acceptance by guards of bees presented in an experiment occurs during the season, with a higher tolerance both towards nestmate and alien bees when nectar abundance is high. The situation we observed differs, however, from the Downs and Ratnieks results in that we did not observe a general shift in the acceptance from summer to autumn, but rather a stronger aggression towards pentane and alkane treated bees during the summer than during the autumn, while aggression towards alkene treated bees did not change.

Despite a few results in which differences are not statistically significant, we found that there was generally a differential importance of alkene and alkane supplementation on acceptance by nestmates. This result is therefore in agreement with what has already been reported by Breed (1998a,b). In fact, the lack of an effect on the acceptance by nestmates has also been reported by Breed (1998a, b) for several alkanes, some of which occur naturally in the cuticular hydrocarbon mixture (*n*-C_{20}, *n*-C_{23}, *n*-C_{25}, *n*-C_{29}) and some of which have a shorter chain than those occurring

naturally (*n*-C_{12}, *n*-C_{14}, *n*-C_{15}, *n*-C_{17}). However, as already noted, two other alkanes of medium chain length (*n*-C_{16} and *n*-C_{18}) were found to affect recognition, as did both of the two alkenes tested (9-$C_{21:1}$ and 9-$C_{23:1}$). The lack of an effect by *n*-alkanes is, however, probably not due to a lack of perception, as Getz and Smith (1987) demonstrated that bees can be trained to differentiate between mixtures of *n*-C_{23} and *n*-C_{25} in the proboscis extension reflex (PER) protocol. This result was recently corroborated by Châline *et al.* (2005), who tested bees' ability to learn and discriminate between individual alkanes and alkenes found in the cuticular hydrocarbon profile. Using a PER protocol, they found that the bees were able to perceive both classes of compound, but that they preferentially learnt the alkenes over the alkanes and would discriminate for them.

A majority of correlation and removal and replacement studies on cues used in nestmate recognition have focused on cuticular hydrocarbons rather than on oxygenated cuticular lipid components which occur generally as minor constituents. We previously found that modifying the cuticular lipid composition by applying linear alkanes in *Polistes dominulus* does not affect acceptance into the colony, whilst the supplementation of alkenes and methyl-branched alkanes leads to aggression and rejection (Dani *et al.*, 2001). A study conducted on the ant *Cataglyphis niger* (Lahav *et al.*, 1999) has shown that the nestmate recognition cues are indeed contained within the hydrocarbon fraction, but not in the more polar fraction of the cuticular lipid extracts. However, a different picture seems to be emerging for the honeybees, for which oxygenated constituents, especially compounds acquired from the comb wax (Breed *et al.*, 1995, 2004a), have also been shown to be used as recognition cues (see Breed, 1998a,b). Breed *et al.* (1992) have shown that workers kept in a container containing long chain esters, such as those found in the queen faeces, were attacked by sister workers. A similar effect has been reported for some free fatty acids (Breed and Stiller,1992; Breed 1998a; Breed *et al.*, 2004a), although other compounds with similar structures did not have any effect. Using the PER protocol, Fröhlich *et al.* (2001) found that bees were unable to discriminate between cuticular hydrocarbon fraction of nestmate drones and workers, while they could be trained to discriminate between the more polar fractions of the cuticle extracts. The authors suggested that, although differences between the hydrocarbon fractions exist (Fröhlich *et al.*, 2000), the mixtures of cuticular hydrocarbons may be too complex for the bees to detect differences. In the context of nestmate recognition, however, our experiments show that bees are able to detect differences due to supplementation of one or more hydrocarbons, provided the applied compounds are alkenes.

It is also worth noting that in our GC-MS analysis the bees were extracted with pentane, the solution evaporated and then redissolved in heptane and the solutions were analysed without any purification or fractionation. Only occasionally have we found oxygenated compounds (Dani *et al.*, 2004), with the exception of (*Z*)-11-eicosen-1-ol and some free fatty acids, in contrast with the results of some previous authors (Blomquist *et al.*, 1980; Fröhlich *et al.*, 2001). Although our extraction and analytical method may have led us to underestimate the presence of oxygenated compounds, the very infrequent presence of aliphatic esters in our samples compared with that reported by previous authors is remarkable and needs further investigation.

Linear alkanes, methyl-branched alkanes and alkenes sharing the same chain length show very distinctive physical and chemical properties, such as molecular conformations and melting points (Gibbs and Pomonis, 1995). Gibbs (2002) has hypothesized that the presence of saturated and unsaturated hydrocarbons in the cuticular lipid mixture leads to phase separations with patches of microscopic solid areas, mainly constituted by alkanes, and of liquid areas, mainly constituted by alkenes, distributed on the cuticle surface and changing with the temperature. The same author has also hypothesized that the liquid areas may be more accessible to chemoreceptors. Such a model could support our finding of a differential importance in nestmate recognition of the alkane and alkene hydrocarbon classes.

Acknowledgements

Funding for this work was obtained through the research network 'Insects' of the University of Copenhagen, Firenze, Keele, Lausanne, Oulu, Regensburg, Sheffield, and the ETH Zürich, financed by the European Commission via the Research Training Network established under the Improving Human Potential Programme (HPRN-CT-2000-00052); through the Italian MIUR and the University of Florence (COFIN 2001 'Animal communication: an integrated study on chemical communication in social insects), and through the Italian MIPAF, within the project AMA (Api, Miele, Ambiente). The authors thank E.D. Morgan for reading and commenting on the manuscript.

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Accepted April 18, 2005