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Journal
International Journal of Comparative Psychology, 28(1)

ISSN
0889-3675

Author
Nakajima, Sadahiko

Publication Date
2015

DOI
10.46867/ijcp.2015.28.00.02

INTERNATIONAL JOURNAL OF COMPARATIVE PSYCHOLOGY

2014, Vol. 28
Dani Brunner, Editor
Peer-reviewed

Swimming in Flavored Water Leads to Avoidance of that Flavor in Laboratory Rats (*Rattus norvegicus*)

Sadahiko Nakajima
Department of Psychological Science, Kwansei Gakuin University, Japan

This article consists of two experiments reporting conditioned flavor avoidance (or taste aversion) in laboratory rats that swam in the flavored water. A statistically reliable effect was demonstrated in Experiment 1 by using a simple conditioning procedure with sweet (sodium saccharin) water. Compared with control rats that had no swimming experience or those that swam in tap water, experimental rats showed avoidance of the sweet water in the choice test between it and tap water, if they had swum in the sweet water for 20 min over four days. Rinsing the rats off with tap water after the swimming had no effect on this flavor avoidance learning. This finding suggests that tasting the sweet water during swimming was critical. Experiment 2 confirmed the flavor avoidance learning in swimming rats by a differential conditioning procedure with sour (citric acid) and bitter (denatonium benzoate) solutions. Although the effect was relatively small in the two experiments reported here, this new procedure may contribute to future research concerning Pavlovian conditioning due to its procedural simplicity.

Running in an activity wheel (e.g., Lett & Grant, 1996) or swimming in a water pool (e.g., Nakajima & Masaki, 2004) leads to Pavlovian conditioned avoidance of the flavored water consumed before the activity in the laboratory rat (see Boakes & Nakajima, 2009, for a review). In this experimental preparation, the flavor is a conditioned stimulus (CS) and the activity an unconditioned stimulus (US). There are some advantages of employing these kinds of activity-based flavor avoidance learning in Pavlovian conditioning studies[1].–Among others, the most notable merit is that these preparations are easy to use. No expensive apparatus or "high-tech" equipment is necessary for conducting these learning experiments. In addition, laboratory assistants with minimum training can execute flavor avoidance experiments, because they do not have to master special skills such as intraperitoneal injection of drugs.

Reflecting on the technologically simple nature of activity-based flavor avoidance learning posed a much simpler procedure for establishing flavor avoidance learning in rats: swimming in flavored water. As thirsty rats drink flavored water while swimming, this procedure provides rats with the CS and US at the same time. The present article

[1] I use the term "conditioned flavor avoidance" (or "flavor avoidance learning") rather than "conditioned taste aversion" (or "taste aversion learning") in the present article, simply because "flavored water" sounds more natural than "taste water" and the measure of conditioning was the amount of intake rather than disgust reactions (Parker, 2003). But, these terms are actually exchangeable in the context of this article.

Please send correspondence to Dr. S. Nakajima, Department of Psychological Science, Kwansei Gakuin University, Nishinomiya, 662-8501, Japan (Email: nakajima@kwansei.ac.jp).
https://doi.org/10.46867/ijcp.2015.28.00.02

reports two experiments conducted with this new and simple preparation for yielding Pavlovian conditioned flavor avoidance in laboratory rats.

A shortcoming of this procedure is that we cannot control or measure the amount of flavored water consumed by a rat during the conditioning phase. Estimation of the consumption by computing a pre-post difference in the amount of flavored water in the pool is virtually impossible, because the rat sponges the water with its fur and it urinates in the pool.

Despite such a technical concern, the present research may contribute to the field of animal learning by proposing a simple modus operandi to study Pavlovian flavor conditioning in rats. Notably, this procedure turns the setting from conventional, forward conditioning (i.e., a flavor CS followed by a swimming US) to simultaneous conditioning (i.e., a concurrent presentation of a flavor CS and a swimming US). According to Pavlov (1927) and many textbooks on conditioning and learning (e.g., Barker, 1997; Chance, 2003; Hall, 1976), simultaneous conditioning is an inferior procedure to establish substantial and stable conditioned responses; however, this is not always the case (see Molet & Miller, 2014, for theoretical discussions). Thus, the present research might contribute to existing knowledge on successful simultaneous conditioning.

Experiment 1

The aim of this experiment was to explore the possibility of flavor avoidance learning in rats swimming in flavored water. This experiment consisted of two experimental groups and two control groups. Rats in the first experimental group swam in pools filled with sweet water. The putative CS and US for this group were the sweet flavor and the swimming activity, respectively, with these stimuli paired in the temporal relationship of simultaneous conditioning. However, this procedure also provides an opportunity for backward conditioning (i.e., the swimming US followed by the flavor CS), because the rats retrieved from the pools might have licked their damp fur. In order to control this factor, rats in the second experimental group were rinsed off with tap water after swimming in sweet water. The two control groups were rats thus that had no swimming opportunity (i.e., neither the flavor CS nor the swimming US was presented for these rats) and rats that swam in tap water (i.e., the US-only control).

Method

Subjects

The subjects were 32 experimentally naïve male rats of the Wistar strain (Jbc: Wistar) purchased from a local supplier (Keari Co. Ltd, Osaka, Japan) a week before the experiment. They were nine weeks old with a mean weight of 315.9 g (range: 285–375 g), measured on the day before adaptation training began. The animals were housed in individual hanging home cages of the vivarium on a 12:12 h light–dark cycle (lights on at 0800 h) at 22 °C and 55% humidity. They were maintained on an ad-lib food (rat chow) schedule, but water was restricted to the daily sessions in the experimental room.

Apparatus

The experimental sessions were conducted in a conventionally illuminated room where eight drinking cages, six swimming pools, and eight polycarbonate boxes were located. The drinking cages were

copies of the home cages (20 × 25 × 18.7 cm, w × l × h). Tap water or sweet flavored water (0.2% sodium saccharin) of 22 °C was provided via a glass bottle with a metal spout inserted from the cage ceiling. The end of the spout was 16.5 cm above the cage floor. When two bottles were used, they were separated 8 cm apart. The swimming pools were blue–gray plastic garbage containers, the inner dimensions of which were 34 cm diameter at the bottom, 43 cm diameter at the top, and 48 cm high; they were filled to a height of 33 cm with 35 L tap water or sweet water at a room temperature of 22 °C. Any floating fecal matter was removed from the pools between the squads of rats, and the pools were cleaned up at the end of each daily training. The polycarbonate boxes (24 × 40 ×18 cm, w × l × h) contained tap water of 8 L (per box) for rinsing a group of eight rats off after swimming in the saccharin pools (see below), and each of the boxes was used once per day so that each rat was rinsed off with fresh tap water at 22 °C.

Procedure

All experimental sessions were conducted at the same time on successive days, as follows. The starting time of each squad of eight rats was 1020, 1045, 1110, or 1135 h.

Adaptation. On Day 1, each rat was adapted to drinking tap water from a bottle for 15 min. The tap water in the bottle was replaced with sweet water of 15 min on Day 2 in an attempt to reduce neophobic reaction to this solution to be tested later. On Days 3–4, two bottles were concurrently presented for 15 min per day: the left bottle was empty and the right bottle contained tap water on Day 3, but the locations of the bottles were interchanged on Day 4. This empty-vs-water bottle adaptation was conducted with the intention of increasing the sensitivity of the choice test at the end of the experiment (cf. Dragoin, McCleary, & McCleary, 1971). The rats were then assigned to one of four groups of eight rats each, matched for water and saccharin intake and bodyweight. This assignment was conducted within each squad, so that each squad of eight rats was formed with two rats from each treatment group.

Training. All the groups then received a four-day training treatment (Days 5–8). Rats of Group SacSwim were allowed to swim in sweet-water pools (20 min per day). Rats of Group SacSwim-Rinse were given identical treatment, but they were rinsed off with fresh tap water in polycarbonate boxes shortly after swimming in the sweet-water pools. Rats of WaterSwim were allowed to swim in tap water pools for the same duration. All of these three groups of rats were lightly dried with towels for a short time before returning to their home cages. Finally, rats of Group NoSwim were kept in the home cages on these days with daily handling for weighing. In summary, Groups SacSwim and SacSwim-Rinse were the critical experimental groups of this experiment, and they were contrasted with Groups WaterSwim and NoSwim, the control rats. The hypothesis investigated was that rats in the experimental groups would acquire conditioned flavor avoidance, which should be reflected in a weaker preference for sweet water compared with the control groups.

Testing. Two-bottle choice testing was administered on the next two days (Days 9–10) for all rats. One bottle contained the sweet water, while the other contained tap water: the left-right positions of the bottles were counterbalanced across rats and days in each group.

Measurement and Analysis

The amount of fluid intake was measured by weighing each bottle before and after the drinking period with an electric balance (BJ-1500, Sartorius Japan, Tokyo) to the nearest 0.1 g. In the choice test, the relative, rather than absolute, intake of the sweet water was adopted as the index of flavor avoidance in order to correct for any possible between-group variations in total fluid intake. Specifically, the preference ratio was calculated as the ratio of target sweet water intake to total fluid intake (sweet water + tap water): the lower the ratio value, the stronger the avoidance estimated. All statistical decisions of this study are based on an alpha level set at $p < 0.05$.

Rats immersed in water release “alarm substance,” a type of pheromone, into the water, which affects the rats’ swimming performance (Abel, 1991a, 1991b; Abel & Bilitzke, 1990). Although the Wistar strain does not reliably react to this substance (Abel, 1992), we cannot easily dismiss the possibility that alarm substance released by the rats affects rats from the later squads, because the pool water was not changed after each squad in this experiment. Thus, the data were initially inspected in terms of experimental order (i.e., squads); this factor had no significant main or interactive effects in any data set,

implying that the alarm substance played no critical role in the present experiment. Therefore, this factor was not included in the later analyses.

Results

Figure 1 depicts the choice test results. One can observe conditioned flavor avoidance on the first test day: target preference ratios of Groups SacSwim and SacSwim-Rinse were low, compared with those of Groups WaterSwim and NoSwim. This impression was statistically supported by a 4 (group) × 2 (day) analysis of variance (ANOVA), which yielded a significant group × day interaction, $F(3, 28) = 3.92$, $p = 0.019$, $\eta_p^2 = 0.30$. The main effects of group and day were nonsignificant, Fs < 1. One may attribute the group × day interaction to the unexpectedly low preference ratio of Group NoSwim on the second test day, but subsequent simple main effect analyses of the interaction using the pooled error terms revealed that the effect of group was significant on the first test day, $F(3, 56) = 3.20$, $p = 0.03$, $\eta_p^2 = 0.15$, but not on the second test day, $F < 1$.

Although post hoc comparisons of data from the first test day using Ryan's procedure failed to reveal any significant paired group contrasts, a planned comparison yielded a significant difference between the combined data of the two experimental groups (SacSwim and SacSwim-Rinse) and the combined data of the two control groups (WaterSwim and NoSwim), Welch's $t(24) = 3.06$, $p = 0.005$, two-tailed, $r = 0.53$.

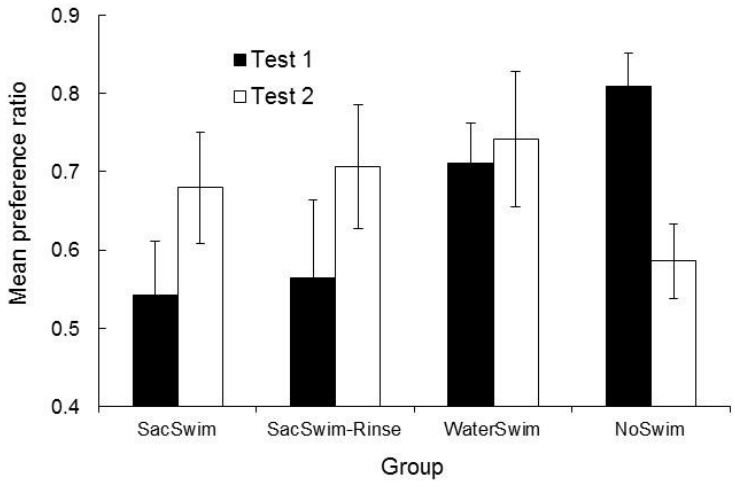

Figure 1. Test performance of rats in Experiment 1, as the mean target preference ratio, in the form of x / (x + y), where x is the intake of the target flavor (sweet water) and y is that of tap water. Error bars indicate standard errors. In the prior training phase of four days, Groups SacSwim and SacSwim-Rinse swam in sweet water for 20 min per day. Immediately after each swimming session, Group SacSwim-Rinse was rinsed off with tap water. Group WaterSwim swam in tap water of the same duration, and Group NoSwim was kept in the home cages in the training phase.

The mean (± SE) total amounts of fluid intake (sweet water and tap water) were 19.9 ± 1.0, 19.0 ± 1.1, 20.8 ± 1.1, and 18.0 ± 0.6 g, for the SacSwim, SacSwim-Rinse,

WaterSwim, and NoSwim, respectively, on the first test day, and the corresponding values were 20.6 ± 1.0, 19.3 ± 1.0, 21.0 ± 1.2, and 20.3 ± 0.6 g, on the second test day. A 4 (group) × 2 (day) ANOVA yielded a significant main effect of day, $F(1, 28) = 9.78$, $p = .004$, $\eta_p^2 = 0.26$, but the main effect of group, $F < 1$, and their interaction, $F(3, 28) = 2.88$, $p = 0.054$, $\eta_p^2 = 0.24$, were not significant.

Discussion

The test results indicate that, as expected, swimming in flavored water leads to flavor avoidance learning in laboratory rats, because rats with experience of swimming in sweet water avoided sweet water more than did control rats that swam in tap water or did not swim. Parenthetically, backward conditioning played no critical role in the present setting, because rinsing the rats after their swim had no effect.

Despite the positive outcome, the present experiment has a few shortcomings. First, the amount of conditioning effect was quite small. The mean target preference ratio was greater than 0.5 in Groups SacSwim and SacSwim-Rinse, suggesting that sweet water was drunk more than tap water even in these experimental groups. Thus, readers must recognize that *flavor avoidance* is defined in terms of relative (rather than absolute) unwillingness of taking a target flavor (i.e., sweet water) in the experimental rats compared with the control rats showing unconditioned strong preference for the sweet water.

Second, the conditioning effect was short-lived and feeble. A statistically reliable group effect was observed in only the first test session. In addition, although the planned comparison confirmed that rats in the two experimental groups showed a lower flavor preference ratio than rats in the two control groups, post hoc analysis of the original ANOVA failed to find significant paired contrasts between the four groups of rats.

Third, this experiment failed to demonstrate that the observed avoidance was specific to the trained flavor. Therefore, the rats that swam in flavored water might have shown avoidance reaction to any type of flavored water. Although there is no rationale for such unspecified flavor avoidance, this possibility is worth considering.

Finally, the amount of exposure to sweet water was not equated between the experimental and control rats. The total duration of exposure to sweet water was 95 min (15 min in the adaptation phase and 20 min × 4 times in the conditioning phase) for Groups SacSwim and SacSwim-Rinse, while it was only 15 min in the adaptation phase for Groups WaterSwim and NoSwim. Usually, familiarization to a target flavor increases its preference; thus, this discrepancy in the amount of exposure would have acted against the detection of conditioned flavor avoidance in this experiment. Therefore, matching the amount of flavor exposure between experimental and control conditions is more suitable to demonstrate flavor avoidance learning.

Experiment 2

In considering the shortcomings of Experiment 1, another experiment was conducted to ensure flavor avoidance learning in swimming rats. Experiment 2 employed a differential conditioning procedure to confirm flavor specificity and to equate the amount of exposure to a target flavor between experimental and control conditions. Specifically, half of the rats swam in water flavored with one of two tastants (sour or bitter) and drank water flavored with the other tastant from the bottles provided, while the activity-tastant combinations were reversed for the remaining rats. The number of subjects per group was increased to 12 to increase statistical power.

Method

Subjects

Twenty-four male Jbc:Wistar rats were employed in Experiment 2. Although these animals had been subjects of an unrelated experiment, they were maintained under *ad libitum* food and water conditions in their home cages without any special treatment for three weeks before Experiment 2. These rats had no experience of swimming and were naïve to the flavors employed here. In addition, the treatments used in the present experiment were orthogonal to the rats' previous histories. The rats were 13 weeks old with a mean weight of 477.0 g (range: 413–538 g) on the first day of the adaptation phase. The housing and maintenance conditions were identical to those of Experiment 1. Standard rat chow was always available in the home cages, but water was restricted to the daily sessions in the experimental room.

Apparatus

The experimental room had now 12 drinking cages, 12 pools, and no polycarbonate boxes. The pools were filled with sour water (0.1% citric acid solution) or bitter water (25 ppb denatonium benzoate solution) of 35 L. All other details were identical to those of Experiment 1.

Procedure

All experimental sessions were conducted on successive days, as follows.

Adaptation. On the first two days (Days 1–2), each rat was adapted to drinking tap water from a bottle for 20 min: the sessions were conducted on two squads of 12 rats each, with the first starting at 1120 h and the second at 1145 h. The two-bottle, empty-vs.-water adaptation of Experiment 1 was not administered in Experiment 2. Preexposure to the target flavor was not necessary, because the design of Experiment 2 equated the number of exposures to the target and control flavors in the next training phase. The rats were then assigned to one of two groups of 12 rats each, and matched for water intake and bodyweight.

Training. All rats received a four-day conditioning treatment (Days 3–6) of 30 min starting at 1130 h in a single squad of 24 rats. On Days 3 and 5, rats in Group SourPool swam in pools of sour water, while rats in Group BitterPool drank sour water from the bottle. On Days 4 and 6, the former drank bitter water from the bottles while the latter swam in pools of bitter water. In other words, swimming and drinking days were alternated with the flavored waters designed for each group. The rats' average intakes (± SEs) of sour and bitter solutions from the bottles were 11.9 ± 0.6, 17.7 ± 0.6, 17.7 ± 0.7, and 18.7 ± 0.7 g, respectively, for Days 3 (sour), 4 (bitter), 5 (sour), and 6 (bitter).

Testing. Two-bottle choice testing of 20 min was administered on the next day (Day 7) for all rats. One bottle contained the sour water, while the other contained the bitter water; the left-right positions of the bottles were counterbalanced across rats in each group. The session was conducted on two squads of 12 rats each with the first starting at 1120 h and the second at 1145 h, as in the adaptation days. Each squad consisted of six rats each from the two groups.

Measurement and Analysis

The measurement and analyses methods were similar to those of Experiment 1. Because the two flavored water bottles were concurrently presented in the choice test, the preference ratio was calculated as the ratio of sour-water intake to total fluid intake (sour water + bitter water): the lower the ratio value, the stronger the sour-water avoidance estimated. This index was adopted rather than the ratio of the target flavor of swimming to the total fluid intake, because the rats had a strong unconditioned bias in preference between the sour and bitter flavors employed here. The hypothesis tested was that there would be a low ratio (strong sour avoidance) observed in Group SourPool compared with Group BitterPool. The test squad factor was not included in the following analyses, because this factor had no main or interactive effect on the results in the preliminary analyses.

Results and Discussion

The choice test clearly showed that Group SourPool avoided the sour bottle more than did Group BitterPool (Figure 2). This observation was statistically supported, $t(22) = 2.21$, $p = 0.038$, two-tailed, $r = 0.43$. The average (± SE) total amounts of fluid intake (sour water and bitter water) were 16.8 ± 0.4 and 21.0 ± 0.8 g for Groups SourPool and BitterPool respectively, and was statistically significant, Welch's $t(18) = 4.64$, $p < 0.001$, two-tailed, $r = 0.74$. Inspection of the intake data of the two flavors suggest that the greater intake in Group BitterPool was due to the rats' general preference for bitter water over sour water. This bias could have prevented expression of conditioned bitter avoidance in Group BitterPool, while the unconditioned dislike of sour water could have facilitated conditioned sour avoidance in Group SourPool.

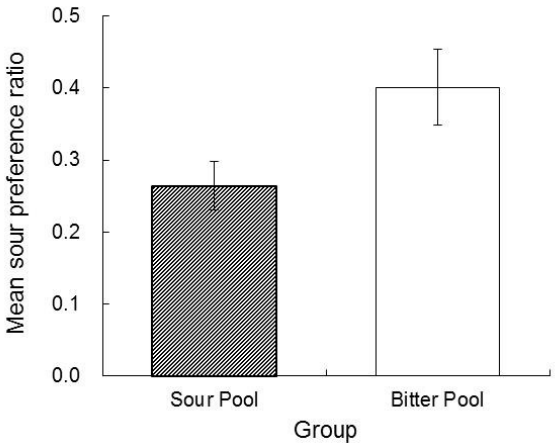

Figure 2. Test performance of rats in Experiment 2, represented by mean sour preference ratio in the form of x / (x + y), where x is the intake of sour water and y that of bitter water. Error bars indicate standard errors. On two of the four-day training phase, Groups SourPool and BitterPool, respectively, swam in sour and bitter water for 30 min. The rats were also given the alternate flavored water for 30 min through the bottles in the remaining two training days.

General Discussion

The purpose of this study was to develop an easy procedure to establish flavor avoidance learning based on swimming activity. This objective was partially accomplished because statistically significant effects were obtained in two experiments with disparate conditioning procedures with different flavor cues: simple conditioning with sweet (sodium saccharin) flavor in Experiment 1, and differential conditioning with sour (citric acid) and bitter (denatonium benzoate) flavors in Experiment 2. The attempt, however, was not fully satisfactory, because the effects obtained in these experiments seem not as robust as expected from our previous reports on conditioned flavor avoidance produced by forward conditioning procedures (Masaki & Nakajima, 2004a, 2004b, 2005, 2006, 2010; Nakajima, 2004; Nakajima & Masaki, 2004).

For example, the means of saccharin preference ratio, where was observed in the forward conditioning groups having four conditioning trials of 15-min saccharin intake followed by 20-min swimming, range from 0.13 to 0.44 with the grand mean (± SE) of 0.32 ± 0.04 (based on the mean values of seven proper groups from Masaki & Nakajima, 2005, Experiments 3 and 4; Masaki & Nakajima, 2010, Experiments 1–5). Compared to these scores, 4-trial training of 20-min swimming in saccharin water resulted in much weaker saccharin avoidance in Experiment 1 of the present research (see the performance of Groups SacSwim and SacSwim-Rinse shown in Figure 1). Furthermore, the effect was statistically reliable only on the first test day in Experiment 1, while it was stable across two test days in our aforementioned studies. The weak and feeble flavor avoidance observed in Experiment 1 is partly due to latent inhibition caused by exposure to the target flavor solution (on Day 2) prior to the conditioning treatment (see Lubow, 2009, for latent inhibition in flavor avoidance learning). However, this factor was absent in Experiment 2, where the conditioning effect was still small.

As aptly pointed out by Rescorla (1981), simultaneous conditioning suffers from some non-temporal factors that degrade associative learning. If we apply his argument to the present experimental setting, then the smallness of effect observed in this study may have been due to insufficient perceptual processing of flavors and swimming in training, perhaps due to the large stimulus interaction among them (e.g., mutual masking) or divided attention in the rats. One may also attribute the smallness of effect to the generalization decrement of conditioned flavor avoidance, because the test situation substantially differed from the training situation; the rats had to drink flavored water from the bottles instead of drinking flavored water around their bodies in the pools.

Of course, the temporal factor may have also affected the associative learning observed here. It is worthy to mention here that the temporal relationship between a target CS and the activity US is critical to determine the polarity of conditioned responses at least with a wheel-running US. In running-based flavor learning, forward (i.e., CS-US) and backward (i.e., US-CS) conditioning procedures, respectively, yielded conditioned flavor avoidance and preference (Hughes & Boakes, 2008; Salvy, Pierce, Heth, & Russell, 2004). A similar relationship can be observed in conditioning with a place CS and a running US: the CS-US sequence produces place avoidance (Masaki & Nakajima, 2008), while a US-CS sequence results in place preference (Belke & Wagner, 2005; Lett, Grant, Byrne, & Koh, 2000; Lett, Grant, & Koh, 2001, 2002; Lett, Grant, Koh, & Smith, 2001). Notably, a study has reported that simultaneous presentation of a

place CS and a running US led to place preference in golden hamsters (Antoniadis, Ko, Ralp, & McDonald, 2000). On the other hand, the flavor avoidance, rather than preference, was demonstrated in the present study by a simultaneous presentation of a flavor CS and a swimming US. This discrepancy might be due to the difference in the US activity type (swimming vs. running), the CS type (flavor vs. place), and/or species (rat vs. hamster). Otherwise, as Masaki and Nakajima (2008) discussed within the opponent process theory of motivation (Schull, 1979; Solomon, 1980; Solomon & Corbit, 1974), running-based place preference by simultaneous conditioning (Antoniadis et al., 2000) might be a product of their subjects' long history of wheel running. If this were the case, then simultaneous flavor-swimming might have led to flavor preference, instead of avoidance, with a long history of swimming before the conditioning.

The largest enigma of swimming-based flavor avoidance learning is its underlying physiological mechanism. We have claimed that energy expenditure caused by swimming or running activity yields flavor avoidance in rats (e.g., Nakajima & Masaki, 2004). This hypothesis is now shaky, because the energy expenditure seems neither necessary nor sufficient at least for running-based flavor avoidance learning (Nakajima, 2011; Nakajima, Kumazawa, Ieki, & Hashimoto, 2012). On the other hand, a growing body of evidence is accumulating to show that gastrointestinal discomfort (e.g., nausea) is critical for running-based flavor avoidance learning (Dwyer, Boakes, & Hayward, 2008; Eccles, Kim, & O'Hare, 2005; Nakajima & Katayama, 2014; Nakajima, Urata, & Ogawa, 2006). Future work will reveal whether this is also the case for swimming-based flavor avoidance learning. Elucidation of the underlying physiological mechanism perhaps gives a hint of this learning phenomenon in rats, and it possibly answer why the procedure employed in the present study was weakly effective.

Before closing the article, it may be well to briefly discuss ecological significance of swimming for rats. Although swimming is a natural escape behavior from water to a stable place (e.g., Glaser, 1910; Morris, 1981), it can also be a positively reinforced instrumental activity. For example, wild brown rats (*Rattus norvegicus*) living in wetlands swim and dive to catch food, such as mollusks, inhabiting the river bottom (e.g., Gandolfi & Parisi, 1973). Galef (1980) has demonstrated that this diving behavior is trainable with chocolate pellets (and socially transmittable) in wild and laboratory rats, if they were young. In the present study, thirsty rats placed into a water pool could have produced preference for the flavor cues associated with the swimming activity. This rewarding property of swimming activity might be a reason why conditioned flavor avoidance was weak in the present study. The rat-in-flavored-water procedure, thus, departs from other conditioned flavor avoidance preparations, that are based on the rat's ability to identify and avoid dangerous substances. This unique property of the new procedure merits further scrutiny.

Acknowledgments

This study was supported by JSPS KAKENHI grants (C21-530779 and C24-530931) to the author.
The author declares no conflict of interest.

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Financial Support: This study was supported by JSPS KAKENHI grants (C21-530779 and C24-530931) to the author.
Conflict of Interest: The author declares no conflict of interest.

Submitted: *July 29th, 2014*
Resubmitted: *November 15th, 2014*
Accepted: *December 20th, 2014*