

Influence of Queen and Diet on Nestmate Recognition and Cuticular Hydrocarbon Differentiation in a Fission-Dispersing Ant, *Aphaenogaster senilis*

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In social insects, colony fission is a particular mode of dispersal by which an adult colony splits into two or more independent nests. In the monogynous ant *Aphaenogaster senilis*, field data suggest that new queens may be produced in queenless daughter nests after nest relocation. Because workers do not fly, colony fission limits dispersal distance, leading young sister colonies to compete together and with the mother queen. In the present study we analysed the effects of queen loss and diet change on nestmate recognition. Queenright colonies were separated into two queenless and one queenright fragments. One queenless group received the same food as the queenright group, while the other queenless group received a different diet for 150 days. Recognition bioassays revealed that aggression between queenright and queenless former nestmates increased progressively until day 20, when they could no longer be reunited. Different diets also induced aggression between orphaned groups. Chemical analyses indicated that cuticular hydrocarbon profiles were already different between groups after 5 days. Overall, our results are in accordance with the graded model of nestmate recognition and suggest that the loss of the mother queen progressively leads to the independence of the related nests after fission. This may also allow queenless ants to merge again with their mother colony during a short time window after fission.

Key words: Colony fission, *Aphaenogaster senilis*, cuticular hydrocarbons, food, recognition bioassay, queen

INTRODUCTION

Nestmate recognition is a behavioral process by which social animals discriminate between members of their own colony and conspecific aliens. It is particularly well developed in ants, bees, and wasps, in which it allows colony insularity to be maintained against competitors, and played an important role in the evolution of eusociality (Wilson and Hölldobler, 2005).

The nestmate recognition process involves a recognition template (probably neural) and recognition signature, mostly cuticular hydrocarbons (CHCs) that may function according to two non-exclusive modes (Hefetz, 2007; Lenoir et al., 2001a; Lenoir et al., 1999; Liu, 2000; Vander Meer and Morel, 1998). In some species, workers behave peacefully as long as the recognition signature of encountered individuals remains close to their own template. Over this threshold of tolerance, workers show full aggression. This seems to

happen, for example, in *Camponotus cruentatus*, in which alien workers engage in full aggression with each other irrespective of the geographic distance between their nests (Boulay et al., 2007a). Alternatively, the graded model suggests that the behavioral response can be proportionate to the magnitude of the difference between the template and the signal. Therefore, an intermediate aggressive response can be observed among nestmates after a prolonged period of separation (Boulay and Lenoir, 2001; Lenoir et al., 2001b). For example, at the end of the hibernation period, the multiple nests that compose polydomous colonies of *Cataglyphis iberica* reestablish contact, during which time workers engage in slight aggression against each other (Dahbi and Lenoir, 1998).

A graded response is also expected in species that reproduce by colony fission, whereby mature colonies split and give rise to two or a few independent daughter colonies that disperse over a short distance. During the first weeks or months after fission, related colonies contain workers that once belonged to the same nest but currently live in different physical and social environments. This includes different queens and diets, which may trigger a progressive divergence of the chemical signatures (Liang et al., 2001; Liang and Silverman, 2000; Vander Meer and Alonso, 2002).

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Nevertheless, workers with a common fission past as in *Cataglyphis cursor* might never be as aggressive as those coming from different populations (Nowbahari et al., 1990).

In the ant *Aphaenogaster senilis* Mayr, the queen exerts a strong inhibitory effect on the production of other queens and on worker reproduction of males (Boulay et al., 2007b; Ichinose and Lenoir, 2009; Ledoux, 1971, 1973). Orphan workers lay haploid eggs and compete for male production (Ichinose and Lenoir, 2009). Field and laboratory observations suggest that colony founding occurs during nest relocation when a group of workers leaves their mother nest with young totipotent larvae from which they rear their future queen (Boulay et al., 2007b). Soon after fission, each colony should become independent and reject former nestmates. However, in the field, small groups of workers using a new nest may rejoin their mother colony, because the new nest location is not entirely suitable or because of a dramatic environmental change, although we do not yet have field observations confirming this hypothesis. Nevertheless, the possibility that workers reunite with former nestmates following fission is suggested by the low level of aggression between colonies that have recently undergone fission (Ichinose et al., 2005). This indicates that the acquisition of a new recognition label is progressive and does not happen very fast.

The aim of our study was to determine the effect of the queen and diet on the nestmate recognition process and colony chemical profile after an experimentally provoked split of the colony in *A. senilis*. We hypothesized that the longer workers were separated, the less they would tolerate each other, and that this would be correlated with cuticular hydrocarbon divergence. Aggression would be amplified by the absence of the queen and access to different food.

MATERIALS AND METHODS

Model species and laboratory conditions

Queenright colonies of *Aphaenogaster senilis* were collected in May 2002 in Doñana National Park in Southern Spain (Andalusia). They were kept in the laboratory at 28°C and provided with live worms (fly larvae maggots) and Beehappy, a sugared solution for bumblebees (Koppert, Cavaillon, France), three times a week and pieces of orange fruit once a week.

Behavioral data

On 3 July 2002, three groups of 250 workers and 10 small larvae were established from each of three queenright colonies. One group contained the queen and was fed a regular diet of worms/orange/Beehappy (queenRight, diet 1; hereafter the R group). A second group was kept queenless and fed the same diet (Orphan, diet 1; O group). The third group was kept queenless and received a different diet composed of Mealworms/apple/10% sucrose (orphan, diet 2 Mealworm; M group).

Recognition bioassays were conducted by introducing one worker (the intruder) among a group of three workers (the receivers), previously kept in a plastic container for 5 minutes. From the time of introduction of the intruder, the frequency of four types of interaction between the receivers and the intruder was recorded during 10 min: 1) peacefully resting in contact, 2) antennal contact, 3) allogrooming, and 4) aggression. Aggression included opening mandibles and biting or seizing the intruder. In addition, the latency to the first aggression in each encounter was measured. If no aggression was observed in a given encounter, the latency was considered to be 10 min. The frequency of each behavioral category and the

latency to aggression were standardized to have a mean of 0 and a unit SD. An aggression index was calculated from the arcsine-transformed coordinate of the data on the first factor of a principal component analysis (PCA) performed on the five standardized behavioral variables; for details, see Ichinose et al. (2005).

For each colony, the following combinations of intruder/receiver encounters were performed: control RR (control: a queenRight worker confronted with her R nestmates with the regular diet 1), RO (queenRight versus Orphan nestmates with the same diet 1), RM (queenRight with diet 1 versus orphan nestmates with Mealworm diet 2), and OM (Orphan with diet 1 versus orphan nestmates with Mealworm diet 2). Reciprocal intruder/receiver encounters were conducted, but their results were pooled since there was no difference between them. Tests were conducted on days 5, 10, 15, 20, 30, 45, 60, 90, and 150 after separation. Differences in the aggression index among the four types of encounter were tested by means of repeated-measure ANOVA. The Bonferroni correction was employed to avoid the Type I error inflation, and the significance level was 0.0056 for nine observation days.

Chemical data

Three workers were randomly collected from each nest on days 0, 5, 10, 15, 20, 30, 45, 60, 90, and 150 (a total of 120 workers). As previously, we analysed thorax hydrocarbons (Ichinose et al., 2005; Ichinose and Lenoir, 2009). Although some variation was observed between head, thorax, and gaster comparable to that in other ant species, preliminary experiments indicated that the thorax is representative of the mean whole-body cuticular signature and is sufficient to induce colony-specific recognition. Individual thoraces with legs were dipped in 1 ml pentane for 5 min to extract CHCs. Samples were then evaporated and preserved at -20°C. Before analysis, dry extracts were dissolved in 50 µl of pentane containing 50 ng of C20 as an internal standard. Dissolved extract (5 µl) was injected into a FID gas-chromatograph (VGM250Q coupled with a TurboChrome Workstation) equipped with an apolar DB-5 fused-silica capillary column. The temperature injector was set at 220°C and the detector at 310°C. Helium was used as the carrier gas at a rate of 2 ml/min. The temperature was programmed at 150°C for 2 min, 5°C/min for 30 min, and 12 min at 300°C. The identity of the eluted compounds was previously determined (Boulay et al., 2007b; Ichinose et al., 2005; Ichinose and Lenoir, 2009; Lenoir et al., 2001c), and we used a total of 32 peaks in our study: all 29 quantifiable peaks previously identified by Lenoir et al. (2001b), and peaks 35, 36, and 37. Differences in CHCs were estimated by the square root of Mahalanobis distances obtained from discriminant analyses of these peaks.

RESULTS

Workers' behavior (resting, antennal contacts, aggres-

Table 1. Repeated-measures ANOVA testing the effects of queen presence and food difference in five behavioral categories. In bold, Significant values are in bold font. Nest confrontations are queenright control (RR), queenright vs. orphan with the same diet (RO), queenless vs. queenless with different diet (OM), and queenright vs. queenless with different diet (RM).

Behavioural category	Confrontation		Day		Confrontationx Day	
	$F_{3,72}$	P	$F_{8,72}$	P	$F_{24,72}$	P
Resting	6.282	<0.001	0.439	0.894	0.739	0.798
Antennal contact	7.386	<0.001	2.108	0.046	1.297	0.198
Allogrooming	2.355	0.079	1.187	0.319	1.305	0.193
Aggression	31.072	<0.001	3.353	0.003	1.332	0.176
Aggression latency	19.132	<0.001	2.513	0.018	1.428	0.125

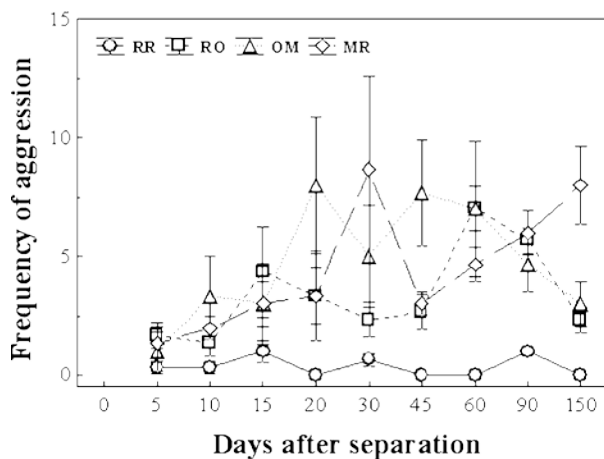


Fig. 1. Temporal changes in the frequency of aggression (mean±SEM for 5 minutes) in worker pairs: control (queenright and queenright nests with the same diet, RR), queenright vs. orphan with the same diet (RO), queenless vs. queenless with different diet (OM), and queenright vs. queenless with different diet (RM).

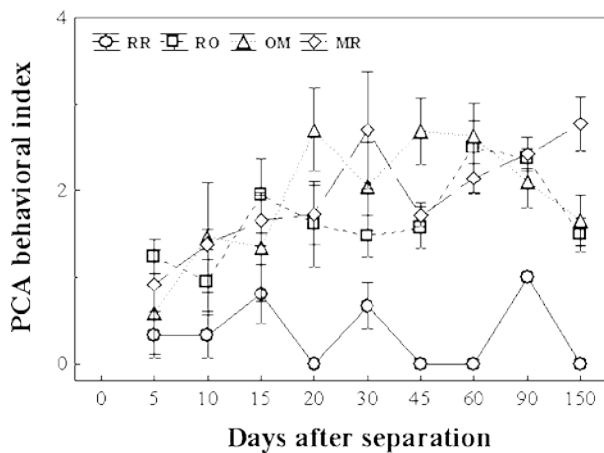


Fig. 2. Behavioral indices (mean±SEM) obtained from a principal component analysis for all behaviors (see text for details) in worker confrontation types: control (queenright and queenright nests with the same diet, RR), queenright vs. orphan with the same diet (RO), queenless vs. queenless with different diet (OM), and queenright vs. queenless with different diet (RM).

Table 2. Univariate ANOVA of the PCA behavioral indices for four worker confrontation types on each observation day. Significant values after Bonferroni correction (significance level=0.0056) are in bold font.

	df	MS	F	P	MS	F	P	MS	F	P
		5 day			20 day			60 day		
Confrontation	3	0.069	0.130	0.939	2.441	13.701	0.002	2.618	15.281	0.001
Error	8	0.531			0.178			0.171		
		10 day			30 day			90 day		
Confrontation	3	0.209	0.294	0.829	1.627	3.731	0.061	0.942	54.508	0.0001
Error	8	0.711			0.436			0.017		
		15 day			45 day			150 day		
Confrontation	3	1.124	2.106	0.178	3.444	12.019	0.002	2.809	20.754	0.0001
Error	8	0.534			0.287			0.135		

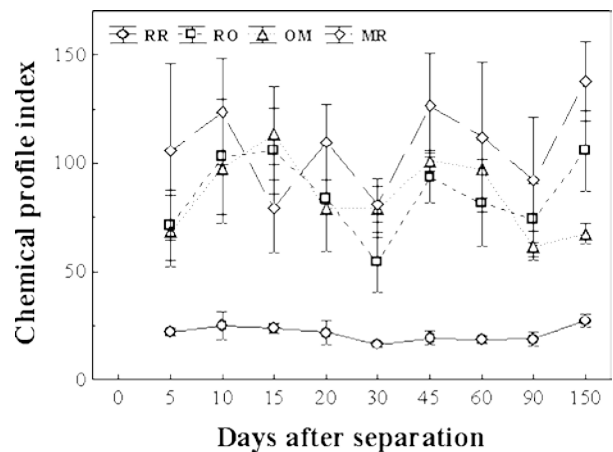


Fig. 3. Chemical profile index (square root of Mahalanobis distance) obtained from a discriminant analysis (mean±SEM) of the proportions of cuticular hydrocarbons: control (queenright and queenright nests with the same diet, RR), queenright vs. orphan with the same diet (RO), queenless vs. queenless with different diet (OM), and queenright vs. queenless with different diet (RM).

sion, and aggression latency, but not allogrooming) changed according to the type of encounter and as a function of the time of separation (Table 1). The aggression index was low for control encounters (RR) and did not differ significantly through time. By contrast, the aggression index increased progressively in the three other groups and became significantly different from that of the RR after 20 days of separation (Fig. 1). There was no significant difference among the RO, OM, and RM encounters, suggesting that separation per se, queen removal, and different diet provoked a significant increase in aggression.

In all three types of encounter involving separated nestmates, PCA behavioral indices increased asymptotically with time until the 20th day, and then remained constant (Fig. 2). This contrasted with the index for control RR encounters, which remained low. The behavioral index was significantly different both between types of encounter ($F_{3, 64}=45.544$, $P<0.001$) and between days ($F_{8, 64}=3.235$, $P=0.004$), but the interaction of these two factors was not significant ($F_{24, 64}=1.310$, $P=0.195$). Univariate ANOVAs conducted on behavioral indices at different days revealed that aggression in RO, OM, and RM tests became significantly different from RR tests at day 20. Differences were marginally significant at day 30 ($P=0.06$) and always remained significant after 45 days (Table 2). These results indicate that workers changed their nestmate recognition behavior toward separated old nestmates with time. Separation, the loss of the queen, and food difference all had a major impact on recognition.

Chemical profile distances between control workers (RR) were logically always low and the variation was very small. Distances between separated groups RO, OM, and RM were higher and the variation was higher (Fig. 3). The distances

were significantly different among the types of encounters ($F_{3, 64}=14.045$, $P<0.001$), but not among observation days ($F_{8, 64}=1.284$, $P=0.268$), and the interaction was not significant ($F_{24, 64}=0.569$, $P=0.937$). The RR distances were significantly different from the others ($P<0.01$), but no significant differences were detected among RO, OM, and RM (Tukey's test, $P>0.05$). These results indicate that 1) the chemical profiles were already significantly different between separated workers after 5 days, which is more rapid than behavioral modification, and 2) separation per se, loss of the queen, and the food differences all had a significant effect on differentiation of the workers' profile.

DISCUSSION

Our study revealed that separated workers of *A. senilis* become aggressive toward their previous nestmates in relation to the queen's presence and differences in diet. A simple effect of separation per se could also be involved, although it was not possible here to separate its effect. The queen effect can be explained at least partly by the development of ovaries in orphaned workers, which induces discrimination and policing (Ichinose and Lenoir, 2009). Food differences between orphan groups also induced the development of aggression. Accordingly, the behavioral index showed that the differentiation of worker behaviors depended on the duration of separation. The index significantly changed after 20 days, increased until the 30th day, and changed little thereafter (Fig. 2). The chemical profile, however, changed rapidly (5 days), and the differences subsequently remained stable (Fig. 3). This pattern of nestmate recognition in *A. senilis* corresponds more to the graded than to the threshold model and confirms previous experiments (Ichinose et al., 2005). Since all cohabitant workers in a colony possess their own, common gestalt odor (Soroker et al., 1995), the set of odors will progressively shift if the queen and workers are removed and/or a new diet is offered. Accordingly, worker behaviors will change in a time-dependent manner.

Cuticular hydrocarbon profiles of individual members in colonies are generally influenced by the presence of the queen (Abdalla et al., 2003; Lahav et al., 2001; Provost et al., 1993; Vander Meer and Alonso, 1998). Therefore, our results are congruent with previous ones. The role of the queen on colony odor is also very important as it homogenises the odors and has a role in the social motivation and tolerance of alien conspecifics. This was previously observed in the ants *Cataglyphis cursor* and *Camponotus fellah* (Berton et al., 1992; Boulay et al., 2002).

Our results also show that differences in diet induce the development of aggression between separated nestmates. It is well known that environmental factors are involved in the modification of chemical profiles in the colony, e.g., nest materials (Pickett et al., 2000), anything around the nest (Chen and Nonacs, 2000; Pirk et al., 2001), and food (Liang and Silverman, 2000; Sorvari et al., 2008), but this is not a general rule; for example, in *Formica fusca*, different diets play only a small role in producing the colony odor (Wallis, 1962). Environmental cues are added to individual hydrocarbons by grooming or trophallaxis, and the gestalt chemical profile specific to the colony will be modified (Boulay et al., 2000; Lenoir et al., 2001c; Soroker et al., 1995).

The differentiation of both worker behavior and the chemical profile in *A. senilis* was time dependent after separation. This also suggests a simple effect of separation, as in other species such as *Formica aquilonia* and *Oecophylla smaragdina* (Newey et al., 2009; Sorvari et al., 2008). In *A. senilis*, the chemical profile of workers changes rapidly in the early days after separation (i.e., in 5 to 10 days), during which time their behavior did not significantly alter, until 20 days (Table 2). This rapid modification (one to two days) of the cuticular hydrocarbon profile has been observed in new egg layers of *Streblognathus peetersi* (Cuvillier-Hot et al., 2005). This suggests that worker recognition is not completely determined by the accordance of the chemical profiles between individuals alone. This may be explained by the template plasticity memory (Errard et al., 2006). From this viewpoint, in the fission reproductive biology of *A. senilis*, nests that have recently undergone fission are less aggressive between themselves than towards alien nests (Ichinose et al., 2005). The progressive separation of the chemical profiles may facilitate reunification of the separated parties if the separated ants need to coalesce again with the mother nest.

ACKNOWLEDGMENTS

This study was performed during Katsuya Ichinose's stay in France with support from a fellowship from the French Government. We thank the authority of the Doñana National Park for allowing ant collection, Arnold Fertin and Guileric Fraquet for help in maintaining ant colonies, and Jean-Philippe Christidès for gas chromatographic analyses. Jacqueline Minett revised the English.

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(Received January 29, 2009 / Accepted June 30, 2009)