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Effects of food deprivation on conditioned orthonasal olfactory preferences with caloric and non-caloric reinforcers

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ABSTRACT

Three experiments were conducted to investigate Conditioned Olfactory Preferences using orthonasal inhalation, which is a less explored perceptual pathway compared to retronasal inhalation. In these experiments, odors were impregnated onto plastic disks to prevent the subjects from consuming or tasting them. The reinforcers used were a sucrose solution (Caloric groups) and a saccharin solution (Non-Caloric groups). The influence of nutritional deprivation was analyzed, with unrestricted access to food throughout the procedure in Experiment 1, food restriction during the conditioning phase in Experiment 2, and limited access to food during the test phase in Experiment 3. The results revealed conditioned preferences using both sucrose and saccharin as reinforcers. Furthermore, dietary restriction reduced the conditioned preference induced by saccharin, but not the preference induced by sucrose. These findings are discussed in light of the potential differences between orthonasal and retronasal presentation of odors during conditioning.

1. Introduction

From an evolutionary perspective, the ability of organisms to acquire eating habits based on the safe, beneficial or dangerous properties of novel food is a characteristic with a relevant value for survival (Rozin, 1976, 1977). This learning has features that allow the organism a correct adaption to environmental conditions when competing for food resources, either with members of their own species (intraspecific competition) or with individuals belonging to other species (interspecific competition). From a functional approach, the efficacy in the selection of nutritious food together with the ability to regulate consumption significantly increases the survival opportunities in environments where food is often scarce (Overmann, 1976; Palmerino, 1981).

In ordinary situations, an omnivorous animal such as the rat (Rattus norvegicus) can have access to new substances, and it is common in this situation to taste them in reduced quantities, a phenomenon known as *neophobia* (Rzóska, 1953; Barnett, 1956; Carroll et al., 1975). If the consumption of a new food is not followed by harmful consequences, there will occur a progressive increase in intake, a process named *habituation to neophobia* (Siegel, 1974; Domjan, 1976). The mechanisms that underlie habituation or attenuation of the neophobic response have been widely discussed (see Domjan, 2018), and their dependence on associative processes continues to be a subject of analysis (Kalat and Rozin, 1973; De la Casa and Díaz, 2013).

Eventually, the consumption of a fluid or food may be followed by aversive consequences, such as discomfort, abdominal pain, diarrhea, etc., and on these occasions an aversive conditioning will develop on the basis of gustatory and olfactory perception (Garcia et al., 1989). Conditioned aversions to either taste or odor have been extensively analyzed (Garcia et al., 1955; Garcia and Koelling, 1966; Garcia et al., 1966; see also, Bures et al., 1998), and their basic principles, as well as the mechanisms and structures that support them, are well established (Garcia, 1990; Bernstein, 1999; Welzl et al., 2001; Bermúdez-Rattoni, 2004; for a review see, Reilly and Schachtman, 2009).

However, the nature of food learning is not always defensive, since the formation of conditioned associations also plays a relevant role when foods are followed by appetitive or healthful consequences (Rozin, 1976; Booth, 1985; Sclafani, 1991). The selection of food based on positive reinforcement has been explained through two mechanisms: a nutritional factor, and a hedonic experience (Capaldi, 1992). In *flavor-nutrient* (or *flavor-calorie*) learning, an association is established between a novel flavor and a substance with nutritional or caloric properties (Mehiel and Bolles, 1984; Sclafani, 1990). On the other hand, *flavor-flavor* (or *flavor-taste*) learning occurs by associating a novel flavor and a substance that does not have nutritional properties, but does have

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a hedonic value (Holman, 1975; Fanselow and Birk, 1982). In addition to the caloric and/or hedonic value of the food consumed, other processes and mechanisms involved in intake can induce an increase in consumption, such as those that are activated when supplying a deficit present in a sick or malnourished organism (Zahorik et al., 1974), or those related to recovery from a state of malaise induced by drugs (Green and Garcia, 1971).

The association of any of these consequences with the aroma and/or savor of food is what has been described in the scientific literature as *conditioned flavor preferences* (Sclafani, 1995; Birch, 1999). Both defensive and appetitive learning in food selection have often been approached from a Pavlovian perspective, albeit with specific properties (Garcia et al., 1989; Booth, 1990). Thus, initially neutral stimuli such as tastes, odors, or a mixture of both (*flavors*) will acquire the properties of a conditioned stimulus (CS) when followed by appetitive or aversive post-ingestive consequences (the unconditioned stimulus or US). This process, or set of processes, is possibly complemented by instrumental factors derived for the value of food as a reinforcing stimulus (e.g, Capaldi and Sheffer, 1992; Capaldi, 1996). Consequently, conditioned flavor preferences integrate an extensive and complex learning system related to the effects that the consumption of alimentary substances has on any organism, including both congenital, physiological and learned aspects (Birch, 1999; Sclafani, 2001; Yeomans, 2006).

Just like in the case of conditioned taste aversion (e.g., Garcia et al., 1989; Freeman et al., 2009), conditioned preferences possess a set of unique characteristics compared to other learning paradigms, such as the ability to be established after a reduced number of associations (Díaz et al., 2004). Furthermore, conditioned preferences can be established despite introducing long delays between flavors and post-ingestive nutritional consequences (Capaldi and Sheffer, 1992) and have demonstrated high resistance to extinction (Albertella and Boakes, 2006; Delamater, 2012), particularly when conditioning is based on a hedonic factor (Harris et al., 2004; Díaz and De la Casa, 2011). Additionally, it has been shown that the motivational state of individuals can partially determine the extent to which associations based on hedonic or nutritional properties are acquired and expressed (Harris et al., 2004; González et al., 2010).

Alongside taste, smell is a multifunctional discriminative cue that, in the natural environment, can facilitate the search for and identification of prey, edible substances, or spoiled food, and can modulate avoidance or preference based on the consequences its ingestion generates in the organism (Rozin, 1982; Bartoshuk, 1990). Several studies support the establishment of odor-conditioned preferences, both when presented alone and in combination with sapid stimuli (Capaldi and Hunter, 1994; Lucas and Sclafani, 1995). Specifically, when inducing preferences for olfactory cues in combination with taste stimuli, two procedures are commonly employed in the literature: the dissolution of aromas and substances (such as saccharin or sugar) in the same fluid, known as flavors (Boakes et al., 2007), or the presentation of a scent impregnated on a disk attached to the bottle containing the gustatory properties (Holder, 1991).

In the first procedure, which is commonly employed in researching these topics, odors diluted in a flavored fluid stimulate both gustatory cells and olfactory receptors through a retronasal route. Using this method, numerous learning phenomena have been replicated, including the acquisition of associations based on caloric content (Capaldi and Hunter, 1994; Boakes et al., 2007), hedonic flavor-flavor associations (Fanselow and Birk, 1982), conditioned avoidance (Boakes et al., 2012), sensory preconditioning (Lyn and Capaldi, 1994), latent inhibition (De la Casa et al., 2009; García-Burgos et al., 2013), the US-preexposure effect (Gil et al., 2014, 2021), and odor-taste and taste-odor potentiation effects (Capaldi and Privitera, 2008).

The second technique involves presenting odors through the orthonasal pathway, where the uptake of odorous molecules directly stimulates the olfactory bulb without reaching the taste receptors on the tongue. Using this procedure, evidence of associations between odors and nutritional consequences has been obtained even in the absence of taste stimulus cues (Lucas and Sclafani, 1995). Additionally, this method has shown the blocking of taste by odor and odor by taste (Holder, 1991). Furthermore, preferences conditioned to orthonasal odors have been found to persist even after a month has passed between conditioning and testing, indicating an associative process capable of establishing long-term memory traces (Torquet et al., 2014).

However, regardless of whether odors are presented mixed with flavors or separately, one of the main areas of interest has been to analyze whether the nutritional or hedonic properties of the reinforcer can determine changes in the characteristics of acquired learning (Capaldi, 1996; Myers and Sclafani, 2006). To understand the importance of flavor-nutrient and flavor-flavor connections in the development of flavor preferences, researchers have attempted to develop techniques and procedures that allow each association to be analyzed independently. Some of these procedures include the use of artificial sweeteners without caloric content (Holman, 1975, 1980), the taste reactivity test (Grill and Norgren, 1978), intragastric infusion of substances with caloric or nutritional value (Sclafani et al., 1990), or methods for devaluate stimuli (Dwyer, 2005). In this regard, water and/or food restrictions have been a common resource. The modulation by the subject's motivational state has been a relevant factor for both the acquisition and expression of conditioned flavor preferences (Harris et al., 2000; González et al., 2010), the expression of latent inhibition (García-Burgos et al., 2013), and the extinction of preferences conditioned by the sensory or nutritional aspects of flavors (Delamater, 2007).

There have been only a few attempts to analyze the motivational modulation of conditioned preferences using a retronasal flavor procedure. For instance, Fedorchak and Bolles (1987) found that deprivation during testing only increased preferences that were mediated by caloric reinforcement (such as ethanol or sucrose) and, conversely, did not enhance those that were developed through sweet but non-caloric reinforcement (like saccharin). On the other hand, Capaldi et al. (1994) demonstrated that conditioned preferences after pairing an odor with sucrose were expressed more strongly if food restriction was implemented during the acquisition phase. This result did not occur when the reinforcer was saccharin. Finally, Harris et al. (2000) reported that preexposure to sucrose reduced the level of conditioned preferences for an odor reinforced with the same substance, regardless of the level of food deprivation of the animals throughout the procedure. However, preexposure to saccharin reduced the expression of conditioned preferences for sucrose when the rats were allowed ad libitum access to food during both conditioning or testing phases, but not when they were food deprived in both phases.

These results led Harris et al. (2000) to propose that during the acquisition phase, hungry rats develop both an odor-taste association and an odor-calorie association, whereas satiated rats would only develop the odor-taste association. Therefore, hungry rats during conditioning should respond differently depending on food restriction at testing: hungry rats at testing would be more sensitive to the odor-calorie association, but satiated rats at testing would attend to the odor-taste association. Conversely, rats that were satiated during conditioning would only respond to the odor-taste association during testing, regardless of the level of deprivation at this stage. However, more recent studies have found evidence that rats that were satiated during conditioning could form both types of associations (e.g., with sucrose), although food deprivation would be necessary for calorie-mediated learning expression at testing (García-Burgos et al., 2013; González et al., 2010, 2015).

In order to analyze potential differences and similarities between preferences conditioned to orthonasal and retronasal odors we conducted three experiments. We utilized the experimental protocol employed by Torquet et al. (2014) to obtain conditioned preferences using orthonasal odors and nutritional reinforcement. Our focus was on analyzing the relevance of the nutritional versus hedonic properties of the reinforcer (sucrose or saccharin) in the establishment of conditioned

preferences to orthonasal odors. Additionally, we aimed to determine the extent to which the state of nutritional deprivation during the conditioning or the test phase could influence the acquisition or expression of this phenomenon with distal odors. To achieve these objectives, we carried out three experiments. In Experiment 1, one group of rats was repeatedly presented with two odors (banana and peppermint), with one odor (odor A+) paired with a sucrose solution and the other odor (odor B-) paired with water. Another group received the same odors but paired with saccharin or water. All animals were water-deprived throughout the experiment but had unrestricted access to food. In Experiment 2, we employed the same procedure as in Experiment 1, but in addition to water deprivation, the rats were food-deprived during the conditioning phase. Finally, in Experiment 3, the animals were food-deprived during the test phase.

Based on the reported findings, we expected to observe odor preferences regardless of the reinforcer for those animals without food restrictions, since the hedonic or sensory factor would gain more relevance in preferences (Exp. 1). Conversely, in Experiment 2, where food deprivation was implemented only during the conditioning phase, we anticipated higher levels of preferences in those animals reinforced with sucrose as compared to those reinforced with saccharin, since the nutritional properties of sucrose would gain relevance for hungry animals. Finally, if the expression of preferences depends on the motivational state during the test phase, we expect that deprived animals at testing would exhibit more preference for the odor associated with the nutritional component (Exp. 3).

2. Experiment 1

The main objective of this experiment was to induce odor conditioned preferences through the orthonasal pathway using both nutritional-caloric (sucrose) and non-caloric (saccharin) reinforcement. In this experiment, the animals had limited access to water but unrestricted access to food throughout the entire duration of the study. Given the conditions of caloric satiety maintained throughout the procedure, we anticipated that the impact of the hedonic factor would be more pronounced. Therefore, we expected the acquisition and expression of learning to be relatively similar for both substances (sucrose and saccharin), as reported in previous studies (e.g., Harris et al., 2000).

2.1. Materials and methods

2.1.1. Subjects

16 male Wistar rats (n = 8), participated in the first experiment. The rats had previously participated in an experiment evaluating startle responses, but they did not have experience with any of the stimuli or contexts used in this experiment. The mean weight at the start of the experiment was 473 g. (Range 385 – 613 g), and for this experiment as well as the subsequent ones, the rats were matched in terms of weight to form the groups. The animals were housed in groups of 2 in type IIIH plexiglas cages, with wood shavings as bedding and other materials available in the cages (pieces of fabric, cardboard and wood, stones, etc). The vivarium was maintained on a 12:12 h light-dark cycle (lights on at 07:00 h), and all behavioral testing was conducted during the light period of the cycle. The animals had free access to food across the entire duration of the experiment. Seven days before to initiate the experimental treatment all animals were placed on a water deprivation schedule (with 45 additional min/day access to water after the experimental treatment) that was maintained across the entire duration of the experiment. All experimental procedures were approved by the Ethics Committee for Animal Research, University of Seville (Protocol CEEA-US2020–15/2), and were conducted in accordance with the guidelines established by the EU Directive 2010/63/EU for animal experiments, and the Spanish R.D. 223/1988.

2.1.2. Apparatus and stimuli

All experimental sessions were conducted in eight Plexiglas cages, 425 × 266 x 185 cm., located in an experimental room, different to the vivarium, illuminated by a single 54-W fluorescent white light on the ceiling. In each experimental cage, a metal grid was placed in the area through which the mouthpieces of the bottles with tastes and odors were inserted. Thus, the animals could not contact the paper filters that were impregnated with the odors. Two odors were used in the experiment: banana aroma and peppermint aroma (counterbalanced), from a powder composition (Dallant, S.A.) that was diluted in tap water (10% w/v). The tastes used were saccharin (0.35% w/v) and sucrose (3.5% w/v) diluted in water. The saccharin or sucrose solution was presented in 200 ml glass bottles equipped with a sipper tube. A 3 cm (Ø) disk of porous paper was impregnated with the corresponding odor (0.15 ml) and inserted into a plastic disk measuring 3 cm (Ø) x 2 cm (h), which surrounded the bottle sipper tube. Fig. 1 shows a picture of one of the bottles used in the experiments.

During the experimental sessions access was allowed to two bottles containing water with their respective tastes and odors depending of the experimental treatment. All fluids were provided at room temperature. The amount of fluid intake was measured by calculating the difference between the weight of the bottle before and after fluid presentations.

2.1.3. Procedure

After 7 days of water restriction, the experiment started with five days of context habituation. On each of these days the animals were introduced in the cages located in the experimental room where they remained for 15-min with access to water in the same bottles that would be used in the test stage. Immediately after each session, for this and the remaining experimental stages, the animals had access to an additional 45 min period of water, until completing one-hour of daily water access. On days 6–7 two additional 15-min sessions with access to water were conducted, but in this case the disk of one bottle was impregnated with the banana odor and the other with peppermint. On days 8–9 two additional habituation sessions were conducted with one bottle containing sucrose + banana and the other sucrose + peppermint for the Caloric group, or saccharin + banana and saccharin + peppermint, for the Non-caloric group, respectively. These sessions were introduced to check for potential interactions between flavors and tastes that could differentially affect fluid intake. The positions of the bottles were counterbalanced each day.

Fig. 1. Detail of the bottle used in the experiments: A disk of porous paper was impregnated with 0.15 ml of the corresponding odor solution and inserted into the round cap, which was attached to the bottle. The bottles were inserted through the grid of the experimental cages in such a way that the animals could drink from the bottle spout but could not lick the disk.

The odor conditioning stage took place from days 10–17. Each day a 15-min trial was conducted with one odor consistently presented with water+sucrose (Caloric group) or with water+saccharin (Non-caloric group), and the alternative odor paired with plain water. For half of the animals, the conditioned odor (scent A) was banana and the neutral odor (scent B) was peppermint, while for the other half the relationship was reversed. As in the pre-test sessions, the position of the bottles changed every day.

The day following the conditioning stage, animals were submitted to the test phase that comprised two trials (days 18–19). For this stage, the animals had access to two bottles containing water, one with a disk containing the reinforced odor, and the other with the non-reinforced odor. As in the previous phase, the position of each bottle was changed across days for each subject.

2.2. Results

Analysis of consumption during pretest sessions. During the last 2 days of the adaptation phase to the experimental context, the animals consumed an average of 17.5 ml (SD = 4.43) from both bottles. For those pre-test sessions with the animals drinking water in presence of the odors, mean consumption from the bottle with the banana and the mint odor was 8.49 ml (SD = 5.05) and 8.53 ml (SD = 7.18), respectively, a difference that was non-significant, $t(14) = .79$, $p > 0.4$.

Regarding pre-test sessions with sweet tastes + odors, a 2 × 2 ANOVA (Odor x Taste) conducted on mean intake revealed a significant main effect of Taste, $F(1,12) = 72.41$, $p < 0.001$, $\eta^2 = 0.86$, with the animals drinking more fluid containing sucrose than saccharin (Mean = 37.39 ml, SD = 7.98 and Mean = 9.69 ml, SD = 4.38, respectively). Thus, while the addition of sucrose to odors increased consumption, saccharin produced a neophobic response. Neither the main effect of Odor nor the interaction was significant, Fs (1,12)< 1.

Analysis of consumption during conditioning phase. Fig. 2 depicts mean consumption across trials for the A+ and B- odors as a function of Taste (Caloric vs. Non-caloric). As can be seen in the figure, consumption in the odor-reinforced bottle (A+) was greater than in the unreinforced odor bottle (B-), and more specifically, the consumption of A+ when the bottle contained sucrose was greater than when it contained saccharin.

A 8 × 2 × 2 mixed ANOVA (Trials x Odor: A+ vs. B- x Taste: Caloric vs. Non-caloric), revealed significant main effects of Odor and Taste, $F(1,14) = 82.8$, $p < 0.001$, $\eta^2 = 0.86$, and $F(1,14) = 76.88$, $p < 0.001$, $\eta^2 = 0.85$, respectively. The main effect of Odor was due to the higher consumption of A+ as compared to B- (Mean = 13.25 ml, SD = 7.93, and Mean = 2.23 ml, SD = 1.62, respectively). The main effect of Taste reflects the higher overall consumption of the Caloric group compared to the Non-caloric group, (Mean = 21.92 ml, SD = 3.89, and Mean = 9.04 ml, SD = 1.45, respectively).

The Odor x Taste interaction was also significant, $F(1.14) = 36.38$, $p < 0.001$, $\eta^2 = 0.72$, reflecting higher consumption of A+ when it was paired with sucrose as compared when it was paired with saccharin (Mean = 20.12 ml, SD = 4.76, and Mean = 6.38, SD = 2.07, respectively), and the absence of differences of B- consumption. No more main effects or interactions were significant (all ps>0.1).

Analysis of consumption during test phase. To analyze fluid consumption in presence of each odor during the test period the analysis was performed on total amount consumed in the two days. Fig. 3 represents mean consumption in each bottle (containing only water), during the two days of testing, as a function of the odor associated with the sweet solutions or water during conditioning (A+ associated with sweet taste, sucrose or saccharin, vs. B-, associated with water), and the properties of the sweet solution during conditioning (Caloric: sucrose vs. Non-caloric: saccharin). As can be seen in the figure, both the Caloric and the Non-caloric group consumed significantly more water from the bottle with

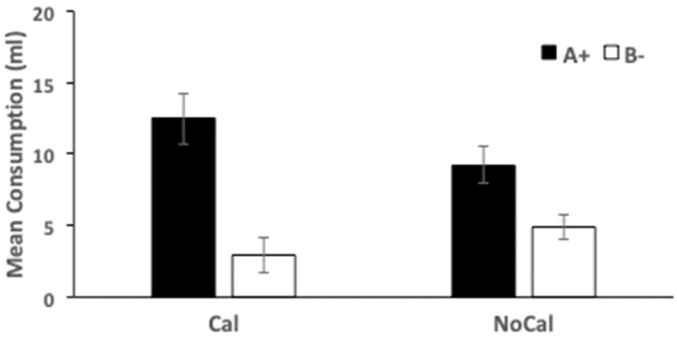

Fig. 3. Mean consumption of water (ml.) as a function of odor (A+ vs. B-), and US (Saccharin vs. Sucrose). A+ : Odor paired with sucrose (Cal Group), or saccharin (NoCal Group). B-: Odor paired with Water. The animals had free access to food during the entire duration of the experiment. Error bars show SEM.

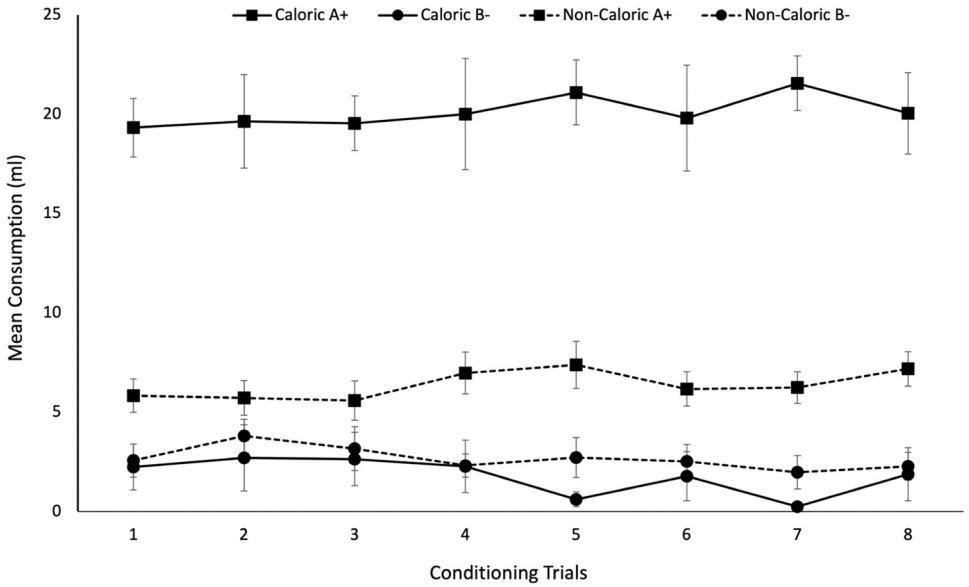

Fig. 2. Mean consumption of water (ml.) as a function of odor (A+ vs. B-), and US (Non-Caloric: Saccharin vs. Caloric: Sucrose) across conditioning Trials. A+ : Odor paired with sucrose (Cal Group), or saccharin (NoCal Group). B-: Odor paired with Water. Error bars show SEM.

odor A+ that from bottle with odor B-.

A 2 × 2 ANOVA with main factors Odor (A+ vs. B-) x Taste (Caloric vs. Non-Caloric) revealed a significant main effect of Odor, $F(1,14)= 25.72$, $p < 0.001$, $\eta^2 = 0.66$, due to the higher consumption of water in those bottles with the A+ odor as compared to those with the B- odor, reflecting on overall preference for the odor paired with the sweet fluid irrespective of its caloric value. Neither the main effect of Taste nor the 2-way interaction was significant, $F(1,14)< 1$, and $F(1,14)= 3.53$, $p > 0.08$, $\eta^2 = 0.2$, respectively. Therefore, as we expected, odor preference conditioning appeared irrespective of the US presented with the aroma during conditioning (sucrose or saccharin).

In order to check potential differences that could have been masked for the analyses of absolute drinking level, we calculated preference ratios using the formula A+ / (A+ + B-). The mean preference ratios for the bottle with A+ odor as a function of taste appear in the upper section of Table 1. A one-way ANOVA conducted on mean preference ratio as a function of taste (Caloric vs. Non-caloric) revealed the absence of significant differences, $F(1,14)= 3.89$; $p >0$ .06, $\eta^2 = 0.21$.

3. Experiment 2

In this experiment, the animals were food-deprived during the conditioning stage, but had unrestricted access to food during the test phase. We hypothesized that food deprivation during the acquisition phase would enhance the influence of nutritional reinforcement and diminish the impact of hedonic reinforcement on conditioning, as indicated by previous studies (Capaldi et al., 1990; Capaldi et al., 1994). This increased significance of the nutritional component was expected to lead to a higher level of preference for the conditioned odor in the Caloric Group (sucrose) compared to the Non-caloric Group (saccharin).

3.1. Materials and methods

3.1.1. Subjects

16 male naïve Wistar rats (n = 8) participated in this experiment with a mean weight at the beginning of the experiment was 313 g (Range 270–382 g). Housing and maintenance conditions were similar to that described for Experiment 1, except for all the animals had limited access to food during the conditioning phase. Food deprivation, consisting in allowing access to food 45 min daily after the experimental treatment, began the day before conditioning, and was maintained throughout the conditioning stage.

3.1.2. Apparatus, stimuli, and procedure

All materials and procedures were the same as described for Experiment 1, except for the food-restriction regime that was implemented before conditioning stage.

Table 1
Mean preference ratios for the bottle with A+ odor [volume of A+/ (volume of A+ + volume of B-)] in the two-bottle tests during the testing stage as a function of Groups (Caloric vs. Non-caloric) for each Experiment.

Experiment 1	
Group	**Preference ratio A+**
Caloric	0.83 (SD =.17)
Non-Caloric	0.64 (SD =.21)
Experiment 2	
Group	**Preference ratio A+**
Caloric	0.73 (SD =.23)
Non-Caloric	0.55 (SD =.26)
Experiment 3	
Group	**Preference ratio A+**
Caloric	0.82 (SD =.20)
Non-Caloric	0.54 (SD =.26)

3.2. Results

3.2.1. Analysis of consumption during pretest sessions

During the initial phase of adaptation to odors, mean consumption of water from the bottle with the banana and mint odors during pre-test sessions was 11.13 ml (SD = 5.96) and 8.25 ml (SD = 5.10), respectively, a non-significant difference. $t(14) = .42$; $p >0$ .4. In the sessions of adaptation to sweetened substances (taste + odors), a 2 x 2 ANOVA (Odor x Taste) conducted on mean intake revealed a significant main effect of Taste, $F(1,12)= 148.51$, $p < 0.001$, $\eta^2 = 0.93$, due to the higher consumption for the sucrose as compared to the saccharin fluid (Mean = 29.94 ml, SD = 4.52 and Mean = 8.29 ml, SD = 1.46, respectively). Neither the main effect of Odor nor the interaction was significant, Fs $(1,12)< 1$.

3.2.2. Analysis of consumption during conditioning phase

Fig. 4 depicts mean consumption across trials for the A+ and B- odors as a function of Taste (Caloric vs. Non-caloric). As can be seen in the figure there were a reduction in consumption of the bottle with the A+ odor containing sucrose (Caloric A+) in the fifth conditioning trial. In addition a general reduction in consumption was observed as compared to Experiment 1, attributable to the food deprivation state implemented in this stage. However, and similar to that observed in Experiment 1, consumption in the odor-reinforced bottle (A+) was higher than in the unreinforced odor bottle (B-), and consumption of A+ when the bottle contained sucrose was greater than when it contained saccharin.

A 8 × 2 × 2 ANOVA (Trials x Odor: A+ vs. B- x Taste: Caloric vs. Non-caloric), revealed significant main effects of Odor and Taste, $F(1,14)= 334.61$, $p < 0.001$, $\eta^2 = 0.96$, and $F(1,14)= 70.56$, $p < 0.001$, $\eta^2 = 0.83$, respectively. The main effect of Odor was due to a higher consumption in presence of odor A+ (sucrose or saccharin) as compared to odor B- (Mean = 9.10 ml, SD = 5.11, and Mean = 0.98 ml, SD = 0.44, respectively). The main effect of Taste reflects higher consumption for those rats in the Caloric group (sucrose) as compared to the Non-caloric group (saccharin), (Mean = 14.55 ml, SD = 2.48, and Mean = 5.60 ml, SD = 1.72, respectively). The main effect of Trials was also significant, $F(7,98)= 4.76$, $p < 0.001$, $\eta^2 = 0.25$, due to an overall increase in consumption across conditioning trials.

All 2-way interactions were significant, Fs> 2.53, ps<0 .05, $\eta^2 > .15$. The Trials x Odor interaction was due to a high and incremental consumption across trials from those bottles with the A+ odor (sucrose or saccharin), and a low and steady consumption of the fluid associated with B-. The Trials x Taste interaction was due to an increase in consumption across trials that was higher for sucrose than for saccharin. Finally, the Odor x Taste interaction reflects higher consumption of sucrose than saccharin from bottle with A+ , and similar consumption of water from bottle with B-. The 3-way interaction was non-significant, $F(7,98)= 2.05$, $p > 0.05$, $\eta^2 = 0.13$.

Analysis of consumption during test phase. Fig. 5 represents mean consumption at testing, as a function of odor (A+, associated with sweet taste, sucrose or saccharin, vs. B-, associated with water), and the properties of the substance used as a reinforcer for that group of animals (Taste: Caloric vs. Non-caloric). As can be seen in the figure, when food deprivation occurred during conditioning, but free feeding was possible during testing, the Caloric group consumed more water from the bottle with scent A+ than from the bottle with scent B-, however, the Non-caloric group showed a similar consumption of both bottles.

A 2 × 2 ANOVA with main factors Odor (A+ vs. B-) and Taste during conditioning (saccharin vs. sucrose) revealed a significant main effect of Odor, $F(1,14)= 4.93$, $p < 0.05$, $\eta^2 = 0.26$, due to the higher consumption of water in those bottles with the A+ odor as compared to those with the B- odor (Mean = 12.72 ml, SD = 6.26, and Mean = 7.06 ml, SD = 5.28, respectively). Neither the main effect of Taste nor the 2-way interaction was significant, $F(1,14)< 1$, and $F(1,14)= 2.18$, $p > 0.1$, $\eta^2 = 0.14$, respectively.

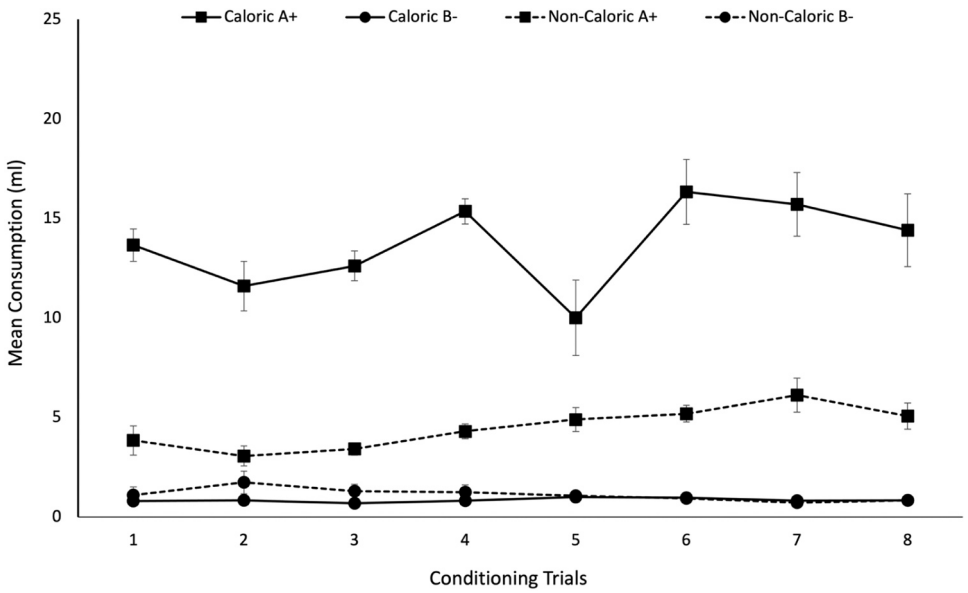

Fig. 4. Mean consumption of water (ml.) as a function of odor (A+ vs. B-), and US (Non-Caloric: Saccharin vs. Caloric: Sucrose) across conditioning Trials. A+ : Odor paired with sucrose (Cal Group), or saccharin (NoCal Group). B-: Odor paired with Water. Error bars show SEM.

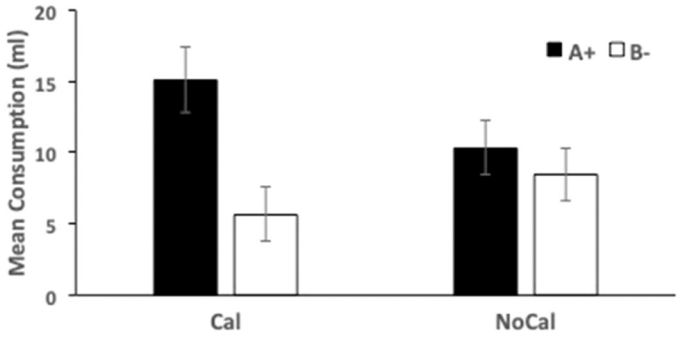

Fig. 5. Mean consumption of water (ml.) as a function of odor (A+ vs. B-), and US (Saccharin vs. Sucrose). A+ : Odor paired with sucrose (Cal Group), or saccharin (NoCal Group). B-: Odor paired with Water. The animals were food-deprived during conditioning and had free access to food at testing. Error bars show SEM.

In spite of the lack of interaction, an inspection of Fig. suggests a higher consumption of fluid in bottle with odor A+ as compared to bottle with odor B- for those animals reinforced with sucrose indicating a differential effect of A+ vs. B- across groups. Considering the relatively high effect size of the interaction it can be concluded that the experiment was underpowered to detect such a difference due to the small sample size.

As described for Experiment 1, we calculated preference ratios for the A+ odor (mean preference ratios for the bottle with A+ odor as a function of taste appear in the middle section of Table 1. A one-way ANOVA comparing mean preference ratios as a function of taste (Caloric vs. Non-caloric) revealed the absence of significant differences, $F(1,14)= 2.26$; $p >0 .13$, $\eta^2 = 0.13$.

4. Experiment 3

In this experiment the animals were submitted to the food restriction schedule at testing. We expected that under conditions of food restriction at test stage, caloric-based conditioning would be more intense than non caloric-based conditioning (see for instance, Fedorchak and Bolles, 1987). This prediction assumes that during the conditioning phase, when the animals are in a state of nutritional satiety, they are capable of acquiring both an odor-taste association and an odor-calorie association if the odor is reinforced with a sweet and caloric substance such as sucrose (García-Burgos et al., 2013; González et al., 2010, 2015).

4.1. Materials and methods

4.1.1. Subjects

16 male Wistar rats (n = 8) participated in this experiment with a mean weight at the start of the experiment of 479 g. (Range 377 – 562 g). Before this experiment, the rats were submitted to a study assessing startle responses, but they had no prior experience with any of the stimuli or environments utilized in this current experiment. Housing conditions were the same as in the previous experiment. The fluid deprivation program began 7 days before the start of the adaption phase and was maintained throughout the experiment. A food deprivation schedule, access to food 45 min each day after the experimental treatment, was initiated the last day of conditioning, and maintained throughout testing phase.

4.1.2. Apparatus, stimuli, and procedure

All material and procedures were the same as described for Experiment 1, except for the food-restriction regime that was implemented before the testing stage.

4.2. Results

Analysis of consumption during pretest sessions. During the initial phase of adaptation to odors, mean consumption of water from the bottle with the banana and mint odors during pre-test sessions was 9.84 ml (SD = 5.21) and 5.63 ml (SD = 5.89), respectively, a non-significant difference, $t(14) = 1.58$ $p > 0.1$. In the sessions of adaptation to sweetened substances (taste + odors), a 2 x 2 ANOVA (Odor x Taste) conducted on mean intake showed a significant main effect of Taste, $F(1,12)= 53.75$, $p < 0.001$, $\eta^2 = 0.82$, reflecting higher consumption of the sucrose as compared to the saccharin fluid (Mean = 35.2 ml, SD = 7.26 and Mean = 11.51 ml, SD = 5.00, respectively). Neither the main effect of Odor nor the interaction was significant, Fs $(1,12)< 1$.

Analysis of consumption during conditioning phase. Fig. 6 depicts mean consumption across trials for the A+ and B- odors as a function of Taste (Caloric vs. Non-caloric). As can be seen in the figure, there were a progressive increase across trials of consumption in the bottle with the

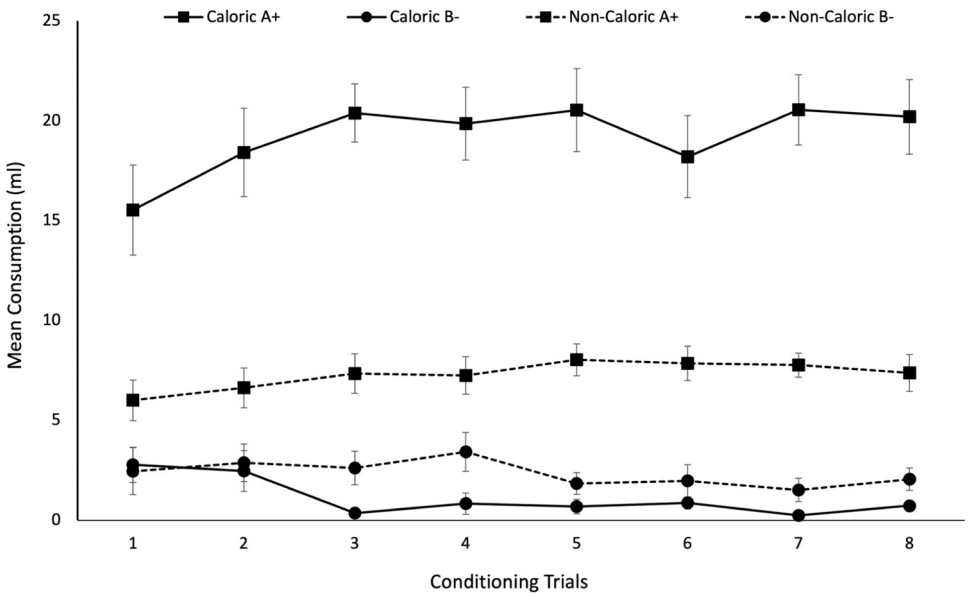

Fig. 6. Mean consumption of water (ml.) as a function of odor (A+ vs. B-), and US (Non-Caloric: Saccharin vs. Caloric: Sucrose) across conditioning Trials. A+ : Odor paired with sucrose (Cal Group), or saccharin (NoCal Group). B-: Odor paired with Water. Error bars show SEM.

A+ flavor containing sucrose. In addition, and consistent with the remaining experiments, consumption in the odor-reinforced bottle (A+) was higher that in the non-reinforced odor bottle (B-), and A+ consumption was higher when the bottle contained sucrose compared to when it had saccharin.

A 8 × 2 × 2 ANOVA (Trials x Odor: A+ vs. B- x Taste: Caloric vs. Non-caloric), revealed significant main effects of Odor and Taste, $F(1,14)= 90.84$, $p < 0.001$, $\eta^2 = 0.87$, and $F(1,14)= 41.74$, $p < 0.001$, $\eta^2 = 0.75$, respectively. The main effect of Odor reflects higher consumption of fluid presented with odor A+ as compared to B- (Mean = 13.24 ml, SD = 7.21, and Mean = 1.73 ml, SD = 1.55, respectively). The main effect of Taste reflects higher consumption in the Caloric group as compared to Non-caloric group, (Mean = 20.33, SD = 4.48, and Mean = 9.62, SD = 1.39, respectively). The main effect of Trials was also significant, $F(7,98)= 2.65$, $p < 0.05$, $\eta^2 = 0.16$, due to an overall increase in consumption across conditioning trials.

The Trials x Odor, and Odor x Taste interaction were significant, $F(7,98)= 5.46$, $p < 0.001$, $\eta^2 = 0.28$, and $F(1,14)= 29.65$, $p < 0.001$, $\eta^2 = 0.68$, respectively. The Trials x Odor interaction was due to an increase in consumption across trials for the fluid (saccharin or sucrose) presented with the odor A+ , and a progressive reduction of water consumption presented with odor B-. The Odor x Taste interaction was due to higher consumption of fluid associated with A+ when it was paired with sucrose as compared to fluid paired with saccharin (Mean = 19.21 ml, SD = 5.03, and Mean = 7.28 ml, SD = 2.17, respectively), and the absence of differences of water consumption associated with odor B-. No more interactions were significant (ps>.07).

Analysis of consumption during test phase. Fig. 7 shows mean consumption of water in presence of each scent during testing as a function of odors (A+, associated with sweet taste, sucrose or saccharin, vs. B-, associated with water), and the caloric or hedonic properties during conditioning. As can be seen in the Figure, there were an overall reduction in consumption with respect to previous Experiments, that is commonly observed when the animals are food-deprived (Verplanck and Hayes, 1953; Bolles, 1961; Fitzsimons and Le Magnen, 1969). In spite of this general reduction of intake, the animals drank more water from the bottle with odor A+ associated during conditioning to sucrose, whereas those exposed to saccharin showed no preference for neither of the two odors.

A 2 x 2 ANOVA with main factors Odor (A+ vs. B-) x Taste (Caloric vs. Non-caloric) revealed a significant main significant effect of Odor, $F(1,14)= 7.19$, $p < 0.05$, $\eta^2 = 0.34$, due to the higher consumption of water in those bottles with the A+ odor as compared to those with the B- odor (Mean = 2.80 ml, SD = 2.12, and Mean = 1.36, SD = 1.71, respectively). The main effect of Taste during conditioning was non-significant, $F(1,14)< 1$. Finally, the 2-way interaction was significant, $F(1,14)= 6.46$, $p < 0.05$, $\eta^2 = 0.32$. Post-hoc comparisons ($p < .05$) revealed that the interaction was due to higher consumption of water from the bottle with A+ as compared to B- odor for those animals conditioned with sucrose. The difference was non-significant for those animals that received saccharin during conditioning.

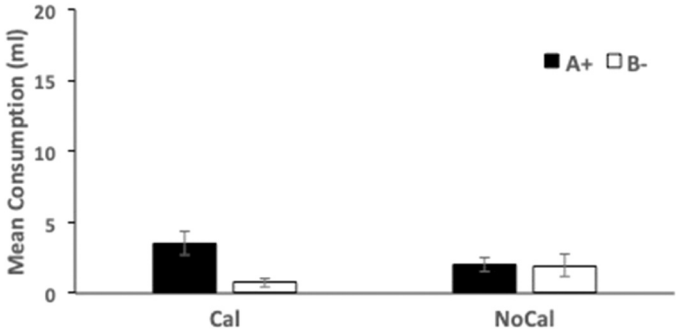

Fig. 7. Mean consumption of water (ml.) as a function of odor (A+ vs. B-), and US (Saccharin vs. Sucrose). A+ : Odor paired with sucrose (Cal Group), or saccharin (NoCal Group). B-: Odor paired with Water. The animals were food-deprived at testing and had free access to food during conditioning. Error bars show SEM.

A one-way ANOVA comparing mean preference ratios as a function of taste (Caloric vs. Non-caloric), that are depicted in the lower section of Table 1, similar to that described for previous experiments revealed the absence of significant differences, $F(1,14)= 4.07$; $p > .06$, $\eta^2 = 0.22$.

5. General discussion

The results obtained in our study revealed the existence of conditioned preference for odors when using both nutritional and hedonic reinforcers with aromas presented through the orthonasal route. Therefore, during the test phase of Experiment 1, the animals exhibited an increase in consumption from the bottle with the aroma associated

with sweet fluids, regardless of whether the reinforcement was caloric or non-caloric. These findings seem to confirm that, in both cases, the animals acquired an appetitive association with distal odors, without tasting the diluted odor along with the sweet substance. Furthermore, our data confirmed that the acquisition and expression of learning depend on the motivational state of the animals during the conditioning or test stages since the conditioned preference using saccharin as a reinforcer was not expressed when the animals were food-deprived during the acquisition stage (Experiment 2) or at testing (Experiment 3). However, preferences were evident in all cases when the reinforcing substance was sucrose, regardless of deprivation conditions.

Regarding the first objective of our study, to analyze the possibility of inducing conditioned preferences to orthonasal odors using substances with hedonic or palatable characteristics (saccharin) and additional nutritional and caloric properties (sucrose), the data from Experiment 1 replicates the results of previous studies that used odors diluted in a fluid solution and satiated rats (Harris et al., 2000; Gil et al., 2014, 2021). The preference for the odor associated with saccharin observed in the Non-caloric group is presumably related to its hedonic value, as its consumption can be perceived as pleasant or palatable (Young, 1948; Holman, 1975). Alternatively, the reinforcing capacity of an artificial sweetener lacking in calories could have an evolutionary and phylogenetic origin because the sweet taste is typically associated with the calories and nutrients provided by the consumption of sugary foods (Young, 1966; Fedorchak et al., 1997; Ramirez, 1990).

On the other hand, the reinforcing value of sucrose may be attributed to its sensory and hedonic properties, its nutritional and caloric content, or a combination of these factors (Capaldi, 1996; Fedorchak, 1997). In this regard, the acquisition of flavor preferences based on nutritional reinforcement appears to support strong associations that are positively correlated with high caloric density (Mehiel and Bolles, 1984). As a result, when both types of associations (flavor-flavor and flavor-nutrient) coexist, as is presumably the case with sucrose, the nutritional component seems to have a greater influence on behavior (Mehiel and Bolles, 1988). However, when the caloric value of two solutions is equivalent, the one with higher palatability will facilitate the conditioning of flavor preferences (Warwick and Weingarten, 1994; Myers and Hall, 2000). Therefore, when both features are present in the same meal, the CS may acquire its properties primarily due to its association with the nutritional component of the US. Conversely, the hedonic factor becomes more relevant when the nutritional factor is absent.

The second objective of our study was to assess the effects of food deprivation on conditioned preferences during the acquisition (Exp. 2) or the test phase (Exp. 3). Our results demonstrate that the motivational state plays a significant role in determining whether the acquisition and expression of conditioned preferences are influenced by the hedonic or sensory component of food, or by its nutritional consequences, in line with previous results (e.g., Capaldi et al., 1994). Thus, when orthonasal odors were paired with saccharin, conditioned preference based on the hedonic value of the reinforcer was more effective when the animals were not food-deprived throughout the experiment. However, when the animals were food deprived at conditioning (Exp. 2) or testing (Exp. 3) preference only appeared when the US was sucrose but not when it was saccharin. These findings may appear contradictory to previous studies that employed diluted odors and saccharin as the reinforcer, as the state of food deprivation during acquisition (Capaldi et al., 1994) or testing (Fedorchak and Bolles, 1987) did not affect preferences conditioned.

Additionally, it is important to take into account that, in Exp. 3, the food-deprived rats consumed substantially less fluid compared to those with unrestricted access to food in Exp. 1 and 2. As a result, it cannot be ruled out that the expression of conditioning was diminished in the Caloric group, or possibly even absent in the Non-caloric group, due to a significant reduction in overall consumption. It would be necessary in future studies to introduce a control condition that mitigates the impact of food restriction on fluid intake.

Some arguments and theoretical interpretations, such as those that have proposed the existence of flavor-nutrient and flavor-taste associations, can provide some clues to interpret our results. For instance, it has traditionally been considered that caloric learning, based on flavor-nutrient (or flavor-calorie) associations, generates an expectation of reinforcement based on the predictive relationship established between the CS and the delayed post-ingestive consequences of nutrient assimilation (Campbell et al., 1988). However, this does not preclude the occurrence of learning based on food palatability (Drucker et al., 1994). On the other hand, the role of evaluative conditioning (De Houwer et al., 2001) in non-caloric preferences learning has been emphasized, where simultaneous (and non-predictive) presentation of the CS and the US would promote changes in the affective valence of the CS (Rozin and Zellner, 1985). Consequently, approach or avoidance responses to the CS are influenced by the hedonic experience and the establishment of a flavor-taste association (also referred to as flavor-flavor) (Havermans and Jansen, 2011).

Based on this differentiation, we can anticipate that when the retronasal modality is used the simultaneous presentation of an odor and a taste at conditioning would favor the flavor-taste association (affective/evaluative learning) due to the hedonic experience, while the flavor-nutrient association would only be established if ingestion is followed by caloric consequences (predictive learning). We have represented these differences in Fig. 8. With the retronasal procedure, food deprivation during conditioning and/or testing would make more relevant the nutritional component, but learning based on food palatability would not be affected (see Fedorchak and Bolles, 1987; Capaldi et al., 1994; Harris et al., 2000). However, some recent studies have found evidence that satiated rats reinforced with caloric/hedonic substances (e.g., sucrose) can result in both flavor-taste and flavor-nutrient associations, although the latter would only modulate the conditioned response if the rats are tested in a state of food deprivation (García--Burgos et al., 2013; González et al., 2010, 2015). In summary, the available data seem to confirm that when diluted odors are used to study conditioned preferences, the motivational state would only modulate predictive or nutritional learning, but not affective or evaluative learning, where the expectation of reinforcement gives relevance to the hedonic evaluation of the stimulus.

On the contrary, with the orthonasal procedure employed in our experiments, the sequence of odor, taste, and post-ingestive consequences could invariably lead to an expectation of reinforcement and thus to predictive learning, whether of a hedonic nature -odor-taste-, or of a nutritional nature -odor-calorie- (see Fig. 8). Our results revealed

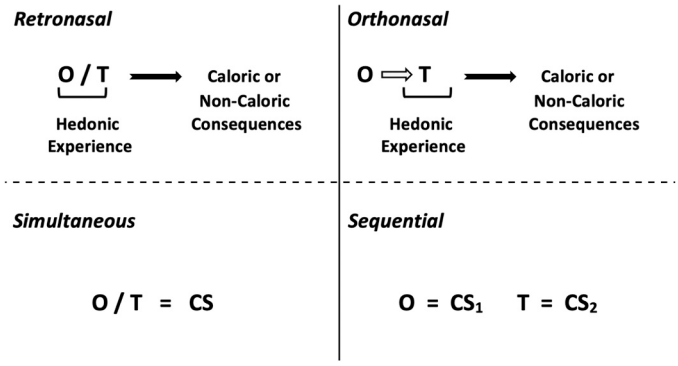

Fig. 8. In the retronasal pathway (left section), the conditioned odor gains hedonic value and predictive capacity with a caloric US due to the simultaneous presentation of odor and taste within the same sensory modality, but it only obtains hedonic value with a non-caloric US. In the orthonasal pathway (right section), the conditioned odor predicts both sweet taste and nutritional value with a caloric US, but lacks hedonic value due to separate odor and taste presentation. However, the conditioned odor will only predict the sweet taste with a non-caloric US in the orthonasal pathway.

that preferences learning is consistently acquired and expressed when an orthonasal odor is followed by a sweet and caloric reinforcer (sucrose), regardless of the subjects' motivational state during conditioning and testing. The use of sucrose as the US facilitates the simultaneous development of odor-taste and odor-calorie associations in both food-deprived and non-food-deprived rats, and the motivational state determines which of the two associations controls the response.

Using saccharin as a reinforcer, on the other hand, interferes with the acquisition and expression of preference learning due to food deprivation. This suggests that with the orthonasal procedure, the sequential presentation of the odor and saccharin would lead to predictive learning based on hedonic reinforcement (odor-taste), which is susceptible to modulation by the motivational state. Thus, if the food restriction is introduced during conditioning, the biological relevance of a sweet substance lacking caloric value, such as saccharin, would be lower, reducing its capacity to generate significant learning. Furthermore, if the animal is food-deprived during test, it is unlikely that the acquired conditioned preferences based on hedonic learning would be expressed with the orthonasal procedure, since this type of learning is predictive and, therefore, sensitive to interference by the motivational state.

In summary, if we assume, based on the evidence obtained in the retronasal procedure, that food deprivation would only affect predictive and sequential learning and not affective or evaluative learning, conditioned preferences in the orthonasal procedure would be influenced by the motivational state related to food access. This would not be the case with the retronasal procedure, where the relevance of the hedonic factor diminishes the influence of food deprivation.

Thus, in the orthonasal procedure, odor could function as a direct signal (CS1) of caloric reinforcement, followed by nutrient assimilation (US), or as an indirect predictive cue through its association with taste (CS2). At the same time, odor could also function as a predictive cue signaling the arrival of the hedonic experience induced by the sweet taste of sucrose, or other sweet substances like saccharin, which lacks caloric properties. At the time of testing, one or the other association could acquire control of behavior, depending on the state of food deprivation in this final phase of the procedure.

Finally, it is worth noting that when odors and tastes are paired, whether diluted in the same solution (retronasal procedure) or separately (orthonasal procedure), it can be observed facilitation or competition phenomena, such as blocking, sensory preconditioning, overshadowing, or potentiation (e.g., Holder, 1991; Garcia et al., 1985; Batsell and Paschall, 2009). However, due to the lack of appropriate controls, we cannot draw any conclusions in this regard, and the influence of these phenomena on our results remains unclear.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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