Evaluation of the combined temperature and relative humidity preferences of the Colombian terrestrial salamander Bolitoglossa ramosi (Amphibia: Plethodontidae)

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<td>Amphibian; Bolitoglossa ramosi; Hydric environment; Plethodontid salamander; Thermal selection; Urodela</td>
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Evaluation of the combined temperature and relative humidity preferences of the Colombian terrestrial salamander *Bolitoglossa ramosi* (Amphibia: Plethodontidae)

C.A. Galindo\(^a\), E.X. Cruz\(^b\) and M.H. Bernal\(^c\).

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Evaluation of the combined temperature and relative humidity preferences of the Colombian terrestrial salamander Bolitoglossa ramosi (Amphibia: Plethodontidae)

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Abstract

Temperature and humidity are critical factors for terrestrial lungless salamanders, as their body temperatures are largely determined by the environmental temperature and require moisture to sustain cutaneous respiration. Herein, we evaluated the preference of Bolitoglossa ramosi (Brame and Wake 1972) between a high temperature and a high relative humidity, the influence of temperature on the relative humidity preferences, and the influence of the relative humidity on the thermal preferences. This study was performed in a field location in the municipality of Líbano, Tolima, Colombia. There, on different nights, we collected 84 adult salamanders and carried out the preference experiments, using aluminum troughs with different thermal and relative humidity gradients. We found that between the high temperature and high relative humidity, salamanders preferred the high relative humidity. However, B. ramosi selected high temperatures when the gradient had a high relative humidity, and low temperatures when the gradient had a low relative humidity. These results show that B. ramosi is able to
thermoregulate and hydroregulate. Nevertheless, hydorregulation seems to be more important than thermoregulation as these salamanders always selected the high relative humidity gradients while their thermal selection relied on the hydric environment.

**Key words:** Amphibian; *Bolitoglossa ramosi*; Hydric environment; Plethodontid salamander; Thermal selection; Urodela.
Due to their ectothermy and permeable skin, environmental temperature and humidity are critical factors for the survival of terrestrial amphibians. Ambient temperature largely determines their body temperature (Navas et al. 2008; Köhler et al. 2011), whereas humidity is important to avoid the evaporative water loss through the skin and to keep their hydric state (Shoemaker et al. 1992). Both conditions, body temperature and hydric state, are known to strongly affect the physiological and behavioral performance of amphibians (Jørgensen 1997; Navas et al. 2008; Anderson and Andrade 2017). Amphibians are able to thermoregulate behaviorally to maintain their body temperatures into a preferred range where the biological activities can be carried out successfully (Hutchison and Dupré 1992; O’Connor and Tracy 1992; Raske et al. 2012; Balogová and Gvoždík 2015; Strickland et al. 2016). On the other hand, they avoid excessive water loss by selecting microhabitats that allow the animals to balance water loss with water uptake, or to limit activity to periods with favorable conditions (Wells 2007; Winters and Gifford 2013). Therefore, terrestrial amphibians should prefer habitats with an optimal combination of temperature and humidity to maximize the intake of energy and regulate their hydration state (Preest and Pough 1989; Titon et al. 2010; Köhler et al. 2011; Mitchell and Bergmann 2016), and because the interaction between these two factors influences the regulation of each of them (Anderson and Andrade 2017). For example, high environmental temperatures result in higher evaporative water loss in terrestrial amphibians, and physiological performance decreases when an organism becomes dehydrated (Preest and
Pough 1989, 2003; Rogowitz et al. 1999; Anderson and Andrade 2017). As a consequence, salamanders increase their resistance to water loss at warm temperatures and, conversely, decrease it under cool temperatures (Winters and Gifford 2013; Riddell and Sears 2015). Despite the importance of these interactions in amphibians, the study of thermal and humidity preferences has generally considered them separately (Spotila 1972; Hutchison and Dupré 1992; Wells 2007). Recently, Mitchell and Bergmann (2016) tested for interactions between environmental temperature, moisture and organismal hydration on temperature and moisture preferences in the green frog *Lithobates clamitans*. In this work, they found that frogs selected environmental conditions that minimized cutaneous evaporative water loss, hydroregulating more stringently than thermoregulating.

Plethodontid salamanders are a very interesting biological model to study the interaction between temperature and humidity preferences due to their particular physiology. They are strongly dependent on the ambient temperature, as their body temperature is equal to the air or substrate temperature (thermoconforms) (Brattstrom 1963; Catenazzi 2016; Lunghi et al. 2016), and they are among the tetrapods with the lowest metabolic rate (Chong and Mueller 2012). Moreover, they are unique among terrestrial vertebrates in having completely lost their lungs relying mainly on the skin surface for gas exchange, which causes high rates of desiccation principally when they are in air with a lower vapor pressure than that of their skin (Feder and Londos 1984; Feder and Burggren 1985; Gifford...
Consequently, salamanders require moisture to sustain cutaneous respiration (Spotila 1972; Gatz et al. 1975; Feder 1983), and their activities are generally nocturnal and limited to moist habitats to minimize water loss (Feder 1983; Peterman and Semlitsch 2014; Gifford 2016). Additionally, terrestrial salamanders of the family Plethodontidae are generally philopatric (Kleeberger and Werner 1982), exhibiting minimal dispersal (Liebgold et al. 2011; Basile et al. 2017), which makes them extremely dependent upon suitable temperature and moisture microclimates to successfully forage and meet their energy requirements (Fraser 1976). Although it has been found that through a season or year, cave salamanders are able to actively search habitats with suitable conditions of temperature and moisture (Lunghi et al. 2015), while terrestrial salamanders are often more abundant in areas of dense cover that retain moisture (O'Donnell et al. 2014).

*Bolitoglossa ramosi* (Brame and Wake 1972) is a plethodontid salamander endemic to Colombia, with a distribution restricted to the eastern slope of the Central Cordillera, between 1 200 and 2 000 m altitude (Palacio et al. 2006). This species is listed as Least Concern by the IUCN (International Union for Conservation of Nature) because it is common and with presumed large populations (Castro et al. 2004). *B. ramosi* is mainly arboreal, as are the majority of tropical plethodontids (McEntire 2016), nocturnal, with direct development and limb regeneration (Arenas et al. 2017), and commonly found on top of leaves, small trees or on the ground, mostly during cloudy nights or after rain events when
the air relative humidity is high and the environmental temperature is low. However, there are few studies on its biology, and particularly on its thermal and hydric relations. Among them, Cruz et al. (2016) found a positive and significant relationship between the body temperature and the substrate and air temperatures, demonstrating that this species is a thermoconformer, as it has been also reported in other plethodontid terrestrial species of the genus *Hydromantes* (Lunghi et al. 2016). They also reported that the body temperature showed a higher thermal dependence on the substrate than the air temperature, indicating that *B. ramosi* presents a thigmothermic regulation.

In this study we tested if: (1) *Bolitoglossa ramosi* would prefer a high relative humidity rather than a high temperature, in a combined temperature and relative humidity gradient; (2) the temperature would have any effect on the relative humidity preferences; and (3) the relative humidity would have any effect on the thermal preferences. We hypothesized that between these two environmental variables, salamanders would prefer the high relative humidity because they depend on moisture to gas exchange, and are particularly abundant at a high relative humidity in the field. In addition, we also hypothesized that under an upper uniform experimental temperature or relative humidity, salamanders would select the high relative humidity to avoid water loss, and the high temperature to increase their physiological performances, respectively.

**Materials and methods**
Experimental animals

The present study was carried out with 84 adult salamanders collected by hand (mean of snout-vent-length= 32.47 ± 1.58 mm, range: 29.10 - 35.70 mm), between November and December 2015, from different places of a pre-mountain forest patch in the municipality of Libano (04º54’36.4” N, 75º03’43.7” W, 1750 m altitude) Tolima, Colombia. This region has an annual mean temperature of 20.5°C, a precipitation of 2262 mm, and an air relative humidity between 85 and 100% (Cortolima 2009). After capturing the animals, from 18:00 to 20:00 hours, they were taken to a field location near the collection sites (approximately 1000 meters straight-line distance), where the experiments were conducted during the same night, between 20:00 and 24:00 hours. Salamanders were used only once and later released at the site of capture. We studied adults (males and females, indistinctly) because they were abundant and easy to collect in several distant places, which decreased the possibility of recapturing the same salamander for the experiments, as they have low dispersal (Liebgold et al. 2011; Basile et al. 2017).

Temperature and relative humidity gradients

We built a combined thermal and relative humidity gradient for each of two rectangular aluminum troughs of 200 cm (length) x 7 cm (width) x 5 cm (depth), placed in parallel inside a greater aluminum channel of 280 cm (length) x 15 cm (width) x 15 cm (depth) which was partially filled with dechlorinated water. We
used dry ice and a 200-W conventional aquarium thermostat heater (ReSun – Sunlike-200) at opposing ends of the greater aluminum channel, in order to obtain a gradient from 15 to 30°C on the surface of the two experimental troughs. Additionally, along the experimental troughs, we positioned a uniform organic substrate with great capacity to retain water (“peat”), which was from partially to totally hydrated (with dechlorinated tap water), so as to provide a relative humidity gradient on the substrate surface between 65% and 95%. After each trial, we moved and sprayed the substrate with dechlorinated tap water to ensure the levels of relative humidity required. Seven sensors of digital thermo-hygrometers (Extech 44550, precision of ±1°C) were placed on the surface of the substrate at intervals of 2°C and 4% RH through the gradients. We selected these thermal and relative humidity gradients according to the daily environmental ranges recorded in the microhabitat of the salamanders studied (diurnal hours: 18-23°C, 60-89% RH; night hours: 17.1-21.2°C, 73-99% RH).

Thermal and relative humidity preference experiments

In order to assess the temperature and relative humidity preferences of the salamanders, and our three hypotheses, we carried out three experiments. In the first, we simultaneously placed 9 salamanders in each of two combined thermal and relative humidity gradients, where the end of the first gradient was 15°C-65% RH and the opposite end was 30°C-95% RH, while the end of the second gradient was 15°C-95% RH and the opposite end was 30°C-65% RH. We repeated this experiment the next night with another 9 individuals per gradient (collected before...
releasing the animals previously studied to avoid pseudoreplication), for a total of 36 different salamanders (9 X 2 X 2). Later on, in the second experiment, we used 6 salamanders (at the same time) for each of two gradients of relative humidity between 65% and 95%, one of them at a uniform temperature of 22 ± 0.5°C and the other at 25 ± 0.5°C. We also repeated the same experiment the following night with 6 other salamanders per gradient (collected before releasing the animals previously studied), for a total of 24 (6 X 2 X 2). Finally, in the third experiment, we placed 6 salamanders simultaneously in each of two thermal gradients from 15°C to 30°C, one of them at a uniform relative humidity of 75 ± 1.5% and the other at 95 ± 1%. As in the previous experiments, the third experiment was repeated the next night with another 6 individuals per gradient, for a total of 24 different salamanders (6 X 2 X 2). We did not test individual salamanders in the gradient so as to increase the number of data obtained per trial, and because previous essays indicated that salamanders always began to individually explore their new environment and later stopped anywhere.

Prior to the trials, we kept the salamanders for 30-45 minutes in plastic containers at the environmental temperature, with wet soil and leaf litter from the capture site, which were sprayed with dechlorinated water to ensure the salamanders were hydrated. Then, we randomly placed three groups, with the same number of salamanders, in the middle and at each end of the gradient, and allowed them to explore and become habituated for 30 minutes before we began the recordings. We kept the gradient in a dark room to eliminate possible light cues, as Bolitoglossa ramosi is primarily nocturnal. However, a dim red light from a
headlamp allowed for continuous visibility of each salamander. We used the scan
method (Lehner 1996) to record the temperature and relative humidity at the point
where each salamander (at the middle of the body length) was found in the
gradient every 30 minutes (three measurements per salamander).

Statistical analyses

For all statistical analyses, we used the mean of the three data points
collected for each salamander in the gradient to eliminate pseudoreplication. The
intra-individual coefficient of variation for these data was less than 14% in almost
all cases (97.6%), indicating a low dispersion around the mean (Zar 1999). Then,
we separately compared the temperature and relative humidity selected by the
salamanders between the two parallel gradients, using the Mann–Whitney U test
as data were not normally distributed (Shapiro-Will test, \( P < 0.01 \)) and variances
were not homogenous (\( F \) test, \( P < 0.01 \)). All statistical analyses were conducted in
SPSS Statistic 19.0 (Inc., Chicago, Illinois).

Results

In experiment 1, in the gradient from 15°C-65% RH to 30°C-95% RH, the
salamanders selected a place with a mean temperature of 28.4°C ± 1.02°C
(median = 28.7°C), and 92% ± 2.14% of relative humidity (median = 93.7%) (\( n =
18 \)). In the parallel gradient, from 15°C-95% RH to 30°C-65% RH, the
salamanders preferred a mean temperature of 17.2°C ± 1.17°C (median =
17.2°C), and 93.6% ± 2.32% of relative humidity (median = 92.7%) (n = 18) (Fig. 1). Between the two experimental gradients, salamanders showed a significant difference in their preferences for temperature (Mann–Whitney $U = 324$, $P = 0.001$, $n = 36$), but not for relative humidity (Mann–Whitney $U = 119.5$, $P = 0.181$, $n = 36$) as it was higher than 90% in both gradients (Table 1).

In experiment 2, where the gradient had a uniform temperature of 22°C, the salamanders were found at a relative humidity between 91% and 94%, with a mean of 93% ± 1.12% RH (median = 93%) (n = 12). In the parallel gradient, at a uniform temperature of 25°C, the salamanders selected a relative humidity from 90% to 93%, with a mean of 92.4% ± 0.95% (median = 92.7%) (n = 12) (Table 1) (Fig. 2). Between the two gradients at the two different uniform temperatures, there was not a significant difference in the relative humidity preferred by the salamanders (Mann–Whitney $U = 39.5$, $P = 0.60$, $n = 24$).

Finally, in experiment 3, in the gradient with a uniform relative humidity of 95%, the salamanders selected a wide range of temperatures, between 18.2°C and 29.2°C with a mean of 24.9°C ± 4.55°C (median = 27.2°C) (n = 12). In the parallel gradient, at a uniform relative humidity of 75%, the salamanders now selected temperatures from 16.7°C to 20.6°C with a mean of 17.9°C ± 1.11°C (median = 17.9°C) (n = 12) (Table 1), (Fig. 3). Herein, the salamanders showed significant differences between the temperatures selected at the two different relative humidity gradients (Mann–Whitney $U = 127.5$, $P = 0.001$, $n = 24$).
Discussion

Amphibians are highly sensitive to changes in environmental variables such as temperature and humidity (Buckley and Jetz 2007; Wells 2007; Tracy et al. 2012). One mechanism by which amphibians can respond to these changes is across behavioral regulation to optimize physiological processes associated with survival activities (Strickland et al. 2016). In this study, the salamander Bolitoglossa ramosi selected both a high temperature (28.4°C) and a high relative humidity (92%) when this option was offered in the same gradient, which could be explained because both high temperature and high relative humidity increase the physiological performance in amphibians (Preest and Pough 2003; Kingsolver and Huey 2008; Köhler et al. 2011; Titon and Gomes 2017). However, in the parallel gradient, where the salamanders were faced with a selection between a high temperature with a low relative humidity, and a low temperature with a high relative humidity, they now chose a humidity greater than 90% even at a low temperature (17.2°C) (Fig. 1). Since the salamanders preferred a high relative humidity in both experimental gradients, hydroregulation seems to be more important than thermoregulation for B. ramosi, as has been found in other anurans (Brattstrom 1979; Tracy et al. 1993; Mitchell and Bergmann 2016) and urodeles (Spotila 1972; Feder 1982). This result was confirmed in experiment 2, where the salamanders always chose a relative humidity greater than 90% in both gradients at the two different constant temperatures (Fig. 2). A high hydric environment could be principally preferred for plethodontid salamanders as they lose water at
accelerated rates and depend on skin surface for effective respiration (Feder 1983; Jørgensen 1997; Gifford 2016).

In the gradient with a high uniform relative humidity (experiment 3), we found that Bolitoglossa ramosi selected temperatures greater than those in the low relative humidity gradient (Fig. 3). This result agrees with some studies that report that plethodontid salamanders are able to thermoregulate behaviourally along experimental thermal gradients (Spotila 1972; Feder and Pough 1975; Sievert and Andreadis 2002; Camp et al. 2014), but that the hydric requirements limit their thermoregulation (Feder 1982; Camp et al. 2014). According to our field data, we detected a slight thermal variation in the places where we observed the salamanders at night (bromeliads, upper sides of leaves, under leaf litter, moist soil), from 17.1°C to 21.2°C. Then, we postulate that although B. ramosi might thermorregulate and select warm temperatures (until 29.7°C), this salamander must accept the available temperatures instead of showing thermal preferences because of the limited thermal diversity in its microhabitat. In addition, lunglessness forces plethodontid salamanders to remain moist and limit their thermoregulation (Feder 1982). By contrast, in the experimental gradient with a lower uniform relative humidity, the salamanders chose low temperatures (Fig 3), which is defined in ectotherms as behavioural hypothermia and is explained as a strategy to reduce evaporative water loss or energy consumption (Malvin and Wood 1991; Tracy et al. 1993; Köhler et al. 2011). These preferences of B. ramosi found in the thermal and humidity gradients are in agreement with the habitat preferences showed for other terrestrial salamanders (O'Donnell et al. 2014;
Lunghi et al. 2015). Nevertheless, contrary to the limited thermal variation found in the field for this tropical species, temperate species show strong seasonal variation in microhabitat selection, such as preferences to relatively cold and humid sectors in summer but not during winter (Lunghi et al. 2015).

The geographic distribution and habitat preferences of *Bolitoglossa ramosi* have been poorly studied; however, this species has been reported in the Andean mountains of Colombia, occupying restricted forest patches composed of tall trees, epiphytes and variable vegetation on the forest floor that maintain good humidity conditions in the environment. According to our observations, *B. ramosi* is principally arboreal, which is a trait that is highly conserved or has evolved repeatedly in plethodontid salamanders, as it suggests adaptive benefits, particularly in tropical forests where diverse plants (e.g. bromeliads) provide moist microclimates for nesting, foraging opportunities and shelter (McEntire 2016). The preference of *B. ramosi* for a high relative humidity, around 90% (Fig. 2), is concordant with the high humidity available in the field during night hours when they were more surface active and abundant (above 85%). This result also agrees with the preferences of *Bolitoglossa paraensis* (Correa et al. 2012) and several species of salamanders that selected a relative humidity of the air correlated with that of the microhabitat chosen in nature (Spotila 1972; Wells 2007). On the contrary, the thermal preference of *B. ramosi* for high temperatures is not linked to its low and narrow microhabitat temperatures. It is unknown if this thermal preference might be related to its physiological maxima, as reported in larval stream salamanders (Strickland et al. 2016), or because species select...
temperatures at which metabolic function is optimized when given the choice
(Huey and Bennett 1987; Angilletta et al. 2006). The information on thermal
physiology is increasingly used to understand the species responses to
environmental changes (Lunghi et al. 2016). However, due to the notable
importance of humidity for B. ramosi, and plethodontid salamanders, it is also
fundamental to know more about the hydric tolerances and hydroregulation of
these species, particularly in a changing world affected not only by the rising of
temperature, but also for the alteration of the hydric environment (Cruz-Piedrahita
et al. 2018).

In conclusion, we found that Bolitoglossa ramosi might thermoregulate and
hydroregulate, as the salamanders were able to select between thermal and
relative humidity gradients. Particularly, with respect to our three hypotheses, we
found that: (1) B. ramosi prefers a high relative humidity rather than a high
temperature; (2) the temperature does not affect the preference of B. ramosi for a
high relative humidity; and (3) the relative humidity affects the thermal preferences
of these salamanders, as they selected high temperatures when there was a high
relative humidity, but low temperatures at the low relative humidity gradient. Little
is currently known on the ecology of neotropical salamanders (Correa et al. 2012),
and remains to be investigated to what extent the local distribution and abundance
of populations or species of salamanders are influenced by the reduction in
shadow and humidity refuges caused by deforestation or other anthropogenic
effects. Furthermore, there is also a lack of studies on the physiology of
salamanders (Gifford 2016), which are necessary to assess the impacts of local or

global threats to the conservation of these vertebrates.

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Beatriz”, in Líbano, Tolima, Colombia.
References


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**Figure legends**

**Figure 1.** Box plot of thermal (white boxes) and relative humidity (gray boxes) preferences of the salamander (*Bolitoglossa ramosi*) in the experiment 1. Box limits indicate lower and upper quartiles (Q1, Q3), whiskers indicate the range of the distribution excluding outliers, and the solid line indicates the median.

**Figure 2.** Box plot of relative humidity preferences of the salamander (*Bolitoglossa ramosi*) in two gradients with a uniform temperature of 22°C and 25°C. Box limits indicate lower and upper quartiles (Q1, Q3), whiskers indicate the range of the distribution excluding outliers, and the solid line indicates the median.

**Figure 3.** Box plot of temperature preferences of the salamander (*Bolitoglossa ramosi*) in two gradients with a uniform relative humidity of 75% and 95%. Box limits indicate lower and upper quartiles (Q1, Q3), whiskers indicate the range of the distribution, and the solid line indicates the median.
Fig. 1.

44x36mm (300 x 300 DPI)
Fig. 2.

44x35mm (300 x 300 DPI)
Fig. 3.

44x36mm (300 x 300 DPI)
Table 1. Percentage of individuals of *Bolitoglossa ramosi* that selected different ranges of relative humidity (RH) and/or temperature (T) in the three study experiments.

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<th>Experiment 2</th>
<th>Experiment 3</th>
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<td>at uniform RH</td>
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<td>Gradient 1: 15°C95%RH to 30°C65%RH</td>
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<th>Range of T (°C)</th>
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<th>Experiment 2</th>
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**Note:** - Data were not collected under these conditions