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The nature of training in flavor preference learning determines the underlying associative structure

Ana González, Jesús Sánchez, Isabel de Brugada [*]

University of Granada, Department of Experimental Psychology, Centro de Investigación Mente, Cerebro y Comportamiento (CIMCYC), Spain

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Pairing a palatable flavor (US) with an initially neutral flavor cue (CS) results in an acquired conditioned preference for the latter. Two main associations have been proposed to explain the acquisition of flavor preferences: Flavor-Flavor and Flavor-Nutrient learning. Although the hedonic reaction triggered by US consumption has also been suggested as a possible additional component underlying acquired flavor preference, this issue has received little attention. Here we explored whether the amount of training to the CS-US compound can favor the formation of a Flavor-Hedonic reaction association using rats as subjects and sucrose as the US. We expected that the more exposure to the CS-US compound, the stronger the S-R type association. Since S-R associations are not sensitive to devaluation procedures, we used a Sensory-Specific Satiety procedure to devalue the US after conditioning and then measured preferences for the CS. On Experiment 1 with a short restrictive training (classic procedure), preference for the CS was decreased after devaluation of the US compared to the control condition. On Experiment 2, with short unrestrictive training, preference for the CS was again weakened. Experiment 3 with a long unrestrictive training, rats expressed preference for the CS regardless of the devaluation procedure. These results suggest that, as with an instrumental paradigm, extensive training in flavor preference learning undermines the US devaluation effect.

1. Introduction

Associative learning has been shown to play a very important role in acquiring flavor preferences (Martin, 2016). In the same way that animals learn to avoid a food after having suffered from aversive consequences post-ingestion (conditioned taste aversion), they can acquire a conditioned preference to initially neutral flavors after pairing with a palatable taste (flavor preference conditioning). Both learning processes have an adaptive utility so that animals can avoid harmful or poisoned foods or learn which flavors could provide them with energy sustenance in a food-scarce environment (Harris et al., 2000).

Acquisition of flavor preferences has been traditionally studied by pairing an appetitive taste (US) with a neutral flavor cue (CS), a case of Pavlovian conditioning. However, in the literature, it has been proposed that such conditioning may represent a special or unique form of Pavlovian learning, given certain particularities of the phenomenon (e.g., Delamater, 2012). From a Pavlovian perspective, an organism can learn simultaneously from multiple components of the US (e.g., sensory, hedonic, motivational) rather than this being a unitary process (e.g., Delamater, 2012; Delamater and Oakeshott, 2007; Hall, 2002; Konorski, 1967). In this sense, the acquired flavor preference has been explained in terms of two main possible associations that can be learned in an overlapping or independent way: Flavor-Flavor and Flavor-Nutrient Learning. Flavor-Flavor Learning refers to the learning that occurs when a neutral cue is associated with the palatable taste of the US (e.g., sweet taste of sucrose or saccharin) (e.g., Fanselow and Birk, 1982; Gil et al., 2021). Flavor-Nutrient Learning is demonstrated when the CS is paired with the motivational properties of the US (e.g., the caloric content of Sucrose) (e.g., Azzara and Sclafani, 1998; Palframan and Myers, 2016). Both Flavor-Flavor (sensory-based) and Flavor-Nutrient (motivational-based) associations have been well dissociated in the literature and have been the focus of many types of research. These two associations have been examined by using non-caloric tastes (e.g., Fanselow & Birk, 1989; Gil et al., 2014; Gil et al., 2021), changing motivational states during training, testing, or extinction (e.g., Capaldi et al., 1994; Harris et al., 2000; Harris et al., 2004), using sham feeding procedures (Bonacchi et al., 2008) or intragastric infusions (e.g., Ackroff et al., 2012; Myers, 2007; Myers and Sclafani, 2001).

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[*] Correspondence to: CIMCYC, Campus de Cartuja s/n, Granada 18011, Spain
E-mail addresses: gonzaleza@ugr.es (A. González), jesusflea@ugr.es (J. Sánchez), dbrugada@ugr.es (I. de Brugada).

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A less studied proposal to explain preference learning, has been based on a Flavor-Hedonic reaction association (Harris et al., 2004; Delamater, 2012). This learning involves a link between a stimulus and the hedonic response elicited by the US (Stimulus-Response association; S-R). Thus, it represents a scenario in which the conditioned taste is preferred not because it activates the sensory representation of the US or because triggers a general central motivational state similar to that evoked by the US but because it automatically triggers the hedonic response produced by the US. In this regard, hedonic responses are shared among multiple stimuli with the same valence and are therefore evoked independently of the representation of a single US. Since this association is not governed by a specific US representation, this acquired preference should be insensitive to changes in the current value of the US. Therefore, if the US loses its reinforcing value (for example, through Conditioned Taste Aversion or Sensory-Specific Satiety), the CS will continue to elicit hedonic responses rather than reduce them as a consequence of devaluation.

This third proposal was suggested by Harris et al. (2004) based on the results of the third experiment of their study. On this experiment, authors first trained thirsty rats by pairing an initial neutral flavor cue with a sucrose solution. After this procedure, a group of rats underwent several extinction trials in which they were presented with the flavor cue alone, while another group did not receive the extinction treatment. Next, half of each group of rats was injected with LiCl after drinking sucrose, while the remainder did not receive the devaluation treatment. The results revealed that the rats did not only show resistance to extinction of their conditioned preference (extinguished and not devalued group) but also kept intact their preference after the devaluation procedure (extinguished and devalued group). However, the rats that did not receive extinction trials and had undergone the conditioned aversion procedure (non-extinguished and devalued group), expressed a devaluation effect by reducing their preference for the CS+. These results suggested that 1) acquired preferences are sensitive to devaluation procedures after training, and 2) following an extinction procedure the sensitivity to devaluation is abolished. To explain these results, the authors argued that extinction may have weakened the Flavor-Flavor association, while the remaining preference that was still expressed after devaluation (extinguished-devalued group) was the result of an association between the flavor cue and the US-hedonic reaction. Thus, these authors provided a tentative interpretation of their results in terms of the expression of a possible S-R association.

Delamater (2007); Experiment1 and 2) also reported a similar pattern of results but suggested an alternative explanation. In Experiment 1, thirsty rats underwent a training procedure in two which different flavored cues were paired with the same US separately. After this, all rats were presented with one of the flavors on several extinction trials (without the US). Half of the rats were then subjected to a conditioned taste aversion to the US (Devalued Group) while the other half of the rats received unpaired presentations of the US and the LiCl injections (Non-devalued Group). Rats in the non-devalued group preferred the non-extinguished flavor over the extinguished one, showing evidence of extinction, unlike the study by Harris et al. (2004). However, similar to Harris et al. (2004), rats in the devalued Group showed the opposite pattern of results, preferring the extinguished over the non-extinguished flavor. In Experiment 2, these results were replicated by employing a full within-subjects design. Delamater (2007), in line with Harris et al. (2004), argued that when thirsty rats undergo an extinction procedure, the Flavor-Flavor association is weakened, as seen in the non-devalued group. But, unlike Harris et al. (2004), he proposes that after US devaluation, the preference for the extinguished over the non-extinguished CS+ is due to the possibility that the latter activates the sensory representation of the US to a greater extent. Likewise, a previously extinguished CS+ is preferred after US-devaluation not because it automatically elicits the hedonic response of the US but because it weakly retrieves its sensory attributes.

Another study that challenges this hypothesis is that of Dwyer et al. (2009), who found that after repeated testing of the CS+ in extinction, the hedonic responses to the CS+ disappeared while the conditioned preference was preserved in consumption. So, these studies seem to suggest that the presumed immunity to extinction that characterises this paradigm is not the result of a Flavor-Hedonic response association as suggested by Harris et al. (2004). Therefore, according to this approach, there seems to be no evidence that a S-R link can control acquired flavor preferences, at least after an extinction procedure or with the training procedures used in these studies.

However, this does not detract from the fact that certain training conditions still may favor the formation of a Flavor-Hedonic reaction association. From the associative learning framework, it has been suggested that during the initial stages of acquisition, the learned content is flexible and guided by the anticipation of obtaining a certain outcome. As learning is consolidated, it becomes much more rigid, automatic, or stimulus-driven (e.g., Adams and Dickinson, 1981). And along these lines, the traditional view of associative learning regarding instrumental behavior is the belief that S-R associations develop over lengthy training procedures because of Thorndike's (1911) Law of Effect (e.g., Adams, 1982; Tricomi et al., 2009; Dickinson, 1985; Killcross and Coutureau, 2003). In this sense we hypothesize that the training conditions established in most experiments do not favor the formation of such an association as the acquisition of flavor preferences has traditionally been studied using short training procedures and restricted access to the target solutions. Thus, we propose that this association would not govern performance after the extinction of the Flavor-Flavor association (as Harris et al., 2004 argued), but after a long-lasting training procedure which overexposes animals to the target compounds. To elucidate this question, it is therefore necessary to investigate how different regimes of exposure during training (minimal vs extended) affect the sensitivity to the US devaluation procedures.

There have been previous studies that have addressed this hypothesis with other classical conditioning procedures different than those of flavor preference learning. Unlike instrumental learning, there is no evidence of a relationship between overtraining and S-R learning in Pavlovian learning (e.g, Holland, 1990, 2005; Holland et al., 2008). Despite this apparent resistance to S-R learning in the Pavlovian paradigm, evidence points to the fact that the representation of learned content, rather than the associative structure, may change as training is extended. In the initial stages of learning the CS may activate a mimetic perceptual representation of the US while with overtraining a more abstract and motivationally-based representation of the US would be activated (Holland, 1990, 1998, 2005, 2008). Both types of representation would evoke US sensory-specific attributes but may differ in their very nature and thus, in the way individuals experience it. This notion was described in Hollanďs experiments (e.g., 1998, 2005; Holland et al., 2008) in which rats were trained by pairing tones to food with different conditioning regimes (short or extended). These experiments showed how overtrained rats expressed an intact devaluation effect (conditioned taste aversion to the US and testing the CS+) but weakened mediated conditioning (conditioned taste aversion to the CS+ and testing the US) compared to minimally trained rats. So, Holland interpreted these results to mean that, in the initial stages of learning, the learned content resembles the sensation of the US when it is experienced ("hallucination") and with training this experience would dissipate and become something similar to an expectation of obtaining the US itself ("image") (Holland et al., 2008; Delamater, 2012).

In the case of flavor preference learning, to our knowledge, the effect of overtraining on the sensitivity to devaluation procedures has not yet been studied. And given its distinctiveness from other classical learning phenomena, it is possible that overtraining with this paradigm could result in a S-R association. Given this hypothesis, in the present study we test the effect of US devaluation on the expression of a conditioned preference after manipulating the amount of exposure to the CS-US compound during training. For the general training procedure, we used a within-subject design in which rats had access to a neutral flavor

(CS+) that was paired with a highly palatable taste (US) and a different neutral flavor that was presented alone (CS-). Animals had free access to standard chow pellets during the whole procedure, narrowing the chances that the S-R association will develop on the basis of motivational rather than hedonic responses. To produce devaluation, we used a Sensory-Specific Satiety procedure. Sensory-Specific Satiety is defined as the temporary loss of the specific hedonic value of the sensory properties of food after its ingestion (e.g., Rolls et al., 1981). This effect is defined as sensory-specific, occurring without the need for metabolic feedback and specifically affecting the sensory properties of the ingested food. Concerning the experimental series, on Experiment 1, we first demonstrate the basic US devaluation effect by training rats with a short and "classic" training procedure in which they have restricted access to the target compounds for 10 days. Then, in Experiments 2, we study the effect of a 6 day-unrestricted training. Finally, on Experiment 3, rats were trained with a 12 day-unrestrictive training procedure. We expect to find that in shorter training procedures the experimental subjects would express the US devaluation effect, while extended training procedures would show insensitivity to the US-devaluation effect or an attenuation of the latter.

2. Experiment 1

Experiment 1 was conducted to show the US-devaluation effect after a typical training procedure in flavor preference learning (restricted amount and time during CS-US exposure). This experiment employed a within-subject training procedure. Rats were trained for 10 days, 20 minutes each and were given a limited amount of the solutions. The training procedure consisted of pairing a neutral flavor cue (CS+) with a sucrose solution (US) and by presenting another neutral flavor cue (CS-) with plain water. After that, rats started the pre-feeding-choice test cycles that lasted 2 days, being pre-fed with sucrose (Pre-fed condition) or water solution (Control condition), after which the preference for the two flavors was assessed (CS+ vs. CS-). Pre-feeding cycles were implemented with a within-subject design so that half of the rats received the sweet solution on the first day of testing and water on the next, while the other half of the rats received the opposite arrangement. We expected a devaluation effect to occur if rats reduced their preference for the CS+ solution when they had been pre-fed with sucrose solution compared to the case in when they had received only water.

3. Methods

3.1. Subjects and apparatus

Sixteen naïve male Wistar rats with a mean weight of 294 g (max: 340gr – min: 263gr) and supplied by Janvier Labs were used in the present experiment. Rats were individually housed in translucent plastic cages (35 × 12 × 22 cm) with wood shavings as bedding. They were maintained on a 12-h light/dark cycle for the whole procedure, starting the light cycle at 8:00 am. The experimental solutions were prepared every day with tap water and presented to animals in centrifuge tubes (50 ml capacity) with stainless steel, ball-bearing-tipped spouts. All tubes were placed in the middle of the front metal cover of the cages in the sessions in which just a single bottle was presented to avoid the effects of any side preferences during the choice tests. Consumption was measured by weighing tubes before and after each procedure. The flavored solutions were composed of 0.05 % Vanilla or Almond aroma (CS+) (Manuel Riesgo, Madrid) and 10 % domestic sucrose (US) or 0.05 Vanilla or Almond aroma (CS-) (Manuel Riesgo, Madrid) diluted with water. The neutral flavor paired with the sucrose was counterbalanced across rats, with half receiving vanilla paired with sucrose and the other half receiving almond paired with sucrose. The Ethics Committee for Animal Research at the University of Granada and the General Department of Agriculture and Animal production of the Andalusian Regional Government (05/11/2020/125) approved all the procedures described in this paper . These procedures were classified as low severity according to European guidelines. Animals were monitored daily by those responsible for animal welfare in the research center.

3.2. Procedure

One day before the beginning of the experiment, the water bottles were removed at 4:00 pm, and access to water or experimental solutions was restricted to two daily sessions (10:00 am- 4:00 pm). On the first two days of the experimental procedure, rats had access in the morning to 30 minutes of unrestricted water to habituate them to the experimental schedule and experimental tubes. In turn, these two sessions served also to measure the baseline consumption of the animals. On the afternoon sessions of the first two days, the rats had unrestricted access to water for 30 minutes.

From day 3rd to 15th, rats underwent the training procedure. This procedure consisted of 20 minutes access to 10 ml of the sweet solution (CS+) or the flavor alone (CS-) in both the morning and afternoon sessions. The presentation of both solutions was counterbalanced across morning and afternoon sessions. Half of the animals received the CS+ or CS- solutions with the following order across sessions: ABBA, and the other half received the opposite pattern: BAAB.

On Day 7, the training procedure was interrupted to assess whether the rats had acquired a preference (baseline). To habituate the animals to the new schedule (testing phase), they received two water two-tube training sessions on the morning of days 7 and 8. This habituation procedure consisted of two 20-min sessions in which rats were offered two water bottles on both sides of the cage. After 10 minutes, the experimenter changed the position of the tubes to acclimatize animals to this procedure on the final tests (the position of the tubes was switched on the tests since it helps to abolish any effects of position bias). This procedure was adapted from (Farabi et al., 2022). On the 9th day, animals were given both CSs presented in water in the same fashion as the habituation procedure described previously. After this test, the rats underwent four more training sessions to establish the CS+ preference. These four training sessions were divided into two (Days10–11 and 13–14), followed by a preference test (Days 12 and 15). At the end of the training procedure, the rats had 10 days of training in which they were exposed 10 times to each solution (CS+ and CS-) and were tested for their preference over 3 days.

Once this procedure ended and preference for the CS+ was stablished, rats underwent the Pre-feeding cycles for 2 days with one day of rest in between in which they were given just plain water. This pre-feeding consisted of presenting 10 ml of a 10 % sucrose (Pre-fed condition) or water (Control condition) solution for 10 minutes. After 30 minutes from the beginning of this Pre-feeding phase, all rats received a two-bottle test in which they were exposed to the CSs in the same manner as described previously. The order in which the rats received both conditions (Pre-feeding/Control) was counterbalanced across the two days. Half received the sucrose solution on the first day, and the other half received the water solution. A devaluation effect will be observed if the preference for CS+ over CS- is reduced after the presentation of the US. This will not occur when animals receive water, as the lack of sweetness will not affect the preference of the CS+, serving as a control.

3.3. Data analysis

General linear model null hypothesis testing analyses were conducted, assuming a rejection level of $p < 0.05$, using Greenhouse–Geisser corrections for mixed factorial analysis of variance when needed. Partial eta squared, and Cohen's d tests were used to measure effect sizes. To assess the conditioned preference in all the experiments, both direct consumption and preference ratios were calculated. Preference ratios were calculated by the following formula: (Consumption of the CS+) / (Consumption of the CS- + Consumption of the CS+). The resulting score ranges from 0 to 1, with values below 0.5 indicating a preference for the CS- and those above 0.5 indicating a preference for the CS+. All ratios were then analyzed using a one-sample t-test to assess whether these differed from chance level (0.5). These statistical criteria were adopted for all the experiments.

3.4. Transparency and openness

This study have not been preregistered. The data of all the experiments are available in the APA's repository on the Open Science Framework (OSF): https://osf.io/s3f96/files/osfstorage/65f43a46e5e51c127abc5db9

4. Results and discussion

Fig. 1 (panel-a) shows rats' consumption across the 10 days of training for both CS+US and CS- solutions. This figure shows how consumption of the CS+US solution is higher than for the CS-, except for the first day of training. A repeated-measures ANOVA with Day (1−10) and CS solution (CS+US vs. CS-) as within-subject factors was conducted to analyze consumption during training. This analysis revealed a main effect of CS $F(1,15)= 120.59$, $p<0.001$; $\eta_p^2 = 0.88$ reflecting higher total consumption of the CS+US over the CS-. A Greenhouse-Geisser correction was applied to the factor Day, and for the interaction, Day*CS since Mauchly Tests revealed that the assumption of sphericity was violated. After applying Greenhouse-Geisser correction, this analysis revealed no significant effect of either Day $F(1.77, 23.43)= 2.57$, $p= 0.10$, $\eta_p^2 = 0.14$ or the interaction CS*Day $F(1{,}56, 23{,}43)= 2.69$, $p= 0.09$, $\eta_p^2 = 0.15$.

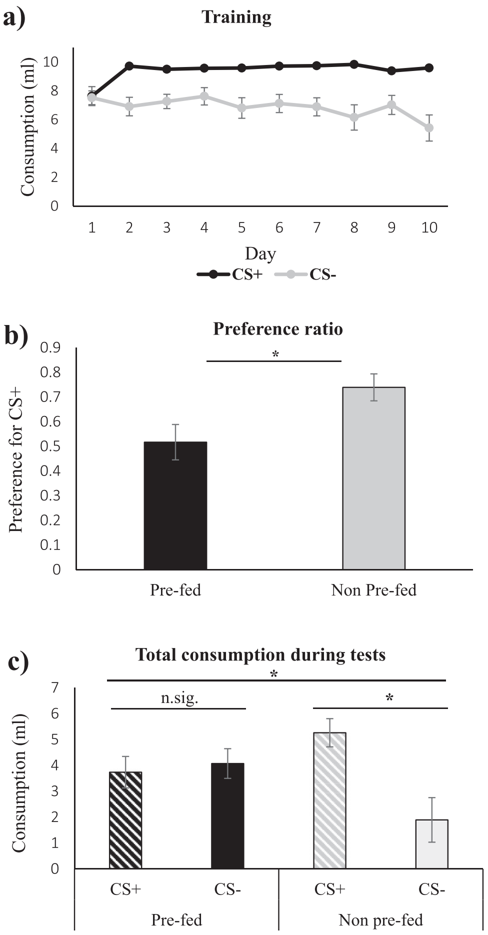

Fig. 1. *Experiment 1. Consumption and relative preference during training and testing. Note.* a) Total consumption (ml) of both CSs across the training days. b) Preference ratio for CS+ when animals were pre-fed with sucrose (Pre-fed) or were just given water (Non pre-fed), and c) Direct consumption (ml) of both CSs across conditions (Pre-fed and Non pre-fed).

The Preference of the CS+ over the CS- across the three initial repeated preference baseline tests was measured through a preference ratio. The three tests were analyzed with a repeated-measures ANOVA with Day as a within-subject measure (1−2−3). This analysis revealed no significant differences across the three tests $F<1$. Thus, an average of the three preference ratios was calculated for each rat, and this mean was compared with 0.5 using a One sample t-test. The results revealed that these differed significantly from 0.5 $t(15)=2.39$, $p= 0.03$, $d= 3.36$ ($M= 0.60$, $SE= 0.04$).

The pre-feeding phase was analyzed to assess differences in the total consumption of water and sucrose. A paired samples t-test revealed that consumption of water was significantly lower than that of sucrose $t(15) =3.44$, $p= 0.004$, $d=0.86$ (M Sucrose= 9.62, $SE= 0.09$; M Water = 7.96, $SE= 0.48$). Water consumption during pre-feeding in the control condition is used to balance the total consumption of the two conditions on testing. A lower total intake during this phase and condition is not expected to impact preference testing due to its familiarity, lack of taste, and calories. Similarly, a Pre-feeding phase without water in the control condition could have been carried out, but the test intakes may have been lower in the pre-feeding condition. If this situation had occurred, it is possible that a drop in preference was due to a floor effect or a reduction in the motivational state of the animals, by, for example, not being thirsty on the test.

Preference tests across the pre-feeding/control conditions were assessed with preference ratios and direct consumption. The data from this stage are displayed in Fig. 1 (panel-b and c). Inspection of this figure suggests that the preference for the CS+ was modified after rats had been pre-fed with sucrose. A paired samples t-test was carried out to compare the CS+ preference ratio during both conditions (pre-fed vs. control). This analysis revealed a significant difference between the measures $t(15)$=-2.84, p=0.01, $d= -0.71$. Both preference ratios were compared with the 0.5 chance level using a One-sample t-test. Only those rats that had not been pre-fed with sucrose (control condition) showed significant differences $t(15)= 4.37$, $p<0.001$ $d= 3.38$, whereas the pre-fed condition did not differ from chance: $t(15)=0.23$, $p=0.81$, $d= 1.80$ (see Fig. 1, panel-b).

Direct consumption during testing was analyzed with a repeated-measures ANOVA with CS (CS+ or CS-) and Pre-feeding (pre-fed or control) as the within-subject factors. The results revealed that the factors CS $F(1,15)= 2.88$, $p=0.11$, $\eta_p^2= 0.16$ and Pre-feeding $F(1,15)= 1.81$, $p=0.19$, $\eta_p^2= 0.10$ did not reach significance. However, the interaction CS*Pre-feeding was significant $F(1,15)= 6.42$, $p=0.02$, $\eta_p^2= 0.30$. This interaction was analyzed using a simple main effects analysis revealing that in the Pre-fed condition, there were no differences in total consumption for both CSs ($F<1$), but for the control condition, there were significant differences $F(1,15)= 13.24$, $p=0.002$, $\eta_p^2= 0.46$, with consumption of the CS+ being higher than that of the CS- (See Fig. 1, panel-c).

Therefore, the US-devaluation effect was observed in the present experiment when using a typical training procedure with a limited quantity and time of exposure to the target compounds. When rats were pre-fed with a sucrose solution, this decreased their preference for its associate CS+ over the CS-. Thus, in line with previous results in the literature, rats' preferences for the CS+ appear to be governed by the US representation, updating its value on tests. In the next experiment, we will increase the amount of time and access to the CS-US compound during training to assess whether, under these conditions, rats still express the US-devaluation effect.

5. Experiment 2

In Experiment 1, we found an effect of devaluation by using a short and restrictive training procedure (typical training procedure in flavor preference learning). In the present experiment, we wanted to analyze the effects of overexposure to the CS-US compound during training. We expected that continuous access to a palatable solution would not only result in an acquired flavor preference for the CS+ but also strengthen the association between the CS+ and the hedonic response to the US. On Experiment 2, rats were trained for six days (3 days with CS+ and 3 days with CS-) with unrestricted access to the solution for 6 hours. In contrast to Experiment 1, under these circumstances we expected to find no effect of US devaluation on the CS+ preference.

6. Methods

6.1. Subjects and apparatus

Sixteen male, naïve Wistar rats with an average weight of 273 g (max: 290 – min: 250) supplied by Janvier Labs were used as experimental subjects. Animals were kept under the same healthcare conditions as in Experiment 1. The flavored solutions were also the same as those described in Experiment 1.

6.2. Procedure

One day before the beginning of the experiment, the water bottles were removed at 4:00 pm. After water deprivation, the next day rats had access to water from 10:00 am to 4:00 pm (6 hours). This session was carried out to assess baseline consumption and habituate rats to the schedule of the training sessions.

On day 2, animals started the training procedure that lasted 6 days. The training procedure consisted of a daily 6-hour exposure to the CS+US or the CS- solution (3 days each). Half of the animals received the CS+ and CS- solutions with the following order across sessions: ABBAAB, and the other half received the opposite pattern: BAABBA. During this procedure, animals had 15 minutes of access to water at 4:00 pm after the CS+US or CS- bottles were removed.

From the afternoon of day 8, the experimental sessions were divided into two as in Experiment 1 and animals only had access to water at the afternoon session during a 30-minute period (10:00 am-16:00 pm). To habituate the animals to the new schedule, they received two water two-tube training sessions as in Experiment 1. After this procedure, on Day 11, animals were tested for the CS+ preference in the same way as explained in Experiment 1. Finally, on days 12 and 14, animals started the pre-feeding-choice test cycles. These cycles were also identical to those in Experiment 1.

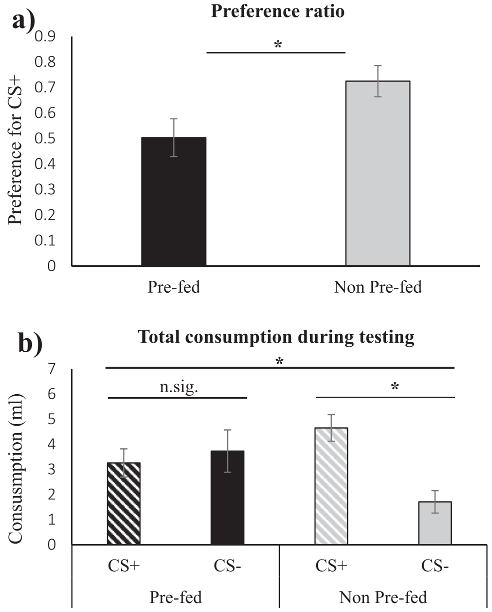

Fig. 2. *Experiment 2. Consumption and relative preference during testing. Note.* a) Preference ratio for CS+ when animals were pre-fed with sucrose (Pre-fed) or were just given water (Non Pre-fed). b) Direct consumption (ml) of both CSs across conditions (Pre-fed and Non Pre-fed).

7. Results and discussion

To analyze consumption during the training phase, a repeated measures ANOVA was conducted with Day (1−3) and CS solution (CS+US/CS-) as within-subject factors. As in Experiment 1, this analysis revealed a significant effect of CS $F(1,15)= 48.31$, $p<0.001$, $\eta_p^{2}= 0.76$, $MSE= 34.07$ with a higher total consumption of the CS+US (total mean across the 3 days: $M=76.50$, $SE=3.6$) than the CS- (total mean across the 3 days: $M=51.73$, $SE=2.28$). The factor Day $F(2,30)=2.12$, $p=0.13$, $\eta_p^2=0.12$ and the Day*CS interaction $F(2,30)= 2.85$, $p= 0.07$, $\eta_p^2=0.16$, $MSE= 42.14$ were not significant.

The initial preference for the CS+ over the CS- after training was measured through a preference ratio. A One sample t-test was carried out to compare the preference ratios with 0.5. This analysis revealed that the ratios differed significantly from 0.5, $t(15)=5.03$, $p< 0.001$, $d= 3.90$ ($M= 0.74$, $SE= 0.04$).

Data from the Pre-feeding phase were analyzed to assess differences in total consumption for both water and sucrose solution. A Paired samples t-test revealed again that consumption of water was significantly lower than sucrose $t(14) =3.89$, $p= 0.002$, $d=1.04$ (Mean Sucrose $M= 9.52$, $SE= 0.23$; Mean Water $M= 8.00$, $SE= 0.30$).

During the pre-feeding-choice test cycles, two rats had to be removed from the analysis since on one of the days of testing (after the pre-fed condition), one tube of each rat was spilled, making it impossible to determine the amount consumed. The direct consumption data and preference ratios across conditions are illustrated in Fig. 1 (panel-a and b). The results seem to show an attenuation of the CS+ preference across conditions. First, both preference ratios were submitted to a paired samples t-test comparing the pre-fed and control conditions. This analysis revealed significant differences across conditions $t(13)=-4.28$, $p<0.001$, $d= -1.15$. Both ratios were tested against the chance level of 0.5 with a one sample t-test, revealing significant differences for the control condition ratio $t(13)=3.43$, $p=0.004$, $d= 2.96$, and non-significant differences for the pre-fed ratio $t(13)= 0.42$, $p=0.967$, $d= 1.71$ (See Fig. 1, panel-a). Further, a repeated measures ANOVA was carried out to assess direct consumption during pre-feeding/control conditions with CS (+/-) and Pre-feeding (control/Pre-fed) as within-subject factors. This analysis revealed non-significant differences according to the main factors CS $F(1,13)= 1.54$, $p=0.23$, $\eta_p^2= 0.11$ and Pre-feeding $F<1$. However, the interaction Pre-feeding*CS $F(1,13)= 15.34$, $p=0.002$, $\eta_p^2= 0.54$, $MSE=2.65$ reached significance. A simple main effects analysis was conducted to explore the source of this interaction, revealing that only rats in the control condition showed a preference for the CS+ $F(1,13)=10.88$, $p=0.006$, $\eta_p^2= 0.45$, $MSE= 5.57$; whereas those in the Pre-fed condition did not $F<1$.

As in previous literature, we found a US-devaluation effect with the present procedure (short and unrestricted exposure during training). Despite having continuous access for 6 hours to the target solutions, rats still expressed the US-devaluation effect. Thus, we can confirm that with this procedure, rats still expressed a preference mediated by the US representation. However, although the rats had continuous access to the target solutions in this experiment, they were only exposed to the CSs for 3 days. For this reason, we decided to extend the training procedure by doubling the number of days of exposure for both solutions before testing. In this way, following the logic employed in the instrumental paradigm, the longer the training, the more automatic it becomes and the less guided by the US representation.

8. Experiment 3

In Experiment 3, we doubled the total training days from 6 to 12 days to increase the amount of exposure to the CS-US compound. We expect that by giving animals more opportunities to pair a flavor cue (CS+) with sucrose (US), learning will become much more rigid and automatic, leading to an association that is insensitive to devaluation methods.

9. Methods

9.1. Subjects and apparatus

Sixteen non-naïve male Wistar rats with an average weight of 472 g (max: 529 – min: 397) supplied by Janvier Labs took part in the present experiment. The rats were naïve to all the stimuli used in the present experiment. Animals were kept under the same conditions as in Experiment 1, and the flavored solutions were also the same as those used previously. In the present experiment, bottles with metal stoppers were used instead of tubes to prevent the rats from nibbling on the rubber stoppers of the tubes during the 6-hour training sessions. Once this phase was finished, we used the same tubes as in Experiments 1 for the remaining experimental sessions.

9.2. Procedure

As in Experiments 1, rats were water-deprived at 4:00 pm the day before the experimental procedure, with a baseline session given on Day 1.

On Day 2, animals started the training procedure that lasted 12 days. This procedure consisted of a daily 6-hour exposure to the CS+US or the CS- solution (6 days each) as explained in Experiment 1. The order in which animals received the CS+ and the CS- solution was the same as in Experiments 1 (ABBAABBAABBA / BAABBAABBAAB). Once the bottles were removed, the rats also had access to 15 minutes of water (4.00 pm) throughout the entire experimental procedure.

The rest of the procedure was the same as the previous experiment.

10. Results and discussion

As Fig. 3 (panel-a) shows, animals consumed higher amounts of the CS+US solution than the CS- throughout the whole training procedure. A repeated-measures ANOVA was conducted with Day (1−6) and CS (CS+US/CS-) as within-subject factors to analyze these data. This analysis revealed a significant effect of CS $F(1,15)= 109.89$, $p< 0.001$, $\eta_p^{2}=$ 0.88, $MSE=$ 271.88 and Day $F(5,75)=2.38$, $p=0.04$, $\eta_p^2=0.13$, $MSE=84.53$ although the Day*CS interaction did not reach significance $F(5,75)= 1.26$, $p= 0.29$, $\eta_p^2=0.07$. These results confirm that the rats consumed more of the CS+US solution during training, as in previous experiments. Moreover, consumption increased across days, possibly due to the habituation of a neophobic response that was present on the first day of training.

A one-sample t-test was conducted to assess whether the initial preference for the CS+ over the CS- was significant compared to chance level. This analysis revealed significant differences from the 0.5 value, $t(15)=9{,}50$, $p< 0.001$, $d= 5.92$ ($M= 0.83$, $SE= 0.03$), thus indicating that the training procedure had been effective.

Consumption during the Pre-feeding phase was analyzed to assess differences in total consumption of the water and sucrose solution. A paired samples t-test revealed again that consumption of water was significantly lower than sucrose $t(15) =2.98$, $p=0.009$, $d= 0.74$ (M Sucrose= 9.32, $SE= 0.10$; M Water = 8.05, $SE= 0.40$).

Preference ratios after pre-feeding are shown in Fig. 2 (panel-b). This figure suggests that rats expressed a preference for the CS+ in both the pre-fed and control conditions. A Paired samples t-test revealed non-significant differences between both preference ratios $t(15)=-1.54$, $p=0.14$, $d= -0.38$. An analysis of one sample t-test was carried out for comparing both ratios to the chance level, showing significant differences in both conditions to the 0.5 value: pre-fed $t(15)= 2.75$, $p= 0.01$, $d=2.5$; control $t(15)= 7.2$, $p< 0.001$, $d= 4.65$. Direct consumption data on the tests are displayed in Fig. 2 (panel-c). These data were analyzed with a repeated measures ANOVA with CS and Pre-feeding as the within-subject factors. This analysis revealed only a main effect of CS $F(1,15)= 45.96$, $p<0.001$, $\eta_p^{2}=$ 0.75, $MSE=$ 2.37 whereas Pre-feeding $F(1,15)= 2.49$, $p=0.14$, $\eta_p^{2}=$ 0.14 and the CS*Pre-feeding interaction did not reach significance $F(1,15)= 3.29$, $p=0.09$, $\eta_p^{2}=$ 0.18. As seen in Fig. 2 (panel-c), these results indicate, in general, that rats drank more of the CS+ than the CS- regardless of condition.

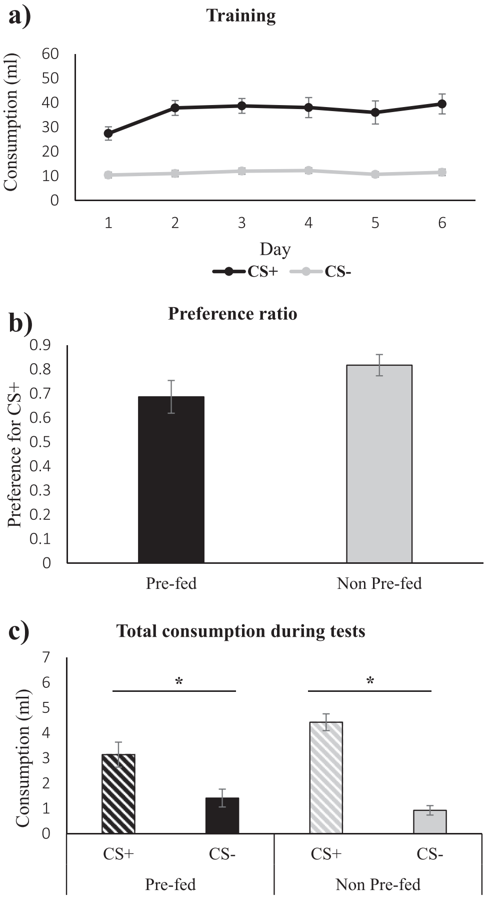

Fig. 3. *Experiment 3. Consumption and relative preference during training and testing. Note.* a) Total consumption (ml) of both CSs across the training days. b) Preference ratio for CS+ when animals were pre-fed with sucrose (Pre-fed) or in the control condition (Non Pre-fed). c) Direct consumption (ml) of both CSs across conditions (Pre-fed and Non Pre-fed) .

The results of Experiment 3 show that after an extended training in which rats had access to the CS-US compound for several hours and days, they expressed a conditioned preference that was resistant to the devaluation procedure. These results suggest that the CS+ preference was not mediated by the US representation, with rats expressing a conditioned preference that was persistent when rats had been pre-fed with sucrose. Instead, these data suggest that rats automatically elicit consumption behavior when presented with CS+. However, an apparent decrease in total CS+ consumption was observed when animals were under the pre-fed condition. Although the rats in the pre-fed condition showed this tendency to consume less (albeit not significant) and the preference was still intact, it is possible that the reduction in total consumption may be reflecting an underlying devaluation effect. Nevertheless, we suggest two main accounts that could also explain this finding. First, during the pre-feeding cycles, animals consume more in a total of the US solution than water in the control condition. Therefore, it is possible that the animals were less thirsty and consumed less overall in the pre-fed test condition. Also, in addition to taste, sucrose has calories, so, during pre-feeding the rats may have changed their motivational state leading to general satiety, which could also have caused a reduction in total intake.

11. General discussion

Experiment 1 demonstrated the US devaluation effect with a short and restricted access training procedure (10 days per each CS solution). Rats showed a reduction in the sucrose paired CS+ preference over the unpaired CS- when they had been previously pre-fed with a sucrose solution. Experiment 2 replicated those findings with a short and unrestrictive training procedure (3–6 hour exposure to each CS). In Experiment 3 we extended the length of the training procedure by doubling the number of sessions given in Experiment 1. We found that the US-devaluation effect disappeared, since unlike the previous experiments, the rats still preferred the CS+ over the CS- after pre-feeding with sucrose.

The results from Experiments 1 and 2 appear to be consistent with those of previous studies, which found that the acquisition of a conditioned preferences is mediated by the representation of the US after a short training procedure (Stimulus-Stimulus) (e.g., Delamater et al., 2006; Delamater, 2007; 2011; Dwyer, 2005; Harris et al., 2004; Holmes et al., 2016). As the US is sucrose, the content of the representation that is activated could be motivational (Flavor-Nutrient) and/or sensory (Flavor-Flavor) in nature. Although we can not completely rule out that both associations mediate preference, it seems more plausible that a sweet-based association is governing preference. Firstly, the fact that animals reduced their preference when sweetness had been devalued seems to indicate that at least part of the conditioned response is driven by sweetness. Moreover, during the training and testing procedure the animals were not food-deprived, which may had hindered the possibility to form or express associations based on caloric consequences (Harris et al., 2000).

On Experiment 3, when animals were trained with an extended unrestricted access procedure, the pattern of intake during the test changed. Rats still expressed a conditioned preference even though sucrose had been devalued, suggesting that the CS+ preference was not sensitive to a US-devaluation procedure. Thus, the presentation of the CS may automatically elicit a conditioned response regardless of the current state of the US (Stimulus-Response). Consequently, the present results support the associative learning view that overtraining can lead to S-R learning in flavor preference learning.

Nevertheless, approaches to studying S-R learning with Pavlovian cues have rarely succeeded with some exceptions (e.g., Holland, 1981; Holland and Rescorla, 1975; Pool et al., 2019; Watson et al., 2022). In fact, as discussed in the introduction, some experiments focused on studying overtraining effects in S-S/S-R dissociation showed no change in the sensitivity to the devaluation procedures (e.g., Holland, 2005). To our knowledge, this is the first attempt to study the effect of different training regimes to assess the role of S-R learning in the acquisition of flavor preferences. In this vein, flavor preference learning has been described as a special case of Pavlovian learning (Delamater, 2012), especially because of the difficulties in producing extinction of acquired preferences (but see Hall, 2022). This has given rise to explanations of this phenomenon based on approaches that differ from the classical associative learning theory such as evaluative conditioning (e.g., De Houwer et al., 2001) or configurational learning (e.g., Pearce, 2002). So, based on these experiments, it is possible that the effects of overtraining on devaluation may also represent a unique feature of this paradigm compared to other Pavlovian learning procedures.

In this respect, the notion that Pavlovian learning might be subject to the effects of an S-R link has been largely abandoned since the advent of the more cognitive models of associative learning and the realization that associative learning arises from complex associations rather than solely between stimuli and responses. So, the dissociation of stimulus-stimulus (S-S) and stimulus-response (S-R) theories of behavior has mostly focused on instrumental, rather than Pavlovian learning. However, recently, Thrailkill et al., (2018) (see also Bouton et al., 2020; Bouton, 2021) applied Pearce and Hall's (1980) model, which was developed in a Pavlovian learning context, to instrumental learning and habit formation (S-R). According to this theory, as the CS becomes a good predictor of a US, attention to the CS declines, along with its salience and associability. This is because our ability to process stimuli is limited, and therefore, when a CS perfectly predicts its consequences, we process it automatically. Thraikill et al. (2018) propose that through this process, the conditioned response may also be elicited in an automated fashion when the CS is present. These authors draw parallels with operant learning, establishing that during training, when a particular discrete stimulus and an instrumental response become highly predictable from the reinforcer (S-R-O), a similar process can occur, encouraging the development of a behavioral habit (S-R without O processing). From this perspective, if an individuals' attention to a predictive stimulus and an instrumental response decrease, the behavior will be automatically triggered when a stimulus sets the occasion. Thus, extending this interpretation to the present results, a tentative explanation based on Pearce and Hall's model (1980) emerges. It is possible that during the training procedure, when rats are exposed for long periods to the CS and the US, the CS reaches an asymptotic level of learning. Therefore, no more can be learned about it, leading to a decrease in attention to this cue. Thus, the CS might be expected to produce an automatic conditioned response irrespective of the representation of the US. To test this, future studies should aim to manipulate the magnitude of the predictive relationship between the CS and US by partially pairing the two events during training. If the preference produced by this training procedure is sensitive to the devaluation procedure, this would constitute further evidence for this hypothesis.

In the literature, flavor preference learning has been suggested as one of the factors involved in overeating by determining food likes and dislikes, food choices and total intake in today's environments (Yeomans, 2012). Most today's societies are featured by the omnipresence of the obesogenic environments that expose us to a wide variety of foods that share very similar sensory properties but have different calorific content. Extensive exposure to these inconsistent predictive sensory cues could affect Flavor-Nutrient learning, a process that regulates food intake. For example, a study by Hardman et al. (2015) showed that the number of varieties of pizzas of a given flavor (pepperoni) available in UK supermarkets totaled 71 different units. Further, in this study, the authors revealed that among these 71 different pizzas brands, which probably taste very similar but differ in certain sensory attributes, the variability in calories ranged from 500 to 2000 kcals on a standard size pizza, depending on the brand. Concerning this, it has been argued that individuals may lose the ability to anticipate the ideal portions of each food based on its nutritional properties (conditioned satiety) due to a continuous inconsistency between sensory cues and caloric load. This inconsistency has been suggested as a major problem for intake regulation by hindering Flavor-Nutrient learning and leading to overconsumption (For a review, see Martin, 2016; Yeomans, 2012). On the other hand, others have argued that this exposure to sensory variety could generate an increase in discrimination between sensory cues, leading to more effective Flavor-Nutrient learning (Palframan and Myers, 2016) that may finally result in overeating, for example, through the disruption of Sensory-Specific Satiety (González et al., 2018). Another consequence of massive exposure to high-palatable foods could be the formation of S-R associations between flavor and hedonic reactions to food such as those observed in our study. In this sense, the development of a preference for a CS+ insensitive to a US revaluation process could encourage excessive food intake such as that shown in hedonic hunger (eating without a real physiological need). In this regard, the present study has explored whether it is possible for S-R learning to occur in flavor preference learning by manipulating different parameters of the experimental procedure, such as the amount of access to the CS-US compound or the length of training. And in fact, we have observed that the length of training and the amount of exposure to the

CS-US compound is important. It should be noted that the type of exposure that has been found to trigger S-R learning (Experiment 3) is characterized by unrestricted access for many hours to the CS-US compound, which could hinder its translation to real-life settings—particularly if we consider that humans, as omnivores, have limited eating or drinking periods throughout the day. Nevertheless, it remains a possibility that with an extended but not massive training, in a way that is more comparable to real life, S-R learning may eventually occur. Although this possibility has not been addressed in these experiments, it could be explored in the future, given its implications.

We should consider several limitations of this study. First, in the present experiment, only one taste — sucrose — was used as the US. Specifically, sucrose is characterized by its highly hedonic taste but is also a source of calories. Future research should study whether, under the same parameters used in these experiments, other USs of different characteristics are sensitive to the manipulations used in this study. Considering this, our experimental design used water as the control condition for Sensory Specific Satiety devaluation. A more complete design should include another group of rats in which another nutrient is used as the US (i.e.: maltodextrin) and sucrose is the control substance during pre-feeding. By doing this, we could address the effects of different USs with different reinforcing properties on the manipulations used here. A final caveat of the present study is the fact that the inferences made here are based on a comparison between the results of two separate experiments. These conclusions will be strengthened with a between-subjects design in which two different groups are subjected to different training regimes.

Finally, only relative preference and direct consumption were analyzed as a principal measure. Assessing the pattern of consumption of animals gives an indirect assessment of hedonics and can be inexact being altered by many factors (Berridge, 1996; Riordan and Dwyer, 2019). To assess hedonic reactions, more precise measures should be employed such as analyzing the pattern of hedonic reactions or licking microstructure analysis. In this vein Delamater (2012) argues the difficulty that entails to determine which type of response is governing S-R performance after a devaluation procedure (S - hedonic R vs S - motivational R associations) (See Hall, 2002; for a similar appreciation). Keeping this in mind, we have assumed that the response that supports the preference is hedonic, but it is still conceivable that the effect found in the present study may be governed by other types of responses that can be elicited by the US such as those motivational. However, it is also worth noting that throughout the procedure animals were provided with free food, which reduces the likelihood that the association being generated is one based on a response originating from a state of arousal elicited by the calories of the nutrient.

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CRediT authorship contribution statement

Isabel De Brugada: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Jesús Sanchez:** Writing – original draft, Methodology, Investigation. **Ana González:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declared no potential conflicts of interest concerning the research, authorship, or publication of this article.

Data availability

I have share the link to the data in the manuscript

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