

Rapid learning in the common midwife toad, *Alytes obstetricans*, under an appetitive stimulus

Miriam Moreno-Orosa¹, Jaime Bosch^{1,2,*}

¹ IMIB-Research Unit of Biodiversity (CSIC-Universidad de Oviedo-Principado de Asturias), Gonzalo Gutiérrez Quirós s/n, Edificio de Investigación 5ª planta, 33600 Mieres, Spain

² Centro de Investigación, Seguimiento y Evaluación, Parque Nacional Sierra de Guadarrama, 28740 Rascafría, Spain.

*Correspondence: jaime.bosch@csic.es

Received: 27 January 2022; returned for review: 02 March 2022; accepted: 28 June 2022.

Studies of response learning of amphibians are scarce compared to other vertebrates. We conducted a simple experiment to evaluate response learning in a primitive anuran species, the common midwife toad (*Alytes obstetricans*) under an appetitive stimulus. We hypothesized that, despite the primitive character of the studied species, the animals would be able to rapidly associate a particular arm of a basic T-shaped experimental arena with the reward, and that the existing male parental care of the species would provide males greater cognitive abilities than females. The response obtained by 12 males and 13 females was statistically different than such expected by chance, suggesting a learning process. The sex of the animal, as well as the average time spent to select the correct choice, were key during the learning process. Contrary to expectations, females showed higher learning capacities than males, perhaps as a consequence of the elaborate mate selection process and the competition between females to access males in this group of amphibians. The obtained results indicate that a rapid learning capacity is highly conservative in the phylogeny, having evolved from early tetrapods to those existing today in reptiles, birds, and mammals.

Key words: amphibians; appetitive stimulus; archaeobatrachia; learning.

Learning occurs when an experience leads to a permanent relative change in the reaction to a situation (PEARCE, 2008). Information about previous experiences is stored as memories in the neural networks of the brain, allowing subsequent behaviour modification (LEWIS *et al.*, 2020). Among vertebrates, the simplest brain organisation resides in the oldest phylogenetic groups (fish and amphibians), whose brains lack a neocortex (MUZIO *et al.*, 2017). Therefore, although studies on the cogni-

tive capacity of amphibians are still scarce in comparison with other vertebrates (MACPHAIL, 1987), the phylogenetic position of the Amphibia class as basal tetrapods makes their study fundamental to the understanding of the evolution of cognition and the development of the nervous system.

Spatial learning acquired by orientation (or turning response) is based on egocentric strategies where attention is focused on the animal itself and not on the spatial

relationships between the elements that make up its environment (MUZIO *et al.*, 2017). This is a type of associative learning where the orientation response is a form of instrumental or operant conditioning (MACKINTOSH, 2002), whose learning unit is the response-reinforcer association, and not stimulus-response. Thus, the occurrence of reinforcement will increase the probability that the response will be repeated, giving rise to the acquisition process of conditioning (WILLIAMS, 1987). Orientation skills in amphibians have been studied through the use of different experimental designs, with mazes being the most commonly used to test the ability to locate in space towards a specific direction, either to receive a reward (appetitive stimulus) or to flee from a dangerous situation (aversive stimulus).

The study of associative learning in amphibians has shown that the strategies used to locate themselves in space are very varied, as they are able to orient themselves spatially by following olfactory cues such as the odour of the breeding site (OLDHAM, 1967), local visual cues within a maze (LIU & BURMEISTER, 2017), or even geometric cues (i.e. the shape of the space; SOTELO *et al.*, 2015). In addition, it has been shown that anurans can orient themselves spatially in a T-shaped structure using egocentric signals, a type of orientation based on a body-centred turn, which is an internal self-referenced cue (e.g. BRATTSTROM, 1990; DANERI *et al.*, 2011; KUNDEY *et al.*, 2016). The results of these studies suggest that amphibians are capable of learning under different conditions, and of exhibiting a great variety of responses to the presented stimuli (GRUBB,

1976). For this reason, the study of learning in amphibians, as the first tetrapods to colonize the terrestrial environment, is of great importance in developing our understanding of the events associated with modifications in brain organization in the rest of the vertebrates (SOTELO & MUZIO, 2015).

Here, we evaluate whether a primitive anuran amphibian, the common midwife toad (*Alytes obstetricans*), is able to learn a simple task after a brief period of training, and if other variables, such as sex, weight, or physical condition, influence the learning process. We hypothesize that the study species will exhibit the ability to learn simple tasks very quickly under the stimulus of a reward and, due to its male parental care behaviour, that the learning ability will be higher in males. This prediction could be supported by the adaptive specialization hypothesis of LIU & BURMEISTER (2017), which establishes that sex differences in learning are determined by differences in cognitive demands between sexes required to solve ecological problems.

We used captive-born midwife toads from the Captive Breeding Centre for Threatened Amphibians of Guadarrama Mountains at the Sierra de Guadarrama National Park (Madrid, Spain). There, 55 adults (30 sexed and 25 unsexed individuals) were kept in a room at 21°C (a high temperature that prevents the development of the amphibian chytrid fungus) with a year-round light-dark cycle of 12/12 hours, and were fed weekly *ad libitum* with live crickets. Adult animals were individually marked with a pit-tag and housed in small groups of 4-5 individuals in medium-sized boxes for reproduction, whereas

juveniles were housed individually in smaller boxes to avoid competition among animals and to maximise their growth-rate before being released into the field. Housed animals were therefore accustomed to people, and no defensive or fear response behaviours were observed in these animals, meaning that they could be easily handled without affecting their natural behaviours.

The experimental sessions were conducted at night in March-April 2021 from 11:00 PM to 4:00 AM, which is the period of greatest activity for the toads. We use a T-shaped arena (each compartment 41.5 cm long and 15 cm high) with two arms at the end of the entrance aisle, right or left. A night vision camera with infrared light sensors placed at the bifurcation of the entrance corridor provided a constant view of the toads without interfering with their movements. Before each session, the subjects were kept individually for at least two hours in a container located within the room where the experiment was conducted, and in complete darkness for acclimatization purposes. We used live small-sized crickets as an appetite stimulus, and not water as other authors proposed (e.g. MUZIO, 1995; DANERI *et al.*, 2007), because the study species is very resistant to dehydration. We used tweezers to increase handling accuracy and minimize the time taken to provide rewards, and small-sized crickets to prevent the subject from becoming satiated during the experimental sessions, which would have affected the efficacy of food as an appetite stimulus.

For each subject, a specific arm (right or left) of the arena was randomly assigned as the reward arm for the whole experi-

ment. In each trial, the toad was carefully deposited at the release point at the beginning of the entrance corridor, and the arena was covered with a hard surface to avoid external visual clues. We scored a choice only when the toad reached the end of one arm (right or left) of the arena; otherwise, the trial was considered null. It was also considered null when the end of one arm was not reached within 15 minutes of releasing the toad, or when the toad remained motionless for more than 10 minutes. If the toad reached the end of the reward arm, it received a cricket under red light and was returned to its individual container, otherwise it was returned without receiving a reward. After each trial, the arena was cleaned with 70% ethyl alcohol to eliminate chemical clues, and the entire arena was sprayed with distilled water.

The training phase of a toad ended when it achieved four correct choices (not necessarily consecutive) or performed up to 15 trials, all during a single session. When a toad concluded its training phase, and after a 10-minute break, the learning evaluation phase was conducted during the same session. The learning evaluation phase comprised four trials, regardless of whether the toad reached the end of the reward arm or the other one, or performed a null trial. During both the training phase and the learning evaluation phase, the toad received a cricket only upon reaching the end of the rewarded arm. For every trial, we recorded the time from the release of the toad to the end of the trial (choice time; in minutes), and the selected arm for non-null trials. The learning score of every animal was calculated as the ratio between

the number of correct choices and the number of valid trials during its learning evaluation phase. Thus, if a toad selected the reward arm in all four trials, the score would be 4/4 (the maximum score). If a toad performed two correct choices, one incorrect choice and one null trial, the score would be 2/3. Choice time values were standardized within individuals by expressing every value as the proportion of the highest time value of its valid trials, in order to compensate for inter-individual differences. Two averaged choice times were calculated for each toad during its learning evaluation phase: when selecting the reward arm, and when selecting the opposite arm. Finally, the weight (in grams) of the tested toad was recorded.

JMP 13.0 software was used to perform the statistical analyses. A one-tailed Student's *t*-test was used to test whether the learning score was greater than the value expected by chance of 0.5. A general linear model was used to analyse if the studied variables were modulating the learning process. For this, the learning score was considered as the dependent variable, while the rest of the continuous variables and the sex were considered as independent variables. A correlation analysis was used to check that the independent variables were not closely correlated with each other, and three different ANOVAs were used to check that these variables did not differ significantly between sexes. Finally, the distribution of the residuals of the linear model was tested for normality by using a Shapiro-Wilk test in order to check that the requirements of this statistical analysis were met.

Twenty-five (12 males and 13 females)

out of 30 tested toads (13 males and 17 females) completed the experiment. The mean learning score obtained in the experiment was statistically higher than expected by chance (mean \pm SD: 0.64 ± 0.24 , $t = 2.90$, $P = 0.0039$), indicating that the rewarded arm was preferably chosen.

The correlation analysis indicated that the independent variables to be included in the linear model were independent from one each other (correlation coefficient lower than $|0.2279|$) and did not differ significantly between sexes ($F_{1,24} < 0.72$, $P > 0.4033$ in all cases). The linear model performed by relating the learning score obtained in the experiment with the independent variables was statistically significant ($F_{4,20} = 4.44$, $P = 0.0099$), explaining 36.4% of the variation observed in the response of the animals. The model complied with the requirements for this type of analysis, since its residuals were normally distributed ($W = 0.96$, $P = 0.6752$). The sex of the subjects ($F_{1,20} = 2.81$, $P = 0.0108$) and the choice time when selecting the rewarded arm ($F_{1,20} = 2.14$, $P = 0.0449$) contributed significantly to the model. The learning score was significantly higher in females than in males (0.62 vs. 0.48), and the choice time when selecting the rewarded arm was positively correlated with the learning score. That is, animals that obtained higher learning scores took more time to reach the rewarded arm.

Our results indicate that common midwife toads are able to learn a simple route very quickly under the stimulus of a reward, contributing with new data to the limited knowledge about the learning abilities of amphibians. In fact, BRATTSTROM (1990) already demonstrated that the close-

ly related species, the oriental fire-bellied toad (*Bombina orientalis*), learned very rapidly when it was slightly dehydrated and water was used as reinforcement in a very similar T-maze experiment. However, 80% of the fire-bellied toads needed three days to reach the established criterion (70% of correct choices out of 10 trials/day) in learning to go to the right, and 100% reached that criterion by day four. In another similar experiment with tiger salamanders (*Ambystoma tigrinum*), the number of attempted trials required to accumulate three consecutive days of making a correct choice on the first attempted trial of the day (with a maximum of 10 trial attempts/day) was, on average, 143, and was completed over a mean of 47 days (KUNDEY *et al.*, 2016).

Toads' sex was found to be the most important variable in learning ability, and contrary to our initial hypothesis, learning ability was significantly higher in females than in males. Although some studies indicate that complex social and spatial behaviours in anurans, such as parental care, might be related to higher learning ability (e.g. LIU *et al.*, 2019), in our case study male parental care does not seem to endow males with higher learning abilities. On the contrary, the sexual selection process of female toads, and maybe their ability to discern the quality of males through their call, or the competition between females for access to males (the most limited resource in this case), could act to increase the learning capabilities of females of this species. Indeed, for the genus *Alytes* there is an extensive literature on the discerning abilities of females towards different characteristics of male songs (e.g. BOSCH &

MÁRQUEZ, 2001; BOSCH *et al.*, 2003), and fights between females to breed with males have also been described (VERRELL & BROWN, 1993; BUSH & BELL, 2010).

After sex, the time taken to select the rewarded arm was the most relevant variable to explain the learning score, with a positive correlation between these two variables. Our initial hypothesis was that there would be a negative relationship between the time taken to select the rewarded arm and the learning score; however, our results seem to indicate that individuals invested some time during the experiment to decide which was the rewarded direction, so that successful decisions required more time.

Although the experiment was extremely simple and involved only two possible alternatives (right or left), the obtained results seem to indicate that the study species may have a relatively rapid learning capacity, being able to successfully orient towards the rewarded arm in more than 50% of the trials. On the other hand, it is also possible that the conditions of captivity had somehow reduced the learning capacity of our study individuals. It is also possible that the elevated degree of inbreeding in our study animals (ALBERT *et al.*, 2015), or the favourable living conditions without exposure to predators and in an *ad libitum* feeding regime, might have conditioned our results. Therefore, it is not possible to discard the possibility that, if the same experimental method were replicated with wild animals, the obtained results would be different.

New and more complex learning experiments are necessary to delimit with accuracy the learning capacities of amphibians.

In agreement with the results of previous studies, our results seem to indicate that certain cognitive skills, such as a fast learning capacity and memory, were already present in the first tetrapods, and evolved along the phylogeny into those present today in reptiles, birds and mammals.

Acknowledgement

Daniela Gómez contributed to the original idea and design for the study. We thank Juan A. Vielva, director of the *Centro de Investigación, Seguimiento y Evaluación* of the Guadarrama Mountains National Park and all people working at the Park for the facilities and resources provided to complete this work. We thank Jon Bielby and Gail Stride for corrections to the English grammar in this article.

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