

**Universidade de São Paulo  
Instituto de Biociências  
Departamento de Fisiologia  
Programa de Pós-Graduação em Ciências Biológicas-Fisiologia Geral**

**Interação do comportamento e fisiologia dos anuros em resposta ao estresse térmico e hídrico: uma abordagem para entender a vulnerabilidade dos anuros às mudanças climáticas**

**Estefany Caroline Guevara Molina**

**São Paulo-SP  
2019**

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**Interaction of behavior and physiology of anurans in response to thermal and hydric stress: an approach to understand the vulnerability of anurans to climate change**

**Estefany Caroline Guevara Molina**

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**Orientador: Prof. Dr. Fernando Ribeiro Gomes**

**Co-orientador: Dr. Agustín Camacho Guerrero**

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Prof. Dr. Fernando Ribeiro Gomes  
Orientador

*Em memória da minha linda  
avó Carmen, ela começou este  
sonho comigo, me mostrou a  
beleza de persistir sob a  
tempestade e me ajudou  
acreditar que tudo é possível  
se lutarmos por isso.*

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**RESUMO**

Nesta tese de mestrado foi atualizado o modelo de termorregulação proposto por Heath (1970), integrando os efeitos do nível de hidratação sobre o comportamento de termorregulação e a tolerância térmica da Rã touro, *Lithobates catesbeianus* (Capítulo I). Para o comportamento de termorregulação foram medidas as temperaturas corpóreas preferenciais (pelas suas siglas em inglês, PBT) de indivíduos hidratados e desidratados, e como tolerância térmica, foram medidas a temperatura voluntária máxima e a temperatura crítica máxima (pelas suas siglas em inglês, VTMax e CTMax, respectivamente) em grupos de indivíduos com diferentes níveis de hidratação. O capítulo II utiliza as informações levantadas no capítulo I para avaliar os efeitos do nível de hidratação sobre o tempo de perda da função locomotora de indivíduos de *L.catesbeianus* expostos a sua VTMax. O conjunto de dados dos capítulos I e II apontam que a desidratação afeta negativamente não só o comportamento de termorregulação e tolerância térmica desta espécie, mas também o tempo necessário para os indivíduos perderem sua função locomotora ao serem expostos a sua VTMax. Nossos dados sugerem que a desidratação é uma variável importante que deve ser incluída para avaliar os efeitos das altas temperaturas e secas nos ectotermos de pele úmida. A integração temperatura-desidratação e seus efeitos nestes organismos podem ser incluídos em modelos de distribuição mecanicistas para atualizar a vulnerabilidade climática deles nos cenários atuais e futuros das mudanças climáticas.



**INTRODUCTION**

Climate change is increasing the frequency of stressful climatic conditions for organisms such as high environmental temperatures and droughts in many regions of the world (Barnett et al., 2005; Bates et al., 2008). These conditions have a great influence on the geographic distribution, behavior and physiological functions of animals, increasing their extinction rate worldwide (Malcolm et al., 2006; Post et al., 2008; Tewksbury et al., 2008; Ceballos et al., 2015). Wet skin ectotherms, such as anurans, are one of the groups of vertebrates most affected by high environmental temperatures and droughts (Stewart, 1995; MacNally et al., 2009, 2014, 2017). This can be due to its (1) low dispersion capacity, (2) need for humid conditions and/or water bodies for reproduction (Lips et al., 2005; Pounds et al., 2006), and (3) lack of morphological characteristics such as high thermal inertia or impermeable skin to protect them from overheating and drying (Tracy and Christian, 2005; Peterman et al., 2013). However, the combined effects of these conditions and the time they can support them have been evaluated for some taxa and this information in general is sparse (Beuchat et al., 1984; Preest and Pough, 1989; Moore and Gatten, 1989; Malvin and Wood, 1991; Tracy and Christian, 2005; Mitchell and Bergmann, 2016; Anderson and Andrade, 2017). The lack of integrating studies of the impacts of these conditions on the physiology and behavior of organisms makes it difficult to assess their climatic vulnerability. The interactive effects of temperature and drought also matters for animal conservation studies under current climate change scenarios (McMenamin et al., 2008).

To understand the combined effects of high environmental temperatures and low water availability on wet skin ectotherms, it is necessary to know how these conditions influence the thermoregulatory behavior and their thermal tolerance limits (Williams et al., 2008). A thermoregulation model proposed by Heath (1970) explains how ectotherms, through behavior, show "proportional" responses by changing their body posture to maintain their body temperature ( $T_b$ ) within a range of preferred body temperatures (PBT). Staying in the PBT range optimizes multiple physiological functions (e.g. locomotion, digestion, development, reproduction) (Licht, 1965; Stevenson et al., 1985; Hertz et al., 1993; Navas and Bevier, 2001; Angilletta et al., 2002; Tracy et al., 2010; Berger et al., 2011; Fontaine et al., 2018). Heath's model also argues that when the environmental temperatures increase and the  $T_b$  of these organisms exceed their PBT range, they may present another type of behavioral response called "all-or-none". This behavioral response implies a decision to either sustain the stressful thermal situation or quickly retract to avoid prompt mortality. The Voluntary Thermal Maximum (VTMax) represents an "all-or-none" behavioral response for such situations (Cowles and Bogert, 1944; Camacho and Rusch, 2017). At their VTMax, the individuals need to cool their body and reduce their  $T_b$ , so they move to a colder place, even at the cost of exposing themselves to a greater risk of predation (Camacho et al., 2018). If the animal cannot prevent its  $T_b$  from increasing further, it will reach its Critical Thermal Maximum (CTMax) (Cowles and Bogert, 1944), losing the locomotor response and dying from heat

shock (Cowles and Bogert, 1944). Added to this, when environmental temperatures rise, the rates of evaporative water loss also increase, potentially accelerating the dehydration of individuals and impairing their performance (Preest and Pough, 1989; Moore and Gatten, 1989). In the case of anurans, there is a dynamic relationship with hydration level, swiftly losing body water and rehydrating, or strongly cooling down through body water evaporation (Wolcott and Wolcott, 2001; Prates and Navas, 2009; Tracy et al., 2010; Anderson et al., 2017). Studies have shown that dehydration may alter their thermoregulatory behavior (PBT) and thermal limits (CTMax) (e.g. Mitchell and Bergmann, 2016; Anderson and Andrade, 2017). The combined effects of temperature and dehydration on thermoregulatory behavior, thermal limits and the performance of organisms, indicate that there is an associated time of tolerance to these conditions, before they begin to present irreversible damage (e.g. loss of locomotor function and death).

Two methods of measuring thermal tolerance, called the static and dynamic, can estimate the temperature level that animals can tolerate and the time they can support such exposure before loss locomotor function and death (Lutterschmidt and Hutchison, 1997; Cooper et al., 2008). The static method introduced by Fry et al. (1942), uses high or low constant stressful temperatures to estimate the time for a final lethal temperature to lead to 50% of the measured population dying from exposure (i.e. thermal death curves) (Fry, 1947, 1967). The dynamic method introduced by Cowles and Bogert (1944) estimates by using a gradual exposure at a controlled rate, the final temperature (i.e. CTMax) that leads to a functional collapse (i.e. loss of locomotor function, muscle spasms and death). The use of lethal high temperatures or the CTMax is too stressful for organisms and kills them quickly, making it difficult to assess their climatic vulnerability. The Voluntary Thermal Maximum is a temperature below the CTMax and represents a behavioral response of the animals to avoid overheating, reaching its CTMax and dying (Camacho and Rusch, 2017; Camacho et al., 2018). Despite being less used for estimating climatic vulnerability in ectotherms and especially in anurans, VTMax might be an advantageous measure for that purpose because it is more likely to occur before, and integrates behavioral thermoregulation, as recommended in for evaluations of climatic vulnerability (Williams et al., 2008). In this sense, the VTMax could be used to estimate the time to loss locomotor function (TLLF) under high temperatures, in order to better assess the climatic vulnerability of species, without killing individuals. Since other factors, such as dehydration, affect the performance, thermal tolerances and thermoregulation of ectotherms (Preest and Pough, 1989; Moore and Gatten, 1989; Mitchell and Bergmann, 2016; Anderson and Andrade, 2017), its effect on the TLLF also matters. However, the lack of integrating the effects of dehydration on the behavioral thermal tolerance of anurans (i.e. VTMax), makes it difficult to assess the climatic vulnerability of these organisms under stressful thermal and hydric conditions.

This thesis's main objective was to evaluate if hydration level affects the thermoregulatory behavior, thermal tolerance and the time to loss locomotor function in anurans. We used the Bullfrog (*Lithobates catesbeianus*) as study model. Bullfrog is an invasive species, considered one of the 100 most dangerous in the world for global diversity (Lowe et al., 2000). We developed two chapters. In the first chapter, we updated the Heath's thermoregulation model (1970) by integrating the effects of dehydration of *L. catesbeianus* in its PBT, VTMax and CTMax. In the second chapter, we assessed the effects of dehydration on the TLLF of Bullfrogs exposed to its VTMax. Both chapters intend to offer relevant physiological information that integrates the effects of temperature and dehydration on thermoregulatory behavior, thermal tolerance limits and TLLF of bullfrogs. Therefore, this information can be included in mechanistic distribution models to update the climatic vulnerability of this invasive species and further predict of its invasive patterns worldwide. Thus, we intend for our study to be applicable to other wet skin ectotherms in order to assess their climatic vulnerability and better inform conservation strategies of this globally endangered taxa.

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CHAPTER I

1. **Effects of dehydration on thermoregulatory behavior and thermal tolerance limits of Bullfrogs (*Lithobates catesbeianus*, Shaw, 1802).**

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## 1.1. Abstract

Foreseeing the effects of high environmental temperatures and drought on populations requires understanding how these conditions will influence the thermoregulatory behavior and thermal tolerance of organisms. Heath (1970) developed a model of behavioral thermoregulation in which ectotherms show fine-tuned (proportional) thermoregulation responses and all-or-none responses to avoid overheating. While scattered evidence suggests that dehydration alters the performance and thermoregulation of ectotherms, these effects have not been used to update such a model. To do that, we evaluated the effects of hydration level (HL) on the behavioral thermoregulation and physiological thermal limits of the “Bullfrog” (*Lithobates catesbeianus*), a model organism and important invader species. To examine the effects of dehydration on proportional responses, we compared the Preferred Body Temperatures (PBT) of frogs with free access to water with other frogs having restricted access to water. To observe the effect of dehydration on all-or-none responses, we measured the Voluntary Thermal Maximum (VTMax) at different hydration levels (100%, 90%, 80% of body weight at complete hydration). To understand the effect of dehydration on physiological thermal tolerance, we also measured the CTMax of frogs at the same hydration levels. Our results update Heath’s thermoregulation model showing disproportionately larger reductions on the PBT than on all-or-none responses and on the thermal limits. Besides, severely dehydrated individuals may lose their VTMax. We suggest that the observed dehydration effects should be included in mechanistic models of species distribution in order to improve climatic vulnerability assessments.

**Keywords:** thermal tolerance, invasive species, integration, hydration level, anurans.



## 1.2. Introduction

The global increase in environmental temperature is also causing droughts across many regions of the world (Barnett et al., 2005; Bates et al., 2008). These stressful climatic conditions have a great influence on geographical distribution, behavior and physiological functions of animals, showing also pervasive consequences for their life history (Malcolm et al., 2006; Post et al., 2008; Tewksbury et al., 2008; Ceballos et al., 2015). Ectothermic animals with relatively small mass and wet skin (e.g. anurans, mollusks) lack morphological traits such as high thermal inertia or an impermeable skin to protect them from overheating and drying. However, their ability to select suitable microenvironments to maintain adequate thermal and water balance have allowed them colonizing very hot and arid areas (Wygoda, 1984; Buttemer and Thomas, 2003; Tracy and Christian, 2005; Young et al., 2005; Cartledge et al., 2006; Tracy et al., 2014).

To understand the combined effects of stressful climatic conditions (e.g. high environmental temperatures and low water availability); we need to know how these conditions influence the thermoregulatory behavior and thermal limits of ectotherms (Williams et al., 2008). Heath (1970) established a behavioral thermoregulation model in which ectotherms finely tune their body temperature ( $T_b$ ) by changes in posture and microhabitat selection. This behavior, termed “proportional responses”, allow them to keep their body temperatures within a range of preferred body temperatures (i.e. PBT). The PBT optimizes multiple physiological functions (Licht, 1965; Heath, 1970; Stevenson, 1985; Hertz et al., 1993; Angilletta et al., 2002; Tracy et al., 2010), including locomotor performance (Bennet, 1990; Navas et al., 1999; Stevenson et al., 1985; Deere and Chown, 2006), feeding rates and digestive efficiency (Kingslover and Woods, 1997; Wang et al., 2002; McConnachie and Alexander, 2004; Fontaine et al., 2018), rates of development and growth (Berger et al., 2011) and reproduction (Navas and Bevier, 2001; Symes et al., 2017). However, when environmental conditions force the animal to an increase in  $T_b$  exceeding its PBT range, individuals may need to quickly retract from a thermally stressful situation to avoid prompt mortality. According to Heath's model, this situation is faced by all-or-none responses (Heath, 1970). The Voluntary Thermal Maximum (VTMax) represents an all-or-none response for such situations (Cowles and Bogert, 1944; Camacho and Rusch, 2017). At their VTMax, cooling down typically becomes imperative and animals forcefully move to a colder place, even at the cost of exposing themselves to increased predation risk (e.g. Camacho et al., 2018). If the animal cannot avoid the temperature of its body from increasing, it will reach its Critical Thermal Maximum (CTMax) (Cowles and Bogert, 1944), losing its locomotor response and dying from heat shock (Cowles and Bogert, 1944; Rezende et al., 2014).

When environmental temperatures rise, the rates of evaporative water loss also increase, potentially accelerating the dehydration of individuals and impairing their performance (Prest and Pough, 1989; Moore and Gatten, 1989; Plummer et al., 2003). Wet skinned ectotherms, like anurans, present a

dynamic relationship with their hydration level, swiftly losing body water and rehydrating, or strongly cooling down through body water evaporation (Wolcott and Wolcott, 2001; Prates and Navas, 2009; Tracy et al., 2010; Anderson et al., 2017). Studies have shown that dehydration may alter their thermoregulatory behavior (PBT) and thermal limits (CTMax) (e.g. Mitchell and Bergmann, 2016; Anderson and Andrade, 2017). However, how these traits respond in combination to dehydration remains poorly documented.

Updating Heath's model to account for dehydration is not only relevant for understanding thermoregulation and thermal tolerance but also to supports state-of-the-art models of climatic vulnerability. An integrating model will demonstrate the response mechanisms of the animals under recurrent stressful conditions resulting of the current climate change, such as high environmental temperatures and the potential risk of dehydration. Here, we used the Bullfrog (*Lithobates catesbeianus*) (Shaw, 1802) to test if hydration levels affect its PBT, VTMax and CTMax. Apart from being a model organism with commercial interest, this North American anuran ranks among the 100 most dangerous invasive species (Giovanelli et al., 2008; Ficetola et al., 2010; Nori et al., 2011; IUCN, 2015). Thus, we intend to understand the response mechanisms of this species under high temperatures and dehydration. Mechanistic models of geographic distribution could use the traits described by Heath's model to predict climatically unsuitable areas (Kearney and Porter, 2004; Carlo et al., 2018). Thus, the update of Heath's model should support the development of better forecasts of invasion of this species, as well as the predictions of climatic vulnerability of other wet skin ectotherms.

### 1.3. Materials and Methods

#### 1.3.1. Obtaining and maintenance of individuals

Between June to November in 2017, 128 juvenile individuals of *Lithobates catesbeianus* (Shaw, 1802) were commercially obtained from the Santa Clara Frog Pond (Santa Isabel municipality, São Paulo, Brazil). Specimens were kept in the vivarium of the Physiology Department of the Institute of Biosciences, University of São Paulo, Brazil. Each individual was kept in a plastic box that is 19 cm high by 33 cm long for 2-3 days before recording their respective measurements. All terrariums had access to water, shelter and photoperiod established in the vivarium with 13h of light and 11h of darkness (13L: 11D). The temperature of the vivarium ranged between 21°C-24°C, similar to the place where the animals were obtained. The animals were fed cockroaches immediately after the experiments and euthanized two days after the measurements, following humane guidelines (decapitation of sedated individuals, using a solution of Benzocaine, 0.1g/l) according to Comissão de Ética no Uso de Animais (CEUA) of the Institute of Biosciences, University of São Paulo, Brazil. For all experiments, body temperature was registered every 10 s by attaching an ultrathin T-type thermocouple (model 5SRTC/1 mm in diameter, omega ®) to the groin of each individual with surgical tape. Since the individuals of

the pilot experiments were easily removed the thermocouple initially located in the cloaca. We tested if the cloaca and inguinal temperatures were similar in those individuals. Since both temperatures vary in the same way as a function of time (Fig. A1), we chose the inguinal temperatures to avoid over stressing the animals. The thermocouple was factory calibrated and connected through a FieldLogger PicoLog TC-08 to a computer. All the experiments were made in a climatic chamber with controlled conditions of temperature and relative humidity ( $\bar{x}$ =18.5°C, 67.1%, N=34). Each thermal index was measured using different individuals, in order to avoid residual effects of previous experiments. If an individual died before 24h of any procedure were discarded from the analysis (see results). The ethics committee of the Biosciences Institute at the University of São Paulo approved all procedures for animal handling and euthanasia (CEUA N° 289/2017).

### 1.3.2. Hydration levels of individuals

To obtain the HL of 100%, the animals remained in a small box with water *ad libitum* for one hour. Before beginning the experiment, each 100% hydrated individual was emptied of its bladder to obtain their standard body mass. To obtain the HLs of 90% and 80%, the same procedure to hydrate the individuals to 100% was applied. Then its bladder was emptied, and each individual was placed inside a mesh bag in front of a fan with an air velocity of approximately 1m per sec, and weighed every 5-10 min until obtaining the desired HLs (e.g. Titon and Gomes, 2017).

### 1.3.3. PBT measurements

Four artificial gradients were constructed with rectangular plastic boxes (19 cm width by 60 cm long). A 1 mm thick aluminum sheet that is 14 cm wide by 56 cm long was placed on the lower part of each box. This aluminum sheet was heated from below at one end with a 60 W incandescent bulb. The other end was cooled with frozen gel bags. In this way, we had artificial gradients with an average temperature of 20°C (s.d. 10; upper: 10.38°C; lower: 42.32°C; 4320 records). We estimated these temperatures on eight gypsum models imitating the shape and size of the frogs and placed them in a gradient. The models were separated from one another by a distance of 6-7 cm. Each model had a type T thermocouple attached to record the temperatures along the gradients. Temperature of each model in a gradient was recorded every 10 s for 90 min between 10:30-12:00h (Fig. A2).

We analyzed the PBT of individual bullfrogs in the described gradients as a function of dehydration and access to water. For that, we separated two groups: The control group (CG) with free access to water, and the water-restricted group (WRG) which did not have access to water. For the CG, 14 Petri dishes of six cm in diameter were filled with water at room temperature ensuring constant access to water (Fig.1A). All individuals were hydrated to a 100% level before the experiments. Once each individual was placed within a gradient, their body temperature was recorded every 10 s, with the help of the

thermocouple attached to the groin of each individual, and its body mass was recorded every 30 min. When a WRG individual reached, a body weight of 80% its initial one, both CG and WRG individuals had their body masses recorded for the last time and the experiment ended. The PBT measurements were made in groups of four individuals per day (two in CG, two in WRG) for two weeks in November 2017, totaling 32 individuals (16 in the CG and 16 in the WRG).

#### **1.3.4. CTMax measurements**

The effects of dehydration on the CTMax were assessed in July 2017 by creating three independent groups of 15 individuals with specific HLs (100%, 90% and 80% in relation to the previously defined standard body mass). First, the CTMax was measured in a group of 100% hydrated individuals, and then in another group of 90% hydrated individuals and then a third group of 80% hydrated individuals.

The CTMax measurements were carried out inside an aluminum container covered with an acrylic lid and heated within a thermal bath. A T-type thermocouple was placed inside the aluminum container to register surface temperature and check for the maintenance of heating rate ( $0.75^{\circ}\text{C}/\text{min}$ , Fig. 1B). The heating rate was controlled with a dimmer connected to the power source. The average start body temperature of individuals was  $20^{\circ}\text{C}$  (s.d. 1.87; upper:  $17.21^{\circ}\text{C}$ ; lower:  $23.80^{\circ}\text{C}$ ;  $N=45$ ) and the aluminum container was  $19.39^{\circ}\text{C}$  (s.d. 2.17; upper:  $14.38^{\circ}\text{C}$ ; lower:  $22.50^{\circ}\text{C}$ ;  $N=45$ ). Each individual was heated in the thermal bath until it attempted to escape. From then onwards, the specimen was turned belly up with the help of forceps to check for its righting response. This procedure was repeated every 30 s, until the individual lost the righting response. At that time, the individual's body temperature was recorded as its CTMax, and it was immediately weighted and cooled off in water at room temperature.

#### **1.3.5. VTMax measurements**

We also measured the VTMax for another 15 individuals per each hydration level, in August 2017. For that, individuals were independently heated within a metallic cylindrical box, wrapped in a thermal resistance for homogenous heating (Fig. 1C). A T-type thermocouple was placed inside the box and adhered to the surface, to register temperature and check for the maintenance of heating rate ( $0.5^{\circ}\text{C}/\text{min}$ , Fig. 1C). The heating rate was controlled with a dimmer connected to the power source. In turn, the box had a half-opened, easily movable plastic lid, so that the individual could exit the box at will (Fig. 1C). The average start body temperature of individuals was  $20.03^{\circ}\text{C}$  (s.d. 1.38; upper:  $17.31^{\circ}\text{C}$ ; lower:  $23.47^{\circ}\text{C}$ ;  $N=37$ ) and the metallic cylindrical box was  $21.19^{\circ}\text{C}$  (s.d. 1.16; upper:  $19.50^{\circ}\text{C}$ ; lower:  $24.42^{\circ}\text{C}$ ;  $N=37$ ). Individuals were heated independently until they left the box. At that moment, their body temperatures were recorded as their VTMax, their body mass was measured, and they were taken to a container with water at room temperature for recovery.

### 1.3.6. Statistical analysis

We calculated and present in order, the average, the standard deviation (s.d.), the minimum value and the maximum value (range), as well as the total number of independent observations (N) for all the measured indices.

The effect of HL (measured as body mass) and group (CG, WRG) on the PBT was evaluated using mixed linear models in R Vr. 3.2.2 (R Core Team 2014) (nlme package, "lme" function) (Bates et al., 2011). Specimen identity was coded as a random variable, while HL and group entered as fixed effects. To assess for statistical interactions between the two fixed factors we compared the Akaike information criterion (AIC) of models including and excluding the interaction, differences of two units in AIC were considered as statistically significant (Wang and Qun, 2006).

For CTMax and VTMax, the respective unidirectional ANOVAs followed by the Tukey test were performed to evaluate differences among hydration levels. The statistical analyses were performed in the SPSS Vr Software. 22.0 (Pardo and Ruiz, 2002) and graphed in SigmaPlot Vr. 11.0.

## 1.4. Results

### 1.4.1. Effects of dehydration on PBT

The average PBT of the CG individuals was 28.51°C (s.d. 0.42; range: 17.59°C-36.47°C; N=16), whereas the average PBT of the WRG individuals was 22.69°C (s.d. 0.42; range: 14.83°C-33.66°C; N=16) (Fig. 2A). CG individuals maintained a hydration level above 90% and showed little effect of dehydration on PBT while WRGs maintained lower hydration levels and therefore preferred lower temperatures (Fig 2B).

The interactive model had the lowest AIC value. The selection criteria for the best model was the one with the lowest AIC value, where model I had an AIC=993.0272 (Degrees freedom (df) = 5) and model II had an AIC=991.0538 (df = 6). With a difference of 1.97 AIC units, we chose model II as a better representation of variation in the PBT. Results for both models are shown in Table 1. Both models reflect the interaction between HL and the groups, in the thermoregulatory behavior, where the hydrated animals maintained a preference towards higher temperatures for a longer time but at the same time a higher level of hydration, different from what is shown with animals with water restriction.

### 1.4.2. Effects of dehydration on CTMax

We found significant differences in the CTMax across the three HL groups ( $p < 0.001$ ;  $df = 44$ ; Tukey test  $p < 0.005$ ) (Fig. 3A). The average CTMax of the individuals with HL 100% was 36.82°C (s.d. 0.77;

range: 35.60-38.95°C; N=15), while CTMax for individuals with HL 90% was 35.50°C (s.d. 0.80; range: 34.11-37.14°C; N=15) and CTMax for individuals with HL 80% was 34.63°C (sd. 0.41; range: 34.01-35.46°C; N=15). All the individuals survived the experiment.

### 1.4.3. Effects of dehydration on VTMax

We also found significant differences in the VTMax between the three hydration level groups ( $p < 0.001$ ; d.f.=36; Tukey test  $p < 0.001$ ) (Fig. 3B). The average VTMax was 35.89°C (s.d. 0.16; range: 34.56-36.77°C; N=15) for HL 100% individuals, 33.60°C (s.d. 0.34; range: 30.21-35.06°C; N=15) for HL 90% individuals and 31.29°C (s.d. 0.43; range: 30.05-33.11°C; N=7) for HL 80% individuals. Eight individuals at 80% HL failed to exit the chamber and died.

## 1.5. Discussion

Our study aims to understand how anurans integrate thermoregulation thermal limits during dehydration. In accordance with Heath's proportional responses, bullfrogs finally adjust their position in the gradient in order to maintain the  $T_b$  within a PBT range. Further, they proportionally adjusted their position in the gradient to their HL, lowering their PBT as they dehydrated. For a fully hydrated ectotherm, maintaining high body temperature below the VTMax benefits several processes, such as growth in mollusks species (Díaz et al., 1996; Díaz et al., 2000; Díaz et al., 2011) and locomotion in anurans (Moore and Gatten, 1989; Anderson and Andrade, 2017; herein). However, studies have shown that dehydration decreases optimal temperature for locomotion (Beuchat et al., 1984; Preest and Pough, 1989; Titon et al., 2010; Titon and Gomes, 2015, 2017). Besides from bringing dehydrated animals, closer to a new thermal optimum, selection of lower temperatures reduces rates of evaporative water loss (Mitchell and Bergmann, 2016; Anderson and Andrade, 2017). Our constant monitoring allowed us to evidence proportional adjustments of PBT in response to dehydration. These proportional changes have been previously suggested by studies comparing groups with different hydration levels (Jaremovic and Rollo, 1979; Moore and Gatten, 1989; Preest and Pough, 1989; Anderson and Andrade, 2017). Thus, we can conclude that lowering mean body temperatures in response to dehydration is widespread, at least across wet skinned ectotherms.

We discovered that dehydration not only can decrease the VTMax in anurans, but also that around half of individuals may lose this all-or-none response, and die. The VTMax constitutes a last behavioral resort to avoid reaching the thermal limits, even overcoming the risk of facing a potential predator. This observation highlights the dangers of experiencing a combination of high temperatures and low hydration levels for anuran populations. Wild anuran populations often maintain a hydration level above 90% even in dry season (Tracy et al., 2014). However, dangerously low HL and high temperatures might be underreported for anurans. Too high temperatures and too low water availability might explain the

absence of dehydration prone species in insolated forest fragments (e.g. Waitling and Braga, 2015) and the limits for geographical distribution (Schwarzkopf and Alford, 2002; Tingley and Shine, 2011; Florance et al., 2011; Brown et al. 2011; Letnic et al., 2015; Titon and Gomes, 2017).

Our study highlights the importance of knowing the effects of dehydration on both behavioral responses and thermal limits. In bullfrogs, dehydration lowered proportional responses ( $0.44^{\circ}\text{C}/1\%$  of standard body mass lost) more than an all-or-none response (VTMax) ( $0.23^{\circ}\text{C}/1\%$  of standard body mass lost), and the thermal limit lowered the least (CTMax) ( $0.10^{\circ}\text{C}/1\%$  of standard body mass lost). Such a different responsiveness makes sense in the light of the literature and our observations: Dehydrated amphibians exhibit a reduction in maximal locomotor performance and optimal temperatures for locomotion (Beuchat et al., 1984; Preest and Pough, 1989; Titon et al., 2010; Titon and Gomes, 2015, 2017) and some of the severely dehydrated individuals lost their VTMax. These facts suggest the combined effects of high temperatures and dehydration mean a double jeopardy for anurans. Not only due to impaired locomotion, but also due to impaired perception of thermal risk. By adjusting their PBT more strongly, the bullfrogs keep themselves far from dangerous levels of body temperature and hydric state (a purported “hydrothermal” safety margin). In agreement with that idea, *Rhinella schneideri* also lowered more the PBT than the CTMax, ( $0.13^{\circ}\text{C}/1\%$  and  $0.06^{\circ}\text{C}/1\%$  of standard body mass lost, respectively, Anderson and Andrade, 2017). Unfortunately, previously published evidence on this topic is largely fragmented and made use of different methodologies, precluding the observation of general patterns in hydrothermal margins (Shoemaker et al., 1985; Dupré and Crawford, 1986; Ladyman and Bradshaw, 2003; Mitchell and Bergmann, 2016; Crowley, 1987; Plummer et al., 2003). Thus, we encourage future studies including stressful climatic conditions on thermoregulatory responses and thermal limits of ectotherms. We hypothesize that groups commonly facing more severe dehydration should present stronger proportional responses.

Our update to Heath's thermoregulation model should be included in mechanistic distribution models. For example, NicheMapper (Kearney and Porter, 2009) allows to use PBT, VTMax and the CTMax and species body mass as fixed values, but these parameters decrease in response to dehydration, and can even disappear (e.g. VTMax). The effects of dehydration on proportional and all-or-none responses have not been explored in mechanistic models that make inferences about the effects of climate on activity patterns, phenology and geographical distribution of species (Kearney et al., 2008; Bartelt et al., 2010; Nowakowski et al., 2017; Oyamaguchi et al., 2018). With this study, we provide the rationale and data to incorporate these effects and improve climatic vulnerability assessments of species.

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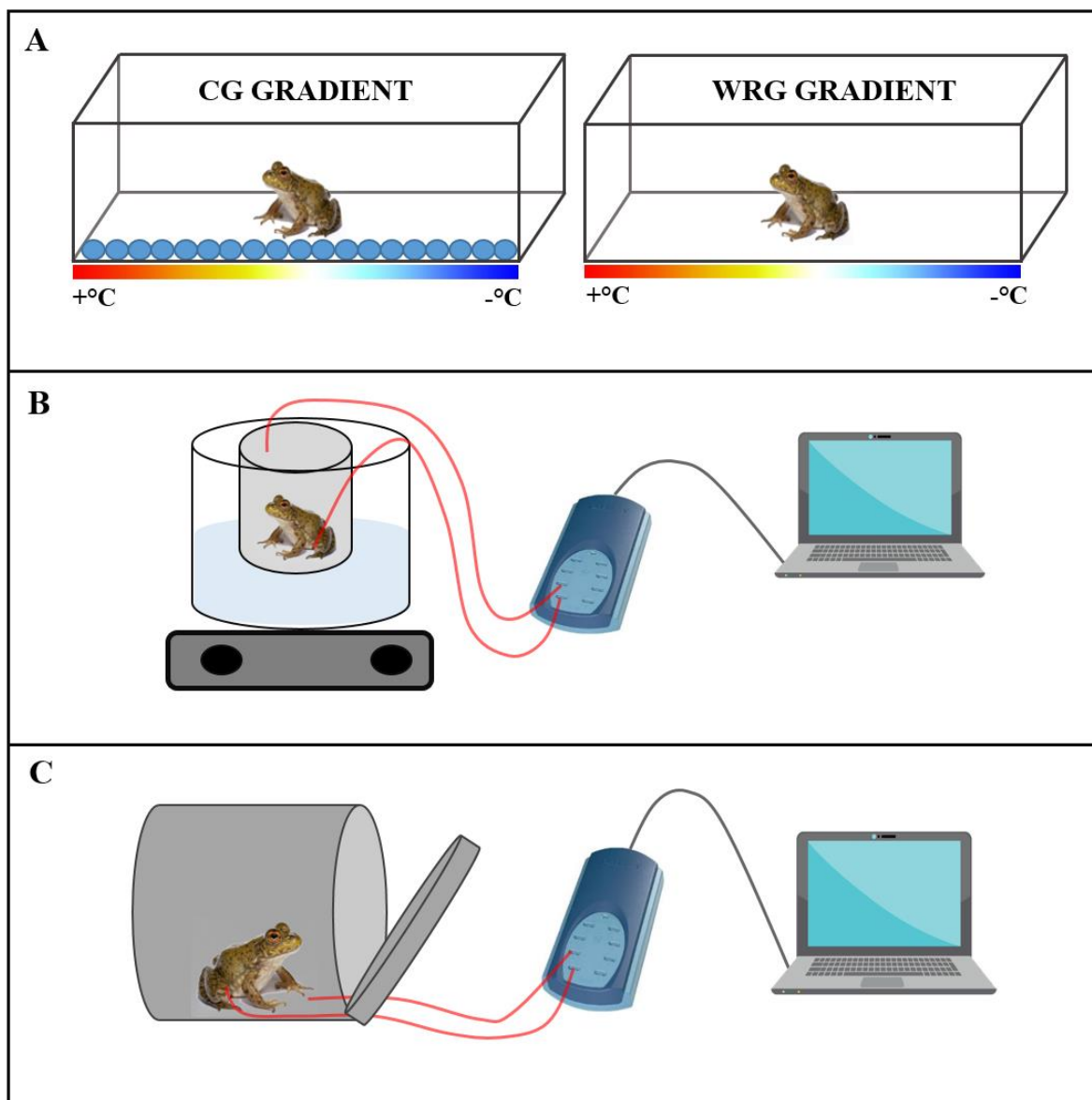
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## 1.7. Figures

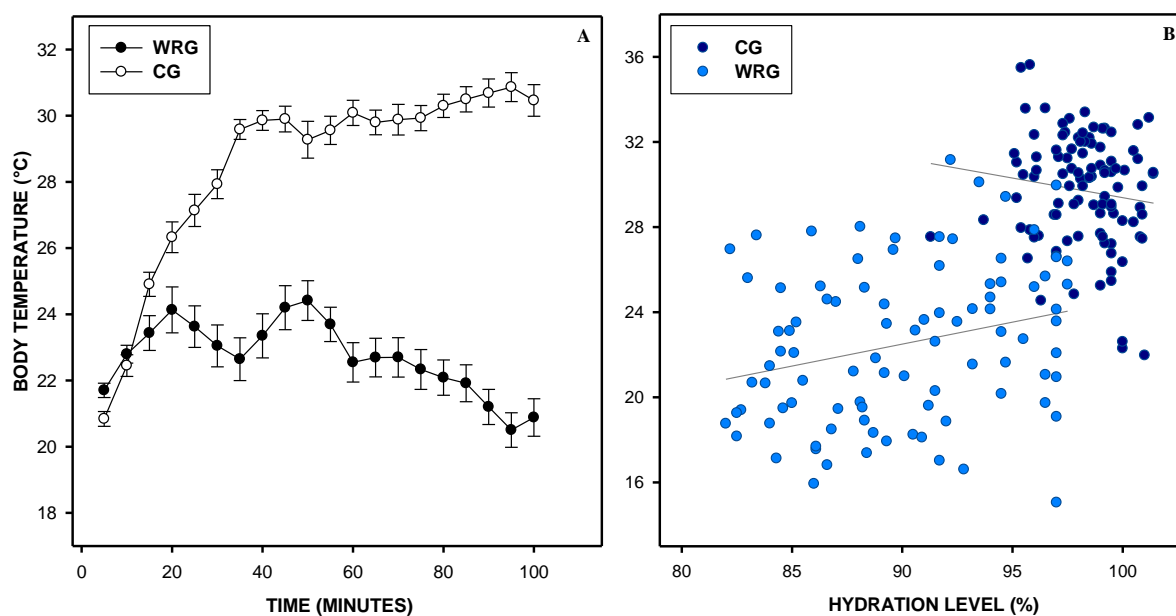
Figure 1. Scheme of the machinery used for thermoregulatory behavior measurements and thermal limits in *Lithobates catesbeianus*.

(A) Thermal gradients used for measuring the PBT. B) A thermal bath for measuring CTMAX measurement method. C) A can-system for measuring the VTMax.



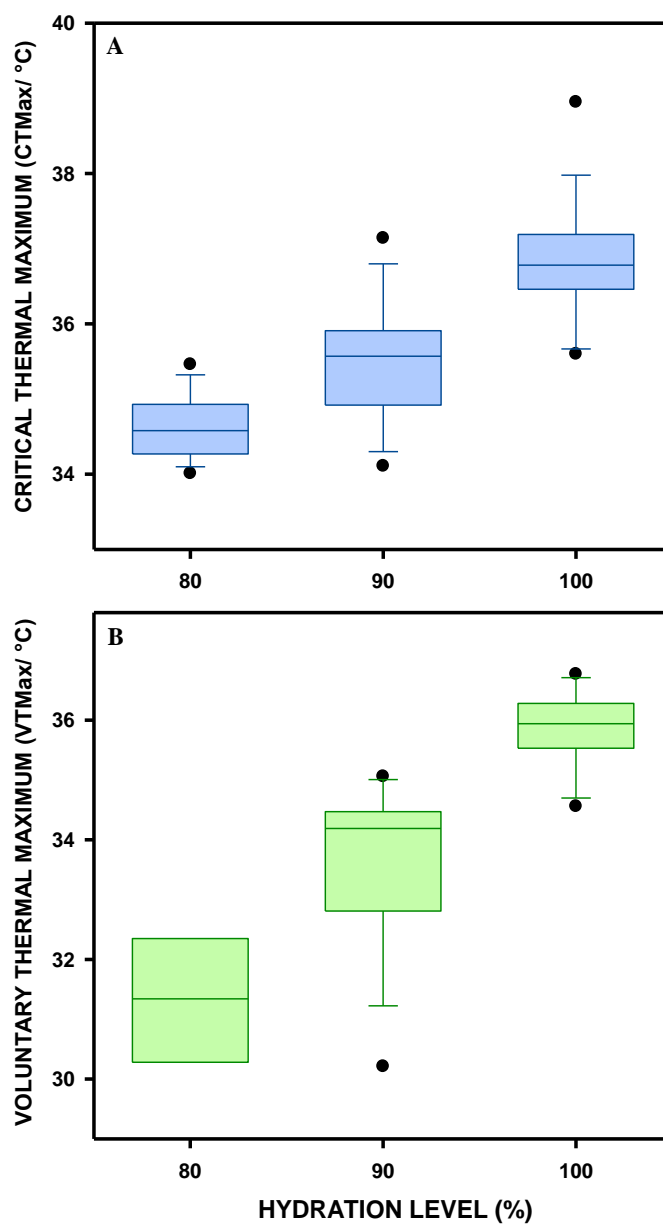
**Figure 2. Preferential temperature (PBT) of *Lithobates catesbeianus* under experimental conditions (WRG, 16 individuals) and controlled conditions (CG, 16 individuals).**

A) Both experimental groups started with similar temperatures but, as the time passes, WRG individuals started exhibiting lower PBT. B) Effect of hydration level on the preferential temperature of *Lithobates catesbeianus* for CG and WRG. Access to water during the experiment allowed maintaining a hydration level above 90% and the majority of individuals remained at higher temperatures over time.



**Figure 3. Exposure of individuals of *Lithobates catesbeianus* in three levels of hydration, to its thermal limits.**

A) Relationship between the Hydration Level (HL) and the Critical thermal maximum ( $p=0.000$ ;  $N=15$  for each level). B) Relationship between hydration level and the voluntary thermal maximum ( $p=0.000$ ;  $N=7$  for 80%,  $N=15$  for 90% and 100% of original body weight).



## 1.8. Table

**Table 1. Effect of hydration level and group (WRG, CG) on the preferential temperatures of *L. catesbeianus*.**

The selection criteria for the best model was the one with the lowest AIC value, where model I had an AIC = 993.0272 (df = 5) and model II had an AIC = 991.0538 (df = 6).

Model	Variable	Value	Std.Error	df	t-value	p.value
<b>I</b>	<b>Intercept</b>	15.778884	6.060125	161	2.600290	0.0102
	<b>Hydration Level</b>	0.141783	0.061630	161	2.300528	0.0227
	<b>Group (WRG and CG)</b>	-5.943362	0.817796	30	-7.267533	0.0000
<b>II</b>	<b>Intercept</b>	59.15017	19.711156	160	3.000847	0.0031
	<b>Hydration Level</b>	-0.29994	0.200696	160	-1.494495	0.1370
	<b>Group (WRG and CG)</b>	-53.30175	20.536057	30	-2.595520	0.0145
	<b>Hydration Level*Group</b>	0.48605	0.210595	160	2.308001	0.0223

## 1.9. References

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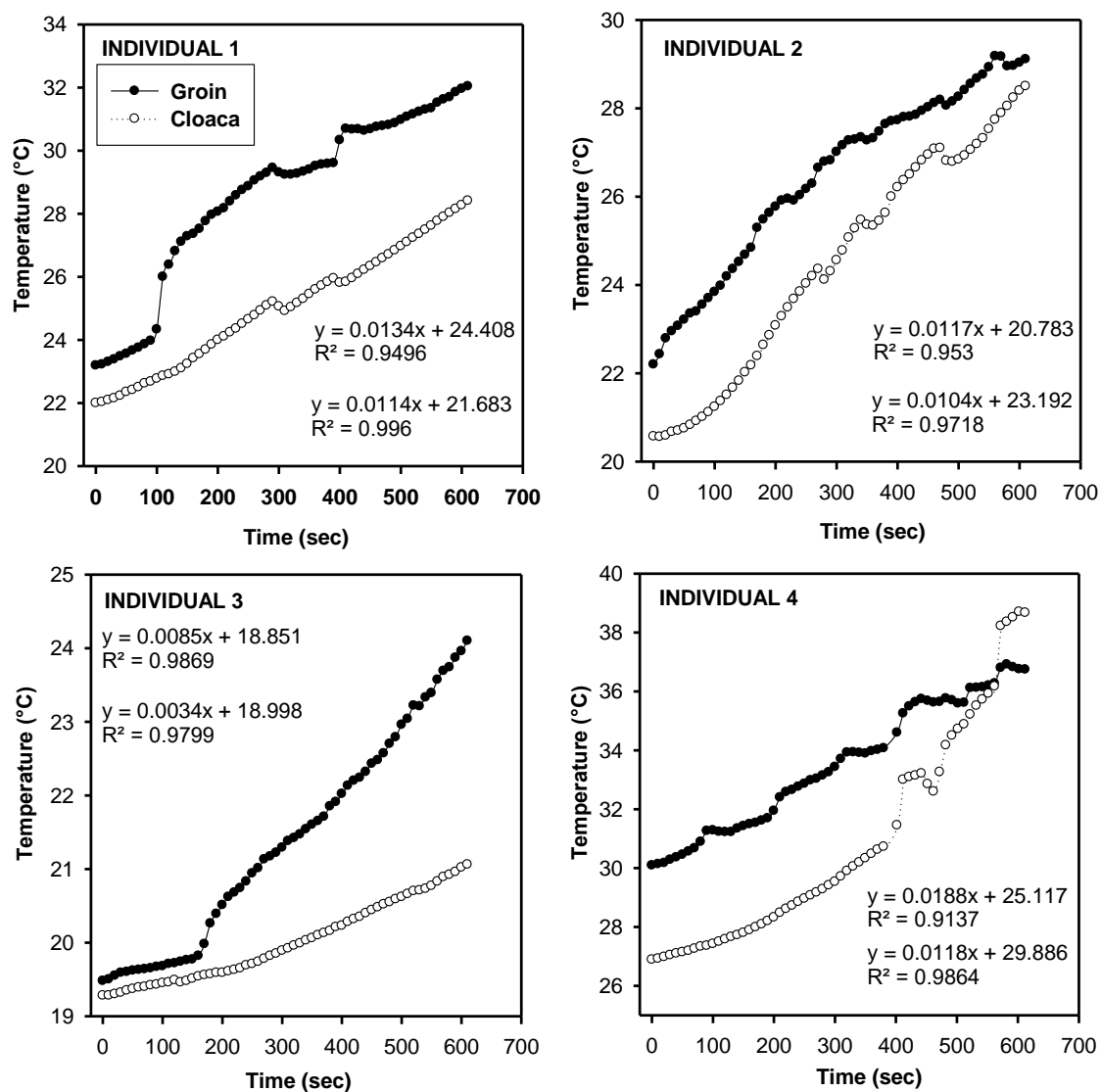
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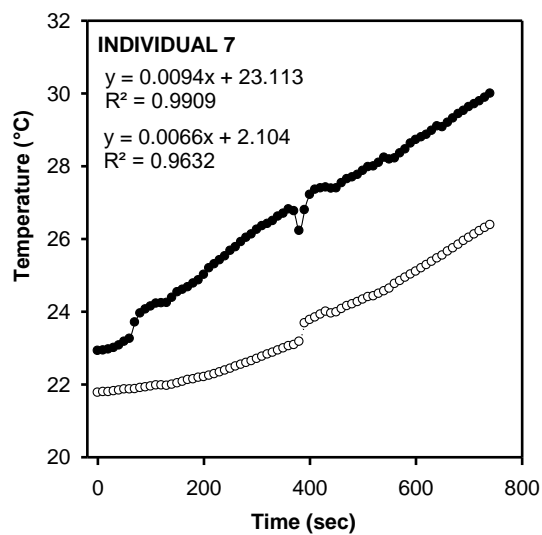
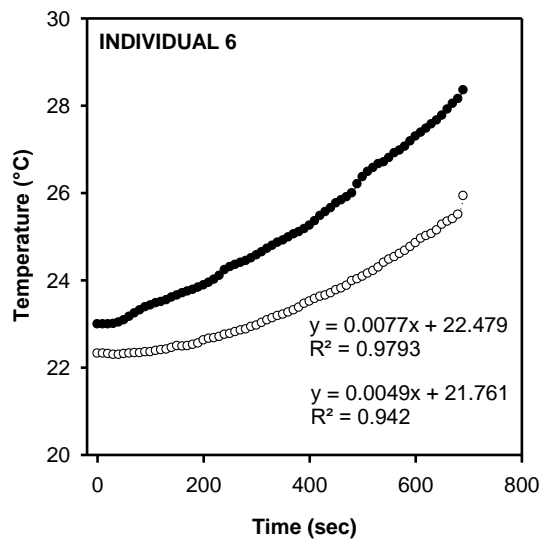
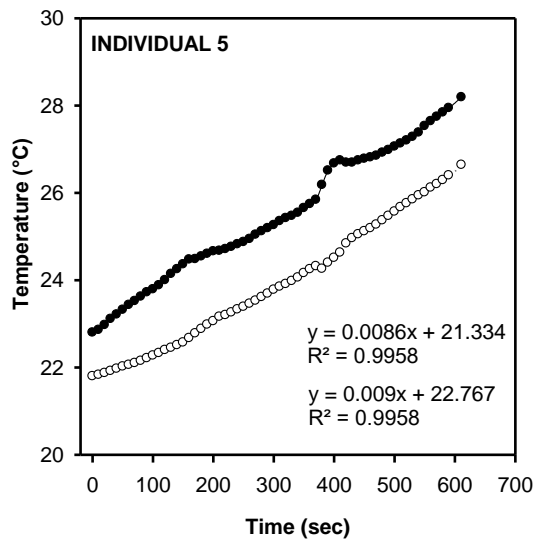
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## 1.10. Appendix

## Appendix 1. Calibration of the cloaca and groin temperatures in seven pilot individuals measured over time.

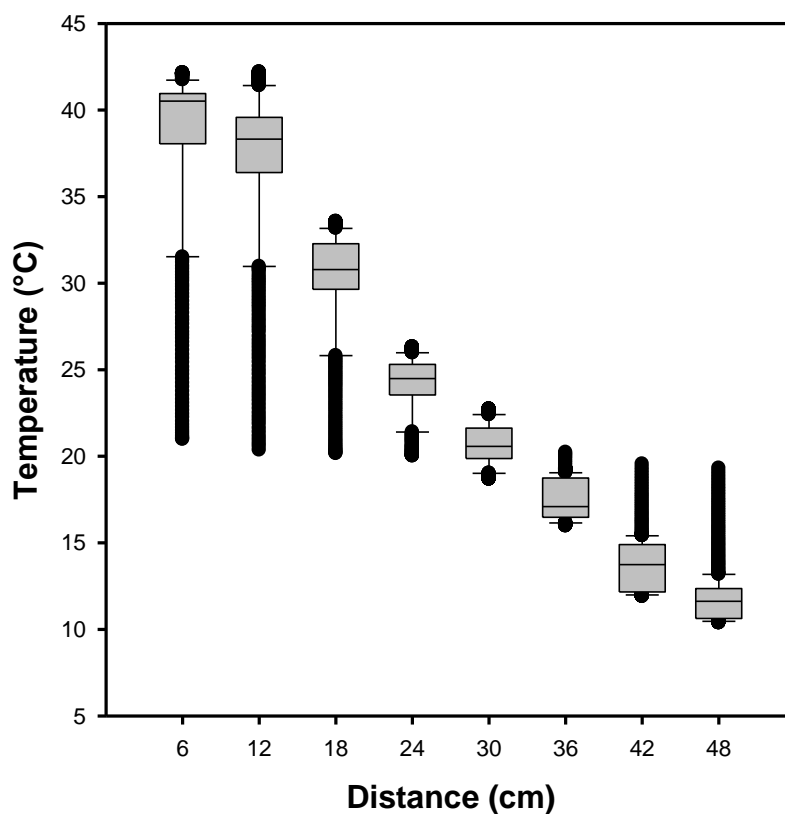
The individuals were heated at the same heating rate (0.5°/ min). The temperatures of the groin and cloaca of the individuals vary in the same way as a function of time ( $p = 0.812$ ). However, differences in the intercept of the two temperatures are maintained over time ( $p = 0.041$ ).





**Appendix 2. Minimum and maximum temperatures exhibited by eight gypsum models located along an experimental gradient during 90 min.**

The distance was measured from the higher temperature end to the lower temperature end of the gradient. The models were separated from each other by a distance between 6-7 cm.



**CHAPTER II**

**2. Effects of dehydration on the time to loss locomotor function in the invasive frog *Lithobates catesbeianus* (Anura: Ranidae).**

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**Keywords:** locomotion, temperature, dehydration, thermal tolerance, invasive frog.

## 2.1. Abstract

Two dimensions may describe the thermal tolerance of an organism: the level of temperature they withstand and the time they resist at each level of stressful temperature. Thus, knowing both dimensions is necessary for the characterization of climatic vulnerability of such organisms. Furthermore, as other factors as dehydration may affect not only thermal tolerance but also thermoregulation, the effect of such factors on the time to lose the locomotor function (TLLF) also matters. Herein, we evaluated the effects of dehydration on the TLLF of frogs exposed to its voluntary thermal maximum (VTMax). We exposed individuals of *Lithobates catesbeianus* at different HLs (80%, 90%, and 100% of fully hydrated weight) to its VTMax, also obtained for this HLs. Dehydration decreased the TLLF of frogs across the different HL (31.50 min, 80%; 179 min, 90%; 243.50 min, 100%), suggesting an exponential negative dehydration effect on TLLF. Based on these results, we suggest ways to include the effects of dehydration on mechanistic models of climatic vulnerability.

**Keywords:** locomotion, temperature, dehydration, thermal tolerance, invasive frog.



## 2.2. Introduction

Two dimensions describe the thermal tolerance of organisms under stressful climatic conditions. The “Press” that indicates the level of climate pressure (e.g. high environmental temperature) they withstand and the “Pulse” that indicates the time that species support at each level of this pressure (Harris et al., 2018). Two experimental methods take into account these dimensions to estimate the thermal tolerance of species. These methods are the static and dynamic (Lutterschmidt and Hutchison, 1997; Cooper et al., 2008). The static method introduced by Fry et al. (1942), uses high or low constant stressful temperatures to estimate the time for a final lethal temperature to lead to 50% of the measured population dying from exposure (i.e. thermal death curves) (Fry, 1947, 1967). The dynamic method introduced by Cowles and Bogert (1944) estimated using a gradual exposure at a controlled rate, the final temperature (i.e. CTMax) that leads to a functional collapse (i.e. loss of locomotor function, muscle spasms and death). Despite these methods have been widely used in vertebrates (Bennet and Judd, 1992; Mora and Maya, 2006; Sunday et al., 2010; Turriago et al., 2015) and invertebrates (Mitchell and Hoffmann, 2010; Rezende et al., 2014; Hangatner and Hoffmann, 2016); using lethal high temperatures or the CTMax is too stressful for organisms and kills them quickly, making it difficult to assess their climatic vulnerability. There are other temperatures of ecological importance below the CTMax, which are more likely to occur first, and that result as an alternative to be included in the assessments of climatic vulnerability of species (Williams et al., 2008).

The Voluntary Thermal Maximum is a temperature below the CTMax and represents a last behavioral resource for animals avoiding to overheat, reach its CTMax and die (see Camacho and Rusch, 2017; Camacho et al. 2018). As a parameter to be included in estimations of climatic vulnerability, the VTMax also integrates the behavior of the organism, as recommended in studies of climatic vulnerability (Williams et al., 2008). Also, is measurable in experimental conditions, and allows the humane recovery of individuals instead of killing them, like happens when estimating lethal temperatures. In this sense, the VTMax could be used to estimate the “Pulse” of the thermal tolerance under this temperature, in order to improve the climatic vulnerability assessments of species. This “Pulse” can be estimated as the Time to Loss Locomotor Function (TLLF) of the animals exposed to this temperature, without killing it. Since other factors such as dehydration affect the thermal tolerance and thermoregulation of ectotherms (Mitchell and Bergmann, 2016; Anderson and Andrade, 2017), its effect on the TLLF also matters. In fact, dehydration negatively affects the VTMax of *Lithobates catesbeianus*, and the most dehydrated individuals lose their response to it and die (Chapter I). However, the lack of integrating the effects of dehydration on the behavioral thermal tolerance of anurans (i.e. VTMax), makes it difficult to assess the climatic vulnerability of these organisms under stressful thermal and hydric conditions.

In this sense, we evaluated the effects of dehydration on the TLLF in frogs exposed to its VTMax. We used *Lithobates catesbeianus*, a species for which we already know the effects of dehydration in their

PBT, VTMax and CTMax (Chapter I). Estimating the TLLF at the frogs' VTMax, appears as an ecologically relevant measure of the thermal tolerance time of organisms. In addition, given the effects of dehydration in the VTMax, knowing their impacts on the time to loss locomotor function under this temperature, can help to understand the climatic vulnerability of species in integrated thermal and hydric stress conditions.

## 2.3. Materials and Methods

### 2.3.1. Obtaining and preparing animals for experiments

During October of 2017, 60 juvenile individuals of *Lithobates catesbeianus* were purchased in the Santa Clara Pond, municipality of Santa Isabel, São Paulo, Brazil. These individuals were kept in the vivarium of the Department of Physiology of the Institute of Biosciences, University of São Paulo, Brazil (IB/USP). Each one was placed in a plastic box with 19 cm high by 33 cm long for 2-3 days before the experiments. All boxes allowed access to water and shelter. Photoperiod was established in the vivarium as 13L:11D and temperatures varied from 21°C to 24°C, a range of variation similar to which animals were previously exposed in the Santa Clara Pond. Individuals ate cockroaches (three units/individual) after the experiments. Animals were sedated by immersion in a solution of 0.1g of benzocaine/one liter of water and decapitated at the next day of experiments. All experimental procedures followed were applied in accordance to the Ethic Committee of the Biosciences Institute of University of São Paulo (Protocol n° 289/2017).

### 2.3.2. Determining hydration levels

We obtained three independent groups of 20 individuals with specific hydration levels (80%, 90%, and 100% of fully hydrated weight). To obtain the individuals 100% hydrated, they were placed in individual boxes with water *ad libitum* for one hour. Before the experiment, each hydrated individual was emptied of its bladder to obtain their standard body mass. To obtain HLs of 90% and 80%, individuals were 100% hydrated, and its standard body mass recorded. Then, each individual was placed inside a mesh bag in front of a fan (airspeed 1m/s), and weighed every 5-10 min until obtaining the desired HL.

### 2.3.3. Estimating the TLLF of individuals

We analyzed the TLLF of individual bullfrogs at the three different HLs. Individuals were exposed to median of VTMax obtained for each HL. The TLLF of 50% of a measured population (i.e. TLLF50) can be used as the time necessary for the median of the VTMax to lead to a loss of locomotor function in half of the population (Camacho et al., 2018). Thus, the median of VTMax calculated for *L. catesbeianus* at these HLs were 35.94°C (HL: 100%), 34.19°C (HL: 90%) and 31.34°C (HL: 80%) (Chapter I). Then, individuals at their respective HL were placed in individual boxes in a BOD Incubator

(Bio-Oxygen Demand) with humidity and temperature control. In the BOD, the initial temperature was established as the one corresponding to the median of the VTMax for the respective HL of individuals. A gypsum model with size and shape similar of individuals was placed in other box and was heated simultaneously and identically. It had a T-type thermocouple (model 5SRTC/1 mm in diameter, omega ®) attached with surgical tape to it. The thermocouple was factory calibrated and connected through a FieldLogger PicoLog TC-08 to a computer to record and control the temperatures experienced by individuals during experiments. When individuals reached its median VTMax, the time counting was initiated and each individual was rotated with its box twice every five minutes. The turns were repeated until each individual showed inability to move which indicated a loss of their locomotor function. Once an individual lost the locomotor function, was removed from the BOD and placed in a box with water for recovery. When half of the individuals in each population measured (N=10 individuals / each HL) lost their locomotor function, the experiment ended and the TLLF50 was obtained for each hydration level.

#### 2.3.4. Statistical analysis

For all groups (HL), we calculate the TLLF50 average with its standard deviation and the minimum and maximum values (range) of TLLF50. A one-way ANOVA was performed followed by the Tukey test to compare the TLLF50 between the three HLs. This same analysis was also applied to evaluate the effect of the median of VTMax for each HL, in the TLLF50 obtained in the same HLs. The statistical analyzes were performed in R Vr. 3.2.2 (R Core Team 2014) and graphed in SigmaPlot Vr. 11.0.

#### 2.4. Results

We found a negative effect of dehydration in TLLF50 in the three HLs, with significant differences between the three hydration level groups ( $p < 0.001$ ;  $df. = 29$ ; Tukey test  $p < 0.001$ ) (Fig. 1). Also, we found a negative effect of median VTMax in TLLF50 of each HL, with significant differences between the three hydration level groups ( $p < 0.001$ ;  $df. = 2$ ; Tukey test  $p < 0.001$ ) (Fig. 1). The TLLF50 of individuals 100% hydrated was  $\bar{x} = 243.50$  min (s.d. 57.01; range: 100-290 min), for individuals 90% hydrated was  $\bar{x} = 179.00$  min (s.d. 76.22; range: 5-265 min) and for individuals 80% hydrated was  $\bar{x} = 31.50$  min (s.d. 28.48; range: 5- 95 min).

#### 2.5. Discussion

Our study shows the effects of dehydration on the "Pulse" of the thermal tolerance of a wet skin ectotherm. The more dehydrated individuals are, the lower the TLLF at a stressful temperature. Our results shown a fall that suggest an exponential negative effect of hydration level and median VTMax on the TLLF (Fig. 1a-b). According to this, individuals who lost 20% of the standard body mass for the

experiment (i.e. 80% hydrated) took a few minutes to lose their locomotor response when exposed to its VTMax, while at hydration levels >90%, it took them more than two hours to lose it. In previous experiments, we found that *Lithobates catesbeianus* may lose the perception of overheating and the ability to respond to its VTMax at very low hydration levels (HL 80%) (Chapter I). The loss of motor coordination could be caused by a neural dysfunction during the acute thermal shock in which the animals were exposed (Lutterschmidt and Hutchison, 1997; Hoffmann et al., 2013). Our results could indicate that this loss of motor coordination occurs more quickly due to the dehydration effects and its relationship with VTMax of individuals (i.e. the more dehydrated, the lower its VTMax), which makes them more sensitive to thermal shock (Chapter I).

The higher level the "Press" (e.g. high environmental temperatures), the shorter the time to death of individuals (Harris et al., 2018) which affects the persistence of organisms in space and time (Odum, 1959; Harris et al., 2018). The traditional methods of thermal tolerance (static and dynamic) use high lethal temperatures or the CTMax of individuals which leads to an almost linear decrease in their thermal tolerance over time, killing them quickly (Smith, 1957; Santos et al., 2011). In this way, it is difficult to make "safe" estimates of the interactions between "Press" and "Pulse" to assess the climatic vulnerability of organisms in space and time. We show a safe method to understand this interaction "Press" and "Pulse", by measuring the time of loss of locomotor function, using a temperature that is not lethal and that is below the CTMax of organisms (i.e. VTMax). The VTMax is an ecologically relevant alternative to be applied for some reasons: First, it leads to a behavioral response of the animals to avoid overheating, reaching its CTMax and dying (Camacho and Rusch, 2017; Camacho et al., 2018). Second, this behavioral response can help the human recovery of individuals under experimental conditions, decreasing its body temperature and rehydrating them; and third, it is likely that the animals will first face this temperature in their environments before its CTMax, which may call attention to its climatic vulnerability. For wet skin ectotherms, such anurans, these advantages are particularly important. Given the importance of temperature-hydration interaction to optimize their physiological functions (Moore and Gatten, 1989; Preest and Pough, 1989, 2003; Köhler et al., 2011; Mitchell and Bergmann, 2016; Anderson and Andrade 2017), its VTMax can be combined with dehydration effects to indicate the time until the loss of the locomotor function in stressful thermal and hydric conditions that they can experience in their environments.

The effects of combined high temperatures and dehydration on the "Pulse" of the thermal tolerance of *L. catesbeianus*, is particularly relevant information to update its distribution models (Ficetola et al., 2007; Giovanelli et al., 2008; Nori et al., 2011). Mechanistic distribution models can include the time that a species can tolerate in stressful thermal and water conditions before presenting population declines (see Kearney et al., 2009, 2010). Based on our methods and results, we suggest that this physiological

information can include in these models to assess the climatic vulnerability of this and other anuran species and better inform the conservation strategies of these endangered taxa.

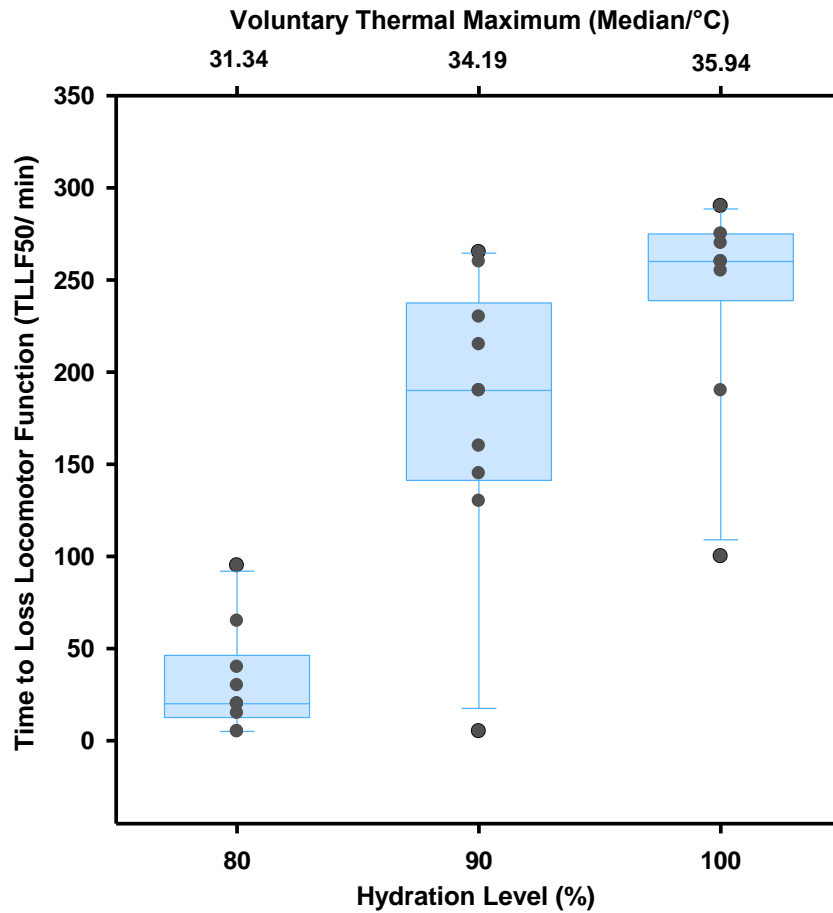
## **2.6. Acknowledgments**

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## 2.7. Figures

**Figure 1. Combined effects of dehydration and VTMax on the TLLF50 of *Lithobates catesbeianus*.**

Relationship between the VTMax and Hydration Level (HL) with the TLLF50. The fall suggests an exponential negative effect of temperature and hydration level on the TLLF50.



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## CONCLUSÕES GERAIS

Nossos resultados destacam os efeitos importantes da desidratação sobre o comportamento termorregulatório e na tolerância térmica de ectotermos de pele úmida, como anuros. Além disso, demonstramos os efeitos da desidratação sobre o tempo para a perda da função locomotora dos indivíduos expostos à tolerância térmica comportamental (i.e. VTMax). Nossa atualização do modelo de termorregulação de Heath (1970) integrando os efeitos da desidratação, não só é relevante para a compreensão da termorregulação e tolerância térmica destes organismos a condições térmicas e hídricas estressantes, mas também para apoiar os modelos mais avançados de vulnerabilidade climática dos organismos. Além disso, adicionar os efeitos da desidratação sobre o tempo para a perda da função locomotora em uma temperatura estressante, deve apoiar o desenvolvimento de melhores previsões de vulnerabilidade climática das espécies. Os efeitos da desidratação sobre as respostas proporcionais (PBT), tudo ou nada (VTMax), limites críticos (CTMax) e o TLLF, não foram exploradas ainda em modelos mecanicistas que fazem inferências sobre os efeitos do clima (e.g. temperaturas ambientais altas e secas) na atividade, fenologia e distribuição geográfica das espécies. Portanto, futuras abordagens podem utilizar nossas informações para incluí-las nesses modelos e prever a vulnerabilidade climática da Rã touro, bem como atualizar suas previsões de invasão, com importantes efeitos ecológicos. As informações teóricas e metodológicas apresentadas nos dois capítulos podem ser aplicadas a outros ectotermos de pele úmida, para os quais é necessário melhorar as avaliações de sua vulnerabilidade climática sob cenários atuais e futuros de mudanças climáticas.