

Research article

Odour convergence and tolerance between nestmates through trophallaxis and grooming in the ant *Camponotus fellah* (Dalla Torre)

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Summary. Social isolation provides a useful tool to study nestmate recognition in ants. In *Camponotus fellah*, reintroduction of 10-day isolated (IS) workers to their colony resulted in intensive trophallaxis and grooming, while longer isolation periods generally provoked rejection of the IS ants. In the first experiment the behaviour of queenless (QL) and queenright (QR) workers towards 10-day IS workers was tested. Trophallaxis of QL or QR with IS workers was of similar magnitude, but was significantly higher than that among the QL or QR, or that between QL and QR workers. Allogrooming was mostly initiated by the resident non-isolated ants (QL or QR) possibly because they detected a slight mismatch between the IS ant's odour and their own template, which represents the group odour. It appears that the presence/absence of the queen did not affect nestmate recognition cues of workers.

The second experiment demonstrated that 20-day IS workers were strongly aggressed by colony guards, irrespective of whether they were QL or QR. However, if they were permitted to exchange trophallaxis and grooming with 5 young nestmates (companion ants) for 5 days before reintroduction to their colony, aggression was greatly reduced, irrespective of the origin of the companion ants (QR or QL). Chemical analysis showed a significant divergence between the hydrocarbon profiles of IS and both QL and QR groups, but a prior contact of the IS workers with companion ants resulted in re-convergence of their profile with that of the colony. These results demonstrate that nestmate recognition cues are exchanged between workers via trophallaxis and grooming and that they are not dominated by queen cues, two conditions that fulfil *Gestalt* nestmate recognition signals requirements.

Key words: Nestmate recognition, trophallaxis, grooming, postpharyngeal gland, hydrocarbons.

Introduction

Nestmate discrimination is a cognitive process in which a worker accepts or rejects encountered conspecifics after comparing their external signature with its own template. In ants, several lines of evidence indicate that the nestmate recognition signal may consist of an assemblage of cuticular hydrocarbons (HCs) (Lahav et al., 1999; Thomas et al., 1999; Wagner et al., 2000; Review in Vander Meer and Morel, 1998 and Lenoir et al. 1999). Depending on the species, recognition cues may be produced equally by all workers, e.g. *Cataglyphis niger* (Lahav et al., 1998), or may be supplied predominantly by the queen, e.g. several *Camponotus* species (Carlin and Hölldobler, 1986, 1987). However, recent studies with *Camponotus fellah* indicate that the queen may affect colony insularity by controlling worker aggressiveness and social motivation rather than through direct production of colony recognition cues (Boulay et al., 2003). Irrespective of whether recognition cues are queen- or worker-derived, achieving a uniform colony odour system depends on inter-individual exchanges for cue homogenisation. However, in a queen-derived system it is predicted that recognition cue exchanges will be mainly directed from the queen to the workers surrounding her (i.e. brood- and queen-tending ants) and consequently to workers involved in more peripheral tasks (i.e. guards and foragers). In contrast, a worker-derived model does not assume specific directions of cue transfer since all colony members are supposed to be involved in the production of the signal.

In many species, nestmates were demonstrated to exchange HCs via trophallaxis and/or allogrooming, using their postpharyngeal gland (PPG) as a mixing organ (Boulay et al., 2000b; Lenoir et al., 2001; Soroker et al., 1994, 1998). Lahav et al. (1998) demonstrated that at least in *C. niger*, queens exhibit lower HC biosynthesis than workers but tend

to accumulate higher amounts of HCs in their PPG through receiving more than they give, which furnished strong biochemical evidence against the queen-derived hypothesis in this particular species.

Recent findings in *C. fellah* emphasized the importance of social interactions for integration of a worker in the colony. Workers individually isolated from the colony for up to 20 days have distinct PPG and cuticular HC profiles compared to their non-isolated nestmates. When reintroduced into the mother colony, these workers are generally aggressed by a group of residents, probably because intruders' cuticular HC composition does not match the guards' template. On the other hand, workers isolated for a shorter duration (3–10 days) engage in intensive trophallaxis upon return, which has been interpreted as a way to swiftly reacquire the colony odour (Boulay et al., 2000a). Trophallaxis, however, also has an appeasing effect (Heinze, 1996), which is not necessarily related to cue transfers, and could be motivated by the need to reduce the guards aggressiveness.

Although absence of contact with other nestmates (i.e. social isolation) or with the queen (i.e. queen deprivation) may occur rarely in nature, they provide useful paradigms to study the dynamics and origin of the nestmate recognition signal. The aim of the present study was to test whether trophallaxis and grooming promote the recognition cue exchange necessary for colony reintegration and to dissociate between the respective roles of the queen and the workers in the formation of the signal. The worker-derived model supposes long trophallaxis and allogrooming between isolated (IS) and queenless (QL) or queenright (QR) workers when compared to that between QL and QR workers. Moreover, IS workers that experience trophallaxis with QL or QR nestmates should then be accepted in both QL and QR nests. In contrast, the queen-derived model predicts that, in order to acquire rapidly the postulated queen signal, both IS and QL workers should engage in long trophallaxis and grooming with QR workers and that interactions with QL workers should promote IS workers reintegration in QL but not in QR nests.

Materials and method

Origin and maintenance of stock colonies

Source colonies of *C. fellah* containing one queen, at least 500 workers and a large brood were obtained from rearing newly mated queens collected in Tel Aviv (Israel) between 1997 and 1999. They were reared in a temperature controlled room ($29 \pm 2^\circ\text{C}$) under 12:12 h light-dark conditions. Colonies were installed in artificial plaster nests allowing direct observations of intra-nest activities. Each nest was connected to a foraging area (through a Tygon tube). Stock colonies were normally reared under equal diet of dead insects (mealworms, flies and moths) and carbohydrates (20% w/v sucrose solution or honey) supplied twice a week.

Experiment 1

Eight colonies composed of 500 to 1500 workers were divided equally into QL and QR groups with the same quantity of brood. In addition, 4 to 5 workers from each of the colonies were individually isolated in Petri dishes (3×0.5 cm). Although only 24 IS workers were later tested in

this experiment, more individuals were initially isolated to compensate for the possible death of more than a third of the workers during the isolation period. All the ants (QR, QL and IS) were reared under the same conditions of light and temperature as stock colonies and had permanent access to sugar water (20% w/v) but not to dead insects which can affect the cuticular HC composition (Liang and Silverman, 2000).

Dyadic encounters were conducted 10 days after the colonies were divided and consisted of 6 types of reunion in which an 'intruder' individual (IS, QL or QR) was encountered with a 'resident' nestmate (QR or QL). All ants were individually identified by colour marks applied on the thorax. Prior to each encounter, each tested ant was placed in a separate clean test tube (1×10 cm) closed with a cotton plug. After the ants had acclimated (i.e., when they had stopped moving rapidly in the tube for about 5 min), the test tubes were opened and carefully connected to allow contact between the workers. The duration of trophallaxis and grooming was recorded for 10 min using an automatic event recorder. The directionality of grooming was noted, (i.e., intruder performing grooming or being groomed), but not that of trophallaxis, which was too equivocal.

Experiment 2

Five colonies were equally divided into QR and QL groups while 200 workers were isolated as described in experiment 1 (although only 109 workers were used in the behavioural and chemical tests, about twice this number were initially isolated to ensure sufficient sample size after isolation). On day 20 post-separation, three sets of IS workers were marked with a dot of paint. Workers from set 1 were reintroduced directly into either the QR or the QL parts of their original colony. IS workers from sets 2 and 3 were confined for 5 additional days with 5 companion ants taken from either a QL or a QR group, respectively. Companion ants were brood-tenders with visible fat body reserves, selected from the brood pile. Brood-tenders generally express low aggressiveness and so were unlikely to attack the IS workers. Nonetheless, the extremities of their mandibles were slightly clipped to prevent possible fatal biting at first contact. The companion ants were replaced daily to enhance HC composition update. IS workers housed with QL and QR companion ants are referred to as IS(QL) and IS(QR), respectively. Two subsets of 10 IS(QL) and 10 IS(QR) were randomly chosen to record their trophallaxis and allogrooming events with the companion ants 5 times daily for 5 min at intervals of at least 30 min. On day 25, IS(QL) and IS(QR) workers were individually introduced into the QL and QR parts of their original nest for behavioural observations, with a delay of at least 3 h between the introduction of two ants into the same nest. The interactions of QL and QR resident ants with IS workers (day 20), and with IS(QL) and IS(QR) workers (day 25) were recorded for five periods of 5 min every 25 min from the moment of introduction. During each 5-min period, snapshots of residents' interactions with intruder ants were recorded every 10 s. The behaviours recorded included antennal exploration, allogrooming, trophallaxis and aggression (flexion of the abdomen with/without opened mandibles and bite).

For one colony, PPG HC-contents were sampled on day 20 post-separation for IS workers ($n = 7$) and on day 25 post-separation for QL ($n = 6$), QR ($n = 6$), IS(QL) ($n = 6$) and IS(QR) ($n = 6$) workers. The analysis of the PPG content was preferred to that of the cuticle since both have very similar profile (Boulay et al., 2003), and to avoid possible contamination from the colour marks of the individual ants.

Chemical analysis

Chemical analyses were performed using workers from one colony only. After the ants were killed by freezing (-20°C), their PPG were dissected in distilled water and immersed in 0.5 ml of pentane. The solutions were stored at -20°C until analysis. For analyses, the samples were evaporated to dryness and re-dissolved in 50 μl of pentane, of which 1 μl was then injected into the gas chromatograph equipped with a DB-5 fused silica capillary column (temperature programmed from 100°C to 280°C at $3^\circ\text{C}/\text{min}$). The identity of the eluting compounds was pre-

viously determined by gas chromatography coupled to mass spectrometry and published by Boulay et al. (2003).

Statistics

In experiment 1, trophallaxis durations and summed durations of given and received allogrooming of intruder ants were compared using two-way ANOVA (factor 1: intruder treatment (IS, QL or QR); factor 2: resident type (QL or QR)). Inter-group differences were tested with Newman-Keuls post hoc test. For each type of dyadic encounter, the durations of given and received grooming were compared with Student t-test for dependent variables. In experiment 2, frequencies of allogrooming and trophallaxis between companion and IS ants during the 5 day period of contact were compared with one-way ANOVA for repeated measures (factor 1: companion type (QL or QR); repeated factor: day (1 to 5)). Frequencies of recorded behaviours during ant reintroduction into QR and QL nests were compared using two-way ANOVA (factor 1: intruder type (IS, IS(QR) or IS(QL)); factor 2: residents type (QR or QL)).

For the discriminant analysis we used peaks that represented more than 1% of the total HCs (see Table 2 for list of peaks used in the analysis). By using a forward stepwise discriminant function analysis we selected the peaks that were most significant for between-groups discrimination ($F > 1$; marked in bold in Table 2). The resulting 7 peaks served for constructing the scatter plot in Figure 4, and calculating the Squared Mahalanobis distances between the centroids of the groups. Comparison between the groups was done by ANOVA.

Results

Experiment 1

Trophallaxis involving IS intruders was significantly longer than that involving either QL or QR intruders (Fig. 1; Two-way ANOVA, intruder type: $F_{2,73} = 13.15, p < 0.0001$; Newman-Keuls test: $p = 0.018$ and $p = 0.012$ for IS vs QL and QR, respectively). In contrast, the duration of trophallaxis was not affected by resident type (Two-way ANOVA, resident type: $F_{1,78} = 0.002, p = 0.960$). More specifically, IS intruders always performed a large amount of trophallaxis with QR or QL residents while trophallaxis between QR and QL ants was low. These latter ants spent the majority of time in self-focused activities.

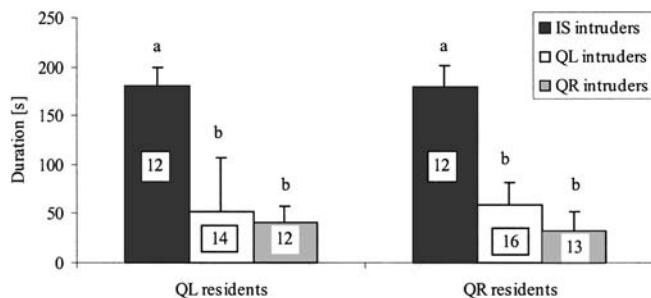


Figure 1. Mean duration (\pm SEM) of trophallaxis between intruder ants (IS, QL and QR) and resident ants (QL and QR) in dyadic encounters, after 10 days of isolation. Numbers in the bars indicate sample size. Different letters denote significant differences (Newman-Keuls post hoc test, $p < 0.05$)

The cumulative duration of grooming (given plus received) was not affected by resident type (Table 1; Two-way ANOVA, resident type: $F_{1,73} = 0.25, p = 0.618$) but was significantly affected by the intruder treatment (Two-way ANOVA, intruder treatment: $F_{2,73} = 3.28, p = 0.043$): grooming acts were significantly more numerous with IS than with QR intruders (Newman-Keuls test: $p = 0.027$). They were intermediate with QL intruders and not significantly different from IS or QR ants (Newman-Keuls test: $p = 0.145$ and $p = 0.246$, respectively). The direction of grooming was also affected by the type of intruder. IS intruders were significantly more groomed by their QL or QR counterparts than vice versa (Table 1; t-test, $p = 0.036$ and $p = 0.0003$). In contrast, the duration of given and received grooming was almost equal in the dyadic encounters involving QL or QR intruders (see Table 1 for detailed statistics).

Experiment 2

Twenty-day isolated ants confronted with 5 non-isolated nestmates (companion ants) engaged in frequent trophallaxis irrespective of whether the companion ants were QL or QR (Fig. 2a, Two-way ANOVA, companion type: $F_{1,18} = 1.08, p = 0.31$). However, the frequency of trophallaxis significantly declined from day 3 on (Two-way ANOVA, repeated factor: $F_{4,72} = 18.17, p < 0.001$). Allogrooming was also significantly more frequent on the first day of contact than on

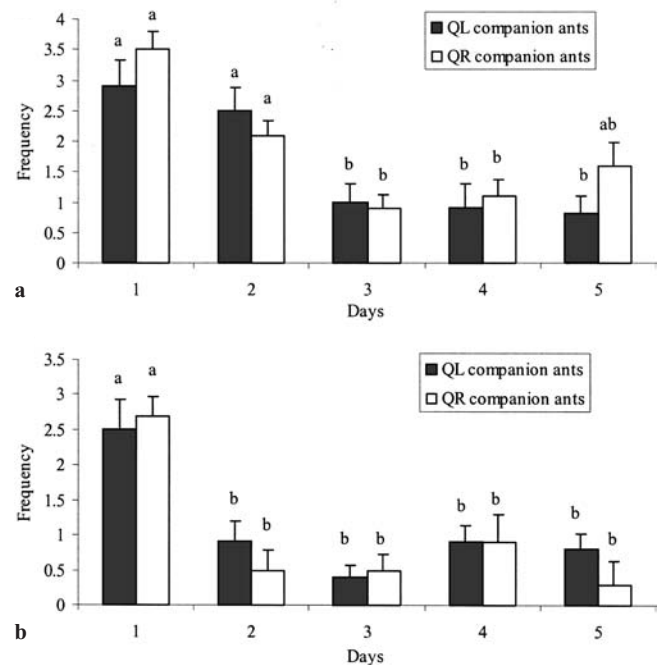


Figure 2. Mean frequency (\pm SEM) of trophallaxis (a) and allogrooming (b) between IS workers and their 'companion' ants (QL and QR). Observations were made on subsets of 10 IS-QL and 10 IS-QR encounters and repeated daily for 5 days. Companion ants were replaced every day. Different letters denote significant differences (Newman-Keuls post hoc test, $p < 0.05$)

Table 1. Durations (\pm SEM) of allogrooming and its direction in dyadic encounters between intruder and resident ants in experiment 1. The first in each pair ant is grooming the second ant, e.g. IS-QL = the 10-day isolated ant is grooming the QL resident, whereas QL-IS = allogrooming in the opposite direction. When two ants of a similar type are encountered e.g., QL-QL, the intruder ant is marked with an asterisk. P values are given by Student t test for dependent variables. Sample sizes are given on Fig. 1.

	IS intruders	Duration	p	QL intruders	Duration	p	QR intruders	Duration	p
QL resident	IS-QL	11.11 \pm 4.66	<u>0.036</u>	QL*-QL	17.58 \pm 4.55	0.28	QR-QL	12.53 \pm 5.92	0.06
	QL-IS	44.4 \pm 18.6		QL-QL*	20.50 \pm 5.10		QL-QR	18.50 \pm 8.80	
QR resident	IS-QR	10.47 \pm 2.29	<u>0.0003</u>	QL-QR	24.40 \pm 12.70	0.09	QR*-QR	5.93 \pm 2.29	0.68
	QR-IS	41.70 \pm 9.20		QR-QL	17.47 \pm 8.32		QR-QR*	5.50 \pm 1.70	

the following days (Fig. 2b Two-way ANOVA, repeated factor: $F_{4,72} = 16.78$, $p < 0.001$) and was not affected by companion ant access to the queen (Two-way ANOVA, companion type: $F_{1,18} = 0.61$, $p = 0.65$).

The 5-day period of contact with 5 nestmates strongly increased the probability of IS workers being reaccepted into their nest. Resident workers accepted both IS(QR) and IS(QL) with only a few aggressive interactions, whereas IS ants were significantly more aggressed (Fig. 3a; Two-way ANOVA, intruder type: $F_{2,72} = 12.24$, $p < 0.0001$). In contrast, the type of resident did not affect significantly their aggressiveness towards the intruders (Two-way ANOVA $F_{1,72} = 0.50$, $p = 0.48$). Interestingly, aggressions towards IS(QR) and IS(QL) workers were not significantly different (Newman-Keuls test: $p = 0.88$ and $p = 0.93$ for QR and QL residents, respectively).

The frequency of non-aggressive resident behaviours was not significantly different whether the intruders were IS,

IS(QR) or IS(QL) (Fig. 3b–d; Two-way ANOVA, intruder factor: $F_{2,72} = 1.89$, $p = 0.15$; $F_{2,72} = 1.06$, $p = 0.35$ and $F_{1,72} = 0.06$, $p = 0.94$ for antennal exploration, grooming and trophallaxis respectively). Nor was the frequency of these behaviours different between QR and QL residents (Two-way ANOVA, resident factor: $F_{1,72} = 0.08$, $p = 0.79$; $F_{1,72} = 1.63$, $p = 0.20$; $F_{1,72} = 0.05$, $p = 0.82$ for antennal exploration, grooming, trophallaxis and aggression respectively).

Discriminant function analysis showed that IS workers were significantly distinguishable from the other groups (IS(QR), IS(QL), QR and QL) on the basis of the relative percentage of their PPG HC-peaks (Fig. 4). Table 2 lists the 16 peaks, representing 19 HCs, which were unequivocally and repeatedly identified in our chromatographic conditions. Seven variables (peaks) were included in the model generated by a stepwise analysis, out of which 6 were methyl-branched compounds or mixtures. The squared Mahalanobis distance between the centroid of the IS group and the

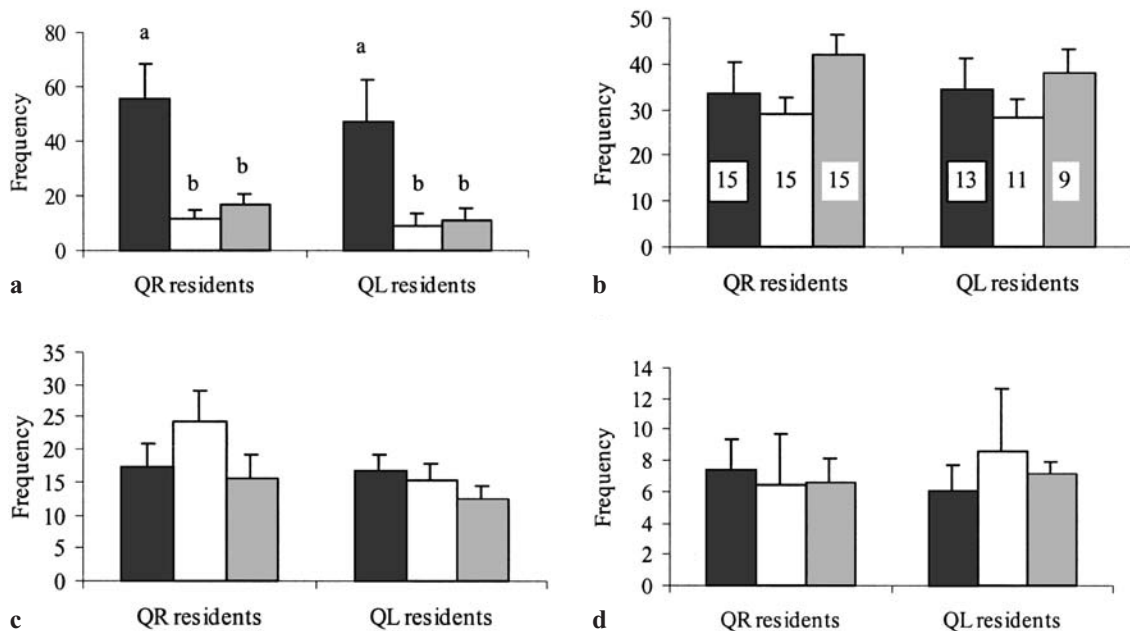


Figure 3. Mean frequency (\pm SEM) of aggressive interactions (a), antennation (b), grooming (c) and trophallaxis (d) between QR or QL resident ants and IS (black bars), IS(QL) (white bars) or IS(QR) (grey bars) ants. Numbers in the bars of fig. 3b indicate sample size. Different letters denote significant differences (Newman-Keuls post hoc test, $p < 0.05$).

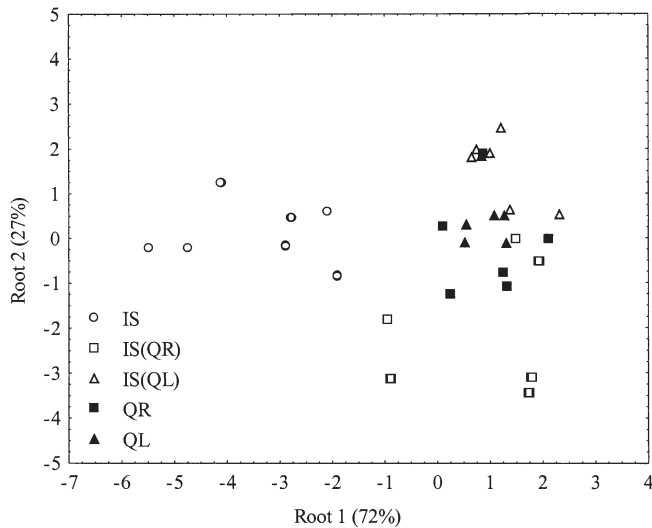


Figure 4. Scatter plot generated by the discriminant analysis, based on the 7 major discriminating PPG HC-peaks (see table 2 for peak identity), of IS, IS(QL), IS(QR), QL and QR ants

Table 2. PPG HC peaks (arranged according to F values) used in the discriminant function analysis. Peaks with higher F values better discriminate between groups. Only peaks with $F > 1$ were included in the model and used to calculate between-groups Mahalanobis distances. Wilks' Lambda: 0.7529 approx. $F_{28,73} = 2.7548$ $p < 0.0003$

Products	F	p	Wilk's λ
3-MeC28	8.5531	0.0003	0.2041
7-MeC25	7.5495	0.0007	0.1890
11-+13-+15-MeC29	7.1211	0.0009	0.1825
C27	5.1878	0.0049	0.1534
11-+13-+15-MeC31	2.3081	0.0934	0.1100
3-MeC₂₉ + 7,15-DMC₂₉	1.6149	0.2095	0.0996
3-MeC27	1.1498	0.3622	0.0926
7,15-DMC31	0.8188	0.5290	0.0706
C25	0.5384	0.7093	0.0676
C26	0.5384	0.7093	0.0697
7-MeC27	0.4784	0.7512	0.0731
11-+13-MeC27	0.3809	0.8195	0.0684
3,7,9-TMC31	0.3151	0.8643	0.0704
8-MeC28	0.2831	0.8850	0.0741
C28	0.1396	0.9654	0.0711
C29	0.0793	0.9877	0.0642

centroids of all other groups ranged from 22.8 (IS-QL) to 28.2 (IS-IS(QL)) whereas squared Mahalanobis distances between the other groups were never higher than 15.3 (in the case of IS(QL)-IS(QR)). Paired comparisons involving the IS group indicated that between-group distances were always significantly higher than within-group distances (ANOVA, $6.84 < F < 8.44$, $p < 0.0001$). This indicates that IS ants were significantly discriminated from all the other groups. No other difference was significant except that between IS(QR) and IS(QL) (ANOVA, $F = 4.2$, $p = 0.005$).

Discussion

Heritable nestmate recognition cues in ants can originate either from the workers or the queen, with the two not being mutually exclusive. Social isolation provides an interesting experimental method to differentiate between these sources and to understand how colony odour homogeneity is obtained. Short-term colony deprivation was previously shown to stimulate social interactions, particularly trophallaxis, when the ant is reunited with nestmates (Boulay et al., 2000a). There may be several reasons for this enhanced trophallaxis, including social deprivation (Boulay et al., 2000b), appeasement (Heinze, 1996), or PPG-HC exchanges (Lenoir et al., 2001). Our first experiment confirms these results and indicates that, in contrast to IS workers, QL workers do not seek trophallaxis and grooming when reunited with another QL or QR nestmate. Thus, 10 days of isolation are sufficient to stimulate behaviours that promote HC exchanges both with the QR and QL groups, whereas queen absence as such does not seem to induce this process. Analysis of the PPG contents provides corroborating evidence, showing that after 25 days the HC-profiles of QR and QL workers are still indistinguishable, while that of the IS ants is clearly distinguishable. Analysis of the direction of grooming also gives interesting indications of the importance of queen substances as recognition signals. If the queen produces relevant signals for nestmate recognition, it is predicted that individuals away from the queen's influence will seek to actively recharge themselves with these signals by intensively grooming the QR ants, but not the QL ants. This prediction was not met by the current behavioural observations, which revealed an equal level of grooming direction between QL workers and QR workers. Moreover, IS workers groomed the QR workers about 4-fold less than they were groomed by the latter, suggesting that the IS workers did not seek queen substances that may have been carried by their QR nestmates. This also indicates that grooming in the non-isolated resident (QR or QL) ants is stimulated after detecting a slight mismatch between the IS workers' odour and their own template, which represents the group odour. This differs from isolation-induced trophallaxis, which is actively requested by the IS ants and apparently has a motivational basis (Boulay et al., 2000b).

The chemical results demonstrate that isolation-induced trophallaxis and grooming help to restore the HC profile, our model system for colony odour. The PPG HC-profile of 20-day IS workers could be discriminated from QR and QL groups. Out of the 7 peaks that better discriminated between the groups (as determined by forward stepwise discriminant analysis), 6 were methyl-branched alkanes, suggesting that these compounds changed more rapidly than the linear alkanes, and may have been the cause for the increased aggression towards these ants. In social wasps, nestmate recognition can be altered by supplementing the test animal with methyl-branched but not linear alkanes, providing direct evidence of their role in colony insularity (Espelie et al., 1994; Dani et al., 2001). In ants, an indirect indication is supplied by tramp species such as *Tetramorium bicarinatum*, which

possess only linear alkanes in their epicuticle, and correspondingly are highly permissive to alien conspecifics (Astruc et al., 2001). On the other hand, the profile of IS(QR) and IS(QL) workers intimately overlapped the profiles of QR and QL workers. This indicates that the intensive trophallaxis and grooming observed during the 5-day reunion with brood tenders were sufficient to reconstitute the colony HC-profile. Moreover, the fact that trophallaxis and grooming behaviour in these experiments declined considerably after two days, suggests that a shorter period may be enough for HC-update. Companion ants were replaced daily, thus excluding the possibility that reduction in grooming was due to a progressive habituation after 3 days of cohabitation. Although we can not exclude the possibility that reduced trophallaxis was due to the IS workers having been in social contact for 3 days (i. e. no longer socially deprived), we rather attribute it to the fact that companions added on day 3 were not able to detect odour differences in the IS ants. This is not surprising in view of earlier results that have shown a rapid distribution of radioactive HC among members of groups of 11 *C. fellah* workers (Lenoir et al., 2001). Although in the present study we analysed the PPG HC-profile, cuticular HCs are likely to behave in a similar way due to frequent autogrooming, ensuring homogenisation between the PPG and the cuticle (Soroker et al., 1995; Hefetz et al., 2001). Therefore, the greatly reduced aggression of both QR and QL resident ants towards IS(QL) or IS(QR) ants is probably due to the reacquisition of the colony HC profile.

If workers carry queen compounds that specifically affect nestmate recognition, this signal should disappear progressively in workers that are kept away from the queen. Since no evidence suggests that the postulated queen signal will disappear faster in QL than in IS ants, QL companion ants should not be able to recharge the IS with queen substances. Under this hypothesis, it is predicted that IS(QR) ants should be accepted by QR workers whereas the IS(QL) should be aggressed at the same level as IS ants, or at least at an intermediate level. Nonetheless, the results of the second experiment proved the contrary, demonstrating that no queen signal is particularly relevant for nestmate recognition in this species. However, this does not mean that the queen is irrelevant for colony insularity. As recently demonstrated, the presence of the queen in colonies of *C. fellah* stimulates high worker aggressiveness independently of cue production (Boulay et al., 2003), which corroborates the results of a parallel study in *Solenopsis invicta* (Vander Meer and Alonso, 2002).

Given the striking congruency between HC profile variations and the possibility for workers to reintegrate into their colony, our results emphasize the likely role of HCs as part of the nestmate recognition signal in *C. fellah*. We further confirm that workers are the cue originators, as well as the importance of worker-worker trophallaxis and grooming for colony odour homogenisation, two necessary requirements for a Gestalt recognition signal.

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