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Research article

Early learning of volatile chemical cues leads to interspecific recognition between two ant species

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Abstract. Nestmate recognition in social insects generally involves matching a label to the template that is acquired through the early learning of non-volatile cuticular hydrocarbon cues. However, a possible role of the volatile chemical cues that exist in the nest, and which may also affect template formation, has not been studied. We investigated this possibility using experimental mixed-species groups composed of the two ant species *Manica rubida* and *Formica selysi.* The experimental set-up either allowed full contact between workers of the two species or interspecific contact was hindered or prohibited by a single or a double mesh. After three months, workers of *M. rubida* ants were selected as focal ants for aggression tests including the following target ants: *F. selysi* workers from the same mixed-species group (for each of the three rearing conditions) or from a single-species group (control). Workers of *M. rubida* were always amicable towards their group-mates, irrespective of the experimental group (contact, single or double mesh). However, *M. rubida* that were not imprinted on *F. selysi*, expressed high levels of aggression towards the non-familiar *F. selysi* workers. The finding that *F. selysi* workers in the mixed-species groups appeared familiar to their *M. rubida* group-mates even without physical contact between them, suggests that the volatile cues produced by *F. selysi* affected nestmate recognition in *M. rubida.* In an attempt to identify these volatile cues we performed SPME analysis of the head space over groups of *F. selysi* workers. The findings revealed that *F. selysi* Dufour's gland constituents, with undecane as the major product, are released into the head space, rendering them likely candidates to affect template formation in *M. rubida.* Analysis of Dufour's gland secretion of *F. selysi* revealed a series of volatile alkanes, including undecane as a major product. These alkanes were not present in the glandular secretion of *M. rubida*, whose secretion was mainly composed of isomers of farnesene. We therefore hypothesize that callow *M. rubida* workers in the mixed-species groups had become imprinted by the above alkanes (in particular undecane, being the major heterospecific volatile in the head space) and incorporated them into their own template.

Keywords: Early learning, interspecific tolerance, volatile chemicals, *Manica rubida*, *Formica selysi*, Formicidae, Dufour's gland, undecane.

Introduction

Social insects have evolved a highly developed recognition system that enables them to behave altruistically towards nestmates but reject alien conspecifics (Hölldobler and Wilson, 1990). Such discrimination is largely based on chemical cues, primarily the cuticular hydrocarbons (CHCs) that constitute a colony-specific odour shared by all colony members (reviewed by Lenoir et al., 1999). Behavioural and chemical studies have demonstrated that this common odour blend requires the exchange of CHCs *via* trophallaxis and/or allogrooming, and that social isolation generally provokes rejection of the isolated individuals due to the lack of such ongoing exchanges (Soroker et al., 1994, 1998; Boulay et al., 2000, 2004; Lenoir et al., 2001). Similarly, experiments with artificial mixed-species groups showed that the species-specific recognition odour of the associated individuals is mutually modified through acquisition of the heterospecific odour components, thus exhibiting a mixed profile (Bagnères et al., 1991a; Hefetz et al., 1992). This chemical flexibility was also demonstrated in

xenobiotic and parasitic associations of ants (Lenoir et al., 1997; D'Ettorre et al., 2002), providing evidence of the ability of social parasites such as slave-making ants to match their CHC profiles to those of different host species.

However, species association can also exist when each species retains its own species-specific or even colony-specific recognition pheromones. This is, for example, the case with arboreal ant gardens that constitute two or three co-inhabiting species, which share the same trails without aggression but in which each species keeps its specific cuticular chemistry (Orivel et al., 1997). In parabiotic associations of ground-dwelling ants each species tends to keep its specific profile without eliciting interspecific aggression, therefore dismissing chemical mimicry as the basis of the peaceful co-existence (Errard et al., 2003). Similarly, inter-specific coexistence between the fungus-growing ants *Cyphomyrmex* and *Wasmannia auropunctata*, was explained by a dear-enemy phenomenon that may have resulted from a process of odour habituation (Grangier et al., 2007). To explain these phenomena, we hypothesised that lack of interspecific aggression between parabiotic associations results from familiarisation with the allospecific colonial odour. This may involve learning of the non-volatile heterospecific CHCs in species that have mutual contacts regularly, and/or in the absence of contacts, through learning of the heterospecific nest volatiles.

This hypothesis is supported by findings that both inter- and intra-specific recognition in two *Atta* species are mediated by alarm pheromone constituents, as well as substances from abdominal exocrine secretions (Hernandez et al., 2006). Akino and Yamaoka (2000) suggested that in *Lasius fuliginosus* volatiles act as a transient cue at short distances, while non-volatiles might serve as definitive signals for recognition of nestmates. Nest volatiles were also implicated in *Camponotus fellah* nestmate recognition (Katzav-Gozansky et al., 2004, 2008). However, the chemical nature of the volatile cues still remains uncertain.

Previous studies regarding the ontogeny of nestmate recognition in ants have shown the existence of a sensitive or critical period just after emergence, during which the young adults can be accepted into foreign nests (Jaisson, 1987, 1991). During this period, it was shown that the interactions (antennal contacts, allogroomings, trophallaxis) of the young adults with their host ants are essential for subsequent recognition of the introduced ants as nestmates. This critical period was also observed in artificially mixed-species groups, which can be formed only with callow ants (Fielde, 1903; Plateaux, 1960; Errard and Jaisson, 1984; Jaisson, 1991).

Generally, ants develop species-specific and colony-specific CHCs only after the first hours of their adult life (on cuticular chemical insignificance of young ants, see Lenoir et al., 1999), facilitating their integration into their colonies. During this period of early adult life (chemical integration; Lenoir et al., 1999), specific chemical cues may be transferred from older workers to the callow ants, thus imprinting them with the colony odour in which they emerged (Jaisson, 1991). The existence and/or acquisition of similar chemical profiles between individuals sharing the same nest forms the basis of a peaceful coexistence. Finally, the general hypothesis has been that within this familiarization and/or habituation period, the young ants are able to learn the odour of their nearest social environment, which strongly influences the recognition of colonial membership (Wilson, 1971; Hölldobler and Michener, 1980; Carlin, 1989; Stuart, 1992; Errard, 1994b, Errard et al., 2006).

The exact process of cue learning during the sensitive period still remains unclear. It is generally considered to be acquired through familiarization with nestmate cuticular hydrocarbons following inter-individual and/or nest surface contacts. However, familiarization with nestmate volatile chemical cues, without any contact taking place, may also occur.

The research reported here was designed to test inter-individual recognition in ants through volatile odour familiarization during their early social experience. To investigate the role of such volatile cues, we composed mixed-species groups of *Manica rubida* with *Formica selysi* under three rearing conditions: full contact, limited contact or no contact, by separating the two species by a single- or double-mesh screen, respectively. *Manica rubida* was selected as the focal species, taking advantage of the fact that its CHC blend is very distinctive from that of *F. selysi* (Hefetz et al., 1992). The behavioural observations were supplemented by chemical analyses of the transferred CHCs onto the focal ants, of the volatile substances in the group head space, and of their glandular origin.

Material and methods

Ant colonies and formation of experimental groups

Three colonies of *Manica rubida* (Myrmicinae) and three colonies of *Formica selysi* (Formicinae) were collected from the same habitat (Morillon, French Alps, altitude 700 m) in July 2002. The colonies were reared in the laboratory in blackened nesting tubes (180×17 mm) placed in a plastic box (280×275×85 mm) that also served as a foraging arena, and regularly fed with the same diet of honey and mealworms *ad libitum.* These constituted the parent-colonies from which the experimental groups were prepared. To prepare the mixed-species groups, callow workers of each species, less than 5 hours post-emergence, were relocated to plastic boxes (80.5×40 mm) and kept as described above. Three experimental groups were constructed: the two species were reared in a single compartment enabling full contact between all ants (n = 2); the two species were separated into two compartments by a single mesh that allowed limited contact between the ants (n = 6); or separated by a double mesh (inter-mesh distance: 0.6 mm) preventing such contact but allowing volatile compounds to pass between compartments (n = 6). Mixed-species groups were composed of 10–15 workers of each species. The single-species control groups were composed of 20 workers, also removed upon emergence from the same natal nest (two *M. rubida* and two *F. selysi* groups). Another mixed-species control group was constructed with 3-month-old workers in which both species were separated by a double mesh (n= 4). All groups

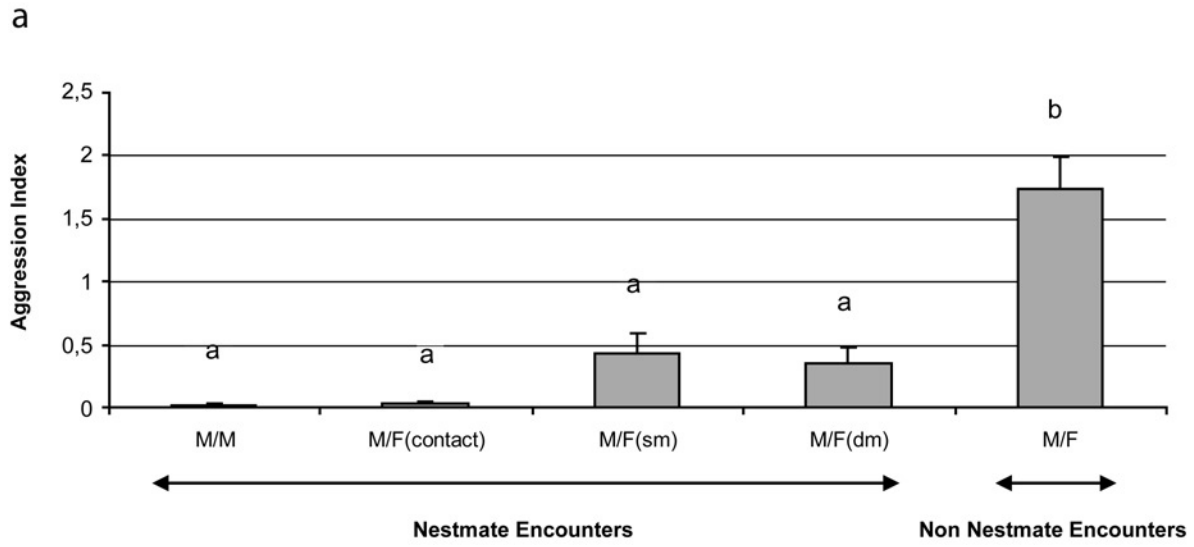

Figure 1a. The outcome of encounters (expressed as Aggressive Index) between workers of *Manica rubida* towards various types of target ant. For abbreviation of the various experimental conditions see Material and Methods. Different letters represent the groups that differed significantly. ANOVA, $F_{4,94} = 10.23$, $P < 10^{-4}$ followed by Newmann-Keuls Post Hoc test.

were kept queenless for at least three months before conducting the aggression tests.

Behavioural assays

The bioassays comprised dyadic encounters between a *M. rubida* worker taken either from a single-species group or from any of the mixed-species groups (for each of the three rearing conditions), and a target ant taken from a single-species group or from the same mixed-species group (group-mate for each of the three rearing conditions). Behavioural observations and scoring were as described in earlier studies (Errard and Hefetz, 1997; Errard et al., 2006). The number of replicate experiments were more than ten (10–28), with each individual tested only once in order to avoid possible effects of familiarization. The results were analyzed using ANOVA followed by Newmann-Keuls post- hoc test (Statistica for Windows 95 ©).

Abbreviations used in the text are as follows (origin of the test ant *M. rubida* / origin of the target ant): M/M – *M. rubida* workers from single-species groups encountering *M. rubida* nestmates (control); M/F (alien) – *M. rubida* workers from single-species groups encountering alien *F. selysi* workers from single-species groups; M/F (contact) – *M. rubida* workers from mixed-species groups, without mesh, encountering *F. selysi* group-mates; M/F (sm) – *M. rubida* workers from mixed-species groups, where the species were separated by a single mesh, encountering *F. selysi* group-mates; M/F (dm) – *M. rubida* workers from mixed-species groups, where the species were separated by a double mesh, encountering *F. selysi* group-mates.

Chemical analysis

Identification of cuticular hydrocarbons of *M. rubida* and *F. selysi* from single- and mixed-species groups was previously reported by Bagnères et al. (1991a) and Hefetz et al. (1992). For the present study we ascertained by GC/MS (Turbomass system (Perkin-Elmer, Norwalk, CT, USA) operated at 70eV) that the CHC profiles of the workers from our laboratory-reared colonies qualitatively matched with those previously reported.

In order to trap *F. selysi* worker-borne volatiles, we separated 20–30 workers from the parent colony in a glass pot, with an SPME fibre (85µm polyacrylate coating, SUPELCO Bellafonte, PA) positioned at the head space in a way that prevented any contact with the ants. After 24 h of the ants' presence in the pot, the fibre was desorbed in the GC/MS injector for 2 min at 250°C in splitless mode, and analyzed using a BP1capillary column that was temperature programmed from 60°C (2 min hold) to 300°C at 5°C/min with a final hold of 20 min.

For analyses of *F. selysi* and *M. rubida* Dufour's gland secretions, 20 workers were cooled for 15 min and then killed by freezing. The glands were cleanly dissected out under a binocular microscope, briefly dried, and immersed in pentane for extraction. The samples were analysed by GC/MS as above.

Results

Behavioural tests

Encounters between *M. rubida* workers and introduced *F. selysi*, both from single-species groups (positive control) were highly aggressive (Fig. 1a, M/F, $AI = 1.06 \pm 0.77$), whereas when encountering nestmates (negative control) the ants briefly antennated and departed peacefully (Fig. 1a, M/M, $AI = 0.01$) (ANOVA: $F_{4,94} = 10.23$, $p < 10^{-4}$). In contrast, in encounters between *M. rubida* from mixed-species groups no aggression was shown towards the *F. selysi* group-mates irrespective of treatment (full contact, limited contact *via* a single mesh barrier, or no contact *via* a double mesh barrier; Fig. 1a, $0.01 \pm 0.01 > AI > 0.43 \pm 0.16$). The aggression levels in these confrontations were not different from that of the negative control (Newman-Keuls, $F_{3,87} = 1.69$; $p = 0.92$; $p = 0.26$; $p = 0.29$). Mutual inspections of the encountering ants (measured as duration of antennal contacts) were always low except for the encounter between group-mates that had had previous contact (Fig. 1b, $F_{4,84} = 17.55$, $p < 10^{-4}$).

We repeated the double-mesh experiment (ants reared in compartmentalized groups without possibility of heterospecific contacts), but used 3-month-old *M.*

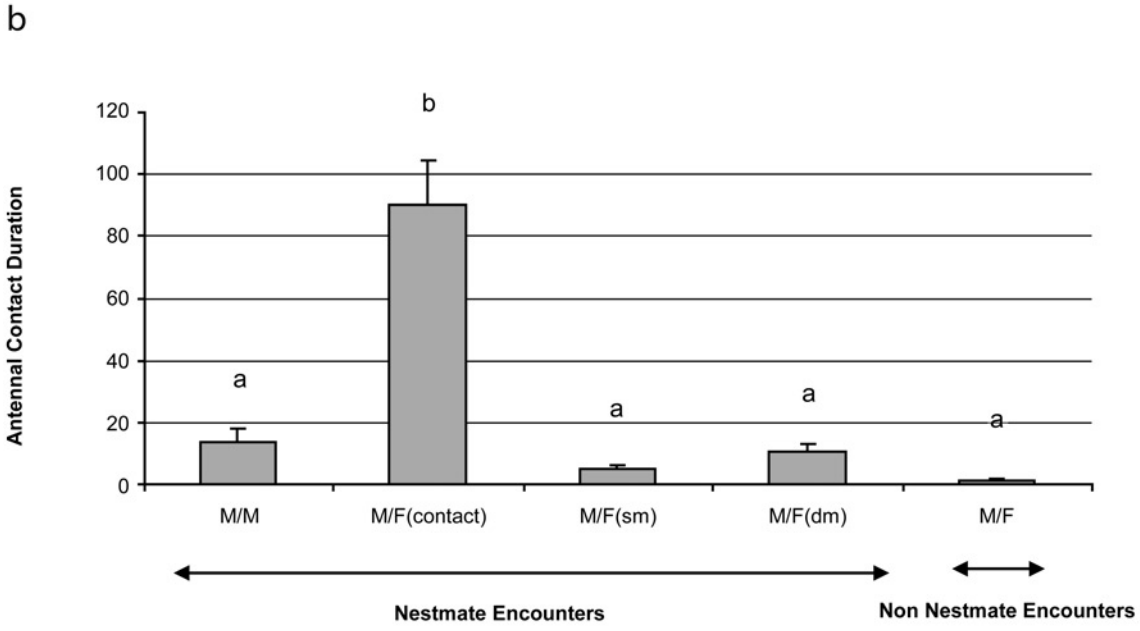

Figure 1b. Average inspection time (expressed as antennal contact duration in seconds) between the two encountered ants. Group abbreviations as described in Material and Methods. Different letters represent the groups that differed significantly. ANOVA, $F_{4,84} = 17.55$, $P < 10^{-4}$ followed by Newmann-Keuls Post Hoc test.

rubida and *F. selysi* individuals, rather than callows. In this case, the confrontations were significantly more aggressive even compared to individuals reared in homospecific groups (positive control) ($AI = 4.33 \pm 1.08$, $AI = 1.73 \pm 1.03$, respectively, $p = 0.001$).

Chemical analyses

Analyses of cuticular profiles of *M. rubida* reared in mixed-species group in full contact (without mesh) revealed that they had acquired about 23.0 ± 3.2 % of heterospecific hydrocarbons (essentially alkenes and alkadienes, which were absent in the single-species control *M. rubida*) from their *F. selysi* group-mates, confirming previous reports (Bagnères et al., 1991a; Hefetz et al., 1992). However, there were no signs of such transfer when *M. rubida* were separated from their *F. selysi* group-mates by either a single or a double mesh.

Discriminant analysis performed on relative proportions of the major hydrocarbon peaks of *M. rubida* workers reared under the four different conditions shows that individuals clustered together, forming three separate groups (Fig. 2; $F_{60,30} = 2.41$, $p < 0.004$). The first discriminant variable separated the ants from the mixed-species groups with full contact (M/F contact) from the other groups (single-species group, M/M; mixed-species group separated by a single mesh, M/F sm; and mixed-species group separated by a double mesh, M/F dm) ($p < 10^{-4}$), but these groups (M/M, M/F sm, M/F dm) did not differ significantly ($p = 0.102$).

Since the familiarization of *M. rubida* workers with *F. selysi* odours in the single- or double- mesh experiments could not be attributed to the acquisition of heterospecific CHCs, we investigated the possibility that it was the volatiles emitted by *F. selysi* that caused the effect. Analysis of the head space over a group of *F. selysi* workers, trapped by the SPME fibre, revealed the presence of both straight-chain and branched alkanes, of which undecane was the major constituent (75 %), accompanied by methyl branched alkanes, the sesquiterpenes E,E- and E,Z-farnesene, and a series of short fatty acids from C5 to C9 (Fig. 3a). Analysis of *F. selysi* Dufour's gland secretion revealed an identical series of hydrocarbons, of which undecane represented 87 % of the total gland content, in addition to several methyl-branched alkanes and at least one farnesene isomer, but not the fatty acids (Fig. 3b). The *M. rubida* Dufour's gland revealed several isomers of farnesene, as described before (Jackson et al., 1990), but there were no traces of the *F. selysi* n-alkanes.

Discussion

This study has shown that *M. rubida* recognized familiar *F. selysi* even when they had been reared behind a single or a double mesh, without any allospecific contact. The tolerance exhibited between the two species reared under such conditions did not differ from that observed when the two species had had full contact, and thus had had the possibility of perception and exchange of the allospecific recognition cues. This was in sharp contrast to the

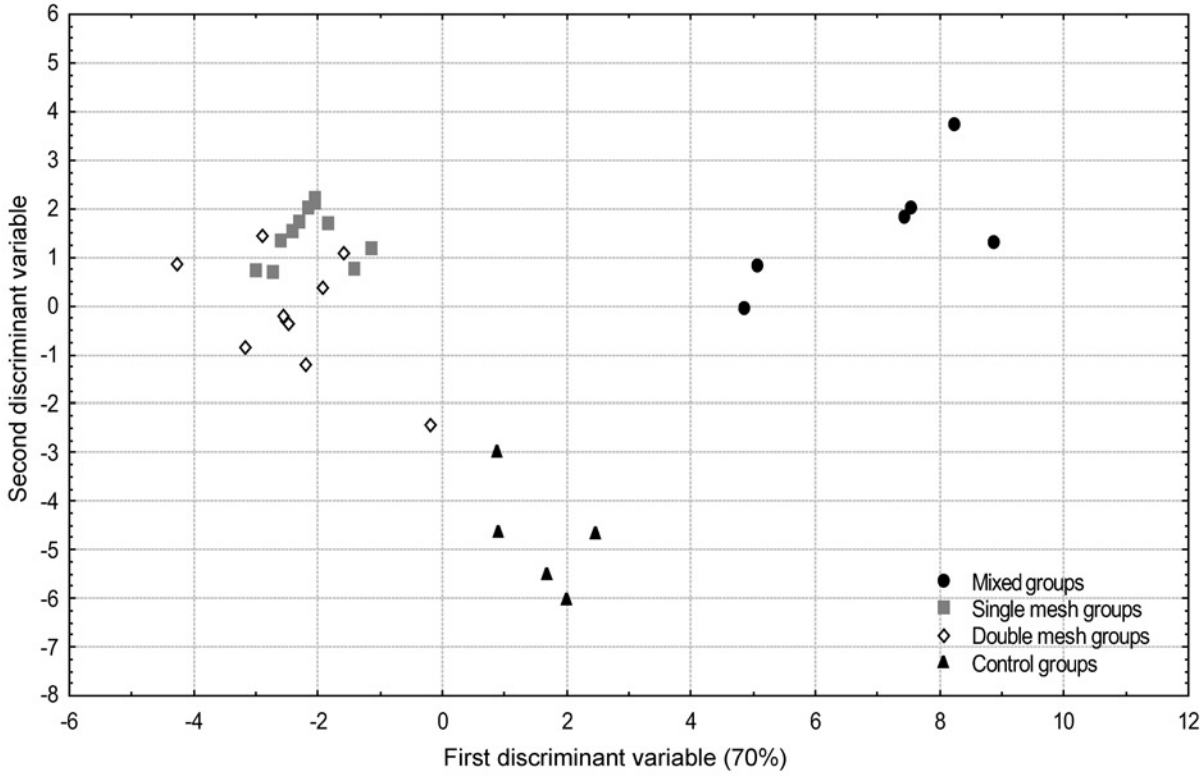

Figure 2. Discriminant analysis (Ward's method, Euclidean distances) conducted on the relative proportions of the 20 major hydrocarbon peaks of the cuticular profiles (C_{23}, 11 Me C_{23}, C_{24}, $C_{25:1}$, C_{25}, 11 Me C_{25}, 5 Me C_{25}, 3 Me C_{25}, C_{26}, 9 Me C_{26}, $C_{27:1}$, C_{27}, 11 Me C_{27}, 5 Me C_{27}, 5,17 diMe C_{27},$C_{28:1}$, 8,12 diMeC_{27}, $C_{29:2}$, $C_{29:1}$, $C_{31:2}$) of *M. rubida* workers from the three experimental groups (6 mixed-species groups: full contact, 11 single-mesh groups: limited contact, 9 double-mesh groups: no contact) and 5 from the single-species control *M. rubida* group.

aggression reported in encounters between *M. rubida* and *F. selysi* reared in single-species group, which generally culminated in the death of the *F. selysi* workers (Errard, 1994a,b).

It was previously reported that the tolerance observed in artificially mixed-species groups of *M. rubida/ F. selysi* can be attributed to mutual perception as well as exchange of the corresponding CHC, consequently creating a uniform group odour (Errard, 1994a; Hefetz et al., 1992; Vienne et al., 1995). However, under our experimental conditions, familiarization could not be explained by CHC exchange leading to a common group-odour, since such chemical transfer was not observed. This was unequivocal, since *F. selysi* possess large amounts of alkenes, which were completely absent in the single-species control *M. rubida* group but were present in *M. rubida* reared in the mixed-species groups. We could not detect any traces of these alkenes when either a single- or a double-mesh screen separated the two species housed together. These findings suggest that *M. rubida* reared in mixed-species groups without any contact with *F. selysi* workers recognized their allospecific group-mates by means of chemical cues other than CHCs. Moreover, the results of the double-mesh condition indicate that these chemicals are volatiles. SPME trapping of the head space revealed a series of compounds that are also found in *F. selysi* Dufour's gland in similar relative proportions. Although we do not as yet have causative proof, we suggest that these volatiles also emanate from Dufour's gland in the mixed-species groups, and occupy the head space of the groups. We do not as yet know the mechanism involved or the relationship between the CHCs and Dufour's compounds, but this finding is in line with previous suggestions that volatile cues too are involved in nestmate recognition (Jaffe, 1983; Hernandez et al., 2002; Katzav-Gozansky et al., 2004). The involvement of Dufour's gland in this process was also implied in studies with the red wood ant *Formica lugubris* (Cherix, 1983), and *Lasius fuliginosus* (Akino et al., 1995a,b), and *Camponotus fellah* (Katzav-Gozansky et al., 2008) in which the majority of the volatile components originate from Dufour's gland, and there was a correlation between levels of intraspecific aggression and qualitative variations in Dufour's gland contents.

Similarly, a number of studies have supported the idea that Dufour's gland contents of several *Formica* species contains species-specific linear hydrocarbons that act as alarm pheromones (Ali et al., 1987; Billen and Morgan, 1998). Likewise, Cherix (1983) reported that in *F. lugubris* Dufour's gland undecane was the major compound, comprising 50 % or more of the secretion.

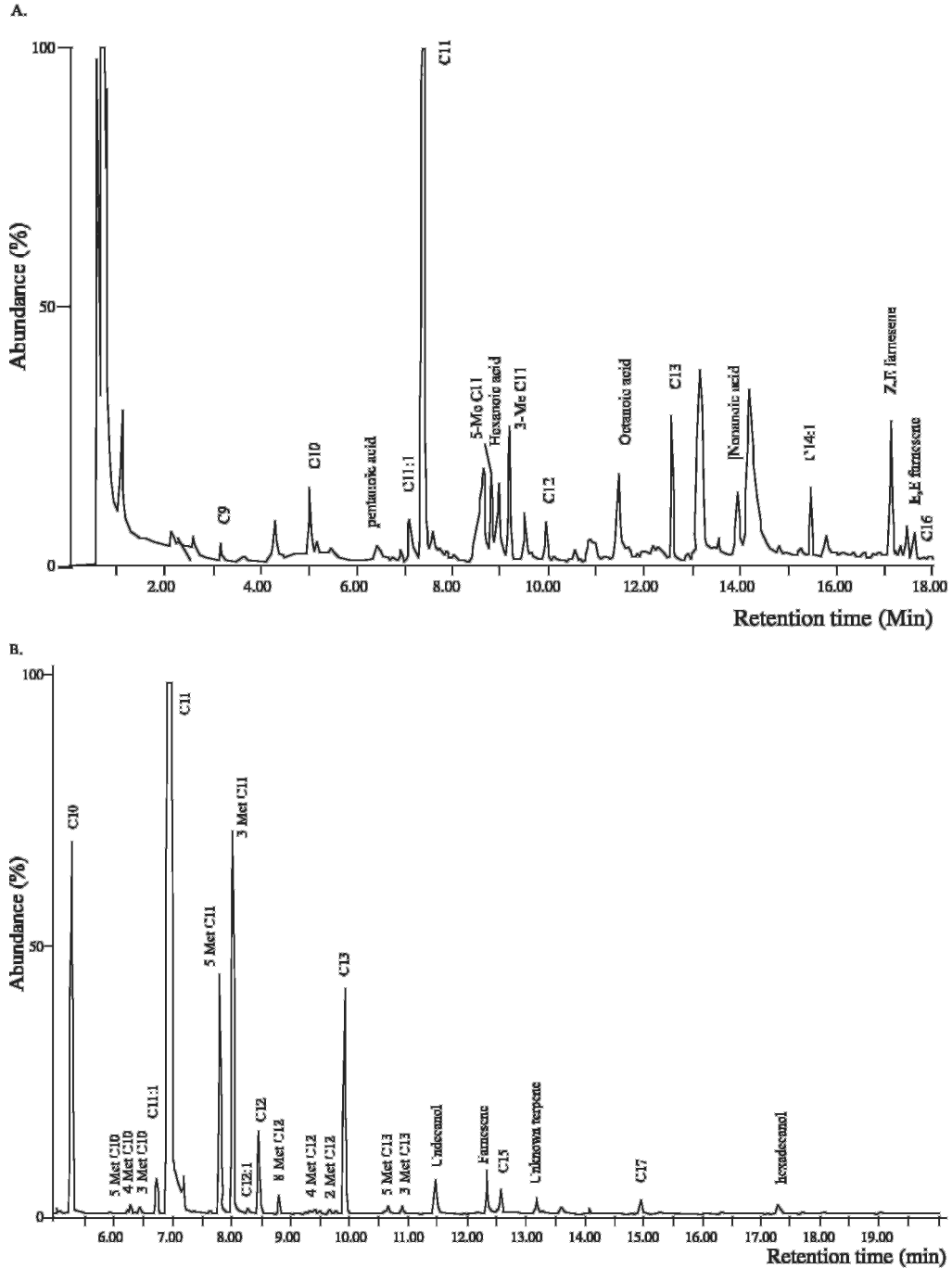

Figure 3. Total ion chromatogram of head space above a group of *F. selysi* workers, collected by means of SPME (A), and of Dufour's gland secretion of *F. selysi* workers (B).

Dufour's gland is by no means the only possible source of volatiles that may affect nestmate recognition. In *Atta laevigata*, the mandibular gland volatiles that act as an alarm pheromone were also reported to affect nestmate recognition (Jaffe, 1982; Jaffe and Marcuse, 1983; Jaffe and Sanchez, 1984; Hernandez et al., 2002, 2006). Those authors confirmed that nestmate recognition is based on cephalic odours and that these odours come mainly from the mandibular gland secretion.

In our case, only Dufour's gland volatiles were detected in the nest headspace. Analysis of *F. selysi* mandibular gland secretion by GC/MS revealed that its components were not present in the nest head space (unpubl. obs.).

A possible hypothesis explaining our behavioural findings is that *M. rubida* individuals could have learned the volatile compounds emitted by *F. selysi* Dufour's gland during the early stages of their life. In this case, inter-specific contact would not have been needed for

tolerance to obtain between the two species, as long as *M. rubida* could familiarize with *F. selysi* volatiles in the head space, and thus act amicably upon the recognition process. This, we suggest, was facilitated by the fact that *M. rubida* workers lack these alkanes, in particular undecane, in their glandular secretions (confirming previous data by Jackson et al., 1990). Such odour familiarization and imprinting must take place early in life, since when older *M. rubida* and *F. selysi* were reared in the same nest but prevented from having heterospecific contact, both species exhibited high aggressiveness. This corroborates earlier reports showing that the creation of mixed-species groups is possible with callow ants but not with older ones (Jaisson, 1987).

The differences noted during our observations seem to support the hypothesis that the perception of learned volatile cues permits a general recognition process that precedes the identification of CHCs by contact. CHC discrimination appeared to require more attention, presumably in order to identify, by contact, the specific and colonial signal that is composed of subtle quantitative variations of these elements.

We hypothesize that when the ants are constrained to cohabit near aliens, this reduces their threshold of reactivity. We suggest that this sensitization may be mediated by the volatile compounds emanating from, for example, the Dufour's glands of the alien *F. selysi.* In the same way, previous studies have shown that associative learning can induce stronger responses than those that are genetically programmed. For example, colonies of the ant *Pheidole dentata* react more strongly to *Solenopsis invicta* invasions than to the invasions of other ant species. In fact, after repeated exposure to *S. invicta*, the *P. dentata* increased their aggressive response (Carlin and Johnston, 1984).

It is interesting to note that similar aspects of odorant chemistry exist in mammals, in which straight-chained aliphatic alkanes, and mainly undecane, can serve as molecular features in the combinatorial coding of odorant information (Ho et al., 2006). Those authors suggested that, in rats, the olfactory system does not respond equally to all aspects of odorant chemistry, functioning as a specific, rather than a general, chemical analysis system. Finally, as the entire animal kingdom seems to be generally equipped for undecane perception, this indicates that *M. rubida* ants which do not have undecane in their environment, may not however have lost the neural circuit that enables discrimination of this olfactory signal, like other ants and other animals. *M. rubida* ants are therefore very sensitive to undecane. It will be interesting to test the hypothesis that young imago *M. rubida* can be familiarized to *F. selysi* by simple exposure to undecane.

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