

RESEARCH ARTICLE

Stimulus-dependent learning and memory in the neotropical ant *Ectatomma ruidum*

Andre J. Riveros^{1,*}, Brian V. Entler² and Marc A. Seid^{2,*}

ABSTRACT

Learning and memory are major cognitive processes strongly tied to the life histories of animals. In ants, chemotactile information generally plays a central role in social interaction, navigation and resource exploitation. However, in hunters, visual information should take special relevance during foraging, thus leading to differential use of information from different sensory modalities. Here, we aimed to test whether a hunter, the neotropical ant *Ectatomma ruidum*, differentially learns stimuli acquired through multiple sensory channels. We evaluated the performance of *E. ruidum* workers when trained using olfactory, mechanical, chemotactile and visual stimuli under a restrained protocol of appetitive learning. Conditioning of the maxilla labium extension response enabled control of the stimuli provided. Our results show that ants learn faster and remember for longer when trained using chemotactile or visual stimuli than when trained using olfactory and mechanical stimuli separately. These results agree with the life history of *E. ruidum*, characterized by a high relevance of chemotactile information acquired through antennation as well as the role of vision during hunting.

KEY WORDS: Classical conditioning, Proboscis extension response, Maxilla labium extension response, Absolute conditioning, Differential conditioning, Color conditioning, chemotactile conditioning, Multimodal learning

INTRODUCTION

Learning and memory are central cognitive processes enabling animals to cope with uncertainty. Bees, for example, must adjust to the variation in the resources offered by the floral market in order to optimize their foraging strategies. Accordingly, bee foragers evaluate flowers and associate features like colors and scents with the quality of their nectar and pollen (Chittka and Raine, 2006; Raguso, 2008). Sensory bias, a byproduct of a long history of co-evolution between plants and bees, facilitates learning of certain colors, such as those of short wavelength (Raine and Chittka, 2007; Russell et al., 2016). Similarly, *Cataglyphis* ants exhibit remarkable visual memory, used to navigate the desert terrain during foraging (Buehlmann et al., 2020; Collet et al., 1992). In a richer sensory environment, leaf cutter ants readily learn to discriminate the best plants for their fungus culture (Saverschek and Roces, 2010; Saverschek and Roces, 2011). Thus, learning and memory support

behavioral phenotypic plasticity corresponding with particularities of life histories and environments.

In the neotropics, sensory-rich environments of the lowland forests are inhabited by the ponerine ant *Ectatomma ruidum*, a widespread species renowned for its learning ability (Schatz et al., 1995, 1999; Franz and Wcislo, 2003; Passera et al., 1994) and spatial fidelity combined with temporal patterns during foraging (Schatz et al., 1995, 1999). Like many ants, *E. ruidum* has impressive navigation and hunting skills, which demand the integration of multiple sensory modalities (Schatz et al., 1997). During foraging, *E. ruidum* is also known to combine these modalities (olfactory, tactile and visual responses) to coordinate hunting and recruitment (Schatz et al., 1997); for example, when strategizing ambushes on stingless and halictid bees (Wcislo and Schatz, 2003). How *E. ruidum* integrates sensory modalities for learning and memory is unknown and often difficult to dissect in field-based experiments, especially considering *E. ruidum*'s complex behavioral patterns in their sensory-rich environments.

Our goal was to dissect the relevance of olfactory, tactile and visual stimuli during associative learning. In ants, learning and memory have been largely studied using free-moving individuals (e.g. Cameron et al., 1998; Dupuy et al., 2006; Oberhauser et al., 2019; Vowles, 1965; Wystrach et al., 2020). However, understanding the mechanisms that underlie learning and memory and the way that animals use stimuli can be enormously enhanced under controlled environments, with control of the information provided during training. In other groups of insects, such as bees, conditioning of the proboscis extension response (PER) has been used for 60 years to study learning and memory under restrained conditions (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Matsumoto et al., 2012; Takeda, 1961). Appetitive olfactory conditioning, which is commonly used, largely replicates the sequence of nectar collection from flowers. Individual bees are presented with a stimulus (e.g. a scent) that is subsequently paired with a reward (sugar water). Honeybees can readily associate the stimulus with the reward, forming long-term memory associations even after a single presentation (i.e. single trial learning; Smith, 1991; Villar et al., 2020).

Guerrieri and d'Ettorre (2010) adapted the PER protocol to study learning and memory in ants under restrained conditions. This new method, the maxilla labium extension response (MaLER), has allowed the exploration of olfactory appetitive and aversive learning in carpenter ants as well as the pharmacological characterization of long-term memory formation (Guerrieri and d'Ettorre, 2010; Guerrieri et al., 2011). Further use of the MaLER has also more recently contributed to the understanding of visual information in wood ants (Fernandes et al., 2018; Fernandes and Niven, 2020). In all cases, restraining individuals has enabled better isolation and control of the information provided for memory formation.

Thus, we used conditioning of the MaLER in an appetitive context to dissect how *E. ruidum* uses four primary sensory

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modalities (olfactory, tactile, chemotactile and visual; Fig. 1). Given the rich chemical environment within the nest and the overall relevance of antennation in ant communication, we expected *E. ruidum* to have strong olfactory responses. Moreover, as a hunter, we expected *E. ruidum* to readily learn visual stimuli. Interestingly, we found that olfactory stimuli led to weak associative responses, unless combined with tactile information (chemotactile), whereas visual stimuli elicited robust responses while learning a single color (absolute learning) or discriminating between colors (differential learning), as predicted. We discuss how the life history features of *E. ruidum* relate to our findings.

MATERIALS AND METHODS

Collection and maintenance of ants

We collected multiple nests of *Ectatomma ruidum* (Roger 1860) in Gamboa, Colon, Republic of Panama (elevation 30 m; 9°07'N, 79°42'W). Nests were opened and transferred to individual plastic containers, and ants were fed daily with termites and sucrose solution. Water was provided *ad libitum*. Colonies were left to accommodate to the new environment for 2 days before starting the experiments.

Training apparatus

The training apparatus has been previously described (Jernigan et al., 2014) and was only adapted to allow the use of a stereoscope. Briefly, the apparatus consisted of a rotatory platform with 12 individual plastic chambers lined with aluminium foil to facilitate light diffusion. Each chamber had an opening that allowed light to be shone from underneath and a frontal opening that allowed presentation of a stimulus (e.g. a scent injected into a clean current of air) as well as the reward (Fig. 1A). Each chamber hosted a single ant restrained in a plastic tube.

Training procedure

General protocol

In all cases, we trained the ants following standard procedures of classical conditioning of the MaLER (Fig. 1B,C). For each experiment, we used ants from the same colony assigned to the corresponding treatments. Specifically, we moved an ant to a chamber for conditioning under a stereoscope and allowed 30 s of accommodation. We then presented a stimulus (the conditioned stimulus, CS) to the ant for 13 s (Fig. 1D). Ten seconds after the onset of the CS presentation, we stimulated the antennae with sugar water (the unconditioned stimulus, US) to elicit the MaLER, and allowed the ant to drink for 3 s. Ants were excluded if they failed at least once to exhibit the MaLER in response to the US ($N=38$), an indication of lack of motivation. Timing was determined following a sound signal from a metronome set to 1 Hz, which further allowed us to record the latency of response and evaluate whether the treatments impacted the speed of the conditioned responses. Once the conditioning procedure ended, we allowed 15 s before moving to the next chamber containing another ant. Thus, the entire procedure lasted approximately 60 s per individual. In all cases, we allowed an intertrial interval of 10 min. Memory retention was tested 24 h after the last training trial was conducted (Fig. 1D). For the retention test, the ants were exposed to the CS for 10 s (following the same time pattern of training) and the response was recorded. To rule out lack of motivation, ants were stimulated with sugar water if they did not exhibit a conditioned response during the test. In all cases, the data show ants that were used for both phases, acquisition and memory testing. We used the procedure described above in the context of absolute or differential conditioning. Below, we describe specific variations introduced in each experiment according with the specific objective.

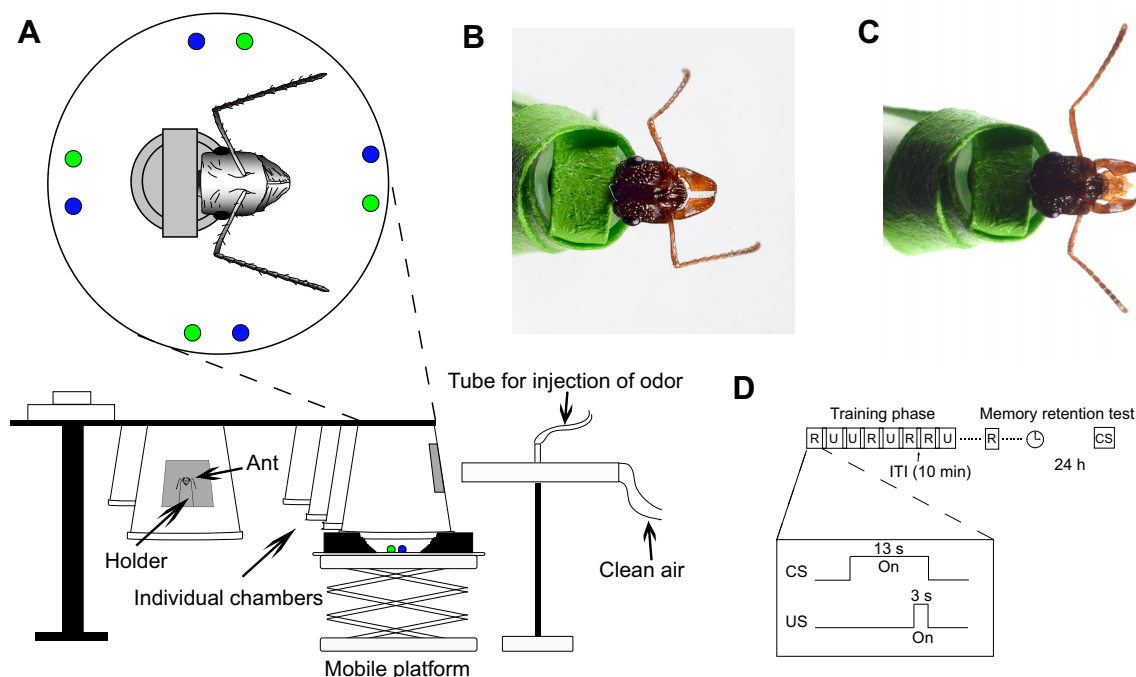


Fig. 1. Experimental setup and conditioning protocol. (A) Diagram of the setup used for training. The inset shows the top view of an ant inside the training chamber. (B) An ant resting before exhibiting a response. (C) Conditioned response with full open mandibles and maxilla labium extended. (D) General protocol for conditioning. CS, conditioned stimulus; US, unconditioned stimulus; R, rewarded trial; U, unrewarded trial; and ITI, intertrial interval.

Experiment 1: absolute conditioning of the MaLER to tactile and olfactory stimuli

The olfactory stimulus consisted of an air current passed through a syringe containing cinnamon powder on filter paper (as a result of this procedure, the specific concentration of cinnamon could not be determined) and injected into a current of clean air. Injection of the scent was manually activated by the experimenter following the time determined with the metronome. The tactile stimulus was delivered as direct antennal stimulation with a strip of clean filter paper. In both cases, ants received five paired presentations of the stimulus and the reward. Presentations followed the general protocol (see above), adjusted for absolute conditioning.

Experiment 2: differential conditioning of the MaLER to bimodal versus unimodal tactile stimuli

For the differential task, the US was paired with antennal stimulation with cinnamon on filter paper (concentration not determined) during the rewarded trials. During the unrewarded trials, ants received antennal stimulation with a clean strip of filter paper. Rewarded (R) and unrewarded (U) trials were presented following a pseudorandom sequence (Training, R, U, R, U, R, U, R). Retention of the associations was tested 24 h after the last training trial.

Experiment 3: associative nature of acquisition of MaLER to a chemotactile stimulus

We tested the associative nature of the conditioned response by training ants in two groups. One group (paired group) followed the general training protocol (see above) and the CS+ and the US were associated. A second group (the unpaired group) received the CS of the first group and the US in alternate trials, thus excluding the association. Aiming to equilibrate the number of total trials relative to the unpaired group, ants in the paired group were exposed to five 'blank' trials. During blank trials, ants were exposed to the training environment for the standard training time (73 s) and then returned to the resting area. Rewarded (R) and blank (B) trials were alternated.

Experiment 4: absolute conditioning of the MaLER to a visual stimulus

We tested whether individuals could learn the association between a colored light (green: wavelength peak $\lambda=520$ nm, blue: wavelength peak $\lambda=462$ nm) and the sucrose reward (see above). As ants could learn the association between changes in light intensity rather than the color, we conducted a test presenting low-intensity light of the color used during training and high-intensity light of the alternative color. Light intensity was adjusted by varying the voltage to a known emission previously recorded using a LI-COR portable spectroradiometer (Jernigan et al., 2014). Intensity was classified as high (green: $29.9 \mu\text{mol m}^{-2} \text{s}^{-1}$, blue: $30.53 \mu\text{mol m}^{-2} \text{s}^{-1}$), medium (green: $15.41 \mu\text{mol m}^{-2} \text{s}^{-1}$, blue: $14.6 \mu\text{mol m}^{-2} \text{s}^{-1}$) or low (green: $7.14 \mu\text{mol m}^{-2} \text{s}^{-1}$, blue: $6.68 \mu\text{mol m}^{-2} \text{s}^{-1}$). Ants were presented with five trials of the paired association using the medium intensity light. During tests, 50% of the ants first received the color used for training (at low intensity) and 50% first received the alternative color (at high intensity). Tests of intensity were conducted after the last trial of training with the same intertrial intervals used during training. We did not conduct a retention test as described in the general protocol as the association might be modified by our test using different light intensities. Latency was determined following a sound signal from a metronome set to 1 Hz. In all cases, the stimulation with light was manually activated by the experimenter following the time determined with the metronome.

Experiment 5: color learning versus tactile learning

Ants were trained following the general protocol (see above) and the specific settings presented in experiments 1 and 4. Latency was determined following a sound signal from a metronome set to 1 Hz. The injection of the scent and the presentation of the colored light were manually activated by the experimenter following the time determined with the metronome.

Data analyses

We used a generalized linear mixed model (GLMM) to test for the effect of the conditioning protocol (depending upon the experiment) (Bos et al., 2014). For the GLMM, we used a binomial structure with a Logit link function; also, we included treatment (conditioning protocol) and trial as fixed effects and individual as a random effect. Differences in retention after 24 h were evaluated using a chi-square test. Latency of response was calculated as the average of at least two conditioned responses. Comparison of response latencies was conducted using Student's *t*-tests if the distribution of data was normal (tested using a Shapiro–Wilk *W* test). When the data did not follow a normal distribution, we used the non-parametric Mann–Whitney (Wilcoxon) test. All the analyses were done using JMP v.14.0 (SAS Institute) with the GLMM Add-in_v4.

RESULTS

Experiment 1: absolute conditioning of the MaLER to tactile and olfactory stimuli

We first evaluated whether *E. ruidum* workers could learn the association between an olfactory or a tactile stimulus and a reward. We collected and restrained ant workers for olfactory ($N=25$) and tactile ($N=26$) conditioning. We conducted the analysis only using ants that were responsive across all trials. Thus, we excluded five ants in the olfactory group. We did not observe any ants exhibiting the MaLER during the first trial. We found that ants exhibited a low performance (barely 30% of the ants exhibited a conditioned response only to the tactile stimulus) while learning either stimulus independently. However, ants exhibited contrasting performance levels when conditioned to the olfactory or the tactile stimuli. Ants conditioned to the tactile stimulus changed their performance across the conditioning trials (GLMM: $F=7.46$, $P<0.0001$; Fig. 2A) and had an overall score significantly different from zero (mean $\pm$ s.e.m.

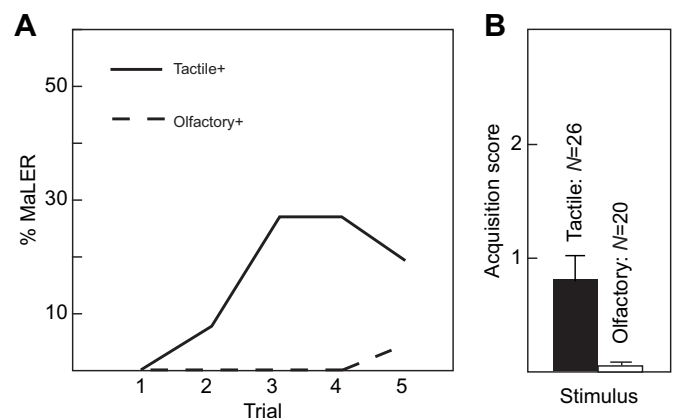


Fig. 2. Associative acquisition of tactile versus olfactory stimuli during an absolute conditioning protocol. (A) Acquisition curves across five paired presentations of stimulus (tactile, olfactory) and reward. MaLER, maxilla labium extension response. (B) Overall learning score (mean $\pm$ s.e.m.). *N*, number of individual ants.

0.81 ± 0.21 , $N=26$, Wilcoxon test $Z=115.5$, $P=0.0001$; Fig. 2B). In contrast, ants trained to the cinnamon scent did not exhibit any significant change in performance across trials (GLMM: $F=0.127$, $P=0.972$; Fig. 2A) and their overall score did not differ from zero (0.05 ± 0.05 , $N=20$, Wilcoxon test $Z=10$, $P=0.5$; Fig. 2B).

Experiment 2: differential conditioning of the MaLER to chemotactile versus unimodal tactile stimuli

Next, we conditioned ants to discriminate between a rewarded chemotactile and a tactile stimulus. We used differential conditioning with the tactile stimulus unrewarded as experiment 1 showed that ants were able to learn an appetitive association using a tactile stimulus.

We collected and restrained 49 ants and trained them for four rewarded and four unrewarded trials after a first rewarded training trial. We did not observe any ants exhibiting the MaLER during the first trial. For final analyses, we only included ants that were responsive across both the acquisition and the retention test ($N=23$).

We found a null response toward the unrewarded tactile stimulus. We did not observe any change in the performance across trials (Fig. 3A) and ants did not exhibit any conditioned response during the acquisition phase, thus giving an overall score of zero (Fig. 3B). In contrast, ants significantly enhanced their conditioned responsiveness toward the bimodal chemotactile stimulus across trials (GLMM: $F=40.6$, $P<0.0001$; Fig. 3A) exhibiting more than 90% MaLER by the fifth trial for the rewarded stimulus. On average, ants responded to three out of the four testing/training trials (2.90 ± 0.24 , $N=23$; Fig. 3B). This high performance decreased during the retention test and reached 70% after 24 h (Fig. 3C). Interestingly, one ant responded to the unrewarded tactile stimulus after 24 h (Fig. 3C).

Experiment 3: associative nature of acquisition of MaLER to a chemotactile stimulus

The results from experiment 2 showed that combining the tactile and the chemical/olfactory stimulus enhanced learning. Moreover, the

ants did not exhibit any generalized response toward the unrewarded tactile stimulus, thus supporting the idea that it is the interaction of cues that enhances learning. Nevertheless, given the conditioned stimulus (the bimodal chemotactile stimulus) and the unconditioned stimulus (sugar water) are delivered to the antennae, it is possible that the responses of the ants observed in experiment 2 derived from a strong appetitive innate response to the chemotactile stimulus and were not real conditioned responses.

Aiming to decouple this effect, we selected two sets of ants and randomly assigned them to one of two treatments: paired and unpaired stimulation. (1) For unpaired stimulation, ants were exposed to 10 trials, receiving only the sugar water stimulus for five trials and only the chemotactile stimulus for the other five trials. (2) For paired stimulation, ants were trained to associate the chemotactile stimulus with a sucrose reward following the general protocol described above. We collected and restrained 48 ants equally distributed between paired and unpaired treatments. We excluded four ants of the paired treatment that were not responsive during at least one of the trials. We did not observe any ants exhibiting the MaLER during the first trial.

We found that the treatment assigned to the ants significantly affected their performance across trials (GLMM: treatment, $F=37.36$, $P<0.0001$; Fig. 4A). Ants conditioned with a paired presentation of the stimulus and the reward increased their performance over trials (GLMM: trial, $F=13.92$, $P<0.0001$; Fig. 4A) and had, on average, a higher learning score than ants presented with the stimulus unpaired with the reward (Wilcoxon test $Z=5.08$, $P<0.0001$; Fig. 4B). After 24 h, we observed a 20% decrease in the conditioned response such that 60% of the ants responded when presented with the chemotactile stimulus (Fig. 4C).

Experiment 4: absolute conditioning of the MaLER to a visual stimulus

We trained ants in an absolute conditioning protocol to learn green or blue colored light. We collected and restrained 64 ants, which were equally assigned to each treatment. All individuals were

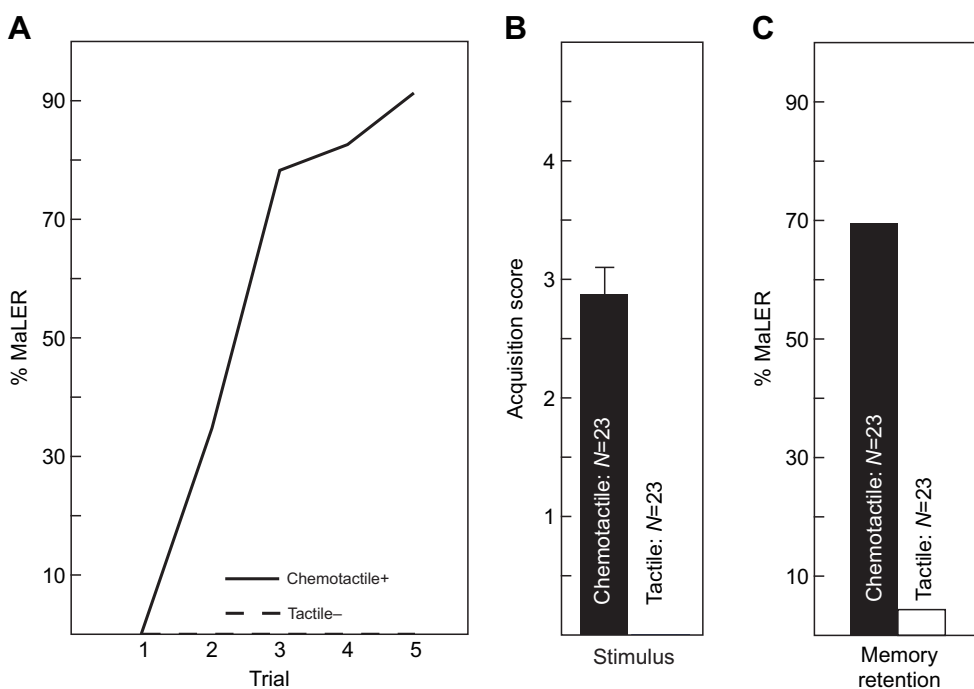


Fig. 3. Associative acquisition and retention of chemotactile versus tactile stimuli during an absolute conditioning protocol. (A) Acquisition curves across five paired presentations of stimulus and reward. (B) Overall learning score (means $\pm$ s.e.m.). (C) Retention 24 h following the last training trial. *N*, number of individual ants.

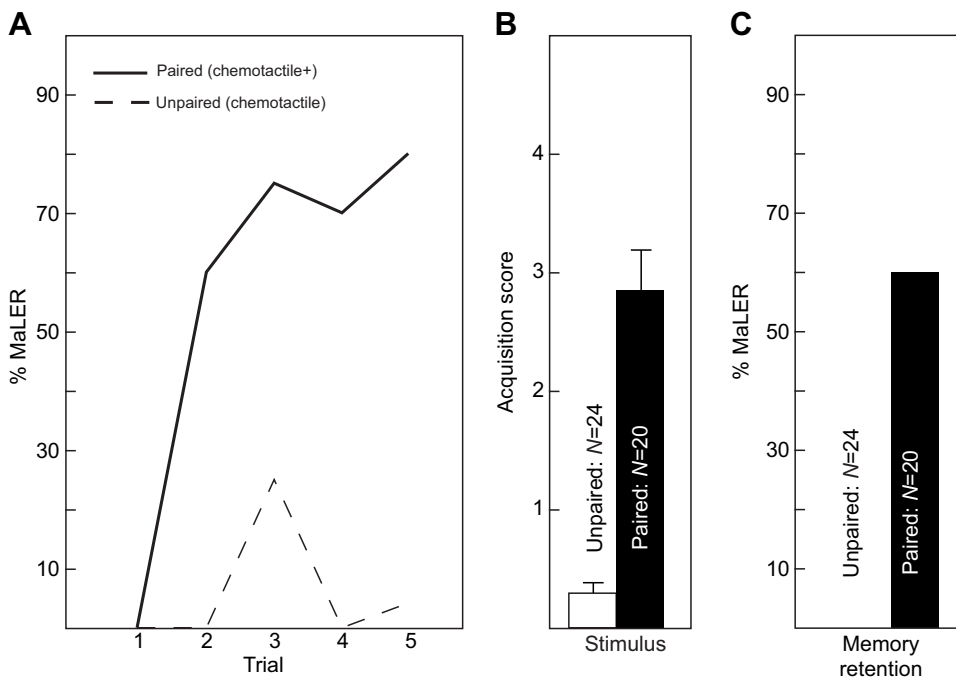


Fig. 4. Responsiveness during acquisition and retention tests toward paired versus unpaired presentation of a chemotactile stimulus and a reward. (A) Acquisition curves across five paired or unpaired presentations of the chemotactile stimulus and reward. (B) Overall learning score (means $\pm$ s.e.m.). (C) Responsiveness 24 h following the last training trial. *N*, number of individual ants.

responsive across all the training procedures and were included for final analyses. We did not observe any ants exhibiting the MaLER during the first trial. We found that ants were able to readily learn the color associations and changed their probability of a conditioned response across trials (GLMM: trial: $F=13.0$, $P<0.0001$; Fig. 5A). The change across trials followed a pattern that could not be distinguished when trained for blue or for green [GLMM: treatment (color): $F=0.18$, $P=0.68$; Fig. 5A], with ants further exhibiting learning scores that did not differ from each other (Wilcoxon test

$Z=0.46$, $P=0.64$; Fig. 5B). Interestingly, ants conditioned to green exhibited faster responses (2.5 ± 0.16 , $N=23$; Fig. 5C) than ants conditioned to blue (3.5 ± 0.15 , $N=23$; Fig. 5C; Student's t -test: $t_{44}=-4.68$, $P<0.0001$).

During the tests using high and low intensity light (see Materials and Methods), the ants exhibited high responsiveness for the wavelength used for training (even if presented at low intensity) and low or no responsiveness for the alternative color (even presented at a high intensity; Fig. 5D).

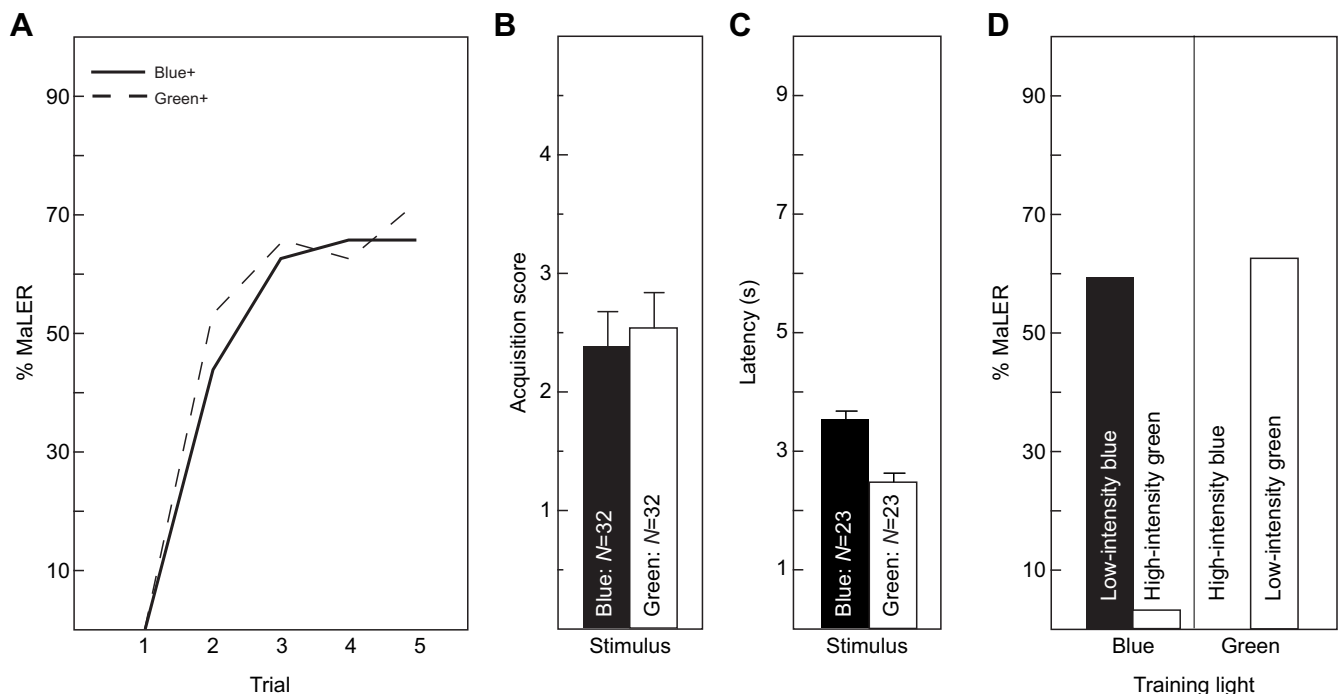


Fig. 5. Acquisition, latency of response and generalization during absolute color conditioning. (A) Acquisition curves across five presentations of green or blue colored light paired with a reward. (B) Overall learning score (means $\pm$ s.e.m.). (C) Mean ($\pm$ s.e.m.) latency of response following presentation of the conditioned stimulus. (D) Test for generalization of the conditioned response. *N*, number of individual ants.

Experiment 5: color learning versus tactile learning

Our first four experiments separately showed that ants readily associate a chemotactile stimulus and a colored stimulus with sugar water. Thus, as a final test we compared the performance of ants randomly assigned to color (green) conditioning versus chemotactile conditioning. We collected and restrained 68 ants and equally assigned them to one of the two treatments. For further analysis, we only included ants that were responsive across all the training and testing procedures (chemotactile: $N=31$; colored light: $N=29$). We did not observe any ants exhibiting the MaLER during the first trial.

Similar to the previous experiments, ants exhibited an increase in performance across trials using both conditioned stimuli (GLMM: trial, $F=26.88$, $P<0.0001$; trial $\times$ treatment, $F=5.54$, $P=0.0012$; Fig. 6A). Ants conditioned to the chemotactile stimulus exhibited a higher performance than ants conditioned to the colored light across trials (GLMM: treatment, $F=8.28$, $P=0.0054$; Fig. 6A) and had an overall higher learning score (Wilcoxon test $Z=2.05$, $P=0.04$; Fig. 6B). However, ants conditioned to green light had shorter latencies than ants conditioned to the chemotactile stimulus (Student's t -test $t_{52}=6.29$, $P<0.0001$; Fig. 6C). When testing for retention after 24 h, we observed a 20% decrease in responses of ants conditioned to the chemotactile stimulus and a 10% decrease in responses of ants conditioned to the green light. We did not observe differences in retention between ants exposed to either stimuli (chi-square test $\chi^2=0.20$, $P=0.653$; Fig. 6D).

DISCUSSION

We evaluated learning and memory performance of *E. ruidum* workers when using chemotactile, olfactory, tactile or visual information under restrained conditions. We found that *E. ruidum* exhibited high performance in both learning and memory. Yet, we observed broad variation correlated with the use of different stimuli. Remarkably, the most contrasting differences in performance were

exhibited between ants trained with an airborne olfactory stimulus versus ants trained with a chemotactile stimulus. The differences in performance generally followed predictions derived from the life history of *E. ruidum* and of ants in general.

Ants typically rely on antennal exploration (i.e. antennation) for behaviors as diverse as nest mate recognition (Neupert et al., 2018), trophallaxis (Cabe et al., 2006), aggression (Paul and Annagiri, 2019) and prey search (Binz et al., 2016). Whereas antennae are major sensory organs for olfactory sensing (Duan and Volkan, 2020; Ryba et al., 2020; Zube et al., 2008), in many cases direct contact is required (Cabe et al., 2006; Sheridan et al., 1996), thus integrating chemical and mechanical information. Like other ants, *E. ruidum* largely relies on antennation for many behaviors inside (e.g. nursing) and outside the colony (e.g. hunting). Aligned with these premises, the ants in our experiments exhibited fastest and highest learning across all modalities when trained with a chemotactile stimulus. Interestingly, there was a consistent decrease of 20% in conditioned responses after 24 h across the three different experiments (Figs 3–6). Such decreases in response during memory retention tests are typically observed in experiments of insect conditioning and likely reflect constraints of the method as well as ecological adaptations of the species. This decrease in response during the retention test was the largest for all modalities and suggests that learning faster did not necessarily imply remembering for longer. We speculate that a strategy to rapidly exploit a resource may require its fast identification but the relevance of remembering may depend on the abundance and/or prevalence of the resource, eventually leading to patch fidelity (Passera et al., 1994; Schatz et al., 1995). In tropical forests, constant available resources that are worth remembering are rare, while more ephemeral resources are common and thus learning without investing in a lasting memory is more advantageous.

Remarkably, olfactory stimulation with the same molecule used as a chemotactile stimulus rendered no conditioned responses,

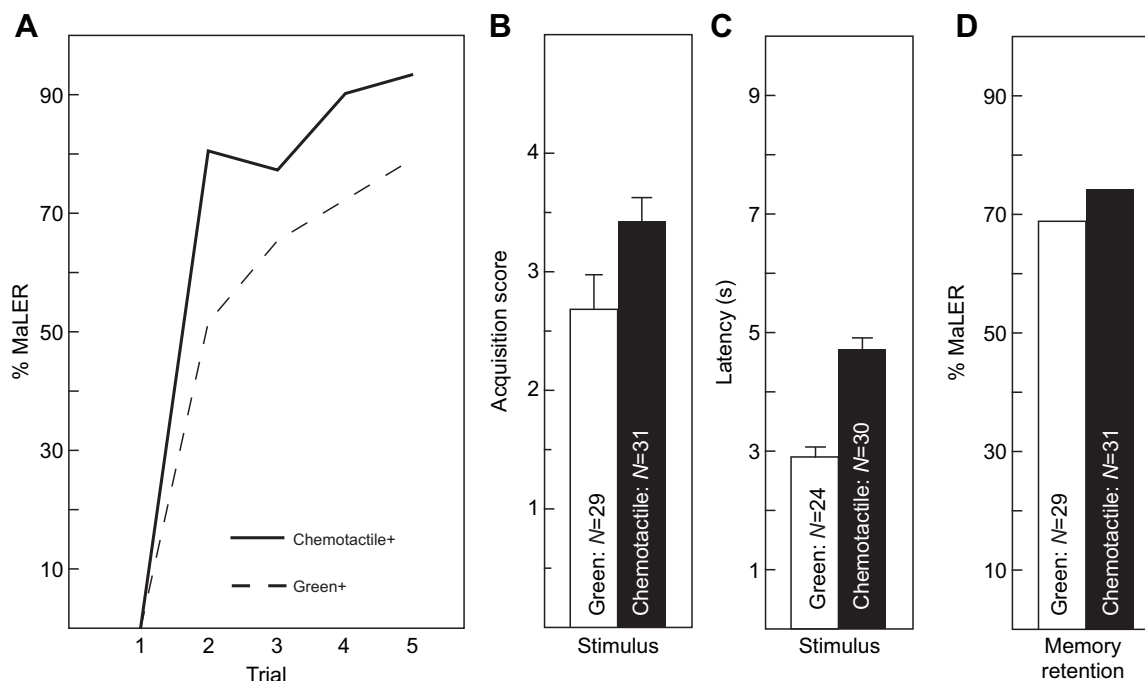


Fig. 6. Acquisition, retention and latency of response during absolute color and chemotactile conditioning. (A) Acquisition curves across five presentations of a green light or a chemotactile stimulus paired with a reward. (B) Overall learning score (means $\pm$ s.e.m.). (C) Mean ($\pm$ s.e.m.) latency of response following presentation of the conditioned stimulus. (D) Conditioned responses 24 h following the last training trial. N , number of individual ants.

highlighting the role of direct contact for resource evaluation. As bimodal information may enhance performance during acquisition (Riveros et al., 2020; Kulahci et al., 2008; Leonard et al., 2012), one may argue that the high performance observed during chemotactile stimulation is due to the enriched stimulus rather than to a synergistic associated value. Our results unfortunately do not enable us to separate these possibilities, which require further investigation along with the exploration of how processing of the same molecule by two different modalities leads to such contrasting results. In a different species of ant, olfactory conditioning led to high performance under restrained conditions (Guerrieri and d'Ettorre, 2010) and in bees tested using the PER protocol, olfactory conditioning typically leads to high acquisition and retention (e.g. Guerrieri et al., 2005, for an olfactory space using multiple odorants). Whether the poor performance observed in *E. ruidum* is due to particularities of the species, a rich olfactory environment or the olfactory stimulus used cannot yet be answered.

Interestingly, and also in agreement with the life history of *E. ruidum*, we observed high levels of performance when ants were conditioned to visual stimuli. As hunters, *E. ruidum* foragers may rely on wide-range or sit-and-wait strategies (Schatz and Wcislo, 1999), showing a relevant role of visual information. Visual stimuli may incorporate features of the prey (e.g. size, color) as well as movement. In our protocol, the ants exhibited high acquisition and retention when trained to either green or blue. However, ants reached higher learning scores and shorter latencies of response when trained to green and did not generalize between colors, as suggested by the tests using contrasting light intensities. Interestingly, the common presence of green in the foliage and blue/purple in flowers is typically used as an argument for the low performance of bees when trained to green and high performance when trained to the short wavelength of blue and purple colors (Menzel, 1985; Jernigan et al., 2014). In *E. ruidum*, such an argument is not supported and certainly further experiments need to be conducted in this regard. New experiments may incorporate the distribution of receptors across *E. ruidum* eyes and electroretinograms. The number of receptors may underlie perception of conspicuousness of a stimulus, in turn affecting acquisition if the stimulus is perceived as being of higher intensity (Mackintosh, 1974).

Most interestingly, further experiments should incorporate motion. Foragers of *E. ruidum* can increase their foraging success on halictid bees by implementing ambush hunting at the nest entrance (Schatz and Wcislo, 1999). One would expect identification of prey and rapid release of a behavioral pattern for catching to be involved. In fact, movement of prey leads to behavioral patterns, such as stinging, that are not observed during collection of suitable immobile prey (Schatz and Wcislo, 1999). Moreover, in the closely related *Ectatomma tuberculatum*, a more visual hunter, conditioning using the MaLER is challenging because of high attention to any object getting close to the head (A.J.R., B.V.E. and M.A.S., unpublished data). Incorporating motion into a protocol of restrained ants is relatively simple and can be adapted from methods already used in bees (Hori et al., 2006) and ants (Fernandes et al., 2018) during conditioning.

Finally, our study shows the reliability of studying *E. ruidum* under restrained conditions using the MaLER as described by Guerrieri and d'Ettorre (2010). Unlike in bees, learning in ants has almost entirely been studied in arenas (e.g. mazes) allowing for free movement of individuals (Cameron et al., 1998; Dupuy et al., 2006; Oberhauser et al., 2019; Vowles, 1965; Wystrach et al., 2020). However, recent years have seen an increase in studies relying on

conditioning under restrained protocols. For example, *Camponotus aethiops* (Guerrieri and d'Ettorre, 2010) and *Camponotus fella* (Guerrieri et al., 2011) have been studied in the context of associative appetitive or aversive learning with olfactory information in protocols of absolute and differential conditioning. Also, a clear behavioral difference has emerged between bees and ants. Despite a large dependence on vision for foraging and navigation, visual conditioning has proven to be more challenging than olfactory conditioning under the PER protocol (Avarguès-Weber and Mota, 2016; Dobrin and Fahrback, 2012; Jernigan et al., 2014; Hori et al., 2006; Mota et al., 2011; Niggebrügge et al., 2009; Sakura et al., 2012; Riveros et al., 2020). This asymmetry in the development of conditioning protocols for visual and olfactory stimuli constrains the study of multimodal integration in bees, despite both sensory modalities greatly representing life history adaptations (Avarguès-Weber and Mota, 2016; Riveros et al., 2020). Our results suggest that visual and chemotactile stimuli can be integrated into conditioning experiments of *E. ruidum*, thus offering an opportunity to study multimodal learning. However, it is an excellent tool that needs to be paired with observations in more natural contexts in order to reach realistic conclusions within the framework of the life history of the species. Remarkably, our results show an important agreement with the life history of *E. ruidum*, thus encouraging the use of the two complementary approaches.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.J.R., M.A.S.; Methodology: A.J.R., B.V.E., M.A.S.; Formal analysis: A.J.R.; Investigation: B.V.E.; Resources: A.J.R.; Writing - original draft: A.J.R., M.A.S.; Writing - review & editing: A.J.R., B.V.E., M.A.S.; Supervision: A.J.R., M.A.S.; Funding acquisition: A.J.R., M.A.S.

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