Determinants of the water seeking response in a T-maze in the fire-bellied toad *Bombina orientalis*

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ABSTRACT

To maintain water balance, terrestrial amphibians are motivated to seek water when dehydrated and to avoid hypertonic solutions that promote water loss to the environment. Thus, dehydration and hypertonic solutions both act to promote positive water balance through opposite appetitive and aversive mechanisms. In one experiment, we tested if appetitive and aversive stimuli interact to facilitate learning of the location of a water source in the fire-bellied toad *Bombina orientalis*. Dehydrated and hydrated toads were placed in a T-maze with accessible distilled water in one arm and hypertonic saline or inaccessible water in the other arm. Maze performance was assessed by measuring time to find the water source and correct first choices over 6 consecutive days of training. Results showed reduced time to find water in dehydrated toads but no reliable improvement in correct first choices that would indicate spatial learning. Hypertonic saline inhibited maze exploration but otherwise had no effect on performance. Because we expected spatial learning for water based on previous work in this species (Brattstrom, 1990 J Herpetol 24:44−47), we conducted a second experiment with longer training, again including dehydrated and hydrated toads. Maze training for four weeks with four weekly trials revealed a rapid reduction in time to find water in dehydrated toads followed later by an improvement in correct first choices suggestive of spatial learning. Additionally, changing the motivational status of toads after the four-week training period showed no evidence that hydrated toads had learned location of the water source during training. Our results suggest that both motivational and learning effects ameliorated water seeking behaviour in this terrestrial amphibian, but that motivation acted much faster than learning. Additionally, toads that experienced no reinforcement during training showed no latent learning effect.

1. Introduction

To maintain adequate water balance, amphibians strictly regulate urine concentration and skin permeability. Freshwater aquatic amphibians face issues of excess water diffusion into tissues and loss of ions to the environment, which are countered by mass production of dilute urine and active uptake of ions through the skin (Wells, 2007). Meanwhile, terrestrial amphibians are more at risk of dehydration, which is countered by water retention and uptake. Water uptake is achieved via the 'water absorption response', were the skin of the pelvic region is pressed against a moist substrate (Stille, 1958). Terrestrial amphibians can tolerate temporary water loss but need to find a water source in their environment where they can rehydrate in order to survive.


Previous research has suggested that dehydrated terrestrial amphibians can learn to find water in a maze when water is used as reinforcement (Brattstrom, 1990 for *Bombina orientalis*; Schmajuk, Segura, & Reboreda, 1980 for *Rhinella arenarum*, formerly *Bufo a.*). Maze and other related spatial learning studies have allowed the study of orientation mechanisms used by amphibians to find water sources, and suggest that orientation mechanisms are conserved in vertebrates (Adler, 1980; Dall'Antonia & Sinsch, 2001; Daneri, Casanave, & Muzio, 2011; Daneri, Casanave, & Muzio, 2015; Janes & Falkenberg, 1980; Liu, Day, Summers, & Burmeister, 2019; Lüddecke, 2003; Sotelo, Bingman, & Muzio, 2015). Amphibians also avoid contact with hypertonic solutions because such contact can draw water away from the body through osmosis (Daneri, Papini, & Muzio, 2007; Muzio et al., 2011). Avoidance of hypertonic solutions is supported by the presence of ventral skin chemoreceptors that detect salts (Hillyard, Goldstein, Tuttle, & Hoff, 2004; Kostinsky, Miller, & Stewart, 2000; Koyama, Nagai, Takeuchi, & Hillyard, 2001).

Little is known about how appetitive and aversive stimuli interact to control performance and learning. Aversive stimuli have been long thought to act as general inhibitors of appetitive behaviour (Dickinson & Pearce, 1977; Konorski, 1967), but more recent work using opposite reinforcers to modify the same learned behaviors found no such effect. For example, Magoon and Critchfield (2008) found no systematic bias of aversive or appetitive reinforcers in humans working for money, and Ilango, Wetzel, Scheich, and Ohl (2010) found that opposite reinforcers interacted to potentiate learning and delay extinction in Mongolian gerbils undergoing an auditory shuttle box avoidance task. This *common-impact* (Farley & Fantino, 1978) or *equivalence* (Ilango et al., 2010) hypothesis is in line with neurophysiology evidence showing that information about rewarding and aversive stimuli converge onto single neurons in decision centers of the mammalian brain (Kim, Shimojo, & O'Doherty, 2006; Morrison & Salzman, 2009). The alternative hypothesis of *differential impact* (Magoon & Critchfield, 2008) posits that one type of reinforcer should have a stronger effect on behavior through inhibition or selective impact.

It is well recognized that central motivational states influence behavior toward stimuli and goals (Bindra, 1978; Salamone & Correa, 2002). The reinforcement that follows reward consumption is also influenced by the motivational state under which a reward is experienced (Bulik & Brinded, 1994; Epstein, Truesdale, Wojcik, Paluch, & Raynor, 2003). Here, we manipulated motivation to seek water by selectively dehydrating or not dehydrating groups of fire-bellied toads to obtain two levels of appetitive reinforcement when these toads were exposed to water. In a first experiment, groups of toads were trained in a simple T-maze in the presence or absence of hypertonic saline to provide aversive stimulation promoting avoidance of the hypertonic saline. Water and hypertonic saline were located in opposite arms of the maze to provide concurrent exposure to appetitive and aversive stimuli expected to drive the same response (i.e. learning where water is located in the maze). We hypothesized a common impact or equivalence of the opposite stimuli on toad spatial learning and predicted that the stimuli would interact to promote spatial learning for water. Thus, a group of toads exposed to concurrent appetitive and aversive stimuli should show more improvement in maze performance over time compared to groups exposed to an appetitive or aversive stimulus alone, or toads exposed to no water balance stimuli in the maze. Unexpectedly, the results of the first experiment did not replicate the finding of rapid spatial learning of the location of a water source in a T-maze by Brattstrom (1990). Therefore, we set out to extend maze training to see if it would provide evidence of spatial learning in the fire-bellied toad under our testing conditions.

In addition to a longer training period, a second experiment investigated the possibility of latent learning of the location of the water source by toads exposed to the maze under a normal hydration status. Latent learning occurs without reinforcement and is expressed immediately under an appropriate motivational state (Tolman, 1948). The test of latent learning involved a change of motivational state after completion of the training period, which was achieved by dehydrating the toads that had previously been hydrated during training. Since latent learning might also depend on the experience of reinforcement under an appropriate motivational state (i.e. incentive learning *sensu* Balleine, 1992), the first test of latent learning was followed by a second one after the newly
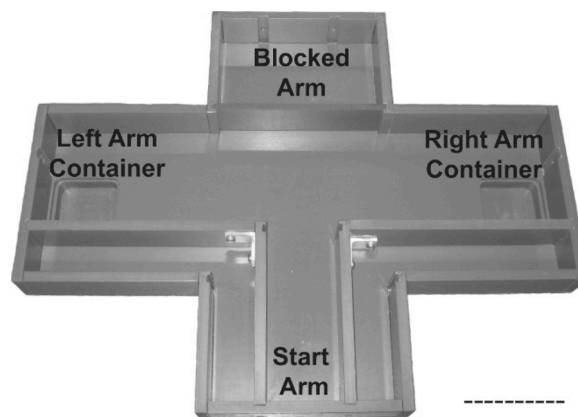
Fig. 1. View of the maze used in the experiments. Toads started trials in the start arm and had to reach water in one of the 2 cm-deep containers at the end of a goal arm. The other goal arm container was filled with a hypertonic saline solution or inaccessible water in the first experiment, and only inaccessible water in the second experiment. During trials, the maze was covered by a transparent sheet of plastic with added external environmental cues (second experiment only) in the form of black squares above the water container on one side and black lines above the other container (not shown). Scale bar on the bottom right is 10 cm and the walls of the maze are 4 cm high.

dehydrated toads would have experienced reinforcement in the maze for the first time. We could not predict if latent or rapid incentive learning would take place due to the paucity of relevant data in non-mammals.

2. Materials and methods

2.1. Animals

In the first experiment, 48 adult fire-bellied toads (*Bombina orientalis*) of mixed sexes were used (mean weight 6.05 g, 95 % CI [5.7, 6.4] at the start of the experiment), while 16 toads were used in the second experiment (mean weight 7.05 g, 95 % CI [6.8, 7.3] at the start of the experiment). All toads were purchased from National Reptile Supply (Mississauga, ON). Holding temperature was kept at 21 °C with a photoperiod of 12:12 h light:dark, with lights on at 7:00 h. Before the experiments (and between weekly training in the second experiment, see below), toads were group-housed in glass terrariums (50 × 26 × 31 cm) with gravel substrate, broken clay pots and flat stones for cover, and had continuous access to water. They were fed crickets (*Acheta domesticus*) lightly dusted with calcium and vitamin powder ad libitum weekly. All experimental procedures were approved by the University of Guelph animal care committee (AUP #3590) under the guidelines of the Canadian Council on Animal Care.

2.2. Maze

Fig. 1 shows the maze used in the experiments. The T-maze configuration was used with a 20 cm long start arm, a 50 cm wide alley with containers at the end of each goal arm, and 4 cm-high walls. During training, the maze was illuminated from above and covered by a rigid sheet of transparent plastic to allow observation while preventing escape of the toads. External environmental cues were added to the transparent sheet in the second experiment to maximise the chance of spatial learning. These cues were black squares located above the water container on one side and black lines above the other container. Each arm of the maze has a container dug into the floor. In the first experiment, one container was filled with distilled water while the other contained either inaccessible distilled water or a solution of 800 mM NaCl. Access to distilled water in one arm was prevented using a perforated plastic cover that fit on top of the container above the water at a level flush with the floor of the maze. The perforations were too small to allow contact with the water inside the container but could allow moisture out. In the second experiment, only inaccessible distilled water was used across the goal side of the maze with the accessible distilled water container.

2.3. Procedure

2.3.1. Dehydration and water uptake

Preliminary attempts to produce spatial learning in the fire-bellied toad based on the methods used by Brattstrom (1990) failed to produce significant dehydration or water uptake in our maze (E. To and J. Kuska, unpublished observations). Therefore, alternative methods were established. To determine effective dehydration and water uptake times, the time course of rehydration was assessed in 6 toads sequentially exposed to 24, 48 and 72 h of dehydration (Fig. 2). This determined that 24 h of dehydration at the beginning of an experiment was not sufficient to produce good levels of water uptake in toads, whereas 48 and 72 h of dehydration were followed by better and similar profiles of water uptake during 30 min of access to water. A period of 48 h of dehydration was chosen to begin subsequent experiments to reduce the risk of extreme dehydration. Water uptake of the dehydrated toads in this preliminary
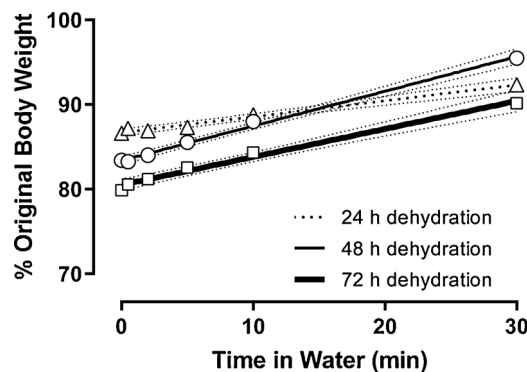

Fig. 2. Time course of rehydration in dehydrated fire-bellied toads. This preliminary experiment showed that 48 and 72 h of dehydration were followed by good profiles of water uptake, as assessed by changes in body mass of individuals after 0.5, 2, 5, 10 and 30 min of water access, but that water uptake was limited after only 24 h of dehydration. A dehydration period of 48 h was chosen for subsequent experiments. A mixed effect analysis with individual as a random factor indicated significant gain of body weight over the 30 min period of water access after 48 h of dehydration ($F_{(3,4)} = 49.7$, $P = 0.004$), with LSD multiple comparisons showing onset of significant weight gain after 5 min. Linear regression lines and associated 95 % confidence intervals are shown to illustrate trends. Triangles and dotted line represent water uptake profile after 24 h of dehydration, circles and thin black line after 48 h of dehydration, and squares and thick black line after 72 h of dehydration.

experiment was evaluated by weighing at set time intervals for a period of 30 min. Significant water uptake only happened after 5 min or more in water following 48 h of dehydration. We determined that a period of 30 min of daily water uptake was needed to maintain constant dehydration levels in toads undergoing daily bouts of dehydration following an original 48-h dehydration period.

Before maze training on the morning of the day beginning a dehydration period, toads were fed in group housing and half an hour later they were removed and dried with a paper towel. The bladders of toads submitted to dehydration were emptied by massaging the abdomen until no urine was expelled. Then, each toad was weighed to obtain a starting body weight (BW) and transferred individually to a 2-L clear plastic box (17 × 12 × 7 cm). Boxes used for dehydration were lined with a dry paper towel and the toads remained in boxes for 48 h before the first day of training. Boxes used to keep toads hydrated were filled to a depth of 0.5 cm with well water to maintain hydration at a high level. A rock allowing toads to stand on a dry surface was added in hydrated boxes for the second experiment because constant contact with water might have been stressful (see Section 3.1 in Results). All toads were returned to their individual boxes after training every day.

During the experimental period, dehydrated toads only obtained water from the maze in the form of an opportunity for 30 min of water uptake each training day. However, when a dehydrated toad did not find water on a given day it was allowed 10 min of water uptake in a separate box outside of the maze to insure it would not reach a dangerous level of dehydration before the next training day. Dehydration and water uptake were inferred from body weight changes. Percent dehydration was calculated as: (*Starting BW* − *BW before training*)/(*Starting BW*) × 100, while percent rehydration following water uptake in the maze was calculated as: (*BW after training* − *BW before training*)/(*BW after training*) × 100.

2.3.2. Maze training

2.3.2.1. First experiment.
Four groups of toads (n = 12 per group) were exposed to different combinations of two factors: dehydration vs. hydration and presence of hypertonic saline vs. inaccessible water in one arm of the maze. The location of distilled water in the goal arm of the maze was counterbalanced across toads to the right or left arm (6 toads on each side per group). To facilitate experimental logistics, dehydrated toads were trained on even-numbered weeks and hydrated toads on odd-numbered weeks. Eight toads were tested each week for a total experimental period of 6 weeks. Training consisted of 6 daily sessions on consecutive days with 3 trials per session. A 1-min interval was used between trials. At the beginning of a session, a toad was dried with a paper towel and weighed. For each trial, a toad was placed at the end of the starting arm, facing toward the center of the maze. It was given 5 min to reach the water container. If it did not move after the first minute, it was prodded manually by the observer to leave the starting arm. When water was reached, the toad was allowed 10 min to stay and rehydrate in the container. If a toad left the container once during this time, it was gently replaced into the container manually. However, if it left the container a second time, it was removed from the maze and the trial ended. After every trial, toads were dried with a paper towel and weighed to assess water uptake. The maze was cleaned with a water-moistened towel between trials to remove potential chemical cues left behind during the previous run of the maze.

2.3.2.2. Second experiment.
Two groups of toads (n = 8 per group) were dehydrated or hydrated as described above. Location of the goal arm of the maze and the type of external cues on the maze cover were counterbalanced across toads. Toads were trained in three separate batches due to logistical constraints. The maze training procedure used in the second experiment was modified to a single daily trial because a tendency for reduced water uptake in later trials of sessions was observed in some toads, which could have influenced the reinforcement experienced by dehydrated toads and interfered with learning. To enable a longer training period, toads were used in four consecutive daily trials per week over a period of four weeks, and then an additional fifth week with two test trials to evaluate latent and incentive learning. Each week included a return to normal housing for feeding and rehydration after the fourth training trial, which lasted two days before the beginning of a new 48-h period of dehydration. Individual toads were identified from week to week by their dorsal marking patterns. Between daily training trials, all toads were put back individually into their 2-L plastic containers. After the 4-week training period, a 48-h dehydration period was conducted before the toads were submitted to two test trials on consecutive days in which they were switched to the opposite motivational state that they had during training. Maze trials were conducted as in the first experiment, except that 30 min in the container was allowed for rehydration. Another modification to the procedures for the second experiment involved a change of location of the maze in the testing room. This was done in an attempt to eliminate the side bias in responding observed in the first experiment (see Section 3.2 in Results).

2.4. Analysis

Maze performance variables were the time to find the water source and correct first choices (i.e. water source found without entry in the wrong goal arm of the maze) during the first trial of sessions in the first experiment, and the same variables for each trial in the second experiment. Water balance variables were the level of dehydration reached before training and the level of rehydration achieved by water uptake in the maze. The time spent in the maze water container was also measured. Data were recorded by an observer sitting near the start arm of the maze. Time to reach water in the goal arm and time spent in the water container were recorded using a digital timer. The number of trials where a toad made no response during a session (i.e. did not enter the wrong arm or reach the water source) was also measured to assess if a tendency to avoid exploring the maze was present. Data from the no response trials was then omitted from analyses of time to find water and correct first choices to avoid biasing results toward poor performance.

Potential changes in the level of dehydration and rehydration during the experimental period were assessed using repeated measures general linear modelling (GLM) analyses with session or trial as a within-subject factor and hydration status and presence of

saline (first experiment only) as between-subject factors. Maze performance variables were analyzed by modeling the effects of session or trial (within-subject), hydration status (between-subject), the presence or absence of hypertonic saline (between-subject, first experiment only), and goal arm side orientation (between-subject) using generalized estimating equations (GEE). GEE is appropriate for analysis of repeated observations with count variables or variables that do not meet the assumptions of parametric statistics (Ballinger, 2004; Pekár & Brabec, 2018). Orientation (whether the correct arm was on the left or right) was added to the models to assess potential side bias. GEE analyses were based on a gamma distribution with log link function (time to find water), negative binomial distribution with log link function (count of correct first choices) or Poisson loglinear distribution (count of no response trials). The negative binomial distribution was used to account for the expectation that half of the toads would show correct first choices due to chance at the beginning of the experiments. An independent working correlation matrix was used with all variables. Tests of the GEE model effects used Wald Chi-square statistics (Type III) with $\alpha = 0.05$. GEE models included the main effect of each factor and all interactions, except those involving side orientation. When needed, exploration of the time course of effects between sessions or trials used least significant difference (LSD) pairwise comparisons (GEE) or the 95 % confidence intervals (CI) of estimated marginal means (GLM repeated). All analyses were conducted in SPSS Statistics 26 (IBM Corporation, Armonk, NY).

3. Results

3.1. First experiment: dehydration and rehydration

Fig. 3 shows the levels of dehydration achieved by toads before training sessions and rehydration levels due to water uptake in the maze. Repeated measures GLM analysis of dehydration levels showed that the assumption of sphericity was violated (Mauchly's test $\chi^2(14) = 58.3, P < 0.001$). Thus, the Greenhouse-Geisser correction was used for tests involving the within-subject factor. The analysis showed that only session (F(3.4) = 14.9, $P < 0.001$, $\eta_p^2 = 0.26$) and hydration status (F(1) = 52, $P < 0.001$, $\eta_p^2 = 0.55$) had significant effects on dehydration levels achieved over the training period (Saline: F(1) = 0.06, $P = 0.82$; all interactions: $P > 0.09$). Because exposure to hypertonic saline had no effect on dehydration levels, the estimated marginal means of dehydration levels for hydration status over training sessions are shown in Fig. 3A to better illustrate trends. The graph shows that dehydrated toads had much higher dehydration levels, as expected, but two other features of the data are noteworthy. First, toads housed in boxes in constant contact with
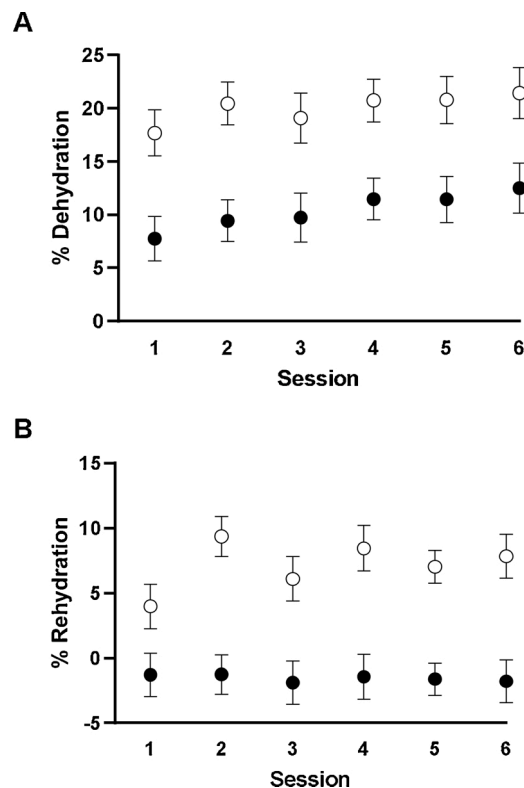

Fig. 3. Toad water balance during the first experiment. A) Percent dehydration at the beginning of sessions and B) percent rehydration achieved after sessions. In both panels, the two groups of dehydrated and hydrated toads were combined for clarity since the presence of hypertonic saline in the maze had no effect on water balance over the experimental period. Values are estimated marginal means and 95 % confidence intervals of repeated measures general linear models of the effects of session and hydration status. Symbols: Dehydrated (white circles) and Hydrated (black circles) toads.

water also had reduced body weights compared to the beginning of the experiment. Mean percent dehydration was 20.2 % [95 % CI: 19.2, 21.1 %] for the dehydrated toads and 10.8 % [95 % CI: 10.1, 11.6 %] for the hydrated toads. Thus, exposure to a dry environment only contributed to about half of the loss in body mass observed during this experiment. Second, dehydration levels slightly increased in both hydrated and dehydrated toads over the training period. The trend for increased dehydration over time in hydrated toads suggests that isolation in a box instead of insufficient water uptake in the maze contributed to increasing dehydration over the six days of training.

The analysis of rehydration levels during sessions also showed violation of the sphericity assumption (Mauchly's test $\chi^2(14) = 49.1$, $P < 0.001$), prompting use of the Greenhouse-Geisser correction. The analysis of rehydration levels showed that session (F(3.6) = 4.2, $P = 0.004$, $\eta_p^2 = 0.09$), hydration status (F(1) = 146, $P < 0.001$, $\eta_p^2 = 0.77$) and the session*hydration status interaction (F(3.6) = 4.1, $P = 0.005$, $\eta_p^2 = 0.09$) were statistically significant (Saline: F(1) = 0.1, $P = 0.75$; all other interactions: $P > 0.13$). The estimated marginal means of rehydration levels for hydration status over training sessions in Fig. 3B show that on average only dehydrated toads used distilled water in the maze to gain body mass. In fact, time spent in the maze water container was proportional to the amount of rehydration only in dehydrated toads (Fig. S1). This data strongly suggests that only dehydrated toads absorbed water and experienced appetitive reinforcement when immersed in distilled water in a maze container. The significant interaction between session and hydration status can be explained by a quick rise in rehydration levels after the first session in dehydrated toads, while rehydration levels were constant in hydrated toads. Fig. 3B additionally shows that hydrated toads on average experienced a negative water balance during sessions. This small loss of 1.6 % body mass on average during sessions might have contributed to the increased dehydration seen over the experimental period in hydrated toads (Fig. 3A); however, it would not explain the similar trend seen in dehydrated toads.

3.2. First experiment: maze performance

Toads did not explore the maze in 42 % of trials (18 %, 13 % and 11 % of first, second and third trial of sessions, respectively). Hydrated toads exposed to hypertonic saline had by far the most no response trials during the experiment (Hydrated-Saline: 83 no responses, Hydrated-No saline: 17, Dehydrated-Saline: 15, Dehydrated-No saline: 5). The GEE model showed that both hydration
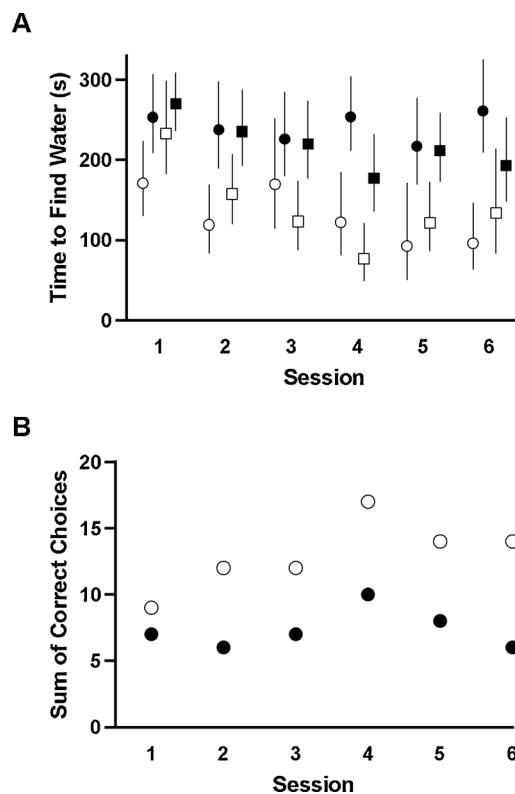
Fig. 4. Maze performance of toads in the first experiment. Performance was assessed using A) time to find water and B) sums of correct choices in the first trial of sessions. Performance is shown for all four groups of toads in panel A but limited to the comparison of dehydrated and hydrated toads in panel B for clarity. Values in panel A are estimated marginal means and 95 % confidence intervals of a generalized estimating equations model of the effects of session, hydration status, presence or absence of hypertonic saline, and goal arm side orientation. Symbols in panel A: Saline-Dehydrated (white circles), Saline-Hydrated (black circles), No Saline-Dehydrated (white squares), and No Saline-Hydrated (black squares) toads. Symbols in panel B: Dehydrated (white circles) and Hydrated (black circles) toads.

status and the presence of hypertonic saline were associated with a sustained high number of no response trials during training (Hydration status: $\chi^2(1) = 8.1$, $P = 0.004$, Saline: $\chi^2(1) = 10.0$, $P = 0.002$, all other model effects: $P > 0.1$), suggesting inhibitory effects of both factors on maze exploration.

Performance in the maze was evaluated by measuring time to find the water source and the number of correct first choices made during the first trials of sessions. We posited that time to find water would depend more on the motivation to seek water, while correct first choices would indicate learning of the water source location. Time to find the water source showed a rapid and sustained decline with training, especially in dehydrated toads (Fig. 4A). GEE modelling showed a significant effect of session ($\chi^2(5) = 30.3$, $P < 0.001$) and no interaction effect with the other factors (all interactions: $P > 0.05$), suggesting that faster times to find water with training was common to all groups. LSD pairwise comparisons showed that time to find water was already lower on the second training session and remained lower throughout the experiment (LSD session 1 vs all other sessions: $P < 0.01$). A significant effect of hydration status ($\chi^2(1) = 51.5$, $P < 0.001$) is explained by consistently lower times to find water in dehydrated toads over the experiment. Additionally, there was no effect of hypertonic saline on time to find water ($\chi^2(1) = 0.07$, $P = 0.79$), but a clear bias for faster times to find water located in the container on the right side of the maze ($\chi^2(1) = 35.5$, $P < 0.001$).

The number of correct first choices on first trials was higher with dehydration ($\chi^2(1) = 4.2$, $P = 0.04$; Fig. 4B) and showed a bias toward the right side of the maze ($\chi^2(1) = 38.3$, $P < 0.001$). There was also a significant effect of session without interaction effects with other factors (all interactions: $P > 0.3$). LSD pairwise comparisons with the first session showed that the effect of session was limited to a larger number of correct first choices on the fourth session ($P = 0.04$) which was not sustained until the end of training (LSD sessions 5 and 6: $P > 0.22$). Therefore, we conclude that the toads did not learn the location of the water source in this experiment despite showing faster times to find water with training.

3.3. Second experiment: dehydration and rehydration

Fig. 5A shows the levels of dehydration achieved by toads in the second experiment. Repeated measures GLM analysis of dehydration levels with Greenhouse-Geisser correction showed that trial (F(4.4) = 5.1, $P = 0.001$, $\eta_p^2 = 0.27$), hydration status (F(1) = 199, $P < 0.001$, $\eta_p^2 = 0.93$) and the trial*hydration status interaction (F(4.4) = 2.5, $P = 0.046$, $\eta_p^2 = 0.15$) had significant effects on dehydration levels over the training period. Confidence intervals around estimated marginal means of dehydration levels by hydration status show overlap over all training trials in hydrated toads, but significantly higher dehydration levels in dehydrated toads in trials 8, 12, and 14–16 compared to the first trial. Therefore, increasing dehydration levels over the experimental period were limited to
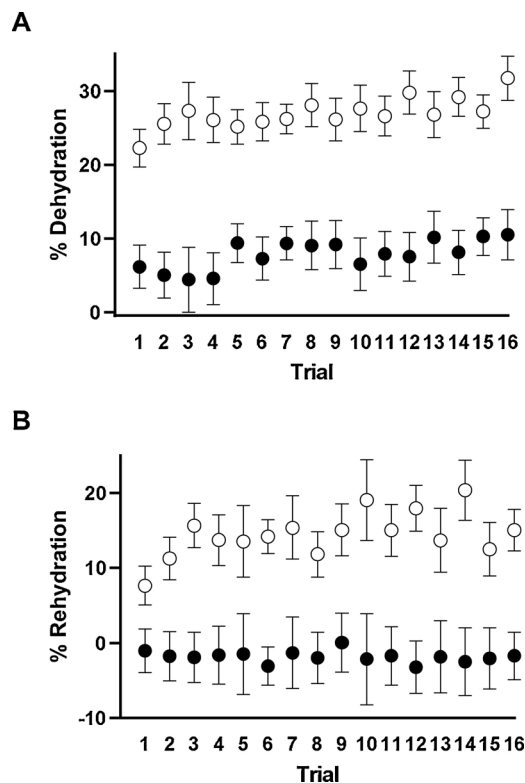

Fig. 5. Toad water balance during the second experiment. A) Percent dehydration at the beginning of trials and B) percent rehydration achieved after trials. Values are estimated marginal means and 95 % confidence intervals of repeated measures general linear models of the effects of trial and hydration status. Symbols: Dehydrated (white circles) and Hydrated (black circles) toads.
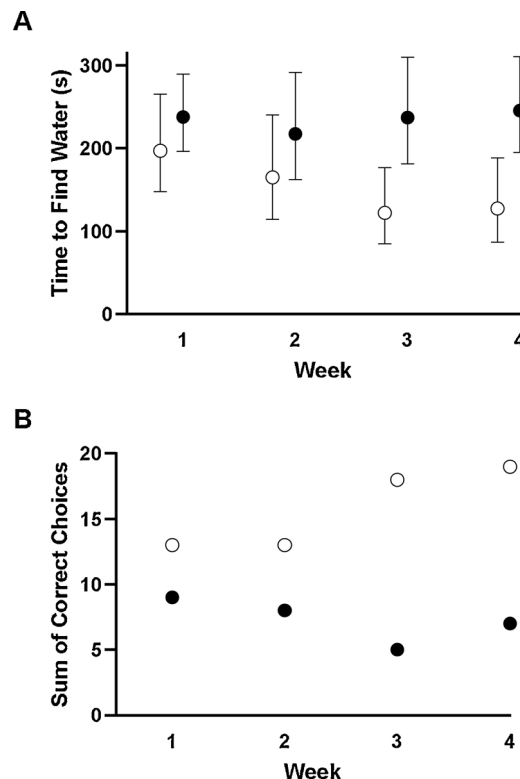
dehydrated toads in the second experiment. Again, toads housed in boxes with constant access to water had reduced body weights compared to the beginning of the experiment. Mean percent dehydration was 27 % [95 % CI: 24.2, 29.8 %] for the dehydrated toads and 7.8 % [95 % CI: 4.7, 11.0 %] for the hydrated toads. Reduced body weights in hydrated toads happened despite provision of an emerging rock for toads to stand on a dry surface in their boxes.

The analysis of rehydration levels during the second experiment showed significant effects of hydration status ($F(1) = 124$, $P < 0.001$, $\eta_p^2 = 0.90$) and the trial*hydration status interaction ($F(5.4) = 2.6$, $P = 0.03$, $\eta_p^2 = 0.16$). Fig. 5B shows that the interaction effect is due to constant values of rehydration over training in hydrated toads but increasing values in dehydrated toads. Confidence intervals around estimated marginal means of rehydration levels in dehydrated toads show significantly higher rehydration levels in trials 3–4, 6–7, 9–12, 14 and 16 compared to the first trial. Confidence intervals overlap throughout the experimental period in hydrated toads. Again, the results suggest that only dehydrated toads absorbed water and experienced appetitive reinforcement in the maze. Hydrated toads on average experienced a loss of 1.8 % body mass during trials in the second experiment compared to a loss of 1.6 % during sessions in the first experiment. Therefore, water loss in the maze does not explain the absence of a significant increase in dehydration during training in hydrated toads of the second experiment.

3.4. Second experiment: maze performance

There were fewer no response trials in the second experiment, where toads did not explore the maze in 5.5 % of trials (Hydrated: 12 no responses, Dehydrated: 2). GEE analysis again showed a sustained higher number of no response trials in hydrated toads during the training period (Hydration status: $\chi^2(1) = 8.4$, $P = 0.004$, Trial: $\chi^2(5) = 3.8$, $P = 0.6$), but inhibition of maze exploration was much reduced in comparison to the first experiment.

Time to find the water source in the second experiment showed a rapid decline limited to the dehydrated toads. To facilitate presentation of this data over the 16 training trials, weekly estimated marginal means are shown in Fig. 6A. GEE modelling showed significant effects of trial ($\chi^2(14) = 612$, $P < 0.001$), hydration status ($\chi^2(1) = 112$, $P < 0.001$) and the trial*hydration status inter-action effect ($\chi^2(14) = 1135$, $P < 0.001$). LSD pairwise comparisons showed that time to find water in dehydrated toads was already lower on the second training trial and remained lower throughout the experiment (LSD trial 1 vs all other trials: $P \leq 0.05$), while time to find water did not significantly change during training in hydrated toads. The significant effect of hydration status is explained by consistently lower times to find water in dehydrated toads, except on the first trial. Additionally, there was again a clear side bias in
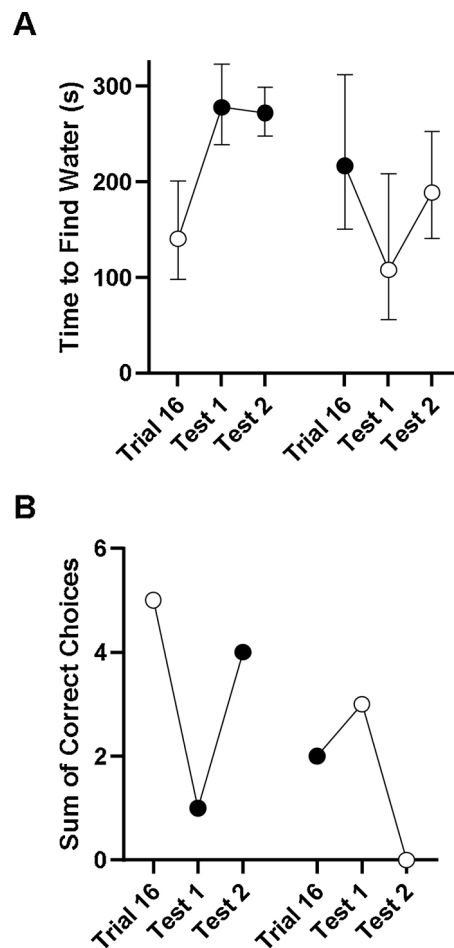

Fig. 6. Maze performance of toads in the second experiment. Performance was assessed using A) time to find water and B) sums of correct choices in training trials. Presentation of the performance data was simplified by showing weekly values. Weekly means in panel A were obtained from estimated marginal means and 95 % confidence intervals of a generalized estimating equations model of the effects of trial, hydration status and goal arm side orientation. Symbols: Dehydrated (white circles) and Hydrated (black circles) toads.

faster times to find water ($\chi^2(1) = 48$, $P < 0.001$), but the bias was toward the left side of the maze in the second experiment.

Weekly sums of correct first choices in the second experiment showed increased numbers in dehydrated toads in the last two weeks of training (Fig. 6B). GEE modelling showed significant effects of trial ($\chi^2(15) = 5296$, $P < 0.001$), the trial*hydration status interaction effect ($\chi^2(13) = 773$, $P < 0.001$) and side ($\chi^2(1) = 10.7$, $P = 0.001$), but not of hydration status ($\chi^2(1) = 0.6$, $P = 0.44$). More correct choices were made toward the left side of the maze. LSD pairwise comparisons with the first trial showed correct choices increasing with training only in dehydrated toads, with significantly higher values at trials 3–4, 11–14 and 16. Correct choices were occasionally significantly lower compared to the first trial in hydrated toads (trials 3, 7, 9 and 15). The early increase in correct first choices at trials 3–4 by dehydrated toads was not sustained until later in training; a pattern similar to the non-sustained increase in correct choices at session 4 in the first experiment. We conclude from this data that dehydrated toads had learned the location of the water source by trial 11 midway through the third week of training.

Maze performance after a change in motivational status was assessed by comparing data on the last trial of training to the first and second tests that followed the motivational change (Fig. 7). GEE analysis of time to find the water source over these three trials only showed a significant trial*original hydration status interaction ($\chi^2(2) = 13.3$, $P = 0.001$). The effects of the other factors were not significant (Trial: $\chi^2(2) = 3.5$, $P = 0.17$, Original hydration status: $\chi^2(1) = 3.1$, $P = 0.08$, Side: $\chi^2(1) = 0.4$, $P = 0.55$). LSD pairwise comparisons with trial 16 showed that previously dehydrated toads increased time to find water once hydrated ($P < 0.001$) and previously hydrated toads decreased time to find water on the first ($P = 0.01$) but not the second test ($P = 0.64$) following dehydration. GEE analysis of correct first choices showed no statistically significant effect (Trial: $\chi^2(2) = 1.2$, $P = 0.55$, Original hydration status: $\chi^2(1) = 0.3$, $P = 0.61$, Trial*Original hydration status: $\chi^2(2) = 3.1$, $P = 0.08$, Side: $\chi^2(1) = 0.01$, $P = 0.92$). Most relevant to the tests of latent and incentive learning, toads that were hydrated during training made only one more correct first choice after

Fig. 7. Performance of fire-bellied toads after a change of motivational status in the second experiment. Performance was assessed using A) time to find water and B) sums of correct choices in two test trials under opposite motivation compared to the last training trial (Trial 16). Values in panel A are estimated marginal means and 95 % confidence intervals of a generalized estimating equations model of the effects of trial, hydration status and goal arm side orientation. The symbols for dehydrated (white circles) and hydrated (black circles) toads track the changes in motivational state. Tests of dehydrated toads switched to a hydrated status are on the left of graphs, while tests of hydrated toads switched to a dehydrated status are on the right.

dehydration, showing no evidence of latent learning. Thereafter, they made no correct first choice on the second test after dehydration, showing no evidence of rapid incentive learning due to the reinforcement experienced in the first test trial after dehydration. Therefore, even though the time to find the water source in the maze quickly changed along with motivational status, spatial learning had no influence on performance during these tests. Interestingly, the side bias observed during training did not influence performance during the tests.

4. Discussion

We could not replicate the finding of rapid spatial learning of the location of a water source in a T-maze by dehydrated fire-bellied toads observed by Brattstrom (1990) despite the fact that dehydrated toads in the present study underwent higher maximal dehydration and had more time to rehydrate following a successful choice in the maze (1800s here vs. 10×30 s previously), which should have produced more appetitive reinforcement. In fact, we observed an opposite pattern of maze performance. While the results of Brattstrom (1990) suggested rapid spatial learning without improvement in time to find water, our results suggest rapid improvement of time to find water followed by delayed spatial learning of the location of the water source. Some differences in experimental procedures between the two studies were inevitable because preliminary observations showed that our subjects did not support well the dehydration procedure with lamps used by Brattstrom (1990) and did not absorb a significant amount of water while sitting for 30 s in distilled water (Fig. 2), which was the length of time used by Brattstrom (1990) for reinforcement in each training trial. The differences between these two related studies are puzzling but could be due to the use of different toad populations, different schedules of reinforcement, or different analytical approaches. Regardless of distinction between the two studies on maze learning in the fire-bellied toad, our results confirmed that this species shows spatial learning in a T-maze.

4.1. Factors influencing maze performance

In the first experiment, groups of fire-bellied toads were trained in a T-maze with or without exposure to a hypertonic saline solution to assess if appetitive and aversive stimuli would facilitate spatial learning. Contrary to our prediction that opposite stimuli would interact to promote learning, punishment of errors by exposure to the saline solution did not influence maze performance. The presence of hypertonic saline in the maze only contributed to reduce maze exploration by the toads (i.e. increased trials with no response). These results do not support the hypothesis that appetitive and aversive stimuli interact to facilitate spatial learning in this species. Instead, it supports the view that aversive experience due to exposure to the hypertonic saline acts by general inhibition of behaviour (Dickinson & Pearce, 1977; Konorski, 1967). Exposure to the saline appeared to be a very aversive event. Toads probing the saline solution with a limb or entering the container immediately withdrew from contact with the solution. Such quick reactions are atypical for these relatively passive animals. If anything, the first experiment showed a selective impact of appetitive reinforcement on water seeking behaviour in line with the hypothesis of *differential impact* of reinforcers (Magoon & Critchfield, 2008). However, the training duration used in the first experiment was not enough to produce reliable spatial learning; therefore, there is a small possibility that additional exposure to aversive punishment could promote learning of the water source location.

Patterns of responding during the second experiment confirmed a rapid effect of dehydration on the time to find water, which preceded a later onset of improved first choices for the water location. We interpret these patterns of maze performance as rapid but directionless effects of motivation on the tendency to run the maze followed later by onset of spatial learning effects on behaviour due to repeated experience of water reinforcement in the maze. Subsequently, the change in motivational status of previously hydrated toads showed no evidence of latent learning of maze features during training and no evidence of a rapid incentive learning effect after toads experienced water reinforcement for the first time under a newly dehydrated state. Thus, the rapid effects of dehydration on water seeking behaviour in toads are likely due to a general energizing effect of this motivational state. This represents a simpler kind of motivational effect than what is seen in amniotes, where there is good evidence that motivational states contribute to learning the incentive value of cues and outcomes of actions (Balleine, 1992; Clayton & Dickinson, 1999; Dickinson & Balleine, 2002). Interestingly, a dissociation of general (energizing) and directed (incentive) influences of motivational states on mammalian behaviour has been proposed (Niv, Joel, & Dayan, 2006). It is possible that a new role of motivational states in incentive learning evolved late in vertebrate phylogeny and is not present in amphibians. A similar conclusion was reached by Muzio et al. (2011) based on the absence of contrast effects in toads adjusting their water seeking behaviour in a runway after shifts in reinforcement. Alternatively, incentive learning might influence some behaviours such as feeding, but not others like water seeking. In support of the latter, prior experience of salt reward is not necessary for salt-seeking under a deprived state in rats (Kriekhaus & Wolf, 1968). The possibility that motivation influences different behaviours through different mechanisms will have to be considered before concluding that incentive learning is absent in amphibians.

4.2. Methodological considerations for maze studies using terrestrial amphibians

A clear side bias was observed in each experiment. We first considered that the side bias toward the right in the first experiment could be due to lateralization of behaviour, which is a well-established phenomenon in anuran amphibians (Malashichev & Robins, 2019). However, the side bias changed for a tendency to go to the left in the second experiment. This change of side preference clearly argues against the possibility of response lateralization in the T-maze. The new maze location in the second experiment provided an orthogonal orientation toward the same corner of the testing room. Toads might have been attracted to a feature in this room corner perceptible through the maze cover, possibly relative darkness in comparison to the more direct illumination coming from above maze

locations. Although side biases persisted during training, no bias was seen in tests after a motivational change in the second experiment, suggesting that side preference was eliminated by extensive maze familiarity.

It is unclear why hydrated toads lost significant amounts of their body mass despite constant contact with or access to water in their individual housing boxes. Considering that the toads were fed before each training week, we assessed if defecation of digested food could be the source of this reduction in body mass. Eight toads were fed and held in dry individual boxes for two days before fecal matter was scooped and weighed. The mass of feces represented on average 0.3 % of initial body mass while total reduction in body mass including feces and dehydration was on average 5 %. Clearly, defecation cannot explain the important reduction in body mass observed in hydrated toads. An alternative explanation could be that the stress of individual housing contributes to the reduction in body mass. However, elevated corticosteroid stress hormones are known to increase salt retention in amphibians (McCormick & Bradshaw, 2006), which would be unlikely to cause dehydration, and in fact could promote the opposite effect by promoting water uptake through the skin (Anderson, Bovo, Eismann, Menegario, & Andrade, 2017). Lastly, the water used to house hydrated toads in individual containers had an osmolarity of 4.2 mOsm/kg (see data for hard water in Platek, Turko, Donini, Kelly, & Wright, 2017), which is well below the osmoneutral point represented by a solution of about 212−225 mM NaCl in *Rhinella arenarum*, a terrestrial amphibian with a comparable life style to the fire-belied toad (Muzio et al., 2011). Because of the latter, we conclude that the well water was not hypertonic and did not contribute to the water loss observed in hydrated toads. The cause of the loss of body mass in hydrated toads remains elusive.

4.3. Conclusion

The behaviour of adult fire-bellied toads in a T-maze revealed that motivation quickly influenced water seeking and that spatial learning of the location of the water source only contributed to water seeking performance after long training. Tests under the opposite motivational state that was under effect during training showed that spatial learning did not happen without appetitive reinforcement. Finally, aversive punishment using the presence of hypertonic saline in the maze only inhibited maze exploration tendency, without an effect on performance of toads that ran the maze. We hope that better knowledge of the determinants of maze performance will promote more investigations of spatial learning and motivation in anuran amphibians. Future studies about the nature of spatial learning representations hold particular promise. For example, manipulation of the location of external environmental cues above the maze could reveal if place learning is used by toads for guidance toward the water source; alternatively, a plus-maze allows starting the toads from the arm of the maze opposite of the start arm used in training, which could reveal if response learning relying on the direction of body turns at the maze choice point is used to find water (Daneri et al., 2011; Tolman, Ritchie, & Kalish, 1946). Spatial learning strategies appear to vary greatly among individuals (Adler, 1980; Dall'Antonia & Sinsch, 2001) and between species of amphibians with different life histories (Liu, Jones, Day, Summers, & Burmeister, 2020). As such, experimental and comparative approaches could help elucidate what aspects of individuality and life history determine spatial learning abilities and strategies. Research efforts should also try to establish if the spatial learning strategies of amphibians vary with the amount of training or stress, as seen in mammals (Kim, Lee, Han, & Packard, 2001; Packard & McGaugh, 1996; Packard & Wingard, 2004).

CRediT authorship contribution statement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lmot.2020.101679.

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