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RESEARCH ARTICLE

Visual associative learning in wood ants

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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 1 h. 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 and 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 and 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 and 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 and Collett, 2002; Durier et al., 2003; Graham et al., 2004; Harris et al., 2005, 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 a PER, and can form a long-term memory of this association (Wittstock et al., 1993). Other species of bees (*Bombus terrestris*, *Melipona quadrifasciata*, *Scaptotrigona depilis*) and flies (*Musca domestica*, *Drosophila melanogaster*) also perform the 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 a proboscis, a similar paradigm can be used, though the response involves other mouthparts. For example, ants (*Camponotus aethiops* and *Camponotus fellah*) perform the maxilla–labium extension reflex (MaLER) (Guerrieri and d'Ettorre, 2010; Guerrieri et al., 2011), and locusts (*Schistocerca gregaria*) perform the palp opening response (POR) (Simões et al., 2011, 2012, 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 and Mota, 2016) using a visual cue as the CS. Although visual plus olfactory cues and olfactory cues alone produce comparable learning rates, using visual cues alone as the CS seems to produce weaker learning (Gerber and 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 and Collett, 2002; Durier et al., 2003; Graham et al., 2004; Harris et al., 2005, 2007). We show for the first time that ants can acquire and retain short- and mid-term visual memories when harnessed, demonstrating visual learning through classical conditioning.

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MATERIALS AND 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 h light:12 h dark cycle and fed with sucrose (33.3%). These conditions were maintained throughout our experiments except that the colonies were starved for at least 2 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 min 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 in 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 2 h prior to training.

Training and testing

Experiments were conducted in a transparent Perspex box (50×50×50 cm) covered in white paper, open to the front to permit experimenter access (Fig. 1A). A camera (eo-13122 mol l^{-1}, Edmund Optics Inc., Barrington, NJ, USA) with a macro lens (EF 100 mm f/2.8 L Macro IS USM, Canon, 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 was kept in darkness. The camera was attached to a computer and recordings were performed using uEye64 software (IDS Imaging Development Systems GmbH, Obersulm, Germany). The visual cue (CS) was a bright blue cardboard rectangle (60×45 mm) attached to the syringe in which the US (sucrose 20%) was manually delivered to the ant (Fig. 1A).

Ants were subjected to paired (N=51) or 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 s. 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 s. Therefore, ants were presented with the CS for 15 s, of which the last 5 s overlapped with the sucrose reward. Ants were subjected to 10 trials, separated by 5 min (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 min. This training consisted of 10 CS presentations and 10 sucrose deliveries, which ensured that ants in the paired and unpaired training groups were subjected to the CS and US the same number of times. The duration of each US and CS presentation was the same as in the paired training: 5 and 10 s, respectively (Fig. 1C). Ants were tested for memory retention either 10 min (paired: N=15; unpaired: N=15) or 1 h (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 s, 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 did not feed on every trial (33 paired and 7 unpaired) were discarded.

MaLER scores

Ants' responses during the presentation of the visual cue in 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 1) (Paul et al., 2002). A positive response was counted every time an ant performed any type of MaLER (Guerrieri and d'Ettorre, 2010) during the CS presentation (data available from figshare: https://doi.org/10.25377/sussex.5794386). 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 a low number of responses and adjusted with a William's correction (Sokal and 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 proportion of ants responding in each training trial and in each retention test between paired and unpaired types, we
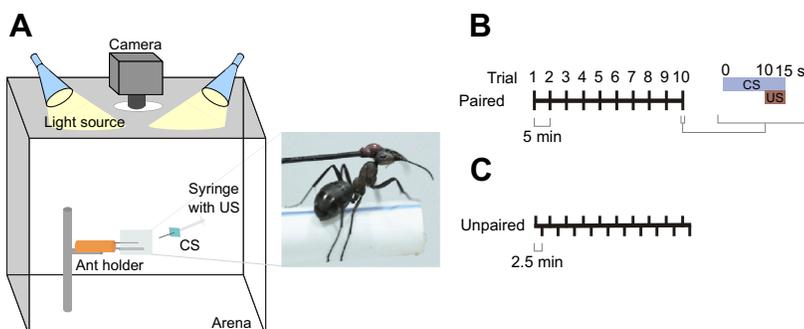

Fig. 1. Experimental set up and training scheme. (A) The ant was placed inside a Perspex box illuminated by two light sources, directly underneath the camera. It was fixed to an insect pin attached to a Plasticine cylinder (orange) supported by a holder, ensuring that it retained a naturalistic stance (see Materials and methods). The conditioned stimulus (CS) and unconditioned stimulus (US) are represented by the blue square (CS) attached to the syringe with the sugar reward (UC). 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. Vertical black lines represent the US and CS, which were temporally dissociated in unpaired training (see Materials and methods).
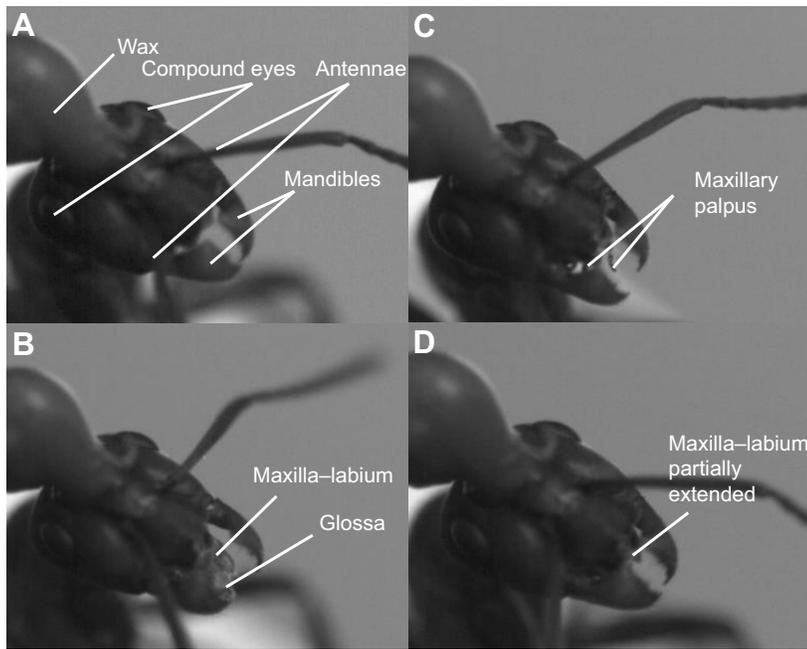
Fig. 2. The maxilla–labium extension response (MaLER) of wood ants. Individual frames from video recordings show ants' mouthpart movements during training. (A) No response; (B) full extension of the maxilla–labium that terminate in the glossa; (C) partial extension with only the maxillary palpus visible; and (D) partial extension of the maxilla–labium structures.

performed *G*-tests of independence, adjusted with William's correction, except for the first trial, which was analysed using Fisher's exact test because of the low number of responses (Sokal and Rohlf, 1995). The responses on the first trial were included in all statistical analysis, to account for spontaneous responses to the CS.

The correlation between the proportion of ants that responded to the CS in the retention tests and the number of times they responded during training was analysed using Spearman's rank correlation. Logistic regression was also performed to determine the number of trials in which ants needed to show a response 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 and 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 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: FEM, FE or PE of the maxilla–labium or maxillary palps (see Materials and 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, 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 10 training trials differed significantly (*G*-test of independence, $N=51$, d.f.$=6$, $G_{adjusted}=17.3$, $P<0.01$). 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. Naive 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 3–4% 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 a significant increase in the occurrence of MaLER in the paired ants, which plateaued at around 50% from the third trial onward (Fig. 4A;
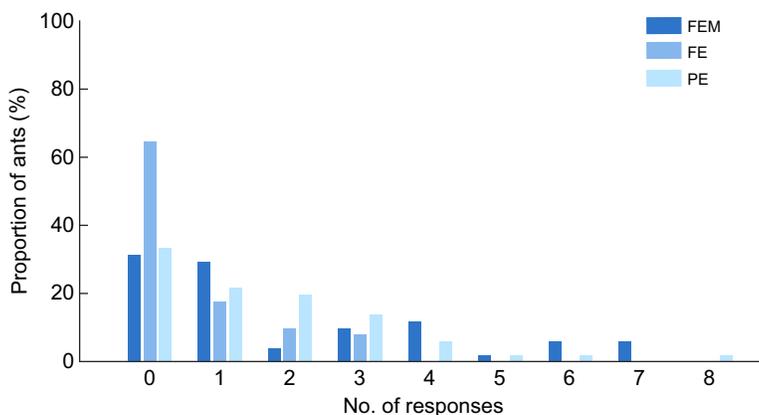

Fig. 3. Consistency in the MaLER to the CS during paired training. Ants ($N=51$) performed full extension with movement (FEM; dark blue), full extension (FE; mid-blue) or partial extension (PE; light blue).
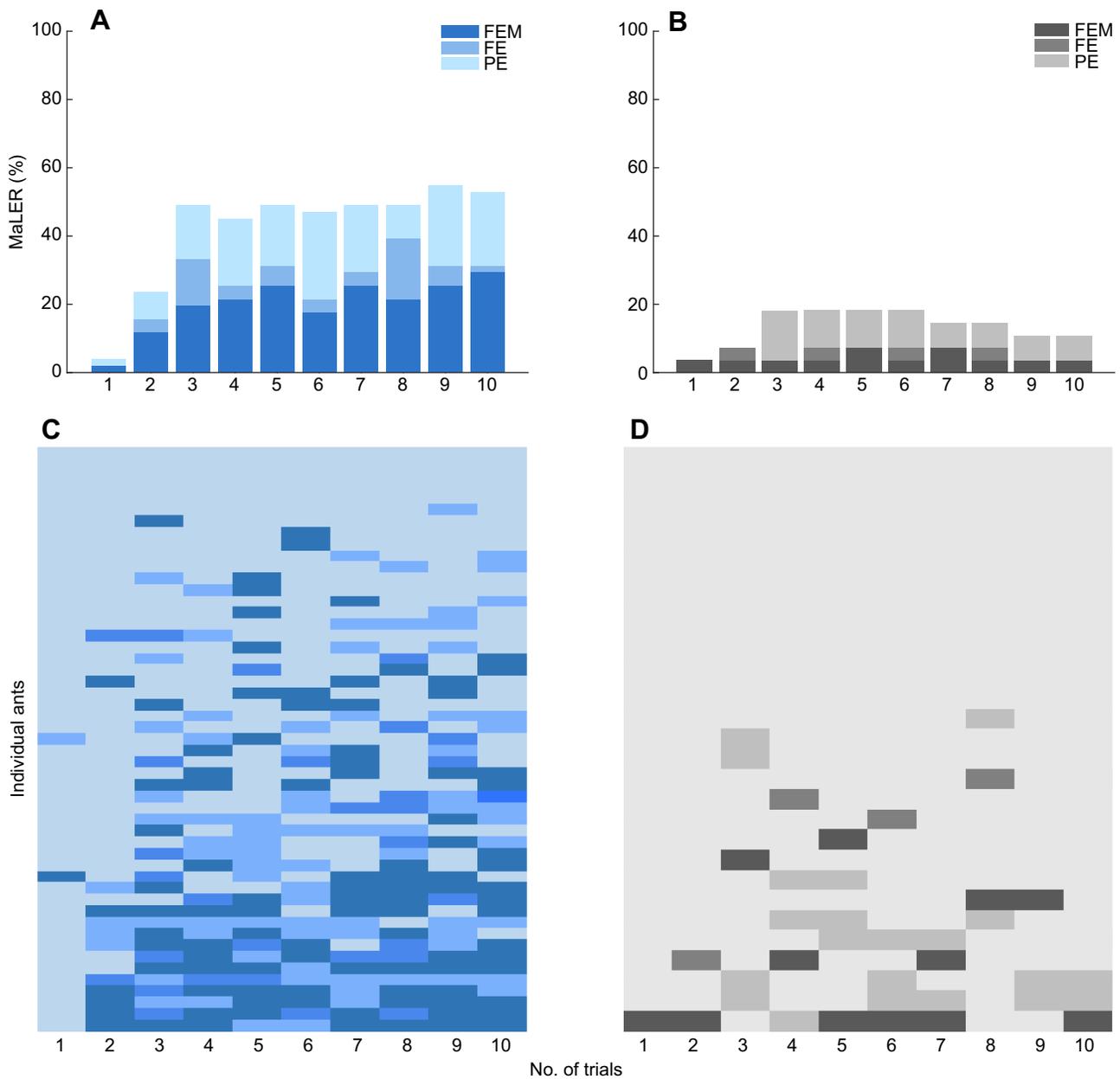
Fig. 4. Wood ants learn to associate a visual cue with a paired sugar reward. (A) The percentage of ants (N=51) performing the MaLER following the CS presentation significantly increased throughout paired training. (B) The percentage of ants performing the MaLER did not increase significantly throughout 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.

logistic regression, $N=51$, d.f.$=507$, $z=5.949$, $P<0.01$). Conversely, there was no increase during training for unpaired ants (Fig. 4B; logistic regression, $N=29$, d.f.$=287$, $z=0.758$, $P=0.45$). 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 during unpaired training (logistic regression, $N=80$, d.f.$=796$, $z=-5.306$, $P<0.01$). 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 of presentation of the CS and US (i.e. whether paired or unpaired).

Paired training evokes short- and mid-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 min or 1 h after the last training trial, which is indicative of a short-term or a mid-term form of memory, respectively (Guerrieri et al., 2011). After 10 min, 53.3% of ants that had received paired training responded during testing compared with just 13.3% of ants that had received unpaired training (Fig. 5A). Likewise, after 1 h, 40% of ants that had received paired training responded during testing compared with 7.48% of ants that had received unpaired training (Fig. 5B). In both tests, the proportion of ants responding to the CS was significantly higher if

Table 1. Comparison of the frequency of ants showing the maxilla–labium extension response (MaLER) between paired and unpaired ants for each trial

Trial	N	d.f.	G (adjusted)	P
1	80	n.a.	n.a.	>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

The number of ants (N), degrees of freedom (d.f.), G-test of independence (G) and P-value are shown. The first trial was analysed with Fisher's exact test.

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

No. of responses in training	N	d.f.	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

The number of ants (N), degrees of freedom (d.f.), logistic regression (z) and P-value are shown (*$P<0.05$, **$P<0.01$).

they had undergone paired rather than unpaired training (Fig. 5; G-test of independence, 10 min test: $N=30$, d.f.=1, $G_{adjusted}=5.5$, $P<0.05$; 1 h test: $N=29$, d.f.=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 the 10 min and 1 h tests, 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 in at least half of the trials, whereas only 18.8% of the ants that failed to respond during testing responded in at least half of the trials during training (Fig. 6A). Indeed, ants that responded during testing also responded significantly more during training compared with ants that did not (logistic regression, $N=30$, d.f.=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 was a higher number of 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 the two tests together, we observed that the percentage of ants that responded during testing correlated with the number of trials on which they responded during training (Fig. 6C; Spearman's rank correlation, $N=10$, d.f.=9, $\rho=0.812$, $P<0.01$). We also examined whether the number of trials on which an individual ant responded during training could predict its response during testing. The probability of performing MaLER during testing increased significantly with the number of trials in which ants responded during training (Fig. 6D; logistic regression, $N=31$, d.f.=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 did not (logistic regression, $N=30$, d.f.=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 MaLER, using a paradigm modified from earlier ones designed for appetitive olfactory classical conditioning (Guerrieri and d'Ettorre, 2010; Guerrieri et al., 2011). We showed that in wood ants, MaLER can be used as the UR in classical conditioning experiments. This response to a visual CS is initially low but increases when paired with an appetitive US, sucrose. After three or more training trials, 50% of the ants respond to the CS before sucrose was given. To ensure ants learnt the intended CS and not any other feature, we used an unpaired control in which all the
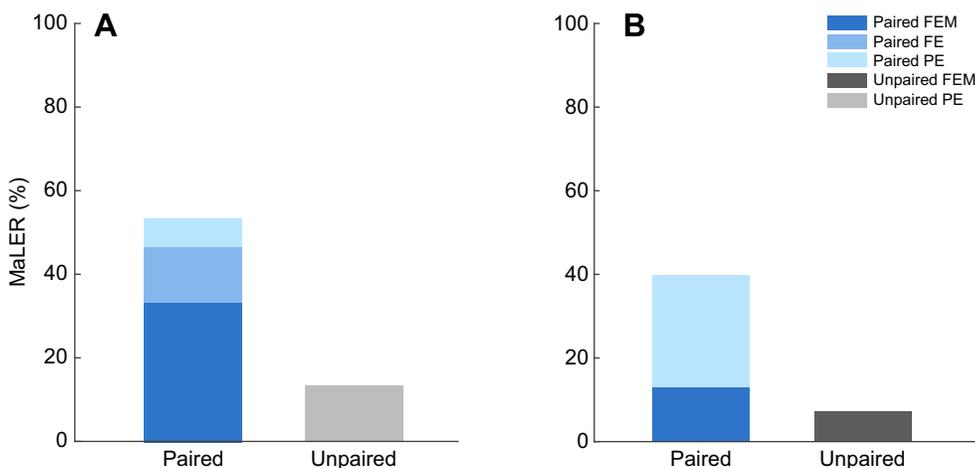

Fig. 5. Wood ants retain appetitive memories for at least 1 h. Ants were tested (A) 10 min (paired: $N=15$; unpaired: $N=15$) or (B) 1 h (paired: $N=15$; unpaired: $N=14$) after the last training trial. The percentage of ants responding to the CS alone after paired training is shown in blue, and the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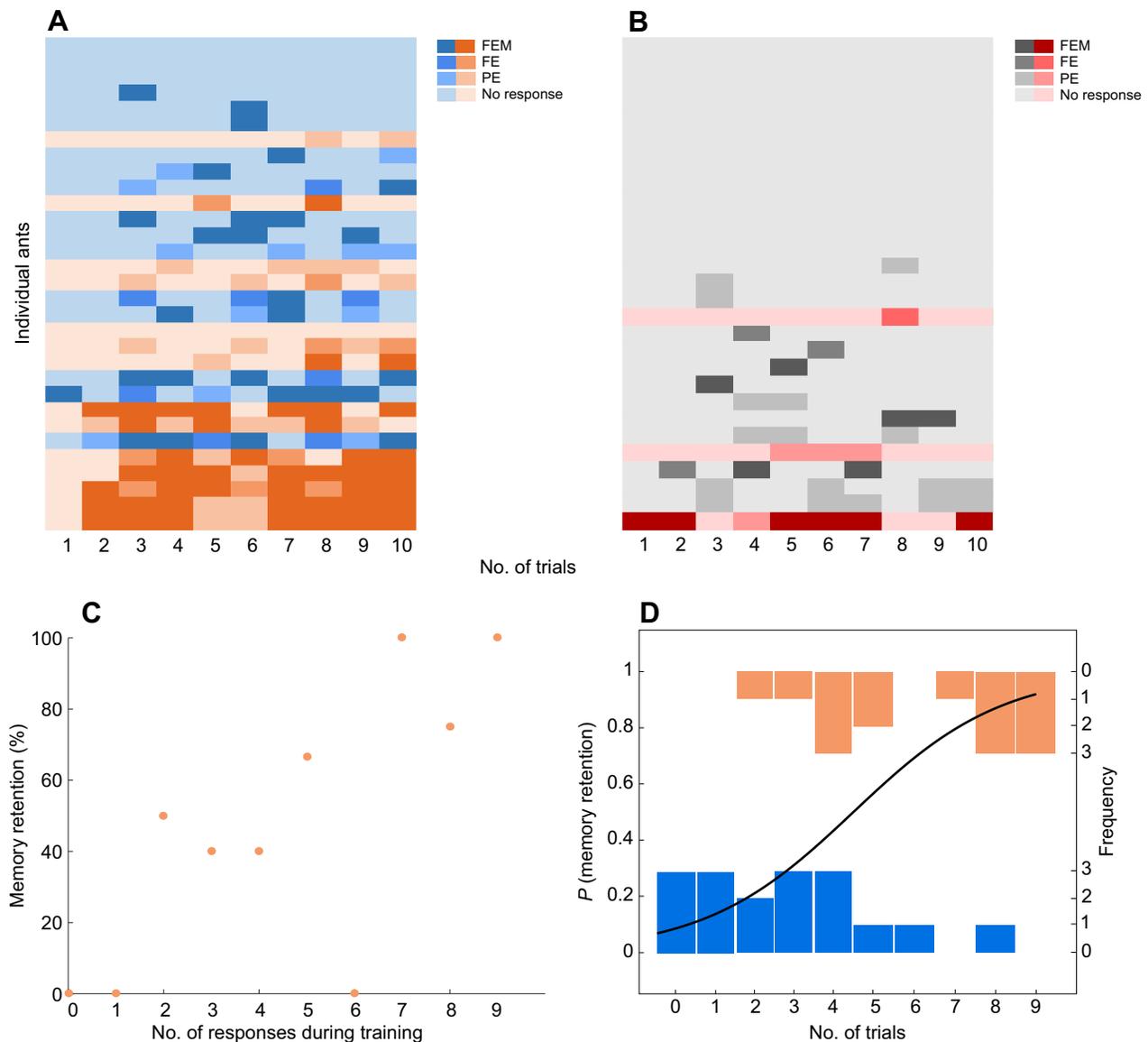
Legend (Fig. 5):
- Paired FEM
- Paired FE
- Paired PE
- Unpaired FEM
- Unpaired PE

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 shown in blue, whereas those that responded are shown in orange. (B) As in A but for unpaired training (*N*=29). Ants that did not respond are shown in grey whereas those that did respond are shown in red. (C) The percentage of paired ants that performed MaLER following 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 (*P*) 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 respond (orange) cluster around higher numbers of responses.

CS and US presentations were temporally dissociated from each other. Because ants in the unpaired training group did not 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 to be the UR because this directly affects how learning and memory are quantified. Our analysis identified different degrees of MaLER expression, in contrast with previous studies that have reported a single type of response (Bitterman et al., 1983; Guerrieri and 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 h. This memory was 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 min or 1 h after training depended upon the number of training trials on which they responded. Previous studies have interpreted these times as short- and early mid-term memory, respectively (Guerrieri et al., 2011). Assessing mid-term retention of memories up to 24 h 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 rate 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 and Mota, 2016). It is possible that higher rates of learning could be achieved in wood ants with 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 with those of honey bees (Perl and Niven, 2016; Jander and 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 and Collett, 2002; Graham et al., 2004; Harris et al., 2005, 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 m (for a review, see Robinson, 2005). Whether searching for sucrose rewards in reduced experimental paradigms or foraging in the natural environment for honeydew, ants are exposed to numerous visual cues, but 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 the 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 and Collett, 2002; Graham et al., 2004; Harris et al., 2005, 2007). However, these studies have used freely moving ants, so 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 ants' 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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SUPPLEMENTARY INFORMATION
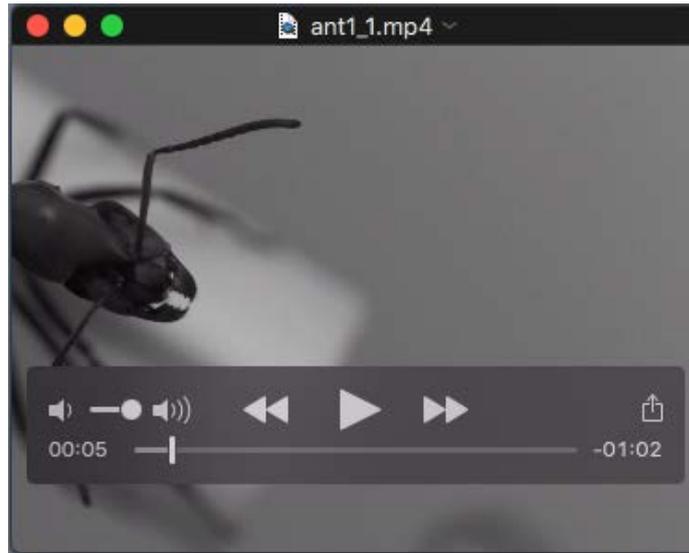
Movie 1. Response to the CS from one ant during paired training, showing the three types of MaLER.
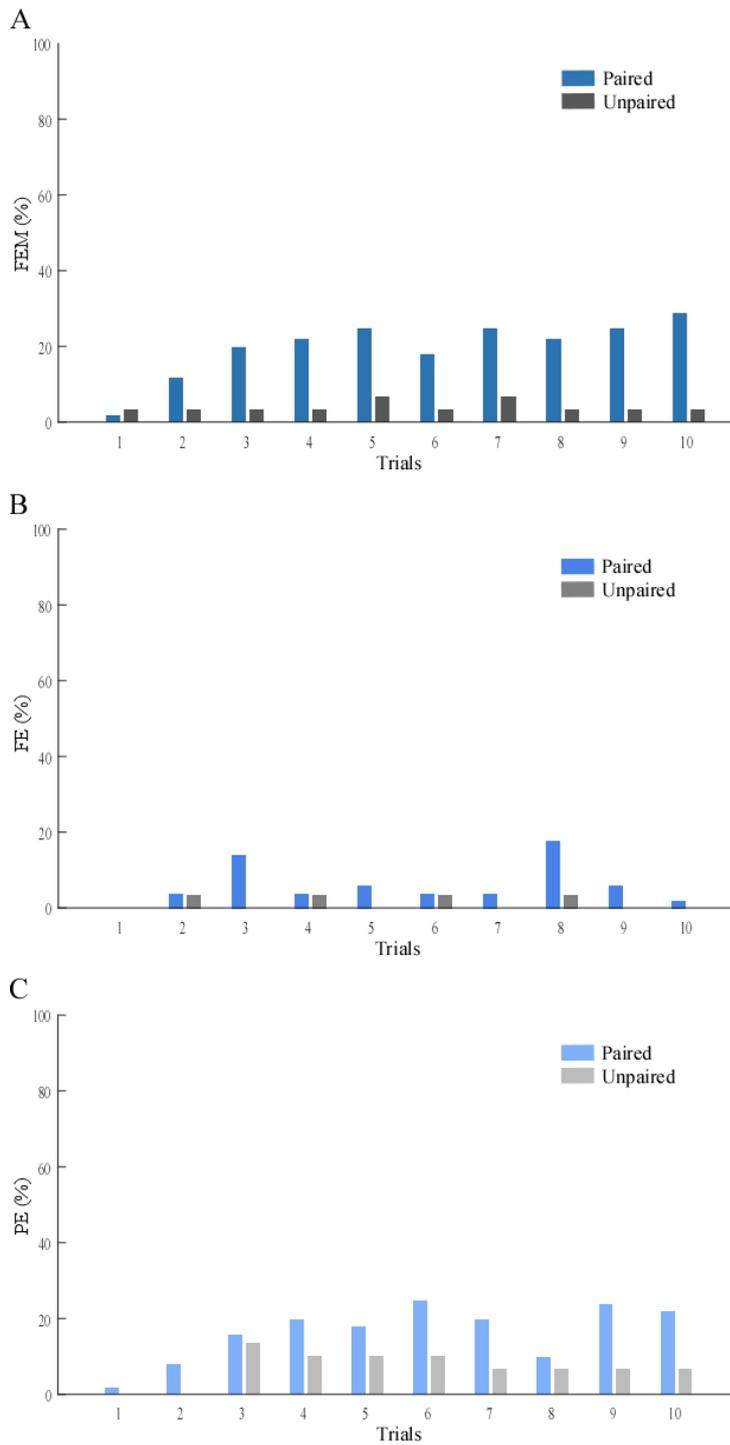
Fig. S1. Learning curves based on each type of MaLER. A) The percentage of paired ants (N=51) performing FEM in response to the CS presentation increased throughout training, but not for unpaired ants (N=29). B) The percentage of ants (paired: N=51; unpaired: N=29) performing FE in response to the CS presentation didn't increase during training for both paired and unaired 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.