



Getting ready for a long bath: skin permeability decreases prior to aquatic breeding in male toads

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Abstract

Vertebrate skin regulates exchanges between the organism and its environment and notably plays a fundamental role in regulating water fluxes. Dynamic changes of skin resistance to water fluxes are expected to occur in species that regularly shift between habitat types especially if these habitats differ in their hydric properties (e.g., terrestrial versus aquatic). We investigated changes of skin permeability using a study system (terrestrial toads) where reproduction induces a transition from terrestrial to freshwater habitats and a prolonged immersion that can last several weeks in males. In this system, the simultaneity between skin shedding and the onset of breeding suggests that the production of new integument layers prior to immersion for reproduction may regulate water influxes. We found that the skin permeability of male toads decreases significantly prior to breeding, suggesting that skin shedding at the onset of breeding regulates water fluxes to alleviate osmotic costs of immersion during reproduction. The continued decrease of skin permeability detected during breeding suggests that additional mechanisms interact with skin structure to further decrease permeability to water during a prolonged immersion. Future studies are required to assess whether changes in skin permeability to water tradeoffs with other skin characteristics (gas exchanges) relevant to aquatic breeding amphibians.

Keywords *Bufo spinosus* · Water relations · Cutaneous evaporative water loss · Habitat · Reproduction

Introduction

Species that shift between habitats during their lifetime must face environmental constraints that can be highly divergent between habitat types. Typical examples of the consequences of such habitat shifts have been thoroughly described in anadromous or catadromous fish. In these species, reproductive migrations between fresh- and seawater are characterized by highly different chemical compositions (e.g., high salt concentration in seawater) and involve significant changes to the osmoregulatory apparatus that allow osmotic balance either in hyperosmotic marine or hyposmotic freshwater habitats

(Edeline 2007; Tseng and Hwang 2008; Bowerman et al. 2017). Other examples have been documented in amphibious species that commute between aquatic and terrestrial habitats (Mazin and de Buffrénil 2001) which diverging physico-chemical characteristics have been shown to influence several traits such as locomotor performance (Bonnet et al. 2005) or environmental tolerance (Brischoux et al. 2013).

Vertebrate skin provides physical protection and regulates exchanges between the organism and its environment, including the regulation of water fluxes (Lillywhite 2006). Accordingly, the resistance of the skin to water passage has been shown to be highly variable and range from very low in aquatic vertebrates to high values in species adapted to xeric environments (Lillywhite 2006). Dynamic changes in skin resistance to water fluxes are expected to occur in species that regularly shift between habitat types, especially if these habitats differ in their hydric properties (e.g., terrestrial versus aquatic). For instance, many amphibians shift between habitats during reproduction with a transition from terrestrial to aquatic sites.

The integument of amphibians is characterized by very little keratin and a thin stratum corneum (Lillywhite 2006). As a

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consequence, transcutaneous water loss is high, and amphibians tend to dehydrate rapidly under dry conditions (e.g., on land, Burggren and Vitalis 2005). Terrestrial species display specific adaptations to reduce dehydration such as an elevated ability to absorb water cutaneously (Bentley 1971; Hillyard et al. 1998) or increased skin lipids (Withers et al. 1984). Yet, many terrestrial amphibian species reproduce in aquatic habitats where mating occurs and eggs and larvae develop (Dodd 2010). In this context, the ability of terrestrial amphibians to absorb water through the skin can become a constraint when they shift to an aquatic lifestyle for reproduction.

During aquatic reproduction, male toads typically experience an osmotic challenge during the prolonged period (several weeks) of immersion in freshwater, presumably due to water influx (Brischoux and Cheron 2019). Despite a significant decrease in plasma osmolality following immersion, breeding toads can maintain a relatively elevated plasma osmolality even after prolonged periods of immersion suggesting that water influx remains limited (Brischoux and Cheron 2019). In this respect, the integument may well play a significant role in regulating water influxes (Bentley 1971; Shoemaker et al. 1992, Boutilier et al., 1992). Because male bufonid toads shed their skin (i.e., production of new integument layers) just prior to the shift to aquatic reproduction (Jørgensen & Larsen 1961), it is likely that the new skin has specific properties that allow for reduction in water fluxes (Wu et al. 2017).

In this study, we assessed the skin permeability (cutaneous evaporative water loss, CEWL) of reproductive and non-reproductive male spined toads (*Bufo spinosus*) in order to test whether a shift from terrestrial to aquatic lifestyle influences skin permeability in this terrestrial species. First, we hypothesized that skin-shedding prior to reproduction is associated with lower skin permeability during aquatic breeding in order to limit water influxes and thus osmotic costs of reproduction (Brischoux and Cheron 2019). Second, irrespectively of reproduction, we expected that there would be regional variation in CEWL depending on the body area of the toads. We predicted that the dorsal area — presumably exposed to more desiccant conditions than the ventral area, which remains in close contact with relatively humid substrate — should display lower CEWL values than the ventral area or the pelvic area that is involved in transcutaneous water intake (Jørgensen 1994).

Material and methods

Study species and sampling

The spined toad (*Bufo spinosus*) is one of the most common anuran species in Western France. As with most toad species, it is characterized by a biphasic lifestyle with breeding occurring in aquatic habitats (ponds) where eggs and

tadpoles develop, while the remaining cycle occurs in terrestrial habitats (Reading and Clarke, 1983, Brischoux et al. 2018). In late winter (February–March), male toads migrate to breeding ponds where they wait for females during several weeks (Brischoux et al. 2018). Females remain only transitorily at breeding sites, and typically return to land within a few hours once mating and egg-laying has occurred (Brischoux et al. 2018). Because aquatic life (immersion at breeding sites) is disproportionately longer in males, we focused our investigations on this sex only.

In order to comprehensively assess skin permeability of toads both during terrestrial and aquatic (reproductive) phases, toads were sampled during three distinct time periods. First, we captured individuals during the non-reproductive period in early October when climatic conditions allow toads to resume activity after aestivation during drier months (summer). During this period, we opportunistically captured individuals that were foraging on roads situated nearby the pond monitored during the breeding season (see below). Within a few days (4–18 October 2019), we were able to collect 19 adult male toads. Second, at the onset of the reproductive period, during male migration to breeding sites, we captured individuals upon their arrival at the breeding pond, but before they actually entered water. To do so, sampling was conducted at night using headlamps to detect male toads located on land and moving in the direction of the pond. These individuals were located within 10 m of the pond edges. Importantly, male toads shed their skin just before reaching breeding ponds (Jørgensen and Larsen 1961), and the individuals that we captured during this time period were all showing remnants of loose old skin suggesting that skin shedding had occurred. Due to the massive migration of male toads to aquatic breeding sites, all individuals ($N=19$) were captured during a single night (29 January 2020). Finally, we captured individuals that were immersed at the breeding pond for a significant time period (i.e., ~3 weeks after the arrival of the first individuals). Sampling was conducted at night using headlamps, and toads immersed in water were captured with a net. Similarly to the previous capture session, all individuals ($N=20$) were captured during a single night (21 February 2020). For clarity, we will refer to these three time periods as “non reproductive”, “arrival at breeding site”, and “aquatic breeding” hereafter.

After capture, individuals were brought back to the laboratory and maintained in plastic containers with a shelter and either a damp substrate (paper towel) for “non reproductive” and “arrival at breeding site” individuals, or water allowing full immersion for “aquatic breeding” individuals. The snout–vent length (SVL) of each individual was measured with electronic calipers (± 0.01 mm), and CEWL measurements (see below) were performed the day following capture.

All individuals were released at their location of capture after measurements.

CEWL measurements

Measurements were carried out at room temperature (20 °C), and toads were acclimated to this temperature for at least 2 h before measurement. We used an AquaFlux AF200 (Biox, London) and the Bioxsoftware AquaFlux 6.2 to calibrate and compute CEWL rate ($\text{g m}^{-2} \text{h}^{-1}$). We used an in vivo nail cap with rubber O-ring (diameter 2.6 mm) to insure a complete seal between the device and the toad's skin. Contact was maintained by gently restraining the toad and applying steady but slight pressure to the probe against the skin. Trials continued until the CEWL reading stabilized ($\pm 0.02 \text{ g m}^{-2} \text{ h}^{-1}$) for 180 s. If any movement (by the toad or the operator) caused a leak in the seal between the probe and the toad (detected as a sudden change in water flux), the trial was repeated. The AquaFlux unit was calibrated at the beginning of each trial. Each measurement was performed in triplicates at each body area (see below), and we used mean values for analyses.

Because we expected regional variation of CEWL depending on the body area of toads (see above), measurements were performed at three different body regions, namely the dorsal area, the ventral area, and the pelvic patch dedicated to cutaneous drinking (Jørgensen 1994). For dorsal measurements of CEWL, we targeted an area situated between the parotid glands because this area contained fewer warts than the remaining dorsal area, thereby allowing a correct seal between the Aquaflux's probe and the toad's skin. For ventral measurements of CEWL, we targeted an area situated between the forelegs. Finally, the CEWL measurements of the pelvic patch were straightforward to perform because this specific area is easily recognizable by its location (pelvis) and color (pinkish, due to high vascularization, in contrast to the creamy white belly of the toads).

Statistical analyses

Relationships among CEWL values measured at different body regions (dorsal, ventral, or pelvic patch) were assessed using linear models. Differences in CEWL among body regions and time periods were assessed using general linear models, as were relationships between toad size and CEWL within body regions and time periods. All analyses were performed with Statistica 12.

Results

Overall, the values of CEWL measured at different body regions (dorsal, ventral, or pelvic patch) were strongly correlated (dorsal–ventral: $F_{1,75} = 579.74$, $r^2 = 0.88$, $p < 0.0001$,

dorsal–pelvic patch: $F_{1,75} = 322.94$, $r^2 = 0.81$, $p < 0.0001$, ventral–pelvic patch: $F_{1,75} = 1176.60$, $r^2 = 0.94$, $p < 0.0001$, Fig. 1), and similar results were found when restricting analyses to the different time periods (all $r^2 > 0.30$, all $p < 0.008$).

CEWL differed significantly across body regions ($F_{2,165} = 15.05$, $p < 0.0001$, Fig. 2) and time periods ($F_{2,165} = 119.40$, $p < 0.0001$, Fig. 2), with no significant interactions ($F_{4,165} = 0.32$, $p = 0.86$). Dorsal CEWL was lower than both ventral CEWL and CEWL measured from the pelvic patch ($p < 0.008$), and ventral CEWL was higher than CEWL measured from the pelvic patch ($p = 0.006$). For all body areas, the CEWL was the highest during the non-reproductive period and was the lowest during aquatic breeding (all $p < 0.0001$).

Toad size did not influence CEWL within body regions and periods (all $p > 0.11$), but we found a significant interaction between the time period and SVL for dorsal CEWL

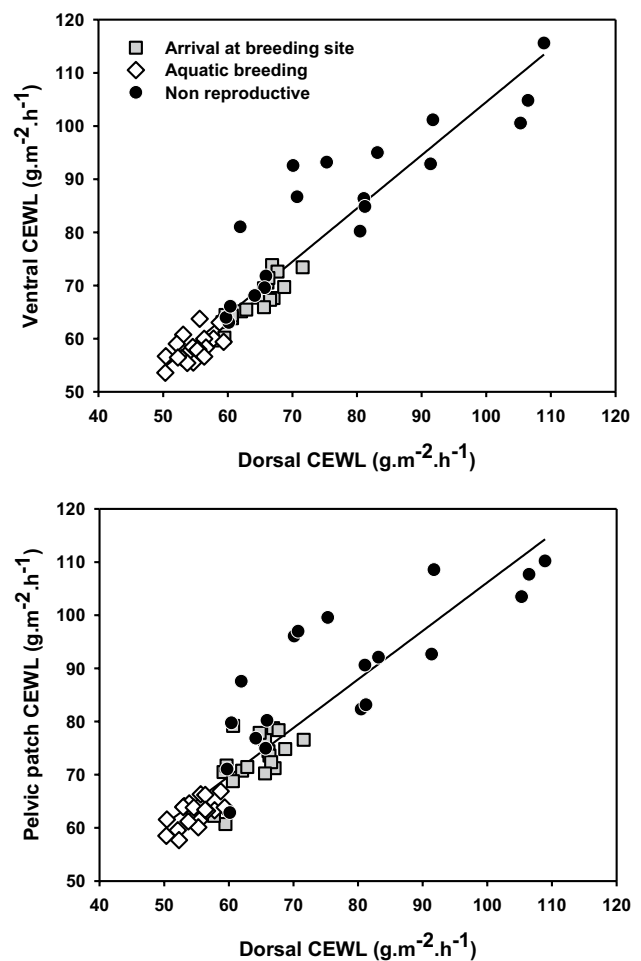


Fig. 1 Relationships between dorsal cutaneous evaporative water loss (CEWL) and ventral CEWL (upper panel) or pelvic patch CEWL (lower panel) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods

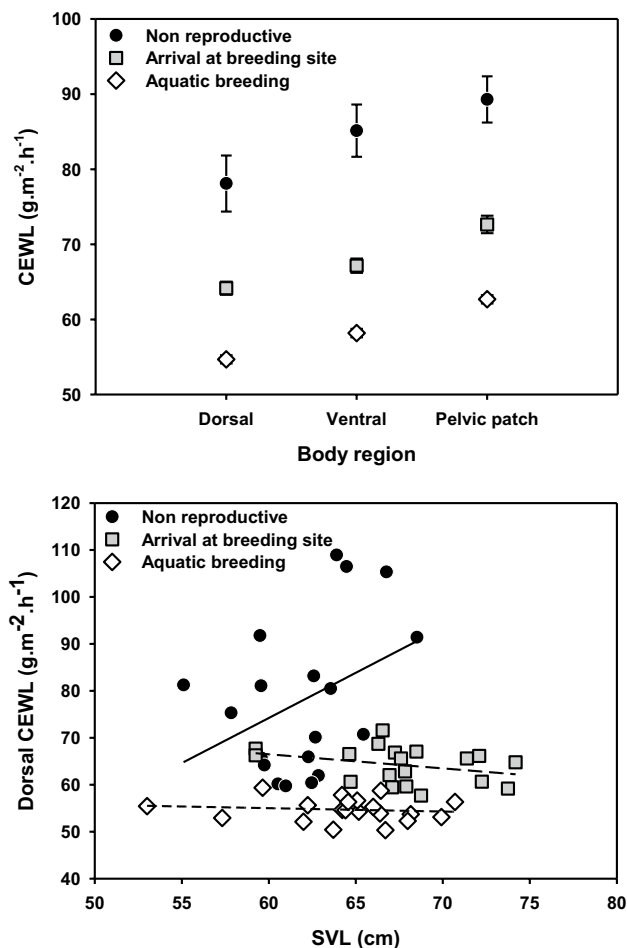


Fig. 2 Cutaneous evaporative water loss (CEWL) measured at three body areas (dorsal, ventral and pelvic patch) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods (upper panel) and relationships between toad size (snout-vent length, SVL) and dorsal cutaneous evaporative water loss (CEWL) during the “non-reproductive” (black circles), “arrival at breeding site” (grey squares) and “aquatic breeding” (white diamonds) sampling periods (lower panel)

($F_{2,52} = 3.71$, $p = 0.03$, Fig. 2) and a similar, but marginal, interaction for ventral CEWL ($F_{2,52} = 2.66$, $p = 0.08$). Interestingly, inter-individual variation of CEWL was almost three times greater during the non-reproductive period (CV ranging from 15.0 to 20.8 depending on body region) than just prior to the arrival at the breeding pond (CV: 5.9–6.9) or during aquatic breeding (CV: 4.0–4.5).

Discussion

We found that the skin permeability of male spined toads decreases significantly prior to breeding. This suggests that the skin shedding that occurs at the onset of breeding (i.e., production of new integument layers with specific properties) may regulate water fluxes in order to alleviate osmotic costs linked to the protracted period of immersion during aquatic breeding (Brischoux and Cheron 2019).

Our results highlighted the regional differences in CEWL, where the dorsal area, which is exposed to more desiccant air, had lower CEWL relative to ventral areas. In addition, the pelvic patch (involved in transcutaneous water intake) had higher CEWL than the two other areas. Yet, the values of CEWL measured at different body areas were highly correlated, suggesting strong regional covariations of this parameter. Interestingly, these regional differences were strongly marked during the non-reproductive period but were also maintained prior to or during aquatic breeding, suggesting that the specific regional properties of skin structure (i.e., keratin, stratum corneum, or lipid layer composition or thickness) were conserved during breeding.

Despite these regional variations, we found that the CEWL of all body areas decreased significantly prior to aquatic breeding and continued to decrease following immersion. Reduced water influxes after shedding may allow individuals to dampen strong deviations in osmotic balance following prolonged immersion in freshwater (Brischoux and Cheron 2019). Importantly, shedding induces a temporary disruption of cutaneous integrity (increased water gain and sodium loss) that lasts a few hours post-sloughing (Jørgensen 1949; Wu et al. 2017). This process lines up particularly well with our field observations, which suggest that skin-sloughing is achieved during terrestrial migration prior to immersion in water. Interestingly, our results highlight that there is a continued decrease in CEWL following immersion in water, suggesting that the affinity for water of the new skin is further reduced during a protracted period of immersion. In addition to possible structural modifications of the skin, a shift to aquatic breeding is likely to involve strong modifications to endocrine regulations (Bentley 1971). In this respect, prolactin is known to influence skin permeability, and notably to induce a reduction in permeability to water (Bentley 1971). Clearly, further studies are required in order to assess how structural skin characteristics (e.g., keratin, stratum corneum, or lipid layer composition or thickness) and endocrine regulation (e.g., prolactin) interact to influence permeability to water during aquatic breeding.

In addition, skin shedding and the subsequent shift to an aquatic lifestyle seem to disrupt the relationship between body size and CEWL. Indeed, during the non-reproductive

period, toad size was positively related to dorsal CEWL and to a lesser extent to ventral CEWL. This result indicates that, during the terrestrial part of their life cycle, smaller individuals — which are presumably more sensitive to dehydration because of a higher surface area to volume ratio — have a lower CEWL which is likely to reduce susceptibility to dehydration. This relationship disappeared during breeding, suggesting that the structural properties of the new skin and/or endocrine regulations interacting to reduce skin permeability are not linked to toad size.

There are some caveats to our investigations notably linked to the fact that we measured CEWL (i.e., water effluxes) rather than water influxes, while aquatic breeding is expected to modify skin permeability to water influxes rather than water effluxes. Yet, both directions of water fluxes are tightly linked and our results are in line with predicted changes. Accordingly, the data on the CEWL of the pelvic patch relative to other body areas suggest that this area, which is dedicated to promote water influxes (cutaneous drinking), also has higher CEWL values and, hence, that the mechanisms responsible for water absorption (e.g., aquaporins, Suzuki et al. 2007) also play a significant role in water loss.

Finally, the breeding strategy of terrestrial toads, and especially the prolonged immersion in water during reproduction, is likely to modify not only water relations but also gas exchanges (Shoemaker et al. 1992, Boutilier et al., 1992, Burggren and Vitalis 2005). For instance, it is likely that submerged breeding toads rely more strongly on cutaneous gas exchange than terrestrial non-reproductive individuals. Future studies should test whether the shift in skin properties that we detected have consequences for the cutaneous gas exchanges of reproductive and non-reproductive toads. In addition, because of the remarkable difference of residence time at breeding ponds between males and females (Brischoux et al. 2018), comparative studies are required to test whether the skin properties of reproductive females undergo similar changes to that of males, in order to assess how divergent reproductive strategies (immersion duration) influence sexual dimorphism in the responses of skin permeability to habitat shifts.

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Author contribution François Brischoux and Olivier Lourdais conceived and designed the study. Marion Cheron and Matthias Renoirt performed data collection. François Brischoux performed data analysis and writing of the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data will be made available upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities under authorizations number 2015–11-20x-01192, 16–392, and R-45GRETA-F1-10.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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