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Original article

## A non-selective serotonin antagonist promotes rapid habituation in the terrestrial hermit crab

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## ABSTRACT

Research has indicated that serotonin (5-HT) modulates non-associative learning in a variety of invertebrate species. Recent work has demonstrated that the terrestrial hermit crab is a suitable animal model for non-associative learning phenomena, including habituation, sensitization, and dishabituation. We examined the potential role of a non-selective 5-HT antagonist, methysergide, in non-associative learning in the hermit crab. We administered methysergide prior to delivering repeated stimulus presentations of a looming visual predator. We found evidence for more rapid habituation relative to a control condition in which crabs did not receive the drug. These results indicate a role for 5-HT in the defensive behavior of the hermit crab and importantly, suggest a conserved role for 5-HT in modulating basic learning processes in invertebrates.

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## 1. Introduction

Non-associative learning represents possibly the most ubiquitous psychological phenomena in the animal kingdom. The types of non-associative learning most commonly studied include habituation and sensitization. Habituation, defined as a learned reduction in animals' reactivity to repeated stimulus presentation (Groves and Thompson, 1970), has been identified and examined in a wide variety of phylogenetically distant species, from the sea snail *Aplysia* (e.g., Castellucci et al., 1970), to the zebrafish (e.g., Wong et al., 2010), to the lab rat (e.g., Davis, 1974), to humans (e.g., Epstein et al., 1992). Sensitization, defined as an increase in general reactivity in response to the presentation(s) of arousing stimuli, has been found to be almost as commonplace (e.g., Brunelli et al., 1976).

Substantial evidence has accumulated regarding the relevant neurochemical systems that are employed in non-associative learning situations. Prior studies of sensitization in invertebrates have implicated the endogenous neurotransmitter serotonin (5-HT) in non-associative learning in invertebrate species [e.g., in *Aplysia* (e.g., Barbas et al., 2003; Brunelli et al., 1976; Glanzman et al., 1989), in the crab *Chasmagnathus granulatus* (Aggio et al., 1996), and in the leech *Hirudo medicinalis* (Burrell and Sahley, 2005; Burrell et al., 2001; Traina et al., 2013; Zaccardi et al., 2004)]. Administration of 5-HT has been found to facilitate the effects of presynaptic transmission (Chitwood et al., 2001) which correlates with an increased likelihood of sensitization. For example, Aggio et al. found that when the crab *Chasmagnathus granulatus* is presented with a visual stimulus of a passing shadow, it responds with a running reaction (an escape response); this response is enhanced with administration of exogenous 5-HT. Exogenous serotonin has also been found to delay the habituation of a leech's swimming response in response to a tactile stimulus (Alkatout et al., 2007). Studies indicate that the administration of drugs that block 5-HT (i.e., antagonists) have the opposite sort of effect, that of increasing the rate of habituation. For example, Zaccardi et al. (2004) found that methysergide, a non-selective 5-HT antagonist, impaired the onset of sensitization of swim induction in leeches and thus promoted the onset of habituation (see also; Crisp and Burrell, 2009; Zaccardi et al., 2012).

There have been a number of studies performed in recent years investigating simple learning in terrestrial hermit crabs (Chan

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et al., 2010a, b; Stahlman et al., 2011; Tran, 2015) and characterizing the environmental and individual factors that govern their defensive behavior. However, there is a lack of experimental work detailing neurochemical functions in these animals in the production of learned behavior<sup>3</sup>. If the role of serotonin in non-associative learning is conserved across species, we should expect that the manipulation of 5-HT receptors would modulate non-associative learning in hermit crabs in much the same way as in other invertebrates. We conducted a single experiment designed to examine the effects of a serotonin antagonist, methysergide, on the hermit crab's withdrawal response. The experiment described in this paper investigated whether the serotonergic system is critical for the expression of simple, non-contingent learning in hermit crabs in a threatening situation.

Auditory stimuli of high intensity have been shown to be sensitizing in a number of species (e.g., Davis, 1974; Stahlman et al., 2011). In keeping with prior literature, we predicted that administration of a 5-HT antagonist (methysergide) would mitigate the impacts of irrelevant acoustic stimulus intensity, such that animals would be less likely to sensitize in the experimental drug condition relative to a control (non-drug) condition. We administered methysergide or vehicle to terrestrial hermit crabs and then presented the animals with acoustic stimuli of either a mild or intense nature, followed by repeated presentations of a looming visual predator (Chan et al., 2010b; Stahlman et al., 2011). We predicted that drug-treated crabs would habituate more quickly to repeated presentation of the simulated predator in comparison with controls.

## 2. Method

### 2.1. Subjects

Twenty-nine experimentally naïve hermit crabs (*Coenobita clypeatus*) with shell aperture lengths of approximately 2- to 5-cm were acquired from a local vendor. The ages and sexes of the crabs were unknown. Prior to the study, and between experimental sessions, crabs were housed in groups of six in four clear plastic tubs (approximately 50 cm × 25 cm × 25 cm) where they were provided with one pellet of Tetrafauna™ Hermit Crab Cakes per crab, and two ceramic water dishes for salt water (1%) and regular drinking water, respectively. The home tubs also contained wet synthetic sponges, coconut fiber substrate (Zoo Med Eco Earth™), and plastic covers. Temperatures were maintained at approximately 24 °C. The humidity levels in the tubs were maintained between 60 and 80%. Each subject was identified by one of six colors of non-toxic nail enamel that was painted on its largest claw (cheliped) and on the animal's shell. Prior to the experiment, and between experimental sessions, the crabs were maintained on a 14 hr/10 hr day-night schedule. Experimental sessions were conducted during the animals' light cycle.

### 2.2. Apparatus

The experiment was conducted in a 61 cm × 61 cm × 43 cm cubicle. The experimental setup consisted of two speakers on either side of a 17-inch LCD computer monitor. This monitor was used to display a visual stimulus of a wingspread hawk (see Fig. 1). The image began as a single pixel at the top-center of the screen, and then descended and expanded at a steady rate for 15-s until the stimulus reached the bottom of the screen at a



**Fig. 1.** An image of the simulated visual predator at its maximum size. The presentation of the image began as a single pixel at the top of the screen, at which point it descended and increased in size at a linear rate for the duration of the trial.

maximum size of screen width of 33 cm (cf. Stahlman et al., 2011). The two speakers were placed 45 cm apart from one another and could broadcast white noise at either a fixed 74 dB sound pressure level (SPL) or fixed 95 dB SPL as measured by a sound meter (RadioShack™ CAT 33-2055) at the location of the subject.

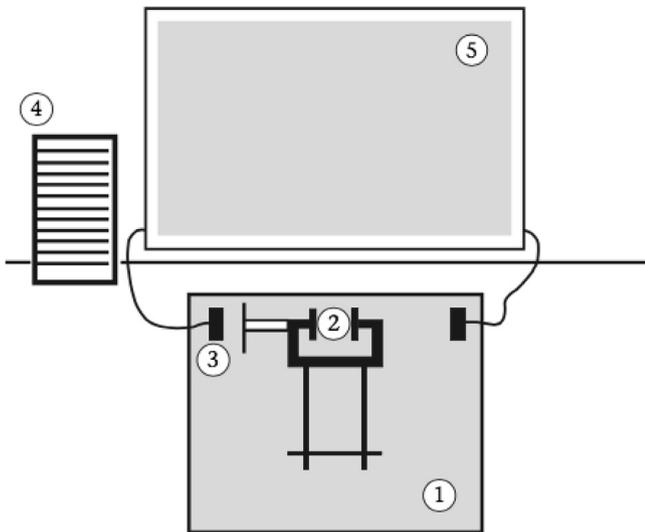
The crab restraint device (CRD) was constructed of a solid wood base (26 cm × 28 cm) and an adjustable C-clamp that was located at the front of the monitor. The C-clamp was attached to two levers that allowed for forward, backward, and vertical movement in order to accommodate different shell sizes and to maintain a consistent distance of 20 cm between each subject and the monitor. We positioned a Logitech C200 webcam so that it was parallel to the shell's long axis, and thus provided the experimenter with a side view of the animal. From this vantage point, the captured image consisted of a small portion of the animal's shell, its legs (only when the animal had emerged), and a royal blue background. A second camera was positioned at an elevated position behind the crab and provided a simultaneous view of both the crab and the monitor. See Fig. 2 for a schematic of the experimental apparatus.

### 2.3. Procedure

Fifty-five hours prior to the experimental session, all crabs were randomly assigned to small tubs (20 cm × 19 cm × 8 cm) where they were pair-housed. Each tub contained coconut-fiber substrate (identical type as the home tub) and a wet sponge. Animals were not provided with food or water.

Two solutions were prepared at the beginning of the day of the experiment. A drug solution was prepared by dissolving 10 mg of methysergide maleate (Sigma-Aldrich, St. Louis, MO) in 45 mL of a saline vehicle. This resulted in a 250 μM solution. The 0.9% vehicle was prepared by dissolving a single sodium chloride tablet (Bioultra, Sigma Aldrich) in 1000 mL of water. Then, 1.5 mL of the drug solution and 1.5 mL of store-bought pineapple juice were pipetted into small circular bowls (approximately 2 cm tall and 6 cm in diameter) and mixed thoroughly with 0.5 g of Tetrafauna Hermit Crab Meal. The same procedure was followed when making

<sup>3</sup> There is a body of biological literature investigating the structure of the serotonergic systems of crabs and closely related invertebrates. Such investigations have found, for example, that 5-HT is a powerful modulator of cardiac function (e.g., Kobayashi, 1987; Yazawa and Kuwasawa, 1992).



**Fig. 2.** Schematic of the experimental apparatus. (1) indicates the wooden base of the CRD; (2) indicates the crab's position; (3) indicates photobeam detectors; (4) indicates the speaker through which the auditory stimulus was played; (5) indicates the computer screen upon which the visual predator was presented. Redrawn from Chan et al. (2010).

the vehicle mixture (1.5 mL of vehicle, 1.5 mL pineapple juice, 0.5 g of Hermit Crab Meal). All crabs were transferred to new tubs (identical to tubs used in the deprivation stage) so that they could be individually housed for the duration of the experiment. Each tub then had the bowl of the appropriate mixture (i.e., either Methysergide or Vehicle) placed within it. The crabs had access to the drug cocktails for four hours<sup>4</sup>; two hours prior to the inception of experimental procedures, each crab was individually picked up and manually placed into the mixture<sup>5</sup>.

After four hours, the drug mixtures were removed from the tubs and experimental sessions commenced. All experimental sessions were conducted under dim incandescent lighting. An individual crab was put onto the back of its shell (i.e., aperture facing up) into the CRD (Chan et al., 2010b). A trial began when the crab emerged from its shell; this was defined to be the point when the crab's eyes were visible in the side camera and the crab was freely moving. Each session began with a 30-s delay, followed by a single presentation of either the Soft or Loud (depending on experimental condition) 50-s auditory stimulus. The crab then received presentations of the predator until a trial occurred in which the crab failed to withdraw during the predator stimulus. Interstimulus intervals varied as a function of the length Presentations of all experimental stimuli were controlled by a Dell<sup>TM</sup> Inspiron 580 desktop computer with code written in Visual Basic 6.0. Experimental procedures took place over two daily sessions that were separated by a 96-hr delay.

We utilized a  $2 \times 2$  mixed factorial design in this experiment. The first factor, manipulated between-subjects, was Drug; there were two levels for this variable, Methysergide (MET) and Vehicle (VEH). The second manipulation was a within-subjects manipula-

<sup>4</sup> Administering the drug via a consumable food mash was noninvasive, which was important since we were seeking to avoid sensitizing the animals to stimuli other than the auditory stimuli which were part of our manipulation. We had earlier conducted a pilot investigation wherein we had injected the drug directly into an exposed leg or the main cheliped, but found that crabs tended to quickly autotomize the affected limb.

<sup>5</sup> We included this step in our design out of an abundance of caution to ensure that all animals came into contact with the mixture. This appears now to be largely unnecessary, as we observed that all animals ambulated to the food dish and consumed the mash during the pre-session period.

tion and was reflective of the intensity of the auditory stimulus, which had two levels: Soft (74 dB) and Loud (95 dB; see Stahlman et al., 2011). Presentation of acoustic stimuli was counterbalanced across crabs, with half the animals receiving Loud trials on Day 1 and their Soft trials on Day 2, the order being reversed for the other animals. The order in which MET and VEH animals were tested was also counterbalanced.

We had two dependent variables (DVs). The first DV was the number of trials it took for the hermit crabs to fail to respond to the presentation of a simulated visual predator. It was indicated by the first trial during which a crab failed to withdraw into its shell in response to the visual stimulus. In this experiment, an anti-predator withdrawal response was defined as the visible withdrawal of the crab's body into its shell (i.e., pulling the body inward towards the shell aperture) during the presentation of the simulated predator. For example, if a hermit crab performed the anti-predator withdrawal response three times (once during each of three different presentations of the visual stimulus), and failed to retract during the fourth stimulus presentation, the crab's score during this session was recorded as 4. Our second DV was the natural logarithm of the time (in seconds) that crabs remained withdrawn in their shells before subsequent trials. Log latencies were used here to normalize scores prior to analysis, as the distribution of scores was positively skewed.

Two crabs (one from each group) underwent molting either during or between experimental sessions, at which point they were eliminated from experimental procedures and their data removed from the analysis. Additionally, four animals (two from each group) failed to emerge from their shells for any trials during each of the two experimental sessions, and thus were removed from data analysis. Thus, analyses were conducted with 12 animals in Group MET and 11 in Group VEH. Data were analyzed using a two-way mixed analysis of variance (ANOVA) with Drug as a between-subjects factor, Auditory as a within-subjects factor, and Crab as a random factor.

## 2.4. Measures

### 2.4.1. Trials to habituate

The ANOVA revealed a main effect for drug,  $F(1, 21.78) = 6.35$ ,  $p = 0.02$ , with MET crabs habituating in significantly fewer trials (mean = 3.45) than VEH animals (mean = 6.22; see Fig. 3). There was not a significant effect of auditory stimulus,  $F(1, 20.98) = 1.52$ ,  $p = 0.23$ , nor was there a significant Drug  $\times$  Auditory interaction,  $F(1, 20.98) = 1.24$ ,  $p = 0.277$ .

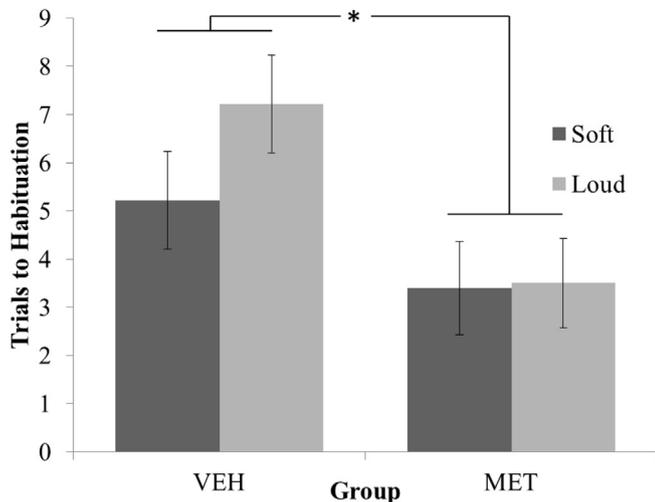
These data indicate that methysergide administration increased the rate of habituation. This is consistent with prior findings that the administration of serotonin antagonists reduces sensitization and promotes habituation in invertebrate species.

### 2.4.2. Latency to emerge

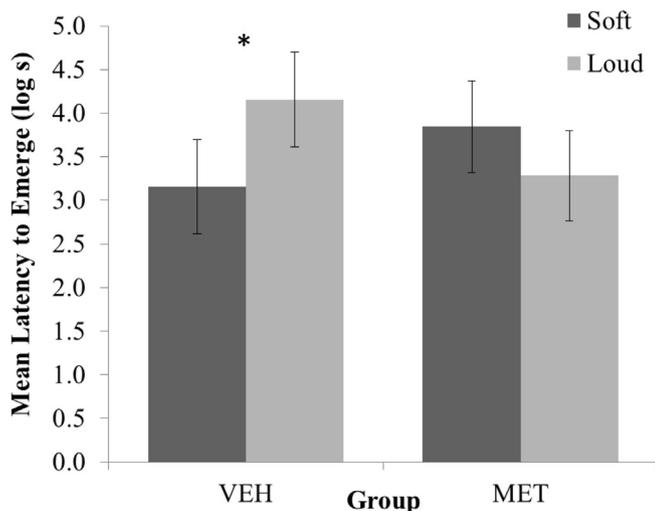
A two-way mixed ANOVA found no significant effect of drug,  $F(1, 21.01) = 0.02$ ,  $p = 0.90$ , and no significant effect of auditory stimulus,  $F(1, 20.18) = 0.90$ ,  $p = 0.35$ . Critically, however, the analysis did reveal a significant Drug  $\times$  Acoustic interaction,  $F(1, 20.18) = 11.52$ ,  $p = 0.003$  (see Fig. 4). A Tukey post-hoc analysis revealed that VEH animals exhibited significantly greater latencies (mean = 4.16) to emerge during Loud sessions as compared to Soft sessions (mean = 3.15),  $p < 0.05$ . No other comparisons were significantly different (all  $ps > 0.09$ ).

## 3. Discussion

It may seem odd that methysergide appears to have differential effects dependent on the nature of the dependent variable mea-



**Fig. 3.** Mean trials to habituation as a function of drug group and auditory stimulus intensity. The asterisk indicates a significant difference. Error bars indicate standard errors of the mean.



**Fig. 4.** Mean log latency for crabs to emerge as a function of drug group and auditory stimulus intensity. The asterisk indicates a significant difference. Error bars indicate standard errors of the mean.

sured. Administration of methysergide resulted in crabs requiring fewer trials to lose the reflexive response to the appearance of the visual predator than in comparison to those crabs that received no drug; however, no such difference manifested when the groups' latencies to emerge were compared. However, this seeming conundrum may be explicable in terms of the chemical systems engaged during each of these patterns – the withdrawal reflex may be mediated by 5-HT to a greater extent than the behavior of emergence. Should this be true, we might expect that administration of a 5-HT antagonist would have a larger effect on the production of withdrawal than on emergence. We did observe that a significant difference in the animals' emergence response (i.e., longer latencies to emerge when less habituated) was eliminated with the administration of methysergide. To our knowledge, no one has expressly investigated this possibility – the neural architecture of *Coenobita* and its relation to behavior is still not well understood (Harzsch and Hansson, 2008). However, the study of other invertebrate species lends clues regarding differential innervation of muscles that underlie rapid escape as compared to other behaviors [e.g., as in crayfish (Edwards et al., 1999) and squid (Otis and

Gilly, 1990)]. Crayfish, for example, engage in escape behaviors of at least two fundamental types: LG (rapid) and non-G (slower) escape responses. Neurochemical modulation of these two classes of response appears to be independent from one another, and 5-HT may be differentially involved in each (Edwards et al., 1999; Krasne et al., 1997). Further investigation using both biological and behavioral assays will be required in order to discover whether such a distinction underlies the withdrawal and emergence behaviors of hermit crabs. Researchers should also consider additional kinds of serotonergic manipulations. The administration of a 5-HT agonist, for example, may result in increased sensitization relative to a vehicle control (see, e.g., Aggio et al., 1996; Traina et al., 2013).

Overall, we obtained strong evidence in support of our central hypothesis. Hermit crabs were quicker to habituate to the repeated presentation of a simulated visual predator when they were under the influence of a 5-HT antagonist, methysergide. Additionally, control crabs were comparatively slow to re-emerge from their shells when sensitized via the presentation of a loud auditory stimulus before trials of the visual predator; this difference was eliminated with administration of methysergide. These results constitute evidence for the role of a serotonergic system in the production of visually mediated withdrawal behavior in terrestrial hermit crabs, and suggests the conservation of a neurobiological mechanism by which a ubiquitous non-associative learning phenomenon is instantiated.

#### Conflicts of interest

The authors declared that there is no conflict of interest.

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