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The use of ephemeral reproductive sites by the explosive breeding toad *Melanophryniscus rubriventris* (Anura: Bufonidae): is it a predator cue mediated behavior?

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Amphibians detect quality signals when selecting reproduction sites. We hypothesize that *Melanophryniscus rubriventris*, an explosive breeding toad that reproduces in small, ephemeral water bodies, is able to select sites without predators. We performed a field experiment simulating oviposition sites, two with predators (tadpoles and bugs) and one control. Contrary to our expectations, we obtained no differences in the number of eggs deposited. We also performed an experiment to test the capability of *M. rubriventris* tadpoles to detect potential predators. Tadpoles could not detect predators, as other species did. *Melanophryniscus rubriventris* is selecting spawning sites following other signals, not predation risk. Identifying selection cues is crucial to protect species that depend on threatened habitats.

Keywords: Bufonid; fresh water; Yungas forests; breeding site; predation

Introduction

Selecting a suitable oviposition site might be crucial for oviparous species without parental care behavior (Refsnider & Janzen 2010). A correct selection could greatly increase offspring survival and consequently the fitness of the individuals (Stearns 1992). Among the huge diversity of reproductive strategies in amphibians, it is possible to find the same diversity of habitat types chosen by the adults to lay eggs, and thus to develop their exotrophic larvae (Haddad & Prado 2005; Wells 2007). As in other taxa with complex life cycles, the existing evidence suggests that adult amphibians are able to select certain oviposition sites so as to minimize risks to their eggs and larvae (Resetarits & Wilbur 1989; Blaustein et al. 2004). Thus, adults of some species can avoid laying eggs at certain sites that may expose their offspring to different risks, from both biotic (e.g. predation, competition), and abiotic factors such as desiccation, temperature variations, salinity or UV radiation (Refsnider & Janzen 2010).

Amphibians breeding at lentic waterbodies are normally distributed across a hydrological gradient, ranging from highly ephemeral ponds to large permanent pools and shallow lakes. The evidence shows a humped pattern in amphibian larval richness, with the greatest number of species reproducing at the middle of this gradient, wherein there is an intermediate pressure from biotic and abiotic limiting factors, and a greater availability of favorable microhabitats for egg deposition and larval development (Wellborn et al. 1996; Babbitt et al. 2003). However, the limiting factors at the extremes of the hydrological gradient, and the signals detected by adults when selecting oviposition sites, are still insufficiently studied in most amphibian species (Refsnider & Janzen 2010). At permanent water bodies, a strong biotic factor (presence of fish predators) is frequently evoked for spawning-site selection by amphibians (Kats et al. 1988; Resetarits & Wilbur 1989; Rieger et al. 2004; Gunzburger & Travis 2005). There is a consensus about the role of predation pressure as the limiting factor at large permanent pools, and a strong pattern of increasing richness of amphibian larvae has been reported in water bodies where fish do not occur (Werner & McPeek 1994; Binckley & Resetarits 2002; Wisenden 2003; Peltzer & Lajmanovich 2004; Werner et al. 2007; Laufer et al. 2009).

In temporary ponds, amphibian larvae are able to coexist with macroinvertebrate predators and competing species through different strategies, mainly behavioral or developmental induced responses (Relyea 2001). In these breeding habitats, predation does not appear to be the strongest driver of oviposition-site choice; many species may choose sites based on a complex set of biotic and abiotic factors such as

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absence of competitors, cannibalism avoidance, microhabitat use, thermal conditions, and hydroperiod length (e.g. Halloy & Fiaño 2000; Murphy 2003; Peltzer & Lajmanovich 2004; Werner et al. 2007). The limitations at the other end of the gradient (e.g. small and ephemeral water bodies) have scarcely been explored (Beja & Alcazar 2003). While the absence of predators and competitors seems advantageous in these small ephemeral ponds, desiccation appeared as the main limiting factor that strongly determines spawning-site selection. Anyway, there are a few species where a positive outcome of the compromise between desiccation risk and predation avoidance was demonstrated (Wellborn et al. 1996; Spieler & Linsenmair 1997).

Melanophryniscus rubriventris (Vellard 1947) is an endemic species of the Andean Yungas forests of Argentina and Bolivia. This toad uses very small and shallow ephemeral water bodies to spawn (average area: 0.38 m^2, 3.78 cm depth; Goldberg et al. 2006). Furthermore, this anuran species displays an explosive reproduction associated with intense rainfall from October to February (Vaira 2005). This reproductive strategy could greatly reduce intraspecific interactions such as competition between cohorts or larval cannibalism (Petranka & Thomas 1995). Goldberg et al. (2006) performed an intensive field study in order to evaluate the oviposition-site selection for *M. rubriventris* in northwestern Argentina. These authors explored the selection of 31 potential breeding sites in relation to ephemeral pond area, depth, connectivity, submerged vegetation cover, daily temperature variation, depth variation, drying rate and presence of conspecific larvae. They were able to determine that breeding pairs choose spawning sites based on physical conditions, particularly selecting overflowed warming ponds. However, in this field observational study the influence of aquatic predator presence could not be determined, due to their absence at the type of ponds selected by *M. rubriventris* for reproduction.

In order to test if reproductive adults of *M. rubriventris* were able to detect and thus avoid water bodies with predators, we performed a field experiment including predators in artificial pools at a reproduction site in northwestern Argentina. We considered that *M. rubriventris* may select ephemeral ponds as a refuge, in order to avoid predation of their eggs and larvae. We therefore explored whether *M. rubriventris* choose to spawn in ephemeral ponds with presence of predators. We would expect that adults would avoid laying eggs if the ephemeral ponds presented predators. If adults always choose predator-free spawning sites, it would be expected that tadpoles would not show mechanisms of predator avoidance.

Two predictions emerge from our hypothesis: adults will only use predator-free ponds to spawn and consequently larvae will not develop mechanisms to recognize chemical cues from potential predators.

Materials and methods

Experimental design

The study was conducted in two stages. The first stage was a field experiment to test spawning-site selection by adults of *M. rubriventris* carried out at Angosto de Jaire (24°01′8.7" S; 65°23′28.1" W, 1703 m asl), Jujuy province, northwestern Argentina. We worked with a native local population of *M. rubriventris*, which was being monitored over 10 years to register occurrence of reproduction (Bonansea & Vaira 2012). The experiment was performed on 25 and 26 January 2012, after a heavy rain on the previous day (c.40 mm). Experimental units consisted of 48 plastic wading pools (20 cm diameter; 4.5 cm depth), which were buried and used to simulate *M. rubriventris* spawning sites. Due to their dimensions, the wading pools were considered a very realistic ephemeral pond model. The containers were filled with dechlorinated tap water to avoid possible inclusion of local predator cues with water from natural water sources.

The experiment had a factorial design with control ponds without predators and two treatment ponds (one with an invertebrate predator and another with a vertebrate predator). Both predators are native to the study area. We selected adult water bugs (*Belostoma* spp.), as they were used as efficient tadpole predators in other studies (e.g. Gunzburger & Travis 2005). We identified under laboratory conditions that *Pleurodema borellii* tadpoles actively prey on *M. rubriventris* eggs and early hatched tadpoles (GL and MV, personal observations); therefore we also used larvae of *P. borellii*, commonly found in temporary or permanent water bodies in the area, as the second tadpole predator.

Treatment ponds consisted of plastic containers where we randomly assigned two water bugs or two *P. borellii* larvae inside a predator cage (a finely perforated transparent plastic cup with lid). Predator density was considered enough given the small water volume, according to previous experiments showing that effective predator cues can be detected independently of their density (Rieger et al. 2004). Control ponds consisted of containers with an empty predator cage inside. Both treatment and control ponds were replicated 16 times. Due to problems during the experiment, such as sudden death of predators or a possible distortion of the absence of predator cues in control ponds because of water entrance from nearby
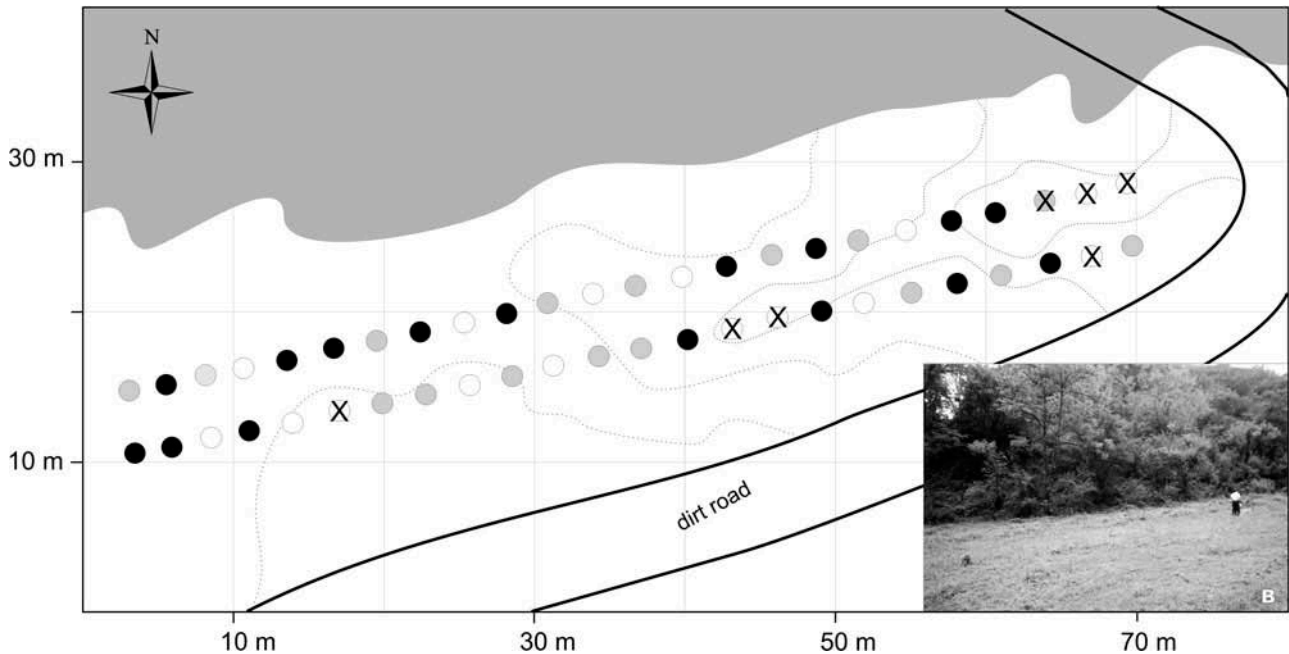
Figure 1. Spatial distribution of plastic wading pools buried to test spawning-site selection by adults of *Melanophryniscus rubriventris* at Angosto de Jaire (24°01′8.7″ S; 65°23′28.1″ W, 1703 m asl), Jujuy province, northwestern Argentina. Empty circles represent control ponds (without predators) and light grey and black circles the two treatment ponds (with water bugs and *Pleurodema borellii* tadpoles, respectively). Slashed circles were those discarded from the analysis. The dark grey region at the top of the map indicates hillside covered by forest behind the open marsh area. Dashed lines indicate level curves. The inset photo is a view of the breeding area from the dirt road.

overflowed ponds, some replicas were discarded and thus not considered for the statistical analysis. Finally, we obtained 16 replicas for *P. borellii* tadpole treatment, 15 for water bug treatment, and 10 for control ponds (Figure 1).

As *M. rubriventris* has repeated explosive reproduction events of three days length, coincident with episodes of heavy rains, the experiment was set a day before rainfalls were expected in the site. Plastic pools were buried in the experimental site on 24 January 2012, at 09.00 h, covering the edges properly with surrounding soil to allow the access of breeding pairs. Then they were filled with water and treatments and controls (with a predator cage or an empty cage) were assigned randomly. Considering that mating of the species is diurnal, occurring mostly during daylight (Vaira 2005), we left the breeding site and returned to record egg deposition the next morning. We recorded the total number of eggs deposited in each container. Subsequently, egg masses were removed and containers were refilled with water. We repeated the same sequence on the second day.

The second stage of the experiments to test predator avoidance by *M. rubriventris* tadpoles took place in the laboratory. Egg masses were collected at a different but nearby locality (Río Los Paños, Jujuy, northwestern Argentina, 24°18′12.34″ S, 65°24′58.10″

W) and kept in plastic containers filled with dechlorinated tap water. Up to one week after hatching, 18 larvae at Gosner's developmental stage 25 (Gosner 1960) were randomly selected to run the experiment. We used rectangular containers of 22 × 6.5 × 5 cm, with a predator cage inside located at one side of the container housing the same type of predators used in field experiments (two *P. borellii* tadpoles or two water bugs). The bases of rectangular containers were marked at the middle by a drawn line in order to visually identify a "predator sector" (where predator cages were set) and "no predator sector" (opposite to the predator sector). As control we used containers with empty predator cages. We then placed first the predators and control and one *M. rubriventris* tadpole per container and allowed to acclimate for 4 h before starting the experiment. All the replicate experiments were done simultaneously. For each of the 18 *M. rubriventris* tadpoles, we made eight sequential records spaced every 4 h registering if the tadpole stayed in the "predator sector" or in the opposite sector of the container. We measured predator avoidance as the number of times (out of eight observations) the tadpole was registered at the "no predator sector" of the container. Each treatment was replicated five times and to control for potential side preferences of the tadpoles, "predator" and "no

predator" sides were set reversed in the different containers. During the experiment both *M. rubriventris* tadpoles and predators did not receive any type of food. The same experimental procedure was performed with tadpoles of *Scinax fuscovarius* at Gosner's stage 25, obtained from an amplectant pair collected at a permanent water body at Rio Zapla, Jujuy province, northwestern Argentina (24° 15′13.35"S, 65°7′49.76" W).

Data analysis

Due to the non-normal distribution of the egg counts data from the field experiments and predator avoidance counts from the laboratory experiment, results were analyzed by non-parametric statistics. We tested the null hypothesis of no effect of presence of predators on the variations of total number of eggs deposited among the treatments ponds (with *P. borellii* tadpoles or water bugs inside) and control ponds. Because the variance of number of eggs deposited was not similar across treatments and control ponds, we analyzed the effect of predator presence on the number of deposited eggs using non-parametric Jonckheere-Terpstra tests (Pirie 1983). Number of eggs was expressed as mean ± 1 SD. We used generalized linear mixed models (GLMMs), with a binomial distribution and a logit-link function, to test associations between tadpole position (e.g. in "predator sector" or not) and the presence of *P. borellii* tadpoles or water bugs, or the absence of predator. These models allow the inclusion of random effects for repeated measures on the same subject; therefore we included the tadpoles as a random effect. This analysis was made for *M. rubriventris* and *S. fuscovarius* tadpoles independently. All statistical analyses were carried out in R software (R Development Core Team 2008) and differences were considered statistically significant for α ≤ 0.05 (Sokal & Rohlf 2011).

Results

Spawning-site selection experiments

On the first day of the experiment we recorded a total of 1634 eggs deposited in different pools. We found a mean of 45.8 ± 92.5 (range 0–371) eggs laid in the *P. borellii* treatment pools, 40.2 ± 61.2 (range 0–217) eggs in the water bugs treatment pools, and 33.8 ± 44.2 (range 0–142) eggs in the control pools. Consequently, we did not find statistically significant differences in the number of eggs deposited among pools (Jonckheere-Terpstra test statistic = 0.51, df = 2, p = 0.61), (Figure 2A). On the second day we recorded a total of 4356 eggs in the different

containers. We found a mean of 94.1 ± 150.7 (range 0–546) eggs laid in the *P. borellii* treatment pools, 135.7 ± 184.5 (range 0–652) eggs in the water bug treatment pools, and 81.6 ± 122.2 (range 0–317) eggs in the control pools. Again we did not find statistically significant differences (Jonckheere-Terpstra test statistic = 0.94, df = 2, p = 0.63) (Figure 2B).

Tadpole predator avoidance experiments

Tadpoles of *M. rubriventris* showed no significant differences in the number of times they were registered in "predator" or "no predator" sectors among the treatments (*P. borellii* or water bugs) and controls (GLMMs χ^2 = 1.41, p = 0.49) (Figure 3A).

Scinax fuscovarius tadpoles presented a significant difference between the frequencies of "predator" and "no predator" observations for the control and the treatments (GLMMs χ^2 = 8.90, p = 0.01) (Figure 3B). In both the *P. borellii* and water bug treatments, tadpoles were recorded twice as frequently in the "no predator" sector than in the other sector.

Discussion

Our results showed that adult *M. rubriventris* do not choose a spawning site based on cues of the presence of predators. While several studies suggest that most amphibians respond actively to predators' or conspecifics' cues (Refsnider & Janzen 2010), our observations suggest that adults of *M. rubriventris* do not show a mechanism of predator avoidance, indicating that other selective pressures, probably linked to physical characteristics related with pond size, hydroperiod length, water temperature, and pond connectivity (Goldberg et al. 2006), may be leading adults to select spawning sites. It might be that adults enhance eggs and larval survival by spawning immediately after early rainfalls in shallow, overflooded and warmer ponds. Also, selecting particular pond locations that ensure connectivity with other ponds could represent a safe site for the development of their larvae. Spawning in small and isolated ponds can cause the death of eggs and tadpoles either by desiccation or by flushing them out during heavy rainstorms.

Our findings also suggest that *M. rubriventris* tadpoles do not detect predators, as *S. fuscovarius* tadpoles did. If we take into account that ephemeral ponds usually do not present predators (Goldberg et al. 2006; Haad et al. 2011; Pereyra et al. 2011), we may assume that *M. rubriventris* tadpoles would not need to develop an adaptive mechanism to avoid predation, as they would seldom or never face this situation. However, since many of the induced
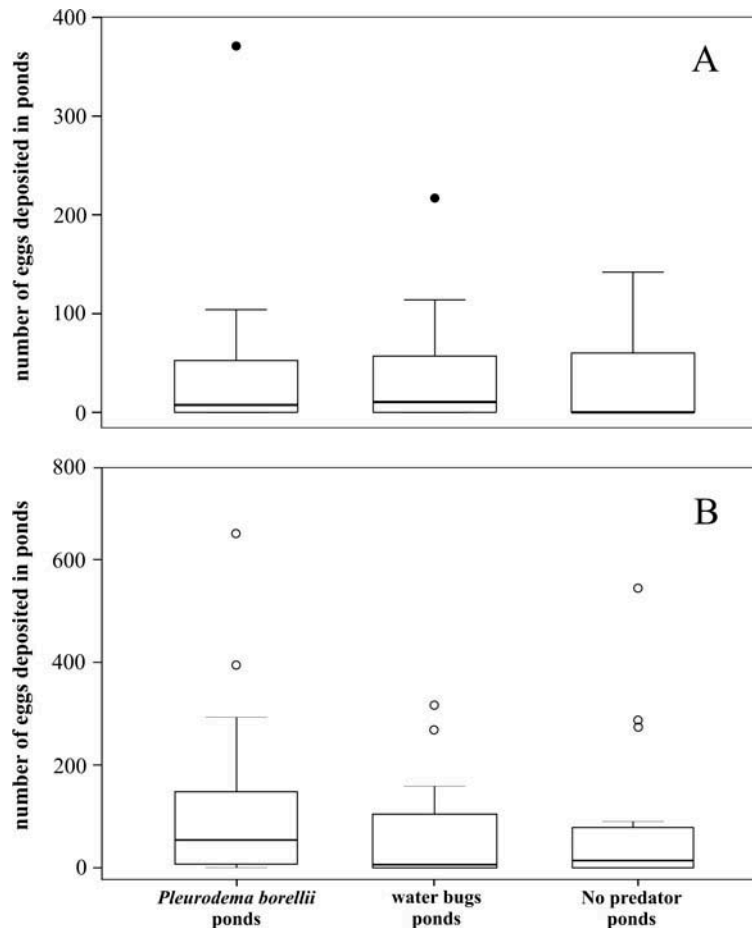
Figure 2. Number of eggs deposited by amplectant pairs of *Melanophryniscus rubriventris* in experimental ponds. Two treatment ponds with *Pleurodema borellii* tadpoles (*n* = 16 ponds) and water bugs (*n* = 15 ponds), and control ponds without predators (*n* = 10 ponds). Differences between treatments were not statistically significant. Data for day 1 (A) and day 2 (B) of the experiment were considered independent events. The bottom and top of the box are the 25th and 75th percentiles. The horizontal line represents the median. Whiskers are the highest still within 1.5 interquartile range (IQR) of the upper quartile. Outliers are represented by a black or white dot.

responses in anuran tadpoles are context dependent, caution should be taken in interpreting our results. It is known that responses of larvae are more pronounced when predators are fed with conspecifics (Laurila et al. 1997).

However, even in the absence of oviposition-site selection by adults to avoid larval predators, spawning sites may still be chosen to indirectly avoid egg or tadpole predation. Ephemeral ponds are very unpredictable, as water levels fluctuate almost daily (Goldberg et al. 2006), preventing potential invertebrate or vertebrate predators, that depend on the availability of standing water, to persist. Therefore, adults selecting such ephemeral ponds to spawn could be indirectly avoiding egg or tadpole predation. Considering that other species of the genus breed in larger temporary ponds, small streams and even

phytotelmata (Haad et al. 2011) it would be very valuable to test the existence of spawning-site selection and predator avoidance mechanisms in such *Melanophryniscus* species.

Our results, together with previous evidence (Goldberg et al. 2006), may indicate that spawning-site choice by the species could be more complex than simply selecting ephemeral ponds. It is likely that adults make a hierarchical selection by first choosing a main area for spawning based on the presence of ephemeral ponds, and then selecting a particular spawning site within that area based on the specific physical pond characteristics (e.g. overflowing water and temperature). By selecting properly, they can avoid ponds more prone to desiccation, and thus ensure suitable conditions for larval development and also minimize predation risks.
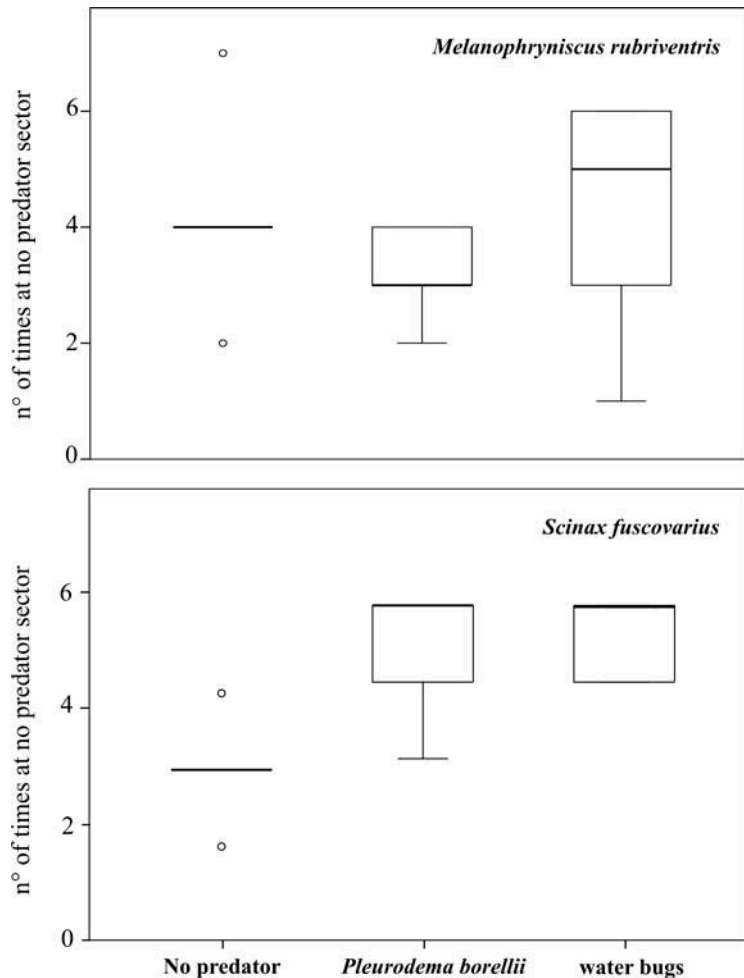
Figure 3. Number of times (out of eight observations) that *Melanophryniscus rubriventris* and *Scinax fuscovarius* tadpoles were registered at the "no predator sector" of containers under different experimental treatments (with *Pleurodema borellii* tadpoles, with water bugs and without predators). The bottom and top of the box are the 25th and 75th percentiles. The horizontal line represents the median. Whiskers are the highest still within 1.5 IQR of the upper quartile. Outliers are represented by a white dot.

Our study highlight avenues for future research into mechanisms controlling non-random choice of oviposition site, especially in the current context of rapid environmental change. Rising temperatures and decreasing precipitation can shorten hydroperiod lengths in breeding habitats (McMenamin et al. 2008), impeding anurans in the selection of ephemeral ponds to spawn, and forcing them to use temporary ponds where predation risks threaten population persistence.

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Disclosure statement

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