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## BEHAVIOURAL RESPONSES OF *ODONTOPHRYNUS AMERICANUS* TADPOLES (ANURA: LEPTODACTYLIDAE) TO FISH CHEMICAL CUES

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**R E S U M E N.** — Las larvas de muchas especies de anfibios viven en ambientes con vegetación y de aguas turbias, donde la visibilidad es muy limitada. En estos ambientes, la utilización de señales químicas para la detección de depredadores podría ser más confiable que la utilización de señales visuales. Las larvas de *Odontophrynus americanus* habitan en las lagunas del valle aluvial del río Paraná Medio, Argentina, donde normalmente se dan condiciones de baja visibilidad. La hipótesis de este trabajo fue que los renacuajos de esta especie pueden detectar a los depredadores y responder subsecuentemente con conducta antipredador ante la percepción de señales químicas de los depredadores. En el presente trabajo se llevaron a cabo tres experimentos de laboratorio para evaluar si los renacuajos de *O. americanus* responden conductualmente al estímulo químico del pez *Astyanax fasciatus*. Las respuestas conductuales fueron evaluadas observando cambios en actividad, utilización de refugio y distribución espacial de los renacuajos. En presencia del estímulo químico del pez, los renacuajos redujeron su actividad en un 27%, incrementaron cinco veces la utilización de refugios, pero no exhibieron una respuesta de elusión espacial al estímulo del depredador. Las respuestas conductuales de los renacuajos de esta especie podrían reducir las probabilidades de encuentro y ataque por parte de los depredadores.

Palabras claves: Mecanismos antipredador, larvas de anfibios, *Odontophrynus americanus*, *Astyanax fasciatus*.

**A B S T R A C T.** — Many amphibian larvae live in turbid and vegetated habitats with very limited visibility. In these habitats, the use of chemical cues for predator detection may be more reliable than visual ones. *Odontophrynus americanus* tadpoles inhabit floodplain ponds of the middle Paraná River, Argentina, where they frequently encounter low visibility conditions. We hypothesize that tadpoles of this species can detect predators and subsequently respond with antipredator behaviour by means of chemical cues from predators. In the present work, we ran three separate laboratory experiments to evaluate whether *O. americanus* tadpoles respond behaviourally to a chemical stimulus of the characid fish *Astyanax fasciatus*. We evaluated behavioural responses by observing changes in activity level, refuge use and spatial distribution of the tadpoles. In the presence of the fish chemical stimulus, tadpoles reduced their activity by 27% and increased refuge use approximately five-fold, but they did not exhibit spatial avoidance from the predator stimulus. The behavioural responses of the tadpoles of this species may reduce the rate of encounter and attack by predators.

Keywords: Antipredator mechanisms, anuran larvae, *Odontophrynus americanus*, *Astyanax fasciatus*.

### INTRODUCTION

The ability of some amphibian larvae to respond behaviourally to waterborne chemical cues from predators is well documented and represents a valuable strategy to reduce predation risk in spe-

cies that co-occur with fishes (Kats *et al.*, 1988; Pearl *et al.*, 2003; Hickman *et al.*, 2004). Common behavioural responses of tadpoles to predators are: reduced activity, increased refuge use and spa-

tial avoidance (Petranka *et al.*, 1987; Semilitsch and Reyer, 1992; Schemidt and Amézquita, 2001). These behavioural changes can diminish predation rates but also generally decrease feeding and reduce growth and size at metamorphosis (Skelly and Werner, 1990). This trade-off between growth rate and predation risk is the central component of the current theoretical approaches that explain the plasticity of antipredator responses (Van Buskirk, 2000).

In larval amphibians, chemical cues often permit discrimination of predator and non-predator heterospecifics more effectively than visual cues, favouring more precise antipredator responses (Kiesecker *et al.*, 1996; Hickman *et al.*, 2004). In turbid water habitats, the use of chemical cues can allow earlier detection of the predator, allowing more successful evasion of predators (Lima and Dill, 1990; Hickmann *et al.*, 2004). Mathis and Vincent (2000) suggested that the priority use of chemical over visual cues in larval salamanders could be explained by the difficulty of detecting cryptic predators, the low visibility in turbid or vegetated habitats, and the poor development of visual systems (myopia).

*Odontophrynus americanus* is a South American leptodactylid frog with markedly terrestrial habits that spawns in puddles and streams (Cei, 1980). Tadpoles are benthic-nektonic and inhabit temporary and semipermanent ponds in the floodplain of the middle Paraná River, east-central Argentina, where they frequently co-occur with fishes (Peltzer and Lajmanovich, 2004). Tadpoles of *O. americanus* are also found in roadside temporary ponds where they rarely encounter predatory fishes. These floodplain habitats are characterized by very turbid water and vegetated margins (*Ludwigia* sp.) where the tadpoles of *O. americanus* are commonly encountered (Lajmanovich, 2000). During high water periods (flood pulse) many fishes move

from the main channel into intermittent drainages and reach the floodplain ponds (Bonetto *et al.*, 1969; Lowe-McConnell, 1987; Junk *et al.*; PAS, unpubl.). *Astyanax fasciatus* is a widespread characid fish that occurs frequently in these habitats (Cordiviola de Yuan, 1992). Although individuals seldom reach 10 cm in length, its voracity and abundance (Ringuelet *et al.*, 1967) make this fish an effective potential predator.

In this study, we examine whether tadpoles of *O. americanus*, change activity level, refuge use, and spatial distribution when presented with chemical cues from this common fish predator. We predicted that *O. americanus* tadpoles, which frequently inhabit turbid waters where visual cues are not available, should respond behaviourally to the chemical cues of *A. fasciatus* by decreasing activity, increasing refuge use and exhibiting spatial avoidance from predator chemical cues.

## MATERIAL AND METHODS

Recently hatched tadpoles of *Odontophrynus americanus* were collected with a dip net at a roadside pond in the west suburbs of Santa Fe city in April 2004 (Santa Fe province, Argentina) (31°39'52" S, 60°44'00" W). Tadpoles were transported to a laboratory for testing. Historically this site belonged to the floodplains of the Paraná and Salado rivers, but currently the pond is isolated from them by water retaining walls. In spite of this, the proximity to the floodplains (less than 1 km) and the nature of the retaining walls allow free migration of adult *O. americanus*. Tadpoles were maintained in cylindrical 25 cm diameter glass aquaria, filled with 6 cm of aged dechlorinated tap water and were fed *ad libitum* with boiled lettuce. Water was replaced every other day.

Specimens of *Astyanax fasciatus* (mean  $\pm$  SD standard length = 6.26  $\pm$  0.62 cm;  $n$  = 10) were collected from a

permanent pond on Los Sapos Island (Santa Fe Province, 2 km west of Santa Fe City, 31°39'58" S, 60°45'09" W), within the flood plain of the Salado River, with a 10 M wide haul. Fishes were kept in a continuously aerated 10 l aquarium and were fed with *Hypsiboas pulchellus* tadpoles collected from the same pond. Throughout the experimental period, animals were kept in controlled lab conditions, at  $25 \pm 2$  °C and on a 14:10 h light: dark photoperiod with fluorescent overhead lamps.

We measured three behavioural responses of tadpoles to fish chemical stimuli in successive experiments: (1) activity level, (2) refuge use, and (3) spatial avoidance from the predator chemical stimulus. In all experiments we used dechlorinated tap water as control and fish conditioned water as stimulus. Water conditioning involved placing 10 fishes in a continuously aerated 15 l aquarium filled with dechlorinated tap water 24 hours before tests started. Fishes were fed just prior to placement into the stimulus collection aquaria but were not fed during the water conditioning period. Tadpoles used in all experiments were between Gosner (1960) developmental stage 28 and 36.

In the first experiment, we tested whether *O. americanus* tadpoles reduced their activity level when exposed to chemical cues from *A. fasciatus*. In this experiment we formed 10 groups of 10 tadpoles choosing each one randomly from the stock available. For each group we measured the activity level first in dechlorinated tap water and subsequently in fish stimulus water. Tadpoles were tested in a cylindrical 25 cm diameter glass aquarium filled to a level of 8 cm with the stimulus water. We placed a group of 10 tadpoles in the aquarium that were allowed to acclimate for 10 min. *Odontophrynus americanus* tadpoles are characterized by moderate activity with rapid short movements separated in time by inactive periods. Since these movements are easily quan-

tifiable, we counted the number of times any tadpole made a swimming movement during a period of 5 min. Once the test finished, we removed the tadpoles carefully with a dip net and thoroughly rinsed the aquarium with tap water. We filled the aquarium with fish conditioned water and gently placed the same group into the aquarium. After a new 10 min acclimation period we again measured the activity level. We considered each group of ten tadpoles as a single replicate and had 10 replicates ( $N = 100$  tadpoles).

In the second experiment, we tested whether groups of tadpoles responded to chemical fish stimulus by increasing the use of artificial refuges. Testing took place in a manner identical to experiment 1, except that we employed a 35 x 17 x 20 cm aquarium and before introducing the tadpoles, we placed eight refuges (uniformly distributed) on the aquarium bottom. The refuges were semi-cylindrical tunnels, each 5 cm long and 3 cm wide, made from longitudinally divided plastic tubes. For each test, we recorded the number of tadpoles inside refuges at 1 min intervals for 10 min. The groups of tadpoles were different from those used in experiment 1. Again, we had 10 replicates (10 groups of ten tadpoles,  $N = 100$  tadpoles).

In the third experiment we measured the spatial avoidance of groups of tadpoles in response to a fish chemical stimulus gradient. We used an aquarium of the same size as in experiment 2 divided longitudinally into 4 equal quadrants via lines drawn on the bottom and walls of the tank. To generate the gradient of fish stimulus, we placed dropping systems 1 cm away from the wall at both ends of the aquarium. In each test we placed one dropping system containing fish conditioned water at one end, and another containing dechlorinated tap water at the other end of the aquarium. Each dropping system consisted of a 50 ml buret regulated to release 1 drop/second. The buret was

placed just above the water surface to reduce the disturbance caused by the drop. To determine the spatial distribution of the tadpoles we scored the number of larvae in each quadrant at 1 min intervals for 10 min. After each test we changed the position of the aquarium or swapped the end corresponding to fish stimulus water with the control stimulus to avoid positional bias. The spatial affinity index *S* (Fishwild *et al.*, 1990) was adapted to estimate the mean quadrant position of the group of tadpoles, and is calculated as follows:

$$S = \frac{1(A) + 2(B) + 3(C) + 4(D)}{N}$$

where A, B, C and D represent the total number of tadpoles scored in each of the four numbered quadrants during a 10 min test and N represents the total number of tadpoles scored in all quadrants. This index ranged from four (maximum avoidance to fish) to one (maximum affinity to fish). Values higher or lower than 2.5 indicate spatial affinity, whereas values around 2.5 indicate no preference for either stimulus. This kind of index is recommended because it includes information from all four quadrants in the aquarium, providing a finer estimate of the mean position of the test tadpoles (Fishwild *et al.*, 1990).

**Statistical Analysis.**— For experiment 1, we calculated the mean number of movements during each test as the response variable. In experiment 2, we calculated the average number of tadpoles inside the refuges during each test. Normality of the data for the three experiments was confirmed with Kolmogorov-Smirnov tests. Since data for the control and fish conditioned water treatments from the same group of tadpoles were not independent, we determined the significance of the differences between these treatments by using paired *t*-tests.

To analyse the spatial distribution data from experiment 3, we compared the data to the null value of 2.5 by means of a one-sample *t* test.

## RESULTS

Tadpoles showed a mean decrease in activity level of 27% in the fish conditioned water treatment ( $57.8 \pm 15.4$ ) relative to the control ( $81.3 \pm 18.7$ ) (paired *t*-test,  $n = 10$ ,  $t = 3.64$ ,  $P < 0.01$ ). The activity in the fish stimulus treatment decreased by  $23.5 \pm 20.44$  movements relative to the control (Figure 1A).

Tadpoles used refuges significantly more often in the presence of a fish stimulus ( $2.33 \pm 1.20$  sheltered individuals) than in control water ( $0.45 \pm 0.27$  sheltered individuals) (paired *t*-test,  $n = 10$ ,  $t = -4.84$ ,  $P < 0.001$ ). On average, for each group of tadpoles, the fish conditioned treatment showed an increase of  $1.88 \pm 1.23$  sheltered individuals (Figure 1B). Although this difference was small in relation to the number of tadpoles, it represents a five-fold increase in the number of sheltered individuals.

Tadpoles did not show spatial avoidance from the aquarium side with chemical fish stimulus ( $2.53 \pm 0.37$ ; one sample *t*-test,  $n = 10$ ,  $t = 0.15$ ,  $P = 0.8830$ ) (Figure 1C). In six groups the spatial affinity index was greater than 2.5, and in the remaining four groups it was lower than 2.5.

## DISCUSSION

Our results show that *O. americanus* tadpoles respond behaviourally to a chemical fish stimulus by changing activity level and microhabitat use. Since tadpoles used in the experiments had no previous exposure to fish stimuli, the antipredator behaviour observed is presumed to be a genetically determined

trait, and thus an evolutionary mechanism in response to predation risk. Some studies have shown that tadpoles raised from eggs in the laboratory were able to respond behaviourally to fish predators (Kats *et al.*, 1988) and to discriminate between predatory and non-predatory fishes (Pearl *et al.*, 2003). Although our data strongly suggest that *O. americanus* tadpoles are able to respond to unfamiliar predators (since tadpoles were obtained from a fishless pond) the potential instinctive nature of this response should be further examined by the collection of fresh eggs in the field and subsequent raising of the tadpoles in a neutral laboratory environment.

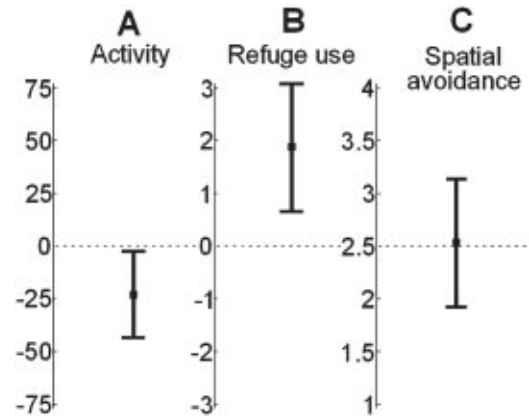
The use of chemical cues to detect predators in *O. americanus* could allow this species to respond behaviourally to fish presence in the turbid and highly vegetated habitats in the Paraná River floodplain. Chemical stimuli may provide aquatic animals with critical information, especially at night, in turbid or in highly vegetated habitats where visual information is not available. This could be essential for detecting cryptic ambush predators (Kiesecker *et al.*, 1996).

Reduction in activity level in larval amphibians has been suggested to be related with decreased predation risk (Skelly, 1994; Schemidt and Amézquita, 2001). This diminution in predation risk can be attributed to a lower encounter rate with predators (Abrams, 1984) and, in cryptic prey, to a reduction in the rates of detection and attack (Skelly, 1994). Fishes are generally thought to preferentially attack active prey, detected either visually or via the lateral line (Bleckman, 1993; Fitzgerald and Wootton, 1993). By lowering activity, *O. americanus* tadpoles could reduce encounter rate with fishes. Also, due to their cryptic coloration, they might reduce the rates of detection and attack, thus reducing mortality risk.

Several studies have documented a change in microhabitat use by larval amphibians in response to predators

(Petranka *et al.*, 1987, Kats *et al.*, 1988, Pearl *et al.*, 2003). The kind of microhabitats available can strongly influence tadpoles' susceptibility to fish predation (Hews, 1995). Hero *et al.* (2001) suggested that the use of a refuge (*e. g.* leaf litter) could be the only defense against predators by palatable species that frequently coexist with fishes. In laboratory aquaria, *A. fasciatus* preyed on tadpoles of *O. americanus* (unpublished data), suggesting that these tadpoles are palatable at least to *A. fasciatus*. In this case, the change in refuge use by tadpoles could reduce their exposure to predators, thus diminishing predation risk.

The lack of spatial avoidance in response to the chemical stimulus indicates that tadpoles do not respond to the gradient generated in the laboratory. Presumably, in the habitats where *O. americanus* occur, chemical gradients



**Figure 1.** Mean responses ( $\pm 1$  SD) of tadpoles in each of the three behavioural tests. (A-B) Mean differences between the control and stimulus treatments in activity level and refuge use respectively. Negative differences indicate a reduction in the variable in the fish stimulus treatment in relation to the control, and positive differences indicate an increment in the values of the variable. The null value of 0 (dashed line) indicates no differences between control and stimulus treatment. (C) Mean values of the spatial distribution index. Dashed line represents the null value of 2.5. Variables used in each behavioural test are described in the text.



are either not a good indicator of the position of a fast moving predator such as *A. fasciatus*, or simply these gradients are never generated. In laboratory observations, tadpoles remain inactive in the bottom of the aquarium until a fish predator is close enough to make a strike. We observed that when this happens, tadpoles flee rapidly to another position. Possibly, at short distances, the combination of mechanical and visual stimuli is a much more efficient indicator of the position of fish predators to trigger spatial avoidance behaviour.

Hickman *et al.* (2004) observed that *Eurycea multiplicata griseogaster* responded to banded sculpins, *Cottus carolinae*, in the field by showing flight and burrowing behaviour. However these responses were not observed in the laboratory. They suggested that these responses were not practical in simplified laboratory chambers. Although we did not perform experiments in the field, we observed that in natural habitats, *O. americanus* tadpoles flee away and take refuge in the leaf litter or in rooted vegetation in response to any physical disturbance like vibrations and sudden changes in light intensity. Consequently, although we did not observe a spatial avoidance response in the laboratory, tadpoles could exhibit this antipredator behaviour in natural habitats, where spatial and refuge possibilities make this response more practical.

The geographic range of *O. americanus* reaches western regions of Argentina where *A. fasciatus* and most of the fishes found in the floodplains of the Paraná River are absent. In these environments tadpoles rarely encounter fish, and the biotic and abiotic characteristics (*e. g.* turbidity, vegetation) of the habitats are very different. Comparisons of the antipredator responses between populations that vary in their encounter rates with fish may reveal interesting patterns of local adaptation to the selective pressures in the river floodplains.

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