

Cutaneous Resistance to Evaporative Water Loss in Brazilian *Rhinella* (Anura: Bufonidae) from Contrasting Environments

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In anurans, cutaneous resistance (R_s) to evaporative water loss (EWL) correlates with ecological features and is influenced by taxonomic position. For example, bufonids are regarded as exhibiting a permeable skin that seems typical for terrestrial anurans. However, this assumption is supported by information on only four bufonid species; therefore, the enormous ecological diversity of the family remains poorly investigated. To assess whether variation in R_s within related bufonids correlates with environmental aridity, we measured area-specific rates of EWL of two Brazilian populations of *Rhinella granulosa* (previously *Bufo granululosus*), one from the Atlantic Forest and other from the semi-arid Caatinga, and compared both with the forest species *R. ornata*. *Rhinella granulosa* from the Atlantic Forest had higher cutaneous resistance than conspecifics from Caatinga and *R. ornata*. *Rhinella ornata* presented the lowest cutaneous resistance values. However, R_s were very close to zero in all three populations. We conclude that enhanced R_s is not part of the suite of traits allowing *R. granulosa* to exploit the Caatinga, and that variation in R_s within bufonids may relate to traits other than water conservation. Some information on microhabitat occupation and ventral skin morphology supports the idea that exceptional abilities for detecting and taking up water may be the key factors enhancing the survival of *R. granulosa*, and possibly other bufonids, in xeric environments.

AMPHIBIANS that lack physiological mechanisms to reduce evaporative water loss (EWL) have been considered 'typical amphibians' (Shoemaker et al., 1992) because most taxa studied to date show little capability to restrict EWL through the skin (Wygoda, 1984). However, some amphibians have EWL rates lower than that of a free water surface (Withers et al., 1984; Young et al., 2005), and may even use changes to the rate of mucous secretion and evaporation to avoid overheating (Lillywhite and Licht, 1975; Tracy et al., 2008). Many morphological and physiological traits confer resistance to EWL, either permanently or during the dry seasons, including cutaneous lipid secretions, as in arboreal hylids (Withers et al., 1984), the production of cocoons in aestivating species (Lee and Mercer, 1967), and possibly some properties of the anatomy of the skin, such as a calcified layer in the dermis of some species (Toledo and Jared, 1993).

In anurans, rates of EWL vary significantly among species in a pattern influenced by both phylogenetic and ecological components. Many arboreal hylid species, for example, exhibit high resistance to water loss (R_s , reported $s\text{ cm}^{-1}$), whereas terrestrial anurans such as bufonids, most of which are ground dwellers, exhibit low resistance (Wygoda, 1984; Young et al., 2005). At narrower taxonomic levels (e.g., family or genus), the evolutionary and ecological correlations of the variation in R_s are still weakly understood (but see Young et al., 2005). So far, this issue has been assessed mainly in the so-called waterproof species, such as arboreal hylids from Australia and South America. Therefore, knowledge about most terrestrial taxa remains very limited, even for those groups presenting very diverse ecological traits. For example, despite the enormous diversity and distribution of the family Bufonidae, only four out of 505 species have had their R_s estimated (Lillywhite, 2006).

Bufonids are usually regarded as having high rates of EWL, with R_s values ranging 0–1.7 $s\text{ cm}^{-1}$ (Withers et al., 1984;

Wygoda, 1984, 1988; Young et al., 2005). Except for *Anaxyrus cognatus* (previously *Bufo cognatus*), which occurs in xeric regions of northern Mexico and southwestern USA, all investigated bufonids occur in humid environments. Since the R_s estimates performed to date poorly represent the ecological diversity of this group, we aimed to measure rates of EWL for species and populations of terrestrial bufonids inhabiting contrasting hydric environments. We focused on *Rhinella granulosa* (previously *Bufo granululosus*), a widespread South American species (Narvaes, 2003). Specifically, we ask if cutaneous resistance to EWL differs between populations from semi-arid and humid environments, and discuss whether or not reduced rates of EWL have favored the colonization of xeric areas by this species. Our approach was to contrast toads from mesic and xeric regions with respect to area-specific rates of EWL, and to compare results with those of agar replicas of each individual toad, which are assumed to lose water freely. For additional comparison with a species from a mesic environment, we also investigated *Rhinella ornata* (previously *Bufo ornatus*), which occurs only in forested settings. We expected that, within the context of relatively high levels of EWL that appear to characterize bufonids, *R. granulosa* from semi-arid regions would exhibit higher R_s than conspecifics from the forest, and higher than *R. ornata*.

MATERIAL AND METHODS

Environments and experimental animals.—The Brazilian Atlantic Forest is a humid environment characterized by dense ombrophilous or semi-deciduous vegetation, mean temperatures of 18–23°C and high precipitation, between 1200 and 1800 mm over the year (INMET, 1990; Morellato and Haddad, 2000). In contrast, the Caatinga is a Brazilian semi-arid environment characterized by xerophytic vegetation, high temperatures (mean 26–28°C), and unpredictable rainfall, usually between 300 and 800 mm over the year. Dry

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Fig. 1. Individual of *Rhinella granulosa* presenting a water conservation posture when kept under dehydrating conditions. Head is kept close to the ground, often touching it, and limbs are retracted close to the body. Eyes are partially closed.

years, sometimes in sequence, occur occasionally (Reis, 1976; INMET, 1990).

Rhinella granulosa from the Atlantic Forest were collected in February 2007 at Ilhéus, Bahia State. Conspecifics from Caatinga were collected in April 2007 at Angicos, Rio Grande do Norte State, also in northeast Brazil. *Rhinella ornata* were collected in December 2006 at an Atlantic Forest area inside the University of São Paulo campus. Because the three groups were collected during the rainy season in their respective areas and because testing began within a week of capture, we do not expect that season of capture and acclimation have influenced the results.

In the laboratory, toads were kept in terraria (80 × 30 × 35 cm) with water available *ad libitum*. Rocks and roofing tiles were used as shelters. They were fed *Tenebrio* larvae and cockroaches every three days. A two-day period of fasting preceded experiments. Photoperiod was 14:10 (L:D) and daily temperature ranged 22–27°C.

Measurements of evaporative water loss.—Before dehydration experiments, toads were taken from the terraria and hydrated for two hours inside plastic containers with 2 cm of tap water. All individuals attained constant mass, indicating that they had become fully hydrated. Toads were patted dry with a paper towel and weighed to the nearest 0.01 g. The maximum body mass attained by a toad was assumed to be its fully hydrated mass (m_h), i.e., the mass of a hydrated toad including water contained in the urinary bladder. After m_h was determined, we tried to empty the urinary bladder of toads through abdomen compression. Because this method was not always successful, we excluded from analyses the data on animals which urinated during the dehydration experiments (this was evident either visually or easily deducible from a drastic change in body mass).

Experiments were performed inside a walk-in climatic chamber set up at 50% relative humidity and 25°C. During dehydration trials, animals were individually kept inside suspended cubical nylon net cages (8 × 8 × 8 cm) in front of a fan set up to produce a wind speed of 0.4 m/s, which, according to preliminary experiments, was the strongest air flow that did not produce undesirable toad activity. Toads in

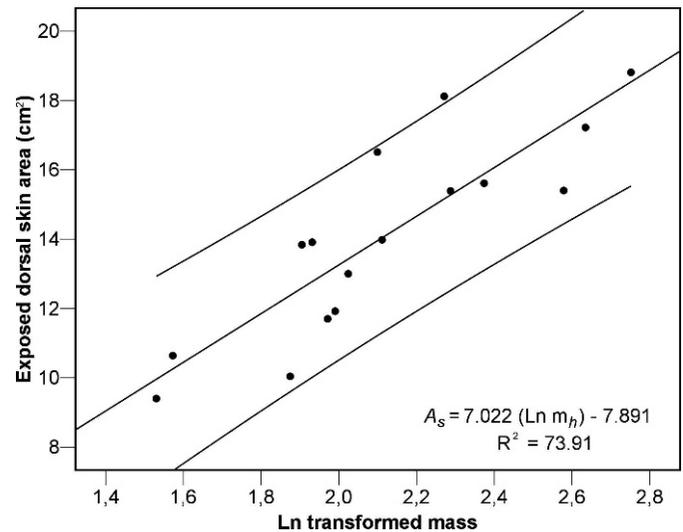


Fig. 2. Relation between exposed dorsal skin area (A_s , reported cm^2) and the natural logarithm (Ln) transformed mass (g) of fully hydrated *Rhinella granulosa* (including urinary bladder water). Confidence intervals (95%) are shown. This relation was used for A_s determination for the three populations considered in this investigation.

trials were weighed every 30 minutes until they reached 90% of m_h , an endpoint that was usually reached between four and five hours. We started measurements after toads exhibited a typical water conservation posture (Heatwole et al., 1969), which usually happened within 20 minutes (Fig. 1). At the end of each trial we measured dorsal skin temperature with a noncontact infrared thermometer (Istrutherm TI-900). We also monitored air temperature and relative humidity using a digital hygro-thermometer (Sunleaves SDMHT600) positioned above the cages. We calculated rates of evaporative water loss from the regression slope of body mass versus time, a trend that was always linear. Every ten minutes during the trial, we assessed activity, position in the chamber, and position relative to airflow. This information was used to place each agar replica (see below) in a position similar to its subject toad. Animals were excluded from the analysis if they were more active during experiments than just minor position adjustments. We performed all experiments between 1000 and 1800 h.

Estimates of exposed dorsal skin.—We interpolated the exposed dorsal skin from an equation relating this variable to m_h using a specific equation for *R. granulosa*. To produce this equation (Fig. 2), we measured the fully hydrated mass (m_h) of 16 individuals, euthanized them with diethyl ether overdose (through inhalation), and froze them in the water conservation posture. We painted all exposed skin with nail polish. Then, we allowed carcasses to thaw and removed the painted skin surface together with the solidified film of nail polish, using a scissor. The film was separated from the skin and discarded. The skin alone was digitized. Images obtained were analyzed in ImageJ software (Rasband, 2008) for determination of area (defined as the exposed dorsal skin area, A_s).

Cutaneous resistance evaluations.—According to Spotila and Berman (1976), total resistance to evaporative water loss (R_T , reported s cm^{-1}) can be calculated as the difference in water vapor density between air and animal divided by the

evaporative water loss:

$$R_T = (SD_S - RH SD_A) \div EWL_{SA}$$

Where SD_S is the saturation vapor density (g cm^{-3}) of water in air at skin temperature, SD_A is the saturation vapor density (g cm^{-3}) of water at air temperature, RH is the relative humidity of the air (expressed fractionally), and EWL_{SA} is the surface area specific rate of water loss ($\text{g cm}^{-2} \text{s}^{-1}$). R_T can be also calculated as the sum of boundary layer resistance (R_B) and cutaneous resistance to water loss (R_S):

$$R_T = R_B + R_S$$

The boundary layer resistance (R_B) is provided by the air surrounding the animal and depends mainly on the topography of its body. It can be determined using agar replicas of the toads, which behave as free-water surfaces and thus lack cutaneous resistance (i.e., $R_T = R_B$). According to equation (2), it is possible to estimate R_S by subtracting R_B (calculated for agar replicas using equation (1) from the R_T value (calculated for live toads using equation [1]).

In this study, we did not measure the contribution of respiratory water loss to the total EWL. However, pharmacological suppression of pulmonary ventilation did not significantly affect evaporative water loss in *Rana pipiens*, *Agalychnis dacnicolor*, or *Bufo marinus* (Bentley and Yorio, 1979) or in *Scaphiopus holbrooki* (Wygoda, 1981). Accordingly, water loss due to respiration proved to be about 100 times smaller than skin loss in *Rana pipiens* (Spotila and Berman, 1976). Given that they include a small pulmonary component, cutaneous resistance values presented in this article should be understood to be "effective R_S " measurements.

Agar replicas.—To obtain agar replicas, toads were immersed upside down in odontological alginate, held by the ventral area, such that the belly and ventral part of the limbs remained at the alginate level. The alginate solidified within 1–2 minutes and the animals were removed with no injury. Alginate templates were then filled with 3% agar (97% water). Each toad had its own corresponding replica. All the experimental procedures described above were conducted with toads and agar replicas in the same way.

Data analysis.—We applied square root transformation on cutaneous resistance values in order to attain homoscedasticity. We used analysis of covariance (ANCOVA) to determine whether fully hydrated mass was a significant covariate of R_S in comparisons among the three populations. Since this was not the case, mass was removed from the analysis, and comparisons were made using one-way ANOVA and Fisher's Least Significant Difference (LSD) as a *post hoc* test. Unless stated otherwise, values presented are means \pm standard deviation. N describes number of individuals.

RESULTS

Rates of evaporative water loss, effective cutaneous resistance, and boundary layer resistance for *R. granulosa* and *R. ornata* are presented in Table 1. The three groups of toads exhibited similar boundary resistances ($F_{2,48} = 0.017$; $P = 0.983$). Fully hydrated mass was not a significant covariate when comparing cutaneous resistance between populations ($F_{2,47} = 0.311$; $P = 0.58$). *Rhinella granulosa* from the Atlantic

Forest had higher cutaneous resistance than conspecifics from Caatinga ($P = 0.002$) and *R. ornata* ($P < 0.001$). *Rhinella granulosa* from Caatinga exhibited higher cutaneous resistance than *R. ornata* ($P = 0.03$). However, R_S values were very close to zero in all three populations.

DISCUSSION

Our data on *R. granulosa* and *R. ornata* are consistent with previous results for bufonids, indicating very low skin resistance to water loss despite environmental aridity. Although statistically significant, differences between groups do not seem large enough to be of ecological relevance, i.e., they probably do not confer differential survival capacities. Also, these small differences may probably be overcome by behavioral traits that influence evaporative water loss in the field, such as postural adjustments or microhabitat selection. Since R_S values were lower than boundary resistance (R_B) and very close to zero in all populations investigated, we consider skin resistance to water loss to be negligible. Furthermore, it is hard to say whether there are actual differences in R_S between the three groups, considering that such low values are comparable to experimental error (determined by inaccuracies in the estimates of surface area or calculations of R_B). Given these considerations, enhancing R_S seems not to be part of the suite of traits allowing *R. granulosa* to exploit the semi-arid Caatinga. Our data seem robust in reinforcing the idea that bufonid toads may colonize more xeric environments with R_S values that are considered low for anurans in general.

The low values reported for these three bufonids contribute to evidence that a strong systematic component influences anuran R_S . Other factors such as climate and life habits (e.g., terrestrial, arboreal) influence R_S , but the relative contribution of each of these factors is difficult to assess at this point. The strongest data set available comes from the genus *Litoria*, in which R_S is influenced by phylogenetic position, so that sister species present similar R_S levels. However, the top values within *Litoria* belong to arboreal species (Young et al., 2005), yet arboreal habits do not predict high R_S in *Platymantis* ceratobatrachids (previously ranids) or *Eleutherodactylus* eleutherodactylids (Rogowitz et al., 1999; Young et al., 2006). The South American genus *Phyllomedusa*, highly arboreal, exhibits the highest R_S for hylids (Wygoda, 1984). Although all species investigated so far occur in semi-arid environments, it is likely that even the forest *Phyllomedusa* exhibit high R_S , since skin glands specialized for lipid secretion occur in *P. distincta*, a forest species (Neves, 2003), and in *P. hypochondrialis* (Barbeau and Lillywhite, 2005), whose distribution comprises mainly forested biomes (Caramaschi, 2006). This diversity in R_S profiles between groups exhibiting similar life habits or habitats may reflect differences among taxa in their use of skin in functional tradeoffs between respiration, reproduction, defense and thermoregulation (Toledo and Jared, 1995).

The array of strategies used by anurans to colonize desert environments is quite diverse and seems to involve both strategies based on water conservation and on water uptake (McClanahan et al., 1994; Navas et al., 2004). Given the high susceptibility to dehydration of *R. granulosa*, and the observation that juveniles are diurnal and active under very hot and dry conditions, water uptake and behavioral hydroregulation may be essential (Navas et al., 2007). Indeed, juveniles retreat to shelters when the ground is

Table 1. Habitat, Fully Hydrated Mass (Mass), Surface Area Specific Rate of Evaporative Water Loss (EWL_{SA}), Total Resistance (R_T), Effective Cutaneous Resistance (R_S), and Boundary Layer Resistance (R_B) for *R. granulosa* and *R. ornata*. Values are mean ± SD.

Species	<i>R. granulosa</i>	<i>R. granulosa</i>	<i>R. ornata</i>
Environment	Caatinga (semi-arid)	Forest	Forest
<i>n</i>	16	16	19
Mass (g)	9.6 ± 4.86	12 ± 4.86	12.6 ± 2.53
EWL _{SA} (mg cm ⁻² h ⁻¹)	34.4 ± 5.28	31.0 ± 3.26	45.9 ± 3.51
R _T (s·cm ⁻¹)	0.53 ± 0.12	0.65 ± 0.07	0.45 ± 0.04
R _B (s·cm ⁻¹)	0.37 ± 0.1	0.36 ± 0.08	0.37 ± 0.07
R _S (s·cm ⁻¹)	0.16 ± 0.13	0.28 ± 0.12	0.08 ± 0.05

too dry, and in thermal gradients appear to hydroregulate more than thermoregulate. In addition, the microhabitats occupied by *R. granulosa* are 5 to 15°C cooler and show 10 to 60% higher RH than exposed ground (Navas et al., 2007), so that microhabitat selection certainly reduces dehydration rates. These behaviors, associated with keen ability to extract water from soils with low water potential or to take advantage of transient sources of water, are likely to contribute to the success of *R. granulosa* in the Caatinga. In terrestrial anurans, the pelvic skin area is highly specialized for water absorption (McClanahan and Baldwin, 1969), and this characteristic is exaggerated in *R. granulosa*, which exhibit remarkable granularity and vascularity in that region (Navas et al., 2007).

Bufofids figure among the most terrestrial amphibians, and exhibit at least 35 species that occur in semi-arid areas all around the world (IUCN, 2008. Global Amphibian Assessment, available at www.globalamphibians.org). It is likely that traits of physiological ecology allowing for the colonization of such environments vary among species, but reduced EWL seems not to be a key factor. Some species such as *Rhinella granulosa* do not seem to rely on extreme avoidance behaviors or reduced EWL, and even so have prolonged activity seasons in the semi-arid Caatinga. Therefore, we expect these animals to have exceptional abilities for detecting and taking up water from the soil. These abilities seem a promising field for future research.

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