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Temperature and dehydration effects on respiratory and cutaneous water flux in the terrestrial toad, *Rhinella schneideri* (Anura, Bufonidae)

LUIS MIGUEL SENZANO CASTRO

Dissertação apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista Júlio de Mesquita Filho, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Zoologia).

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AUTOR: LUIS MIGUEL SENZANO CASTRO
ORIENTADOR: DENIS OTAVIO VIEIRA DE ANDRADE

Aprovado como parte das exigências para obtenção do Título de Mestre em CIÊNCIAS BIOLÓGICAS (ZOOLOGIA), pela Comissão Examinadora:

Prof. Dr. DENIS OTAVIO VIEIRA DE ANDRADE
Departamento de Zoologia / Instituto de Biociências de Rio Claro - SP

Prof. Dr. JOSE EDUARDO DE CARVALHO
Ecologia e Biologia Evolutiva / Universidade Federal de São Paulo - UNIFESP

Profa. Dra. KÊNIA CARDOSO BÍCEGO
Departamento de Morfologia e Fisiologia Animal / FCAV/UNESP

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ABSTRACT

As ectotherms, amphibians may experience wide fluctuations in body temperature and, due to their high skin permeability, terrestrial species face a constant risk of desiccation. These two variables (temperature and dehydration) are centrally relevant for water balance regulation because they directly affect water flux through the integument, *i.e*. skin evaporative water loss (EWL_{Skin}) and water uptake (WU). In addition, as nearly all anurans are lung breathers, the respiratory water loss (EWL_{Resp}) will add to EWL_{Skin}. Although the contribution of EWL_{Resp} to total EWL (EWL_{Total}) is generally assumed to be negligible, the partitioning between EWL_{Skin} and EWL_{Resp} varies among species and is affected by temperature and dehydration. Therefore, we investigated the combined effects of temperature and dehydration on the rates of EWL through the skin and the lungs of the terrestrial toad, *Rhinella schneideri*. Subsequently, we evaluated how water uptake (WU) through the pelvis skin was affected by temperature and dehydration. To this aim, we measured rates of EWL_{Total} and EWL_{Skin} in intact and masked adult toads at 15, 25 and 35 ^{o}C under fully hydrated condition and dehydrated until they have lost 10% and 20% of their initial body mass. Masked toads were able to breath normally during the measurement of EWL_{Skin}; EWL_{Resp} was calculated as EWL_{Total} minus EWL_{Skin}. Rates of EWL were also determined using biophysical agar models of *R. schneideri* specimens, which allowed the estimation of skin resistance (R_s) to evaporation. WU rates were determined by measuring body mass gain against rehydration time of toads placed on a thin film of water. EWL_{Skin} and EWL_{Resp} increased with temperature, however, this effect was much more pronounced for EWL_{Resp} than for EWL_{Skin} and, as a result, the partitioning between cutaneous and respiratory water loss was significantly altered with temperature. Indeed, the contribution of EWL_{Resp} to EWL_{Total} increased from 2.44% at 15 ^{o}C to 8.1% at 35 ^{o}C. This result may be attributed to a limited capacity for EWL_{Skin} regulation combined with a temperature induced increment in pulmonary ventilation resulting from the elevation in metabolic rate with temperature. The contribution of EWL_{Skin} to EWL_{Total} decreased with dehydration which may be related to a loss in skin water content and the subsequent compression of cell layers, which limit water efflux to the environment. Therefore, the relative contribution of EWL_{Resp} augmented with temperature and dehydration accompanied by the corresponding decrease in the relative contribution of EWL_{Skin}. Rates of WU increased with dehydration but not with

temperature, which indicate the central role of the osmotic gradient in driving water flow through the anuran skin.

Como animais ectotérmicos, os anfíbios experimentam longas flutuações na temperatura corpórea e, devido à sua alta permeabilidade cutânea, eles também enfrentam o risco constante da dessecação. Essas duas variáveis (temperatura e desidratação) são muito relevantes no contexto do balanço hídrico dado que elas afetam diretamente o fluxo de água através da pele, ou seja, a perda evaporativa de água (PEA_{Pele}) e a absorção de água (RE). Além disso, dado que a maioria dos anuros possuem respiração pulmonar, a perda de água respiratória (PEA_{Resp}) irá se adicionar à cutânea. Embora a contribuição da PEA_{Resp} para a perda de água total (PEA_{Total}) seja geralmente considerada insignificante, a divisão entre PEA_{Pele} e PEA_{Resp} varia de acordo com as espécies e é afetada pela temperatura e desidratação. Portanto, nós investigamos os efeitos combinados da temperatura e desidratação nas taxas de PEA através da pele e pulmões no sapo terrestre, *Rhinella schneideri*. Posteriormente, avaliamos como a absorção de água através da pele ventral (RE) foi afetada pela temperatura e desidratação. Para isso, medimos as taxas de PEA_{Total} e PEA_{Pele} em sapos adultos intactos e mascarados à 15, 25 e 35 ^{o}C e desidratados até perderem 10% e 20% da massa corpórea inicial. Os sapos mascarados foram capazes de respirar normalmente durante a medição da PEA_{Pele}; PEA_{Resp} foi calculado como PEA_{Total} menos PEA_{Pele}. As taxas de perda de agua também foram determinadas usando modelos de ágar de espécimes de *R. schneideri*, o que permitiu a estimativa da resistência da pele (RP) à evaporação. As taxas de RE foram determinadas medindo o ganho de massa corpórea durante o tempo de reidratação dos sapos colocados sobre uma fina camada de água. PEA_{Pele} e PEA_{Resp} aumentaram com a temperatura, no entanto, este efeito foi muito mais marcado para a PEA_{Resp} do que para a PEA_{Pele} e, como resultado, a divisão entre a perda de água cutânea e respiratória foi significativamente alterada com a temperatura. De fato, a contribuição da PEA_{Resp} para a PEA_{Total} aumentou de 2,44% a 15 ^{o}C para 8,1% aos 35 ^{o}C. Este resultado pode ser atribuído a uma capacidade limitada para a regulação da PEA_{Pele}, combinada com um aumento na ventilação pulmonar induzido pela temperatura devido à elevação na taxa metabólica. A contribuição de PEA_{Pele} para a PEA_{Total} diminuiu com a desidratação, o que pode estar relacionado à perda de conteúdo de água da pele e a subsequente compressão das camadas celulares, o que limita o fluxo de água para o ambiente. Portanto, a contribuição relativa da PEA_{Resp} aumentou com a temperatura e a desidratação, acompanhada da diminuição correspondente da contribuição relativa de PEA_{Pele}. As taxas de RE aumentaram com a

desidratação, mas não com a temperatura, o que indica o papel central do gradiente osmótico na condução do fluxo de água através da pele.

INTRODUCTION

Terrestrial anuran amphibians are capable of performing their routine activities away from standing water, yet they still depend on moisture to rehydrate (Wells 2007). Nonetheless, terrestriality may entails hydric constraints since most amphibians are susceptible to high rates of evaporative water loss (EWL) because of their highly permeable skin (Hillman *et al*. 2009). As a consequence, terrestrial anurans are prone to experience variable degrees of hydration states while active (see Tracy *et al*. 2014). As ectothermic organisms, amphibians may also experience wide fluctuations in body temperature, which mirroring the variation of their thermal environment (Seebacher and Alford 2002, Noronha-de-Souza *et al.* 2015). Due to the fact that EWL rates are influenced by temperature (Tracy 1976), and because evaporation from the skin has a cooling effect on body temperature (Andrade *et al*. 2016), thermoregulation and water balance are highly intertwined in terrestrial anurans (Feder and Burggren 1992, Navas *et al*. 2008). Therefore, the interaction between these two important physiological functions may involve important trade-offs that will vary from species to species, among different organismal conditions, and as a function of the thermal and hydric environment (Rogowitz *et al*. 1999, Seebacher and Alford 2002, Anderson and Andrade 2017).

In general, terrestrial species may withstand large losses of body water (Wells 2007) however, diverse detrimental effects are expected (see Lillywhite 1975, Hillman 1987). Dehydration is also accompanied by a decrease in EWL and an increase in water uptake (WU) through the ventral skin area, the "pelvic patch" (see Anderson *et al*. 2017). This dehydration-induced dynamic on cutaneous water flux is, as expected, influenced by the temperature (Preest *et al*. 1992, Rogowitz *et al*. 1999). Indeed, if relative humidity is fixed, rises in temperature increases the rates of EWL by affecting air capacitance for water vapor. Thus, dehydrated anurans can minimize water loss by behaviourally selecting cooler sites (Tracy *et al*. 1993, Dohm *et al*. 2001, Anderson and Andrade 2017). Finally, although behavioral performance is also negatively impacted by dehydration, the optimal temperature to attain optimal performance is also shifted to low temperatures under more dehydrated states (Anderson and Andrade 2017). These responses illustrate the complex trade-offs involving thermoregulation and water balance in anurans.

Evaporative water loss is not a process that occurs solely through skin surface (EWL_{Skin}) but, for lung breathers, also involves evaporation through the lung surface (EWL_{Resp}) (Mautz 1982, Hillman *et al*. 2009). Nonetheless, since most anurans exchange a substantial fraction of their respiratory gases through the skin and since they have a relatively low pulmonary surface area (Czopek 1965) associated to low rates of ventilation while resting (Burggren and Doyle 1986), EWL_{Resp} is quite commonly assumed to be negligible in these animals (Spotila and Berman 1976, Bentley and Yorio 1979, Wygoda 1981, 1984). However, lung ventilation in anurans can vary considerably among species (Hutchison *et al*. 1968), under different circumstances (Whitford 1973) and in response to environmental factors (Boutilier 1992). For example, lung ventilation is known to be affected by temperature (Kruhøffer *et al.* 1987, Branco *et al*. 1993, Bícego-Nahas *et al*. 2001, Zena *et al*. 2016) and dehydration (Boutilier *et al*. 1979). However, the interactive effects of both of these factors, temperature and dehydration, on the relative contribution of EWL_{Resp} and EWL_{Skin} to total evaporation (EWL_{Total}) is not available. This evaluation is ecologically and functionally meaningful since these two routes of water loss involve different sets of constraints that may be differently affected by changes in temperature and hydration state (Hutchison *et al*. 1968, Geise and Linsenmair 1986, Rogowitz *et al*. 1999, Burggren and Vitalis 2005). Accordingly, we examined the combined effects of temperature and dehydration on the partitioning of water balance between the skin and the lung in the terrestrial toad, *Rhinella schneideri*, under different temperatures and hydration levels. More specifically, we measured EWL rates of intact and masked toads, for the estimation of EWL_{Total} and EWL_{Skin}, respectively. Thereafter, we estimated EWL_{Resp} as the difference between EWL_{Total} and EWL_{Skin}. Finally, we determined the influence of temperature and dehydration on the rate of water uptake (WU) through the pelvic patch of the toads. We chose *R. schneideri* to perform the present study because this species has a terrestrial lifestyle and is broadly distributed in tropical biomes of South America (Haddad *et al*. 2013) and, therefore, it is likely to experience natural fluctuations in body temperature and hydration state.

Our specific hypothesis for the present study includes: 1) the rise in temperature will increase the rates of EWL_{Skin} and EWL_{Resp}; 2) this effect will be more pronounced for EWL_{Resp}, as metabolism and lung ventilation will likely be elevated at higher temperatures; 3) dehydration will cause a temperature-independent decrease in EWL_{Skin} but not on EWL_{Resp}; 4) however, the partitioning between EWL_{Skin} and EWL_{Resp} is not expected to be affected by hydration state; and 5) the WU rates will be leaded by dehydration and not by temperature.

Experimental animals

We collected 14 adults of both sexes of *R. schneideri* (average body mass = 191.7; SD ±78.8 g), 8 in mid-September of 2015 and 6 in late January of 2017, in the municipality of Barbosa, São Paulo State, Brazil (21º15'1,72"S, 49º55'16,75"W; 371 m a.s.l.). Animals were taken to the Laboratory of Comparative Animal Physiology at the São Paulo State University (Rio Claro, SP, Brazil). Animals were kept in captivity in individual plastic boxes (40x30x25 cm), maintained at room temperature of 24.1±0.5 ºC, 50-80% of relative humidity and natural photoperiod. PVC plastic tubes (10 x 20 cm) and plant leaves were provided as shelters. Toads were fed twice weekly on mealworms (*Tenebrio* sp.) and crickets (*Gryllus* sp.); water was freely available all time. Animals were fasted for a minimum of 72 h before experimental trials. All procedures were approved by the UNESP-IB/Rio Claro Animal Ethic and Use Committee (Comissão de Ética no Uso Animal, CEUA; License No 6915; Appendix 3).

Experimental design and dehydration protocol

Experiments were conducted under 3 temperatures (15, 25 and 35 ºC) and 3 hydration levels (100, 90 and 80%), where 100% refers to fully hydrated toads (see details below), and 90 and 80% refers to toads dehydrated until their body mass have equalled 90 and 80% of their fully hydrated body mass. Each toad was exposed to all the 9 combinations of temperature and hydration levels in random order. Toads were subjected to just one treatment per day and, after the EWL and WU measurements, they were allowed to recover for at least 12 hours. After 4 consecutive days of measurements, toads were fed and we allow them a longer recovering period of 3 days. Usually, toads were dehydrated, when it was the case, early in the morning (0600 to 1400 h) and measurements taken in the afternoon (1600 and 2200 h). All toads were initially measured under the intact condition (no mask) for the determination of EWL_{Total} under all temperature and hydration levels. Only after the completion of these measurements, they were measured under the masked condition for the determination of EWL_{Skin} (details provided below).

Before all experimental trials, animals were fully hydrated by placing them individually for 2 h inside a plastic cage (volume 2.2 L) with 0.5 cm water depth. After that period, their urinary bladder was emptied by either gently pressing the abdomen area. Excess of water over

skin was blotted with paper tissue and the weight of the fully hydrated animal recorded (±0.01 g). If that was the case, measurements were made on the fully hydrated toads, otherwise, they were subjected to a dehydration protocol, which consisted of exposing the toads to a wind tunnel at air speed of 3.8 $m.s^{-1}$, until the desired level of dehydration was obtained (within 3 to 7 hours for 90 and 80%, respectively). This wind tunnel was built from a PVC tube (25 cm diameter x 15 cm length) covered with a plastic mesh on both sides and positioned 10 cm away in front an electric fan that blew room air (RH ~65%; temperature 21.3±1.9 ^{o}C) through it.

Once the desired hydration level was attained, animals were placed into the measurement chamber and transferred to a climatic chamber BOD (Eletrolab EL101/2RS) where they were left for 2 hours to acclimate with the experimental temperature. Measurements started immediately after acclimation and proceeded until we identified a minimum period of 15 min of steady-state recordings occurring within 1 to 2 hours of experimentation. Steady-state condition was judged from the visual inspection of the graphic output of the data acquisition software (ExpeData Software, Sable Systems) and confirmed by checking whether the toad was quiescent inside the measuring chamber. In case the animal was agitated and we failed to obtain a steady-state record within 2 h from the beginning of the experimental trial, or in case the animal defecated inside the chamber, the experiment was aborted and data was discarded. For successful trials, immediately after the experiment was interrupted, we measured dorsal skin temperature using an infrared thermometer (±0.1 ^{o}C; 62 Mini IR, Fluke). At this time, we also re-weight the animals to check whether their hydration level was altered. WU rates were determined immediately after EWL measurements (see details below).

Evaporative water loss measurements

Total evaporative water loss (EWL_{Total}) was determined by placing unmasked toads individually inside a circular PVC chamber (vol = 1 L; 15 cm diameter x 6 cm height) connected to an open-flow EWL measurement system (see Appendix 1). In this system, a constant airflow (21.66 cm^3.s) generated and controlled by an integrated air pump, meter, and flow controller (SS-4 Subsampler, Sable Systems) was directed through two drying columns (one of them settled before SS-4) filled with silica gel and then to the PVC chamber containing the animal. The airflow leaving the animal chamber was monitored with a water vapor analyser (RH-300, Sable Systems). The analog output from the airflow unit and the RH meter was acquired via an A/D converter (UI-2 Data Acquisition Interface, Sable Systems) and digitally recorded in a computer at real time with the ExpeData software (Sable Systems). Previously and after any

EWL measurement, we collected baseline RH measurements for the empty chamber, which were later used for the calculation of the respective animal EWL rate. RH of the incurrent airflow was allowed to vary from 0.5 to 1.5%, above which the water-absorbing columns were replaced.

The EWL_{Skin} was measured under identical conditions as described above but on masked toads (see Withers 1977). Masks were built with heat-mouldable plastic sheets modelled on top of plaster of Paris models of the toad´s head; models were obtained from counter-molds created by individual head impressions taken in dental alginate (AvaGel). Masks were hermetically glued on the toad's face 12 h before measurements using cyanoacrylate adhesive (superglue) (Wright and Whitaker 2001), following from the upper mid-bony ridges over the eyes to the bony rim on lower lip. Therefore, the mask covered the nares and kept the toads mouth shut, even though vision and the use of the buccopharyngeal pump were uncompromised for ventilation (Fig. 1a). During EWL measurements, in which the toad was confined inside the measuring chamber, the mask was airtightly connected to secondary and independent air pump (SS-4 Subsampler, Sable Systems) that pulled external air through it (flow rate = 2.5 cm^3.s), which allowed for toad's normal breathing on fresh air. This setup, therefore, excluded the contribution of EWL_{Resp} to the recorded changes in RH and allowed for the isolated measurement of EWL_{Skin}.

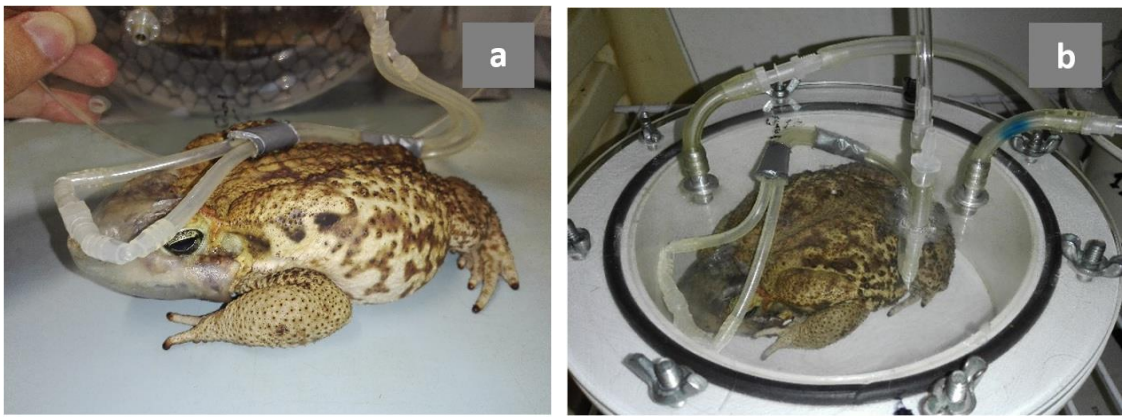

Figure 1. Masked individual of *Rhinella schneideri* showing details of mask placement (a) and inside the EWL measuring chamber (b).

The absolute water loss of both the EWL_{Total} (unmasked toads) and EWL_{Skin} (masked toads) were determined by using the equation, $EWL = (VD_e - VD_i)*F$; where *EWL* is the absolute water loss ($\mu gH_2O.s^{-1}$), *VDe* and *VDi* are the excurrent and incurrent water vapor density (WVD) in the animal's chamber ($\mu gH_2O.s^{-1}$), respectively, and *F* is the airflow ($cm^3.s$) (Withers *et al*. 1982). Respiratory water loss was estimated as, $EWL_{Resp} = EWL_{Total} - EWL_{Skin}$. The partitioning between EWL_{Skin} and EWL_{Resp} was expressed as a percentage of EWL_{Total} ($\%EWL_{Skin}$ and $\%EWL_{Resp}$).

Finally, to estimate EWL_{Skin} by area-specific water loss ($\mu g.cm^{-2}.s^{-1}$), absolute EWL_{Skin} rates ($\mu gH_2O.s^{-1}$) were divided by 2/3 of toad's estimated total skin surface area (A_s). A_s was estimated from body mass as described by Klein *et al.* (2016) specifically for the Bufonidae family: $A_s = 7.956Mass^{0.6772}$. Equating A_s to 2/3 accounts for the fact that only a portion of skin surface area was exposed to air during the experiments (see Withers *et al.* 1982, 1984). To estimate the magnitude of the potential errors in disregarding the contribution of EWL_{Resp} to EWL_{Total}, we also calculated area-specific EWL_{Skin} rates on the basis of EWL_{Total}.

Skin resistance to evaporation

Total resistance (R_T) to evaporation is the combined result of both skin (R_s) and boundary layer resistances (R_b), thereby, in order to estimate *R. schneideri* R_s, it is necessary to determine R_b. This was done by measuring EWL rates from agar models of toads, and as these models lack any R_s and lose water freely, their EWL rates will be solely determined by R_b (Spotila and Berman 1976). Toad's agar models were obtained from the impression of formalin-fixed specimens of *R. schneideri* in dental alginate. Toads alginate casts were filled with 3% agar solution and left to harden (usually within 1 h). After that, we measured the agar models EWL rates under identical conditions as described above for live toads. In total, we measured 23 agar models at each experimental temperature (15, 25, 35 ^oC). Agar models R_b values were then linearly regressed against body (agar) masses and the resulting allometric equation was used to estimate toad's R_b, on the basis of their body masses (see also Table 4). Toad's R_T was calculated from EWL measurements following Spotila and Berman (1976), $R_T = VDD/EWL$; where R_T is the total resistance ($s.cm^{-1}$), *VDD* is the water vapor density (WVD) gradient ($\mu g.cm^{-3}$) between the saturated WVD at the temperature skin surface of animal and the partial WVD at ambient temperature, and *EWL* is the area-specific EWL ($\mu g.cm^{-2}.s^{-1}$). Skin resistance was calculated as, $R_s = R_T - R_b$. In all these calculations, we used EWL_{Skin} data measured

obtained from masked toads, however, for comparison purposes, we also calculated R_s based on EWL_{Total}, assuming a negligible EWL_{Resp}.

Water uptake

Immediately after EWL measurements of intact unmasked toads, we randomly selected 10 individuals per experimental treatment to measure the rates of water uptake through the pelvic patch. To this aim, we placed the animals in individual plastic boxes (1 L) containing 0.5 cm of unchlorinated tap. At 2-min intervals, toads were carefully blotted with paper tissue and weighed (±0.01 g; Marte scale, AD5002) for 6 consecutive times (Titon *et al*. 2010). WU rates were calculated from the regression slope of body mass gain against rehydration time. Animals that presented sudden decreases in body mass, indicative of urination, were excluded from the data set. WU was expressed by skin surface area ($\mu g.cm^{-2}.s^{-1}$) assuming that the ventral region in contact with the water equalled to one-third of total skin surface area (estimated as described above).

Statistical analysis

The effects of temperature and dehydration on EWL_{Total}, EWL_{Skin}, EWL_{Resp}, R_s and WU was evaluated through a two-way repeated measures analysis of variance (two-way RM ANOVA) with temperature and hydration level as factors. On the $\%EWL_{Skin}$ and $\%EWL_{Resp}$, relativized data was firstly arcsine square root transformed. The Holm-Sidak method was employed as a *post-hoc* multiple comparison test to identify differences within treatments whenever was necessary. To assess the potential effect of neglecting EWL_{Resp} on area-specific EWL_{Skin} and R_s estimations, we calculated area-specific EWL_{Skin} and R_s with and without the inclusion of EWL_{Resp}. These rates were then compared among experimental temperatures and hydration levels by performing two separate Generalized Linear Mixed-effect Models (GLMM)("*lme4*" package), one model for area-specific EWL_{Skin} and the other for R_s, where temperature and hydration level were set as fixed factors, the individuals as random factor, and the area-specific EWL_{Skin} and R_s as the response variable to their respective model. The interactions among factors were assessed by Likelihood Ratio tests and we used the *post-hoc* multiple pair-wise comparison Tukey's test ("*multcomp*" package) to test differences within treatment with and without the inclusion of EWL_{Resp}. Data was verified with the Shapiro-Wilk test for normality and the Levene's test for homogeneity of variances. Absolute rates of EWL_{Total}, EWL_{Skin} and EWL_{Resp} was $\log_{10}$ transformed to meet distribution assumptions. All analyses were performed on R (V. 3.3.1; R Development Core Team, 2016) employing RStudio

(V. 1.0.136; RStudio Team, 2016). All data analysis is presented as mean values and error bars indicating ±SD. Differences were considered statistically significant when $P \leq 0.05$.

Temperature rise increased the rates of EWL_{Total} ($F_{2,26}$ = 1147.15, P<0.001; Fig. 2A), and absolute EWL_{Skin} ($F_{2,26}$ = 730.5, P<0.001), and EWL_{Resp} ($F_{2,26}$ = 659.95, P<0.001) (Fig. 2B) (Table. 1). Dehydration progression caused a decrease in the rates of EWL_{Total} ($F_{2,26}$ = 162.2, P<0.001; Fig. 2A) and absolute EWL_{Skin} ($F_{2,26}$ = 143.3, P<0.001); but not in EWL_{Resp} ($F_{2,26}$ = 1.51, P=0.24) (Fig. 2B). No significant interaction was found between temperature and dehydration for absolute EWL_{Total} ($F_{4,52}$ = 0.95, P=0.443), EWL_{Skin} ($F_{4,52}$ = 2.11, P=0.092) and EWL_{Resp} ($F_{4,52}$ = 0.21, P=0.928) (Table 1). The increase in temperature ($F_{2,26}$ = 135.6, P<0.001)

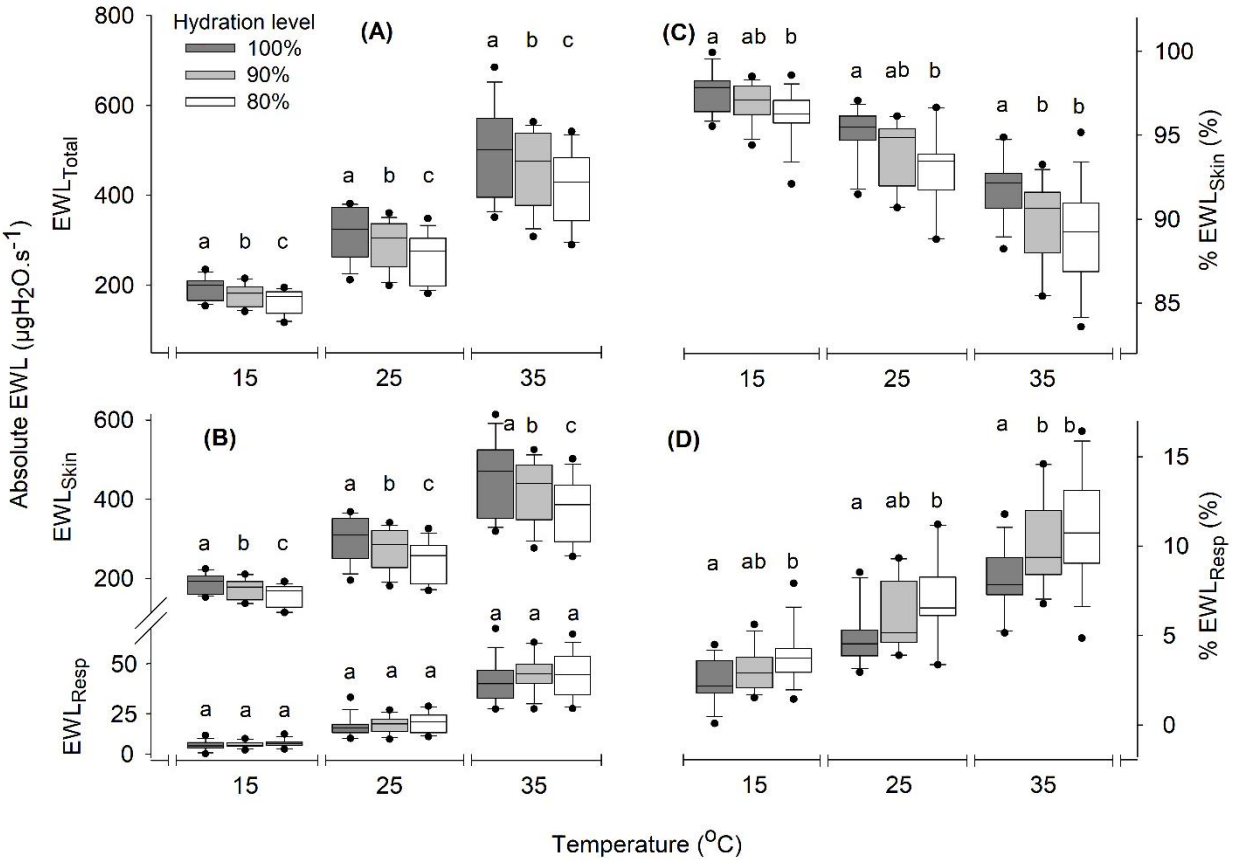

Figure 2. Effects of dehydration and temperature on EWL rates measured on *Rhinella schneideri* toads (n = 14). (*A*) The overall absolute water loss calculated from the whole-animal surfaces (EWL_{Total}), and (*B*) the partitioning into its cutaneous and respiratory components. The relative contribution of both the $\%EWL_{Skin}$ (*C*) and $\%EWL_{Resp}$ (*D*) avenues are expressed as a percentage of the EWL_{Total}. There were significant differences at all temperature comparisons (P<0.05) and different lowercase letters denote significant differences among hydration states (100, 90 and 80%) within each temperature. Boxplot lines indicate medians and the lower and upper borders represent the 25th and 75th percentiles, whiskers ± SD and filled circles are outliers.

or dehydration ($F_{2,26}$ = 7.65, P=0.002) was accompanied by a pronounced reduction in the $\%EWL_{Skin}$ (Fig. 2C), whilst the relative contribution of $\%EWL_{Resp}$ were gradually augmented with both the temperature ($F_{2,26}$ = 135.6, P<0.001) and dehydration ($F_{2,26}$ = 7.65, P=0.002)(Fig. 2D). No significant interaction was found between temperature and dehydration for $\%EWL_{Skin}$ ($F_{4,52}$ = 0.196, P=0.939) and $\%EWL_{Resp}$ ($F_{4,52}$ = 0.196, P=0.939) (Table 1).

Rates of area-specific EWL_{Skin} calculated solely from the skin avenue (Fig. 3A) maintained the expected temperature-induced increase ($F_{2,26}$ = 375.51, P<0.001) and dehydration-induced decrease ($F_{2,26}$ = 71.35, P<0.001) patterns as previously observed in Fig. 2, and it was found a significant interaction effect between both temperature and dehydration factors ($F_{4,52}$ = 10.48, P<0.001; Table 2). The skin resistance (R_s) calculated from area-specific EWL_{Skin} avenue (Fig. 3B) showed a temperature- and dehydration-induced increase ($F_{2,26}$ = 187.8, P<0.001 and $F_{2,26}$ = 131.3, P<0.001, respectively) with a significant interaction between both factors ($F_{4,52}$ = 3.76, P=0.009; Table 2).

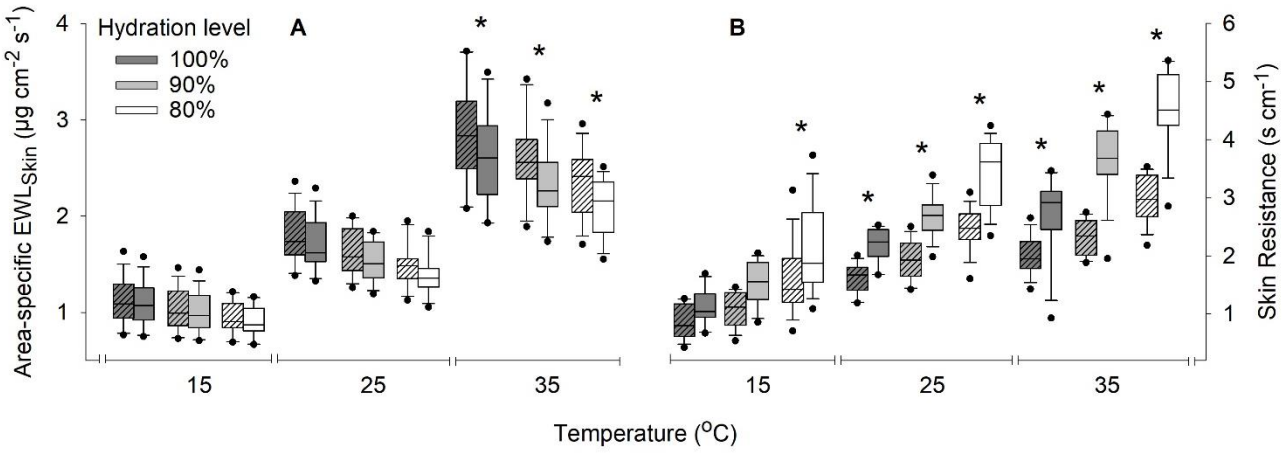

Figure 3. Dehydration and temperature effects on area-specific EWL_{Skin} and skin resistance (R_s) to evaporation of *Rhinella schneideri* toads (n = 14). Filled boxplots are the area-specific EWL_{Skin} on (A), and the R_s on (B), calculated solely from the skin water loss avenue with the use of masked toads. Dashed boxplots are the expected area-specific EWL_{Skin} on (A) and the expected R_s on (B) if calculated from the whole-animal absolute water loss (EWL_{Total}) which includes the pulmonary EWL contribution (EWL_{Resp}) measured on unmasked toads. There were significant differences at all temperature comparisons (P<0.05) and among hydration levels (P<0.05), and asterisks denote significant differences between masked and unmasked toad measurements at a given temperature and hydration level on (A) and (B). Boxplot lines indicate medians and the lower and upper borders represent the 25th and 75th percentiles, whiskers ± SD. and filled circles are outliers.

Our calculations on area-specific EWL_{Skin} based on EWL_{Total} (unmasked toads), showed a significant EWL_{Resp} interaction effect in response to temperature ($X^2_1 = 17.27$, $P<0.001$) but not with hydration level ($X^2_1 = 0.12$, $P=0.673$); however, a significant interaction was found among both factors ($X^2_1 = 30.18$, $P<0.001$) (Table. 3). Thus, the inclusion of EWL_{Resp} led to a small overestimate on rates of area-specific EWL_{Skin} at lower temperatures when compared to actual EWL_{Skin} rates (masked toads), and this difference augmented significantly at 35 ^oC in all hydration levels (Fig. 3A). On the other hand, our calculations on R_s values based on EWL_{Total} resulted in a marked underestimate of actual R_s values (except in 100% and 90% at 15 ^oC; Fig. 3B), underestimate that was significantly increased at higher temperatures ($X^2_1 = 36.71$, $P<0.001$) and higher dehydration levels ($X^2_1 = 13.32$, $P<0.001$). We found a significant interaction among both factors ($X^2_1 = 149.7$, $P<0.001$) (Table. 3).

WU rates were affected by temperature ($F_{2,18} = 19.37$, $P = <0.001$) and dehydration ($F_{2,18} = 60.06$, $P<0.001$). In general, WU rates were increased at higher temperatures and dehydration levels (Fig 4). There was no interaction between temperature and dehydration on WU rates ($F_{4,36} = 1.43$, $P=0.244$; Table 2).

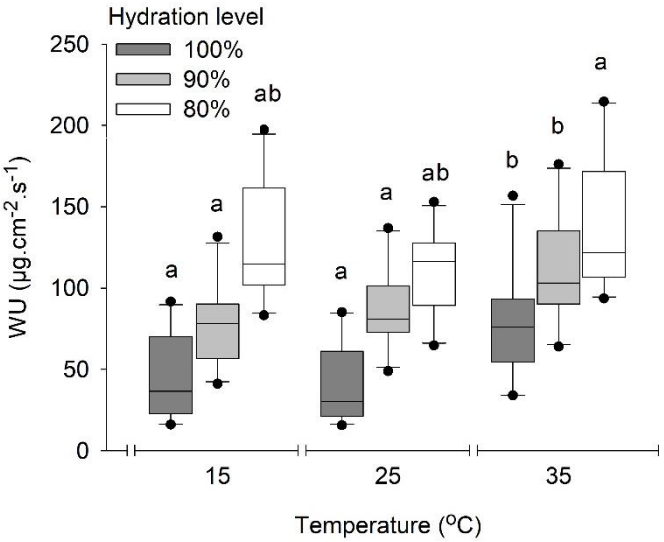

Figure 4. Rates of water uptake in response to different temperatures and dehydration states on *Rhinella schneideri* toads (n = 10). There were significant differences at all hydration state comparisons ($P<0.05$) and different lowercase letters indicate significant differences between different temperatures (15, 25 and 35 ^oC) within each dehydration treatment. Boxplot lines indicate medians and the lower and upper borders represent the 25th and 75th percentiles, whiskers ± SD and filled circles are outliers.

The increment in global evaporative water loss (EWL_{Total}) with temperature is almost universal among anuran amphibians (Cloudsley-Thompson 1967, McClanahan *et al.* 1978, Shoemaker *et al.* 1987, Buttemer 1990, Buttemer and Thomas 2003, Tracy *et al*. 2008). Since water vapor pressure increases with temperature (Dejours 1976, Tracy 1976), increased rates of evaporation will follow, unless animals exhibit concurrent adjustments to promote water conservation (Feder and Burggren 1992, Andrade *et al*. 2016). When partitioned between its respiratory and cutaneous components, it becomes clear that, although temperature increase affected both avenues, EWL_{Resp} was largely more affected than EWL_{Skin}. Indeed, the relative contribution of EWL_{Resp} increased from 2.4% to 8.1% as temperature was elevated from 15 to 35^{o}C, with the corresponding decrease in the contribution of EWL_{Skin} (from 97.5% to 91.8%). In anurans, as in most organisms, metabolism and lung ventilation almost universally increases with temperature (Wells 2007, Wang *et al*. 1999, Lillywhite 2006), a response already documented for *R. schneideri* (Kruhøffer *et al*. 1987, Branco *et al*. 1993, Bícego-Nahas *et al*. 2001, Zena *et al*. 2016). Thus, the temperature induced increase in EWL_{Resp}, relative to EWL_{Skin} in *R. schneideri* can be confidently attributed to a concurrent temperature induced increment in metabolism and lung ventilation (see also Mautz 1982, Hillman *et al*. 2009).

In anurans, EWL_{Total} decreases as dehydration progresses (Thorson 1956, Warburg 1965, Cloudsley-Thompson 1967, Loveridge 1970) a response previously documented in *R. schneideri* (Anderson *et al*. 2017). The effects of dehydration on the area-specific EWL_{Skin} rates, at 25 ^{o}C, are slightly lower than those reported by Anderson *et al*. (2017). This difference may be related to the fact that Anderson *et al*. (2017) did not exclude EWL_{Resp} from the area-specific EWL_{Skin} calculations, as we did in the present study. The partitioning between respiratory and cutaneous EWL under dehydration is poorly known in amphibians. However, it has been pointed out that a reduction in the rates of EWL_{Skin} due to desiccation, may result in the increase of EWL_{Resp} relative contribution (Geise and Linsenmair 1986). Dehydration diminishes water content on integumentary cell layers (Lillywhite 1971, Lillywhite and Licht 1974) and compress the keratinized stratum corneum, which decreases skin permeability (Lillywhite 1975, Lillywhite and Licht 1975, Machin 1969). Also, capillary blood flow adjustments in the dorsal skin (Burggren and Moalli, 1984; Hillman, 1987; Slivkoff and Warburton, 2001; Burggren and Vitalis, 2005) or more tucked postures adopted by dehydrated

toads, may also contribute to lower EWL_{Skin} rates. These factors explain why absolute rates of EWL_{Skin}, as well as its relative contribution to EWL_{Total}, diminished with dehydration in *R. schneideri*. On the other hand, dehydration did not affect the absolute rates of EWL_{Resp} and, as a consequence, its relative contribution to EWL_{Total} increased by the same magnitude in which EWL_{Skin} was diminished. This effect was similar across all temperatures, however, its magnitude was amplified at higher temperatures, which can be explained by the fact that the relative contribution of EWL_{Resp} to EWL_{Total}, in relation to EWL_{Skin}, is already elevated at higher temperatures (see comments above).

Cutaneous rates of EWL in anurans are commonly expressed by unit area of skin surface (area-specific EWL_{Skin}), which is generally calculated on the basis of EWL_{Total} (instead of actual EWL_{Skin}) under the assumption of a "negligible" EWL_{Resp}. This assumption is justified by the fact that the highly permeable skin of anurans is permissive to high rates of evaporative water loss combined to the fact that their relatively low metabolism and lung ventilation, minimizes evaporation via respiration. Our results point out that the error in not partitioning EWL rates into its respiratory and cutaneous component for the calculation of area-specific EWL_{Skin} is indeed relatively low, but not entirely negligible, at lower temperatures. For fully hydrated *R. schneideri* this error averaged in about 1.8% of the EWL_{Skin} rates at 15 ºC. However, as discussed above, the contribution of EWL_{Resp} to EWL_{Total} increases considerably with temperature and, at 35ºC, the error in assuming EWL_{Resp} as negligible results in an overestimation of area-specific EWL_{Skin} of 8.8%. Dehydration caused the relative contribution of EWL_{Resp} to EWL_{Total} to increase (discussed above) and as a consequence, assuming EWL_{Resp} as negligible resulted in greater errors at greater dehydration levels. For example, the overestimation of EWL_{Skin} by not discounting EWL_{Resp}, increased from 3.3% at 15ºC to 12% at 35ºC from fully hydrated to 80% dehydrated toads. The consequences of not discounting EWL_{Resp} for the calculation of area-specific EWL_{Skin} just presented, were also observed for the estimation of skin resistances. Accordingly, at low temperatures and fully hydration, assuming EWL_{Resp} as negligible resulted in moderate underestimation of R_s (about 28.7%), however, this error became more pronounced at higher temperatures and dehydration levels reaching upon to 34% at 35ºC and 80% dehydration. In conclusion, the assumption of EWL_{Resp} as negligible in assessing the amphibian skin permeability should not be considered unrestrictedly and needs to consider the potential factors affecting the partitioning between cutaneous and respiratory routes for water evaporation. Interspecific differences in skin permeability and the degree of

dependence on pulmonary gas-exchange will also affect the partitioning between cutaneous and respiratory evaporative water loss.

Water uptake through the skin in *R. schneideri* increased with dehydration in agreement with previous observations in other anurans (Tracy 1976, Parsons and Mobin 1991, Jørgensen 1997, Uchiyama 2015) and in this same species (Anderson *et al*. 2017). This pattern is related to the dehydration induced increment in body fluid osmotic pressure, which in turn, augments the osmotic gradient between the animal and the hydrating medium. Temperature did not cause *R. schneideri*'s WU rates to increase between 15 and 25ºC, however, a significant increment occurred at 35ºC for fully hydrated and 90% hydrated toads. This result contrasts partially with the notion that temperature does not affect markedly the rates of WU in anurans (*e.g.* Cloudsley-Thompson 1967, Claussen 1969, Tracy 1976). For example, Tracy (1976) showed that, under steady-state conditions, WU in *Rana pipiens* remains virtually constant at most body temperatures. Although uncertain, we suspect that some of the physiological parameters affecting skin permeability may respond differently to temperature changes occurring at different temperature intervals, as is reported to occur in some plethodontid salamanders (Spotila, 1972). This may involve changes in local microcirculatory perfusion of the pelvic patch (Slivkoff and Warburton 2001, Viborg and Rosenkilde 2004, Viborg *et al*. 2006) and constitutive changes in this region water permeability (Willumsen *et al*. 2007), possibly mediated by changes in the expression of aquaporins (Hasegawa *et al*. 2003, Suzuki *et al*. 2007, Saitoh *et al*. 2014).

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TABLES

Table 1. Summary and comparisons of the mean absolute evaporative water loss (EWL) in response to different temperatures and hydration states on *Rhinella schneideri* toads. Rates of the whole-animal surfaces (EWL_{Total}), the partitioning into its cutaneous (EWL_{Skin}) and respiratory (EWL_{Resp}) components, and the respectively relative contribution of both avenues ($\%EWL_{Skin}$ and $\%EWL_{Resp}$) in relation to the EWL_{Total}.

		Absolute EWL ($\mu gH_2O.s^{-1}$)				
Temp.(°C)	*Hydrat.(%)*	*EWL_{Total}*	*EWL_{Skin}*	*EWL_{Resp}*	*$\%EWL_{Skin}$ (%)*	*$\%EWL_{Resp}$ (%)*
15	100	193.48 ± 25.29^{aA}	188.66 ± 24.03^{aA}	4.81 ± 2.61^{aA}	97.55 ± 1.22^{aA}	2.44 ± 1.22^{a}
	90	177.53 ± 24.89^{bD}	172.15 ± 25.17^{bD}	5.38 ± 1.77^{aD}	96.89 ± 1.2^{abD}	3.1 ± 1.2^{ab}
	80	163.97 ± 27.49^{cG}	157.84 ± 27.51^{cG}	6.13 ± 2.13^{aG}	96.16 ± 1.51^{bG}	3.83 ± 1.51^{b}
25	100	317.24 ± 56.92^{aB}	301.93 ± 54.67^{aB}	15.3 ± 5.65^{aB}	95.14 ± 1.59^{aB}	4.85 ± 1.59^{a}
	90	288.34 ± 52.33^{bE}	271.73 ± 51.57^{bE}	16.6 ± 4.46^{aE}	94.1 ± 1.91^{abE}	5.89 ± 1.91^{ab}
	80	262.92 ± 52.84^{cH}	245.07 ± 51.66^{cH}	17.84 ± 5.65^{aH}	93.05 ± 2.28^{bH}	6.94 ± 8.1^{b}
35	100	500.53 ± 99.04^{aC}	460.21 ± 91.51^{aC}	40.32 ± 11.68^{aC}	91.89 ± 1.78^{aC}	8.1 ± 1.78^{a}
	90	457.29 ± 84.49^{bF}	411.85 ± 81.68^{bF}	45.44 ± 10.22^{aF}	89.86 ± 2.54^{bF}	10.13 ± 2.54^{b}
	80	416.45 ± 83.91^{cI}	371.71 ± 81.33^{cI}	44.74 ± 12.35^{aI}	88.99 ± 3.13^{bI}	11.05 ± 3.13^{b}

Factors	*F*	*DF*	*P*	*F*	*DF*	*P*	*F*	*DF*	*P*	*F*	*DF*	*P*	*F*	*DF*	*P*
Temperature	1147.15	2, 26	<0.001	730.5	2, 26	<0.001	649.95	2, 26	<0.001	135.6	2, 26	<0.001	135.6	2, 26	<0.001
Hydration	162.2	2, 26	<0.001	143.3	2, 26	<0.001	1.51	2, 26	0.24	7.65	2,26	0.002	7.65	2, 26	0.002
Temp. x Hydrat.	0.95	4, 52	0.443	2.11	4, 52	0.092	0.21	4, 52	0.928	0.196	4, 52	0.939	0.196	4, 52	0.939

Note. Different letters indicate statistically significant differences between groups. Lowercase letters denote comparisons of hydrations levels (100, 90, 80%) within each temperature. Uppercase letters denote comparisons of each hydration level between temperatures. A - C, 100%; D - F, 90%; G - I, 80%. *F* = F-value; *DF* = Degrees of freedom; *P* = p-value (Two-way RM ANOVA).

Table 2. Summary and comparisons of the mean area-specific EWL of skin (EWL_{Skin}), skin resistance (R_s), and water uptake (WU) rates in response to different temperatures and hydration levels on *Rhinella schneideri* toads.

Temp.(°C)	*Hydrat.(%)*	*EWL_{Skin} ($\mu g.cm^{-2}.s^{-1}$)*	*Rs ($s.cm^{-1}$)*	*WU ($\mu g.cm^{-2}.s^{-1}$)*
15	100	1.09 ± 0.23^{aA}	0.94 ± 0.31^{aA}	43.49 ± 25.96^{aA}
	90	0.99 ± 0.2^{bD}	1.54 ± 0.38^{bD}	77.65 ± 25.09^{bD}
	80	0.9 ± 0.15^{cG}	2.11 ± 0.74^{cG}	126.82 ± 36.48^{cGH}
25	100	1.71 ± 0.27^{aB}	2.05 ± 0.29^{aB}	41.37 ± 25.46^{aA}
	90	1.53 ± 0.2^{bE}	2.67 ± 0.35^{bE}	87.3 ± 25.06^{bD}
	80	1.38 ± 0.21^{cH}	3.43 ± 0.57^{cH}	110.77 ± 26.01^{cG}
35	100	2.61 ± 0.47^{aC}	2.56 ± 0.72^{aC}	78.68 ± 35.23^{aB}
	90	2.33 ± 0.38^{bF}	3.69 ± 0.63^{bF}	110.76 ± 33.76^{bE}
	80	2.09 ± 0.29^{cI}	4.51 ± 0.66^{cI}	138.88 ± 43.09^{cH}

Factors	*F*	*DF*	*P*	*F*	*DF*	*P*	*F*	*DF*	*P*
Temperature	375.51	2, 26	<0.001	187.8	2, 26	<0.001	19.37	2, 18	<0.001
Hydration	71.35	2, 26	<0.001	131.3	2, 26	<0.001	60.06	2, 18	<0.001
Temp. x Hydrat.	10.48	4, 52	<0.001	3.76	4, 52	0.009	1.43	4, 36	0.244

Note. Different letters indicate statistically significant differences between groups. Lowercase letters denote comparisons of hydration levels (100, 90, 80%) within each temperature. Uppercase letters denote comparisons of each hydration level between temperatures. A - C, 100%; D - F, 90%; G - I, 80%. *F* = F-value; *DF* = Degrees of freedom; *P* = p-value (Two-way RM ANOVA).

Table 3. Summary and comparisons of the mean area-specific EWL_{Skin} and mean skin resistance to evaporation (R_s) on *Rhinella schneideri* toads calculated solely from the skin water loss avenue (through the use of masked toads); and also calculated from the whole-animal water loss (unmasked toads) which includes the pulmonary EWL contribution (EWL_{Resp}) measured at different temperatures and hydration levels.

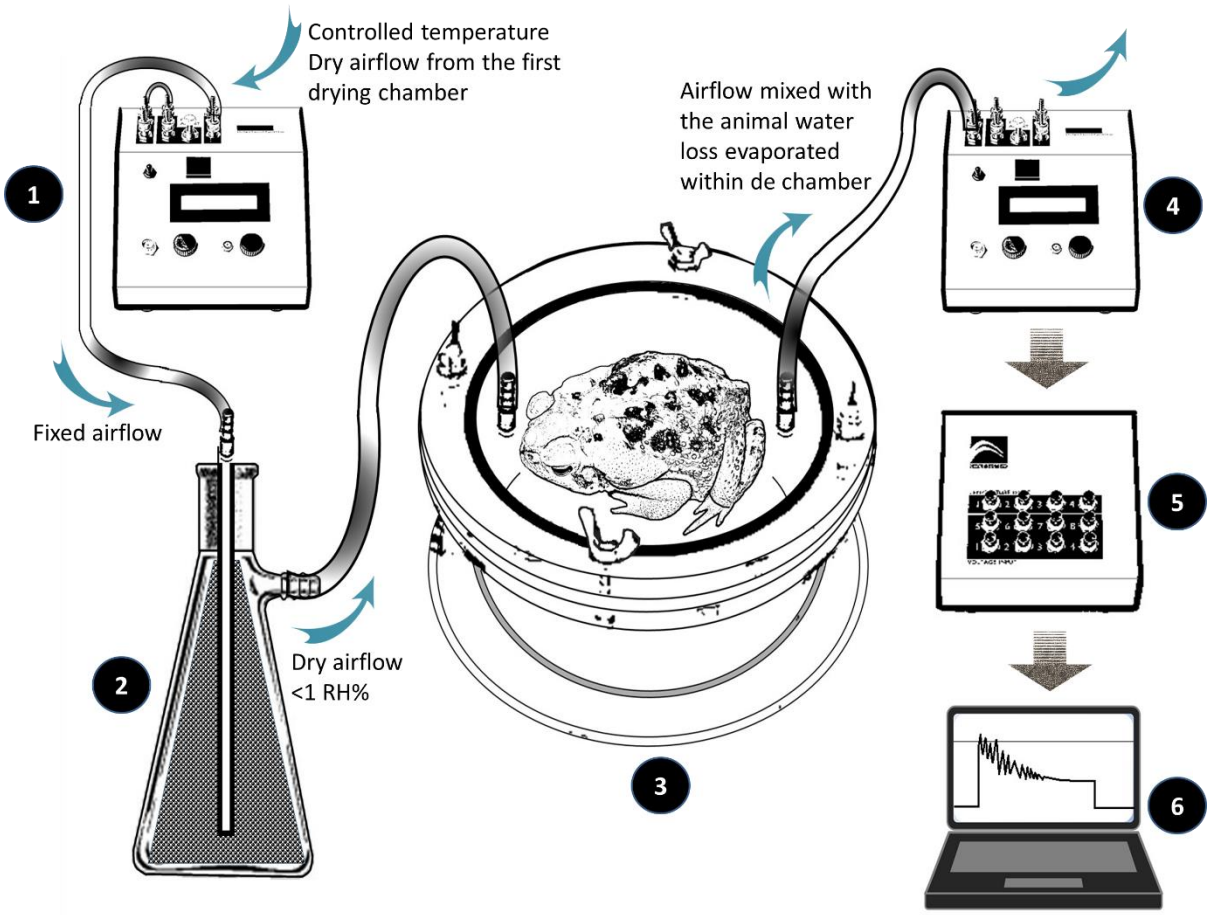
		EWL_{Skin} ($\mu g.cm^{-2}.s^{-1}$)		R_s ($s.cm^{-1}$)	
Temp.(°C)	*Hydrat.(%)*	*Masked toads*	*Unmasked toads*	*Masked toads*	*Unmasked toads*
15	100	1.09 ± 0.23[a]	1.11 ± 0.23[a]	0.94 ± 0.31[a]	0.67 ± 0.29[a]
	90	0.99 ± 0.2[a]	1.02 ± 0.21[a]	1.54 ± 0.38[a]	1.09 ± 0.29[a]
	80	0.9 ± 0.15[a]	0.93 ± 0.16[a]	2.11 ± 0.74[a]	1.59 ± 0.6[b]
25	100	1.71 ± 0.27[a]	1.8 ± 0.28[a]	2.05 ± 0.29[a]	1.45 ± 0.25[b]
	90	1.53 ± 0.2[a]	1.63 ± 0.24[a]	2.67 ± 0.35[a]	1.93 ± 0.32[b]
	80	1.38 ± 0.21[a]	1.48 ± 0.22[a]	3.43 ± 0.57[a]	2.47 ± 0.35[b]
35	100	2.61 ± 0.47[a]	2.84 ± 0.51[b]	2.56 ± 0.72[a]	1.85 ± 0.33[b]
	90	2.33 ± 0.38[a]	2.6 ± 0.43[b]	3.69 ± 0.63[a]	2.33 ± 0.3[b]
	80	2.09 ± 0.29[a]	2.35 ± 0.35[b]	4.51 ± 0.66[a]	2.98 ± 0.4[b]

Factors	X^2	*DF*	*P*	X^2	*DF*	*P*
Temperature	17.27	1	<0.001	36.71	1	<0.001
Hydration	0.12	1	0.673	13.32	1	<0.001
Temp. x Hydrat.	30.18	1	<0.001	149.7	1	<0.001

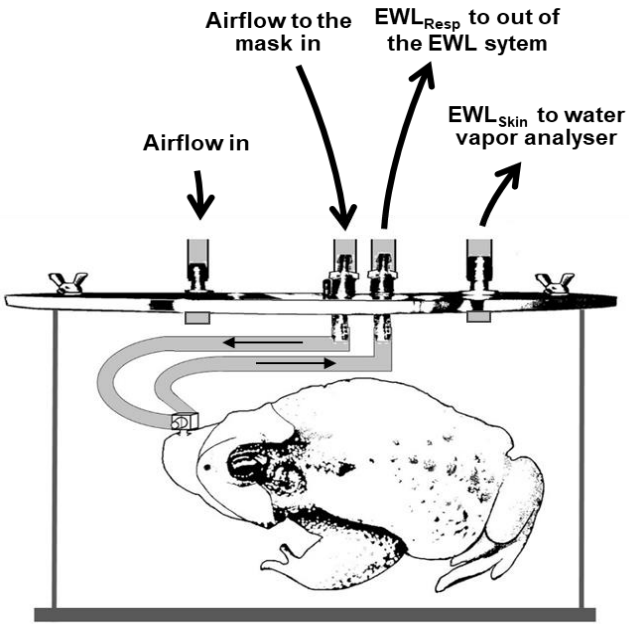
Note. Different lowercase letters indicate statistically significant differences between masked and unmasked toad measurements at a given temperature and hydration level. X^2 = Chi-Square value; *DF* = Degrees of freedom; *P* = P-value (Likelihood Ratio Test).

Table 4. Summary of the linear regression models on each temperature based on the measurement of 23 agar models to estimate toad's R_b on the basis of their body mass.

Temperature (°C)	*slope (β_1)*	*Intercept (β_0)*	r^2	*±95% C.I.*
15	1.630E-03	2.501	0.097	0.16
25	-4.988E-04	1.895	0.035	0.084
35	-3.497E-04	1.412	0.007	0.132



Appendix 1. EWL open-flow system. *1*) mass flow meter pump; *2*) drying chamber filled with silica gel; *3*) plexiglas chamber containing the animal; *4*) water vapor analyser; *5*) Data Acquisition Interface; *6*) data uninterruptedly acquired and graphed in real time on a computer.



Appendix 2. Schematic representation which illustrates the experimental setup of EWL_{Skin} measurements of masked toads inside the plexiglas chamber.

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"JÚLIO DE MESQUITA FILHO"
Campus de Rio Claro

COMISSÃO DE ÉTICA
NO USO DE ANIMAL
CEUA – IB – UNESP - CRC

ANIMAIS DE VIDA LIVRE
DECISÃO CEUA Nº 35/2016

Instituição: **UNESP – IB – CRC**	Departamento: Zoologia
Data de Registro CEUA: 12.09.2016	

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Efeito da desidratação e temperatura no balanço hídrico em duas espécies de anfíbios anuros",** protocolo nº 6915, sob responsabilidade de Luis Miguel Senzano Castro (Pesquisador Responsável) e Denis Otávio Vieira de Andrade (Orientador), que envolve a produção, manutenção e/ou a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794 de 8 de outubro de 2008, do Decreto n 6.899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA).

Subprojeto(s) vinculado(s): ==.==

Colaboradores:

A Comissão de Ética no Uso de Animal - CEUA do Instituto de Biociências da UNESP – Campus de Rio Claro, em sua **30º reunião ordinária**, realizada em **07.12.2016.**

(x)	**Aprovou** o Projeto de Pesquisa acima citado, ratificando o parecer emitido pelo relator.
( )	Desde que **atendidas as pendências** apontadas na reunião (vide anexo), **aprova** o Projeto de Pesquisa acima citado (prazo máximo de 30 dias).
( )	**Referendou** o Projeto de Pesquisa acima citado, ratificando o parecer emitido pelo relator.
( )	Aprovou **retornar** ao interessado para atendimento das pendências encontradas (prazo máximo de 30 dias).
( )	**Não** Aprovou.
( )	**Retirou**, devido à permanência das pendências.

Vigência da autorização	03/03/2016 a 03/03/2018
Finalidade	( ) Ensino (x) Pesquisa Científica
Nº da Solicitação ou Autorização SISBIO	A fazer
Atividade (s)	(x) Captura ( ) Coleta de espécimes ( ) Marcação ( ) Outros
Espécies/Grupos Taxonômicos	Leptodactylus Latrans (rã manteiga) e Rhinella Schneideri (sapo cururu)
Local (is) de realização das atividades	Serão capturados e mantidos no laboratório de Fisiologia Animal Comparada, do Dep. De Zoologia, do Instituto de Biociências da Unesp de Rio Claro

Objetivo Acadêmico:	( ) TCC ( ) Doutorado (x) Mestrado ( ) Outros

Rio Claro,07/12/2016

Prof. Dr. José Paulo Leite Guadanucci
Coordenador

Instituto de Biociências – Seção Técnica Acadêmica
Avenida 24-A nº 1515 - CEP 13506-900 – Rio Claro - S.P. – Brasil - tel 19 3526-4105 - fax 19 3534-0009 - http://www.rc.unesp.br

Appendix 3. Experimental procedures approved by the UNESP-IB/Rio Claro Animal Ethic and Use Committee (Comissão de Ética no Uso Animal, CEUA; License No 6915).