

Accepted for its publication in *Behavioural Processes*, 23rd May 2023

Conditioned place avoidance in the planaria *Schmidtea mediterranea*: A pre-clinical invertebrate model of anxiety-related disorders

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Submitted: 22/03/2023

Abstract

The objective of the present study was to develop a model of avoidance learning and its extinction in planarians (*Schmidtea mediterranea*). Based on previous experiments showing conditioned place preference, we developed a procedure to investigate conditioned place avoidance (CPA) using shock as an unconditioned stimulus (US) and an automated tracking system to record the animals' behaviour. In Experiment 1, we assessed the unconditioned properties of different shock intensities by measuring post shock activity. In two subsequent experiments we investigated CPA using different designs, surfaces as conditioned stimuli (CSs; rough and smooth), and different US intensities (5V and 10V). In general, we observed the successful development of CPA. However, CPA was stronger with higher shock intensities, and we found that, in our preparation, a rough surface is best at entering into an association with the shock than a smooth surface. Finally, we also observed extinction of CPA. The evidence of CPA and its extinction in flatworms validates the planaria as a pre-clinical model for the study of avoidance learning, a hallmark of anxiety disorders.

Author contributions

JAA, JP and GPU developed the study concept. Data collections were performed by JAA and LJ. JAA, GPU and JP performed the data analysis and interpretation of results. LJ and JP drafted the manuscript; JAA and GPU provided critical revisions. All authors read and approved the final version of the manuscript prior to submission and declare no financial interest associated with this research.

Open Practices Statement

Data for the three experiments along with a code-book are available at <https://repository.derby.ac.uk/item/9x86q/avoidance-learning-in-the-planaria>. All animals with valid data and observations were included in the analyses.

Conference Proceedings

The data reported in this article were presented: 1) at the *1 Virtual Symposium of Comparative Psychology and Invertebrate Learning*, 5th June 2020 (Zoom Event), as an oral communication; and 2) at the *XXVI Associative Learning Symposium at Gregynog Hall*, Tregynon, Wales, 10-12 April 2022, as an oral communication.

In response to noxious stimulation that constitutes a threat to life or bodily safety, organisms activate innate defensive behaviours that contribute to protect the individual and remove it from the threat posed by pernicious events of biological significance. Defensive behaviours contribute to survival success and, to be effective, must be sensitive both to the key noxious event and the concurrent situational cues that the animal can potentially use to anticipate the threat. By associating the situational cues with the harmful effects of noxious events, animals can prepare to face them in an advantageous position to minimise the harm or avoid the event altogether (Fanselow, 1994). The experiments reported below assess the nature of defensive responses to a shock stimulus in the flatworm planaria, and whether they can learn to avoid the situational cues that co-occur with a harmful shock event.

Planarians are free-living flatworms that belong to the phylum of platyhelminths, the most distant phylum to vertebrates that present a bilaterally symmetrical nervous system. Interestingly, their neurons are strikingly similar to those of vertebrates, with dendritic spines—a putative physiological locus for learning and memory processes (Kasai et al., 2010) rare among other advanced invertebrates (Petralia et al., 2016). Planarians express all the major neurotransmitters such as serotonin, acetylcholine, the catecholamines, GABA and excitatory amino acids that are also present in mammals (Buttarelli et al., 2008). They are widely used in developmental biology as a model for regeneration, aging, stem cells, the development of tissues and pharmacological research (Newmark & Alvarado, 2002; Pagán, 2014). They have also been used as a model for studying basic learning phenomena (Prados et al., 2013; Prados et al., 2020), substance abuse (e.g., Mohamed Jawad et al., 2018; Raffa et al., 2013; Rawls et al., 2010; Sal et al., 2021) and memory reconsolidation (Turel et al., 2020).

The behavioural repertoire of the planaria includes a range of defensive behaviours in response to a diversity of potentially harmful events. For instance,

planarians exhibit negative phototaxis, manifested as their propensity to avoid illuminated environments and light sources (e.g., Davidson et al., 2011; Paskin et al., 2014). In a recent study, Prados and colleagues (2020) assessed the habituation of negative phototaxis, and their work suggests planaria can use contextual cues (like a sandy rough surface) as a signal for bright illumination—resulting in long-term habituation of exploratory behaviour.

Bright illumination has been used as the conditioned stimulus in conditioned place preference (CPP) tasks with planarians. Zhang et al. (2013) established the preference for a dark over a brightly illuminated section of a petri dish—an instance of the negative phototaxis of the planaria. They subsequently exposed the animals to sucrose in a brightly illuminated dish; final tests revealed a change of preference: animals now showed a preference for the brightly illuminated side of the test petri dish, offsetting the innate negative phototaxis. In a closely related set of experiments, Mohammed Jawad et al. (2018) developed the sucrose driven conditioned place preference paradigm and showed that planarians associated the tactile properties of the experimental context (they used smooth and rough surfaces rather than light as the CSs) with the rewarding effects of sucrose. Both the light-sucrose (Zhang et al., 2013) and the surface-sucrose (Mohammed Jawad et al., 2018) associative learning were found to be disrupted by treatment with a dopamine antagonist, indicating that conditioned place preferences are dependent on the dopamine reward system, in line with the literature in vertebrate animals using appetitively motivated learning tasks. CPP has been shown to develop using a range of reinforcers including the sucrose, as mentioned above, and drugs of abuse like amphetamine (Kusayama & Watanabe, 2000), mephedrone or cocaine (e.g., Hutchinson et al., 2015; Ramoz et al., 2012).

In addition to the appetitive conditioned place preference procedures described above, planarians can also be considered a suitable model for the study of aversively motivated behaviour and avoidance. Avoidance is a hallmark of anxiety disorders and

has regained interest in the last decade due to the potential it has to aid the development of behavioural and pharmacological therapies (Pittig et al., 2018; Urcelay & Prevel, 2019). Planarians display a range of defensive behaviours to potentially harming events that are prone to conditioning, making them appropriate for modelling aversive conditioning and avoidance. For example, Wisenden and Millard (2001) assessed the defensive behaviour of planarians in response to chemical cues from injured conspecifics released by individuals as a signal of predation risk. These signals elicit unconditioned avoidance behaviour. In their experiments, following paired presentations of fish tank water (the CS) with the injury-released chemicals (the US), the animals actively avoided the fish tank water, a learned antipredator behaviour driven by a chemical alarm. More recently, Adams and Byrne (2019) used histamine—a known punisher in vertebrate models—to alter context preferences in planarians. Animals confined and exposed to histamine in the darkened areas of a petri dish subsequently spent more time in the light area of the petri dish during a test—compared to planarians that had also been confined to the dark compartment but exposed to fresh water.

The planarians' defensive systems exhibit anxiety-like characteristics sensitive to the same pharmacological manipulations (i.e., increased GABA and 5-HT transmission) which are mainline treatment of anxiety disorders (i.e., benzodiazepines and SSRIs). Nayak et al. (2016) used a dark/light CPP preparation where planarians pre-treated with cocaine and ethanol were tested during drug withdrawal and compared with untreated control animals. They measured how much time the animals in the different groups spent in the illuminated section of the test environment compared to the dark compartment. They found that planaria in drug withdrawal spent significantly less time in the light compartment than the untreated controls, and this response was inhibited by the administration of an anxiolytic drug, clorazepate, a classic benzodiazepine. Using a similar preparation, Zewde et al. (2017) reported

analogous results: planarians treated with either clorazepate, fluoxetine, ethanol and cathinone S-mephedrone ('Bath Salt'), spent more time in the light than in the dark side of a petri dish than control animals, indicating that these drugs reduce the defensive response to the anxiogenic light stimulus. On the other hand, treatment with FG-7142, an inverse benzodiazepine agonist, had the opposite effect, increasing the anxious response to the light.

Avoidance behaviour, a characteristic feature of anxiety related disorders like post-traumatic stress disorder (PTSD) is modelled in mammals with the use of stressors like predator odour. Using planarians, Cho et al. (2019) found that the characteristic light avoidance response in planarians was enhanced by the presentation of a natural stressor, the predator odour frog extract. However, treatment with the SSRI Fluoxetine—approved to treat depression and anxiety disorders—counteracted the planarian avoidant response.

To recapitulate, planarians show unconditioned avoidance responses to stimuli derived from predators and bright illumination. Avoidance of light is enhanced because of exposure to stressors like withdrawal from drugs or predator odours and these responses are sensitive (sometimes bi-directionally) to pharmacological manipulations like those used in the treatment of anxiety disorders.

The use of drugs or natural rewarding substances like sucrose might have long-lasting effects that also affect receptor and/or motor systems. These continuous forms of stimulation can affect the behaviour (sucrose and nicotine, for example, are known to reduce the animal's activity; see Mohamed Jawad et al., 2018; Sal et al., 2021) and result in physiological alterations for relatively prolonged periods of time. It would be advantageous to use instead discrete stressors with limited or non-existent long-lasting physiological effects. A favoured procedure in rodent avoidance research uses discrete electric shocks as a natural stressor that can be anticipated by, for example, audio-visual situational cues. A mild shock can be presented for a very limited amount of time

(half a second) and the animal's physiological and activity levels readily return to normal once the animal gets familiar with the mildly harmful and predictable event. This, however, involves exposing sentient beings to noxious events that produce pain using costly preparations.

Given that rodents are protected by the UK Animal Act (and similar laws around the world), the use of pain inflicting shocks in rodents are regulated procedures. In line with the principles of the 3Rs (Replacement, Reduction and Refinement; Russell & Burch, 1959), we aim to partially replace the use of rodents with an invertebrate model, the flatworm *Schmidtea mediterranea*. An invertebrate model that displays defensive reactions to noxious events (see Browning & Birch, 2022; Proctor et al., 2013, for recent discussions on the sentience of invertebrate animals) as well as complex patterns of behaviour in standard learning preparations, such as blocking (Prados et al., 2013), will be perfectly aligned with the 3Rs principles while providing a solid model to study behaviour and anxiety-related disorders. Our goal was, therefore, to develop a method suitable to study avoidance behaviour in this species of planarians mirroring the rodent procedures that use a discrete shock and is, therefore, free of long-lasting effects in the organism that might distort the observed behaviour. This will ultimately facilitate the translation of findings in different species and enable the use of planarians as a preclinical model to better understand and change avoidance behaviour.

S. mediterranea have been widely studied for their significant regenerative capabilities (Robb et al., 2007; Saló et al., 2009). Because of this, the genome of *S. mediterranea* has been fully sequenced (Grohme et al., 2018), allowing researchers to employ targeted genetic behavioural manipulations and pharmacological interventions. *S. mediterranea* can also reproduce asexually by fission along their body, usually at the tail end, which separates and grows into a complete, adult, genetically identical planarian to the host. The task used in the experiments reported below adapted a widely used CPP task (e.g., Hutchinson et al., 2015; Mohammed Jawad et al., 2018;

Turel et al., 2020), replacing the rewarding stimulus (i.e., sucrose or cocaine) with discrete shocks administered by and under the full control of the experimenter, free of the ambiguous long-lasting effects of administered drugs and natural rewards.

Experiment 1

Previous research has shown that shocks have aversive properties in different species of planaria (e.g., *Dugesia*); animals respond to the shock by presenting a characteristic shrinking response (e.g., Baxter & Kimmel, 1963; Prados et al., 2013). The aim of this experiment was to assess the unconditioned properties of different shock intensities upon locomotion suppression in the *S. mediterranea*, to establish a benchmark voltage to be used as the aversive unconditioned stimulus (US) in subsequent conditioned place avoidance experiments.

Four groups of planaria received either 0V, 5V, 10V or 15V shocks during a single session. We measured the locomotion of the planaria, before and after the shock treatment, to assess whether the shocks impacted their locomotion activity in an intensity-dependent manner. Establishing the properties of different shock voltages on locomotion suppression will enable the selection of an adequate shock magnitude for its use as the US in subsequent experiments. Following on what has been observed in other species (Blanchard & Blanchard, 1972), we expected that the administration of shocks would temporarily attenuate (i.e., suppress) the locomotion of the planarians.

Method

Subjects. In this and subsequent experiments, we used experimentally naïve *S. mediterranea* sourced from the University of Leicester's colony. Our colony was made up of asexual clones genetically identical, reducing the confounding effects of individual differences on behaviour due to the genetic makeup of the experimental subjects. The colony was kept in *Montjuic water*, a solution of 5 mmol/l NaCl, 1.0 mmol/l CaCl₂, 1.0 mmol/l MgSO₄, 1.0 mmol/l MgCl₂, 1.0 mmol/l KCl and N/A mmol/l

NaHCO₃, that has been shown to be the ideal medium for the animals to healthily grow and develop (see, for example, Brubacher et al., 2014; Sal et al., 2021). Montjuic water was made on-demand and stored for a maximum of 21 days, at which point any remaining water was disposed of and replenished. The incubator was kept at 20°C and a 16/8 hours of light/dark cycle (lights on at 9 AM). Animals were fed ox liver twice weekly followed by water and habitat maintenance (see Turel et al., 2020). Before the start of the experiment, the animals were transferred from the breeding colony and housed in individual recesses (2cm x 3.5cm) of plastic ice-cube trays containing 10ml of Montjuic water. The trays were kept in the same incubator as the colony and were covered with a light-diffusing opaque lid. Over a period of fourteen days the animals were deprived of food and handled (with a soft brush) once per day to replenish the water in the trays and to minimize division of the animals through fission.

In Experiment 1, thirty-two animals were used and randomly assigned to one of four experimental groups, 0V, 5V, 10V and 15V, matched by their levels of activity observed during a baseline pre-training assessment (see the Procedure section below). Five subjects, one each from the 0V, 5V and 10V groups and two animals from the 15V group, were excluded from the analyses for failing to respond during the test session. Therefore, there were 7 animals in groups 0V, 5V and 10V, and 6 animals in group 15V. Four additional animals in Group 15V died midway during the test; for those animals we analysed the data corresponding to the bins (see below the description of the test) where activity was recorded. The 15V shock was deemed as excessively intense and harming, and we stopped using it following this experiment.

Apparatus. The animals were tested in concave watch glass soda lime dishes 100 mm in diameter, 13.5 mm height and 1.2 mm thickness filled with 15ml of Montjuic water. They were tested in groups of up to 16 by using four custom-made wooden boxes that would each hold four dishes. Each box contained a dimmable 30 x 30 cm 24W LED panel as the floor of the box; in the present experiments, the light was set at

60 lux. The dishes were placed on top of the LED panel. A camera on the top centre of the wooden box could simultaneously record the activity of four animals using SharpCap capture software; these videos were subsequently analysed using a video-track system (ViewPoint, Lyon, France) allowing us to automatically register the activity of the four animals in each box during the experimental sessions (see Figure 1; also, Prados et al., 2020, Experiment 2). This is advantageous compared to other procedures where researchers rely on observers' judgements to establish whether a conditioned response has developed (see, for example, Prados et al., 2013). One planarian was placed in each dish using a smooth pencil brush. A variable DC power supply (SkyTronic Ltd., Manchester, UK) and copper electrodes were used to deliver the shocks by presenting the electrodes near the head and tail of the planarian (see Prados et al., 2013).

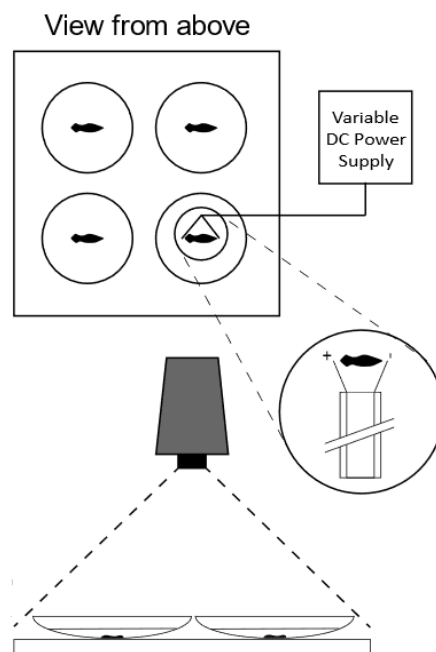


Figure 1. Schematic representation of the apparatus used in this series of experiments.

Procedure. There were three phases in Experiment 1: baseline measurement, shock treatment and test. During the baseline measurement, the locomotor activity of each

planarian was recorded for 10 minutes. Following the baseline measurement, the animals were left to rest for a forty-minute period in the home individual recesses; during the resting time, the researchers assessed the activity levels of the animals during the pre-training test and assigned them to the four experimental groups (0V, 5V, 10V, and 15V) matched by their levels of activity—to ensure there were no differences between the groups before the shock treatment phase.

The shock treatment phase lasted thirty minutes and started with three minutes of acclimation to the dishes followed by the administration of six shocks with an Inter-Trial-Interval (ITI) of 4-minutes. The shocks were administered manually with an approximated mean duration of half a second (following the procedure described by Prados et al., 2013). Animals in groups 5V, 10V and 15V received 5-, 10- and 15-volts shocks respectively during the shock treatment phase; animals in the control 0V group were presented with the electrodes on the same schedule as the other groups, but no shocks were delivered. Following the last shock, the animals were allowed an additional three-minute rest period before a 10-min test in which the motor activity of the animals was again recorded following the same procedure used for the baseline measurement phase.

The activity (locomotion) was measured in centimetres and recorded during the baseline and test phases in bins of 1 minute. ANOVAs with the 1 min Bins as factor carried out on the data of the pre- and post-training tests showed that the factor was not significant, and it did not interact with other factors (indicating that the activity of the animals was very stable during the 10 minutes of the tests). We therefore calculated the average activity over the 10 bins for the pre- and post-training activities (see Figure 2). The analysis of the activity during the pre- and post-tests was conducted with a mixed ANOVA. For all the experiments reported here, the rejection criterion was set at .05 for all statistical tests. Partial eta squared measures were reported as effect sizes

and their 95% confidence intervals [CI] were reported using the software available in Nelson (2016); Cohen's d was provided when a one sample t-test was conducted.

Results and discussion

Figure 2 displays the average locomotor activity during the pre- and post-training tests for the four groups. During the pre-training test all groups showed similar levels of activity; however, during the post-training test the three groups treated with shock showed a sharp reduction in their levels of activity. A mixed ANOVA with the between-subjects factor Group and the within-subjects factor Tests (pre- and post-training tests) revealed significant effects of Group, $F(3,23) = 16.92, p < 0.001, \eta_p^2 = .68, 95\% \text{ CI } [.36, .77]$, Tests, $F(1,23) = 58.06, p < 0.001, \eta_p^2 = .71, 95\% \text{ CI } [.45, .81]$, and a significant interaction Group x Tests, $F(3,23) = 8.66, p < 0.001, \eta_p^2 = .53, 95\% \text{ CI } [.16, .66]$. Post hoc Tukey HSD pairwise comparisons revealed that the control Group 0V differed from the other three groups (5V, 10V and 15 V), ($p < 0.001$). There were no differences between the groups 5V, 10V and 15V.

Further analyses were carried out to analyse the Group x Tests interaction. One-way ANOVAs were carried out on the data of the pre- and the post-training tests, revealing a null effect of Group in the pre-training test, $F(3,26) = 1.80$, and a highly significant effect of Group in the post-training test, $F(3,26) = 29.46, p < 0.001, \eta^2 = .77, 95\% \text{ CI } [.58, .82]$.

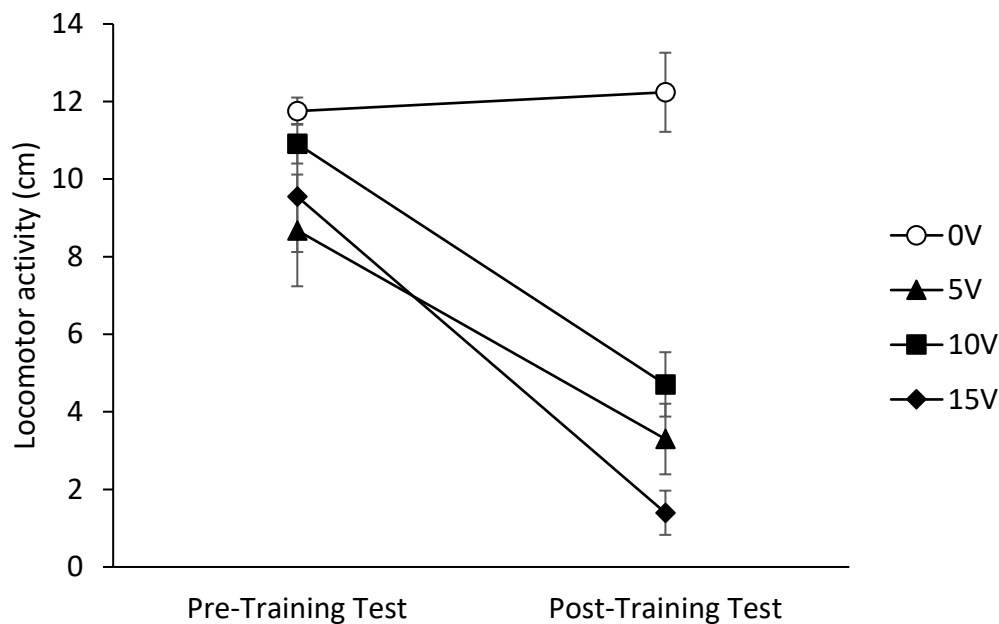


Figure 2. Mean locomotor activity throughout the baseline and post-training test sessions by group (\pm SEM) in Experiment 1.

The goal of this experiment was to identify a safe but suitably aversive level of shock to use in our CPA protocol. Both 5V and 10V groups showed a significant decrease in locomotor activity compared to baseline after receiving six shock presentations, whereas the 0V group displayed no change after six mock trials. The 15V group had a high mortality rate and as such was deemed not suitable for use as an aversive stimulus. This pattern of results is consistent with our expectations and with previous findings in other species in which shock temporarily inhibits locomotion (Blanchard & Blanchard, 1972). Our results suggest that both 5V and 10V safely generated similar levels of locomotion suppression, without large differences between them.

Experiment 2

The focus of this series of experiments was to observe conditioned place avoidance in planaria and develop a reliable protocol for the investigation of aversively motivated learning processes. In Experiment 2, we adapted a conditioned place

preference (CPP) protocol previously used in our laboratory (e.g., Mohammed Jawad et al., 2018; Turel et al., 2020), replacing the rewarding stimulus with a 5V shock to create an aversively motivated paradigm. During a pre-training test, the animals were exposed to two distinctive contexts, a rough and a smooth surface, using a two-sided dish, and their preference was recorded. We then exposed the animals to the preferred context where they received six daily presentations of the aversive 5V shock (on each of 4 days). Animals were also exposed to the non-preferred context but not shocked. A final preference test was then carried out in the two-sided dishes to see whether this treatment reversed the preference expressed in the pre-training test—that is, if animals developed an avoidance response to the context paired with the shock.

The experiment was run in two replications. The only difference between the two replicas was the nature of the housing conditions: in the first replica the animals were housed in the plastic ice cube trays described in Experiment 1; in the second replica, the trays were covered in silicone in an attempt to make the surface of the house cubicles more distinguishable from the smooth experimental context (see Prados et al., 2020, for a discussion of how the similarity between the house and the experimental contexts can affect performance in an habituation task). Two ANOVAs were carried out on the data of the pre-training and the post-training tests including the factor Replica. The Replica factor and all the interactions involving it were non-significant (all $F_s < 1$); therefore, we collapsed the data of the two replicas and report them as a single experiment.

Method

Subjects. Thirty-two experimentally naïve *S. mediterranea* sourced from the University of Leicester's colony were used in this experiment. The flatworms were bred and housed in the same manner as in Experiment 1.

Apparatus. The apparatus and experimental setup remained the same as in Experiment 1. Three types of dishes were used in the present experiment as the experimental contexts: the plain watch glasses described above; a rough-etched dish that provided a distinctive surface that the animals easily discriminate from the smooth surface (e.g., Sal et al., 2021; Turel et al., 2020); and two-sided dishes 50% smooth-plain and 50% rough-etched (bisecting the midline).

Procedure. There were three experimental phases in Experiment 2: pre-training test, training, and post-training test.

Pre-training test. On the first day of the experiment, a context preference test was carried out in the two-sided dishes described above to assess the baseline context preference. The pre-training test had a duration of 30 mins, during which the animals were allowed to freely explore the two-sided dishes. Using the tracking software, the time spent on each surface was measured. A preference ratio (PR) was then calculated: $PR = \text{time in preferred context} / [\text{time in preferred context} + \text{time in non-preferred context}]$. A PR close to 0.5 would be indicative of no preference whereas scores approaching 1 would indicate a strong preference. The animals were assigned to one of two groups Rough (n=16) or Smooth (n=16) based on their basal preference: in the first replica, 9 planarians preferred the rough over the smooth surface and 7 showed a preference for the smooth surface; in the second replica 7 preferred rough and 9 smooth. Following the pre-training test, the animals were allowed two days of rest in their home environment before the onset of the training phase.

Training. This phase took place over four days (days 4 to 7 of the experiment). We conducted two 30-min training sessions per day (a total of eight training sessions) during which each planarian was exposed to the two surfaces (Rough and Smooth, using the dishes described above) and experienced six 5V shocks on their preferred surface (either the rough or smooth). Half of the planaria in each group experienced shocks in the AM session when placed on their preferred surface; the remaining

planaria experienced the shocks in the PM session in their preferred context. In the alternative sessions, the animals were exposed to the non-preferred context in the absence of any shocks. There was an interval of 3.5 hours between the AM and the PM sessions. The distribution of groups among the experimental chambers was counterbalanced so that each run contained animals assigned to both groups (Rough and Smooth). Shocks were given using the same schedule as Experiment 1: a 3 min acclimation period, followed by the presentation of six 5V shocks with an ITI of 4 min and a final 3 min rest period before the end of the session.

Test. The test took place on the final day of the experiment (day 8). The animals were allowed to freely explore the two-sided dishes for 30 min (as in the pre-test session); the time spent on each surface was measured. An avoidance score was then calculated by subtracting the preference ratio shown during the post-training test from the preference ratio showed during the pre-training test. An avoidance score near zero indicates that the animals did not change their preferences —so no avoidance developed. Positive scores are indicative of a change of preference whereby the animals tend to avoid the initially preferred context. A one-sample t-test was carried out to compare the avoidance score with the value of zero for the whole cohort of animals. Subsequent analyses (one-way ANOVA) were performed on the data of the two groups of animals (trained with the rough and smooth surfaces) to analyse the differences between them. We finally carried out one-sample t-tests for the two groups of animals (trained in the rough and smooth contexts) separately.

Results and discussion

During the pre-test, the preference ratio for the whole cohort was 0.60 ($MSE=0.02$); a one sample t-test comparing the observed preference score with 0.5 revealed a significant difference, $t(31) = 5.47$, $p < 0.01$, $d = 0.96$, indicating a reliable basal preference for a particular surface. The preference scores for the two groups (those preferring the Rough or the Smooth context) were 0.59 (0.02) and 0.62 (0.03)

respectively; a one-way ANOVA did not reveal any differences between the two groups, $F < 1$.

Figure 3 shows the mean avoidance score during the post-training test. A score of 0 indicates no change in preference. As mentioned above, avoidance scores above zero suggest the development of avoidance of the context paired with the shock—and therefore a change from the baseline preferences recorded during the pre-training test. Given that the design was balanced (we had an equal number of animals that preferred the rough and the smooth contexts) we first analysed the avoidance score for the whole cohort; visual inspection of Figure 3 (left panel) suggests that there was a significant avoidance response for the whole cohort. A one sample t-test (against zero) supported this impression, $t(31) = 2.46$, $p = 0.01$, $d = 0.43$. However, we informally noticed that the change was driven by the animals that experienced pairings of the rough context with the shock. We therefore present the data for the two groups (rough and smooth) of animals in the right panel of Figure 3; these data strongly suggest that an avoidance response developed for the rough context but not for the smooth one. A one-way ANOVA confirmed this impression, $F(1,30) = 11.09$, $p < 0.01$, $\eta^2 = 0.27$, 95% CI [.04, .48]. Finally, one-sample t-tests (against zero) performed on the data of the groups Rough and Smooth showed that the group Rough developed a significant avoidance response, $t(15) = 3.90$, $p < 0.01$, $d = 0.97$ but not the Smooth group, $t < 1$.

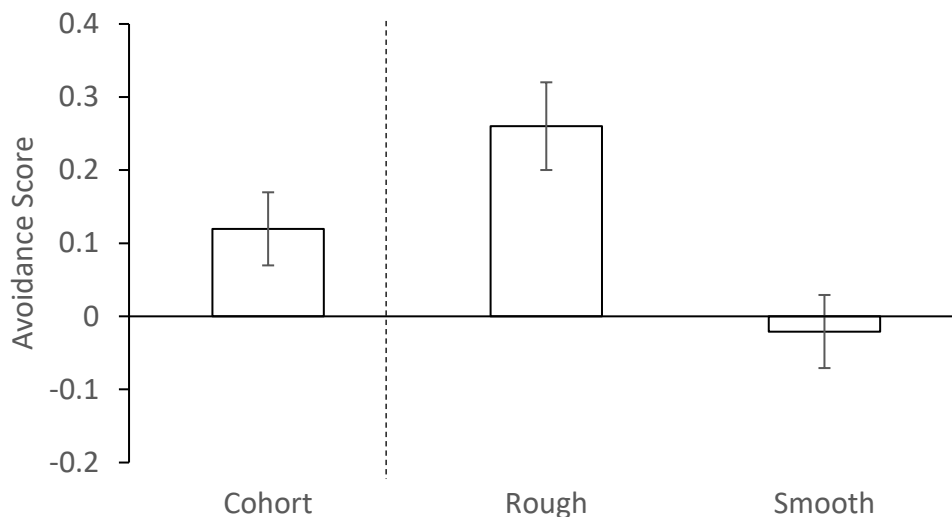


Figure 3. The left panel shows the mean avoidance score (\pm SEM) for the whole cohort of animals in Experiment 2. The two columns in the right panel show the mean avoidance scores (\pm SEM) for the groups given training in the Rough and the Smooth contexts.

Although the analyses of the entire cohort revealed a significant conditioned place avoidance, subsequent analyses made it clear that no conditioning took place within the group trained on the smooth surface. On completion of the first replica, we hypothesized that a reason for the failure to observe avoidance learning in the smooth surface would be the similarity between the smooth surface of the dishes used in the experiment and the smooth surface of the plastic ice cube trays used to house the planarians. Previous work in our laboratory has revealed that the similarity between the house and the experimental contexts affects context related learning. Prados et al. (2020) observed that planarians exhibited long-term habituation of exploratory behaviour when trained in a surface which was clearly dissimilar from the surface of the housing trays. However, when the two surfaces were similar—sandy rough house and sandy glass, or smooth glass and smooth plastic as in the present experiment—the animals failed to develop long-term habituation. Prados et al. (2020) suggested the novel surface (either rough or smooth) was more salient than the relatively familiar context similar to the house one, which would lack salience or associability—an

instance of latent inhibition (e.g., Lubow & Moore, 1959). To address this possibility, here we ran a replica using a modified house tray coated with silicone to make the house and the smooth experimental contexts dissimilar. As mentioned above, this manipulation was not effective, and we obtained perfect replicas of the main outcome: strong avoidance learning in the rough but not in the smooth context. In subsequent experiments we assessed the development of avoidance learning administering conditioning in the highly efficient rough context CS only.

In the present experiment, the observed conditioned avoidance might be due to a change of preferences merely due to the increased familiarity with the two contexts through the training phase: any initial preferences tend to vanish with repeated experience with the contexts (see Mohamed Jawad et al., 2018). Initial dislike for the less preferred context tends to vanish as the animals' experience with this context increases. Although this possibility does not fit with the data of the Smooth group (in which animals retain their initial preference for the smooth context even though they accumulate experience with the rough context), we considered it would be more rigorous to control for this possibility in Experiment 3 by including a Control group that had the same experience with the contexts as the Experimental group but never experienced the shock. The use of this control group addresses the changes in preference due to mere exposure to the surfaces.

Experiment 3

In Experiment 3, we used a design in which animals were randomly assigned to one of two Groups, Experimental and Control. Animals in Group Experimental received pairings of the rough context with shock, and equal exposure to the smooth context but in the absence of shock. The Control group was matched with the Experimental group in terms of the experience with both contexts but never experienced the shock. If the results of the group Rough of Experiment 2 are due to increased familiarity with the contexts, we should observe a change in preference in both groups. However, if the

results of the previous experiments are indicative of the development of an avoidance response, we should observe a change of preference in the Experimental group only (e.g., Bozarth, 1987). Experiment 3 was carried out in two replications which are reported as Experiments 3A and 3B.

Experiment 3A

Method

Subjects. Thirty-two experimentally naïve *S. mediterranea* sourced from the University of Leicester colony were used in this experiment. We randomly allocated sixteen planarians to each experimental group; however, eight planarians died during the experiment, so the final number of planarians for the Experimental and Control groups was ten and fourteen respectively. The animals were bred and housed in the same manner as in Experiments 1 and 2.

Apparatus. The experimental apparatus and husbandry of the animals was the same as in Experiment 2; all planarians were housed in plastic cube trays for ten days before the beginning of the experiment.

Procedure. There were two phases in this experiment, training and test. During the training phase, two daily sessions took place, AM and PM. The training phase lasted four days, so all the animals experienced a total of eight sessions. Animals in the Experimental group were given four sessions in the rough context, where they were exposed to the shock, and four in the smooth context without any shocks (see Figure 4). Animals in the Control group were given the same exposure to the rough and smooth contexts but in the absence of any shocks. In each session, half of the experimental subjects were placed in the rough dishes and exposed to six 5V shocks over a 30 min session with an ITI of 4 min (as in Experiment 2); in the same session, half of the control subjects were also placed on the rough surface and exposed to the electrodes, but no shocks were delivered. The remaining control and experimental

subjects were placed in the smooth surface for the 30 min of the session. The experimental design was fully counterbalanced across sessions so that half of the animals received the shocks (or were exposed to the electrodes) in the AM session, and the other half in the PM session. Furthermore, each experimental chamber accommodated two control and two experimental subjects within each session.

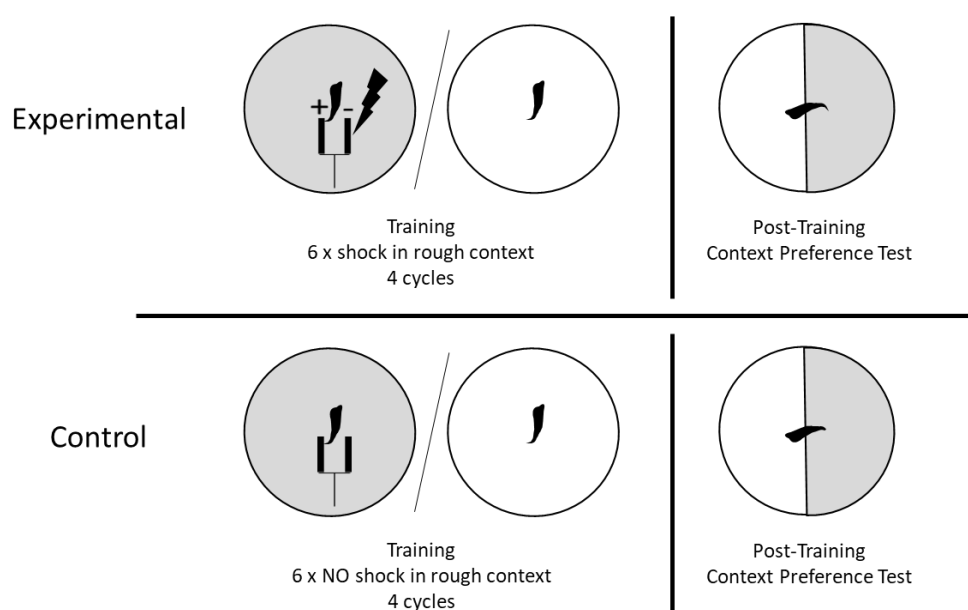


Figure 4. Schematic of training sessions of Experiments 3a and 3b. The Experimental group received shock on the rough context in either the AM or PM session and no shock in the alternate sessions. Control animals received the same exposure and manipulation as those in Experimental group, but no shock when the electrode was presented.

The test trial took place on the final day of the experiment (Day 5). A 30-min preference test session was conducted using the two-sided dishes, roughly equidistant from the time the AM sessions ended and the PM sessions would have begun during training. During the test session, the percentage of time spent in each surface was recorded. A preference ratio for the rough context paired with shock ($\text{time in rough} / [\text{time in target rough} + \text{time in smooth}]$) was calculated. A score of 0.5 is indicative of no preference (equal time spent in the rough and smooth contexts), whereas scores

close to zero are indicative of an avoidance of the rough context. We transformed these scores by subtracting them from 0.5 to obtain an avoidance score in line with the previous experiments — therefore a positive value is indicative of conditioned avoidance. A one-way ANOVA was carried out to compare the avoidance score of the two groups.

Results and discussion

Figure 5 shows the mean avoidance scores for the two groups during the test trial. Initial impressions of the data suggest the development of a conditioned avoidance in the Experimental but not in the Control Group. A one-way ANOVA did not confirm this impression, revealing a non-significant effect of Group, $F(1,21) = 1.94$, $p=0.08$.

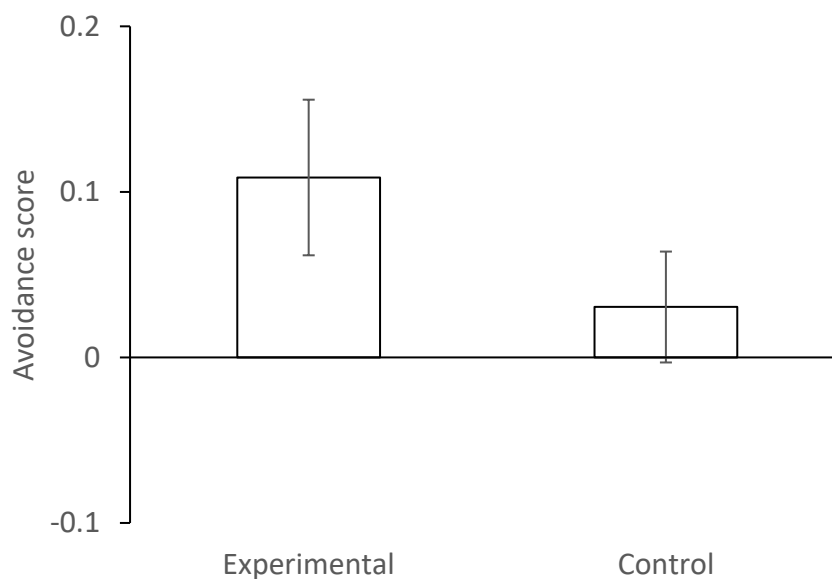


Figure 5. Time spent on each surface during thirty-minute test session on dual surface testing dish (\pm SEM) for both control and experimental groups in Experiment 3A.

Experiment 2 and the present experiment suggest that pairing a rough context with the delivery of shocks results in avoidance learning, although Experiment 3a did not have enough sensitivity to detect significant differences between Experimental and

Control Groups. Experiment 2 provided evidence for the development of avoidance learning in planaria, but the experimental design might be vulnerable to criticism—as discussed above, in the absence of a control group. That is, the change in preference observed following aversive training might be confounded with mere familiarisation with the contexts. The present experiment used a stronger experimental design incorporating a control group. Although following aversive training in the experimental group no reliable statistical differences were observed, the ordinal direction of the means looks in the correct place, which is suggestive but not enough to achieve the significance threshold. This implies that the two experimental groups might have performed differently during the test: only the animals in the experimental group give the impression of been avoiding the context paired with the shock-US.

The absence of differences among groups might be attributable to a lack of power—we lost some animals during the experiment leading to a relatively low sample size, especially in the Experimental group ($n=10$). Another possibility is that the lack of clear differences is due to the use of a relatively mild shock as the US. Experiment 1 indicated that experience with a 5V shock was sufficient to lower locomotor activity. Since there were no differences between the Groups 5V and 10V and given the high mortality of the animals treated with a 15V shock, we decided to be conservative and use the milder shock, 5V, which seems to be effective in establishing some level of avoidance. However, it is well known that the intensity of the US is a key factor in determining the development of a conditioned response, with more intense USs leading to more robust learning. This is the outcome typically observed in rodents using Pavlovian fear conditioning (e.g., Annau & Kamin, 1961) and avoidance tasks (e.g., Kimble, 1955). Indeed, standard associative learning models like the Rescorla-Wagner model (Rescorla & Wagner, 1972) anticipate higher learning rates with higher shock intensities. In Experiment 3B we replicated Experiment 3A but with a higher shock intensity (10V). Another key feature of learned behaviour is that it extinguishes

when the animals are presented with the conditioned stimulus in the absence of reinforcement (Pavlov, 1927). Therefore, in Experiment 3B we also conducted two additional test sessions to assess extinction of the conditioned avoidance response.

Experiment 3B

Method

Subjects. Thirty-two experimentally naïve *S. mediterranea* sourced from the University of Leicester colony were used in this experiment. The animals were bred and housed as described in the Experiment 3A. Four planarias died during the experiment, leaving the final number of subjects in twenty-eight (Group Experimental n=13; Group Control n=15).

Apparatus and Procedure. The apparatus and procedural details replicate those described for experiment 3A; the only change was the intensity of the shock used as the US, which was increased from 5V to 10V in this experiment. In addition, two additional test trials were carried out 24 and 48 hours after the first post-training test session to assess whether exposure to the contexts in the absence of the shock-US resulted in extinction of the avoidance response, as has been observed in conditioned place preference experiments (Mohammed Jawad et al., 2018; Turel et al., 2020). A mixed ANOVA was carried out to compare the avoidance score of the two groups across the three test trials.

Results

Figure 6 shows the mean avoidance score for the two experimental groups throughout three test trials. As stated above, positive values indicate the development of an avoidance response. Initial impressions of the data suggest the development of a conditioned avoidance in the Experimental but not in the Control Group. The conditioned avoidance response, however, seems to decline rapidly after the first test session and the differences between the groups disappeared. A mixed ANOVA with

Group (Experimental vs Control) and Test (1-3) as factors revealed a significant Group x Test interaction, $F(2,52) = 3.66$, $p = 0.03$, $\eta^2 = 0.12$, 95% CI [.01, .27]. There were also a near significant effects of Group, $F(1,26) = 3.39$, $p=0.07$, $\eta^2=0.11$, 95% CI [.03, .33] and Tests $F(2,52) = 3.09$, $p = 0.05$, $\eta^2 = 0.10$, 95% CI [.03, .32]. Further analyses, using one-way ANOVAs, were carried out to assess the Group x Tests interaction. There was a significant effect of Group in Test 1, $F(1,26) = 15.43$, $p < 0.01$, $\eta^2 = 0.37$, 95% CI [.09, .57]. However, the effect of group was not significant in Tests 2 and 3, $F_s < 1$. Analysis of the Tests factor (1-3) in Group Experimental revealed a significant effect, $F(2,24) = 4.39$, $p = 0.02$, $\eta^2 = 0.26$, 95% CI [.02, .43]; the within-subjects contrasts revealed a significant difference between Test 1 and Test 2, $F(1,12) = 16.14$, $p < 0.01$, $\eta^2 = 0.57$, 95% CI [.19, .72]; none of the remaining comparisons were significant, largest $F(1,12) = 3.68$. The same analysis in Group Control did not reveal differences between test sessions, $F(2,28) = 1.34$.

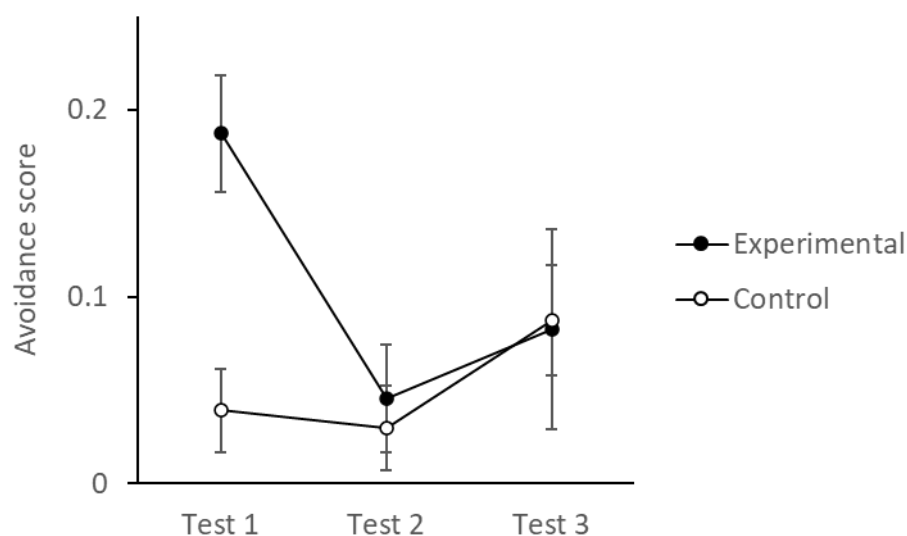


Figure 6. Avoidance scores from tests sessions on dual surface testing dish (\pm SEM) for both Control and Experimental groups in Experiment 3B.

General Discussion

The present study assessed the development of avoidance responses in planarians using a conditioned place avoidance paradigm. Overall, we found that animals tend to actively avoid contextual cues that have been paired with the presentation of an aversive event, a shock unconditioned stimulus (US). Experiment 1 aimed to assess the impact that shocks of different intensities had on the locomotor activity of the animals. Compared to a control group, animals that experienced six shocks of 5V, 10V and 15V intensities showed a significant suppression of locomotor activity; however, although there were no significant differences between the three levels of intensity, the order of suppression is descriptively in line with the shock intensities. At the other end, the 15V shock was found to be harmful (six out of eight animals in this group died before the end of the experiment).

Experiment 2 assessed the development of conditioned place avoidance in a biased design where the animals experienced the 5V shocks in their preferred context. Some animals showed a preference for the rough context whereas others expressed a preference for the smooth context. An analysis of the whole cohort showed that animals developed a significant avoidance response for the initially preferred context which was then paired with the shock. However, informal observation of the animals suggested that this significant avoidance response was driven by those animals that experienced pairings of the rough context and the shock. Additional analyses confirmed that only the animals trained with the rough context developed an avoidance response. Based on previous work from our laboratory (Prados et al., 2020), we assessed whether the similarity of the housing environment (plain smooth plastic) and the smooth (glassy) context, prevented the development of conditioned place avoidance—familiarity with the target smooth surface might be considered an instance of latent inhibition. The comparison between two replicas using a smooth housing context and an altered housing context covered in silicone revealed that this variable

did not have any effect on the outcome of the experiment (as argued above, we decided to report the two otherwise identical replicas as a single experiment).

In a previous report from our laboratory (Prados et al., 2020), we observed that housing the animals in a distinctive rough sandy surface facilitated the expression of long-term habituation learning in a smooth surface similar to the one used here. This suggests that the novelty of the surface is a key factor in promoting learning. The present results suggest that the silicone and glassy contexts are relatively similar; perhaps housing the animals in a highly distinctive context like the sandy one used by Prados et al. (2020) might have improved the chances of observing avoidance learning in the smooth glassy context. However, in the previous work referred to above, we used a different species of planaria, *Dugesia*, which are bigger than the *S. mediterranea* used in the present series of experiments. In pilot experiments we tried the rough-sandy context with *S. mediterranea* and found it difficult to handle the animals in the sand context without damaging them. Hence, after some unsuccessful attempts with the sandy context, we swapped to the silicon context that was safer for the animals.

Although at first sight Experiment 2 might be taken to be a clear demonstration of conditioned place avoidance, the use of a design without a control non-shocked group might be somewhat misleading. The change in preference shown by the animals may simply reflect a smoothening of the initial preferences due to the acquired experience with both contexts during the training phase of the experiment: with higher familiarity, the animals might feel equally at ease in both sides of the test petri dish. This alone can result in a significant change of preferences with the animal's preference rate approaching 0.5 (e.g., Mohammed Jawad et al., 2018). To address this possibility, we ran Experiment 3, where all the animals were trained in the rough context—and equally exposed to the smooth context but in the absence of shock, and with the inclusion of a non-shocked control group. Experiment 3A showed a trend towards the development

of conditioned avoidance in the Experimental group; however, the standard analysis of variance failed to reveal any differences with the non-shocked Control group. An informal analysis of the performance during the tests of each of the two groups separately suggests, however, that the animals in the Experimental group developed a significant avoidance of the trained context whereas this was not the case in the Control group (see Figure 5). The absence of differences between the two groups might have been the consequence of a lack of statistical power (we had a relatively small sample in the Experimental group); alternatively, it might simply reflect the limited effectiveness of the mild shock (5V) used as the US. It is well established that the intensity of the reinforcer is a determinant of the magnitude of the conditioned response (e.g., Annau & Kamin, 1961). Experiment 3B replicated the procedure of Experiment 3A with a more intense shock-US (10V). We also tested the animals thrice, 24, 48 and 72 hours after the completion of training, to assess how well the avoidance learning is retained and whether the experience of 30 min exposure to the surfaces in the absence of the shock contributed to the extinction of the conditioned avoidance response. The results revealed a clear difference between the Experimental and Control groups during Test 1: animals that were shocked in the rough context showed a significant avoidance response compared to the control non-shocked group (which did not show a significant preference for the rough/smooth contexts). In addition, the avoidance response extinguished after the first test, and the two groups behaved equally in the two final tests.

The mere notion that invertebrate animals could represent contextual cues and acquire conditioned responses has been disputed. In vertebrates, it is well established that contexts can act both as discrete cues and as occasion setters (or modulators; see Urcelay & Miller, 2014 for a review). However, Lubow (2010) suggested that due to their limited neural structure, invertebrates might not be able to represent contextual information, and might be unable to establish associations between contextual cues

and a discrete stimulus (like the shock used in the present experiments) or establish associations between two non-biologically relevant stimuli. Empirical data recently reported challenges this somewhat conservative approach. Studies on renewal (e.g., Bouton & Bolles, 1979) in the snail *Cornu aspersum* have established that contextual cues like the photoperiod, defined as the time of the day and changes in illumination, can act as effective retrieval cues for acquisition and extinction memories (Loy et al., 2020). The use of contextual cues as a retrieval cue strongly supports the notion that, despite their relatively simple neural structure, invertebrate animals can represent the contextual cues and use them not only as signals for significant events, but also as occasion setters that modulate the efficacy of the association between other events.

Our results provide experimental evidence to validate the use of our conditioned place avoidance protocol for the study of aversively motivated learning in invertebrates, specifically, *S. mediterranea*. Planarians that experienced repeated pairings of a particular surface and shocks, avoided the surface in a subsequent test (in the absence of shocks) in line with the rodent literature. Conditioned place avoidance paradigms are used in the research of pain in rats given an injection of a nociceptive stimulus (e.g., formalin) and placed in a distinctive context, or given shocks in that context (see, for example, Gao et al., 2004). These paradigms in rodents and planarians constitute a useful tool for the study of avoidance responses, a diagnostic criterion across anxiety-related disorders (anxiety disorder and OCD, for example).

One intriguing aspect of the current experiments is that we only observed robust avoidance learning in animals trained on the rough surface, but little or no evidence of learning in those animals trained on the smooth surface (Experiment 2). Moreover, the addition of the silicon housing environments in one of the replicas of Experiment 2 appeared to have little effect (c.f., Prados et al., 2020). The surprising finding of conditioning only on the rough surface, we speculate, may be driven by the ecological validity of the rough surface for *S. mediterranea*. Smooth surfaces like those of watch

glasses are not typically present in nature, even the smoothest stone to human touch has countless imperfections, that are likely detected by planaria. This reduced ecological validity of the smooth surface, and the inverse for the rough surface, results in a reduced ability for the smooth surface to enter into an association with the shock, much in the same way a rodent fails to associate a tone paired with a lithium chloride injection but can easily associate a flavour with the same lithium chloride injection after one trial. This has been thought to result from the easiness with which flavours and the resulting sickness are associated due to them all stimulating interoceptive sensory systems (e.g., Garcia & Koelling, 1966; Urcelay & Domjan, 2021). General theories of learning can deal with this finding by assuming that the rate of conditioning to the smooth surface is determined by a lower stimulus-specific parameter (e.g., alpha) which determines the easiness with which it enters into an association with the outcome, as has been made the case for attentional theories (Mackintosh, 1975).

As we mentioned above, in previous reports from our lab we have found that smooth surfaces acquire appetitive properties when paired with a reinforcer in conditioned place preferences (e.g., Mohamed-Jawad et al., 2018; Turel et al., 2020). However, in these reports we used *Dugesia* instead of *S. mediterranea*. Dexter et al. (2019), for example, have reported the difficulty in replicating the avoidance learning in single cells reported by Jennings (1906) using *Stentor coeruleous* instead of *Stentor roeseli*. Differences in the species stimulus sensitivity could, therefore, be playing a role in the relatively surprising results reported here.

Overall, we provide clear evidence that *S. mediterranea* and our protocol that mimics well validated rodent protocols is a valid tool for the study of aversive context conditioning and it could be used as promising model to advance the study of anxiety-related disorders, while favouring the use of invertebrates in the study of avoidance learning in line with the principle of the 3Rs.

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