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RESEARCH ARTICLE

Temperature and dehydration effects on metabolism, water uptake and the partitioning between respiratory and cutaneous evaporative water loss in a terrestrial toad

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ABSTRACT

Terrestrial anurans often experience fluctuations in body temperature and hydration state, which are known to influence evaporative water loss through the skin (EWL_{Skin}) and lungs (EWL_{Resp}). These effects arise from associated changes in skin permeability, metabolism and lung ventilation. Herein, we determined the rates of EWL_{Skin} and EWL_{Resp} in the terrestrial toad *Rhinella diptycha* at different temperatures and hydration states. We measured oxygen uptake rates to verify whether alterations in the partitioning between EWL_{Skin} and EWL_{Resp} were associated with metabolism-induced changes in pulmonary gas exchange. We also measured the influence of hydration and temperature on water uptake (WU) through the skin. Finally, as estimates of skin resistance to evaporation (R_s) are usually inferred from total evaporative water loss (EWL_{Total}), under the assumption of negligible EWL_{Resp}, we calculated the potential error in accepting this assumption for different temperature and hydration states. EWL_{Skin} and EWL_{Resp} increased with temperature, but this response was greater for EWL_{Resp}, which was attributed to the temperature-induced elevation in metabolism and lung ventilation. Dehydration caused a decrease in the relative contribution of EWL_{Skin} to EWL_{Total}, mirrored by the concurrent increase in the contribution of EWL_{Resp}, at all temperatures. Thus, R_s increased with dehydration. WU rates were dictated by dehydration with little influence of temperature. The partitioning between EWL_{Skin} and EWL_{Resp} was affected by both temperature and hydration state and, under some conditions, considering EWL_{Resp} as negligible led to significant errors in the assessment of skin resistance to evaporation.

KEY WORDS: Amphibians, Anurans, Thermoregulation, Water balance, Skin resistance, Oxygen uptake

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 entail hydric constraints as most amphibians are susceptible to high rates of evaporative water loss (EWL) because of their highly permeable skin (Hillman et al., 2009). Accordingly, terrestrial anurans are prone to experiencing variable degrees of hydration while active (see Tracy et al., 2014). As ectothermic organisms, amphibians may also experience wide fluctuations in body temperature, which generally mirrors the variation of their thermal environment (Seebacher and Alford, 2002; Noronha-de-Souza et al., 2015). Moreover, from the pioneering insights of the classic study of Tracy (1976), we now appreciate that thermoregulation and water balance are highly intertwined in terrestrial anurans (Feder and Burggren, 1992; Navas et al., 2008; Andrade et al., 2016). Mainly, this is because EWL rates are influenced by temperature while, in turn, evaporation from the skin has a cooling effect on body temperature (Tracy, 1976; Feder and Burggren, 1992; Navas et al., 2008; Andrade et al., 2016). Therefore, the interaction between these two essential 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; Riddell et al., 2018).

In general, terrestrial anurans can withstand large losses of body water (Wells, 2007) even though detrimental effects are expected (Lillywhite, 1975; Hillman, 1987). Dehydration causes body fluids to become more concentrated (Anderson et al., 2017), which may impose an extra burden on the cardiovascular system and compromise aerobic metabolism (Gatten, 1987; Hillman et al., 2000; Withers and Hillman, 2001; Navas et al., 2008). Dehydration is also known to decrease EWL and increase water uptake (WU) through the ventral skin area, the 'pelvic patch' (see Anderson et al., 2017). The dehydration-induced dynamics of cutaneous water flux are, as expected, influenced by temperature (Preest et al., 1992; Rogowitz et al., 1999). Indeed, if relative humidity is fixed, rises in temperature increase the rate of EWL by affecting air capacitance for water vapour. 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 behavioural 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.

Total rates of EWL in terrestrial lung breathers is partitioned between the water evaporated from skin surface (EWL_{Skin}) and that lost from the lung surface (EWL_{Resp}) (Mautz, 1982; Hillman et al., 2009). Nonetheless, as most anurans exchange a substantial fraction of their respiratory gases through the skin, and as they have a relatively low pulmonary surface area associated with low rates of ventilation while resting (Czopek, 1965; Burggren and Doyle, 1986), EWL_{Resp} is quite commonly assumed to be negligible in these organisms (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

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List of abbreviations

A_s	skin surface area
EWL	evaporative water loss
EWL_{Resp}	respiratory water loss
$\%EWL_{Resp}$	relative contribution of EWL_{Resp} to EWL_{Total}
EWL_{Skin}	skin evaporative water loss
$\%EWL_{Skin}$	relative contribution of EWL_{Skin} to EWL_{Total}
EWL_{Total}	total evaporative water loss
Q_{10}	temperature coefficient
R_b	boundary layer resistance
R_s	skin resistance
R_T	total resistance to evaporation
SMR	standard metabolic rate
$\dot{V}_{O_2}$	rate of oxygen consumption
WU	water uptake
WVD	water vapour density

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 on the partitioning of total evaporation (EWL_{Total}) between EWL_{Resp} and EWL_{Skin} remains uncertain. This evaluation is ecologically and functionally meaningful as these two routes of EWL involve different sets of constraints that may be differently affected by changes in temperature and hydration state (Hutchison et al., 1968; Geise and Linsemair, 1986; Rogowitz et al., 1999; Burggren and Vitalis, 2005). Accordingly, we examined the combined effects of temperature and hydration level on the partitioning of EWL between its cutaneous and respiratory components in the terrestrial toad *Rhinella diptycha* (Cope 1862). We also determined the influence of temperature and dehydration on the rate of oxygen consumption ($\dot{V}_{O_2}$) and WU through the pelvic patch of the toads. Finally, on the basis of EWL rates, we estimated skin resistance (R_s) to water evaporation and provide an evaluation of the potential error in estimates of skin permeability assuming EWL_{Resp} as negligible. We chose *R. diptycha* for the present study because this species has a terrestrial lifestyle and is broadly distributed in tropical biomes of South America (Haddad et al., 2013; Vallinoto et al., 2017) and, therefore, it is likely to experience natural fluctuations in body temperature and hydration state.

MATERIALS AND METHODS

Animals

We collected 14 adult *R. diptycha* of both sexes (mean±s.d. body mass 191.7±78.8 g) for EWL measurements, eight in mid-September of 2015 and six in late January of 2017; and 10 individuals (mean±s.d. body mass 235.3±35.6 g) for metabolic measurements in December of 2017. Animals were collected in the municipality of Barbosa, São Paulo State, Brazil (21°15′1,72″S, 49° 55′16,75″W; 371 m a.s.l.) and taken to the Laboratory of Comparative Animal Physiology at the São Paulo State University (Rio Claro, SP, Brazil). All toads were kept individually in plastic boxes (40×30×25 cm), maintained at room temperature (24.1±0.5°C), 50–80% of relative humidity and natural photoperiod. PVC plastic tubes (10×20 cm) and plant leaves were provided as shelters. Toads were fed twice weekly on mealworms (*Tenebrio* sp.) and crickets (*Gryllus* sp.) and water was freely available at all times. Animals were fasted for a minimum of 72 h before experimental trials. The permit for animal collection was issued by the Instituto Chico Mendes da Conservação da Biodiversidade, Brazil (MMA-JCMBIO, no. 22025-1), and all procedures were approved by the UNESP-IB/Rio Claro Animal Ethic and Use Committee (Comissão de Ética no Uso Animal, CEUA; licence no. 6915).

General experimental protocols

Experiments were conducted under three temperatures (15, 25 and 35°C) and three hydration levels (100%, 90% and 80%), where 100% refers to fully hydrated toads (see details below), and 90% and 80% refer to toads dehydrated until their body mass equalled 90% and 80% of their fully hydrated body mass, respectively. Each toad was exposed to all nine combinations of temperature and hydration level in random order. Toads were subjected to just one treatment per day and, after measurements, were allowed to recover for at least 12 h before been subjected to a new treatment. After 4 consecutive days of measurements, toads were fed and were allowed a longer recovery period of 3 days. On the day of an experimental trial, toads were dehydrated in the morning between 06:00 h and 14:00 h, and EWL and WU measurements were taken in the afternoon and evening, between 16:00 h and 22:00 h. All toads were subjected to this same general protocol twice: first, for the determination of total EWL rate, in which the sum of EWL_{Skin} and EWL_{Resp} was measured in animals in an 'intact' condition, i.e. not masked; and second (after completion of the first experimental series), for the determination of EWL_{Skin}, in which toads wore a mask that provided an air supply dedicated to lung ventilation (see details below). EWL_{Resp} was then estimated as the difference between EWL_{Total} and EWL_{Skin}. WU measurements were performed immediately after the measurement of 'intact' total EWL.

At the start of any experimental trial, animals were fully hydrated by placing them individually for 2 h inside a plastic cage (2.2 l volume) with 0.5 cm water depth. After this time, their urinary bladder was emptied by gently pressing the abdominal pelvic area. Excess water on the skin was blotted with paper tissue and the mass of the fully hydrated animal was recorded (±0.01 g). In this way, measurements were made on the fully hydrated toads. Otherwise, toads were subjected to a dehydration protocol, which consisted of exposing them to a wind tunnel until the desired level of dehydration was obtained (within 3–7 h for 90% and 80%, respectively). This wind tunnel was built from a PVC tube (25 cm diameter×15 cm length) covered with a plastic mesh on both sides and positioned 10 cm in front of an electric fan that blew room air (relative humidity ∼65%; temperature 21.3±1.9°C) through it at an air speed of 3.8 m s^{-1}. Once the desired hydration level was attained, animals were placed into the measurement chamber and transferred to a temperature-controlled BOD incubator (Eletrolab EL101/2RS), where they were left for 2 h to acclimate to the experimental temperature. EWL measurements started immediately after acclimation and proceeded until we identified a minimum period of 15 min of steady-state recordings occurring within 1–2 h of experimentation. Steady-state condition was judged from 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 measurement chamber. In cases in which the animal was agitated and we failed to obtain a steady-state record within 2 h of the beginning of the experimental trial, or those in which the animal defecated inside the chamber, the experiment was aborted and the data were discarded. For successful trials, immediately after the experiment finished, we measured dorsal skin temperature using an infrared thermometer (±0.1°C; 62 Mini IR, Fluke). At this time, we also re-weighed the animals to check whether their hydration level was altered.

Metabolic measurements were determined for a different group of toads from those used for EWL measurements. However, these

toads were also measured, in random order, at the same combination of hydration level and temperature as for the EWL measurements. The dehydration procedure was as described above and, after the desired hydration level was attained, animals were transferred to a respirometric chamber located inside an incubator (as above) for temperature control. $\dot{V}_{O_2}$ measurements began at midday and proceeded for 36 h; from this period, we discarded the first 12 h, and used the subsequent 24 h for O_2 consumption calculation. During $\dot{V}_{O_2}$ measurements, animals were kept undisturbed and in complete darkness. Before and after measurements, animals were weighed to control for hydration level, and in the case of defecation or urination, data were discarded.

EWL

EWL_{Total} was determined by placing unmasked toads individually inside a cylindrical PVC chamber (1 l volume; 15 cm diameter×6 cm height) connected to an open-flow EWL measurement system. In this system, a constant airflow (21.66 cm^3 s^{-1}) generated and controlled by an integrated air pump, meter and flow controller (SS-4 Subsampler, Sable Systems) was directed through two drying columns placed at the inlet and outlet of the SS-4 unit. These columns were filled with silica gel and provided an airflow with relative humidity values varying from 0.5% to 1.5%, above which the water-absorbing columns were replaced. The dry air was then directed to the PVC chamber containing the animal while we monitored the excurrent air for water content with a water vapour analyser (RH-300, Sable Systems). The analog output from the airflow unit and the relative humidity meter was acquired via an A/D converter (UI-2 Data Acquisition Interface, Sable Systems) and digitally recorded on a computer in real time with ExpeData software (Sable Systems).

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

Before and after any EWL measurement, we collected baseline relative humidity measurements for the empty chambers. The increment in water content from these baseline values to those attained during measurements was used to calculate the absolute water loss of both the EWL_{Total} (unmasked toads) and EWL_{Skin} (masked toads) by using the equation: $EWL=(VD_e-VD_i)\times F$, where EWL is the absolute water loss (μg H_2O s^{-1}), VD_e and VD_i are water vapour density (WVD) of the excurrent and incurrent air from/to the animal's chamber (μg H_2O s^{-1}), respectively, and F is the airflow rate (cm^3 s^{-1}) (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}).

To express EWL_{Skin} as an area-specific rate (μg H_2O cm^{-2} s^{-1}), absolute EWL_{Skin} (μgH_2O s^{-1}) was divided by 2/3 of the 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 the 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} rate 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 resistance (R_b); therefore, in order to estimate *R. diptycha* R_s, it is necessary to determine R_b. This was done by measuring EWL rate 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 agar models were obtained from the impression of formalin-fixed specimens of *R. diptycha* in dental alginate. Toad alginate casts were filled with 3% agar solution and left to harden (usually within 1 h). After that, we measured the EWL rates of the agar models under identical conditions to those described above for live toads. In total, we measured 23 agar models at each experimental temperature (15, 25, 35°C). Both the R_b and toad R_T were calculated from EWL measurements following Spotila and Berman (1976), who state that: R=VDD/EWL, where R (s cm^{-1}) is the total resistance (R_T) on toads or on agar (R_b), VDD is the WVD gradient (μg cm^{-3}) between the saturated WVD at the skin surface temperature of the animal or agar model and the partial WVD at ambient temperature, and EWL is the area-specific EWL (μg H_2O cm^{-2} s^{-1}). Skin resistance was calculated as: $R_s=R_T-R_b$. In all these calculations, we used EWL_{Skin} data obtained from masked toads; however, for error estimation purposes, we also calculated R_s based on EWL_{Total}, assuming a negligible EWL_{Resp}.

Water uptake

Immediately after EWL measurement of intact unmasked toads, we randomly selected 10 individuals per experimental treatment to measure the rate of water uptake through the pelvic patch. To do this, we placed the animals in individual plastic boxes (1 l volume) containing 0.5 cm of unchlorinated tap water. At 2 min intervals, toads were carefully blotted with paper tissue and weighed (±0.01 g; Marte scale, AD5002) 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 dataset. WU was expressed per skin surface area (μg H_2O cm^{-2} s^{-1}) assuming that the ventral region in contact with the water equalled one-third of total skin surface area (estimated as above).

Oxygen consumption

The rates of O_2 consumption ($\dot{V}_{O_2}$) were determined by using an intermittently closed respirometry system. Animals were placed individually inside seven hermetically sealed respirometry chambers (1 l volume) and put inside a climatic BOD chamber to control for experimental temperature. With the use of a software subroutine (DATACAN V, Sable Systems), we established an output control on a multiple flow-controller unit (Multiplexer, TR-RM8, Sable Systems). Under this setup, each chamber was set

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to be ventilated with external fresh air for a period of 60 min (open phase), followed by 10 min (closed phase) in which the air from the chamber was recirculated through an oxygen analyser (PA-1B, Sable Systems). The open phase allowed the renewal of the air in the chamber, while the decline in the fractional concentration of O_2 along the closed phase was digitally recorded (DATACAN V, Sable Systems) and used for $\dot{V}_{O_2}$ calculations. Therefore, at every 70 min interval, we were able to attain a 10 min $\dot{V}_{O_2}$ measurement for each individual animal. Air humidity was removed from the airstream by placing a silica-gel column between the animal chamber and the inlet of the O_2 analyser unit. As we did not find statistical differences between nocturnal and diurnal $\dot{V}_{O_2}$ rates, we estimated the toad's standard metabolic rate (SMR, ml O_2 kg^{-1} h^{-1}) as the average of all $\dot{V}_{O_2}$ measurements taken during a 24 h period.

Statistical analysis

The effects of temperature and dehydration on EWL_{Skin}, EWL_{Resp}, R_s, WU and $\dot{V}_{O_2}$ were evaluated through a two-way repeated measures analysis of variance (two-way RM ANOVA) with temperature and hydration level as factors. For $\%EWL_{Skin}$ and $\%EWL_{Resp}$, relativized data were first arcsine square root transformed. The Holm–Sidak method was employed as a *post hoc* multiple comparison test to identify differences within treatments whenever necessary. R_b values obtained from the agar models were tested for the influence of body mass (from the toads used to acquire the models) and temperature using a two-way RM ANOVA. 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, individual as random factor, and 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 for differences within treatment with and without the inclusion of EWL_{Resp}. Data were verified with the Shapiro–Wilk test for normality and Levene's test for homogeneity of variances. Absolute rates of EWL_{Total}, EWL_{Skin} and EWL_{Resp} were $\log_{10}$ transformed to meet distribution assumptions. All analyses were performed in R (v3.3.1; http://www.R-project.org/) employing RStudio (v1.0.136; https://www.rstudio.com/). All data are presented as means±s.d., unless indicated otherwise. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

Temperature rises increased the rates of EWL_{Total} (Fig. 1A), absolute EWL_{Skin} and EWL_{Resp} (Fig. 1B) (Table 1). Dehydration progression caused a decrease in the rate of EWL_{Total} (Fig. 1A) and absolute EWL_{Skin} but not EWL_{Resp} (Fig. 1B). Temperature and dehydration significantly affected the partitioning of EWL_{Skin} and EWL_{Resp} (Table 1). Indeed, the increase in temperature or dehydration caused a pronounced reduction in $\%EWL_{Skin}$ (Fig. 1C), with a corresponding increment in $\%EWL_{Resp}$ (Fig. 1D). Rates of area-specific EWL_{Skin}, calculated from

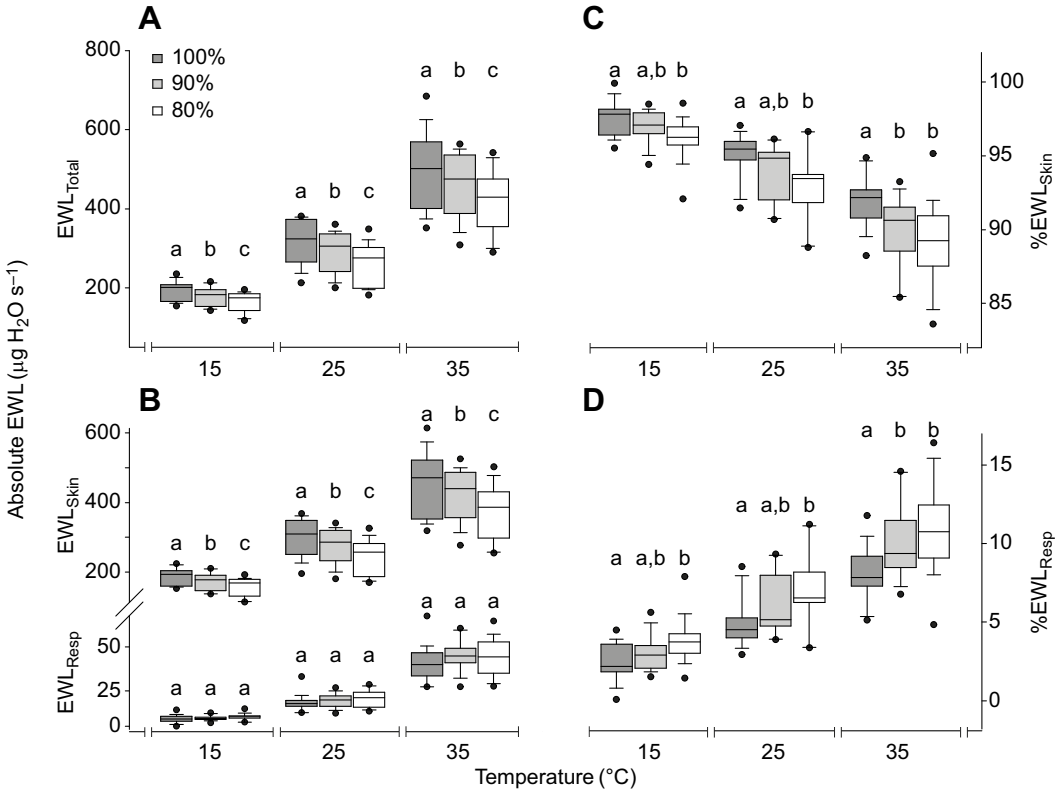

Fig. 1. Effects of temperature and dehydration on evaporative water loss (EWL) rates of *Rhinella diptycha* toads (*n*=14). (A) The overall absolute EWL calculated from the whole-animal surfaces (EWL_{Total}), and (B) the partitioning of EWL_{Total} into its cutaneous (EWL_{Skin}) and respiratory (EWL_{Resp}) components. (C,D) The relative contribution of EWL_{Skin} (C) and EWL_{Resp} (D), expressed as a percentage of 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 are ±s.d. and filled circles are outliers.

Table 1. Summary and comparisons of absolute evaporative water loss (EWL) in response to different temperatures and hydration levels of *Rhinella diptycha* toads

Temperature (°C)	Hydration (%)	Absolute EWL (μg H_2O s^{-1})			%EWL_{Skin} (%)	%EWL_{Resp} (%)
		EWL_{Total}	EWL_{Skin}	EWL_{Resp}		
15	100	193.48±25.29^{a,A}	188.66±24.03^{a,A}	4.81±2.61^{a,A}	97.55±1.22^{a,A}	2.44±1.22^{a,A}
	90	177.53±24.89^{b,D}	172.15±25.17^{b,D}	5.38±1.77^{a,D}	96.89±1.2^{a,b,D}	3.1±1.2^{a,b,D}
	80	163.97±27.49^{c,G}	157.84±27.51^{c,G}	6.13±2.13^{a,G}	96.16±1.51^{b,G}	3.83±1.51^{b,G}
25	100	317.24±56.92^{a,B}	301.93±54.67^{a,B}	15.3±5.65^{a,B}	95.14±1.59^{a,B}	4.85±1.59^{a,B}
	90	288.34±52.33^{b,E}	271.73±51.57^{b,E}	16.6±4.46^{a,E}	94.1±1.91^{a,b,E}	5.89±1.91^{a,b,E}
	80	262.92±52.84^{c,H}	245.07±51.66^{c,H}	17.84±5.65^{a,H}	93.05±2.28^{b,H}	6.94±8.1^{b,H}
35	100	500.53±99.04^{a,C}	460.21±91.51^{a,C}	40.32±11.68^{a,C}	91.89±1.78^{a,C}	8.1±1.78^{a,C}
	90	457.29±84.49^{b,F}	411.85±81.68^{b,F}	45.44±10.22^{a,F}	89.86±2.54^{b,F}	10.13±2.54^{b,F}
	80	416.45±83.91^{c,I}	371.71±81.33^{c,I}	44.74±12.35^{a,I}	88.99±3.13^{b,I}	11.05±3.13^{b,I}

Whole-animal surface EWL rate (EWL_{Total}), partitioning of EWL_{Total} into its cutaneous (EWL_{Skin}) and respiratory (EWL_{Resp}) components, and the relative contribution of the two routes (%EWL_{Skin} and %EWL_{Resp}) to EWL_{Total}. All values are means±s.d.
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%).

absolute EWL_{Skin} (Fig. 2A), increased with temperature ($F_{2,26}$=375.5, P<0.001) and decreased with dehydration ($F_{2,26}$=71.4, P<0.001), with a significant interaction between them ($F_{4,52}$=10.5, P<0.001; Table 2).

We also calculated the area-specific EWL_{Skin} rate, on the basis of EWL_{Total}, therefore accepting the assumption of negligible EWL_{Resp}. In this case, EWL_{Skin} increased with temperature (χ^2_1=17.3, P<0.001), but was unaffected by hydration level (χ^2_1=0.12, P=0.67), although a significant interaction existed between the two factors (χ^2_1=30.2, P<0.001) (Table 3). Thus, using EWL_{Total} to estimate area-specific EWL_{Skin} led to an overestimation of these rates in comparison to the values calculated on the basis of absolute EWL_{Skin} measured in masked toads, i.e. with the exclusion of EWL_{Resp}. This effect was augmented by the temperature rise (P=0.002) and dehydration (P<0.001 for both 90% and 80%) at 35°C (Fig. 2A).

R_b was not affected by body mass ($F_{1,22}$=0.16, P=0.68), but varied with temperature ($F_{2,21}$=306.94, P<0.001). Moreover, the average body mass of the toads used for acquiring the agar models did not differ from the body mass of the experimental animals (P=0.45). Therefore, in order to estimate R_s, we adopted a single averaged R_b value for each experimental temperature (see Table 3). R_s (Fig. 2B) showed a temperature- and dehydration-induced increase ($F_{2,26}$=187.59, P<0.001 and $F_{2,26}$=131.34, P<0.001, respectively), with a significant interaction between the two factors ($F_{4,52}$=3.75, P=0.01; Table 2). R_s values calculated on the basis of EWL_{Total} underestimated the actual R_s values calculated from the absolute EWL_{Skin} for masked toads. This effect was augmented with the temperature rise (χ^2_1=36.69, P<0.001) and dehydration progression (χ^2_1=13.3, P<0.001), with a significant interaction between the factors (χ^2_1=148, P<0.001) (Fig. 2B, Table 3).

WU rates were affected by temperature ($F_{2,18}$=19.37, P<0.001) and dehydration ($F_{2,18}$=60.1, P<0.001). In general, WU rates were increased at higher temperatures and dehydration levels (Fig. 3), with no significant interaction between them ($F_{4,36}$=1.43, P=0.24; Table 2).

$\dot{V}_{O_2}$ rates increased with temperature ($F_{2,18}$=76.49, P<0.001), but were unaffected by dehydration ($F_{2,18}$=0.56, P=0.58) (Fig. 4), with no interaction between these factors ($F_{4,36}$=1.13, P=0.36) (Table 2). Temperature coefficient (Q_{10}) varied from 2.44 to 1.56 for the temperature intervals of 15–25 and 25–35°C, respectively, and was

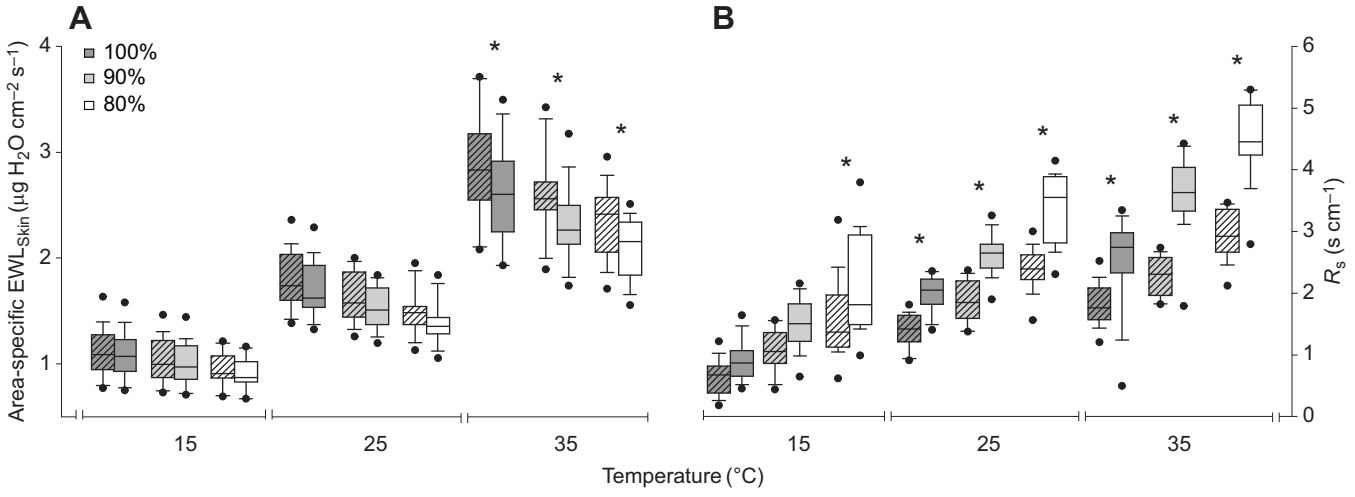

Fig. 2. Temperature and dehydration effects on area-specific EWL_{Skin} and skin resistance (R_s) to evaporation of *R. diptycha* toads (n=14). Solid boxplots are area-specific EWL_{Skin} (A) and R_s (B), calculated from the skin water loss route with the use of masked toads. Dashed boxplots are the expected area-specific EWL_{Skin} (A) and the expected R_s (B) calculated from EWL_{Total}, which includes the pulmonary EWL contribution (EWL_{Resp}) measured in 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. Boxplot lines indicate medians and the lower and upper borders represent the 25th and 75th percentiles, whiskers are ±s.d. and filled circles are outliers.

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Table 2. Summary and comparisons of area-specific EWL_{Skin}, skin resistance (R_s) to evaporation, oxygen consumption ($\dot{V}_{O_2}$) and water uptake (WU) rates in response to different temperatures and hydration levels of *R. diptycha* toads

Temperature (°C)	Hydration (%)	EWL_{Skin} (μg H_2O cm^{-2} s^{-1})	R_s (s cm^{-1})	$\dot{V}_{O_2}$ (ml O_2 kg^{-1} h^{-1})	WU (μg H_2O cm^{-2} s^{-1})
15	100	$1.09±0.23^{a,A}$	$0.91±0.35^{a,A}$	$22.24±9.01^{a,A}$	$43.49±25.96^{a,A}$
	90	$0.99±0.2^{b,D}$	$1.51±0.43^{b,D}$	$23.16±9.9^{a,D}$	$77.65±25.09^{b,D}$
	80	$0.9±0.15^{c,G}$	$2.08±0.8^{c,G}$	$23.15±9.14^{a,G}$	$126.82±36.48^{c,G,H}$
25	100	$1.71±0.27^{a,B}$	$2.01±0.3^{a,B}$	$49.54±16.16^{a,B}$	$41.37±25.46^{a,A}$
	90	$1.53±0.2^{b,E}$	$2.6±0.34^{b,E}$	$52.49±15.32^{a,E}$	$87.3±25.06^{b,D}$
	80	$1.38±0.21^{c,H}$	$3.37±0.56^{c,H}$	$51.58±15.84^{a,H}$	$110.77±26.01^{c,G}$
35	100	$2.61±0.47^{a,C}$	$2.5±0.77^{a,C}$	$79.45±19.87^{a,C}$	$78.68±35.23^{a,B}$
	90	$2.33±0.38^{b,F}$	$3.64±0.67^{b,F}$	$78.55±22.12^{a,F}$	$110.76±33.76^{b,E}$
	80	$2.09±0.29^{c,I}$	$4.46±0.67^{c,I}$	$75.76±20.24^{a,I}$	$138.88±43.09^{c,H}$

Factors	F	d.f.	P	F	d.f.	P	F	d.f.	P	F	d.f.	P
Temperature	375.5	2, 26	<0.001	187.59	2, 26	<0.001	76.49	2, 18	<0.001	19.37	2, 18	<0.001
Hydration	71.4	2, 26	<0.001	131.34	2, 26	<0.001	0.56	2, 18	0.58	60.1	2, 18	<0.001
Temperature×hydration	10.5	4, 52	<0.001	3.75	4, 52	0.01	1.13	4, 36	0.36	1.43	4, 36	0.24

All values are means±s.d.

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%). *P*-values were calculated by two-way RM ANOVA.

not affected by dehydration ($F_{2,18}$=0.18, P=0.83 and $F_{2,18}$=1.86, P=0.18 for the 15–25 and 25–35°C intervals, respectively).

DISCUSSION

The increment in EWL_{Total} with temperature documented in *R. diptycha* 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). As water vapour 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, we found that EWL increased with the rise in temperature through both surfaces in *R. diptycha*; however, EWL_{Resp} showed a steeper increase in comparison to EWL_{Skin}. As a result, while the relative contribution of EWL_{Resp} increased from 2.4% to 8.1%, from 15°C to 35°C, the relative contribution of EWL_{Skin} fell from 97.5% to 91.8%. These changes probably reflect the 3.5-fold elevation of *R. diptycha* oxygen uptake rate within the same temperature interval, which implies an increased rate of lung ventilation (see 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. diptycha* can be 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;

Table 3. Summary and comparisons of area-specific EWL_{Skin} and mean R_s calculated from EWL_{Total} (rather than EWL_{Skin}) measured on unmasked *R. diptycha* toads, which includes the pulmonary EWL contribution (EWL_{Resp}) at different temperatures and hydration levels

		Unmasked toads		
Temperature (°C)	Hydration (%)	EWL_{Skin} (μg cm^{-2} s^{-1})	R_s (s cm^{-1})	R_b (s cm^{-1})
15	100	1.11±0.23	0.65±0.3	2.83±0.35
	90	1.02±0.21	1.06±0.35	
	80	0.93±0.16	1.57±0.66*	
25	100	1.8±0.28	1.39±0.27*	1.86±0.17
	90	1.63±0.24	1.87±0.33*	
	80	1.48±0.22	2.41±0.35*	
35	100	2.84±0.51*	1.8±0.35*	1.39±0.28
	90	2.6±0.43*	2.28±0.32*	
	80	2.35±0.35*	2.94±0.41*	

Factors	χ^2	d.f.	P	χ^2	d.f.	P
Temperature	17.3	1	<0.001	36.69	1	<0.001
Hydration	0.12	1	0.67	13.3	1	<0.001
Temperature×hydration	30.2	1	<0.001	148	1	<0.001

Mean boundary layer resistance (R_b) was calculated from agar models. All values are means±s.d.

Asterisks indicate statistically significant differences between unmasked (this table) and masked toad measurements described on Table 2, measured at a given temperature and hydration level. *P*-values were calculated by the likelihood ratio test.

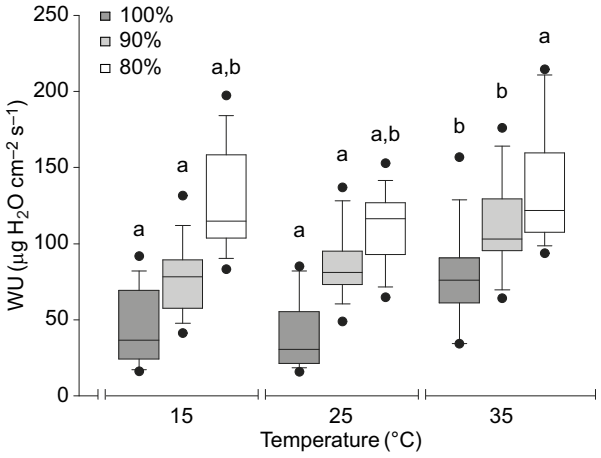

Fig. 3. Rates of water uptake (WU) in response to different temperatures and dehydration levels of *R. diptycha* toads (n=10). There were significant differences at all hydration state comparisons (P<0.05) and different lowercase letters indicate significant differences between different temperatures (15, 25 and 35°C) within each dehydration treatment. Boxplot lines indicate medians and the lower and upper borders represent the 25th and 75th percentiles, whiskers are ±s.d. and filled circles are outliers.

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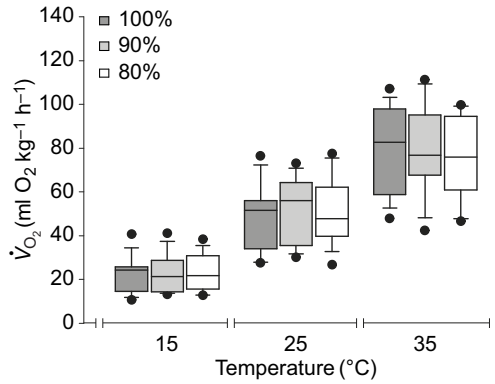

Fig. 4. Temperature and dehydration effects on rates of oxygen consumption ($\dot{V}_{O_2}$) of *R. diptycha* toads (*n*=10). There were significant differences at all temperature comparisons ($P<0.05$) while no significant differences were found among any hydration level within each temperature ($P>0.05$ for all comparisons). Boxplot lines indicate medians and the lower and upper borders represent the 25th and 75th percentiles, whiskers are ±s.d. and filled circles are outliers.

Loveridge, 1970), a response previously documented in *R. diptycha* (Anderson et al., 2017) and also confirmed by the present study. The effects of dehydration on the area-specific EWL_{Skin} rate, at 25°C, reported here were slightly lower than those reported by Anderson et al. (2017), and this difference may be ascribed 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. Dehydration diminishes water content of integumentary cell layers (Lillywhite, 1971; Lillywhite and Licht, 1974) and compresses the keratinized stratum corneum, which decreases skin permeability (Machin, 1969; Lillywhite, 1975; Lillywhite and Licht, 1975). Also, capillary blood flow adjustments in the dorsal skin (Burggren and Moallf, 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 under dehydrated states. In combination, all these factors may explain why the absolute rates of EWL_{Skin}, as well as its relative contribution to EWL_{Total}, diminished with dehydration in *R. diptycha*. In contrast, the absolute rates of EWL_{Resp} were unaffected by dehydration, but, as a consequence of the decrease in EWL_{Skin}, the relative contribution of EWL_{Resp} to EWL_{Total} increased with dehydration (see also Geise and Linsemair, 1986). These results indicate that lung ventilation was not affected by dehydration, which is corroborated by our results showing that *R. diptycha* $\dot{V}_{O_2}$ remained virtually unchanged across all hydration levels. Finally, although the dehydration effects on the partitioning of EWL_{Resp} and EWL_{Skin} were similar across all temperatures, 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}, was already elevated at higher temperatures.

Cutaneous rates of EWL in anurans are commonly expressed by unit area of skin surface (area-specific EWL_{Skin}), which is often 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 EWL, while their relatively low metabolism and lung ventilation minimizes EWL_{Resp}. Our results indicate that the error in using EWL_{Total} for the calculation of area-specific EWL_{Skin} is indeed relatively low at lower temperatures. For example, for fully hydrated toads at 15°C, this error caused a 1.8% overestimation of the EWL_{Skin}. However, as discussed above, the contribution of EWL_{Resp} to EWL_{Total} increases considerably with temperature and, at 35°C and full hydration, the error in assuming EWL_{Resp} as negligible resulted in an overestimation of area-specific EWL_{Skin} of 8.8%. In a similar way, as dehydration caused the relative contribution of EWL_{Resp} to EWL_{Total} to increase, assuming EWL_{Resp} to be negligible resulted in greater errors at greater dehydration levels, and this effect was inflated at higher temperatures. For example, as toads moved from fully hydrated to 80% dehydrated, this error increased from 3.3% at 15°C to 12% at 35°C. The consequences of not discounting EWL_{Resp} for the calculation of area-specific EWL_{Skin} were mirrored by the estimations of R_s. Accordingly, at low temperatures and full hydration, assuming EWL_{Resp} as negligible resulted in an underestimation of R_s by 28.7%, which became more pronounced at higher temperatures and dehydration levels, reaching 34% at 35°C and 80% dehydration. Whether the magnitude of such errors would confound broad ecological interpretations is currently uncertain but, from a methodological standpoint, the consequences of not discounting EWL_{Resp} from the calculation of area-specific EWL_{Skin} resulted in measurable errors in R_s estimations. Therefore, we maintain that the assumption of EWL_{Resp} as negligible should not be accepted without reservation while assessing amphibian skin permeability.

WU through the skin in *R. diptycha* 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 response 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 (Willumsen et al., 2007). Temperature did not cause *R. diptycha*'s WU rates to increase between 15 and 25°C; however, a significant increment occurred at 35°C for fully and 90% hydrated toads. This result contrasts partially with the notion that temperature does not markedly affect the rates of WU in anurans (e.g. Cloudsley-Thompson, 1967; Claussen, 1969; Tracy, 1976). Although uncertain, we suspect that some of the physiological parameters affecting skin permeability respond differently to combined changes in temperature and hydration state, possibly involving adjustments 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's water permeability (Willumsen et al., 2007), mediated by changes in the expression of aquaporins (Hasegawa et al., 2003; Suzuki et al., 2007; Saitoh et al., 2014).

The measured SMR of *R. diptycha* was well within the values predicted for terrestrial anurans on the basis of their body mass (Gatten et al., 1992; Secor and Faulkner, 2002). Also, the SMR of *R. diptycha* was very similar to values previously reported for this species (Glass et al., 1997; Bícego-Nahas et al., 2001). As usual for ectothermic organisms, temperature rise was accompanied by an increment in SMR at the typical rate expected for anuran amphibians (Q_{10}=2.44 and 1.56 for the 15–25°C and 25–35°C temperature intervals, respectively; see Gatten et al., 1992). This temperature-induced increment in SMR was smaller than the temperature-induced increment in EWL_{Resp} (Q_{10}=3.4 and 2.8 for the 15–25°C and 25–35°C intervals, respectively), which casts doubt on our previous interpretation linking the temperature-induced changes in EWL_{Resp} to changes in metabolism and lung ventilation. This mismatch could indicate that temperature elevation caused ventilation rates to increase at a faster pace than metabolism, resulting in greater air convection requirements at higher temperatures. However, this is unlikely as the air convection requirement usually decreases with temperature (Glass et al., 1985; Stinner, 1987; Rocha and Branco, 1997). A more plausible explanation is that water vapour capacity varies

exponentially with temperature and, therefore, strict proportionality between temperature-induced changes in EWL_{Resp} and metabolism/ventilation is not to be expected. Dehydration did not affect the SMR of *R. diptycha* at any temperature, thus suggesting no metabolic costs associated with dehydration under resting conditions (Preest et al., 1992). While dehydration may increase SMR in some frog species (Katz, 1975; Pough et al., 1983), our metabolic results fit well with those reported for other terrestrial bufonids, where SMR was not affected by dehydration (Gatten, 1987; Gil and Katz, 1996; Preest and Pough, 2003; Forster, 2013). Despite the fact that available data are limited, we suspect that the sensitivity of SMR to dehydration may vary among different anuran clades and life histories (Degani and Meltzer, 1988; Gatten et al., 1992; Hillman, 2018).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.M.S., D.V.A.; Methodology: L.M.S., D.V.A.; Software: L.M.S., D.V.A.; Validation: L.M.S., D.V.A.; Formal analysis: L.M.S., D.V.A.; Investigation: L.M.S., D.V.A.; Resources: D.V.A.; Data curation: L.M.S.; Writing - original draft: L.M.S.; Writing - review & editing: L.M.S., D.V.A.; Visualization: D.V.A.; Supervision: D.V.A.; Funding acquisition: D.V.A.

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