

Tribute to R. G. Boutilier: Skin colour and body temperature changes in basking *Bokermannohyla alvarengai* (Bokermann 1956)

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Summary

In amphibians solar basking far from water sources is relatively uncommon since the highly permeable amphibian skin does not represent a significant barrier to the accompanying risk of losing water by evaporation. A South American frog, *Bokermannohyla alvarengai* (Bokermann 1956), however, spends a significant amount of the day exposed to full sun and relatively high temperatures. The means by which this frog copes with potentially high rates of evaporative water loss and high body temperatures are unknown. Thus, in this study, skin colour changes, body surface temperature, and evaporative water loss rates were examined under a mixture of field and laboratory conditions to ascertain whether changes in skin reflectivity play an important role in this animal's thermal and hydric balance. Field data demonstrated a tight correlation between the lightness of skin colour and frog temperature, with lighter frogs being captured possessing higher body temperatures. Laboratory experiments supported this relationship, revealing that frogs kept in the dark or at lower temperatures (20°C) had darker skin colours, whereas frogs kept in the light or higher temperatures (30°C) had skin colours of a lighter hue. Light exhibited a stronger

influence on skin colour than temperature alone, suggesting that colour change is triggered by the increase in incident solar energy and in anticipation of changes in body temperature. This conclusion is corroborated by the observation that cold, darkly coloured frogs placed in the sun rapidly became lighter in colour during the initial warming up period (over the first 5 min), after which they warmed up more slowly and underwent a further, albeit slower, lightening of skin colour. Surprisingly, despite its natural disposition to bask in the sun, this species does not possess a 'waterproof' skin, since its rates of evaporative water loss were not dissimilar from many hylid species that live in arboreal or semi-aquatic environments. The natural history of *B. alvarengai* is largely unknown and, therefore, it is likely that the herein reported colour change and basking behaviour represent a complex interaction between thermoregulation and water balance with other ecologically relevant functions, such as crypsis.

Key words: thermoregulation, basking, skin pigmentation, reflectivity, solar radiation, water balance, frog, *Bokermannohyla alvarengai*.

Introduction

Basking behaviour for thermoregulatory purposes (Ferguson et al., 2003) is a relatively uncommon behaviour in amphibians (Hutchison and Dupré, 1992) due to their usually highly permeable skin, which allows for high rates of evaporative water loss (EWL) (Jameson, 1966; Shoemaker et al., 1992). Although some amphibians can tolerate up to 45% body water loss from evaporation alone (Shoemaker et al., 1992), water loss is thought to be a major constraining factor in many aspects of amphibian ecology (Beuchat et al., 1984). To date, only a few frogs have been described to make effective use of prolonged basking in dry environments for thermoregulatory purposes (Bradford, 1984; Carey, 1978;

Freed, 1980; Lillywhite et al., 1973; Lillywhite et al., 1998; Muths and Corn, 1997; Pearson and Bradford, 1976; Sinsch, 1989; Vences et al., 2002); even fewer have been shown to tolerate prolonged exposure to full sunlight, primarily frogs from the hot, arid regions of South America and Africa (Shoemaker et al., 1987). In the latter case, basking is usually accompanied by remarkably low rates of EWL and the frogs are, therefore, called 'waterproof'; such species belong to the genera *Chiromantis*, *Phyllomedusa* and *Hyperolius* (Drewes et al., 1977; Kaul and Shoemaker, 1989; Shoemaker et al., 1989; Stinner and Shoemaker, 1987; Withers et al., 1982). These species exhibit water loss rates that are 10–100 times lower than rates typically seen in other anurans (Kaul and

Shoemaker, 1989), due mainly to lipids and waxes found in the skin that prevent excessive water loss. Some of these 'waterproof' frogs exhibit a wiping behaviour for applying these lipids over their skin (Barbeau and Lillywhite, 2005; Blaylock et al., 1976; Lillywhite et al., 1997), in addition to a water conserving posture (WCP; Stille, 1958) that serves to seal the frogs' more permeable ventral surface to the substrate to further minimise EWL, while maximising water uptake from ground sources (Pough et al., 1983; Shoemaker et al., 1992).

The interplay between body temperature and water balance may also involve skin colour modifications (Withers, 1995). For example, below 36°C, the skin of *Chiromantis xerampelina* has a mottled, dark grey-brown colour and body temperature closely follows ambient temperature (Kaul and Shoemaker, 1989). On the other hand, at higher ambient temperatures, the skin colour of *Chiromantis* changes to a chalky white colour while the evaporative water loss increases to maintain body temperature lower than ambient (Kaul and Shoemaker, 1989). In another example, King et al. demonstrated that higher temperatures led to lighter skin colours in the green treefrog (King et al., 1994), suggesting that skin colour changes in directions appropriate for maximal solar absorption at low temperatures and maximal solar reflection at high temperatures. Overall, although skin reflectance has been measured in a number of frog species (Carey, 1978; King et al., 1994), the possible role of skin colour change in thermoregulation and water balance of amphibians has seldom been explicitly quantified or demonstrated. Indeed, numerous previous studies have assessed colour changes in frogs qualitatively (Edgren, 1954; Hoppe, 1979; Iga and Bagnara, 1975; King and King, 1991) without emphasising specific colour components or utilising a quantitative approach to assess dynamic changes in skin colour.

The thermoregulatory importance of colour changes in basking anurans might be more effective for 'waterproof' frogs (King et al., 1994; Spotila et al., 1992; Tracy, 1976) since species with high rates of water loss would be cooled down due to evaporative cooling while trying to increase their body temperature by basking, unless heat is gained from the substrate (Lillywhite, 1970). Waterproof species, on the other hand, could conceivably bask in the sun and achieve body temperatures equal to or higher than ambient, since they would not be cooled down by the constitutive levels of evaporative cooling. Skin colour changes in 'waterproof' frogs could then be looked at as a thermoregulatory adaption that has the potential of finely adjusting heat gain in basking frogs. The benefits of such a thermoregulatory strategy may include increased rates of digestion (Freed, 1980; Lillywhite et al., 1973) and an increased ability to ward off infections (Sherman and Stephens, 1998) and parasites (Cagle, 1950).

The general picture emerging is that colour change and basking behavior in amphibians may represent a balance of trade-offs related to the regulation of body temperature and water balance. Complicating the matter, these trade-offs are

likely to be affected by other ecologically relevant functions (e.g. feeding, activity, defense, etc) and also by abiotic factors, especially water availability and ambient temperature. In the present paper, we begin to examine such questions in a little known South American frog, *Bokermannohyla alvarengai*, described by Bokermann in 1956 (formerly *Hyla alvarengai*, now re-classified as *Bokermannohyla alvarengai* by Faivovich et al., 2005). This species spends hours every day sitting on lichen-covered stones fully exposed to the sun and, although highly cryptic against the stones common to their habitat, they become lightly coloured, almost white in appearance, while exposed to full sunlight (Fig. 1; P.C.E., personal observation) (Sazima and Bokermann, 1977). *Bokermannohyla alvarengai* inhabits a montane meadow environment in southeastern Brazil with rocky outcrops and a predominantly herbaceous vegetative cover, suggesting that basking and skin colour changes may play an important role in their body temperature regulation and, possibly, in water balance, due to the relatively exposed nature of their natural environment. Therefore, and more specifically, the objectives of this study were threefold. Firstly, we examined skin colours and skin surface temperatures in frogs found in the field. Secondly, we examined the effect of altering light and temperature on the changes in skin colouration, and determined the time course for these changes. Finally, we examined the whole animal evaporative water loss rates and metabolic rates to determine whether this species could be justifiably considered a 'waterproof' species of frog or not.

Materials and methods

Animals

Juvenile frogs *Bokermannohyla alvarengai* (Bokermann 1956) ($N=7$; mass 2.4 ± 0.6 g, mean \pm s.e.m.; sex undetermined) were collected and studied during the daylight hours between June 4 and 5th, 2005 at Serra do Cipó, Minas Gerais, Brazil ($19^{\circ}15'40''S$, $43^{\circ}33'20''W$, altitude 1385 m). After collection, frogs were transported back to the Jacarezário laboratory, UNESP, Rio Claro in São Paulo State, southeastern Brazil ($22^{\circ}24'S$, $47^{\circ}33'W$) in Styrofoam coolers. Experiments for Series I (i.e. field measurements) were conducted at the location in which the frogs were first found and without any previous disturbance or manipulation. Experiments for Series II and III were conducted within 5 days of the frogs arriving in Rio Claro. Experiments for Series IV and V were conducted 2 weeks following collection from the field. While kept in captivity, frogs were force fed with mealworms every other day, except before experimentation, when food was withheld for 4 days.

Determination of surface temperatures

Surface temperatures of the frogs both in the field and during experimental trials were obtained using a portable infrared thermal imaging camera (Model 7515; Mikron Instruments®, Oakland, NJ, USA).

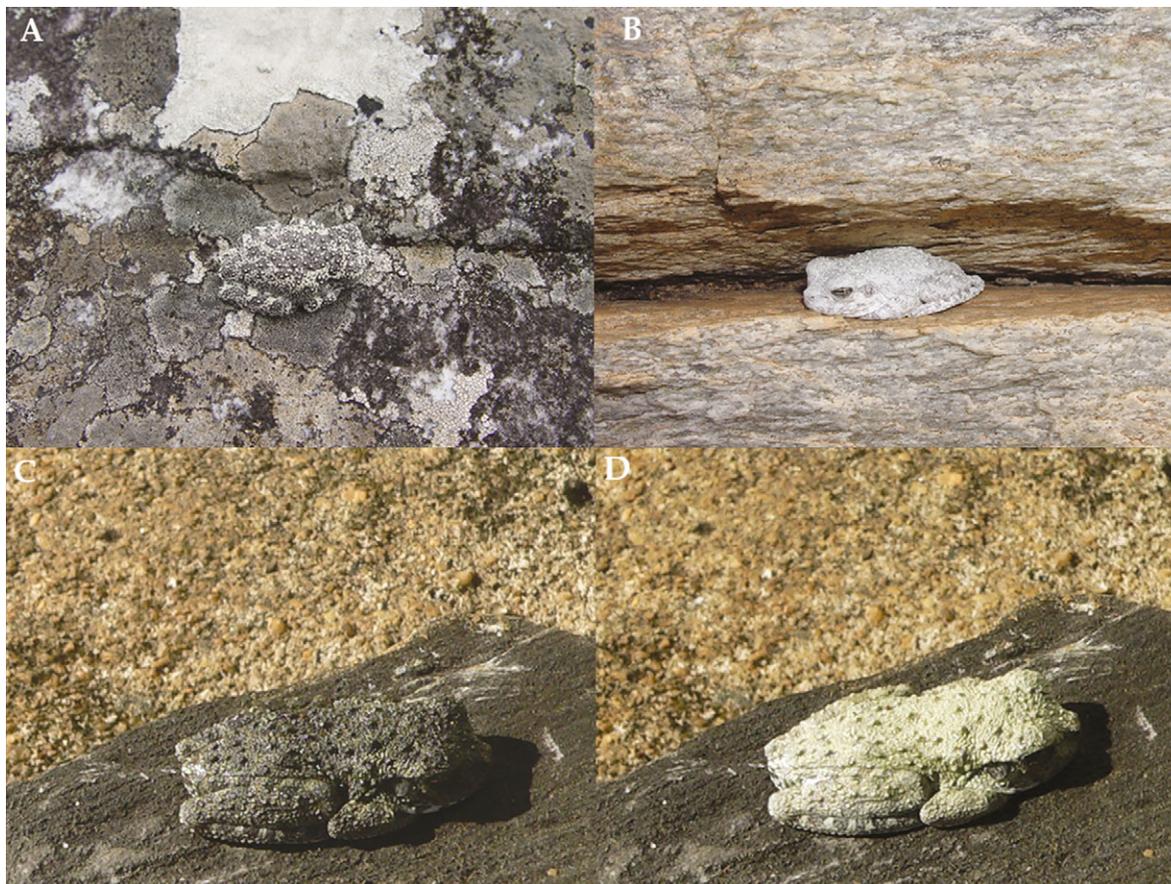


Fig. 1. Digital images of *B. alvarengai* taken under various circumstances. (A) A cryptically coloured individual in the field, (B) a non-cryptic individual in the field, (C) a darkly coloured cool (18°C) frog at the beginning of Series III experiments, and (D) the same frog as in C 1 h later after reaching 28°C. Note the dramatic colour differences between individuals in the field under varying circumstances (A,B) and within the same individual (C,D) at different body temperatures.

Thermal images were subsequently transferred to a computer and analysed using commercial software (MikroSpec RT[®], Mikron Instruments[®]). Regions of interest were outlined and the average temperature of these regions obtained. An emissivity of 0.95 was assumed (Blumberg et al., 2002; Tattersall et al., 2004).

Colour analysis of digital images

A digital camera (Panasonic Lumix Model DMC-FZ10, Secaucus, NJ, USA) was used to acquire images of frogs at specific temperatures. As an initial first step to generate consistently exposed photographs, all pictures of frogs were taken in full sunlight and on days with no overcast cloud cover, and the camera set to auto-expose all images (typically F/stop was 4 and Exposure was 1/300–1/500 s). During the experimental trials (Series II and III) a colour chart was placed within the field of view, consisting of black, white, red, green, and blue colours produced from a colour printer (HP PSC 1315). The colour chart was produced in a graphics program where 'true black' corresponded to a Red, Green, Blue (RGB)

value of (0,0,0); 'true red' corresponded to an RGB value of (255,0,0); 'true green' corresponded to an RGB value of (0,255,0); 'true blue' corresponded to an RGB value of (0,0,255). Digital images were analysed using the Adobe Photoshop[®] histogram tool to determine average red, green or blue of the colour charts. Any deviance in digital images from 'true-black', 'true-red', 'true-green' or 'true-blue' colours were corrected for using a linear calibration, where X_1 and X_2 corresponded to the measured RGB value from the black and red colour charts, and Y_1 and Y_2 corresponded to their 'true' colour values, 0 and 255. Linear calibrations were then developed in similar fashions for the 'true green' and 'true blue' values, and used to correct colours measured from the image files of the frogs. On occasions, grey-scale colour charts (RGB values of 100, 100, 100 or 200, 200, 200) were used to determine whether the digital images obtained with the camera could be corrected with a simple linear calibration or if a curvilinear calibration was required. It was determined that a simple linear calibration was suitable for corrections of the slight deviations in exposure that occurred between images.

After determining each image's unique 0 and 255 calibration correction for each of the three colours, the frog's dorsal skin surface was analyzed using the same histogram tool as above. An area encompassing the entire dorsal surface (excluding any glare) was selected and the average red, green and blue colours in this area were taken and corrected using the linear calibrations mentioned above. Once corrected, grey-scale intensity (where 0 is complete black, and 255 is complete white) was determined by taking an equally weighted average of the red, green and blue colours as an assessment of overall darkness or brightness of the skin.

Experimental protocol

The first series of experiments (Series I) were performed in the field and were designed to test a possible correlation between skin colour and body temperature of the frogs occurring under natural conditions. Following collection from the field, four other series of experiments were performed under more controlled laboratory conditions. Series II was designed to assess the steady state skin colours in frogs at two different temperatures and two different light levels. Series III was designed to track the dynamic changes in skin colour and skin temperature in frogs exposed to the sun. Series IV was designed to determine the average evaporative water loss rates at the range of environmental temperatures experienced by the frogs. Finally, Series V was aimed at determining the frogs' metabolic rates at three different temperatures.

Series I: Field temperatures and colour values

Frogs were located in their natural habitats by active visual search while wandering around the collection area. Once a frog was located, we proceeded to determine the dorsal surface temperature of the frog and rock temperatures using non-invasive infrared thermography. We also took digital photographs of each individual for subsequent skin colour analysis. Finally, ventral temperatures were determined by rapidly turning the frogs over and taking a thermal image (within 5–10 s). It is noteworthy that during the procedure, movements and sound production around the area were kept to a minimum in order to avoid disturbing the animals. In this regard, *Bokermannohyla alvarengai* did not appear to be disturbed by our presence and did not try to escape, in any instance, until the very moment we handled them to take the thermal image from their ventral surface.

Series II: Environmental chamber experiments

In order to examine whether light levels or temperature had effects on skin colouration, we exposed frogs to two different temperatures (20 and 30°C) and two light levels (dark and light). This was accomplished using a temperature controlled environmental chamber furnished with two Philips® TLT fluorescent bulbs (20 W each), which we could turn on or off

according to the condition desired. Frogs were placed on top of rocks similar in colour to stones from their natural habitat that were housed under glass containers and then placed inside the environmental chamber and allowed to stay at either 20°C/Dark, 20°C/Light, 30°C/Dark or 30°C/Light for 1 h. Afterwards, we transferred the stone with the frog to full sun conditions, in order to keep light exposure levels optimal, and took a digital image. The total time taken to transfer frogs from the environmental chamber to the outside of the lab for taking the digital image was less than 20 s, minimising any possible change in temperature or colour. On some occasions, frog surface temperature was checked by thermography and, in all cases, they had come into complete equilibrium with the chamber temperature.

Series III: Sun exposure experiments

Frogs were initially placed on a small rock outside the laboratory the night before measurements were made, and covered with a white, reflective container in order to keep the local environment around the frog cool (~20°C) and dark until the following morning (between the hours of 09:00 h and 12:00 h). In the early morning, frogs were initially shielded from the sun by the shade cover from the building. The seven frogs were arranged in such a way that the sun would begin to reach each rock consecutively at approximately half hour intervals. At the beginning of an experiment, the reflective container was removed and each frog's surface temperature monitored with the thermal imaging camera, while digital images were captured at regular intervals (1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 min). We had previously observed that 30 min would be enough time to warm the frogs by approximately 10°C. Longer periods of time were usually not achieved (for an exception, see below), since frogs would usually retreat beneath the stones away from the sun after reaching temperatures around 30°C, preventing consistent image capture and thermal exposure. In one case, we were able to perform an experiment on one frog and recorded simultaneous changes in skin, rock, and black body (a piece of black electrical tape with high solar absorptivity) temperature as well as digital images of skin colour changes for a complete hour.

Series IV: Evaporative water loss measurements

Whole animal evaporative water loss (EWL) estimates were obtained using custom-built flow-through chambers (100 ml). Frogs were placed inside the chambers and dry gas (79% N₂, 21% O₂), provided by a gas mixing flowmeter (Cameron Instruments, Model GF-3MP, Port Aransas, TX, USA), was pumped through at a fixed rate of 200 ml min⁻¹ (STPD). The gas leaving the chambers became humidified by the frogs, and this humidified gas was measured using a relative humidity meter (Sable Systems RH-200, Las Vegas, NV, USA). The RH meter was calibrated using dry, bottled nitrogen (0% humidity) and water-saturated air at known temperatures (for 100%

humidity). Total EWL rates ($\text{mg H}_2\text{O h}^{-1}$) were determined as the product of the absolute humidity ($\text{mg H}_2\text{O ml air}^{-1}$) leaving the chamber \times air flow rate (ml min^{-1}) $\times 60$ (min h^{-1}). EWL rates were taken as the minimal rates observed during 1 h exposures to four different ambient temperatures (20, 25, 30 and 35°C). During these experiments, the frogs were observed to adopt a nearly complete water conserving posture, and made minimal movements. The EWL rates were further expressed on an exposed skin surface area ($\text{mg H}_2\text{O cm}^{-2} \text{h}^{-1}$) basis by dividing EWL by frog's surface area, estimated on the basis of the equations provided by McClanahan and Baldwin (McClanahan and Baldwin, 1969), multiplied by two-thirds (an estimate of total exposed skin surface, excluding ventral skin).

Series V: Oxygen uptake measurements

Oxygen uptake measurements (\dot{V}_{O_2}) were determined in the dark at 17, 22 and 27°C , conditions achieved by maintaining the animals inside a climatic chamber (FANEM, Sao Paulo, SP, Brazil) during the experiments. Initially, frogs that had been fasting for at least 4 days were weighed, placed inside custom-made glass respirometric chambers (vol. ~ 80 ml), and left to acclimatize at the experimental temperature for at least 4 h. Then, measurements were taken for a period of at least 24 h. The temperature sequence in which experiments were performed was $17^\circ\text{C} \rightarrow 22^\circ\text{C} \rightarrow 27^\circ\text{C}$. To avoid any stress associated with dehydration during the metabolic measurements, we kept a film of water in the bottom of the respirometric chambers throughout the experiments.

Oxygen uptake rates were measured using a computer automated and intermittently closed respirometry setup (Sable System, TR-RM8). This system controls pumps and solenoid valves and was programmed to ventilate the respirometers with fresh air (open phase, 100 ml min^{-1}) for a 90 min period, which was then followed by a 30 min closed phase when the air was recirculated through an oxygen analyzer (PA-1, Sable System). The output from the gas analyzer was collected on a data acquisition system (Sable System, DATACAN V) and \dot{V}_{O_2} was calculated from the rate at which oxygen concentration decreased within the respirometer during the closed phase. The fall in oxygen concentration inside the respirometer was linear and \dot{V}_{O_2} values were calculated as the slope of the O_2 decline, obtained for all the single measurements recorded during the closed phase (1 sample s^{-1} , i.e. 1800 data points sampled over 30 min). This regression usually provided r^2 values greater than 0.9 and the system yielded a \dot{V}_{O_2} measurement every 2 h. Water vapour was absorbed by a tube of Silica gel located at the inflow of the oxygen analyzer, and for the calculation of \dot{V}_{O_2} values we assumed a RQ of 1.

Data analysis

All results are presented as means \pm s.e.m. Series II results were examined using a two-way repeated-measures analysis of variance (RM ANOVA), with temperature (two levels: 20 and

Table 1. Field temperature measurements of *B. alvarengai* at time of collection

Temperature ($^\circ\text{C}$)			
Dorsal (mean)	Ventral (mean)	Ventral leg	Rock
21.8 \pm 1.7	20.8 \pm 1.2 [†]	19.3 \pm 1.1*	21.5 \pm 1.8*

Values are means \pm s.e.m. ($N=7$).
*Significant difference ($P<0.05$) from average dorsal temperature;
[†] $P=0.057$.

30°C) as one treatment and light (two levels: dark and light) as the other treatment. Series III results were examined using a one-way RM ANOVA. Series VI results were examined using a one-way RM ANOVA with temperature (four levels: 20, 25, 30 and 35°C) as the treatment. Series V results were examined using a one-way RM ANOVA with temperature as the treatment. In all cases $P<0.05$ was considered as a significant difference.

Results

Series I: Field temperatures and colour values

On the day of collection from the field, the air temperatures were 18– 19°C , the relative humidity 70–80%, and conditions sunny to slightly overcast. Frogs were collected in mid to late afternoon and were usually found in fully exposed conditions, basking on large, flat, lichen-covered stones. The average dorsal surface temperature of frogs immediately prior to collection was $21.8 \pm 1.7^\circ\text{C}$, which was slightly, and significantly, higher ($P=0.045$; one-tailed paired t -test) than the rock temperature of $21.5 \pm 1.9^\circ\text{C}$ (Table 1). Due to the presence of moisture on the ventral surface, mean ventral temperature ($20.8 \pm 1.2^\circ\text{C}$) was 1°C cooler than dorsal temperature, an effect which was nearly significant ($P=0.057$; one-tailed paired t -test). Skin greyscale values of frogs at the time of collection in the field demonstrated a significant, positive correlation with dorsal skin temperature ($r^2=0.95$; $N=7$, $P<0.05$). Though most frogs blended in with the grey, lichen-covered stones (Fig. 1A), the warmest frog (Fig. 1B) was found on an orange coloured rock, in obvious contrast to its surroundings due to its nearly white colouration. It took, on average, 15 min to collect and process images from each frog in the field, during which time the animals were completely stationary in a water conserving posture. Frogs were apparently undisturbed by our presence and made no attempts to escape.

Series II: Controlled light and temperature experiments

Temperature and light both had significant effects on skin colouration ($P=0.029$ and $P=0.001$, respectively); however, the largest effect (both biologically and statistically) was achieved by the influence of light alone (Fig. 2). For example, skin grey intensity increased by approximately 300% simply through changes in light levels, whereas the overall

percentage change in intensity between 30 and 20°C was approximately 50%. Skin colour values at 20°C in the dark were 59±6, 62±9, 16±6, and 46±6 for red, green, blue and grey, respectively, increasing to 184±7, 212±7, 125±15 and 174±9 in the light. At 30°C, skin colour values were higher overall, starting at 92±10, 102±19, 30±8 and 75±12 for red, green, blue and grey, respectively, increasing to 200±8, 247±2, 155±11 and 201±6 in the light. In all cases, there was no significant interaction ($P=0.18-0.88$) between light level and temperature as performed with two-way RM ANOVA.

Series III: Sun exposure experiments

In general, the frogs would only remain in the sun for 30 min before attempting escape (see below for one exception).

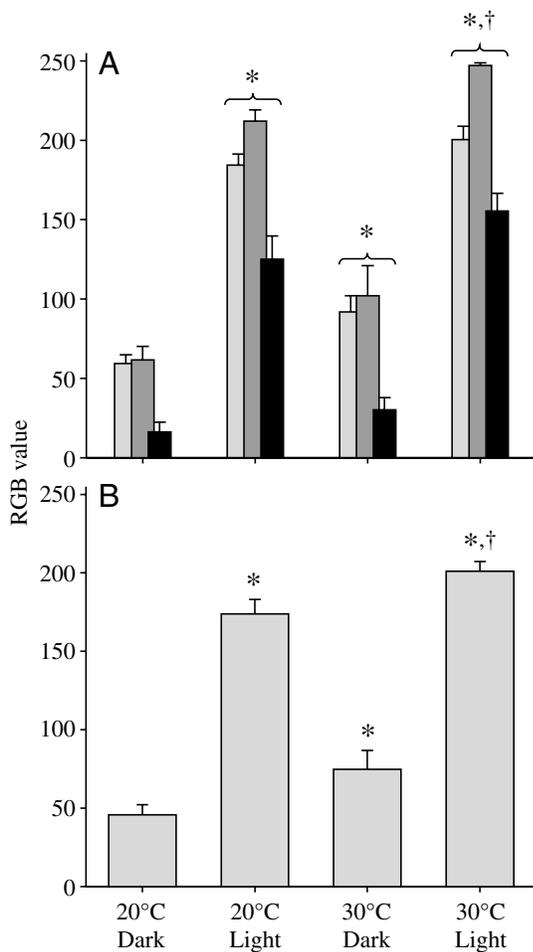


Fig. 2. Skin colour values (A) and grey scale values (B) for frogs exposed to 20 and 30°C in both the dark and the light (Series I experiments) for at least 1 h each. Values are means \pm s.e.m. In A, light grey bars refer to the average red value, dark grey values to the average green value, and black bars to the average blue values. Both light level and temperature had significant effects. *Significant difference from 20°C dark exposed frogs; †significant difference from 30°C dark exposed frogs.

During this time, their surface temperatures increased from an average of 23.3±1.0°C to 31.5±0.7°C. Surface temperatures seemed to follow two distinct rates of change. During the first 5 min, temperature increased linearly and rapidly, after which the rate of temperature change slowed, but still continued to increase (Fig. 3). Meanwhile, the average red, green, blue and grey values increased rapidly over the first 3 min, remained fairly constant for the next 2 min, after which they continued to increase more slowly over the remaining 30 min (Fig. 3). The net result, when comparing skin greyscale intensity against frog surface temperature, was that the average data suggested three phases of warming: (i) the first phase was where rapid changes in skin colour accompany rapid temperature changes; (ii) the second phase was where skin greyscale intensity changed little while temperature continued to rise; and (iii) both skin greyscale intensity and temperature increased linearly until a maximal response was reached. The background colour of the stone upon which the frogs sat was assessed at the beginning (0 min) and end of the experiment (30 min). The RGB and grey-scale intensity values were not significantly different (paired t -test, $P=0.43$), suggesting that the light levels were not dramatically changing throughout these experiments.

The general pattern of frog skin colour change was more clearly revealed in a single experiment performed on one extremely cooperative frog which remained in the sun for 60 min on a separate occasion from the above experiments, allowing for the collection of a variety of variables (Fig. 4). During this particular experiment, we were able to record the rock temperature, frog temperature, black body temperature, and the skin colour changes contemporaneously (which in other instances were precluded by the unwilling cooperation of the frogs). Upon exposure to the sun, black body

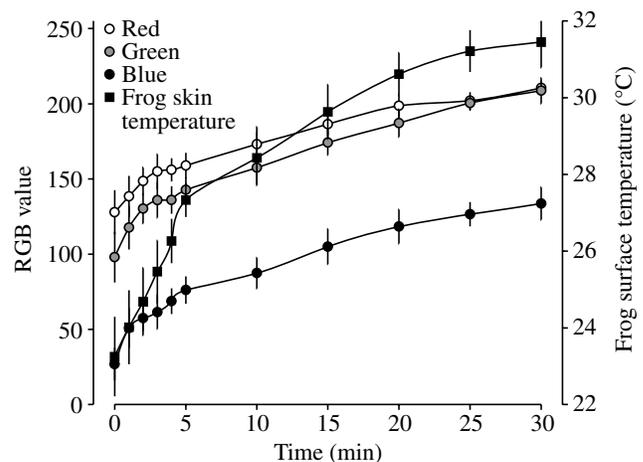


Fig. 3. Changes in skin colour intensity (red, open circles; green, grey filled circles; blue, solid filled circles) during 30 min of exposure to the sun from Series II experiments. Values are means \pm s.e.m. Frog skin temperature is also shown (filled squares) increasing throughout sun exposure.

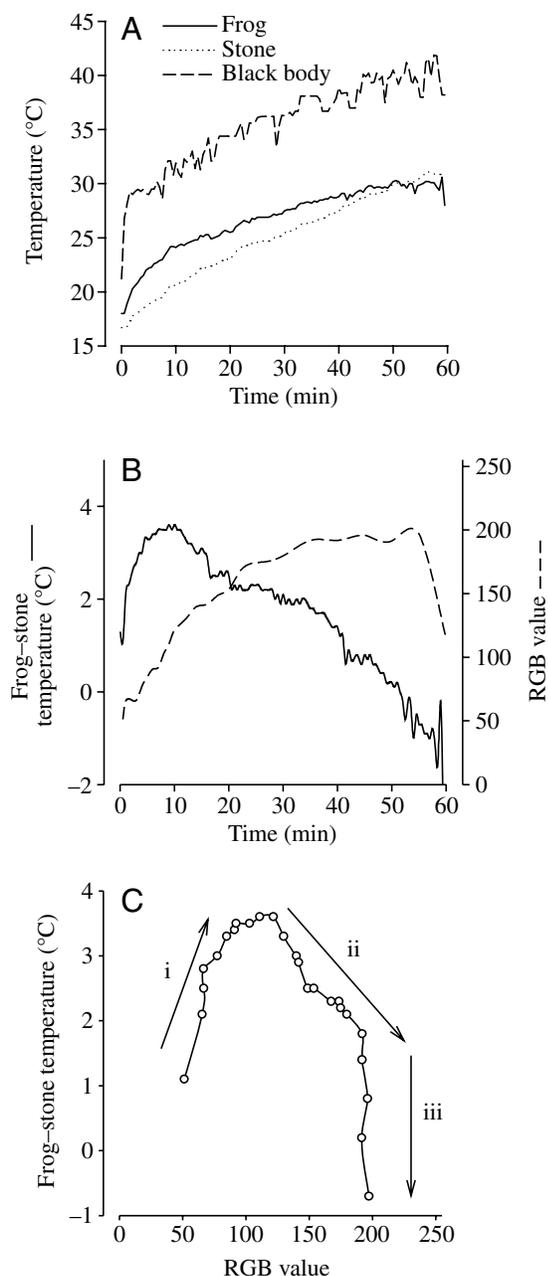


Fig. 4. Sample temperature and grey intensity trace from one frog that willingly basked in the sun for 60 min. (A) Rock temperature (solid line), black body temperature (broken line; the surface temperature of a piece of black electrical tape exposed to the sun, but not in contact with substrate), and frog dorsal surface temperature (dotted line). (B) Changes in the frog-stone temperature difference (solid line) and the skin's greyscale value (broken line). (C) Correlation between the frog-stone temperature difference and the skin greyscale value (open circles). Roman numerals (i-iii) refer to (i) an early phase when the skin is dark and the frog warms up rapidly, (ii) a secondary phase when the skin has lightened up considerably and the frog and stone temperature difference diminishes, and (iii) a late phase where the frog is as light as possible and the frog equilibrates with stone temperature and eventually falls below stone temperature, presumably due to increased evaporative water loss.

Table 2. Rates of evaporative water loss in *B. alvarengai* housed at different ambient temperatures

Temperature (°C)	Evaporative water loss		Q_{10}^c	r (s cm ⁻¹) ^d
	(mg H ₂ O h ⁻¹) ^a	(mg H ₂ O cm ⁻² h ⁻¹) ^b		
20	50.1±6.2	5.13±0.67		13.3±1.9
25	62.1±5.7	6.42±0.78	1.59±0.11	14.3±2.1
30	79.3±2.7	8.55±1.63	1.77±0.30	15.0±2.2
35	108.0±4.6	11.70±2.38	1.86±0.08	14.2±2.0

Values are means ± s.e.m. (N=7).

^aAll water loss rates were significantly affected by temperature.

^bEvaporative water loss rates were also calculated based on two-thirds (i.e. excluding ventral skin) of the estimated total skin surface area from the equation, $SA=9.9M_b^{0.56}$ (where surface area SA is in cm², and M_b is in g) (McClanahan and Baldwin, 1969).

^c Q_{10} values are calculated between adjacent temperatures; temperature did not have a significant effect on Q_{10} ($P=0.53$).

^d r represents the total resistance to water flux, calculated using the formula from Spotila et al. (Spotila et al., 1992).

Table 3. Oxygen uptake rates in *B. alvarengai* housed at different ambient temperatures

Temperature (°C)	Oxygen uptake (ml kg ⁻¹ h ⁻¹) ^a	Q_{10}^b
17	84.5±10.2	
22	117.9±13.5	1.98±0.20
27	167.3±29.4*	2.10±0.56

Values are means ± s.e.m. (N=7).

Mean body mass=2.47±0.39 g (mean ± s.e.m.; N=4).

^aOxygen uptake was significantly affected by temperature; ^b Q_{10} values are calculated between adjacent temperatures.

temperature quickly rose, and then steadily continued to rise over the remaining 60 min (Fig. 4A). The stone surface temperature increased linearly during sun exposure (Fig. 4A). Frog surface temperature, on the other hand, increased rapidly at first, after which its temperature changed more slowly. Frog skin colour (greyscale) changed in a non-linear fashion with time, starting low and increasing rapidly at first, after which the rate of colour change declined until a fairly constant level was reached (Fig. 4B). Correlations between the degree of heating (expressed as frog-stone temperature) and skin greyscale values revealed some interesting trends. As commented above, this particular individual showed an early heating phase when the skin started out dark and the frog warmed up faster than the stone. This was followed by a second phase when the skin had lightened up considerably and the frog and stone temperature difference diminished and began to reverse. The final late phase was where the frog's skin was as light as possible and frog temperature was fully equilibrated with stone temperature being, eventually, lowered slightly below stone temperature (Fig. 4C).

Series IV: Rates of evaporative water loss

Whole animal evaporative water loss rates were significantly affected by temperature ($P < 0.001$), increasing from $50.1 \pm 6.2 \text{ mg H}_2\text{O h}^{-1}$ to $62.1 \pm 5.7 \text{ mg H}_2\text{O h}^{-1}$ to $79.3 \pm 2.7 \text{ mg H}_2\text{O h}^{-1}$ and to $108.0 \pm 4.6 \text{ mg H}_2\text{O h}^{-1}$ from 20°C to 25°C to 30°C and to 35°C (Table 2). The Q_{10} for these rates was 1.59 ± 0.11 (between $20\text{--}25^\circ\text{C}$), 1.77 ± 0.30 (between $25\text{--}30^\circ\text{C}$) and 1.86 ± 0.08 (between $30\text{--}35^\circ\text{C}$).

Series IV: Oxygen uptake measurements

Oxygen uptake rates were clearly affected by temperature, although the difference between the rates measured at 17°C and 22°C did not reach statistical significance. Oxygen uptake increased with temperature with a Q_{10} of ~ 2 ($1.98\text{--}2.10$; Table 3), regardless of the temperature interval considered ($17\text{--}22$ or $22\text{--}27^\circ\text{C}$).

Discussion

Knowledge of *B. alvarengai* natural history is limited to brief comments based on the sporadic observations of a few individuals found in the field (Eterovick and Sazima, 2004; Sazima and Bokermann, 1977). The most remarkable aspect repeatedly observed in this species is the prolonged, stationary basking behaviour associated with changes in skin

colouration. Our field data confirm such observations, since all animals we studied were found fully exposed on the surface of large rocks. In addition, we were able to show that skin colour and body temperature were interrelated for different individuals found at different times of the day under field conditions. Such results strongly support the view of temperature/light induced changes in skin pigmentation, rather than a variation in skin colouration amongst the different individuals, and this was further demonstrated by our documentation of the changes in skin colouration and body temperature under laboratory conditions.

Indeed, we found a positive correlation between frog temperature and skin colour, whether in the field or laboratory conditions, showing that there is a graded response, with temperature playing a cooperative role with changes in light levels. Frogs showed an unequivocal ability to modify the colouration of their skin, simultaneously in response to temperature and illumination, in ways that maximise solar reflectivity at high temperatures and light levels and minimize solar reflectivity at low temperatures and light levels. There was, however, a noticeable difference between the relationship of skin colour and T_b recorded in the field and that measured under laboratory conditions, which may reflect the time component of the response of the skin chromatophores to circulating hormones. Animals caught in the field had hours to equilibrate under full sun and were closer to the maximum skin 'lightness' probable for their given T_b (see Fig. 5), suggesting that they begin to augment skin reflectivity at relatively low ambient temperatures. Under the more artificial laboratorial conditions, cold, darkly coloured frogs warmed up rapidly, as predicted by their low reflectivity, and then gradually lighten and decrease heat gain. It is likely that background colour and illumination interact to some extent in determining skin colour (King et al., 1994). Under controlled, bright light conditions (Series II), we witnessed frog skin colours going lighter than observed in either the field or other experimental conditions, and under completely dark conditions, we witnessed frog skin colours appearing darker than ever observed in cold frogs under minimal light conditions (see Fig. 5). It appears that the Series II experiments define the boundaries of skin colour intensity within which the animals are capable of responding relatively rapidly (within minutes) and according to need. Indeed, adults of *B. alvarengai* observed in the field while engaged in reproductive activities at night always exhibit a dark grayish colour (P.C.E., personal observation).

The biological role of basking and changes in skin colouration under natural conditions in this species are difficult to envisage, particularly considering the lack of information on the natural history of *B. alvarengai*. Such limitations compel us to ground the discussion of our physiological measurements on biological traits actually known for this species; however, some plausible interpretations of the biological significance of basking and colour change can be drawn from our data. Firstly, why do these frogs bask? *B. alvarengai* occurs from southeastern

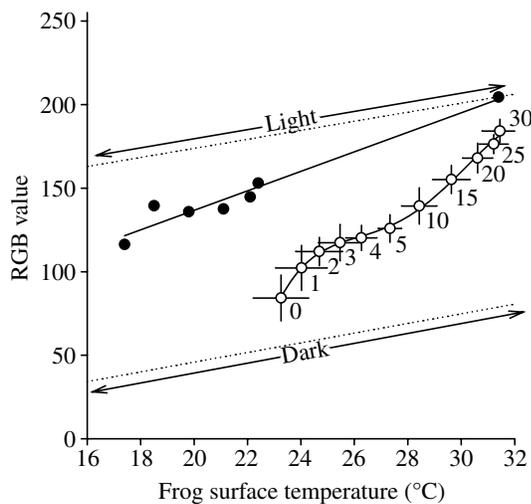


Fig. 5. Correlations between skin colour (grey intensity) and dorsal surface temperature in field and laboratory conditions. Filled black circles refer to values obtained during collection of frogs in the field ($N=7$; linear correlation: $r^2=0.95$). Open circles with error bars refer to the average \pm s.e.m. changes in skin greyscale intensity and temperature during Series II experiments when frogs were allowed to warm up in the sun. Numbers beside these values refer to the elapsed time in minutes (0–30). The two dotted lines, marked Light and Dark, refer to the minimum and maximum possible range of skin colour values defined by Series I experiments from frogs placed in complete dark or light at 20 and 30°C .

Brazil to the south of Bahia state, being restricted to the Espinhaço mountain range at elevations above 1000 m. In such locations, the climate is characterized by a cold and dry winter (April to September, mean winter temperature 10–15°C) and a hot, rainy summer (October to March, mean summer temperature 18–20°C). The climate is tropical, but at elevation. Rainfall varies between 1450 and 1800 mm and approximately 50% of that is concentrated in the months of November, December and January (Nimer, 1989). Therefore, as also noticed for other species of montane frogs (Carey, 1978; Muths and Corn, 1997), basking might confer a number of thermoregulatory benefits for *B. alvarengai*, including increased rates of digestion (Freed, 1980; Lillywhite et al., 1973), accelerated gonadal development (Figueiredo et al., 2001), and increased ability to respond to infections and diseases (Cagle, 1950; Sherman and Stephens, 1998).

A second question that arises from this study is simply that of why skin colour changes? For exposed, basking frogs, two main concerns are likely to be at play: the increased risk of predation from activity in a non-sheltered environment and the potential risk of overheating (in fact temperature elevation also leads to the potential risk of losing excessive water through evaporation, which is discussed below). Interestingly, both factors seem to be balanced by basking *B. alvarengai*. The highly cryptic colouration shown by *B. alvarengai*, together with their low density, may confer a very effective mechanism of avoiding detection by visually oriented predators, particularly at low body temperatures. However, when body temperature is elevated, the lightening of the skin can lead to a very prominent white hue and make the animals truly conspicuous (see Fig. 1). It is likely, therefore, that as body temperature rises, the risk of overheating overcomes the risk of being more perceptible to potential predators and colour change occurs to reduce the absorption of solar radiation. Similarly, Withers suggests that the skin colour in desert tree frogs becomes lighter as a mechanism for increasing reflectance (Withers, 1995), thereby reducing heat gain during dry air exposure. Why do the frogs not simply retreat under the stones as temperature gets too high in nature, as they sometimes did during laboratory experiments? By adopting a lighter colouration, frogs can reduce the rate of heat loading from the sun, allowing for longer periods of time at elevated, but non-lethal body temperatures, and reap the subsequent thermal benefits. Besides, we noticed that most frogs captured in the field were in the water conserving posture and that there was a considerable amount of water underneath them upon capture, possibly condensed from the atmosphere while the frogs were exposed during the early, cool morning hours. Thus, shuttling between sunny (exposed rock) and sheltered locations might cause the frogs to lose the water captured by condensation and lead to increased risk of excessive water loss. Finally, locomotion between microhabitats could make the frogs even more conspicuous to predation (cf. Richardson, 2001) than simply assuming a lighter colouration, in addition to the extra energetic costs of locomotion.

Unfortunately, no quantitative data about the duration or diurnal pattern of basking in *B. alvarengai* is available, and neither is information on possible ontogenetic or seasonal differences in such behaviour. The following discussion is, therefore, based on unpublished data collected over the years by one of the authors (P.C.E.) and on field observations made during our collection of the frogs in the field. Basking individuals have been observed year-round and in all post metamorphic developmental stages (P.C.E., personal observation). It is interesting that even very small froglets (about 1.5 cm snout–vent length) bask for short time periods on rocks close to the streams where they metamorphose. Such froglets can be easily found close to breeding sites from January to April (wet season), since they do not seem to go farther than a few metres from the water. In one instance, six of these froglets were observed to remain in their basking sites on rocks during the morning and all of them left between 12:00 h and 13:00 h, by which time their colouration had changed from a spotted grey to a bright white (P.C.E., personal observation). In the present study, we observed basking in juvenile frogs well into the late afternoon at reasonable distances from the closest water body (~500 to 2000 m, not quantified). As the frogs grow, they seem to disperse farther and become more difficult to locate, with some adults occasionally being observed basking very far from any water body. Adults are consistently found in the vicinity of water bodies only during breeding activities, from October to December, at temporary streams with sandy or rocky bottom (P.C.E., personal observation). Such ontogenetic changes in habitat are likely to reflect differences in the ability to control body temperature and water loss. If that is the case, the observations commented above indicate that such differences are more likely to be related to differences in body size (due to surface area:volume constraints) effects on water loss, rather than by ontogenetic changes in the ability to perform skin colour changes.

Although our measurements were conducted during the dry season, there is no evidence of changes in the properties of the skin or the basking behaviour of *B. alvarengai* throughout the year. In dry season-adapted *Hyperolius viridiflavus*, skin colour changes with temperature in similar ways that occurs in *B. alvarengai*, but during the wet season, the iridophore layer becomes thinner and less organized, and the skin apparently lacks the ability to change colour with temperature (Kobelt and Linsenmair, 1986). It is possible that a similar pattern exists in *B. alvarengai*; however, our measurements would have to be repeated during the wet season to confirm this. In terms of chromatophore function, one striking difference between *B. alvarengai* and the ‘waterproof’ frogs is the temperature range across which these colour changes occur. For example, below 35–36°C in *Hyperolius* and *Chiromantis*, these frogs retain a brownish white colour, only becoming completely white at air temperatures near 40°C (Kaul and Shoemaker, 1989; Kobelt and Linsenmair, 1986). *B. alvarengai*, on the other hand, becomes nearly white at moderately low temperatures, well below 35°C. Whether this relates directly to their higher rates

of water loss, or corresponds to a different range of preferred body temperatures, is unknown. Perhaps the inflection point in the relationship between skin colour and T_b around 28°C (see Fig. 5) corresponds to their preferred T_b , above which further increases in temperature slow down while skin colour continues to lighten.

All amphibians lose moisture across their skin and from other non-cutaneous routes at rates typically much higher than other terrestrial vertebrates. The estimated EWL in this study at 25°C was 62 mg H₂O h⁻¹ or 6.42±0.78 mg H₂O cm⁻² h⁻¹ (Table 2). This value is approximately 44% of that calculated from equations for arboreal hyloid frogs (140 mg h⁻¹ for a 2.6 g frog) (Wygoda, 1984), indicating that water loss rates in *B. alvarengai* are relatively low for a hyloid frog and that there may be some physiological or ultrastructural adaptations in the skin that allow for these moderately low (compared to most amphibians) EWL rates. On the other hand, rates of evaporative water loss in the 'waterproof' frogs can be as low as 0.41 mg H₂O g⁻¹ h⁻¹ (corresponds to approximately 0.3 mg H₂O cm⁻² h⁻¹) (Drewes et al., 1977). These values are at least 20 times lower than those in the present study (compare to Table 2) and, therefore, *B. alvarengai* cannot realistically be considered a 'waterproof' species of frog. A possible reduction in metabolic rate, which could be interpreted as a possible adaptation to reduce respiratory water loss, seems not to be at play in *B. alvarengai* either. This is supported by the fact that the metabolic rates obtained in our study were not lower than the values predicted on the basis of the scaling equation (Gatten, 1992). Furthermore, the Q₁₀ effect on metabolism fits well within the range reported for other anuran species (Gatten et al., 1992) indicating that no special metabolic adjustment occurs when body temperature is elevated and the risk of losing excessive water is augmented.

Basking represents a water balance challenge to most anurans and, accordingly, this behaviour is usually considered to be restricted to individuals with easy access to water (Brattstrom, 1963). Some high altitude anurans use basking only during the mid-morning and early afternoon hours to raise T_b above ambient temperature (Kuhnen, 1997), whereas smaller anuran species in the high neotropics avoid basking altogether, and simply select suitable microhabitats out of the sun (Navas, 1996). In both cases, the high rates of water loss (5.7% body mass per hour) (Pearson and Bradford, 1976) seem to impact the time these frogs can allocate to basking. For *B. alvarengai*, there is no published information available on the circadian and seasonal profile for basking; however, anecdotal observations suggest that this species does stand fully exposed in the sun for long periods of time (P.C.E., personal observation). Besides colour changes, some frogs are capable of regulating water loss to prevent excessive overheating when basking, whereas others seem not to possess this adaptation (Brattstrom, 1979; Buttemer and Thomas, 2003; Lillywhite, 1975; Lillywhite and Licht, 1974; Shoemaker et al., 1987). Our data on EWL rates indicate that *B. alvarengai* fits into this latter category. In fact, the temperature sensitivity of EWL was relatively low (Table 2) and, therefore, there was no evidence

that an increase in EWL at higher temperatures was being used to help to defend core T_b , although it might be that 35°C was not high enough for this response to manifest.

An argument has been made previously on numerous occasions (King et al., 1994; Spotila et al., 1992; Tracy, 1976) that thermoregulatory colour changes would be most adaptive or beneficial in anurans that possess low or limited evaporative water loss. However, there is previous work showing anurans with normal or relatively high rates of EWL that readily change colour (Carey, 1978; Garwood and Welsh, 2005), suggesting that the restriction of thermoregulatory colour changes to 'waterproof' frogs might be too narrow a concept. Perhaps a more parsimonious explanation is that all amphibians have the ability to change colour, whether over the short term or over longer time courses, but only some have the ability to minimize EWL. Depending on the evolutionary history and environmental context of a particular group, both of these adaptations may or may not exist in concert.

In terms of skin colour changes, there is a large literature demonstrating the effect of background colour on the skin darkening responses in anurans (see Tonosaki et al., 2004). Generally speaking, light coloured backgrounds lead to a lightening of the skin and dark coloured backgrounds lead to a darkening of the skin. These responses are mediated *via* central neural control of the pituitary secretions of circulating α -MSH that subsequently evoke changes in the dispersion of the melanosomes in the skin (Oshima, 2001; Tonosaki et al., 2004). These changes in skin colour with respect to illumination and background colour likely serve as a predator avoidance strategy by making amphibians more cryptic (King et al., 1994), though it does not appear that a firm link has ever been made between central thermoregulatory control mechanisms and specific changes in skin colour. Interestingly, these responses to background intensity are observed in aquatic amphibians (Roubos, 1997) that rarely, if ever, emerge from water, suggesting that in most species, background adaptation is the primary reason for pigment dispersal in the skin under varied light conditions. Sorting out the effects and interactions between background colour, illumination and temperature on skin colour changes in *B. alvarengai* has yet to be done, though background colouration, anecdotally, did not appear to exhibit a strong influence.

Perspectives

Little is known at present about the natural history of *B. alvarengai*. Based on the measurements presented here, we speculate that due to their low to moderate rates of water loss, these frogs would forage nocturnally at high relative humidities and low temperatures to minimize water loss and reduce predation risk (see also Sazima and Bokermann, 1977). In the early morning hours, they would find suitable basking sites where they could sit and have water condense over their body (the difference between ventral and dorsal surface temperatures provides suggestive support of this and is crucial to the formation of this condensate). Subsequently, this water

may become trapped beneath them and be conserved during the day through the adoption of the water conserving posture. Heat, and its associated benefits, is gained during basking, and cryptic or disruptive colouration is used to decrease the risk of predation while exposed on the stones. Whenever heat load is increased to levels that could lead to overheating, skin color change comes into play and helps to decrease the absorption of radiant solar energy. Finally, we should add a cautionary note that our interpretations in this paper were reached by bridging our physiological measurement data to unknown ecological traits, therefore, and most desirably, our predictions remain to be verified by further field research.

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