Influence of temperature, size and confinement on testosterone and corticosterone levels in breeding male spined toads (*Bufo spinosus*)

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ABSTRACT

Winter breeding amphibians opportunistically engage in reproductive behaviour when environmental conditions become favourable. In such explosive breeding systems, males strongly compete for reproductive females. Although most research has been oriented on species in which males use mating calls to attract females, many high-density explosive breeding amphibians do not rely on mating calls. In such systems, larger and stronger males are thought to have significant advantages to access reproductive females. Testosterone (T) is expected to increase with the physical attributes that facilitate access to females, while increased corticosterone levels (CORT) are needed to sustain the energetic requirements associated with mating behaviour. In this study, we investigated how environmental temperature, and body size influence testosterone and corticosterone in males of an explosive winter-breeding species with low investment in mating call, the spined toad (*Bufo spinosus*). We found that both baseline CORT and T were positively correlated with environmental temperature. Interestingly, despite a remarkable range of variation in CORT and T, there was no evidence that either hormone was correlated with body size. Finally, we found no effect of confinement (13 h of captivity) on circulating CORT and T levels. This suggests that breeding male toads may be relatively insensitive to stress in order to maintain reproductive effort during their short mating period. Future studies should investigate both the influence of the phenology of breeding and the social interactions on these hormonal levels.

1. Introduction

Most ectotherms lack the ability to produce significant metabolic heat and their body temperature is driven by ambient thermal conditions (Angilletta, 2009). As a consequence, most of the life-history traits of ectothermic vertebrates are influenced by environmental temperature (i.e., embryonic development, growth rates, reproduction, activity, maintenance costs, and survival rates, Huey and Stevenson, 1979). Despite this direct dependency on environmental temperature, some ectothermic vertebrates can undertake critical activities (e.g., reproduction) during seasons characterized by overall adverse environmental conditions (e.g., low temperature during winter).

This is particularly the case for temperate winter-breeding amphibians (Pechmann and Semlitsch, 1986). Indeed, many temperate amphibians experience prolonged larval development in breeding sites that are often temporary and may disappear during warmer and drier seasons (Griffiths, 1997). The winter-breeding strategy is thought to have emerged to optimise larval survival because early breeding should allow transitory ponds to remain optimal (e.g., with water) during the entire larval development while minimizing predation pressure (Diaz-Paniagua, 1992). Despite these phenological benefits to enhance larval development, winter-breeding can impose significant constraints on breeding adults.

Because environmental and especially thermal winter conditions are often suboptimal for ectothermic vertebrates winter-breeding amphibians mate opportunistically when environmental conditions are favourable (Reading, 1998). Indeed, during winter, such favourable conditions are often temporary and may even represent a single, highly unpredictable and ephemeral optimal temporal window for breeding resulting in explosive breeding (Oseen and Wassersug, 2002). Explosive breeding is characterised by large gathering of adult individuals that compete for mates (Duellman and Trueb, 1986). Generally, males disproportionally outnumber females resulting in strong competition for females (Lomann and Madsen, 2010). Because males actively search for females and strongly compete with other males, larger and stronger males are thought to have significant advantages to access reproductive females (Howard and Kluge, 1985; Leary et al., 2005). However, body size is not always an accurate predictor of mating success in many amphibian species, suggesting that other traits may be important (Friedl and Klump, 2005).

At the physiological level, mating is associated with elevated levels of androgens that promote sexual and territorial behaviours (i.e., testosterone, Woolley et al., 2004; Wikelski et al., 2004; Eikenaar et al., 2012; Leary and Harris, 2013) but also with increased levels of corticosterone that promote the mobilization of energy to sustain mate searching and mating (Romero, 2002; Moore and Jessop, 2003; Landys et al., 2006; Eikenaar et al., 2012). Importantly, the interaction between these two hormones has been suggested to elicit specific mating tactics (Knapp, 2004; Leary et al., 2004, 2008; Leary and Knapp, 2014). For instance, non-calling "satellite" mating tactic is associated with low testosterone levels and high corticosterone levels (Leary and Harris, 2013). So far, most research has been directed at understanding the relationships among hormone levels and mating calls in explosive-breeding amphibians. (Leary et al., 2004, 2008; Leary and Harris, 2013; Leary and Knapp, 2014). However, many high-density explosive breeding amphibians do not rely on intense investment in mating calls (Höglund and Robertson, 1988). In such systems, males invest less energy in sexual signalling, and the proximate determinants of mating success may therefore be different.

In this context, environmental conditions and body size are likely to influence testosterone and corticosterone in amphibians that do not rely to a large extent on mating calls. For instance, testosterone is expected to increase with environmental temperature during breeding (Iela et al., 1980; Mendonça and Licht, 1986; Paniagua et al., 1990) and with the physical attributes (e.g., body size) that should facilitate fighting ability, dominance, and therefore, access to reproductive females (Wikelski et al., 2004; Wingfield et al., 1990). Similarly, corticosterone is expected to increase with environmental temperature during breeding as a result of increased metabolic rates, and thus patterns of energy expenditure (Dupoué et al., 2013; Jessop et al., 2016) and this may help to sustain the energetics needs of the mating period (Romero, 2002; Landys et al., 2006, Moore and Jessop, 2003). In addition, in case of a disturbance (i.e., a stressful event), testosterone and corticosterone are expected to display reverse responses. Corticosterone is expected to increase as a response to the disturbance (stress response; Wingfield et al., 1998; Angelier and Wingfield, 2013; Romero and Wingfield, 2015), and to reach elevated levels that can down-regulate testosterone secretion, and therefore, mating behavior (Sapolsky et al., 2000; Moore et al., 2005; Lynn et al., 2010; Narayan et al., 2012, 2013). Such physiological adjustments are thought to enhance immediate survival at the cost of reproduction (Wingfield and Sapolsky, 2003; Moore and Jessop, 2003).

In this study, our aim was to investigate how environmental conditions and body size influence testosterone and corticosterone in explosive breeders with low investment in mating call. To do so, we focused on reproductive males in a winter-breeding amphibian, the spined toad (*Bufo spinosus*). Spined toads are explosive breeders that reproduce in late winter (February-March) when thermal conditions can be constraining. During a usually short and transitory optimal window (e.g., environmental temperature ~10 °C, Reading, 1998), male toads migrate toward breeding sites were they compete for females (Reading and Clarke, 1983, Davies and Halliday, 1977; Gittins et al., 1980; Hoglund and Robertson, 1987). Because males do not rely on mating calls to attract females in this species (Höglund and Robertson, 1988), physical attributes that should influence male-male competition and access to reproductive females are relatively straightforward to quantify (i.e., body size, mass and condition).

We examined variations of baseline testosterone and corticosterone according to several parameters. First, we expected environmental temperature to positively influence both testosterone and corticosterone levels because of the relationship between thermal conditions and metabolism in ectotherms and/or because reproductive activity is stimulated by increasing temperatures (Angilletta, 2009). Because of this potential positive influence of temperature on both hormones, we also expected a positive correlation between baseline corticosterone and testosterone levels (Moore and Jessop, 2003; Eikenaar et al., 2012).

Second, we expected body size or condition and testosterone to be positively related as larger and stronger males may have significant mating advantages (dominance, fighting abilities, access to female, Davies and Halliday, 1979). In addition, under stressful conditions, mimicked by a relatively long restraint protocol (Hopkins et al., 1999, Graham et al., 2012, Narayan et al., 2013), we expected CORT levels to increase (stress response). Because CORT can negatively influence testosterone levels (Greenberg and Wingfield, 1987), we expected confinement to decrease circulating testosterone levels, and thus a negative correlation between stress-induced corticosterone and stress-induced testosterone levels.

2. Materials and methods

2.1. Study species

The spined toad, *Bufo spinosus*, is one of the most common anuran species in western Europe. Toads emerge from hibernation in late winter (February) and migrate towards aquatic breeding sites (ponds, small lakes, ditches, etc., Davies and Halliday, 1979; Reading and Clarke, 1983). Males may remain at the breeding site for several days if climatic conditions are optimal, while females leave after mating and egg-laying (Davies and Halliday, 1979; Reading and Clarke, 1983).

2.2. Captures, sampling and measurements

Sampling took place at the peak of the breeding season (late February – early March 2016) during 4 consecutive nights in sites nearby the Centre d'Etudes Biologiques de Chizé (46°09′N, 0°24′W) in the Deux-Sèvres department, France. Air temperature was recorded at the beginning and the end of each capture session (LM-8000, Reed, Toronto, Canada). Sampling was conducted at night by patrolling slowly along the breeding sites, using headlights to locate male toads.

Upon sighting, each toad (N = 61) was captured with a net and blood-sampled. Blood (~100 µl) was sampled through cardiocentesis using a 1 ml syringe and a 30 G heparinized needle. The blood samples were collected within five minutes after capture (2.58 ± 1.04, range: 0.86–5.16). There was no effect of handling time on CORT or T levels (CORT: p = 0.87; T: p = 0.64) and these samples were considered to reflect baseline CORT and T levels. All individuals were temporarily marked with a small puncture of the foot web in order to avoid recapture and released at the location of capture.

We used a standard confinement protocol to measure the stress response. Toads were placed individually into transparent boxes and brought back to the laboratory. To monitor stress-induced CORT and T levels, we collected another sample of blood on a subsample of toads (N = 20) after 13.10 ± 0.40 h of captivity when CORT levels have been shown to peak in some toad species (Hopkins et al., 1999, Graham et al., 2012, Narayan et al., 2013). Blood was centrifuged and the plasma was separated and stored at −20 °C until assays were processed. Plasma concentrations of T and CORT were determined by radio-immunoassay at the CEBC, as described previously (Lormée et al., 2003). The minimum detectable levels were 0.38 ng.ml^{-1} and 0.33 ng.ml^{-1} respectively for T and CORT and the intra- and inter-assay coefficients of variation were 10.01% and 13.85% and 6.84% and 10.67% respectively for T and CORT (that were run twice both within and between assays).

All individuals were weighed (electronic balance: ± 0.1 g) and their snout-vent length was measured with an electronic calliper (± 0.01 mm) following the initial blood sampling. We quantified a body condition index using residual scores from the linear regression between body size and body mass.

All procedures were approved by French regulations (approval number R-45GRETA-F1-10 to FB).
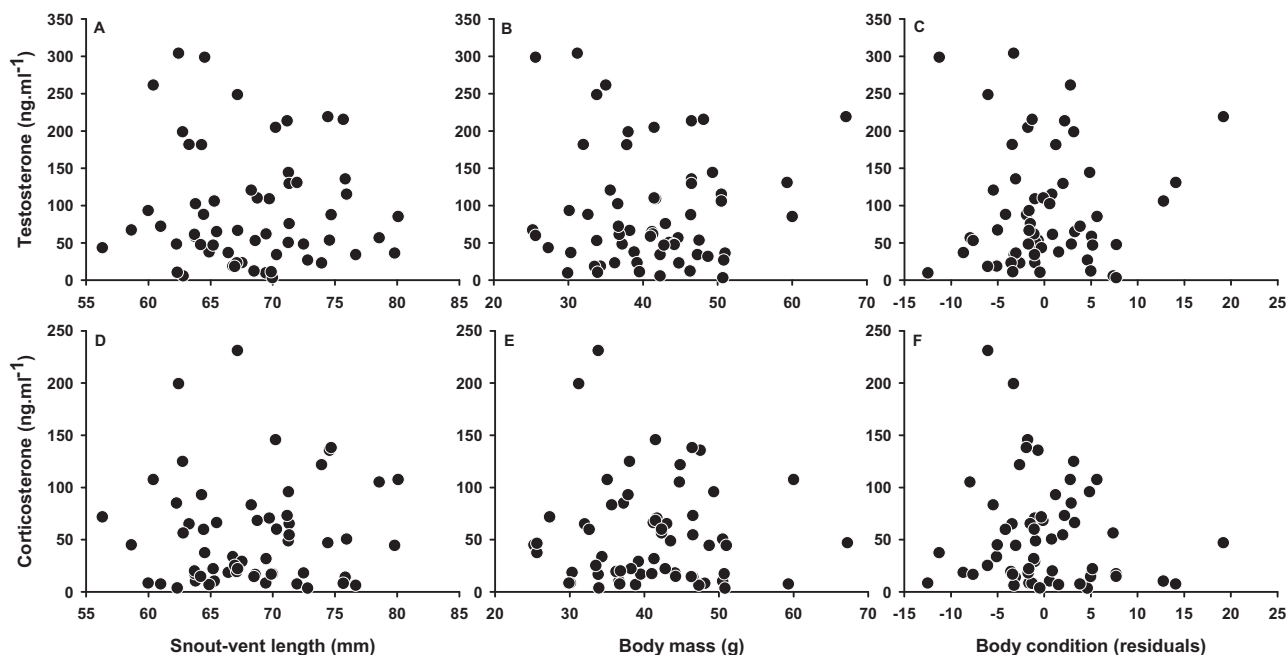
Fig. 1. Relationships between testosterone (upper panels) or corticosterone (lower panels) and toad morphological variables (snout-vent length A–D, body mass B–E and body condition C–F). All relationships were non-significant (all $r^2 < 0.005$, all $p > 0.32$).

2.3. Statistical analyses

Relationships between morphological variables (size, mass and condition) and CORT or T levels were assessed using linear regressions; as were relationships between hormones.

Because many toads were sampled for a given temperature, relationships between air temperature and T or CORT levels were assessed with Spearman rank correlations.

Hormonal responses to confinement were analysed with paired t-tests, and interactions between hormonal responses to confinement and morphological variables (SVL, BM and BCI) were analysed with repeated measures General Linear Models (GLM).

All statistical analyses were performed with Statistica 12.

3. Results

3.1. Body size, mass and condition

There was no relationship between T or CORT levels and body size, mass or condition (all $r^2 < 0.005$, all $p > 0.32$, Fig. 1). Similarly, there was no relationship between temperature and body size, mass or condition (all $r^2 < 0.01$, all $p > 0.45$)

3.2. Temperature

There was a positive relationship between T or CORT levels and air temperature (Spearman rank correlation, $r_s = 0.38$, $p < 0.05$ and $r_s = 0.51$, $p < 0.05$ for T and CORT respectively, Fig. 2). Overall, baseline T and CORT were positively correlated ($F_{1,59} = 21.33$, $r^2 = 0.26$, $p < 0.0001$, Fig. 3).

3.3. Response to confinement

We did not detect an influence of captivity on T or CORT levels (paired *t*-test, $t = 1.23$, $df = 18$, $p = 0.23$ and $t = 0.10$, $df = 18$, $p = 0.92$ respectively for T and CORT, Fig. 4). Hormonal response to confinement was not related to morphological variables (repeated measures GLM, effect of time: all $p > 0.23$, interactions between morphological variables (SVL, BM or BCI) and time: all $p > 0.17$).
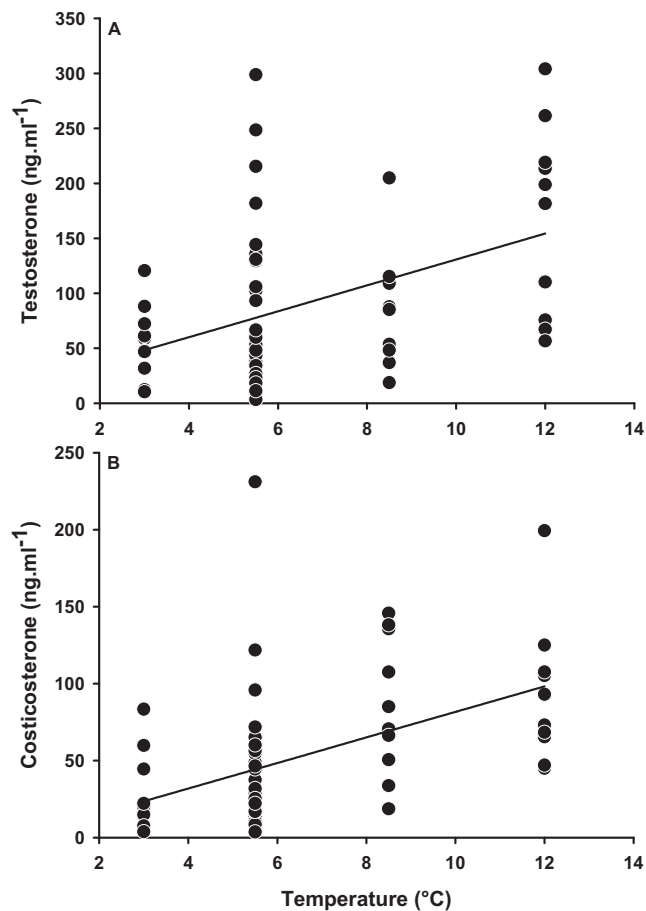

Fig. 2. Relationships between air temperature and testosterone (A) and corticosterone (B) assessed with Spearman rank correlations ($r_s = 0.38$, $p < 0.05$ and $r_s = 0.51$, $p < 0.05$ for T and CORT respectively).
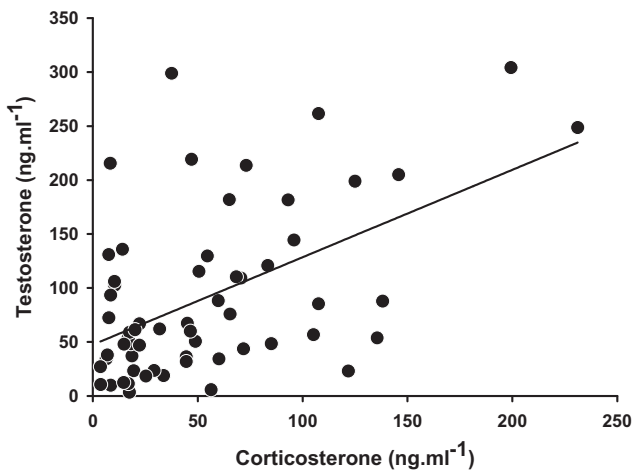
Fig. 3. Relationship between baseline corticosterone and testosterone (linear regression, $F_{1,59} = 21.33$, $r^2 = 0.26$, $p < 0.0001$).
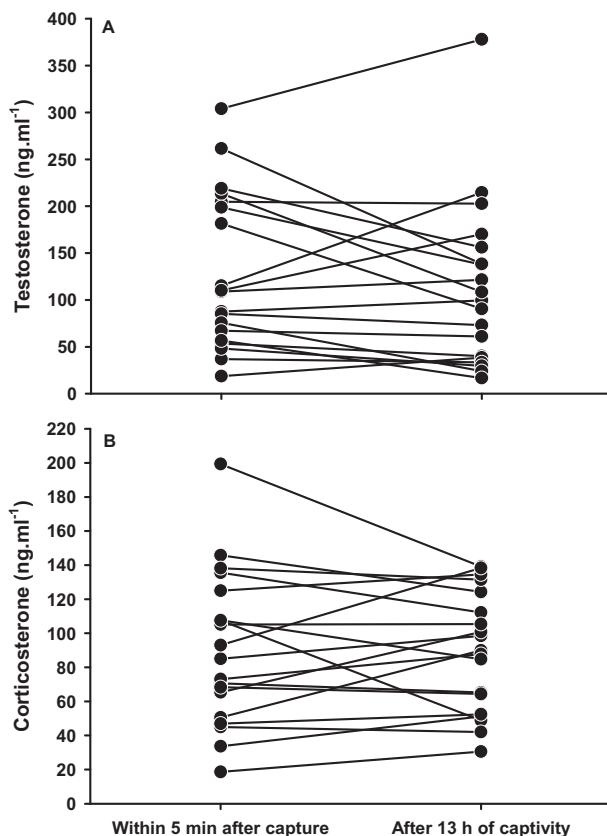

Fig. 4. Effect of confinement (13 h of captivity) on testosterone (A) and corticosterone (B). Each pair of connected dots corresponds to one individual and were analyzed with paired t-tests ($t = 1.23$, $df = 18$, $p = 0.23$ and $t = 0.10$, $df = 18$, $p = 0.92$ respectively for T and CORT).

Stress-induced T and CORT were not correlated ($F_{1,18} = 2.64$, $r^2 = 0.12$, $p = 0.12$).

4. Discussion

Overall, we found that both baseline CORT and T were positively correlated with temperature, suggesting that an increased temperature activates metabolism and hormone secretion. As a consequence, we also found that baseline corticosterone and testosterone were positively correlated. Interestingly, there was no evidence of any effect of body

size, mass and condition on these hormones. Finally, and surprisingly, we found no effect of confinement (13 h of captivity) on circulating corticosterone and testosterone levels.

Although we did not find a significant effect of body size, mass and condition on baseline T and CORT, we reported a remarkably large inter-individual variation in these hormonal levels. Indeed, baseline plasma testosterone ranged from 3.26 to 304.05 ng.ml^{-1}, and baseline plasma corticosterone ranged from 3.60 to 231.15 ng.ml^{-1}. This suggests that T and CORT secretion are under the control of other biotic and abiotic variables. First, social interactions that occurred previously to capture may affect circulating levels of T and CORT (Woolley et al., 2004; Golinski et al., 2014). For example, previous studies have shown that T levels are affected by male-male interactions, and by mating success (Burmeister and Wilczynski, 2001, 2005; Hirschenhauser and Oliveira, 2006). Similarly, specific mating tactics (i.e., dominant versus satellite males) are associated with different CORT profiles (Leary and Harris, 2013). Second, the timing of arrival on the breeding sites may also affect the dynamics of T and CORT regulation through either an effect on body reserves (Moore and Jessop, 2003), or social stimulation by the presence of congeners (rivals and/or mates, Burmeister and Wilczynski, 2001, 2005; Hirschenhauser and Oliveira, 2006). Finally, temperature is probably a strong driver of these hormonal concentrations (see below, Dupoué et al., 2013). Interestingly, another study found that T and CORT levels can be positively correlated with body size or body mass, but under specific circumstances only (Orton et al., 2014). This suggests that the influence of physical attributes on hormonal levels may depend on the environmental context.

We found a positive relationship between environmental temperatures and both CORT and T. Such relationship is likely to be mediated by the thermal dependence of metabolism in this ectothermic species (Angilletta, 2009). Indeed, increased patterns of energy expenditure are tightly linked with CORT (DuRant et al., 2008; Wack et al., 2012; Jessop et al., 2014), and this is likely to explain the positive relationship we found between temperature and baseline corticosterone levels (see also Dupoué et al., 2013; Jessop et al., 2016). Regarding T, reproductive behaviors and physiology are also probably mediated by environmental temperature through an effect on metabolism (Paniagua et al., 1990) and this is likely to explain the positive relationship between T and temperature. Accordingly, environmental temperature appears as the main driver of both CORT and T as compared to other variables (condition, size) during the mating period. Because of this thermal dependence of both T and CORT, we found a positive relationship between these two hormones (see also Moore and Jessop, 2003; Orton et al., 2014; Joshi et al., 2018). Such relationship could be mediated either through a positive effect of CORT on metabolism, and therefore T (sexual behavior and physiology) or through a by-product of the thermal dependence of both hormones (i.e., without any causal relationship between T and CORT). Importantly and contrary to one of our prediction, this result suggests that there is no negative effect of CORT on T during the mating period in male common toads.

Despite the well-known effect of stress on both reproductive and stress hormones (Hopkins et al., 1999; Narayan et al., 2013; Narayan and Hero, 2014), we did not find any evidence of an influence of confinement (13 h of captivity) on both T and CORT (see also Narayan and Gramapurohit, 2016). This suggests that male common toads may be hormonally insensitive to stress during the mating period (Wingfield et al., 1990). Importantly and accordingly, previous studies have found that hormonal stress responses can be dampened when the fitness benefits of maintaining reproductive behavior outweigh its costs (Wingfield and Sapolsky, 2003), as it is the case in species with a very short reproductive time window (Wingfield et al., 1995; Moore and Jessop, 2003; Krause et al., 2015). Such down-regulation of the adrenocortical stress response could explain why our relatively long restraint protocol had no influence on corticosterone levels. For instance, acute stressors do not result in an increased corticosterone secretion in laying turtles, while such an increase is obvious in non-breeding ones

(Jessop, 2001). Similarly, T levels may be unaffected by confinement because of an insensitivity of the HPG axis to chronic stress in males during the breeding period. Supporting this interpretation, Bliley and Woodley (2012) found that repeated acute stressors did not affect T levels or sexual behaviors in another amphibian species.

Alternatively, we cannot totally rule out that our restrain protocol did not elicit a stress response. For instance, hormonal levels could rise during a shorter time window and subsequently decrease and return to normal either through a hormonal feedback or a habituation to stress (Dickens and Romero, 2013). Yet, this is quite unlikely because toads were transferred into a totally new environment (plastic boxes) with no congeners, no mates and no food during the stress protocol. In addition, other studies have found that such a protocol elicit hormonal changes in similar species (Hopkins et al., 1999, Graham et al., 2012, Narayan et al., 2013). Future studies should usefully test for these hypotheses by comparing hormonal responses to confinement during breeding and non-breeding seasons.

To conclude, our results show that CORT and T levels in breeding male Spined toads are primarily driven by environmental temperature and that other variables (size, condition, stress) may have little or no influence on CORT and T levels. Yet, environmental temperature alone can hardly explain the remarkable range of variation we found in both CORT and T. We emphasize that future studies should investigate the individual variation in hormonal levels throughout the breeding season to better understand if the timing of arrival on the breeding site may affect these endocrine levels. In addition, experimental studies should also investigate the effect of social interactions (e.g. both rivals and mates) on these hormonal levels.

Acknowledgments

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