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Olfactive conditioning and use of visual and odorous cues for movement in the ant *Myrmica sabuleti* (Hymenoptera: Formicidae)

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Abstract

Olfactive conditioning was achieved in the ant *Myrmica sabuleti* MEINERT, 1861 using either meat or sugared water as a reward ($P < 0.016$). This conditioning was superior using meat as a reward as had been the case in visual operant conditioning previously obtained in that species. Differential olfactive conditioning was also achieved in *M. sabuleti* using sugared water as a reward ($0.031 < P < 0.016$). This result suggests that these ants may use odours as cues to negotiate their way. Experiments were thus conducted in mazes provided with learned odours: the ants could negotiate their way ($P < 0.001$). They could also do so with alternative presence of visual and odorous cues ($P < 0.001$). Ants were then confronted with visual and odorous cues either in agreement or competitively presented. *Myrmica sabuleti* workers responded primarily to learned odours and secondarily to learned visual cues (correct odours + wrong visual cues: $P < 0.001$, wrong odours + correct visual cues: $P < 0.01$); the result for wrong odours + correct visual cues was still significant because the ants appeared to rapidly stop responding to learned odours when these elements fail to help them navigate.

Key words: *Myrmica sabuleti*, navigation, olfactive conditioning, orientation.

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Introduction

Ants use several strategies to navigate while foraging and returning to their nest. They gauge the distances they walk using odometry (CAMMAERTS 2005). They can memorise some of their locomotion reactions (MACQUART & BEUGNON 2006) and sequences of turns made while walking to the food (REZNIKOVA & RYABKO 1994). They recognise and use their own odorous area markings (CAMMAERTS & CAMMAERTS 1998, 1999, 2001). They can see and use distant elements, as those located in the canopy (SALO 1998) as well as celestial cues (ROSENGREN 1971). Ants (like bees) can also make path integration for coming back to their nest (RONACHER & WEHNER 1995). However, based on the literature, ants essentially use visual landmarks, i.e., cues present along their paths. This ability has been the focus of study in recent years (PASSERA & ARON 2005) and we have also conducted experiments on the subject using *Myrmica sabuleti* MEINERT, 1861 as a model (CAMMAERTS & LAMBERT 2008). The latter work shows that *M. sabuleti* foragers used memorised cues to negotiate their way, combining the "snapshot" and the "sketchmap" models previously elaborated by CARTWRIGHT & COLLETT (1982) and BEUGNON & al. (1996), respectively, and briefly discussed in PASSERA & ARON (2005). But foragers might also use odorous elements present in their environment to find their way. PASSERA & ARON (2005), reviewing ant orientation systems, refer to odours as minor stimuli. Indeed, the use of odours for orientation has received scant attention.

We therefore investigate this issue, using the ant *M. sabuleti* as a model. Specifically, we tested, in mazes, workers of this species that had previously been conditioned to odours. The first step was to attempt olfactive conditioning. This was followed by differential olfactive conditioning in *M. sabuleti* workers using two differently odorous elements. Olfactive conditioning can be obtained in many animals, among others bees and ants (PEARCE 2008). In bees, classical olfactive conditioning has been achieved since a long time: such a conditioning with the "proboscis extension" response is now a paradigm. Operant conditioning is also common in bees, using artificial flowers. All this has already been largely used for research purposes (BITTERMAN & al. 1983, LALOI & al. 1999, DECOURTYE & al. 2003 and references therein). In ants, conditioning experiments are more recent but have also successfully been conducted (PASSERA & ARON 2005, REZNIKOVA 2007 and references therein). Recently, classical olfactive conditioning has been achieved (DUPUY & al. 2006, ESCHBACH & al. 2006, GUERRIERI & al. 2007). Such a conditioning differs from those occurring in the field, which are rather operant ones. In the present work, we did not use classical conditioning but a more natural form of conditioning, resembling operant conditioning. On the species here studied, *M. sabuleti*, visual classical conditioning (CAMMAERTS 2004b), temporal and spatial learning (CAMMAERTS 2004b), spatial conditioning (CAMMAERTS 2004c) (but not yet spatio-temporal learning) as well as visual operant conditioning (CAMMAERTS 2004d) have been obtained. They have been used as a method for several studies on this ant species' visual perception (CAMMAERTS 2004a, 2007, 2008). A comparison between these efforts and the presently reported results is made in the Conclusion and Discussion section.

The second step of the present work consisted in testing ants conditioned to two odorous elements in mazes provided with these elements. Whether the ants used the odour or the sight of these elements was evaluated. Thereafter, the ants were tested in mazes provided with learned odorous sources as well as with learned visual cues. These two elements were differently assembled to determine their relative importance for travelling ants.

This experimental work yields valuable insight into how *M. sabuleti* workers use odorous and visual cues to navigate. This has physiological and ecological implications which will be evoked in the Conclusion and Discussion section.

Material and methods (Figs. 1 - 3)

Collection and maintenance of ants

Olfactive conditioning was tried on six small colonies collected in spring, at Olloy / Viroin (Belgium), each containing a queen, brood and about 250 workers. Differential olfactive conditioning as well as negotiation of a maze provided with cues were studied using four large colonies collected, in spring, at Höhes Martelingen (Grand-Duché de Luxembourg) and two collected, in spring, in Lorraine (France). The ants nested in old quarries, on particular areas with a few stones, nearly no tree and numerous short plants. Each of these six colonies contained one or two queens, brood and about 500 workers. Each experimental colony was maintained in the laboratory, nesting in one to three glass tubes half-filled with water, a cotton-plug separating the ants from the water. The glass tubes were deposited in a tray (28 cm × 43 cm × 7 cm for the smaller colonies; 37 cm × 52 cm × 8 cm for the larger ones) whose borders were covered with talc to prevent escape. The trays served as foraging areas: food was delivered in them, and ants were trained as well as tested using apparatus placed on their bottom (Fig. 3a).

Temperature was maintained at 20°C ± 2°C; humidity was 60% to 80% and constant over the course of an experiment. During the training phases, the lighting, delivered by five OSRAM concentra 60 W lamps, had a constant intensity of 600 lux. While working on ants (e.g., relocating their training apparatus, testing them), the lighting had a constant intensity of 10,000 lux.

Food, delivered twice a week, consisted of sugared water offered in a small glass tube plugged with cotton and of pieces of dead cockroaches deposited on a glass slide. One and / or the other of these food items was withheld four days before an experiment and was not provided during experiments because it served as a reward for training the ants.

Experimental apparatus

To achieve olfactive conditioning as well as differential olfactive conditioning, experimental apparatus were used for training the ants and other identical ones for testing them.

The experimental apparatus used to conduct olfactive conditioning consisted of a glass slide (7.6 cm × 2.6 cm) deposited on the ants' foraging area. During the training phases, a piece of either onion or thyme was set on the two extremities of the glass slide and a piece of dead cockroach (in the presence of onion) or some cotton imbibed with sugared water (in the presence of thyme) was laid in its middle. New glass slides were used for testing the ants: these slides were provided only with either onion or thyme (i.e., no meat or cotton with sugared water).

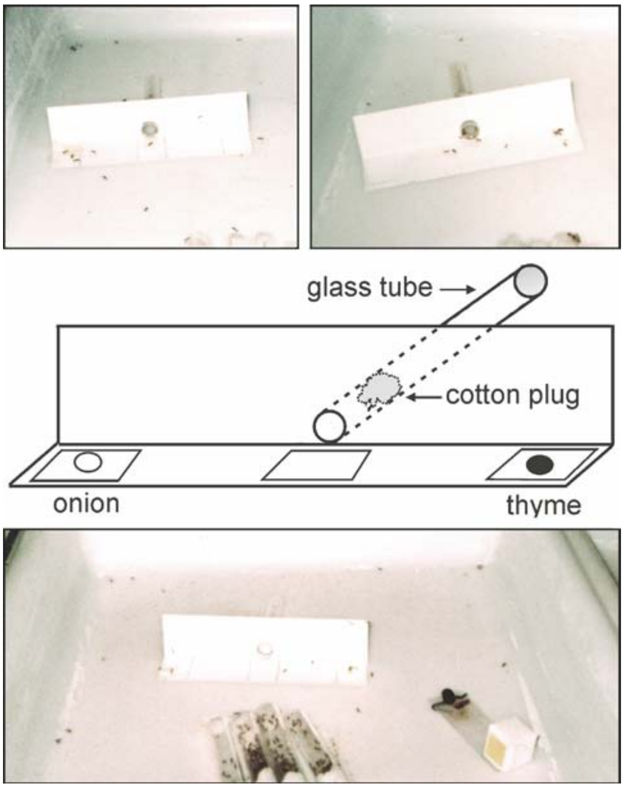

Fig. 1: Olfactive differential conditioning to onion and to thyme, in the ant *Myrmica sabuleti*. Twelve experimental apparatus, as that drawn in the middle of the figure, were built in strong white paper, six for training the ants, six for testing them. To train the ants, either onion or thyme was deposited and the glass tube was filled with sugared water (upper photos). To test the ants, either onion or thyme or the two odorous elements (lower photo) were deposited and the glass tube was empty but still plugged with cotton.

The experimental apparatus used to conduct differential olfactive conditioning consisted of a piece of paper (white extra-strong (Steinbach ®), 6 cm × 14 cm) orthogonally folded along its longest median. It presented consequently a horizontal and a vertical part. A thin glass slide cover slip (2.2 cm × 2.2 cm) was deposited on the two extremities as well as on the middle of the horizontal part. A hole (diameter: 1.2 cm) into which a small glass tube (length: 7 cm; diameter: 1 cm) could be inserted was drilled in the middle of the vertical part very near the longest median (Fig. 1, in the middle). When set into the apparatus, the small glass tube therefore came to lie on the foraging area (Fig. 1). During the training phases, a piece of onion and one of thyme was alternatively deposited on the thin glass slide located on the left and on the right of the experimental apparatus, respectively. At the same time, the small glass tube was filled with sugared water and plugged with cotton (Fig. 1, upper photos). Note that the thin glass slide located in the middle of the horizontal part of the apparatus was positioned directly in front of the opening of the glass tube delivering sugared water. Another identical set of experimental apparatus were used to test the ants. They were provided with onion or thyme or the two odorous elements, depending on the experiment. Here, the small glass tubes never contained sugared water: they were empty but plugged with cotton to prevent entry (Fig. 1, lower photo).

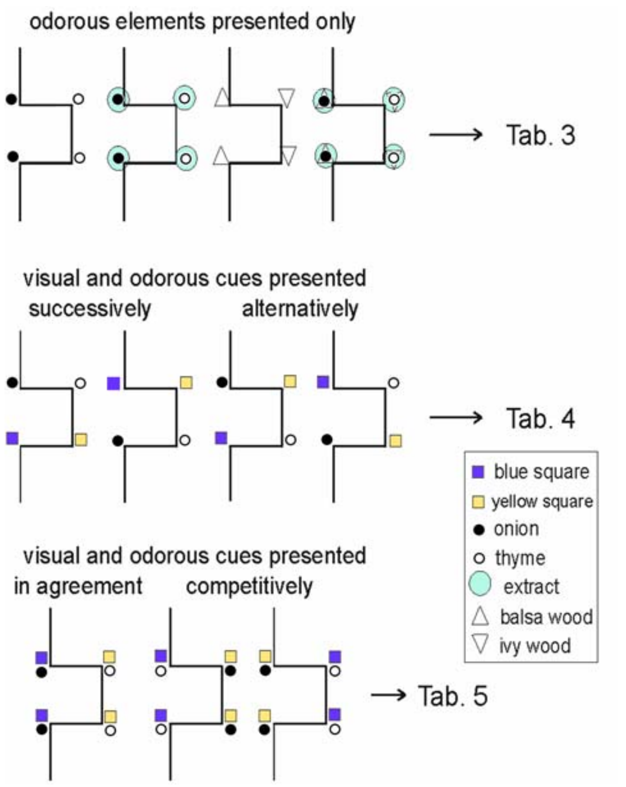

Fig. 2: Cues arrangements in mazes employed for studying the use of odours and visual cues by *Myrmica sabuleti* workers to negotiate their way. The building of a maze is detailed in Cammaerts & Lambert (2008). Such mazes can be seen in Figure 3. In mazes, ants met four points of choices provided with one or two cues as follows. Line 1 of the figure, odorous cues only; from the left to the right: odorous cues only; onion and thyme, aqueous extract of onion and thyme, balsa and ivy wood, balsa and ivy wood imbibed with aqueous extracts of onion and thyme. Line 2, visual or odorous cues at the 4 points of choice; from the left to the right: blue, yellow, thyme, onion; onion, thyme, yellow, blue; blue, thyme, yellow, onion; onion, yellow, thyme, blue. Line 3, visual and odorous cues together at the 4 points of choices; from the left to the right: blue + onion, yellow + thyme, yellow + thyme, blue + onion (all is correct); blue + thyme, yellow + onion, yellow + onion, blue + thyme (visual cues are correct); yellow + onion, blue + thyme, blue + thyme, yellow + onion (odorous cues are correct).

To study the use of odours as cues, the ants of each colony were trained using, at the same time, two different experimental apparatus (Fig. 3, at the border of the ants' tray). One consisted of a folded piece of paper as described above and allowed differential olfactive conditioning to onion and thyme. The other was the type of cube previously used for studying maze negotiation by *M. sabuleti* workers (Cammaerts & Lambert 2008). It consisted of a glass slide on which were laid, on one extremity, a small dead cockroach, and on the other a cube (2 cm × 2 cm × 2 cm) made of white extra-strong paper (Steinbach ®) provided with a blue and a yellow square (1.5 cm × 1.5 cm; strong coloured paper Canson ®) on two opposite faces. The spectra of light reflected by the blue and the yellow paper are given in Cammaerts (2007). During the present study, the ants of each colony were tested in separate (i.e., each colony had its own maze) mazes (having their own bottom) identical to those previously used (Cammaerts & Lambert 2008), but otherwise provided with cues (Fig. 3, b - g). Briefly, the floor width and wall height of such mazes was 2 cm; the distance between two turns (points of choice for the ants) was 4 cm; there was no ceiling to allow the experimenter a good view and for sufficient aeration; the walls were covered with talc to avoid ants' escape. *Myrmica sabuleti* workers lay a trail only after having found food; so, they did not do so when tested in a maze. They might deposit some amount of their Dufour gland content, this giving no information at all as for which direction is correct. However, a sheet of white paper of appropriate shape and dimensions was deposited on the bottom of each maze and was renewed after each experiment. The cues set inside the mazes differed between the experiments. They are presented in Figure 2 and will be detailed below. A few other technical details (e.g., aqueous extracts, use of balsa and ivy) will also be given then.

Experimental protocol

To achieve olfactive conditioning as well as differential olfactive conditioning, we conducted, successively, a control, a 10-day period of training, a first test, a 5-day period of training and a second test.

To train the ants, the appropriate experimental apparatus were deposited on the foraging area of the six used colonies. They were relocated 10 (olfactive conditioning) or 20 (differential olfactive conditioning) times in the course of the first training phase as well as 5 (olfactive conditioning) or 10 (differential olfactive conditioning) times during the second training phase. These relocations helped avoid spatial learning (Cammaerts 2004b) and the establishment of chemical trails (Cammaerts & Cammaerts 1980). Each time, the onion or the thyme was renewed (olfactive conditioning) or was replaced by thyme and onion, respectively (differential conditioning). The reward (piece of cockroach or sugared water) was then renewed if necessary. These reinforcing manipulations were performed every 5 to 25 h, avoiding 12-h and 24-h intervals because ants can acquire temporal learning (Cammaerts 2004b).

To perform the control and each of the two tests, the experimental apparatus used to train the ants were removed from the foraging areas and those designed for tests (those without reward) were presented to the ants. Ant reactions were then quantified as explained below.

For studying the use of visual and odorous elements as cues, we successively performed, on the six used colonies, a control, a 3-day period of training (brief because the ants had just previously been conditioned), a test, and for the first series of experiments, again a 3-day period of training and a second test.

To train the ants, we proceeded exactly as explained here above: we presented to the ants of each colony an apparatus allowing differential olfactive conditioning as well as a cubic experimental apparatus allowing differential visual conditioning. We relocated these apparatus several times and renewed the meat and sugared water if necessary, again avoiding a 12-h or 24-h interval.

To perform a control or a test on one of the six used colonies, the two experimental apparatus designed for training were removed from the foraging area and a maze, provided with appropriate cues, was placed into this area. Ten

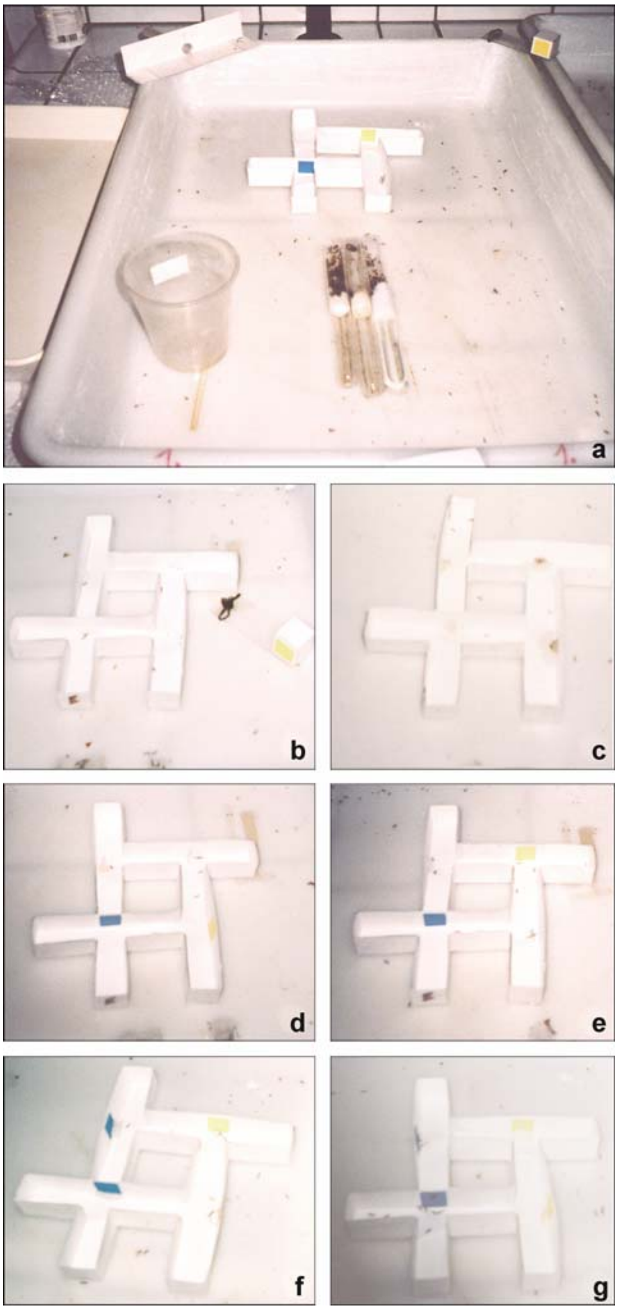

Fig. 3: Some views of the experiments made in mazes. (a) Experimental design; the apparatus used for training the ants can be seen on the borders of the ants' tray; the glass into which ants were isolated after having been tested can be seen in the ants' tray; a maze is lying in the ants' tray. The building of such a maze is defined in Cammaerts & Lambert (2008). (b - g) Tests in mazes provided with (b) odorous elements only; (c) extracts of these elements; (d) visual and odorous cues successively presented; (e) visual and odorous cues alternatively presented; (f) visual and odorous cues presented in agreement; (g) visual and odorous cues competitively presented: visual cues are correct, odorous cues are wrong.

ants were allowed, one by one, to enter the maze or were gently deposited into it near its entrance. Each ant's movement was then observed and quantified as detailed below. After having moved all along the maze, each tested ant was allowed to exit the maze or was gently removed. It was briefly isolated in a polyacetate glass whose borders had been covered with talc in order to avoid testing the same individual twice during the same experiment.

Quantification of ant reactions

Note again that each experiment was simultaneously performed on six colonies.

Olfactive conditioning: the ants on the glass-slide were counted 15 times for each of the 6 tested colonies and the 6 mean values were calculated. The mean of the 6 mean values was also established for the control and each of the two tests (Tab. 1).

Differential olfactive conditioning: the same counts and calculations as above were made, but successively (a) in the presence of onion, on the left-half area of the apparatus; (b) in the presence of thyme, on the right-half area; (c) in the presence of onion and thyme, on the entire area (Fig. 1, Tab. 2). If the ants counted on the entire area were as numerous as the sum of those counted on each of the two halves, then the ants would not have acquired differential conditioning. Some of them would have been conditioned only to go to the right in the presence of onion and other ones to go to the left in the presence of thyme. On the contrary, if the ants counted on the whole area were less numerous than the sum of the two other counts, then differential conditioning has been acquired.

Use of visual and odorous elements as cues: the quantification of movement through the maze was identical to that for studying the negotiation of a maze provided with visual cues (Cammaerts & Lambert 2008). Briefly, for each point of choice travelled by each of the 10 tested ants during one experiment made on one colony, it was noted if the ants made an incorrect (= 0) or a correct (= 1) choice. We took into account only the first ants' response at each point of choice and not their possible second, seldom third ones occurring when the ants turned back on their way along more than 4 cm. This quantification yielded, for each colony, a total number of correct choices among the 40 potential ones, and for the six colonies among the 240 potential ones (Tabs. 3 - 5). The numbers of ants having made 0, 1, 2, 3, and 4 correct choices were also established for each colony as well as for the six colonies on which the same experiment was conducted (Tabs. 3 - 5). For a few experiments, the total number of correct choices was also separately calculated either for the visual and the odorous cues (Tab. 4) or for the first, the second, the third and the fourth point of choice in the maze (Tab. 5).

Statistical analysis

For each of the two kinds of olfactive conditioning trials, the 6 mean numbers of counted ants obtained for each test were compared to the corresponding 6 control mean numbers using the non-parametric test of Wilcoxon (Siegel & Castellan 1988). The tables give the values of N, T and P according to the nomenclature of the latter authors.

As for the use of visual and odorous elements as cues, for each test experiment, the distribution of the numbers of ants having made 0, 1, 2, 3, and 4 correct choices was compared to the corresponding control numbers of ants using the non-parametric χ^2 test. The choices observed for an experiment were considered as being not statistically differ-

Tab. 1: Olfactive conditioning of *Myrmica sabuleti* workers to onion using meat as a reward and to thyme using sugared water as a reward. The table gives (1) the mean numbers of ants present on the experimental apparatus during the control and each of the two tests, (2) the mean values of the previous mean numbers, (3) the results of non-parametric Wilcoxon tests between 6 test mean numbers and the 6 corresponding control mean numbers. N, T, P are used according to the nomenclature of SIEGEL & CASTELLAN (1988).

Olfactive stimuli	**(1) Mean numbers of ants counted for colony**						**(2) Mean**	**(3) Statistics**		
Experiments	**1**	**2**	**3**	**4**	**5**	**6**	**values**	**N**	**T**	**P**
onion										
control	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
test 1	0.67	1.00	0.67	0.40	1.47	0.67	0.81	6	21	0.016
test 2	0.93	1.00	0.67	0.60	1.67	1.33	1.03	6	21	0.016
thyme										
control	0.00	0.33	0.00	0.00	0.22	0.00	0.09			
test 1	1.33	0.87	0.73	0.33	0.40	1.07	0.79	6	21	0.016
test 2	1.33	1.13	0.40	0.40	0.53	0.46	0.71	6	21	0.016

Tab. 2: Differential olfactive conditioning to onion and to thyme using sugared water as a reward. The ants had to go to the right in the presence of onion and to the left in the presence of thyme. The table gives, for the control and the two tests, (1) the mean numbers of ants present on the left part of the apparatus in the presence of onion, on the right part in the presence of thyme and on the whole area in the simultaneous presence of the two odorous stimuli, (2) the mean values of the previous mean numbers, (3) the results of non-parametric Wilcoxon tests performed between 6 test mean numbers and the corresponding 6 control mean numbers. N, T, P are used according to the nomenclature of SIEGEL & CASTELLAN (1988).

Experiments	**(1) Mean numbers of ants counted for colony**						**(2) Mean**	**(3) Statistics**		
Olfactive stimuli	**1**	**2**	**3**	**4**	**5**	**6**	**values**	**N**	**T**	**P**
control										
onion	0.00	0.00	0.07	0.00	0.00	0.00	0.01			
thyme	0.00	0.00	0.07	0.00	0.00	0.00	0.01			
onion + thyme	0.00	0.00	0.07	0.00	0.00	0.00	0.01			
test 1										
onion	0.13	2.47	0.33	0.40	4.40	0.53	1.38	6	21	0.016
thyme	0.00	0.07	1.20	0.27	6.70	0.13	1.40	5	15	0.031
onion + thyme	0.07	1.33	1.53	0.13	6.20	0.67	1.66	6	21	0.016
test 2										
onion	0.00	1.13	1.93	0.73	2.40	0.27	1.08	5	15	0.031
thyme	0.00	0.07	1.60	0.20	1.87	1.00	0.79	5	15	0.031
onion + thyme	0.00	0.07	1.47	0.87	3.95	0.95	1.21	5	15	0.031

ent from the choices obtained during the control experiment when $P > 0.05$.

Results

Olfactive conditioning (Tab. 1)

Both the numerical and the statistical results demonstrate that such conditioning was achieved in *M. sabuleti* workers. The results were better for onion (obtained using meat as a reward) than for thyme (obtained using sugared water as a reward). Ants that acquired olfactive operant conditioning responded rapidly to the learned odour (came onto the apparatus as soon as the odour was presented) but did not stay in the vicinity of that odour and soon moved away from it.

Differential olfactive conditioning (Fig. 1, Tab. 2)

The numerical and statistical results demonstrate that *M. sabuleti* workers were conditioned (a) to approach and to go to the right in the presence of onion; (b) to approach and to go to the left in the presence of thyme; (c) to approach and to go to the right in presence of onion as well as to the left in the presence of thyme. Indeed, the ants responding to the simultaneous presence of the two odorous elements were less numerous than the sum of those responding to each of these two elements separately presented. Nevertheless, they were somewhat more numerous than those responding to either one or the other of the two odorous elements. This was logical because the ants were then doubly stimulated. We effectively observed that, after having re-

Tab. 3: Response of *Myrmica sabuleti* workers in empty mazes (control), in mazes provided with odorous elements (two tests), provided with aqueous extracts of these elements, provided with non-odorous elements (pieces of balsa and ivy wood), provided with the latter elements imbibed with the aqueous extracts. Each experiment was made on 6 colonies; the quantification of the ants' response is detailed in the text. P: results of non-parametric χ^2 tests made between (4th column in bold) the distribution of experimental values and the control distribution. NS: non-significant difference between the two distributions.

Experiments	**Colony**	**Nr. of correct choices**	**Nr. of ants having made**					**P**
			0	**1**	**2**	**3**	**4**	
			correct choices					
control	1	08/40	4	5	0	1	0	
	2	11/40	3	4	2	1	0	
	3	13/40	2	4	3	1	0	
	4	10/40	4	3	2	1	0	
	5	11/40	3	4	2	1	0	
	6	11/40	3	4	2	1	0	
	total	**64/240**	**19**	**24**	**11**	**6**	**0**	
with odorous elements, first test	1	32/40	0	0	3	2	5	< 0.001
	2	33/40	0	0	1	5	4	
	3	33/40	0	0	1	5	4	
	4	31/40	0	0	2	5	3	
	5	33/40	0	0	1	5	4	
	6	30/40	0	0	3	4	3	
	total	**192/240**	**0**	**0**	**11**	**26**	**23**	
idem, second test	**total**	**176/240**	**0**	**3**	**14**	**27**	**16**	< 0.001
with aqueous extracts of the odorous elements	**total**	**187/240**	**0**	**1**	**11**	**28**	**20**	< 0.001
with non-odorous elements	1	12/40	3	3	3	1	0	NS
	2	11/40	3	4	2	1	0	
	3	11/40	2	6	1	1	0	
	4	09/40	3	5	2	0	0	
	5	11/40	4	2	3	1	0	
	6	10/40	4	4	0	2	0	
	total	**64/240**	**19**	**24**	**11**	**6**	**0**	
with non-odorous elements imbibed with the extracts	**total**	**169/240**	**0**	**4**	**15**	**29**	**12**	< 0.001

sponded to one odour and moving away from it, they generally again perceived the same or the other odour and so returned to the experimental apparatus. The same ant could thus be counted several times. This evidently occurred more frequently in the presence of the two odours than in the presence of only one.

Briefly, this experiment proved that *M. sabuleti* workers could acquire differential olfactive conditioning.

The numerical results of the second test were somewhat lower than those of the first test because the ants' motivation differed between the two: the first test was performed four days after having provided meat to the ants, while the second one took place only two days after such a food delivery.

Use of visual and odorous elements for movement (Fig. 3a)

Odorous elements presented only (Fig. 2, 1st line, Tab. 3). When pieces of onion and thyme were appropriately set into the mazes, the ants correctly negotiated these apparatus (Fig. 3b). The results were statistically significant ($P < 0.001$) in the two successive tests.

Although they never had the same shape and dimension during the training phases and the tests, the odorous elements are recognisable pieces of plants. We therefore repeated the maze experiment with aqueous extracts of these plants. Pieces of onion (or of thyme) were placed in hot water (90°C) for 15 min. Cotton balls were dipped into one or the other aqueous solution and were used to appropriately mark the mazes. Tests identical to the previous ones were then performed. The ants correctly negotiated such mazes (Fig. 3c) (statistically significant at $P < 0.001$).

When pieces of two non-odorous (or differently odorous) materials (small pieces of balsa and of ivy wood) were set into the mazes instead of onion and thyme, the ants could no longer correctly solve the task. The global numerical results were identical to the control ones (i.e., non-significant); detailed results (that is those of each experi-

Tab. 4: Response of *Myrmica sabuleti* workers in mazes provided with odorous elements and visual cues, these two elements being differently assembled as shown in Fig. 2, 2nd line. Each experiment was made on 6 colonies; the quantification of the ants' response is detailed in the text. P: results of non-parametric χ^2 tests made between the distribution of experimental values (4th column in bold) and the control distribution (Tab. 3). V, O, S: number of correct choices in front of the visual cues, the odorous ones, all (both) elements, respectively.

Experiments	**Colony**	**Nr. of correct choices**			**Nr. of ants having made**					**P**
		V	**O**	**S**	**0**	**1**	**2**	**3**	**4**	
					correct choices					
the two visual cues preceded the two odorous elements	1	16/20	18/20	34/40	0	0	1	4	5	< 0.001
	2	16/20	15/31	31/40	0	0	3	3	4	
	3	15/20	18/20	33/40	0	0	1	5	4	
	4	15/20	17/31	32/40	0	0	1	6	3	
	5	15/20	18/20	33/40	0	0	1	5	4	
	6	14/20	17/31	31/40	0	1	1	4	4	
	total	**91/120**	**103/120**	**194/240**	**0**	**1**	**8**	**27**	**24**	
the inverse	**total**	**85/120**	**106/120**	**191/240**	**0**	**1**	**10**	**26**	**23**	< 0.001
twice a visual cue preceded an odorous element	1	14/20	17/20	31/40	0	1	1	4	4	< 0.001
	2	14/20	17/31	31/40	0	1	0	6	3	
	3	14/20	17/20	31/40	0	0	2	5	3	
	4	14/20	17/31	31/40	0	0	1	7	2	
	5	14/20	16/20	30/40	0	1	1	5	3	
	6	15/20	19/31	34/40	0	0	1	4	5	
	total	**85/120**	**103/120**	**188/240**	**0**	**3**	**6**	**31**	**20**	
the inverse	**total**	**87/120**	**100/120**	**187/240**	**0**	**6**	**6**	**23**	**25**	< 0.001

ment) are given for comparative purposes (Tab. 3, non-odorous plants).

When pieces of balsa wood imbibed with extract of onion and pieces of ivy wood imbibed with extract of thyme were appropriately deposited in mazes, the ants were again able to find their way rather well ($P < 0.01$). Note that wood is not absorbent and consequently did not retain large amounts of extracts, potentially explaining the relatively weak score.

These various approaches demonstrate the use of odours by *M. sabuleti* workers for negotiating their way. These results occurred in the absence of visual cues. Therefore, additional experiments simultaneously employing both visual and odorous elements were undertaken.

Visual and odorous elements successively or alternatively presented (Fig. 2, 2nd line, Tab. 4). *Myrmica sabuleti* workers correctly negotiated mazes provided with successively (Fig. 3d) or alternatively (Fig. 3e) presented visual and odorous cues (Tab. 4). The results of each of the four experiments were statistically different from the control ones ($P < 0.001$).

Although not identical (194 and 191 vs. 188 and 187), the results of the two experiments involving successive presentation of the visual and the odorous elements did not statistically differ from those of the two experiments using alternative presentation ($0.10 < P < 0.20$).

On the other hand, the ants made fewer correct choices in front of the visual elements (91, 85, 85, 87) than when perceiving odorous elements (103, 106, 103, 100). This different response to visual and odorous cues was analysed based on the following experiments.

Visual and odorous elements presented in agreement or competitively (Fig. 2, 3rd line, Tab. 5). When the encountered visual and odorous elements were in agreement, the ants correctly travelled through the maze (Fig. 3f) ($P < 0.001$). Moreover, they were more successful (higher score) than when encountering only odorous elements (statistical comparison: df = 2; $\chi^2 = 7.549$; $0.02 < P < 0.05$). Thus, even if the previous experiment indicated that odorous elements were more important than visual cues, the visual cues apparently did help the ants to find their way.

When visual cues were correctly set in the mazes while odorous cues were incorrectly deposited (Fig. 3g), the ants' scores were lower (93 / 240) and statistically less significant ($0.001 < P < 0.01$) than when only odorous elements were set (192 / 240; 176 / 240; $P < 001$). Surprisingly, the scores increased from the first to the fourth point of choice (14, 18, 28, 33 correct choices at the first, the second, the third, and the fourth point of choice, respectively). The ants thus soon stopped responding to the incorrect odorous elements and went on responding to the correct visual ones. When the correct cues set in the mazes were the odorous ones, the ants were highly significantly ($P < 0.001$) successful. Nonetheless, their scores (151 / 240) were lower than when visual cues were also correctly presented (199 / 240). During the experiments with correct odorous elements and incorrect visual cues, the ants did not increase their score from the first to the fourth point of choice (39, 36, 35, 41 at the first, the second, the third, and the fourth point of choice, respectively). They continued to be influenced by the incorrect visual cues.

Tab. 5: Response of *Myrmica sabuleti* workers in mazes provided, at each point of choice, with A: correct visual and odorous cues, B: correct visual cues and wrong odorous ones, C: wrong visual cues and correct odorous ones. Each experiment was made on 6 colonies; the quantification of the ants' response is detailed in the text. P: results of non-parametric χ^2 tests made between the distribution of experimental values (4th column in bold) and the control distribution (Tab. 3). S, 1, 2, 3, 4: number of correct choices at all the points of choice and at the point 1, 2, 3, and 4, respectively.

Experiment	**Colony**	**Nr. of correct choices at point(s)**					**Nr. of ants having made correct choices**					**P**
		S	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	
A	1	35/40	9/10	9/10	9/10	8/10	0	0	0	5	5	< 0.001
	2	36/40	9/10	9/10	9/10	9/10	0	0	0	4	6	
	3	34/40	10/10	9/10	8/10	7/10	0	0	0	6	4	
	4	32/40	8/10	8/10	7/10	9/10	0	0	2	4	4	
	5	32/40	8/10	8/10	7/10	9/10	0	0	0	8	2	
	6	30/40	7/10	8/10	7/10	8/10	0	1	1	5	3	
	total	**199/240**	**51/60**	**51/60**	**47/60**	**50/60**	**0**	**1**	**3**	**32**	**24**	
B	1	17/40	3/10	3/10	5/10	6/10	1	3	5	0	1	< 0.01
	2	15/40	2/10	3/10	5/10	5/10	1	4	4	1	0	
	3	15/40	2/10	3/10	5/10	5/10	0	5	5	0	0	
	4	14/40	2/10	3/10	3/10	6/10	3	1	5	1	0	
	5	16/40	3/10	3/10	5/10	5/10	2	2	4	2	0	
	6	14/40	2/10	3/10	5/10	6/10	0	5	4	1	0	
	total	**93/240**	**14/60**	**18/60**	**28/60**	**33/60**	**7**	**20**	**27**	**5**	**1**	
C	1	24/40	6/10	6/10	5/10	7/10	0	3	1	5	1	< 0.001
	2	26/40	6/10	6/10	6/10	8/10	0	1	5	1	3	
	3	27/40	7/10	6/10	6/10	8/10	0	2	1	5	2	
	4	24/40	6/10	6/10	6/10	6/10	0	1	4	5	0	
	5	25/40	7/10	6/10	6/10	6/10	0	1	4	4	1	
	6	25/40	7/10	6/10	6/10	6/10	0	1	4	4	1	
	total	**151/240**	**39/60**	**36/60**	**35/60**	**41/60**	**0**	**9**	**19**	**24**	**8**	

Though ants soon stopped responding to incorrect odorous elements but did not do so for incorrect visual cues, their score in mazes provided with correct visual cues and incorrect odorous elements were lower than scores in mazes provided with incorrect visual cues and correct odorous elements (93 / 240 vs. 151 / 240; df = 3; $\chi^2 = 30.36$; $P < 0.001$).

Consequently, *M. sabuleti* workers primarily respond to odorous elements to find their way, but also secondarily use visual cues. At the same time, they are able to neglect no longer valid odours but continue taking into account, for a time, outdated visual cues. The physiological and ecological implications of this behaviour are commented upon in the Conclusion and discussion section.

Conclusion and discussion

The present work yielded four sets of information. *Myrmica sabuleti* foragers can associate simple locomotor reactions to (= can be conditioned to) odorous elements. They use learned odorous elements as cues to find their way. Moreover, they use odours primarily and visual cues secondarily. Surprisingly, they stop responding to learned odours as soon as these cues become invalid, but fail to do so for obsolete visual cues.

Six comments can be made as for these results.

1. Visual classical conditioning (Cammaerts 2004b), visual spatial (differential) classical conditioning (Cammaerts 2004c) as well as visual operant conditioning (Cammaerts 2004d, 2007, 2008) have already been demonstrated in *M. sabuleti.* The experimental apparatus used in those studies differed from one another and from those used here. Even taking these differences into account, the numerical results of the present olfactive conditioning experiments were somewhat lower than those of the earlier visual conditioning experiments. This reflects the ants' different behaviour in the presence of either visual or olfactive stimuli. After having acquired visual conditioning, the ants moved on and all around the experimental apparatus during some times, while ants that acquired olfactive conditioning quickly approached the odorous elements but soon moved away from them. On the other hand, the same difference was observed while conducting visual as well as olfactive conditioning: it was of better quality when meat instead of sugared water was used as a reward (Cammaerts 2004d, present work) (see also comment 3 here below).

2. As for the maze tests, the higher scores obtained in both the present and a previous work (Cammaerts & Lambert 2008) were (and will always be) less than 100%. This is because, in ants, some random walking always occurs even along a learned trajectory: this strategy allows the discovery of new food sources or other useful elements.

3. The ants preferentially responded to odours over visual cues. This preference might be greater than the results indicate. Firstly, the ants were conditioned to odorous sour-

ces using sugared water as a reward and to visual cues using meat as a reward, and, in the studied species, meat yields better results during conditioning (see above). UDALOVA & al. (2006) showed a similar situation for *M. rubra*. Accordingly, if ants had been conditioned to odours using meat as a reward, their response to such a kind of cues would have been greater. Secondly, the ants rapidly stopped responding to invalid odorous elements, but continued (at least for the duration of the experiment) answering to outdated visual cues. We based our statement of a difference between the ants' response to odorous and visual cues on entire trajectories in mazes, from their beginning until their end. Taking only the first parts of these trajectories into account would have yielded even greater difference.

4. Ant behaviour during the olfactive conditioning test experiments agreed with that exhibited inside mazes provided with no longer valid odorous cues. The ants then soon stopped responding to previously learned odours.

5. When tested in the presence of aqueous extracts of learned odours, *M. sabuleti* foragers often made their choice 1 to 2 cm before reaching the odorous sources, apparently perceiving the odours from a short distance. On the other hand, when confronted with non-odorous elements soaked in learned odours, the ants moved slowly, remaining near the presented elements for lengthier periods: this reflected the small amounts of learned odorous compounds absorbed by such non-odorous elements.

6. Ants' maze running performances were not at all improved by proprioceptive information gained in the maze while being tested. Indeed, we conducted successively the control, the experiments with odours, those with odours and visual cues, those with aqueous extracts of the odorous, then, only after that, those with balsa and ivy, two non odorous plants. The results of this second control were exactly the same as those of the first control: we obtained the same Poisson distribution for the numbers of 0, 1, 2, 3, and 4 correct choices the ants gave (Tab. 3). If the ants had learned the correct path in the maze, the results of the second control would have been statistically different from those of the first control. Consequently, the ants never learned the correct path through the maze when tested. Each time, they only responded to cues with which they had been trained.

Three probable facts can be deduced from the present work.

1. *Myrmica sabuleti* workers' different behaviour towards visual versus odorous cues suggests that the dynamics (and perhaps partly the mechanisms) of visual and olfactive learning and memorising may differ. This physiological hypothesis is currently being tested in ethological experiments.

2. The different reaction to invalid odours and visual cues is probably useful in the natural environment, where visual elements (e.g., stones, branches) are likely to be more stable than odours (e.g., flowers, fruits, odorous plants) which may be more fluctuating and ephemeral. However, odours persist during sufficiently long times for allowing ants to discover some food, recruit nestmates, collect enough food and come back to their nest.

3. *Myrmica sabuleti* always nests in environments provided with odorous plants and never colonises too woody or stony ground. In the Aise valley, for instance, *M. sabuleti* nests only in areas provided with plants such as *Trifolium repens, Galium mollugo, Lotus corniculatus, Daucus carota, Leucanthemum vulgare, Fragaria vesca, Cytisus scoparius.* On the other hand, a morphological study of *M. sabuleti* workers' eyes (RACHIDI & al. 2008) showed that these ants have small eyes, even compared to other *Myrmica* species (*M. rubra* (LINNAEUS, 1758), *M. ruginodis* NYLANDER, 1846). This may help explain why *M. sabuleti* workers preferred odorous cues. This finding was supported by observing *M. sabuleti* workers searching for their way: they stretched out and continuously moved their antennae. Nevertheless, such workers might also use cues located above them since they do perceive black circles presented horizontally above them (CAMMAERTS 2004a).

Comparisons with other experimental works must now be done.

Olfactive classical conditioning was achieved in *Camponotus vagus* (SCOPOLI, 1763) by GUERRIERI & al. (2007). Olfactive differential classical conditioning was also achieved by DUPUY & al. (2006), then by ESCHBACH & al. (2006) for *Camponotus fellah* DALLA TORRE, 1893 and *C. mus* ROGER, 1863, these ants having learned to respond to one odour and to avoid another one. Note that these latter authors termed their experimental apparatus a "maze" although it was a Y olfactometer. Several other studies indicated that ants use odours to approach collecting areas. WOLF & WEHNER (2000) showed that *Cataglyphis fortis* (FOREL, 1902) foragers use odorous compounds transported by the wind, when they are close to the food. EHMER (1999) demonstrated that *Paraponera clavata* (FABRICIUS, 1775) workers correctly approach food flanked by a previously learned odour of pine. *Camponotus pennsylvanicus* (DE GEER, 1773) can discriminate the odour of the sap of the plant on which they customarily found food (HELMY & JANDER 2003). The literature therefore indicates that ants can associate odours with sites of interest, can be attracted by odours previously perceived in association with food. Nonetheless, although many studies have examined the use of visual cues and landmarks by ants (PASSERA & ARON 2005, CAMMAERTS & LAMBERT 2008: references therein), no one has pointed out the sole use of odorous sources as precise cues, followed one after the other, just like landmarks, as we did in the present work.

Let us mention a last idea: odours as cues may be very useful because they can convey abundant information based on potential different mixtures, concentrations and gradients. This calls for studies dealing with different kinds of cues and conducted on several species with differently developed visual and olfactive sensory organs.

Our study is a first step in this direction: it shows that – for an ant with poor vision, nesting in odorous environments – mainly odours help negotiate paths, odours are quickly forsaken when they become obsolete and visual cues are also, but secondarily, taken into account when moving about.

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Zusammenfassung

Olfaktorisches Konditionieren wurde bei der Ameise *Myrmica sabuleti* Meinert, 1861 erzielt, wobei entweder Fleisch oder Zuckerwasser als Belohnung eingesetzt wurde ($P < 0.016$). Das Konditionieren war erfolgreicher, wenn Fleisch als Belohnung verwendet wurde, wie es auch bei visuellem operantem Konditionieren für die Art bereits nachgewiesen ist. Differenzielles olfaktorisches Konditionieren bei *M. sabuleti* wurde ebenfalls erzielt, wobei Zuckerwasser als Belohnung eingesetzt wurde ($0.031 < P < 0.016$). Dieses Ergebnis legt nahe, dass diese Ameisen Geruchsreize verwenden können, um sich räumlich zurecht zu finden. Daher wurden Experimente in Labyrinthen durchgeführt, die mit erlernten Geruchsreizen ausgestattet waren: Die Ameisen fanden sich zurecht ($P < 0.001$). Sie konnten das auch dann, wenn alternativ visuelle Reize und Geruchsreize da waren ($P < 0.001$). Die Ameisen wurden dann mit visuellen Reizen und Geruchsreizen konfrontiert, die entweder übereinstimmten oder nicht. *Myrmica sabuleti* Arbeiterinnen reagierten primär auf erlernte Geruchsreize und sekundär auf erlernte visuelle Reize (korrekte Geruchsreize + falsche visuelle Reize: $P < 0.001$, falsche Geruchsreize + korrekte visuelle Reize: $P < 0.01$); das Ergebnis für falsche Geruchsreize + korrekte visuelle Reize war signifikant, weil die Ameisen offenbar rasch aufhörten, auf erlernte Geruchsreize zu reagieren, wenn diese Reize nicht mehr dabei halfen, sich zurechtzufinden.

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