Journal of Comparative Psychology

Modification of a Prey Catching Response and the Development of Behavioral Persistence in the Fire-Bellied Toad (Bombina orientalis)

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The present report investigated how fire-bellied toads (*Bombina orientalis*) modified their response in a prey catching task in which the attribution of food reward was contingent on snapping toward a visual stimulus of moving prey displayed on a computer screen. Two experiments investigated modification of the snapping response, with different intervals between the opportunity to snap at the visual stimulus and reward administration. The snapping response of unpaired controls was decreased compared with the conditioned toads when hour or day intervals were used, but intervals of 5 min produced only minimal change in snapping. The determinants of extinction of the response toward the visual stimulus were then investigated in 3 experiments. The results of the first experiment suggested that increased resistance to extinction depended mostly on the number of training trials, not on partial reinforcement or the magnitude of reinforcement during training. This was confirmed in a second experiment showing that overtraining resulted in resistance to extinction, and that the pairing of the reward with a response toward the stimulus was necessary for that effect, as opposed to pairing reward solely with the experimental context. The last experiment showed that the time elapsed between training trials also influenced extinction, but only in toads that received few training trials. Overall, the results suggest that toads learning about a prey stimulus progress from an early flexible phase, when an action can be modified by its consequences, to an acquired habit characterized by an increasingly inflexible and automatic response.

Keywords: amphibians, extinction, goal-directed, habit, overtraining

McFarland and Sibly (1975) proposed that behavior "may be regarded as the output of a multivariable adaptive control system" (p. 266) that involves competition between different mutually exclusive possibilities. The nature of this competition is still not well understood, but findings in mammals and birds showed that it has to take into account motivational, emotional, and memory components (Cardinal, Parkinson, Hall, & Everitt, 2002). Memory components are themselves diverse, as there is evidence suggesting that multiple learning and memory systems compete for control over behavior (Kesner & Rogers, 2004; Packard, 2009; Sherry & Schacter, 1987; Toates, 2006; van der Meer, Johnson, Schmitzer-Torbert, & Redish, 2010; White & McDonald, 2002). It is, however, unclear if these many memory systems were inherited from the ancestors of amniote vertebrates, or if they represent adaptive specializations of birds and mammals.

One approach used to discriminate between different forms of control over behavior is to observe the effect of an unexpected disruption of an appetitive reinforcer on a conditioned response, that is, comparing an established response before and after the disruption, which can be achieved by devaluation or omission of the usual reinforcement. Such manipulations have been used to demonstrate that behavioral control varies depending on the phase of instrumental learning in mammals. Instrumental learning is thought to proceed from an early phase when actions are sensitive to their outcome to a later phase when responses become progressively more insensitive to their consequences (Dickinson, 1985). For example, overtraining of an instrumental response in rats can lead to response persistence following reward devaluation (Adams, 1982; Dickinson, 1985). Similarly, extended training in a maze can lead rats to perform misdirected turning responses that do not take into account the current spatial location of a reward (Packard & McGaugh, 1996). Such acquired habits were characterized by Graybiel (2008) as ordered responses performed automatically following a particular context or stimulus, which are relatively resistant to change (see Seger & Spiering, 2011, for a history of the concept). One could surmise that habits are used to diminish the amount of cognitive processing involved in predictable situations, thereby saving energy or allowing allocation toward other functions. Brain lesion experiments have further suggested that distinct neural substrates are involved in action-outcome or stimulus-response behavioral control (Balleine & O'Doherty, 2010; Corbit & Janak, 2007; Killcross & Coutureau, 2003; Seger & Spiering, 2011; Yin & Knowlton, 2006; Yin, Knowlton, & Balleine, 2004; Yin, Ostlund, Knowlton, & Balleine, 2005).

Testing arena and stimulus. The experimental context was a square platform (40 × 40 cm) made of gray PVC, cut to fit between the edges of the computer screen used for display and remain flush against it (see Figure 1). The platform and computer were on top of a larger table. There were no walls around this experimental context; therefore, the toads faced only the computer screen while the experimenter stood behind. The cricket visual stimulus consisted of five live crickets placed in a clear plastic tube against a white background. Crickets were filmed in high definition using a Canon VIXIA HV20 camcorder (Canon; Tokyo, Japan) in standard VGA size (640 × 480) at 30 frames per second. The video clip was displayed using the software VLC Media Player version 0.9.9 (VideoLAN, Paris, France) in a 10 × 3 cm panel on a computer screen (LP 1965 19-in. LCD, Hewlett-Packard, Palo Alto, California) mounted to the wall, with its bottom part resting on the table. The stimulus was centered in the middle of a black screen at the vertical level of the experimental platform.

Procedure. In order to reduce behavioral inhibition in the new experimental context, two shaping trials were conducted before the training trials began. The shaping trials involved administration of a cricket ~10s after the toads were put on the platform. In this experiment, shaping trials did not involve the video stimulus; thus, the toads did not display snapping responses toward the screen at that time. The shaping trials were conducted on 2 consecutive days, 1 day prior to the beginning of the training trials, which were then performed every other day. At the beginning of each training trial, a toad was placed on the square platform facing the screen, 10 cm away from it. The experimental trials began with the display of the video clip and were stopped when a toad had snapped five times at the prey stimulus or when the maximal trial duration of 5 min (300 s) was reached. In either case, the stimulus was immediately removed following the end of the trial. The latency to reach five snaps was calculated automatically and displayed by the VLC player after a touch of the keyboard stopped the trial and removed the stimulus from the screen. The maximal duration of 300 s was also attributed to toads that wandered off the experimental platform twice before the end of the trial. Toads were returned to the starting position by the experimenter following the first wandering off the platform.

Toads of different sizes were evenly distributed between groups at the beginning of the experiment, and equal numbers of toads from each experimental group were mixed together in housing tanks. A conditioned group (*Conditioned*) had toads presented with a cricket reward held with tweezers in front of their snout immediately (within a second) following the fifth snap at the stimulus. Most toads readily accepted and consumed crickets presented this way (those that did not were rejected from analysis). The toads were allowed to consume the cricket plus an additional 5 s on the platform before being returned back to their holding tank. Toads in this group were only rewarded with a cricket if they reached five snaps within the allotted 300 s. A second group of toads (*Outside context*) underwent the same procedure, but upon completion of the task and removal of the stimulus, they were left for 5 sec on the platform before being returned to their holding tank without receiving a cricket. Instead, they were administered (noncontingent on completion of the task) a cricket outside of the experimental context on the days in between trials. These toads received their cricket in an opaque white plastic bin measuring 21 cm in diameter with 14 cm tall walls. A third group of unpaired controls (*5 min before*) received a cricket on the experimental platform 5 min before presentation of the video stimulus. The fourth and last group of unpaired controls (*5 min after*) received a cricket on the experimental platform 5 min following completion of a trial involving presentation of the video stimulus. Toads in the latter two groups always received crickets whether they reached five snaps at the stimulus or not. They were returned to their holding tanks during the 5-min interval between food administration and stimulus presentation or vice versa. Crickets were roughly matched to the size of each toad to account for size variation. All trials and feeding occurred during the light phase at approximately the same time of day for each toad, and testing times of toads from different groups were evenly distributed throughout the day.

Statistics. Latency to reach five snaps was analyzed over all training trials. Latency data was log-transformed (base 10) before analysis to meet the assumption of normality. Repeated measures ANOVA (GLM repeated measures in SPSS Statistics 19; IBM, New York) was used with trial as within-subject factor and group as between-subjects factor. Significant effects of group were followed by the Fisher's least significant difference (LSD) posttest for comparisons between the *Conditioned* group and controls. The assumption of sphericity was never met, but corrections to the degrees of freedom according to Greenhouse–Geisser or Huynh–Feldt did not change the results. Finally, univariate ANOVAs and LSD posttests were performed at each trial to assess the time at which the effects between groups were significant. Alpha was set at 0.05.

Results and Discussion

Four toads were rejected from analysis because they consistently did not accept crickets presented to them during trials. All died shortly after the end of the experimental period; thus, they
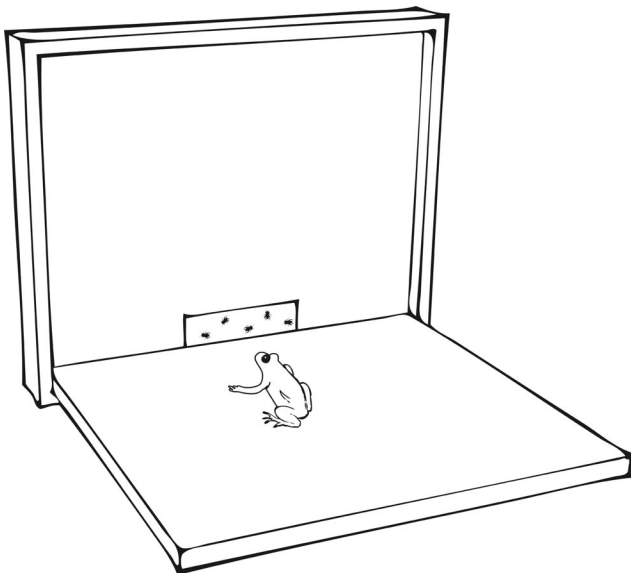

Figure 1. Schematic drawing of the experimental context. A fire-bellied toad is seen attending to the central panel displaying the cricket stimulus on the computer screen. See text for dimensions.

likely got sick during the experiment. One of them was in the *5 min before* group, and three of them were in the *5 min after* group. The latencies to reach five snaps are charted in Figure 2. Analysis of the data for the first training trial showed that, despite great variation, there was no significant difference among groups, $F(3, 24) = 2.34$, $p = .10$. The ANOVA over all training trials showed significant effects of trial, $F(21) = 7.23$, $p < .001$, $\eta_p^2 = 0.23$, interaction between trial and group, $F(63) = 5.59$, $p < .001$, $\eta_p^2 = 0.42$, and group, $F(3, 24) = 27.51$, $p < .001$, $\eta_p^2 = 0.78$. The LSD posttest then showed that the latency in the *Conditioned* group was lower than the *Outside context* ($p < .001$, $d = 1.37$) and *5 min before* ($p = .04$, $d = 0.70$) groups. Trial-by-trial ANOVAs revealed that a significant difference between groups was present at Trial 5, $F(3, 24) = 3.65$, $p = .03$, $\eta_p^2 = 0.31$, and all subsequent trials. At Trial 5, only the *Outside context* group differed from the *Conditioned* group, but at Trials 13, 15, 17, and, 19 through 22, LSD posttests also revealed that the latency to reach five snaps was significantly higher in the *5 min before* group compared with the *Conditioned* group. The low sample size that remained in the *5 min after* group after toad rejections could have prevented the assessment of a subtle difference with the *Conditioned* group. However, visual inspection of the data suggests that the effect was restricted to the *5 min before* group.

Contrary to our expectations, the 5-min interval used here did not clearly suppress the prey catching response. Only a marginal difference was observed between the conditioned toads and the toads rewarded in the experimental context with a 5-min delay. Studies of pigeon autoshaping (Gibbon, Baldock, Locurto, Gold, & Terrace, 1977) and rat conditioned licking (Boice & Denny, 1965), tasks sharing general similarities with the prey catching task

used here, have demonstrated that respective intervals of 32 and 6 s between stimulus and reward were sufficient to prevent the increased responding seen with shorter intervals. Could it be that the temporal coding of events is not as precise in toads in comparison with animals with higher metabolic rates, like mammals and birds? It might be useful for toads to associate all environmental stimuli that occurred 5 min before and after feeding because they encounter prey only at a low rate in natural conditions. A broad time range when learning about the environment has already been observed in illness-induced flavor aversion (Garcia, Ervin, & Koelling, 1966; Rozin & Kalat, 1971), in which rats can learn to avoid food consumed an hour or longer prior to induction of illness. It would be interesting to systematically examine modification of the toad prey catching response using different time intervals between stimulus and reward; however, this is beyond the scope of the present report.

A possible reason for the maintenance of responding despite the 5-min interval between stimulus and reward could be that the toads were fed crickets before and during the experiment, thus priming their response toward a cricket visual cue. However, the same feeding regimen of crickets in toads rewarded outside of the experimental context clearly did not have a great impact on their response toward the visual stimulus, so this explanation should be rejected. The next experiment used much longer stimulus–reward intervals in order to find out whether a convincing demonstration of learning could be obtained with that task.

Experiment 2: Modification of the Prey Catching Response With Longer Intervals Between Stimulus Presentation and Reward

Because the effect on latency to snap seen in the *5 min before* group in the first experiment was weak, it prompted the design of an additional experiment using longer intervals to assess whether control toads fed in the context could diminish their snapping behavior in a stronger fashion when it was not contingent or contiguous with the attribution of a cricket. Sample size was increased to 12 toads per group for this experiment to account for toad rejections and ensure adequate sample size for analysis.

Method

A total of 48 toads were used in Experiment 2 (mean weight: 7.3 g, 95% CI [6.9, 7.7]). The toads were housed in groups of six in the same conditions as described for Experiment 1, except that plastic tanks (28 × 18 × 18 cm) were used. Four groups of 12 toads underwent the same prey catching procedure as in Experiment 1. In fact, the new toads in *Conditioned* and *Outside context* groups experienced exactly the same procedures as the equivalent groups in Experiment 1. Two additional groups of unpaired controls were added. The *Context control* group had toads fed a cricket on the experimental platform on the days in between trials, without the video stimulus playing. The time to cricket administration following placement on the platform was yoked to the response time (time to five snaps) of a paired toad in the *Conditioned* group. Toads in the *Scrambled 1 h* group were fed a cricket on the platform alternately 1 hr before or after presentation of the video stimulus, with order of presentation counterbalanced between toads. These toads were put back into their holding tanks for the
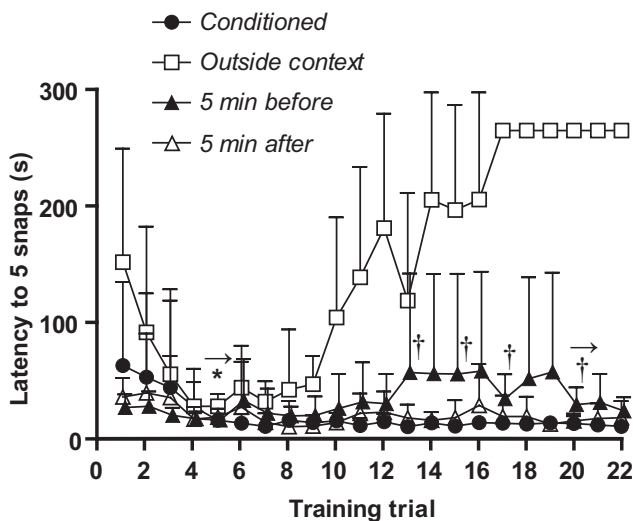
Figure 2. Modification of the snapping response over a 44-day training period. Toads were only submitted to one trial per day, every second day. Latency to reach five snaps at the cricket stimulus is shown (means + 95% CI). Sample sizes are 8 for the *Conditioned* (black circles) and *Outside context* (open squares) groups, 7 for the *5 min before* (black triangles) group, and 5 for the *5 min after* group (open triangles). The asterisk (*Outside context*) and crosses (*5 min before*) indicate significant differences when compared with the *Conditioned* group. Arrows above symbols point in the direction of continuous blocks of statistically significant trials.

time period between food administration and stimulus presentation. This experiment was conducted by the first author in the summer of 2011.

Results and Discussion

Ten toads were rejected from Experiment 2. One died during the experiment, and nine never snapped at the stimulus because they were either lethargic or hyperactive and never paid attention to the stimulus. There was one rejection each in the *Conditioned* and *Outside context* groups, whereas there were four rejections each in the *Scrambled 1 h* and *Context control* groups. The latencies to reach five snaps in Experiment 2 are charted in Figure 3. Analysis of latencies at the first training trial showed that there was no significant difference between groups, $F(3, 34) = 1.52$, $p = .23$. The ANOVA over all training trials showed significant effects of trial, $F(21) = 16.89$, $p < .001$, $\eta_p^2 = 0.33$, interaction between trial and group, $F(63) = 8.23$, $p < .001$, $\eta_p^2 = 0.42$, and group, $F(3, 34) = 33.19$, $p < .001$, $\eta_p^2 = 0.75$. The LSD posttest then showed that the latency in the *Conditioned* group was lower than in all other groups ($p < .001$ for all comparisons; $d = 1.54$ for *Conditioned* vs. *Outside context*; $d = 1.34$ for *Conditioned* vs. *Context control*; and $d = 1.11$ for *Conditioned* vs. *Scrambled 1 h*). Trial-by-trial ANOVAs revealed that a significant difference between groups was present at Trial 6, $F(3, 34) = 3.18$, $p = .04$, $\eta_p^2 = 0.22$, and all subsequent trials. At Trial 6, only the *Outside context* group differed from the *Conditioned* group, but this difference was seen beginning at Trial 8 for the *Context control* group and beginning at Trial 10 for the *Scrambled 1 h* group.
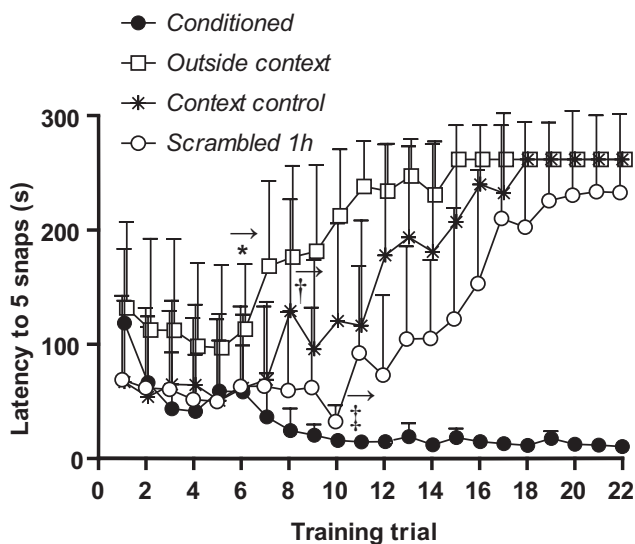
Figure 3. Second experiment on modification of the snapping response. Toads were again submitted to one trial per day, every second day, for a period of 44 days. Latency to reach five snaps at the cricket stimulus is shown (means + 95% CI). Sample sizes are 11 for the *Conditioned* (black circles) and *Outside context* (open squares) groups, and 8 for the *Context control* (stars) and *Scrambled 1 h* (open circles) groups. The asterisk (*Outside context*), cross (*Context control*), and double cross (*Scrambled 1 h*) indicate significant differences when compared with the *Conditioned* group. Arrows above symbols point in the direction of continuous blocks of statistically significant trials.

The results of Experiment 2 showed that 1-hour and 1-day intervals between cricket feeding and presentation of the stimulus resulted in a strongly decreased snapping response toward the cricket stimulus. Learning about the visual stimulus is thus involved in at least some aspects of the observed prey catching response flexibility. However, the group differences that provided evidence of learning were due to two factors: (a) a progressive decrease in latencies in the *Conditioned* groups, and (b) the larger gradual increases in latencies in the control groups. The increased latencies in controls could possibly result from habituation. If this was the case, the learning observed in this task could have resulted from interference with habituation that would normally occur with repeated presentation of the same cricket stimulus. However, the fact that the conditioned groups showed reduction in snapping latencies with training suggests that associations between stimulus and snapping behavior might have taken place. More work is needed to ascertain the learning mechanisms underlying flexible prey catching behavior in this toad.

Another aspect that contributed to unusual learning curves in the first two experiments is that the animals began at intermediate levels of responding. The choice of a task that involved an intermediate level of responding in naïve animals was prompted by the difficulties in motivating amphibians with nonbiologically relevant stimuli or situations. Modification of the intensity of a natural response is therefore an amenable way to study their behavioral flexibility.

Experiment 3: Effect of Partial Reinforcement and Magnitude of Reinforcement on Extinction

The next experiment dealt with extinction of the snapping response, which involved omission of reinforcement following response acquisition. Here, extinction refers to the procedure used and leaves open possibility that the conditioned snapping response will not extinguish, that is, show resistance to extinction. This procedure was used to assess behavioral flexibility or persistence in the face of an unexpected reward change. Papini (2003) termed such a procedure "surprising nonreward" and argued that some vertebrates modify their behavior by learning about their emotional reaction (frustration) to a surprising nonreward event.

Two typical effects used to support the role of frustration in modulating the response during extinction are the partial reinforcement extinction effect (PREE) and the magnitude of reinforcement extinction effect (MREE). Animals displaying the PREE and MREE decrease their response comparatively more in extinction when trained under continuous rather than partial reinforcement in acquisition, or when trained with larger compared with smaller rewards (Amsel, 1958). It is thought that disrupting the anticipated outcome of constant or large rewards obtained in acquisition can result in a negative emotional state called "primary frustration" (Papini, 2008). As extinction proceeds, this frustration state can be paired with the environment to rapidly reduce responding by anticipation of primary frustration: a phenomenon called "secondary frustration." These effects have been termed "paradoxical" because they are contrary to the predictions of the law of effect, which posits that strong associations established by strong reinforcement would be more resistant to change. Interestingly, studies using vertebrates other than mammals or birds could not demonstrate PREE or MREE phenomena (reviewed in Papini, 2003,

2008). For example, Muzio et al. (1992, 1994) showed that Argentine toads that acquired a runway task for water reward extinguished their running response at the same rate whether they were trained under continuous or partial reinforcement. Similarly, variation in the magnitude of water reinforcement did not lead to different extinction rates in the same task (Muzio et al., 1992; Papini, Muzio, & Segura, 1995). A later study from the same group concluded that the absolute number of reinforcements and the spacing of reinforcements in time during acquisition were the most important factors determining response in extinction (Muzio et al., 2006).

The studies in Argentine toads just outlined are, however, the only ones that tested for PREE and MREE in an amphibian. Is extinction of the present prey catching task in the fire-bellied toad modulated by the same factors? The first of three extinction studies presented here tested for the presence of PREE and MREE in the fire-bellied toad using the prey catching task already introduced. Experiment 3 was designed to test whether emotional reaction to surprising nonreward could be involved in flexibility of the snapping response. At the same time, control groups included allowed the possibility to evaluate the effect of the number of reinforced trials on response extinction.

Method

Animals. A total of 50 adult fire-bellied toads of mixed genders were used in this experiment (mean weight: 4.9 g, 95% [CI 4.5, 5.3]). They were housed in groups of 4 to 6 in the plastic tanks and conditions described previously, with one exception: daytime room temperature was 21 °C, but a cooler night was simulated by gradually bringing the temperature down to 17 °C during the night over a period of 1 hour, then bringing it back to the warmer temperature in the morning. The toads were fed crickets ad libitum once a week for at least 6 weeks of acclimatization to the laboratory prior to the beginning of the experiment. During the shaping and acquisition phases of the experiment, feeding was confined to the trials, whereas during extinction, toads were fed a weekly sustenance diet equivalent to three crickets per toad in their holding tank on a day without a trial.

Procedure. The same testing arena and procedure were used as in Experiments 1 and 2, with the following differences. In order to maximize acquisition of the prey catching response, because subsequent extinction was the focus of this experiment, the two shaping trials involved the video stimulus of crickets. In a shaping trial, a toad was rewarded with a cricket immediately following one snap at the video stimulus. This was followed by acquisition training with five groups of toads required to snap five times at the cricket stimulus to complete the trials under different schedules of reinforcement (see Figure 4A). The shaping and acquisition trials were conducted at approximately the same time of day for each toad. The beginning of the training period for the groups with a different number of acquisition trials was organized so that all toads would finish acquisition on the same day. Acquisition was then followed by 22 extinction trials. Extinction trials were stopped after five snaps, 5 min, or two escapes from the platform, but no cricket reward was given at that time. Toads were subjected to only one trial per day and trials were run every other day. The groups, as defined by their schedules of reinforcement in acquisition, were as follows: Groups CR6 and CR12 received continuous
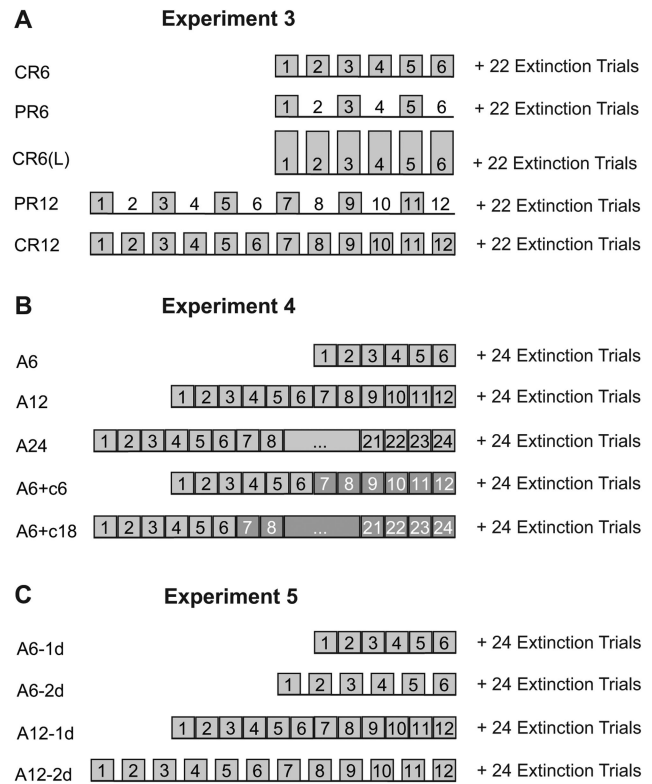
Figure 4. Schedules of reinforcement or reward attribution used in acquisition training before the onset of extinction trials in Experiments 3, 4, and 5. In all panels, abbreviations for groups are listed on the left and reinforced trials are illustrated by light gray squares or rectangles with black numbering. Empty spaces between squares/rectangles indicate that there were 2 days between trials, whereas no space indicate that trials were conducted on consecutive days. In Panel A, nonreinforced trials are left blank and the tall gray rectangles illustrate trials with twice the normal amount of reinforcement in group CR6(L). In Panel B, trials represented in darker gray with white numbering indicate that reward was attributed in the context without the video stimulus playing. The number of extinction trials is shown on the right. There were 2 days between extinction trials in all cases.

reinforcement of one cricket per trial for 6 or 12 acquisition trials before the onset of extinction, whereas Groups PR6 and PR12 received partial reinforcement of one cricket reward only on half of the acquisition trials for 6 or 12 trials. Partial reinforcement involved alternation of rewarded and nonrewarded trials, with counterbalancing of the type of trial at the beginning of acquisition within groups. Finally, Group CR6(L) received two crickets, instead of one, for six continuously reinforced acquisition trials with large rewards. The size of cricket rewards was matched to the size of the toads, and the reward size was maintained in Group CR6(L) so that these toads would receive twice as much food at the end of an acquisition trial as in the other groups. This experiment was conducted in two phases by the same experimenter (Juntaro Ikura): 20 toads were used from February to May 2009, whereas 30 were used later in May to July 2009. In both phases, each group had an equal number of toads.

Statistics. Potential differences among groups during extinction were assessed after transformation of the latencies to reach

five snaps in the extinction trials into ratios. This was done to normalize baseline responding across animals. The ratios calculated were response latency in an extinction trial divided by latency at the last acquisition trial for each animal. The ratios were then log-transformed and a repeated measures ANOVA was calculated with trial as the within-subject factor and group as the between-subjects factor, as in Experiments 1 and 2. This was followed by the LSD posttest to determine differences between groups. One-sample t tests comparing extinction data with the maximal trial duration of 300 s were also conducted to determine at which trial a group's response would not be significantly different from the maximal trial duration, an event that was termed "full extinction." This was done to (a) assess possible differences in the onset of full extinction between groups, and (b) exclude the extinction trials influenced by the ceiling of maximal trial duration from the curve fitting procedure used for presentation of the extinction data. As just alluded to, curve fitting was used for presentation of the extinction data to allow better visualization of the general trends between groups. It was done by fitting straight lines on the log-transformed extinction ratio data for each group using linear regression in GraphPad Prism version 5.01 (GraphPad Software, La Jolla, California), with the constraint that the curves originated at zero, that is, the ratio value at the last acquisition trial. This procedure generated curves that did not significantly deviate from linearity, as shown by nonsignificant results of runs tests in all instances.

Results and Discussion

Nine toads were rejected because they did not acquire the task (five in Group PR6, three in Group PR12, and one in Group CR12). The pattern of rejection suggests that this task was more difficult to acquire under partial reinforcement. There was a significant difference between groups in latency to reach five snaps at the last acquisition trial, $F(4, 36) = 3.40$, $p = .018$, $\eta_p^2 = 0.25$, which required response ratios (extinction to last acquisition) to normalize baseline responding at the beginning of extinction for further analysis.

The ANOVA over all extinction trials showed significant effects of trial, $F(21) = 58.04$, $p < .001$, $\eta_p^2 = 0.62$, interaction between trial and group, $F(84) = 1.81$, $p < .001$, $\eta_p^2 = 0.17$, and group, $F(4, 36) = 3.58$, $p = .015$, $\eta_p^2 = 0.29$. The LSD posttest then showed that the latency ratios in extinction were significantly higher in Group CR6 compared with Groups CR12 ($p = .001$, $d = 0.89$) and PR12 ($p = .04$, $d = 0.55$). Note that the comparison between Groups CR6 and CR6(L) was close to statistical significance ($p = .082$). Significantly higher latency ratios were also observed in Group PR6 compared with Group CR12 ($p = .012$, $d = 0.88$). There were no other significant differences between groups. The significant interaction is difficult to interpret because, as Figure 5 shows, different extinction rates led to divergent responses between groups early during extinction, but later the responses were brought closer together due to the limit on trial duration. Because the effect of group appeared sufficient to explain the data, the meaning of the interaction effect was not explored further in this experiment.

Figure 5A shows the untransformed data for Experiment 3 along with the results of the "full extinction" analysis. Latencies to reach five snaps in Group PR6 did not differ from the 300-s maximal
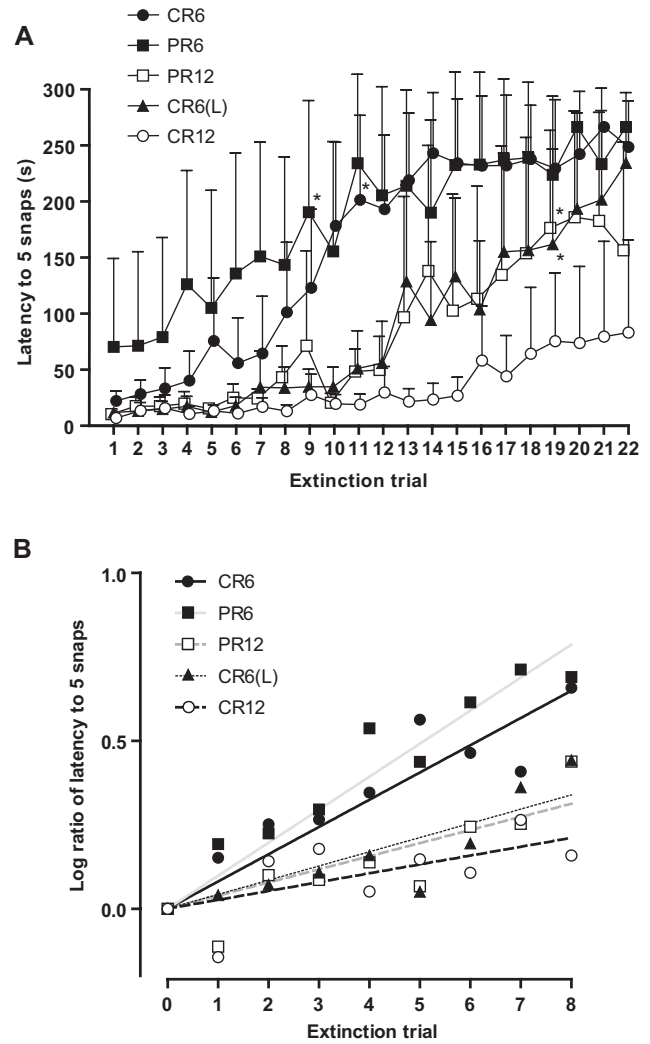
Figure 5. Extinction of the snapping response in Experiment 3. (A) Latency to reach five snaps (mean + 95% CI) over the 22 extinction trials. Asterisks indicate the first nonsignificant difference compared with maximal trial duration for each group, as assessed by the one-sample t test. Symbols are CR6 = black circles; PR6 = black squares; PR12 = open squares; CR6(L) = black triangles; and CR12 = open circles. (B) Linear regression of the log-transformed latency to five snaps ratios (extinction latency divided by latency at last acquisition trial) for each group over extinction Trials 1 through 8. The curves outline general extinction trends across groups before maximal trial duration diminished extinction slopes in some groups. Group symbols are the same as in Panel A, with the addition of distinct line patterns to help distinguish between different curves (CR6 = black line; PR6 = gray line; PR12 = gray hatched line; CR6[L] = black dotted line; CR12 = black hatched line).

trial duration for the first time at Trial 9, $t(6) = 1.57$, $p = .169$, whereas this happened at Trial 10 for Group CR6, $t(8) = 2.21$, $p = .058$, and at Trial 19 in both Groups PR12, $t(6) = 2.33$, $p = .059$, and CR6(L), $t(8) = 2.26$, $p = .054$. The latencies to reach five snaps were significantly lower than maximal trial duration at all extinction trials in Group CR12. Figure 5B shows the result of the linear regression analyses including only the first eight extinction trials, that is, before response in any of the groups could be

influenced by the limit on trial duration. Extinction rates in the different groups appear to make two major clusters: PR6 and CR6 with high extinction rates, and the three other groups with low extinction rates. However, the pattern of onset of full extinction across groups suggest that Groups PR12 and CR6(L) show an intermediate level of response extinction, and Group CR12 shows the lowest response extinction.

Together, these analyses suggest that an important factor determining extinction of the prey catching response in Experiment 3 was the number of acquisition trials, not the number of reinforcements per se, as seen by differences restricted to comparisons between groups that had 12 instead of 6 acquisition trials (when excluding the large rewards group), regardless of the schedule of reinforcement. The magnitude of reinforcement also determined response in extinction to some extent, because Group CR6(L) showed a low response extinction rate similar to the groups that had 12 acquisition trials. These results show that the phenomena of PREE and MREE did not affect the prey catching response in extinction in accordance with the conclusion of previous observations that frustration does not modulate behavior in amphibians (Muzio et al., 1992, 1994; Papini, 2003; Papini et al., 1995).

Interestingly, Muzio and colleagues (2011) recently argued that the absence of learning phenomena suggesting emotional reactions following surprising reward changes, including the absence of PREE, indicates that habit formation is the predominant learning mode in amphibians. However, Argentine toads tested for PREE and MREE quickly extinguish their runway response once reinforcement is withheld (Muzio et al., 1992), thereby displaying a behavioral flexibility incompatible with the concept of habit used in the present study (cf. Graybiel, 2008; Seger & Spiering, 2011). If the increased persistence in extinction observed after more acquisition trials in Experiment 3 represents the onset of a habit, further training could eventually lead to an inflexible prey catching response. The next experiment involved a test of this idea, and thus the possibility that the role of emotions (or absence of a role) can be dissociated from the control that habits exert over behavior.

Experiment 4: Effect of Overtraining and Rewards in Context on Extinction

The second experiment on extinction of the prey catching response focused on the effect of increased acquisition training and the role of associations between food reward and experimental context, instead of response reinforcement. Because the results of Experiment 3 suggested that more acquisition trials produced an increased resistance to extinction, the hypothesis that overtraining of the prey catching response would lead to increased resistance to extinction was investigated by submitting a group of toads to even more acquisition training before extinction than in the previous experiment. Two relevant studies in amphibians used runway learning for water or food reward. In the first study, Muzio and colleagues (2006) used Argentine toads (*Rhinella arenarum*) as subjects. Toads that received 30 runway acquisition trials showed a faster running response early during extinction compared with toads that received only 10 acquisition trials. This phenomenon was explained by the fact that the toads increased their ability to obtain water as training proceeded, thus experiencing more reinforcement and a stronger drive to respond after longer training, but it could also suggest that a habit developed with longer training. In

a second study, Shibasaki and Ishida (2012) used a similar runway task in which fire-bellied newts (*Cynops pyrrhogaster*) were rewarded with food for running to a goal box. Newts that received either 25 or 75 acquisition trials showed no difference during extinction. The runway task that was used in these studies, however, might not have been ideal to investigate habit formation because rats tested in a similar fashion to run an alley for food showed no effect of the extent of acquisition training on the response in extinction (Wagner, 1961). Such runway tasks might offer limited opportunity for the animals to display flexible behavior.

Another factor that determines response extinction in rats, pigeons, and goldfish that underwent instrumental training for food is exposure to nonresponse contingent rewards in the experimental context (Adams, 1982; Igaki & Sakagami, 2004; Nevin, Tota, Torquato, & Shull, 1990). Adams (1982), for example, showed that the amount of exposure to a reward, but not response–reward pairings, was responsible for the development of behavioral persistence, as seen by the development of a relative insensitivity following reward devaluation (Experiment V in that study). He proposed that what is learned about the reward can change as training proceeds, possibly its affective value or new associations between reward and environmental cues through second-order conditioning, which would be insensitive to reward devaluation. In order to tease apart the contributions of the experimental context from those of the stimulus and response to the development of a persistent prey catching response during extinction in the toad, groups of toads were administered rewards in the experimental context without the cricket stimulus being played after an acquisition period. This manipulation abolished snapping behavior during these trials, preventing associations of the reward with both the video stimulus and the snapping response.

Method

Animals. A total of 40 adult fire-bellied toads of mixed genders were used in this experiment (mean weight: 6.1 g, 95% CI [5.7, 6.5]). They were housed in groups of 10 (2 toads from each group, see the following section) in the glass tanks and conditions described for Experiments 1 and 2. The toads were fed crickets ad libitum once a week for 2 weeks of acclimatization to the laboratory prior to the beginning of the experiment. Again, during the shaping and acquisition phases of the experiment, feeding was confined to the trials, whereas during extinction, the toads were fed a sustenance diet once a week in their holding tank on a day without a trial.

Procedure. The procedures for shaping, acquisition, and extinction trials were the same as in Experiment 3. However, the shaping and acquisition trials were conducted on consecutive days and a new type of trial (context trial) was utilized in some groups, whereby a cricket was administered following a delay in the experimental context without the video stimulus playing. The delay before cricket administration in context trials depended on the time it took a matched toad, in a different group that was undergoing more extensive acquisition training, to reach five snaps at the cricket stimulus (i.e., a yoking procedure). Figure 4B shows the different schedules of reinforcement/reward used for the five groups in this experiment. The groups were as follows: toads in Groups A6, A12, and A24 received reinforcement of one cricket

per trial for snapping five times at the video stimulus for 6, 12, or 24 acquisition trials before the onset of extinction, whereas toads in Groups A6 + 6 and A6 + 18 were submitted to 6 or 18 context trials after being reinforced for snapping five times at the cricket stimulus for the first six acquisition trials. Each toad in Group A6 + 6 was matched with a toad in Group A12, whereas toads in Group A6 + 18 had a match in Group A24. Due to the yoking procedure, toads in Groups A12 and A24 were always used before the toads in the groups with context trials to allow calculation of delays before reward administration in these trials. In this experiment, there were 24 extinction trials conducted every other day to allow for comparison with the extinction responses seen in Experiment 3. The beginning of the training periods was organized so that all groups would finish training on the same day before the onset of extinction. This experiment was conducted in the summer of 2010 by the first author. The data was analyzed as in Experiment 3.

Results and Discussion

One toad in Group A6 + 18 died during the extinction period; it was rejected from the analysis. There was a significant difference between groups in latency to reach five snaps at the last acquisition trial, $F(4, 35) = 4.45$, $p = .005$, $\eta_p^2 = 0.29$, which again supported the use of ratios in which response in extinction was divided by response at the last acquisition trial to normalize baseline responding. Response at the last acquisition trial (sixth) was used to compute the ratios in groups that had context trials.

The ANOVA over all extinction trials showed significant effects of trial, $F(23) = 20.7$, $p < .001$, $\eta_p^2 = 0.38$, interaction between trial and group, $F(92) = 2.18$, $p < .001$, $\eta_p^2 = 0.20$, but not group, $F(4, 34) = 2.39$, $p = .07$. As Figure 6 shows, the ratios of snapping

Figure 6. Extinction of the snapping response in Experiment 4. Linear regression of the log-transformed latency to five snaps ratios over all extinction trials show that the 3 groups that received 6 reinforced trials during acquisition have very similar extinction responses. Groups that received more reinforced trials show less response extinction. Group symbols are A6 = open circles/black line; A12 = black triangles and hatched line; A24 = open squares and gray line; A6 + c6 = black circles and dotted line; and A6 + c18 = black square and gray hatched line.

latencies in extinction clustered closely together in the three groups that had six acquisition trials, regardless of whether they had subsequent context trials or not. It is important to note that, in contrast to the results of Experiment 3, full extinction was not achieved in any group at any time during the extinction period. The extinction curves shown in Figure 6 were thus calculated using all 24 extinction trials. The divergence of extinction curves through the extinction period best explains the significant interaction between trial and group in this experiment. In order to explore this interaction further, follow-up univariate ANOVAs were conducted a posteriori at the first and last extinction trials. There were no significant differences between groups at the first extinction trial, $F(4, 34) = 2.21$, $p = .09$, but a significant difference was present at the last extinction trial, $F(4, 34) = 8.78$, $p < .001$, $\eta_p^2 = 0.56$. The LSD posttest at this trial showed that all groups with 6 acquisition trials (A6, A6 + 6, and A6 + 18) had higher ratios of snapping latencies than Groups A12 and A24, but did not differ among themselves (A6 vs. A12, $p = .02$, $d = 0.35$; A6 vs. A24, $p < .001$, $d = 0.74$; A6 vs. A6 + 6, $p = .96$; A6 vs. A6 + 18, $p = .33$; A6 + 6 vs. A12, $p = .02$, $d = 0.24$; A6 + 6 vs. A24, $p < .001$, $d = 0.70$; A6 + 6 vs. A6 + 18, $p = .35$; A6 + 18 vs. A12, $p = .002$, $d = 0.69$; A6 + 18 vs. A24, $p < .001$, $d = 1.01$). Groups A12 and A24 also did not differ significantly ($p = .09$),despite the fact that the snapping response in Group A24 changed very little from the level seen at the end of acquisition. In fact, follow-up paired t tests showed that the latencies to reach five snaps did not differ between the last acquisition and last extinction trials in Group A24, $t(7) = 0.92$, $p = .39$, whereas they differed significantly in Group A12, $t(7) = 4.23$, $p = .004$, $d = 1.36$. This suggests that toads in Group A24 were the only ones that displayed a complete resistance to extinction within the 48-day period that was investigated.

This minimal change in snapping response through the extinction period extends the findings of Experiment 3 by showing that overtraining produced a sustained behavioral persistence in the toads. Further, there was no effect of rewards in context following acquisition trials on subsequent extinction of the prey catching response. This pattern of result also suggests that there is no effect of a delay between the last acquisition trial and the onset of extinction, as the toads in Groups A6 + 6 and A6 + 18 had their last acquisition Trials 6 and 18 days earlier than the other groups before the onset of extinction.

The lack of effect of exposure to rewards in the experimental context in toads stands in contrast to the findings observed in other vertebrates, in which increased exposure to rewards in the context during training produced resistance to reward devaluation (Adams, 1982; Igaki & Sakagami, 2004; Nevin et al., 1990). This difference might be due to the use of massed trials within training sessions in prior studies compared with the widely spaced trials used in the present study. Massed trials resulted in much lower intervals between successive rewards in the context than what the toads experienced, and this is known to promote context conditioning (Mustaca, Gabelli, Papini, & Balsam, 1991). Nevertheless, the context trials were expected to contribute somewhat to promotion or disruption of the acquired snapping response, which did not happen. It is possible that when toads learn a new response, it becomes canalized early during training and thereafter new associations are difficult to establish.
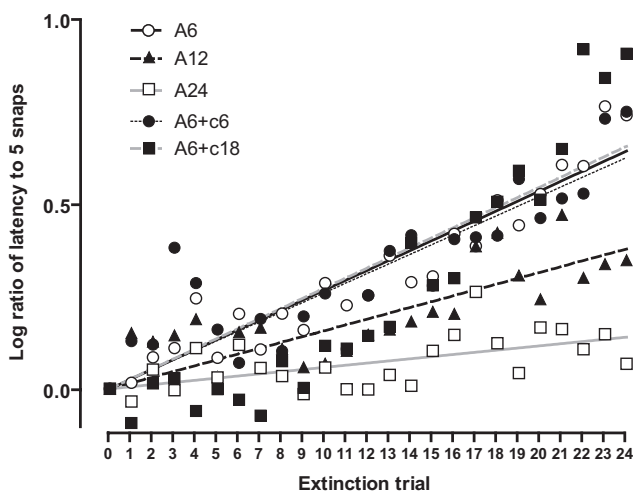
In contrast to extinction results in Experiment 3, none of the groups reached full extinction in Experiment 4. This was despite the fact that a group with only six acquisition trials was present in both cases. Hunger levels might have been higher in the toads that spent a shorter period in the laboratory before beginning the trials due to the shorter acclimatization period used in Experiment 4. This could have led to a stronger reinforcing effect of food rewards, especially at the beginning of training. Alternatively, a shorter interval between acquisition trials in Experiment 4 (1 day instead of 2) could have resulted in more resistance to extinction. This possibility was investigated in the next experiment.

Experiment 5: Effect of Trial Spacing During Training on Extinction

The difference in onset of full extinction observed between Experiments 3 and 4 may be due to the use of different intervals between trials in acquisition. This is supported by work with Argentine toads showing that time elapsed between reinforced trials in acquisition is an important factor determining response in extinction (Muzio et al., 2006). Work in rats also supports the view that shorter interval between reinforcers during training leads to increased insensitivity to reward devaluation (Experiment II in Adams, 1982). Experiment 5 thus included both the temporal distribution and number of acquisition trials as factors to determine their respective contribution to the persistence of the prey catching response in extinction in the fire-bellied toad. If the results in Argentine toads and rats apply to the present prey catching task, extinction should be slower with shorter intervals between reinforced trials.

Method

Animals. A total of 32 adult fire-bellied toads of mixed genders were used in this experiment (mean weight: 6.3 g, 95% CI [5.6, 6.9]). They were housed in groups of 5 to 6 in the plastic tanks and conditions described for Experiments 1 and 2. The toads were fed crickets ad libitum once a week for 2 weeks of acclimatization to the laboratory prior to the beginning of the experiment. Feeding was as described in Experiments 3 and 4.

Procedure. The procedures for shaping, acquisition, and extinction trials were the same as in Experiments 3 and 4. The two shaping trials were completed on consecutive days and there was a pause of 1 day before the beginning of acquisition trials. Figure 4C shows the different schedules of reinforcement used for the four groups in this experiment. The groups were as follows: toads in Groups A6–1d and A12–1d received reinforcement of one cricket per trial for snapping five times at the video stimulus for 6 and 12 acquisition trials that took place on consecutive days before the onset of extinction, whereas toads in Groups A6–2d and A12–2d had a delay of 1 additional day between their 6 and 12 reinforced trials compared with the other groups. Care was taken so that each toad was used at approximately the same time of day for all trials. The beginning of the training periods was organized so that all groups would finish training on the same day before the onset of extinction. There were 24 extinction trials scheduled every other day for all groups to ensure comparison with Experiments 3 and 4. This experiment was conducted in the summer of 2010 by the first author. The data was analyzed as in Experiments 3 and 4.

Results and Discussion

One toad in Group A12–2d died during the extinction period and one toad in Group A6–2d never acquired the task. They were both rejected from the analysis. There was no significant difference between groups in latency to reach five snaps at the last acquisition trial, $F(3, 26) = 1.82$, $p = .17$. Nevertheless, ratios of response in extinction divided by response at the last acquisition trial were again used to normalize baseline responding across individual toads.

The ANOVA over all extinction trials showed significant effects of trial, $F(23) = 19.87$, $p < .001$, $\eta_p^2 = 0.43$, interaction between trial and group, $F(69) = 2.23$, $p < .001$, $\eta_p^2 = 0.21$, and group, $F(3, 26) = 4.22$, $p = .015$, $\eta_p^2 = 0.33$. The LSD posttest then showed that the latency ratios in extinction were significantly higher in Group A6–2d compared with all other groups (A6–2d vs. A6–1d, $p = .042$, $d = 0.45$; A6–2d vs. A12–2d, $p = .003$, $d = 0.81$; A6–2d vs. A12–1d, $p = .007$, $d = 0.71$), but there were no other significant differences between groups. Figure 7 illustrates the extinction curves for each group. As in Experiment 4, full extinction was not achieved in any group at any time during the extinction period, so the extinction curves were obtained using all 24 extinction trials. Follow-up univariate ANOVAs conducted a posteriori at the first and last extinction trials to explore the interaction effect found that there was no significant difference between groups at the first extinction trial, $F(3, 26) = 1.02$, $p = .40$. There was a significant difference between groups at the last extinction trial, $F(3, 26) = 7.25$, $p = .001$, $\eta_p^2 = 0.46$. However, as opposed to the result of overall effect of group, the LSD test at the last extinction trial showed significant differences only between groups that had 6 acquisition trials compared with those that had
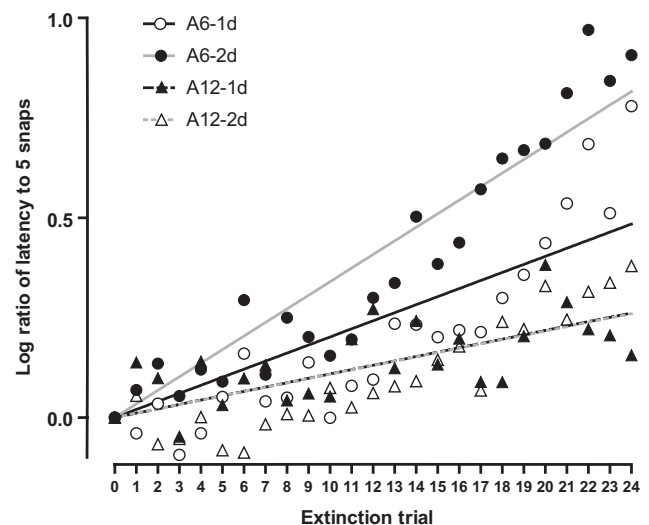
Figure 7. Extinction of the snapping response in Experiment 5. Linear regression of the log-transformed latency to five snaps ratios over all extinction trials show that the 2 groups that received 12 reinforced trials during acquisition have very similar extinction responses, as their curves overlap completely. Groups that received less reinforced trials show more response extinction. Group symbols are A6-1d = open circles/black line; A6-2d = black circles and gray line; A12-1d = black triangles and hatched line; A12-2d = open triangles and gray hatched line.

12 acquisition trials, not between Groups A6–2d and A6–1d. This can be explained by the fact that extinction values in Group A6–1d had suddenly increased to a level very close to the values of Group A6–2d by the end of extinction. In fact, the extinction curve in Group A6–1d showed an almost significant deviation from linearity (runs test, $p = .067$). Despite the latter, the overall effect of group described above supports the conclusion that Group A6–1d had a significantly lower latency to reach five snaps than Group A6–2d during extinction.

Muzio and colleagues (2006) have already shown that the time between reinforced trials in acquisition is an important determinant of the response in extinction in Argentine toads performing a runway task, with shorter intervals between reinforced trials producing response persistence. The present results add an important observation to these findings, in that if a response has become more automatic with extended training, the time between reinforced trials has no effect on subsequent extinction. This is clearly seen by the superposition of the extinction curves for Groups A12–2d and A12–1d in Figure 7. On the other hand, the groups that received less acquisition training showed that a longer interval between reinforced trials produced faster extinction of the prey catching response, as was observed in Argentine toads. This experiment also again confirmed the effect of increased acquisition training on extinction observed in Experiments 3 and 4 by showing that groups that received more acquisition trials showed more persistent responding in extinction.

General Discussion

Methodological Considerations

Because the toads used here are animals with a low metabolism that require only limited food for sustenance, widely spaced trials with 1 or 2 days between cricket rewards were used to prevent the development of satiety. Although this could impede making direct comparisons with similar work done in mammals and birds, in which training sessions involving many rewards are typically used, this procedure had the clear advantage of restricting the investigation to the effects of long-term memory. Another aspect that restricts direct comparison with many studies conducted in mammals and birds is the use of extinction to disrupt the appetitive reinforcer instead of reinforcer devaluation procedures. Despite the fact that reinforcer devaluation and extinction engage different mechanisms, they can both be used to discriminate between different forms of behavioral control. Extinction was used here because it could be easily implemented with the present task involving a single response and visual stimulus. It could also be compared with previous studies of extinction done with the Argentine toad (e.g., Muzio et al., 1992; Schmajuk et al., 1980). Many studies have used extinction instead of other means of reward devaluation to study the effects of reward changes on behavior in popular laboratory animals. For example, extinction has been used to define the concept of "behavioral momentum," which incorporates response rate and resistance to change into a single construct (Nevin & Grace, 2000). Extinction was also used to show that prolonged cocaine usage results in a change from goal-directed to habitual control of drug-seeking behavior (Zapata, Minney, & Shippenberg, 2010), which is relevant to the present study. Future work involving methods for specific and rapid re-

ward devaluation of competing responses is needed to validate the specificity of the effects observed in toads.

Are Some Responses More Susceptible to Habit Formation?

Running for food or water reward is not something an amphibian is likely to do in a natural setting. For example, the typical prey catching response of amphibians involves orientation toward a potential prey, followed by slow approach, fixation, and attack (Eibl-Eibesfeldt, 1951; Ewert et al., 2001). The foraging sequence of rodents also does not normally involve high-speed running, but cautious exploration of their environment, prey detection, and attack, followed by extensive manipulation of the prey (Timberlake & Washburne, 1989). It has often been proposed that animals learn more easily about stimuli of greater biological significance (Bolles, 1970; Mackintosh, 1975; Pearce & Hall, 1980; Seligman, 1970). What is unclear is whether an increased associability would also facilitate the formation of persistent habits. Learning involving biologically relevant cues and responses could indeed produce behavioral persistence. This is suggested by sexual conditioning in male quails, in which the conditioned response to a realistic stimulus of a female head is resistant to extinction when compared with a less realistic stimulus (Krause, Cusato, & Domjan, 2003; see Domjan, Cusato, & Krause, 2004, for review). If one compares the present results with studies of overtraining of runway responses in amphibians and rats, in which overtraining of running responses did not result in persistent running (Muzio et al., 2006; Shibasaki & Ushida, 2012; Wagner, 1961), it appears that the effect of biological significance on learning not only applies equally across vertebrates but also might facilitate the development of behavioral persistence.

Learning and Memory Systems in a Toad?

The modification of the prey catching response in a toad outlined in the present study is reminiscent of changes that occur in mammalian instrumental learning. In the early stages of instrumental learning, actions are sensitive to changes in reward, but automatic responses insensitive to reward changes can develop with extended training (Dickinson, 1985). The prey catching response was clearly driven by a biologically relevant stimulus (the cricket video clip), as the toads would not be motivated to behave in artificial situations. Although amphibians seem restricted to learning about what stimulus to respond to (Suboski, 1992), the present results suggest that basic cognitive processes provide enhanced flexibility at least in the early phase of learning about a new stimulus/situation in these animals. In other words, purposive actions and learned habits could compete for behavioral control in amphibians, but quick habit learning might easily be mistaken for innate responding. In order to highlight these cognitive processes, a task that enables demonstration of flexibility is required. We argued that runway learning is likely not adequate to do so in amphibians or rodents. Consideration of the subject's natural behavioral repertoire is thus crucial.

It will be interesting to explore the neural substrate of the different phases of learning proposed in the present study, especially because studies involving brain lesions in toads already showed that there is a dissociation of learning and memory sys-

tems for acquisition and extinction of the runway running response (Muzio et al., 1993, 1994). The relatively simple brains of amphibians could prove useful to highlight the basic workings of different learning and memory systems in vertebrates.

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