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Differential conditioning and long-term olfactory memory in individual *Camponotus fellah* ants

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SUMMARY

Individual *Camponotus fellah* ants perceive and learn odours in a Y-maze in which one odour is paired with sugar (CS+) while a different odour (CS–) is paired with quinine (differential conditioning). We studied olfactory retention in *C. fellah* to determine whether olfactory learning leads to long-term memory retrievable 24 h and 72 h after training. One and 3 days after training, ants exhibited robust olfactory memory through a series of five successive retention tests in which they preferred the CS+ and stayed longer in the arm presenting it. In order to determine the nature of the associations memorized, we asked whether choices within the Y-maze were driven by excitatory memory based on choosing the CS+ and/or inhibitory memory based on avoiding the CS–. By confronting ants with a novel odour *vs* either the CS+ or the CS– we found that learning led to the formation of excitatory memory driving the choice of the CS+ but no inhibitory memory based on the CS– was apparent. Ants even preferred the CS– to the novel odour, thus suggesting that they used the CS– as a contextual cue in which the CS+ was embedded, or as a second-order cue predicting the CS+ and thus the sugar reward. Our results constitute the first controlled account of olfactory long-term memory in individual ants for which the nature of associations could be precisely characterized.

Key words: long-term memory, learning, conditioning, olfaction, ant, *Camponotus fellah*.

INTRODUCTION

Associative learning allows the connection of events in an individual's environment in order to make it predictable. It consists of the acquisition of new information through individual experience so that adaptive responses can be produced when facing known situations. Information acquired is stored in the nervous system from where it can be retrieved whenever it is appropriate. Storage and retrieval of acquired information constitute the basis of biological memory. Memory is a dynamic process organized in at least two different forms, short-term memory (STM) and long-term memory (LTM), which exhibit different temporal courses and distinct underlying molecular processes (McGaugh, 2000; Kandel, 2001). While STM is generally labile and independent of protein synthesis, LTM is stable and dependent on *de novo* synthesis of proteins.

Invertebrates have played a pivotal role in the understanding of behavioural and neural mechanisms of learning and memory (Giurfa, 2007b). Decades of research on insect learning have established some of these animals as standard models for the study of learning and memory (Menzel et al., 2007). Examples of this are the fruit fly *Drosophila melanogaster* (Gerber et al., 2004; Davis, 2005; Fiala, 2007) and the honeybee *Apis mellifera* (Giurfa, 2007a), which offer the advantages of learning simple and complex associations and having a relatively simple nervous system that allows associative phenomena to be retraced to the cellular and molecular level in different kinds of laboratory preparations.

Apart from bees and flies, few insects have reached a similar status in learning and memory studies. This is regrettable as such studies would certainly benefit from an across-species comparative dimension allowing the appreciation of commonalities and species-specific mechanisms underlying experience-dependent plasticity. With this in mind, we recently developed a controlled learning assay, which allowed us to study olfactory learning in ants of the genus *Camponotus* on an individual basis (Dupuy et al., 2006). Ants were trained to forage in a Y-maze in which two odours had to be discriminated. One odour was positively reinforced with sucrose solution (positive conditioned stimulus or CS+) while the other was negatively reinforced with quinine solution (negative conditioned stimulus or CS–). After a training session of 24 trials, ants of two species, *C. fellah* and *C. mus*, learned to differentiate the two odours. In non-reinforced tests performed 5 min after the training, they consistently chose the odour previously reinforced with sucrose solution and spent more time searching in the arm of the maze presenting this odour. These results thus showed for the first time that individual ants perceive and learn odours in controlled laboratory conditions (Dupuy et al., 2006). This demonstration converges with recent work on *Camponotus* ants, which described the neuroanatomy of the olfactory circuit (Zube and Rössler, 2008; Zube et al., 2008) and measured neural responses to odours in the olfactory pathways using electrophysiological techniques (Yamagata et al., 2005; Yamagata et al., 2007). Taken together, these findings indicate that these ants could become a suitable and established model for the study of olfactory learning at both behavioural and cellular levels.

In order to further characterize the ants' performance in the differential olfactory conditioning mentioned above (Dupuy et al., 2006) two critical questions have to be answered: (1) does olfactory learning in the Y-maze lead to long-term memory? and (2) what is the nature of the associations learned by the ants and driving their

choice of odorants? In other words, do ants learn to choose the CS+, do they learn to avoid the CS–, or do they use both kinds of associations when choosing within the Y-maze?

With respect to the first question, in our previous work (Dupuy et al., 2006) we performed non-reinforced retention tests almost immediately after the last training trial (5 min). We thus concluded that STM was established in our protocol but whether olfactory memory can reach longer durations remained to be studied. In choosing the intervals to perform LTM tests, one can focus on results obtained in the honeybee, the only social hymenopteran for which memory phases have been accurately characterized (Menzel, 1999). Honeybees learn to associate odorants and a reward of sucrose solution in the laboratory (Takeda, 1961; Bitterman et al., 1983). One conditioning trial (i.e. a single pairing of an odorant and sucrose reward) leads to a mid-term memory (MTM) that can be retrieved 1–12 h after conditioning and to an early long-term memory (e-LTM) that can be retrieved 24–48 h after conditioning. After that, memory vanishes and retention performances therefore decrease. Three conditioning trials, on the other hand, lead to a stable late long-term memory that can be retrieved 72 h or more after conditioning (l-LTM) and that may last for the entire life (Menzel, 1999). Thus, retention tests performed 24 h and 72 h postacquisition constitute a valid approach for a first characterization of LTM in *Camponotus* ants.

The second question aims at understanding what ants do really learn within the maze: to choose the CS+, to avoid the CS–, or both. This question can be answered by training the ants in the differential conditioning protocol and then presenting them with a novel and neutral odour. The ants are thus subjected to non-reinforced tests in which they have to choose between the CS+ and the novel odour, and between the CS– and the novel odour. Preference for the CS+ over the novel odour reveals the presence of excitatory learning of the CS+ while preference for the novel odour over the CS– reveals the presence of inhibitory learning resulting in explicit CS– avoidance [we use the terms 'excitatory' and 'inhibitory' *sensu* Pavlov, who defined excitatory and inhibitory learning as learning leading to the active production and suppression of a stimulus response, respectively (Pavlov, 1927)]. Experiments using this rationale have been performed in free-flying bees trained to discriminate visual targets in a Y-maze and have allowed characterization of the nature of associations learned by the bees (Horridge and Zhang, 1995; Giurfa et al., 1999; Giurfa, 2004).

Here we studied whether olfactory learning leads to stable LTM retrievable at 24 h and 72 h after the last acquisition trial in *Camponotus fellah* ants, and determined whether our differential conditioning protocol leads to the formation of excitatory, inhibitory or both kinds of olfactory memory.

MATERIALS AND METHODS

Insects

Camponotus fellah (Dalla Torre 1893) colonies were reared in the laboratory. Experiments were done in Toulouse, France, with individuals of two different colonies. Each colony was composed of around 1000 workers and one queen, and was maintained at nearly constant temperature (25±3°C), humidity of 70±20% and natural light/dark cycles. Colonies were placed in closed plastic containers (9±7.5 cm and 8 cm high), which were connected by a tube to an open plastic container with fluon-painted walls, which was the external foraging arena. Fluon was used to prevent ants from escaping from the container.

The arena contained a vertical wooden stick on which experimental ants could be collected and put back after each Y-maze visit. Animals could move freely within the nest and had access to fresh water. Between experiments, ants were fed with honey-water and chopped crickets. They were deprived of sugar a week before the onset of experiments and maintained under sub-feeding conditions during the experiments in order to enhance their appetitive motivation to respond to the sucrose solution offered in the Y-maze. Ants used for conditioning experiments were immobilized by cooling and individually marked with white acrylic paint on the thorax.

Experimental set-up

Ants were individually trained to discriminate between two odours, octanal and limonene [Sigma-Aldrich Chimie (Lyon, France)], while foraging in a Y-maze. Only one ant at a time was present in the maze (Fig. 1). The maze was 1.9 cm heigh and its entrance channel and arms were 8 and 6 cm in length, respectively. Arms were separated by 90 deg. The maze was placed on a rectangular supporting base (13.5 cm×14.5 cm) from which it could be removed to be cleaned. The base was supported by four acrylic cylinders (10 cm height), which allowed experimental manoeuvring from below. The maze could be partially covered/uncovered by a removable glass plate (10 cm×15 cm) that left the entrance channel free (Fig. 1).

The procedure was similar to that described previously (Dupuy et al., 2006). Briefly, in each arm, a 10 µl micropipette tip containing a piece of filter paper (0.1 cm×2.7 cm) soaked with 15 µl of odour was inserted in a hole on the floor. The tips had their bottom sealed and their top covered with a plastic net hood. Each tip was placed at a point 1.5 cm into the arm entrance so that ants entering an arm experienced the odour emanating from it. In each arm, reinforcement (sucrose solution 30%, weight/weight, or quinine solution 0.3%) was placed 3.5 cm after the odour tip (Fig. 1). In this way, ants first experienced the odour and then the reinforcement. Sucrose solution was chosen as the appetitive reinforcement as ants actively forage for it (Dupuy et al., 2006). Quinine was chosen as the aversive reinforcement as it is explicitly avoided by several insects such as bumblebees (Chittka et al., 2003) and fruit flies for which it is a common aversive unconditioned stimulus (Fiala, 2007).

An air stream filtered by active charcoal and humidified by water was driven from the back wall of each arm by means of plastic tubes, resulting in a laminar air flow (1.6 cm s^{-1}) reaching the arm intersection. It allowed the odours to be driven towards the decision area of the maze and prevented the direct contact of the odour with the reinforcement. Thus, odours were not present in the solution transported by the forager in its crop, a fact that ensured that apart from the trained forager, all other ants were naive for the conditioned odours. A glass plate partially covered the maze and allowed better concentration of odours (Fig. 1). It was removed once the ant found the sucrose solution. An air extractor was situated above the maze in order to eliminate the odours escaping from the maze throughout the experiment.

Procedure

Pretraining

This stage had the aim of allowing the ant to become accustomed to foraging in the Y-maze. The maze presented neither odorants nor air stream, just the sucrose solution in the intersection of the two arms. The walls of the maze were painted with fluon. Each ant was carefully placed on a piece of cardboard to enable it to be carried from the nest to the pre-training maze. After drinking the sucrose solution for the first time, the ant was gently removed from the maze by means of the cardboard and transported to the top of the

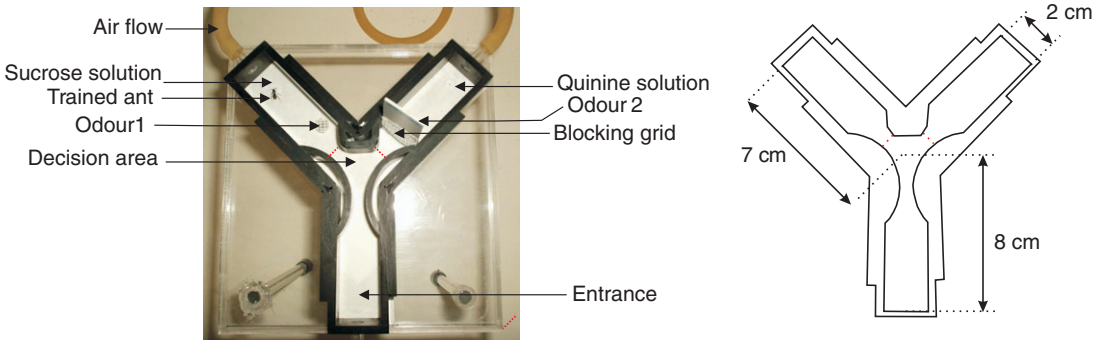

Fig. 1. Top view of the acrylic Y-maze used for conditioning ants in an olfactory discrimination task. Each ant was gently transported on a piece of cardboard from the nest to the entrance zone of the maze, where it was released. The ant moved towards the decision area, delimited by imaginary red lines, where it had to choose between two odours. Each odour was delivered in a micropipette tip, covered by a net hood in order to avoid direct contact. Odours were changed from one arm to the other in a pseudorandom sequence. A mild air flow, coming from the back walls of the maze, ensured odour diffusion. Odour detection at the decision area and/or arm entrance was followed by the reinforcement assigned to each odour (sugar solution or quinine solution). A 1.5 μl droplet of reinforcement was put on small plastic squares (0.5 cm×0.5 cm) positioned close to the back wall of each arm. Due to the spatial arrangement of odour and reinforcement, ants therefore experienced first the odour and then the reinforcement (forward pairing).

vertical wood stick from where it could find its way back to the nest. After 4 or 5 min approximately, the ant resumed its foraging activity by coming back to the vertical stick. In its absence, the Y-shaped filter paper covering the floor of the maze was replaced by a new one. For each ant, this entire procedure was repeated three times before the training was started.

Training

Individual ants were conditioned using a fresh Y-maze, similar to the one used for pre-training, but with the air stream connectors at the end of the arms and without fluon on the walls. After pre-training, ants did not try to escape from the maze but entered the arms searching for food. Two odours, octanal and limonene, were presented, each in a different arm of the maze. For one group of ants, octanal was positively reinforced (the CS+) while limonene was negatively reinforced (the CS–); for another group, this was reversed (i.e. limonene+ *vs* octanal–). Ants were trained during 16 visits (16 trials) and only one ant was present in the maze at a time. Only foragers sufficiently motivated to regularly visit the maze were used for the experiments. Odour-reinforcement position was switched between arms following two pseudorandom sequences (Dupuy et al., 2006): RLRRLLRLLRLRRLRLLRLRRLRL and its mirror alternative (where R and L indicate the side of the sucrose reward). These sequences varied from ant to ant and ensured that ants did not associate the reward with any particular arm (Dupuy et al., 2006). Between trials, the Y-shaped filter paper covering the floor of the maze was changed and the glass plate, the maze and its base cleaned with alcohol and dried with hot air by a hair dryer. This cleaning procedure was repeated systematically after each visit to the maze to avoid orientation by means of pheromones. Special care was taken to always eliminate all possible traces of alcohol that could affect the ant's choice.

In each trial, the first choice of the ant could be correct (CS+ choice) and thus lead to sucrose solution or incorrect (CS– choice) and thus lead to quinine solution. If the choice was correct, we immediately blocked the entrance to the negative arm (for details, see Dupuy et al., 2006). If the ant chose the incorrect arm, a wrong choice was recorded; the ant was then free to move to the positive arm and obtain the reward therein while the negative arm was blocked. Once the ant drank the sucrose solution and left the arm in the direction of the transporting cardboard placed at the entrance channel, access to both arms was blocked thus preventing further uncontrolled olfactory experiences. The ant was then brought back again to the vertical wood stick by means of the piece of cardboard piece. For each ant, data from four consecutive trials were pooled in a block (i.e. four blocks per acquisition curve), which allowed analysis of acquisition in terms of the proportion of correct choices per block.

Experiment 1: assessing olfactory memory

After the completion of training, each ant was subjected to five consecutive retention tests performed under extinction conditions (i.e. no reinforcer was provided in the maze) in order to assess the presence of olfactory memory resulting from the training. Two variables were recorded: (1) its first odorant choice and (2) the time spent in each arm during 2 min. The position of the odorants was changed from one visit to the next following the sequence used for the training. The Y-shaped filter paper covering the floor of the maze was changed between tests and the maze and its base cleaned as explained above. Once the experimental ant had completed the protocol ending with the last retention test, it was removed from the set-up and from the colony.

Two different groups of ants were independently trained and tested. For one of them, retention was evaluated 24 h after the last acquisition trial ('24 h group'); for the second group, retention was evaluated at 72 h ('72 h group'). For the two groups and for each test, we calculated the percentage of animals that chose the correct odorant and the percentage of time spent in the correct arm of the maze.

Experiment 2: characterizing the nature of learned associations

We determined whether excitatory, inhibitory or both kinds of association mediate the ants' choice in our differential conditioning protocol. Two groups of ants were trained over 12 trials following the procedure described above, and then tested without reinforcement immediately after training (i.e. in the visits to the maze following the last training visit). For both groups, ants were first presented with octanal *vs* limonene, the odorants used during the training (control test), in order to verify acquisition. The second test differed between groups. For one group (Group CS+), the CS+ was presented against a new odorant, 2-octanone, while for the other

group (Group CS–) the CS– was presented against the new odorant. Excitatory learning of the CS+ is revealed if ants of Group CS+ prefer the CS+ to the novel odour. Inhibitory learning of the CS– is revealed if ants of Group CS– prefer the novel odour to the CS–, thus avoiding the negatively reinforced odour. We did not perform the two tests in a sequence in the same group of ants because preliminary results showed that non-reinforced experiences on the novel odorant could dramatically affect its choice in the second test. 2-Octanone was chosen as the novel odorant because it has a vapour pressure comparable to that of limonene and octanal (*ca*. 2 mmHg at 20°C) and because it has a different functional group (ketone) from the trained odours, octanal (aldehyde) and limonene (terpene). Experiments on olfactory discrimination in bees have shown that the functional group of chemical molecules is a critical variable allowing stimulus differentiation (Guerrieri et al., 2005). Moreover, this dimension seems to be universal in facilitating olfactory discrimination among a number of species (Haddad et al., 2008).

Statistical analysis

During acquisition, we recorded the first choice of the experimental ant (correct or incorrect). Data were regrouped in blocks of four visits each, which allowed calculation of the proportion of correct choices per block during conditioning. In Experiments 1 and 2, learning performance was analysed along four and three blocks of training, respectively.

Variation in performance along the blocks of trials and between-odours contingencies was evaluated by means of two-factor ANOVA (block×odour contingency) for repeated measures. Tukey test was used for *post-hoc* comparisons. When necessary, data were transformed for normality using the arcsin squareroot transformation. We determined whether the proportion of correct choices of each block was higher than a theoretical level of 50% by means of a one-queued *t*-test. Similarly, a one-queued binomial test was used to establish whether the proportion of correct choices of a single visit was higher than 50%. Comparisons between groups of ants were performed using repeated measures ANOVA.

In the retention tests, two variables were recorded: the first choice and the time spent in each arm of the maze. A one-queued binomial test was used to determine whether the proportion of first choices was higher than 50%. The time spent in each arm of the maze was used to calculate the relative time (%) spent in the correct arm with respect to the total time spent in both arms. A one-queued t-test was used to determine whether the percentage of time in the correct arm was higher than 50%.

RESULTS

Experiment 1: assessing olfactory memory

Ants were trained to discriminate between the odorants octanal and limonene and then tested either 24 h (N=19) or 72 h (N=21) after the last acquisition trial. Independently of which odour was rewarded or punished (limonene+ *vs* octanal– or limonene– *vs* octanal+), both groups learned to discriminate between the odorants and exhibited similar acquisition performances. Their results were therefore pooled and are presented as two learning curves in Fig. 2A. Both the 24 h and 72 h group significantly improved their performance along the four blocks of trials (24 h group: $F_{3,54}$=13.54; P<0.0001; 72 h group: $F_{3,60}$=3.70; P<0.02). The two acquisition curves did not differ significantly ($F_{1,114}$=3.60; P=0.07), a result that was expected given that training proceeded in an identical manner in the two groups. Nevertheless, we analysed the data of the 24 h and the 72 h groups separately because afterwards they were tested at different times.

Comparison of performances between training blocks in the 24 h group showed that the first block differed significantly from the other three blocks (Tukey test; P<0.001 in all three comparisons). For the 72 h group, the first block was significantly different from the fourth block (Tukey test: P<0.03). Other comparisons between blocks were non-significant. For the 24 h group, performance in all blocks was significantly different from 50% (one queued *t*-test; first block: t_{18}=3.83; second block: t_{18}=7.91; third block: t_{18}=11.91; fourth block: t_{18}=26.19; P<0.005 in all cases), thus suggesting that along the four trials of the first block, ants learned to discriminate between the odorants. For the 72 h group, a similar result was found (first block: t_{20}=4.32; second block: t_{20}=5.97; third block: t_{20}=8.77; fourth block: t_{20}=10.28; P<0.001 in all cases). To verify this hypothesis, we analysed the ants' performance within the first block of four trials (see inset in Fig. 2A). Indeed, for the 24 h group, the proportion of correct choices did not differ from 50% during the first three visits of the first block but became significantly different from a random choice in the fourth visit (one queued binomial test: P<0.001). For the 72 h group, the proportion of correct choices did not differ from 50% during the first two visits of the first block but became significantly different from a random choice in the third and fourth visit (binomial test: P<0.05 in both cases). These results show, therefore, that in both groups learning was extremely fast as it had already occurred within the first block of trials. They also show that at the beginning of conditioning, ants in both groups were naive to the conditioned odours as no odour preference was visible in the first and second trials (see inset of Fig. 2A).

Both 24 h and 72 h after the last acquisition trial, retention was independent of odour contingency (limonene+ *vs* octanal– or limonene– *vs* octanal+), so that in each group (24 h and 72 h) the results of the two subgroups of ants were pooled for both variables considered (Fig. 2B: first choice; Fig. 2C: percentage of time spent in the arm with the correct odour). Analyses performed on these variables showed that a robust olfactory memory guided ants' choices both 24 h and 72 h after the last acquisition trial. In the 24 h group, ants more frequently chose the correct odour in the five consecutive tests without reinforcements. In all tests but the third, performance attained 79%; in the third test, it reached 84%. In all cases, these values were significantly different from 50% (binomial test; P<0.01 for all five tests). Similarly, ants spent more time in the arm of the maze presenting the positive odour (first test: t_{18}=5.42; second test: t_{18}=3.81; third test: t_{18}=3.56; fourth test: t_{18}=3.32; fifth test: t_{18}=3.39; P<0.001 for all five tests).

In the 72 h group, ants also more frequently chose the correct odour in three of the five tests (first, second and fifth tests). In these tests, performance attained 76%, 71% and 81%, respectively, and was significantly different from 50% (binomial test; P<0.04 for all three tests). In the third and fourth tests, performance reached 62% and 67%, respectively, and did not differ from 50% (P=0.19 and P=0.10, respectively). The fact that retention was still significant in the last test shows that despite a lack of significance in the third and fourth tests, olfactory memory still guided the ants' choices. This conclusion was confirmed by an analysis of the time spent in the positive arm of the maze. Performance attained 67%, 65%, 62%, 60% and 72% in the first, second, third, fourth and fifth tests, respectively. This performance was significantly different from 50% in all tests (first test: t_{20}=3.36; second test: t_{20}=2.73; third test: t_{20}=2.19; fourth test: t_{20}=1.86; fifth test: t_{20}=6.44; P<0.05 for all five tests). These results show therefore that *C. fellah* ants efficiently learned olfactory discrimination and were able to retrieve the learned information 24 h and 72 h after learning. Moreover, they demonstrate that the memories formed are particularly resistant to extinction

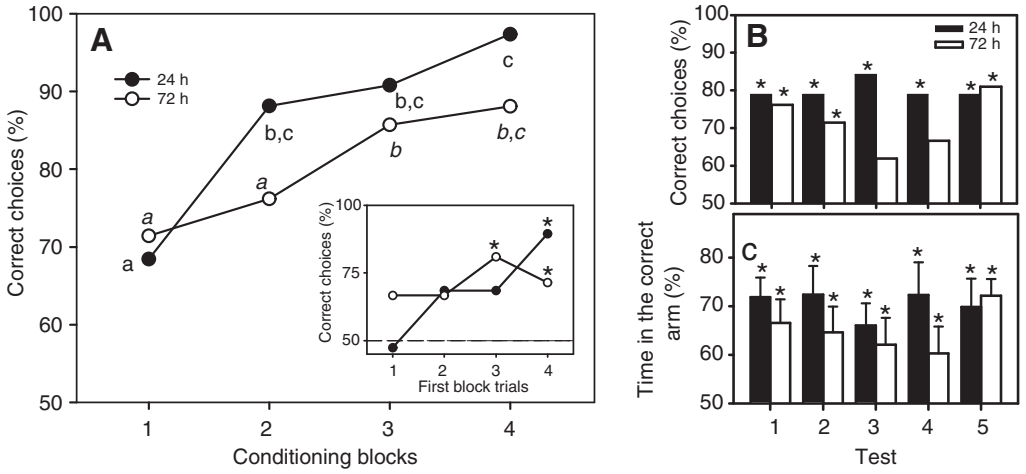

Fig. 2. Discriminative learning and retention of *Camponotus fellah* ants trained to distinguish octanal from limonene. (A) Acquisition curves of two groups of ants (24 h, *N*=19 and 72 h, *N*=21) trained in parallel and tested for retention 24 h and 72 h after the last acquisition trial. Curves represent the pooled performance (percentage of correct choices, i.e. choice of the odour associated with sucrose) of ants trained with both contingencies (i.e. odour A+ *vs* odour B– and *vice versa*) along four blocks of four visits to the maze. Different letters indicate values that differ significantly within each acquisition curve. Both groups learned the olfactory discrimination during training. Inset: performance in the first four training trials showing fast learning within the first training block. Asterisks indicate significance with respect to a 50% choice level. (B) First choice in five consecutive tests without reinforcement performed 24 h or 72 h after training. Asterisks indicate significant differences in performance with respect to a 50% choice level. Both groups of ants remembered and preferred the odour previously associated with sucrose. (C) Percentage of time spent in the correct arm (with respect to the time spent in both arms) during five consecutive tests without reinforcement performed 24 h or 72 h after training (means + s.e.m.). Asterisks indicate significant differences in performance with respect to a 50% choice level. Both groups of ants spent more time in the arm presenting the odour previously rewarded therefore showing robust long-term memory.

given the fact that they could be retrieved after several consecutive non-reinforced tests.

Experiment 2: characterizing the nature of learned associations

In this experiment, training was reduced to three instead of four blocks given the fast acquisition shown by ants in the previous experiment. Two groups of ants were trained to discriminate limonene from octanal and then subjected to a retention test with the trained odorants and to a second test differing between groups. For Group CS+ (*N*=18), the previously rewarded odorant (the CS+) was presented against a novel odorant, 2-octanone; for Group CS– (*N*=11), the previously punished odorant (CS–) was presented against 2-octanone. Although training was identical in the two groups, we present the acquisition curves separately (Fig. 3A) to refer test performances to acquisition levels reached at the end of training.

The two groups behaved similarly irrespective of odorant contingency (limonene rewarded *vs* octanal punished or *vice versa*) so that for each group a single learning curve is presented (Fig. 3A). For both groups, the performance during blocks of training did not change significantly (Group CS+: $F_{2,34}$=2.56; *P*=0.09; Group CS–: $F_{2,34}$=2.20; *P*=0.22). This was not due to an absence of learning as shown by the high percentage of correct choices in all three blocks of trials (between 67% and 79%). In fact, in all blocks, ants were significantly above a random choice of 50% (Group CS+, first block: t_{17}=2.49; second block: t_{17}=4.61; t_{17}=4.75; *P*<0.025 for all three blocks; Group CS–, first block: t_{10}=3.19; second block: t_{10}=2.89; t_{10}=5.22; *P*<0.01 for all three blocks). Thus, as in the previous experiment, learning was extremely fast and had probably already occurred within the first block of trials. Analysis of the performance within the first block (see inset in Fig. 3A) showed that for Group CS+ this was indeed the case as the percentage of correct choices was different from a random choice in the third and fourth trials (binomial test; *P*<0.05), thus showing significant learning within the first block of trials. For Group CS–, performance was random in the first trial (binomial test; *P*=0.27), differed from a random choice in the second and third trials (binomial test; *P*<0.03 in both cases) but decayed to a non-significant level in the fourth trial (binomial test; *P*=0.5). Despite this decay, performance increased again (67%) and became significant in the next visit (first trial of the second block; not shown; binomial test; *P*<0.05). These results underline again that the first experiences in the maze triggered a fast learning process in *C. fellah* ants.

To characterize the memories established in these experimental circumstances, we analysed the test performance of Group CS+ and Group CS–. Both groups remembered the associations learned during the training. Group CS+ chose the rewarded odorant in 81% of the cases and Group CS– in 82% of the cases (Fig. 3B). Both values were significantly different from 50% (binomial test; *P*<0.001). Furthermore, both groups spent more time in the correct arm of the maze (the arm presenting the CS+; *F*ig. 3C; 70% for Group CS+, t_{17}=2.49, *P*<0.025, and 68% for Group CS–, t_{10}=3.94, *P*<0.005).

When ants of the CS+ Group were afterwards confronted with the CS+ *vs* the novel odorant 2-octanone, they preferred the CS+. Although the tendency in the percentage of choices for the CS+ was not significant (56%; binomial test, *P*=0.40), the percentage of time spent in the arm with the CS+ (71%) was highly significant (t_{17}=3.32; *P*<0.005), thus showing that ants were guided by the memory of the odour previously rewarded. Ants of the CS– group displayed a surprising behaviour: when confronted with the CS– *vs* the novel odorant 2-octanone, they preferred the CS– even if this odorant was paired with quinine during conditioning. Although the percentage of choices for the most preferred odour was clearly biased towards the CS– (72%), this proportion was non-significant

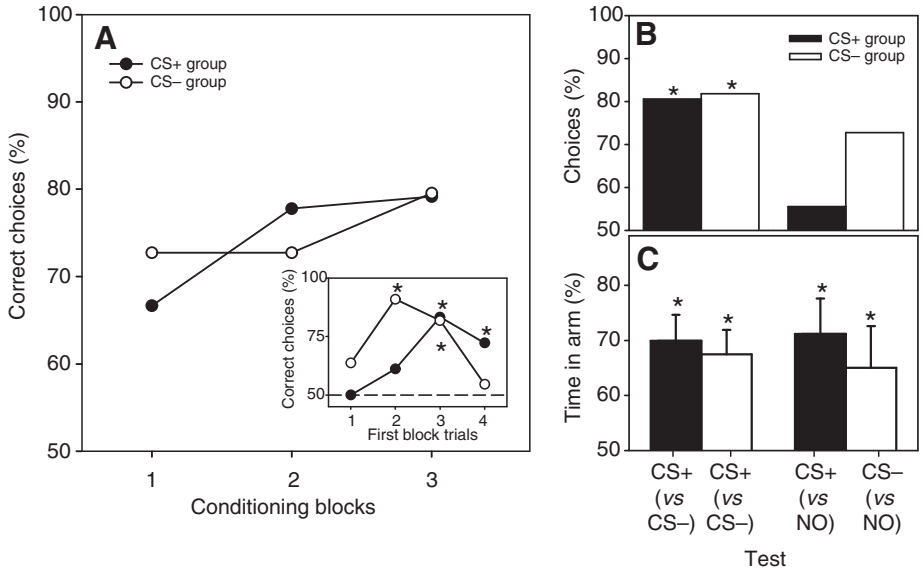

Fig. 3. The nature of associations established by *C. fellah* ants trained to distinguish octanal from limonene. (A) Acquisition curves of two groups of ants (CS+ group, *N*=18 and CS– group, *N*=11) trained in parallel and tested after the last acquisition trial. Curves represent the pooled performance (percentage of correct choices, i.e. choice of the odour associated with sucrose) of ants trained with both contingencies (i.e. odour A+ *vs* odour B– and *vice versa*) along three blocks of four visits to the maze. Both groups were above random level during training. Inset: performance in the first four training trials showing fast learning within the first training block. Asterisks indicate significance with respect to a 50% choice level. (B) First choice in two consecutive tests without reinforcement. In the first test, both the CS+ and CS– group were presented with the CS+ *vs* the CS– to verify learning. Both groups significantly preferred the CS+. In the second test, group CS+ was presented with the CS+ *vs* a novel odour (NO) while group CS– had the CS– *vs* the NO. Group CS+ showed a non-significant tendency to prefer the CS+ to the NO. Group CS– preferred the CS– to the NO. Asterisks indicate significant differences in performance with respect to a 50% choice level. (C) Percentage of time spent in the correct arm (with respect to the time spent in both arms) during the same two consecutive tests without reinforcement (means + s.e.m.). Asterisks indicate significant differences in performance with respect to a 50% choice level. Both groups of ants spent more time in the arm presenting the CS+ when it was presented against the CS–. Group CS+ spent more time searching for the CS+ when presented *vs* the NO while Group CS– spent more time searching for the CS– when it was presented *vs* the NO.

(binomial test, P=0.11), probably because of the sample size. The time spent in the arm with the CS– (67%) was nevertheless significant (t_{10}=2.19; P<0.05), thus showing that ants explicitly chose the odorant that was negatively reinforced.

DISCUSSION

The present work shows that olfactory learning induces long-term memory formation in *Camponotus* ants. To our knowledge, this constitutes the first controlled account of olfactory memory in ants in which the presence of memory has been verified 72 h after training and in which the nature of the associations driving the ants' choice was precisely characterized. Investigations performed on groups of ants on an ecological scale have the advantage of studying the ants' foraging behaviour in a natural context but cannot make claims on memory duration as the precise experience of individuals can hardly be controlled in such experiments. In such circumstances, it is impossible to define whether an individual's response is driven by information acquired individually or by simply following the group. The latter option can also be the basis of complex collective decision processes, which obviously have nothing to do with individual learning and memory (Theraulaz et al., 2003). Biological memory belongs indeed to an individual's register and, as such, it is stored in the central nervous system from where it can be retrieved. Assessing memory duration and memory phases therefore requires the careful control of an individual's experience, something that was achieved in our experimental laboratory protocol (Dupuy et al., 2006).

After being trained to discriminate an odorant positively reinforced from an odorant negatively reinforced, ants were able to retrieve the learned information 24 h and 72 h after training, thus showing that besides STM in the range of minutes – shown in our previous work (Dupuy et al., 2006) – they have the capacity to store and retrieve olfactory information in the long term (in the range of days). In the honeybee, the only social hymenopteran for which memory phases have been accurately characterized (Menzel, 1999), one pairing of an odorant with sucrose (i.e. one conditioning trial) leads to an e-LTM that can be retrieved 24–48 h after conditioning while three conditioning trials lead to a stable l-LTM that can be retrieved 72 h or more after conditioning. Similar retrieval capacities have been found here although we did not study the effect of the number of trials on memory duration, a problem that can easily be addressed by our protocol in future work in which retention is measured after a variable number of acquisition trials. Caution is, however, required when comparing our results with those of the honeybee. In bees, the differentiation between e-LTM and l-LTM is not simply based on a behavioural account (i.e. when memories are retrieved and how many trials are required to see such retrieval) but also refers to a distinct molecular basis. Indeed, e-LTM depends on protein synthesis, but from already available mRNA, without *de novo* transcription, while l-LTM depends on transcription (Schwaerzel and Müller, 2006). Induction of both forms of LTM requires activation of a cAMP-dependent protein kinase (PKA) mediated by nitric oxide (NO) (Müller, 2000). In the case of *Camponotus* ants, whether such a molecular distinction applies remains so far unknown. It should be possible, however, to inject protein synthesis inhibitors into the ant brain and determine which kind of memories are unaffected by this treatment. We predict that memories retrieved 24 h after training will be intact as they would

correspond to e-LTM while memories retrieved 72 h after training will be severely affected as they would correspond to l-LTM. In the absence of such an experiment, we will restrict our terminology to a temporal domain and call e-LTM the memories guiding the ants' choices 24 h after training and l-LTM the memories mediating choice behaviour 72 h after training.

Leaving apart reports on ant memory based on group performances (see above), the use of long-term, olfactory memory has previously been proposed in the context of ant social recognition. In this case, memorized odour cues are produced by the ants themselves and are used to facilitate social recognition. This raises an important difference with respect to our findings as the odours memorized by *C. fellah* did not belong to the colony but characterized food sources whose properties had to be discriminated. Olfactory memory in a social context has been reported in the case of *Pachychondyla villosa* ant queens (Dreier et al., 2007). By quantifying the level of aggression between pairs of familiar or unfamiliar queens over time, it was shown that unrelated founding queens of *P. villosa* and *P. inversa* store information on the individual identity of other queens and can retrieve it from memory after 24 h. Contrary to the experiments performed in this work, neither the reinforcer nor the specific cues learned by these queens were identified. Differences in the cuticular hydrocarbon profile may provide the necessary information to be memorized in order to keep the discrimination (Dreier et al., 2007). Memorization of colonial or species olfactory identity has been proposed for other ant species but the specific cues entering into the memory traces are unclear even in experiments conceived to test individual and not group performances (e.g. Nowbahari, 2007; Leonhardt et al., 2007). More problematic are the claims of the presence of long-term olfactory memory in ants based on serious misconceptions about insect memory. It has recently been argued, for instance, that *Cataglyphis cursor* ants can establish a LTM of the individual odour of a heterospecific ant *Camponotus aethiops* as they can 'discriminate it from the odour of an unfamiliar individual after at least 30 min, which is considered to be LTM for an insect' (Foubert and Nowbahari, 2008). Obviously, the claim that a 30 min period corresponds to LTM in insects is wrong (Menzel, 1999).

Our work uncovers the nature of the associations learned by ants in the Y-maze. Originally conceived as a differential conditioning protocol, with one odour rewarded with sucrose and an alternative odour punished with quinine, our training procedure yielded a result that was unexpected at a first sight but that can be explained based on the choice dynamics of ants. In principle, a differential conditioning protocol should lead to the establishment of excitatory memory traces, resulting from experience with the positively reinforced stimulus (CS+), and inhibitory memory traces, resulting from experience with the negatively reinforced stimulus (CS–). We therefore expected that ants should prefer the CS+ to a novel odorant, and the novel odorant to the CS–. Although ants did indeed prefer the CS+ to 2-octanone, they also preferred the CS– to 2-octanone.

Preference of the CS– to 2-octanone was not due to intrinsic repellent properties of 2-octanone, which may overrun the inhibitory learning induced by the CS–. In *C. fellah*, 2-octanone is not known to induce any behavioural effect (see http://www.pherobase.com/) although in another *Camponotus* species, *C. schaefferi*, 3-octanone, but not 2-octanone, acts as an alarm pheromone for workers (Duffield and Blum, 1975). In *C. fellah*, preliminary observations did not show repellence to this substance. Moreover, results of the CS+ group allow us to discard this hypothesis: if the novel odorant 2-octanone had exerted a repellence stronger than the inhibitory CS– learning, results from the test confronting the CS+ *vs* 2-octanone should have shown an amplified preference for the CS+ (summation of CS+ preference and 2-octanone aversion), compared with that observed in the test confronting CS+ *vs* CS–. This was never the case (Fig. 3).

Furthermore, preference for CS– over 2-octanone cannot be explained by stating that 2-octanone acquired an inhibitory value due to a non-reinforced exposure during a previous test as this preference was recorded in independent groups (groups CS+ and CS–) so that ants were exposed only once to the novel odorant. Moreover, although perceptual similarity can affect olfactory discrimination (Deisig et al., 2002), similarity between the trained octanal and the novel 2-octanone did not affect the results as test performances were the same irrespective of whether octanal was associated with sucrose or quinine. Note also that for honeybees, where this information is available, octanal and 2-octanone are perceived as highly dissimilar (Guerrieri et al., 2005) as bees trained with 2-octanal respond to it in 96% of cases while they only respond to 2-octanone in 28% of cases.

We therefore suggest that the preference for the CS– to 2-octanone results from the fact that ants had little contact with the quinine solution during training with the CS–, and that this contact was usually restricted to the very first trials. In other words, the potential aversive effect of quinine might have been overrun by the positive experiences with sucrose. In this scenario, the odorant paired with quinine, rather than acting as a CS–, may act instead as a contextual cue in which the positively reinforced odorant is embedded. Furthermore, the 'negative' odour may also act as a second-order stimulus in a second-order conditioning process. In second-order conditioning, animals that have learned that a given conditioned stimulus (CS_1) predicts the unconditioned stimulus (US), also learn, through pairings of a second conditioned stimulus (CS_2) with the CS_1, that CS_2 predicts CS_1, which in turn predicts the US. A similar phenomenon may be occurring in the Y-maze, thus leading the ants to prefer the CS– to the novel odorant. Second-order conditioning has been shown in honeybees both in the olfactory (Bitterman et al., 1983; Menzel, 1990; Hussaini et al., 2007) and visual modality (Grossmann, 1971), thus making this explanation plausible. In any case, the CS– would always be less attractive than the CS+, given the close connection (spatial and temporal) between the sucrose reward and the CS+. This factor may explain why CS+ is preferred to CS– and why CS– is preferred in turn to the novel odour (Fig. 3B,C). Only in the case of the first choice did CS+ ants not exhibit a clear preference for the CS+ over the novel odour (Fig. 3B; not significant). For the second variable, the percentage of time spent in the CS+ arm, ants significantly preferred the CS+ to the novel odour as expected in the case of excitatory CS+ memory driving their choice.

A different explanation could be provided to account for the lack of inhibition produced by the CS–. It may simply be that quinine is not an efficient negative reinforcer for ants, unlike vertebrates. Recent findings on honeybee taste support this idea (de Brito Sanchez et al., 2006; de Brito Sanchez et al., 2007). Behavioural and electrophysiological explorations of gustatory sensilla on the antennae of bees failed to detect any response to bitter substances, which would be perceived as being not so different from water. The fact that the bee genome (The Honeybee Genome Consortium, 2006) has so far revealed the presence of only 10 gustatory receptor genes in bees (*vs* 68 in the fruit fly) (Robertson and Wanner, 2006) supports the idea that bees, and probably other social Hymenoptera, do not necessarily have a developed sense for bitter substances. In this scenario, bitter gustatory input would not be an effective negative reinforcer for ants and would not support the formation of inhibitory memory associated with the CS–.

The use of olfactory memory seems to be particularly adaptive in the case of Mediterranean *C. fellah* ants, which are mostly nocturnal (A. Hefetz, personal communication) and may thus rely on olfactory rather than visual cues. Moreover, these ants do not seem to use a clear trail pheromone but tend to forage alone, even if on some occasions they may use group recruitment. Under these circumstances, the establishment of olfactory LTM as shown by our work is particularly useful in helping the return to profitable feeding sites. If memory phases have a specific ecological correlate, related, for instance, to the foraging dynamics of an animal (Menzel, 1999), one could predict that ants relying mostly on visual cues and using pheromone trails to return to feeding sites would exhibit more labile olfactory memory when compared with *C. fellah*. This hypothesis could easily be tested using the procedures described in our work.

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