Running Head: expression of acquisition and extinction memories

Heat shock disrupts expression of excitatory and extinction memories in planaria: interaction with amount of exposure.

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Abstract

In planarians, as seen in rodents, natural reinforcers (sucrose) and drugs of abuse support Conditioned Place Preference (CPP), which is a form of Pavlovian learning to examine the rewarding effects of natural reinforcers and drugs of abuse. Using this preparation, we have previously observed acquisition, extinction and reinstatement of sucrose CPP. In the present experiments, we used planaria to investigate the amnestic effects of Heat Shock (HS, a known stressor in planaria) following different amounts of CPP extinction sessions. Experiment 1 showed that planarians developed a CPP response to a sucrose-paired surface. Heat shock, when given in conjunction with exposure to the sucrose-paired surface, produced amnesia as assessed by a subsequent sucrose reinstatement test. We interpreted that the amnesic effect of HS was due to HS affecting the dominant excitatory memory at the time of HS exposure. Thus, we hypothesized that after extensive extinction training (10 exposures), HS would lead to recovery from extinction (when the new inhibitory memory is dominant at the time of HS exposure). Experiment 2 explored this possibility and showed that given HS following 10 extinction sessions had no amnestic effect on the excitatory CPP response. In Experiment 3, we hypothesized that 16 extinction sessions would produce a stronger (and hence dominant) extinction inhibitory trace, which then would be vulnerable to HS. We observed that HS impaired the expression of the extinction memory following 16 exposures. These results reveal different effects of HS on CPP memories depending on the amount of extinction, and are fully consistent with the literature using rodents and humans. In addition, they suggest that planaria is a promising pre-clinical model to assess fundamental memory processes.

Key Words: conditioned place preference; reinstatement; extinction; reconsolidation; planaria

1. Introduction

Following the reactivation of previously acquired memories, these become transiently susceptible to the effects of amnestics (Misanin, Miller & Lewis, 1968). Memory reactivation destabilizes the original memory trace, making it vulnerable to the effects of amnestics, similar to the vulnerability to amnestics observed immediately following training (McGaugh, 1966). The process following memory reactivation that leads to re-stabilization of memory traces has been called "reconsolidation" following the notion that the reactivated memory again undergoes a round of consolidation, although it should be noted that this phenomenon has also been interpreted as retrieval failure (Miller & Matzel, 2006). Regardless of the interpretation of the findings, amnesia following memory reactivation has been observed across a variety of species including *Aplysia* (Cai et al., 2012; Lee et al., 2012), *Caenorhabditis elegans* (Rose and Rankin, 2006), honeybees (Stollhoff et al., 2005), snails (Sangha et al., 2003), crabs (Pedreira et al., 2002), fish (Eisenberg et al., 2003), rodents (Gruest et al., 2004; Kida et al., 2002; Misanin et al., 1968; Nader et al., 2000) and humans (Hupbach et al., 2007; Kindt et al., 2009). Because of this generality, the phenomenon seems to be conserved across species, and has been considered a promising candidate for the treatment of clinical conditions such as post-traumatic stress disorder, anxiety, and drug addiction (Beckers & Kindt, 2017; Milton & Everitt, 2012). That is, amnesia following reactivation opens the possibility that maladaptive memories can be updated following reactivation, to attenuate their negative influence on behaviour (Lee et al., 2017).

Despite the relevance of this phenomenon for understanding the dynamic nature of memory processes in general, and translating these into potential treatments for psychiatric disorders, there have been discrepancies concerning the exact parameters and conditions that lead to impairments when amnestics are administered together with memory reactivation. For example, some have observed that expression of memory is impaired by inhibition of protein synthesis before or immediately after the retrieval (Debiec et al., 2002; Kida et al., 2002; Milekic & Alberini, 2002; Nader et al., 2000), but other researchers found that reconsolidation does not depend on protein synthesis

inhibition (Lattal & Abel, 2001). Such controversial findings suggest that there are 'boundary conditions' for reconsolidation to occur, which impact the retrieval, destabilization and subsequent expression of memory. Findings by Eisenberg and colleagues (2003) and Suzuki and colleagues (2004) suggested that the number of reactivation events, age, and strength of memory are key variables that control the effects observed in subsequent behaviour following memory reactivation with the administration of amnestics. This suggests that conflicting results in the field may be accounted for by the use of different parameters, which obviously limits the translation of these findings into the clinic. In addition, recent attempts to replicate well known protocols did not consistently observe the same findings (Schroyens, Kindt & Beckers, 2017; Schroyens, Alfei, Schnell, Luyten & Beckers, 2019), so despite the popularity of this field, there is a pressing need to establish not only the generality of these phenomena across species, but also the exact conditions under which they are observed.

In nonhuman animals, memory reactivation can be achieved by presenting the conditioned stimulus (CS) in the absence of the unconditioned stimulus (US) used during training in standard Pavlovian conditioning preparations. Non-reinforced presentations of the CS can reactivate the existing excitatory memory trace; but if the CS presentation is sufficiently long or the CS is presented multiple times, it can lead to extinction (i.e., reduction in the conditioned response). Contemporary explanations of extinction suggest it results in new inhibitory learning which is context dependent (Bouton, 2004; see Urcelay, 2012 for a review). The transition from reactivation of the excitatory trace to the formation of a new inhibitory trace (i.e., extinction) has led to opposite findings in terms of the effects of a given amnestic. For example, Eisenberg et al. (2003) trained medaka fish in a fear conditioning task. Following training, they gave 1, 5 or 10 presentations of the CS immediately followed by administration of the anaesthetic MS222 (an amnestic). Administration of MS222 together with one exposure to the CS led to amnesia of the fear memory 24 hours later; however, application of MS222 three hours after retrieval (one extinction trial) had no effect on memory. This pattern of results is consistent with the notion that the amnestic agent impairs the retrieved excitatory memory affecting its reconsolidation. Treatment with MS222 after ten presentations of the CS attenuated the

expression of extinction memory, revealing strong responding on a subsequent test. This finding was interpreted in terms of MS222 having an amnesic effect on the extinction memory (Eisenberg et al., 2003). Similar findings have been reported in rats using other preparations and drugs (Suzuki et al., 2004; Lee, Milton & Everitt, 2006).

Whilst the interpretation of the opposite findings observed when manipulating the number (or amount) of CS presentations—amnesia of the excitatory memory with few CS exposures, and amnesia of the extinction memory following multiple CS exposures—seems straightforward, recent studies have suggested a more complicated picture. For example, Briggs and Olson (2013) trained rats using an inhibitory avoidance task, and twenty four hours later administered different amounts of exposure to the CS (15 sec, 6 min or 12 min). Immediately after exposure, they gave an injection of either cycloheximide (an amnestic) or vehicle. When tested twenty four hours later, the groups exposed to 15 secs or 12 mins showed a pattern consistent with the findings described above—namely, that the amnestic has opposite effects depending on the amount of CS presentation. More challenging was the pattern observed in the groups exposed to the CS for 6 mins. In these groups, no differences were observed between groups administered the amnestic and the vehicle. The insensitivity to amnestics following an intermediate amount of exposure to CS has also been observed in rats using fear conditioning (Alfei et al., 2015; Cassini et al., 2017; Merlo et al., 2014; 2018), appetitive conditioning (Flavell & Lee, 2013) and in humans using fear conditioning (Sevenster, Beckers & Kindt, 2014) thus showing that the phenomenon has some generality and is replicable across laboratories. In addition, the three phenomena (amnesia of excitatory memory, insensitivity to the amnestic, and amnesia for extinction memory) show that humans and rodents have an exquisite sensitivity to parameters such as the amount of exposure to the CS, which can lead to opposite effects of amnestics, or indeed no effect at all.

Amnesia following memory reactivation has shown good generality in terms of preparations and species (Nader, 2016), and the phenomenon has been observed in a number of invertebrate species (see above). However, there are (to the best of our knowledge) no reports investigating amnesia following reactivation in planaria. Planaria represent an ideal invertebrate model to study the

generality of these phenomena because morphological, electrophysiological and pharmacological features of the planaria's primitive nervous system closely resemble those of the brain of vertebrates (Sarnat & Netsky, 1985). For example, at the level of individual neurons, planarians' neurons express dendritic spines, which are a putative memory storage site critical for learning, and not found in other invertebrates such as the nematode *C elegans* (Petralia, Wang, Mattson, & Yao, 2016). This reflects planarians' putative position at the evolutionary base of all bilaterian animals. Determining whether these phenomena are observed in planaria would address the question of whether similar mechanisms are observed across phyla, and hence was an additional motivation of the experiments reported here.

In the present study we investigated whether the amount of CS exposure (operationalised as the number of extinction training sessions) had an effect on reconsolidation of excitatory and inhibitory memories in planarians exposed to an amnestic agent (a heat shock, as in the study by Rose & Rankin, 2006, in *C elegans*). The Animals were trained in a conditioned place preference (CPP) task using an unbalanced design (Amaning-Kwarteng et al., 2017; Hutchinson et al., 2015; Mohammed Jawad et al., 2018). In this preparation, planarians are placed in a petri dish with two distinctive surfaces (plastic and sand) and allowed to freely move for a period of time (a pre-conditioning test). The time spent in each of the two surfaces is recorded and their basal preference determined. The animals are then given CPP training in which the non-preferred surface is paired with a rewarding substance (10% sucrose); animals are also given an equal number of training trials with the alternative preferred surface in the presence of water. Following 4 cycles of training trials with the two surfaces, the animals' preference is assessed again 24-hours after the last training trial (a post-conditioning test). Planarians typically show a significant change in their preference from the pre- to the post-conditioning test (a CPP response), displaying a higher preference for the initially non-preferred surface that was paired during training with the rewarding agent. The fact that this change in preference can be observed 24 hours after the last cycle of training trials indicates the establishment of a long-term memory linking the surface (the CS) with the rewarding effects of the rewarding agent (the US). Repeated exposure to the surface-CS in the absence of the US results in the extinction of

the CPP response (Amaning-Kwarteng et al., 2017; Mohammed Jawad et al., 2018); furthermore, one single exposure to the rewarding agent following extinction has been reported to reinstate the CPP response (Mohammed Jawad et al., 2018).

The experiments reported here made use of this CPP protocol to assess the effect of an amnestic agent on the expression of the CPP response following different levels of extinction training. As mentioned above, the amnestic agent used in our experiments was the exposure to a heat shock immediately after a reminder (exposure to the CS-surface). Exposure to heat leads to the production of Heat Shock Proteins (HSP) that disrupt *de novo* protein synthesis interfering with the memory re-consolidation process; Rose and Rankin (2006) reported persuasive evidence that heat shock delivered immediately after memory reactivation interferes with later memory recall in *C elegans*. Following short extinction training, we can expect the acquisition excitatory memory to dominate over the inhibitory learning that develops during extinction training; in other words, the animals are likely to preferentially retrieve the excitatory memory during the exposure to the CS. However, with increased levels of extinction training, the inhibitory learning can be expected to strengthen and be preferentially retrieved during reactivation. Therefore, we hypothesized that a heat shock would disrupt the expression of the excitatory memory (CPP response) if presented after a relatively short extinction training (4 extinction trials; Experiment 1); however, if the heat shock is presented following long extinction training (10 or 16 trials; Experiments 2 and 3 respectively) the amnestic agent would be more likely to affect the now predominant inhibitory learning that develops during extinction.

In the three experiments reported, 24 hours after the last extinction trial the animals were orthogonally assigned to one of four groups depending on two variables: a) heat shock *vs.* no heat shock (control) treatment; and b) reinstatement (exposed to sucrose) *vs.* control (exposed to water) treatment. Immediately after heat exposure (or control) treatment, planarians were exposed to the test petri dish (with the two surfaces, rough and smooth) in order to reactivate the putative memory trace in the presence of activated heat shock proteins. Twenty four hours later, planarians from these two groups were further allocated to either the sucrose reinstatement or control condition. We had,

therefore, four groups in the last phase of the experiment: Heat Shock-Sucrose; Heat Shock-Water; No Heat Shock-Sucrose; and No Heat Shock-Water.

2. Method

2.1. Animals

Three hundred and fifty-two large brown planaria (*Dugesia)* were purchased from Blades Biological Ltd. (Kent, UK; Catalogue #LZC 031). The number of animals allocated at the beginning of each experiment was: Exp 1, *n* = 96; Exp 2, *n* = 128; Exp 3: *n* = 128. The animals were kept in a refrigerated incubator at 20° C with a light-dark cycle of 9/15 hours in a 1 ml/l solution of *Aquasafe*® (*Tetra*, UK). Aquasafe removes toxins and chlorine from tap water making it safe for fish and other freshwater creatures. The animals were fed raw chicken meat twice per week and the water of the aquarium was changed after every feeding. One week before the experiments began, planarians were food deprived and individually re-housed for the duration of the experiment in plastic ice cube trays filled with 5 ml of treated water.

2.2. Materials

Plastic petri dishes, 9 cm in diameter, were used as the different experimental contexts. The dishes could have a smooth surface (matted plain plastic), a rough surface (sand glued to the dish using transparent silicone), or a split surface (half smooth and half rough). Throughout the experiments, the animals could be exposed to treated water or a 10% sucrose solution. For the heat shock exposure, two digital dry bath heaters and Eppendorf tubes were used; aquatic temperature was measured using a digital water thermometer. During the experimental sessions, the animals' locomotor activity was tracked by using a video-track system (*ViewPoint*, Lyon, France).

2.3. Procedure

All experiments had five different phases. 1) pre-conditioning test (1 day); 2) conditioned place preference training (CPP; 8 days); 3) post-conditioning test and extinction of CPP (Exp 1: 4 days, Exp 2: 10 days, and Exp 3: 16 days); 4) heat-shock and memory reactivation (1 day); and 5) CPP reinstatement test (2 days; see Figure 1).

-- Figure 1 around here --

2.3.1. Pre-Conditioning Test

Each planarian was placed into the midline of the split petri dishes (half smooth and half rough) filled with 9 ml of treated water and allowed to freely move for 30 min. The time spent on each surface of the dish was recorded, and a preference score was calculated for each animal by dividing the time spent in the less preferred side by the total time of the session. Seventy-two planaria that had unclear (very close to .5) or absolute preferences (values close to 0 or 1) in the pre-conditioning test were not selected for the experiments. For each animal, the less preferred surface was paired with the sucrose reward during the subsequent conditioning place preference sessions—an unbalanced CPP design (e.g., van der Kooy, 1987).

2.3.2. Conditioned place preference (CPP) training

The animals were given eight daily 30-min sessions in which they were alternately exposed to the sucrose reward in their less preferred surface (smooth or rough) and treated water in their preferred surface. On the first day of the training, half of the animals were exposed to sucrose in their less preferred surface for 30 min whilst the other half received treated water in their preferred surface. A day later, animals which were exposed to sucrose on the first day received treated water in their preferred surface, and those that received water on the first day were exposed to sucrose for 30-min session. This alternating cycle was repeated four times (see Figure 1), a standard procedure in CPP experiments in the planaria (e.g., Mohammed Jawad et al. 2018). The order in which the animals were exposed to each context was counterbalanced across animals. During the experimental sessions, the locomotor activity of the animals was recorded. The treated water in the animals' ice cube tray home was changed on each day following exposure to sucrose to avoid contamination.

2.3.3. Post-conditioning test and extinction of CPP

Following the completion of the conditioning phase, on the tenth day of the experiment, each animal was tested in the split petri dish with treated water. During the 30 min session we recorded the locomotor activity of the animals and the time spent on each of the two surfaces. A *change of preference score* was then calculated for each animal by subtracting the preference score in the pre-

conditioning test from the preference score observed in the post-conditioning test. A change of preference score of zero would indicate no change in preference; on the contrary, any positive value (and hence different from zero) would reveal a change in preference, an index of conditioned place preference (CPP). This procedure was then repeated on consecutive days to monitor the extinction of the CPP response following the procedure developed by Mohammed Jawad et al. (2018). Animals that did not exhibit a positive score during the Post-conditioning test (n = 81) and hence did not show reliable learning, were excluded from the rest of the experiment. After this exclusion, sixty-one animals continued the experiment in Experiment 1; sixty-nine animals remained in Experiment 2; and sixty-nine animals were continued in Experiment 3.

In the experiments reported below, different levels of extinction of CPP were used: the extinction phase lasted 4 days in Experiment 1; 10 days in Experiment 2; and 16 days in Experiment 3. At the end of the extinction period, the subjects were assigned to two groups: Heat Shock and No Heat Shock matched by their extinction performance.

2.3.4. Heat-Shock exposure and memory reactivation

On the day following the completion of the extinction phase, the animals in the Heat Shock were individually put into an Eppendorf tube filled with 1 ml of treated water and placed into the dry bath heaters set at 32° C for 20 min. A water thermometer was used to measure the aquatic temperature in the Eppendorf tubes throughout this session. The aquatic temperature was stable and consistent across all Eppendorf tubes in the dry bath heaters. Animals in the No Heat Shock were treated in the same way but the dry bath heaters were kept at room temperature. Five min after the heat exposure, planarians were transferred to the split petri dishes for a 30 min memory reactivation trial.

2.3.5. CPP Reinstatement Test

During the final phase of the experiment, the animals in each group were divided into two sub-groups. Half the animals in each group (Heat Shock and No Heat Shock) were exposed to a 10% sucrose solution for 30 min in a distinctive glass petri dish 5 cm in diameter; the other half of the

animals were exposed to treated water. Twenty-four hours after the exposure to sucrose or water, a final CPP test was carried out in the split petri dishes.

2.4. Data Analysis

Each of the three experiments in this study was run in two replications, and replication as a factor did not have a significant effect in any of the analyses. Therefore, it will not be considered further. One-sample *t*-tests were performed on the data from the pre-conditioning test against a theoretical value of 0.5 to determine whether the animals showed a preference for one of the surfaces (rough or smooth); and on the change of preference score data from the post-conditioning test to determine whether the procedure was successful in establishing a CPP response. We performed within-subjects ANOVAs on the data of the extinction trials to determine if there was a significant decrease in the preference score. Finally, we assessed the data from the reinstatement test by comparing each group to a theoretical zero using one-sample *t*-tests. Because evidence for excitatory and inhibitory learning depended on change scores being different from 0 (or not), we used Bayesian analyses to determine evidence for the unidirectional (one-sided) alternative BF_{+0} or null BF_{0+} hypotheses (Rouder, Speckman, Sun, Morey & Iverson, 2009; van Doorn et al., 2019) using JASP (JASP Team, 2019). We used JASP's default prior distribution (Cauchy Scale: 0.707). Bayes factors between 1 and 3 are considered to be weak, between 3 and 10 are moderate, and Bayes factors over 10 are interpreted as strong evidence. Error percentages reported with the results indicate their numeric robustness based on the accuracy of the Bayes factor analyses. Lower values are indicators of greater numerical stability of the results. Error percentages below 10% can be ignored, and those below 20% are acceptable (van Doorn et al., 2019; Goss-Sampson, 2020). All other analyses were conducted using IBM SPSS Statistics for Windows, Version 26.0.

3. Results

3.1 Pre-conditioning Test

In the three experiments, the animals showed a preference score of 0.4 (Experiment 1), 0.35 (Experiment 2) and 0.36 (Experiment 3) for the less preferred surface. One-sample *t*-tests revealed

that these preference scores significantly differed from 0.5 (chance level), smallest $t(68) = -16.139$, $p < .001$, Cohen's $d = 4.69$.

3.2 Conditioned place preference (CPP) training

During the training phase, we recorded the levels of activity (distance covered during the 30 min session). The animals showed lower levels of activity in the context in which they were exposed to sucrose relative to the context in which they were exposed to water; sucrose-induced hypo-locomotion is consistent with previous reports (e.g., Mohammed Jawad et al., 2018). In Experiment 1, the animals covered a mean distance of 119.08 cm ($SD = 41.37$) in the trials in which they were exposed to sucrose in the less preferred context, and 305.09 cm ($SD = 77.05$) in the trials in which they were exposed to water in the preferred context. In Experiment 2 they covered 122.58 cm ($SD = 41.61$) in sucrose and 262.65 cm ($SD = 71.65$) in water. In Experiment 3, 121.95 cm ($SD = 41.52$) in sucrose and 294.47 cm ($SD = 85.46$) in water. Three Repeated Measures ANOVAs were carried out on the data of the three experiments, revealing a significant effect of Context (i.e., sucrose *vs.* water), smallest $F(1, 136) = 380.46$, $p < .001$, $\eta^2_p = .74$.

3.3 Post-conditioning test and extinction of CPP

The preference scores of the subjects in the post-conditioning test (Extinction Day 1) and the rest of the extinction phase (Extinction Days 2-4 in Experiment 1; Extinction Days 2-10 in Experiment 2; and Extinction Days 2-16 in Experiment 3) were subtracted from the preference scores observed in the pre-conditioning test in order to examine: 1) whether animals developed a conditioned preference for the context paired with sucrose during the conditioned place preference phase of the experiment; and 2) whether this preference extinguished throughout the non-reinforced extinction test trials (see, for example, Mohammed Jawad et al., 2018; Ouyang et al., 2017).

Any positive value in E1 (the first extinction test trial, see Figures 2-4 A) would be indicative of a change of preferences in favour of the initially less preferred surface paired with sucrose during the training. We observed significant positive change in preference score in the three experiments: the mean change in preference score was 0.098 ($SD = .123$), $t(60) = 6.230$, $p < .001$, Cohen's $d = .79$, in

Experiment 1; 0.19 (*SD* = .165*)*, *t*(68) = 10.015, *p* < .001, Cohen's *d* = 1.2, in Experiment 2; and 0.14 (*SD* = .106*)*, *t*(68) = 10.925, *p* < .001, Cohen's *d* = 1.31, in Experiment 3.

The initial change in preference score (E1) gradually diminished approaching the level of 0 by the end of the extinction phase in the three experiments. A Repeated Measures ANOVA was performed on the data of the extinction phase for each of the three experiments. A significant decrease in the change of preference score was revealed in Experiment 1 throughout the four extinction trials, *F*(3, 180) = 4.581, *p* = .006, η^2_p = .071 (see Figure 2A). A paired-samples *t*-test revealed that animals spent significantly less time in the sucrose-paired surface in the last extinction trial (*M* = .019, *SD* = .132) than in the first extinction trial (*M* = .098, *SD* = .123), *t*(60) = 3.992, *p* < .001, Cohen's *d* = 0.62. In Experiment 2, a significant decrease was observed over 5 blocks of two extinction trials, *F*(4, 272) = 5.531, *p* = .001, η^2_p = .075 (see Figure 3A); a paired-samples *t*-test revealed that animals spent significantly less time in the sucrose-paired surface in the last block of extinction trials (*M* = .091, *SD* = .124) than in the first block of extinction trials (*M* = .171, *SD* = .149), *t*(68) = 4.134, *p* < .001, Cohen's *d* = 0.58. The same pattern was observed during the four blocks of four extinction trials in Experiment 3 (see Figure 4A), *F*(3, 204) = 9.557, *p* < .001, η^2_p = .123. A paired-samples *t*-test revealed that animals spent significantly less time in the sucrose-paired surface in the last block of extinction trials (*M* = .073,*SD* = .113) than in the first block of extinction trials (*M* = .121, *SD* = .112), *t*(68) = 3.896, *p* < .001, Cohen's *d* = 0.42.

3.4 Heat-Shock exposure effect on CPP

The effect of heat shock on the animals' CPP response (the change in preference score) was assessed immediately after the exposure to heat during the 30 min session which also served as a memory reactivation (see Table 1). In Experiment 1, a mixed two-way ANOVA was conducted with a between-subjects factor, Group (Heat shock *vs.* No Heat Shock), and a within-subjects factor, Test, comparing the last extinction trial with the Heat-Shock test trial (the memory reactivation trial). The analysis showed that there were no significant differences between the groups and the test trials, largest *F*(1,59) = 2.483, *p* = .120, η^2_p = .040, suggesting that the heat shock did not affect the change in preference score during memory reactivation. Consistent with findings in rodents (Nader, Schafe &

Le Doux, 2000), exposure to an amnestic did not exert any effect in short-term memory expression. The same output was revealed by the same analysis carried out on the data of Experiment 2, largest $F(1,67) = 1.08$, $p = .302$, $\eta^2_p = .016$, and Experiment 3, largest $F(1,67) = .647$, $p = .424$, $\eta^2_p = .010$.

-- Table 1 around here --

3.5 CPP Reinstatement Test

3.5.1 Experiment 1

Following the exposure to heat shock and the CPP test, animals in the sub-groups Sucrose were exposed to sucrose whereas animals in the Water condition were exposed to treated water, all in a distinct context (i.e., glass petri dish; see Mohammed Jawad et al., 2018). We used this reinstatement manipulation to assess the effect of Heat Shock on CPP excitatory and extinction memories. The CPP response was assessed in the final reinstatement test (see Figure 2B). A visual inspection of Figure 2B reveals that the animas with no heat shock history show evidence of CPP response—excitatory memory retrieval; however, the animals exposed to sucrose (Group No Heat Shock-Sucrose) showed a higher level of responding than those exposed to water (Group No Heat Shock-Water). The animals in the Heat Shock condition did not show evidence of CPP response (retrieval failure of excitatory memory). One-sample *t*-tests were carried out to establish whether the animals' change preference scores differed from '0' in the reinstatement test. Only the group No Heat Shock-Sucrose was found to be significantly different from '0', $t(13) = 2.580$. $p = .023$, Cohen's $d = .69$.

Bayesian analysis found that the alternative unidirectional hypothesis (BF_{+0}) predicting a difference in the CPP response in the reinstatement test session for the Group No Heat Shock-Sucrose was 5.72 times more likely than the null hypothesis which was that there was no difference between the change score of the Group No Heat Shock-Sucrose and '0'. This result indicates moderate evidence in favour of H_+ (BF_{+0}). The error percentage is < 0.001%, which indicates great stability of the numeric robustness of the analysis.

The null hypothesis (BF_{0+}) predicting no difference in the CPP response of the Group No Heat Shock-Water was found to be 1.57 times more likely than the alternative hypothesis which predicted

the change score of Group No Heat Shock-Water different from zero. That shows weak evidence in favour of H_0 (BF_{0+}) than H_+. The error percentage of this analysis is .012%, indicating great stability of the numerical algorithm used to compute the result.

Again, the null hypothesis (BF_{0+}) predicting no difference in the CPP response of the Heat Shock-Sucrose group was 3.27 times more likely than the alternative hypothesis which predicted that there was a difference in the CPP response of the Group Heat Shock-Sucrose. This result displays moderate evidence in favour of H_0 (BF_{0+}) relative to H_+. The error percentage is < .001% which means great stability of the Bayes factor calculation. Finally, the null hypothesis (BF_{0+}) predicting no difference in the CPP response of the Heat Shock-Water group was found to be 3.65 times more likely than the alternative hypothesis which predicted that a difference in the CPP response of this group. This indicates moderate evidence in favour of H_0 (BF_{0+}) over H_+. The error percentage is .002%, which is evidence of high stability of the numerical algorithm that was used to obtain the result.

-- Figure 2 around here --

These results of Experiment 1 suggest that sucrose presentation can effectively reinstate the CPP response in the control No Heat Shock condition; however, the animals given heat shock and exposed to sucrose (a reinstatement treatment) failed to retrieve the CPP excitatory memory. These data strongly suggest that presentation of a heat shock at the time of memory reactivation had an amnestic effect on the predominant excitatory memory.

The same analysis were performed on the data from Experiments 2 and 3, independently reported below for clarity.

3.5.2 Experiment 2

The data of the reinstatement test for Experiment 2 are displayed in Figure 3B. As it can be observed in the Figure, both groups which experienced the sucrose reinstatement manipulation showed evidence of CPP, irrespective of whether they experienced Heat Shock or not. In other words, with 10 CS exposure sessions, heat shock had no effect on the CPP memory when assessed with the reinstatement manipulation. One-sample *t*-tests were carried out to compare the change score of preference of each group with ‘0’ zero in order to detect the shift in preference. The change in

preference score of the group treated Heat Shock-Sucrose was significantly different from '0' zero, $t(20) = 2.911$, $p = .009$, Cohen's $d = .64$ whilst the change in preference score of the Group No Heat Shock-Sucrose was marginally different from '0', $t(12) = 2.028$, $p = .065$, Cohen's $d = .56$. Bayesian analyses found that the unidirectional hypothesis predicting a difference in the CPP response (BF_{+0}) in the reinstatement test for the Group No Heat Shock-Sucrose was 2.52 times more likely than the null hypothesis that there was no difference between the change score of the Group No Heat Shock-Sucrose and zero. This result indicates anecdotal evidence in favour of H_+ (BF_{+0}) over H_0. The error percentage is < 0.001%, which indicates great stability of the numerical algorithm.

The null hypothesis (BF_{0+}) predicting no difference in the CPP response of the Group No Heat Shock-Water was found to be 3.77 times more likely than the alternative hypothesis which predicted a difference in the CPP response of Group No Heat Shock-Water. That shows moderate evidence in favour of H_0 (BF_{0+}) than H_+. The error percentage of this analysis is .012%, suggesting strong stability of the algorithm. Additionally, the (BF_{+0}) unidirectional hypothesis predicting a difference in the CPP response of Heat Shock-Sucrose group in the reinstatement test session was 11.34 times more probable than the alternative hypothesis predicting no difference. This indicates strong evidence in favour of H_+ (BF_{+0}) over H_0. The error percentage is < 0.001% which reveals great stability. Finally, the null hypothesis (BF_{0+}) predicting no difference in the CPP response of the Heat Shock-Water group was found to be 3.32 times more likely than the alternative hypothesis which predicted that a difference in the CPP response of the Heat Shock-Water group. This indicates moderate evidence in favour of H_0 (BF_{0+}) over H_+. The error percentage is .005%, which reveals great stability of the numerical algorithm that was used to acquire the result.

-- Figure 3 around here --

Taken together, the present results suggest that exposure to the rewarding agent sucrose reinstated the acquisition memory in the two groups exposed to sucrose, independently of whether they had been exposed to a heat shock (Groups Heat Shock-Sucrose and No heat Shock-Sucrose). Although the reinstatement in Group No heat Shock-Sucrose was weaker, the overall pattern is

similar in both groups. Clearly, heat shock treatment had no amnestic effect on the animals' CPP acquisition memory expression in Experiment 2 when extinction sessions were extended to ten.

3.5.3 Experiment 3

The data of the reinstatement test for Experiment 3 are displayed in Figure 4B. As it can be observed in the Figure, the groups that did not experience Heat Shock showed a similar pattern to that observed in similar groups in Experiments 1 and 2. The groups that experienced Heat Shock showed evidence of excitatory memory regardless of the reinstatement manipulation. That is, consistent with our expectations based on previous findings, the heat shock attenuated the expression of the extinction memory, and thus revealed evidence of the retrieval of the excitatory CPP memory regardless of sucrose reinstatement.

One-sample *t*-tests were conducted to compare the change scores of each group with '0' to examine any change in preference. Consistent with prior findings, the extinguished CPP response of planaria in Group No Heat Shock-Sucrose was reinstated; a one-sample *t*-test revealed that the CPP response of this group was significantly different from '0', $t(16) = 3.225$, $p = .005$, Cohen's $d = .78$. Group No Heat Shock-Water did not show any evidence of excitatory learning, $t(16) = .749$, $p = .465$, Cohen's $d = .18$. Bayesian analyses revealed that the alternative unidirectional hypothesis (BF_{+0}) predicting a difference in the CPP response in the reinstatement test session in the Group No Heat Shock-Sucrose was 18.2 times more probable than the null hypothesis. This indicates strong evidence in favour of H_+ (BF_{+0}) over H_0. The error percentage is < 0.001%, which indicates great stability of the numerical algorithm. The null hypothesis (BF_{0+}) predicting no difference in the CPP response of the Group No Heat Shock-Water was found to be 3.13 times more probable than the alternative hypothesis. This result shows moderate evidence in favour of H_0 (BF_{0+}) than H_+. The error percentage of this analysis is .004%, suggesting great stability of the algorithm.

A different pattern was observed in the groups that experienced Heat Shock. The change scores of animals in Group Heat Shock-Sucrose were marginally different from '0', $t(17) = 1.961$, $p = .066$, Cohen's $d = .46$ and the change scores in Group Heat Shock-Water were not different from '0', $t(16) = 1.726$, $p = .104$, Cohen's $d = .42$. When collapsed, the two Groups that received Heat Shock

after 16 extinction sessions showed evidence of excitatory memory as their scores differed from '0', $t(34) = 2.528$, $p = .016$, Cohen's $d = .43$. Additionally, the unidirectional hypothesis (BF_{+0}) predicting a difference in the CPP response of the Group Heat Shock-Sucrose was 2.22 times more probable than the null hypothesis predicting no difference in CPP response. This indicates anecdotal evidence in favour of H_+ (BF_{+0}) over H_0. The error percentage is < 0.001%, which reveals great stability of the Bayes factor calculations used to obtain this result.

Finally, the unidirectional hypothesis (BF_{10}) predicting a difference in the CPP response of the Heat Shock-Water group found to be 1.59 times more probable than the alternative hypothesis predicting no difference. This shows anecdotal evidence in favour of H_+ (BF_{+0}) than H_0. The error percentage is .002%, which indicates great stability of the numerical algorithm that was used to obtain this result. Together, for these two groups collapsed, the alternative unidirectional hypothesis (BF_{+0}) of these scores being higher than '0' was 5.6 times more likely than the null hypothesis predicting no difference in the CPP response. This result indicates moderate evidence in favour of H_+ (BF_{+0}) than H_0. The error percentage is < 0.001%, which reveals great stability of the analysis.

-- Figure 4 around here --

To conclude, the results of Experiment 3 show that, after a long extinction period (16 sessions), animals that received sucrose priming showed reinstatement of the CPP response, as in Experiments 1 and 2. Importantly, exposure to heat shock eliminated the sucrose reinstatement effect, presumably because heat shock attenuated the expression of extinction memory, revealing excitatory learning regardless of the reinstatement treatment.

4. General Discussion

The objective of this study was to assess the effect of an amnesic (heat shock) on the expression of sucrose CPP in the planaria, after varying amounts of exposure to extinction training. The present study showed conditioning, extinction and reinstatement of sucrose place preference, and provided the first evidence of amnesia for a reactivated memory (a.k.a., reconsolidation) in planarians. In addition, the results in this study revealed that the effect of heat shock on reactivated conditioned place preference (CPP) memories depends drastically on the amount of exposure (i.e.,

extinction training) administered prior to the amnestic. After a short period of exposure (4 extinction trials, Experiment 1), heat shock produced amnesia of the excitatory CPP response, eliminating the reinstatement effect produced by sucrose exposure. When heat shock was administered following an intermediate extinction protocol (10 extinction trials, Experiment 2), it had no effect as both groups (Heat Shock-Sucrose and Heat Shock-Water) showed a reinstatement effect. However, when the heat shock was administered after a long extinction protocol (16 extinction trials, Experiment 3), it attenuated the expression of extinction thus revealing CPP regardless of reinstatement with sucrose.

The acquisition of CPP response to sucrose (and drugs of abuse such as cocaine) and the extinction of such CPP have been recently shown in planaria (Amaning-Kwarteng et al., 2017; Mohammed Jawad et al., 2018). Thus, the present results are consistent with these reports in that we also observed acquisition of CPP using sucrose as a reinforcer, and extinction by exposure to the testing petri dish in the absence of sucrose. In addition, as seen in rodent studies (Rescorla & Heth, 1975), the extinguished CPP response is reinstated by exposure to the rewarding drug/agent in planaria. Only one study demonstrated reinstatement following extinction by the exposure to sucrose (Mohammed Jawad et al., 2018), but this report did not make use of a control group given CPP training and exposure to sucrose before the final test. The present experiments extend those findings and add generality with the use of a control group that did not receive sucrose during the reinstatement session, suggesting that the reinstatement effect is reliable and not the result of merely handling the animals.

Heat shock has been previously found to disrupt memory expression of long-term habituation in the nematode *C elegans*. That is, when heat shock was administered immediately after a reminder of the habituated stimulus, it interfered with the expression of long-term habituation (Rose & Rankin, 2006). Similarly, the results of the present study showed that heat shock exposure following a short extinction protocol resulted in amnesia, as it prevented the reinstatement of the CPP response. Our results are not only consistent with the invertebrate literature, but also with findings in rodents. For example, Misanin and colleagues (Misanin et al., 1979) trained rats in a one-trial passive avoidance task, and immediately after training immersed rats in 45° C water for 11 minutes. When tested 24

hours later, animals that received the hyperthermia treatment showed profound amnesia compared with control animals which did not experience the hyperthermia treatment (also see Ahlers & Riccio, 1987). Thus, the amnestic effect of heat shock reported in the present experiments is consistent with observations in invertebrates.

When the number of extinction sessions was increased to 10 sessions in Experiment 2, there was no effect of heat shock on the CPP memory, as both groups which received the sucrose reinstatement showed evidence of CPP. This insensitivity to the effects of amnestics has been widely documented in rodents (Alfei et al., 2015; Briggs & Olson, 2013; Cassini et al., 2017; Flavell & Lee, 2013; Merlo et al., 2014; 2018) and humans (Sevenster, Beckers & Kindt, 2014). Thus, the insensitivity to amnestics with intermediate lengths of exposure has now been observed in different species (now including invertebrates; also see Merlo, Santos, Pedreira & Merlo, 2019 for evidence in Crabs) and in both appetitive and aversive settings thus suggesting that the phenomenon has generality. Because we did observe an effect of heat shock in Experiment 1, and Experiment 2 used similar parameters for heat shock exposure, it is difficult to account for the findings of Experiment 2 by posing that heat shock was ineffective. Rather, it seems to be the case that memory reactivation immediately after training targets the most recent (excitatory memory) but as exposure increases a new inhibitory trace becomes established. At an intermediate state in which neither excitatory nor the inhibitory memories are dominant, the amnestic has no effect upon memory reactivation. As it has been suggested, prediction error is a putative marker of this period of insensitivity to amnestics (Alfei et al., 2015; Sevenster, et al., 2014). When the excitatory memory is reactivated for the first time, a large discrepancy between what is expected and experienced will occur, and this will target the excitatory memory. Upon repeated exposures, prediction error will diminish and it may be the absence of prediction error with intermediate levels of exposure that results in insensitivity to the amnestic.

Prediction error alone, however, is insufficient to explain the results of Experiment 3, where heat shock has an effect on the expression of extinction. As suggested by Eisenberg et al. (2003), the net result of memory reactivation may be the sum of multiple interacting memory traces evoked by the CS presentation. Excitatory traces depend on the intensity of the US used during CS-US pairings, and

the amount of CS alone exposure during reactivation. Because extended CS alone exposure following excitatory learning results in extinction, and this is best captured as new inhibitory learning (Bouton, 2004), whichever memory trace becomes vulnerable to disruption depends on two competing processes: CS-US or “excitatory” vs CS-NoUS or “inhibitory” traces. The outcome of this competition is determined by various parameters such as the strength of original training (Suzuki et al., 2004), and/or amount of extinction sessions (Eisenberg et al., 2003; Lee et al., 2006). In other words, there is an inverse correlation between the stability of the trace and trace dominance. In the present study, the amount of extinction trials played a critical role in determining the outcome of the competition between the excitatory and inhibitory memory traces. Together, these findings reveal that the maintenance and formation of excitatory and inhibitory memory traces depends on the amount of extinction training. One possibility to formalize the trace dominance hypothesis is to use a temporal weighting rule (TWR), according to which the weight of different experiences (excitatory or inhibitory) are dynamically averaged by animals over time in order to minimize the uncertainty (Devenport et al., 1997; Devenport, 1998). According to Devenport and colleagues, dynamic averaging as the outcome of the TWR can explain complex behaviours in which different interfering memories (such as the ones manipulated here) interact for their expression.

Duvarci et al., (2006) found that an amnesic after extinction training decreased responding during a subsequent test, a result that in principle is opposite to the findings of Experiment 3 and many others mentioned above. The results of these experiments speak about these discrepancies as we also saw behaviour indicative of extinction in Experiment 1 after only 4 sessions of exposure (a result that replicated Mohammed Jawad et al., 2018, and other unpublished results from our laboratory). Despite observing behaviour indicative of extinction, Heat Shock administration impaired the excitatory memory, which is consistent with the observations of Duvarci and colleagues (2006). In that study, using a within-subjects design rats received training with two CSs. Twenty-four hours after training, they gave one 30s exposure to one CSr and a 600s exposure to the alternative CSe, each in a different context. They observed extinction in the latter condition, so that by the last 30 secs of the 600 sec exposure, rats did not freeze to the presentation of the CS. Immediately after these CS

presentations, they administered intra BLA anysomicin and tested a day later. The observed amnesia (relative to a control group which received Vehicle) to both CSs, irrespective of the amount of exposure. In Experiment 1, we also observed significant extinction, and consistent with their findings we also observed amnesia after Heat Shock administration. Our subsequent experiments, in particular Experiment 3 where we observed amnesia for extinction, suggest that the parameters used by Duvarci and colleagues (2006) were insufficient to establish the extinction memory as the dominant trace, in particular considering that extinction was given all in one session and by massed exposure to the CSe. This raises the intriguing possibility that, unlike what has been previously proposed (Dudai, 2004), to establish which trace is dominant based on behaviour during exposure may not be the best indicator of which mnemonic trace is being targeted by the amnesic.

The Bayes factors suggested only moderate or sometimes weak evidence for the different tests in Reinstatement due to the low statistical power. However, the pattern observed throughout the data in all three experiments is consistent with previous findings in the literature and thus our expectations. Overall, the pattern across the experiments in the present study shows that multiple memories (excitatory and inhibitory) simultaneously interact for their expression, and the dominant trace that is retrieved becomes susceptible to modification. This indicates that planarians, despite relying on a relatively simple neural network, can deal with multiple long-term memories and the interaction of these shows similar characteristics to those seen in vertebrates. From a comparative perspective, these similar findings suggest that previous observations in vertebrates are not unique to these, and hence that planaria is a good model to study reconsolidation processes. Furthermore, from a translational perspective, the planaria is a promising model that could provide further insights for amnesia research. Because one species of planaria, the *Schmidtea Mediterranea,* has been fully sequenced (Grohme et al., 2018), the present findings bolster the notion that planaria can be used to understand the molecular machinery sub serving the interaction of multiple memory processes as those observed in the present experiments. Besides, planaria appears an important evolutionary model that would make a significant contribution to the learning and memory research in order to understand how such complex cognitive processes evolved through the animal kingdom.

In summary, the present results contribute to our knowledge on post-reactivation amnesia in the planaria, and suggest that as observed in vertebrates the amount of reactivation determines the effect of amnestics. Consistent with previous findings in other species, these findings show excitatory and inhibitory memories interact to determine the fate of retrieved memory, and planaria might bring some further insights to the alternations in memory traces after reactivation.

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		Change in Preference Score Mean (*M*) and Standard Deviation (*SD*)			
		Last Extinction		Heat Shock Test	
	Groups	Mean (*M*)	Standard Deviation (*SD*)	Mean (*M*)	Standard Deviation (*SD*)
Experiment 1	Heat shock	.002	.12	.048	.18
	Control	.037	.15	.054	.14
Experiment 2	Heat shock	.088	.15	.113	.25
	Control	.102	.15	.143	.24
Experiment 3	Heat shock	.073	.17	.030	.27
	Control	.075	.15	.071	.15

Table 1. Mean and standard deviation of the change scores of the groups in their less preferred side over the final extinction session and the test session following heat shock exposure.

Figure 1. Summary of experimental designs with the five stages. 1) Pre-conditioning test (the numbers refer to the initial preference for the two surfaces (rough/shaded and smooth); 2) CPP training: the animals were given pairings of the less preferred surface with sucrose—a rewarding agent; 3) Post-conditioning and extinction tests, carried out to assess the post-conditioning preferences in the absence of the rewarding agent; there were 4, 10 and 16 trials in Experiments 1, 2 and 3 respectively; 4) Heat-shock exposure and test to assess the immediate effect of the amnestic event on the performance of the animals during memory reactivation; 5) CPP reinstatement test in which animals are independently exposed to the rewarding agent 24 hours before a final preference test.

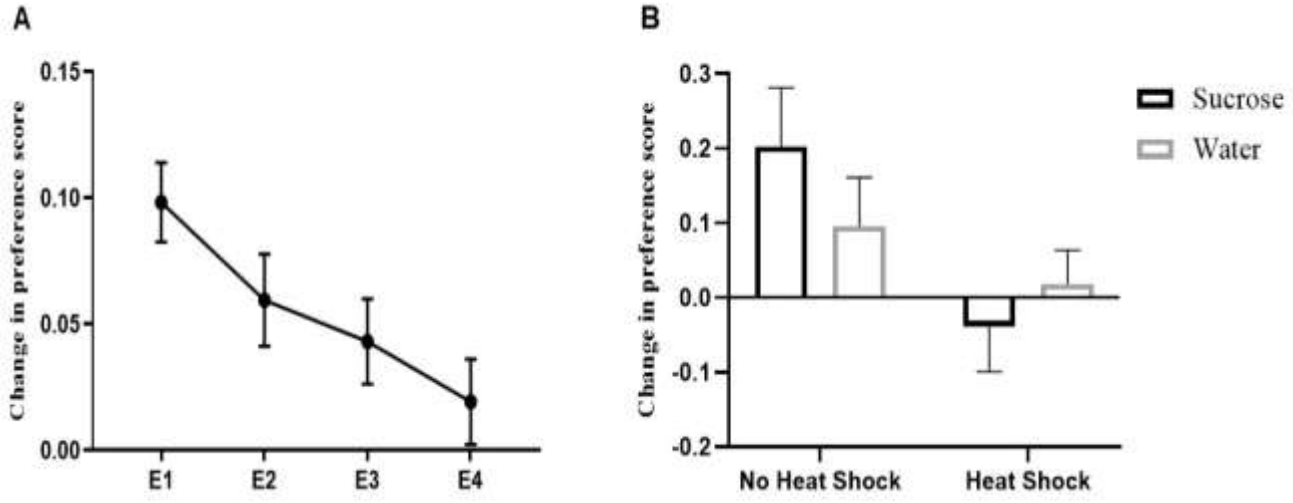

Figure 2. Experiment 1. 2A. Mean of the change scores (± SEM) of the subjects in their less preferred surface were presented over the extinction phase (4 sessions). **2B.** Mean of the change scores (± SEM) of the groups in their less preferred side in the reinstatement test after sucrose exposure were presented. Each bar represents the mean of change in groups' preference scores, and sample size of groups differed between 14-16 (n = 61 in total).

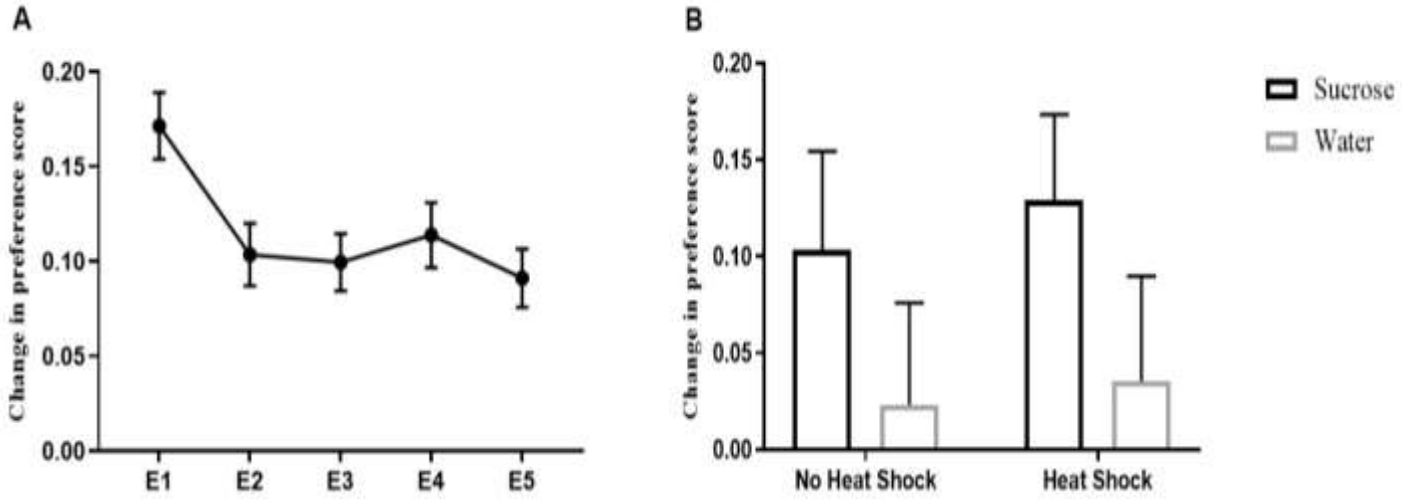

Figure 3. Experiment 2. 3A. Mean of the change scores (± SEM) of the subjects in their less preferred surface were presented over five blocks of two extinction sessions (total: 10 sessions). **3B.** Mean of the change scores (± SEM) of the groups in their less preferred side in the reinstatement test after sucrose exposure were presented. Each bar represents the mean of change in groups' preference scores, and sample size of groups differed between 13-21 (n = 69 in total).

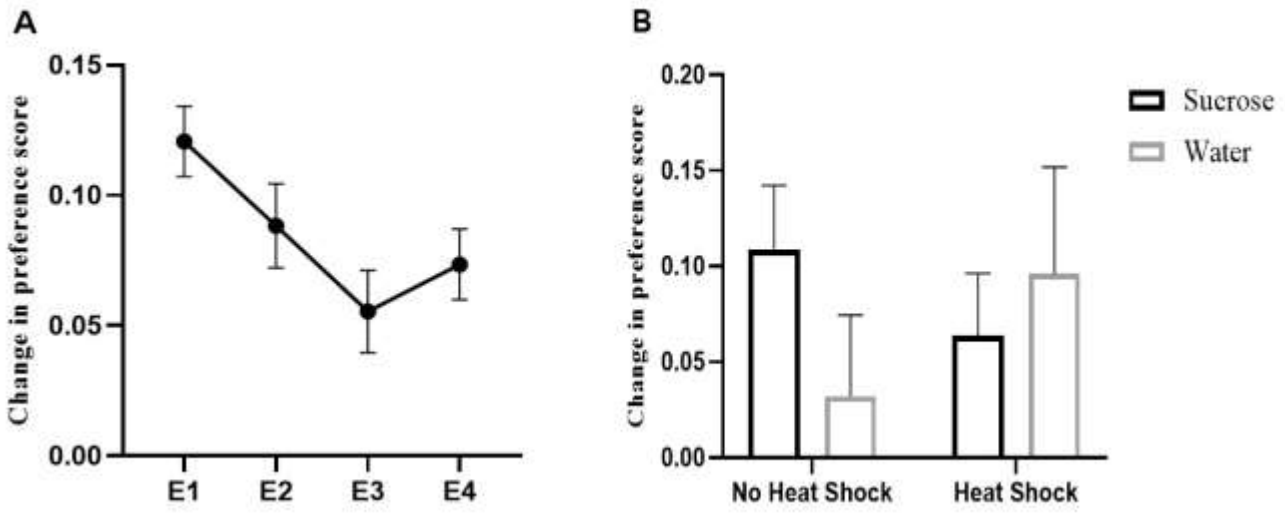

Figure 4. Experiment 3. 4A. Mean of the change scores (± SEM) of the subjects in their less preferred surface were presented over four blocks of four extinction sessions (total: 16 sessions). **4B.** Mean of the change scores (± SEM) of the groups in their less preferred side in the reinstatement test after sucrose exposure were presented. Each bar represents the mean of change in groups' preference scores, and sample size of groups differed between 16-17 (n = 69 in total).