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RESEARCH ARTICLE

Long-term olfactory memories are stabilised *via* protein synthesis in *Camponotus fellah* ants

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SUMMARY

Ants exhibit impressive olfactory learning abilities. Operant protocols in which ants freely choose between rewarded and non-rewarded odours have been used to characterise associative olfactory learning and memory. Yet, this approach precludes the use of invasive methods allowing the dissection of molecular bases of learning and memory. An open question is whether the memories formed upon olfactory learning that are retrievable several days after training are indeed based on *de novo* protein synthesis. Here, we addressed this question in the ant *Camponotus fellah* using a conditioning protocol in which individually harnessed ants learn an association between odour and reward. When the antennae of an ant are stimulated with sucrose solution, the insect extends its maxilla–labium to absorb the solution (maxilla–labium extension response). We differentially conditioned ants to discriminate between two long-chain hydrocarbons, one paired with sucrose and the other with quinine solution. Differential conditioning leads to the formation of a long-term memory retrievable at least 72 h after training. Long-term memory consolidation was impaired by the ingestion of cycloheximide, a protein synthesis blocker, prior to conditioning. Cycloheximide did not impair acquisition of either short-term memory (10 min) or early and late mid-term memories (1 or 12 h). These results show that, upon olfactory learning, ants form different memories with variable molecular bases. While short- and mid-term memories do not require protein synthesis, long-term memories are stabilised *via* protein synthesis. Our behavioural protocol opens interesting research avenues to explore the cellular and molecular bases of olfactory learning and memory in ants.

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

INTRODUCTION

Insects have played a pivotal role in the study of learning and memory. They learn simple and complex associations and possess a relatively simple nervous system that allows the retracing of associative phenomena to the cellular and molecular levels in different kinds of laboratory preparations (Giurfa, 2007a). While research on insect learning and memory has mostly focused on fruit flies *Drosophila melanogaster* (reviewed in Berry et al., 2008) and bees (for reviews, see Giurfa, 2007b; Leadbeater and Chittka, 2007), more recent studies have attempted to understand the processes underlying learning and memory in other species, such as crickets (Matsumoto and Sakai, 2000; Matsumoto and Mizunami, 2002a; Matsumoto and Mizunami, 2002b) and ants (Schilman and Roces, 2003; Dupuy et al., 2006; Kleineidam et al., 2007; Josens et al., 2009; Provecho and Josens, 2009). The picture emerging from these studies suggests that the organisation of memory follows common principles across a broad spectrum of species including both invertebrates and vertebrates (Thompson, 1986). Indeed, in all cases, memory is organised in at least two different forms; short-term memory (STM) and long-term memory (LTM). These show different temporal courses and distinct underlying molecular processes (Kandel, 2001); while STM is generally labile, content limited and independent of protein synthesis, LTM is robust, long lasting and depends on protein synthesis.

In the honeybee, *Apis mellifera*, such memories have been well characterised (Menzel, 1999) using a Pavlovian conditioning protocol; the olfactory conditioning of the proboscis extension reflex (PER). In this protocol, a harnessed hungry bee is exposed to an odorant followed by a sucrose reward delivered to the antennae and then to the proboscis. The bee learns the association between the odorant (the conditioned stimulus or CS) and sucrose (the unconditioned stimulus or US) and, afterwards, extends its proboscis to the odorant alone because it predicts reward delivery (Takeda, 1961; Bitterman et al., 1983). Different memory forms are established following this associative learning and depending on the experience gathered. One conditioning trial (i.e. a single pairing of an odorant and sucrose reward) leads to a mid-term memory (MTM) that can be retrieved 1–12 h after conditioning and to an early long-term memory (e-LTM) that can be retrieved 24–48 h after conditioning. After that, memory vanishes and retention performances decrease. Three conditioning trials, in contrast, lead to a stable late long-term memory (l-LTM) that can be retrieved 72 h later and that may last for the bee's entire life (Menzel, 1999). While e-LTM depends on protein synthesis, but from already

available mRNA without *de novo* transcription, l-LTM depends on transcription (Schwärzel and Müller, 2006).

Camponotus ants are suitable organisms for addressing the question of the mechanisms underlying the formation of LTM. Freely walking *Camponotus* ants placed in a Y-maze learn to discriminate an odour associated with sucrose solution from another odour associated with quinine solution (Dupuy et al., 2006). Three days later, ants still prefer the appetitive odour (Josens et al., 2009). Given that the retention period of 3 days corresponds to l-LTM in honeybees (Menzel, 1999), Josens and colleagues reasoned that retention observed in ants 3 days after conditioning also corresponds to the equivalent of a l-LTM (Josens et al., 2009). They nevertheless underlined the need to be cautious with this interpretation as l-LTM is not simply defined in terms of a behavioural account (i.e. when memories are retrieved and how many trials are required to observe such retrieval) but also refers to a distinct molecular basis. Specifically, whether repeated olfactory experiences in *Camponotus* ants lead to a protein synthesis-dependent memory remains unknown. Here, we asked whether memories retrieved 1 h, 12 h and 3 days (72 h) after conditioning depend on protein synthesis.

We used a new conditioning protocol (Guerrieri and d'Ettorre, 2010), which relies on the fact that a hungry harnessed ant extends its maxilla–labium when its antennae are stimulated with a sucrose solution. This response, called the maxilla–labium extension reflex (henceforth MaLER), is analogous to PER in honeybees (Takeda, 1961; Bitterman et al., 1983). MaLER can be elicited by odours if these are appropriately paired with sucrose solution (Guerrieri and d'Ettorre, 2010). This protocol therefore offers the advantage of combining immobilisation of the ants with associative learning performances and can be helpful in determining the nature of LTM in ants. We used long-chain hydrocarbons as conditioned stimuli as they were recently shown to be learned efficiently by ants in an appetitive context (Bos et al., 2010), and asked whether ants store this information in a LTM from where it can be retrieved 72 h after conditioning. Furthermore, we studied whether such a putative l-LTM is protein synthesis dependent using a protein synthesis blocker, cycloheximide (CHX), which inhibits translation and which was given to the ants prior to conditioning. We determined the effect of CHX ingestion on learning and retention at intervals of 1, 12 and 72 h post-conditioning. Our aim was to study whether CHX exerts a selective impairment of retention at 72 h, consistent with a protein synthesis dependence of the underlying memory.

MATERIALS AND METHODS

Animals

Workers of three colonies of *Camponotus fellah*, Emery, were reared under standard laboratory conditions (28°C, 12 h:12 h L:D, 65% relative humidity). Colonies were kept inside artificial nests, which consisted of two plastic boxes interconnected by a plastic hose. One of the boxes was covered with an opaque lid, and thus kept in the dark, and paved with a plaster template divided into chambers. The other box, which was uncovered, represented the foraging arena, where food (honey solution 50% v/v and mealworms) was supplied. Colonies were deprived of sucrose 15–20 days before starting the experiments, but received *ad libitum* water and mealworms.

Experimental setup

On the first day of each experiment, individual workers were collected from the foraging arena of one of the three artificial colonies used. A different colony was chosen every day to supply ants. Medium-sized workers were used as they are mostly foragers in *Camponotus* species (Dupuy et al., 2006), thus ensuring a higher motivation to respond with MaLER to the sucrose used as reward in our experiments. Ants were cold anaesthetised and harnessed in a holder made of an inverted 0.2 ml microtube (Eppendorf, Hamburg, Germany) cut at its apex. The ant's head was passed through the apical hole of the tube and kept in position with a piece of adhesive tape placed behind the collum (neck), so that it could only move its antennae and mouthpieces (Guerrieri and d'Ettorre, 2008). After 1 h recovery from anaesthesia, ants were individually fed with 1 µl of sucrose solution (10% w/w). For half of the animals (experimental group), the solution also contained 1 µg of the protein synthesis blocker CHX (Sigma-Aldrich, Copenhagen, Denmark). For the other half (control group) only the sucrose solution was delivered. After 3 h of subsequent rest, ants were tested for MaLER upon antennal stimulation with sucrose solution. Non-reactive individuals (less than 10% on average) were discarded.

Unconditioned and conditioned stimuli

We performed a differential conditioning in which one long-chain hydrocarbon was paired with 50% w/w sucrose solution (appetitive unconditioned stimulus or US+) while another hydrocarbon was paired with 10 mmol l^{-1} quinine solution (aversive unconditioned stimulus or US–) (Fig. 1A). The concentration of quinine was derived from conditioning experiments performed on freely walking *C. fellah* and *C. mus* ants (Dupuy et al., 2006).

Docosane (*n*-C_{22}) and octacosane (*n*-C_{28}) (Sigma-Aldrich) acted as conditioned stimuli (CS). They were chosen because this kind of odour is well learned in an appetitive context (Bos et al., 2010) and because their chain lengths are dissimilar enough to favour discrimination and differential responses (Deisig et al., 2002). In spite of their low volatility, these hydrocarbons can be detected without physical contact with the antennal receptors (Brandstaetter et al., 2008), i.e. *via* olfaction. Therefore, the protocol used constituted a case of olfactory conditioning.

Each hydrocarbon was dissolved in pentane (0.01 mg ml^{-1}). Before conditioning began, 10 µl of the solution was poured onto the tip of a glass rod (stimulation rod), using a microsyringe (Nanofil, WPI Inc., Aston, Stevenage, UK). The rod was held with its tip downwards until the pentane completely evaporated, keeping the droplet of hydrocarbon solution ≤3 mm from the tip.

Conditioning

The conditioning phase consisted of six CS+ trials (pairings of one CS with the US+) and six CS– trials (pairings of the other CS with the US–), presented in a pseudorandom sequence (the same stimulus was never presented more than twice consecutively). Within each group (CHX and control), for half of the ants, *n*-C_{22} represented the CS+ and *n*-C_{28} the CS–, while for the other half the opposite was the case.

Each conditioning trial lasted 1 min. Twenty-five seconds after being placed under a stereomicroscope, the ant received the CS followed by the US (5 s each). The CS was presented by approaching and gently touching the antennae with the tip of the stimulation rod (Fig. 1A,B).

Presentation of the US started 3 s after the onset of the CS, thus leading to a 2 s overlap during which the animal received both stimuli. The US was presented to the ant on the fine tip of a manually pulled capillary tube, which was directly applied on the mouth parts (Fig. 1A). The ant typically displayed a MaLER to sucrose but not to quinine. The inter-trial interval was 10 min as the 10 ants in a group were tested in series.

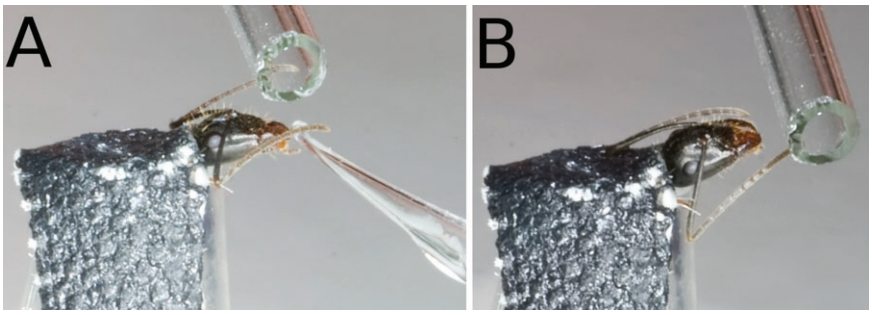

Fig. 1. Conditioning and test setup. (A) Harnessed ants were individually stimulated on the antennae (conditioned stimulus, CS) and on their mouthparts (unconditioned stimulus, US). (B) During the test, ants were stimulated only on their antennae with the rewarded CS (CS+), the aversive CS (CS–) or the novel (N) stimulus. Ants extended their maxilla–labium when presented with CS+, as shown in the picture.

Memory retention tests

Retention tests were performed in which the CS+, the CS– and a novel (N) long-chain hydrocarbon (n-C_{19}) were presented without the US in a random sequence varying from ant to ant (Fig. 1B). The order of presentation was balanced across individuals. Tests were performed 1, 12 or 72 h after conditioning. These intervals were chosen because they correspond to the typical distinct memory phases in the honeybee: respectively, early MTM (e-MTM), late MTM (l-MTM) and l-LTM (Menzel, 1999). Each retention test lasted 1 min and consisted of a 5 s hydrocarbon presentation 25 s after placement of the ant under the stereomicroscope. The inter-test interval was 10 min. After a complete test run was finished, the response to sucrose was tested. Only those individuals that still responded to sucrose stimulation were included in the statistical analyses (95% of all tested individuals).

Statistics

In conditioning trials, as well as in retention tests, we quantified conditioned responses (CRs), i.e. responses (MaLER) to the CS in the absence of the US. All results are provided as the mean probability (±s.e.m.) of displaying a CR in a given trial, within a given group. To detect possible variations in CR probability during trials or tests, we used repeated-measures analysis of variance (ANOVA), which can be used in the case of dichotomous data if certain experimental conditions are met (Lunney, 1970), which was the case here.

Within each group, we used a two-way repeated-measures ANOVA to compare the acquisition curves for the CS+ and the CS–, and one-way repeated-measures ANOVA to compare the response levels elicited by the three hydrocarbons in the retention tests. *Post hoc* comparisons were done by means of Scheffé's contrasts.

The responses to the CS+ in the tests for CHX and control groups were compared by one-way ANOVA. Given the fact that the same data sets were used for two different comparisons, the level of significance was adjusted to 0.025. All statistical tests were performed with Statistica (Statsoft, Maisons-Alfort, France).

RESULTS

Conditioning

Acquisition curves did not differ whether n-C_{22} or n-C_{28} represented the CS+ or CS– ($P>0.1$ in all cases), so results were pooled and presented as a CS+ *vs* CS– differentiation irrespective of the hydrocarbon used. This was valid for all six groups of ants, which were treated identically before the tests. These six groups correspond to each of the three memories tested, i.e. e-MTM, l-MTM, l-LTM, and for the two treatments applied: control (which received only the sucrose solution before conditioning) and CHX treated (which received CHX and sucrose solution before conditioning). In all cases, ants could discriminate between the CS+ and the CS– (control groups: e-MTM: $N=39$, $F_{1,76}=22.57$; l-MTM: $N=40$, $F_{1,78}=27.32$; l-LTM: $N=35$, $F_{1,68}=28.16$; $P<0.0001$ in all cases; CHX groups: e-MTM: $N=39$, $F_{1,76}=18.14$; l-MTM: $N=39$, $F_{1,76}=24.96$; l-LTM: $N=36$, $F_{1,68}=29.34$; $P<0.0001$ in all cases). Thus, there was no effect of CHX on acquisition (Fig. 2A,C,E).

Retention tests

One hour after conditioning (Fig. 2B), both control and CHX ants differentiated significantly between CS+, CS– and N (control: $F_{2,76}=5.03$; $P<0.009$; CHX: $F_{2,76}=8.82$; $P<0.0004$). In both control and CHX groups, the response levels to CS+ were higher than those to CS– (control: $P<0.02$; CHX: $P<0.007$) and N (control: $P<0.05$; CHX: $P<0.001$). The response levels to CS+ in the test did not differ significantly between control and CHX groups ($F_{1,76}=0.05$; $P>0.8$).

Twelve hours after conditioning (Fig. 2D), both control and CHX ants differentiated significantly between CS+, CS– and N (control: $F_{2,78}=5.93$; $P<0.004$; CHX: $F_{2,76}=4.12$; $P<0.02$). In both control and CHX groups, there were more responses to CS+ than to CS– ($P<0.025$ in both cases). However, only in the control group was the response level to CS+ higher than that to N (Control: $P<0.02$; CHX: $P>0.05$). The response to CS+ in the test did not differ significantly between control and CHX groups ($F_{1,77}=0.01$; $P>0.9$).

Seventy-two hours after conditioning (Fig. 2F), control and CHX groups yielded different responses. Control ants responded significantly more to the CS+ than to the CS– and to the N ($F_{2,68}=11.86$; $P<0.0001$), thus showing intact retention. There were more responses to CS+ than to CS– ($P<0.001$) and to N ($P<0.003$). Conversely, responses of CHX ants exposed to CS+, CS– and N did not differ significantly ($F_{2,70}=1.75$; $P>0.1$). Responses to CS+ in the test were significantly more frequent in the control group than in the CHX group ($F_{1,69}=8.25$; $P<0.006$).

DISCUSSION

Our results show that olfactory memories established by ants in an appetitive framework go through different phases, some of which are mediated by protein synthesis-independent processes while others clearly depend on protein synthesis. In particular, we found that feeding ants with CHX just before conditioning affects neither acquisition nor retention at 1 or 12 h post-conditioning. However, CHX suppressed olfactory retention at 72 h post-conditioning, thus demonstrating that olfactory memories retrievable 3 days after training in *C. fellah* ants fulfil both the temporal and the molecular requirements of l-LTM. As in other insects [e.g. honeybee (Menzel, 1999); *Drosophila* (Davis, 2005)], earlier memories do not require protein synthesis, confirming the duality verified across vertebrates and invertebrates concerning the different molecular bases of early and late memories. These common principles in the mechanisms of memory formation have been stressed in many studies on different animals (Dudai, 1996; DeZazzo and Tully, 1995; Rose, 1991).

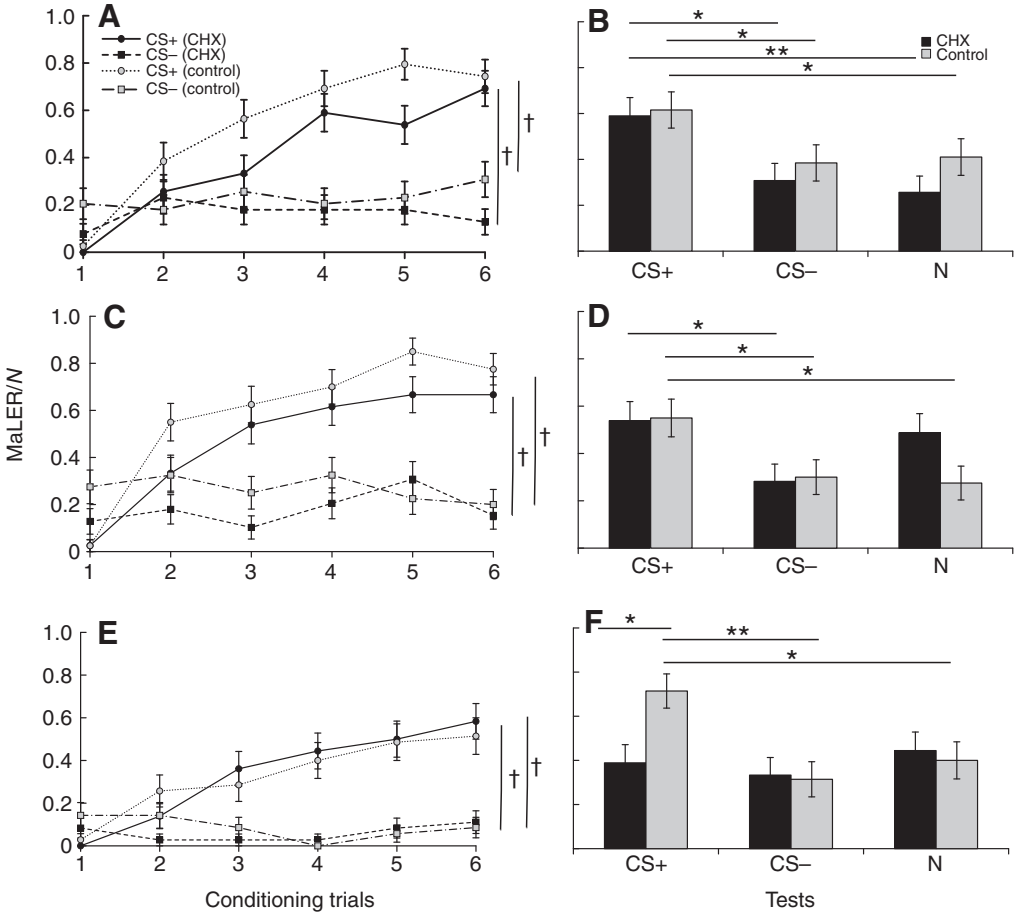

Fig. 2. Acquisition and discrimination in all the assayed groups. (A,C,E) The response probabilities (means ± s.e.m.) to rewarded conditioned stimuli (CS+, circles) and to aversive conditioned stimuli (CS–, squares) in the course of six conditioning trials. Vertical lines with daggers indicate significant differences between curves (ANOVA; $P<0.0001$ in all cases). (B,D,F) The responses given to CS+, CS– and the novel stimulus (N) in the retention tests. (A and B) Results for early mid-term memory tests (ants tested 1 h after conditioning). Control, $N=39$; cycloheximide (CHX), $N=39$. (C and D) Results for late mid-term memory tests (12 h after conditioning). Control, $N=40$; CHX, $N=39$. (E and F) Results for late long-term memory tests (72 h after conditioning). Control, $N=35$; CHX, $N=36$. Horizontal lines over the bars indicate significant differences in Scheffé's *post hoc* tests (*$P<0.025$; **$P<0.01$). MaLER, maxilla–labium extension reflex; N, number of ants tested.

Overall, we found no effect of treatment with CHX on acquisition, showing that the capacity to associate odours with their respective reinforcements was unaffected by the protein synthesis blocker. This result also demonstrates that STM, in the range of a few minutes (here 10 min) is unaffected by CHX treatment and is thus protein synthesis independent. Indeed, in any learning curve involving several trials separated by a few minutes, the second trial actually constitutes a STM test for what has been learned in the first trial (and so on). The fact that acquisition curves were identical in the CHX and control groups shows that protein synthesis is not required for STM formation. The retention test at 1 h post-conditioning, which corresponds to an early phase of MTM, was not affected by blockade of protein synthesis either. Similarly, after 12 h, which corresponds to a later phase of MTM, no effect of CHX was found. We can, therefore, conclude that MTM is homogeneous in the sense that neither earlier (1 h) nor later phases (12 h) require protein synthesis. Finally, LTM, assessed through the 72 h retention tests, showed a clear dependency on protein synthesis. In the honeybee, two forms of LTM have been identified: e-LTM (between 24 and 48 h), with protein synthesis-independent retention, and l-LTM (>48 h), with protein synthesis-dependent retention (Schwärzel and Müller, 2006). One can therefore assume that, as in honeybees, 72 h retention is also dependent on gene transcription, while 24 h retention, which was not tested in this study, would depend on protein synthesis only. Future experiments should confirm this assumption.

All in all, this scenario confirms that ant memories undergo an early consolidation phase which allows long-term storage of only those memories that are crucial to the animal and which will not be subjected, once consolidated, to interference by conflicting or coincident information (Menzel, 1999). This consolidation process leading to a protein synthesis-dependent phase requires time, as shown in this and many other works, so before reaching this state, the behaviour of ants is under the control of intermediate memories of higher susceptibility to amnesic treatments.

In honeybees, it has been suggested that the temporal structure of the foraging cycle provides a framework for understanding the sequence and properties of memory phases (Menzel, 1999; Menzel, 2001). In this scenario, memory phases correspond to the phases in which a foraging bout is structured. For instance, honeybee choices within a patch of flowers of the same species follow each other quickly and would be guided by STM; interbout intervals, in contrast, can vary from several minutes to months. In the first case, when a bee comes back from the hive to the same patch after some minutes, late forms of STM may guide the forager's choice; if it comes back the next day after several hours of inactivity, MTM may operate. If, however, return occurs after several days or even months (e.g. when an overwintered bee flies out for the first time in the spring), LTM would steer the bee's choice. In the case of ants, we suggest that the similarities in memory structure with honeybees are based on phylogenetic relatedness and similarities between foraging cycles. Ants, like bees, are central place foragers, always returning to the central place of their nest. *Camponotus fellah* ants forage on unpredictable insects but also on more predictable extrafloral nectaries. Individual foragers trained to collect sucrose solution in a Y-maze return regularly (every 10 min on average) to the maze (Dupuy et al., 2006; Josens et al., 2009) to collect sucrose

and in this context develop memories that may guide their choice several days after training (Josens et al., 2009). From this ecological perspective, field studies characterising foraging dynamics of *C. fellah* ants from a memory-based approach would be welcome. Similarly, invasive studies should reveal whether the structures, circuits and molecular cascades underlying memory formation in ants are the same as those in bees or whether species-specific adaptations exist.

In summary, our protocol allows exploration of other aspects of the cellular and molecular bases of olfactory learning and memory in ants. For instance, ants constitute an extremely diverse group in which species exhibit different life styles and ecological adaptations. For those species feeding on nectar, we could conceive of comparative studies in which, depending on foraging cycles, different memory dynamics could be found (Menzel, 1999). Species living in biotopes in which encounters with nectar reward are less frequent might need less repetitive experiences to trigger molecular cascades leading to LTM formation, or, in contrast, may never consolidate information acquired into LTM because of the rareness of rewarding events. These and other hypotheses are now experimentally accessible *via* our conditioning protocol, thus raising the status of ant studies in the field of invertebrate learning and memory.

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