Larval memory affects adult nest-mate recognition in the ant *Aphaenogaster senilis*

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Prenatal olfactory learning has been demonstrated in a wide variety of animals, where it affects development and behaviour. Young ants learn the chemical signature of their colony. This cue-learning process allows the formation of a template used for nest-mate recognition in order to distinguish alien individuals from nest-mates, thus ensuring that cooperation is directed towards group members and aliens are kept outside the colony. To date, no study has investigated the possible effect of cue learning during early developmental stages on adult nest-mate recognition. Here, we show that odour familiarization during preimaginal life affects recognition abilities of adult *Aphaenogaster senilis* ants, particularly when the familiarization process occurs during the first larval stages. Ants eclosed from larvae exposed to the odour of an adoptive colony showed reduced aggression towards familiar, adoptive individuals belonging to this colony compared with alien individuals (true unfamiliar), but they remained non-aggressive towards adult individuals of their natal colony. Moreover, we found that the chemical similarity between the colony of origin and the adoptive colony does not influence the degree of aggression, meaning that the observed effect is likely to be due only to preimaginal learning experience. These results help understanding the developmental processes underlying efficient recognition systems.

1. Introduction

Prenatal experience may structure the nervous system, determine body organ function and shape behaviour to ensure and enhance an individual’s survival in the immediate and longer-term postnatal period [1]. In particular, prenatal learning of chemosensory stimuli in mammals may determine food preferences after birth as flavours of the mother’s diet pass rapidly into the amniotic fluid and reach the fetus [2]. For instance, rabbits exposed as fetuses to the odour of cumin are selectively attracted as neonates to this odour [3]. In parasitoid insects, host chemical cues learned during preimaginal stages may influence host-searching ability in adults. For example, larvae of *Hyssopus pallidus* wasps exposed to fruit odour at early preimaginal stages significantly increased the adult response to frass from fruit-fed caterpillars [4]. In a variety of animal taxa, prenatal olfactory learning appears to be crucial for several important decision-making processes and for the expression of certain behaviours, including maternal recognition and attachment, habitat choice and social behaviour [5–9]. The widespread occurrence of this phenomenon suggests that prenatal learning of chemosensory stimuli by simple exposure may affect ontogeny of behaviour in many animal groups.

The anatomical organization of the insect olfactory nervous system shares many fundamental similarities with that of mammals, suggesting the presence of analogous mechanisms for olfactory perception, discrimination and learning [10]. However, the parallel between pre/neonatal stages in vertebrates and pre/neomaginal stages in insects is complex owing to the scarcity of experimental behavioural studies on insect embryos. Some important insights came from studies on preimaginal olfactory learning in formicine ants. Isingrini
et al. [11] introduced small and large larvae of Cataglyphis cursor ants in an alien adoptive colony where they developed until the pupal stage; at this point, they were transferred back to their original colony. After eclosion, workers originated from the small larvae group preferred to care for brood belonging to the adoptive colony rather than for their sister brood, but this was not the case for the large larvae group. The authors inferred that the first instars of larval life might be the more sensitive to cue learning or the total duration of the exposure to the colony odour might have a significant effect on brood recognition. In another study, Camponotus floridanus ants spent their larval stage and their young adult stage in two different unrelated colonies: these experimental ants preferred to care for brood belonging to the colony in which they had spent time as larvae rather than for brood belonging to the other colony [12]. It thus appears that the preimaginal experience has an effect on brood recognition. Even queen recognition might be affected by larval experience, since callow workers of Cataglyphis cursor adopted by a new alien queen for two months were still attracted by their mother queen [13].

Effective recognition systems are essential to structure social interactions in a variety of taxa [14–16]. Nest-mate recognition is one of the major features of eusocial insects, and therefore the study of recognition mechanisms allows understanding their advanced social organization. Recognition cues are chemicals, namely a mixture of cuticular hydrocarbons that are qualitatively similar in a given species but can vary in their relative amounts between colonies [17–19]. Nest-mate recognition would thus result from the matching of the chemical ‘label’ present on the body surface of the encountered individual (cuticular hydrocarbon profile) with a neural ‘template’ of the evaluator (referent colony odour) [20,21]. The degree of mismatch between the label and the template generally results in a graded response, from body investigation to threat and overt aggression [22,23]. Indeed, the degree of aggression exhibited by ants reared in experimentally mixed groups decreases when the degree of chemical proximity between colonies increases [24]. Studies on artificially mixed colonies of different ant species showed that allo-specific members are recognized as nest-mates; this implies that familiarization to different odours may affect the odour template [24–26].

The recognition mechanism is essentially based on cue learning and for discrimination to take place, the template has to be acquired first. There is evidence that young ants learn their colony odour template just after eclosion [27–30]. However, to date, no study investigated the possible impact of cue learning during early developmental stages on discrimination between adult individuals. Since preimaginal learning of odour cues used in brood recognition has been demonstrated earlier [11,12], here we investigated whether exposure to an alien colony odour during the larval stage might affect nest-mate recognition at the adult stage, thus in a novel adult–adult context. We transferred larvae of the ant Aphaenogaster senilis for a period of time into an ‘adoptive colony’ of the same species to test whether adult ants, proceeding from these adoptees, would treat differently individuals belonging to the familiar, adoptive colony compared with alien, completely unfamiliar individuals. We also investigated, for the first time in the preimaginal learning context, whether the chemical similarity between colonies had a modulating effect on aggression showed by the adoptees.

2. Material and methods

(a) Colonies collection and rearing laboratory conditions
Aphaenogaster senilis is a monogynous Myrmicinae ant distributed in the northern Mediterranean area. Nine mature queenright colonies were collected in March 2011 in Banyuls-sur-Mer (France). The ants were kept under laboratory conditions (temperature 24 ± 4°C; relative humidity 50–60%; 12 L: 12 D cycle) housed in artificial nests consisting of spherical plastic boxes (12.5 cm diameter) with a plaster floor. A hole gave access to the foraging area, a larger plastic box (18 × 25.5 cm) containing the nest. The plaster was regularly moistened and the ants were fed three times per week with insects, a mix of apple and honey, and water ad libitum.

(b) Experimental set-up
Nine experimental groups, containing a queen and 100 workers, were formed from each original colony. Workers were chosen randomly from both the nest and the foraging area, and were marked with a dot of enamel paint on the thorax to recognize them from newborns. From these experimental colonies, three were selected as ‘parent colonies’, three as ‘adoptive colonies’ and three as ‘alien colonies’ (see electronic supplementary material, table S1). According to the number of larval instars of A. senilis determined by Boulay et al. [31], larvae of two different developmental categories (‘small larvae’ between stage 2 and stage 3, and ‘large larvae’ between stage 3 and presprenymph) were selected from the three ‘parent colonies’. About 60 small and 60 large larvae were transferred into each ‘adoptive colony’, whose own brood at the same development stage was previously eliminated (figure 1). Large larvae spent between 4 and 14 days in the ‘adoptive colony’ before pupation, whereas small larvae experienced adoption for 17–35 days. We thus obtained two experimental groups, in which larvae differed for the duration of the adoption experience. The transferred larvae developed in the ‘adoptive colony’ for the time necessary to become pupae. Once the adopted pupae’s eyes were clearly pigmented, these pupae were transferred back to their ‘parent colony’. Soon after eclosion, each experimental ant was marked individually on the thorax in order to know its exact age. After 10 days of adult life, the aggressive response of these experimental ants was measured in dyadic encounters with adult individuals from the ‘adoptive colony’, from the ‘parent colony’ or from an ‘alien colony’ (completely unfamiliar). Each ant was therefore tested only once.

The ‘control colonies’ (see electronic supplementary material, table S1) were treated in the same way except for the actual transfer of larvae into an adoptive colony: the small and large larvae from these control colonies were just removed and replaced back into their parent colony. The aggressive response of these control ants towards individuals belonging to two different alien colonies (alien 1 and alien 2), and to their own colony, was then measured.

(c) Aggression bioassays
The behavioural test consisted in a dyadic encounter in a circular arena (4.5 cm diameter) coated with Fluon and lined with a filter paper bearing the parent colony odour (i.e. previously left in the nest for 24 h). In this condition, the arena was likely to be perceived as home territory by the focal ant. Before the start of the aggression test, the focal ant and the stimulus ants were placed in the arena, separated by a cylinder and left for 5 min to acclimatize. The test began when the cylinder was removed, allowing the ants to interact for 3 min, during which we recorded the duration of four different behaviours of the focal ant using the software ETHOLOG: v. 2.25 [32]. Each behaviour was given an arbitrary score related to increasing aggression: 0, investigate with antennae; 1, antennal...
Figure 1. Schematic overview of the experiment: 1, small and large larvae were transferred from the parent colony to the adoptive colony; 2, larvae developed into pupae; 3, pupae were transferred back into the parent colony; 4, pupae developed into adult ants; 5, these experimental ants encountered, in aggression bioassays, either (a) parent, (b) adoptive or (c) alien individuals.

/bootbox; 2, open mandibles; 3, bite. Overall aggression index for each test were calculated using $\text{AI}_i = \left( \sum_{t=1}^{T} \text{AI}_i \times t_i \right) / T$, in which $\text{AI}_i$ is the overall aggression level, $\text{AI}_i$ and $t_i$ are the aggression score and the duration of the $i$th behaviour, and $T$ is the total interaction time [24].

(d) Chemical analyses
In order to investigate the possible correlation between the chemical profile of the colonies and the observed aggression, we analysed the ants’ cuticular hydrocarbons by gas chromatography coupled with mass spectrometry (GC–MS; for details on the chemical analyses, see the electronic supplementary material). Compounds were identified on the basis of their mass spectra and retention time, and compared with standards and published results.

(e) Statistical analyses
(i) Behavioural data
The aggression index represents the response variable. The variables considered to potentially affect the aggression response of ants were ‘treatment’ (control, experimental small larvae and large larvae) and ‘encounter’ (parent, adoptive and alien). These variables were used as fixed factors with three levels. The effects of these predictor variables on the response (aggression index) were analysed using linear mixed models (LMM), carried out with the package lme4 [33] of the program R v. 2.8.1 [34]. The respective full model included the interaction of ‘treatment’ and ‘encounter’. We used colony of origin as random factor to correct for possible intrinsic behavioural differences among colonies. The response variable (aggression index) showed a right-skewed distribution and thus it was square-root-transformed prior to analysis in order to normalize it.

Moreover, we tested whether the differences in aggression index among the three encounter classes (parent, adoptive and alien) within the group of small larvae and the large larvae were modulated by the chemical distance between the colony of origin and the adoptive colony. For this, we used linear models (LM), again with square-root-transformed response, and tested for the interaction between ‘encounter’ (three levels) and ‘chemical distance’ (covariate).

We made sure that the residuals of models were adjusted to a normal distribution by visually checking normal probability plots, and we checked for homogeneity of variances by plotting the fitted values versus the residuals. $p$-values were calculated using likelihood-ratio tests based on changes in deviance when each term was dropped from the full model.

(ii) Chemical data
The area of 29 regularly occurring peaks (representing cuticular hydrocarbons) was calculated using Chem Station software (Agilent Technologies, Waldbronn, Germany). Before multivariate analysis, each peak area was transformed (according to Lenoir et al. [35]) and then analysed by principal components analysis (PCA) to reduce the number of variables (8 PCs explaining 91.2% of variance) subsequently used in a discriminant analysis (DA). We carried out DA to determine whether predefined groups (colonies) could be discriminated on the basis of their chemical profiles, and to assess the degree of chemical similarity among groups (Mahalanobis chemical distances). The correct classification of individual samples to the respective groups was verified. All the statistical analyses of chemical data were performed using Statistica v. 6.0 for Windows (Stat Soft Inc., Tulsa, OK).

3. Results
There was a significant interaction between treatment (control, small larvae and large larvae) and encounter (parent, adoptive and alien) indicating that the differences in aggression among the three encounter categories were modulated by the treatment (LMM: $\chi^2 = 24.22$. $p < 0.001$; see electronic supplementary material, table S2 for full model outputs). Post hoc analysis within the different treatments revealed a significant influence of the types of encounters on the aggressive response of ants ($p < 0.001$). Within all treatment groups, we found a significantly lower aggression index in encounter situations with parent ants than with aliens or individuals from the adoptive colony (post hoc comparisons in figure 2a–c). In the treatment group with longer adoption period (small larvae), ants were significantly more aggressive towards alien individuals than towards individuals from the adoptive colony (post hoc comparisons in figure 2c). However, this difference was not significant in the treatment group with shorter adoption period (large larvae; figure 2b). Ants from the control group were equally aggressive towards individuals from the two alien colonies (figure 2a). Post hoc analysis across treatments within the same encounter class revealed that ants
from different treatments reacted differently to unfamiliar alien individuals. Both the small and large larvae groups showed as adults significantly lower aggression towards alien individuals than the control group (small larvae versus control: \( p < 0.001 \); large larvae versus control: \( p < 0.01 \); small larvae versus large larvae: \( p = 0.24 \)).

The chemical data showed that certain colonies were more chemically similar than others (see electronic supplementary material, table S3). We thus tested whether the differences in the aggression index among the three encounter classes within the treatment group of the small larvae and large larvae were modulated by the chemical distance between the ‘parent colony’ and the ‘adoptive colony’ (see below). However, a non-significant interaction between these two predictor variables (encounter \times \text{chemical distance}) was found (LM: \( F_{2,126} = 0.52, p = 0.59 \)), indicating no such modulation. The same applies to the large larvae group (\( F_{2,95} = 0.29, p = 0.75 \)).

A typical chemical profile of *A. senilis* workers contains linear, methylated and dimethylated alkanes and alkenes [36] (electronic supplementary material, figure S1). The results of the DA (electronic supplementary material, figure S2) show that cuticular hydrocarbon profiles were colony-specific in all nine colonies (Wilks's lambda: 0.013; \( F_{64,16} = 2.81; p < 0.001 \), with 81.39% of individuals correctly classified (range: 100–50%). However, there is some overlap and certain colonies appear to be more chemically similar than others (see electronic supplementary material, table S3; Mahalanobis distances between colonies pairs). In particular, we considered chemical distance between ‘parent colonies’ and ‘adoptive colonies’. Colony pairs 7–4 show the shortest chemical distance (2.93), colonies 6–3 the highest distance (12.85) and colonies 8–9 an intermediate distance (6.08); but this did not influence the aggression response (see above).

### 4. Discussion

Our results show that odour familiarization during preimaginal life can affect nest-mate recognition abilities at the adult stage in the ant *A. senilis*. This is the first time that preimaginal experience has been investigated in Myrmicinae, a major subfamily of Formicidae, characterized by high evolutionary divergence and abundance of social parasitic species. Adult ants that develop as larvae into an ‘adoptive colony’ adjust their aggression level towards adult conspecifics on the basis of the familiarization period with social odour.

Although previous studies in experimentally mixed groups of ants showed that the degree of aggression decreases with increased cuticular hydrocarbon proximity [24], we found that the degree of chemical similarity between paired colonies (parent and adoptive) does not affect the degree of aggression towards familiar, adoptive individuals; thus, our results are likely to be due only to the experimental treatment. This is the first study considering other factors that may be involved in the efficacy of preimaginal olfactory learning (i.e. the chemical distance among colonies).

Ants transferred as larvae from the first stages (small larvae) into an adoptive colony, and thus left in that colony for longer time, familiarize with the odour of the adoptive colony and show as adults a lower aggression level towards adoptive individuals than towards aliens. However, ants transferred as last larval stage (large larvae) into an adoptive colony do not familiarize with odours of that colony to the same extent as small larvae, but they treat adoptive individuals more similarly to aliens once they reach adulthood. These results are consistent with previous results reported by Isingrini *et al.* [11] concerning brood recognition, which typically happens inside the colony. We now demonstrate that preimaginal olfactory learning is important in the context...
of colony defence, namely adult–adult recognition, which is fundamental to maintain cooperation inside the nest, and to reject potential competitors, predators and parasites.

Olfactory learning capacity appears to be influenced by the exposure time to chemosensory stimuli. Adopted ants developing from small larvae had a prolonged preimaginal odour experience compared with those developing from large (i.e. older and closer to pupation) larvae, the larva size being inversely related to the duration of experience. Alternatively, it could be possible that olfactory learning is better achieved in certain sensitive early developmental stages, as already hypothesized by Isingrini et al. [11], related to the degree of development of the nervous system.

Adult ants coming from both the adopted small and large larvae group showed lower aggression towards alien con-specifics than the control group, although within treatments aggression against aliens was always the highest. This effect could be due to a broadening of the recognition template in adoptees. Indeed, preimaginal experience in the adoptive colony could have contributed to the formation of a wider template, which includes cues from the adoptive colonies (learned during larval life) and cues from the parent colony (learned during the first 10 days after eclosion). A broader recognition template would increase the probability of acceptance errors [19] and thus explain the observed result.

The olfactory pathway of adult Hymenoptera is well investigated [37,38] and has been recently reviewed extensively [39,40]. We know how the nervous system is organized but we do not know at which level of the olfactory system the odour quality ‘nest-mate’ or ‘non-nest-mate’ is attributed to the brain representation (the template). The mushroom bodies, regions of neuropile, are connected with higher associative functions (learning and memory), and play a key role in the neuronal control of adaptive behavioural modifications [41–43] and in the processing and storage of chemosensory information [44]. We tested the adoptees’ recognition capacity in adults at the age of 10 days; this implies that ants were able to store the cues learned during preimaginal life in their long-term memory. A possible brain site for the storage of preimaginal memory could be the mushroom bodies. Very little is known about structural modification of ants’ brain during the development and about larval olfactory systems. Gronenberg et al. [45] showed in Camponotus floridanus that proliferative cells or mitotic cell stages are present in young larvae and they found no signs of cell proliferation after the second larval stage. This suggests that neurogenesis is complete in young pupae and no new Kenyon cells (intrinsic neurons of the mushroom body) that might contribute to mushroom body growth are produced in adult ants. The connection between larval and adult experience could be a consequence of the survival of larval neurons during metamorphosis, enabling the persistence, in the adult brain, of memories formed during the larval stage [46–48]. A radical change in neuronal design from the larval to the adult system is not very likely, considering that the nervous system metamorphosis is largely parsimonious [49]. Memory has to start at a time probably determined by the maturation of the nervous system. It may be possible that mechanisms crucial during adult life (such as nest-mate recognition) are established before adulthood to ensure their functioning at the right time. Moreover, it has been established that the structure of the central nervous system is partly under the control of activity within the system [50,51]. It may be that neural activity generated by larval use of memory also exerts an influence on the structural development of those parts of the central nervous system involved in the template formation process.

Although adoptees are able to distinguish between familiar and alien individuals, our results show that olfactory preimaginal learning does not affect the adoptees’ ability to recognize nest-mates (individuals of the parent colony that were encountered upon eclosion). Memory for familiar cues learned during preimaginal life appears not to be erased but possibly updated by an imprinting-like phenomenon soon after emergence [29]. This is different from the results of Isingrini et al. [11], where preimaginal experience had a stronger effect on brood recognition: at the age of 5 days ants preferred to care for familiar larvae (from the adoptive colony) instead of kin larvae (from the parent colony). However, this preference declined with age: workers tested at the age of 25 days did not show a clear preference between familiar and parent larvae. This suggests that a post-hatching experience in the parent colony may alter the effect of preimaginal learning, but without reversing the preference learned early in life [11]. In our study, we tested workers at the age of 10 days and we thus assume that any possible effect of post-hatching experience is taken into account.

Memory involves costly protein synthesis [52,53] and thus would not always necessarily be adaptive. Ozaki et al. [54] suggested a sensory adaptation model as a proximate mechanism of nest-mate recognition that focuses on olfactory sensilla present in the antenna as processing organs for nest-mate recognition cues. On the other hand, there is evidence that habituation at the level of the antennal lobe might play a role in the nest-mate recognition process [55–57]. According to these two models, the template is decentralized and thus localized somewhere else than higher brain centres. We propose that the decentralized and centralized models might not be mutually exclusive. Both habituation and sensory adaptation can take place when continuous exposure to the stimuli occurs. Our results show that some important information is processed during the larval stages, retained across the pupal stage and then integrated into the adult nervous system, even if no more contact with the stimuli (specific hydrocarbons of the ‘adoptive colony’) occurs. We hypothesize that habituation and/or sensory adaptation, being less costly, could partly replace, during the adult life of ants, the use of long-term memory to discriminate nest-mates. However, the ants should be efficient in discrimination even when they are not in constant contact with the relevant stimuli, such as when they are distant from the colony to forage. In this case, the use of memories could play a key role in the discrimination process.

Further studies of ants’ nervous system development and of the mechanisms that govern the preimaginal learning process are needed to improve our understanding of how ants acquire the recognition template in order to clarify the mechanisms underlying recognition. The discovery of larval memories for nest-mate recognition should stimulate future studies investigating the developmental processes that spawn cognitive behaviours.

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