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Visual associative learning in wood ants

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SUMMARY STATEMENT

Ants form an associative memory of a visual cue paired with a reward through classical conditioning. This allows visual associative learning to be studied in fixed rather than free moving ants.

ABSTRACT

Wood ants are a model system for studying visual learning and navigation. They can forage for food and navigate to their nests effectively by forming memories of visual features in their surrounding environment. Previous studies of freely behaving ants have revealed many of the behavioural strategies and environmental features necessary for successful navigation. However, little is known about the exact visual properties of the environment that animals learn or the neural mechanisms that allow them to achieve this. As a first step towards addressing this, we developed a classical conditioning paradigm for visual learning in harnessed wood ants that allows us to control precisely the learned visual cues. In this paradigm, ants are fixed and presented with a visual cue paired with an appetitive sugar reward. Using this paradigm, we found that visual cues learnt by wood ants through Pavlovian conditioning are retained for at least one hour. Furthermore, we found that memory retention is dependent upon the ants' performance during training. Our study provides the first evidence that wood ants can form visual associative memories when restrained. This classical conditioning paradigm has the potential to permit detailed analysis of the dynamics of memory formation and retention, and the neural basis of learning in wood ants.

INTRODUCTION

Learning and memory formation in insects has been studied extensively, most typically in foraging individuals from colonies of eusocial Hymenoptera (bees or ants) or adult fruit flies (Giurfa, 2007; Davis, 2005). Learning associations between appetitive or aversive stimuli and the characteristics of the environment in which they occur enhances foraging efficacy (Dukas & Bernays, 2000; Simões *et al.*, 2013). Indeed, foraging insects display learning abilities that include the categorization of visual stimuli (Zhang *et al.*, 2004), contextual (Dale *et al.*, 2005) and sequence learning (Collett *et al.*, 1993), interval timing (Boisvert & Sherry, 2006) and sameness-difference rules (Giurfa *et al.*, 2001), amongst others (Chittka and Niven, 2009).

Visual memories are of particular importance to foraging insects including honey bees (*Apis mellifera*) (e.g. Cartwright & Collett, 1983), desert ants (*Cataglyphis* spp.) (e.g. Collett *et al.*, 1992), wood ants (*Formica rufa*) (e.g. Nicholson *et al.*, 1999) and bumble bees (*Bombus terrestris*) (e.g. Gumbert, 2000), which use them to navigate towards a feeder. In wood ants, visual memories of landmarks can enable ants to locate the position of a food source or the nest but can also provide directional cues along the entirety of the route (Collett & Collett, 2002; Durier *et al.*, 2003; Graham *et al.*, 2004; Harris *et al.*, 2005; Harris *et al.*, 2007).

Although experimental paradigms with freely moving animals can allow them to reproduce behaviours they naturally display in the wild, experimenters typically lack control over the specific cues being learnt. Furthermore, for species in which optogenetic techniques are lacking, it is not possible to investigate the neural circuits underpinning learning and memory in detail unless the animals are fixed to permit electrophysiology. These issues can be overcome to some extent by the development of classical conditioning paradigms for appetitive learning in restrained insects (e.g. Takeda, 1961; Bitterman *et al.*, 1983) that reproduce an experimental paradigm for studying learning and memory developed more than 100 years ago by Pavlov (1897).

In appetitive classical conditioning, a conditioned stimulus (CS) is paired with an unconditioned stimulus (US), such as food or water, which evokes a strong, unconditioned response (UR). The majority of these studies in insects are based upon a paradigm developed originally for honey bees (Takeda, 1961; Bitterman *et al.*, 1983). In this paradigm, the honey bee performs the proboscis extension reflex (PER) when presented with sucrose (US) paired with a CS, typically an odour. After only three trials, bees learn to associate the CS with the US, responding to the CS alone with PER, and can form a long-term memory of this association

(Wittstock & Menzel, 1993). Other species of bees (*Bombus terrestris*, *Melipona quadrifasciata*, *Scaptotrigona depilis*) and flies (*Musca domestica*, *Drosophila melanogaster*) also perform PER to a CS after pairing it with an appetitive US (Fukushi 1976; Fukushi, 1979; Laloi *et al.*, 1999; Chabaud *et al.*, 2006; McCabe *et al.*, 2007). In insects that lack proboscis a similar paradigm can be used, though the response involves other mouthparts. For example, ants (*Camponotus aethiops* and *C. fellah*) perform the maxilla-labium extension reflex (MaLER) (Guerrieri & d’Ettorre, 2010; Guerrieri *et al.*, 2011), and locusts (*Schistocherca gregaria*) perform the palp opening response (POR) (Simões *et al.*, 2011; Simões *et al.*, 2012; Simões *et al.*, 2013).

Most of these studies on associative memories using classical conditioning approaches on restrained insects use an odour as the CS, with only a few exceptions in flies (Fukushi, 1976) and honey bees (Hori *et al.*, 2007; Niggebrügge *et al.*, 2009; Balamurali *et al.*, 2015; Avarguès-Weber & Mota, 2016) using a visual cue as the CS. Although combining visual and olfactory cues can produce learning rates as high as olfactory alone, using visual cues alone as the CS seems to produce weaker learning (Gerber & Smith, 1998).

In this study, we developed a visual appetitive learning paradigm through classical conditioning in the wood ant, *Formica rufa*. Wood ants have been shown to form visual memories while navigating and rely upon them while foraging (Collett & Collett, 2002; Durier *et al.*, 2003; Graham *et al.*, 2004; Harris *et al.*, 2005; Harris *et al.*, 2007). We show for the first time that ants can acquire and retain short- and longer-term visual memories when harnessed, demonstrating visual learning through classical conditioning.

METHODS

Animals

Experiments were performed using wood ants (*Formica rufa* L.) from two different colonies. Colonies were collected from Ashdown Forest, Sussex, UK (N 51 4.680, E 0 1.800) in June and August 2016 and maintained indoors for 5 months prior to training, during which they were kept at 21 °C, under a 12 L : 12 D cycle and fed with sucrose (33.3%). These conditions were maintained throughout our experiments except that the colonies were starved for at least two days before the start of training. On the day of training, ants were selected from the surface of the colony and placed in a box with a glass slide upon which was a drop of sucrose solution (20%). Only those ants that started to feed were selected for subsequent training, thereby ensuring that ants chosen were motivated to feed. To prevent ants becoming satiated,

potentially reducing their motivation to learn during training, they were removed from the box as soon as they started to feed.

Harnessing

Ants were placed in the freezer for 1-2 minutes and then harnessed individually at the back of their head to an insect pin (Austerlitz Insect Pin®) with low melting point wax. The insect pin was then fixed in modelling clay (Plasticine®). The ant was allowed to stand on a custom-made plastic holder embedded into the same modelling clay, thereby ensuring it maintained a typical standing posture and that the legs were free to move (Fig. 1A). Ants were left fixed in a dark room for at least two hours prior to training.

Training and testing

Experiments were conducted in a transparent Perspex box (50 x 50 x 50 cm) covered in white paper, open to the front to permit experimenter access (Fig. 1A). A camera (eo-13122M, Edmund Optics Inc., Barrington, USA) with a macro lens (EF 100 mm f/2.8 L Macro IS USM, Cannon, Surrey, UK) was placed directly above the ant, viewing it through a hole in the upper surface of the box. This ensured that the ant's head stayed within the camera's field of view throughout the whole experiment. To reduce extraneous visual input, the box was back lit by two 26 W light sources, and the rest of the room kept in darkness. The camera was attached to a computer and the recordings performed using uEye64 software (IDS Imaging Development Systems GmbH). The visual cue (CS) was a bright blue cardboard rectangle (60 x 45 mm) attached to the syringe in which the US (sucrose 20%), manually delivered to the ant (Fig. 1A).

Ants were subjected to a paired (N=51) or an unpaired (N=29) training. These two types of training were conducted in parallel, in a randomized order. During paired training, the syringe plus cardboard was held a few millimetres in front of the ant for about 10 seconds. The syringe with a drop of sucrose was then moved next to the mouthparts so ants could detect its presence and feed on it for about 5 seconds. Therefore, ants were presented with the CS for 15 seconds, of which the last 5 seconds overlapped with the sucrose reward. Ants were subjected to 10 trials, separated by 5 minutes (Fig. 1B). During unpaired training, the CS (cardboard plus syringe) and the US (drop of sucrose from the syringe) were presented to the ants but temporally dissociated from each other. Between the presentation of either the US or the CS, ants rested for 2.5 minutes. This training consisted of 10 CS presentations and 10 sucrose deliveries, which ensured both paired and unpaired ants were subjected to the CS and US the same number of times. The duration of each US and CS presentations were the same as in the

paired training, of 5 and 10 seconds respectively (Fig. 1C). Ants were tested for memory retention either 10 minutes (paired: N=15; unpaired: N=15) or 1 hour (paired: N=15; unpaired: N=14) after the last trial. In these tests, the visual cue (cardboard plus syringe) was presented to the ant for about 10 seconds, and their response recorded. The US (drop of sucrose) was then delivered to ensure ants were still motivated to feed and respond. The ants' response was recorded in every trial and test. For all the cases, the duration of the CS and US presentations varied slightly because they depended on how long ants took to start feeding. Ants that didn't feed on every trial (33 paired and 7 unpaired) were discarded.

Maxilla-Labium Extension Reflex (MaLER) scores

Ants' response during the presentation of the visual cue during training trials and tests were recorded and separated into three types of behaviour: Full Extension with Movement (FEM) as if feeding; Full Extension without movement (FE); or Partial Extension (PE) of the maxilla-labium or maxillary palps (Fig. 2; Movie S1) (Paul *et al.*, 2002). A positive response was counted every time an ant performed any type of MaLER (Guerrieri & d'Ettoire, 2010) during the CS presentation (data available at Sussex Research Online). If ants were extending or moving their mouthparts spontaneously just prior to a trial, we postponed training or testing for a few seconds. Ants that continuously moved their mouthparts were excluded from our analysis.

Statistical analysis

The distribution of the number of responses from each type of MaLER was analysed using a G-test for replicated goodness-of-fit with a prior expectation of one third of the responses from each type. This was performed with grouped classes (one, two and three or more responses) to avoid classes with low number of responses and adjusted with a William's correction (Sokal & Rohlf, 1995).

The influence of the type of training (paired or unpaired) and of trial number in the ant's response to the CS during training was analysed using Logistic Regression with Mixed Effects (Bates, 2010). To compare the proportions of ants responding in each training trial and in each retention test between paired and unpaired types, we performed G-tests of Independence, adjusted with William's Correction, except for the first trial which was analysed using a Fisher's Exact test due to the low number of responses (Sokal & Rohlf, 1995). The responses on the first trial were included in all statistical analysis, to account for spontaneous response to the CS.

The correlation between the proportions of ants that responded to the CS in the retention tests with the number of times they responded during training was analysed using a Spearman's rank correlation. Logistic regression was also performed to determine how many trials on which ants needed to respond during training to predict whether they would respond in the test (Bates, 2010).

G-tests of independence were performed in Excel, using bespoke code (Sokal & Rohlf, 1995). All other statistical analysis was performed using R (RStudio v1.0.143). For the logistic regression, the 'lme4' package was used (Bates, 2010).

RESULTS

The wood ant Maxilla-Labium Extension Reflex (MaLER) can be conditioned to a visual stimulus

During each paired training trial, ants were presented with a visual stimulus (CS) and sucrose (US). On those training trials in which paired ants responded, they did so with one of three different types of response that varied in the degree to which the mouthparts were extended and moved: Full Extension with Movement (FEM); Full Extension without movement (FE); or Partial Extension (PE) of the maxilla-labium or maxillary palps (see Methods). Typically, ants performed FEM or PE more often than they performed FE. In most cases, ants showed little consistency among trials in the type of MaLER with which they responded, though a few ants did consistently perform FEM or PE (Fig. 3). The distribution of the occurrence of each of the three MaLER types across the ten training trials differed significantly (G-test of Independence, $N=51$, $df=6$, $G_{adjusted}=17.3$, $p<0.01$). Consequently, we display the three types of MaLER separately in subsequent figures, though we considered the MaLER as a single response when analysing responses to the CS. Statistical analysis and learning curves for each type of MaLER are included in the supplementary information (Fig. S1, Table S1).

Ants were exposed to 10 paired ($N=51$) or unpaired ($N=29$) training trials. Naïve ants showed a low tendency to perform MaLER when presented with the visual stimulus (Fig. 4); on the first paired or unpaired trial, prior to training, just 3 to 4% of the ants performed MaLER. There was no significant difference in the spontaneous MaLER rate between paired and unpaired naive ants (Table 1). Increasing the number of training trials led to significant increase in the occurrence of MaLER in the paired ants, which plateaued at around 50% from the third trial onward (Fig. 4A; Logistic regression, $N=51$, $df=507$, $z= 5.949$, $p<0.01$). Conversely, there was no increase during training for unpaired ants (Fig. 4B; Logistic Regression, $N=29$, $df=287$, $z=0.758$, $p=0.45$). Individual ants showed substantial variation in the number and type of

MaLER that they displayed during training (Fig. 4C,D). Considering all trials together, the proportion of ants that responded to the visual cue was significantly higher during paired than unpaired training (Logistic Regression, $N=80$, $df=796$, $z=-5.306$, $p<0.01$). After the first trial, each subsequent trial differed significantly between paired and unpaired ants (Table 1). Taken together, these results suggest that ants associated the visual cue with the sucrose reward contingent upon the timing the CS and US were presented (*i.e.* whether paired or unpaired).

Paired training evoked short- and longer-term appetitive memories

To assess whether wood ants had formed a memory following training, we tested them by presenting the CS alone. Each ant was tested either 10 minutes or 1 hour after the last training trial, which is indicative of short- or a longer-term form of memory, respectively (Guerrieri *et al.*, 2011). After 10 minutes, 53.3% of ants that had received paired training responded during testing compared to just 13.3% of ants that received unpaired training (Fig. 5A). Likewise, after 1 hour, 40% of ants that had received paired training responded during testing compared to 7.48% of ants that received unpaired training (Fig. 5B). In both tests, the proportion of ants responding to the CS was significantly higher if they had undergone paired rather than unpaired training (Fig. 5; G-test of Independence, 10 minutes test: $N=30$, $df=1$, $G_{adjusted}=5.5$, $p<0.05$; 1 hour test: $N=29$, $df=1$, $G_{adjusted}=4.42$, $p<0.05$). Each test was followed by the US (sucrose) to ensure that the ants were still motivated to feed. For both tests, every ant fed. Thus, following paired but not unpaired training, ants acquired short-term and mid-term visual associative memories.

We assessed whether those ants that responded to the presentation of the visual stimulus (CS) with MaLER during training were the same subset of ants that subsequently responded during testing. We pooled both the 10 minute and 1 hour tests together, separated ants that responded during testing from those that did not, and determined whether they had responded during training (Fig. 6A, B); 64.3% of the paired ants that responded during testing also responded during training at least half of the trials, whereas only 18.8% of the ants that failed to respond during testing responded on at least half of the trials during training (Fig. 6A). Indeed, ants that responded during testing also responded significantly more during training compared to ants that didn't (Logistic Regression, $N=30$, $df=306$, $z=3.775$, $p<0.01$). In contrast, only three ants of the 29 ants that had undergone unpaired training responded during testing, and just one of those three ants had performed MaLER on at least half the trials during training. Therefore, ants' behaviour during training was similar to that during testing, however,

there were a higher number responses during paired training and testing than during unpaired training and testing.

Next, we analysed whether the number of trials on which an ant responded during paired training was correlated with their response during testing. Combining both tests together, we observed that the percentage of ants that responded during testing correlated with their number of trials on which they responded during training (Fig. 6C; Spearman's Rank Correlation, $N=10$, $df=9$, $Rho=0.812$, $p<0.01$). We examined whether the number of trials on which an individual ant responded during the training could predict its response during testing. The probability of performing MaLER during testing increased significantly with the number of trials on which ants responded during training (Fig. 6D; Logistic Regression, $N=31$, $df=29$, $z=2.79$, $p<0.01$). Furthermore, ants that responded on four or more trials during training were significantly more likely to respond during testing than those that had not (Logistic Regression, $N=30$, $df=29$, $z=2.725$, $p<0.01$; Table 2), which is indicative of a threshold during training for short- and mid-term memory formation.

DISCUSSION

Our aim was to develop a classical conditioning paradigm to analyse the acquisition and retention of visual associative memories in restrained wood ants. Here, we have shown that this is possible by making use of the maxilla-labium extension reflex (MaLER), using a paradigm modified from earlier ones designed for appetitive olfactory classical conditioning (Guerrieri & d'Ettorre, 2010; Guerrieri *et al.*, 2011). We showed that in wood ants MaLER can be used as the unconditional response (UR) in classical conditioning experiments. This response to a visual conditioned stimulus (CS) is initially low but increases when paired with an appetitive unconditioned stimulus (US), sucrose. After three or more training trials, 50% of the ants respond to the CS before sucrose is given. To ensure ants learnt the intended CS and not any other feature, we used an unpaired control in which all the CS and US presentations were temporally dissociated from each other. Because unpaired ants didn't increase their responsiveness to the CS, we conclude that the key visual feature learnt by paired ants was the intended CS.

In classical conditioning experiments, it is important to have a detailed description of the behaviour we consider the UR because this directly affects how learning and memory is quantified. Our analysis identified different degrees of MaLER expression that contrasts with previous studies that have reported a single type of response (Bitterman *et al.*, 1983; Guerrieri

& d’Ettorre, 2010) but is similar to the palp opening reflex of desert locusts, which also includes flicking, palpation or outward opening of the maxillary palps (Simões *et al.*, 2011).

Our experiments also showed that wood ants can form a memory of the association between the visual CS and the US, and retain it for at least 1 hour. This memory is contingent upon the timing of the CS and US presentations; ants that were trained with unpaired presentations of CS and US did not show memory formation. Furthermore, the propensity of an individual ant to retain a memory of the CS/US association for 10 minutes or 1 hour after training depends upon the number of training trials on which they respond. Previous studies have interpreted these times as short- and early mid-term memory, respectively (Guerrieri *et al.*, 2011). Assessing longer-term retention of memories up to 24 hours was not possible because restrained ants did not survive long enough for testing. This may indicate that restrained animals are subjected to high levels of stress, which is known to negatively influence learning (Bateson *et al.*, 2011). However, our method of restraint allows ants to adopt a natural stance and to move their limbs and antennae freely (Fig. 1A), whilst ensuring that the CS and US presentations as well as the number of trials and the inter-trial interval are controlled, and that associative memories are acquired only by Pavlovian association. Thus, it seems unlikely that restraint adversely affects the rates of learning we report.

Despite differences in methodology, our training paradigm produced similar rates of learning (~50%) to previous studies using classical conditioning of a visual cue performed with honey bees (Hori *et al.*, 2007; Balamurali *et al.*, 2015; Niggebrügge *et al.*, 2009). Hori *et al.* (2007) and Balamurali *et al.* (2015) achieved a learning rates of ~40-50% in restrained honey bees. Niggebrügge *et al.* (2009) achieved higher response rates of ~60-80% with a visual cue in restrained honey bees but ablated the bees’ antennae, in contrast to our methodology and that of Balamurali *et al.* (2015) in which the antennae were intact (Avarguès-Weber & Mota, 2016). It is possible that higher rates of learning could be achieved in wood ants were the antennae ablated. Yet despite the different paradigms for producing visual classical conditioning, wood ants, like honey bees, can form associative memories. This ability of wood ants, despite their relatively small compound eyes and visual systems compared to those of honey bees (Perl & Niven, 2016; Jander & Jander, 2002), emphasises that this is not restricted to insects with a substantial investment in vision, but is more widespread among the Hymenoptera.

Previous studies of learning in wood ants have employed freely moving animals (e.g. Collett & Collett, 2002; Graham *et al.*, 2004; Harris *et al.*, 2005; Harris *et al.*, 2007). In these experiments, ants move in a specific direction to acquire sucrose, which reflects their natural

foraging strategy; ants leave the nest and walk to locate aphids, which are a source of honeydew, a sugar-rich secretion upon which they feed. To feed directly on aphid honeydew, wood ants forage through cluttered environments along foraging trails that can extend for 100 metres (for review see Robinson, 2005). Whether searching for sucrose rewards in reduced experimental paradigms or foraging in the natural environments for honeydew, ants are exposed to numerous visual cues. Yet how the sequences of visual cues encountered upon a foraging route are associated with the final reward from the feeder or an aphid remains unclear. It is unlikely that our learning paradigm, in which a single visual cue is presented briefly prior to obtaining a reward, captures the process of learning and memory formation along foraging routes fully. Instead, it is more akin to the final moments of foraging immediately before the reward is received. Whether sequences of visual cues with increased duration between the cues and a reward that more closely resemble natural foraging can be learnt within our experimental paradigm remains unclear. Nevertheless, our study provides the first evidence that wood ants can form visual associative memories even when restrained and lacking the context of navigating through the environment.

Wood ants have been used extensively as a model system for studying navigation and visual learning in insects, producing many insights into mechanisms underpinning these behaviours (Collett & Collett, 2002; Graham *et al.*, 2004; Harris *et al.*, 2005; Harris *et al.*, 2007). However, these studies have used freely moving ants so that insights into the neural circuits underlying the formation of memories in wood ants are restricted to insights that can be inferred from behavioural tests (e.g. Fernandes *et al.*, 2015). Our study provides a method for analysing visual learning in restrained ants, which opens up new experimental possibilities for determining the neural basis of navigation and visual learning in wood ants. Although removed from wood ant's natural foraging strategies, our paradigm permits control over the timing and duration of learnt cues and rewards, as well as the number of trials individual ants are subjected to, allowing a more detailed understanding of how precisely wood ants form the visual memories upon which their foraging depends.

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Author contributions A.S.D.F., C.B. and J.E.N. conceived the project. A.S.D.F. and J.E.N. designed and performed the experiments and analysis. A.S.D.F. and J.E.N. wrote the paper with input from C.B.

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Figures

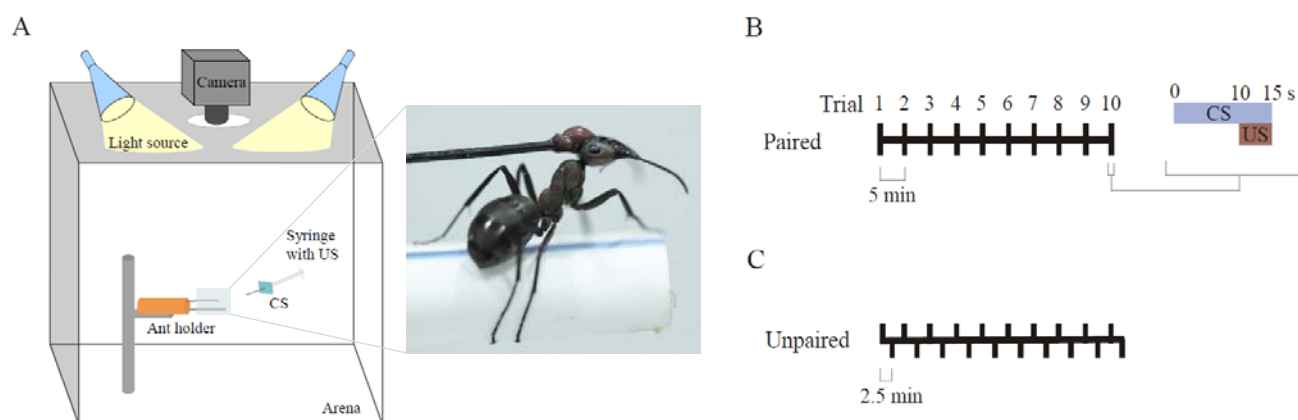


Fig. 1. Experimental set up and training scheme. A) The ant was placed inside a Perspex box illuminated by two light sources, directly underneath the camera. It was fixed to an insect pin attached to a Plasticine cylinder (orange) supported by a holder, keeping a naturalistic stance (see Methods). The conditioned stimulus (CS) and unconditioned stimulus (US) are represented by the blue square attached to the syringe with the sugar reward, respectively. *Inset.* A close-up view of an ant in the holder. The ant's head is fixed with wax. Ants were subjected to two types of training, B) paired or C) unpaired.

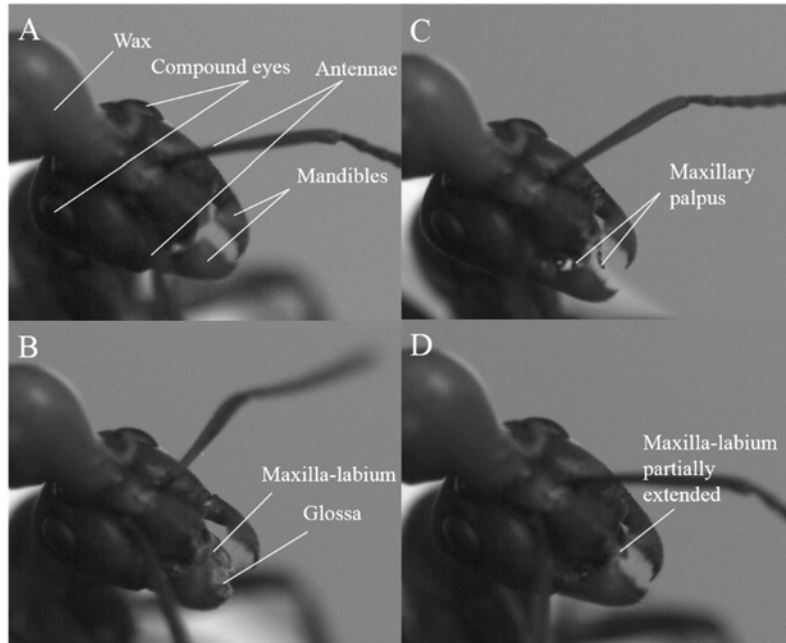


Fig. 2. The Maxilla-Labium Extension Response of wood ants. Individual frames from video recordings show ants' mouth part movements during training. A) no response; B) full extension of the maxilla-labium that terminate in the glossa; C) partial extension with only the maxillary palpus visible; and D) partial extension of the maxilla-labium structures.

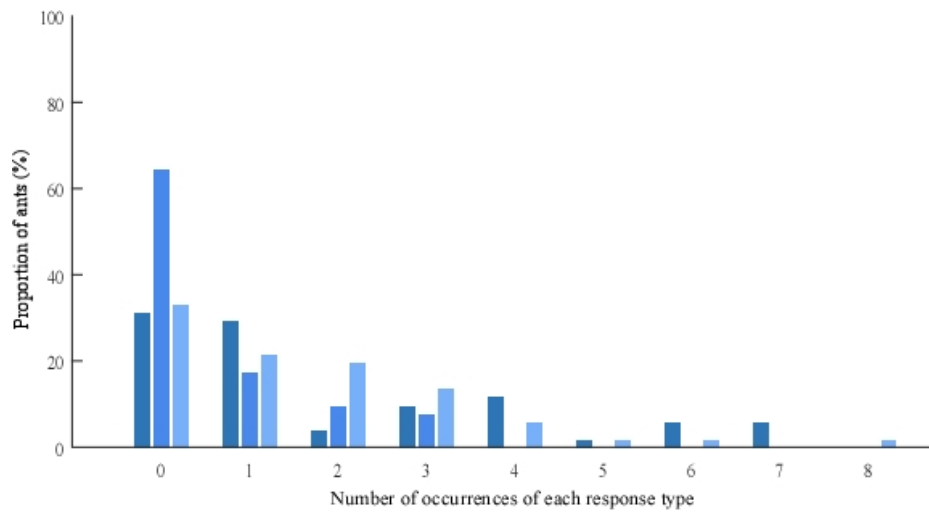


Fig. 3. Consistency in the Maxilla-Labium Extension Response (MaLER) to the CS during paired training. Ants (N=51) perform full extension with movement (FEM; dark blue), full extension (FE; mid blue) or partial extension (PE; light blue).

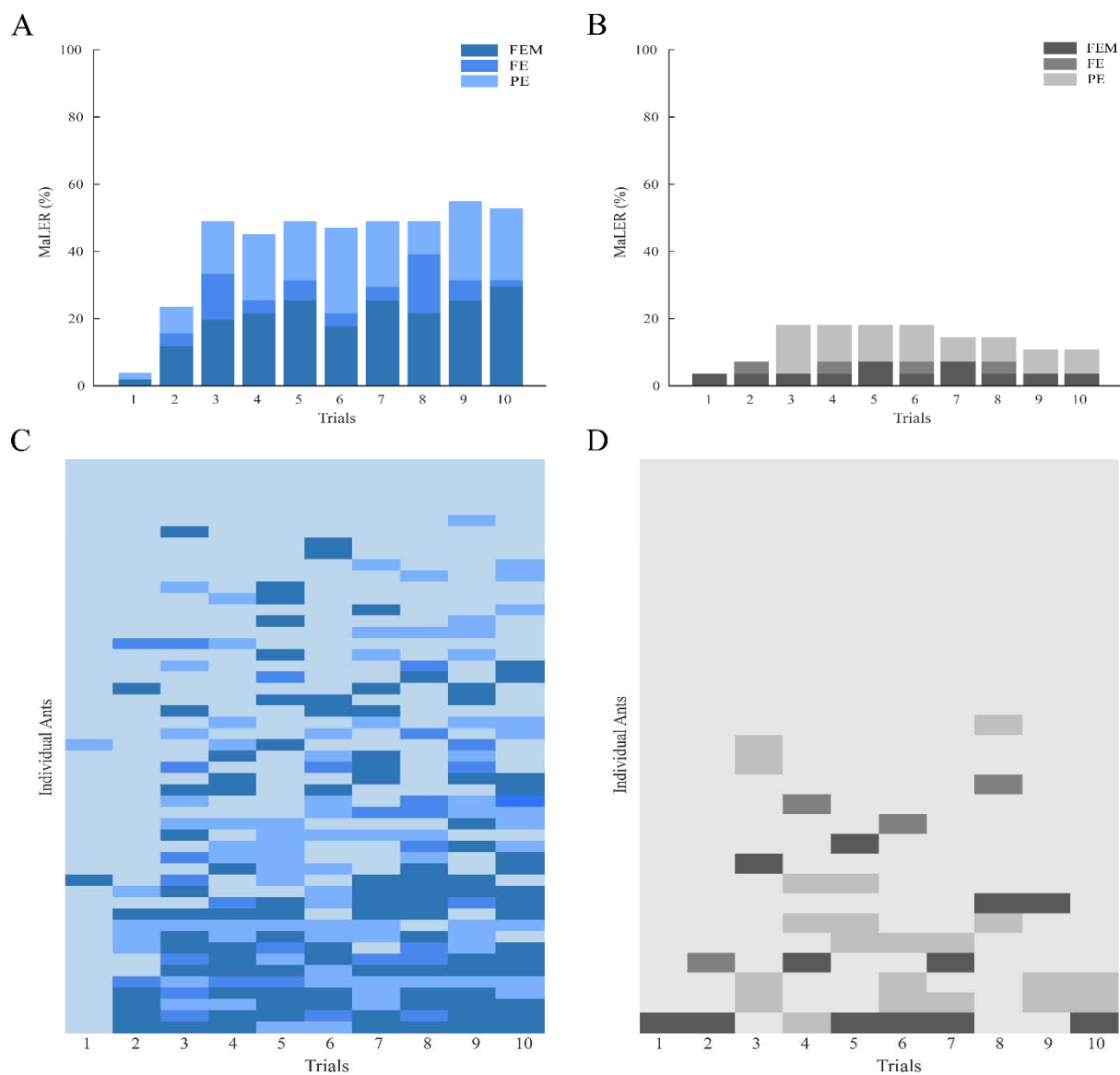


Fig. 4. Wood ants learn to associate a visual cue with a paired sugar reward. A) The percentage of paired ants (N=51) performing MaLER in response to the CS presentation significantly increased throughout training. B) The percentage of ants performing MaLER did not increase significantly throughout unpaired training (N=29). C) Individual performance of paired ants, and D) unpaired ants during training. The three types of MaLER are represented in dark (FEM), medium (FE) and light (PE) blue or grey.

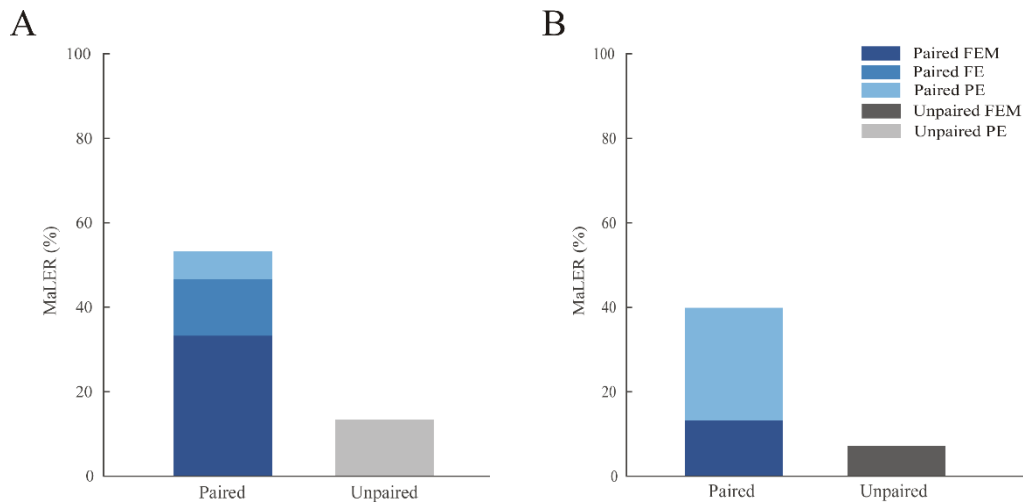


Fig. 5. Wood ants retain appetitive memories for up to 1 hour. Ants were tested A) 10 minutes (paired: N=15; unpaired: N=15) or B) 1 hour (paired: N=15; unpaired: N=14) after the last training trial. The percentage of ants responding to the CS alone after paired training is shown in blue. Ants responding to the CS alone after unpaired training are shown in grey. The three types of MaLER are represented in dark (FEM), medium (FE) and light (PE) tones.

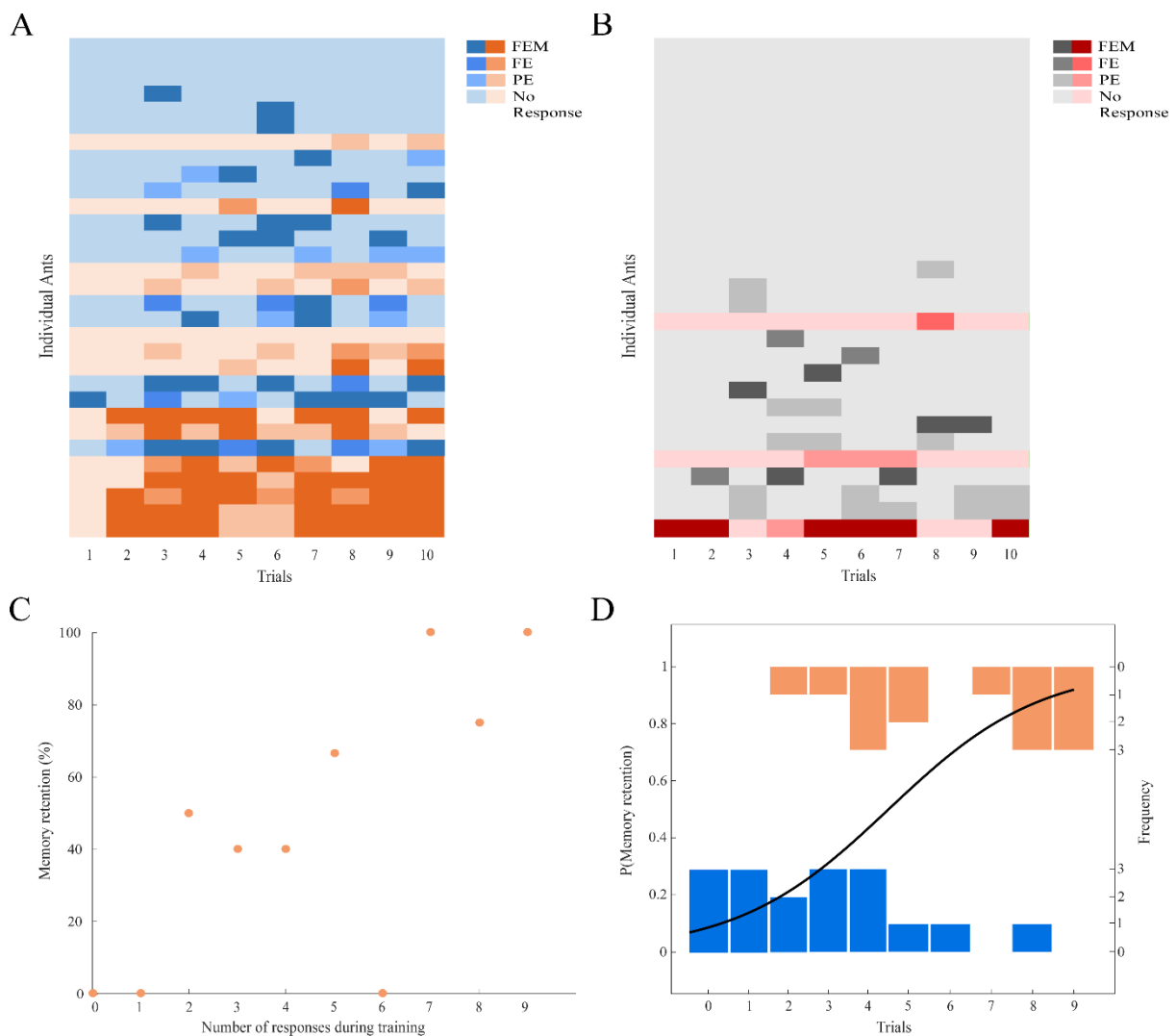


Fig. 6. Wood ants' responses during memory tests are predicted by their behaviour during training. A) Responses of individual ants during paired training (N=30). Those that did not respond during subsequent testing are blue, whereas those that responded are orange. B) As in A but for unpaired training (N=29). Ants that did not respond are grey whereas those that did are red. C) The percentage of paired ants that perform MaLER in response to the CS in the tests correlates significantly with the number of times they responded to the CS during training. D) The more trials on which an ant responded during paired training, the higher the probability of responding in the test (black line). Ants that did not respond during testing (blue) cluster around lower numbers of responses during training while ants that did (orange) cluster around higher number of responses.

Tables

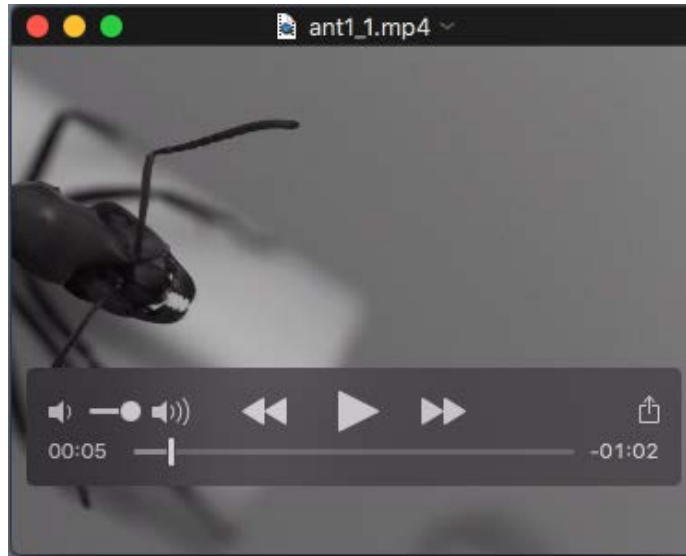
Trial	N	df	G (adjusted)	P
1	80	na	na	>0.1
2	80	1	3.86	<0.05
3	80	1	8.41	<0.01
4	80	1	6.63	<0.01
5	80	1	8.41	<0.01
6	80	1	7.5	<0.01
7	80	1	10.69	<0.01
8	80	1	11.76	<0.01
9	80	1	17.13	<0.01
10	80	1	17.13	<0.01

Table 1. Comparison of the frequency of ants showing MaLER responses between paired and unpaired ants for each trial. The number of ants (N), degrees of freedom (df), G-test of independence (G) and p-value are shown. The first trial was analysed with a Fisher's exact test.

Number of responses in training	N	df	z	p
1 or more	31	30	0.008	0.994
2 or more	31	30	0.007	0.994
3 or more	31	30	0.009	0.993
4 or more	31	30	2.752	0.006**
5 or more	31	30	2.774	0.006**
6 or more	31	30	2.392	0.017 *
7 or more	31	30	2.554	0.011*
8 or more	31	30	2.29	0.022*
9 or more	31	30	0.008	0.994

Table 2. The probability of ants responding during memory tests is predicted by the number of training trials on which they responded to the CS. The number of ants (N), degrees of freedom (df), Logistic regression (z) and p-value are shown.

SUPPLEMENTARY INFORMATION



Movie S1. Response to the CS from one ant during paired training, showing the three types of MaLER.

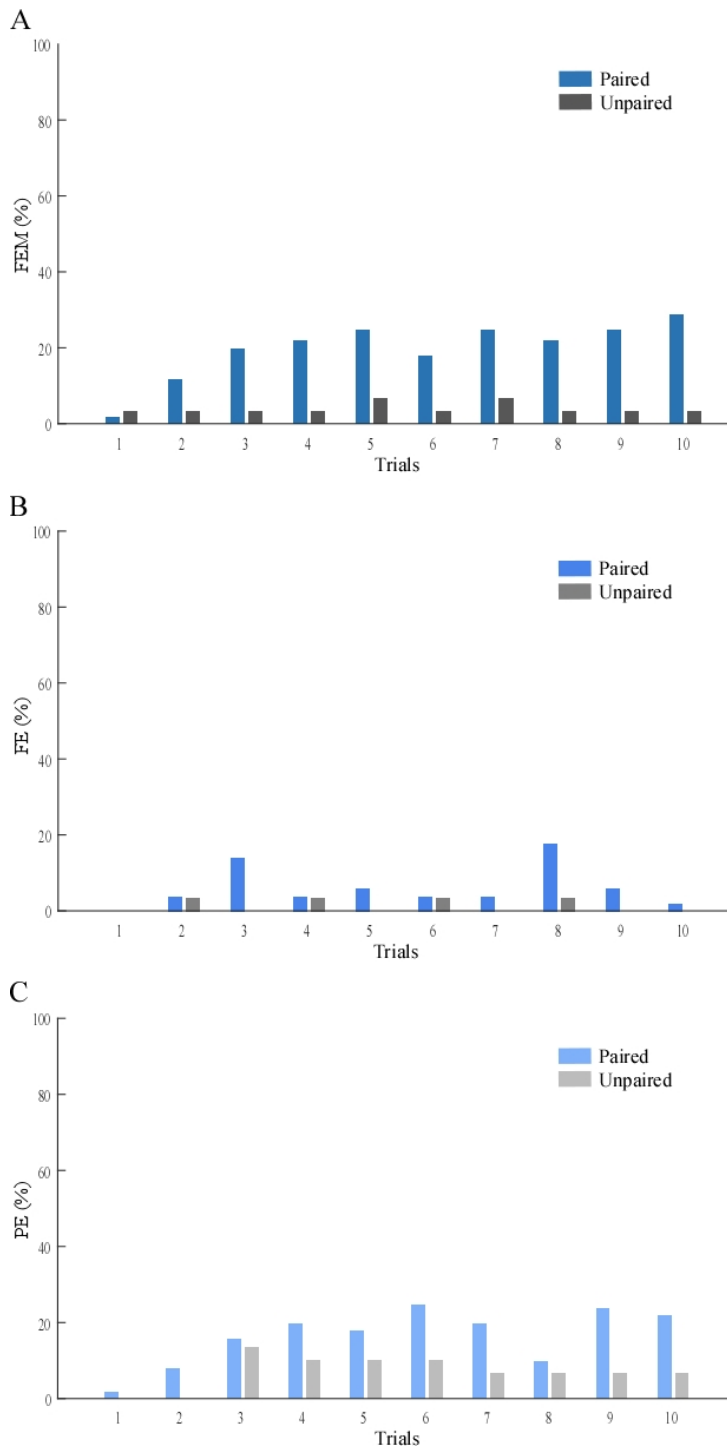


Fig. S1. Learning curves based on each type of MaLER. A) The percentage of paired ants (N=51) performing FEM in response to the CS presentation increased throughout training, but not for unpaired ants (N=29). B) The percentage of ants (paired: N=51; unpaired: N=29) performing FE in response to the CS presentation didn't increase during training for both paired and unpaired ants. C) The percentage of paired ants (N=51) performing PE in response to the CS presentation increased throughout training, but not for unpaired ants (N=29).

Type of training	Type of MaLER	N	df	z	p
Paired	FEM	51	50	3.868	0.0001**
	FE	51	50	0.835	0.404
	PE	51	50	2.975	0.0029**
Unpaired	FEM	29	28	0.129	0.8977
	FE	29	28	0.349	0.7267
	PE	29	28	0.839	0.402

Table S1. Influence of training in ants' responses to the CS, for each type of MaLER. The number of ants (N), degrees of freedom (df), Logistic regression (z) and p-value are shown.