

## Collective operant conditioning and circadian rhythms in the ant *Myrmica sabuleti* (Hymenoptera, Formicidae)

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### Abstract

*Myrmica sabuleti* workers were trained to find their meat food near one or several olfactory or visual cues. They were then singly tested in a Y apparatus to assess their performance in responding to the learned cue. They rapidly responded to a non-pheromonal odour, acquiring 10% of their performance on the task in 8 h and the last 24% in 4 d. When no longer trained, they lost 18% of their performance in 7 h and all of it in about 3 d. This indicates a rather short-term olfactory memory. Their olfactory sensitivity and response ability followed a circadian rhythm, namely stronger at early night and weaker during the day. When they have to associate several odours to their food site, they consider each odour separately. Their performance in responding to an odour decreased linearly with the number of odours encountered. These ants slowly memorised a visual cue. They gave nearly no correct response during the first 1.5 d and acquired 25% - 30% of their performance in the next 6 d. They slowly stopped responding to a memorised visual cue: they continued responding to it for 2 d then lost 20% of their performance in the next 3.5 d. After that, they maintained 10% of their performance. This demonstrates a long-term visual memory. Their visual sensitivity and response ability had a circadian rhythm (stronger during the day, weaker at early night). After having been trained to find their food near several visual cues, the ants gave entire responses in the presence of all the cues as well as correspondingly reduced responses in the presence of only parts of the cues. This ant species therefore sees all the visual cues globally, as a whole picture, and shows only a fraction of its performance when confronted with each single visual cue.

**Keywords:** ant, circadian rhythm, memory, olfaction, vision

### Introduction

While foraging and thereafter returning to their nest, ants must orient themselves. Ants' navigation is a relatively old subject of interest (COLLETT *et al.*, 2003; CHENG *et al.*, 2008; NARENDRA, 2007 and references therein). Ants estimate the distances they walk using odometry (CAMMAERTS, 2005a). WITTLINGER *et al.* (2006, 2007) presented a mechanism for such an odometry. Ants memorise some of their motor patterns (MACQUART *et al.* 2006). They also use their own odorous area markings (CAMMAERTS & CAMMAERTS, 1998, 1999, 2001). They can see and use distant visual cues (e.g. those located in the canopy) (SALO, 1998). Basically, however, they use visual cues located along their way (landmarks) (PASSERA & ARON, 2005). They also associate odours to useful sites (EHMER, 1999;

WOLF & WEHNER, 2000; HELMY & JANDER, 2003). A previous study (CAMMAERTS & RACHIDI, 2009) showed that, at a collective level, foragers of the ant *Myrmica sabuleti* Meinert 1861 use primarily odours and secondarily visual cues to orientate themselves in a maze. The tested ants soon stopped responding to no longer valid odours but continued responding to obsolete visual cues. What underlies such a collective response remained unknown. Do the olfactory and visual learning mechanisms differ, for instance, in terms of dynamic and/or capacity to memorise more than one cue? Answering this question was the first aim of the present work.

On the other hand, we aimed to enlarge our knowledge on olfactory and visual learning in ants, specifically at a collective level. Learning has been intensively studied in vertebrates (PEARCE, 2008) as well as in invertebrates, above

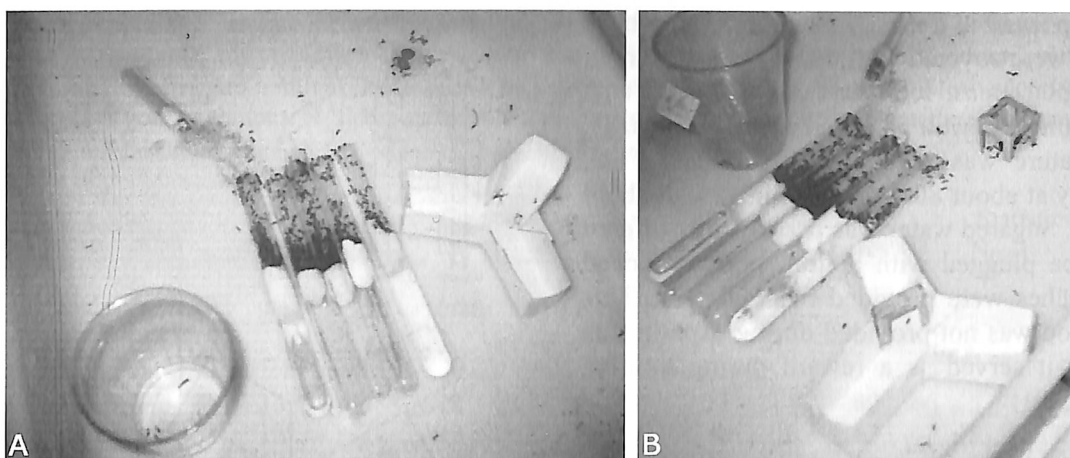


Fig. 1. Set-up designed to study the dynamics of acquisition and loss of olfactory and visual conditioning. Training involved delivering meat either surrounded by pieces of fennel (olfactory training, A) or under a green hollow cube (visual training, B). Tests were performed in a Y apparatus (positioned in the ants' tray), with the cue (fennel or green) in one arm. Each time, 20 ants of six colonies were tested, one by one in the course of time (once during the day, once at early night): they had to choose between the arm with and without cue.

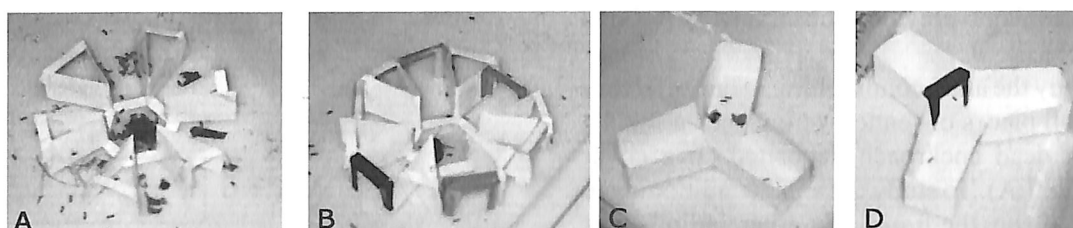


Fig. 2. The ants of five colonies were trained to find their food near multiple olfactory (A) or visual (B) cues then were tested in Y apparatus with either one olfactory (C) or one or multiple visual cues (D) in one arm. This experiment revealed how ants memorise and how many cues they can memorise at a time.

all in bees (GIURFA, 2007). Theories explaining learning have been proposed (RESCORLA & WAGNER, 1972, PEARCE & BOUTON, 2001). In ants, olfactory as well as visual learning have also been studied already, although to a lesser extent than in bees. Past work has dealt with either ants' olfaction (EHMER, 1999; HELMY & JANDER, 2003) or, more often, their vision (DURIER *et al.*, 2003; GRAHAM *et al.*, 2007). Such studies have been conducted either on a society (VOSS, 1967; KRETZ, 1979) or, more recently, on animals singly tested (DUPUY *et al.*, 2006; ESCHBACH *et al.*, 2006; NARENDRA *et al.*, 2007; GUERRIERI *et al.*, 2007; JOSENS *et al.*, 2009). The samples have been rather small and the visual as well as the olfactory conditioning have never been studied consecutively in the same species and individuals, using the same protocol and under the same experimental conditions. Here, we tried to close this gap.

Therefore, we conducted experiments on *M. sabuleti* workers in order to determine the dynamics of the acquisition and loss of a response

to olfactory and visual elements, the number of olfactory and visual cues the ants can learn and if they learn these two kinds of cues in the same way. We studied olfactory as well as visual memory consecutively on the same population, at a collective level, using large samples (80, 100 or 120 individuals for each test). Doing so, we aimed to better understand our results on *M. sabuleti* workers' navigation (CAMMAERTS & RACHIDI, 2009) and to provide more information about ants' collective olfactory and visual learning.

## Material and Methods

### Collection and maintenance of ants

The experiments were conducted on six colonies collected at Hôhes Martelingen (G-D Luxembourg) and in Lorraine (France). They were demographically similar, containing a queen, brood and about 500 workers. They were maintained in artificial nests made of one to three glass tubes half-filled with water, a cotton-plug separating the ants from the water. The glass tubes

were deposited in trays (37 X 52 X 8 cm) whose borders were covered with talc. The trays served as foraging areas: food was delivered and ants were trained as well as tested in them (Figs. 1). Temperature was maintained at  $20^{\circ} \pm 2^{\circ} \text{C}$ , humidity at about 80% and the lighting at about 600 lux. Sugared water was permanently offered in a tube plugged with cotton; pieces of dead cockroaches were provided twice a week. This meat food was not provided during experiments because it served as a reward during training (Figs. 1, 2).

### Experimental processes

Ants were collectively trained using training apparatus deposited in the ants' tray, each colony having its own apparatus. This training is an operant conditioning, identical to that previously used (CAMMAERTS & RACHIDI, 2009) so that the results could be analysed in the same way and helped explain previous observations (above reference).

To study the ants' conditioning to one olfactory cue, small pieces of fennel were placed around a piece of dead cockroach deposited on a cover glass (Fig. 1 A). To study the ants' conditioning to one visual cue, the frame of a green hollow cube was placed over a piece of dead cockroach deposited on a cover glass (Fig. 1 B). The hollow cubes were made of strong green paper (Canson®) and glued like the black hollow cubes used for studying the ants' adaptation to light intensity (CAMMAERTS, 2005b) (Fig. 3, hollow cube). The wavelength spectra reflected by the green paper are given in CAMMAERTS (2007) and CAMMAERTS & CAMMAERTS (2009).

To study the ants' learning of several olfactory or visual cues, a hexagonal experimental apparatus was used (Fig. 2 A, B). Each was made of white extra strong paper as detailed in Fig. 3 (sub-title: hexagonal apparatus). A piece of cockroach was placed in the centre of the apparatus and either an olfactory or a visual cue could be set in each of its six corridors. The ants could reach the food (= the reward) only by moving through the six corridors and so perceiving the presented cues. Depending on the tested colonies, two to six different cues, either odorous or visual, were deposited in two to six of the six corridors. Precisely, colonies labelled 1, 2, 3, 4 and 5 were trained in the presence of 2, 3, 4, 5 and 6 cues respectively. The corridors not provided with a cue were closed (made

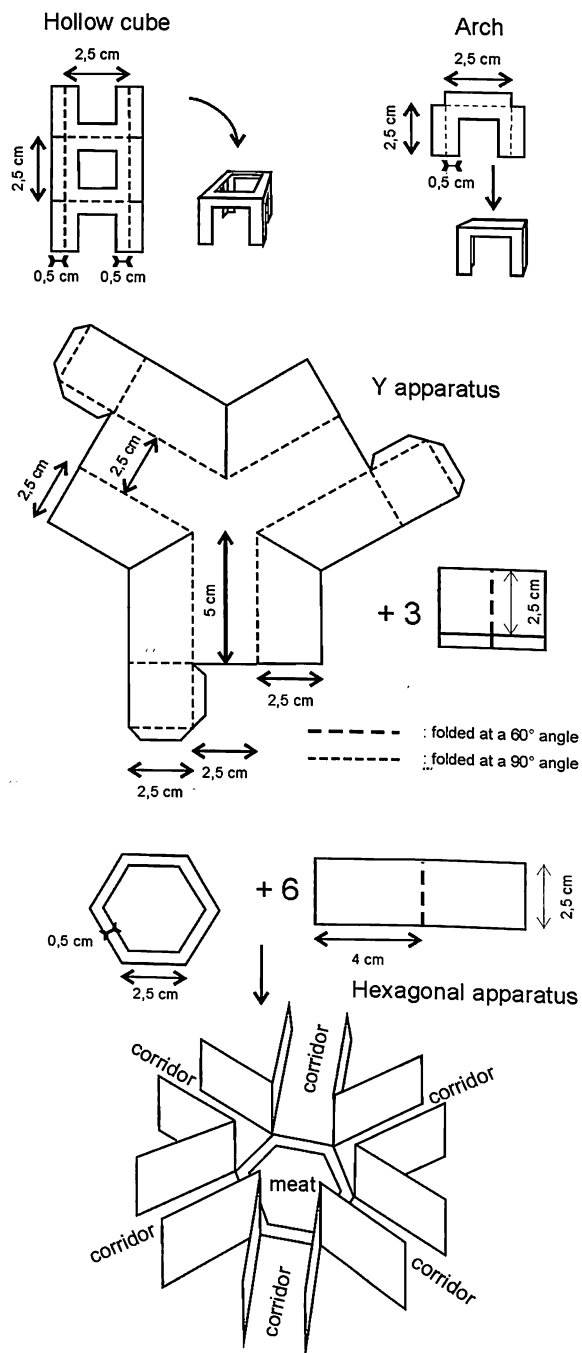


Fig. 3. Experimental apparatus. - The hollow cube (green paper) was used to train the ants to the presence of a green cue near the food. - The paper arch was set in an arm of a Y apparatus to assess the response to one or several visual cues. - The Y apparatus helped assess the response to an olfactory element or to one or multiple visual cues, the elements being set in one arm of the Y. - The white paper hexagonal apparatus consisted of a horizontal hexagon with six folded sheet of paper (4 x 2.5 cm) vertically attached to the six corners. This yielded six corridors into which one olfactory or visual cue could be set.

Table 1. Numbers of ants (among 20 each in six colonies) going to the left vs to the right in a Y-maze: before any experiment (**control**), towards fennel (an odorous cue) while always finding their food near such a cue (**acquisition**) or after cue removal (**loss**). *Clock* as well as real spent times are given. Mean scores are mean percentages of correct choices. N T P: results of non-parametric Wilcoxon tests between the six test and control numbers (nomenclature after SIEGEL & CASTELLAN, 1989).

Experiments	Colonies						Mean scores%	Statistics		
	1	2	3	4	5	6		N	T	P
<b>Control</b>										
Left/right	9/11	9/11	10/10	10/10	10/10	10/10	58/62			
<b>Conditioning acquisition</b>										
23h 30' 8 h	13	11	12	12	13	11	60	5	15	0.031
13h 30' 22 h	13	11	13	12	14	12	63	5	15	0.031
22h 30' 31 h	14	13	15	12	14	14	68	6	21	0.016
13h 30' 46 h	14	14	14	13	15	14	70	6	21	0.016
23h 55.5 h	14	14	16	16	15	15	75	6	21	0.016
14h 30' 71 h	17	15	16	15	16	16	79	6	21	0.016
23h 79.5 h	16	18	16	17	17	17	84	6	21	0.016
16h 30' 97 h	17	16	17	17	17	16	83	6	21	0.016
23h 104.5 h	17	17	18	16	18	17	86	6	21	0.016
14h 30' 119 h	17	16	15	18	16	16	82	6	21	0.016
23h 30 128 h	16	18	16	17	17	16	83	6	21	0.016
14 h 142.5 h	18	16	16	15	16	16	81	6	21	0.016
<b>loss</b>										
22h 30 7 h	14	11	14	15	13	11	63	5	15	0.031
14 h 22.5 h	13	12	13	11	14	11	62	6	21	0.016
22 h 30 31 h	12	14	11	13	13	12	63	6	21	0.016
14 h 41.5 h	13	12	11	12	13	11	60	6	21	0.016
22 h 30 55 h	12	11	10	14	14	11	60	4	9	0.125
13 h 30 70 h	11	10	10	10	12	11	53	3	6	0.125
23 h 30 80 h	10	11	12	10	10	11	53	3	3 ± 0.32	
14 h 30 95 h	11	11	11	10	10	8	51	2	-	-
23 h 30 104 h	9	10	12	9	11	10	51	2	-	-

inaccessible for the ants) with a piece of white extra strong paper. The six different odorous cues were pieces of onion, thyme, fennel, sage, mint and tarragon. Pieces of vegetables were used for being olfactory cues as natural as possible and for being efficient at least during 8 to 14 h. The six different visual cues were arches made of strong paper (Fig. 3, arches), the paper being white with black lines, white with black points, black, orange, blue-green, or blue-violet. *M. sabuleti* workers can discriminate black elements (CAMMAERTS, 2004a, 2008) as well as colours (CAMMAERTS, 2007b). The wavelength spectra reflected by the coloured papers are given in CAMMAERTS (2007) and CAMMAERTS & CAMMAERTS (2009).

Ants were singly tested in a Y-apparatus (Fig. 1 A, B, Fig. 2 C, D), each colony having its own apparatus. These apparatus were built of strong white paper (Fig. 3, Y apparatus). Their borders were covered with talc to prevent ant escaping. In them, the ants deposited no trail since they were

not rewarded but they might let any other chemical traces. So, as a precaution, the floor of each Y-apparatus was changed between each test. During a control, the Y was empty. During a test, the Y was provided either with one olfactory cue or with one or several visual cue(s). New pieces of the plants used to train the ants as well as identical but new arches made of the same papers were used to test the ants. It was checked that several visual cues could be seen by ants, at their point of choice in the Y-apparatus. The cues were placed in the left or in the right arm of the Y, alternately from one test to the next and from one colony to the next.

To conduct a control or a test on a colony, 20 foragers of that colony were transferred one by one into the Y apparatus, near its entrance. Each one was observed until it turned either to the left or to the right of the Y, its choice being recorded

Table 2. Legend identical to that of Tab. 1, except that the cue was a green hollow cube (a visual cue).

Experiments	Colonies						Mean scores%	Statistics		
	1	2	3	4	5	6		N	T	P
<b>Control</b>										
Left/right	9/11	10/11	12/8	9/11	11/9	10/10	61/59			
<b>Conditioning acquisition</b>										
22h 30' 6.5 h	10	9	12	11	10	9	51	5	7	> 0.5
14h 30' 22.5 h	13	11	9	13	11	11	57	5	15	0.031
22h 30 h	14	10	10	12	13	9	57	6	17	0.109
14h 30' 46.5 h	13	12	12	12	14	13	63	6	21	0.016
22h 30' 54.5 h	12	12	11	15	12	13	63	6	21	0.016
14h 30' 70.5 h	13	13	15	12	14	13	67	6	21	0.016
23h 79 h	14	12	13	12	13	15	66	6	21	0.016
14h 30' 94.5 h	14	14	15	14	13	14	70	6	21	0.016
22h 30' 102.5 h	14	14	12	14	15	13	68	6	21	0.016
13h 30' 117.5 h	14	15	16	15	12	15	73	6	21	0.016
22h 30' 126.5 h	14	14	13	14	14	14	69	6	21	0.016
14h 30' 142.5 h	16	15	15	17	17	14	78	6	21	0.016
23 h 151 h	14	17	15	15	15	15	76	6	21	0.016
15 h 167 h	16	17	16	15	18	15	81	6	21	0.016
23 h 175 h	13	16	16	16	15	15	76	6	21	0.016
14h 30' 190.5 h	16	15	15	16	17	16	79	6	21	0.016
23 h 199 h	14	16	15	16	14	16	76	6	21	0.016
15 h 215 h	16	17	16	15	17	15	80	6	21	0.016
23 h 227 h	13	17	17	14	15	15	76	6	21	0.016
15 h 239 h	18	15	16	15	17	15	80	6	21	0.016
<b>loss</b>										
22h 6 h	14	15	13	15	15	15	73	6	21	0.016
12 h 20 h	16	14	14	16	16	16	77	6	21	0.016
22 h 30 h	13	15	13	15	12	15	69	6	21	0.016
13 h 30 45.5 h	13	14	13	14	14	13	68	6	21	0.016
22 h 30 54.5 h	12	13	14	12	13	14	65	6	21	0.016
14 h 30 70.5 h	12	13	14	12	11	14	63	6	21	0.016
23 h 79 h	10	13	12	12	13	12	60	6	19.5 ± 0.047	
14 h 30 94.5 h	12	10	11	12	12	14	59	6	19	0.047
22 h 30 102.5 h	10	12	11	11	10	12	55	5	13	0.094
12 h 116 h	11	11	12	13	13	12	60	4	10	0.063
23 h 127 h	12	11	10	11	12	10	55	3	6	0.125
14 h 30 142.5 h	14	11	10	12	10	12	58	5	15	0.031
22 h 30 150.5 h	12	11	10	12	10	12	56	5	15	0.031
14 h 166 h	13	11	12	11	14	11	60	4	10	0.063
22 h 30 174.5 h	12	12	11	10	12	10	56	4	10	0.063
14 h 30 190.5 h	12	13	13	12	12	11	61	6	21	0.016

(and only its first choice). Then, it was removed from the Y and transferred into a polyacetate cup whose rim had been covered with talc, until 20 congeners were tested.

Each test was performed on several series of 20 ants, i.e. on 80, 100 or 120 individuals.

### Experimental protocol

[A] First series of experiments, aiming to precise the acquisition and the loss of an olfactory

or a visual conditioning (Fig. 1)

1. The olfactory or the visual cue was duly set near the meat food, at a given recorded time, given in Tabs 1 and 2. It was relocated once or twice in 24 h, never periodically, in order to avoid spatial as well as temporal learning by the ants (CAMMAERTS, 2004b) and food was renewed when necessary.

2. Then, at successive given recorded times, 20 ants of each colony were tested once during the day and once at early night (not more often

because a test is in itself an experiment of extinction which reduces the acquired conditioning).

3. Three to four days after the test score of the population no longer increased, the olfactory or the visual cue was removed from the foraging areas, but testing was conducted as previously, day and night, until the test score of the population no longer decreased.

4. For the visual learning only, colony labelled 6 was then tested again every 7 days during two months in front of the previous learned visual cue, without having been trained again during that time period.

[B] Second series of experiments, aiming to precise the amount of olfactory or visual cues the ants of a colony can memorise and the underlying process (Fig. 2)

Note that *M. sabuleti* workers can discriminate different odours and visual cues since, when differentially conditioned to two visual cues and two different odours, they correctly negotiated a maze provided with the four elements (CAMMAERTS & RACHIDI, 2009).

1. Training was started on colony one with 2 cues, on colony two with 3 cues, on colony three with 4 cues, on colony four with 5 cues and on colony five with 6 cues. As previously, the training apparatus was relocated once or twice in 24 h and food was renewed when necessary.

NB: Colony 6 was omitted from this study: it was used to investigate the time period during which the ants can retain a visual cue previously associated to food (above section [A] 4).

2. After a 4-d period (olfactory training) or a 6-d period (visual training), 20 ants of each colony were tested in the presence of each cue. This test experiment was made twice for colony one so that, ultimately, 80 ants were tested. It was also made twice for colony two so that a total of 120 ants were tested. For colony three, four and five, the experiment was not repeated, the total numbers of tested ants being 80, 100 and 120 respectively.

3. Moreover, for the visual training, 20 ants of each of the five used colony were tested in front of all the cues presented (that is 2, 3, 4, 5 and 6 cues for colony one, two, three, four and five, respectively). A maximum of 6 arches could be inserted, one behind the other, in one arm of a Y maze and could be seen by an ant staying at the choice point.

4. In a complementary and repeated experiment, 20 ants of colony five were tested in the presence

of 2, 3, 4 and 5 among the 6 visual cues permanently surrounding the food site.

5. In a supplementary experiment conducted twice, 20 ants of colony two were tested in front of 2 among the 3 visual cues they encountered while collecting food.

### Quantification of ant responses and statistical analyses

Trained ants had continuous access to training equipment during days and nights. Consequently, ants' training could not be quantified. However, 2 to 10 ants consistently stayed or moved near the training apparatus. These ants were foragers, so those used for the tests. Since we tested each time more than 80 individuals, if any tested ant had not come previously to the training apparatus, this would have had minimal impact on the results.

Ants' response was quantified while performing control and test experiments. Each of these experiments performed on a colony involved recording the number of ants having turned to the left and to the right in the Y-apparatus (control) or towards the cue and towards the other direction (test) (Tabs 1, 2). The experiments performed on the six colonies (first study) or on several series of 20 ants of one colony (second study) enabled calculating the mean proportion of ants that turned towards the cues (% of correct responses; Tabs 1, 2).

For the acquisition and the loss of an olfactory or a visual conditioning, Wilcoxon non-parametric tests (SIEGEL & CASTELLAN 1988) were performed between the six numbers (a number per colony, see above methodology) of ants having turned towards the cue during the test and the six corresponding control numbers. The response was considered as being not significant at  $P > 0.05$ . Since each test is an extinction experiment, our assessment is contaminated with some unmeasured amount of extinction, but slightly because each ant was tested only for a few seconds and then returned in front of the training apparatus. Moreover, any slight extinction would contaminate all curves equally: the curves are thus comparable and the conclusions still robust. We previously studied 'artificial' extinction, presenting the stimuli without the reward (CAMMAERTS, 2004b, c). In the present work, we assessed natural forgetting by halting the presentation of the cue. Forgetting seems to be more difficult to quantify than learning, and many attempts have already been made to measure the

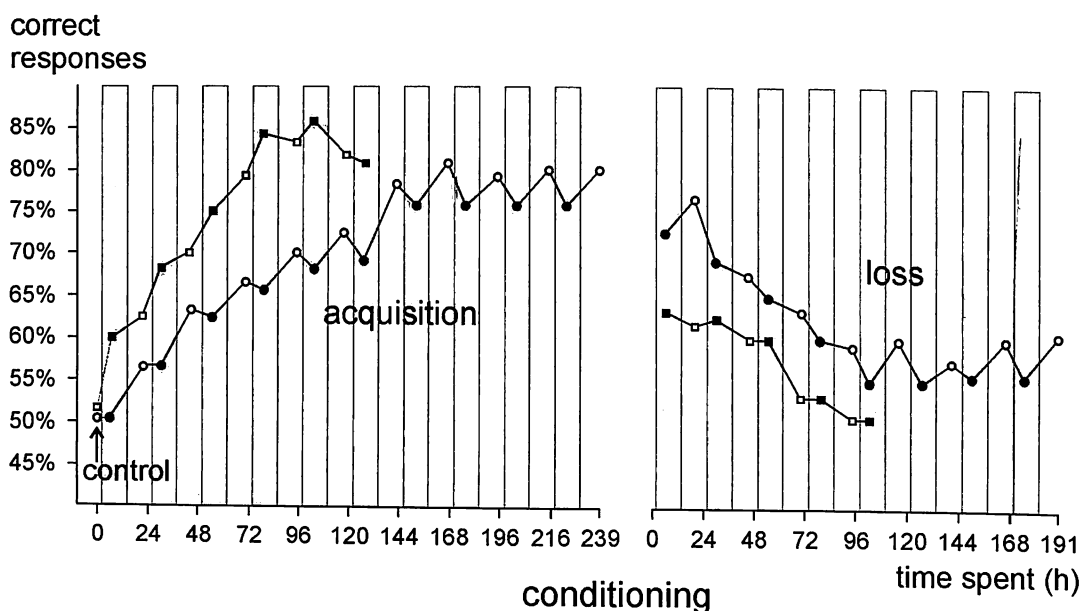


Fig. 4. Acquisition and loss of an olfactory (squares) and a visual (circles) conditioning. While the ants (of six colonies) were finding their food near fennel or under a green hollow cube (Fig. 1), 20 ants of each colony were tested, one by one, in the course of time, once during the day and once at early night, in a Y apparatus. Grey indicates night. There is no gap between acquisition and loss: the zeros of the loss graphs are the last points of the acquisition graphs. Note clear differences between the two dynamics (olfactory and visual): an initial increase or decrease for the olfactory conditioning vs initial latency period for the visual one; a different final score (higher for olfactory cue); a long-lasting memory for the visual conditioning.

nature of this function (e.g. WIXTED & EBLESEN, 1991, 1997).

Ants might initially prefer one of the two arms of the Y apparatus (for instance if they earlier found their food to their left or right). The Y mazes might be asymmetrical as for ant's preference. So, before training the ants, we conducted a control experiment using empty Y apparatus and then compared our results to those obtained during that control. It appeared that the control results were statistically identical to the theoretical ones (10 each time).

For the amount of olfactory and visual cues ants can memorise, non-parametric 2 X 2 contingency table chi-square tests were made between the total numbers of ants having turned to the cue(s) or to the other direction, and the total corresponding control numbers. Again, the responses were considered to be not significant at  $P > 0.05$ .

## Results

### First series of experiments

#### Acquisition and loss of an olfactory conditioning (Tab. 1, Fig. 4)

After having been trained to an odour during 8 h, the ants already responded to that odour with a mean score of 60%. This quick initial increase of

the test score of the population might be an artefact. We checked this by a complementary experiment performed on two untested colonies (in total: 40 ants): after a control response of 50%, the score of this new ant population equalled 55, 57.5, 57.5 and 60% after 2, 4.5, 6 and 8 h, respectively.

The ants' score then increased over a 4-d period, thereafter permanently equalled 81 - 85%. When the odour was no longer presented, the score of the population decreased rapidly to a value of 63% over a 7-h period and became similar to the control value after 3.5 d. These dynamics can be described according to the 'Monod law'.

for the acquisition:  $C = 0.5 + \alpha t \times (\beta / \alpha t + \beta)$

for the loss,  $C = 0.5 + 0.3 - \alpha t \times (0.3 / \alpha t + 0.3)$

with C: % of correct response

0.5 = initial value of C

t = time

$\alpha$  = increase of C for a unit increase of t, if the total increase of C was not limited

$\beta$  = maximum increase of C (= 0.3)

During the acquisition, maintenance, and loss of an olfactory conditioning, the ant population responded to the odour better at early night than during the day: a circadian rhythm thus probably

affected the ants' olfactory sensitivity and/or response ability.

### First series of experiments

#### Acquisition and loss of a visual conditioning (Tab. 2, Fig. 4)

Ants trained to a visual cue responded to that cue only after a latency period of about 35 h. The score of the population then slowly increased during 6 days, reaching 75 - 80% at early night and during the day respectively. The ants kept then that final score. When the cue was no longer presented, the population maintained its final score for about 30 h; thereafter, the score values slowly dropped to 60% over a 4.5-d period. Surprisingly, the ants kept that final, statistically significant score. It even reached a somewhat higher value later on. Indeed, colony 6 tested during two months following their partial loss of visual memory presented a score of 60, 70, 75, 65, 65, 70 and 65% after 15, 22, 29, 36, 42, 50 and 60 d, respectively. The three year lifespan of *Myrmica* sp. workers (CAMMAERTS, 1977) ensures that most tested foragers were active foragers during the training period. The dynamics of the acquisition and loss of a visual conditioning might be described by a logistic function resembling:  $C = \beta 0.5 / 0.5 + (\beta - 0.5) e^{-\alpha t}$

with C = % of correct response

0.5 = initial value of C

t = time

$\alpha$  = increase of C for an unit increase of t, if the increase of C was not limited

$\beta$  = maximum increase of C (= 0.75: experimental results)

and an additional factor ( $\lambda$ ), accounting for the latency period, should be added.

During the acquisition, maintenance, and loss of the visual conditioning, the responses were better during the day than at early night. This circadian rhythm, identical for all colonies, observed in all experiments, affecting the ants' association between food and a visual cue, may concern the ants' use of vision and/or visual learning ability. It was the inverse of that observed in front of an odour. This is commented in the 'Discussion' section.

### Second series of experiments

#### Amount of olfactory cues ants can memorise and underlying process (Fig. 5 A)

The ants of colony one, trained to onion and thyme, exhibited, in front of each of these two

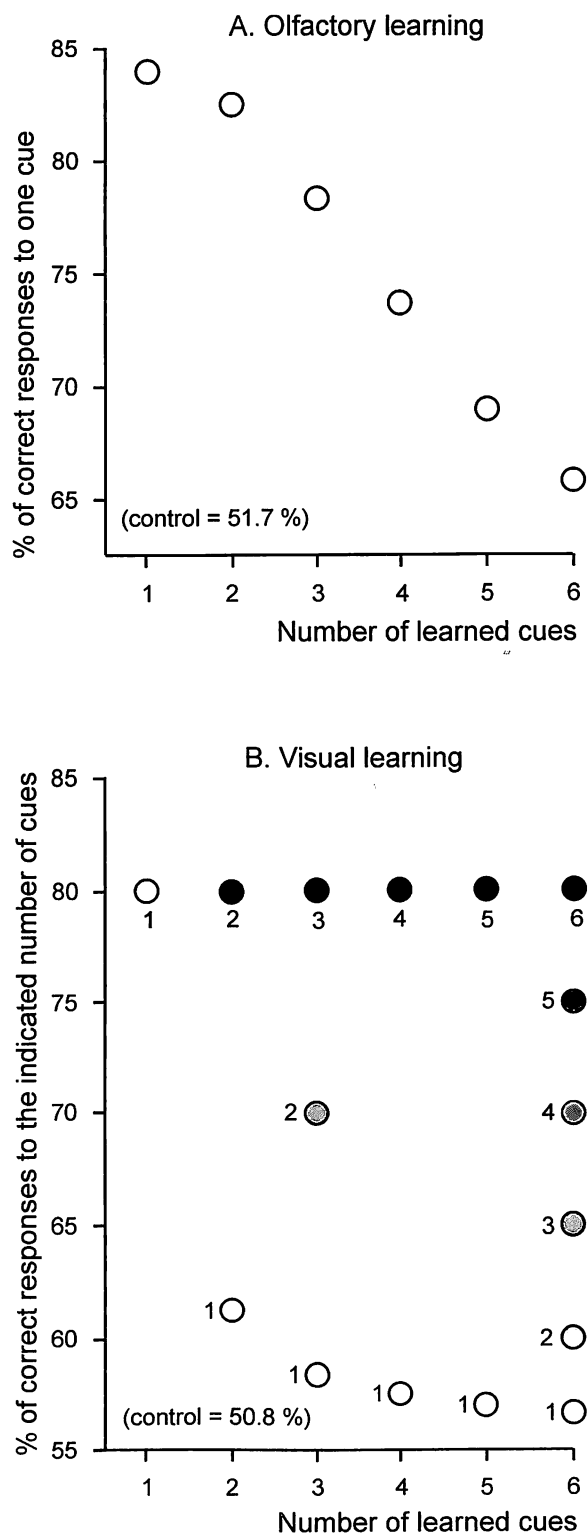


Fig. 5. Ants' olfactory and visual learning of multiple cues. The ants of five colonies were trained to 2, 3, 4, 5 or 6 visual or olfactory cues using the hexagonal apparatus. They were then tested in a Y apparatus in the presence of one or the other of these previously encountered cues (empty circles), all the encountered visual cues (black circles) as well as two, three, four and five visual cues (gradually grey circles). The numbers of cues presented during the tests are given near each circle.



separately presented cues, a mean score of 82.5% (based on four experiments (see the protocol above described);  $N = 80$ ; 16/4, 16/4, 17/3, 17/3;  $P < 0.05$ ). Colony two, trained to onion, thyme and fennel, responded to these cues separately presented with a mean score of 78.3% (six experiments;  $N = 120$ ; 16/4, 16/4, 17/3, 15/5, 15/5, 15/5;  $P < 0.05$ ). Colony three, whose food was presented during four days near onion, thyme, fennel and sage, then responded to these odours separately presented with a mean score of 73.8% (four experiments;  $N = 80$ ; 15/5, 15/5, 15/5, 14/6;  $P < 0.05$ ). Colony four, trained to 5 odours (onion, thyme, fennel, sage, mint), showed a mean score of 69% when responding to these odours individually presented (five experiments;  $N = 100$ ; 14/6, 14/6, 14/6, 13/7, 14/6;  $P < 0.005$ ). Finally, ants of colony five having had their food surrounded with 6 odours (onion, thyme, fennel, sage, mint, tarragon) still significantly responded to each of these odours separately presented (mean score of 65.8% based on six experiments;  $N = 120$ ; 13/7, 13/7, 14/6, 13/7, 13/7, 13/7;  $P < 0.05$ ). Such responses may reflect memorizing of each odour individually, whereby this memory decreased linearly with the number of odours having been encountered near the reward. These results and their extrapolation indicate that the ants of a colony collectively memorise one and two odours with an equally high score, can memorise 3 to 6 odours with linearly decreasing scores, may very weakly memorise 8 to 10 odours, and might be able to memorise simultaneously about 10 to 11 odours, but probably no more (see 'Conclusion - Discussion').

## Second series of experiments

### Amount of visual cues ants can memorise and underlying process (Fig. 5 B)

A green cue was here omitted because this cue was seen by ants during the first study and ants retain 10% of their visual performance.

Ants of colony one, simultaneously trained to 2 visual cues (black lines, black points), exhibited a mean score of 61.3% in front of each of these two cues (based on four experiments;  $N = 80$ ; 12/8, 12/8, 13/7, 12/8;  $P < 0.05$ ). Colony two, trained in the simultaneous presence of 3 visual cues (black lines, black points, full black), responded to each of these cues (separately presented) with a mean score of 58.3% (six experiments;  $N = 120$ ; 12/8, 12/8, 11/9, 12/8, 11/9, 12/8;  $P < 0.05$ ). Colony three, having found their food surrounded with 4 visual cues (black lines, black points, full black,

orange), showed a mean score of 57.5% when tested in front of each of these cues (four experiments;  $N = 80$ ; 12/8, 11/9, 11/9, 12/9;  $P < 0.05$ ). Colony four, concurrently trained to 5 visual cues (black lines, black points, full black, orange, blue-green), showed a mean score of 57% when confronted with each of these cues presented one by one (five experiments;  $N = 100$ ; 11/9, 11/9, 12/8, 12/8, 11/9;  $P < 0.05$ ). Finally, ants of colony five, simultaneously accustomed to 6 cues (black lines, black points, full black, orange, blue-green, violet-blue), exhibited a mean score of 56.6% when tested in front of each cue presented one by one (six experiments;  $N = 120$ ; 12/8, 11/9, 11/9, 11/9, 12/8, 11/9;  $P < 0.05$ ). These results and those obtained using odorous cues show different patterns (Fig. 5 A, B). So, olfactory and visual memory may involve different physiological mechanisms.

The ants of the five used colonies were tested in front of all the visual cues that had surrounded their meat food during training: each colony (that is twice 20 ants) responded with a mean score of 80% (so the population's test score equalled 80%). On the other hand, 20 ants of colony five were tested twice in front of 2, 3, 4 and 5 cues among the six visual cues they had to memorise. Their respective responses were 12/8 and 12/8, 13/7 and 13/7, 14/6 and 14/6, 15/5 and 15/5. In the absence of any cue, the population control score equals about 50% (Tabs 1, 2). If the results 'all the cues = 80%' and 'no cue = 50%' are graphically represented for each of the five used colonies and linearly joined, the theoretically expected responses to one cue equals those experimentally obtained, that is 61.3%, 58.3%, 57.5%, 57.0% and 56.6% for ants of colony one, two, three, four and five trained in front of 2, 3, 4, 5 and 6 cues, respectively. Moreover, the experimental values obtained when testing ants of colony five (conditioned to 6 cues) in the presence of 2, 3, 4 and 5 cues also equal those theoretically (graphically) expected. This agreement between theoretical and experimental values was checked by performing a supplementary experiment. Ants of colony two, whose meat food was surrounded with 3 cues, were then tested in front of 2 cues: the resulting mean score was 70% (two experiments;  $N = 40$ ; 14/7, 14/7). This is the theoretical value expected based on a linear graphical representation of the ants' scores '50% in front of no cue' and '80% in front of all the cues'.

## Discussion

### Protocol

Submitted to classical conditioning (reward offered immediately after stimulus presentation), insects (bees, ants) acquire their learning much more quickly than ants we tested (NARENDRA *et al.*, 2007; DUPUY *et al.*, 2006, LALOI *et al.*, 1999). Here, ants moved freely and experienced an "operant-like" conditioning, approaching from time to time the training apparatus and the reward. This feature probably explains why these ants learned more slowly than insects individually submitted to classical conditioning.

Contrary to previous works (CAMMAERTS, 2004a, 2005b, 2007a, 2007b, 2008), here, we tested the ants in an apparatus different from that used to train them because, this time, the training apparatus were inappropriate to quantify responses. Responses may thus have been influenced by this different apparatus (NARENDRA, 2007; CHENG, 2005; COLBORN *et al.*, 1999; COLLETT *et al.*, 2002, 2003). However, the here observed performances were nearly of the same quality and occurred nearly at the same speed as those previously obtained (see above references of CAMMAERTS).

Though individual 'learning' underlies the studied collective association between cue(s) and reward, the assessed variables (laps of times, scores) could only be applied to the collective response. Another methodology (for instance assessment in the course of successive trials performed on isolated ants) could allow quantifying the ants' individual learning *sensu stricto* (GALLISTEL *et al.*, 2004).

### The results

Our work clarifies previous results on *M. sabuleti* workers' use of odours and visual cues when foraging and yields new information on olfactory and visual learning in ants.

First, it shows that *M. sabuleti* workers quickly respond to an odour permanently presented with their food and quickly stop responding when the odour is removed. In contrast, a visual element is more slowly memorised, this requiring an initial latency period of 35 h. If it is removed, after a latency period of 30 h, it is slowly and incompletely forgotten in 4.5 d. Ants permanently retain 10 - 20% of their visual performance. This explains the navigation system of *M. sabuleti* revealed by CAMMAERTS & RACHIDI (2009).

Second, the "day" and "night" experiments

pointed out clear and different circadian rhythms for olfactory and visual abilities: olfaction works better at night, vision at day.

The experiments involving the simultaneous training with several olfactory or visual cues lead to the following interpretation. The ants' performance in responding to learned odours decreased linearly with the number of odours presented. Each odour is probably memorised individually. In contrast, the ants' response to learned visual cues geometrically decreased. Several visual cues are probably memorised globally, each cue being a part of the ants' visually memorised environment.

Concerning olfaction, the fact that the ants soon stop responding to a no longer presented odour agrees with the fact that, in mazes, they soon stop responding to obsolete odours (CAMMAERTS & RACHIDI, 2009). This is useful since, on field, an odour can change or quickly disappear. But an odour can be used, for a time, to travel as shown by our previous work on *M. sabuleti* and as demonstrated by STECK *et al.* (2009) in *Cataglyphis fortis*.

Concerning vision, the 6-d training period necessary to acquire an optimal score had been presumed in earlier studies on ant visual perception (f.i. CAMMAERTS, 2004a, 2005b, 2007a, 2007b, 2008). I then estimated that ants must be tested after a 6- and a 9-d period of conditioning, and not earlier.

The latency period following visual cue removal explains the fact that, in mazes provided with obsolete visual cues, ants continued responding to these no longer valid cues (CAMMAERTS & RACHIDI, 2009). Such 'fidelity' is in agreement with the fact that, on field, visual elements are likely to persist unchanged. Furthermore, significant responses to visual cues no longer seen during months may explain the often observed route fidelity in ants (ROSENGREN, 1971, SALO & ROSENGREN, 2001).

The global memorisation of several cues agrees with previous results on visual perception of different numbers of elements. These different numbers were accurately perceived, but assessed globally, without being actually counted (CAMMAERTS, 2008).

Concerning both olfactory and visual learning, *M. sabuleti* workers forgot their entire olfactory response in 3.5 d as well as 10% of their visual score in about 4 d but retain 10%. ROSENGREN (1971), NARENDRA *et al.* (2007) and ZIEGLER & WEHNER (1997) revealed a stronger memory in

ants but, in those studies, the ants had been trained to a pool of many cues for weeks or even months.

The probable different mechanisms underlying ants' olfactory and visual learning have been here revealed at a collective level. Long lasting individual marking, individual training and testing should be undertaken for elucidating this problem at an individual level.

#### *Ants versus other insects*

Our results are in agreement with what is known about bee learning. These hymenopterans quickly learn odours, but more slowly learn colours. Individually trained, they reach a score of 100% correct responses for odours and 90% for colours. Moreover, in bees, it is easy to change the learning from one odour to another, but it is difficult to do so with colours, shapes and landmarks (GOULD, 1982 and references therein). Learning in bees (essentially olfactory learning) has been largely studied by GIURFA and collaborators (f.i. GIURFA, 2007 and references therein).

In bees, the steps for odour learning are probably simple because the axons coming from the antennae are rather directly connected to the appropriate alpha lobe. In contrast, the steps for visual learning may be more complex. The information collected by the eyes has a more complicated route to the appropriate zones of the brain (GOULD, 1982).

#### *Circadian rhythms*

The two discovered circadian rhythms reflect the fact that: vision is better during the day and is then indeed more useful, whereas olfaction works better at early night, when it may provide more information than vision. On the other hand, the observed visual circadian rhythm contradicts the known adaptation of ant eyes to light intensity during the course of the day. The visual threshold changes with light intensity, being higher under high light intensity and lower under low light intensity, both for overall light (CAMMAERTS, 2005b) and for different colours (CAMMAERTS & CAMMAERTS, 2009). The circadian rhythms we discovered are therefore not those of the sensorial organs (eyes, antennae) but rather those of particular parts of the brain. They were not acquired during the life of the animals because the tested ants were born from queens themselves born in the laboratory. These queens and tested ants were continuously maintained in a room without windows, joined with another windowless

room. The temperature, light and humidity were constant. Thus, it seems that the tested ants, or more precisely parts of their brain present innate circadian rhythms.

Circadian rhythms have been studied in many invertebrates and vertebrates (LANIER, 1999). They affect molecular, cellular, physiological and behavioural levels (MENAKER *et al.*, 1997). Chemical and genetic factors have been evoked to explain them. Genetic studies on *Drosophila melanogaster* show that several genes are involved in the underlying mechanisms. Circadian rhythms appear to be controlled by rhythmic expression of clock genes in specific brain neurons (LEE *et al.*, 1996). Clock genes have also been observed outside the nervous system (WEBER, 1995). The present study reveals the possible existence of different circadian rhythms in two different peripheral zones of an ant's brain, the olfactory and the optic lobes. This supports recent approaches to the subject (GIELBULTOWICZ, 2000; MASANORI & YOSSHIHIKO, 2008; DOLEZEL *et al.* 2008) and echoes those obtained by DECKER *et al.* (2007), who used differential conditioning to study the acquisition of olfactory memory in the cockroach *Leucophaea maderae*. That insect's ability is regulated by a circadian system where learning is better at early night and deficits in memory performances occur at other circadian phases.

#### **Conclusion**

The present work defines the dynamics of the acquisition and the loss of *M. sabuleti* foragers' olfactory and visual conditioning, their collective performances, their probable system for reaching such performance and the circadian rhythms of their olfactory and visual learning abilities. It points out the mechanisms underlying the previously found fact that *M. sabuleti* foragers primarily rely on odours and only secondarily on visual cues to navigate.

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