



## Effect of water deprivation on baseline and stress-induced corticosterone levels in the Children's python (*Antaresia childreni*)



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### ABSTRACT

Corticosterone (CORT) secretion is influenced by endogenous factors (e.g., physiological status) and environmental stressors (e.g., ambient temperature). Heretofore, the impact of water deprivation on CORT plasma levels has not been thoroughly investigated. However, both baseline CORT and stress-induced CORT are expected to respond to water deprivation not only because of hydric stress per se, but also because CORT is an important mineralocorticoid in vertebrates. We assessed the effects of water deprivation on baseline CORT and stress-induced CORT, in Children's pythons (*Antaresia childreni*), a species that experiences seasonal droughts in natural conditions. We imposed a 52-day water deprivation on a group of unfed Children's pythons (i.e., water-deprived treatment) and provided water ad libitum to another group (i.e., control treatment). We examined body mass variations throughout the experiment, and baseline CORT and stress-induced CORT at the end of the treatments. Relative body mass loss averaged ~10% in pythons without water, a value 2 to 4 times higher compared to control snakes. Following re-exposition to water, pythons from the water-deprived treatment drank readily and abundantly and attained a body mass similar to pythons from the control treatment. Together, these results suggest a substantial dehydration as a consequence of water deprivation. Interestingly, stress-induced but not baseline CORT level was significantly higher in water-deprived snakes, suggesting that baseline CORT might not respond to this degree of dehydration. Therefore, possible mineralocorticoid role of CORT needs to be clarified in snakes. Because dehydration usually induces adjustments (reduced movements, lowered body temperature) to limit water loss, and decreases locomotor performances, elevated stress-induced CORT in water-deprived snakes might therefore compensate for altered locomotor performances. Future studies should test this hypothesis.

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### 1. Introduction

Organisms cope with environmental variations using behavioural and physiological adjustments that are largely mediated by endocrine regulation (McEwen and Wingfield, 2003; Romero et al., 2009; McEwen and Wingfield, 2010). For instance, when an environmental change occurs, the Hypothalamic–Pituitary–Adrenal (HPA) axis is activated which results in a rapid and important release of corticosterone (CORT) in the blood (Wingfield et al., 1998; Wingfield, 2003; Wingfield et al., 2011; Wingfield, 2012, 2013). This increase in circulating CORT levels can activate and/or inhibit specific physiological and behavioural responses to allow the organisms to cope with potentially challenging situations (Sapolsky et al., 2000; Romero, 2004; Landys et al., 2006). Diverse environmental factors, such as variations of temperatures (Tyrrell and Cree, 1998; Cree et al., 2003; Romero and Wikelski, 2006; Dupoué et al., 2013), food availability (Woodley et al.,

2003; Jenni-Eiermann et al., 2008), predation pressure (Engh et al., 2006; Remage-Healey et al., 2006; Berger et al., 2007), or anthropogenic disturbances (Wikelski et al., 2001; Romero and Wikelski, 2002; Partecke et al., 2006; Crino et al., 2011), have all been shown to affect CORT levels in vertebrates (Sapolsky et al., 2000; Romero, 2002; Landys et al., 2006).

Surprisingly, the potential effect of one of the most essential resources – water – has not been investigated in this framework (Schmidt-Nielsen, 1997). Further, water acquisition and maintenance of water balance remain overlooked in ecological field studies (see Bonnet and Brischoux, 2008 for a review). Yet, deviation of the osmotic balance, especially dehydration, is a typical example of a particularly challenging situation for many organisms. Dehydration triggers behavioural and physiological adjustments to limit water loss and/or to restore water balance. Typical examples involve major changes in activity (Pough et al., 1983; Crowley, 1987; Lorenzon et al., 1999; Bonnet and Brischoux, 2008; Lillywhite et al., 2008a; Davis and DeNardo, 2009; Hetem et al., 2010), shifts in thermoregulatory strategies (Crowley, 1987; Bradshaw, 1997; Lorenzon et al., 1999; Angilletta et al., 2010; Köhler et al., 2011), or reduced metabolic rates (Kennett and Christian, 1994; Tieleman et al., 2002; Muir et al., 2007). Prolonged

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dehydration might lead to severe physiological alteration and ultimately to death (Whitehead et al., 1996). Thus, acute or long-lasting dehydration can be considered as life-threatening. In addition, CORT is presumed to act as a mineralocorticoid, and thus to play a role in the maintenance of osmotic balance (Duggan and Lofts, 1978; Dauphin-Villemant and Xavier, 1986; Bentley, 2002; McCormick and Bradshaw, 2006). Taken together, these elements suggest that water deprivation can significantly affect secretion of CORT, not only as a result of a stressful physiological state (i.e., dehydration) but also because CORT can play a role in osmotic balance.

Snakes are well suited to investigate such questions. First, limited dispersal ability regularly exposes snakes to local conditions (Aragon et al., 2010). Therefore, they would be liable to face difficulties to find fresh water during drought periods. Second, snakes display marked behavioural and physiological adjustments to limit water loss when dehydrated (Silveira et al., 1992; Ladyman and Bradshaw, 2003; Ladyman et al., 2006). Third, several snakes species inhabit (at least temporarily) environments where fresh water acquisition represents a major challenge, such as wet-dry tropics, deserts or oceanic areas. Finally, snakes are particularly tolerant to strong deviation of their water balance, and thus allow creating marked contrasts under experimental conditions.

In this study, we investigated the influence of water deprivation on both baseline CORT and stress-induced CORT in a snake species from Australian wet-dry tropics, the Children's python (*Antaresia childreni*). We experimentally created a hydric contrast between two groups of snakes (control versus water-deprived) to examine the consequences of water deprivation on CORT secretion. Specifically, we expect baseline CORT to be higher in water-deprived snakes, both as a result of hydric stress and the putative mineralocorticoid role of CORT (Duggan and Lofts, 1978; Dauphin-Villemant and Xavier, 1986; Bentley, 2002; McCormick and Bradshaw, 2006). In response to an additional stressor (capture and stress protocol), we expected both groups to show a similar stress response. However, the maximal stress-induced CORT level should be higher in water-deprived snakes, as a result of an already elevated baseline level (Dupoué et al., 2013).

## 2. Materials and methods

### 2.1. Study species and husbandry

Children's pythons (*A. childreni*) are medium-sized (up to 1.2 m snout vent length, 600 g body mass) constricting snakes that occur in Australian wet-dry tropics, areas characterised by a seasonal alternation of wet (rainfall > 200 mm per month from December to March) and dry (<5 mm per month from June to August) periods (Wilson and Swan, 2003). During dry seasons, pythons can face significant water deprivation. Snakes used in this study (15 females and 12 males) were part of a captive colony of Children's pythons maintained in the Centre d'Etudes Biologiques de Chizé, France. Snakes were housed individually in plastic cages (35.5 × 63 × 15 cm) containing a shelter and displaying a thermal gradient (25 to 35 °C) to allow free thermoregulation. We kept experimental animals fasted one month before experiments and checked that females were in a non-reproductive state to

avoid any influence of digestive activity or reproductive state on CORT levels.

### 2.2. Experimental protocol

Pythons were assigned randomly within sex to the control or water-deprived hydric treatment (N = 14 and 13 respectively). In each treatment, we distributed males and females evenly (N = 6 males in each treatment, N = 8 and 7 females for control and water-deprived group respectively; Table 1). Individuals from the water-deprived group were exposed to a 52-day period of water deprivation while snakes from the control group were provided water ad libitum.

Snakes from both experimental groups were similar in terms of body mass (BM) (ANOVA with treatment as the predictor and BM as the dependent variable, males:  $F_{1,10} = 0.2$ ,  $P = 0.694$ ; females:  $F_{1,13} = 0.2$ ,  $P = 0.699$ ) and snout-vent length (SVL) (same design than above but with the SVL as the dependent variable, males:  $F_{1,10} = 0.2$ ,  $P = 0.666$ ; females:  $F_{1,13} = 0.4$ ,  $P = 0.519$ ).

Although not as precise as total body water or plasma osmolality quantifications, BM is an integrative variable to assess dehydration in squamate reptiles (see DeNardo et al., 2004; Lillywhite et al., 2008a,b, 2012). We recorded BM weekly (eight measures over the experiment). These data were used to compare the pattern of BM loss through time (henceforth "Time") between our two experimental treatments. At the end of treatment, water-deprived snakes were provided water ad libitum immediately after the blood sampling (see below). We subsequently recorded snake BM 4 and 6 days after re-exposition to water. These additional measures were used to calculate the amount of water drunk in water-deprived snakes, as well as the corresponding proportion of their BM deficit (%; see Lillywhite et al., 2008a).

### 2.3. Blood sampling and CORT assay

Pythons were randomly selected in order to mix sexes and experimental treatments during the blood sampling sessions. All blood samples were drawn by cardiocentesis using a 1 mL syringe and a 27-gauge needle that was heparinized. A first blood sample (150 µL) was collected within approximately 3 min post capture (mean ± SE, 2.12 ± 0.13 min, range: 1.02–3.67 min) in order to measure baseline CORT level (Romero and Wikelski, 2001). We used a standardized capture/restraint stress protocol, slightly modified from Wingfield (1994) (Dupoué et al., 2013): snakes were placed in a transparent plastic box (35 × 25 × 12.5 cm) without any refuges at room temperature (mean ± SE, 23.2 ± 0.1 °C). A second sample (150 µL) was collected 1 h later (mean ± SE, 61.88 ± 0.24 min, range 60.80–66.25 min). Preliminary investigations on this species suggested that this protocol allowed initiating the CORT stress response (Dupoué et al., 2013). Because body temperature can affect baseline CORT in reptile (Tyrrell and Cree, 1998; Cree et al., 2003; Dupoué et al., 2013), we recorded snake skin surface temperature with an infrared thermometer (Fluke 572 CF, Germany) before each blood sampling. Skin surface temperature should be closely related to core body temperature in this medium sized species (Dupoué et al., 2013) and the close focus of the thermometer (1 cm Ø) allows precise measurements.

**Table 1**  
BM variation over the experiments for the two experimental treatments (control versus water-deprived). Initial BM and final BM correspond to the BM at the beginning and the end of the experiment. Absolute (g) and the relative (%) mass loss correspond to the difference between initial and final BM. Absolute (g) and relative (%) amount of water drunk was calculated in water-deprived snakes as the difference of BM before and after re-hydration. Deficit (%) corresponds to the relative proportion of water drunk compared to mass loss. Table shows means (± SE). See text for details.

| Sex | Treatment          | Initial BM (g) | Mass loss  |            | Water drunk |           |            | Final BM (g) |
|-----|--------------------|----------------|------------|------------|-------------|-----------|------------|--------------|
|     |                    |                | g          | %          | g           | %         | Deficit %  |              |
| F   | Control (8)        | 484.8 ± 22.3   | 12.6 ± 2.4 | 2.6 ± 0.7  | –           | –         | –          | 473.4 ± 21.4 |
| F   | Water-deprived (7) | 495.5 ± 13.1   | 49.9 ± 4.6 | 10.1 ± 1.4 | 36.8 ± 3.4  | 8.4 ± 1.0 | 76.3 ± 8.7 | 482.3 ± 10.7 |
| M   | Control (6)        | 413.5 ± 27.8   | 23.9 ± 2.6 | 5.9 ± 0.5  | –           | –         | –          | 387.7 ± 26.3 |
| M   | Water-deprived (6) | 397.9 ± 26.7   | 49.1 ± 3.6 | 12.7 ± 1.0 | 26.5 ± 4.1  | 8.2 ± 1.7 | 55.1 ± 9.8 | 375.2 ± 24.3 |

Immediately after sampling, the blood was centrifuged 3 min at 3000 g. The plasma was collected and stored at  $-28^{\circ}\text{C}$ . Plasma CORT assays were performed at the CEBC by following a well-established radioimmunoassay protocol (see Lormée et al., 2003 for details). Samples were run in two assays (intra-assay variation: 7.07%, inter-assay variation: 9.99%). All blood samples were collected between 8:00 and 18:00 h and we took into account this factor in our analyses since CORT can be subjected to daily variations in reptiles (Dauphin-Villemant and Xavier, 1987; Cree et al., 2003).

Preliminary examinations of the data revealed that two individuals (water-deprived males) displayed extremely high baseline CORT (41.05 and 35.56  $\text{ng}\cdot\text{mL}^{-1}$  respectively). Baseline CORT of these individuals was  $>390\%$  higher than those of the other experimental individuals, and even  $>240\%$  higher than stress-induced levels of the other experimental individuals. In addition, these two snakes displayed stress-induced values lower than their baseline levels. Importantly, none of them showed any other behavioural or physiological peculiarity (e.g., BM loss was similar to other individuals). As a consequence, we removed these individuals from our CORT analyses, although in practice, including them in our analyses did not change statistical effects.

#### 2.4. Statistical analyses

All analyses were performed using R software (R Development Core Team, version 2.13.1). We used linear models (lm, package stat) and linear mixed model (lme, package nlme) when analyses required us to take snake identity into account as a random factor. We checked that all our models respected normality (Shapiro–Wilk test, all  $P > 0.05$ ).

We assessed the effect of the experiment on the pattern of BM loss with linear mixed models with BM as the dependent variable, initial BM as linear covariate, and experimental treatment (control vs. water-deprived), time, sex and interaction between these three parameters as explanatory factors. Individual identity was set as a random factor. BM was compared between experimental treatments for each sex at each weighing time by using post hoc tests on the interaction (Treatment  $\times$  Time  $\times$  Sex) with no adjustments on P-value (package lsmeans). Adjusted values of BM presented in figures were calculated using lsmeans function (package lsmeans).

We analysed the effect of the experiment on baseline CORT levels with linear models with baseline CORT levels as the dependent variable, body temperature and time of day as linear covariates, and experimental treatment, sex and the interaction between treatment and sex as explanatory factors. Stress-induced CORT was analysed as the same design

as above but with baseline CORT added as a linear covariate. The difference between stress-induced CORT and baseline CORT level was assessed using a linear mixed model with CORT level as dependent variable, and sample (baseline or stress-induced), experimental treatment and the interaction between sample and treatment as explanatory factors. Individual identity was set as a random factor. CORT levels were compared between samples for each treatment using post hoc tests on the interaction (Sample  $\times$  Treatment) with no adjustments on P-value.

### 3. Results

#### 3.1. BM variation

A significant mass loss was observed over the experiment (Fig. 1, Tables 1 & 2), and BM loss was dependent on the initial BM (Table 2) and experimental treatment (Fig. 1, Table 2). Water-deprived snakes lost mass at a higher rate than control snakes (post hoc,  $P < 0.05$ ; Table 1, Fig. 1). We found a significant effect of the sex on BM variations (Fig. 1, Tables 1 & 2) with males losing a higher proportion of BM than females (Fig. 1, Tables 1 & 2).

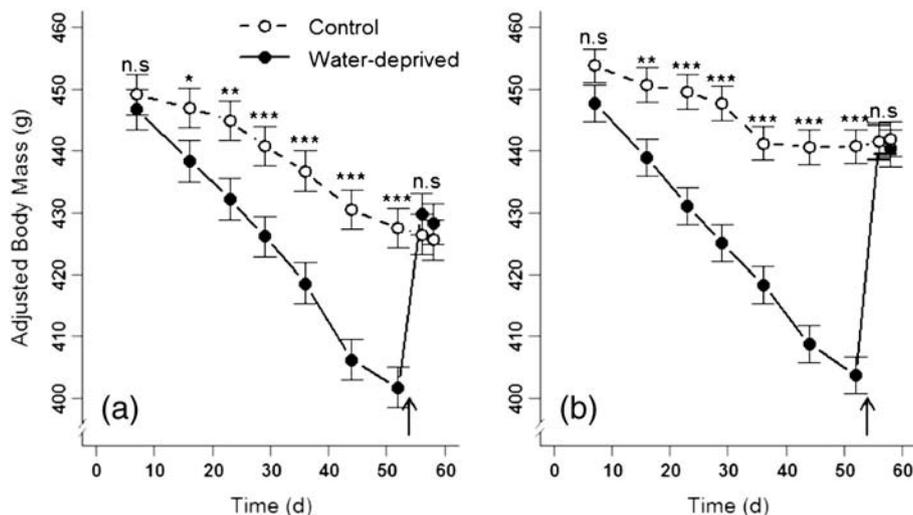
During the re-hydration phase, water-deprived snakes were observed to drink readily and abundantly despite the disturbance associated with blood sampling and capture/restraint stress protocol. These individuals rapidly gained mass and eventually attained BM similar to control snakes (post hoc, males:  $P = 0.454$ ; females:  $P = 0.987$ ; Table 1, Fig. 1). Overall, the amount of water drunk accounted for 69.0% of the BM deficit accumulated during the water deprivation (SE: 6.9%, range: 31.9–106.3%; Table 1).

#### 3.2. CORT levels

Baseline CORT level was influenced solely by body temperature and time of the day (Table 3). We did not detect any effect of the sex, treatment or their interaction on baseline CORT level (Table 3).

Stress-induced CORT level was significantly influenced by baseline level and treatment (Table 3, Fig. 2). Indeed, snakes from the water-deprived treatment showed a stress-induced CORT level significantly higher than control individuals (Table 3, Fig. 2). We did not detect any influence of sex, body temperature, time of the day or interaction between sex and treatment on stress-induced CORT level (Table 3).

Stress-induced CORT levels were significantly higher than baseline CORT levels in pythons from both treatment (post hoc, control:  $P = 0.022$ ; water-deprived:  $P < 0.001$ ; Fig. 2).



**Fig. 1.** Effect of the experimental treatment on BM (mean  $\pm$  SE, control snakes: open circles, dashed line and water-deprived snakes: filled circles, full lines) in a) males and b) females. BM values were adjusted to initial BM. Arrows indicate the end of the dehydration period, and subsequent access to water ad libitum for water-deprived snakes. Significant effects of experimental treatment are symbolized: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  and n.s. (non-significant).

**Table 2**

Statistical model used to examine BM loss in control and water-deprived snakes. Individual identity was treated as random factor and initial BM was treated as linear covariate. See text for details.

| Factor                 | df | F         | P       |
|------------------------|----|-----------|---------|
| Intercept              | 1  | 143,324.6 | <0.0001 |
| Initial BM             | 1  | 3595.8    | <0.0001 |
| Time                   | 8  | 102.7     | <0.0001 |
| Treatment              | 1  | 39.5      | <0.0001 |
| Sex                    | 1  | 4.7       | 0.042   |
| Time × Treatment       | 8  | 39.3      | <0.0001 |
| Time × Sex             | 8  | 6.5       | <0.0001 |
| Treatment × Sex        | 1  | 1.5       | 0.233   |
| Time × Treatment × Sex | 8  | 0.5       | 0.845   |

#### 4. Discussion

In this study, we investigated the effects of water deprivation on baseline and stress-induced CORT levels in the Children's python (*A. childreni*). Our experiment induced mass loss in both treatments, but this loss was significantly higher in water-deprived snakes (Fig. 1). Physiological (i.e., mass gain) and behavioural (i.e., drinking behaviour) patterns during re-hydration strongly suggest that most of the mass deficit was due to water loss (Fig. 1, Table 1), as suggested in other snake species (Lillywhite et al., 2008a,b, 2012). Overall, water-deprived snakes lost >10% of their initial BM, and although we did not measure total water volume or plasma osmolality, it likely resulted in a substantial dehydration (DeNardo et al., 2004). Interestingly, the dehydration caused by our experimental treatment influenced stress-induced but not baseline CORT levels.

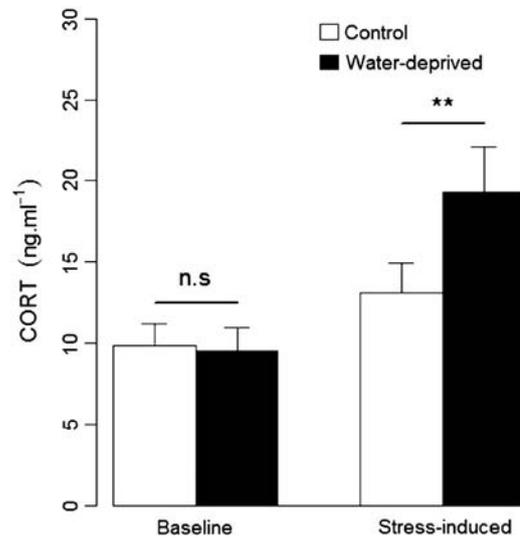
Regardless of treatments, males tended to lose mass more rapidly than females (Table 1, Fig. 1). This pattern likely reflects sexual dimorphism in body plans and allocation strategies (Bonnet et al., 1998; Lourdais et al., 2006; Stahlschmidt et al., 2011). Indeed, *A. childreni* is a typical capital breeder (Lourdais et al., 2006). Prior to reproduction, females accumulate large lipid body reserves that allow sustaining the whole reproductive process (from vitellogenesis to egg brooding) during which these females cease feeding (Lourdais et al., 2013). In contrast, males are more muscular, and accumulate less fat stores (Lourdais et al., 2006). Therefore, during a fasting episode, males should rely on protein (muscle) catabolism and lose more mass while females should presumably rely on their lipid reserves. Still, even if males lost more mass, dehydration accounted for a significant portion of BM loss (Fig. 1, Table 1), with a similar pattern of rehydration between sexes.

We predicted baseline CORT levels to be higher in water-deprived snakes both as a result of hydric stress and the mineralocorticoid role of CORT. However and interestingly, we found similar baseline CORT levels between treatments suggesting alternative hypotheses. First, this pattern may reveal a putative caveat in our experimental design, insofar as the dehydration induced may not have triggered a physiologically stressful situation. This seems unlikely as water deprivation

**Table 3**

Statistical model used to examine baseline and stress-induced CORT levels in control and water-deprived snakes. "T<sub>b</sub>" indicates body temperature. See text for details.

| Variable       | Factor          | df | F    | P      |
|----------------|-----------------|----|------|--------|
| Baseline       | T <sub>b</sub>  | 1  | 12.7 | 0.002  |
|                | Time of day     | 1  | 9.0  | 0.007  |
|                | Treatment       | 1  | 0.0  | 0.958  |
|                | Sex             | 1  | 1.4  | 0.256  |
|                | Treatment × Sex | 1  | 0.0  | 0.938  |
| Stress-induced | Baseline        | 1  | 34.6 | <0.001 |
|                | T <sub>b</sub>  | 1  | 1.9  | 0.183  |
|                | Time of day     | 1  | 0.4  | 0.559  |
|                | Treatment       | 1  | 9.4  | 0.007  |
|                | Sex             | 1  | 4.1  | 0.058  |
|                | Treatment × Sex | 1  | 2.7  | 0.116  |



**Fig. 2.** Comparison of baseline and absolute stress-induced (60 min post handling) CORT levels between pythons (*A. childreni*) from control (open bars) and water-deprived (filled bars) experimental treatment. Bars represent means ( $\pm$ SE) of CORT levels ( $\text{ng}\cdot\text{mL}^{-1}$ ). Significant effects of experimental treatment are symbolized: \*\*  $P < 0.01$  and n.s. (non-significant).

occurred over a prolonged duration (>50 days), during which water-deprived snakes lost a significant proportion of their BM (>10%, Table 1). Relative BM loss was 2 to 4 times higher in water-deprived individuals (Table 1). In addition, the eagerness with which water-deprived snakes restored their water balance during the rehydration phase (we observed immediate drinking behaviour once water was available) strongly suggests that these individuals were in negative water balance. More likely, we suggest that water deprivation, at least at the level of dehydration induced by our experiment, does not trigger a HPA response resulting in baseline CORT level elevation. Although it is unknown whether more severe water deprivation would induce such a response, our results do not support mineralocorticoid role of CORT. Previous studies examining the relationship between CORT and hydric constraints reported conflicting results. For instance, water deprivation significantly increases baseline CORT levels in pregnant female, but not in male common lizards (Dauphin-Villemant and Xavier, 1986), suggesting complex interactions with sex and reproductive status on CORT levels. Actually, it seems that CORT is mostly involved in salt balance regulation (Holmes and McBean, 1964; Duggan and Lofts, 1978; Bradshaw and Rice, 1981; Bradshaw et al., 1984), rather than water balance per se (Bradshaw and Rice, 1981; Dauphin-Villemant and Xavier, 1986). Future studies should experimentally investigate the role of CORT in water versus salt balance regulation in order to clarify the mineralocorticoid function of CORT.

Why then did we find an increased stress-induced CORT in water-deprived snakes? The increase of stress-induced CORT concentration in pythons from the water-deprived treatment could be the result of a decrease of body fluids but this explanation seems unlikely since baseline CORT was not affected by water deprivation. More likely, we hypothesise that the reason probably lies in the behavioural and physiological adjustments known to occur in these taxa. Squamates reduce dehydration by reducing their movements (Davis and DeNardo, 2009) and secluding themselves in humid microenvironments such as subterranean caverns, burrows or underneath large rocks until environmental conditions improve (Bonnet and Brischox, 2008; Lillywhite et al., 2008a). They also display a thermal depression and reduced metabolic rates (Ladyman and Bradshaw, 2003; Ladyman et al., 2006). All of these adjustments probably aim at reducing additional transcutaneous and ventilatory water loss. These water-saving strategies (especially lowered temperature and metabolism) likely reduce performances, including anti-predator responses (locomotion, defensive

behaviour) (Angilletta, 2009). In addition, dehydration has previously been shown to decrease locomotor performances in amphibians and lizards (Moore and Gatten, 1989; Wilson and Havel, 1989; Prates et al., 2013). To our knowledge, such effects have not yet been investigated in snakes and further studies are required to test for the effects of water deprivation on locomotor performances in snakes. According to our results, we hypothesise that the high CORT stress response we found in water-deprived snakes might positively compensate the alteration of performances by allowing these individuals to rapidly mobilize reserves, to increase metabolic rate, and thus to efficiently retaliate to a potential threat. Accordingly, CORT is known to significantly influence the metabolic rates in snakes (Guillette et al., 1995; DuRant et al., 2008; Preest and Cree, 2008), and the CORT stress response is known to increase when snakes are exposed to cold temperature (Bonnet et al., 2013; Dupoué et al., 2013).

More generally, our results show that baseline CORT might not respond to water deprivation (at least at the level of dehydration induced by our experiment), as it does for other vital resources (e.g., temperature and/or food availability, Jenni-Eiermann et al., 2008; Woodley et al., 2003). The role of CORT as a mineralocorticoid still needs to be clarified, and future studies should investigate CORT differential functions relative to water versus salt balance, two intricate aspects of the osmotic equilibrium (McCormick and Bradshaw, 2006). For instance, CORT manipulation of individuals kept in contrasted conditions (control versus water-deprived) should help to clarify these issues. Finally, the increased stress-induced CORT found in water-deprived snakes suggests a stronger stress response in reaction to lower performances, and such hypothesis can be directly tested.

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