



# Dealing with hot rocky environments: Critical thermal maxima and locomotor performance in *Leptodactylus lithonaetes* (Anura: Leptodactylidae)

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Environmental temperature has fitness consequences on ectotherm development, ecology and behaviour. Amphibians are especially vulnerable because thermoregulation often trades with appropriate water balance. Although substantial research has evaluated the effect of temperature in amphibian locomotion and physiological limits, there is little information about amphibians living under extreme temperature conditions. *Leptodactylus lithonaetes* is a frog allegedly specialised to forage and breed on dark granitic outcrops and associated puddles, which reach environmental temperatures well above 40 °C. Adults can select thermally favourable microhabitats during the day while tadpoles are constrained to rock puddles and associated temperature fluctuations; we thus established microhabitat temperatures and tested whether the critical thermal maximum (CTmax) of *L. lithonaetes* is higher in tadpoles compared to adults. In addition, we evaluated the effect of water temperature on locomotor performance of tadpoles. Contrary to our expectations, puddle temperatures were comparable and even lower than those temperatures measured in the microhabitats used by adults in the daytime. Nonetheless, the CTmax was 42.3 °C for tadpoles and 39.7 °C for adults. Regarding locomotor performance, maximum speed and maximum distance travelled by tadpoles peaked around 34 °C, approximately 1 °C below the maximum puddle temperatures registered in the puddles. In conclusion, *L. lithonaetes* tadpoles have a higher CTmax compared to adults, suggesting a longer exposure to extreme temperatures that lead to maintain their physiological performance at high temperatures. We suggest that these conditions are adaptations to face the strong selection forces driven by this granitic habitat.

**Keywords:** CTmax; thermal tolerance; tadpoles; granitic rocks; hot environment

## INTRODUCTION

From all environmental conditions, temperature is probably the most influential variable that controls animal performance (Hutchison, 1961). In ectothermic animals, environmental temperature profoundly affects protein tertiary structure and thereby physiology, which further constraints habitat preferences and ecological performance (Hutchison, 1961; Viña, 2002; Gunderson & Stillman, 2015). Individuals exposed to thermal stress often die because of cardiac dysfunction (Viña, 2002). In many amphibians, however, heat stress causes rapid desiccation due to their wet and permeable skins, which are in turn necessary to facilitate cutaneous gas exchange (Hutchison, 1961; Tracy, 1976; Duarte et al., 2012). Extreme temperatures may also affect locomotor performance and associated foraging and anti-predator behaviour, which eventually cause a decrease in fitness (Wilson & Franklin, 1999; Wilson et al., 2000; Herrel & Bonneaud, 2012).

Previous studies have addressed the existence of

an optimal range of temperatures for frog activity (e.g. Noland & Ultsch, 1981) and its relationship with the mean environmental temperature and the lethal temperature range (Wilson & Franklin, 1999; Wilson et al., 2000; Herrel & Bonneaud, 2012). Also, optimal temperature is known to vary between species and development stages (Navas et al., 2010; Katzenberger, 2014; Turriago et al., 2015). Although most studies regarding amphibians in extreme heat conditions have been addressed to the palearctic regions (Hillman et al., 2009), recent information about subtropical and tropical regions has been published (Duarte et al., 2012; Katzenberger, 2014; Gutierrez-Pesquera et al., 2016). However, studies about tropical species have been focused primarily on tadpoles (Simon et al., 2015; Gutierrez-Pesquera et al., 2016). Increasing information about extreme environments in tropical regions is highly important because mean optimal values are closer to the limit values of the optimal distribution range in most animals (Lillywhite & Navas, 2006). To thrive under extreme thermal conditions, frogs are expected to show behavioural and physiological adaptations,

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such as protein polymorphisms or phenotypic plasticity (Hoffman, 2003; Somero, 2004; Denver, 1997; Somero, 2010; Brown, 2013 Gunderson & Stillman, 2015).

*Leptodactylus lithonaetes* (Anura: Leptodactylidae) is found on granitic outcrops in northern South America (Heyer, 1995). These habitats essentially lack any shadow, except for a few shrubs and grasses in rock puddles (see Fig. 1) thus, temperatures are considerably higher compared to the surrounding forest (IDEAM, 2018). However, adults appear to have better opportunities for thermoregulation by microhabitat selection compared to tadpoles: adults forage, call and mate at night, but during the daytime, they are hidden most likely in rock crevices. Tadpoles, on the other hand, are constrained to the small and shallow puddles and exposed to midday temperatures. Due to the positive correlation between maximum habitat temperature and maximum critical temperatures (Miller & Packard, 1977; Compton et al., 2007); if tadpoles are exposed to higher temperatures than adults, they should present higher values of critical thermal maximum (CT<sub>max</sub>). Moreover, because temperature changes affect the power output, contractile and relaxation rates of muscles (Rome, 1983; Hirano & Rome, 1984; Fleming et al., 1990; Franklin & Johnston, 1997), locomotor performance of tadpoles might be adapted to their thermal environment and should be

higher at more suitable temperatures.

The aims of this study were thus (1) to establish whether puddle temperatures are indeed higher than the microhabitats allegedly chosen by adults, (2) to estimate CT<sub>max</sub> of adult and larvae *L. lithonaetes* and compare it with other anuran species, (3) to test the prediction that tadpoles have higher CT<sub>max</sub> values than adults and (4) to estimate the effect of temperature on locomotor performance of tadpoles and compare it with environmental temperatures experienced in the field.

## MATERIALS AND METHODS

### Study species

*Leptodactylus lithonaetes* (Heyer, 1995) is a moderate sized and cryptic frog. Females (snout-vent length (SVL): 55-78 mm) are slightly bigger than males (SVL: 45-71 mm) (Heyer, 1995). Adults forage, call and mate during the night; breeding pairs lay eggs in foam nests built near small puddles, on granitic or sandstone rocks, where tadpoles complete their development. Presumably, the breeding season takes place between May-August, which corresponds to the rainy season in the area (IDEAM, 2018). However, detailed information about the breeding behaviour and the ecology and development of the larvae of *L. lithonaetes* still needs to be assessed. The species occurs below elevations of 1250 m, more often on outcrops surrounded by savannah and gallery forests, in south-west Venezuela and eastern Colombia (Heyer, 1995; Heyer & Heyer, 2001).

### Study sites

Our study was conducted in November 2015 at the Bojonawi Nature Reserve (6° 06' N, 67° 29' W, 50 m.a.s.l.), about 9.5 km south of Puerto Carreño (Vichada), Colombia. *Leptodactylus lithonaetes* is abundant on a granitic outcrop, about 0.27 km<sup>2</sup> in area, just at the edge of the Orinoco River. During three previous field trips, we spotted adults foraging at night on the exposed rock and calling from the puddles or from within the associated vegetation (Fig. 1). In the puddles, tadpoles of *L. lithonaetes* were observed foraging in small groups of about 8-10 individuals. To estimate maximum values and diurnal variation in environmental temperature, we registered water temperatures every hour during three consecutive days. Measurements were taken with an analogue thermometer ( $\pm 0.5$  °C) in the centre of the puddles (n=12) at a depth of approximately 10 cm. Puddles differed in area but not in depth, and we only included puddles with tadpoles present. To estimate maximum values and diurnal variation in rock temperatures, we obtained thermal images by pointing an Infrared Camera (FLUKE® Ti32. Fluke Corporation. Everett, WA, USA) within 1 m of the surface. We took thermal images every hour from 0730 to 1700 h during four days and registered the temperature of 135 haphazardly chosen spots.

As a post hoc analysis, we included grass cushions temperatures due to the possibility that adults occupy this microhabitat during the day. However, because these measurements were not considered in our initial hypothesis, we obtained the data from thermal images of rocks in which the grass cushions were also visible.



**Figure 1.** Image of a representative puddle inhabited by *L. lithonaetes* tadpoles. These puddles are formed after rain fills the hollows dispersed over the granite rock outcrops.

In total, we obtained 60 measurements from grass cushions temperatures. These measurements were taken approximately every hour from 0800 h to 1200 h during one day.

### CTmax and locomotor performance

We collected seven adult frogs by hand and 16 tadpoles with a net. Tadpoles were collected from three different puddles to reduce the probability of close relatedness between them. All adults and six randomly selected tadpoles were used to estimate Critical Thermal Maxima (CTmax) whereas the remaining ten tadpoles were used to estimate the thermal dependence of locomotor performance. All experiments were conducted in a research station within 600 m of the site where the animals were captured. For CTmax experiments, animals were captured between 0900–1200 h and measured between 1400–1900 h, giving them at least 2 hours of acclimation to the environmental temperature (about 28 °C). Locomotor performance experiments were conducted between 0845–0930 h.

CTmax is operationally defined as the mean temperature at which an animal loses its locomotor performance and the ability to escape from conditions that otherwise would cause death (Hutchison, 1961). Following Stebbins (1954) and Hutchinson (1961), we placed each individual (tadpole or adult) in a small plastic jar containing 50 mL of tap water, which itself was placed in a water bath heated at a rate of approximately 1 °C/min. Every 2 minutes we stimulated the tadpole and the adults with a soft push. In addition, when the adults halt their reaction to the stimuli, they were placed face up to be sure that they lost completely their locomotor abilities. Then we measured the water temperature with an analogue thermometer ( $\pm 0.5$  °C). We then registered the water temperature at which animals lost their ability to swim (adults and tadpoles) and recover their body posture (adults). At the end of each experiment, the plastic jar containing the individual was removed from the setup and slowly cooled to avoid injuries or death due to thermal stress. All animals survived the experiments, looked healthy after some hours and were thus released at their capture sites.

The locomotor performance of tadpoles was measured by placing each individual in an 18 x 13 x 6.5 cm (H x W x D) plastic aquarium with a length scale at the bottom. To induce tadpoles to swim, we dropped a small pebble close to their tail from a height of 33 cm. For each tadpole, we conducted three trials in ascendant order at each of the experimental temperatures (25, 28, 31, 34 and 37 °C). The temperature values were chosen randomly according to the natural range of variation of the temperatures reached in the puddles ( $31.01 \pm 2.46$ ; see Fig. 2) plus two “extreme” temperatures (25 and 37 °C).

Two consecutive trials were separated by at least 10 min to avoid potential fatigue or habituation effects. All trials were recorded from the top of the aquarium with a Sony HDR-HC71080i video camera fixed to a supporting structure to avoid camera movement. Tadpole movements in the recordings were video tracked with the software Tracker® (<http://www.opensourcephysics.org>).

Open Source Physics, Boston MA, USA) to estimate the maximum speed (m/s), maximum acceleration (m/s<sup>2</sup>) and maximum distance (m) travelled during escape responses. Our three proxies for locomotor performance allegedly relate to tadpoles ability to escape from potential predators (Watkins, 1996; Bursik, 2000; Wilson et al., 2005).

### Statistical analysis

Difference in CTmax values between tadpoles and adults of *L. lithonaetes* were analysed using an Exact Mann-Whitney-Wilcoxon Test available in R package “coin” (Hothorn et al., 2008) due to the non-parametric distribution of the dependence variable (ordinal data). To test the effect of temperature on each estimate of locomotor performance, we conducted Linear Mixed Models (LMM). Given that the effect of temperature on physiological performance is typically non-linear and generally resembles an asymmetrical bell-shaped curve, we smoothed the models using LOESS smoothing function. All statistical analyses and graphs were conducted on R 3.2.3 software (R Development Team 2016 Vienna, Austria. <https://www.r-project.org/>) and conducted at a significance level of  $\alpha = 0.05$ .

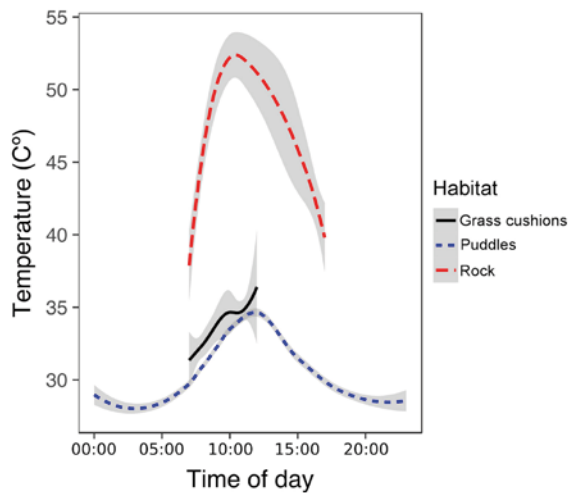
## RESULTS

Mean water temperature in the puddles varied throughout the day between 27.5–35.75 °C, peaking at 12:00 h ( $35.75 \pm 0.25$ ; mean  $\pm$  standard deviation (SD)). Mean grass cushion temperatures varied throughout the day between 27.1–40.5 °C, peaking at 11:00 h ( $33.7 \pm 2.3$ ). Mean rock temperatures exhibited a much wider range of daily variation, between 36–52.54 °C, and peaked at 10:45 h with  $52.54 \pm 6.59$  (see Fig. 2). The maximum temperature we registered on the rock was 68.3 °C. The critical thermal maximum differed significantly between tadpoles and adults ( $U = 0$ ,  $Z = -3.25$ ,  $P < 0.01$ ). The CTmax was about 2.6 °C higher for tadpoles ( $42.33 \pm 0.81$ , mean  $\pm$  SD,  $n = 6$ ) compared to adults ( $39.71 \pm 0.75$ ,  $n = 7$ ). Two out of three proxies for locomotor performance in tadpoles were significantly affected by temperature: they swam faster and longer distances at higher temperatures between 31 °C (LMM<sub>distance</sub>:  $\beta = 0.04$ ,  $SE = 0.01$ ,  $P < 0.01$ ) and 34 °C (LMM<sub>speed</sub>:  $\beta = 0.09$ ,  $SE = 0.03$ ,  $p = 0.01$ ; LMM<sub>distance</sub>:  $\beta = 0.055$ ,  $SE = 0.01$ ,  $P < 0.01$ ); above this peak value, performance dropped with increasing temperatures (Fig. 3).

## DISCUSSION

The CTmax of tadpoles was generally very high in *L. lithonaetes* in comparison to other species larvae from extreme heat environments in tropical regions, such as *Physalaemus* ssp. ( $37.9 \pm 0.91$ ), *P. diplolistris* ( $40.7 \pm 0.45$ ) and species from several tropical habitats ( $40.9 \pm 0.22$ ) (Simon et al., 2015; Gutierrez-Pesquera et al., 2016). Noland and Ultsh (1981) interpreted high CTmax in *Anaxyrus (Bufo) terrestris* as an adaptation to living in shallow ephemeral puddles, in contrast to *Lithobates (Rana) pipiens*, which inhabits permanent ponds and exhibits CTmax about 2 °C lower. The choice of warmer



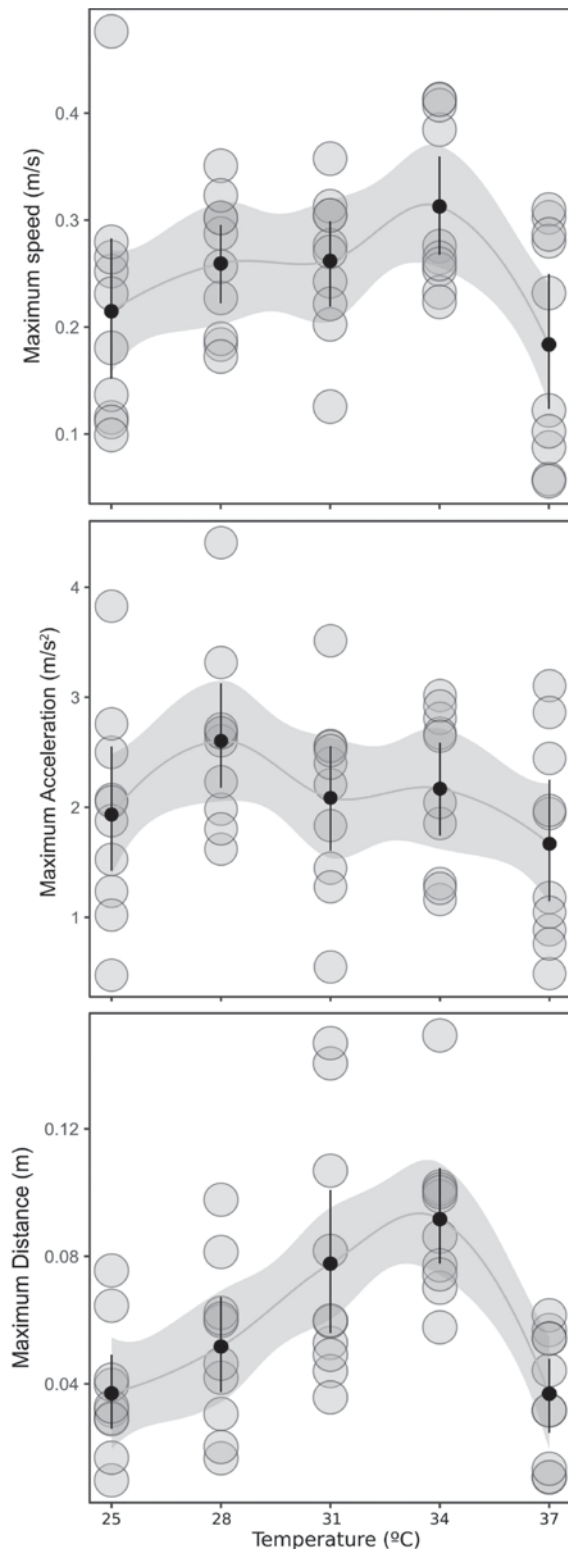


**Figure 2.** Daily rock (red dashed line;  $n = 135$  measurements), grass cushions (black solid line;  $n = 60$  measurements) and water (blue dashed line;  $n = 12$  puddles) temperatures recorded at the site of study. Water temperatures were recorded in puddles where tadpoles of *L. lithonaetes* were found. Shaded region indicates 95 % confidence interval.

puddles for oviposition and larval development has been in turn interpreted as an adaptation to complete development in a shorter period of time (Berven et al., 2007). Indeed, the availability of appropriate breeding sites for *L. lithonaetes* at the study site is profoundly affected by rains. During both the dry and the rainy seasons, puddles may dry out after a few days or a few weeks without rain, due to high water evaporation on hot rocky substrates; the low probability of tadpole survival to puddle desiccation may favour the evolution of rapid developmental rates.

In the tropics, however, the presence of *L. lithonaetes* tadpoles in hot puddles could also reflect the advantages of lower predation and competition (Wassersug, 1975; Abe & Neto, 1991): shallow, ephemeral and thermally challenging puddles are known to be colonized by a lower number of species, compared to permanent ponds (Holt, 1977; Sih et al., 1985). In fact, just in a few rock puddles, *L. lithonaetes* tadpoles were found co-existing with one species of libellulid dragonfly and one of belostomatid bug, both alleged predators of anuran larvae (Heyer et al., 1975). Also, the tadpoles were almost exclusively found in the puddles formed on the exposed rock, despite cooler puddles and ponds were available in the forest and grassland habitats, less than 50 meters away from the hot rocky habitat.

In other anurans, tolerance limits of tadpoles are reduced during the metamorphosis stage (stages 43-44; Gosner, 1960; Cupp, 1980; Noland & Ultsh, 1981), which may reflect the adjustment for a new life form. However, the adaptation of tadpoles to high temperatures may also be reflected in the CTmax of the adult frogs (Hoppe, 1978). Contrary to our expectations, water temperatures were significantly lower to rock temperatures and comparable to grass cushions temperatures (Fig. 2). Our data suggest that the thermal profile experienced by adults was generally higher than that experienced by tadpoles. Nevertheless,



**Figure 3.** Effect of water temperature on locomotor performance of *L. lithonaetes* tadpoles. Grey lines denote loess smoothness of the model. Shaded regions and vertical black lines indicates 95 % confidence intervals ( $n = 10$  individuals).

*L. lithonaetes* tadpoles were more tolerant of higher temperatures than adults. These results could reflect tadpole adaptations to extreme puddle temperatures during the dry season due to high water evaporation rates. However, our results should be treated carefully

because (1) we did not measure puddle temperatures during the dry season and (2) we are assuming that adults hide in rock and grass cushions during the day. Adults are rarely observed during the day thus, it is not completely certain that these are the only microhabitats chosen by them. Tadpoles are instead restricted to water bodies just a few centimetres in depth and therefore have arguably poorer opportunities to thermoregulate by microhabitat selection. If this argument holds, the microhabitat preferences of the adults would explain why tadpoles have higher thermal tolerances compared to adults. A telemetry study where information is collected on thermal conditions experienced by adults would provide crucial evidence to corroborate this hypothesis.

As we expected, maximum distance of locomotor performance in *L. lithonaetes* tadpoles peaked between 31–34 °C (Fig. 3), which coincided well with a range of ecologically relevant temperatures for this species (Fig. 2), like has been observed in *Rhinella (Bufo) granulosus* and *Hyperolius viridiflavus*, species that inhabit arid environments (Reviewed by Navas et al., 2008). Muscle force is known to increase with temperature in a non-linear fashion, possibly because temperature reduces the accumulation of inorganic phosphates (Rome & Swank, 1992; Ranatunga, 1998; Navas et al., 2006; Allen et al., 2008). Burst power and resistance of frog muscles also depend strongly on temperature (Phillips et al., 2006; James et al., 2012). The asymmetry of the locomotor performance curves can be explained by the “warmer is better” hypothesis (Bennett, 1987) for at least two of the three measured traits: the locomotor performance is improved by higher body temperatures, since aerobic speed is highly temperature dependent via stamina consumption. The locomotor responses we elicited here probably represent the ability of tadpoles to escape from predators effectively and therefore, should peak within the range of temperatures that tadpoles experience in the field. However, to evaluate the relationship between the optimal temperature and the maximal locomotor performance in *L. lithonaetes* tadpoles, an intra and interspecific comparison is needed.

Acclimation may play a role in an animals CTmax and locomotor performance (Hutchison, 1961; Brattstrom, 1968; Wilson & Franklin, 1999; Wilson et al., 2000), a plastic response that we did not study here. We acknowledge that most of the studies in this research area use longer acclimation periods under known regimes temperatures, which facilitate comparisons between populations and/or species. However, tropical amphibians are likely to have limited metabolic acclimation capacities (Feder & Lynch 1982; Navas et al., 2008; but see Brattstrom, 1968) and our main objective was to show differences in high-temperature tolerance between tadpoles and adults. Therefore, we consider that our results are ecologically relevant because i) the animals were acclimated to the thermal profile of their natural environment and ii) the range of temperatures, i.e. thermal profile, experienced by tadpoles and adults (puddle vs. grass cushion temperatures, respectively) was comparable. Moreover, it is unlikely that the difference between rock and puddle temperatures is responsible for the lower CTmax of adults since, to our knowledge, all the research in amphibians

has shown that acclimation temperature has either no effect or a positive effect on CTmax (Hutchinson & Ferrance, 1970; Simon et al., 2015). Thus, our results suggest that the observed differences in CTmax between tadpoles and adults are likely due to actual physiological differences between stages rather than a different short-term acclimation. A follow-up study using a standardised acclimation period would be useful to control the effect of exposure to previous thermal profiles on the CTmax. Further research topics include the biochemical and molecular adaptations that allow tadpoles to tolerate higher temperatures than adults (Feder & Hofmann, 1999; Allen et al., 2008; Boussau et al., 2008), and the extent and importance of behavioural thermoregulation in the temperature regimes experienced by adults (Brattstrom, 1979; Brown, 2013). Both sources of information would provide substantial and more conclusive evidence for a case of adaptation in the thermal tolerances of *L. lithonaetes* tadpoles.

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