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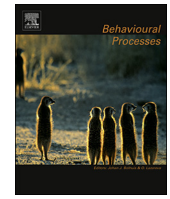

Classical conditioning in earthworms employing an odorous conditioned stimulus

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ABSTRACT

The study of associative learning in invertebrates has become an essential tool for exploring their cognitive abilities and for studying the biological bases of learning and memory. In this experiment with earthworms, we paired a rose odour conditioned stimulus (CS) with a bright light unconditioned stimulus (US). After these pairings, the head retraction response — which is initially elicited by the bright light — is eventually evoked by the rose odour. This is the first demonstration of classical conditioning of an odour in earthworms (vibrations are usually employed as CSs), using a procedure that enables us to rule out an explanation in terms of pseudo-conditioning.

1. Introduction

In this paper we present the first experiment that shows a classical conditioning effect in earthworms (*Aporrectodea longa*) employing an odorous CS. Odorous cues, like tactile stimuli (i.e. vibrations) are part of the ecological niche of earthworms, where vibrations and odours become potential cues for detecting predators or food (McManus and Wyers, 1979; Zirbes et al., 2011). Earthworms are specialized for chemosensory as well as tactile contact with their environment (Darwin, 1881; Laverack, 1960). Although both could be potentially employed as CSs in the study of classical conditioning in earthworms, so far only vibration has been employed as a CS (Dyal, 1973).

In fact, Ratner and Miller (1959a) provided the first experimental demonstration of classical conditioning in earthworms using a vibration as the CS. They showed that after 100 trials in which a vibration was followed by a light, the experimental group showed a significant increase in the withdrawal responses in comparison with control groups (see also Peeke et al., 1967). Ratner et al. employed this technique to evaluate different parameters affecting classical conditioning, such as decerebration and the inter-trial interval (Ratner and Miller, 1959b; Ratner and Stein, 1965). Moreover, inter-stimulus interval (Herz et al., 1967; Ratner and Miller, 1959a, 1959b; Wyers et al., 1964), extinction (Herz et al., 1967; Ratner and Miller, 1959a), partial reinforcement (Peeke et al., 1965; Wyers et al., 1964), temperature (Herz et al., 1964) and amount of training (Peeke et al., 1965) effects have also been studied with this technique. More recently, Watanabe et al. (2005) employed a similar experimental setting to study the role of mRNA in memory consolidation following classical conditioning in earthworms.

Odours have been used as cues in the study of instrumental learning (McManus and Wyers, 1979) but these do not appear to be appropriate stimuli for inducing classical conditioning. Abramson and Buckbee (1995) failed to find classical conditioning when employing an olfactory paradigm. In their experiments, they paired an odour CS (rose scent) with an odour US (n-Butanol scent). Earthworms find the smell of n-Butanol to be aversive and thus produced a rapid defensive withdrawal response. In Experiment 1 a paired group (CS-US) was compared with the following standard control groups: backward (US is presented prior to the CS), unpaired (CS and US are presented in a pseudorandom sequence), US-only, CS-only, and blank (no stimuli were presented). The course of training revealed that the paired and backward groups showed higher levels of responding than the other control groups. The performance of the backward group did not differ significantly from the paired group, which indicates the presence of sensitization or pseudo-conditioning. Additional experiments confirmed this fact. When comparing responding (on an extinction test) of the paired group with the backward group in Experiment 2; with US-only groups in Experiment 3; and with a group in which a new odour was interpolated between the CS and US in Experiment 4; the results lead the authors to conclude that it is not possible to obtain classical conditioning when using an olfactory paradigm. The behaviour of earthworms appears to instead depend on sensitization, which is a non-associative learning mechanism.

However, it is possible that the sensitization effect found in these experiments is specific to this particular experimental paradigm. The training apparatus employed by Abramson and Buckbee consisted of an

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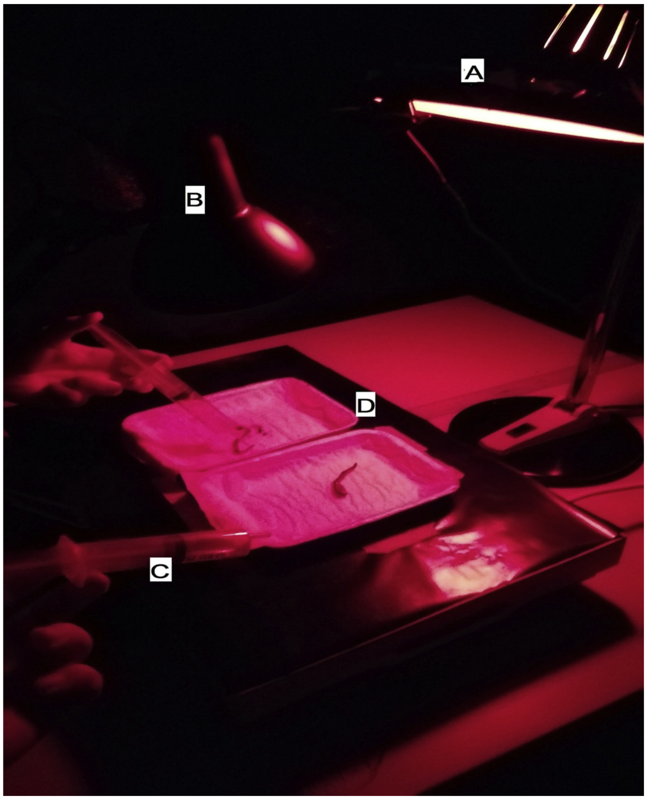

Fig. 1. Experimental apparatus: A) red ambient light; B) light-US; C) syringes with odour-CS; D) polystyrene trays with experimental subjects.

uncovered tray in which the earthworm is situated (see Abramson and Buckbee, 1995; Fig. 1). The CS and US odours were administered with two 20-cc. syringes, with one *immediately* followed by the other. Although an exhaust fan was working during the experiment to vent odours, it remains possible that the CS and US odours were perceived together, encouraging a sensitization effect.

In this experiment, we aimed to test the possibility that an odour could serve as a CS by using a procedure that prevents the exacerbation of sensitization produced by the use of two odours. In particular, we paired an odour CS with the standard bright light US (as opposed to a second odour) employed in the study of classical conditioning in earthworms.

2. Method

2.1. Animals

Fifty-four earthworms (*Aporrectodea longa*) were employed. They were housed together in standard flowerpots (70 × 33 × 35 cm) filled with garden soil mixed with the food (different varieties of chopped lettuce) and with a humidity level of 80%. The earthworms were between 8–15 centimeters in length.

2.2. Apparatus

Fig. 1 illustrates the experimental apparatus. Two subjects of the same group were run at the same time. The subjects were placed on a platform where two polystyrene trays (18 × 12 x 2 cm) were located and covered with a wet paper sheet. The light stimulus to be used as the US (1550 lx, 3-seconds in duration) was provided by a desk lamp centered over the platform at a height of 24 cm. The odour was administered by means of a 10 cc plastic syringe. It was prepared by placing a drop of attar or roses (*Rhade Shyam*) onto a piece of 1 cm^2 filter paper secured to the tip of the plunger of the syringes with a thumbtack. The odour was delivered by injecting it in front of the earthworm's head, at a distance of 1–2 cm.

2.3. Procedure

The earthworms were randomly divided into three groups: Paired (experimental) group, Backward group, and Unpaired control group. Two researchers ran the experiment in a dark room illuminated with red light, which is imperceptible to earthworms. First, the earthworms were taken from the flowerpots, cleaned with distilled water and finally placed onto the trays where they remained for a 2-minute adaptation period prior to training.

The "Paired" group received 20 CS-US pairings, with an ITI of 60 s. After the presentation of the odour-CS, the light-US was presented immediately. The "Backward" control group received the same number of presentations but the pairings of the CS and US were given in the reverse order to that of the Paired group. In the "Unpaired" control group, the stimuli were presented pseudorandomly. The same stimuli could never be presented more than two times in succession. In this last group the number of trials was 40 in total (20 CS and 20 US). This arrangement ensured that the experience was similar with both stimuli, but in order to equate the time of training, the ITI was reduced from 60 to 30 s. The researchers recorded the number of retraction responses (minimum 0.5 cm.) elicited in the presence of the light US.

Following the training phase, the trays were replaced and the subjects were relocated to new trays where they waited for 2 min before the test. The test consisted of 10 extinction trials where the odour-CS was presented. The retraction responses were video-recorded, and these recordings were then analyzed by a researcher who was blind to the training procedure that the worms had received.

2.4. Statistical analysis

Retraction responses were evaluated with a Generalized Linear ANOVA considering an underlying negative binomial distribution (log link). The rejection criterion was established at $p < .05$.

3. Results and discussion

Fig. 2A displays the proportion of responses recorded throughout the 20 training trials for groups Paired, Backward, and Unpaired, with all groups showing a similar level of responding to the US. Although all groups showed a decline in the retraction response throughout the course of training, a 3 × 20 (Group X Trial) ANOVA GLM conducted on these data revealed no significant effects, χ^2s < = 11.76, *ps* > 0.05.

Fig. 2B shows the proportion of retraction responses recorded throughout the test trials. Responding in the group that received CS-US pairings was higher than that of the Backward and Unpaired control groups, which did not differ between them. A 3 × 10 ANOVA GLM revealed only a significant effect of group: χ^2 (2) = 10.431, $p < 0.05$. A post-hoc *t*-test comparison revealed significant differences between the Paired group and the rest of the groups, $p <= 0.04$; that did not differ between them, $p > 0.05$. No main effect of trial, χ^2 (9) = 3.610, or interaction effects, χ^2 (18) = 5.435, were found, *ps* > 0.05.

The results found in this experiment constitute strong evidence of classical conditioning in earthworms with an odour CS. The fact that responding to the CS was greater in Group Paired than in Group Unpaired allows us to rule out alternative explanations in terms of pseudoconditioning and sensitization. This conclusion is further strengthened by the observed performance of Group Backward, which differed from Group Paired but not Group Unpaired. Moreover, the use of a bright light US makes a contribution towards the standardization of behavioural techniques, since this method is similar to that employed in conditioning experiments in which vibration is used as the CS. We are now in the position of being able to conduct experiments to study cue

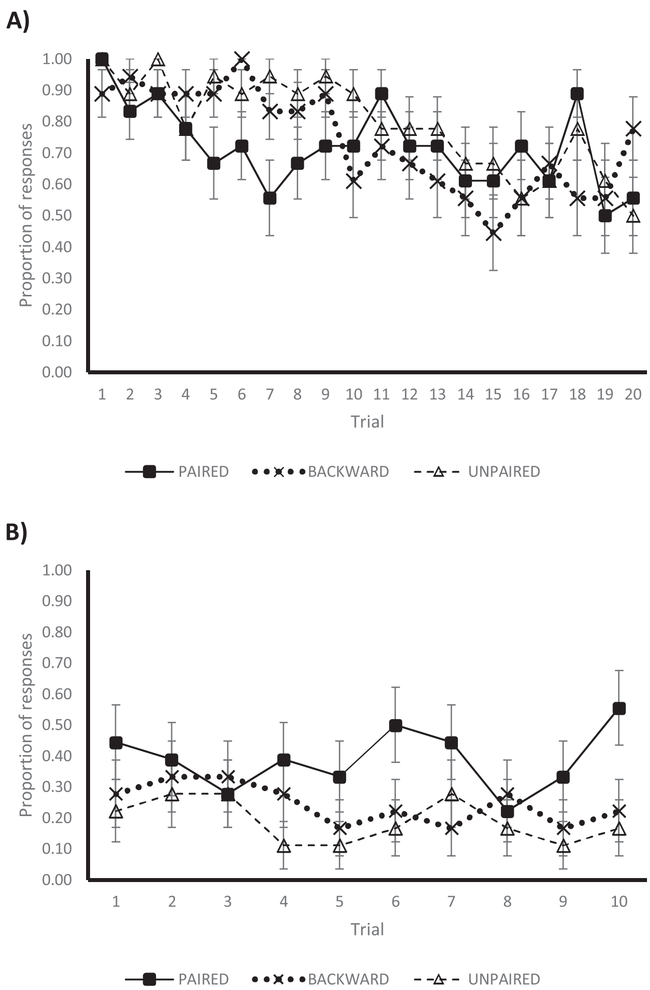

Fig. 2. Proportion of responses throughout the trials for the Paired, Backward and Unpaired groups. 2A) Proportion of responses to the US throughout 20 training trials; 2B) Proportion of responses to the CS throughout 10 test trials. Vertical bars represent SEMs.

competition in learning situations (unless two CSs are necessary), which is a theoretically relevant issue for evaluating associative learning theories as well as understanding the evolution of cognition (de Wall and Ferrari, 2010).

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