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Common Toads (*Bufo arenarum*) Learn to Anticipate and Avoid Hypertonic Saline Solutions

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Toads (*Bufo arenarum*) were exposed to pairings between immersion in a neutral saline solution (i.e., one that caused no significant variation in fluid balance), followed by immersion in a highly hypertonic saline solution (i.e., one that caused water loss). In Experiment 1, solutions were presented in a Pavlovian conditioning arrangement. A group receiving a single neutral-highly hypertonic pairing per day exhibited a greater conditioned increase in heart rate than groups receiving either the same solutions in an explicitly unpaired fashion, or just the neutral solution. Paired toads also showed a greater ability to compensate for water loss across trials than that of the explicitly unpaired group. Using the same reinforcers and a similar apparatus, Experiment 2 demonstrated that toads learn a one-way avoidance response motivated by immersion in the highly hypertonic solution. Cardiac and avoidance conditioning are elements of an adaptive system for confronting aversive situations involving loss of water balance.

Keywords: heart-rate conditioning, avoidance conditioning, hypertonic saline reinforcer, toads

Most of the experiments concerning aversive conditioning in vertebrates use peripheral pain induced by electric shock as the reinforcer (Brush, 1971). Although orderly functions have been reported between electric shock parameters and acquired responses, for example, in rats (Campbell & Masterton, 1969), electric shocks have proven relatively ineffective to study learning in anuran amphibians (for a review, see Macphail, 1982; Muzio, 1999; Suboski, 1992). In a particularly striking demonstration of this failure, McGill (1960) exposed seven leopard frogs (*Rana pipiens*) to electric shocks in a shuttle box and found not only that escape latencies increased (rather than decreased), but also that all seven frogs died either in the course of the experiment or shortly after it had been discontinued. Boice (1970) reported more encouraging results in a one-way avoidance experiment with four anurans species. On the assumption that shuttle box performance depends on the natural repertoire of behaviors, it was expected that species that exhibit active behavioral strategies in their natural environment would show better performance than normally inactive species. For each species, the performance of an escape-avoidance group was compared with that of a yoked control receiving the same amount of shock. In the two active species (*Bufo woodhousei* and *R. clamitans*), avoidance responses were more frequent in the experimental than in the yoked control group, whereas the two passive species (*R. pipiens* and *Scaphiopus hammondi*) exhibited no avoidance at all. Still, the two species that exhibited one-way avoidance performed at relatively low levels, reaching 50% and 10% avoidance after 200 training trials (*B. woodhousei* and *R. clamitans*). Crawford and Langdon (1966) also reported increased one-way avoidance behavior within a series of 20-trial sessions, but little or no change across sessions in an experiment with *B. terrestris*. Only one previous experiment has been conducted in *B. arenarum* in a two-way escape learning situation with electric shock (Schmajuk & Segura, 1980). In this study, toads showed increased escape behavior after 50 trials.

Alternatives to electric shock have been used to produce efficient escape learning in a variety of species. In rodents, for example, the Morris water maze has been used extensively in studies of escape conditioning (e.g., Gerlai, 2001). A similar task was implemented by Bilbo, Day, and Wilczynski (2000) in a study with leopard frogs (*R. pipiens*). Frogs were released into a circular tank with water maintained at 30 °C. Because these frogs prefer cooler temperatures, they learned to step on a visible platform, producing variable but generally decreasing escape latencies. Although pilot studies had apparently shown that these frogs are unable to learn this escape task when the platform is hidden (as it is usually done in the Morris water maze task with rodents), this type of task may offer a viable alternative to study aversive learning in amphibians. In a similar fashion, aversive learning is particularly effective in rodents when chemical stimuli are used, as in the flavor aversion procedure (Bernstein, 1999). Flavor aversion has been used effectively in lizards (Day, Crews, & Wilczynski, 1999). However, it is surprising to note that two amphibian species

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(the toad *B. paracnemis* and the newt *Pachytriton breviceps*) receiving pairing of a novel food with lithium chloride showed no evidence of aversion to flavor (Paradis & Cabanac, 2004).

As described below, the aversive learning experiments reported in this article make use of the toad's ability to respond to fluid salinity, a response system with clear ecological value in amphibians. Water salinity is an important source of physiological stress for amphibians because of the risk for dehydration. However, many species of Amphibia, both Anura and Urodela, are capable of tolerating a moderately saline environment (Balinsky, 2005). Furthermore, some anuran species are adapted to breed in brackish environments, showing variation in tolerating water salinity (Gõmez-Mestre & Tejedo, 2003).

The two experiments reported here were designed to exploit the toad's (*B. arenarum*) sensitivity to detecting the composition of fluids through their ventral skin, also used for absorption during the process of rehydration (Christensen, 1974; Reboreda, Muzio, Viñas, & Segura, 1991; Schmajuk & Segura, 1982). Absorption of water has been shown to be an efficient reinforcer in appetitive learning experiments. For example, speeds of acquisition and extinction of a runway response are directly related to the amount of deionized water available at the goal box (Muzio, Segura, & Papini, 1992). Sodium solutions can also be used to generate different behavioral effects (Loza Coll, 1998; Pistone Creydt, 2000). Because the concentration of sodium chloride (NaCl) in the toad's internal milieu is approximately 115 mM (Reboreda et al., 1991), solutions of NaCl ranging between 0-250 mM (hypotonic to slightly hypertonic solutions) are associated with weight gain through rehydration and to the emission of appetitive behaviors, such as approach and contact with these solutions. In contrast, NaCl solutions ranging between 350–1000 mM (highly hypertonic solutions) lead to weight loss and are correlated with aversive behaviors, such as escape responses. Between these two extremes, the 300 mM of NaCl solution (a moderately hypertonic solution) leads to neither weight gain nor weight loss and is thus considered a "neutral solution" for variation in weight (Loza Coll, 1998). In addition to its reinforcing properties, NaCl solutions of different molarities result in different degrees of absorption or loss of fluids that can be assessed in the difference between pretrial and posttrial weights. Previous research indicates that absorption of water in the runway training situation improves with practice (e.g., Muzio et al., 1992), which suggests that learning plays a role in fluid balance. Changes in weight during training trials may be used as an index of the functional value of learning (Domjan, 2005; Hollis, 1982). For example, by comparing absorption of water in toads receiving either paired or unpaired trials, it is possible to determine the extent to which the presence of a signal for a highly hypertonic solution resulting in dehydration allows the toad to compensate for and prevent loss of water.

This paper reports the results of two experiments on the ability of toads to detect the concentration of several saline solutions, using a neutral NaCl solution as an anticipatory signal for a highly hypertonic (aversive) NaCl solution. This procedure yielded evidence of avoidance learning in amphibians, a phenomenon difficult to obtain in these animals. The effectiveness of saline solutions to induce avoidance training is probably related to its greater ecological relevance compared to that of other aversive reinforcers commonly used in past experiments. Conditioning was assessed in cardiac acceleration (Experiment 1) and avoidance behavior (Experiment 2). The use of heart rate as a measure of anticipatory responding in toads is uncommon, but its effectiveness suggests that it may be a profitable procedure to measure aversive conditioning in amphibians.

Experiment 1: Autonomic Conditioning

A pilot experiment had shown that cardiac acceleration was the response to the inescapable exposure to a hypertonic solution (800 mM of NaCl). Such a response would be a prerequisite for the escape behavior ensuing after exposure to a dehydrating solution. By arranging a sequential presentation of two solutions, one neutral and the other hypertonic, the present procedure allows for an assessment of conditioning in an autonomic (cardiac) response. This technique also permits an evaluation of the functional significance of Pavlovian conditioning in the context of water balance.

Method

Animals. The animals were 18 male, sexually mature, experimentally naive toads (*Bufo arenarum*). These animals were captured in ponds around Buenos Aires, Argentina, treated with antibiotics to prevent bacterial and parasite infestations, and kept in group cages with running tap water during the initial 2 weeks after arrival in the laboratory. Standard weights (the weight of a hydrated animal with its urinary bladder empty; Ruibal, 1962) were obtained the day before pretraining. The weights varied between 80 and 140 g and were not statistically different across groups, $F < 1$. The vivarium was kept at a constant temperature (21–23 °C) and humidity (48%–52%), and were subject to a 16:8 hr light: dark cycle (lights on at 03:00 hr). Toads were transferred to individual cages before the start of the experiment. Toads were at about 80% of their standard weights at the start of each pretraining and training session.

Apparatus. The experimental device was a black Plexiglas chamber (0.15 × 0.15 × 0.20 m, L × W × H) connected to a hydraulic system. This system consisted of an external recipient full of the specific solution connected to the chamber by a flexible tube that allowed for the rapid presentation and draining of the solution during the trial. The chamber had a wire mesh floor. When the solution was presented, the toad's ventral skin made contact with it. Toads sat on a 0.5-cm-deep fluid container. Containers were filled from the bottom. The chamber was covered with a translucent Plexiglas lid that allowed for constant observation of the animals through a mirror. Cardiac responses were registered with a pressure transducer (S72-25 Model, Coulbourn Instruments, Allentown, PA) connected to a recording device linked to a personal computer. Training was carried out in an experimental room kept at a constant temperature and humidity (21–23 °C, 50%). Masking white noise (20 Hz–30 kHz) was present during training trials.

Procedure. Two days before the start of the experiment, all toads were anesthetized with ether and a permanent cannula was surgically implanted in the dorsal aorta. This cannula was connected to the pressure transducer to record heart rate during training trials. All toads received two 5-min trials (1 per day) of pretraining. Five minutes before the start of each pretraining trial, the toads were taken to the experimental room. During these trials, the animals were free to move about in the experimental chamber. No stimuli were presented during these two pretraining trials.

Training started on the following day. Four training trials, one per day, were administered to all animals. One hour before the start of each trial, toads were placed in individual plastic containers without access to water; the container was moved to the experimental room about 15 min before the start of the trial. Toads were randomly assigned to one of three groups ($n = 6$). Toads assigned to Group Paired received the following sequence of events: (a) habituation to the chamber, 120 s; (b) presentation of a neutral solution (300 mM of NaCl) as the conditioned stimulus (CS), 120 s; (c) draining of the chamber, 30 s; (d) presentation of a highly hypertonic solution (800 mM of NaCl) as the unconditioned stimulus (US), 120 s; (e) draining, 30 s; and (f) final washing with deionized water, 180 s. Toads assigned to Group CS-only received the same sequence of events as the Group Paired, except that in step (d), they were exposed to the neutral solution (300 mM of NaCl) instead of the hypertonic one. Toads assigned to Group EUP (explicitly unpaired) received the same sequence as Group Paired, except that in step (c), a period of 180 s was interpolated between the CS and the US, instead of the 30 s used in the other groups. No solution was present during this period. The delay in 30 s for pairing and the delay in 80 s for preventing pairing were values derived from preliminary studies.

Each of the four trials started when a toad was placed in the experimental chamber, with the cannula connected to the registration device to measure the cardiac response (beats/min). Scores of heart rate were accumulated in blocks of 10 s. Uptake of water was also recorded by subtracting the weight of each toad after the trial from its weight before the trial. The difference was then divided by the standard weight of that animal and multiplied by 100 to provide a relative measure of water uptake adjusted for body weight. These two dependent variables were subjected to analysis of variance (ANOVA) with repeated measures, followed by pairwise comparisons of the groups based on the least significant difference (LSD) test. In all cases, for these comparisons significance was evaluated by setting the alpha value at the 0.05 level.

Results and Discussion

Figure 1 presents the cardiac response recorded for each group in each of the four trials. Each data point is the average heart rate for any given group over a block of 10 s. The main data come from the three periods, including the CS period, the draining that followed, and the reinforcer (US) periods. Trial 1 exhibited the following features. Presentation of the neutral saline solution (CS) generated an initial increase in the cardiac activity for all three groups, returning to the basal levels during the last 30 s. The analysis indicated a significant group by time effect, $F(22, 165) = 2.60$, $p < .001$, a significant decrease of heart rate in time, $F(11, 165) = 9.46$, $p < .001$, but a nonsignificant group effect, $F < 1$. Because of the significant interaction, individual one-way analyses were computed on the last three 10-s blocks of the CS period to verify whether there were group differences at the end of this period. Nonsignificant effects were found in all three blocks (block 210: $F < 1$; block 220: $F(2, 15) = 1.64$, $p = .22$; block 230: $F(2, 15) = 2.34$, $p = .13$). During the draining period of 30 s, none of the factors of the Group x Time analysis yielded significant differences (Group: $F < 1$; Time: $F(2, 30) = 2.37$, $p = 0.11$; Interaction $F < 1$). The presentation of the highly hypertonic saline solution (US) generated an initial increase in the cardiac activity for Groups Paired and EUP; there was also an increase for Group CS-only, suggesting that the mere draining and replenishment of the container with the neutral solution was enough to affect heart rate. However, heart rate decreases faster in Group CS-only than in the other two groups. The statistical analysis of the entire US period showed a significant group by time interaction, $F(22, 165) = 3.69$, $p < .001$, but nonsignificant effects for groups, $F(2, 15) = 3.51$, $p < .06$, and time, $F < 1$. The analysis on the last

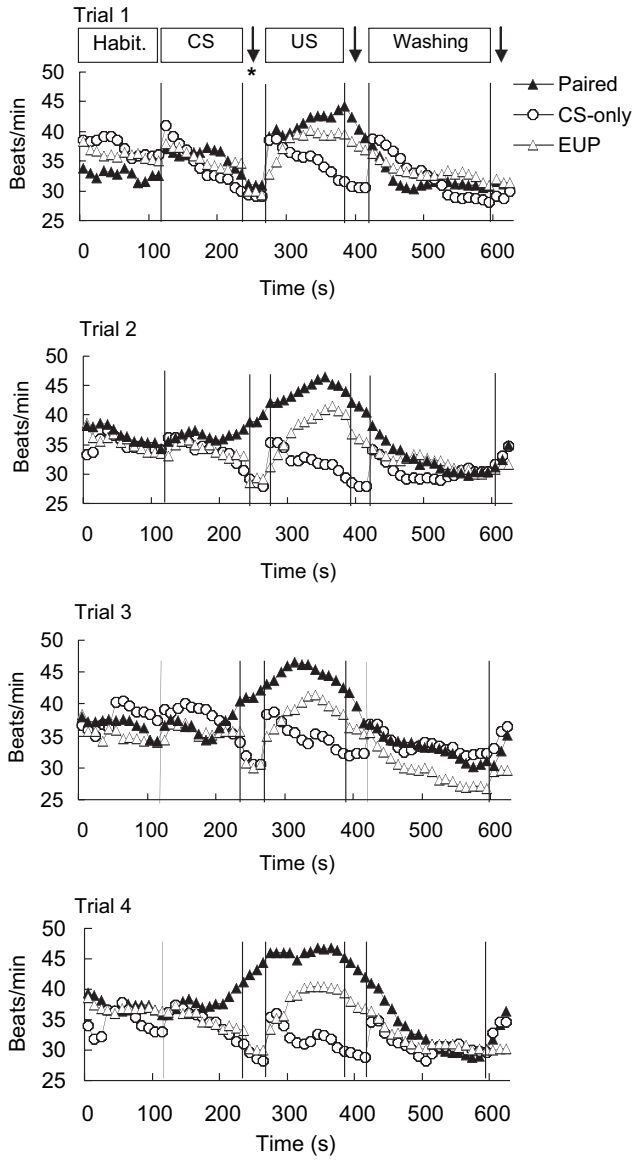

Figure 1. Mean cardiac response (beats per minute) during Trials 1–4, from top to bottom. The first 2 min of the trial were left to reach the heart rate basal level before exposure to the stimulus solutions. Solid lines separate the sequence of events as follows: Habit: habituation; CS: conditioned stimulus (300 mM of NaCl solution); US: unconditioned stimulus (800 mM of NaCl solution); Washing: draining the saline solution and replacing it with deionized water; arrows: the period of 30 s during which the saline solution was discharged from the container; an asterisk indicates the interpolation of 180 s between the CS and US for Group EUP.

10 s of the US period revealed a significant difference between groups, $F(2, 15) = 17.9$, $p < .001$. Post hoc pairwise comparisons corroborated that Group CS-only was significantly lower than Group Paired, $p < .001$; all other comparisons were also significant, all ps $< .05$. During the final washing period, heart rate recovered to baseline for all the groups.

The effects observed in heart rate during Trials 2 and 3 were the same, with one major exception: Heart rate in Group Paired increased in the 30 s of draining just before the introduction of the highly hypertonic solution. This increase extended into the CS period during Trial 4, as shown in Figure 1.

A similar set of analyses were calculated on the data of Trial 4. For the CS period, the analysis yielded a significant group by time interaction, $F(22, 165) = 4.14$, $p < .001$, and a significant change across time blocks, $F(11, 165) = 2.50$, $p < .007$. The difference between groups was nonsignificant, $F < 1$. Given the significant interaction and the fact that functions diverged during the last 30 s of the CS period, independent one-way analysis were computed on the last three blocks of 10 s. There were no group differences in block 210, $F(2, 15) = 2.83$, $p > .09$, but significant group effects in blocks 220 and 230 (block 220: $F(2, 15) = 6.79$, $p < .008$; block 230: $F(2, 15) = 8.07$, $p < .005$). Post hoc pairwise comparisons confirmed that heart rate was significantly higher for the Group Paired than for the Group CS-only (block 220: $p = .003$; block 230: $p = .002$). Heart rate was also significantly higher for the Group Paired than for the Group EUP (block 220: $p = .013$; block 230: $p = .008$). The group difference that emerged during the final 20 s of the CS period was maintained and enhanced during the discharge period that followed. The Group x Time analysis yielded a significant interaction effect, $F(4, 30) = 5.49$, $p < .002$. The groups were also significantly different, $F(2, 15) = 19.29$, $p < .001$, but there were no changes across time, $F < 1$. Post hoc pairwise tests demonstrated that the source of the group effect was the difference between the Group Paired and the other two groups, both ps < 0.001, which, in turn, did not differ from each other, $p < .64$. It is interesting to note that pairing experience also affected the heart rate during exposure to the hypertonic solution (US). The increase in heart rate was highest for Group Paired, intermediate for Group EUP, and lowest for Group CS-only-exposed again to the neutral solution. The analysis showed a significant group by time interaction, $F(22, 165) = 3.52$, $p < .001$, and a significant group effect, $F(2, 15) = 11.03$, $p < .002$; no differences were found across time blocks, $F < 1$. Post hoc pairwise comparisons revealed significant differences between all pairs of groups, all ps $< .05$. Heart rate recovered to baseline levels during the final washing period.

A comparison between the scores of Trials 1 and 4 for the three groups was conducted. Trial x Time repeated-measures ANOVAs were computed on the last three blocks of 10 s from the CS period and draining period for each of the three groups. For the Paired group, the analysis yielded the following results. During the last 30 s of the CS period was a significant trial-by-time effect, $F(2, 20) = 11.42$, $p < .001$, but nonsignificant effects for trial, $F(1, 10) = 3.75$, $p > .08$, and for time, $F < 1$. Because of the significant interaction, individual one-way analyses were computed on the last three blocks of 10 s of the CS period to determine whether there were trial differences at the end of this period. There was a significant effect only for the last block (block 230: $F(1, 10) = 6.94$, $p < .03$). Nonsignificant effects were found in the other two blocks (block 210: $F(1, 10) = 1.19$, $p > .29$; block 220: $F(1, 10) = 4.25$, $p > .07$). The trial difference that emerged during the final 10 s of the CS period was maintained and enhanced during the draining period at 30 s. The Trial x Time analysis yielded a significant interaction effect, $F(2, 20) = 5.07$, $p < .02$. The trials were also significantly different, $F(1, 10) = 15.16$, $p < .003$, and the time showed also significant differences, $F(2, 20) = 3.84$, $p < .04$.

For Group EUP, the analysis of the last 30 s of the CS period showed a significant trial-by-time effect, $F(2, 20) = 5.22$, $p < .02$, but nonsignificant effects for trial and time, Fs < 1. Because of the significant interaction, individual one-way analyses were computed on the last three blocks of 10 s of the CS period to determine whether there were trial differences. Nonsignificant effects were found in all three blocks (block 210: $F < 1$; block 220: $F(1, 10) = 1.68$, $p = .22$; block 230: $F(1, 10) = 1.51$, $p = .25$). During the discharge period there were nonsignificant effects for trial, $F < 1$, for time $F(2, 20) = 3.39$, $p > .05$, and for the interaction, $F(2, 20) = 1.61$, $p > .23$.

For Group CS-only, the analysis of the last 30 s of the CS period only indicated a significant time effect, $F(2, 20) = 8.44$, $p < .003$, but nonsignificant effects for trial and time, Fs < 1. During the draining period of 30 s were nonsignificant effects for trial, $F < 1$, for time $F(2, 20) = 3.03$, $p > .07$, and for the interaction, $F(2, 20) = 1.97$, $p > .16$. From a global standpoint, the difference observed in the scores of the Paired but not in those of the other two groups indicates that the paired condition learned to anticipate the reinforcer.

Figure 2 shows data on water uptake for the three groups across trials. The dependent variable reflects changes in weight for the entire trial. Notice, first, the variations in weight recorded after Trial 1. Inescapable exposure to the highly hypertonic saline solution resulted in a greater weight loss for the Groups Paired and EUP than for the Group CS-only. Nonetheless, the latter group, which was never exposed to the highly hypertonic solution, showed some loss. Interesting changes occurred across the subsequent trials. Group Paired, for example, went from net weight loss to almost no loss in Trial 4, despite extensive exposure to the highly hypertonic solution. In contrast, Group EUP, exposed to the same amount of highly hypertonic solution, exhibited no indication of a compensatory response and continued to lose weight across all four trials. Finally, Group CS-only maintained and slightly increased the average weight across trials. A Group $\times$ Trial repeated-measures ANOVA provided support for this description of the results in a significant interaction, $F(6, 45) = 4.30$, $p < .003$. There were also significant differences across groups, $F(2, 15) = 8.23$, $p < .005$, and across trials, $F(3, 45) = 8.57$, $p < .001$. One-way analyses for each trial indicated that the source of the overall interaction effect was in group differences in Trials 3 and 4, Fs$(2, 15) > 37.33$, all ps $< .001$. Post hoc LSD tests demonstrated significant differences for all pairwise comparisons, all ps $< .008$, except for the difference between Groups Paired and CS-only on Trial 3, $p < .053$.

The cardiac data recorded during Trial 4 revealed the Pavlovian conditioning of an autonomic response. The difference between Groups Paired and EUP, coupled with the use of a single trial per day, eliminates the possibility that changes in responding to the CS are the result of nonassociative processes such as sensitization or pseudoconditioning (Papini, 1998). The difference between

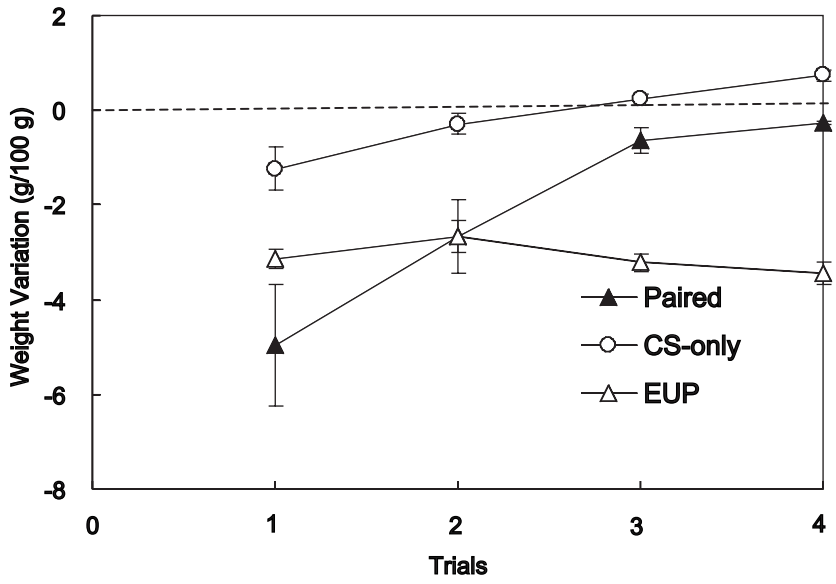

Figure 2. Amount of variation in body weight corrected for individual differences in body weight as a function of training trials in each of three groups. The dashed line represents no variation in body weight. Group Paired received temporally contiguous exposure to the neutral solution (CS) and the highly hypertonic solution (US). Group CS-only received exposure to the neutral solution in both periods. Group EUP received exposure to both CS and US solutions, but a temporal delay of 180 s was interpolated between the two. Means and confidence intervals ($\alpha = .5$) are plotted.

Groups Paired and CS-only indicates that changes during the CS are not the simple result of the various changes in circulation of fluid in the conditioning chamber. Toads are able to predict the impending presentation of an inescapable, aversive, highly hypertonic solution and demonstrate this learning as an anticipatory increase in the heart rate during the CS and discharge periods immediately before the reinforcer period. As the results of the water uptake demonstrate, the adaptive function of this type of Pavlovian conditioning is to enable the toad to compensate for the loss of water that will result from inescapable exposure to a highly hypertonic solution. After only four trials, the toads in the Group Paired were able to prevent water loss just as efficiently as a group never exposed to the highly hypertonic solution (Group CS-only). In contrast, the temporal separation between CS and US in Group EUP generates a constant loss of water during the trial.

Experiment 2: One-Way Avoidance Conditioning

The goal of Experiment 2 was to determine whether conditions similar to those leading to autonomic conditioning in Experiment 1 also support the development of one-way avoidance conditioning. In Experiment 2, toads were allowed free movement in the conditioning chamber. We hypothesized that the Pavlovian contingency implemented in Experiment 1 leads to the acquisition of an internal aversive state (revealed in Experiment 1 as changes in heart rate), that can provide reinforcement for instrumental responses of escape and avoidance (Mowrer, 1947). Toads received one daily trial of shuttle-avoidance training (for spaced-trial avoidance training, see Portavella, Salas, Vargas, & Papini, 2003; Portavella, Torres, Salas, & Papini, 2004). The warning signal (WS) and reinforcer were the same CS and US used in Experiment 1. The shuttle response consisted in the option of moving from the chamber associated with the highly hypertonic solution to a safe compartment never paired with the aversive solution. This safe compartment was added to the basic apparatus used in the previous experiment. Failure to respond led to contact with the aversive saline solution.

Method

Animals. The animals were 17 male toads, obtained and maintained as described in the previous experiment. They were sexually mature and experimentally naïve. The standard weights varied between 70 and 137 g and were not statistically different across groups, $F < 1$. Other conditions of maintenance were as described in Experiment 1.

Apparatus. The experimental device was a two-chamber, one-way shuttle box. Both Plexiglas chambers ($0.15 \times 0.15 \times 0.20$ m, L × W × H) were connected to a hydraulic system that allowed for the presentation and draining of the appropriate solutions during the trial. The chambers were separated by a sliding door and a barrier (15×3 cm, L × H). A shuttle response required that the toad cross over the barrier, from the chamber paired with the highly hypertonic solution to the safe chamber, using its four limbs. The chambers were covered with translucent Plexiglas lids. The experimenter recorded the shuttle response by direct observation via a mirror positioned above the chambers. The experimental room was kept at a constant temperature (21–23 °C) and humidity (50%). Background white noise was present during training trials.

Procedure. Toads received two 5-min pretraining trials, one per day. Toads were taken to the experimental room 5 min before the start of each trial. During the trial, the animals were able to

freely move about the shuttle box. No stimuli were presented during these two pretraining trials.

Training started on the following day and continued for a total of 15 acquisition trials and 6 extinction trials (1 trial per day). Toads were randomly assigned to one of three groups. The toads assigned to Group Avo ($n = 8$) received the following sequence of events: (a) habituation to the chamber, 120 s; (b) exposure to the neutral solution (300 mM of NaCl, the WS) for a maximum of 120 s (an avoidance response was possible during this period); (c) if the toad did not cross to the safe compartment, then the neutral solution was drained, 30 s (an avoidance response was possible during draining); and (d) if the toad continued in the unsafe compartment, then the highly hypertonic solution (800 mM of NaCl, the reinforcer) was presented for a maximum of 120 s (an escape response was possible during this period).

The toads assigned to Group WS-only ($n = 5$) received the same sequence of events as that of Group Avo, except that in step (d), the compartment was filled again with the neutral solution. Finally, the toads assigned to Group EUP ($n = 4$) received the same sequence of events as those in Group Avo, except that a period of 180 s was interpolated between steps (b) and (d); this period included the 30 s of draining described in step (c). During all the trials and for the three groups, the safe compartment was filled with the neutral solution. (Time of access of 30 s)

In preparation for each daily trial, the animals were placed in an individual plastic container without water 1 hr before the start of the trial. The container was transferred to the experimental room about 15 min before the start of the trial. Each trial started by placing the toad in the unsafe chamber, with the sliding door closed. The sequence of trial events described above followed. The latency to shuttle to the safe compartment was recorded for each trial. A shuttle response occurring within 150 s of exposure to the WS was an avoidance response (i.e., 120 s of exposure to the WS plus 30 s of draining). Notice that for Group EUP, there were an additional 150 s before the presentation of the highly hypertonic solution. The recording of water uptake and other procedural details were as described in the previous experiment.

Results and Discussion

Figure 3, top panel, shows the performance of the three groups in their response latency. A value of zero in the ordinate corresponds to the initiation of step (2) in the sequence of trial events (i.e., exposure to the neutral solution). The dotted line signals the maximum latency for an avoidance response (150 s). In their response during the WS period (initial 150 s), it is clear that only toads in Group Avo developed and maintained an avoidance response; none of the other two groups ever performed below the dotted line. A comparison between Groups Avo and WS-only indicates that the avoidance response was not triggered by mere exposure to the neutral solution. However, toads exhibited a relatively stable degree of activity in this situation, as demonstrated by the latencies of Group WS-only: they were stable across the 21 trials and also consistently below maximum trial duration of 300 s. A comparison between Groups Avo and EUP demonstrates that the mere exposure to both the neutral and highly hypertonic solutions is not enough to cause the development of avoidance behavior—there must be temporal contiguity between the two. In addition, the relatively gradual extinction observed in Group Avo provides evidence for associative learning. Finally, although Group EUP did not exhibit avoidance behavior during the initial 150 s of the trial, it did show a substantial amount of virtual "avoidance," because of the absence of the reinforcer, during the interval between the WS and the reinforcer, followed by extinction of the behavior.

A Group × Trial repeated-measure ANOVA for the 15 acquisition trials yielded significant effects for all three factors: groups, $F(2, 14) = 30.20$, $p < .001$; acquisition trials, $F(14, 196) = 3.51$, $p < .001$; and their interaction, $F(28, 196) = 1.71$, $p < .02$. Post hoc pairwise comparisons indicated that Group Avo produced significantly lower latencies than the other two groups, ps $< .001$, whereas Group WS-only produced, in turn, significantly lower latencies than Group EUP, $p < .02$. A similar analysis of the extinction trials showed significant effects across groups, $F(2, 14) = 17.9$, $p < .001$, and a significant extinction of performance, $F(5, 70) = 9.46$, $p < .001$, but the interaction was nonsignificant, $F(10, 70) = 1.58$, $p < .14$. Post hoc pairwise tests indicated that Groups Avo and WS-only did not differ from each other, $p > .08$, but they both differed from Group EUP, ps $< .002$.

Measurements of water uptake showed that exposure to the hypertonic solution caused the toads to lose weight. Figure 3, bottom panel, shows that Group WS-only, which was never exposed to dehydration, actually gained a small amount of weight during the trial as a result of exposure to the neutral solution. In contrast, Group EUP, the group exposed to the highly hypertonic solution in the largest number of trials, exhibited consistent weight loss throughout the acquisition trials. Group Avo showed loss in Trial 1 but thereafter had either no change or a slight increase in weight. The two panels of Figure 3 provide striking mirror image functions for response latency and weight change in Groups Avo and EUP.

A Group × Trial repeated-measure ANOVA for variation in weight indicated significant differences for all factors: groups, $F(2, 14) = 22.04$, $p < .001$; trials, $F(14, 196) = 2.49$, $p < .003$; and their interaction, $F(28, 196) = 1.74$, $p < .02$. Furthermore, post hoc pairwise comparisons confirmed that all groups differed significantly from each other, all ps $< .005$. These effects disappeared during extinction, when the conditions of training were the same for all the toads. None of the factors in a Group × Trial repeated-measures ANOVA achieved a significant level, all Fs < 1.51.

General Discussion

These results demonstrate that toads easily acquire aversive conditioning when the reinforcer stimulus is exposure to a highly hypertonic solution causing dehydration. Experiment 1 demonstrated such conditioning in a Pavlovian situation involving acceleration of the heart rate as the conditioned response. This measure showed that the toads have an expectation of the impending presentation of the US. Experiment 2 showed that toads can acquire an avoidance response that prevents exposure to the highly hypertonic solution when an instrumental contingency is available. These studies provide clear demonstrations of aversive conditioning in an amphibian, a learning modality that had been particularly troublesome to demonstrate in previous studies (Macphail, 1982; Muzio, 1999; Suboski, 1992).

These demonstrations of aversive conditioning have some limitations. For example, in Experiment 1, although significant evi-

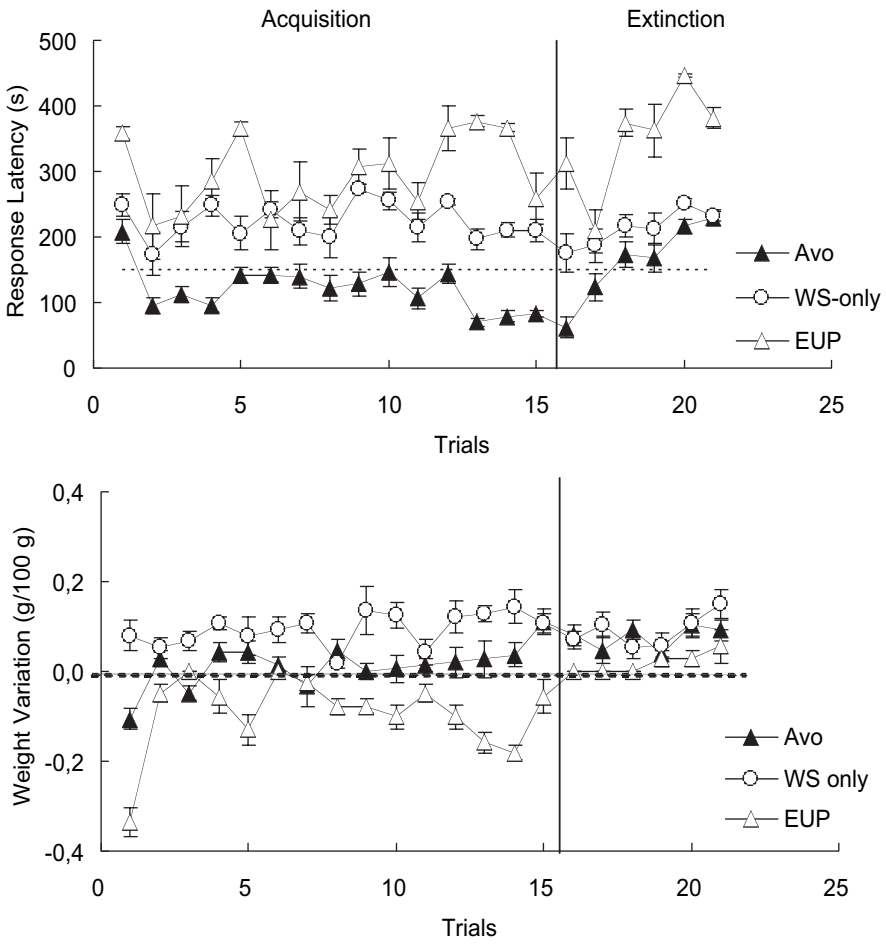

Figure 3. Top panel: latency to move to the safe compartment of a shuttle box in Group Avo (avoidance), WS-only (warning signal only), and EUP (explicitly unpaired). The WS was 300 mM of NaCl solution, whereas the reinforcer was 800 mM of NaCl solution. Toads could avoid (by moving to the safe compartment during the initial 150 s of the trial, marked with a dashed line) or escape (by moving during the presentation of the highly hypertonic solution). Bottom panel: variations in body weight corrected for individual differences in body weight across trials. Means and confidence intervals ($\alpha = .5$) are plotted. The dashed line represents no variation in body weight.

dence of anticipatory acceleration in heart rate was detected after only four trials, it remains unknown whether such changes would have persisted for a larger number of trials. These limitations in training were the result of a cardiac recording technique that is essentially an acute procedure, given the difficulty in maintaining a clean cannula during extended periods of time. In Experiment 2, a stronger demonstration of avoidance learning could be provided with the use of a master-yoked design in which pairs of toads are exposed to the same amount of WS and reinforcer across trials (see Portavella et al., 2003). Furthermore, toads in Group EUP actually had a signal reliably paired with the presentation of the highly hypertonic solution, namely, the absence of water during the delay between the WS and the reinforcer. Despite the presence of a signal, toads rarely crossed to the safe compartment before the highly hypertonic solution was presented. Apparently, then, exposure to the neutral solution was a more efficient WS than exposure to no solution at all. The differential effectiveness of various signals is common in conditioning experiments and is usually interpreted as reflecting functional constraints on the equipotentiality of signals during conditioning (Domjan, 2005). Examples of selective associations have been reported with a variety of signals and reinforcers (e.g., Weiss, Kearns, Cohn, Panlilio, & Schindler, 2005). The case may be that solution-solution associations have a more direct influence on autonomic and skeletal outputs in toads than associations involving a nonsolution CS for the highly hypertonic solution US.

Some potentially interesting applications of the procedure implemented in these experiments may be cited. The data show that exposure to the highly hypertonic solution causes dehydration, an event that is hypothesized to be the effective reinforcing stimulus in these situations. Because the molarity of the saline solution can be manipulated to vary from hypotonic to highly hypertonic, with an effectively neutral value of approximately 300 mM, this reinforcing stimulus dimension opens the way to study both appetitive and aversive conditioning within the same situation and under similar conditions.

A second example involves the coordination between autonomic and skeletal responses to maintain fluid balance. The cardiac pattern of acceleration of heart rate observed in Experiment 1 is the one expected for a situation involving exposure to an aversive stimulus likely to induce an escape response. An increase in blood circulation to the musculature enables the animal to face the metabolic demands involved in the production of an active response to escape from the aversive stimulus. As shown in Experiment 2, toads quickly developed an avoidance response that effectively prevented contact with the highly hypertonic solution. Taken together, these results suggest that toads have two available pathways to cope with an aversive situation involving dehydration. First, toads can use a behavioral strategy involving escape and avoidance responses that minimize contact with the hypertonic solution, much like they use an approach strategy to maximize contact with a hypotonic solution (e.g., Muzio et al., 1992). Measurements of water uptake demonstrate that the escape or approach behavior causes the appropriate outcome, namely, either preventing water loss or facilitating water gain. Second, when an escape route is not available, toads have the ability to develop a compensatory response that effectively prevents water loss, even after extensive exposure to the highly hypertonic solution. Similar compensatory conditioned responses leading to tolerance have been described in organisms exposed to a variety of drugs (e.g., Siegel, Baptista, Kim, McDonald, & Weise-Kelly, 2000). Although the proximate brain mechanisms underlying the toad's compensatory response have not been studied, the functional outcome is well known from other studies.

Several lines of evidence point to the adaptive advantage of having access to a stimulus signaling the impending presentation of either appetitive or aversive reinforcers (Domjan, 2005). Hollis (1982, p. 3) suggested, for example, that Pavlovian CSs "enable the animal to optimize interactions with the forthcoming biologically important event (US)." Experiments show that males gain a reproductive advantage when the environment provides signals predicting the presentation of either another male, in a defensive context, or of a receptive female, in a reproductive context (Domjan, Blesbois, & Williams, 1998; Gutiérrez & Domjan, 1996; Hollis, Dumas, Piyusha Singh, & Fackelman, 1995; Hollis, Pharr, Dumas, Britton, & Field, 1997). In a similar fashion, close temporal contiguity between CS and US in Experiment 1 provided conditions that allowed toads to prevent and largely eliminate water loss otherwise induced by inescapable exposure to the highly hypertonic solution.

Unlike the conclusions drawn from previous research (Macphail, 1982; Muzio, 1999; Suboski, 1992), the present results suggest that aversive learning can be rapid and efficient in amphibians when saline solutions of different molarities are used as signals and reinforcers.

References

Balinsky, J. B. (2005). Adaptation of nitrogen metabolism to hyperosmotic environment in Amphibia. *Journal of Experimental Zoology, 215,* 335–350.

Bernstein, I. L. (1999). Taste aversion learning: A contemporary perspective. *Nutrition, 15,* 229–234.

Bilbo, S. D., Day, L. B., & Wilczynski, W. (2000). Anticholinergic effects in frogs in a Morris water maze analog. *Physiology and Behavior, 69,* 351–357.

Boice, R. (1970). Avoidance learning in active and passive frogs and toads. *Journal of Comparative and Physiological Psychology, 70,* 154–156.

Brush, F. R. (1971). (Ed.), *Aversive conditioning and learning.* New York: Academic Press.

Campbell, B. A., & Masterton, F. A. (1969). Psychophysics of punishment. In B. A. Campbell & R. M. Church (Eds.), *Punishment and aversive behavior* (pp. 3–42). New York: Appleton-Century-Crofts.

Christensen, C. U. (1974). Adaptations in the water economy of some anuran amphibia. *Comparative Biochemistry and Physiology A, 47,* 1035–1049.

Crawford, F. T., & Langdon, J. W. (1966). Escape and avoidance responding in the toad. *Psychonomic Science, 6,* 115–116.

Day, L. B., Crews, D., & Wilczynski, W. (1999). Spatial and reversal learning in congeneric lizards with different foraging strategies. *Animal Behavior, 57,* 395–407.

Domjan, M. (2005). Pavlovian conditioning: A functional perspective. *Annual Review of Psychology, 56,* 179–206.

Domjan, M., Blesbois, E., & Williams, J. (1998). The adaptive significance of sexual conditioning: Pavlovian control of sperm release. *Psychological Science,* **9,** 411–415.

Gómez-Mestre, I., & Tejedo, M. (2003). Local adaptation of an anuran amphibian to osmotically stressful environments. *Evolution, 57,* 1889–1899.

Gerlai, R. (2001). Behavioral tests of hippocampal function: Simple paradigms complex problems. *Behavioural Brain Research, 125,* 269–277.

Gutiérrez, G., & Domjan, M. (1996). Learning and male-male sexual competition in Japanese quail (*Coturnix japonica*). *Journal of Comparative Psychology, 110,* 170–175.

Hollis, K. L. (1982). Pavlovian conditioning of signal-centered action patterns and autonomic behavior: A biological analysis of function. *Advances in the Study of Behavior, 12,* 1–64.

Hollis, K. L., Dumas, M. J., Piyusha Singh, & Fackelman, P. (1995). Pavlovian conditioning of aggressive behavior in blue gourami fish (*Trichogaster trichopterus*) winners become winners and losers stay losers. *Journal of Comparative Psychology, 109,* 123–133.

Hollis, K. L., Pharr, V. L., Dumas, M. J., Britton, G. B., & Field, J. (1997). Classical conditioning provides paternity advantage for territorial male blue gouramis (*Trichogaster trichopterus*). *Journal of Comparative Psychology, 111,* 219–225.

Loza Coll, M. (1998). *Behavior of the toad Bufo arenarum exposed to external solutions of different osmolarity.* Unpublished master's thesis, Universidad de Buenos Aires—Buenos Aires, Argentina.

Macphail, E. M. (1982). *Brain and intelligence in vertebrates.* Oxford, UK: Clarendon Press.

McGill, T. E. (1960). Response of the leopard frog to electric shock in an escape-learning situation. *Journal of Comparative and Physiological Psychology, 53,* 443–445.

Mowrer, O. H. (1947). On the dual nature of learning: A reinterpretation of "conditioning" and "problem-solving." *Harvard Educational Review, 17,* 102–150.

Muzio, R. N. (1999). Aprendizaje instrumental en anfibios. *Revista Latinoamericana de Psicología, 31,* 35–47.

Muzio, R. N., Segura, E. T., & Papini, M. R. (1992). Effect of schedule and magnitude of reinforcement on instrumental learning in the toad, *Bufo arenarum. Learning and Motivation, 23,* 406–429.

Papini, M. R. (1998). Classical conditioning. In G. Greenberg & M. Haraway (Eds.), *Comparative psychology: A handbook.* New York: Garland.

Paradis, S., & Cabanac, M. (2004). Flavor aversion learning induced by lithium chloride in reptiles but not in amphibians. *Behavioural Processes, 67,* 11–18.

Pistone Creydt, V. (2000). *Associative learning in the toad Bufo arenarum: Reward alternation using solutions of different salinity.* Unpublished

master's thesis, Universidad de Buenos Aires—Buenos Aires, Argentina.

Portavella, M., Salas, C., Vargas, J. P., & Papini, M. R. (2003). Involvement of the telencephalon in spaced-trial avoidance learning in the goldfish (*Carassius auratus*). *Physiology and Behavior, 80,* 49–56.

Portavella, M., Torres, B., Salas, C., & Papini, M. R. (2004). Lesions of the medial pallium, but not of the lateral pallium, disrupt spaced-trial avoidance learning in goldfish (*Carassius auratus*). *Neuroscience Letters, 362,* 75–78.

Reboreda, J. C., Muzio, R. N., Viñas, M. C., & Segura, E. T. (1991). β-adrenergic control of water permeability of the skin during rehydration in the toad *Bufo* arenarum. *Comparative Biochemistry and Physiology C, 100,* 433–437.

Ruibal, R. (1962). The adaptive value of bladder water in the toad, *Bufo cognatus. Physiological Zoology, 35,* 218–223.

Schmajuk, N. A., & Segura, E. T. (1980). Behavioral changes along escape learning in toads. *Acta Physiologica Latinoamericana, 30,* 211–215.

Schmajuk, N. A., & Segura, E. T. (1982). Behavioral regulation of water balance in the toad. *Herpetologica, 38,* 296–301.

Siegel, S., Baptista, M. A. S., Kim, J. A., McDonald, R. V., & Weise-Kelly, L. (2000). Pavlovian psychopharmacology: The associative basis of tolerance. *Experimental and Clinical Psychopharmacology, 8,* 276–293.

Suboski, M. D. (1992). Releaser-induced recognition learning by amphibians and reptiles. *Animal Learning and Behavior, 20,* 63–82.

Weiss, S. J., Kearns, D. N., Cohn, S. I., Panlilio, L. V., & Schindler, C. W. (2005). Cocaine and selective associations: Investigations into a biological constraint on learning with drug self-administration and shock-avoidance as reinforcers. *International Journal of Comparative Psychology, 18,* 112–125.

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