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TITLE:

Visual Classical Conditioning in Wood Ants

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SHORT ABSTRACT:
We present a protocol for the classical conditioning of harnessed ants that permits researchers to study visual learning and memory formation at a level of analysis not possible with freely moving individuals.

LONG ABSTRACT:
Several species of insects have become model systems for studying learning and memory formation. Although many studies focus on freely moving animals, studies implementing classical conditioning paradigms with harnessed insects have been important for investigating the exact cues that individuals learn and the neural mechanisms underlying learning and memory formation. Here we present a protocol for evoking visual associative learning in wood ants through classical conditioning. In this paradigm, ants are harnessed and presented with a visual cue (a blue cardboard), the conditional stimulus (CS), paired with an appetitive sugar reward, the unconditional stimulus (US). Ants perform a Maxilla-Labium Extension Reflex (MaLER), the unconditional response (UR), which can be used as a readout for learning. Training consists of 10 trials, separated by a 5-minute intertrial interval (ITI). Ants are also tested for memory retention 10 minutes or 1 hour after training. This protocol has the potential to allow researchers to analyze, in a precise and controlled manner, the details of visual memory formation and the neural basis of learning and memory formation in wood ants.

INTRODUCTION:

Insects have been extensively used as models for studying learning and memory formation[1]. A particularly successful vein of research involves using classical conditioning of restrained animals, which allows precise control over the cues being learned and permits researchers to investigate the neural mechanisms underpinning learning and memory. The majority of studies have focused on the appetitive classical conditioning of honeybee workers, *Apis mellifera*. The honeybee workers are trained to associate a CS with a US that evokes the UR. In this paradigm, originally developed by Takeda[2] and Bitterman *et al.*[3], the UR is the Proboscis Extension Reflex (PER), the US is sugar, and the CS is an odor. Bees learn the association between the CS and the US and can form a long-term memory of this association.

The original paradigm using the PER as the UR has been used to unravel the neural pathways underpinning olfactory learning in bees[4]. It has been modified in several ways to test learning of different sensory stimuli, including visual stimuli[5-7], to incorporate aversive learning using suppression of the PER[8] or the Sting Extension Reflex (SER)[9] as the UR, and to test learning in other species, such as bumblebees[10] and fruit flies[11]. Although memory formation with several modalities has been studied through classical conditioning, visual learning is still difficult to observe using this approach, even in species that show a high degree of visual learning ability during foraging, such as honeybees.

Recent studies have applied a similar approach to insects that do not have a proboscis, such as locusts, which perform the Palp Opening Response (POR)[12], and ants, which perform the Maxilla-Labium Extension Reflex (MaLER)[13]. This has already revealed phase-specific learning abilities that match the phase-specific feeding strategies of the two different desert locust phenotypes, the gregarious and the solitarious forms, re-enforcing the idea that memory formation needs to match ecological needs[14]. Furthermore, studies on olfactory learning in ants has shown similarities between ants and honeybees in memory formation and retention, with long-term memory retention, 72 hours after training, being dependent on protein synthesis[15].

These adaptations of the original paradigm have allowed learning and memory formation to be studied with many modalities and in several model species. This is essential for identifying mechanisms of memory formation common to the insects but also for identifying differences that reflect the particular ecology of learning and memory in different species. The main goal of the protocol we describe here is to provide a way to perform classical conditioning experiments using a visual conditional stimulus with a widely studied ant species, *Formica rufa*. It is issued from our study of visual learning in wood ants[16], which is also an adaptation of visual classical conditioning paradigms.

PROTOCOL:

1. Maintaining *Formica rufa* Colonies

Note: The wood ant (*Formica rufa*, L.) colonies used for this study were collected from Ashdown Forest, Sussex, UK (N 51 4.680, E 0 1.800). Within the UK, *F. rufa* colonies should be collected between July and August. A good proportion of the nest must be removed, including several hundred workers and brood, to ensure the colony is sustained and active for longer periods of time (up to one year). Permission should be sought from the concerned authorities before nest removal.

1.1. House the wood ant colonies in a wide box (around 135 cm x 70 cm x 35 cm) with walls covered with polytetrafluoroethylene (PTFE) resin (*e.g.*, Fluon) to prevent ants from escaping, at 21°C, under a 12-h light:12-h dark cycle.

1.1.1. Provide sucrose (333 g/L) and water to the ants, using water dispensers. To ensure the ants' diet is rich in protein, feed the colony with frozen crickets 2x a week. Spray the nest with water every day to keep it humid.

1.1.2. To improve the sanitary conditions of the colony, add pine resin when necessary; pine resin reduces parasitism in wood ant colonies[17]. Place a small container (around 15 cm in diameter and 5 cm deep) inside the nest so that ants can deposit any dead colony members in it.

1.2. Keep ants under the afore-mentioned conditions for at least 2 weeks after collecting before attempting any experiments. Remove the sucrose from the nest to starve the ants 2 d prior to the experiments, thereby improving their motivation.

2. Selecting and Harnessing Ants

Note: For harnessing ants, a custom-made holder is needed. This can be constructed using modeling clay and a cardboard with a cut opening attached to it horizontally. The upper surface of the cardboard should be partially coated with wax, permitting insect pins to be attached during fixing. Careful handling is necessary for every step of this protocol (including maintenance, transport, and experiment), but in particular when harnessing ants, to avoid subjecting them to high levels of stress prior to training.

2.1. To select ants that are motivated to eat, place a slide with a drop of sucrose (200 g/L) inside a small box (around 14 cm x 8 cm x 5 cm) with walls covered with PTF resin to prevent escape. Do not select ants that are carrying wood or dead ants from the nest. Instead, take ants that are crawling up the walls of the nest, because these are more likely to be foragers trying to leave the nest to search for food.

2.2. Place each ant in the box and wait to see if it feeds on the sucrose drop. If it does, transfer it immediately to another empty box to prevent satiation.

2.3. Transfer each ant to a separate tube. Place each tube in the freezer for 1 - 2 min, or in crushed ice for up to 5 min, to immobilize the ant.

2.4. Place the immobilized ant in the holder, through the cut on the cardboard, by the joint between its head and thorax. Ensure the antennae remain on the side of the head, using insect pins embedded in the wax layer on top of the cardboard.

2.5. Use a custom-made heating element to wax the tip of an insect pin to the ant's head, parallel to the cardboard. During this procedure, do not touch the antennae with the heating element wire to avoid incurring any damage to them.

2.6. After the wax dries, remove the insect pins holding the antennae and carefully remove the ant from the holder.

2.7. Fix the insect pin holding the ant in a modeling clay cylinder and fix a custom-made plastic holder beneath it, ensuring the ant maintains a typical standing posture and that the whole body is free to move except the head.

2.8. Keep the harnessed ants in darkness for at least 2 h prior to training.

3. Training and Testing

3.1. Set-up, conditioned stimulus, and unconditioned stimulus

3.1.1. Conduct the experiments in a white acrylic box (50 x 50 x 50 cm), open at the front to permit access for the experimenter. Record the ant's behavior with a camera.

3.1.1.1. Place the camera with a macro lens directly above the ant, through a hole in the upper surface of the box.

3.1.1.2. To reduce any extraneous visual cues, keep the room in darkness except for a light source pointing directly to the top of the acrylic box (two 26-W lights).

3.1.2. Use as a visual cue (the CS) a bright blue cardboard rectangle (60 x 45 mm) attached at its center to a needle, connected to a syringe (2 mL) with which the US (sucrose 200 g/L) is manually delivered to the ant (**Figures 1A** and **1B**).

Note: The sucrose solution can be made with any white sugar, provided that it does not have color and odor when dissolved in water.

3.1.3. Conduct two types of training in parallel, paired and unpaired (see the explanation below), in a randomized order.

Note: In this study, we did not pay attention to the nature of the visual cue. Only the association between the cue and the reward were taken into account. Color and shape were not tested, and it is likely that a CS with other features would produce similar results. Nevertheless, the blue color was picked because ants from the same genus have been shown to be sensitive to wavelengths that cover the blue color[18].

3.2. Paired training

Note: In paired training, the CS and US are presented in every trial, associated with each other (**Figure 1C**).

3.2.1. Start recording the ants 10 s before presenting the CS to ensure that ants are not spontaneously performing MaLER before the presentation. If ants do perform MaLER during this period, postpone the trial for a few seconds. If any ant shows this behavior continuously, exclude it from the analysis.

3.2.2. Move the syringe + CS in front of the ant for ~10 s, with the tip of the needle kept between the ant's face and a maximum of 5 mm above and to each side. During this time, move the tip of the needle as close as possible to the ant's head but without touching the antennae (**Figure 1B**).

Note: Motion was included when presenting the CS because it has been shown to play a role in visual associative learning in honeybees[6].

3.2.3. Apply pressure to exude a single drop of sucrose from the syringe tip and place the drop of sucrose next to the antennae and mouthparts to allow the ant to detect the sucrose. Allow the ant to feed for about 5 s.

Note: The amount of sucrose ants consumed in each trial was not controlled in these experiments. The drop of sucrose on which ants feed should be large enough to allow them to freely feed for about 5 s.

3.2.4. Repeat this procedure 10x, with an ITI of 5 min.

Note: In these experiments, ants were always turned to the right, with their right eye close to the open side of the acrylic box. Therefore, the CS always approached the ants from their right side. Though this does not invalidate learning, these experiments can be conducted by turning half of the ants to the left and half to the right, to avoid any possible lateralization effect.

3.3. Unpaired training

Note: This training consists in presenting to the ants either the CS or the US separately, these two stimuli being, thus, dissociated from each other over time (**Figure 1D**).

3.3.1. Start recording the ants 10 s before each trial.

3.3.2. Present the CS in the same manner as in paired training but do not deliver the sucrose to the ant.

3.3.3. After 2.5 min, deliver the US directly to the mouthparts (also touching the antennae) using a syringe without the CS attached.

3.3.4. Start with a CS trial and repeat this procedure 10x for each type of trial, intercalating them with an ITI of 2.5 min.

3.4. Testing

Note: A test should be conducted only 1x, either 10 min or 1 h after the last training trial; a test in which the ant is presented with the CS but not with the US could cause the extinction of the associative memory formed previously.

3.4.1. Start recording the ants 10 s prior to testing.

3.4.2. Present the CS to the ant for about 10 s.

3.4.3. Ensure that the ant is still motivated to feed by delivering sucrose after the test.

4. Data Collection and Analysis

4.1. Record the ant's behavior, from above, 10 s before each trial and during the CS and US presentations. Ensure that all trials and tests are recorded for posterior analysis.

4.2. Using the recordings made during training and testing, score the ants' responses during the 10 s of CS presentation.

4.3. Separate the ants' responses during the CS presentation into three types of behavior, depending on the extension and movement of the mouthparts: Full Extension with Movement (FEM) as if feeding, Full Extension without movement (FE) or Partial Extension (PE) of the maxilla-labium or maxillary palps (**Figures 2A** - **2D**). For analysis, group all MaLER types as a single response (**Figure 2E**).

4.4. Exclude any ant that did not feed in every trial and test.

Note: For analysis, a statistical test that takes individual ants into account is recommended, thereby accounting for variability within individuals. When classifying ants' responses during training in a binary manner (1 for positive responses and 0 for no response), logistic regression with mixed effects is advisable[16,19]. For comparing the proportion of ants responding in each training trial or test, a *G*-test or Fisher's exact test is recommended, depending on the number of ants analysed[16,20.]

REPRESENTATIVE RESULTS:

During classical conditioning experiments, the CS must not induce a spontaneous response in the animals. In the experiments we conducted, only 3% - 4% of the ants performed MaLER in response to the visual cue on the first trial, prior to training. Ants that were undergoing a paired training performed increasingly more MaLER in response to the CS (**Figure 3A**; logistic regression, N = 51, degrees of freedom (df) = 507, z = 5.949, $p < 0.01$). The percentage of paired ants that respond to the CS plateaued around 50%, from the third trial onwards. On the contrary, unpaired ants showed no significant increase in MaLER during training (**Figure 3B**; logistic regression, N = 29, df = 287, z = 0.758, p = 0.45). The occurrence of MaLER in response to the visual cue was significantly higher during paired than unpaired training (logistic regression, N = 80, df = 796, z = -5.306, $p < 0.01$), which was true for every trial except the first (**Table 1**).

For examining their short- and a mid-term memory[15], the ants were tested either 10 min or 1 h after the last training trial. For both tests, the proportion of ants performing MaLER in response to the CS was significantly higher when they had undergone paired rather than unpaired training (**Figures 3C** and **3D**; **Table 1**).

During training, individual ants showed substantial variation in the number and type of MaLER that they displayed (**Figures 4A** and **4B**). Only 14% of paired ants responded in every trial from the second or third trial onward, while most ants alternated between trials in which they responded and in which they did not. On those training trials in which ants responded, the degree to which they extended and moved their mouthparts varied. Therefore, we divided MaLER into three different types: FEM, FE, or PE. Typically, ants performed FEM or PE more often than FE. However, only a few ants performed consistently the same type of response; in most cases, ants showed little consistency on the type of MaLER they performed (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental set-up and training scheme. (**A**) Fix the ant with wax to an insect pin attached to a modeling clay cylinder (orange) and place a custom-made holder underneath it to permit a naturalistic stance. Place it inside a white acrylic box illuminated by two light sources, directly underneath a camera. Use as the conditioned stimulus (CS) a cardboard (blue square) attached to the syringe that delivers the unconditioned stimulus (US), a sugar reward. The **inset** shows a close-up view of an ant in the holder. (**B**) The tip of the syringe needle is moved as close as possible to the ant's head but without touching the antennae as shown in this schematic. Train ants through either a (**C**) paired or (**D**) unpaired training. This figure has been modified from Fernandes *et al.*[16].

Figure 2: The Maxilla-Labium Extension Response of wood ants. Individual frames from video recordings show ants' mouth part movements during training. (**A**) This panel shows no response. (**B**) This panel shows a full extension (FE) of the maxilla-labium that terminates in the glossa. (**C**) This panel shows a partial extension (PE) with only the maxillary palpus visible. (**D**) This panel shows a partial extension (PE) of the maxilla-labium structures. (**E**) Ants undergoing paired training (N = 51) perform a full extension with movement (FEM; dark brown), an FE (mid-brown) or a PE (light brown). This figure has been modified from Fernandes *et al.*[16].

Figure 3: Wood ants form associative memories of a visual cue paired with a 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). Ants were tested (**C**) 10 minutes

(paired: N = 15; unpaired: N = 15) or (**D**) 1 hour (paired: N = 15; unpaired: N = 14) after the last training trial. The percentage of ants responding to the CS during and after paired or unpaired training are represented in brown or grey, respectively. The three types of MaLER are represented in dark (FEM), medium (FE), and light (PE) tones. This figure has been modified from Fernandes *et al.*[16].

Figure 4: Individual performance of ants during training. These panels show the individual performances of (**A**) paired ants and (**B**) unpaired ants. The three types of MaLER are represented in dark (FEM), medium (FE), and light (PE) brown or grey. This figure has been modified from Fernandes *et al.*[16].

Table 1: Comparison of MaLER responses to the CS between ants that had undergone paired and unpaired training, for each trial and test. The number of ants (N), degrees of freedom (df), *G*-test of independence (G) and *p*-value are shown. The first trial was analyzed with a Fisher's exact test. This table has been modified from Fernandes *et al.*[16].

DISCUSSION:

Classical conditioning is one of the most well-established paradigms to study learning and memory. The protocol we presented here is an adaptation of the paradigm designed for honey bee workers[2,3] and subsequently used with several other species, such as bumblebees, fruit flies, which also use PER as a readout for learning[10,11], and locusts and ants, which use POR and MaLER, respectively[12,13]. Using this protocol, it is possible to train harnessed wood ants to learn the association between a visual cue and a sugar reward and analyze the retention of this short- (10 min) and middle-term (1 hour) memory[16].

In any behavioral experiment, it is necessary to take into account critical steps that can minimize the variability in the animals' responses. In the protocol presented here, several measures are taken to minimize variability before and during training. Prior to the start of experiments, the colony needs to be starved for at least two days and ants should be selected based on their willingness to eat from a sugar drop in the holding box. Selecting ants in this way is intended to maximize the chance of training ants that are motivated to feed. Careful handling is also an important consideration because it can help to reduce stress levels, which disrupts learning if it is too intense[21]. To this end, ants should be anesthetized with cold to stay motionless whilst being harnessed, because any movement (for escaping) during this procedure could be a source of stress. Furthermore, the contact between the ant and the wax should be minimal, avoiding contact between the antennae and the hot wax or wire, which could cause damage. Although these observations have not been analyzed formally, the antennae seemed to move with a specific pattern during learning.

During experiments, careful delivery of the sugar is also important to keep ants motivated. Again, whilst this has not been analyzed formally, abrupt food delivery seemed to cause additional stress to the ant, which in turn led to a lack of motivation and learning. Furthermore, the sucrose delivered during the training should be of a reduced concentration (200 g/L) to avoid satiation before the end of the training and testing. This allows MaLER to be a good candidate unconditional response because, together with a low spontaneous performance of this response to the visual cue, it also does not saturate over trials. Lastly, contrary to most classical conditioning studies[2,3,5-13], we trained one ant at the time until the end of the experiment, leaving it in place between trials rather than removing it to test another ant. Training several ants together seemed to produce more variable results, which may be due to an increase in stress and/or conflict between visual information caused by the complete change of the scenery. To reduce the duration of each experiment, we used a 5-minute ITI instead of the 10-minute ITI used in most classical conditioning studies[16]. Although all these considerations should help ensure that the ants are motivated to feed and learn during training, some variability cannot be avoided. We recommend using ants that seem to have normal social, appetitive, and locomotion behavior and excluding ants from the analysis the moment they fail to feed on a training trial or a test.

The nature of the CS was not tested in this study. Although we have used a blue visual stimulus because ants of the same genus are sensitive to these wavelengths[18], other colors might also be learned in association with a reward. Further experiments would be required to fully characterize the colors being seen and learned in this set-up. This is also true for different shapes and sizes of the visual cue. We have not tested if ants' spatial resolution would be sufficient for distinguishing the visual stimulus presented here at the distance from the ants' eyes it was presented at. Although wood ants' compound eyes have been described in terms of size and number of facets[22], to our knowledge, their spatial resolution has not been fully described yet. However, this has been calculated for *Melophorus magoti*[23]. A similar characterization of the wood ants', or other tested insects' eyes would contribute to a clear investigation of the features of the visual cue being observed and learned by the animals. Furthermore, we included motion when presenting the visual stimulus to the ant because it has been shown to play a role in honeybee associative learning during classical conditioning[6]. However, this was also not tested in this study and, due to the different movement nature of flying insects compared to walking insects, differences between honeybee and ant visual classical conditioning could be observed.

On a final note, we were unable to examine long-term memory retention because ants did not survive being harnessed for such long periods after training. However, in subsequent sets of experiments, we have kept ants alive and motivated to eat and learn when harnessed and left them overnight in a dark and humid environment (placing a box over them). Therefore, this paradigm could be used to unravel long-term memory retention of wood ants, in addition to short- and middle-term memory.

With this simple procedure adapted from general classical conditioning paradigms, it is possible to study the acquisition and retention of visual memories in harnessed wood ants, which have been studied widely in paradigms using free-moving animals. This paradigm has the potential to be used for analyzing the neural basis of visual learning in a very well-established model for insect navigation.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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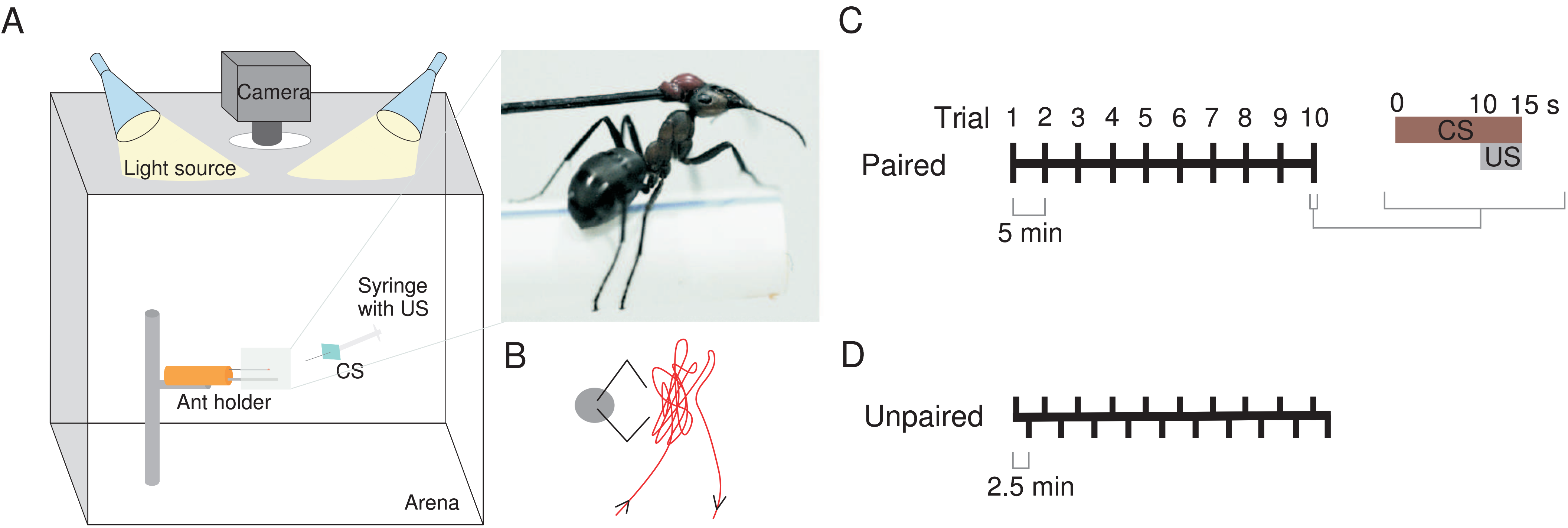

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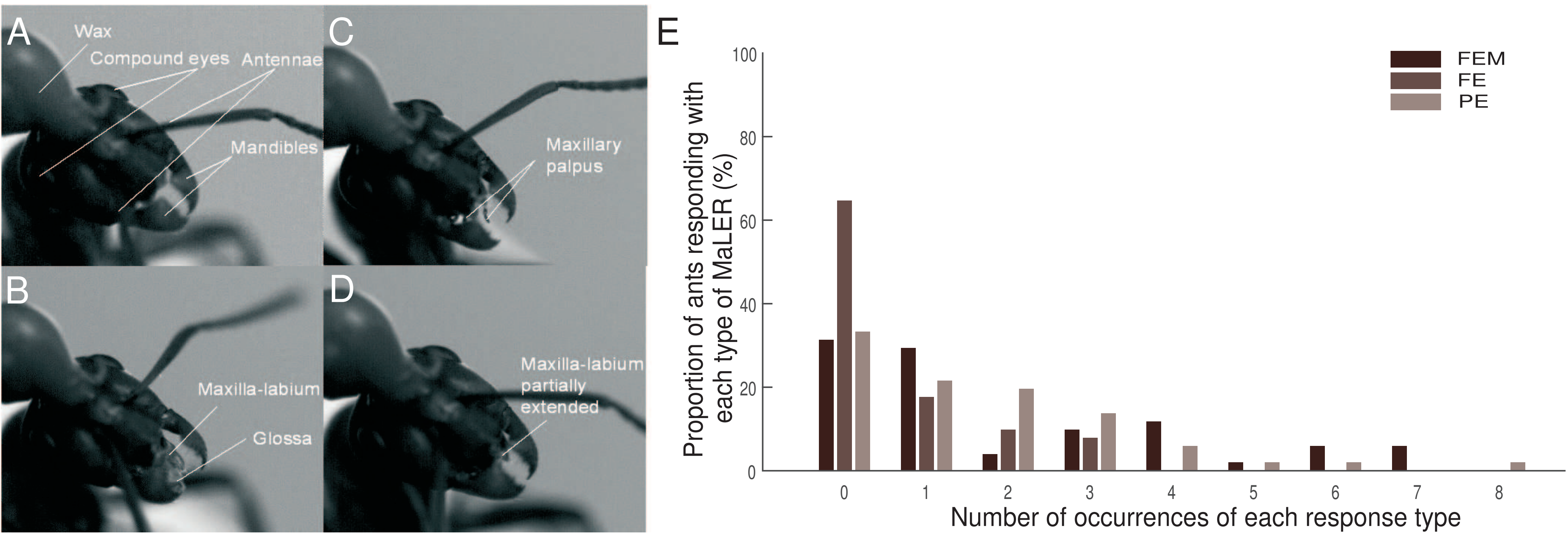

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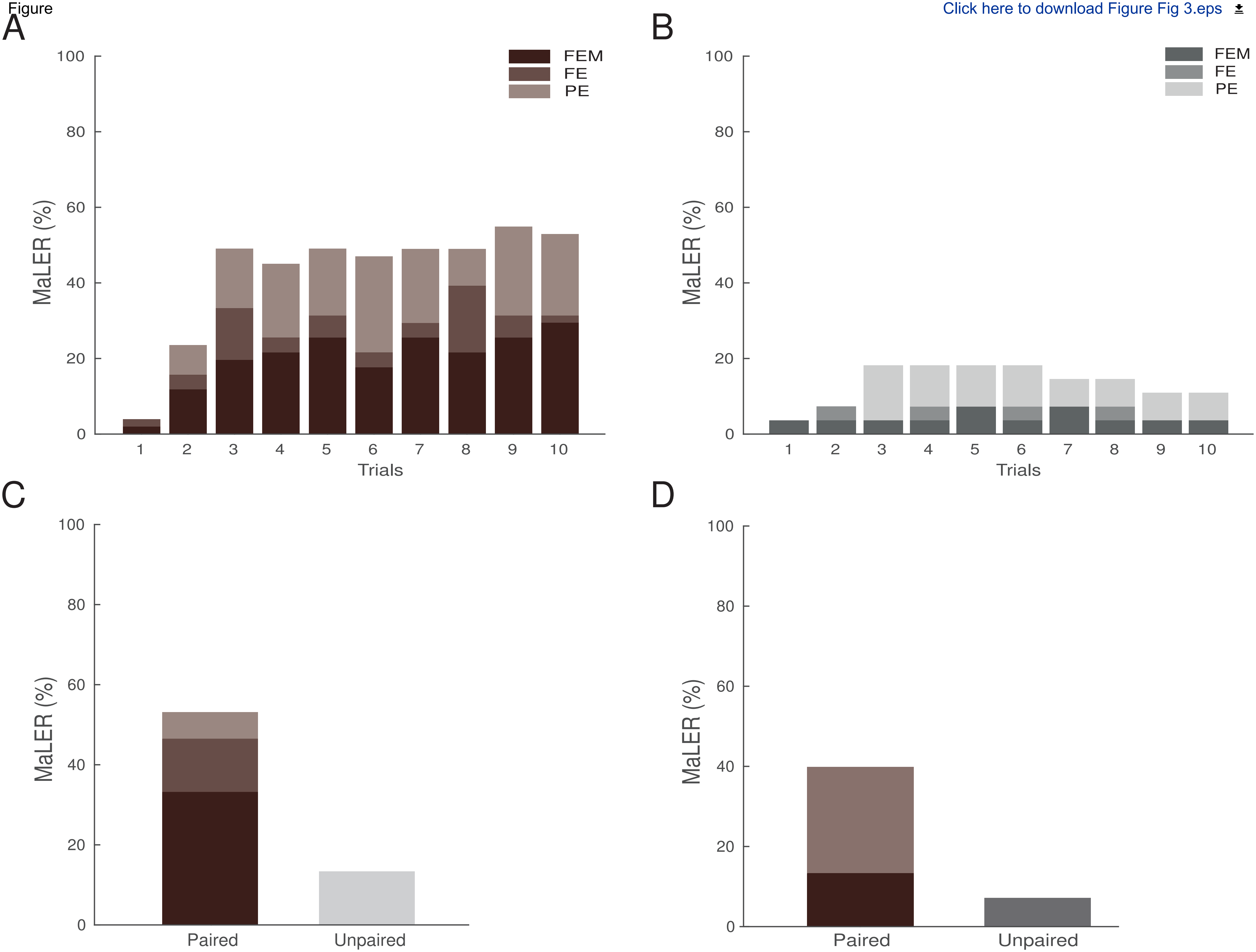
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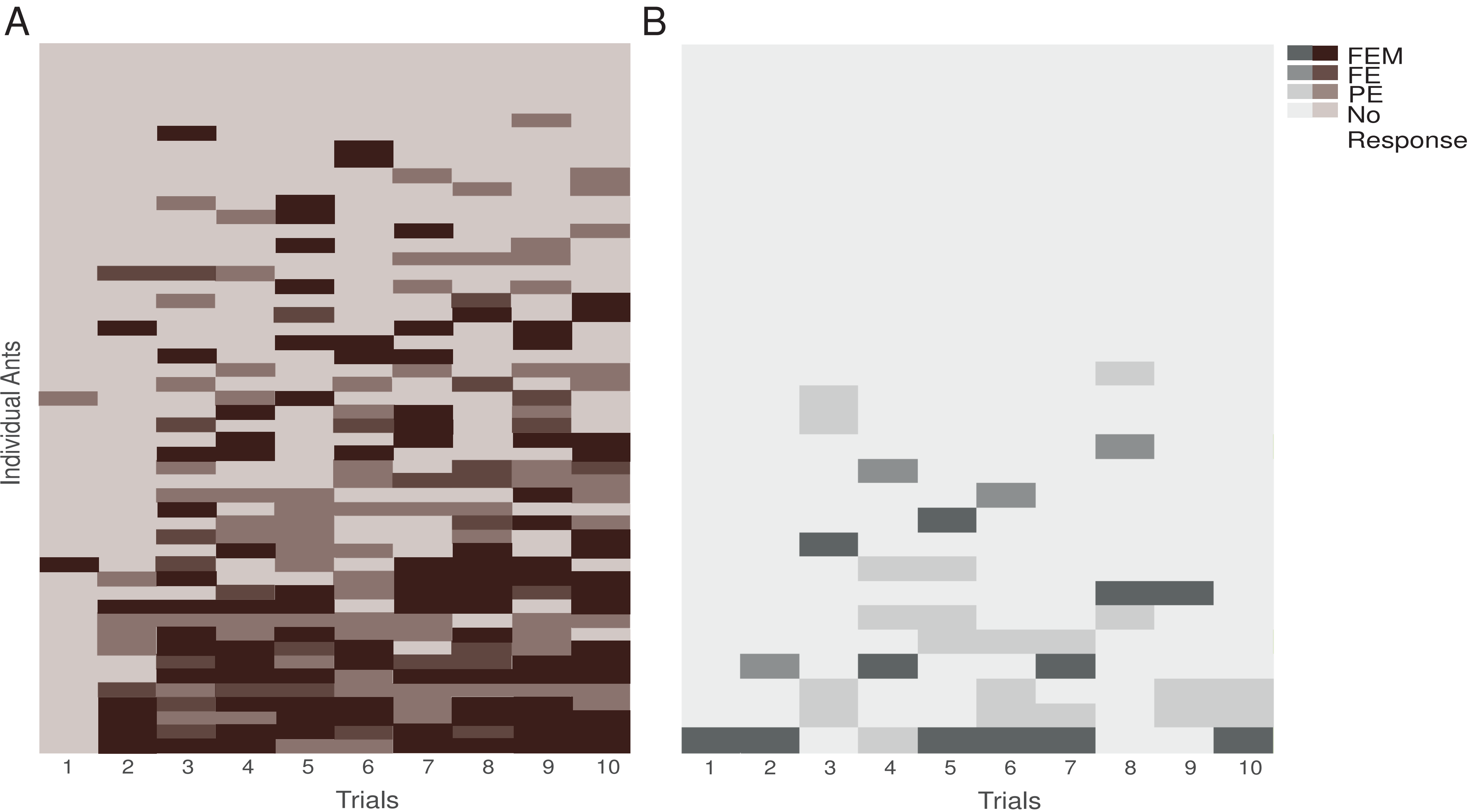

Table 1

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
10 min	59	1	5.5	<0.05
1 h	59	1	4.42	<0.05

Click here to download Table of Materials JoVE_Materials.xlsx

Name of Material/ Equipment	Company
Fluon	Blades Biological Ltd, Edenbridge, UK
Crickets	Blades Biological Ltd, Edenbridge, UK
Natural Pine Rosin/Resin	Minerals-water Ltd, Rainham, UK
Austerlitz Insect Pin	Fine Science Tools GmbH, Heidelberg, Germany
High speed camera	Edmund Optics Inc., Barrington, USA
Macrolens	Cannon, Surrey, UK
Software	IDS Imaging Development Systems GmbH
Blue Cardboard	john smith's at Union Store, University of Sussex
Syringe	Fisher Scientific LTD, Loughborough, UK
Needle (0.5 x 16 mm)	Fisher Scientific LTD, Loughborough, UK

Catalog Number	Comments/Description
ACS 109; ACS 112; ACS 114	For preventing insects from scaping
LZJ 217	Given to the ant colonies as protein sour
500g	Given to the ant colonies for sanitation
26000-40	For harnessing ants
eo-13122M	MaLER recordings during training and tes
EF 100 mm f/2.8 L Macro IS USM	MaLER recordings during training and tes
uEye64	MaLER recordings during training and tes
JACK-PJM41358	Constitutes de conditional stimulus
BD Plastipak 300185case; Product Code.12369289	US and CS (attached) presentation
BD Microlance 300600; Product Code:10442204	US and CS (attached) presentation

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Title of Article: VISUAL CLASSICAL CONDITIONING IN WOOD ANTS

Author(s): A. SOFIA D. FERNANDES, C. L. BUCKLEY, J. E. NIVEN

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AUTHOR:

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Institution:	UNIVERSITY OF SUSSEX
Article Title:	VISUAL CLASSICAL CONDITIONING IN WOOD ANTS
Signature:	
Date:	26/04/2018

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MS # (internal use):

UNIVERSITY OF SUSSEX

Dr. Ronald Mayers
Journal of Visualized Experiments
50 Westminster Bridge Road
London, SE1 7QY
UK

20th June 2018

Dear Editors

We thank the editorial board and reviewers for their generous comments on the manuscript. We have edited it to address all concerns raised, which have indubitably improved it.

We believe that our manuscript is now suitable for publication in the *Journal of Visualized Experiments*, but we remain open to any other suggestions that might be raised in order to improve its quality.

Please find accompanying this letter the edited manuscript and figures.

Many thanks for your consideration

Ana Sofia David Fernandes
(on behalf of Dr. Chris L. Buckley and Dr. Jeremy Niven)

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Editorial comments:

We thank the editorial board for all suggestions for improving our manuscript.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please revise lines 220-226 and 235-241 to avoid textual overlap with previously published text.

We thank the editorial board for these suggestions. We have changed the lines 246-248 to "Ants that were undergoing a paired training performed increasingly more MaLER in response to the CS (Fig. 3A; Logistic regression, N=51, df=507, z= 5.949, p<0.01). The percentage of paired ants that respond to the CS plateaued around 50%, from the third trial onwards. On the contrary, unpaired ants showed no significant increase in MaLER during training (Fig. 3B; Logistic Regression, N=29, df=287, z=0.758, p=0.45). The occurrence of MaLER in response to the visual cue was significantly higher during paired than unpaired training (Logistic Regression, N=80, df=796, z=-5.306, p<0.01), which was true for every trial except the first (Table 1)."

Current lines 259-266 were changed to "Only 14% of paired ants responded in every trial from the second or third trial onwards, while most ants alternated between trials in which they responded and in which they did not. On those training trials in which ants responded, the degree to which they extended and moved their mouthparts varied. Therefore, we divided MaLER in three different types: Full Extension with Movement (FEM); Full Extension without movement (FE); or Partial Extension (PE) of the maxilla-labium or maxillary palps. Typically, ants performed FEM or PE more often than FE. However, only a few ants performed consistently the same type of response; in most cases, ants showed little consistency on the type of MaLER they performed (Fig. 4C)."

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The article from which all figures were redrawn was published under JEB's Green Open Access Scheme. Therefore, the authors hold the copyrights and are free to reuse the figures. We have added the appropriate citation to all figures.

4. Please submit the figures as a vector image file to ensure high resolution

throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

All figures will be re-submitted as vector images (eps format).

5. Figure 2: Please label panels C and D. In the uploaded Figure 2, there are only panels A and B. Please revise the figure or figure legend to ensure that they are consistent.

We thank the editorial board for pointing this out. Figure two has been corrected.

6. Table 1: Change “hr” to “h”.

We thank the editorial board for these suggestion. This change has been done in Table 1.

7. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available.

We have removed all trademarks from the Table of Equipment and Materials.

8. Please provide an email address for each author.

The email addresses for the remaining authors have been added:
** Email Address: C.L.Buckley@sussex.ac.uk
*** Email Address: J.E.Niven@sussex.ac.uk

9. Please revise the Introduction to include a clear statement of the overall goal of this method.

We thank the editorial board for this suggestion. We have added a sentence on current line 100 describing the main goal of this protocol: “The main goal of the protocol we describe here is to provide a way to perform classical conditioning experiments using a visual conditional stimulus with a widely studied ant species, *Formica rufa*.”

10. Please use SI abbreviations for all units: L, mL, μL, h, min, s, etc.

We have corrected all units to SI abbreviations.

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We thank the editorial board for this suggestion. We have changed all sentences to passive voice.

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For example: Plasticine®, Blades Biological Ltd, Minerals-water Ltd, Austerlitz Insect 143 Pin®, Plasticine®, Edmund Optics Inc., Cannon, etc.

We have removed all commercial language from the manuscript.

13. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We changed all sentences to the imperative tense added them as a note.

14. 2.1/2.2/3.1: Please break up into substeps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We thank the editorial board for these suggestions. We have broken up step 2.1 in two different steps, previous 2.2 in three steps and 3.1 in 3.1.1 and 3.1.2.

15. Line 228: Please format Guerrieri et al., 2011 as a superscripted numbered reference.

This reference has been changed to the appropriate format.

16. Line 241: There is no panel C in Figure 4. Please revise the text.

We thank the editorial board for pointing this out. We have changed the text accordingly (current line 266).

17. References: Please include volume and issue numbers for all references.

We have added the volume and issue numbers to all references.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Learning and memory is an interesting topic in invertebrate cognition and ants are one of the most suitable organisms to study any kind of learning paradigms based on different sensor modalities.

Although honeybees gained the most attention in conditioning experiments through PER and SER, some recent studies put ants forward as an alternative model with which a MaLER is used for evidence of conditioning.

The authors present here an interesting protocol in wood ants which is well written with details and has the potential to provide a source for researchers willing to do similar experiments in the same or other ant species.

We thank the reviewer for all important suggestions for improving our manuscript.

Major Concerns:

I have no major concern about the organization and language of the manuscript. Some minor comments are below

Minor Concerns:

TITLE

Short but informative enough.

When I first read the authors of the manuscript, I imagined A. Sofia and D. Fernandes as two separate contributors - a missing comma between two - considering the writing style of the remaining authors (First letter of the name and surname in full. However, then I realized that I was wrong. However, may it be better to use A.S.D. Fernandes to prevent confusions?

We thank the reviewer for this suggestion. Although the reviewer is right, the author's name has been written as "A. Sofia D. Fernandes" in other papers previously published so we decided to keep the same name for consistency.

Abstract

line 7: I recommend the use "of ants are harnessed" instead of "ants are fixed" (see also below for restained). Which one is better defining the situation?

We thank the reviewer for this suggestion. We have changed the words “restrained” and “fixed” for “harnessed (current line 45 of Short Abstract and 57 of Long Abstract).

Key words:

OK

Short Abstract

OK

Long Abstract

Line 59; "... ants are fixed" ... ants are restrained" as in short abstract?

We thank the reviewer for this suggestion. We have changed the words “restrained” and “fixed” for “harnessed (current line 45 of Short Abstract and 57 of Long Abstract).

Introduction

Line 74: "focussed" or "focused"

We thank the reviewer for correcting this typo (current line 73).

Line 74: The use of honeybee workers is OK but which species? For instance, when you mention wood ants you specify the species since there are several species all which are considered as wood ants. Similarly, I recommend the use of Apis mellifera at least at the first appearance in the text.

We thank the reviewer for this suggestion. We added the species name in the first appearance of “honeybee” in the text (current line 73).

Line 79: "long-term memory" instead of "long term-memory"

We thank the reviewer for correcting this typo (current line 78).

Line 84: is there something wrong with the end of the sentence following the part "as the UR,"?

We thank the reviewer for this question. We have clarified the end of this sentence by changing it to "…as the UR, and to test learning in other species, such as bumblebees[10] and fruit flies[11]" (current lines 82 and 83).

Line 100: second "insect" at the end of the sentence is not needed I think.
We thank the reviewer for this suggestion. We have removed the word "insect" on current line 99.

Protocol

Line 111: Why did the author(s) suggest the removal of a whole colony from the field, especially the colony of a wood ant species with its pronounced big size? Is there a necessity for it or a special aim? I have my own experiences with wood ants. I have kept ants in my laboratory and never transported a whole colony to the laboratory because 1- it is a big work, 2- the colony will require a large space in the lab. to construct their colonies and to forage, 3- a small proportion of a mound with several hundred workers and brood survived up-to 2 years without the queen.
The reviewer is right about this aspect, though in our experience with wood ants we do not usually keep nests for longer than a year as their activity decreases significantly over such long periods of time, which seems to largely decrease their motivation for food foraging and learning. We have changed the text to "A good proportion of the nest must be removed, including several hundred workers and brood, to ensure the colony is sustained and active for longer periods of time (up to one year)" (current lines 111-113).

Line 134: "dead other ants"??
We have removed the word "other" from this line (137).

Line 162: "…to the top of the" what? Something seems to be missing.

We have corrected this to "the top of the Perspex box" (current line 164).

Line 164: "Use as a visual cue (CS) a bright blue cardboard rectangle (60 x 45 mm) attached"
Use as a visual cue (CS), a bright blue cardboard rectangle (60 x 45 mm), attached…"
Or
Use as a visual cue (CS) - a bright blue cardboard rectangle (60 x 45 mm) - attached…"
1-The visual cue used is a blue cardboard. The selection of this colour may

need explanation because researchers willing to use this or a modified version of the protocol will need to select a visual cue for their test animals. However, a visual of a blue cardboard, or any other color, or another shape and/or pattern cue can lead to different sensations depending on the sensory abilities of the species. A species may not see a colour, or create an internal representation of the colour cue based on either its chromatic or achromatic properties but can readily respond to a solid visual cue or a pattern.

2-Do we pay attention to the nature of the visual cue or just to the association? I mean do the ants learn the rectangle shape or the colour? Why 60x45 mm size was selected? Does this size and the placement of the rectangle provide enough visual angle to the eyes of the restrained ants?

We thank the reviewer for raising these key issues. In this study, we have not tested for colour preference or discrimination. The shape and size of the CS was also not tested here. Therefore, though ants successfully associated this visual stimulus to a sucrose reward, it is possible that stimuli with other features would produce similar results. To explain this, we included a note in the step 3.1, "In this study we did not pay attention to the nature of the visual cue. Only the association between the cue and the reward were taken into account. Colour and shape were not tested and it is likely that a CS with other features would produce similar results. Nevertheless, the blue colour was picked because ants from the same genus have been shown to be sensitive to wavelengths that cover the blue colour[18]."

We have also added a note about the motion produced during the CS presentation, on step 3.2, "Motion was included when presenting the CS because it has been shown to play a role in visual associative learning in honeybees[6]."

Furthermore, we have included a brief discussion about these issues in the lines 306 to 321: "The nature of the CS was not tested in this study. Though we have used a blue visual stimulus because ants of the same genus are sensitive to these wavelengths[18], other colours might also be learnt in association with a reward. Further experiments would be required to fully characterize the colours being seen and learned in this set up. This is also true for different shapes and sizes of the visual cue. We have not tested if ants' spatial resolution would be sufficient for distinguish the visual stimulus presented here at the distance from the ants' eyes it was presented at. Though wood ants' compound eyes have been described in terms of size and number of facets[22], to our knowledge, their spatial resolution has not been fully described yet. However, this has been calculated for *Melophorus magoti*[23]. Similar characterization of the wood ants', or other tested insects, eyes would contribute to a clear investigation of the features of the visual cue being observed and learned by the animals. Furthermore, we included motion when presenting the visual stimulus to the ant because it has been shown to play a role in honeybee associative learning during classical conditioning[6]. However, this was also not tested in this study and due to the different movement nature of flying insects compared to walking insects, differences between honeybee and ant visual classical conditioning could be observed."

Line 165: "...to the syringe.." or "...to a syringe.."
The volume of the syringe may be given for researchers to use the precise or most suitable size for their experiments and to prevent delivery of excessive food to the experimental animals. I recommend specifying the amount of food delivered each time.
We thank the reviewer for this comment. We have added the volume of the syringe on step 3.1.2 and a note on step 3.2 explaining that the volume of food intake was not controlled and that the drop of sucrose should be large enough to allow the ant to feed on it for the ~5 seconds of the US presentation.

Line 182: ten times or 10 times?

This line was changed from "ten times" to "10 times" (current line 193).

Will the authors let us know about the details of behavioral outcomes of the training from beginning to the last trial? I expected to read something to guide me to monitor how the paired and/or unpaired training affected or not ants MaLER responses? The researchers can be informed about FEM, FE and PE also in this part of the protocol in order to help them to track the ongoing of their conditioning.

As far as I understand from the training and testing details, experimenters will have to monitor ants' behavior during training and testing using the video camera.
I suggest the author(s) to include a step about video recording during training. How will the experimenter perform the recording? Will he/she record for a defined period and then evaluate the scenes in a computer connected to the cam recorder or will he/she do the evaluation simultaneously during applications in training and testing?
We thank the reviewer for these suggestions. We hope we improved the protocol on recording and scoring MaLER with our changes in point 4. of the protocol:

" 4. Data collection and analysis

4.1. Record the ant's behaviour, from above, 10 sec before each trial and during the CS and US presentations. Ensure that all trials and tests are recorded for posterior analysis.

4.2. Using the recordings made during training and testing, score ants' responses during the 10 sec of CS presentation.

4.3. Separate the ants' responses during the CS presentation into three types of behaviour, depending on the extension and movement of the mouth parts: 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. 2A, B, C, D). For analysis, group all MaLER types as a single response (Fig. 2E).

4.4. Exclude any ant that did not feed in every trial and test."

Representative results

Line 237: FEM, FE and PE were already explained in the previous sections. I think use of abbreviations will be enough here.

We have changed this line for abbreviations only (currently 263).

Discussion

Line 248: "....locusts and ants, which use either POR or MaLER, respectively." May it be better if revised as;
"....locusts and ants, which use POR and MaLER, respectively."

We have changed the word "or" for "and" in this line (currently 272).

Line 248: I am not sure about the use of Fixed, restrained or harnessed. All three terms appeared in the text.
The author(s) will provide uniformity fot their use, I am sure.

We thank the reviewer for this suggestion. We changed these words for "harnessed" to be consistent.

Line 252: "... this paradigm,"
Which paradigm? In the paradigm presented here...

We have changed this line to "In the protocol presented here" (currently 277).

Line 256: One of the most common problems with alive animals is the stress that the researchers cause in their test animals. This is also common for ants, as stated here, because they are very sensitive to be alarmed with a number of disturbances, even they are at small magnitudes. The authors underline the importance of careful handling, but I suggest them to include at least a phrase in the protocol about careful handling particularly for those who will perform their first experiments with ants, thus naive to ant studies. Although the protocol warns readers to be careful, with the term handling I understand all steps during the experiments including even their storages, feding in the lab. nest, transportation etc.

We thank the reviewer for this suggestion. We have included a note in step 2 of the protocol: "Careful handling is necessary in every step of this protocol (including maintenance, transporting and experimenting), but in particular when harnessing ants to avoid subjecting them to high levels of stress prior to training."

References

There is not a common style for writings of journals of the cited references.

For instance,

2, Journal of Insect Physiology, all starting in capitals

4, Annual review of neuroscience, only the first word with capital starting

We have capitalized the name of the journals appropriately.

Some corrections for the references,

REF3, Journal of Comparative Psychology, 97, 107.

Journal of Comparative Psychology, 97, 107-19

REF7, apis mellifera l.

Apis mellifera L.

REF11, 1335-1348

1335-1348.

REF16, The acceptance of the paper seems to be in 2017 but publication is listed in JEB as 2018.

Please do not forget to correct in-text citations if 2017 is changed as 2018

We have corrected these references and in-text citations.

FIGURE- TABLE LEGENDS

Figure 1 Legend;

Line 350; Placed it - place it.

We thank the reviewer for this comment. The text was changed accordingly.

Line 354 Fernandes at.al.
Fernandes et al. (see also figures 2 and 3 and Table 1

We thank the reviewer for this comment. This was changed in all figure and table legends.

Figure 2 Legend;
I suggest the author(s) to use abbreviations FEM, FE and PE in line 358 in B, C and D in their first appearances in the legend. Then for E, only abbreviations can be used.
We thank the reviewer for this comment. This legend was changed as suggested.

Line 361 Fernandes at.al.
Fernandes et al.
This was changed in all figure and table legends.

Table 1 legend,
The use of "paired and unpaired ants" seems a bit strange. Can the authors provide another statement?
We thank the reviewer for this comment . We have changed the Table legend to "Comparison of the frequency of MaLER responses to the CS between ants that had undergone paired and unpaired training, for each trial and test."

The resolutions of the figures were poor which made it impossible for me to make critics on them in their current form. I am sure better resolutions will be provided if the paper is accepted.
We have now submitted figures in .eps format with higher resolution.

Reviewer #2:

Dear Authors,
Your work is excellent, though you do not set up a new technique, but only ameliorate an already existing technique (used for instance at the University Paul Sabatier, Toulouse, France).
In general, the writing is good. However, I have some propositions of corrections which could improve your paper.

We thank the reviewer for all important suggestions for improving our manuscript.

How do you know where (at which distance, at which height, with which orientation) you must present the cue to the ants?
We thank the reviewer for pointing out this important issue. We have clarified the CS presentation by including a figure (Fig. 1B), by explaining it in the step 3.2.2 "Move the syringe + CS in front of the ant for ~10 sec, with the tip of the needle kept between the height of the ant's face and a maximum of 5mm above. During, move the tip of the needle as close as possible to the ant's head but without touching the antennae (Fig. 1B)." and by adding a note on step 3.2 "In these experiments, ants were always turned to the right, with their right eye closed to the open side of the Perspex box. Therefore, the CS always approached the ants from its right side. Though this does not invalid learning, these experiments can be conducted by turning half of the ants to the left and half to the right, to avoid any possible lateralization effect."

The long abstract could be a little shortened. Several words can be deleted.
We thank the reviewer for this suggestion. We have not changed the abstract because we do consider all information there necessary, but we are willing to reduce it if there's any precise information the reviewer considers it should be omitted.

Table 1: its title is not easy to understand since you do not give the frequencies which are compared.
We thank the reviewer for this comment. We have changed the table legend to "Table 1. Comparison of MaLER responses to the CS between ants that had undergone paired and unpaired training, for each trial and test."

Short abstract: line 3, replace 'in' by 'with'
We thank the reviewer and followed this suggestion (current line 47).

Introduction:

Line 72: learnt? Probably 'learned'

Learnt was replaced by learned, as suggested by the reviewer (current line 71).

Line 74: 'work' may be 'works'

"Work has" was replaced by "studies have" (current line 73).

Line 88: that lack: try to find another word (humans do not lack wings; they are simply without wing)

"Lack" was replaced by "do not have" (current line 87).

Line 93: write either 'a work' or 'works'

"Work" was replaced by "studies" (current line 92).

Line 99: write 'for identifying'

"Understanding" was replaced by "identifying" (current line 98).

Line 100: 'is based' ??? probably better with 'is issued from'

"Based on" was replaced by "issued from" (current line 102).
Lines 101, 102, 103: can be deleted
The last sentence of the introduction was deleted, as suggested by the reviewer.

Protocol

1. write: 'within the UK,'

We followed this suggestion, which is found in line 110.
Last line: 'where' is probably wrong

"where approptiate" was removed from this sentence (current line 113).

1.1. Line 116: replace 'is' by 'was'

Line 117: write 'fed with frozen'
We thank the reviewer for these suggestions. However, we are recommending the reader to something rather than explaining what we did, and therefore we consider more appropriate to keep the word "is" rather than "was" and "feed" rather than "fed" (current line 118).

Last line: write: 'can deposit in it the dead colony members'

"Dispose of" was replaced by "deposit in it" (current line 122).

1.2 last line: replace 'them' by 'the ants'

“Them” was replaced by “the ants” (current line 125).

2.

2.1 line 132: proposition: replace 'to feed' by 'to eat'

“Feed” was replaced by “eat” (current line 135).

2.2, 2.3, 2.4: OK

3

3.1 line 162: 'directly to the top of the' ? something is probably lacking

We have corrected this to “the top of the Perspex box” (current line 164).

Line 166: at this step of the reading, the readers do not know what you mean by 'paired' and 'unpaired'. You must here give the explanation, or write 'see below the explanation'.

“See the explanation below” was added (current line 169).

Figure 1: OK

3.3 unpaired training

Line 185, 186: write: Change consisted in presenting to the ants either the CS or the US, these two stimuli being thus dissociated from each other over time (Fig. 1C)

We thank the reviewer for this comment. We have changed this note to “This training consists in presenting to the ants either the CS or the US separately, these two stimuli being thus dissociated from each other over time (Fig. 1C).”

Figure 1C: OK

3.3. 1, 2, 3: all is OK

3.4 Note. Line 196: write: 'is presented with the CS'

The word “with” was added in this line.

3.4.1, 2, 3: all is OK

4 4.1, 4.2: OK

Figure 2: there is a problem. There are no figure C, D, E. At my mind, they are present but the lettering is not present. Write it, please

We thank the reviewer for this comment and corrected the figure accordingly.

Note: OK

Representative Results

Line 218, 219: write 'During .... Experiments, the CS must not induce spontaneous response in the animals.

We thank the reviewer and followed this suggestion (current lines 244-245).

Line 222: replace 'There was, however, no' by 'On the contrary, there was no ....'

We thank the reviewer and followed this suggestion (current line 249).

Line 228, 229: write: 'For examining their short and middle term memory, the ants were tested 10 minutes as well as one hour after the last training trail.

We have changed this sentence to "For examining their short- and a mid-term memory[15], the ants were tested either 10 min or 1 hr after the last training trial.". We decided to keep the word "or" instead of "as well as" because the ants were only subjected to one of the tests (current lines 254-255).

Figure 3 A. B. C. D: OK

Line 236: write: 'one of the three different'

Line 239: write: 'more often than FE'

We thank the reviewer for these comments. This whole paragraph has been re-written as a suggestion from the editorial board.

Figure 4 A, B: OK

!!! Figure 4C: there is no figure 4C and no legend for figure 4C. Please, could you solve this problem?

We thank the reviewer for pointing this out. We have changed the text accordingly.

Discussion

Line 250: replace 'retention of this memory at least 1 hour' by 'the retention of this learning (i.e. the individuals' short and middle lasting memory) for at least one hour.

We thank the reviewer for this suggestion. We have rephrased this line to "retention of this short- (10 min) and middle-term (1 hour) memory16." (current line 275).

Line 251: add a comma after' experiment'

Line 256: replace 'to feed' by 'to eat'

Line 257-258: proposition for the end of the sentence: help reducing stress which disrupts learning if being intense.

We thank the reviewer and followed these suggestions.

Line 259: 'this' is not clear; does it signifies 'immobilized' or 'fixed'. Proposition: To this end, fixed ants should stay motionless, because any movement (for escaping) was a cause of stress.

We rephrased this to "To this end, ants should be anaesthetised with cold to stay motionless whilst being harnessed because any movement (for escaping) during this procedure could be a source of stress." (current lines 283-285).

Line 262: change 'vibrate'. If the antennae were vibrating, it was not a normal behavior but a stressing one. Write: seemed to move normally

We changed this to "seemed to move with a specific pattern during learning". This is because antenna did seem to change the movement patterns when touching sugar and later in training when the CS was presented, though this hasn't been analysed (current line 287).

263-267: excellent
267-273: good idea! But not well written. Proposition: we trained one ant, until the end of the experiment, learning .....ant. Training ..... increase of stress and/or some conflict between visual information caused .... studies.

274-280: please shorten, and simply write that you cannot avoid idiosyncrasy, and advise to use only ants having a normal social, appetitive and locomotion behavior.

We combined these two paragraphs and followed the reviewer's suggestions:

"During experiments, careful delivery of the sugar is also important to keep ants motivated. Again, whilst this has not been analysed formally, abrupt food delivery seemed to cause additional stress to the ant which in turn led to lack of motivation and learning. Furthermore, the sucrose delivered during training should be of reduced concentration (20 g/L), to avoid satiation before the end of training and testing. Lastly, contrary to most classical conditioning studies2, 3, 5-13, we trained one ant at the time until the end of the experiment, leaving it in place between trials rather than removing it to test another ant. Training several ants together seemed to produce more variable results, which may be due to an increase in stress and/or conflict between visual information caused by the complete change of the scenery. To reduce the duration of each experiment, we used a 5 minute ITI instead of the 10 minute ITI used in most classical conditioning studies16. Although all these considerations should help ensure that ants that are motivated to feed and learn during training, some variability cannot be avoided. We recommend using ants that seem to have a normal social, appetitive and locomotion behaviour and excluding ants from the analysis the moment they fail to feed on a training trial or a test.” (current lines 289-305).

Line 281-286: I understand, but '24 hours' still concerns the middle memory. Myrmica ruginodis memorizes visual cues during more than 2 months (= 60 months for humans).

We agree with the reviewer and changed “24h after training” for “such long periods after training”. We still believe that with the proper maintenance ants could survive harnessed long enough to test for long-term memory, though these hasn’t been tested yet (current lines 323).

Line 281: replace 'to test for long' by ' to examine long'

We replaced “to test for” by “to examine” (current line 322).

Line 283: to feed ◊ to eat ?
Line 284: write: 'a box above them
Line 286: medium ◊ middle

We thank the reviewer and followed these suggestions.

line 290-291: of visual learning. Stop here, your technique cannot examine navigation

We thank the reviewer for this comment. However, we are not implying that this technique can be used to examine navigation, but we do consider important to mention that these species is a stablished model for navigation, which typically involves visual learning, and therefore a protocol that allows studying visual learning in harnessed animals is important.

line 296: write 'commenting a previous
We thank the reviewer for this suggestion and have changed the text accordingly.

References
OK excellent

(however, I have made so many works studying and using ants' conditioning (I am making my 190th work), and you cite none of my works). This is not at all important.

We thank the reviewer for this comment and we are open to any suggestions for references that might be relevant to this protocol.

Hoping having been useful to you, hoping you could still improve you paper, I wish you courage and all the best in your work and your life.

An anonymous referee.

Reviewer #3:

Manuscript Summary:

This paper yields the protocol of the experiment for ants' associative learning between a visual cue and reward, especially to reduce variation in redundant responses. It describes how to stabilize ants' responses to the stimulus in detail, regarding selecting ants in a nest, cooling ants in the freezer, shortening inter-trial-interval and so on. These descriptions could contribute very much to the researchers studying associative learning by using ants.

We thank the reviewer for all important suggestions for improving our manuscript.

Major Concerns:

This paper deals with conditional stimulus, cardboard, and unconditional stimulus, reward of sugar, and notes that how to select animal behavior as response to the stimulus. While they here select MaLER as response to the stimulus, they note that the candidate of response should be the behavior which are not voluntary action and could be logistically saturated through trials. Such discussion is general and useful for various researchers in associative learning.

We thank the reviewer for this comment. We had underlined this by adding in the discussion the current lines 292 to 295: "Furthermore, the sucrose delivered during training should be of reduced concentration (200 g/L), to avoid satiation before the end of training and testing. This allows MaLER to be a good candidate unconditional response because, together with a low spontaneous performance of this response to the visual cue, it also does not saturate over trials."

Minor Concerns:

In Figure 2B the meaning of horizontal axis is unclear. Spell out.

We thank the reviewer for this comment. We have re-written this axis to make it clearer.

Although Figure 3B shows variation of response in the unpaired training. Since there are two types of unpaired condition, CS only and US only. Is Figure 3B shows unpaired data altogether? Time series of CS should be distinguished from those of US in the graph.

We thank the reviewer for pointing this out. There is only one type of unpaired training, which includes both CS and US presentations. However, they are dissociated from each other, so the CS is presented without being followed by the US and 2.5 min later the US is presented directly to the mouth parts without the CS. To clarify this, we changed the Note on step 3.3 to "This training consists in presenting to the ants either the CS or the US separately, these two stimuli being thus dissociated from each other over time (Fig. 1C).“

Reviewer #4:
This manuscript as presented is polished, but I do have 3 main questions/omissions that arose in my mind upon reading, that require a clearer explanation to make the video really useful to researchers:

We thank the reviewer for all important suggestions for improving our manuscript.

Major points:
(1) Line 164 Use as a visual cue (CS) a bright blue cardboard rectangle
Given that this whole methodology is about classical conditioning, surprisingly little time is expended discussing the conditional stimulus. How did you decide its characteristics - you do not seem to have any references with respect to the wood ant visual system / brain? Does it need to be bright blue - what if I like red? What ecological relevance does this have - does it need to have? What other dimensions would be suitable - you use 60 x 45mm but could people do something else? Could researchers show videos instead? You should discuss a little bit about the visual system of the subject (wood ants) - e.g. what is their eyes' spatial resolution, in relation to the presentation of the stimulus - size, distance away, the pattern on the stimulus if any. This would seem to be a crucial consideration for scientists using different species of ants, which have wildly varying visual capabilities. If the ant can't see the stimulus properly, it's a waste of time. If I have a different experimental subject - say I work with Temnothorax ants - what would be the steps to go through in creating a suitable CS? Is there a good general reference on insect/ant vision I could consult, and a process I could follow? This seems important for making the video *useful* - less a restatement of what you have done, more the parameters of what others might do

We thank the reviewer for raising these key issues. In this study, we have not tested for colour preference or discrimination. The shape and size of the CS was also not tested here. Therefore, though ants successfully associated this visual stimulus to a sucrose reward, it is possible that stimuli with other features would produce similar results. To explain this, we included a note in the step 3.1, "In this study we did not pay attention to the nature of the visual cue. Only the association between the cue and the reward were taken into account. Colour and shape were not tested and it is likely that a CS with other features would produce similar results. Nevertheless, the blue colour was picked because ants from the same genus have been shown to be sensitive to wavelengths that cover the blue colour[18]."

We have also added a note about the motion produced during the CS presentation, on step 3.2, "Motion was included when presenting the CS because it has been shown to play a role in visual associative learning in honeybees[6]."

Furthermore, we have included a brief discussion about these issues in the lines 306 to 321: "The nature of the CS was not tested in this study. Though we have used a blue visual stimulus because ants of the same genus are sensitive to these wavelengths[18], other colours might also be learnt in association with a reward. Further experiments would be required to fully characterize the colours being seen and learned in this set up. This is also true for different shapes and sizes of the visual cue. We have not tested if ants' spatial resolution would be sufficient for distinguish the visual stimulus presented here at the distance from the ants' eyes it was presented at. Though wood ants' compound eyes have been described in terms of size and number of facets[22], to our knowledge, their spatial resolution has not been fully described yet. However, this has been calculated for *Melophorus magoti*[23]. Similar characterization of the wood ants', or other tested insects, eyes would contribute to a clear investigation of the features of the visual cue being observed and learned by the animals. Furthermore, we included motion when presenting the visual stimulus to the ant because it has been shown to play a role in honeybee associative learning during classical conditioning[6]. However, this was also not tested in this study and due to the different movement nature of flying insects compared to walking insects, differences between honeybee and ant visual classical conditioning could be observed."

(2) Lines 172-173 If ants do perform MaLER during this period, postpone the trial for a few seconds.
219-220 only 3 to 4% of the ants performed MaLER in response to the visual cue on the first trial, prior to training
What does one do with these ants, do you exclude them, because it seems like one should?

We thank the reviewer for pointing this out. However, we do not agree that these ants are excluded from the analysis, because removing naïve responses from the analysis skews learning to a higher significance than it should, given that the first trial is falsely represented as 0%. Because we take into account individual ants in our analysis, the spontaneous responses in the first trial are considered. We think that naïve responses to the CS should always be considered in classical conditioning experiments.

Line 173 'If any ant shows this behaviour continuously, exclude it from the analysis.' - it seems like an excessively high threshold for removing them. 'Postpone the trial for a few seconds' seems to be inviting false positives. Could you be a bit stricter?

We thank the reviewer for this comment and we do agree that ideally one should be as strict as possible in order to avoid false positives. However, to our knowledge, insects do perform these behaviours spontaneously (either POR in locusts or PER in bees) thought training, and therefore we are not able to exclude all of them. Because the same procedure is applied to both paired and unpaired ants and we still observe an increase of MaLER in paired training but not in unpaired training, we consider that any false positives would be taken into account in both groups, and therefore any differences between them would be indicative of learning. Nevertheless, we are open to any suggestions on this for future experiments.

(3) Lines 206-207 The distribution of the different types of behaviour will differ from each other during training but can be grouped as a single response.

This was not explained clearly to my satisfaction, if they're being grouped as a single response, then why bother (203-204) 'separating them into three types of behaviour'? Perhaps there's a good reason that could be more flagged up clearly in the video. One would think there's some kind of significance to the variable responses.

We thank the reviewer for pointing this out. We did observe differences in the number of times ants would respond with each of the types of MaLER. However, the distinction was made purely to inform researchers following this protocol that there are different extents to which ants can show MaLER, which we consider useful when analysing ants' responses to the CS. We haven't analysed these different responses to a greater extent. Nevertheless, the reviewer is right that there might be some kind of significance to this variable responses and it would be interesting to analyse it, though for the purpose of quantifying learning we do not consider necessary.

Minor points:

(1) You talk about using 33.3%, 20% sucrose - how to produce? 10 seconds of the video could be on that? (is it literally, go to the supermarket and buy some granulated sugar, and mix 1 cup sugar to 2 cups water, etc?) Usually these things are discussed in terms of molars? (e.g. 1 mol solution)

We thank the reviewer for pointing this out. All sucrose concentrations were changed to mass/volume (g/L). We have also added the following note in step 3.2.1 "The sucrose solution can be made with any white sugar, provided that it does not have colour and odour when dissolved in water."

Abstract: Mention what kind of visual stimulus one can use with your setup?

We thank the reviewer for this comment. When mentioning the visual cue in the abstract, we have added "(a blue cardboard)", in current line 58.

Training consists of, not in?

We thank the reviewer for noticing this and we have changed the text accordingly (current line 61).