

Pond drying cues and their effects on growth and metamorphosis in a fast developing amphibian

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Abstract

The hydroperiod of breeding habitats imposes a strong selection on amphibians and pond-breeding species usually exhibit a high degree of plasticity in the duration of larval period. However, the potential for phenotypic plasticity in fast developing species was investigated only in a small number of anurans, and the specific response to environmental cues such as low water versus decreasing water level, as well as the effects of such cues on particular developmental stages, are even less understood. In this context, we investigated the plastic response to pond desiccation in a neotropical species (*Ceratophrys stolzmanni*) by raising tadpoles in three water level treatments: constant high, constant low and decreasing. The growth rates were the highest reported for amphibian tadpoles (up to 0.3g/day) and the time to metamorphosis was short in all treatments, with the fastest developing tadpole metamorphosing in only 16 days after egg deposition. Individuals from the constant high water level treatment had a higher growth rate than those in the other two treatments, whereas decreasing and constant low water levels had similar effects on development, speeding up metamorphosis. In turn, this involved a cost as these tadpoles had a lower body size and mass at metamorphosis than the ones raised in constant high water levels. The

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final stages of metamorphosis, when tadpoles are the most vulnerable, were shorter in tadpoles exposed to a decreasing water level, allowing them to leave water quickly. Our experiment demonstrates that phenotypic plasticity is maintained even in environments devoid of permanent aquatic habitats. Ceratophryid tadpoles are able to shorten their developmental time when they perceive a risk of desiccation and react similarly to cues coming from the two unfavorable water conditions showing their adaptation to ephemeral and unpredictable breeding habitats.

Keywords: amphibians, growth, life-history traits, metamorphosis, Pacific horned frog, phenotypic plasticity, pond drying, tadpoles

Introduction

A primary goal of life-history theory is to predict patterns in the age and size of individuals at ontogenetic switch points, such as hatching, metamorphosis and reproductive maturity (Stearns & Koella, 1986; Rowe & Ludwig, 1991). In amphibians, phenotypic plasticity is a ubiquitous characteristic affecting almost all life-history traits, especially those related to reproduction and larval development (Wells, 2010; Urban, Richardson & Freidenfelds, 2014). Optimal time of metamorphosis is determined by a trade-off between opportunities for growth and risks of mortality in aquatic and terrestrial habitats (Wilbur & Collins, 1973; Werner, 1986; Rudolf & Rödel, 2007). In most species, the time and size at metamorphosis are highly plastic and both depend on a multitude of factors such as water level (Michimae & Emura, 2012), larval density (Goater, 1994; Newman, 1998), predators (Orizaola *et al.*, 2013), temperature (Gómez-Mestre & Buchholz, 2006; Walsh, Downie & Monaghan, 2008), food availability (Morey & Reznick, 2000; Rudolf & Rödel, 2007), and the complex interaction between them (Grözinger *et al.*, 2014). Phenotypic plasticity is seen as particularly adaptive in unpredictable habitats where individuals die if they are unable to metamorphose before the pond dries (Newman, 1992; Padilla & Adolph, 1996). On the other hand, an accelerated development can result in mortality before metamorphosis, a pattern that was associated with an oxidative stress (Gómez-Mestre, Kulkarni & Buchholz, 2013) and, when correlated with the smaller body size at metamorphosis, can have a large impact on individual fitness afterwards (Morey & Reznick, 2000; Altwegg & Reyer, 2003).

Plasticity of life-history traits in response to desiccation is known from several taxonomic groups, and response to desiccation risk could depend on various factors, such as a decreasing water level or just permanently low water level, but these factors are rarely tested simultaneously (Denoël, 2003; Richter-Boix, Tejedo & Rezende, 2011; Charbonnier & Vonesh, 2015). In amphibians, previous studies have focused mainly on two types of environments: deserts, where the only available aquatic habitats are ephemeral and amphibians need to have extreme adaptations to pool desiccation risks, meaning short larval development time, or in more moderate environments, with a mosaic of temporary and permanent aquatic habitats, where a plastic response to desiccation should be favored by selection (Richter-Boix *et al.*, 2011). However, it was

suggested that, because of inherent physiological constraints, the two strategies do not occur together (Perotti, Jara & Úbeda, 2011), and species with extremely short larval duration should only be able to show a low degree of plasticity and reduced size at metamorphosis (Richter-Boix *et al.*, 2011).

In this context, we tested the extent of phenotypic plasticity to pond drying in a species living in an environment where permanent water bodies lack. We used as a model a frog from the family Ceratophryidae, inhabiting a xeric environment and belonging to a clade presumed to have a high larval growth rate combined with a short larval period, as a result of a diet shift towards cannibalism (Richter-Boix *et al.*, 2011). By manipulating two cues of pond drying, we aimed at determining if: (1) tadpoles express phenotypic plasticity in both growth and developmental times until metamorphosis, (2) cues from a decreasing water level treatment and those from a low water depth treatment have similar effects, (3) faster development is correlated with smaller size at metamorphosis, and (4) mortality rates vary among the different treatments. Also, because tadpoles are particularly vulnerable after their forelimbs emerge (Wassersug & Sperry, 1977), we were interested to determine if they were able to accelerate the exit to the terrestrial habitat in response to perceived desiccation risk, an effect rarely monitored in experiments (Walsh, 2010). We hypothesize that tadpoles should be able to adequately respond to desiccation risk and that both low water and decreasing water levels induce a faster development and metamorphosis at a cost of a smaller size at metamorphosis and higher mortality rates.

Materials and Methods

Study species and location

The Pacific horned frog *Ceratophrys stolzmanni* is a fossorial species endemic to the Tumbesian dry forest of Ecuador and Peru (Ortiz, Almeida-Reinoso & Coloma, 2013). The species is active only during the rainy season, spending the rest of the year burrowed underground (Angulo *et al.*, 2004). It is an explosive breeder; reproductive events being triggered by heavy rains. For egg-laying, females use temporary (i.e., lasting up to two months) and ephemeral (i.e., that can dry up in a few days) aquatic habitats (pers. obs.).

The study area is located in Arenillas Ecological Reserve, El Oro Province, south-western Ecuador (03°34'S; 80°08'E, 30 m a.s.l.), one of the last remnants of lowland tropical dry forests in the world and one of the few remaining habitats for the Pacific horned frog (Ortiz *et al.*, 2013). The annual precipitation average is 676 mm, but with large variations between years (between 65 and 2647 mm, Huaquillas meteorological station, 45 years records), and distributed almost exclusively during the four months of the wet season (January – April; 515 mm on average), while the dry season has at least 4 months with less than 10 mm monthly precipitation (Espinosa *et al.* 2016). Because of the extended dry season, there are no permanent water habitats, even longer-lasting ponds drying up in a few weeks.

Experimental setup

The experiment was conducted between 23 January and 16 February 2015, in a field laboratory (an abandoned military building, with large windows without glass, covered only with mosquito mesh). On 23 January, we collected a pair of *C. stolzmanni* from the reserve (300 m from the laboratory). Immediately after capture, we placed the pair in a large enclosure (80 x 50 cm) with 5 cm water depth and some aquatic vegetation and allowed them to lay their eggs. On the next morning, we removed the adults and vegetation and allowed the clutch to hatch. We used a single sibship to minimize genetic variation among individuals.

About 36 h after egg laying, 36 similarly sized tadpoles, all in G25 developmental stage (Gosner, 1960 - mouthparts evident, spiracle present), were randomly assigned to one of three water level treatments: constant high (water volume: 2000 ml; water depth: 7.7 cm), constant low (water volume: 500 ml; water depth: 2.2 cm), and decreasing (water level starting at 2200 ml, water depth: 8.4 cm and decreasing daily by 200 ml for the first 6 days, i.e. by 0.7 cm, then by 100 ml for the next 6 days i.e. by 0.4 cm, and 25 ml i.e. by 0.1 cm until the 19th day (when it was stopped because the last tadpole from this treatment had finished its metamorphosis) – final water volume: 250 ml; water depth: 1.2 cm. The decreasing water level regime was designed to induce a strong cue of pond drying as early as possible in the developmental period while allowing tadpoles enough time to complete metamorphosis. Tadpoles were individually raised in rectangular plastic containers (21x15cm, 12 cm height), containing local well water. Tadpoles were maintained individually to avoid the effects of competition and cannibalism risk (Ortiz et al., 2013, Semlitsch & Caldwell, 1982). The water depth conditions are in the range of natural breeding habitats encountered in the area, starting min. 4 cm - max. 110 cm at egg-laying, and varying according to evaporation and/or rain (pers. obs.). Therefore, although the water level of ponds often decreases faster at the end of drying, all patterns of water level variations can occur in natural conditions. All experimental containers were placed on the same table, and position was randomly changed daily to avoid any effects due to position. Since the experiment was set in an open air enclosure located in the species' habitat, temperature (24 - 31° C) and lighting (12:12 L:D) conditions were natural and similar to what the tadpoles would be exposed in the wild. Temperature was identical among containers (less than 1°C difference at any time, with no constant pattern among containers from various treatments). Throughout the experiment, water was changed daily and a mixture of “Gisis Alimento completo para *Tilapia*” (protein content 32%) and “Purina Pro Plan Kitten” (protein content 40%) was provided as food *ad-libitum*. In the later developmental stages, containers were checked every two hours for accurate detection of metamorphosing individuals. To avoid froglet drowning, once they reached the G42 developmental stage, a small amount of floating grass was provided in the container on which they could climb, but they were otherwise maintained in the same conditions of water level as required by treatment, until reaching G45.

We recorded time until two stages of development: G42 (emergence of at least one forelimb) - based on recommendations of Walsh (2010), while for the purpose of our study, we considered the time from G42 to G45 important (tail reduced to a stub, mouth angle at level of posterior margin of the eye), because at this stage tadpoles can avoid mortality caused by the drying of the pond as they can survive in the terrestrial environment. Because larval period is short, we chose to work with a precision of hours instead of days for more accuracy. Body size of individuals (snout-vent-length: SVL, i.e. from the tip of the snout to the posterior end of the cloaca) and body mass were measured in G42 and G45, using a dial calliper (0.1 mm precision) and a My Weigh 300Z portable scale (0.1 g precision), respectively.

Individual growth rates were defined as metamorphic weight at G42, in mg, divided by the length of larval period, in days (Richter-Boix *et al.*, 2011). The relative body mass loss during metamorphosis was calculated as percent of G42 mass lost until G45. One tadpole from the constant low water treatment was excluded from the analysis because it was not able to metamorphose (at the age of 650 h it was in G35 stage, while the last tadpole from the same treatment had metamorphosed at the age of 445 h). All individuals were released at the end of the experiment in their native habitat.

Statistical analyses

All analyses were conducted using R version 3.2.2, with an *a priori* level of significance of 0.05. Since age-related data were not normally distributed (Shapiro-Wilk test) even after attempted transformation, analyses concerning age were made using non-parametric Kruskal-Wallis ANOVA (K.W.) tests, followed by Nemenyi post-hoc tests (PMCMR package). To conform to normality and homoscedasticity assumptions, body mass was log-transformed, and all morphometric parameters were analyzed using one-way ANOVA followed by Tukey-HSD tests (stats package) when a significant effect was detected. We investigated the effect of treatment (water level: three conditions) on age at G42 and G45 and on the interval between these two stages, size and mass of individuals in G42, as well as estimated growth rates until G42. Analysis was computed only on size and mass in G42 stage, because these traits were highly correlated with the ones in G45 (SVL: $r^2 = 0.92$, $df = 33$, $P < 0.001$; mass: $r^2 = 0.92$, $df = 33$, $P < 0.001$). The mean \pm SE values of all traits at the two developmental stages can be found in Table S1.

Results

Survival and time to metamorphosis

Survival to metamorphosis was 100% in all treatments. Tadpoles reached G42 as early as 366 h (15 days) and were able to leave the water (G45) 396 h (16.5 days) after egg-deposition (or 330 h and 360 h respectively, from the onset of the experiment, i.e., G25). The latest tadpole to metamorphose reached G45 stage 630 h (26 days) after egg-deposition (Table S1).

The water level treatment had a significant effect on the duration of the larval stage (K.W. G42: $\chi^2 = 14.58$, $df = 2$, $P < 0.001$; G45: $\chi^2 = 18.65$, $df = 2$, $P < 0.001$). Tadpoles from the constant high water level reached the stages G42 and G45 significantly later than tadpoles from the other treatments (Fig. 1a). The fastest development was experienced by tadpoles exposed to decreasing water levels, which were on average 394 h (16 days) old in G42 and 424 h (17 days) old in G45, but the differences between them and the tadpoles from constant low water level were not significant. Tadpoles exposed to decreasing water levels showed a reduction of 11.9% in time to G42 and 13.1% to G45 compared to control, while for constant low water level the reduction was of 9.9% and 10.2%, respectively.

Looking specifically at the time interval between G42 and G45, tadpoles exposed to decreasing water level treatment were able to make the transition from G42 to G45 much quicker (Fig. 1b) compared to both constant water level treatments (36% quicker than tadpoles in constant high and 16% quicker than the ones in constant low; K.W. $\chi^2 = 18.89$, $df = 2$, $P < 0.001$).

Growth and size at metamorphosis

Treatment had a strong impact on size at metamorphosis (G42): tadpoles raised in constant high water level were both heavier and larger than the ones exposed to desiccation risk (ANOVA: body mass $F_{2,32} = 26.26$, $P < 0.001$; SVL $F_{2,32} = 33.94$, $P < 0.001$; Table S1). Tadpoles constantly exposed to a low water level had the smallest body size (an average 79 % lighter than tadpoles from constant high water level); however, the differences in size between the low and decreasing water level treatments were not statistically significant (Fig. 1c). There was also a significant effect of treatment on the estimated growth rate (ANOVA: $F_{2,32} = 16.27$, $P < 0.001$). The tadpoles from the constant water level treatment experienced a higher growth rate than the ones from the other two treatments (Fig. 1d).

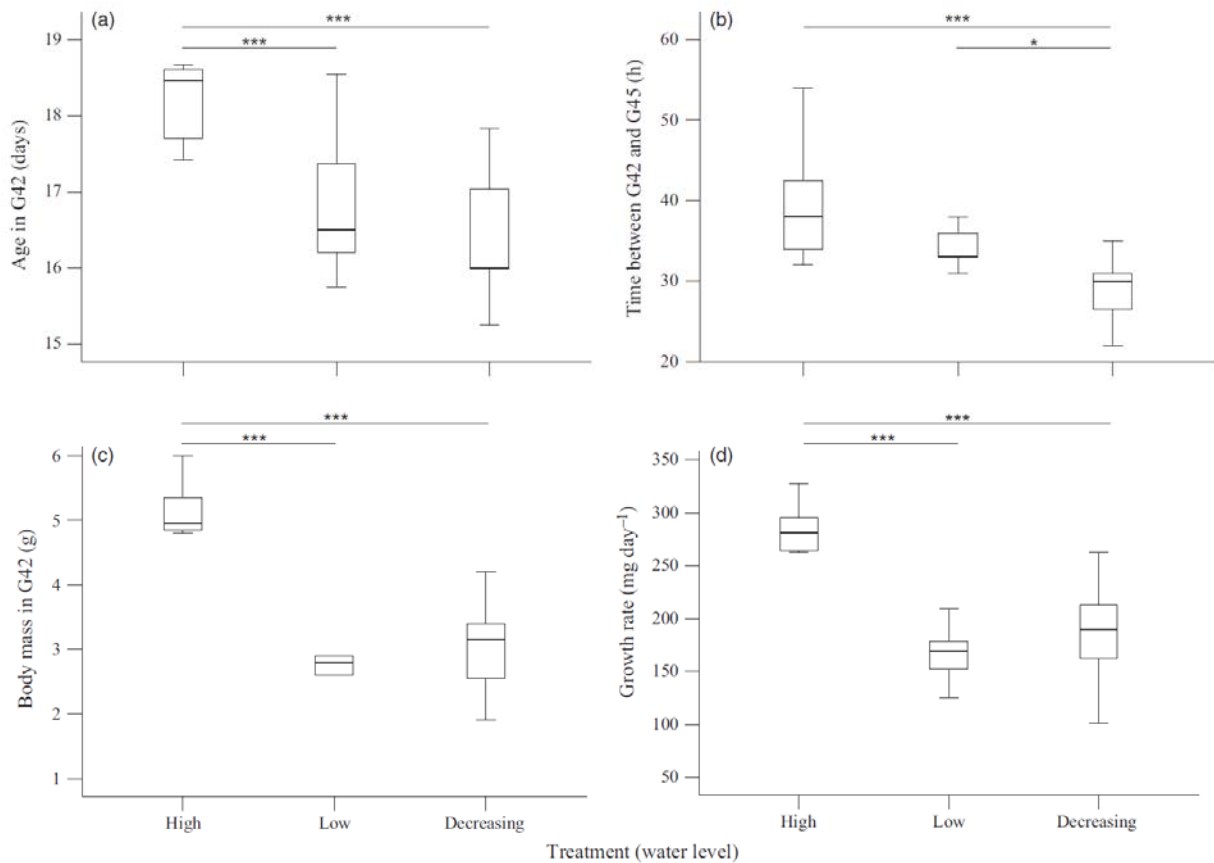


Figure 1 Life-history traits of Pacific horned frog tadpoles raised in different water level treatments: constant high water level, constant low water level, decreasing water level. (a) age of tadpoles in G42 developmental stage; (b) interval of time necessary for tadpoles to pass from G42 to G45 developmental stage (i.e., the last stages of metamorphosis); (c) tadpole body mass in G42; (d) estimated tadpole growth rate until G42. Box plots show the median (dark midline) and spread of values, with boxes encompassing percentiles 25 - 75 and whiskers denoting minimum-maximum value not higher than 1.5 times the interquartile range. * $P < 0.05$, *** $P < 0.001$.

Discussion

Drying and metamorphosis

Our study proves that *C. stolzmanni* tadpoles show phenotypic plasticity by being able to time their metamorphosis in response to water level availability. Tadpoles raised in constantly high levels of water metamorphosed later and at a larger size than tadpoles from decreasing and constantly low

water treatments. Our results are in accordance with Wilbur & Collins' model (1973) for amphibian metamorphosis, which assumes that tadpoles encountering favourable conditions postpone metamorphosis, capitalizing on the opportunity for additional growth, while tadpoles exposed to hostile conditions develop quicker and leave the water. Compared to other species that use ephemeral habitats and are able to metamorphose quickly, such as *Scaphiopus couchii* (Morey & Reznick, 2004) and *Isthmohyla pseudopuma* (Crump, 1989), *C. stolzmanni* tadpoles experienced a higher degree of variability in the larval developmental period among different treatments.

Tadpoles exposed to a decreasing water level were able to go through the late metamorphic stages (i.e., from G42 to G45) quicker than tadpoles kept in constant water levels (both high and low). In these final larval stages, tadpoles are considered highly vulnerable because of lower locomotor abilities compared to both tadpoles and juvenile frogs (Wassersug & Sperry, 1977). As a result, selection should favour an acceleration of development when mortality risks are high, as observed by Van Buskirk & Saxer (2001) in the presence of predators. Downie, Bryce & Smith (2004) compared 12 species of frogs and found that longer retention of tail was associated with juveniles remaining in the water, while rapid tail loss was correlated with an early emergence from the water. For *C. stolzmanni* tadpoles faced with a high risk of pond desiccation, the ability to go from G42 to G45 stage at a higher speed allowed them to more rapidly pass to the terrestrial habitat.

The degree of plasticity in developmental time is usually correlated with the unpredictability of breeding habitats (Morey & Reznick, 2004; Richter-Boix *et al.*, 2011). In our experiment, although *C. stolzmanni* tadpoles were capable of varying their larval period by more than 12% according to water availability, the time to metamorphosis was overall fairly short. In the study area, during the year 2015 we have noticed that *C. stolzmanni* used for egg-laying habitats ponds with hydroperiods varying between 2-3 days and slightly more than 2 months.

The minimum larval duration was shorter in our experiment compared to the one reported for the species by Ortiz *et al.* (2013) (20 days). Other ceratophryid species, such as *Chacophrys pierotti* and *Lepidobatrachus llanensis* are also known to complete their larval development very rapidly, in as few as 15 days (Quinzio, Fabrezi & Faivovich, 2006). The short period to metamorphosis places *C. stolzmanni* along with the New World spadefoot toads, as one of the fastest tadpoles to metamorphose (Richter-Boix *et al.*, 2011). Previous research showed that maternal or genetic effects can impact metamorphosis timing (Laugen, Laurila & Merilä, 2002; Loman, 2002). In our study, we used a single sibship to minimize variation, but we expect our results to be representative for the studied population, because no permanent aquatic habitats are present in the study area (pers. obs.) and therefore water acts as a limiting, temporary and unpredictable resource. However, future work should compare responses among sibships and populations to determine the basis of the plastic responses.

Environmental cues of pond drying

Our results show that cues from a constant low water and those from a decreasing water level act similarly as factors eliciting the acceleration of metamorphosis of the studied species. The effect

of continuous exposure to low water level on larval duration has been rarely tested and has produced diverse results. In *Hoplobatrachus chinensis* tadpoles from constant low treatment differed only from the ones exposed to rapidly fluctuating water levels, but not decreasing or constant high (Fan, Lin & Wei, 2014); in *Physalemus pustulosus*, the response to constant low was intermediate between constant high and decreasing (Charbonnier & Vonesh, 2015); in *Rana temporaria* and *Hyla pseudopuma* the constant low had the longest larval period in comparison to both constant high and decreasing (Crump, 1989; Merilä *et al.*, 2000). In contrast, in *Pelobates syriacus* no significant difference in larval duration between constant high and constant low water level treatments were found (Székely, Tudor & Cogălniceanu, 2010). The fact that tadpoles of *C. stolzmanni* reacted similarly to cues of a very shallow tank and a decreasing water level can be considered to be adaptive as in the study area both habitats are similarly detrimental in being at high risk of desiccation. Accelerated development in response to pond drying can be regulated by various environmental cues, such as increased temperature, decreased water volume, crowding, or changes in concentration of solutes (Brady & Griffiths, 2000). In our experiment, there were no temperature differences between treatments. Because water was changed daily, it can be expected that the concentration of chemical compounds stayed similar across treatments, though small differences cannot be excluded. The most probable explanation is therefore that the shallow water level prompts acceleration of metamorphosis either by triggering precocious activation of metamorphosis controlling hormones (Denver, Mirhadi & Phillips, 1998) or indirectly by reducing foraging activity and decreasing growth rates (Laurila & Kujasalo, 1999), which in later developmental stages speeds up metamorphosis (Denver *et al.*, 1998). Another proxy for desiccation can be an increase in tadpole density (Denver, 1997) and future studies should investigate its effects on *C. stolzmanni* growth and development.

Age versus size at metamorphosis

According to Rudolf & Rödel (2007), species that are under strong time constraints (e.g. that use ephemeral and temporary breeding habitats) have fixed developmental rates, which means that lower growth rates are correlated with smaller size at metamorphosis. On the other side of the spectrum, species that breed in more permanent ponds have flexible developmental rates, and a low growth rate results in metamorphosis at a later age but at a larger size than the one predicted by fixed development. As a species adapted to reproduce in ponds with short hydroperiod, *Ceratophrys* tadpoles that needed to accelerate their metamorphosis as a result of stressful environment (in our case, a decreasing or low water level) had a reduced body size (both in terms of SVL and body mass) compared to tadpoles from constant high water levels, reflecting a trade-off between growth and development. The spectrum of body sizes in froglets (i.e., just after metamorphosis) that were obtained in this experiment are within the range observed in natural conditions (average SVL = 34.0 mm, range = 23.8 – 47.9 mm; average body mass = 3.9 g, range = 1.2 – 11.9 g, $n = 72$; D. Székely, pers. obs.). A low body size at metamorphosis is expected to

be costly, as it was reported in the literature to affect various individual fitness components in the terrestrial habitat (Morey & Reznick, 2001; Cabrera-Guzmán *et al.*, 2013; Tarvin *et al.*, 2015).

Growth rate and unpredictable habitats

With an average of 186 mg/day (decreasing water level treatment) and 269 mg/day (constant high water level treatment) and a maximum of 327 mg/day, *C. stolzmanni* tadpoles from our experiment have, to our knowledge, the highest mean growth rate reported for anuran larvae (see Richter-Boix *et al.*, 2011 for a review). High values of estimated growth rates were encountered in *Spea hammondi* (59 and 96 mg/day for decreasing and constant water level, respectively: data retrieved by Richter-Boix *et al.*, 2011 on the basis of Denver *et al.*, 1998) and *Pelobates syriacus* (158 mg/day in fast decreasing and 177.5 mg/day in constant high: Székely *et al.*, 2010). Ephemeral habitats are frequently associated with low quality or diminished food and crowded conditions. For facultative anurophagous tadpoles, such as those of *Ceratophrys* species (Schalk *et al.*, 2014), having a bigger size might be advantageous by providing access to a nutritious, high protein food source (other tadpoles), which, in other anuran species, results in shorter larval periods and increased mass at metamorphosis (Kupferberg, 1997; Babbitt & Meshaka Jr., 2000) and presumably reduces competition. The high larval growth rate seems to be a characteristic of the Ceratophryidae family, partially explained by their capacity to feed on exceptionally large prey (Fabrezi *et al.*, 2016). For instance, in an amphibian assemblage from semi-arid Argentinean Chaco region consisting of 18 species, accelerated growth rates were encountered in four ceratophryid species (*Chacophrys pierotti*, *Ceratophrys cranwelli*, *Lepidobatrachus laevis* and *L. llanensis*), all using ephemeral ponds for reproduction (Fabrezi, 2011).

Conclusions

Our study showed that *C. stolzmanni* tadpoles have very fast developmental rates while still maintaining their ability to respond plastically to the desiccation of breeding habitats by speeding up their development as a reaction to diverse cues on water availability. Therefore, our results provide insights into the understanding of the ecology and persistence of amphibian species living in risky unpredictable habitats.

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