ANIMAL BEHAVIOUR, 2006, 72, 1081–1091
doi:10.1016/j.anbehav.2006.03.011

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Individual olfactory learning in *Camponotus* ants

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(Received 28 July 2005; initial acceptance 7 October 2005; final acceptance 3 March 2006; published online 23 August 2006; MS. number: 8635R)

We studied olfactory learning in two ant species, *Camponotus mus* from Argentina and *Camponotus fellah* from Israel. To this end, we established an experimental laboratory protocol in which individual ants were trained to associate odours with gustatory reinforcers. Ants were trained individually to forage in a Y-maze in which two odours had to be discriminated. One odour was positively reinforced with sucrose solution and the other was negatively reinforced with quinine solution. After a training session of 24 trials, ants of both species learned to differentiate the two odour pairs, the structurally dissimilar limonene and octanal, and the structurally similar heptanal and 2-heptanone. In nonreinforced tests, ants consistently chose the odour previously reinforced with sucrose solution and spent more time searching in the arm of the maze presenting this odour. Learning performances were more robust in the case of limonene versus heptanal. These results thus show for the first time that individual ants perceive and learn odours in controlled laboratory conditions.

Learning constitutes an experience-dependent change in an individual's behaviour, which determines that animals that had the experience behave differently from those that did not (Rescorla & Holland 1976; Shettleworth 1994; Pearce 1997). Although there are several forms of learning, which vary in their informational content, their neural substrates and in the way information is acquired, they all have in common that learning occurs at the level of the individual. Even when groups of animals adopt novel adaptive responses, such changes are strictly based on the information acquired by individuals and are, therefore, also cases of individual learning. Social learning is appropriately defined, therefore, as individual learning occurring within a social context (Heyes 1993; Brown & Laland 2003). Furthermore, learning is associated with memory, which is the capacity of storing information in a given physiological substrate, from which it can be recovered in appropriate circumstances to generate adaptive responses (Tulving & Craik 2000). Individual animals, not groups, have brains (or equivalent neural structures) enabling information storage and retrieval as a result of learning.

A common problem in biological approaches of learning and memory is the difficulty in distinguishing between changes in behavioural performance and the nature of the learned information driving such changes (Rescorla 1988). Although focusing on changes in performance is crucial for any learning study, knowing, in addition, which associations underlie such changes may be enlightening. In that sense, experimental protocols that not only demonstrate changes in performance but also allow the study of associations driving behaviour are welcome. Such protocols require a precise control of the individual's experience with the stimuli that are to be learned. To this end, and because learning is an individually based phenomenon, individual and not mass training needs to be used in learning protocols. Mass training precludes control of individual experience because animals trained as a group may either respond based on their learning of the experimental situation or simply follow other individuals of the group. Even if animals are tested individually after mass training, it is impossible to know how much experience they gathered during such training, especially if instead of being identified, they are haphazardly chosen from a group.

Insect models have contributed considerably to the study of learning and memory because they combine behavioural plasticity and experimental accessibility, at

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0003–3472/06/$30.00/0

both behavioural and cellular levels (Carew & Sahley 1986; Menzel & Müller 1996; Belvin & Yin 1997; Dubnau & Tully 1998; Heisenberg et al. 2001; Menzel 2001; Waddell & Quinn 2001; Giurfa 2003; Greenspan & van Swinderen 2004). Experimental accessibility is related to the fact that learning experiments with insects are amenable to conditions in which the individual and the stimuli used can be controlled. Nevertheless, the number of insect models in which such conditions have been met remains small. Besides the honeybee, *Apis mellifera*, and the fruit fly, *Drosophila melanogaster*, only cockroaches, *Periplaneta americana* (Mizunami et al. 1998; Sakura et al. 2002; Watanabe et al. 2003; Pinter et al. 2005; Watanabe & Mizunami 2006) and crickets, *Gryllus bimaculatus* (Matsumoto & Mizunami 2000, 2002a, b, 2004, 2005) have provided useful insights into behavioural and cellular mechanisms of learning and memory. Other insect models are thus necessary to allow comparative approaches focusing on commonalities and species-specific particularities of learning.

From this perspective, ants are an interesting model for comparative studies on learning and memory as they constitute a varied group with a great diversity of life histories, ecological interactions and novel evolutionary adaptations (Hölldobler & Wilson 1990). Experience-dependent changes in performance have been repeatedly demonstrated in ants in different behavioural contexts. However, such changes do not necessarily reveal the specific cues and/or reinforcers guiding the ants' behaviour. Although ants can learn to solve a variety of problems such as orienting and navigating in more or less complex environments (e.g. *Cataglyphis*: Wehner et al. 1996; Wehner 2003; *Formica*: Schneirla 1941, 1943; Durier et al. 2004; Graham et al. 2004) or visiting feeding places at specific times of the day (Harrison & Breed 1987; Schatz et al. 1999), it is sometimes difficult to establish what is learned in such cases. For instance, when *Cataglyphis* ants learn that a specific landmark indicates the nest entrance, it is difficult to define the reinforcer underlying this performance. Whenever an *Ectatomma* ant is at the right place at the right time in a forest to acquire a food reward (Harrison & Breed 1987; Schatz et al. 1999), we may certainly say that it has learned a temporal reinforcement schedule, but it is difficult to identify the sensory cues and associations mediating such learning at any moment of the animal's choice. From this perspective, relating orientation performances to the framework of well-established learning paradigms such as classical or operant conditioning, or identifying the specific associative links underlying spatial problem solving, may be helpful. In addition, mass training protocols should be abandoned and terms such as 'colony learning' (e.g. Johnson 1991; Johnson et al. 1994) should be used with caution.

Olfactory cues are important in most aspects of the life of ants, such as foraging, communication, larval grooming, nest defence and localization, social control and nestmate recognition. Apart from studies on interspecific recognition, experimental studies related to olfactory perception in ants have been carried out mostly from a navigational (Ehmer 1999; Wolf & Wehner 2000, 2005) or a foraging perspective (Roces 1990, 1994; Johnson 1991). These studies did not quantify individual acquisition or retention performances but some suggested that ants could indeed learn odours in an appetitive context of food search, that is, when actively searching for food. Leaf-cutter ants, *Acromyrmex lundi*, learn the odour of food introduced into the nest by other foragers so that their choice is later guided by these olfactory cues (Roces 1990, 1994). However, the nature of the associations established and of the reinforcements involved remains unclear. Carpenter ants, *Camponotus pennsylvanicus*, seem also to learn the odour of a substrate or of an air stream associated with food (Helmy & Jander 2003). However, this result was obtained by using mass training, so the influence of collective aspects on decision making cannot be excluded (Theraulaz et al. 2003).

Ants relying heavily on olfactory cues have well-developed olfactory centres in their brains (Gronenberg 1999a,b), which, in some species, are proportionally larger than those of honeybees. Such centres are particularly large in species of the genus *Camponotus*, reflecting the importance of olfactory cues for these ants. Furthermore, *Camponotus* ants are nectivorous, which implies that sucrose solution could be used experimentally as a reinforcer in conditioning experiments. Therefore, it seems possible to use these ants for studying olfactory learning and perception. What is lacking from this perspective is a clear-cut experimental demonstration that an individual ant forager can learn odours while searching for food in a simplified laboratory environment. Using such an environment allows us to control not only individual performances but also the cues and reinforcers that ants can learn to solve a discrimination problem. We trained two species of the genus *Camponotus*, *C. mus* from Argentina and *C. fellah* from Israel, to forage in a Y-maze in which two odours, one positively reinforced with sucrose solution and the other negatively reinforced with quinine solution, had to be discriminated.

METHODS

Study Insects

The colonies of *C. mus* and of *C. fellah* were all reared in the laboratory. Experiments with *C. mus* were done in Buenos Aires, Argentina, with individuals of six different colonies reared in the same conditions. Experiments with *C. fellah* were done in Toulouse, France, with individuals of two different colonies. Queenright colonies of *C. mus* were reared at a temperature of $26 \pm 3^{\circ}$C and a humidity of $70 \pm 20\%$. Each colony was placed in a plastic open container (20×30 cm and 20 cm high), with walls covered with fluon to prevent ants from escaping, and exposed to external light conditions visible through the laboratory windows. The container included the nest and a surrounding foraging arena. Queenright colonies of *C. fellah* were reared at similar temperature, humidity and illumination conditions as *C. mus*. They were placed in a plastic container (9×7.5 cm and 8 cm high) composed of six chambers made of plaster and covered by a glass plate, which were connected by a tube to a second plastic container (7×6 cm and 9 cm high), which was the external foraging arena. The walls of this arena were covered with fluon to prevent ants from escaping. Each foraging arena

contained a vertical wooden stick on which ants could be collected or put back.

Both ant species were fed with insects as a protein source (cockroaches for *C. mus* and crickets and larvae of *Tenebrio molitor* for *C. fellah*), and with water ad libitum delivered in cotton wool. Carbohydrates were provided in the form of honey water droplets, which were put on a small plastic plate placed in the arena. During the experiments, a limited amount of honey water (approximately 0.1 ml) was provided daily to enhance the ants' appetitive motivation to respond to the sucrose solution offered in our experiments. Ants used for conditioning experiments were immobilized by cooling and individually marked with white acrylic paint on the thorax.

Experimental Set-up

We trained marked ants to forage, one at a time, in an acrylic Y-maze 1.9 cm high (Fig. 1a). The entrance channel and the arms of the maze were 8 and 6 cm long, respectively. The arms were separated by 90°. The maze was placed on a rectangular supporting base (13.5 × 14.5 cm) from which it could be removed to be cleaned. The base was supported by four acrylic cylinders (10 cm high), which allowed experimental manoeuvring from below. The maze could be partially covered/uncovered by a removable glass plate (10 × 15 cm) that left the entrance channel free (Fig. 1a). The floor of the maze was covered by a piece of Y-shaped filter paper. We replaced this paper by a clean one after each visit of an ant to the maze to avoid the use of pheromonal trails.

The entrance to each arm was defined as the intersection of both arms (dashed lines in Fig. 1 defining an unmarked decision area in the maze). In each arm, a 10-µl micropipette tip containing 15 µl of odour and a piece of filter paper (0.1 × 2.7 cm) was inserted in a hole in the floor specially created for this purpose. The tips were sealed at their bottom and covered with a plastic net hood at their top. Each tip was placed 1.5 cm from the arm entrance, so that ants entering an arm experienced the odour emanating from it. In each arm, reinforcement (sucrose or quinine solution; see below) was placed 3.5 cm after the odour tip (Fig. 1a), so that ants first experienced the odour and then the reinforcement. 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. This allowed the odours to be driven towards the decision area of the maze and prevented direct contact between the odour and the reinforcement. This is important because odours must not be carried into the nest in the solution transported by the forager in its crop, as the rest of the ants have to be naïve for the odours used in the maze. The glass cover (see above) allowed better concentration of odours and was removed once the ant found the sucrose solution.

A semicylindrical PVC grey wall (34.6 cm high, 40.6 cm in diameter) was positioned around the maze and 14 cm from its base. This wall prevented the ants from using external visual cues to guide their choices. We ensured that illumination coming from artificial lamps and laboratory windows was symmetrical (with respect to the left and right arms of the maze) and homogeneous. An air extractor was situated 28 cm above the maze to eliminate the odours escaping from the maze throughout the experiment.

Stimuli

Olfactory stimuli were used to condition individually marked ants within the maze. We used two pairs of chemical substances (Fig. 1b); in one pair, the odorants were limonene (98% purity) and octanal (99% purity), which are structurally dissimilar; in the other pair, the odorants were heptanal (95% purity) and 2-heptanone (98% purity), which are structurally more similar. These odorants have been used regularly in behavioural and

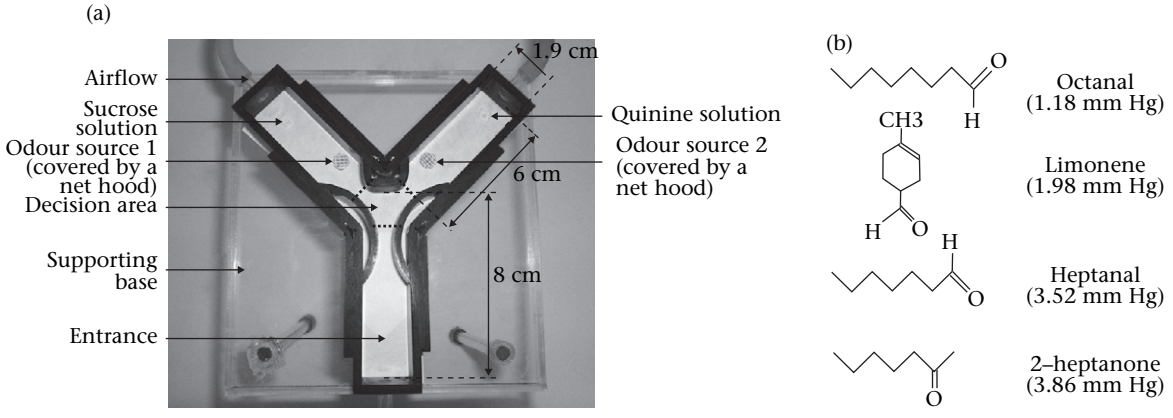

Figure 1. (a) Top view of the acrylic Y-maze used for conditioning ants in an olfactory discrimination task. Each ant was transported to the entrance zone of the maze, where it was released. The ant moved towards the decision area, delimited by the dashed lines on the figure, where it had to choose between the two odours. The airflow 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). Owing to the spatial arrangement of odour and reinforcement, ants therefore experienced first the odour and then the reinforcement (forward pairing). See Methods for further details. (b) Odours used for conditioning. The values in parentheses indicate the corresponding vapour pressures. Ants were conditioned to discriminate octanal from limonene and heptanal from 2-heptanone. The first discrimination involved structurally dissimilar odours, the second one, structurally similar odours. Odours within a pair had comparable vapour pressures to avoid differences caused by diffusion within the maze.

physiological studies on olfactory learning and discrimination in other social Hymenoptera such as honeybees (e.g. Guerrieri et al. 2005a, b). These studies showed that the functional group and the number of carbons of a molecule are critical variables in olfactory perception (Guerrieri et al. 2005a). Limonene, a terpene with 10 carbons, and octanal, an aldehyde with eight carbons, are expected to be perceptually dissimilar whereas heptanal, an aldehyde with seven carbons, and 2-heptanone, a ketone with seven carbons, should be more similar. All chemicals were obtained from Sigma-Aldrich (Lyon, France). In all cases, 15 μl of pure substance were applied inside the micropipette tip containing a filter paper. Odours diffused from the tip into the arm of the maze and the decision chamber, so that each arm was characterized by a distinctive odour. Odours of each conditioning pair had similar vapour pressures (mm Hg; 25°C; limonene: 1.98; octanal: 1.18; heptanal: 3.52; 2-heptanone: 3.86) thus ensuring comparable diffusion within the maze. The micropipette tips were renewed during the experiment every eight visits (approximately 1 h) or if the ant walked on one of them.

Both *Camponotus* species are nectivorous and collect nectar from extrafloral nectaries. We therefore used 30% (weight/weight) sucrose solution as a positive reinforcer. As a negative reinforcer, we used quinine solution. Quinine solution has been used as an aversive stimulus in learning experiments with fruit flies and bees (Tompkins et al. 1979; Chittka et al. 2003). Based on preliminary assays, we chose 3% (weight/weight) quinine solution for *C. mus* and 0.3% quinine solution for *C. fellah*. The amount of both solutions (sucrose and quinine) provided in each trial was 1.5 μl and was delivered by means of Eppendorf micropipettes (0.5–10 μl) on small plastic squares (0.5 × 0.5 cm) positioned close to the back wall of each arm. The amount of sugar solution was determined in previous control experiments and was chosen to keep the motivation of the foraging ant high as evidenced by its frequent and prompt returns from the nest to the vertical wooden stick in the foraging arena, from where it was collected and transported to the maze (see below).

Procedure

Pretraining

For each experiment, an ant was individually marked with a colour spot on its thorax and trained to forage for sugar solution in a Y-maze. The maze had no odorants or airstream but just the sucrose solution in the middle of the decision area. This maze was used exclusively for pretraining purposes. Minor and media ants were chosen because they usually forage for food (R. Josens, personal observations; see also Hölldobler & Wilson 1990, pp. 323, 336). After being marked, the ant was carefully placed on a piece of cardboard serving to carry it from the arena to the pretraining maze. After drinking the sucrose solution for the first time, the ant was gently removed from the maze. To this end, we waited until it climbed on to the piece of cardboard placed at the entrance channel of the maze. As the walls of the maze were painted with fluon to prevent the ants from escaping, the piece of cardboard was the only exit from the Y-maze. We then grasped the piece of cardboard, with small forceps, and gently placed the ant on the top of the vertical wooden stick. From there, the ant walked down to the ground of the arena and then into the nest chamber, where it could exchange food with its nestmates without being disturbed.

Usually, a motivated forager came back to the vertical stick after approximately 4 or 5 min. During this time, we replaced the Y-shaped filter paper covering the floor of the maze by a new one. The ant was then collected at the stick on the same piece of cardboard previously used for transporting it to the maze, and brought back to the maze entrance. Three such pretraining visits were allowed before we started the conditioning session. After these visits, the ants became accustomed to use the piece of cardboard as a carrier to and from the maze and most of them stopped trying to climb on to the walls of the Y-maze.

Acquisition

Individual ants were conditioned with a fresh Y-maze, similar to the previous one used for pretraining, but with the airstream connectors at the end of the arms and without fluon on the walls. After pretraining, ants did not try to escape from the maze but went directly into the arms searching for food, so the fluon became unnecessary. Training followed a differential conditioning procedure in which one odour was positively reinforced while another odour was negatively reinforced. Each ant was trained on 24 visits to the maze. Only foragers motivated enough to visit the maze 24 times in succession were used and only one ant was present in the maze at a time. For each odour pair (octanal versus limonene and heptanal versus 2-heptanone) we used two groups of 10–11 ants. For one group, one odour was reinforced positively with sucrose solution and the other odour was reinforced negatively with quinine solution; for the other group, the contingencies were reversed.

When the ant entered the maze in the first conditioning trial, it experienced the presence of the two different odorants. The first choice of the ant could be correct, that is, choice of the odour leading to the sucrose solution (the 'positive' arm), or incorrect, that is, choice of the odour leading to the quinine solution (the 'negative' arm). If the choice was correct, we immediately blocked the entrance to the negative arm by means of a plastic net (1.8 × 1.9 cm) with an external frame (2.1 × 5.8 cm) allowing the odour to reach the decision area, but not allowing the ant to experience the negative reinforcement. If the choice was incorrect, the ant was free to move to the positive arm while the negative arm was blocked as explained above. Once the ant drank the sucrose solution and left the arm in the direction of the piece of cardboard placed at the entrance channel, we blocked access to the positive arm by means of another plastic net to prevent the ant from going back to the positive arm and thus from experiencing the correct odour without positive reinforcement. The ant was then brought back again to the vertical wooden stick by means of the piece of cardboard. If the ant tried to climb on to the walls of the maze, which

did not have fluon on them, we immediately put a glass plate on top of the maze to prevent the ant from escaping.

The position of the odours and of their associated reinforcers in the two arms was switched between trials following two pseudorandom sequences that varied from one ant to the next. The sequence RLRRLLRLLRLRR LRLLRLRRLRL and its reversed alternative (with R and L, right and left, respectively, indicating the side of the sucrose reward) were used as they ensured that ants did not associate the reward with any particular arm. 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 provided by a hair dryer. We repeated this cleaning procedure systematically after each ant visit to the maze to avoid orientation by means of pheromones left during the previous visit. We took care to eliminate all possible traces of alcohol that could affect the ant's choice.

Retention tests

We also tested single individuals for memory retention. Immediately after the last conditioning trial, the ant received two retention tests carried out under extinction conditions (i.e. no reinforcer was provided in the maze). In each test, the ant was presented with the two odorants and we recorded two variables: its first choice and the time spent in each arm during 2 min. At the end of the 2 min, sucrose solution was provided on the positively reinforced odour. In the next test, the positive and negative odorants in the arms were transposed. Between tests, the Y-shaped filter paper covering the floor of the maze was changed and the maze and its base cleaned as explained before. Once the experimental ant had completed the experimental protocol ending with the last retention test, it was removed from the set-up and from the colony.

Statistical Analysis

During acquisition, we recorded the first choice of the experimental ant. These data were regrouped in six blocks of four visits each, which allowed us to calculate the proportion of correct choices per block during conditioning. Blocking is a common procedure in learning experiments. Variation in performance along the six blocks of trials and between-odours contingencies was evaluated by means of two-factor ANOVA (block $\times$ odour contingency) for repeated measures. We applied this ANOVA after transforming the proportion of correct choices per block for normality using the arcsine square-root transformation. Within each *Camponotus* species, we compared acquisition between groups by using a two-factorial ANOVA (block $\times$ odour pair) for repeated measures. For post hoc comparisons we used a Tukey test. The proportion of correct choices for each block of four visits could be compared to a theoretical proportion of 50% with a Mann–Whitney test. Within each block, the proportion of correct choices in a single visit was compared to a 50% proportion by means of a chi-square test.

In the retention tests, two variables were recorded: the first choice and the time spent in each arm of the maze. Within each retention test, we used a binomial test to compare the proportion of first choices to a random (50%) choice. The time spent in each arm of the maze was used to calculate the relative time (%) spent in the correct arm during the test with respect to the total time spent in both arms. Percentages were transformed by log(time + 1) for normality and compared to a 50% theoretical level with a t test. To compare the test performances of the two groups of ants, we used a 2×2 chi-square test for the first choice and a t test for the relative time in the correct arm.

No direct comparisons between species were attempted because we did the experiments in different places at different times, thus precluding quantitative comparative analyses.

RESULTS

Learning in *C. mus*

Limonene versus octanal

Camponotus mus ants trained with limonene versus octanal behaved similarly, independently of the odour contingency (two-factor repeated measures ANOVA block $\times$ odour contingency, odour contingency effect: $F_{1,18} = 0.96$, NS), so we pooled the results of the two groups. Ants learned to choose the odour reinforced with sucrose solution. The proportion of correct choices increased significantly along the acquisition blocks (each block corresponds to four consecutive visits to the maze; $F_{5,90} = 4.61$, $P < 0.001$; Fig. 2a). Performance in the first block differed significantly from that in the fourth to the sixth blocks (Tukey test: $P < 0.05$ in all three cases). The proportion of correct choices in the first block of trials was not different from a theoretical value of 50% (Mann–Whitney U test: $U = 170$, $N_1 = 20$, NS).

In the tests without reinforcements, the performance was independent of odour contingency, so we pooled the results of the two groups of ants for both variables considered, first choice (chi-square test: test 1: $\chi^2_1 = 0.39$, NS; test 2: $\chi^2_1 = 2.4$, NS) and time spent in the arm with the correct odour (t test: test 1: $t_{18} = 1.71$, NS; test 2: $t_{18} = 0.16$, NS). In both tests, ants preferred the odour previously rewarded with sucrose solution, thus showing that their choice was not guided by remote cues from reinforcers but by the odours previously learned. Significant performance was observed both for the first choice (binomial test: test 1: $P < 0.01$; test 2: $P < 0.05$; Fig. 2a) and for the time spent in the positive arm with respect to the total time in both arms (t test: test 1: $t_{38} = 8.17$, $P < 0.001$; test 2: $t_{38} = 9.79$, $P < 0.001$; Fig. 2b). Thus, *C. mus* ants learned to discriminate limonene from octanal efficiently during conditioning and such learning was clearly reflected by the tests without reinforcements.

Heptanal versus 2-heptanone

Camponotus mus ants were also trained to discriminate the structurally similar odours heptanal and 2-heptanone. Ants performed similarly, independently of the odour contingency (block $\times$ odour contingency, odour contingency effect: $F_{1,20} = 3.67$, NS), so we pooled the results

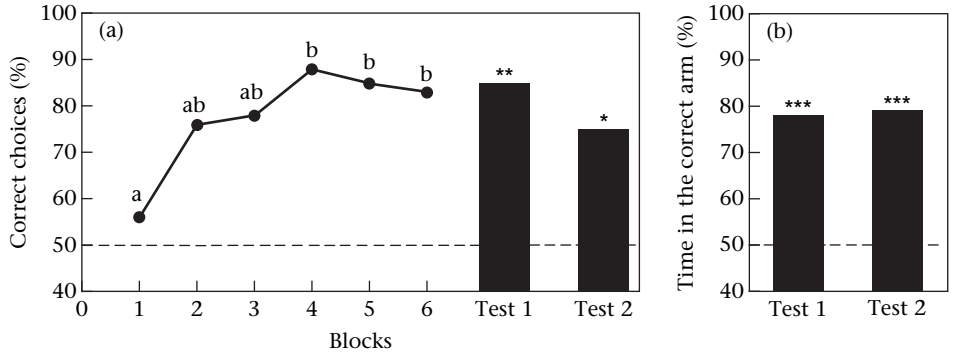

Figure 2. Discriminative learning of *Camponotus mus* trained to distinguish octanal from limonene. The dashed line at 50% indicates random choice between correct and incorrect odours. (a) Acquisition curve representing the pooled performance (percentage of correct choices that correspond to a sucrose-reinforced arm choice) of ants trained with both contingencies (i.e. odour A+ versus odour B− and vice versa) along six blocks of four visits to the maze ($N = 20$). Different letters indicate values that differ significantly within each acquisition curve. The first choices in two subsequent tests without reinforcement ($N = 20$) are also shown. (b) Percentage of time spent in the correct arm with respect to the time spent in both arms in tests 1 and 2. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

of the two groups. However, comparison of performances of both groups (heptanal+ versus 2-heptanone− and heptanal− versus 2-heptanone+) yielded a P of 0.07, thus suggesting that learning may vary depending on odour contingency. Even if not significant, *C. mus* showed a tendency to learn the pair heptanal+ versus 2-heptanone− better than the reversed contingency.

Ants learned to discriminate the rewarded from the punished odour, even if the odours involved in this discrimination, heptanal and 2-heptanone, were similar. The proportion of correct choices increased significantly along the acquisition blocks ($F_{5,100} = 2.76$, $P < 0.05$; Fig. 3a). Performance in the first block differed significantly from that of the fifth block (Tukey test: $P < 0.05$) and was marginally nonsignificant with respect to that of the sixth block ($P = 0.08$). The proportion of correct choices in the first block of trials was different from 50% (Mann–Whitney U test: $U = 154$, $N_1 = 21$, $P < 0.05$). Within this first block of trials, none of the four visits yielded a significant deviation from a random choice (chi-square test: first and second visits: $\chi^2_1 = 0.73$, NS; third and fourth visits: $\chi^2_1 = 2.91$, NS). This means that ants were choosing randomly between odours in their first four visits but that the progressive increase in correct choices (from 59 to 68% correct choices) resulted in a cumulative significant effect for the first block of trials.

When ants were tested without reinforcements, performance was independent of odour contingency for the first choice, so we pooled the results of the two groups of ants for this variable (chi-square test: test 1: $\chi^2_1 = 0.4$, NS; test 2: $\chi^2_1 = 0.29$, NS). The time spent in the arm with the correct odour differed between groups of ants for test 1 (t test: $t_{19} = 2.85$, $P < 0.05$) but not for test 2 ($t_{19} = 0.52$, NS). As the response trend was nevertheless clearly coincident in both tests, we decided to pool the data of the two groups of ants in both test 1 and test 2. Results of all tests were consistent: ants always preferred the positive odour, thus showing that their choice was indeed guided by the odours previously learned. Significant performance was found both for the first choice (binomial test: test 1: $P < 0.05$; test 2: $P < 0.001$; Fig. 3a) and for the time spent in the correct arm with respect to the total time in both arms (t test: test 1: $t_{40} = 4.51$, $P < 0.001$; test 2: $t_{40} = 13.66$, $P < 0.001$; Fig. 3b). Thus, *C. mus* ants learned

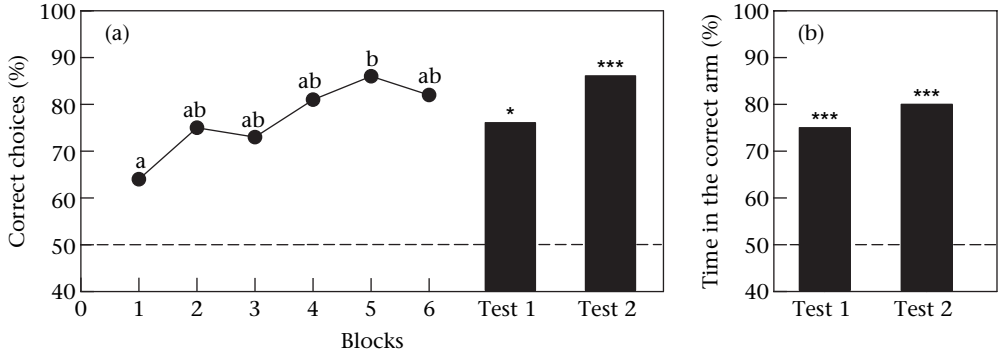

Figure 3. Discriminative learning of *Camponotus mus* trained to distinguish heptanal from 2-heptanone. The dashed line at 50% indicates random choice between correct and incorrect odours. (a) Acquisition curve representing the pooled performance (percentage of correct choices) of ants trained with both contingencies along six blocks of four visits to the maze ($N = 22$). Different letters indicate values that differ significantly within each acquisition curve. The first choices in two subsequent tests without reinforcement ($N = 21$) are also shown. (b) Percentage of time spent in the correct arm with respect to the time spent in both arms in tests 1 and 2. *$P < 0.05$; ***$P < 0.001$.

to discriminate heptanal from 2-heptanone efficiently, and chose the appropriate odour in the nonreinforced tests.

Comparison between pairs of odours

Results from both odour pairs, limonene versus octanal and heptanal versus 2-heptanone, show that *C. mus* ants learned to discriminate between odours with positive and negative outcomes. Acquisition was similar for both odour pairs (two-factor) repeated measures ANOVA, block × odour pair, odour pair effect: $F_{1,40} = 0.001$, NS; Figs 2a, 3a). The tests without reinforcements were also similar for both odour pairs (Figs 2a,b, 3a,b). For the first choice, performance was the same for both odour pairs in both test 1 (2×2 $\chi^2_1 = 0.51$, NS) and in test 2 (2×2 $\chi^2_1 = 0.75$, NS). Performance was also the same for the time spent in the arm with the correct odour both in test 1 (t test: $t_{39} = 0.69$, NS) and in test 2 ($t_{39} = 0.24$, NS). These results therefore show that learning was not affected by the odours used. Ants learned to discriminate both odour pairs similarly even though one pair involved structurally dissimilar odours and the other involved structurally similar odours.

Learning in *C. fellah*

Limonene versus octanal

Camponotus fellah ants were trained with the same two pairs of odours as *C. mus*. When trained with limonene versus octanal, they performed similarly, independently of the odour contingency (two-factor repeated measures ANOVA block × odour contingency, odour contingency effect: $F_{1,18} = 0.012$, NS), so we pooled the results of the two groups. The ants learned to discriminate limonene from octanal. The proportion of correct choices increased significantly along the acquisition blocks ($F_{5,90} = 5.25$, $P < 0.001$; Fig. 4a). Performance in the first block differed significantly from that in the third to the sixth blocks of trials (Tukey test: $P < 0.05$ in all four comparisons). The proportion of correct choices in the first block of trials (61%) was not different from 50% (Mann–Whitney U test: $U = 140$, $N_1 = 20$, NS).

In the tests without reinforcements, the performance was independent of odour contingency, so we pooled the results of the two groups of ants in tests 1 and 2 for both variables considered, first choice (chi-square test: test 1: $\chi^2_1 = 2.22$, NS; test 2: $\chi^2_1 = 2.22$, NS) and time spent in the arm with the correct odour (t test: test 1: $t_{18} = 1.42$, NS; test 2: $t_{18} = 0.34$, NS). In all cases, *C. fellah* ants preferred the odour previously reinforced with sucrose. Both the first choice (binomial test: tests 1 and 2: $P < 0.001$; Fig. 4a) and the time spent in the arm with the correct odour (t test: test 1: $t_{38} = 14.52$, $P < 0.001$; test 2: $t_{38} = 9.89$, $P < 0.001$; Fig. 4b) were significantly different from a random choice. Thus, *C. fellah* ants learned to discriminate limonene from octanal efficiently and chose these odours accordingly in the nonreinforced tests.

Heptanal versus 2-heptanone

Camponotus fellah ants were also trained to discriminate heptanal from 2-heptanone. In this case, the two groups with reversed contingencies differed significantly (block × odour contingency, odour contingency effect: $F_{1,18} = 10.49$, $P < 0.01$) because ants learned to discriminate heptanal+ from 2-heptanone− better than the reversed contingency. This difference coincides with the nonsignificant tendency to discriminate heptanal+ from 2-heptanone− better in *C. mus* ants. Results of both groups, heptanal+ versus 2-heptanone− and heptanal− versus 2-heptanone+, are thus presented separately in Fig. 5.

Camponotus fellah ants learned to discriminate heptanal+ from 2-heptanone−. The proportion of correct choices increased significantly along the acquisition blocks ($F_{5,45} = 5.75$, $P < 0.001$; Fig. 5a). Performance in the first block differed significantly from that in the fourth to the sixth block (Tukey test: $P < 0.01$ in all three comparisons). The proportion of correct choices in the first block of trials was different from 50% (Mann–Whitney U test: $U = 20$, $N_1 = 0$, $P < 0.01$). In this block, ants reached a level of 73% correct choices, owing to their strong initial preference for heptanal (80% in the first visit and 70% in the three

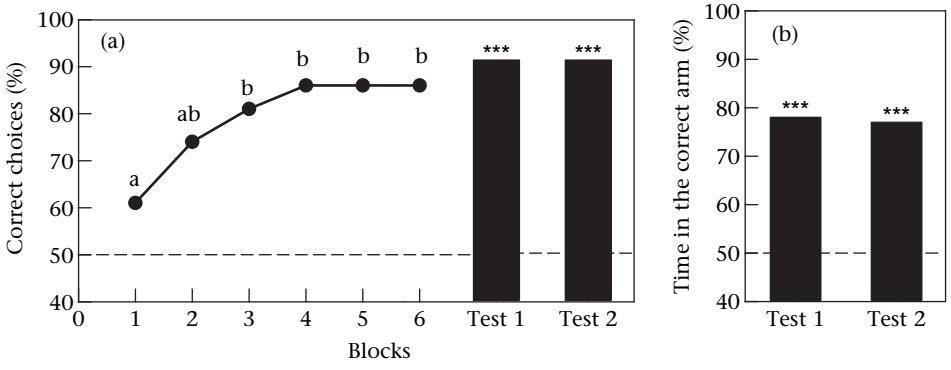

Figure 4. Discriminative learning of *Camponotus fellah* trained to distinguish octanal from limonene. The dashed line at 50% indicates random choice between correct and incorrect odours. (a) Acquisition curve representing the pooled performance (percentage of correct choices) of ants trained with both contingencies along six blocks of four visits to the maze ($N = 20$). Different letters indicate values that differ significantly. The first choices in two tests without reinforcement are also shown. (b) Percentage of time spent in the correct arm with respect to the time spent in both arms in tests 1 and 2. ***$P < 0.001$.

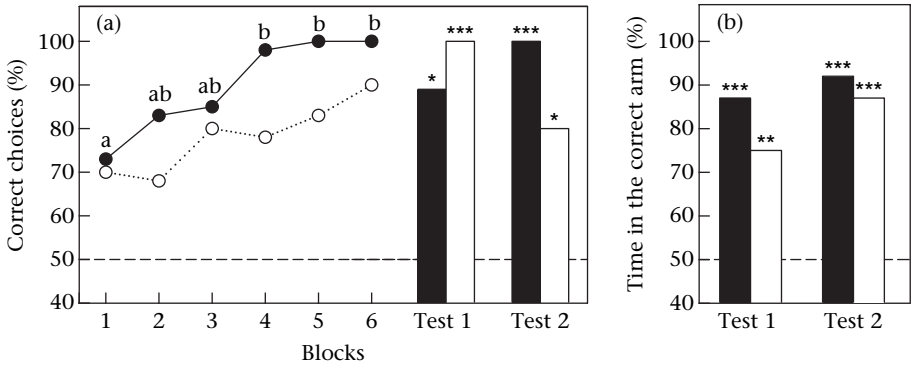

Figure 5. Discriminative learning of *Camponotus fellah* trained to distinguish heptanal from 2-heptanone. The dashed line at 50% indicates random choice between correct and incorrect odours. (a) Acquisition curves for heptanal+ versus 2-heptanone− (●; $N = 10$) and 2-heptanone+ versus heptanal− (○; $N = 10$). Different letters indicate values that differ significantly. The first choices in two tests without reinforcement are also shown. (b) Percentage of time spent in the correct arm with respect to the time spent in both arms. (■: heptanal+ versus 2-heptanone−, $N = 9$; □: 2-heptanone+ versus heptanal−; $N = 10$). *$P \leq 0.05$; **$P < 0.01$; ***$P < 0.001$.

successive visits of the first block). Despite this initial preference, the increase in correct choices along the blocks of training was significant and shows that ants improved odour discrimination along training.

In the nonreinforced tests, ants had a significant preference for the correct odour heptanal, both in their first choice (binomial test: test 1: $P < 0.05$; test 2: $P < 0.01$; Fig. 5a) and in the relative time spent in the correct arm (t test: test 1: $t_{16} = 7.18$, $P < 0.001$; test 2: $t_{16} = 17.13$, $P < 0.001$; Fig. 5b).

For 2-heptanone+ versus heptanal−, we did not observe a significant increase in correct choices along the conditioning blocks ($F_{5,45} = 1.97$, NS; Fig. 5a). An analysis of performance for the first block of trials revealed that choice was already different from 50% (Mann–Whitney U test: $U = 25$, $N_1 = 10$, $P < 0.05$). This difference was observed for all blocks of training ($P < 0.01$ in all cases). This suggests that even if there was no significant progression along the blocks of trials, the ants' choice was not random but clearly biased towards 2-heptanone, the odour reinforced with sucrose solution. In all four trials of the first block, the proportion of correct choices was not different from 50% (chi-square test: trial 1: 60%, $\chi^2_1 = 0.4$; trial 2: 70%, $\chi^2_1 = 1.6$; trial 3: 80%, $\chi^2_1 = 3.6$; trial 4: 70%, $\chi^2_1 = 1.6$; NS in all cases); thus the first block yielded a significant deviation from 50% owing to the accumulation of nonsignificant deviations towards 2-heptanone. Thus, in the first four visits ants chose randomly between the odours but learned very rapidly to choose the rewarded 2-heptanone and kept their choice along the acquisition blocks.

Camponotus fellah ants showed a significant preference for the correct odour 2-heptanone in the nonreinforced tests, both in their first choice (binomial test: test 1: $P < 0.001$; test 2: $P = 0.05$; Fig. 5a) and in the relative time spent in the correct arm (t test: test 1: $t_{18} = 3.05$, $P < 0.01$; test 2: $t_{18} = 9.66$, $P < 0.001$; Fig. 5b).

Comparison between the three odour combinations

Comparison between the three acquisition curves of Figs 4a and 5a shows that they differed significantly (two-factor repeated measures ANOVA block × odour pair, odour pair effect: $F_{2,37} = 5.48$, $P < 0.01$). Ants reached higher acquisition levels when trained with heptanal+ versus 2-heptanone−. As explained above, acquisition was different from that corresponding to 2-heptanone+ versus heptanal− ($F_{1,18} = 10.49$, $P < 0.01$). It was also different from that corresponding to the pooled acquisition of octanal versus limonene ($F_{1,28} = 9.24$, $P < 0.01$). The curves of octanal versus limonene and of 2-heptanone+ versus heptanal− did not differ significantly ($F_{1,28} = 0.15$, NS). The tests without reinforcements yielded no significant differences between the three groups, for either test 1 or test 2, for the first choice (Kruskal–Wallis test: test 1: $H_2 = 1.10$, NS; test 2: $H_2 = 2.01$, NS). For the time spent in the correct arm, no differences between groups were found for test 1 ($F_{2,36} = 1.46$, NS). For test 2, however, significant differences between groups were found ($F_{2,36} = 5.86$, $P < 0.01$), which reflected the better performance for the group that experienced heptanal+ versus 2-heptanone−, a result consistent with the differences detected between acquisition curves (see above).

DISCUSSION

Our results show that individual ants perceive and learn odours in controlled laboratory conditions. Two different species, *C. mus* and *C. fellah*, from different continents learned to discriminate two pairs of odours, in which one odour was positively reinforced with sucrose solution while the other was negatively reinforced with quinine solution. In all experiments, we used only one ant at a time to control individual experience and to avoid the spurious effects underlying mass training. The amount of experience that each ant gathered with the odours and the reinforcers used in our study was therefore known and was the same for all ants.

Although learning in our experimental design was clearly associative, as indicated by the tests without reinforcement, it is difficult to determine at this stage whether classical or operant associations were guiding the ants' choice. In the first case, the ants would learn to

associate the presence of an odour with the reinforcers used. In the second case, odours would act as discriminative stimuli indicating when to make an arm choice, an action that would be followed by the appropriate reinforcer. In either case, however, odour cues played an essential role in driving the ants' behaviour and were integrated in different associations with the gustatory reinforcers. It is possible that ants in the Y-maze learned both operant and classical associations. The fact that ants could move freely in the Y-maze and that their choice was followed by an appetitive or an aversive reinforcer underlines the operant nature of this experimental design. Learning both classical and operant associations in the same experimental context has been well established in the fruit fly *D. melanogaster* (Brembs & Heisenberg 2000; Brembs 2003). The relative associative strengths of the two olfactory stimuli presented in the Y-maze remain to be determined. The question is whether ants learned to choose the positively reinforced odour or whether they learned to avoid the negatively reinforced odour. Ants could also learn both the excitatory and the inhibitory nature of each odour. This issue could be answered by an experiment in which ants are trained following the same differential conditioning protocol with two odours, and then tested with three odours instead of two. Besides presenting the two odours on which the ants were trained, the experiment would include a third, unknown and neutral odour presented against both the positive and the negative odours. If the ants prefer the neutral odour to the negative one, this would mean that they explicitly learned to avoid the negatively reinforced odour. If they prefer the positive odour to the neutral one, this would imply that they also explicitly learned to choose the positively reinforced odour. Experiments using this rationale have been done with free-flying bees trained in visual discrimination tasks in a Y-maze (Horridge & Zhang 1995; Giurfa et al. 1999). Bees, like the ants in our study, had to discriminate rewarding from nonrewarding stimuli in a Y-maze (visual stimuli in the case of bees) and their performance showed that they learned both excitatory and inhibitory associations. They explicitly learned to choose the rewarding stimulus but also avoided the nonrewarding stimulus (Horridge & Zhang 1995; Giurfa et al. 1999). The question of whether ants learned olfactory stimuli in the Y-maze on a similar basis remains to be answered.

Our results raise the question of the nature and extent of the olfactory memories established in our protocol. Our tests were carried out just after the last acquisition trial, following the natural sequence of the ants' visits to the maze. This sequence was defined by the ant, and not by the experimenter, because the ants were walking freely. However, visits to the maze were usually interspersed with intertrial intervals of approximately 5 min for a motivated ant. This means that at least 5 min after the last acquisition trial, a stable and robust memory was available which was evident in the tests without reinforcement in which ants always chose the previously rewarded odour. Whether olfactory memories can last for longer remains to be studied.

Although ants could learn to discriminate efficiently both pairs of odours used (the structurally dissimilar limonene and octanal and the structurally similar heptanal and 2-heptanone), learning performances were more robust for limonene versus octanal. In this case, no difference between groups was found for both *C. mus* and *C. fellah*, which learned similarly the discriminations limonene+ versus octanal− and limonene− versus octanal+. Using more similar odours (heptanal and 2-heptanone) yielded a different result as ants then showed odour-dependent performances. This dependency was marginally nonsignificant in *C. mus* but was significant for *C. fellah*, which learned to discriminate heptanal+ from 2-heptanone− better than heptanal− from 2-heptanone+, despite the two odours having comparable vapour pressures and thus similar expected diffusions within the maze. This difference may be because 2-heptanone is an alarm pheromone in several ant species (*Conomyrma pyramica*: Blum & Warter 1966; *Forelius foetidus*: Scheffrahn et al. 1984; *Iridomyrmex pruinosus*: Blum et al. 1966; Crewe & Blum 1971; *Atta* sp. Moser et al. 1968; Hughes et al. 2001; *Diacamma indicum*: Morgan et al. 2003) and may thus be difficult to associate with a food reward. Although no data are available for the *Camponotus* species used in our study, 3-octanone has been identified as an alarm pheromone in another *Camponotus* ant, *C. schaefferi* (Duffield & Blum 1975), thus suggesting that learning asymmetries in our experiments may be determined by the intrinsic value of the substances used. Asymmetries in olfactory learning and discrimination have been shown recently in the honeybee (Guerrieri et al. 2005a) and seem to be a general feature of several invertebrate olfactory systems as suggested by our study on *Camponotus* ants.

Throughout our study, we explicitly avoided direct comparisons between the two species used, *C. mus* and *C. fellah*, because we did the experiments at different times of the year and in two different continents, with two different intensities of negative reinforcer (0.3% and 3% quinine solution for *C. fellah* and *C. mus*, respectively). Despite this restriction, it is possible to observe striking similarities between the species as both efficiently learned the olfactory discriminative tasks with comparable dynamics. It seems, therefore, that olfactory learning follows general across-species principles, at least for the *Camponotus* species considered.

In a natural context, olfactory orientation towards the food source has been described for various ant species. Foragers of the leaf-cutting ants *Atta cephalotes* and *Acromyrmex octospinosus* orient upwind to odour stimuli to reach the proximity of the food source (Littledyke & Cherrett 1978). In the same way, the ant *Cataglyphis fortis* optimizes its approach to a known food source by picking up the food odour and steering an upwind course until it reaches the food (Wolf & Wehner 2000, 2005). It seems, therefore, that some ant species use olfactory cues from the food source when searching close to the feeding site. This search strategy does not necessarily imply a learning process because orientation could be based on natural preferences for some odour types commonly associated with food sources. However, olfactory learning has been suggested in various ant species (*A. cephalotes* and *A. octospinosus*: Littledyke & Cherrett 1978; *Lasius niger*: Beckers

et al. 1994; *Camponotus pennsylvanicus*: Helmy & Jander 2003) but these studies did not explicitly demonstrate learning at the individual level. In one study (Roces 1990), recruits of leaf-cutting ants *Acromyrmex lundi* were conditioned to the odour of the food carried by a scout worker, but later studies could not confirm this (Fowler & Schlindwein 1994; Howard et al. 1996). Using a new experimental procedure in controlled laboratory conditions, our study has shown for the first time without doubt that individual *Camponotus* ants perceive and learn odours in the appetitive context of food search.

In a natural context, *Camponotus* foragers use pheromone trails to mark the path to a food source (Hölldobler & Wilson 1990). What is, therefore, the adaptive value of learning odours in a foraging context as shown by our study, when pheromonal trail information could be sufficient to reach the food? In fact, olfactory cues emanating directly from the food sources will enhance the chance of encountering and recognizing such sources in successive foraging trips. Ants may use pheromonal cues to reach the proximity of the food source and, close to it, they would be guided by the olfactory (and probably visual) cues learned in direct association with the food reward.

Acknowledgments

We are indebted to three anonymous referees for comments on the manuscript and to Alain Lenoir for providing the colonies of *C. fellah*. We also thank him, Abraham Hefetz and Luc Passera for helpful advice on *C. fellah* biology. Gérard Latil provided valuable help in keeping the colonies in the laboratory. This study was possible thanks to the support of the cooperation programmes EcosSud and CONICET-CNRS and ANPCYT. Fabienne Dupuy and Roxana Josens thank the social-insect research team at the University of Buenos Aires and the research team at the University of Toulouse, respectively, for much help and support.

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