



Duration of extinction trials as a determinant of instrumental extinction in terrestrial toads (*Rhinella arenarum*)

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Abstract

Instrumental learning guides behavior toward resources. When such resources are no longer available, approach to previously reinforced locations is reduced, a process called extinction. The present experiments are concerned with factors affecting the extinction of acquired behaviors in toads. In previous experiments, total reward magnitude in acquisition and duration of extinction trials were confounded. The present experiments were designed to test the effects of these factors in factorial designs. Experiment 1 varied reward magnitude (900, 300, or 100 s of water access per trial) and amount of acquisition training (5 or 15 daily trials). With total amount of water access equated in acquisition, extinction with large rewards was faster (longer latencies in 900/5 than 300/15), but with total amount of training equated, extinction with small rewards was faster (longer latencies in 100/15 than 300/15). Experiment 2 varied reward magnitude (1200 or 120 s of water access per trial) while holding constant the number of acquisition trials (5 daily trials) and the duration of extinction trials (300 s). Extinction performance was lower with small, rather than large reward magnitude (longer latencies in 120/300 than in 1200/300). Thus, instrumental extinction depends upon the amount of time toads are exposed to the empty goal compartment during extinction trials.

Keywords Instrumental extinction · Reward magnitude · Duration of extinction trials · Toads

Introduction

Instrumental learning guides behavior toward resources important for survival and reproductive success, allowing animals to procure access to food, fluids, and potential mates. When such resources fail to occur, approach responses weaken and animals switch to a search mode likely to bring them in contact with new sources of reward. This process is called extinction (Todd et al. 2014). In extinction, animals evaluate the new environmental conditions to decide whether to persist using a previously successful behavior. The conditions under which a behavior was

acquired determine the decision to stop responding during extinction when the response fails to reach the resource.

Although the extinction of instrumental behavior is a pervasive phenomenon among vertebrates, extinction learning in amphibians and mammals is not always dependent upon the same factors (Muzio et al. 2011; Papini 2002, 2003, 2006). Whereas in mammals the relationship between reward magnitude and extinction persistence is indirect (i.e., more reward in acquisition leads to faster extinction), in amphibians this relationship is direct (i.e., more reward during acquisition leads to slower extinction).

Species differences in instrumental extinction have been mainly observed in what Amsel (1992) called the paradoxical reward effects, that is, when seemingly poorer training conditions in acquisition (e.g., small reward) actually yield more behavior in extinction compared to seemingly richer acquisition environments (e.g., large reward). For example, in experiments with mammalian species, the widely spaced training of an instrumental response paired with a large reward tends to lead to faster extinction compared to that of a response paired with a small reward (Papini et al. 2001; Wagner 1961). This phenomenon, called the magnitude

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of reinforcement extinction effect (MREE), suggests an inverse relationship between reward magnitude and persistence in extinction. According to Amsel (1992), the MREE is paradoxical in that more reward leads to less behavior in extinction. In this sense, the appetitive extinction (as in other surprising changes in reward effects) is accompanied in mammals by changes that can be described as emotional (e.g., Papini et al. 2015). In amphibians, however, the relationship between reward magnitude and extinction persistence is direct, that is, more reward leads to more behavior in extinction—a reversed MREE (Muzio et al. 1992; Papini 2014). Thus, extinction in amphibians is consistent with a nonemotional interpretation of the effects of surprising changes in reinforcement conditions, unlike the case of mammals exposed to similar situations (Muzio et al. 1992). For instance, toads learned to approach a location where water was accessible more readily with larger reward magnitudes, but when shifted to extinction their performance either converged rapidly or remained directly related to the reward magnitude received in acquisition (Muzio et al. 1992, 2011; Papini et al. 1995). In these experiments, reward magnitude was manipulated by allowing toads to sit for different amounts of time on a goal container with accessible water. As toads “drink” by absorption through a patch of ventral skin (Christensen 1974), water uptake is then proportional to the time that their ventral skin is in contact with water. By weighing animals before and after the single daily trial, the magnitude of rehydration (the reward) could be directly determined. As expected, weight variation closely matched the time allotted for water access in a trial (Muzio et al. 1992; Papini et al. 1995). A potential problem with the procedure used in these experiments is that by keeping constant the time in the goal compartment from acquisition to extinction, animals also differed in the duration of extinction trials (i.e., amount of exposure time to extinction cues in the goal compartment while water was not accessible). Therefore, a reversed MREE may result either from differential reward magnitudes in acquisition or from differential duration of extinction trials.

Another factor affecting instrumental extinction is the amount of acquisition training. In rats, extinction tends to be faster after a relatively larger amount of practice than after limited practice, a phenomenon referred to as the overtraining extinction effect (OEE; North and Stimmel 1960; Senkowski 1978). The OEE effect, also reported in turtles (Ishida and Papini 1997), reflects an indirect relationship between amount of acquisition training and extinction persistence—a paradoxical effect in Amsel’s (1992) terms. In amphibians, once again, the opposite result has been reported. Toads that have received 10 acquisition trials (one trial per session) in a runway task extinguish faster than toads that have received 30 acquisition trials (Muzio et al. 2006). Therefore, this reversed OEE seems to reflect a

direct relationship between amount of acquisition training and extinction persistence (as opposed to what is observed in mammals). But one problem with this observed reversed OEE is that in addition to receiving different amounts of training, groups in this study differed in terms of the total amount of reward received in acquisition trials. This may simply be a special case of a reward magnitude effect, although not on a trial basis, but on the basis of the complete sequence of acquisition training.

The present experiments were designed to dissociate the effects of reward magnitude, amount of acquisition training, and duration of extinction trials on instrumental extinction in terrestrial toads. As previously stated, the effects of reward schedules on instrumental extinction have uncovered phenomena showing functional differences across vertebrate species. A complete understanding of such behavioral differences requires a systematic approach to the determinants of extinction in a non-mammalian species. The accumulation of information on extinction in amphibians since the 1980s (e.g., Muzio et al. 1992; Schmajuk et al. 1981) promises to fill this gap. Thus, the present research has theoretical value for an understanding of the comparative basis of learning and cognition in vertebrates.

Experiment 1

Experiment 1 pitted the amount of acquisition training against the total amount of reward at two levels for each comparison to determine the source of greater persistence in extinction after overtraining in acquisition. This was achieved with the four-group design described in Table 1. Acquisition training was followed by 15 extinction trials in which duration of extinction trials was the same as the time allowed for reward during acquisition trials. Keeping constant the time in the goal compartment from acquisition to extinction minimized the effects of stimulus

Table 1 Design of experiment 1

Group	Reward magnitude/trial (s)	Acquisition/extinction trials	Total reward in acquisition (s)	Goal time per trial in extinction (s)
100/15	100	15/15	1500	100
300/5	300	5/15	1500	300
300/15	300	15/15	4500	300
900/5	900	5/15	4500	900

Reward magnitude was manipulated in terms of the time (s) allowed animals to absorb water through the ventral pelvic skin. Pairwise comparisons allowed variation in one parameter (e.g., number of acquisition trials: 15 or 5) while holding the other constant (e.g., total reward in acquisition: 4500 s), and vice versa. This design gives rise to four target comparisons (see text for details)

generalization decrement on extinction performance, but it introduced an unequal exposure to extinction-associated cues across groups. This issue is considered in Experiment 2.

Two possible outcomes were predicted. First, if overtraining increased persistence in extinction due to a greater total amount of reward in acquisition, then the groups designated in Table 1 as 900/5 and 300/15 (each having a total of 4500 s of access to water), and Groups 300/5 and 100/15 (each having a total of 1500 s of access to water) should show similar extinction performance. Second, if the effect of overtraining on extinction was due to additional response training, then Groups 300/15 and 100/15 (15 trials of acquisition training) should exhibit greater persistence in extinction than Groups 900/5 and 300/5 (5 trials of acquisition training), respectively.

Method

Subjects

Thirty-eight naïve adult male terrestrial toads (*Rhinella arenarum*) captured in ponds around Buenos Aires, were used as subjects. This species is not listed as threatened (IUCN 2017). Animals were maintained according to the US National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. Upon arrival in the laboratory, all animals were placed in group cages with running tap water for at least the following 2 weeks. The vivarium was kept at a temperature between 21 and 23 °C, and under a 16:8 h light/dark cycle (lights from 03:00 to 19:00 h). As usual, reflexes were tested to detect possible neurological disorders (including the vestibulo-ocular, oculo-cephalic, amplexus, unken, and licking reflexes, among others; for a complete list of these reflexes see Muzio 1995). Subjects were treated with antibiotics and antiparasitic medication mixed with the meal. The antibiotic medication feeding regimen lasted 7 days; during this period, every toad received a daily feeding ration of insectivore bird ground (dissected insects) and a live mealworm larva. A dose of antiparasitic medication was also administered on the first day. This feeding regime ended about 1 week before the start of training. The standard weight (weight of the hydrated animal after the urinary bladder has been emptied; Ruibal 1962) of each animal was recorded 2 days before the start of the experiment. Standard weights varied between 57.5 and 179.7 g and did not differ significantly across groups ($P > 0.85$). Toads were dehydrated at 80% of their standard weight at the beginning of each pretraining and training trial. During the experiment, animals were kept in small, dry, individual plastic cages.

Apparatus

Animals were trained in a runway built with Plexiglas. The runway had a start compartment (20 × 12 × 20 cm; $L \times W \times H$), an alley (60 × 12 × 20 cm; $L \times W \times H$), and a goal compartment (20 × 12 × 20 cm; $L \times W \times H$); two guillotine doors separated the alley from the start and goal compartments. The walls and the doors of the runway were black. Each part of the runway had a translucent cover with a 40-W light on it. In the goal compartment, there was a Plexiglas container with a metallic mesh in the base. The container always had water, but was only available if the level of the water was higher than the metallic mesh. This allowed for any cues from water to be matched across rewarded and nonrewarded trials.

Procedure

Each animal received one trial per day, 7 days per week, all at about the same time during the day (between 08:00 and 13:00 h) and during the light portion of the daily cycle. Animals were matched by pretraining response latency and randomly assigned to one of four groups: 100/15 ($N = 10$), 300/5 ($N = 10$), 300/15 ($N = 9$) and 900/5 ($N = 9$). In group labels, the first number refers to the duration of access to deionized water after each acquisition trial, a way of manipulating reward magnitude (100, 300, or 900 s), whereas the second number refers to the amount of acquisition training (5 or 15 acquisition trials, one per day). Table 1 describes the major features of the design used in Experiment 1. All the trials in Experiments 1 and 2 were run by the same experimenter.

All animals received two trials of pretraining, one per day. During pretraining, animals were placed in the start compartment with the guillotine door closed. The container in the goal compartment was filled with deionized water. After 30 s, the door was opened and animals could freely move for 10 min. Three 1-ml drops of deionized water were placed on the alley's floor, one next to the start door, the other midway into the alley, and a third next to the goal door. In the second pretraining trial, only the last two drops were placed. During training trials, the floor was dry. During acquisition trials, animals were placed in the start compartment and the guillotine door was raised after 30 s. Two dependent variables were registered: (1) Running latency (in seconds): Time from the moment the animal had its four legs in the alley and out of the start compartment, to the moment it entered the goal compartment with its four legs. This variable was recorded by the manual operation of a digital timer (1-s units) and transformed to its \log_{10} to improve normality and allow for the use of parametric statistics. (2) Weight variation (g/100 g): The weight of every subject (in grams) was registered before and after each trial to estimate water consumption. The difference between these two

weights was divided by the standard weight and multiplied by 100 to provide a relative measure of water uptake corrected for individual differences in body weight. These two dependent variables have been reported in similar previous studies (e.g., Muzio et al. 1992).

In each trial, after the guillotine door was raised, the animal had a maximum of 180 s to move into the alley and a maximum of 180 s to enter the goal compartment once in the alley. A maximum latency of 180 s was assigned (1) when the animal failed to leave the start compartment after 180 s or (2) when the animal left the start compartment before the maximum 180 s, but failed to enter the goal compartment within the following 180 s. In incomplete trials, toads were gently guided to the goal compartment where they received the scheduled outcome, either access to the deionized water in acquisition trials or to no water in extinction trials. The time that each animal spent in the goal compartment during extinction trials was the same as during acquisition trials; water was present, but it was inaccessible to the animal. The apparatus was cleaned at the end of each pretraining and training trial to control for odor traces.

After each trial, animals were transferred to dry cages where they remained until the next day. At least 30 min after each daily trial, animals that had lost weight during the trial were given access to deionized water, whereas those that had gained weight were kept dry. This procedure ensured that animals would be at 79–81% of their standard weight at the start of each trial.

The results for each dependent variable and for acquisition and extinction trials were analyzed as follow. Runway latency was examined using Practice (15 or 5 acquisition trials) \times Total Reward (4500 or 1500 s of access to water) \times Trial mixed factorial analysis of variance (ANOVA). ANOVA's assumptions were tested with Box's test of equality of covariance matrices and Mauchly's test of sphericity. Statistical significance was set at the 0.05 level for all tests. In one case in which sphericity was violated, the Greenhouse–Geisser correction was reported. Weight variation violated equal variance and normality in some of the groups; therefore, this dependent variable was analyzed using single-factor nonparametric Kruskal–Wallis tests and Mann–Whitney pairwise comparisons were used when appropriate. The significant level for Mann–Whitney tests was set at the 0.008 level using a Bonferroni correction (0.05 divided by 6, the total number of pairwise comparisons). All statistical tests were computed with the IBM SPSS v. 21 package.

Results

Altogether, four comparisons were of interest in this experiment. Two comparisons involved groups equated in terms of the amount of acquisition training, but receiving different

reward magnitudes: Groups 300/15 versus 100/15 with 15 training trials and Groups 900/5 versus 300/5 with 5 training trials. Two other comparisons involved the same total amount of reward, but distributed in either 5 or 15 training trials: Groups 900/5 versus 300/15 with a total of 4500 s of access to water and Groups 300/5 versus 100/15 with 1500 s of access to water.

The results for runway latency are presented in Fig. 1. Since groups differed in the number of acquisition trials, two different tests were run to examine acquisition behavior. One analysis included the initial 5 trials of acquisition for all groups, whereas the other analysis included the final 5 acquisition trials only for groups that received 15 acquisition trials. The Practice \times Total Reward \times Trial analysis of the initial 5 acquisition trials revealed a main trial effect [$F(4.14, 21.55) = 21.55; P < 0.001; \eta^2 = 0.38$]. None of the interactions and main effects was significant ($P_s > 0.07$). The second analysis comparing Groups 300/15 and 100/15 terminal acquisition (trials 11–15) failed to detect a group difference, $p > 0.11$. There were no detectable effects for trials or for the interaction, $P_s > 0.40$.

In the Practice \times Total Reward \times Trial ANOVA for the extinction phase, the triple interaction and the Reward \times Training interactions were nonsignificant ($P_s > 0.70$). In contrast, Practice \times Trial [$F(3, 102) = 3.93, P < 0.02, \eta^2 = 0.10$] and Time \times Reward [$F(1, 34) = 9.17, P < 0.01, \eta^2 = 0.21$] interactions were significant. A posteriori LSD comparisons within the Practice \times Trial interaction, comparing the 5-trial condition versus 15-trial condition, showed that animals receiving 5 acquisition trials exhibited higher runway latencies in trials 3 and 4 ($P_s < 0.03$), but were not different on trials 1 and 2 ($P_s > 0.60$). A posteriori LSD comparisons within each condition indicated that animals that received 15 acquisition trials did not increase runway latencies along the 4 extinction trials ($P_s > 0.07$),

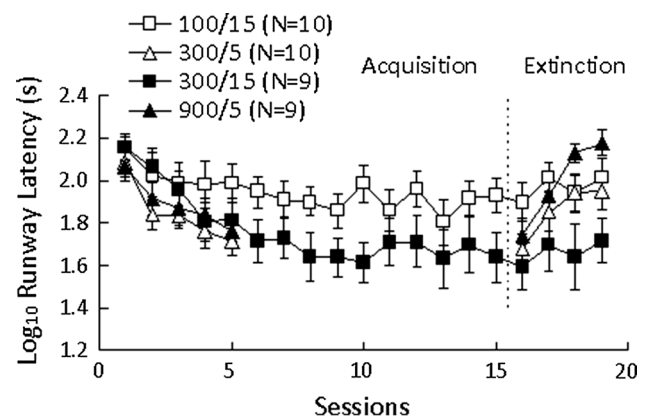


Fig. 1 Running latency (Log_{10} s) during acquisition or extinction for each of the groups of Experiment 1. Means and standard errors are plotted. The dash line separates acquisition than extinction trials

while animals in the 5 acquisition trials condition reported higher runway latencies in extinction trials 2–4 relative to extinction trial 1 ($P_s < 0.005$). In this case, extinction trial 2 also differed from trial 4 ($P < 0.03$) but not from trial 3 ($P > 0.055$), and extinction trial 3 did not differ from trial 4 ($P > 0.60$).

A posteriori analysis of the Time \times Reward interaction indicated that the group that received 15 trials and 4500 s of total reward was the one that showed on average lower runway latencies ($P < 0.01$) with no differences between the remaining groups ($P > 0.19$).

Figure 1 suggests that groups receiving 15 acquisition trials maintained in extinction about the same response latencies reached during the final acquisition trials, whereas groups receiving 5 acquisition trials increased their runway latencies during extinction. To assess this possibility, a linear regression using the last acquisition trial and the 4 extinction trials was computed for each animal. The slopes of these functions were averaged for each group and are presented in Fig. 2. The mean slope provides a description of the extinction rate for a given group. Because the data satisfied equal variance and normality assumption, a two-way ANOVA Reward \times Training was calculated for these slopes. It indicated no evidence of interaction or Reward effect ($P_s > 0.30$), but showed a Training effect [$F(1, 34) = 8.47$, $P < 0.01$, $\eta^2 = 0.20$] indicating that groups receiving 15 trials exhibited lower slopes than groups receiving 5 trials, independently of the amount of total reward.

Figure 3 shows the mean weight variation for each group during the last two acquisition trials. Animals that had access to water for either 900, 300, or 100 s per trial had differential variations in body weight. There was clear evidence that the three reward levels used in this experiment effectively

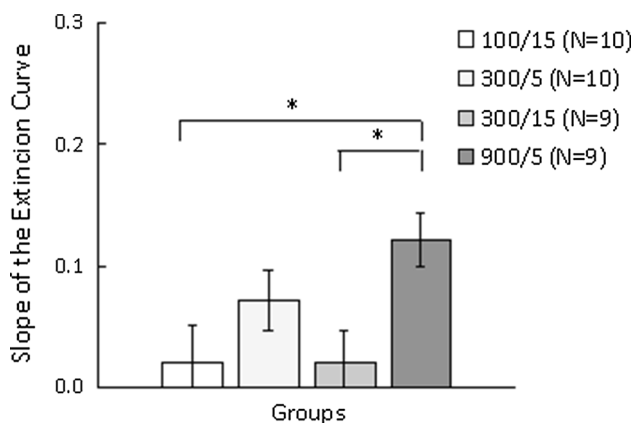


Fig. 2 Mean slope of the extinction phase for each of the groups of Experiment 1. Slope was calculated in each individual by a linear regression considering last acquisition trial and the four extinction trials as Y values and the numbers 1, 2, 3, 4 and 5 as X values. Error bars denote standard errors of the means. Asterisks denote significant differences ($*P < 0.05$)

changed body weight. A statistical comparison yielded a significant group effect, Kruskal–Wallis, $\chi^2 = 32.30$, $P < 0.001$, $\eta^2 = 0.87$. Mann–Whitney pairwise comparisons showed that all three conditions differed from each other, $U_s = 0.00$, $P_s < 0.001$. The terminal performance of Groups 300/15 and 300/5 did not differ, $P > 0.04$ (considering that the Bonferroni correction dropped the alpha level to 0.008), despite a slight increase across trials in weight variation in the former group.

Discussion

Experiment 1 was designed to provide data on a reversed OEE effect reported in previous experiments with amphibians—more acquisition training leading to slower extinction (Muzio et al. 2006)—by pitting two alternative accounts against each other. First, if this reversed OEE was due to a greater total amount of reward in acquisition, then matched groups should extinguish at equal rates and groups receiving 1500 s of total reward should extinguish faster than groups receiving 4500 s. As suggested by a factorial analysis, no evidence of Trial \times Reward interaction or Trial \times Reward \times Practice interaction was found. This result must be interpreted as evidence contrary to this first prediction.

Second, if this reversed OEE was due to additional response training, then groups with a greater amount of acquisition training should extinguish slowly relative to groups with fewer acquisition trials. In fact, a Practice \times Trial interaction was observed, indicating a difference in extinction performance between groups receiving 15 and 5 acquisition trials. While groups with 5 trials exhibited a fast extinction after four nonrewarded trials, groups that received 15 acquisition trials showed only an

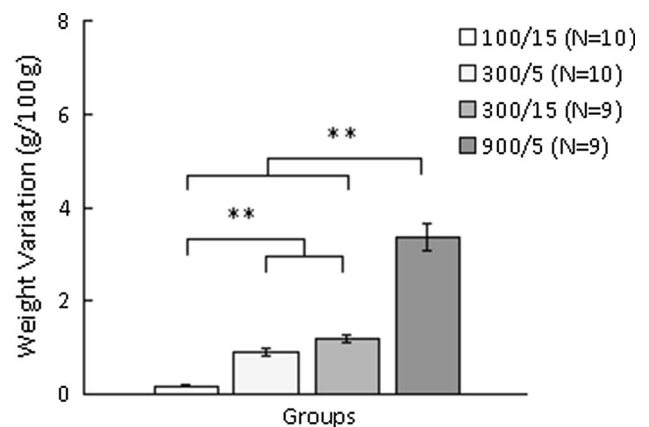


Fig. 3 Weight variation (g/100 g) for each group during the last two acquisition trials of Experiment 1. Means and standard errors are plotted (** $P < 0.01$)

incipient change in response that resulted statistically non-significant (see Fig. 2).

As a whole, the results of Experiment 1 suggest that, in amphibians, the OEE depends strongly on the amount of practice, while total amount of reward received does not explain resistance to extinction in overtrained animals.

Although the statistical analysis failed to detect it, Fig. 1 suggests that Groups 300/15 and 100/15 differed in runway performance at the end of acquisition training. It had been previously observed that animals that receive small rewards exhibit poorer runway performance than animals that receive larger rewards. Such an effect was detected in the analysis of the extinction trials by the Reward \times Practice interaction. While Groups 300/15 and 100/15 showed little increase in their runway latencies, Group 300/15 had on average smaller values than Group 100/15.

As is usual in experiments on learning in animals, an attempt to manipulate one factor across groups usually ends up varying more than that factor. In this case, equating groups in terms of total acquisition reward also produced inequalities in terms of duration of extinction trials. This was due to keeping the time the animal was enclosed in the goal box constant from acquisition to extinction. Thus, toads in Groups 900/5 and 300/15 were matched in terms of total reward, but each extinction trial for the former (900 s) lasted the same amount of time as 3 such trials for the latter (each 300 s in length). This leads to results that apparently contradict previous findings. For example, in Experiment 1 faster extinction after training with a large reward (Group 900/5) than after training with a small reward (Group 300/15) looks like a regular MREE. Such MREE would be similar to what has been observed in rats (e.g., Papini et al. 2001; Wagner 1961). In contrast to these results, previous experiments with toads (Muzio et al. 1992; Papini et al. 1995) have consistently provided evidence for a reversed MREE (i.e., slower extinction after training with large reward than small reward). But, is this apparent MREE due to differences in reward magnitude or to differences in the duration of extinction trials?

Experiment 2

Experiment 2 was designed to unconfound the relationship between reward magnitude and duration of extinction trials in the goal compartment. Table 2 describes the design. Two groups matched in terms of reward magnitude (either 1200 or 120 s of access to water per trial) were subsequently exposed to different duration of extinction trials. These reward magnitudes were chosen to maximize the difference between them (a 10-to-1 ratio, rather than the 3-to-1 ratio used in Experiment 1; see Table 1). Additionally, two groups matched in duration of extinction trials were included to independently assess the effects of reward magnitude (Groups 120/300 vs. 1200/300 in Table 2). The amount of training was equated, and it was short since Experiment 1 suggested that most behavioral changes occurred after relatively little acquisition. If the apparent MREE observed in Experiment 1 was caused by reward magnitude, then Group 1200/300 should extinguish faster than Group 120/300. However, if the apparent MREE was caused by duration of extinction trials, then Groups 120/300 and 1200/1200 should extinguish faster than groups 120/120 and 1200/300, respectively.

Method

Subjects and apparatus

Forty-seven experimentally naive, adult, male toads were obtained and maintained as described in the previous experiment. Standard weights varied between 53.8 and 144.7 g, and were not statistically different across groups ($P > 0.53$). Other conditions of maintenance and the apparatus used were the same as in Experiment 1.

Procedure

Animals were pretrained as described in Experiment 1. Afterward, toads were matched by pretraining response latency and randomly assigned to one of four groups:

Table 2 Design of experiment 2

Group	Reward magnitude/trial (s)	Acquisition/extinction trials	Total reward in acquisition (s)	Goal time per trial in extinction (s)
120/120	120	5/4	600	120
120/300	120	5/4	600	300
1200/300	1200	5/4	6000	300
1200/1200	1200	5/4	6000	1200

Reward magnitude was manipulated in terms of the time (s) allowed animals to absorb water through the ventral pelvic skin. Pairwise comparisons allowed variation in one parameter (e.g., total reward in acquisition: 6000 or 600 s) while holding the other constant (e.g., goal time/trial in extinction: 300 s), and vice versa. This design gives rise to three target comparisons (see text for details)

1200/1200 ($N = 12$), 1200/300 ($N = 11$), 120/120 ($N = 12$), and 120/300 ($N = 12$). In group denominations, the first number refers the time of access to water in each acquisition trial (either 1200 or 120 s), whereas the second number refers to the duration of each extinction trial in the goal compartment without access to water (either 1200, 300, or 120 s). All animals received 5 acquisition trials, and 4 extinction trials, one per day. All other procedural details were as described in the previous experiment. As in Experiment 1, when equal variance and normality could not be assumed, the dependent variable was analyzed using non-parametric Kruskal–Wallis tests, with Mann–Whitney tests for pairwise comparisons.

Results

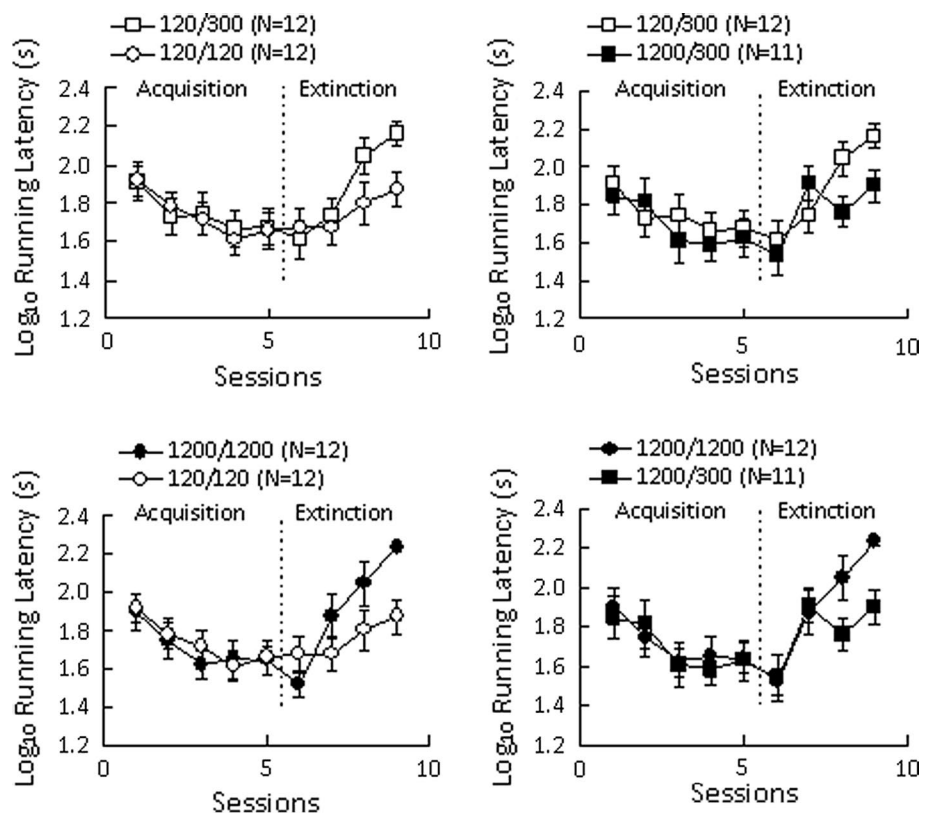
Figure 4 shows the running latency during acquisition or extinction for each of the groups of Experiment 2. During acquisition, a Group \times Trial ANOVA yielded a Trial effect, $F(4, 172) = 17.07, p < 0.001, \eta^2 = 0.28$, but no Group or interaction effects, $P_s > 0.80$. An analysis of extinction data detected a Group by Trial interaction, $F(9, 129) = 2.68, P < 0.008, \eta^2 = 0.16$. Pairwise LSD tests between Groups for each Trial found nonsignificant differences for extinction Trials 1–3, $P_s > 0.05$. However, all comparisons of interest were significant for Trial 4. Group

120/300 exhibited significantly higher running latency than Group 120/120, $P < 0.006$, while Group 1200/1200 exhibited higher running latency than Group 1200/300, $P < 0.003$. Moreover, Group 1200/1200 showed higher extinction latencies than Group 120/120, $P < 0.002$, and Group 120/300 extinguished significantly faster than Group 1200/300, $P < 0.02$.

Extinction rate was estimated as in Experiment 1 calculating the slope of the extinction curve (Fig. 5). Since the data did not satisfy the equal-variance assumption, nonparametric statistics were used. A Kruskal–Wallis test revealed a significant group effect, $\chi^2 = 10.87, P < 0.02, \eta^2 = 0.24$. Pairwise comparisons revealed a difference only between Groups 120/120 and 1200/1200, $U = 11.00, P < 0.001$, but not between Groups 120/300 and 120/120, 120/300 and 1200/300, or 1200/1200 and 1200/300, $P_s > 0.05$.

Figure 6 shows the mean weight variation for each group during the last two acquisition trials. A Kruskal–Wallis analysis yielded a significant difference between groups, $\chi^2 = 35.48, P < 0.001, \eta^2 = 0.77$. Pairwise Mann–Whitney tests showed that whereas groups receiving the same reward magnitude did not differ from each other ($P_s > 0.08$), each of the comparisons between groups receiving 1200 versus 120 s of water access was significant ($U_s = 0.00, P_s < 0.001$).

Fig. 4 Running latency (Log_{10} s) during acquisition or extinction for each of the groups of Experiment 2. Means and standard errors are plotted. The dash lines separate acquisition than extinction trials. Although the four groups were run together, results are plotted in pairs in order to facilitate the comparisons



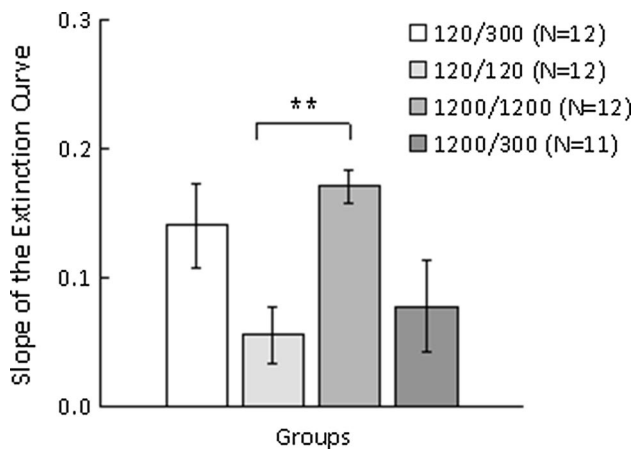


Fig. 5 Mean slope of the extinction phase for each of the groups of Experiment 2. Slope was calculated in each individual by a linear regression involving the last acquisition trial and the four extinction trials as Y values and the numbers 1–5 as X values. Error bars denote standard errors of the means. Asterisks denote significant differences (** $P < 0.01$)

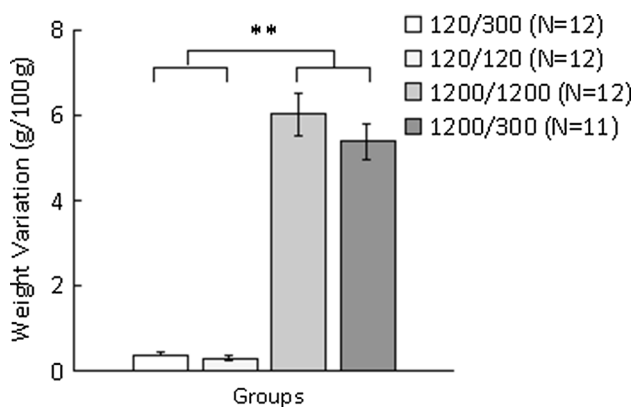


Fig. 6 Weight variation (g/100 g) for each group during the last two acquisition trials of Experiment 2. Means and standard errors are plotted (** $P < 0.01$). Comparisons (see text for details)

Discussion

Four pairwise comparisons were of interest in Experiment 2. One comparison involved groups equated in terms of duration of extinction trials, but trained with different reward magnitudes in acquisition: Groups 1200/300 versus 120/300 with 300 s of exposure to the goal compartment, but either 1200 or 120 s of access to water per trial. This comparison revealed that groups with equal duration of extinction trials showed a reversed MREE; that is, slower running in extinction with small rather than large reward.

Two other comparisons involved the same reward magnitude during acquisition, but different duration of extinction trials: Groups 120/300 versus 120/120 with either 300

or 120 s of exposure to the goal compartment and Groups 1200/1200 versus 1200/300 with either 1200 or 300 s of duration of extinction trials. In both cases, with reward magnitude constant, extinction was faster with longer duration of extinction trials. The higher extinction latencies in Group 120/300 than 120/120 could be due to stimulus generalization decrement, whereas the higher extinction latencies in Group 1200/1200 than 1200/300 could not be accounted for in the same terms. Furthermore, the two groups receiving the same time of exposure to the goal compartment in acquisition and extinction (Groups 1200/1200 vs. 120/120) also provided an assessment of the effects of stimulus generalization decrement on extinction performance. The difference between these groups was also significant, showing that duration of extinction trials and reward magnitude have larger effects on extinction running speed than on generalization decrement.

Thus, behavior showed that extinction learning was: (1) directly related to reward magnitude in acquisition (i.e., reversed MREE) (2) directly related to time of exposure to extinction cues during extinction trials, and (3) apparently little affected by stimulus generalization decrement.

As in Experiment 1, terminal acquisition performance and dehydration in the goal compartment were dissociated. Whereas behaviorally there was no evidence of a group effect, weights increased significantly more after 1200 s of exposure to water than after 120 s (Fig. 4). The dissociation between instrumental behavior and weight variation during acquisition trials is puzzling. Taking advantage of the large samples used in both experiments, correlations were computed between three variables as they occurred during the last two trials of acquisition: time of access to water (from 120 to 1200 s), weight variation (g/100 g), and running latency (\log_{10}). As expected, weight variation correlated significantly with access to water, $r(83) = 0.93$, $P < 0.01$, two-tailed, $r^2 = 0.86$. However, there was no evidence of a correlation between running latency and access to water or weight variation, $r_s(83) < 0.12$, $P_s > 0.05$, two-tailed, both $r^2_s = 0.01$. Thus, terminal acquisition performance and reward magnitude do not seem to relate in any obvious way. The effects of reward magnitude on instrumental behavior are seen clearly during extinction, when reward is removed from training.

General discussion

Terrestrial toads such as *R. arenarum* depend on daily access to water to avoid dehydration. As a result, these animals quickly learn to find sources of water and to respond to the content of the fluid, especially in terms of its salinity, to avoid dehydration (Daneri et al. 2007; Puddington et al. 2016). The present experiments aimed at identifying the

factors that regulate the toads' behavior in situations involving the omission of water reinforcement, as is the case during appetitive extinction. Additionally, these experiments were designed to dissociate the contribution of reward magnitude, amount of acquisition training, and duration of extinction trials on instrumental extinction in terrestrial toads. As mentioned in the introduction, a complete understanding of the behavioral differences shown across vertebrates in the effects of reward schedules on instrumental extinction requires a systematic approach to the determinants of extinction in a non-mammalian species. Thus, the evidence accumulated over the last decades in our laboratory on extinction processes in this amphibian contributes to an understanding of the comparative basis of learning and cognition in vertebrates.

The results of the present research can be summarized in the following conclusions. First, there was good evidence that varying the time of access to water produced different levels of weight variation. Whereas this has traditionally been interpreted as inducing different levels of reward value, there was no clear evidence that these levels affected acquisition performance. Previous experiments with more extensive practice have found such relationship to be quite orderly, with terminal latencies to be monotonically related to time of water access (e.g., Muzio et al. 1992). In the present experiments, most groups had limited amounts of acquisition training (e.g., 5 daily trials), a factor that might explain the absence of clear effects of reward magnitude on running latencies.

Second, although these variables for the most part did not affect acquisition performance, they did produce orderly effects on extinction. With total water access equated across groups in Experiment 1, smaller rewards per trial, but distributed over 15 trials (Group 300/15) led to lower extinction latencies than larger rewards per trial, but distributed over 5 trials (Group 900/5). Whereas this resembles a MREE, two facts work against this interpretation. On the one hand, a comparison of Groups 300/5 and 100/15, also matched in total water access, but differing in terms of the distribution of rewards across trials, failed to replicate this effect. On the other hand, Experiment 2 suggests that this presumed MREE was likely related to the amount of time of exposure to extinction cues, rather than to reward magnitude in acquisition. With reward magnitudes differing by a 10-to-1 ratio, groups with equal duration of extinction trials (Groups 1200/300 vs. 120/300) demonstrated a reversed MREE (i.e., lower extinction latencies after large-reward acquisition than after small-reward acquisition).

After a series of experiments involving various shifts in reward magnitude and two instrumental tasks, Muzio et al. (2011) argued that toads acquire new instrumental behaviors via habit learning, rather than in terms of incentive expectancies. Nothing in the present results contradicts

that conclusion. However, a separate series of experiments on extinction after partial reinforcement training suggested that nonreinforced trials had minimal impact on extinction performance and that the relevant factor was the distribution of rewarded trials in time (Muzio et al. 2006). Thus, toads trained under 50% partial reinforcement receiving a reward every 2 days on average extinguished faster than toads training under 100% continuous reinforcement receiving a reward every day, but at the same rate as a 100% continuous reinforcement group receiving a reward every 2 days on average. Follow-up experiments introducing a retention interval between the end of acquisition and the beginning of extinction showed a substantial decrement in behavior at longer intervals after similar acquisition performance (Puddington et al. 2013). The conclusion favored after these experiments was that toads are sensitive to the decay of long-term memories encoding reward information, with little weight being assigned to nonrewarded trials. The present experiments suggest that there is, after all, a role for nonreinforcement during extinction learning in toads. As shown in Experiment 2, with reward magnitude equated, extinction was a function of time spent in the goal compartment in nonrewarded trials (Groups 1200/1200 > 1200/300 and 120/300 > 120/120, where ">" refers to the length of running latencies in extinction).

To our knowledge, the role of duration of extinction trials has not been studied in mammals in a design similar to that used in Experiment 2. Manipulations of the time of exposure to extinction cues are found mainly in experiments on memory reconsolidation. In Pavlovian fear conditioning, short exposure to the CS alone after acquisition generally leads to a reconsolidation process that strengthens the response, whereas multiple CS-only trials lead to extinction (Suzuki et al. 2004). A recent review reports no studies manipulating exposure to nonreward cues in appetitive instrumental learning (Reichelt and Lee 2013). Thus, this is an area in which more behavioral studies are needed.

In summary, the instrumental behavior of toads in a seemingly simple training situation (i.e., approaching a source of water) is under the control of several factors. The present experiments highlighted reward magnitude, amount of acquisition training, and duration of extinction trials, whereas previous experiments emphasized the decay of long-term memories of rewarding events. Thus far, none of these experiments with terrestrial toads seem to require an emotional response induced by reward omission to understand instrumental learning. Moreover, experiments done in other amphibian species, such as newts (*Cynops pyrrhogaster*), using solid food as reward instead of water, are also consistent with a nonemotional interpretation (Shibasaki and Ishida 2012), unlike the case with mammals exposed to similar situations (Papini et al. 2015). Future

experiments could extend these findings using a more extensive parametric manipulation of these factors.

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