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Thermal ecology of the post–metamorphic Andean toad (*Rhinella spinulosa*) at elevation in the Monte Desert, Argentina

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Abstract

Rhinella spinulosa is an anuran toad species distributed latitudinal and altitudinal (1200 to 5000 meters) from Peru to Argentina, inhabiting mountain valleys in the Andes.

Considering the broad range of habitats where they live, it is important to understand the thermal physiological mechanisms, thermal tolerances and physiological adaptations for

surviving in rigorous environments. We investigated the thermal parameters (field body temperature, selected body temperature, locomotor performance in field and laboratory conditions, and thermal extremes) during diurnal activity for a population of juvenile, post-metamorphosed toads (*Rhinella spinulosa*) from the Monte Desert of San Juan, Argentina. Post-metamorphic toads are active from approximately 1100 to 1900 (in contrast to nocturnal adult toads). Our findings show that these toads have a wide thermal tolerance range, ranging from a critical thermal maximum of 36.9 °C to crystallization temperatures below 0 °C. During their active period, toads always showed suboptimal thermal conditions for locomotion. Despite the suboptimal condition for the locomotion, diurnal activity is likely to confer thermal advantages, allowing them to search for food and increase digestion and growth rates. We also found that the toads are capable of super-cooling, which prevents mortality from freezing when the environmental temperatures drop below 0 °C. The environmental temperatures are below zero at night, when toads are inactive and take refuge under rocks. In summary, this toad population demonstrates high thermal plasticity, as shown by a relatively high level of activity sustained over a wide range of ambient temperature (~ 35 °C). These thermal adaptations allow this species of juvenile toads to inhabit a wide range of altitudes and latitudes.

Key Words— critical thermal maximum; crystallization temperature; field body temperature; locomotor performance; supercooling.

1. Introduction

Temperature plays a key role in the regulation of physiological and behavioural function of ectotherms (Angilletta, 2009). Seasonal and daily activity, locomotor performance, digestion rate, growth rate, oxygen consumption, and gas exchange routes are all affected by body temperature (Lillywhite et al., 1973; Huey and Stevenson, 1979; Choi et al., 2000; Witters and Sievert, 2001; Marvin, 2003; Viscor, et al., 2003; Navas et al., 2008).

Post-metamorphic (juvenile) toads in the genus *Bufo* exhibit diurnal activity while the adults are generally nocturnal. Apparently, the diurnal activity of juvenile toads is related to cannibalism avoidance, illumination level and the availability of insect preys (Kinney and Fisher, 1978; Hagman and Shine, 2008; Pizzatto et al., 2008). The change in activity pattern is also considered advantageous from a thermoregulatory point of view (Seymour, 1972; Lillywhite et al., 1973; O' Connor and Tracy, 1992; Tracy et al., 1993; Lambrinos and Kleier, 2003; Navas et al., 2007). Diurnal patterns of juvenile toads had not been well studied, but it is possible that diurnality provide benefits such as increased rate of digestion, growth and avoidance of predators (Lillywhite et al., 1973; Brattstrom, 1979; Huey and Stevenson, 1979; Rome et al., 1992).

Extreme environments are characterized by rapid fluctuations of environmental temperatures (Warner, 2004). Daytime activity exposes organisms to temperatures near ambient thermal safety margins as well as high evaporation rates. The efficiency in maintaining stable body temperatures is constrained by the poor control that toads have over dehydration (Carey, 1978). This constraint is most pronounced in small juvenile toads (Tracy et al., 1993).

On the other hand, locomotion is key for toads to escape predators, for dispersal and to find sites with favourable thermal conditions (Arnold, 1983; Irschick and Garland,

2001). Therefore, locomotion in harsh environments should be highly plastic to allow individuals to cope with high variation in ambient temperature. The thermal physiology of juvenile Andean toads during their diurnal activity period has been poorly studied.

We investigated the thermal parameters during diurnal activity of a population of juvenile toads of *Rhinella spinulosa* from the Monte Desert of San Juan, Argentina.

Rhinella spinulosa occurs throughout a large altitudinal and latitudinal geographic range from Andean Peru to Argentina, also inhabiting Bolivia and Chile (Ceï, 1979; Veloso and Navarro, 1988; Frost, 2014). This specie can reach altitudes up to 5000 m (Gallardo, 1987; Veloso and Navarro, 1988; Lavilla and Ceï, 2001). During daily activity the environmental temperatures change quickly, therefore, the toads experience large thermal amplitude (~30 °C). We studied the thermal extremes (critical thermal maximum, crystallization temperature) to evaluate the thermal tolerance of this specie. We hypothesized that juvenile Andean toads would exhibit a moderate critical thermal maximum and very low crystallization temperature, associated with diurnal environmental temperatures. Additionally, to understand the locomotor constraints during diurnal activity, we compared their locomotor performance in laboratory and field conditions. Finally, to understand the thermal constraints of juvenile Andean toads during their activity period, we measured their selected body temperature and field body temperature, and calculated the *db* index following the Hertz methodology (1993).

2. Methods

2.1 Environment and experimental animals

The Monte Desert is characterized by an arid climate with a mean annual temperature of 17.3 °C, a mean maximum temperature of 25.7 °C, a mean minimum temperature of 10.4 °C, and a mean annual rainfall of 89 mm (most of which falls during

the wet season in austral summer months) (Cabrera, 1976). The study area is located 100 km south of San Juan City (31.9044 S; 68.7098 W; elevation 1200 m).

We collected juvenile toads for laboratory studies on May 19th and 20th of 2013 in an open environment characterized by limited vegetation, bare soil with random rocks coverage and permanent water (Fig. 1). *Rhinella spinulosa* has explosive metamorphosis, ensuring that all post-metamorphic (juvenile) individuals were of a similar age. Also, at the time of metamorphosis, the adult's are inactive, eliminating the possibility of sampling them. 80 toads were captured and transported immediately to the laboratory to measure their critical thermal maximum ($N = 15$), crystallization temperature ($N = 15$), selected body temperature ($N = 15$) and locomotor performance ($N = 35$). Field experiments were performed on previous days (the 17th, 18th and 19th).

2.2 Calibration of infrared thermometer

We used a rapid and non-invasive infrared thermometer to measure body temperature throughout the experiment (Rowley and Alford, 2007). We used seven toads for calibration (snout–vent length, SVL = 1.6 ± 0.04 cm, weight = 0.94 ± 0.09 g). Each toad was placed on an aluminum plate heated at a rate of 0.4 °C/min. The initial temperature was 5 ± 0.5 °C and the maximum temperature was 29 ± 0.5 °C. We measured the cloacal temperature (T_{cloacal}) with a digital thermometer (TES 1312, TES Electrical Electronic Corp., Taipei, Taiwan, ± 0.1 °C), connected to a 30-gauge thermocouple positioned ~5 mm inside the cloaca and fixed with superglue (La Gotita, Poxipol Argentina). Simultaneously, the dorsal temperature (T_{dorsal}) was measured with the infrared thermometer. These temperatures were recorded every 15 minutes for 3 hours. We performed a linear regression model ($r^2 = 0.99$; $P < 0.00001$; $N = 84$; $T_{\text{cloacal}} = -0.4286 +$

$0.9886 * T_{\text{dorsal}}$) to adjust the values obtained from the infrared temperatures with the cloacal temperatures.

2.3 Determination of field body temperatures and environmental parameters

We validated that dorsal surface temperatures measured in the field were suitable measures of the body temperatures (T_b) of the juvenile toads ($N = 285$). We also measured body size (SVL) to the nearest mm with a transparent ruler. We measured the microenvironment temperature using the substrate temperature (T_s) at the site of capture (using a TES TP-K03 substrate probe) and the air temperature (T_a) at 1 cm from the soil surface (using a TES TP-K02 gas probe with a digital thermometer TES 1312TES Electrical Electronic Corp., Taipei, Taiwan, ± 0.1 °C). Finally, we measured the relative humidity (Rh) and air temperature at 5 cm above the soil with a data logger (Hobo RH/Temp, Onset[®]) every five minutes. Substrate and water temperatures were recorded by two data loggers (iButton DS1922L, Maxim Corporation, USA) every five minutes.

2.4 Determination of thermal tolerances

Immediately after capture, toads were transported to the laboratory to measure their thermal tolerances. We measured the critical thermal maximum and crystallization temperature of 30 toads that were acclimated for two days at 25 °C with a photoperiod of 12:12. Critical thermal maximum (CT_{max}) was determined using the method described by Hutchison (1961). Individual toads ($N= 15$) were placed in a glass container (15 x 20 cm) with 150 ml of water at ambient temperature (25 ± 1 °C). An electric heating mantle raised the temperature of the water bath at a rate of ~ 1 °C min^{-1} . Water temperature acted as an indicator of body temperature because of their small body size (~ 0.78 cm^3). We quantified CT_{max} as the water temperature at which the toads showed muscle spasms.

Most ectotherms will remain unfrozen as they are progressively cooled until some limit to supercooling (the crystallization temperature, CT) is reached. Their body temperature will subsequently rebound, as a result of ice nucleation to a temperature. This abrupt rise in temperature due the liberation of the fusion heat represents the onset of the exothermic reaction (Claussen and Costanzo, 1990).

We dried toads ($N= 15$) with a paper towel, and then placed each one in a glass container (15 x 20 cm) at ambient temperature (25 ± 1 °C) inside a chiller's bath(Thermo NESLAB RTE 7, Newington, NH) with ethylene-glycol. A 30-gauge thermocouple positioned against the abdomen provided a continuous recording (1 s) of body temperature on a data logger (PP 222; Pico logic, UK). The CT was determined using a decreasing temperature rate of 0.5 °C min^{-1} .

All toads used for the determination of extreme thermal physiological parameters (CT and CT_{max}) survived the experiment.

2.5 Determination of selected body temperature

To determine their selected body temperature (T_{sel}), toads ($N= 15$) were acclimated for two days at 25 °C with a photoperiod of 12:12. These toads were only used for this experiment. A thermal linear gradient was generated in the floor of the terrarium (180 cm long, 60 cm wide and 60 cm high). The terraria is built with aluminum sheets with a system of metal tubes in direct contact with the floor. The warm end of the terrarium was maintained by a heat source, that consisted of hot water pumped from a container using an electrical pump (ATMAN II, Model AT-301, China). Inside the container, an electric heater (400W), connected to a digital thermostat (AG, model TC-120L, San Juan, Argentina), maintained the temperature at 50 ± 1 °C. The cold end of the terrarium was maintained by the circulation of cold water using an electrical chiller (Thermo NESLAB

RTE 7, Newington, NH). To avoid dehydrating the toads, the floor of the terraria were covered by a moistened cotton cloth.

The terrarium was divided into 5 cm wide sections with a height of 15 cm to avoid interactions between neighbouring animals (Light et al., 1966). The thermal gradient was linear and ranged between 50 ± 1 °C to 8 ± 1 °C. To determine T_{sel} , individuals were subjected to this gradient for nine consecutive hours (1100 to 2100 hours). The body temperature was measured with an infrared thermometer (TES 1326s, TES Electrical Electronic Corp., Taipei, Taiwan, ± 0.1 °C) placed in contact with the dorsal skin of toad (every 30 minutes). We calculated the *db* index ($db = T_{sel} - T_b$), following the methodology proposed for Hertz et al. (1993), as the absolute deviation from the T_b and T_{sel} for each individual (individual variation).

2.6 Determination of locomotor performance in the laboratory and the field

Locomotor performance was measured as the total distance covered during 5 min of forced locomotion. We divided the total distance covered (cm) by the SVL of toad (cm) to estimate locomotor performance (Titon et al., 2010) at different temperatures (0, 5, 10, 15, 20, 25 and 30 °C). We measured 35 toads acclimated for two days at 25 °C with a photoperiod of 12:12. Toads were kept in plastic containers at target experimental temperatures (0, 5, 10, 15, 20, 25, and 30 °C) for at least 20 min before testing. The arena consisted of a circular aluminium tank (perimeter 79 cm with 10 cm high walls). Using a temperature-controlled jumping arena, we counted the number of laps covered by each individual over a 5 min test period. The appropriate temperature for each test was maintained by pumping water through the metal tubes using an electrical chiller (Thermo NESLAB RTE 7, Newington, NH) and monitored using a digital thermometer (TES 1312,

TES Electrical Electronic Corp., Taipei, Taiwan, ± 0.1 °C). Toads were induced to jump by tapping them with a wooden stick.

In the field, we measured the locomotor performance at different activity times from ~1000 to 1900. We used 5 toads, for each of the nine periods (1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800 and 1900), totalizing 45 toads measured. The temperature used for these experiments was the substrate temperature, recorded every five minutes with a data logger (iButton DS1922L, Maxim Corporation, USA). We only used each individual once to reduce the manipulation stress.

2.7 Data Analysis

We calculated the mean and standard error for all the thermal parameters. We analysed relationships using non-parametric Spearman correlations. We applied the Mann–Whitney U test to evaluate differences between the thermal parameters of the species studied because the data were not normally distributed and variances were not homogenous.

We used the Pearson regression to create a linear model of the relationship between dorsal temperatures measured by infrared thermometer and cloacal temperatures. We tested for data normality with the Shapiro-Wilk test. The PAST version 9.4 (Hammer et al., 2001) statistical package was used for statistical analysis.

3. Results

We detected differences between environmental temperatures (Wilcoxon pair test, $Z= 12.2$, $P< 0.001$) such that T_a (16.4 ± 0.19 °C) was higher than T_s (12.7 ± 0.13 °C). The mean relative humidity (Rh) was 36.8 ± 0.3 %. The activity period of post–metamorphosed

toads range from 1100 until 1900 hours (Fig. 2). The mean body size (SVL) was 1.5 ± 0.01 cm. The average T_b of 11.5 ± 0.12 °C was positively correlated with T_s (Spearman, $r = 0.68$, $P < 0.00001$, $N = 285$) and T_a (Spearman, $r = 0.26$, $P < 0.0006$, $N = 285$; Fig. 3).

The T_{sel} had a mean of 27.2 ± 2.8 °C, significantly higher than T_b observed in the field (Mann Whitney $U = 5.37$, $P < 0.00001$) (Fig. 3). The mean db index of post-metamorphosed Andean toads was 12.7 ± 2.7 °C.

Under field conditions, *R. spinulosa* had a maximum locomotor performance at 1500 hr, with a mean of 682.5 ± 45.1 SVLs, obtained when substrate temperature was equal to 23.1 °C (Fig. 4a). Locomotor performance was strongly associated with the substrate temperature (Spearman, $r = 0.76$, $P < 0.00001$; Fig. 4b). Under laboratory conditions, *R. spinulosa* had a maximum locomotor performance at 25 °C, with a mean of 927.2 ± 46 SVLs (Fig. 5). Locomotor performance decreased significantly at temperatures higher than 25 °C and lower than 15 °C (Kruskal-Wallis: $H_{(6, 36)} = 33.1$, $P < 0.000001$).

The mean critical thermal maximum (CT_{max}) was 36.9 ± 0.14 °C and the mean temperature of crystallization was -1.28 ± 0.14 °C. All individuals showed an exothermic reaction.

4. Discussion

The focal population of juvenile Andean toads shows a diurnal activity pattern similar to that observed for other *R. spinulosa* populations (Lambrinos and Kleier, 2003; Espinoza and Quinteros, 2008). We found that the substrate temperature largely determined the field body temperature during a diurnal activity period. Conduction probably played an important role in the gain of body temperature. These juvenile of *R. spinulosa* toads have a dark dorsal surface and yellow or orange feet (Lambrinos and Kleier, 2003). It is likely that the melanic skin of post-metamorphosed *R. spinulosa* helps

to improve the body heating in rigorous environmental conditions, as observed for other anuran species such as *Rana temporaria* and *Melanophryniscus rubriventris* (Vences et al., 2002; Sanabria et al., 2014).

Body temperature is also likely to be affected by heat loss through evaporation (Tracy, 1976). High air temperatures and dry air would facilitate a high rate of water loss through the skin, resulting in a great amount of heat loss. Under field conditions we observed that the juvenile of *R. spinulosa* always chose humid sites close to water bodies. This probably allows them to simultaneously maintain water balance and moderate body temperatures (Brattstrom, 1963). Similar behaviour was observed in juvenile toads of *Rhinella arenarum* in the lowlands of the Monte Desert (Sanabria et al., 2003). Lambrinos and Kleier (2003) found that juvenile of *R. spinulosa* used dry places for basking, but they were always very close to water or shade. In the studied population, toads always foraged in wet substrates or near water bodies. Small body size probably limits dispersal, and is also likely to be constrained by evaporative water loss.

Espinoza and Quinteros (2008) described thermal and hydric benefits of basking aggregations of juvenile toads in populations of *R. spinulosa* in northern Argentina. In our study population, we did not observe aggregations of toads during their diurnal activity period, but observed aggregation behaviour during inactive periods (overnight), when the toads congregated under rocks during low environmental temperatures. Nocturnal temperatures form a strong barrier to movement/activity for small toads (Lambrinos and Kleier, 2003).

Selected body temperature (T_{sel}) is considered the "ideal" body temperature an animal tries to achieve in the field (Labra et al., 2008). This is the body temperature at which physiological processes such as digestion, swimming performance, and locomotion are optimized (Wells, 2007; Navas et al., 2008). Field body temperatures of the studied *R.*

spinulosa population were below the selected body temperatures. Also, the *db* index showed a great deviation between T_{sel} and T_b , which suggests that animals always experience thermal environmental constraints (Hertz et al., 1993). Limitations to thermoregulation include those imposed by the thermal environment, predation risk and water balance (Huey and Slatking 1976; Herczeg et al., 2008). These limitations restrict the juvenile toads from obtaining a T_b close to the T_{sel} . Sanabria et al. (2011; 2012) observed a similar result for the *db* index in *R. arenarum* and *O. occidentalis* during the dry season (autumn) where the toads experienced in the field thermal constraints and a homogenous environment with a lack of adequate temperatures for thermoregulation.

The CT_{max} observed in the juveniles of *R. spinulosa* had a mean value close to 42.8 °C, which was in the range of temperatures obtained for other species such as *R. arenarum* (Sanabria et al., 2012) and *O. occidentalis* (Sanabria et al., 2013a) adults, and *Pleurodema nebulosum* juveniles (Sanabria et al., 2013b) of the Monte desert; and *Rhinella granulosa* in Caatinga, Brazil (Navas et al., 2007). However, the locomotor performance curve under laboratory conditions had a maximum performance at 25 °C. The temperature of maximum locomotor performance was close to the T_{sel} . Locomotor performance decreased rapidly at 30 °C, which was close to the CT_{max} .

Juvenile toads showed thermal coadaptation for the T_{sel} and maximum performance under laboratory conditions (Navas et al., 2007). Their locomotor performance in field conditions was correlated with changes in the substrate temperature during their primary activity period. The locomotor-vs-temperature curve under field conditions showed correspondence between maximum locomotor activity and maximum substrate temperature (1500 hours). At this time period the environmental substrate temperature was ~24 °C. Environmental temperature changed during the day, primarily as the solar ray angle changed over the ground. Consequently, the locomotor performance

changed during the activity time of post-metamorphosed toads (from ~1100 to ~1900; Fig. 5). The locomotor performance decreased rapidly until the end of activity at 1900 when the mean substrate temperature reach ~15.5 °C. It is likely that juvenile toads exhibit diurnal activity because solar radiation improves the thermal conditions for locomotion and finding and capturing prey (Child et al., 2008).

Juvenile of *R. spinulosa* probably forage during diurnal activity hours under suboptimal body temperatures. However, field body temperatures may be closer to the selected temperature for very short periods of time when insolation is high. The prevailing temperatures during this activity period allows toads to explore micro-environments, providing access to relatively larger areas in search of food.

The overnight temperature drops quickly and can reach temperatures close to 0 °C. Juvenile Andean toads showed supercooling with crystallization temperatures on the order of -1.5 °C. The post-metamorphosed toads probably experience temperatures below 0 °C during early hours of activity and overnight, therefore it is likely that the toads have a mechanism that allows them to tolerate temperatures below 0 °C. Supercooling is an efficient mechanism to avoid the freezing of tissues and also minimizes a lot of stressors that cause injury and are potentially lethal (Costanzo, 2012). The best known mechanism to avoid freezing for toads is the generation of plasma glucose that allows tissues to dehydrate and prevents damage from ice generation (Costanzo and Lee, 2013). In this case, glucose acts as a cryoprotectant in high enough concentrations to significantly depress the freezing point and reduce the amount of ice formed during freezing (Storey, 1997). Juvenile Andean toads are likely to experience negative temperatures, and the production of plasma glucose may operate to avoid mortality by freezing. Our data indicate that the diurnal activity of juveniles of *R. spinulosa* confers benefits, allowing them to increase their metabolic and digestion rate, and growth. *R. spinulosa* is a specie with wide

latitudinal and altitudinal distribution (Ceï, 1979; Veloso and Navarro, 1988). Our data show that *R. spinulosa* is a thermos-generalist specie, with ecological, behavioural, and physiological plasticity in that allows it to survive in adverse microclimatic conditions. Specifically, we uncovered plasticity in the high *db* index and broad locomotor performance curve. More research is needed to understand the role of diurnal activity and the antifreezing mechanism in *R. spinulosa*.

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Figure legends

Figure 1: A) The collection site is a typical dry environment of Monte Desert. Situations in which we found toads during sampling: A) individuals on wet surfaces with free water less

than 1 mm deep; B) Individuals on moist bare soil without free water; C) Individuals with body parts on the water.

Figure 2: Temperatures and relative humidity for the study site during the experiment. The grey bar shows the activity period and the black bar shows the inactivity period of post-metamorphosed Andean toads *Rhinella spinulosa*.

Figure 3: Frequency of body temperatures of metamorphosed toads in the field (black bars). The substrate temperature (continuous line) is higher than the air temperature (dotted line). The mean body temperature of juvenile toads in the field (grey arrowhead) was less than the selected body temperature (black arrowhead).

Figure 4: A) Variation in the locomotor performance under field conditions during the activity of metamorphosed toads. The horizontal dotted line shows the maximum locomotor performance under laboratory conditions. The locomotor performance is expressed as the result of the relativization of the distance covered by the toad in five minutes and the animal snout vent length (Locomotor performance = Distance Moved / SVL; Titon et al., 2010). B) Variation of substrate temperature during experiments of locomotor performance. The boxes indicate the period of day with clouds and sun.

Figure 5: Variation in locomotor performance under laboratory conditions of metamorphosed toads. The arrowheads show the critical thermal maximum (CT_{max} , black) and the crystallization temperature (CT , grey). The arrows show the mean body temperature in the field (T_b , black) and the selected body temperature (T_{sel} , grey). The dotted vertical lines show the minimum and maximum T_b recorded for toads during field

activity. The locomotor performance is expressed as the result of the relativization of the distance covered by the toad in five minutes and the animal snout vent length (Locomotor performance = Distance Moved / SVL; Titon et al., 2010). Mean value of the thermal parameter in brackets.

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