

## Classical conditioning, temporal learning and spatial learning in the ant *Myrmica sabuleti*

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CAMMAERTS, M.-C., Classical conditioning, temporal learning and spatial learning in the ant *Myrmica sabuleti*. *Biologia, Bratislava*, 59: 243–256, 2004; ISSN 0006-3088.

After working for 30 years on ethological, physiological and chemical problems concerning ants, we predicted that conditioning and/or learning may be obtained in these animals. Therefore, the first thing we made was to test three types of learning in foragers of the ant *Myrmica sabuleti*: temporal learning, spatial learning and classical conditioning. Workers were able to perform temporal learning of a particular period of the day after a 12-day training period. This initial learning extinguished after 3 days in the absence of food. A second subsequent temporal learning was more rapid and more efficient. Moreover, its extinction took longer. The workers were also able to exhibit spatial learning of a feeding place precisely located in their foraging area. After extinction of this first spatial learning in a few experiments in the absence of food, a second subsequent learning performed under the same training conditions was more rapid and generally more efficient, and its extinction took a little longer, as previously found for temporal learning. Classical conditioning was possible in *M. sabuleti*, when the conditioned stimuli (visual: green triangle; olfactive: onion) were presented either a few minutes before, just before, or at the same time as the unconditioned stimulus (liquid sugared food). Such conditioning failed if the conditioned stimulus was presented, then removed, 15 min before the unconditioned stimulus, or, if it was presented after the unconditioned stimulus. In the three cases where conditioning occurred, the ants' responses extinguished after a few experiments. Second subsequent conditionings were more rapid and generally more efficient, and extinguished more slowly. The workers discriminated yellow and blue from green, but generalised the response they gave to a triangle, to a square and a rectangle; they generalised their response to onion, to leek and garlic, but discriminated cabbage and Belgian endive from onion. Thus, in summary, classical conditioning as well as temporal and spatial learning can be obtained in the ant *M. sabuleti* using precise experimental protocols. This discovery allows us to go further and to study, in the same species, spatial conditioning, visual perception, and operant conditioning (works respectively published and in press).

Key words: ants, classical conditioning, *Myrmica sabuleti*, spatial learning, temporal learning.

### Introduction

Some animal species can encode the time at which

biological events occur. Such an ability has been documented in social insects (FOURCASSIÉ et al., 1999). In this respect, temporal learning has been

demonstrated in two Neotropical ants, *Paraponera clavata* (F., 1775) and *Ectatomma ruidum* (Roger, 1860) (HARRISON & BREED, 1987; SCHATZ et al., 1993, 1994, 1999b) and in the Mediterranean ant *Cataglyphis cursor* (Fonscolombe, 1846) (SCHATZ et al., 1999a). Such learning could not be shown in ants from colder climates (REICHLER, 1943; DOBRZANSKI, 1956; FOURCASSIÉ & TRANIELLO, 1994). This led CRUDEN et al. (1983) to suggest that temporal learning ability was restricted to tropical ant species. This is why, in the present work, we tested this hypothesis and attempted to demonstrate temporal learning in *Myrmica sabuleti* Meinert, 1860, an ant living in a relatively cold climate.

Many animal species with navigation abilities can learn where to look for food (BIEBACH et al., 1989; REEBS, 1996). This spatial information learning exists in insects (BEUGNON & LACHAUD, 1992; BEUGNON et al., 1996). Ant foragers often use landmarks in order to orient themselves, even if they use a trail pheromone. Species exhibiting this phenomenon include, *Formica fusca* L., 1758 (MÖGLICH & HÖLLDOBLER, 1975), *Pogonomyrmex badius* (Latreille, 1802) (HÖLLDOBLER & WILSON, 1970; HÖLLDOBLER, 1976), *Pheidole militicida* Wheeler, 1915 (HÖLLDOBLER & MÖGLICH, 1980) and *Atta cephalotes* (L., 1758) (HÖLLDOBLER & WILSON, 1986). Thus we can assume that spatial learning is possible at least in some species. Indeed, the learning of a sequence of visual patterns has been found in the ant *Cataglyphis cursor* (CHAMERON et al., 1998; SCHATZ et al., 1999c). Spatio-temporal learning was tested in this ant species: it failed, but again, spatial learning occurred (SCHATZ et al., 2000). Maze-learning has also been shown to occur in some ants (in *Formica pallidefulva* Latreille, 1802, *Formica polyctena* Foerster, 1850, etc see HÖLLDOBLER & WILSON, 1990, p. 366), which implies spatial learning ability. All these considerations made us attempt spatial learning in the ant *M. sabuleti*.

In addition to learning due to a habituation process, animals can learn by association and the simplest form of this is classical conditioning, also named Pavlovian conditioning (MANNING & DAWKINS, 1998). Conditioning has been shown in many vertebrate species, as well as several invertebrate species, especially honeybees, the latter studies still proceeding (LALOI et al., 2000 and references therein). The conditioned stimuli usually used in honeybees are chemical and to a lesser extent, visual. Ants also perceive odours and are sensitive to visual cues, but until now there has

been no evidence of the occurrence of classical conditioning in this hymenoptera group. ("Olfactory conditioning" considered by ROCES (1990) is not classical conditioning; this is discussed in the "Conclusion and Discussion" Section). Therefore, we examined whether classical conditioning was possible in the ant *M. sabuleti* using a chemical and a visual conditioned stimulus. Note that, usually, classical conditioning is associated with the response of an individual. However, in the present work it would be associated with the response of a group of animals (foraging workers) as a whole. If classical conditioning seems to occur in *M. sabuleti*, we should also define several of its characteristics.

## Material and methods

### *Collection and maintenance of ants*

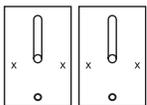
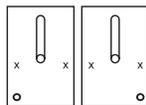
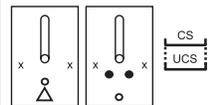
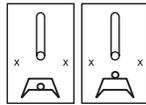
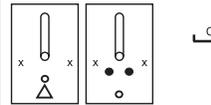
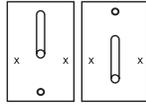
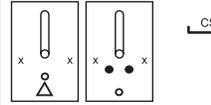
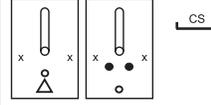
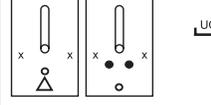
A very large colony of *M. sabuleti* was collected in Lorraine (France), at Pont-St. Vincent (48°36'03" N, 06°05'00" E) and divided into five groups of two fragments, each fragment containing 250 workers, one queen and brood. Each fragment was maintained in an artificial nest made of a glass-tube half-filled with water, a cotton-plug separating the ants from the water. Each glass-tube was deposited into a polyethylene basin (7 × 28 × 42 cm) serving as a foraging area. Each week, to the left and right of the nest entrance (Fig. 1), half a cockroach was placed on a glass slide.

### *Planning of the experiment (Fig. 1)*

We studied a group of two fragments of the colony with regard to, successively, temporal learning or spatial learning, food being placed to the right or left of the nest entrance. Then we studied classical conditioning by simultaneously presenting the conditioned stimulus (CS) and the unconditioned stimulus (UCS). Finally we observed the ants' discrimination and generalisation of stimuli.

We performed experiments pertaining to spatial learning on a second group of two fragments of the colony, food being placed on the lower or upper stage of a construction (see below). Then we studied classical conditioning by successively presenting the CS and the UCS. A third group of two fragments was used to research spatial learning by placing food in front of or behind the nest, then classical conditioning by presenting the UCS 5 min after the removal of the CS. A fourth group of two fragments were used in a classical conditioning experiment by presenting the UCS 15 min after the removal of the CS. Finally we performed a classical conditioning experiment on a fifth group of two fragments by presenting the CS after the removal of the UCS.

When successive experimental studies had to be performed on the same fragment of the colony, we ensured (i) that the ants' responses obtained during a study were extinguished before beginning the next study and (ii) that four weeks elapsed between the

Groups of nests	Studies:			
	Temporal learning	Spatial learning	Classical conditioning	
I				+ discrimination generalization
II				
III				
IV				
V				

Symbols:

 : nest tube; x : piece of cockroach, provided once a week;  
 : green triangle;  : droplet of sugared water;  : onion piece;  
 : tower with sugared water on its lower or upper stage;  
I to V : five groups of the same society, each of two fragments;  
CS: conditioned stimulus (triangle or onion);  
UCS: unconditioned stimulus: sugared water;  
5 or 15 min: latency time between CS and UCS

Fig. 1. Experimental planning of the studies conducted on fragments of one *Myrmica sabuleti* society.

study of temporal learning and that of spatial learning, as well as between the study of spatial learning and that of classical conditioning. We expected that these precautions would avoid a possible influence of one training procedure on the following one.

#### Temporal learning

During 12 successive days, the presence of workers in two fragments of the colony was observed during a 15 min period on a glass slide placed 10 cm in front of the nest entrance (Fig. 1). This was always performed at the same time of day, i.e. between 1:00 and 1:15

a.m., this time frame being chosen for personal logistic convenience, and because at that time the ants were not very active (i.e. not foraging, searching for food). During each of these 12 successive daily observations, we began by counting the ants coming onto the glass slide during one minute before food was deposited on it and repeated this twice with a 3 min time lapse. For one fragment of the colony we called the recorded numbers of ants  $a_1, a_2, a_3$  and  $a'_1, a'_2, a'_3$  for the other fragment. We then performed similar counts, but with food (a droplet of 65% sugared water [Beehappy<sup>®</sup>, diluted three times]) placed on the slide, and called the recorded number of ants  $n_1, n_2, n_3$  for one fragment and  $n'_1, n'_2, n'_3$  for the other. Then we counted again, in the same way, the ants appearing after the removal of the food, identified as  $b_1, b_2, b_3$  for one fragment and  $b'_1, b'_2, b'_3$  for the other. It should be noted that the recorded numbers of ants relate to their visits on the slide, each ant being theoretically able to leave and return to the slide, possibly several times.

For each of the 12 observations, the following temporal learning index  $TL$  was calculated:

$$TL = 2 \times (n_1 + n_2 + n_3 + n'_1 + n'_2 + n'_3) \times (a_1 + a_2 + a_3 + a'_1 + a'_2 + a'_3 + b_1 + b_2 + b_3 + b'_1 + b'_2 + b'_3)^{-1}$$

The value of this index is greater the more numerous the ants' occurrences on the glass slide at the time corresponding to that of feeding. The value of the  $TL$  index varies from zero (an equal number of ants on the glass slide at the time of feeding as well as before and after that time, i.e. absence of learning) to infinite (no ants just before and after the correct time of feeding, i.e. perfect temporal learning, with no anticipation and no continuation).

When performing the 12<sup>th</sup> presentation of food, the  $TL$  index was calculated twice: once without giving food, in order to estimate the ants' temporal learning (see results) and a second time with food, for obtaining the 12<sup>th</sup> and last value of  $TL$  (Fig. 2).

During two days we counted approximately every hour, the ants (from the two fragments of the colony) appearing on the glass-slide during one minute. Each day without food represented an extinction experiment. During these days, ants appearing on the food place during one minute were counted at the time of the day at which temporal learning reinforcements had been made. After total extinction of the ants' response, a second temporal learning session, consisting of six experiments only, was performed and the  $TL$  index calculated each time. Then, experiments of extinction were performed again; we counted, each day, the ants appearing on the food place during one minute at the time we chose for temporal learning, until the complete extinction of the ants' response.

A possible anticipation of response by the ants was investigated by calculating for each temporal reinforcement, the 'anticipation' variable  $AV$ .

$$AV = a_1 + a_2 + a_3 + a'_1 + a'_2 + a'_3 - (b_1 + b_2 + b_3 + b'_1 + b'_2 + b'_3).$$

The variable  $AV$  is greater the more numerous the ants responding by anticipation.

#### Spatial learning

A droplet of liquid sugared food was presented during 15 min, on a glass slide situated in a precise place in the foraging area of six fragments of the colony. This was done 12 times, twice in 24 h, but never at the same time to avoid temporal learning. The precise place was either to the right or to the left of the foraging area (depending on the colony fragment) at 10 cm from the nest entrance, either on the lower or the upper stage of a platform with two stages (*ibidem*) or at 10 cm in front of or behind the nest (*ibidem*) (Fig. 1). The ants present (and probably eating) on the glass slide at the end of each minute (of the 15 min of food presentation) were counted, and the mean of the 15 values was calculated for each of the 12 presentations (Fig. 5). The trail pheromone of *M. sabuleti* consists of 3-ethyl-2,5-dimethylpyrazine (EVERSHED et al., 1981) and is rather volatile (CAMMAERTS et al., 1993). Thus even if the ants laid a trail between their nest and the training place, this trail would have disappeared completely before the subsequent presentation and would not be able to lead the ants to the food place. After having performed the 12 food presentations, we counted the ants for 3 min (actually, the number of ant visits) appearing during one minute onto a glass slide deposited at the place for which the spatial learning has been pursued and onto another one deposited at the opposite place (respectively, to the right and the left from the nest entrance, or at the lower and the upper stages of the platform, or in front of and behind the nest). This procedure was chosen because the numbers of ants coming onto the correct area have to be compared to the numbers of ants going onto an equivalent area, identically distant from the nest entrances. These counts allowed the percentage of ants' visits onto the "correctly placed" glass slide (i.e. the slide situated at the place for which the learning had been tempted) to be calculated. The counts and percentage calculations were made until the ants' learning extinguished completely.

Then, a second session of spatial learning reinforcements, consisting of only six experiments was performed, during which the ants present on the glass slide at the end of each minute were again counted and the mean of the 15 values established. Then, the percentages of ants appearing on the "correct" food place were evaluated without giving them food (this evaluation was done by the numbers of ants appearing during 3 min – see above) and this experiment of extinction was repeated until the ants' responses extinguished once again.

#### Classical conditioning

The unconditioned stimulus (UCS) was a droplet of liquid sugared water (Beehappy<sup>®</sup>, diluted three times) deposited on a glass slide placed in front of the nest entrance at a 15 cm distance. The conditioned stimulus (CS) was either (for five fragments of the colony) a

green triangle (base 4 cm; height 3 cm) placed vertically (in fact, a tetrahedron with one of its faces horizontal, and another one vertical) 3 cm behind the food, or (for five other fragments of the colony) two pieces of onion placed 3 cm in front of the food, on its right and left (see Fig. 1).

Five kinds of presentation of the CS and the UCS were attempted, each kind on a group of two fragments of the colony (Fig. 1): simultaneous presentation, immediately successive presentation, presentation of the UCS 5 min after the removal of the CS, presentation of the UCS 15 min after the removal of the CS, presentation of the CS after the removal of the UCS. Each time 12 successive presentations were made (2, each lasting 15 min, during 24 h, but never at the same time, in order to avoid temporal learning), during which the ants present on the glass slide (and probably feeding) at the end of each minute were counted, and the mean of these counts calculated. It should be noted (see spatial learning) that if the ants laid a trail during one presentation, this trail would have vanished and become ineffective before the subsequent presentation. After the 12 presentations, control counts were made of the ants arriving during 10 min onto the glass slide, neither the UCS nor the CS being present. Counts were then made of the ants present on the slide during 10 min while the UCS was not present but the CS was correctly deposited on the foraging area. These last experiments represented the first experiments of extinction. They were repeated until the ants' responses extinguished completely. Then, second sessions of conditioning, consisting of only five experiments were performed, and each time the mean numbers of the ants present on the glass slide were calculated. Then we evaluated the ants' responses and their extinction in the same way as during the first extinction session (based upon the numbers of ants present during 10 min). On one of the five groups of two fragments of the colony (Fig. 1), a third session of conditioning experiments was performed in order to estimate the ants' discrimination and generalisation of stimuli (see below).

#### *Ants' discrimination and generalisation of stimuli*

The ants conditioned to a green triangle successively received a yellow triangle, a blue triangle, a green square and a green rectangle. Those conditioned to onion successively received leek, garlic, cabbage and Belgian endive (witloof). Each time the ants' response was quantified by their number coming, during 10 min, onto the food place when the food was not given. Between each of these presentations of new stimuli, an experiment of reinforcement to either the green triangle or onion was conducted (more precisely, the usual CS was presented together with the UCS).

#### *Statistical analysis*

Numerical results were statistically analysed using non-parametric tests (SIEGEL, 1956; SIEGEL & CASTELLAN, 1988). The Wilcoxon (matched-pairs) signed-ranks test was used to compare the six or 12 numerical values obtained in the course of the first and second sessions of learning or conditioning. This test

was also used to compare the values obtained in the course of the first and second sessions of extinction. The binomial test was used to compare the ants' control responses to their responses at the "correct" place. The binomial one-tailed test (or the  $2 \times 2$  Chi-square goodness-of-fit test) was used to compare control responses to the responses of ants at the 'correct' time or in the presence of the CS. The tests were, of course, always made on the numerical values, even when the results are, for reasons of clarity, illustrated in percentages. Two values or series of values were considered as not significantly different when  $P > 0.05$ .

## Results

### *Temporal learning*

At the beginning of the first learning attempt only a few ants were present on the food and the calculated temporal learning index  $TL$  was a low value. Then, in the course of the 12 successive presentations of food at a given time of the day, the  $TL$  index increased from 0.46 to 4.00 (Fig. 2). Some anticipation of response occurred during the first training period: the variable  $AV$  increased from  $-9$  to  $+3$ . This shows that the ants' response was decreasingly due to the detection of the odour of the sugared water, recruitment or disturbance caused

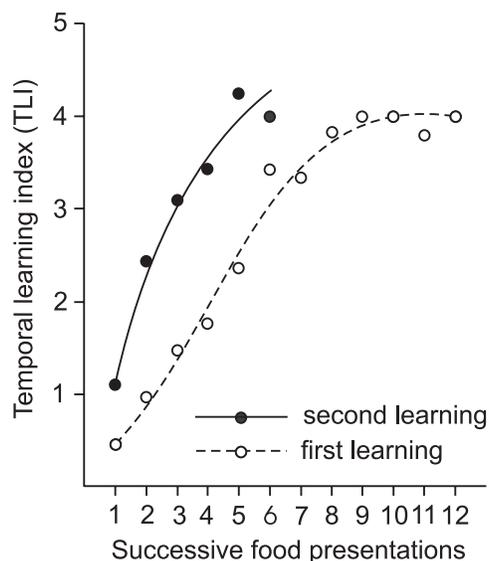


Fig. 2. Changes in the temporal learning index  $TL$ , assessing the ants' temporal learning. First learning was made of 12 presentations, second learning of only 6. Differences between the two learnings are significant ( $P < 0.01$ , Wilcoxon test). Calculation of  $TL$  index is explained in 'Material and Methods'.

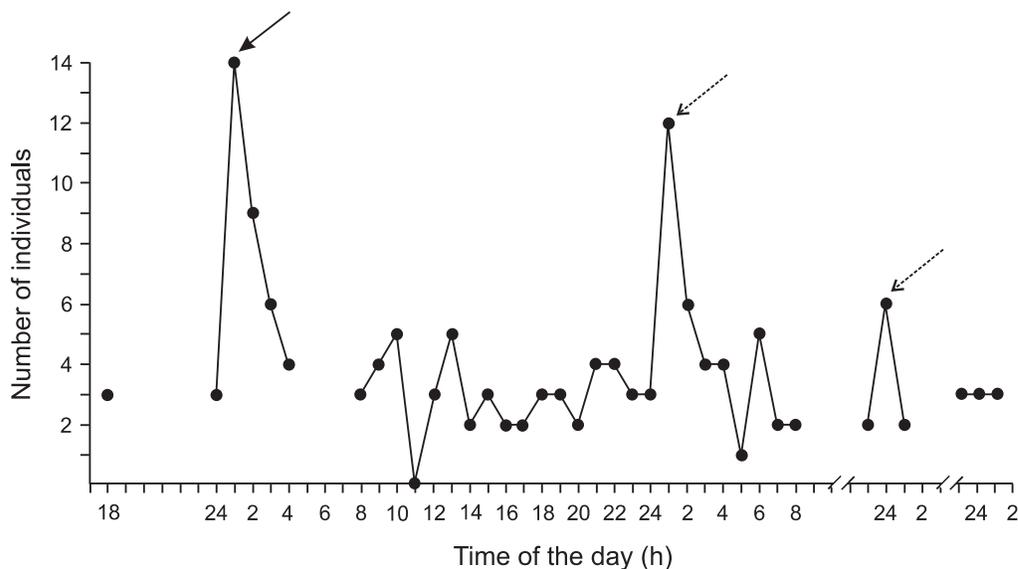


Fig. 3. Extinction of the ants' first temporal learning. The ants coming during one minute onto the food place were counted nearly each hour during one and a half days, then at three different times (11:00 p.m., 12:00 p.m. and 1:00 a.m.) during the two following days. These counts reveal the ants' ability to acquire temporal learning (full arrow) at 1:00 a.m., as well as the extinction of learning (dashed arrows). The ants' learning has been acquired thanks to 12 reinforcement experiments, the last one having occurred one day before the counting corresponding to the first peak (full arrow).

by the food presentation. When measured without giving food, after the training period, the value of the *TL* index was 3.45 and that of the *AV* variable, 3. These values statistically differed from the initial ones (being  $TL = 0.46$ ;  $AV = -9$ ) ( $P < 0.01$ ). The ants thus learned to arrive at the food at a precise time of the day, acquiring however a slight anticipation of response which, in our opinion, does not invalidate the ants' temporal learning. The acquisition of such learning was confirmed by the counts of the ants present on the food place throughout the day (Fig. 3): they were numerous only at the time for which the learning experiment was performed. During each count exactly the same experimental procedure was performed and all the environmental factors (e.g. light intensity, temperature, substrate vibration, humidity) were maintained at a constant level. Therefore, the behaviour of ants at 1:00 a.m. can be explained by only one factor: the fact that the ants learned what time of the day it was. This learning (more precisely, the ants' first learning) extinguished after 3 days (Fig. 3). A second learning session, consisting of only six experiments was then performed: the *TL* index was initially larger than at the beginning of the first learning session (1.10 instead of 0.46), then increased more rapidly and reached

somewhat larger values than in the course of the first learning session (Fig. 2). The differences between the first and second learning sessions were statistically significant ( $P < 0.01$ ). The fact that the second learning had a higher value than the first was confirmed by the observation that more ants arrived on the food place at the correct time (28 instead of 14) and by the fact that the ants' response extinguished more slowly (7 to 8 days instead of 3 days) (Fig. 4). The differences between the first and the second learning sessions were statistically significant ( $P < 0.01$  or  $P < 0.025$ ). However, the ants' anticipation of response was not higher after the second learning session than after the first one ( $AV = 2$  after the second learning session). Note that these results cannot be due to the deposit of some trail pheromone by the ants (see Material and Methods).

#### Spatial learning

During the first learning experiments the mean numbers of ants eating at a specific time during the 15 min of food presentation generally increased in the course of the 12 successive food presentations (Fig. 5a). Then, the percentages of ants present on the correct food place (i.e. the place for which the learning experiment was tested: see

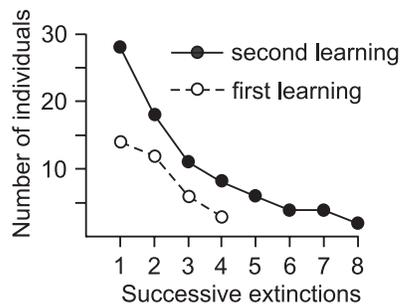


Fig. 4. Extinction of the ants' first and second temporal learnings. The number of ants (N) coming during one minute onto the food place was recorded each day at the time during which the learnings have been carried out. Since no food was then given, each count consisted of an experiment of extinction.

'Material and Methods') were 88.9% for the right side and 85.7% for the left side of the nest entrance, 75% for the lower stage and 87.5% for the upper stage of the construction and 78.1% for a place situated in front of and 92.2% for a place situated behind the nest. Let us recall that these results cannot be due to deposit by the ants of some of their trail pheromone (see Material and Methods). Statistically, these results differed from a 50% score ( $P < 0.01$ ). The ants thus learned to come for food at a precise place within their foraging area. The ants' responses (i.e. their first learning) could be extinguished in a few experiments (Fig. 5b). We noted that the ants had little inclination to go on the lower stage of the construction. In front of such a construction, the ants preferred to go up (thus to the upper stage) and, only afterwards visited the lower stage. In the course of the second session of learning, consisting of only six experiments, the ants eating at a specific time during the 15 min of food presentation were generally more numerous than those observed during the first learning experiments (Fig. 5c). The second session of learning produced somewhat higher scores than the first one, and needed a few more experiments to extinguish (Fig. 5d). The differences between the first and the second learning were statistically significant ( $P < 0.01$  or  $P < 0.025$ ).

#### Classical conditioning

We have explained that no trail pheromone deposit by the ants can persist long enough to lead the ants onto the food place after several hours. Therefore, if the ants still go to the food place, it must be for other reasons than a certain quantity

of pheromone deposit. The results are summarised graphically in Fig. 6.

#### Simultaneous presentation of the CS and the UCS

The mean numbers of ants eating at a specific time increased slightly during the course of the 12 successive presentations. The four control counts made just after these presentations were 0, 1, 0 and 1 ant. Twenty-five (green triangle) and 10 (onion) workers were then counted in the presence of the CS. These results statistically differing from the control counts ( $P < 0.01$ ), we can confirm that the attempted conditioning succeeded. Five (green triangle) and two (onion) experiments were necessary to extinguish the ants' first conditioning. During the second session of conditioning the number of ants feeding increased more rapidly. Although only five reinforcements were made, the second session of conditioning gave slightly higher values than the first one and the extinction of the conditioning took a little longer. The differences between the first and the second conditioning were statistically significant ( $P < 0.01$  or  $P < 0.025$ ). A third session of conditioning, consisting of 5 reinforcements, was performed (the results are not illustrated) to evaluate the ants' discrimination and generalisation of stimuli (see last paragraph of this section).

#### Successive presentation of the CS and the UCS

The number of ants present on the food place (at a specific time) increased during the course of the 12 successive presentations, though their number largely varied according to the degree of satiation of the tested colonies. Control counts were then four and zero ants, while counts in the presence of the CS were 34 (green triangle) and 9 (onion) ants. These results were statistically significant ( $P < 0.01$ ). Thus, conditioning obviously occurred. Seven (green triangle) and five (onion) presentations of the CS which were not followed by a food presentation, allowed the ants' first conditioning to be extinguished. A second session of conditioning, although consisting of only five reinforcements, yielded higher scores and conditioning needed more experiments for extinguishing ( $P < 0.01$  or  $P < 0.025$ ).

#### Presentation of the UCS 5 min after the removal of the CS

The number of ants present around the food at a specific time of each food presentation somewhat increased in the course of the 12 successive presentations. Then, 0, 5, 1 and 3 ants were successively counted in the absence of the CS (i.e. as a control),

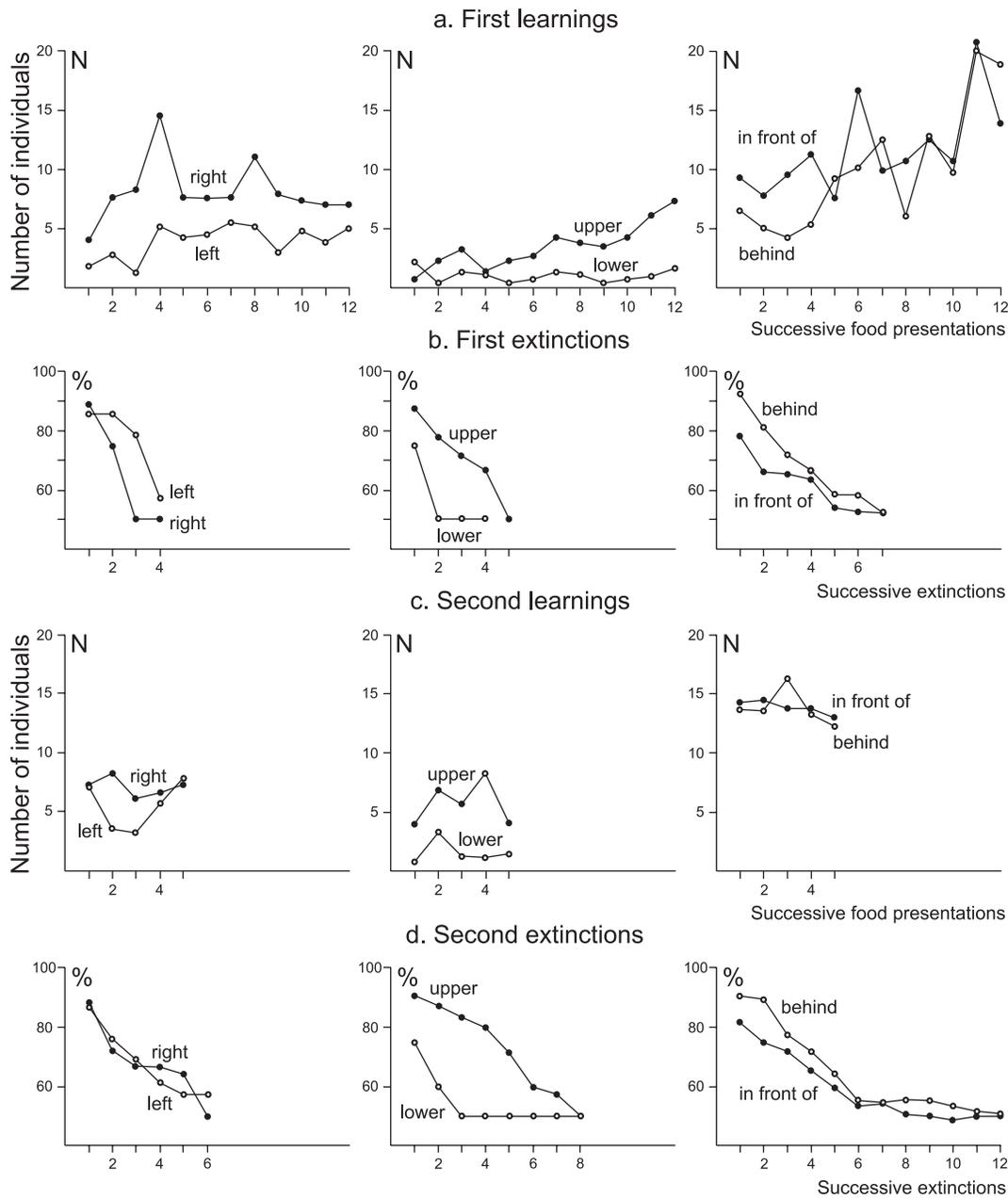
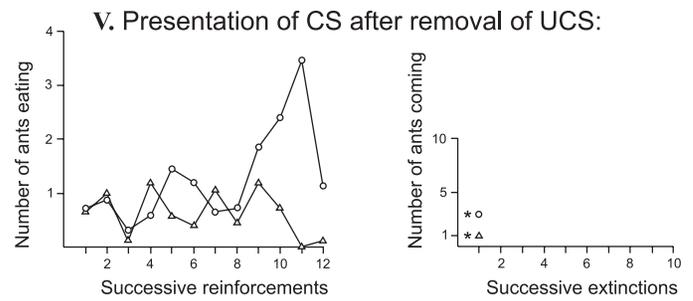
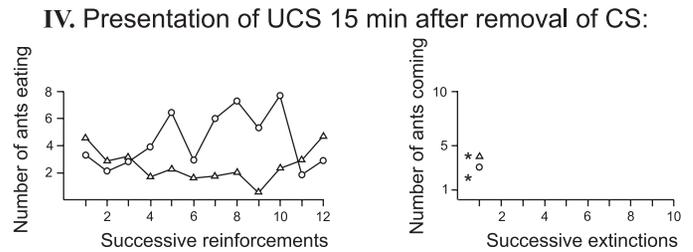
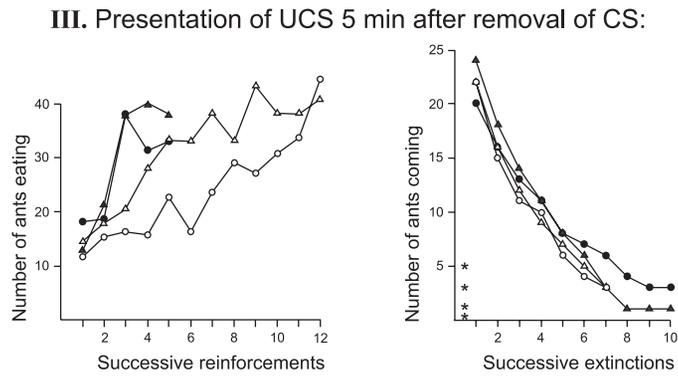
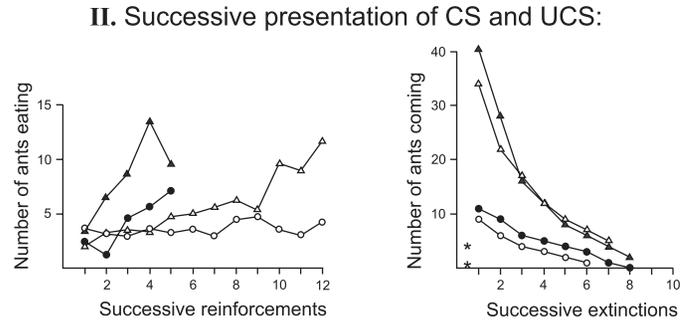
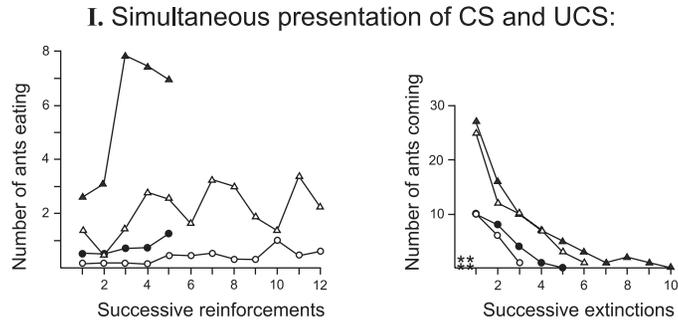


Fig. 5. Spatial learning in *Myrmica sabuleti*. N – mean number of ants eating at a time of the 15 min of food presentation. % – percentage of ants coming on the food place for which the learning experiments have been carried out. Right, left, lower, upper, in front of, behind indicate that the places for which the learning experiments have been carried out were situated to the right or the left of the nest entrance, on the lower or the upper stage of a construction, or in front of or at the back of the nest (see Fig. 1).

and 22 (green triangle) and 22 (onion) workers were counted in the presence of the CS. Control and experimental counts statistically differed ( $P < 0.01$ ). We can thus deduce that ants had been conditioned. Seven extinction experiments (for the green triangle and for the onion) were necessary to




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First conditionings:  $\blacktriangle$  : CS = green triangle,  $\circ$  : CS = onion

Second conditionings:  $\blacktriangle$  : CS = green triangle,  $\bullet$  : CS = onion

\* : control

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Fig. 6. Classical conditioning in *Myrmica sabuleti*. CS – conditioned stimulus (a green triangle or 2 pieces of onion). UCS – unconditioned stimulus (a droplet of sugared liquid food). Y-axis: mean number of ants eating at a given time or coming onto the food place during a period of 10 min, the CS then being presented. Control values: number of ants coming onto the food place during a period of 10 min, the CS being not presented. See Fig. 1 for the design of these experiments.

Table 1. Efficiency of conditioned stimuli (CS), visual (green triangle) or olfactive (piece of onion), the unconditioned stimulus (UCS) being a droplet of sugared water presented either simultaneously, immediately after or 5 min after the CS.

CS and UCS presentations	Simultaneous		Immediately successive		With a 5 min delay between the two presentations	
	Visual	Olfactive	Visual	Olfactive	Visual	Olfactive
Nature of CS:						
Number of ants responding to the CS after a first conditioning	25	10	34	9	22	22
Number of ants responding to the CS after a second conditioning	28	10	41	11	24	20
Number of experiments necessary to extinguish the first conditioning	5	2	7	5	7	7
Number of experiments necessary to extinguish the second conditioning	7	3	8	6	8	9

extinguish these first conditionings. Second conditioning experiments, though consisting of only five reinforcements, produced somewhat better responses, and the extinction of these responses took a little longer than the extinction of the first conditionings, two observations statistically significant ( $P < 0.025$ ).

*Presentation of the UCS 15 min after the removal of the CS*

The mean numbers of ants eating at a specific time did not increase in the course of the 12 successive presentations. Control counts were then four and three ants and counts in the presence of the CS were four and two ants. These counts obviously did not differ significantly. So, in this case the ants' conditioning experiments failed.

*Presentation of the CS after the removal of the UCS*

In the course of the 12 successive presentations, no increase in the numbers of ants present on the food place at a time was observed. One and three ants were then counted in the absence of the CS, then again one (green triangle) and three (onion) ants were counted in their presence. These results did not obviously differ. Therefore, we can deduce that in the present case no conditioning of ants occurred.

*Efficiency of the visual and the olfactive stimuli*

We estimated that the ants' conditioning was of better quality (and the CS more efficient) when the number of ants responding to the CS after their first and second conditioning was higher and when the experiments necessary to extinguish the

ants' first and second conditionings were more numerous. We made this evaluation for the three cases in which conditioning succeeded. Results corroborated the presumptions we formed in the course of our experiments: the visual CS used appeared the more efficient if presented just before the UCS (Tab. 1, column 3 *vs* columns 1 and 5) while the olfactive CS used appeared the more efficient if presented a short time (5 min) before the UCS (Tab. 1, column 6 *vs* columns 2 and 4).

*Ants' discrimination and generalisation of stimuli (Tab. 2)*

No ant conditioned to a green triangle responded to a yellow or a blue triangle and the counts corresponding to these stimuli did not differ statistically from the control values. *Myrmica sabuleti* workers can thus discriminate yellow and blue from green. On the contrary, they responded to a green square and a green rectangle and counts in the presence of these two stimuli differed statistically from the control ones ( $P < 0.01$ ). The ants reacted to both a square and a rectangle as they reacted to a triangle after having been trained: they are thus unable to discriminate between the three shapes presented. The ants conditioned to onion responded significantly to leek and garlic (difference with controls:  $P < 0.01$ ). The ants thus exhibited the response acquired in the presence of onion, in the presence of the two other odorous plants: they are therefore unable to discriminate between the three plants. In contrast, they did not respond to cabbage or Belgian endive. *Myrmica sabuleti* workers thus discriminated these two latter odorous plants from onion.

Table 2. Ants' discrimination and generalisation of stimuli. The ants conditioned to a green triangle (or to pieces of onion) received successively four other visual (or olfactory) stimuli, and were, after each of these four tests, conditioned again to the initial conditioned stimulus. The ants' responses to each stimulus were quantified by their numbers arriving during 10 min onto the food place, the stimuli being presented, but not the food. Control values (number of ants coming in the absence of the conditioned stimuli) were 0, 1, 0 and 1 ant.

Ants initially conditioned to a green triangle		Ants initially conditioned to onion	
CS presented	Ant's response	CS presented	Ant's response
Initial CS	25	Initial CS	10
Initial CS, 2 <sup>nd</sup> time	27	Initial CS, 2 <sup>nd</sup> time	10
Yellow triangle	0	Leek	4
Blue triangle	0	Garlic	4
Green square	22	Cabbage	0
Green rectangle	23	Belgian endive	0

## Discussion and conclusions

The present work deals with temporal learning, spatial learning and classical conditioning in the temperate-cold climate ant, *Myrmica sabuleti*.

After 12 food presentations, this ant species was able to exhibit temporal learning at a specific time of the day, which extinguished after three days. A second successive learning experiment, consisting of six food presentations, gave better scores and extinguished after seven days. The ants' temporal learning is thus clear and its characteristics are similar to those of any learning, i.e. second learning is of better quality than the first one, with a longer extinction period. However, a slight anticipation of response exists, a fact usually observed when trying to obtain temporal learning (e.g., HARRISON & BREED, 1987; SCHATZ et al., 1993, 1994, 1999a). It would have been more convincing to demonstrate temporal learning of ants at two different training times (e.g. 1:00 and 4:00 a.m. or 10:00 p.m. and 1:00 a.m.). However, early in the morning (about 4:00 a.m.) and during the evening (about 10:00 p.m.) the ants became active, generally foraging and searching for food (personal observation). For this reason, we consider that showing temporal learning at 1:00 a.m. is more valuable. Temporal learning could not be demonstrated in 15 ants species living in a similar temperate-cold climate, i.e. in several species of *Formica*, *Lasius* and *Leptothorax*, in *Myrmica scabrinodis* Nylander, 1846, and in *Tetramorium caespitum* (L., 1758) (REICHLER, 1943; DOBRZANSKI, 1956; FOURCASSIÉ & TRANIELLO, 1994). It has been postulated that temporal learning is useful (and hence, possible) in ants nesting in tropical climates, because these ants must avoid

foraging during the warmest hours of the day (CRUDEN et al., 1983). Indeed, such learning has been found in two Neotropical ants species, and in one Mediterranean one (HARRISON & BREED, 1987; SCHATZ et al., 1993, 1994, 1999b). However, temporal learning has now been shown in *M. sabuleti* (present work). It could thus be useful to attempt temporal learning experiments in other ant species from cold, Mediterranean and warm climates, for testing the hypothetical relation between the ants' usual habitat and their temporal learning ability. In fact, the ability of temporal learning is helpful to a species for it allows it to maximise the efficiency of its activities with the periodic variations of the biotic and abiotic factors of the environment. Three levels of complexity in temporal learning are distinguished by FOURCASSIÉ et al. (1999) in their clear and complete survey of the subject. The temporal learning obtained in the present work on *M. sabuleti* corresponds to the second level of complexity considered by these authors.

*Myrmica sabuleti* can also learn to visit a food place situated at a precise location (six different locations have been tried) within their foraging area. The first learning extinguished in a few experiments. The second learning sessions, although consisting of fewer experiments than the first ones, yielded higher responses, and presented slower extinction. These trends are typical characteristics of learning, but they had not previously been shown in ants. Other insects, such as cockroaches, seem also to learn the locations of specific food resources in their home range (DURIER & RIVAULT, 2001). It would be interesting to attempt, in *M. sabuleti*, a spatio-temporal learning, that is to test if the species can learn to visit a place at a given time,

and on another place at another given time. Such a spatio-temporal learning has been attempted in *Cataglyphis cursor*, without success (SCHATZ et al., 2000). It has been demonstrated successfully in *Ectatomma ruidum* by SCHATZ et al. (1999b), the foragers having learned where, when, and for how long a time period some food was available, in laboratory and field conditions.

Classical conditioning was demonstrated, in *M. sabuleti*, by presenting either a visual (green triangle) or an olfactory (onion) CS and some liquid sugared food as UCS, in three circumstances: when the CS presentation occurred a short time before, just before, or together with the UCS presentation. Classical conditioning failed when the UCS was presented 15 minutes after removal of the CS, or was presented before the CS. In the three cases where conditioning occurred, a few experiments were necessary to extinguish it. Second conditioning, although obtained using fewer reinforcements than the first ones, were generally of better quality and extinguished more slowly than the first ones, two facts common to any conditioning but which had not previously been shown in ants. The visual CS used appeared to be more efficient if presented just before the UCS; the olfactory CS used was more efficient if presented a short time before the UCS. However, having used only one visual and one olfactory stimulus, we cannot generalise our conclusions and state that any visual CS would be more efficient than any olfactory one. The workers of *M. sabuleti* discriminate yellow and blue from green, but appeared to be unable to discriminate between a triangle and a square or a rectangle. They responded to leek and to garlic as they did to onion, thus being unable to discriminate between leek and onion, and between garlic and onion. However, they could discriminate cabbage and Belgian endive from onion.

Cockroaches are also able to associate landmark cues to the presence of food resources (DURIER & RIVault, 2000). Moreover, conditioning experiments have been successfully made on honeybees for many years (WIGGELSWORTH, 1967; BOGDANY, 1978 and references therein; HAMMER & MENZEL, 1995) as well as on wasps (e.g., JANDER, 1998). In honeybees, a visual CS must be presented just before (i.e. during the seconds preceding) the UCS to obtain efficient conditioning (MANNING & DAWKINS, 1998); when presented together with the UCS, conditioning fails. This difference between the conditioning of ants and bees may be due to differences between the manner with which they associate perceived elements of their environment and with which they

use cues to orientate themselves towards food (BOGDANY, 1978).

ROCES (1990) demonstrated what he termed “olfactory conditioning” during the recruitment process of *Acromyrmex lundii* (Guérin-Méneville, 1838). However, our opinion is that the ants’ behaviour studied by ROCES did not result from classical conditioning. There are no replicated presentations of a CS and a UCS to the same animals and later, the association by these animals of the two stimuli. Indeed ROCES (1990) gave odour-impregnated flakes to scout ants and proved that recruited ants then statistically picked up identically odour-impregnated papers. The recruited ants thus perceived a stimulus (not a CS then a UCS or a reward) and subsequently responded to that particular stimulus. In fact, the author clearly showed that recruited ants correctly respond to scout ants, bringing back to the nest the same odorous food than that carried back by the scouts. This may be common in species using recruiting and recruited individuals; ROCES (1990, 1994) perfectly demonstrated it. However it is not classical conditioning, nor learning; it may rather be some imprinting mechanism as that obtained by JAISSON (1975) in *Formica polyctena*.

Meticulous conditioning experiments (similar to those carried out on honeybees: see references above) should be performed to teach us about ants’ perception of visual and olfactory stimuli. Different forms, dimensions, slope of objects, distances at which they are presented, different colours and odours should be used as CS. In honeybees, simple classical conditioning was also used to investigate a complex odour recognition (LALOI et al., 1999). In addition, complex learning by association (using complex CS and/or complex CS presentations) was proved to be possible (DEISIG et al., 2001; KOMISCHKE et al., 2001). The role played by each antenna (during the training period and for obtaining the conditioned responses) has been studied experimentally (SANDOZ & MENZEL, 2001; SANDOZ et al., 2001). However, since foraging depends on learning by association ability, research is pursued on the influence of Gaucho<sup>®</sup> (a widely used insecticide) on the honeybees’ learning ability (PHAM-DELÈGUE, 2001). In contrast, learning and conditioning studies of ants are not very advanced, though numerous studies exist on ants’ ability to forage, to use landmarks and to return to their nest (HÖLLEDOBLER & WILSON, 1990, SCHATZ et al., 1999b, c). However, there are articles showing route fidelity in ants (ROSENGREN, 1971; ROSENGREN & FORTELIUS, 1986 on *Formica* spp.; QUINET et

al., 1997 on *Lasius fuliginosus* (Latreille, 1798)). Experiments on conditioning should allow to precise the kind of visual and olfactory cues (landmarks, parts of plants, shadows, pebbles, odours) the ants may associate with a place where they find food. This would give insight into how foragers can localise and memorise places where food can be found and may complement studies on ants' spatial orientation.

Basically, spatial and temporal learning on the one hand and conditioning on the other, are similar processes and can thus be studied in the course of the same experimental work. Thus, since *M. sabuleti* can be conditioned to a stimulus and acquire spatial learning, an interesting future aim may be to attempt, in that species, a complex spatial conditioning. It could be determined if *M. sabuleti* workers are able to associate a precise stimulus with a given place, and another stimulus with another place. Using individual marking of ants, such a complex spatial conditioning could determine if conditioning in this ant species is individual.

If complex spatial conditioning appears possible in *M. sabuleti*, experiments could be extended to learning of a complex trajectory discontinuously marked by cues previously used as CS in the process of complex spatial conditioning. This new step in conditioning, which we propose to attempt in *M. sabuleti*, might succeed, since the ant *Cataglyphis cursor* can effectively learn a sequence of visual patterns (CHAMERON et al., 1998; SCHATZ et al., 1999c).

#### Acknowledgements

We are sincerely grateful to D. CAMMAERTS and B. HERVIER-MARTINEZ who gave us judicious advices. We very much thank R. CAMMAERTS for helping us to count the ants during temporal learning experiments, for establishing the figures and improving our paper. Many thanks are also due to B. SCHATZ and G. BEUGNON (University of Toulouse), to N. MANGAN (Cambridge University) and to an anonymous referee who provided a lot of helpful comments and valuable remarks for improving the draft of our paper. We want also to thank C. MELOT who helped us for statistically analysing our results and Ms P. HEZEMANS who corrected the English version of our manuscript.

#### References

BEUGNON, G. & LACHAUD, J-P. 1992. La représentation chez l'insecte: au commencement était l'image. *Psychol. Française* **37**: 21–28.  
 BEUGNON, G., PASTERGUE, I., SCHATZ, B. & LACHAUD, J-P. 1996. Cognitive and behaviouristic

approaches of space and time information processing in insects. *Behav. Process.* **35**: 55–62.  
 BIEBACH, H., GORDIJN, M. & KREBS, J. R. 1989. Time-and-place learning by garden warblers, *Sylvia borin*. *Anim. Behav.* **37**: 353–360.  
 BOGDANY, F. J. 1978. Linking of learning signals in honeybee orientation. *Behav. Ecol. Sociobiology*. **3**: 323–336.  
 CAMMAERTS, M-C., CAMMAERTS, R. & DEJEAN, A. 1993. Propriétés physiques et éthologiques des pistes de six espèces de Myrmicines. *Actes Coll. Insectes Soc.* **8**: 95–102.  
 CHAMERON, S., SCHATZ, B., PASTERGUE-RUIZ, I., BEUGNON, G. & COLLETT, T.S. 1998. The learning of a sequence of visual patterns by the ant *Cataglyphis cursor*. *Proc. Roy. Soc. London, Ser. B* **265**: 2309–2312.  
 CRUDEN, R. W., HERMAN, S. M. & PETERSON, S. 1983. Patterns of nectar production and plant-pollinator coevolution, pp. 80–125. In: BENTLEY, B. & ELIAS, T. (eds), *The Biology of Nectaries*, Columbia University Press, New York.  
 DEISIG, N., LACHNIT, H., HELLSTERN, F. & GIURFA, M. 2001. Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learning and Memory* **8**: 70–78.  
 DOBRZANSKI, J. 1956. *Badania nad zmyslem czascu u mrowek*. *Folia Biologica, Krakow* **4**: 385–397.  
 DURIER, V. & RIVAULT, C. 2000. Learning and foraging efficiency in German cockroaches, *Blattella germanica* (L.) (Insecta: Dictyoptera). *Anim. Cognition* **3**: 139–145.  
 DURIER, V. & RIVAULT, C. 2001. Effects of spatial knowledge and feeding experience on foraging choices in German cockroaches. *Anim. Behav.* **62**: 681–688.  
 EVERSHEED, R. P., MORGAN, E. D. & CAMMAERTS, M-C. 1981. Identification of the trail pheromone of the ant *Myrmica rubra* L., and related species. *Naturwissenschaften* **67**: 374–385.  
 FOURCASSIÉ, V. & TRANIELLO, J. F. A. 1994. Food searching behaviour in the ant *Formica schaufussi* (Hymenoptera, Formicidae): response of naïve foragers to protein and carbohydrate food. *Anim. Behav.* **48**: 69–70.  
 FOURCASSIÉ, V., SCHATZ, B. & BEUGNON, G. 1999. Temporal information in social insects, pp. 261–276. In: DETRAIN, C., DENEUBOURG, J.-L. & PAS-TEELS, J. M. (eds) *Information Processing in Social Insects*, Birkhauser, Basel, Boston, Berlin.  
 HAMMER, M. & MENZEL, R. 1995. Learning and memory in the honeybee. *Journal of Neuroscience*. **15**: 1617–1630.  
 HARRISON, J-M. & BREED, M. D. 1987. Temporal learning in the giant tropical ant, *Paraponera clavata*. *Physiol. Entomol.* **12**: 317–320.  
 HÖLLDOBLER, B. 1976. Recruitment behavior, home range orientation and territoriality in harvester ants, *Pogonomyrmez*. *Behav. Ecol. & Sociobiol.* **1**: 3–44.

- HÖLLDOBLER, B. & MÖGLICH, M. 1980. The foraging system of *Pheidole militicida* (Hymenoptera: Formicidae). *Insectes Soc.* **27**: 237–264.
- HÖLLDOBLER, B. & WILSON, E. O. 1970. Recruitment trails in the harvester ant *Pogonomyrmex badius*. *Psyche* **77**: 385–399.
- HÖLLDOBLER, B. & WILSON, E. O. 1986. Nest area exploration and recognition in leafcutter ants (*Atta cephalotes*). *J. Insect Physiol.* **32**: 143–150.
- HÖLLDOBLER, B. & WILSON, E. O. 1990. *The Ants*. Springer-Verlag, Berlin-Heidelberg, 732 pp.
- JAISSON, P. 1975. L'imprégnation dans l'ontogénèse des comportements de soins aux cocons chez la jeune Fourmi rousse (*Formica polyctena* Forst.). *Behaviour* **52**: 1–37.
- JANDER, R. 1998. Olfactory learning of fruit odors in the eastern yellow jacket, *Vespula maculifrons* (Hymenoptera, Vespidae). *J. Insect Behav.* **11**: 879–888.
- KOMISCHKE, B., SANDOZ, J.-C., MALUN, D. & GIURFA, M. 2001. Side-specificity of configural olfactory learning in honeybees, p. 214. In: Proceedings of the 2001 European Congress of the IUSSI, Henry-Ford-Building of the Free Univ. Berlin, Berlin.
- LALOI, D., GALLOIS, M. & PHAM-DELÈGUE, M.-H. 2000. Performance d'apprentissage olfactif chez l'abeille: variations en fonction de l'âge des ouvrières et étude comparative des réponses des reines et des mâles. *Actes Coll. Insectes Soc.* **13**: 95–104.
- LALOI, D., ROGER, B., BLIGHT, M. M., WADHAMS, L. J. & PHAM-DELÈGUE, M.-H. 1999. Individual learning ability and complex odour recognition in the honeybee, *Apis mellifera* L. *J. Insect Behav.* **12**: 585–598.
- MANNING, A. & DAWKINS, M. S. 1998. *An introduction to animal behaviour*. Cambridge University Press, 450 pp.
- MÖGLICH, M. & HÖLLDOBLER, B. 1975. Communication and orientation during foraging and emigration in the ant *Formica fusca*. *J. Comp. Physiol.* **101**: 275–288.
- PHAM-DELÈGUE, M.-H. 2001. La Gaucho® est-il l'ennemi des abeilles? *La Recherche* **347**: 70–73.
- QUINET, Y., DE BISEAU, J.-C. & PASTEELS, J.-M. 1997. Food recruitment as a component on the trunk-trail foraging behaviour of *Lasius fuliginosus* (Hymenoptera: Formicidae). *Behav. Process.* **40**: 75–83.
- REEBS, S. G. 1996. Time-place learning in golden shiners (Pisces: Cyprinidae). *Behav. Process.* **36**: 253–262.
- REICHLER, F. 1943. Untersuchungen über Frequenzrhythmen bei Ameisen. *Z. Vergl. Physiol.* **30**: 227–256.
- ROCES, F. 1990. Olfactory conditioning during the recruitment process in a leaf-cutting ant. *Oecologia* **83**: 261–262.
- ROCES, F. 1994. Odour learning and decision-making during food collection in the leaf-cutting ant *Acromyrmex lundii*. *Insectes Soc.* **41**: 235–239.
- ROSENGREN, R. 1971. Route fidelity, visual memory and recruitment behaviour in foraging wood ants of the genus *Formica* (Hymenoptera, Formicidae). *Acta Zool. Fenn.* **133**: 1–105.
- ROSENGREN, R. & FORTELIUS, W. 1986. Ortstreue in foraging ants of the *Formica rufa* group. Hierarchy of orienting cues and long-term memory. *Insectes Soc.* **33**: 306–334.
- SANDOZ, J.-C., GALIZIA, C. G. & MENZEL, R. 2001. Side-specific olfactory learning in the honey bee: behaviour and optical learning, p. 264. In: Proceedings of the 2001 European Congress of the IUSSI, Henry-Ford-Building of the Free Univ. Berlin, Berlin.
- SANDOZ, J.-C. & MENZEL, R. 2001. Side-specificity of olfactory learning in the honeybee: Generalization between odors and sides. *Learning and Memory* **8**: 286–294.
- SCHATZ, B., BEUGNON, G. & LACHAUD, J.-P. 1994. Time-place learning by an invertebrate, the ant *Ectatomma ruidum*. *Anim. Behav.* **48**: 236–238.
- SCHATZ, B., LACHAUD, J.-P. & BEUGNON, G. 1993. Apprentissage temporel chez la ponérine *Ectatomma ruidum* Roger. *Actes Coll. Insectes Soc.* **8**: 9–15.
- SCHATZ, B., BEUGNON, G. & LACHAUD J.-P. 1999a. Apprentissage temporel chez la fourmi Méditerranéenne *Cataglyphis cursor* (Hymenoptera, Formicidae). *Actes Coll. Insectes Soc.* **12**: 101–104.
- SCHATZ, B., LACHAUD, J.-P. & BEUGNON, G. 1999b. Spatio-temporal learning by the ant *Ectatomma ruidum*. *J. Exp. Biol.* **202**: 1897–1907.
- SCHATZ, B., CHAMERON, S., BEUGNON, G. & COLLETT, T. S. 1999c. The use of path integration to guide route learning in ants. *Nature* **399**: 769–772.
- SCHATZ, B., LACHAUD, J.-P. & BEUGNON, G. 2000. Tentative d'apprentissage spatio-temporel chez la fourmi *Cataglyphis cursor* (Hymenoptera, Formicidae). *Actes Coll. Insectes Soc.* **13**: 157–161.
- SIEGEL, S. 1956. *Non parametric statistics for the behavioral Sciences*. Mc Graw-Hill Kogakusha Ltd, Tokyo, 312 pp.
- SIEGEL, S. & CASTELLAN, N. J. JR. 1988. *Non-parametric statistics for the behavioural sciences*. McGraw-Hill book Company, 396 pp.
- WIGGELSWORTH, V. B. 1967. *The principles of insect physiology*. Methuen & Co, London, 741 pp.

Received September 26, 2002

Accepted November 13, 2003