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# Caffeine-based flavor preference conditioning in the rat

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**Abstract**
The massive consumption of caffeine-containing beverages has prompted many studies involving human participants that have obtained caffeine-based increases in liking for a flavor. However, few studies have succeeded in obtaining caffeine-based flavor preference learning in rats. The main aim of the present study was to examine the conditions under which such learning can be detected. Three experiments differed mainly in terms of the base solution to which caffeine was added. Using a base of maltodextrin and saccharin, Experiment 1 found modest increases in flavor preferences in both food- and fluid-restricted rats. Experiment 2 found a strong caffeine-based flavor preference when water, but not saccharin, was used as the base. Whereas the first two experiments used a within-subject design, in which one flavor was paired with caffeine and a second flavor was not, Experiment 3 used a between-subject design with fluid-restricted rats given almond-flavored water containing caffeine in the Paired condition but not in the Unpaired condition; caffeine-based flavor preference learning was again found. In Experiments 1 and 2 post-conditioning exposure to the flavor alone produced a decrease in preference. In summary, the main achievements of this study were to extend the conditions under which caffeine-based flavor preferences can be detected in rats and demonstrate that such learned preferences are subject to extinction.

**Keywords** Associative learning · Flavour preference learning · Caffeine · Rat · Extinction

## Introduction

The consumption of sugar-sweetened beverages (SSBs) such as soft drinks and energy drinks has been linked to the increasing prevalence of obesity (Han & Powell, 2013; Hu & Malik, 2010). Research in rodents related to SSBs has primarily focused on the role of their sweet taste and high energy content (e.g., Kendig, Fu, Rehn, et al., 2018). Caffeine's contribution to flavor preferences has received relatively limited empirical attention, despite its presence in a large proportion of SSBs (Fredholm, Bättig, Holmén, et al., 1999).

As comprehensively reviewed by Temple, Bernard, Lipshultz, et al. (2012a), the properties of caffeine and its behavioral effects change as a function of concentration. Physiologically, the effect of caffeine arises from its ability to increase adrenaline release, inhibit dopamine re-uptake, and block adenosine receptors (Ferre, Ciruela, Borycz, et al., 2008; Fredholm et al., 1999). At low concentrations, caffeine intake can result in elevated alertness, positive affect, and improved cognitive and motor skills (McLellan, Caldwell, & Lieberman, 2016). At higher concentrations, caffeine can become aversive by promoting feelings of anxiety, disrupting bowel and urination processes, and increasing heart-rate and blood pressure (Temple, Bernard, Lipshultz, et al, 2012a). It can also serve as a negative reinforcer by alleviating withdrawal symptoms such as headaches, tiredness, and irritability (Temple, Bernard, Lipshultz, et al, 2012a).

Many studies of caffeine-based flavor learning using human participants have found increased liking for novel flavors that have been repeatedly paired with caffeine (Chambers, Mobini, & Yeomans, 2007; Mobini, Elliman, & Yeomans, 2005; Tinley, Durlach, & Yeomans, 2003; Tinley, Durlach, & Yeomans, 2004; Yeomans, Durlach, & Tinley, 2005; Yeomans et al., 2000a; Yeomans, Mobini, & Chambers, 2007; Yeomans, Spetch, & Rogers, 1998). Whereas in all of these studies the participants were adults, similar results were obtained when the participants were adolescents (Temple, Ziegler, Graczyk, et al., 2012b).

Yeomans et al. (2007) found that the combination of an artificial sweetener, aspartame, and caffeine produced greater liking for a novel flavor than produced by either alone, while bitterness from either quinine or caffeine reduced liking. Furthermore, Yeomans et al. (2000b) reported that liking for caffeine-based flavors can extinguish, in that

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their participants who were given repeated exposure to the flavor after the caffeine had been removed showed reduced liking for the flavor.

In contrast to studies using human participants, many early attempts to obtain caffeine-based flavor preference learning in rats were unsuccessful. Instead, they tended to find an aversion to a flavor after it had been paired with caffeine (Brockwell, Eikelboom, & Beninger, 1991; J. K. Smith, Neill, & Costall, 1998; Steigerwald, Rusiniak, Eckel, & O'Regan, 1988; Vitiello & Woods, 1975; White & Mason, 1985). Nevertheless, two groups have reported success in obtaining flavor preferences based on caffeine.

Using a within-subjects design, Fedorchak, Mesita, Plater, and Brougham (2002) gave their rats either caffeinated (CS+) or caffeine-free (CS-) flavored solutions. These solutions were given in the home cages for four 23-h sessions of each flavored solution and were the only source of fluid available to the rats during this conditioning stage. There followed 24-h preference tests between the CS+ and CS- flavored solutions in the absence of caffeine. These tests revealed a preference for the CS+ solution when low concentrations (0.125 mg/mL and 0.25 mg/mL) of caffeine were used during the conditioning stage but an aversion to the CS+ solution when higher concentrations (0.5 mg/mL and 0.75 mg/mL) were used. This study also examined the role of hunger by restricting chow in one group. Hungry rats consumed more of the caffeinated solution during conditioning and showed a greater preference for the caffeinated solution. However, in the absence of caffeine, hungry rats' preferences for the CS+ flavor were not detectably different from those of non-deprived rats. This suggests that an animal's motivational state may have a limited impact on flavor-caffeine learning.

As noted by Myers and Izbicki (2006), the procedure used by Fedorchak et al. (2002) requires rats to consume caffeine solutions that may be unpalatable, since no other fluids are made available. The latter factor may cause rats to drink small amounts throughout the day, which in turn could produce variability in caffeine's physiological effects, depending on individual drinking patterns. The different method adopted by Myers and Izbicki (2006) avoids this problem. Their hungry rats were placed in drinking chambers for 30-min sessions in which they were given solutions consisting of a highly palatable, low-energy base solution, 2% maltodextrin and 0.2% saccharin, to which either the CS+ or the CS- flavor was added, with caffeine added to the solution containing the CS+ flavor. Because the solutions were readily consumed by all rats, good control over dosage was obtained. The study examined a range of caffeine concentrations ranging from 0.07 to 0.25mg/mL in volumes between 15 and 40 mL. Myers and Izbicki (2006) found that the lowest concentration of caffeine produced a modest flavor preference (mean 65.3%), the medium concentration produced no significant preference, and the higher concentration produced a mild aversion (mean 39.2%).

The experiments on human flavor learning that were cited earlier obtained increased liking for caffeine-associated flavors only in participants who were habitual caffeine consumers or in those who were exposed to caffeine in the experiment prior to the conditioning stage (e.g., Tinley, Yeomans, & Durlach, 2003). Whether exposing rats to caffeine prior to the conditioning stage was needed to obtain a caffeine-based flavor preference was a question addressed by both Fedorchak et al. (2002) and Myers and Izbicki (2006). Neither study found that prior exposure to caffeine had a detectable effect on flavor preference learning when a low concentration of caffeine was used. Although, as noted above, humans' liking for a caffeine-based flavor can extinguish (Yeomans et al., 2000a), neither Fedorchak et al. (2002) nor Myers and Izbicki (2006) addressed the question of whether preference for a caffeine-associated flavor would extinguish when caffeine was removed. This question is of some interest because flavor preferences acquired by rats when flavors are paired with sucrose (e.g., Albertella & Boakes, 2006) or even with subsequent intra-gastric infusions (e.g., Drucker, Ackroff, & Sclafani, 1994) can be unusually resistant to extinction.

The main aim of the present study was to test whether previous results on caffeine-based learning in hungry rats would extend to thirsty rats. To the extent that experiments in which rats are given caffeine can be considered translational, it seems that humans are less likely to consume caffeine-containing beverages in the absence of food when they are hungry than when they are thirsty. A second aim was to examine whether such preferences would extinguish when a flavor was no longer paired with caffeine.

## Experiment 1

The method used in this experiment followed closely that of Myers and Izbicki (2006), including the use of a base solution made up of 2% maltodextrin and 0.2% saccharin. The main differences were, first, to include a comparison between rats that were food-deprived and those that were fluid-deprived and, second, to examine the effects of an extinction procedure.

The design is outlined in Table 1. Following a conditioning stage in which caffeine was added to the CS+ flavored base solution but not to the CS- base, rats were given a two-session test between the CS+ or CS- flavors added to the base in the absence of any caffeine. The extinction stage consisted of repeating these flavor preference tests. Finally, two caffeine preference tests were given with the aim of detecting whether rats would avoid or prefer solutions containing caffeine in the absence of either flavor. In the first of these tests the choice was between caffeine in base and base alone. This was

**Table 1** Design of the three experiments in this study

| | | | | |
|---|---|---|---|---|
| **Experiment 1** | | | | |
| **Groups** n=12/group | **Conditioning** | **Repeated tests in extinction** | **Caff Pref Test 1** | **Caff Pref Test 2** |
| **Hungry** | 6 × CS+ in base +caffeine | 8 × CS+ in base vs. CS- in base | Caff +base vs. Base only | Caff+ water vs. Water only |
| **Thirsty** | vs. 6 × CS- in base | | | |
| **Experiment 2** | | | | |
| **Groups** n=8/group | **Conditioning** | **Pref test** | **Extinction** | |
| **Saccharin** | 6 × CS+ in base +caffeine | CS+ in base vs. CS- in base | N/A | |
| **Water** | vs. 6 × CS- in base | | 10 × Pref tests | |
| **Experiment 3** | | | | |
| **Groups** n=16/group | **Conditioning** | **Preference test** | | |
| **Paired** | 6 × Almond in water + caff vs. 6 × water only | Almond in water vs. Water only | | |
| **Unpaired** | 6 × Almond in water vs. 6 × caff in water | | | |

"Base" refers to the solution to which a flavor and/or caffeine was added. Following Myers and Izbicki (2006), a maltodextrin plus saccharin solution served as the base in Experiment 1. In Experiment 2 the base was a saccharin solution in the Saccharin group and water only in the Water group. In Experiment 3 only water was used as a base

CS+ refers to the flavor paired with caffeine and CS- refers to the flavor not paired with caffeine in the within-subjects design of Experiments 1 and 2

followed by a choice between caffeinated water and non-caffeinated water.

## Method

### Subjects

Twenty-four experimentally naïve female Sprague-Dawley rats were obtained at 6 weeks of age from ARC Perth. On arrival, they weighed between 190 and 222 g and were housed in groups of four in large plastic cages (37 × 53 × 25 cm). These cages normally contained two 1-L water bottles. They were situated within a colony room maintained on a reverse 12-h light/dark cycle, with lights off at 1000 h. They remained housed under these conditions throughout the experiment. Rats were weighed twice each week to check that no unpredicted weight loss occurred.

Following 3 days of handling, animals were allocated to Hungry and Thirsty conditions on a cage basis, matching for body weight. For those in the Hungry condition (n=12), daily access to food was progressively reduced from 6 h, to 4 h, and finally to 2 h. On the following day the total 2-h chow consumption by the four rats in each cage was then measured. The specific amount per cage was placed in each home cage when rats were returned after an experimental session. Every 2 weeks, chow was made available for 2 h and intake was measured; the new intake was then prescribed for the next 2 weeks.

For rats in the Thirsty condition, chow was always available but daily access to water in the home cages was progressively reduced from 4 h to 2 h to 1 h to 30 min, and remained at 30 min for the rest of the experiment. This access was given after rats returned from a daily session or at about the same time of day on days when no session was run.

### Apparatus

Twelve acrylic cages measuring 23 × 35 × 19 cm, with paper chip bedding covering the floor and steel wire lids, served as drinking chambers in a laboratory that adjoined the colony room. Fluids were presented in plastic bottles with stainless-steel ball-bearing spouts, inserted between wires of the cage lids.

### Solutions

Solutions were mixed with tap water and weighed before and after each drinking session. Two flavors were used: One was

imitation vanilla (1% w/v) and the second was almond essence (0.5% w/v) (both Queen brand). These flavors were added to a base solution of 0.2% (w/v) saccharin (sodium saccharin salt hydrate; Sigma S-1002) and 2% (w/v) maltodextrin (Myopure). A caffeinated version of each solution was made by adding 0.07 mg/mL of caffeine to 15 mL resulting in a 1.05mg caffeine dose (Envirofields pure caffeine anhydrous powder). It may be noted that this concentration was that found by Myers and Izbicki (2006) to produce a flavor preference and is similar to that of many popular sodas.

### Procedure

Sessions were conducted between 1030 and 1230 h, 6 days per week. Sessions lasted 30 min during the initial pre-training and conditioning stages, whereas sessions lasted 10 min during the subsequent extinction and test stages. Rats were allocated to receive either almond or vanilla as their CS+ flavor, matching for body weight immediately prior to the start of the experiment, and were run in two counterbalanced squads of 12. During the conditioning stage rats were given approximately 15 mL of their allocated solution; thus, if they consumed all 15 mL of a caffeine-containing solution, they would receive a dose of 1.05 mg of caffeine. During two-bottle test sessions rats were given effectively unlimited access to both solutions.

The experiment began with two sessions designed to make the base solution familiar by giving the rats 15 mL of this solution without any flavoring. During the 12-session conditioning stage rats received six sessions in which they were given their CS+-flavored base solution plus caffeine alternating with six sessions in which they were given their CS- flavored base solution without caffeine. This sequence started with a CS- session. In the first CS+ that followed, it was discovered that bottle spouts for six rats had been blocked and so a supplementary conditioning session was run for these rats.

After the conditioning stage, there were two sessions in which rats were trained with two bottles, each containing only the base solution, i.e., without any added flavors. These were to familiarize the rats with the test procedure and to assess side preferences. In these sessions, rats were first given one bottle till drinking started and then the second until they had also sampled this; both bottles were then inserted into the chamber for a further 10 min. No rats displayed a position preference (> 80% to one side) over these two sessions.

During the repeated tests rats were given a choice between the two flavors, each added to the base solution and in the absence of any caffeine; thus, this succession of tests served as an extinction procedure. The position of the flavors was alternated from session to session and so test data were averaged over two successive sessions. Unlimited access to the two solutions was given during these tests. A total of four pairs of such tests were conducted. The test sessions were conducted daily, except for the last pair. These followed a 2-day break in which rats remained in their home cages with their motivational conditions maintained as usual.

In the final stage, two additional tests each consisting of two sessions were conducted. As noted above, these were to measure preference for caffeine in the absence of any added flavor. In the first such test rats were given a choice between the base solution alone and the base containing caffeine. In the final test the choice was between water alone and water to which caffeine had been added. The design of Experiment 1 is summarized in Table 1.

### Data analysis

Statistical analyses were undertaken using SPSS Version 26. Significance was set at $p < .05$. Greenhouse-Geisser correction was applied if sphericity was violated in repeated-measures analyses but the uncorrected degrees of freedom were reported.

Intake data during conditioning were averaged across pairs of sessions to calculate rats' intake of CS+ and CS- solutions. These six two-session intake averages were then analyzed using a 2 × (6) mixed ANOVA, with Motivation (Hungry or Thirsty) as the between-subjects factor and sessions as the within-subjects factor.

Preference ratios were calculated by dividing the CS+ intake by the total intake from both bottles. Since flavor positions were reversed across pairs of tests, rats that displayed side preferences on both days would average their overall preference to a 50% preference ratio. One-sample t-tests were used to compare test preferences with 50%.

A comparison of preferences during extinction between the Hungry and Thirsty groups was carried out, provided that the difference in total intakes on test did not exceed 20% of the groups' mean intake. These data were analyzed using a 2 × (4) mixed ANOVA, with Motivation as the between-subjects factor and extinction test (Tests 1–4) as the within-subjects factor.

## Results

### Conditioning

The 2 × (6) ANOVA applied to intakes during conditioning revealed a main effect of Session, $F(5, 110) = 2.61$, $p = .03$, but no effect of Motivation or interaction between Motivation and Session, $F$s < 1. In fact, mean intakes of the groups – Hungry, 14.7 mL and Thirsty, 14.8 mL – indicated that most rats on most occasions drank all of the 15 mL available. Trend analyses failed to detect either linear or quadratic trends across sessions, $p$s > .10.

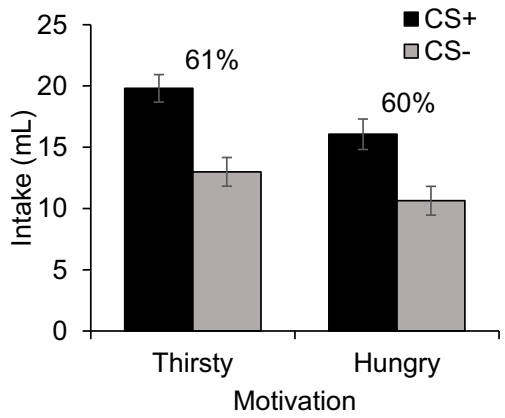


**Fig. 1** Experiment 1. Mean (± SEM) intakes during the initial two-session preference test between base solutions containing either CS+ (black) or CS- flavor (grey) but no caffeine. Intakes were averaged over the first two test days in which left and right positions of flavors were reversed. Preferences (CS+ intakes/CS+ intakes + CS- intakes × 100) are also shown

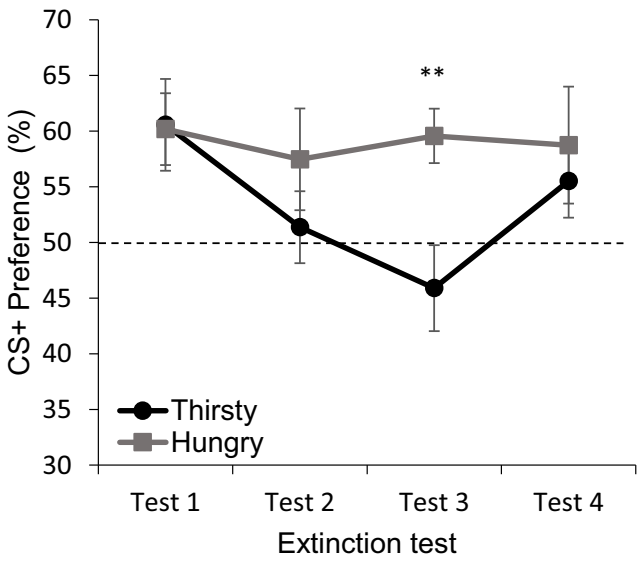


**Fig. 2** Experiment 1. Mean (± SEM) preference for the CS+ flavor as a percentage of total intake (CS+ and CS- flavors) in the four extinction tests. **Thirsty vs. Hungry, p < .01. NB. There was a 2-day break when rats remained in their home cages between Tests 3 and 4

### Extinction

As seen in Fig. 1, the initial preference test suggested that both groups preferred the caffeine-associated flavor to the non-caffeine flavor. A 2 × 2 ANOVA was conducted on fluid intakes during this preferences test, with Motivation (Hungry vs. Thirsty) as the between-subjects factor and Flavor (CS+ vs. CS-) as the within-subjects factor. This found a main effect of Flavor, whereby, averaged across Motivation conditions, intakes of the CS+ flavor were greater than those of the CS- flavor, $F(1,22) = 16.3$, $p < .001$. There was also a main effect of Motivation, whereby fluid intakes by Thirsty rats were greater in than those by Hungry rats, $F(1,22) = 18.89$, $p < .001$. There was no interaction between the two factors, $F<1$, indicating that preferences for the CS+ over the CS- flavor were not detectably different between Hungry and Thirsty rats. The one-sample *t*-tests confirmed a CS+ preference (i.e. > 50%) for both the Thirsty group, $t(11) = 3.28$, $p = .007$, and the Hungry group, $t(11) = 2.47$, $p = .03$.

The group difference in total intakes during extinction did not exceed 20%, the criterion specified above (Hungry: mean 28.9 mL; Thirsty: mean 33.9 mL). Thus, valid comparisons of preferences for the CS+ flavored base could be made between the two groups. The preferences are shown in Fig. 2, where these preferences tended to show a decline and then a small recovery in the Thirsty group, whereas preferences remained essentially unchanged in the Hungry group.

The 2 × (4) ANOVA on preferences during extinction failed to find main effects of Test or Motivation or an interaction effect, *p*s > .10. This indicated that across all four tests, CS+ preference did not differ between groups and preferences did not change across tests. However, inspection of Fig. 2 suggests a group difference at Test 3, and therefore post hoc independent sample t-tests were conducted at each test, with corrections to avoid inflating the family-wise error rate (FER). These confirmed that in Test 3 CS+ preference was significantly higher in Hungry than in Thirsty rats, $t(22) = 2.99$, $p = .007$, but not in the other three tests, *p*s > .10.

Additional t-tests comparing preferences against 50% during extinction in the Hungry group confirmed that they showed a significant preference for CS+ flavor in all four extinction tests, *p*s < .05. Meanwhile, the Thirsty group showed a CS+ preference in the first extinction test but this preference was no longer greater than 50% in extinction tests 2–4 (*p*s > .10).

### Caffeine preference tests

In the first caffeine test, preferences for the caffeinated base solution did not differ from 50% in either group, indicating that groups were indifferent between the caffeinated and non-caffeinated base solution, *p*s > .10. However, in the second test, the Thirsty group showed a preference (59.3%) for caffeinated water relative to non-caffeinated water, $t(11) = 2.80$, $p = .02$. No such preference for caffeinated water was found in the Hungry group (51.8%), $p > .10$. Group differences in caffeine preferences in the second test were not analyzed due to the large difference in total test intakes between the Hungry (3.1 mL) and Thirsty (11.8 mL) groups.

## Discussion

This experiment confirmed that the procedure introduced by Myers and Izbicki (2006) can detect caffeine-based flavor preferences, and it extended their results to fluid-deprived rats.

The strength of these preferences was, however, modest; the possible reason is discussed in the *General discussion*. As for extinction, there was evidence from the Thirsty group that flavor preferences extinguished over the first three pairs of test sessions; the reappearance of a preference in the fourth pair may be an example of spontaneous recovery in that Test 4 was carried out after a break of 2 days. The lack of evidence for extinction in the Hungry group was unexpected and we have no explanation for this surprising outcome; we can only speculate that the effect might have been detected if the extinction stage had been much longer.

An additional finding was that the thirsty rats revealed a preference for the taste of caffeine when it was added to water, but not when added to the maltodextrin-plus-saccharin base solution. This result suggests that the high palatability of this base solution, especially for the hungry rats, made it difficult to detect caffeine-based preferences in two-bottle tests in which both bottles contained the base solution. The finding of a preference for caffeine in water suggests that during the conditioning stage the consequences of caffeine ingestion had increased preference for both the CS+ flavor and the taste of caffeine, presumably a very mild bitter taste at this low concentration.

## Experiment 2

The main aim was to test whether stronger caffeine-based flavor preferences than those found in Experiment 1 could be obtained when other base solutions were used. We chose solutions that are known to be less palatable than the saccharin-plus-maltodextrin base used in Experiment 1 and by Myers and Izbicki (2006). We reasoned that the use of less palatable solutions would reduce flavor learning to both CS+ and CS- that could have reduced the size of caffeine-based preferences detected in Experiment 1. This experiment followed the basic design of Experiment 1, but with one group of rats given a saccharin solution and a second group water only as bases; see Table 1 for a summary of the design. The second aim was again to examine whether such preferences would extinguish. All rats in this second experiment were fluid-deprived.

### Method

#### Subjects

Sixteen female Sprague-Dawley rats were originally obtained from ARC Perth to serve in a Pavlovian conditioning experiment in which they were exposed when food-restricted to auditory and visual stimuli paired with food pellet deliveries but were not exposed to any other flavors. When subsequently transferred to the present experiment these females were 12–13 weeks old and weighed between 290 and 340 g. They were housed four to a cage under similar conditions to the rats in Experiment 1. Fluid restriction was introduced progressively for all rats, as in the previous experiment, and they were then given 30-min access to water in their home cages immediately following a session in the drinking chambers. Prior to the start of the experiment, eight rats (from two cages) were allocated to the Saccharin group and the other eight to the Water group on a cage basis, matching for bodyweight.

#### Apparatus and solutions

The procedures were performed in the drinking chambers described for Experiment 1. A solution of 0.2% saccharin was used as the base for rats in the Saccharin group, while there was no base, i.e., only water, for the Water group. Within each group rats were allocated to either 0.5% almond essence or 1% imitation vanilla flavoring as their CS+ flavor, again matching for body weight. Without palatable additives rats consume significantly less solution and hence the volume of the solution was decreased from 15 mL to 10 mL for Experiment 2. During the conditioning stage a concentration of 0.105 mg/mL of caffeine was added to the solution containing the CS+ flavor. Thus, if rats consumed the entire 10 mL available, they would receive the same caffeine dose of 1.05 mg as in Experiment 1.

#### Procedure

The basic procedure was similar to that of Experiment 1 and is summarized in Table 1. One difference in procedure was that in the present experiment a single squad of all 16 rats was run each day. Following two sessions in which the rats were given their unflavored base solution, i.e., either saccharin solution or water, in the drinking cages, the conditioning stage consisted of 12 sessions in which a CS+ flavored solution containing caffeine alternated with a solution containing the CS- flavor that did not contain caffeine. After two-bottle training, an initial two-session preference test was given between the CS+ and CS- flavors, in water for the Water group and in the saccharin solution for the Saccharin group.

Since only the Water group showed a preference for the CS+ flavor in the initial test, only this group was given an extinction test. The extinction stage was similar to that in Experiment 1, in that it consisted of repeated two-session choice tests between the CS+ - flavor in water (without added caffeine) and the CS- flavor in water. These tests were repeated until the mean CS+ preference had declined to 50%.

## Results

### Conditioning

As shown in Fig. 3, during the conditioning stage intakes in the Saccharin group were greater than those in the Water group. It may be noted that the Saccharin group consumed the maximum of 10 mL in the final three conditioning sessions and thus these rats then received the full dose of 1.05 mg, whereas the lower intakes in the Water group meant that these rats received about two-thirds of the full dose.

A 2 × (2) × (6) ANOVA of these intakes, with Group (Saccharin vs. Water) as the between-subjects factor, and Session (1–6) and Caffeine-content (caffeinated CS+ flavor vs. non-caffeinated CS-flavor) as the within-subjects factors, revealed main effects of Group, $F(1, 14) = 59.87$ , $p < .001$, and of Session, $F(5, 70) = 9.10$, $p < .001$. Levene's test of equality of error variance was significant in CS+ intakes in Session 4, and both CS+ and CS- intakes in Session 5, $ps < .05$. Although this indicated violation of the homogeneity of variance assumption, *F*-tests are generally robust enough against this violation with equal group sizes (Blanca, Alarcón, Arnau, Bono, & Bendayan, 2018). Nevertheless, only the main effects are interpreted here. The Group main effect confirmed greater intakes in the Saccharin group relative to the Water group. There was also a linear trend, which reflected the increase in intakes across sessions, $F(1,14) = 59.24$, $p < .001$. There was a Session effect and an interaction between Session and caffeine content, $F(5, 70) = 4.18$, $p = .002$. There was no obvious reason for the anomalous data from Session 4.

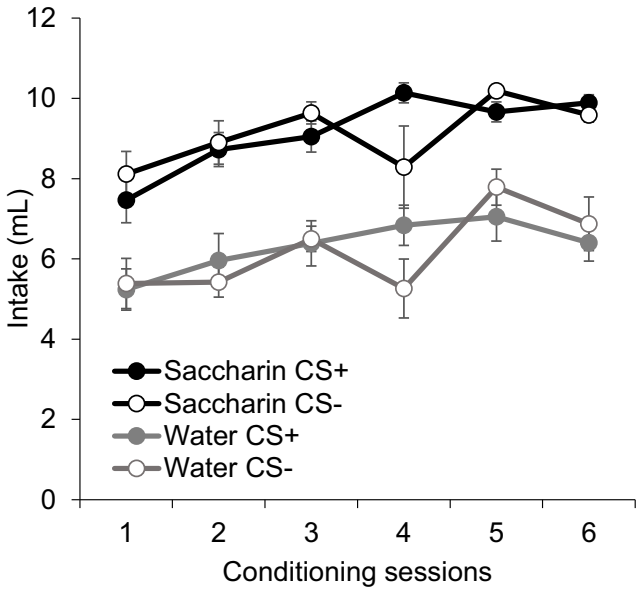


**Fig. 3** Experiment 2. Mean (± SEM) intakes of caffeinated CS+ solutions (closed circles) and caffeine-free CS- solutions (open circles) by the Water (gray) and Saccharin groups (black) during the conditioning stage

### Preference test

As suggested by Fig. 4, preference for the CS+ flavor was greater in the Water than in the Saccharin group. To assess whether there was a preference, t-tests against a comparison value of 50% were conducted separately for each group. This found the Water group showed a CS+ flavor preference, $t(7) = 4.76$, $p = .002$, whereas no such preference was shown by the Saccharin group, $p > .10$. Since the Saccharin group drank considerably more of both solutions during the test, this made a statistical comparison of preferences between the groups invalid and was not conducted.

### Extinction

Total fluid intakes by the Water group remained essentially constant over the extinction stage, ranging from means of 7.2 mL to 9.0 mL per session over the six two-session tests. The preferences measured in these tests are shown in Fig. 5, where it may be seen that preference for the caffeine-associated (CS+ ) flavor declined steadily. Indeed, t-tests confirmed that CS+ preferences were significantly greater than 50% (i.e., there was a CS+ preference) in tests 1–5 ($ps < .05$) but not in test 6, $p > .10$.

## Discussion

The main aim of this second experiment was achieved in that the caffeine-based CS+ preferences displayed by the Water group, as seen in Fig. 4, were considerably stronger than those

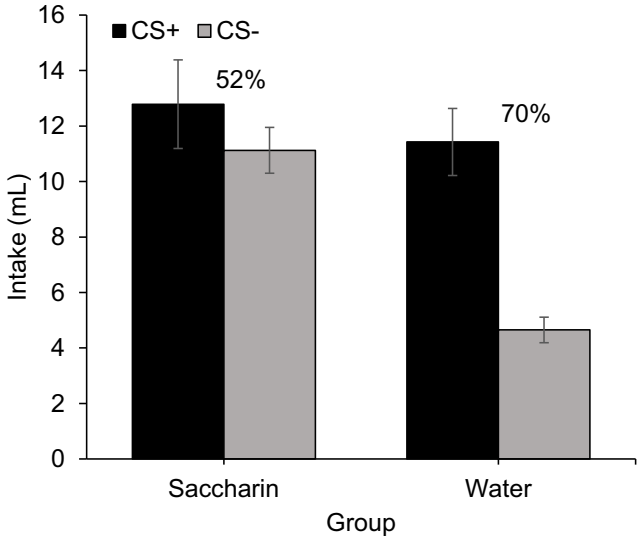


**Fig. 4** Experiment 2. Mean (± SEM) intakes and flavor preferences in the Water and Saccharin groups during the first two-session two-bottle choice test between CS+ and CS- flavors in the absence of caffeine. In the Water group the flavors were added to water and in the Saccharin group the flavors were added to 0.2% saccharin. CS+ preferences as a percentage of total intake are shown on top of the columns

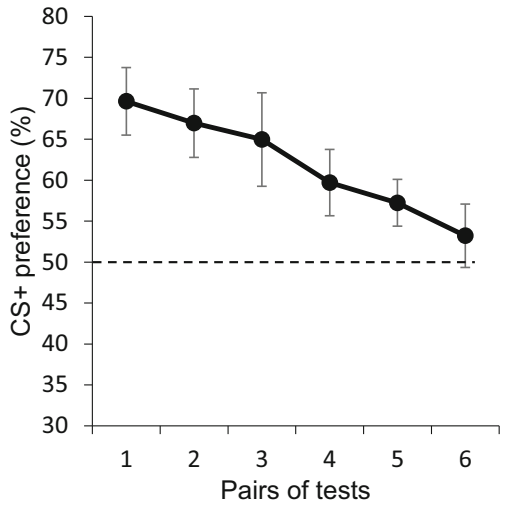


**Fig. 5** Experiment 2. Mean (± SEM) preference for the CS+ flavor as a percentage of total intake (CS+ and CS- flavors) over the six pairs of tests in the Water group. NB: Extinction tests were not performed in Saccharin group following the failure to detect a preference for CS+ in Test 1

obtained in Experiment 1. A further aim, that of obtaining further evidence for extinction of such preferences, was also achieved, as shown in Fig. 5.

On the other hand, no caffeine-based flavor preference was detected in the Saccharin group. It may be noted that intake of the CS- flavor in the Saccharin group was large (see Fig. 3), thus suggesting that an increase in the palatability of both flavors by association with saccharin may have masked any possible caffeine-based preference; the same point may apply to the weak preferences found in Experiment 1 when the base of maltodextrin-plus-saccharin was used. In hindsight, it seems that a choice test for the Saccharin group between the CS+ and CS- flavors when both were presented in water might have revealed a preference for the CS+ in this group. If such a water-based test had revealed a caffeine-based preference, we would not have abandoned the planned extinction test for this group.

Another possible reason for obtaining stronger caffeine-based preferences in the Water group was that, because of these rats' smaller intakes during the conditioning stage, they received lower doses of caffeine than rats in the Saccharin group. The effectively full dose of 1.05 mg received by Saccharin group was found to be optimal for obtaining caffeine-based conditioning of a flavor by Myers and Izbicki (2006), but then their rats were males and heavier than the females used in the present experiment.

## Experiment 3

The aim of this experiment was to test for the generality of the effects of caffeine found in the previous experiments. A major change was to use a between-subject design, whereby one group, Paired, were given sessions in which rats were given an almond-flavored caffeine solution and a second group, Unpaired, were given sessions of unflavored caffeine solutions that alternated with sessions in which they were given almond-flavored water; see Table 1. Furthermore, whereas female rats were used in the first two experiments, this final experiment used males. Following the finding of stronger caffeine-based preferences in the Water group in Experiment 2, only solutions in water were used here.

The flavor used in this experiment was 0.5% almond essence. In previous, but unrelated, experiments (Badolato, Hall & Boakes, in press) we have found this to be somewhat less preferred than the 1% vanilla also used in Experiments 1 and 2, with unconditioned controls showing a preference relative to water of about 40%, that is, very lightly aversive.

### Method

#### Subjects

Thirty-two male Sprague Dawley rats had previously participated in a running wheel experiment (Boakes & Wu, 2020) but had no prior experience of any flavors other than that of their regular chow. At the start of the present experiment, they were 11–12 weeks old and weighed between 367 and 510 g. Housing conditions were the same as in the previous experiments. All rats were subjected to the same progressive water restriction as previously, starting on the first day at 4 h and finally 30 min for the rest of the experiment. Access to chow was unrestricted.

#### Apparatus and solutions

Sixteen drinking chambers were the acrylic cages used in the previous experiments. The target flavor was a 0.5% concentration of almond (Queen brand). The concentration of caffeine was 0.105 mg/mL in a 10-mL serve. Thus, if a rat consumed all the caffeine-containing solution it was offered, it would receive a dose of 1.05 mg, as in Experiments 1 and 2.

### Procedure

Six sessions were given each week, starting at 1100 h, with all sessions 20 min long, except where noted below. Rats were run in two squads of 16.

***Water pre-training*** **(Sessions 1–4)** All rats first received four sessions of water training in the drinking chambers to habituate them to drinking in this context. On completion of this training rats were weighed and, matching for weight, half were assigned to the Paired condition and the other half to the Unpaired condition.

**Conditioning (Sessions 5–16)** Rats within the Paired condition were given six sessions of the almond-flavored caffeine solution, alternating with six sessions of water. Rats in the Unpaired condition were given six sessions of almond-flavored water alternating with six sessions of caffeinated water.

**Two-bottle training and Test 1 (Sessions 17–20)** All rats were given two 10-min sessions of water-only presentations in two bottles. Over the next two 10-min sessions two-bottle preference tests were given between almond-flavored water and unflavored water, in the absence of any caffeine, with almond on the left in Session 19 and on the right in Session 20.

## Results

Preferences obtained from the test are shown in Fig. 6. A 2 × (2) ANOVA of fluid intakes was conducted with Conditioning (Paired vs. Unpaired) as the between-subjects factor and Fluid (Water vs. Almond) as the within-subjects factor. While there were no main effects of either factor, there was an interaction between Conditioning and Fluid, $F(1,30) = 11.02$, $p = .002$. Inspection of Fig. 6 suggested that this was due to greater intakes of the almond-flavored water than unflavored water by the Paired than by the Unpaired rats. Indeed, when almond preferences were compared between Paired and Unpaired groups with an independent samples t-test, the Paired group had higher almond preferences than the Unpaired group, $t(29) = 3.80$, p = .001.

## Discussion

This experiment was successful in achieving its aim of detecting a difference between rats given a pairing of almond with caffeine and those for which almond signaled the absence of caffeine. It may be noted that the preference for almond shown by the Paired group were weak and avoidance of this flavor by the Unpaired group was fairly strong. This almost certainly reflects the use of almond rather than vanilla as the target flavor in this experiment. In other studies in this laboratory, we have found that the 0.5% almond solution used here is somewhat aversive – around 40% preference – under control conditions (e.g., Badolato, et al., in press).

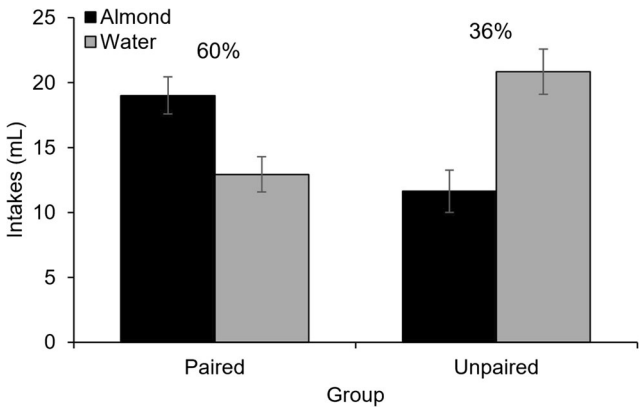


**Fig. 6** Experiment 3. Average (± SEM) intakes of almond-flavored water and unflavored water in the preference test. Preferences for almond as a percentage of total fluid intake (almond + water) are shown on top of the columns

## General discussion

The main finding from this study was that caffeine-based conditioned flavor preferences can be obtained over a range of motivational and training conditions. In Experiment 1, in which caffeine was added to a highly palatable, energy-containing base solution, only modest preferences were detected in both food- and fluid-restricted rats. Experiment 2 found stronger preferences in fluid-restricted rats when caffeine was simply added to flavored water during the training stage. Instead of the within-subject design used in both the first two experiments and in the two previous studies that have obtained caffeine-based flavor preference learning, Experiment 3 used a between-subject design that also demonstrated caffeine-based flavor learning.

It may be noted that the rats in the present experiments had no exposure to caffeine prior to the training stage and that all used a low concentration of caffeine. Thus, the results lend further support to the suggestion by Myers and Izbicki (2006) that at very low doses caffeine produces an inherently positive effect. This suggestion was partly based on results from their Experiment 3 revealing that the degree of flavor preference conditioning based on a low dose was unaffected by whether the rats had extensive prior exposure to caffeine or none at all; on the other hand, prior exposure was needed in order to obtain conditioning of a flavor preference when a flavor was added to a higher concentration of caffeine. These researchers proposed that, when a higher dose is used with rats without prior experience of caffeine, conditioned flavor avoidance may result from both its increasingly bitter taste and negative consequences (Myers & Izbicki, 2006). It may be noted that studies using human participants have typically used higher concentrations of caffeine than that used here; for example, 0.5 mg/mL in Yeomans et al. (2005).

A further, and novel, effect found in the present study was that rats can develop a preference for the taste of caffeine. Presumably this would not have been found if a higher concentration of caffeine had been used. This effect may be similar to the development of human liking for coffee despite its initially unpleasant taste.

Most studies of caffeine-based flavor learning in human participants have used ratings of liking for a flavor as the main outcome measure. When rats are used in such experiments, the two-bottle preference test used in

caffeine experiments such as the present ones does not provide a direct measure of liking. The degree of preference for a caffeine-associated flavor depends on what it is compared with. The low preferences found in Experiment 1 were obtained when a CS+ flavor, which presumably had become associated with the highly palatable base solution as well as with the positive effects of caffeine, was compared with a CS- flavor that had also become associated with the base solution. It seems that the added preference for the CS+ contributed by caffeine was relatively small. The same argument can be applied to the failure in Experiment 2 to detect a caffeine-based flavor preference in the Saccharin group. In contrast, when there is no base solution and simply water is used, this problem is at best minor; few studies have succeeded in obtaining increased preference for a flavor accompanying a reduction in fluid deprivation, i.e., alleviation of thirst, and the reported effects have been small (e.g., Revusky, 1968). This would account for the high preferences for the CS+ flavor found in the Water group of Experiment 2. Given the limitations of two-bottle preferences testing, more direct measures of liking for a caffeine-associated flavor might have been obtained from either oral taste reactivity tests or from microstructural analyses of lick patterns (e.g., Davis & Smith, 1992; Dwyer, 2012).

A secondary aim of these experiments was to test for extinction of caffeine-based flavor preferences. Following previous precedents, such as testing for extinction of sucrose-based flavor preferences in food-restricted rats (e.g., Harris, Shand, Carroll, & Westbrook, 2004), in the extinction stage of Experiments 1 and 2 the rats were given repeated two-bottle choice tests between CS+ and CS- flavors in the absence of access to caffeine. As shown in Fig. 3, in Experiment 2 rats in the Water group became increasingly indifferent between the two flavors. This result suggested that caffeine-based flavor preferences in rats are sensitive to extinction, just as human studies have found that liking ratings for a caffeine-associated flavor decline with repeated post-training exposure to the flavor in the absence of caffeine (e.g., Yeomans et al., 2000b). With regard to everyday consumption of sodas, it seems that the caffeine that is added to almost all such drinks is needed both to increase liking for their flavors and to sustain such liking.

The general conclusion from this series of experiments is a methodological one. Future researchers who plan to study caffeine-based flavor preferences in rats are advised to use the simplest of the methods employed in the present study; that is, the animals need to be fluid restricted and given a solution of the target flavor in water, to which an appropriately low concentration of caffeine has been added. Use of palatable base solutions may hinder detection of the sought-after effects.

## Declarations

This study was partly funded by an Australian Research Council Discovery Project grant to K.B. Rooney and R.A. Boakes, DP17010392, and partly by the School of Psychology, University of Sydney. Experiments 1 and 2 were reported in a Psychology Honours thesis by LF, who ran all three experiments. LF and RAB designed the experiments and together wrote the paper, in collaboration with SR, who also performed most of the statistical analyses. We are grateful to Martin Yeomans for his comments on an early draft of this paper. There are no conflicts of interest. The experimental procedures were approved by the University of Sydney Animal Ethics Committee under Protocol #1082. Prior to publication the data are available on request to RAB and subsequent to publication will become openly available on the internet.

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