

## Research



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**Author for correspondence:**  
François Brischoux  
e-mail: francois.brischoux@gmail.com

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## Physiology

# Osmotic 'cost' of reproduction in breeding male toads

François Brischoux and Marion Cheron

Centre d'Etudes Biologiques de Chizé, CEBC UMR 7372 CNRS—La Rochelle Université, 79360 Villiers en Bois, France

FB, 0000-0002-5788-1326

Shifts between habitats during reproduction can induce costs that are independent of the reproductive effort and that often apply to both sexes. Such shifts can also illustrate physiological costs complementary to those involving energetic currencies. In this study, we investigated osmotic consequences of reproduction in a context where reproduction induces a shift from terrestrial habitats to freshwater environments. During reproduction, toads migrate to breeding ponds where males remain for several weeks, while females leave shortly after egg-laying. We assessed plasma osmolality of male spined toads during the whole reproductive period (approx. 30 days) in conjunction with markers of individual condition. We found that osmolality decreases during the protracted period of immersion in freshwater during reproduction, presumably through water influx as indicated by body mass changes. Hormonal markers of metabolism and sexual activity were positively correlated with osmolality. Recent research has highlighted hydric 'costs' of reproduction when access to water is limited. Our study adds to this growing field of investigation, yet with an opposite perspective, where water availability linked to reproduction provokes hyperhydration rather than dehydration.

## 1. Introduction

Trade-offs among components of reproductive effort involve both ecological and physiological costs [1]. Among physiological costs, direct costs (i.e. energy and nutrient demands) have attracted considerable attention, probably because they involve the currencies that satisfy demands of the reproductive event itself [2–4]. Yet, physiological costs of reproduction can also be indirect [2–4]. These indirect physiological costs can be further divided into optional compensatory or consequential costs. Compensatory costs involve reduced investment into components of physiology (e.g. immune system or oxidative shielding) to increase resource allocation to reproduction, while consequential costs are inevitable consequences (e.g. hyperthermia or bone loss) of the reproductive event [4].

This latter category is particularly interesting because these consequential costs are largely independent of the reproductive effort (i.e. the number of offspring produced [5]). In this respect, the consequences of shifts between habitats during reproduction are remarkable because they often apply to both sexes, contrarily to other costs that tend to affect mainly the maternal organism [4]. For instance, moving to reproductive grounds can require an energetically demanding migration for both males and females [6–8]. In addition, large-scale movements to reproductive grounds may also increase susceptibility to predation in both males and females [9,10]. Importantly, shifts between habitats during reproduction also illustrate other physiological processes linked to consequential costs of reproduction. This is typically the case when the reproductive habitat differs strongly from the habitat used in the non-reproductive life of organisms. For instance, euryhaline European eels shift from freshwater to marine environments to reproduce [11]. In addition to the other migratory costs mentioned above, such

a shift involves remodelling of the osmoregulatory apparatus that aim to maintain the osmotic balance in the hyperosmotic marine environment [12–14].

Yet, such osmotic consequences of reproduction are likely not restricted to shifts to hyperosmotic habitats. Indeed, reproductive habitat changes can also involve migration to hypotonic environments such as freshwater habitats (e.g. anadromous fish [15]). In this study, we investigated osmotic consequences of reproduction in a context where reproduction induces a shift from terrestrial habitats to freshwater environments. Many amphibian species are biphasic and live most of their life on land but reproduce in freshwater environments where eggs and larvae develop [16]. On land, many amphibians dehydrate rapidly and display specific adaptations to evade dehydration which are exemplified by their ability to absorb water cutaneously and using their urinary bladder as a water reserve [17,18]. Accordingly, skins of aquatic species have lower permeability to water than those of terrestrial species [17]. Yet, this remarkable ability for water absorption through the skin may become a constraint when terrestrial amphibians are shifting to aquatic environments during reproduction. Spined toads (*Bufo spinosus*) are particularly well suited to investigate potential osmotic consequences of reproduction. On land, this species can be found in a wide variety of habitats including relatively xeric environments [19]. Their ability to absorb water cutaneously is well developed and has been previously coined as ‘cutaneous drinking’ [20]. During reproduction, toads migrate to breeding ponds where males remain for several weeks, while females leave shortly after egg-laying [21], suggesting that possible osmotic consequences of reproduction may apply more strongly to males than to females. In this study, we assessed plasma osmolality of male spined toads during the whole reproductive period to test whether a protracted immersion in freshwater dilutes body fluids. We conjointly monitored physiological markers, such as body condition and hormonal levels (corticosterone (CORT) and testosterone (T) which are pertinent markers of metabolism and sexual activity during reproduction in this species [22]), in order to test whether a change in plasma osmolality influences markers of individual condition.

## 2. Methods

### (a) Study species and sampling

The spined toad, *B. spinosus*, is one of the most common anuran species in Western Europe. Reproduction occurs in late winter (February) and toads massively migrate towards aquatic breeding sites [21], usually within a few days [22]. Males remain at the breeding site for several weeks, while females leave shortly after mating and egg-laying [21]. As a consequence, we focused our investigations on male toads specifically.

Sampling took place between early February and mid-March 2019 in a pond situated nearby the Centre d’Etudes Biologiques de Chizé (46°09’ N, 0°24’ W) in France, during five sessions covering a period of 32 days. Importantly, this sampling period spanned the whole breeding period, from the arrival of the first breeding males (first session) to departure of the last breeding males (last session). Sampling was conducted at night using headlights to locate male toads at the breeding site. Upon sighting, each toad (total  $N = 100$ , 19–21 different individuals per sampling sessions) was captured with a net, and a blood sample was collected (approx. 100  $\mu$ l) via cardiocentesis using a 1 ml syringe and a 30-G heparinized needle [22]. Blood samples were collected within 5 min of capture ( $2.73 \pm$

1.07 min, range: 1.13–5.00) in order to quantify baseline hormonal levels [22]. All individuals were weighed ( $\pm 0.1$  g), and their snout–vent length was measured with electronic calipers ( $\pm 0.01$  mm). We quantified a body condition index using residual scores from the linear regression between body size and body mass [22]. All individuals were marked with a small puncture on the foot web with a medical trocar (a different web per sampling session) in order to avoid multiple sampling of the same individuals across sessions (for ethical reasons). This marking procedure is temporary (lasting approx. two months, FB and MC 2018, personnel observation) and less invasive than toe-clipping. Recaptures of marked individuals showed that toads captured during the first session were still present at the reproductive pond during the last sampling session. All individuals were released at their location of capture after measurements and blood collection. A final session performed 39 days after the arrival of the first individuals verified that all toads had left the breeding pond.

### (b) Osmolality and hormonal assays

Blood was centrifuged, and the plasma was separated and stored at  $-20^{\circ}\text{C}$  for one month until assays were conducted. Plasma osmolality ( $\text{mOsmol kg}^{-1}$ ) was measured from 10  $\mu$ l aliquots on a Vapro2 osmometer (Elitech group). Plasma concentrations of T and CORT were determined by radioimmunoassay at the CEBC, as described previously [23]. The minimum detectable levels were 0.30 and 0.28  $\text{ng ml}^{-1}$ , respectively, for T and CORT, and the intra- and inter-assay coefficients of variation were 13.16% and 17.56% and 10.66% and 11.77%, respectively, for T and CORT (that were run twice both within and between assays).

## 3. Results

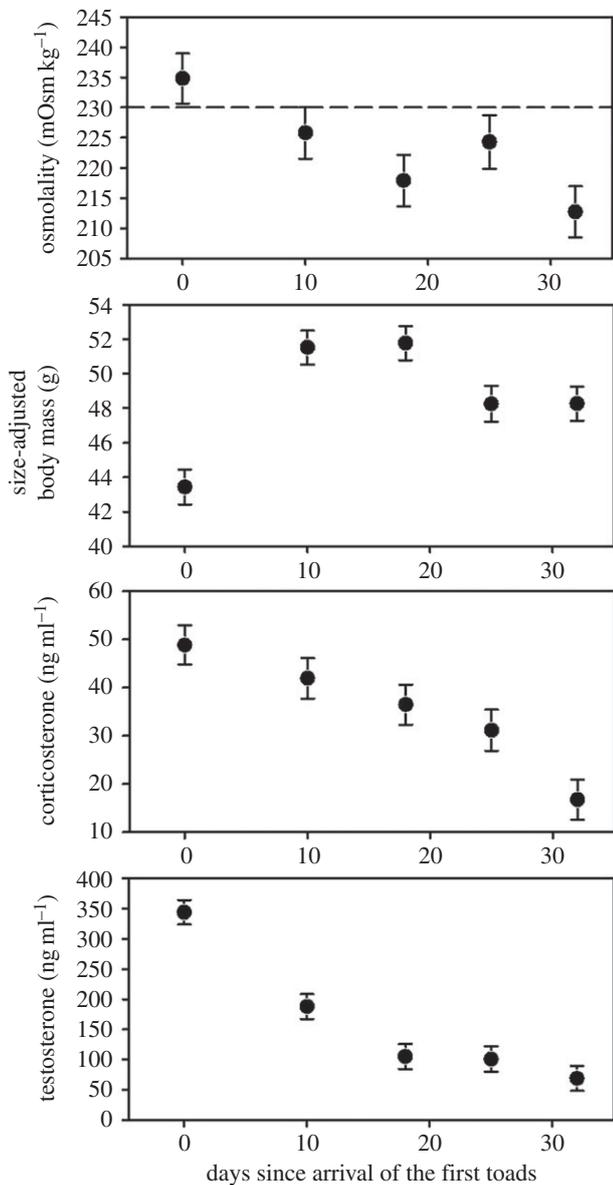
Osmolality was significantly different between sessions ( $F_{4,95} = 3.95$ ,  $p = 0.005$ ; figure 1) and decreased with the time elapsed since the arrival of the first individuals at the breeding site ( $F_{1,98} = 12.35$ ,  $p = 0.0006$ ,  $r^2 = 0.10$ ).

Body condition was also significantly different between sessions ( $F_{4,94} = 11.19$ ,  $p < 0.0001$ ; figure 1) but was only marginally negatively related to the time elapsed since the first session ( $F_{1,98} = 3.31$ ,  $p = 0.07$ ,  $r^2 = 0.02$ ). A closer examination of the data showed that the body condition of toads sampled during the second and third sessions was higher than that of toads sampled during all the other sessions (Fisher’s LSD, all  $p < 0.009$ ; figure 1). Body condition was not correlated to osmolality across field sessions ( $F_{1,98} = 0.006$ ,  $p = 0.94$ ) but was positively correlated to osmolality during the first ( $F_{1,19} = 7.38$ ,  $p = 0.01$ ,  $r^2 = 0.24$ ) and the last session ( $F_{1,18} = 4.76$ ,  $p = 0.04$ ,  $r^2 = 0.16$ ).

CORT levels were different between sessions ( $F_{4,95} = 8.60$ ,  $p < 0.0001$ ; figure 1) and decreased with the time elapsed since the first session ( $F_{1,98} = 32.31$ ,  $p < 0.0001$ ,  $r^2 = 0.24$ ). CORT levels were positively related to osmolality across sampling sessions ( $F_{1,98} = 6.51$ ,  $p = 0.01$ ,  $r^2 = 0.05$ ; figure 2) but not within sessions (all  $p > 0.19$ ). Similarly, T levels were different between sessions ( $F_{4,95} = 26.61$ ,  $p < 0.0001$ ; figure 1) and decreased with the time elapsed since the first session ( $F_{1,98} = 93.32$ ,  $p < 0.0001$ ,  $r^2 = 0.48$ ). T levels were positively related to osmolality across sampling sessions ( $F_{1,98} = 13.62$ ,  $p = 0.0003$ ,  $r^2 = 0.11$ , figure 2) but not within sessions (all  $p > 0.11$ ).

## 4. Discussion

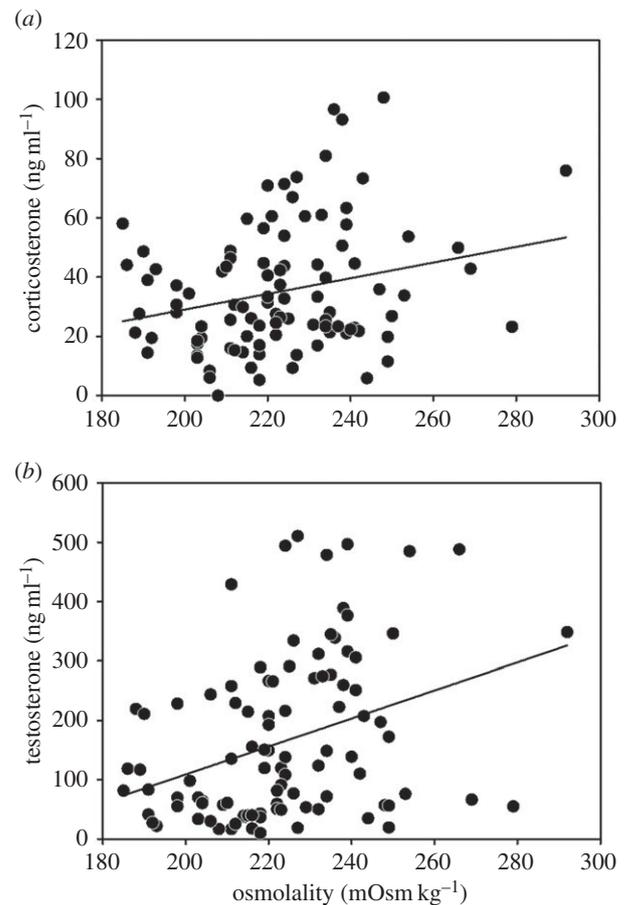
The most salient result of our study shows that male toads cannot remain normosmotic during the protracted period of



**Figure 1.** Plasma osmolality, size-adjusted body mass, corticosterone levels and testosterone levels in relation to the time since arrival of the first toads at the breeding site. Dashed line in the upper panel represents normosmolality in toads (approx.  $230 \text{ mOsm kg}^{-1}$  [17]). Each sampling session represents 19–21 individuals, and data are presented as mean  $\pm$  s.e.

immersion in freshwater during reproduction. Indeed, upon arrival at their reproductive pond, male toads displayed plasma osmolality similar to normosmolality in terrestrial amphibians (e.g. approx.  $230 \text{ mOsmol kg}^{-1}$  [17]). During the following weeks of immersion in water (up to 32 days for our study site), plasma osmolality decreased and attained the smallest values during our last sampling session (mean approx.  $210 \text{ mOsmol kg}^{-1}$ , minimum value  $188 \text{ mOsmol kg}^{-1}$ ). Despite the overall decrease in osmolality we detected, male breeding toads were clearly not osmoconformers as they maintained elevated plasma osmolality (greater than  $200 \text{ mOsmol kg}^{-1}$ ) even after prolonged periods of immersion in freshwater [24]. Future studies are required to elucidate the mechanisms underlying the slow decrease of osmolality we detected [25].

Our results on body condition further suggest that the decrease of osmolality may have occurred through water influx rather than osmolyte efflux [24]. Male toads significantly gained mass between the first and the second session (approx.



**Figure 2.** Corticosterone (a) and testosterone (b) in relation to plasma osmolality across sampling sessions. Each dot represents an individual ( $N = 100$ ).

8 g representing 20% of the initial body mass). The reasons why body mass plateaued during the following days or even decreased during the last sampling sessions may be linked to the reproductive anorexia occurring in breeding male toads. During breeding, male toads forgo feeding in order to maximize time spent in their aquatic breeding habitat and thus mating opportunities [26]. In this case, any further mass gain linked to water influx (as suggested by the overall decrease in osmolality we found) would be obscured by the mass loss linked to the metabolic demands of organismal maintenance, rivalry and mating in the breeding pond. Alternatively, but not exclusively, reproductive anorexia may induce protein catabolism resulting in elevated levels of urea [27]. In turn, excreting this metabolic waste could allow toads to remove excess water [28], thereby resulting in decreased body mass during the last sampling sessions. This hypothesis dovetails relatively well with the slight increase of osmolality visible during the fourth sampling session which also corresponds to body mass loss in sampled toads. Clearly, future studies should investigate the temporal dynamics of urea, as well as other organic osmolites and ions, during the breeding season.

The temporal dynamics of the two hormonal markers we monitored seem to be linked to osmolality (see also [24]). CORT, a marker of metabolism and activity during reproduction in this species [22], continuously decreased during the reproductive period in male toads and was correlated with osmolality. This trend was even stronger for T (a marker of sexual activity [22]) for which levels were almost halved between the first and the second sampling sessions. Similarly to CORT, T was positively correlated with osmolality. Yet, we cannot entirely rule-out that these temporal dynamics of

hormones are independent of osmolality (i.e. naturally decreasing across the reproductive period, but see [29,30]). Future studies are required to assess if osmolality and hormonal levels are functionally linked [24], or if both variables simply depend on time within the breeding season. Importantly, these decreasing hormonal levels could bear consequences for the ability of the earliest toads to compete with latecomers for access to mating opportunities. Future studies should investigate whether the timing of arrival on a breeding site, and thus plasma osmolality and hormonal levels, influences male–male competition for access to females.

Whether such decreased osmolality bears consequences for male toads remains complicated to assess. At the cellular level, hyposmolality is well known for provoking alterations of cell volume (swelling), but this is transitory and counter-balanced by intracellular osmolytes extrusion [31], a likely process in hyposmotic toads [24]. Additionally, short-term hyposmolality has been shown to increase brain volume [32], affect neuronal functioning and increase seizure susceptibility [33–35], none of which seems plausible in our system where male toads can spend several weeks in a chronic, hyposmotic state. Alternatively, hyposmolality may even be beneficial during the protracted reproductive period. In humans, hyposmolality has been shown to affect energetic metabolism and results in protein sparing and increased lipolysis [36]. If a similar process holds true in breeding toads (but see above), chronic hyposmolality may allow toads to preserve their protein structure. This would allow for sustained muscular abilities needed to repel rivals and during mating (amplexus) when males clasp females with

their arms for prolonged periods prior to gamete release [26]. Future studies should investigate whether hyposmolality influences the energetic sources (proteins versus lipids) in breeding toads in order to test this hypothesis.

More generally, recent research has highlighted the importance of water balance during reproduction in tetrapods [37–43]. Yet, all of these studies used systems that focussed on limited access to water to explore the hydric ‘costs’ of reproduction. Our study adds to this growing field of investigation, yet with an opposite perspective, where water availability linked to reproduction provokes hyperhydration rather than dehydration. Clearly, our results militate for such field of research to encompass a larger framework.

**Ethics.** This work was approved by the French authorities (R-45GRETA-F1-10).

**Data accessibility.** Data are available as electronic supplementary materials.

**Authors’ contributions.** F.B. conceived and designed the study, M.C. and F.B. collected the data, F.B. and M.C. conducted the analyses, interpreted the data, designed the figures and wrote the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Competing interests.** We declare we have no competing interests.

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