PRIMARY RESEARCH PAPER

Behavioral responses of benthic and nektonic tadpoles to the presence of a benthic predator

Yasmim Caroline Mossioli de Souza [ORCID] · **Fabiane Santana Annibale** [ORCID] · **Rodolfo Mei Pelinson** [ORCID] · **Denise de Cerqueira Rossa-Feres** [ORCID]

Abstract We experimentally tested whether the presence of a free benthic predator (Odonata naiads) alters the displacement time, the position occupied in the water column, and the proportion of food consumed by benthic and nektonic tadpoles. The presence of predators did not influence the displacement time or the proportion of food consumed by any of the two species. In the presence of predators, benthic tadpoles avoided the benthic microhabitat, increasing their time in the middle of the water column. This behavior was unexpected since the previous studies indicate that the morphology of benthic tadpoles restricts them to the bottom of water bodies. We, thus, hypothesize that such a drastic behavior change was a consequence of the real risk of predation to which the tadpoles were exposed. Our results are in accordance with the threat-sensitivity hypothesis, in which prey behave flexibly when exposed to different degrees of predation threats. Nektonic tadpoles, however, slightly increased their permanence in the water column in the presence of the same benthic predators. Therefore, we provide support for the hypothesis that predators induce greater behavioral changes in prey that exhibit patterns of microhabitat use similar to theirs.

Handling editor: Gary Bucciarelli

Y. C. M. de Souza (✉)
Graduate Program in Biodiversity, São Paulo State University – UNESP, São José do Rio Preto, SP 15054-000, Brazil
e-mail: mossioli.souza@unesp.br

F. S. Annibale
Universidade Virtual do Estado de São Paulo – UNIVESP, Bady Bassit, SP 15115-000, Brazil

R. M. Pelinson
Instituto de Biociências, Letras e Ciências Exatas (IBILCE), São Paulo State University – UNESP, São José do Rio Preto, SP 15054-000, Brazil

D. d. C. Rossa-Feres
Department of Biological Sciences, São Paulo State University – UNESP, São José do Rio Preto, SP 15054-000, Brazil

Keywords Feeding behavior · Indirect interactions · Induced defense · Occupied depth · Trade-off

Introduction

Tadpoles are surrounded by predators from both outside (e.g., spiders and birds; Jara, 2008; Martins et al., 2021) and inside waterbodies (e.g., Odonata naiads and fish; Mettler et al., 2021; Iglesias-Carrasco et al., 2022). The distribution of tadpoles and their predators in the aquatic environment is affected by several factors, including luminosity, temperature, oxygen levels, vegetation cover, depth, type of substrate,

hydroperiod, as well as quality and availability of nutrients (Beiswenger, 1977; Noland & Ultsch, 1981; Collins & McIntyre, 2015; de Melo et al., 2018; Kloh et al., 2023). Thus, microhabitats have attributes that can favor the occurrence of both prey and predators. Predator–prey interactions lead to adaptations of both predators and prey, as the former improve their capture ability, and the latter develop anti-predatory strategies (e.g., Van Buskirk, 2001; Teplitsky et al., 2005). Some prey responses are behavioral, for example, tadpoles typically use refuge (e.g., Babbitt & Tanner, 1998; Hartman & Lawler, 2014) and reduce foraging in the presence of predators (e.g., Rae & Murray, 2019; Mamede & Nomura, 2021). Others are morphological, such as changes in width and length of the tail and the body (e.g., Relyea et al., 2021; Sergio et al., 2021).

Organisms that live in the bottom of water bodies, such as benthic tadpoles (Altig & Johnston, 1989; McDiarmid & Altig, 1999), respond to the presence of predators usually with immobility or reduced swimming activity (e.g., Azevedo-Ramos et al., 1992; Schalk, 2016). At midwater, these organisms swim more actively, like nektonic tadpoles (Altig & Johnston, 1989; McDiarmid & Altig, 1999), and use aquatic vegetation as a refuge against predators, which increases their chances to survive (e.g., Kopp et al., 2006). Thus, predator–prey interactions can lead to important behavioral adjustments for both preys and predators. However, species less likely to interact, such as those that inhabit different microhabitats, may have a weak influence on each other's behavior.

In this study, we tested the hypothesis that predators have a greater impact on the behavior of prey species that inhabit the same microhabitats as their predators, compared to prey species that occupy different microhabitats. More specifically, we tested how a free benthic predator (Odonata naiads) altered the displacement time, the position occupied in the water column, and the proportion of food consumed by benthic and nektonic tadpoles. Because a previous study has found that benthic tadpoles tend to remain at the bottom of water bodies regardless of the presence of benthic predators (de Souza et al., 2022), we predicted that these tadpoles would reduce their activity rates as a strategy to avoid predation and, hence, reduce their food consumption. Nektonic tadpoles, on the other hand, can avoid these predators by foraging

at different water depths (Altig & Johnston, 1989; McDiarmid & Altig, 1999), so we predicted they would be less affected by the presence of the benthic predator.

Materials and methods

Species, collection, and acclimation

We selected tadpoles of two species, one benthic, *Physalaemus nattereri* (Steindachner, 1863), and one nektonic, *Scinax fuscovarius* (Lutz, 1925), both common and abundant at the study region (e.g., Vasconcelos et al., 2011). Benthic tadpoles of *P. nattereri* present a globular body, low fins, small and dorsal eyes (Rossa-Feres & Nomura, 2006), and occur at the bottom, near, or among the vegetation of shallow water bodies (Schulze et al., 2015). Nektonic tadpoles of *S. fuscovarius* present a compressed body, high fins, a flagellum at the tip of the tail, and big, lateral eyes (Rossa-Feres & Nomura, 2006). They occur, generally, at midwater of deep water bodies (60 cm of depth), usually next to or among vegetation (Schulze et al., 2015). Despite differences in morphology and habit, these species have a similar diet, composed mainly of algae. However, *P. nattereri* tadpoles also consume Ciliophora protozoa and Acari (Rossa-Feres et al., 2004).

We also selected Odonata naiads, from species of *Micrathyria* Kirby, 1889, as they are efficient predators of tadpoles (e.g., Gascon, 1989; Arribas et al., 2018). They usually remain inactive, wait until the prey approaches, and execute a quick blow (Pritchard, 1965). This behavior can be considered as a "sit and wait" foraging strategy (Wellborn et al., 1996).

Tadpoles and Odonata naiads were collected with wire mesh (32 cm diameter; 3 mm mesh size) on February 25, 2019, and March 11, 2019, in two temporary ponds located in a pasture (pond 1: 20° 50′ 48.7″ S, 49° 28′ 27.4″ W and pond 2: 20° 50′ 50.3″ S, 49° 28′ 29.5″ W) in the city of Mirassol, southeast of Brazil. Tadpoles and Odonata naiads were separately transported in plastic bags containing water from the ponds where they were collected and accommodated in isothermal expanded polystyrene boxes to avoid overheating.

Collections were performed with authorization from the Institute of the Environment and Renewable

Natural Resources (IBAMA) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio)—System of Authorization and Information on Biodiversity (SISBio) license number: 18163-1 to D.C.R.-F. Maintenance of tadpoles in the laboratory and the experimental design were approved by the Ethics Committee on the Use of Animals—CEUA (no 187/2018), in accordance with the National Animal Experimentation Control Council (CONCEA).

Animals were acclimatized in the laboratory for 2–5 days before the beginning of the experiment. During this period, tadpoles and Odonata naiads were kept in polyethylene aquaria filled with constantly aerated dechlorinated water. The experiment was conducted in a controlled photoperiod (12 h of light and 12 h of darkness) and air temperature (28 °C–30 °C), which kept the water temperature around 26 °C, simulating the natural condition of the ponds in which the animals were collected. Tadpoles were fed ad libitum with a commercial food based on algae and krill (Sera Micron®), and Odonata naiads were fed with tadpoles that would not be used in the experiments.

Prey sample sizes were as follows: *P. nattereri* ($n=40$) and *S. fuscovarius* ($n=40$), and for the predator *Microthyria* sp. ($n=100$). We selected tadpoles and Odonata naiads with similar sizes (*P. nattereri*: 19.36 ± 2.86 mm; *S. fuscovarius*: 24.73 ± 2.30 mm; and *Microthyria* sp.: 17.63 ± 2.30 mm; data presented as means $\pm$ SD). Odonata naiads had adequate size to predate tadpoles, as evidenced in preliminary tests (YCMS, personal observation). We also selected tadpoles in developmental stages 26–33 (Gosner, 1960), in which the major changes in tadpole morphology are related to body growth, not to developmental changes (McDiarmid & Altig, 1999).
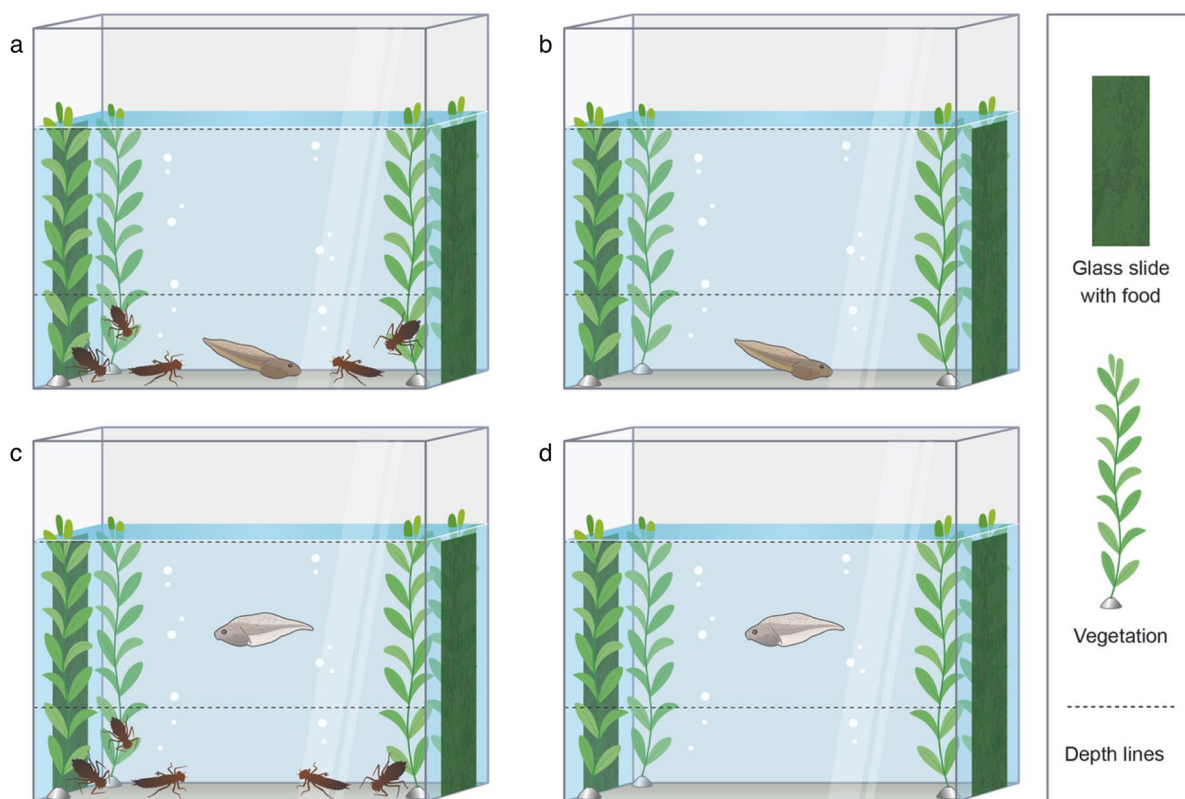
Experimental design

We performed the experiments on February 28, 2019, and March 3, 2019, with *S. fuscovarius* tadpoles, and on March 14, 2019, and March 17, 2019, with *P. nattereri*. We used glass aquaria (30 cm × 20 cm × 30 cm, $l \times w \times d$) filled with 12 L of dechlorinated water. We covered three faces of each aquarium with a blue plastic sticker to reduce possible stress that other colors could cause and to avoid visual contact among individuals (Maia & Volpato, 2013). The uncovered face of the aquaria (30 cm × 30 cm) was marked with two horizontal dashed lines to delimit water depths:

bottom (15–10 cm of depth) and midwater (10–0 cm of depth); the depth was measured starting from top to bottom. These dashed lines facilitated the determination of tadpoles' position along the water column. To test the influence of predator on the foraging behavior of tadpoles, we prepared a mixture of water and the same commercial food used during tadpoles' acclimatization (Sera Micron®) at a concentration of 100 mg mL^{-1} (e.g., Venesky et al., 2013). We brushed 18 mL of this solution on the upper part of the glass slides (29 cm × 19 cm), making a uniform layer of food over an area of 20 cm × 5 cm of the slide (Fig. 1). We placed the slides on laboratory benches and waited for 24 h to use them. This period was long enough for the mixture to dry naturally and become firmly adhered to the slide surface.

The treatment corresponded to an aquarium with five free predators, one tadpole, either of *P. nattereri* or *S. fuscovarius*, two slides with food (one at each extreme of the aquaria), and four artificial plants. The plants were included to simulate aquatic vegetation that nektonic tadpoles of *S. fuscovarius* use in natural microhabitats (e.g., Schulze et al., 2015). The control had the same items, except for the predators (Fig. 2). We utilized free predators to create more realistic ecological dynamics for the experiment (e.g., Underwood, 2009). Predators have specific behaviors when hunting prey, and, by being free, they can exhibit these behaviors, making predator–prey interaction more realistic (e.g., Lima, 2002). Each trial was replicated 20 times per species. Thus, in total, there were 20 control and 20 treatment trials for each species.
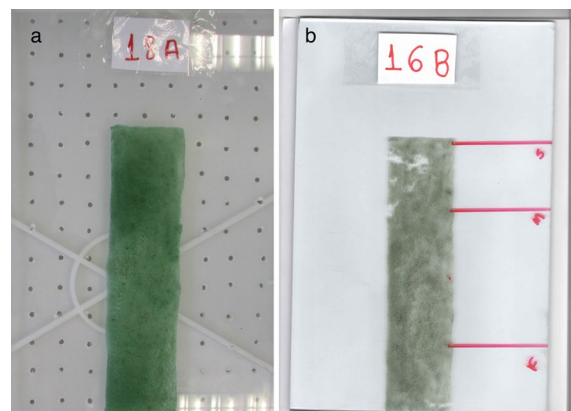
Fig. 1 Glass slide with food (**a**) and glass slide after the food were consumed by *S. fuscovarius* tadpole (**b**)

Fig. 2 Simplified experimental design. Treatments corresponded to an aquarium with five predators and a benthic (**a**) or a nektonic tadpole (**c**). Controls corresponded to an aquarium without predators and a benthic (**b**) or a nektonic tadpole (**d**)

The experiment started with the addition of a focal tadpole in each aquarium. We waited for 5 min to start observing the tadpoles' behavior and to minimize potential stressful behaviors that could result from handling the individuals. Observations were performed between 10:00 and 11:00 h, the period of the day when tadpoles were determined to be more active (YCMS, personal observation). The time to observe tadpole behavior was established with the aim of preventing tadpoles acclimating to the predators' presence or degradation of chemical cues in water (e.g., Peacor, 2006; Ferrari et al., 2007). Both situations could reduce the accuracy of predation risk assessment by the tadpoles and influence their behavioral response. Each aquarium was individually observed for 180 s, always by the same person (YCMS) positioned 80 cm away. At this distance, neither predators nor tadpoles exhibited stress signals, such as agitation, which indicated that the observer's presence did not influence their behavior. The observer was sitting in a chair positioned in front of the aquarium. The height and distance of the observer to the aquarium were the same to ensure that the observation was performed in a standardized way. The experiment ran for a total of 3 h after the observation period to quantify food consumption. Then, the slides with food were removed and left to dry naturally for 24 h for further analyses of food consumption. Due to logistical reasons, only 10 treatment units and 10 control units were observed per day for each species. Thus, the experiment had a total duration of 4 days, two for tadpoles of *S. fuscovarius* and two for tadpoles of *P. nattereri*. Tadpoles and Odonata naiads were used only once for each trial, and the observation order of control and treatment groups was randomized.

After the experiment, tadpoles were euthanized by immersion in 10% lidocaine anesthetic solution and preserved in solution of 1:1 alcohol 70% and formaldehyde 15%. Odonata naiads were conserved in alcohol 70%. We deposited the preserved specimens

at the Amphibians Scientific Collection (DZSJRP Amphibia-Tadpoles) from the Department of Zoology and Botany of UNESP, campus of São José do Rio Preto, Brazil (DZSJRP 0003.01, 0361.01, 0003.INV, and 0361.INV).

Data analysis

To evaluate predator influence on the displacement time of the tadpole (Supplementary Material, Table 1), we quantified the time in seconds, considering only whole numbers, that the tadpole swam during the observation period (180 s). We used a chronometer to pause the time when tadpoles stopped displacing. As such, we considered the displacement time of each tadpole as the sum of all moments that tadpoles displaced during the observation period.

To evaluate predator influence in the position occupied in the water column by the tadpole (Supplementary Material, Table 1), we noted the depth occupied by the tadpole in the water column during the observation period (180 s). However, the tadpoles did not change their initial depth along the water column during the observation period. Therefore, we considered this a binary variable: bottom or column.

To evaluate predator influence on the proportion of food consumed by the tadpoles (Supplementary Material, Table 1), we quantified the percentage of food removed from the glass slides offered to tadpoles. For this, we removed the slides after 3 h of the experiment and left them on laboratory benches to dry naturally. Then, the slides were digitized (HP LaserJet M1132) and analyzed using the Particle Analysis tool from ImageJ® software (Schneider et al., 2012). This tool counts pixels in a selected area and provides the proportion of particles in the image, or, in our case, the proportion of food removed from the slides by each tadpole (e.g., de Souza et al., 2022).

Statistical analysis

To test whether predators differentially influenced the behavior of benthic and nektonic tadpoles, we used generalized linear models with an adequate probability distribution for each response variable (Zuur et al., 2009). In all analyses, we included the day that the tadpoles were submitted to the experiment as an explanatory variable. Because displacement time was measured as the number of seconds (i.e., a discrete variable),

we modeled it using the negative binomial distribution. The position occupied in the water column and the proportion of food consumed were modeled using the binomial and beta distributions, respectively. We assessed the fit of our models by visual inspection of randomized quantile residuals (Dunn & Smyth, 1996). We, then, tested for the effect of the day when the trials were conducted and presence of predators through likelihood ratio tests (Zuur et al., 2009). All models were fitted using the glmmTMB function from the "glmmTMB" package (Brooks et al., 2017) in the R software (R Core Team, 2022).

Results

Predators remained mostly at the bottom of the water column (Supplementary Material, Fig. 1). Four *P. nattereri* tadpoles were consumed by predators during the experiment (Supplementary Material, Video 1). *Scinax fuscovarius* tadpoles used the vegetation to support themselves, attaching the oral disk to the tip of the leaves (Supplementary Material, Fig. 2). They also consumed food along the water column (Supplementary Material, Fig. 3a and 3b).

For both species, the presence of predators did not influence the displacement time of tadpoles, but the day when trials were conducted did (*P. nattereri*, day 1: 21.18 ± 5.50 s and day 2: 0.00 ± 0.00 s; Table 1 and Fig. 3a; *S. fuscovarius*, day 1: 22.90 ± 40.07 s and day 2: 0.00 ± 0.00 s; Table 1 and Fig. 3b). Also, predators led to some changes in tadpoles' spatial distribution. When predators were present, 18% of *P. nattereri* tadpoles occupied the water column whereas none moved away from the bottom when predators were absent (Table 1 and Fig. 3c). For *S. fuscovarius*, 86% of the tadpoles occupied the water column when predators were present, whereas in the absence of predators, only 60% did (Table 1 and Fig. 3d).

Unexpectedly, none of the benthic tadpoles consumed food during the whole experiment. The proportion of food consumed by *S. fuscovarius* was similar in the presence and absence of predators (Table 1 and Fig. 4).

Discussion

We corroborated our hypothesis that prey with similar habits to their predators would present greater

Table 1 Likelihood ratio tests for the effect of the experimental day (i.e., temporal blocks), presence of predators, and their interaction for *P. nattereri* and *S. fuscovarius* separately

|	*P. nattereri*			*S. fuscovarius*		
	DF	X^2	P	DF	X^2	P
Displacement time						
Day	**1**	**6.807**	**0.009**	**1**	**16.655**	**< 0.001**
Predator	1	0.163	0.687	1	0.232	0.630
Interaction	1	0.000	1.000	1	1.000	1.000
Proportion of tadpoles in the water column						
Day	1	0.166	0.684	1	1.238	0.266
Predator	**1**	**5.117**	**0.024**	1	3.324	0.068
Interaction	1	0.000	1.000	1	2.613	0.106
Proportion of food consumed						
Day				1	1.365	0.243
Predator				1	0.018	0.894
Interaction				1	0.184	0.668

DF degrees of freedom

Significant effects are highlighted in bold type
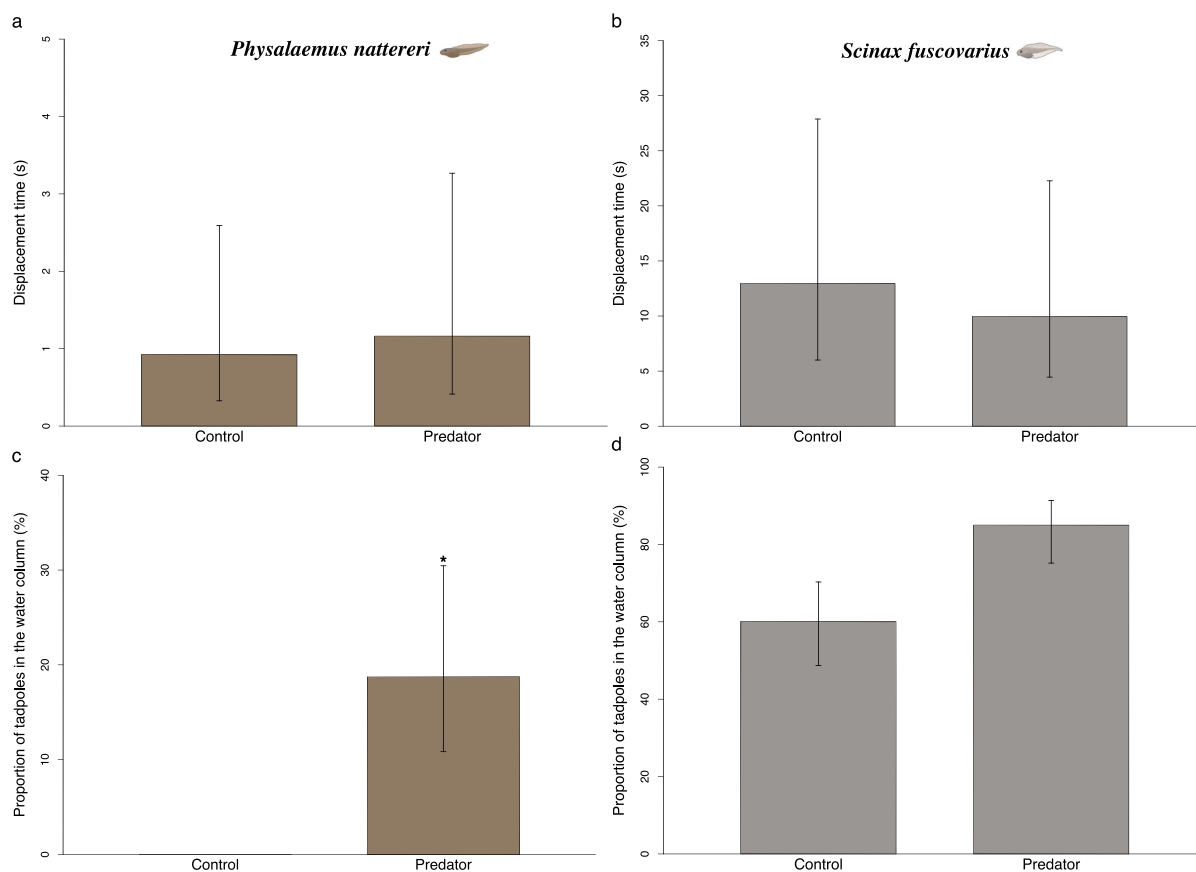
Fig. 3 Mean displacement time of **a** *P. nattereri* and **b** *S. fuscovarius* tadpoles in response to the presence or absence of predators between experimental treatments. Mean proportion of tadpoles in the water column for **c** *P. nattereri* and **d** *S. fuscovarius* in response to the presence or absence of preda- tors between experimental treatments. Error bars are standard errors. Mean values and standard errors were estimated from generalized linear models. Significant treatment highlighted with an asterisk
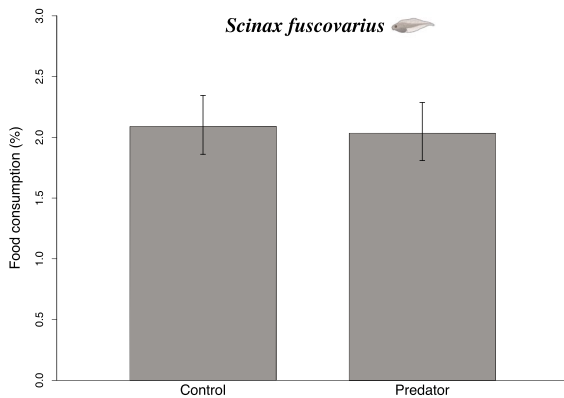
Fig. 4 Mean proportion of food consumed by *S. fuscovarius* tadpoles in response to the presence or absence of predators between experimental treatments. Error bars are standard errors. Mean values and standard errors were estimated from generalized linear models

behavioral changes than those with different habits. We observed that the presence of benthic predators significantly increased the use of the water column by benthic *P. nattereri* tadpoles, which is unusual for this species, whereas nektonic tadpoles of *S. fuscovarius* only marginally increased their use of the water column. However, the presence of predators did not affect the activity levels of tadpoles of either species, nor affect food consumption of *S. fuscovarius* tadpoles. *P. nattereri* tadpoles did not consume food during the experiment.

The presence of predators led tadpoles of *P. nattereri* to increase their permanence in the water column. This was unexpected considering their restrictive morphology (e.g., globular body and low fins; Altig & Johnston, 1989; McDiarmid & Altig, 1999) and physiology (e.g., negative buoyancy; Gee & Waldick, 1995; Tu et al., 1999). Furthermore, field observations show that benthic tadpoles tend to remain at the environment's bottom (e.g., do Prado et al., 2009; Schulze et al., 2015). As tadpoles of *P. nattereri* remained at the bottom in the absence of predators, their change in water column use can be understood as a predator avoidance strategy. Greatly differing from this result, when exposed to the same but caged predators (*Micrathyria* sp.), tadpoles of *P. nattereri* did not exhibit displacement and remained immobile (de Souza et al., 2022). In this study, the high number of predators and the real risk of predation may have represented stimuli for anti-predatory behavior,

forcing tadpoles to move to other depths of the water column. The different behaviors of *P. nattereri* when exposed to caged and free predators are in accordance with the threat-sensitivity hypothesis, in which prey behave flexibly toward different degrees of predator threats (e.g., Helfman, 1989; Bishop & Brown, 1992).

Several studies have shown that behavioral changes, such as the decrease in activity level, are a common anti-predatory response of benthic tadpoles (e.g., Ramamonjisoa et al., 2019; Scribano et al., 2020). Contrary to this expectation, in this study, the displacement time of *P. nattereri* tadpoles did not differ in the presence or absence of benthic predators. However, it is possible that the change in water column use by *P. nattereri* tadpoles helped them to avoid predators with no detriment to their activity levels, as suggested by the threat-sensitivity hypothesis.

Benthic tadpoles did not consume food either in treatment or in control groups. Swimming up and down to rasp food from substrate surfaces may represent an energetic challenge for tadpoles, as suggested by Annibale et al. (2019). Thus, the position that we chose to offer food for tadpoles may have constrained *P. nattereri*'s feeding behavior. Furthermore, we observed (DCRF and FSA) that *P. nattereri* tadpoles consume more food at nighttime, as such, it is possible that the period, when the trials were conducted, also affected tadpoles feeding behavior.

Corroborating our hypothesis, the presence of benthic predators did not affect the displacement time or the food consumption rates of nektonic tadpoles. The slight increase in the permanence of nektonic tadpoles in the water column when predators were present may reflect a weak influence of the benthic predator on behavioral aspects of the nektonic tadpoles. This is likely due to the low encounter rate between these organisms in microhabitats, since they have distinct habits (benthic vs nektonic) in natural conditions. This hypothesis is also in accordance with the threat-sensitivity hypothesis, in which prey behave flexibly toward different degrees of predator threats (e.g., Helfman, 1989; Bishop & Brown, 1992). As such, our study demonstrates empirically that predation pressure is strong on prey with habits similar to those of their predators, but weak on those with different habits.

The literature about the predator–prey interaction is extensive, especially on morphological and behavioral changes induced by predators on tadpoles (e.g.,

McCollum & Van Buskirk, 1996; Relyea, 2001; Jara & Perotti, 2010; Gazzola et al., 2023). Even so, our knowledge of how such relationships influence species distributions within the same habitat is limited to inferential studies, and behavioral aspects of Neotropical tadpoles are still little explored (e.g., Rossa-Feres et al., 2015; Annibale et al., 2023). Thus, our study contributes to a better comprehension of how the coexistence between predators and prey influences species' spatial distribution. For example, tadpoles that are restricted to microhabitats where their morphology does not favor better locomotor performance, such as benthic tadpoles of *P. nattereri* that migrate to the water column to avoid predators, may be less capable of exploring available food sources or may not have access to the best food sources. Therefore, it is possible that these individuals reach metamorphosis with smaller sizes or experience slower development. Moreover, the behavioral repertoire of *P. nattereri* tadpoles highlights the limited knowledge about how these tadpoles assess the risk of predation, indicating a need for further experimental studies.

Data availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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