Abstract  The present study investigated how snapping behavior toward familiar and unfamiliar prey is modified by reward omission and aversive conditioning in the fire-bellied toad *Bombina orientalis*. Toads were trained to snap at cricket images by rewarding them with live crickets. The task was learned, and the learning criterion (10 snapping responses within 2 minutes) was reached in all individuals investigated. Subsequent reward omission did not alter the frequency of snapping to the familiar cricket stimulus. Snapping decreased only in some individuals, when a mild foot shock was applied at snapping. However, at presentation of images of hitherto unfamiliar meal worms and foot-shock application at snapping to the stimulus, the majority of toads diminished snapping significantly. Snapping responses decreased more rapidly, when snapping at meal worms was not rewarded or a footshock was applied uncorrelated to the presentation of or snapping at meal worms. These results demonstrate that in toads familiarity and unfamiliarity of prey stimuli are important factors in aversive learning, because well-trained responses to familiar stimuli become immune against reward omission. Furthermore, at presentation of unfamiliar stimuli, omission of reward and uncorrelated footshock had a stronger aversive effect than correlated footshock [Current Zoology 57 (6): – , 2011].

Keywords  Negative conditioning, Food preference, Amphibian, Response extinction

In contrast to teleosts, birds and mammals, information about the learning capacities of amphibians (frogs, salamanders, caecilians) is inconsistent. The long dominating view was that their behavior, especially their feeding behavior, is reflex-like or instinctive (Tinbergen, 1951). However, later studies gave clear evidence for learning in amphibians. These studies covered different aspects of learning such as predator learning in amphibian embryos using negative conditioning (Mathis et al., 2008; Ferrari and Chivers, 2009), instrumental learning by water reinforcement (Schmujak et al., 1980; Muzio et al., 1993), reward conditioning in a place-preference task (Presley et al., 2010), learning of neighbor-stranger discrimination in the auditory domain (Bee and Gerhardt, 2001), anticipatory feeding in frogs (Van Bergeijk, 1967) and specificity of non-reward learning in toads (Cervantes-Perez et al., 1991).

While frogs and salamanders quickly acquire food preferences by positive reinforcement, aversion conditioning using electric shock as negative reinforcer, which in mammals yield reliable results, so far either failed or led to inconsistent results (McGill, 1960; Crawford and Langdon, 1966; Russek, 1969; MacPhail, 1982; Suboski, 1992; Cabanac, 1999). This might be due to the fact that in amphibians autonomic responses such as alteration of heart rate, increase in body temperature or in skin conductance are not reliably related to fear and stress, as is the case in amniotes.
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(Cabanac, 1999; Cabanac and Cabanac, 2000, 2004). Freezing such as defensive crouch often occurs in response to highly aversive stimuli such as stronger electric shocks, which is then misinterpreted as “non-learning”. As for avoidance learning, Paradis and Cabanac (2004) suggested that conditioned taste aversion is absent in amphibians, and this appeared to be confirmed by studies in our lab: although showing aversive reactions, Bombina kept feeding on food made unpalatable using diverse chemicals (Lindemann, 2000). Recently, however, Daneri et al. (2007) demonstrated that aversive learning can be rapid and efficient in the toad Bufo arenarum, when saline solutions of different molarities are used as signals and reinforcers. Thus, more data on aversion learning or tolerance to “frustration” in amphibians are needed.

An important aspect of instrumental conditioning is the effect of omission of reward. Like an aversive stimulus, omission of reward can cause extinction of appetitive responses and elicit aversive reactions. Papini (2003) argued that at omission of reward non-mammalian vertebrates including amphibians exhibit an “allocentric” type of response in the sense that stronger and more consistently applied positive reinforcers lead to a slower response extinction, while in mammals an “egocentric” type is found in the sense that extinction occurs faster under these conditions, whereas weaker and irregularly applied positive reinforcers lead to a slower extinction rate.

The present study addresses learning in the context of feeding behavior of the toad Bombina orientalis focusing on the effect of omission of reward and of negative conditioning (electric footshock). The training procedure consisted of an appetitive instrumental learning task, in which snapping at a video of moving crickets was reinforced by the delivery of familiar food, i.e. crickets. Afterwards, omission of reward was studied under two conditions, viz., at presenting either the familiar video cricket stimulus or a video of mealworms hitherto unfamiliar to the toads. Aversive learning was studied by combining omission of reward with a mild electric footshock when presenting videos of either the familiar or unfamiliar prey. We also studied the effects of correlated vs. uncorrelated “punishment” by applying the footshock either at every snap or in a random fashion. The latter was carried out to test for context or place conditioning in relation to feeding of amphibians. Extinction was studied by omitting the footshock at presentation of the two types of prey videos after correlated or uncorrelated application of footshock. Because some individuals appeared to consistently learn faster than others, we tested the data for “fast” and “slow learners”.

1 Material and Methods

1.1 Animals

Toads (Bombina orientalis; n=45) were bred at our institute or purchased at a commercial dealer (Tropeninstitut Hamburg, Germany). Animals were housed in groups in terraria (37 x 20 x 30 cm) with permanent access to water and kept at 22–24°C with an artificial day-night rhythm of 12 hours. Young adult (20–30 mm snout-vent length) and adult (40–50 mm SVL) male and female toads were used for the experiments; individuals were identified by their pattern of color spots. Animals were fed three live crickets Gryllus bimaculatus per individual dispensed in the terraria once a week. In the training period, animals were fed only in the behavioral test. The experiments followed the guidelines of the animal welfare laws and were approved by the Animal Care and Use Committee of the state Bremen, Germany.

1.2 Experimental setup

During the experiments, toads were situated in a test glass box (20 x 20 x15 cm), which was located in front of the monitor at a distance of 0.5 cm (Fig. 1). The test box had opposite removable panes. The pane facing the monitor was removed during sessions; the opening was covered with a transparent sheet to prevent animals from snapping directly onto the monitor. The floor of the test box was covered with a moistened conductor plate (diameter 15 cm), through
which a mild foot shock (intensity 0.8–1.3 V; duration 500 ms) could be applied by a stimulus generator (fabricated in the university shop) in the conditioning period of the experiments. The strength of the foot shock was adjusted to the threshold for minimal bodily responses of an individual. While receiving a mild electric shock, toads appeared to be undisturbed and continued to monitor the moving prey stimuli on the screen.

1.3 Stimuli

Twelve to 15 crickets sorted by size (body length of 7–8 mm) were placed in a glass tube (9 cm length; diameter 2.5 cm) and videotaped from a lateral view using a digital camcorder (Sony TRV900E, Sony Corp.). Mealworms *Tenebrio molitor* of roughly 2.5 cm length and placed in a petri dish were filmed from a dorso-lateral view. The area in which the cricket and the mealworm population moved was matched. Either the cricket or the mealworm video was presented by a video recorder (Sony EV-C2000E Video Hi8 cassette recorder) on a color TV (Sony Trinitron KV-14M1D).

1.4 Training procedure

Each toad was manually transferred to the test box and placed centrally in front of the monitor; only the cricket videotape was displayed on the screen. Time of testing started at first orientation of the toad towards the crickets on the screen. The toad was rewarded with a live cricket immediately after snapping at the cricket video stimulus. On the second and third day reward was given only after every second and fifth snap, respectively. Thereafter, the toad was rewarded either after ten snaps or after 2 min in case of less than ten responses. The criterion of 10 snaps was reached, when at least ten responses occurred within 2 min in the three last training sessions. Thereafter, sessions were stopped after ten snaps or after 2 minutes. Initially, the training sessions were performed twice a week and then continued once a week, until 15 sessions were completed. Data were recorded by a self-made program, which was started manually by key stroke at first orientation to a stimulus. Each snap was fed into the computer by key stroke; the program read out number, time points and total time of snaps.

1.5 Conditioning

In the conditioning tests, toads were not rewarded when snapping at a stimulus on the screen. A total of 15 sessions were performed for each toad of the different groups. At first, sessions were carried out daily for seven days, and thereafter only once a week. Retention was tested by performing the last session four weeks after the fourteenth test session. In the first group, called C-NR (cricket video – no reward), the cricket video was presented, and toads were no longer rewarded for snapping. In the second group of toads, referred to as C-F (cricket video – footshock), the cricket video was presented, and the footshock was applied immediately after each snap toward a stimulus. In the third group of toads, M-NR (mealworm video – no reward), the mealworm video and reward omission were combined. In the fourth group, M-F (mealworm video - footshock), toads were confronted with the mealworm video and received a footshock at each snap to the stimulus. In toads of the fifth group, M-NcF (mealworm video – non-correlated footshock), the mealworm video was presented, and a footshock was applied randomly, i.e., 10 shocks uncorrelated with the presentation or the snaps of individuals. An overview of groups is given in table 1. During conditioning, toads that initially did not respond to the mealworm stimuli with 10 snaps were excluded.

1.6 Extinction

After the fifteenth session of conditioning, in six consecutive weekly sessions footshock was omitted in groups C-F, M-F and M-NcF.

1.7 Statistical analysis
In the training, conditioning and extinction period, the number of snaps was analyzed using the software PASW Statistics (Version 18, SPSS Inc., Chicago, Illinois, USA). Data of individuals and groups were tested by means of non-parametric tests, because they were not normally distributed (Shapiro-Wilk test). The number of snaps of individuals was compared between the training period (after reaching the criterion; i.e., 10 snaps per 2 minutes) and the early (first week; daily sessions seven times) and late (following weeks; sessions once a week) conditioning period using the Wilcoxon signed-rank test. Individuals of a group were assigned to fast or slow learners depending on a significant decrease in number of snaps in the early or in the late period of conditioning, respectively. The classification depends on the point in time, when a statistical significant decrease of number of snaps in the conditioning compared to the training period occurred. Individuals that did not show a significant decrease were assigned non-learners. The number of snaps during late conditioning was also compared with those during extinction. Significant differences between groups were tested by using the Mann-Whitney-U test. Differences in values at $P < 0.05$ were considered as significant.

### 2 Results

#### 2.1 Training with live cricket rewards

Animals readily snapped at cricket stimuli presented on a screen; they sat close to the screen and snapped at one of the stimuli on the screen. The criterion (10 snaps during 2 minutes) was reached in the second to fourth session except for one individual that reached the criterion after the sixth session. One individual did not orient to the cricket stimuli and was excluded. The average time for ten snaps at a cricket stimulus on the screen was 23 s (range 11.8–58 s) across all individuals and all groups tested.

#### 2.2 Conditioning

**Group C-NR** (Fig. 2)

All animals of this group continued to snap ten times to cricket stimuli after omission of reward (live cricket). Moreover, the average snapping time of this group of 15.5 s (range 11.8–21.3 s) in the training period slightly decreased to 14.9 s (range 11.4–18.3 s) during conditioning. Individual snapping times across conditioning sessions ranged from 6.9 to 41.7 s. The seven toads of this group were designated non-learners.

**Group C-F** (Fig. 3)

Application of a footshock at snapping towards the cricket stimulus reduced the number of snaps within 2 min in most individuals. One toad displayed significantly fewer number of snaps in the early conditioning period, and this behavior was sustained in the late period (6.2 on average for all sessions; $P < 0.007$). This toad was assigned a fast-learner. Four toads significantly reduced snapping only during the late period, in which the number of snaps decreased to 7.7 (SEM ±1.4; $P < 0.001$) on average. These toads were considered slow-learners. The remaining five toads did not reduce snapping at the stimulus and were considered non-learners.

**Group M-NR** (Fig. 2)

Two toads initially did not respond to the stimulus and were excluded; nine toads of this group initially responded to presentation of the mealworm with ten snaps (Table. 1). This snapping behavior diminished with repeated presentation under reward omission. Five toads snapped 10 times in the first three sessions of the early conditioning period within 86 s on average. Thereafter, in the early period of conditioning they reduced snapping significantly to an average number of 3.8 snaps ($P < 0.000$), and are thus regarded as fast learners. In the late period, snaps further decreased to 0.8 snaps on average. The remaining four toads snapped 10 times within an average time of 56 s over the early period and displayed significantly lower snaps (on average 4.8 snaps; SEM ±1.9; $P < 0.000$) only in the later period. They were classified as slow-learning animals.
Group M-F (Fig. 3)
Application of a footshock at snaps to a mealworm stimulus reduced the number of snaps in the majority of individuals. Seven toads, considered fast learners, significantly diminished snapping (5.5 snaps on average; \( P < 0.000 \)) in the early period, and an average number of 1.7 snaps were released in the late period. Four toads reduced snapping to an average number of 6.8 only in the late period (\( P < 0.001 \)); they were considered slow learners. One toad (not shown in Fig. 3) continued snapping 10 times within an average time of 45 s during conditioning, regardless of the footshock. It was considered a non-learner.

Group M-NcF (Fig. 4)
Application of an uncorrelated footshock at presentation of the mealworm video significantly reduced snapping behavior. In the early conditioning period, five toads initially snapped 10 times in the first four sessions, but then decreased the average number of snaps to 2.2 (\( P < 0.000 \)). These fast-learning toads further lowered the average number of snaps to 0.7 snaps in the late period. Another two toads reduced the number of snaps to 2.8 on average (\( P < 0.003 \)) only in the last period of conditioning, and were considered slow-learners. One toad initially did not snap at the mealworm stimulus and was excluded.

Retention
In the last session of the conditioning period performed four weeks after the second-last session, the average number of snaps of toads of groups and learning types did not differ significantly from those in the late conditioning period minus the last session (Wilcoxon test). However, the number of snaps differed considerably between individuals (Figs. 3, 4; compare slow-learners).

2.3 Extinction
Starting with the week following the fifteenth session of conditioning, extinction was tested during six weeks in those groups, in which toads received footshocks in the conditioning period (C-F, M-F, M-NcF). During extinction, fast and slow learners of these groups did not significantly change the average number of responses compared with late conditioning (Wilcoxon test).

2.4 Differences between fast- and slow-learners of groups (Fig. 5)
In slow and fast learners, the average number of snaps during conditioning as well as those during extinction was compared only between groups that included at least four individuals.

Conditioning
Fast-learning toads not rewarded at mealworm presentation (M-NR) as well as those receiving an uncorrelated footshock (M-NcF) exhibited a significantly lower number of snaps than fast learners receiving a footshock during snapping at the mealworm stimulus (M-F) (\( P < 0.02 \)). Fast learners of group M-NcF and of M-NR did not differ significantly in their number of snaps. Slow-learning toads of group M-NR displayed a significant reduction (\( P < 0.01 \)) in number of snaps when compared to toads of group C-F. Slow learners of group M-F and M-NcF did not show significant differences to slow learners of the other groups.

Extinction
During extinction, fast learners of group M-NcF exhibited a significantly greater reduction (\( P < 0.001 \)) in number of snaps than toads of group M-F. Slow learners of group M-F exhibited a significantly greater reduction in numbers of snaps (\( P < 0.02 \)) than those of group C-F, while slow learners of group M-NcF did not differ significantly in their number of snaps compared to the other two groups of slow learners.

3 Discussion
Our data demonstrate that in the toad *Bombina orientalis* feeding behavior and prey preferences can be influenced by omission of reward and aversive conditioning using electric footshock. However, in both cases the magnitude of the learning effect depends on the stimulus situation.

### 3.1 Omission of reward

The first and somewhat surprising finding is that omission of reward had no effect on the frequency of snapping toward the cricket video or the snapping time: Average snapping time of 15.5 seconds for 10 snaps in the training period of this group did not increase as a consequence of reward omission, as one would expect, but slightly decreased. This finding is best explained by the familiarity of the toads with the prey stimulus. All toads were fed crickets and rewarded with crickets during training. Apparently, toads had formed a stable positive experience of crickets that remains unaffected even after a prolonged absence of feeding success. Many amphibians can stay without feeding over long periods up to one year (Dimmitt and Ruibal, 1980).

### 3.2 Familiarity of prey

The situation changes, when omission of reward is combined with the presentation of a hitherto unfamiliar prey stimulus, viz., mealworms. Initially, all animals snapped at this stimulus, but with a much longer latency. Toads eagerly snapped at the cricket video stimulus with a mean latency of 1.5 s, whereas at presentation of the mealworm video snapping latency increased to 8.3 in fast learners and to 5.6 s in slow learners. We attribute this significant increase in snapping latency to the lack of familiarity of the mealworm. We cannot exclude that differences in attractiveness of the two prey types also play a role, but this was out of focus of the present study. Counterbalance of variables of the two prey types could be achieved only for the area in which the prey moved. Control of movement pattern of a prey is counterproductive in the present paradigm, since this is an important feature used by amphibians for prey identification, and the specific set of prey features forms the basis of both familiarity and attractiveness. In a study on quantity discrimination in salamanders using standardized computer-animated prey (Krusche et al., 2010), a standardization of movement pattern led to a failure in visual discrimination. Amphibians are known to be sensitive to differences in visual prey features such as movement pattern, shape, velocity, size, and orientation (Ewert, 1976; 1984; Roth et al., 1998). Many frogs including *Bombina orientalis* and salamanders in general respond immediately to fast and jerkily moving prey objects, as is typical of most insects including crickets, compared to slowly and smoothly moving ones such as mealworms or earthworms (Roth et al., 1998). A lower degree of familiarity of the prey may make the prey preference more sensitive to omission of reward. Another study in our lab revealed that toads trained with mealworms in the same paradigm and rewarded with crickets continued to snap at mealworms after omission of reward (Heidorn, unpublished results). Taken together, prey familiarity appears to have an important effect on feeding behavior in toads.

### 3.3 Prey Experience

Our findings are consistent with Papini’s concept of an “allocentric” effect of reward omission supposed to be typical of amphibians (Papini, 2003): the stronger and more regular the reward experience, the more resistant against reward omission is this experience, as is the case with crickets. In the case of mealworms, there is no reward experience at all, and the consequence of reward omission should be a dramatic decrease in snapping activity. However, this is apparently counteracted by an “innate” prey scheme that leads most amphibians to snap at moving elongate or compact stimuli even without any prey experience, although this scheme can be modified by experience (Ewert, 1976, 1984; Brzoska and Schneider, 1978; Luthardt and Roth, 1979). Thus, the degree of reduction of snapping activity in the case of mealworm presentation can be understood as a “compromise” between experience of reward omission and strength of prey scheme.
3.4 Stimulus-footshock association

Although omission of reward had no effect on snapping at the familiar cricket stimulus, application of a footshock correlated with snapping at the cricket video reduced snapping in 5 out of 10 toads. However, reduction is stronger in the case of snapping at the mealworm video. Here, in 11 out of 12 toads application of the footshock led to an intermediate to strong decrease in snapping rate. The effect was strongest, when the footshock was applied uncorrelated to the snapping at the mealworm video; all 9 toads tested significantly reduced their snapping activity. As in the previous experiment with omission of reward, the effect of footshock was stronger when combined with the unfamiliar compared to the familiar prey stimulus, probably again due to the lack of familiarity or the lower attractiveness of the mealworm. The differences between the effects of correlated and uncorrelated footshock may be explained by the difference between the stimulus-footshock association vs. the context (test box)-footshock association. In the first case, the toad gains the experience that the stimulus is unfavorable, whereas in the latter case the toad may learn that the context of feeding is unfavorable. The latter could be considered a kind of negative context or place conditioning. Frogs easily develop preferences for places, where food is to be expected, and it has been shown recently that such a place preference is under the control of the dopaminergic reward system present in amphibians, too (Presley et al., 2010).

3.5 Differences in speed of learning

In our experiments, fast and slow learners could easily be distinguished. Fast learners reduced their snapping activity earlier and more profoundly in all experimental settings, and the differences were largest in the case of mealworm stimuli and uncorrelated application of footshock. These learning differences could be caused by differences either in feeding motivation or in a greater efficiency of learning and memory formation. Bilbo et al. (2000) demonstrated that in an experimental paradigm similar to the Morris water maze leopard frogs *Rana pipiens* learned to reach a platform using visual cues, and this learning success could be disrupted by application of the anticholinergic drugs atropine sulfate.

3.6 Learning and feeding strategies

Our data reveal a remarkable asymmetry between appetitive and aversive learning in feeding behavior in amphibians: Toads easily learn by positive reinforcement to snap at artificial prey stimuli, as was shown during the training period. Once established, such a food preference remains relatively stable and is not or only slightly modified by negative experience either in the form of lack of success (omission of reward) or unpleasant experience (mild footshock). In times when availability of food is restricted, a tolerance for food scarcity combined with increased resistance to harmful food experience may be advantageous. Many amphibians are known for having a very large prey spectrum (Werner et al., 1995), while others specialize on prey such as collembolans of low nutritive value (many plethodontid salamanders, Roth, 1987) or even noxious preys such as ants (many bufonids and dendrobatoids specialize on ants; Isacch and Barg, 2002; Saporito et al., 2004), because both types of prey are abundant and almost continuously available. Both feeding strategies are supported by fast positive reinforcement learning and diminished food avoidance learning: animals easily switch to the most nutritious prey when available, but continue to feed on unfavorable and even unpalatable food, if there is no choice.

In conclusion, amphibians clearly exhibit the ability for classical and instrumental conditioning including context conditioning. There is a strong asymmetry between positive and negative conditioning: whereas frogs and salamanders quickly learn prey and context/place preferences, but once consolidated, these memory traces are relatively robust against negative reinforcers such as reward omission and punishment. Such negative reinforcers have a much greater effect on unstable prey experience. This appears to be a highly advantageous feeding strategy for animals with a very broad and highly fluctuating prey spectrum including noxious and defensive prey and long periods of starvation.
References


Fig. 1 Schematic illustration of the test setup

A toad sits in front of the screen, on which the cricket video is presented.
Fig. 2 Three individual toads representing learning types found in the group of toads, in which snaps at stimuli were not rewarded in the conditioning period.

Toads confronted with a cricket video (C-NR) showed no change in number of snaps despite reward omission, while toads of group M-NR confronted with a mealworm video during conditioning exhibited a significant reduction in snaps at early or late conditioning. Open circles indicate numbers of an individual of group C-NR, black and open squares represent those of a fast-learning and a slow-learning individual of group M-NR, respectively.
Fig. 3 Four representatives of the major learning types among the toads receiving a footshock at snaps to stimuli during conditioning

Toads of group C-F were confronted with a cricket video and those of group M-F with a mealworm video. In group C-F, mainly slow- and non-learners were found, while the majority in group M-F belonged to fast- and slow-learners. Open circles indicate a non-learning and black squares a fast-learning individual; open or gray squares represent slow-learning individuals.
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Fig. 4 Representatives of the two learning types found in the group (M-NcF), receiving a mild footshock uncorrelated to the snaps at mealworm stimuli during conditioning

Black squares represent a fast-learning individual, open squares a slow-learning individual.

Fig. 5 Average number of snaps in fast (black bars) and slow (gray bars) learners in the conditioning and in the extinction period

Toads were not rewarded at snaps to a stimulus of a mealworm (M-NR) or cricket (C-NR) video. All toads of group C-NR were non-learners and are therefore not shown in the diagram. During conditioning, toads of the other groups received a footshock at snaps to the cricket (C-F) or mealworm stimulus (M-F), or received a footshock uncorrelated to snaps at the mealworm stimulus (M-NcF). Footshock was omitted during extinction. Significant differences (horizontal line *; \( P \leq 0.02 \)) in average number of snaps were tested among fast and among slow learners in groups containing at least four individuals. Vertical bar indicates standard error of the mean.
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<td>M-NcF</td>
<td>Presentation of mealworm video Reward omission Footshock not correlated with presentation or snap</td>
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