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SHORT COMMUNICATION

Aversive conditioning and memory in the harvester *Mischonyx squalidus* (Arachnida: Opiliones)

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Abstract. Aversive conditioning is a form of associative learning. Here, we investigated learning and memory capacity of the harvester *Mischonyx squalidus* (Roewer, 1913), to examine the formation of short (STM) and long-term memories (LTM). First, we trained harvesters to associate an aversive stimulus (shock) with a neutral stimulus (tea odor). Each subject went through three consecutive trials, in which pairings between the stimuli lasted for 3s, with intervals of 1 (STM) and 30 (LTM) min. Subsequently, to test memory, we introduced the subjects in a choice arena where they could choose between staying in either of two areas: impregnated with tea chemicals or blank. We did this immediately after the conditioning phase or 24h after it. Both protocols were successful for STM within the same-day test, but not for LTM on the following day. In summary, we provide a new method to train aversive conditioning in harvesters and evidence for short-term memory.

Keywords: Learning, negative stimulus, shock platform, cognition

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The study of cognition in arachnids has developed incredibly in the last decade. Numerous works have been published showing simple forms of learning abilities to complex problem solving (Peckmezian & Taylor 2015; Jakob & Long 2016; Cross & Jackson 2019; Mannino et al. 2023). Most of these studies have dealt with spiders, whereas other arachnids have received less attention over the years (but see Santer & Hebets 2009; Wiegmann et al. 2016; Gaffin et al. 2022; Lehmann et al. 2022). Among these other arachnids, the order Opiliones is the 3rd in number of described species in the class Arachnida, with 6782 living and 61 extinct species (Kury et al. 2025). Learning and memory have been shown with experiments involving associative learning (Santos et al. 2013; Costa et al. 2016), both studies using a positive stimulus and testing only for short-term memory. However, being able to learn and memorize from a previous threatening experience can be fundamental to survival (Gabriel & Golightly 2014; Wystrach et al. 2020), so one could expect long-term memory. Because a biologically relevant threatening experience can be hard to reproduce, laboratory-controlled stimuli as shock are widely used since they can trigger physiological and neurological responses associated with nociception (Perisse et al. 2023).

In some cases, learning can occur faster and with longer-lasting memorization when involving an aversive situation (Perry et al. 2013). For example, aversive conditioning is a special form of associative learning that includes an unconditioned stimulus (negative stimulus in this case) associated with a neutral stimulus. Aversive conditioning protocols can be a good option when investigating learning from a negative stimulus and for long-term memory. Different protocols have been used to evoke short-term memory (STM) and long-term memory (LTM) in invertebrates (Amano & Maruyama 2011). Typically, STM protocols use massed training with no/or a very short interval between the trials and it induces memories that are independent of protein synthesis. The LTM protocols use spaced training with a longer interval between the trials. This interval is fundamental for the occurrence of protein synthesis and memory consolidation (Fulton et al. 2005; Mery & Kawecki 2005).

In this study, we investigate the learning and memory capacity of the harvester *Mischonyx squalidus* (Roewer, 1913) (Gonyleptidae) under an aversive conditioning protocol, using two different protocols to test the formation of short and long-term memories.

Mischonyx squalidus appears as *Mischonyx cuspidatus* or *Ilhaia cuspidata* in previous papers (see Gueratto et al. 2021). We collected males of *M. squalidus* under rocks and trunks at the Parque Ecológico do Tietê (−23.507722, −46.547899), in the city and State of São Paulo in July 2019. We used a single sex to exclude the influence of sex and because we found more males than females. We maintained them in individual terraria (10 × 5 × 5 cm height), fed them twice a week with damp dog food, and provided water *ad libitum* in a damp cotton ball. We maintained the temperature at 24° C with ambient light.

Shock is known to trigger nociception responses and it is widely used for learning and memory proposes in a large group of taxa: Mollusks (Sherff & Carew 2009); Nematodes (Amano & Maruyama 2011); Insects (Flies: Mery & Kawecki 2005); Arachnids (Spiders: Bednarski et al. 2012); and others (more examples reviewed in Perry at al. 2013). However, it is common to have problems with the shock apparatus when it is not customized to the species being studied or in some machine models that have inconsistent voltage or problems with conductivity. For that reason, we built a specific shock platform following Peckmezian & Taylor (2015).We made the following adjustments and calibrations to customize it to harvesters: (1) we added the switch to open or close the electrical circuit as we wanted; (2) we included the rubber to guarantee that the ventral part of the opisthosoma would stay in contact with the platform; and (3) we ran preliminary tests with different voltages to guarantee that we were using an aversive stimulus without harming the animals or hampering their movements.

For the neutral stimulus, we chose to use chemicals since harvesters rely on chemicals in a great variety of biological tasks (Willemart et al. 2009; Dias et al. 2020). The experiment consisted

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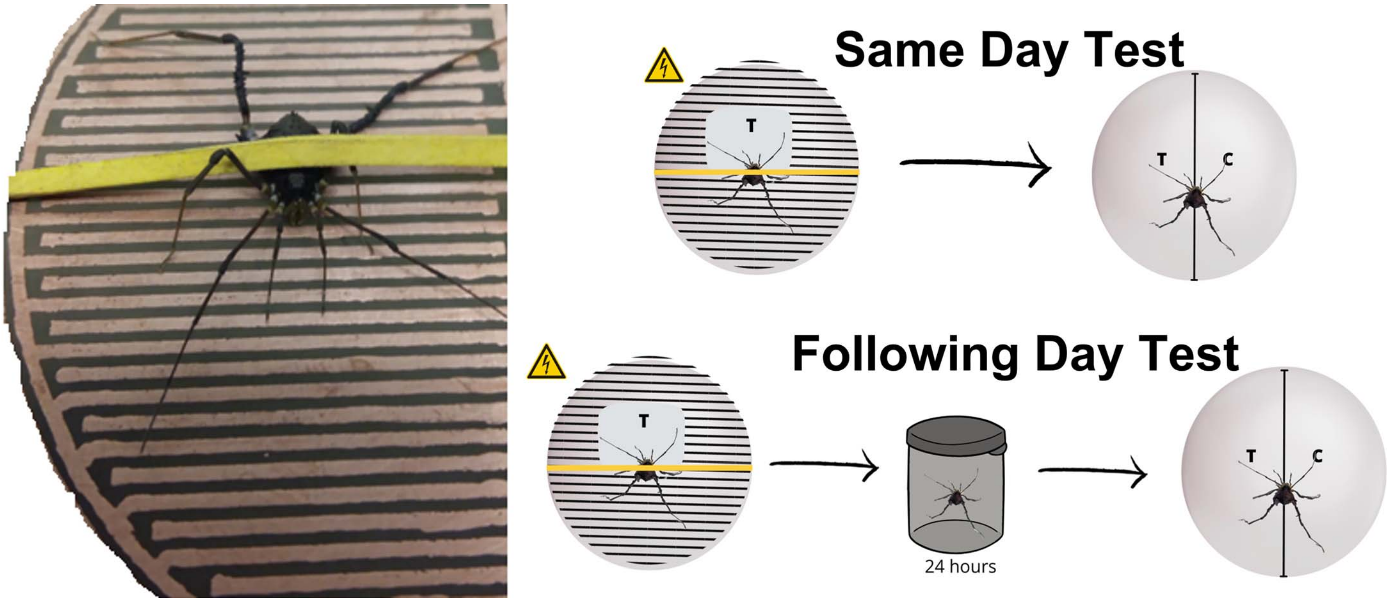

Figure 1.—Set up of the shock platform, with its copper base with parallel negative and positive strips (left) and basic procedure of the experiment (right). T = treatment (tea); C = blank (control).

of having a harvester tied to a shock platform + a chemical (conditioning phase) and subsequently submitting the same individual to a choice arena, where it could stay on the side with the same chemical previously experienced in the shock platform or the control side (test phase). For the shock platform, we used a copper base (10 × 5 cm) with parallel strips, spaced 1.5 mm from each other, alternating a positive and a negative bar (Fig. 1). We designed the pattern using Adobe Photoshop CS5.5 (Adobe Systems, San Jose, CA, U.S.A.) and after that printed on toner transfer film. The film was transferred using a hot iron to a copper board. To prevent the animals from falling off the platform, we used a rubber band to tie the harvester to the platform with the abdomen and legs touching two neighboring strips (therefore a positive and a negative), guaranteeing the electrical circuit was completed (Fig. 1). Using a switch, we determined when the shock was released and its duration. For this experiment, we were interested in testing two different protocols: one with a short interval between the shocks (1 min) to test STM formation and a second one with a longer interval (30 min) between the shocks to test LTM formation (see Brembs 2003; Mery & Kawecki 2005; Amano & Maruyama 2011 for similar protocols to test memory).

Conditioning phase.—For the aversive conditioning protocols, we paired the harvesters with a chemical stimulus (neutral stimulus), securing the animal to the platform with a rubber band and applying a shock (the combination of the two being the aversive stimulus). For the chemical stimulus, we used filter paper left 24 h in contact with mate tea dried leaves. These had been previously used in experiments with harvesters because they detect it but are not attracted or repelled by it (Santos et al. 2013). To ensure the neutrality of the tea leaf stimulus, we conducted a control experiment in which we presented to the test subjects a blank filter paper alongside a filter paper previously impregnated for 24 hours with the odor of mate tea leaves. We then recorded the time spent by each individual on each side over a period of 10 minutes. The harvesters spent 117 s (median; min = 0s, max = 500s) in the half with tea and 168 s (median; min = 0 s, max = 600 s) in the blank side ($P = 0.938$; W = 66; $n = 20$), a non-significant difference.

The procedure consisted in placing the animals with the ventral part of the body in contact with the platform but with the pedipalps, legs I and II (sensory legs – Gainett et al. 2017) in contact with a sample of the filter paper impregnated with tea chemicals (Fig. 1). To provide an aversive stimulus, we used 20-volt shock applied on the copper platform described above. We tried higher voltage, but it hampered the animal's movements. Lower voltages triggered no observable response by the harvesters. Each animal went through 3 consecutive trials for the learning trials with pairings between the chemical and the shock for 3s. We used a sample of 30 males, with 15 individuals randomly assigned to each treatment.

Test phase.—After training, we submitted the animals to a test phase. We placed the animals in a circular arena (18 × 6 cm) in which the halves were covered with a filter paper substrate with or without tea chemicals. The animals were acclimated for 2 min under a transparent cup (8 cm diameter) and then released to move freely in the arena. We recorded the animals for 5 min (plus the 2 minutes of acclimatization), and later scored the time spent in each half. When we moved the animals to the test arena, some animals ($n = 9$) performed a freezing behavior that sometimes lasted more than 2 min of acclimation. In these cases, we started counting the time spent in one half when the animal moved any part of the body. We tested animals of both treatments, STM and LTM, in two stages: the first test was right after the conditioning phase (same-day test); and the second test was on the following day, 24 h after the conditioning phase (following-day test).

For statistical analysis, we performed a Linear Mixed-Effects Model (LMM) using the time that animals spent on each side as our response variable and day (same day – following day), treatment (tea or blank) and group (STM or LTM) as our fixed effects variables (lmer, package LME4). To account for repeated measures, we added individuals' ID as a random effect. After we concluded the analysis, we performed a type III ANOVA using Satterthwaite's method to compare the interactions between the variables tested. All

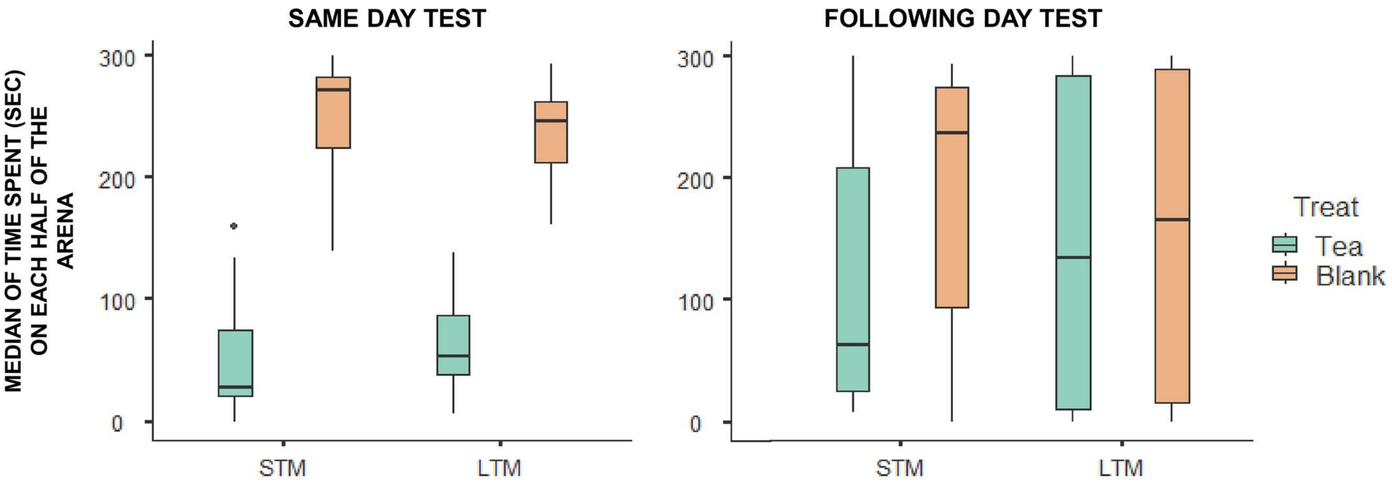

Figure 2.—Time spent on the treatment and control halves in a two-choice arena after the harvester *Mischonyx squalidus* (Arachnida, Opiliones) has been subjected to an aversive stimulus (shock) associated with a neutral stimulus (tea). STM and LTM = Short-term and long-term memory protocols, respectively. Horizontal lines, from top to bottom, correspond to upper quartile, median and lower quartile. The vertical lines correspond to min and max values.

statistical analyses were performed using ''R'' software, version 3.6.2 (www.r-project.org).

For the same-day test, individuals trained in STM spent 249 ± 51 s (mean ± SD) in the blank half, and 52 ± 50 s in the half with tea chemicals (mean ± SD). Individuals trained in LTM spent a mean of 238 ± 40 s in the blank half (mean ± SD) and 62 ± 40 s in the half with tea chemicals (mean ± SD). For the following-day test, individuals trained in STM spent 184 ± 119 s in the blank half and 116 ± 119 s in the half with tea chemicals (mean ± SD). For the individuals trained in LTM, the time spent on the blank half was 150 ± 128 s and 151 ± 128 s in the half with tea chemicals (mean ± SD).

The results of our LMM followed by the ANOVA showed that the treatment effect was significant (F(1, 112) = 47.61, $P < 0.001$), indicating an influence of the aversive learning process on avoiding the tea-associated area. However, the main effects of group (F(1, 112) = 0.00, $P = 0.996$) and day (F(1, 112) = 0.0002, $P = 0.988$) did not present any significance, suggesting that neither the type of training (STM vs. LTM) nor the testing day in isolation were differentiating factors in the learning process.

In addition, the interaction between treatment and day was significant (F(1, 112) = 24.11, $P < 0.001$), showing that the effect of learning varied significantly over time. This suggests that while animals effectively learned to avoid the tea-associated area during the same-day test, tea avoidance was not retained or effectively translated into long-term memory, as evidenced by the random choice behavior observed in both groups on the following day (Fig. 2). The lack of significant interactions between treatment and group (F(1, 112) = 1.2123, $P = 0.273$), as well as the three-way interaction among treatment, group, and day (F(1, 112) = 0.8615, $P = 0.355$), also support the conclusion that the different types of training designed to elicit short-term and long-term memory did not lead to observable differences in long-term associative learning outcomes. In sum, we have shown associative learning using an aversive stimulus and short-term memory irrespective of the time elapsed between the two given stimuli. However, we did not observe long-term memory. Finally, we have developed a successful protocol using aversive stimuli that applies to Opiliones.

We found that the tested harvesters retain the information acquired immediately before the training, both in STM and LTM protocols. Though expected, most of the work on learning in invertebrates has been conducted in insects (Mizunami et al. 2004; Giurfa 2013). However, mechanisms in insects and other taxa are not necessarily the same, calling for studies in a broader range of taxonomic groups. Harvesters learn to associate a stimulus with a shelter (Santos et al. 2013), habituate to a predatory stimulus (Pagoti et al. 2024), and may alter their food choice because of learning (Costa et al. 2016). We are now one step further, showing that an aversive stimulus also triggers associative learning but that the memory does not last in either STM or LTM protocol. We do not know enough about harvester′s brain to speculate about why that is. It has been previously reported that only one in six different training schedules has produced LTM in flies (Yu et al. 2006). Differences at the molecular, cellular, and/or systems level between harvesters and flies explain the absence of long-term memory in these harvesters (see discussion in Davis 2011). Because it is known that performance in acquiring memory increases as the number of training trials increases (Yu et al. 2005), running more than 3 trials (as we did) may be a follow-up of our study.

The aversive conditioning protocol used in this study is a cheap and easy way to access learning and STM in harvesters similar to what has been used in other arachnids (Peckmezian & Taylor 2015, 2017). Since harvesters learned to avoid the place with the conditioned chemical stimulus within just a few short trials pairing the aversive stimulus and a chemical, such a protocol has been successful and can be used in future studies.

The present study was the first that used an aversive protocol to study learning in harvester showing STM. Also, we developed a shock platform that might be adapted in future learning studies in Opiliones. Moreover, the use of a broad methodology and protocols could support comparative studies with other taxa. Finally, we hope that the described protocol and material will be an incentive to carry out more learning studies in harvesters, as in jumping spiders (Long et al. 2015; Jakob & Long 2016; De Agrò 2020; Winsor et al. 2020).

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