

Are glucocorticoids good indicators of pregnancy constraints in a capital breeder?



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ABSTRACT

Pregnancy imposes a substantial energetic demand on the mother (i.e. metabolic costs of pregnancy) and is often associated with modified maternal behavior and increased physical burdens that make females more vulnerable to predation. The Hypothalamic-Pituitary-Adrenal axis plays a fundamental role in reproduction through hormonal control of energy regulation and parental care. Therefore, evaluating the changes in baseline and stress-induced glucocorticoid levels in response to pregnancy may provide a robust tool to assess not only the constraints of gestation but also the way females may adjust to these constraints. In this study, we measured baseline and stress-induced corticosterone (CORT) concentrations in pregnant and non-pregnant aspik vipers (*Vipera aspis*), which are capital breeders. We also measured muscle condition (tail width) and locomotion performance (traction force) because these are robust proxies of protein mobilization associated with fasting. Baseline CORT concentration increased significantly over time in pregnant females, while they were lower and stable in non-reproductive females. Pregnant females had lower muscle condition at the onset of the study and tail width was negatively correlated with CORT concentration in this group. Contrary to our prediction, the stress response was not attenuated in pregnant females, but was proportional to baseline CORT concentration. Our results suggest that baseline CORT variations are closely related to energy constraints and structural protein mobilization in this capital breeder.

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1. Introduction

Reproduction induces major physiological and behavioral changes, especially in viviparous species due to the additional constraints imposed by pregnancy (Clutton Brock, 1991). Supporting embryonic development imposes significant energy demands on the mother, resulting in resource mobilization and altered condition after parturition (Bleu et al., 2012; Foucart et al., 2014; Schultz et al., 2008). Moreover, pregnancy is often associated with important shifts in maternal behavior, including habitat use and foraging activities (Bernardo, 1996; Bull and Shine, 1979; Neumann et al., 1998; Viérin and Bouissou, 2001). While these behavioral changes are usually required to optimize embryonic development, they can compromise female energy balance or increase predation risk (Bull and Shine, 1979). Finally, maternal morphological changes (i.e. increased physical burden due to embryos) are dramatic and enhance vulnerability to predation

(Le Galliard et al., 2003; Plath et al., 2011; Schwarzkopf and Andrews, 2012; Shaffer and Formanowicz, 1996). Quantifying these constraints (energy demand, behavioral changes and vulnerability) and clarifying their dynamics during pregnancy is essential to better understand the life history transition to viviparity. These constraints are also associated with hormonal changes, and it is essential to consider their proximate role in regulating pregnancy.

Neuroendocrine systems play a fundamental role in reproduction through hormonal control of parental investment such as the regulation of energy allocation and parental behavior (Brunton et al., 2008; Ricklefs and Wikelski, 2002; Sinervo and Licht, 1991; Wingfield and Sapolsky, 2003). In vertebrates, glucocorticoid hormones (primarily corticosterone, CORT, in birds, reptiles and rodents) have received a specific attention because they are thought to mediate the trade-off between investment in current versus future reproductive episodes (Angelier and Wingfield, 2013; Wingfield and Sapolsky, 2003). Glucocorticoids play an important role in energy mobilization and moderate circulating CORT concentration (hereafter [CORT]) may help breeders to sustain the energetic demands of reproduction (Romero, 2002; Sapolsky et al., 2000; Landys et al., 2006). Glucocorticoid secretion

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can be dramatically upregulated in response to stressors, and this adrenocortical stress response shifts investment away from reproduction and redirects it toward survival (Wingfield and Sapolsky, 2003; Angelier and Wingfield, 2013; Wingfield et al., 1998). However, this adrenocortical response can be downregulated by parents to optimize parental effort and reproductive performances (Wingfield and Sapolsky, 2003). For instance, the CORT stress response is usually minimized or suppressed in pregnant mammals (Atkinson and Waddell, 1995; Brunton et al., 2008; Johnstone et al., 2000) and this has been interpreted as a way to avoid the deleterious effect of elevated [CORT] on embryonic development (Field and Diego, 2008).

Viviparity has emerged on repeated occasions in squamates reptiles (at least 115 independent transitions, see Blackburn, 2006) and this group provides a unique opportunity to examine the dynamic link between specific constraints of pregnancy and maternal glucocorticoid secretion. Most lizards and snakes are lecithotrophic, and nutrient transfer after ovulation is absent or limited (Blackburn and Sidor, 2014; Van Dyke et al., 2014). Still, pregnancy imposes specific maternal energetic demands to facilitate growing embryonic oxygen needs (Birchard et al., 1984) and process embryonic waste (Van Dyke and Beaupre, 2011) resulting in significant metabolic costs of pregnancy (Dupoué and Lourdais, 2014). Maternal thermoregulation is essential to optimize reproduction (Lorioux et al., 2013a, 2013b; Shine, 2004), but also results in increased exposure and predation risk (Mathies and Andrews, 1995; Shine, 2003). Finally, the physical burden of developing embryos is known to impair locomotor abilities in pregnant squamates and to increase predation risk (Johnson et al., 2010; Le Galliard et al., 2003; Olsson et al., 2000; Shine, 1980). Therefore, pregnancy is associated with substantial energy demand and increased exposure to predators and these aspects should affect baseline [CORT] and the CORT stress response. Modulation of CORT secretion has been demonstrated in non-avian reptiles during reproduction, although there is considerable variation in how the adrenocortical response is modulated (Cartledge and Jones, 2007; Smith et al., 2012). For example, baseline [CORT] is higher in gravid females than in non-gravid females in tuatara (*Sphenodon punctatus*), but they exhibit a dampened stress response compared to non-gravid females and males (Anderson et al., 2014). In the parthenogenic lizard *Cnemidophorus uniparens*, circulating [CORT] declines during vitellogenesis and ovulation (Grassman and Crews, 1990), while no variation is observed between reproductive stages in the common gecko, *Hoplodactylus maculatus* (Gierling and Cree, 1995). Several studies have found that increased baseline [CORT] are associated with pregnancy (Taylor et al., 2004; Dauphin-Villemant and Xavier, 1986) while other studies report no differences between pregnant females and non-breeders (Graham et al., 2011). Yet the dynamics of baseline and stress-induced CORT concentration during pregnancy remain largely understudied.

We examined the impact of pregnancy on baseline and stress-induced [CORT] in the aspic viper, *Vipera aspis*. This species mobilizes its energy stores (mainly body fat) to support reproduction (i.e. capital breeder, Lourdais et al., 2002b) and often fasts during pregnancy (Lourdais et al., 2002a). We collected multiple blood samples in pregnant and non-pregnant vipers to compare [CORT] between breeding statuses and over the course of pregnancy. We also measured changes in muscle size and locomotor performances to better understand their specific links with [CORT]. Specifically, we tested the following predictions:

- 1) Baseline [CORT] should be higher in pregnant than in non-pregnant females and should increase throughout pregnancy because of increasing energetic constraints (Romero, 2002; Kenagy et al., 1999; Reeder et al., 2004; Reeder and Kramer, 2005).

- 2) Increased energy demands should translate into altered musculature and locomotor performances in pregnant females because of structural protein mobilization (Lourdais et al., 2004). Importantly, baseline [CORT] should be linked with morphological and performance costs due to pregnancy because elevated baseline [CORT] is usually associated with protein catabolism and reduced body condition (reviewed in Sapolsky et al., 2000; Landys et al., 2006).
- 3) Since pregnant vipers maintain active behavioral thermoregulation even under stressful events (i.e. predation risk, see Lorioux et al., 2013b), the CORT stress response should be attenuated in pregnant females relative to non-pregnant females to allow the expression of maternal behavior and/or minimize embryos exposure to elevated [CORT] (Wingfield and Sapolsky, 2003; Cree et al., 2003).

2. Materials and methods

2.1. Study species and breeding

We used a long-term captive colony of aspic vipers (*Vipera aspis*), a small medio-European venomous snake. Females are typical capital breeders and store energetic resources over several months before reproducing (Bonnet et al., 1998; Jonsson, 1997; Lourdais et al., 2002b; Naulleau and Bonnet, 1996). Pregnancy is associated with higher preferred body temperature and increased basking times (Lorioux et al., 2013a; Naulleau, 1979; Saint Girons, 1952) compared to males and non-reproductive females. During this period, females often reduce feeding and decrease their home range (Lourdais et al., 2002a; Naulleau and Bonnet, 1996).

Snakes were bred following a previously described protocol (Lorioux et al., 2013a). After mating, 39 snakes from the colony were transferred and assigned to one of the twelve experimental cages (1 m × 0.75 m × 0.4 m). Because of space limitation, snakes were housed in combination of either 3 or 4 individuals. No agonistic behavior was observed in co-housed individuals (Lorioux et al., 2013a). A 75 W light bulb was placed on one side of the cage, creating a thermal gradient (18–41 °C) in the cage. On the opposite side (cold section), an opaque PVC shelter was provided facing the light source. This refuge allowed snakes to hide, and was also used as nocturnal shelter (Lorioux, Pers. Obs.). Snakes were allowed to bask for 6 h per day, between 10 a.m. and 4 p.m. Room temperature was held constant at 18 °C. Vipers are low frequency feeders and fasting often occurs, notably during pregnancy. To avoid confounding effects of nutritional balance, all females were unfed until the completion of the experiment. Water was supplied *ad libitum*.

2.2. Reproductive status and pregnancy monitoring

At the end of the mating period we assessed female reproductive condition using ultrasonography (Micromaxx, SonoSite, Inc., Bothell, WA, USA). Twenty-three females were non-reproductive (NR), i.e. females that never entered vitellogenesis, and 16 females had enlarged follicles.

Ultrasonography was used to monitor embryonic development and to estimate embryonic stages in pregnant females (Hubert and Dufaure, 1968; Lorioux et al., 2013b). Five females initially ovulated eggs and had detectable embryos but subsequently withdrew from reproduction. In two females, embryos were initially detected but disappeared after eight weeks and only yolk mass remained until the completion of the experiment. For the 3 other females, we observed a complete disappearance of the yolk mass and initially detected embryos. These 5 females that failed reproduction are identified as “RF” females. The 11 pregnant females (P, i.e. females that completed pregnancy and produced at least one

viable offspring), were monitored by regular ultrasonography throughout pregnancy and the number of developing embryos was determined.

At the end of pregnancy, cages were inspected daily to detect parturition. The different components of the litters (viable neonates, stillborns, and undeveloped ova) were counted and weighed (± 0.1 g). Mean number of viable neonates (i.e. fecundity) was 5.8 ± 2.6 per female. Only two females produced one stillborn each.

Mean SVL (Snout-to-vent length) were 54.5 ± 1.0 cm, 54.9 ± 0.8 cm, and 53.2 ± 1.8 cm for P, NR and RF females respectively and did not differ statistically (ANOVA, $F_{2,39} = 0.388$, $p = 0.681$). Initial body masses were 169.1 ± 10.4 g, 164.3 ± 7.0 g, and 158.4 ± 8.5 g for P, NR, and RF females respectively, and were not statistically different (ANOVA, $F_{2,37} = 219.29$, $p = 0.820$). Initial body condition (i.e. residuals from the regression of body mass at the onset of the experiment against SVL) did not differ between groups (ANOVA, $F_{2,39} = 0.035$, $p = 0.965$).

2.3. Experimental design

2.3.1. Pregnancy stages

Using ultrasonography, we identified relevant time periods to collect blood samples as well as morphometric and locomotor data (see Lorioux et al., 2013a, and 2013b for details). In aspic vipers, a pre-ovulation ecdysis occurs and provides a reliable index for the onset of gestation (Lorioux et al., 2013a). Specifically we considered four time periods in pregnant females:

S1 (late vitellogenesis): mean \pm SE: 5.7 ± 2.2 days before ovulation. Large follicles with circular shape are observed.

S2 (early pregnancy): 29.8 ± 2.4 days after ovulation, 34% of total development. Distinctively ovulated eggs with ovoid shape. The embryos are distinguishable with a mean size of 1.2 ± 0.3 cm. Estimated developmental stage according to Hubert and Dufaure (1968): 23–24.

S3 (mid pregnancy): 52.5 ± 1.8 days after ovulation, 58% of total development. Embryos mean size = 2.1 ± 0.5 cm; the embryos exhibit a typical spiral body shape. Estimated developmental stage: 30–31.

S4 (late pregnancy): 77.5 ± 1.8 days after ovulation, 86% of total development. The fetuses are uncoiled and not measurable; remaining vitellus is estimated at 5–10% of the conceptus. Estimated developmental stage: 39–40.

Typical gestation length in the aspic viper is 82–90 days under our experimental thermal conditions (Lorioux et al., 2013a; this study). Based on these time periods, we obtained equivalent measurements for NR and RF females. We added a fifth blood sample for P females only, 8 days after parturition.

2.3.2. Baseline and stress-induced CORT concentrations

All blood samples were drawn by cardiocentesis using a 1 mL syringe and a 27-gauge needle. In order to measure baseline circulating [CORT], the snake was rapidly removed from the experimental cage and a first blood sample (200–250 μ l) was collected within approximately 3 min after capture (Romero and Reed, 2005) (mean \pm SE, 190 ± 69 s, range: 87–462 s (Romero and Reed, 2005)). [CORT] increases following a stressor may not be detectable before 10 min in some reptile species (Romero, 2002) and no correlation was found between baseline [CORT] and sampling time (all females and sessions included, $p = 0.384$, $r^2 = 0.005$), therefore all samples were considered.

We used a standardized capture/restraint stress protocol, slightly modified from Wingfield (1994) as detailed in Dupoué et al. (2013). Vipers were placed in a transparent plastic box (35 \times 25 \times 12.5 cm) without any refuges and transferred to a

climatic chamber (Vötsch Industrietechnik, VP 600, Balingen, Germany) at $25 \text{ }^\circ\text{C} \pm 0.1 \text{ }^\circ\text{C}$, where they remained for 1 h. A second blood sample (150 μ l) was collected 1 h later in order to assess stress-induced CORT concentration (mean sampling time \pm SE, 206 ± 29 s, range: 89–664). The female was then placed in its initial cage and the blood sample was immediately centrifuged at 3000g during 5 min. Plasma was collected and stored at $-28 \text{ }^\circ\text{C}$. Plasma CORT assays were performed at the CEBC by following a well-established RIA protocol (see Lormée et al., 2003 for details). Samples were run in two assays (intra-assay variation: 7.07%, inter-assay variation: 9.99%). Cross-reaction was 9% with 1-deoxycorticosterone and less than 0.1% with other plasma steroids. The total amount of blood collected represented less than 1% of total body mass and did not visibly impact the animals.

2.3.3. Morphology and performances

2.3.3.1. Tail base width. Epaxial muscles have been previously measured in snakes, notably in the dorsal region in muscular species (Lourdais et al., 2013, 2005a). For safety reasons we slightly modified the technique and measured the width of the tail basis. In vipers, the tail is composed of longitudinal muscles but no fat tissues as described in some lizards. Tail width can show significant emaciation, especially in post-parturient females (Lorioux, personal observation). Measurements were made with a caliper that applies constant pressure (e.g. a spessimeter; Absolute digimatic Mitutoyo, Japan) at approximately 1 cm posterior to the cloaca. A permanent mark was made on the skin with a cautery unit (Bovie Medical corporation, Clearwater, USA) to repeat the measure within session (triplicates) and among sessions (four sessions). The relative coefficient of variation was low (0.6%, 0.7%, 0.8%, and 0.7% for sessions 1, 2, 3, and 4 respectively). We used the mean value of triplicates per session for each individual, as a measure of tail base width in the analyses.

2.3.3.2. Locomotor performances. We evaluated locomotor performances as the ability of vipers to escape a potential predator. We measured snake traction force with a dynamometer (Force Gauge 200N, Fisher scientific Bioblock, Illkirsch, France) as detailed in Lourdais et al. (2004). Each female was handled after inserting the first quarter of the body in a transparent tube and firmly maintained by the operator. The snake tail was taped to the dynamometer and the snakes were stimulated to provoke body contractions. Before each run, females were placed in climatic chambers (LMS LDT, Sevenoaks, Kent, United Kingdom; stability $\pm 1 \text{ }^\circ\text{C}$) at $25 \text{ }^\circ\text{C}$. For each session, we performed three consecutive trials of 30 s each, separated by a 10 s resting period. We considered the maximal value among the 3 consecutive trials per session in the analyses, since it provides a rather good estimate of strength (see Lourdais et al., 2005a).

2.4. Statistical analyses

All statistical analyses were performed using R (2.12.1 Development Core Team 2010). We used general linear mixed models (GLMM) to test the influence of reproductive status and session on baseline [CORT], stress-induced [CORT], tail base width and traction force. Female identity was treated as a random factor, and reproductive status and session as fixed factors (lme models, package nlme). Hormonal data were log-transformed to meet normality assumptions. We examined the relation between baseline and stress-induced [CORT] in a GLMM treating female identity as a random factor, reproductive status and session as fixed factors and baseline [CORT] as a linear covariate. The relationships between tail base width and [CORT] (baseline and stress-induced) were examined in a GLMM procedure treating reproductive status as fixed factor, tail base width as a linear covariate, and

female identity as a random factor. Variation in tail base width was analyzed using a GLMM with reproductive status, session, SVL, the interaction term “reproductive status \times session” as co-factors, and female identity as a random factor. Maximal traction force was analyzed using a GLMM with reproductive status, session, tail base width, SVL, and the interaction term “reproductive status \times session” as co-factors, and female identity as a random factor. Two-by-two comparisons were analyzed using Tukey's *post hoc* tests.

We also wanted to test for the effect of fecundity on different aspects (i.e. [CORT], locomotor performances). Because fecundity was not related to maternal SVL in our sample ($F_{1,10} = 0.176$, $p = 0.683$) absolute values were used in our models. Significance was determined at $\alpha = 0.05$ for all tests. Values are presented as mean \pm SE.

3. Results

3.1. Changes in CORT concentration

Baseline [CORT] significantly influenced by reproductive status (Table 1) with P females exhibiting the highest concentration (30.3 ± 2.9 , 17.6 ± 1.7 , and 18.9 ± 3.0 ng/mL respectively for P, NR, RF females). A significant interaction was detected between reproductive status and session (Table 1; Fig. 1a). While baseline [CORT] was similar between NR and P females for the first session P females exhibited increased [CORT] for the 3 other sessions (Fig. 1a). Baseline [CORT] was not affected by session for NR and RF females (Fig. 1a).

Stress-induced [CORT] was higher in P females compared to NR and RF (100.9 ± 7.0 , 75.3 ± 4.9 , and 73.7 ± 7.7 ng/mL respectively for P, NR, and RF females). A significant interaction was also detected between pregnancy status and session (Table 1). Specifically, stress-induced [CORT] was higher in P females compared to NR females in sessions 3 and 4 but not in sessions 1 and 2 (Fig. 1b). When treating baseline [CORT] as a covariate, we found that stress-induced [CORT] was positively related to baseline [CORT] ($F_{1,87} = 9.15$, $p = 0.003$) but was not influenced by reproductive status (interaction term $F_{2,107} = 0.67$, $p < 0.53$), session (interaction term $F_{1,87} = 0.67$, $p < 0.42$) or the combination of status and session (interaction term $F_{3,87} = 0.82$, $p < 0.49$).

Table 1

Statistical models used to examine changes in [CORT], tail base width and traction force changes during reproduction. DF: degree of freedom; SS: sum of squares.

Variable	Factors	DF	SS	F ratio	P value
Baseline CORT (ng/mL)	Intercept	1	99	119.39	<0.001
	Status	2	36	4.49	0.018
	Session	3	99	1.01	0.393
	Status \times session	6	99	2.70	0.018
Stress-induced CORT (ng/mL)	Intercept	1	101	372.11	<0.001
	Status	2	36	7.01	0.003
	Session	3	101	3.59	0.016
	Status \times session	6	101	3.40	0.004
Tail base width (mm)	Intercept	1	104	5990.53	<0.001
	Status	2	35	7.29	0.002
	Session	3	104	86.43	<0.001
	SVL	1	35	24.17	<0.001
	Status \times session	6	104	4.73	<0.001
	Status \times SVL	2	32	3.31	0.049
Traction force (kg)	Intercept	1	100	546.92	<0.001
	Status	1	35	46.64	<0.001
	Session	3	100	4.89	0.003
	Tail base width	1	100	24.44	<0.001
	SVL	1	35	2.55	0.119
	Status \times session	6	100	1.45	0.202

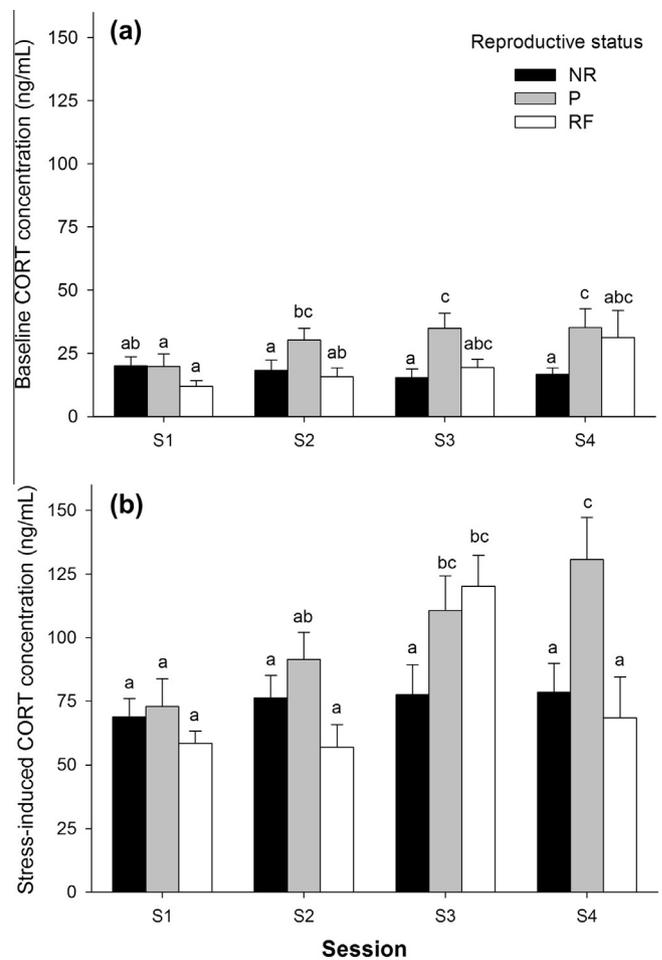


Fig. 1. Effects of reproductive status and session on baseline [CORT] (a), and the stress response (b) in pregnant (P), N = 11, non-reproductive females (NR), N = 19, and females that failed reproduction (RF), N = 5. S1: late vitellogenesis, S2: early pregnancy, S3: mid-pregnancy, S4: late pregnancy. Each bar of points represents mean \pm s.e. Bars not connected by the same letter are significantly different (Tukey's *post hoc* test $p < 0.05$).

3.2. Changes in tail base width and performances

Tail base width was influenced by reproductive status, session and the interaction term (Table 1, Fig. 2a). We also found a significant influence of SVL in interaction with reproductive status (Table 1). That is, tail base width was positively related to SVL in NR females for each session (all p values < 0.001), while no relation was found for R or RF females for any session (all p values > 0.05). P females had a lower tail width than NR females and this difference existed at the onset of the study (Table 2, Fig. 2a). A decrease in tail width over sessions was observed in NR females (*post hoc* tests, all p values < 0.001). A similar pattern was found in P females (all p values < 0.001 except session 2 not different from session 3; see Fig. 2a) while values were stable for RF females (all p values > 0.082 ; Fig. 2a).

Maximal traction force was influenced by reproductive status, session, and tail base width, but not by SVL or the interaction between status and session (Tables 1 and 2; Fig. 2b). Traction force was positively correlated with tail base width for P ($r = 0.539$; $F_{1,45} = 18.436$, $p < 0.001$) and NR females ($r = 0.415$; $F_{1,81} = 16.840$, $p < 0.001$), and negatively correlated with tail base width for RF females ($r = -0.596$; $F_{1,17} = 9.376$, $p = 0.007$). Maximal traction force was significantly lower in pregnant females when compared to non-reproductive ones (Table 2, Fig. 2b), RF females had lower traction force than NR females (Table 2) but were not different

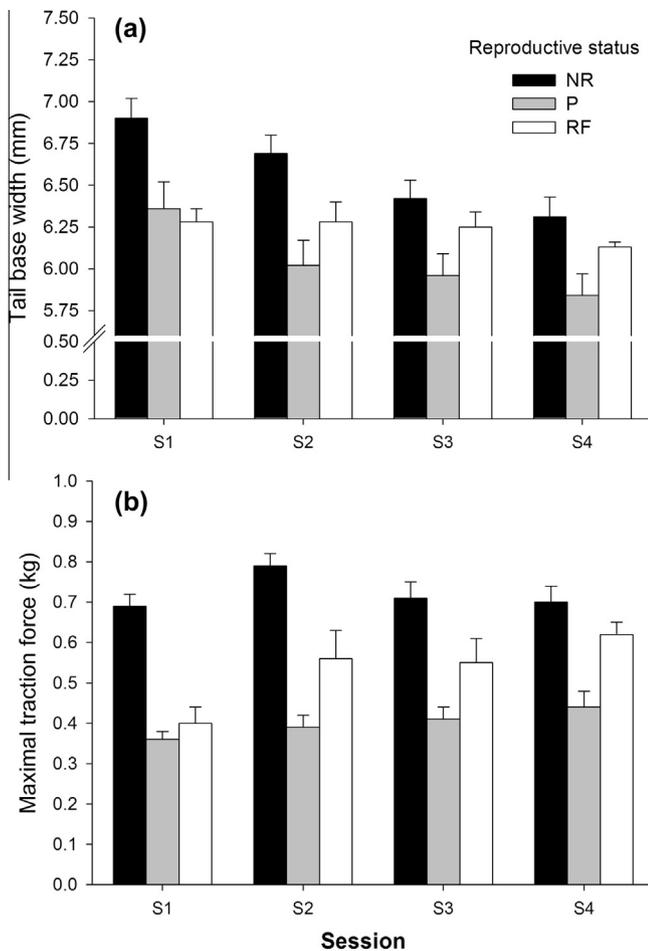


Fig. 2. Effects of reproductive status and session on tail base width (a), and maximal traction force (b). P: pregnant females; NR: non-reproductive females; RF: females that failed reproduction. S1: late vitellogenesis, S2: early pregnancy, S3: mid-pregnancy, S4: late pregnancy. Each bar of points represents mean \pm s.e. Differences between groups are described in Table 2 and in Section 3.

Table 2

Detailed inter-status comparisons (within each session S1–S4) for tail base width and traction force. P values were obtained from Tukey's *post hoc* tests.

Variable	Session	P vs NR	P vs RF	NR vs RF
Tail base width (mm)	S1	<0.001	0.800	0.012
	S2	<0.001	0.375	0.051
	S3	0.007	0.291	0.351
	S4	0.005	0.248	0.287
Maximal traction force (kg)	S1	<0.001	0.445	0.002
	S2	<0.001	0.036	0.015
	S3	<0.001	0.174	0.052
	S4	<0.001	0.015	0.583

from P females (Table 2). Traction forces in RF females were higher in sessions 2, 3 & 4 than in session 1 (*post hoc* tests all p values < 0.009; Fig. 2b). This pattern was also found in P females but the changes in traction force were much less pronounced (*post hoc* tests all p values < 0.046; Fig. 2b). In NR females no clear pattern was detected with only a significant difference in traction forces between session 1 and 3; *post hoc* test p < 0.043; Fig. 2b). In pregnant females, maximal traction force was negatively correlated to fecundity ($r = -0.53$; $p = 0.003$).

3.3. Proximate determinant of CORT concentrations

When combining all sessions, we found that baseline [CORT] was not significantly related to tail base width ($F_{1,104} = 1.058$,

$p = 0.306$), but we found a significant interaction with reproductive status (interaction term $F_{2,104} = 5.177$, $p = 0.007$; Fig. 3). The relationship between [CORT] and tail base width was significant and negative among P females ($r = -0.439$; $F_{1,45} = 10.772$, $p = 0.002$) but not for NR ones ($r = 0.012$; $F_{1,79} = 0.012$, $p = 0.915$), and RF ones ($r = -0.137$; $F_{1,16} = 0.305$, $p = 0.588$). Stress-induced [CORT] was significantly related to tail width ($F_{1,105} = 5.338$, $p = 0.028$), with a significant interaction with reproductive status ($F_{2,105} = 3.127$, $p = 0.048$; Fig. 3). The relation between stress-induced [CORT] and tail base width was significant and negative among P females ($r = -0.401$; $F_{1,46} = 8.801$, $p = 0.005$) but not for NR ones ($r = -0.099$; $F_{1,79} = 0.702$, $p = 0.405$), and RF ones ($r = -0.159$; $F_{1,16} = 0.413$, $p = 0.530$).

In P females, baseline and stress-induced [CORT] before parturition (S4) were not influenced by fecundity (baseline: $r = -0.110$, $F_{1,9} = 0.047$, $p = 0.736$; stress-induced: $r = 0.385$, $F_{1,9} = 2.733$, $p = 0.243$). We found that stress-induced [CORT] was significantly lower after parturition (130.7 ± 16.6 vs 80.5 ± 12.0 ng/mL, respectively for S4 and *post partum* values, *post hoc* test: $p = 0.008$). This was not the case for baseline [CORT] (36.9 ± 7.1 ng/mL vs 26.2 ± 5.9 ng/mL respectively for S4 and *post partum* values, *post hoc* test: $p = 0.201$).

4. Discussion

Pregnancy represents an extremely demanding state (Clutton Brock, 1991) since it directly affects energy mobilization and exposure to predation (McEwen and Wingfield, 2003). Glucocorticoids have multiple effects on reproduction and our results in a capital breeder suggest that CORT concentrations are closely related to maternal energy mobilization. Moreover, the stress-induced CORT response does not seem to be down-regulated during pregnancy in the aspiv viper.

4.1. Changes in baseline CORT concentration, musculature and performance

We detected a progressive increase in baseline [CORT] over time in pregnant females while non-reproductive females had lower and stable baseline [CORT]. These results are consistent with previous findings in mammals demonstrating an increase in cortisol concentration throughout pregnancy (Allolio et al., 1990; Jensen et al., 2002; Reeder and Kramer, 2005). They support the idea that gestation imposes specific energy requirement notably at late gestational stages. While nutrient allocation is absent after ovulation in a lecithotrophic species, females still have to support increasing embryonic demands for oxygen that rise notably during the second part of development. Pregnancy affects maternal physiology with modified blood oxygen affinity, and increased heart and ventilation rates (Birchard et al., 1984; Ingermann et al., 1991; Keller-Wood et al., 2012; Van Dyke and Beaupre, 2011). In our study species, maternal metabolic rate increases by 40% between early and late pregnancy and the “cost” of supporting embryo represents 14% of maternal metabolism at late pregnancy stages (Dupoué and Lourdais, 2014).

Our study was conducted under food deprivation, which is frequent in low frequency feeders (McCue, 2007). A relationship between baseline [CORT] and energy imbalance has been demonstrated in other species including reptiles, birds, and mammals, notably when proteins are mobilized (Romero and Wikelski, 2001; Challet et al., 1995; Cherel et al., 1992; Lynn et al., 2010; Angelier et al., 2015). Although we were expecting baseline [CORT] to increase also in non-pregnant females as they were fasting through the experiment, we did not find any effect of session in this group. However, the relationship between baseline [CORT] and body condition is often non-linear with [CORT] starting to

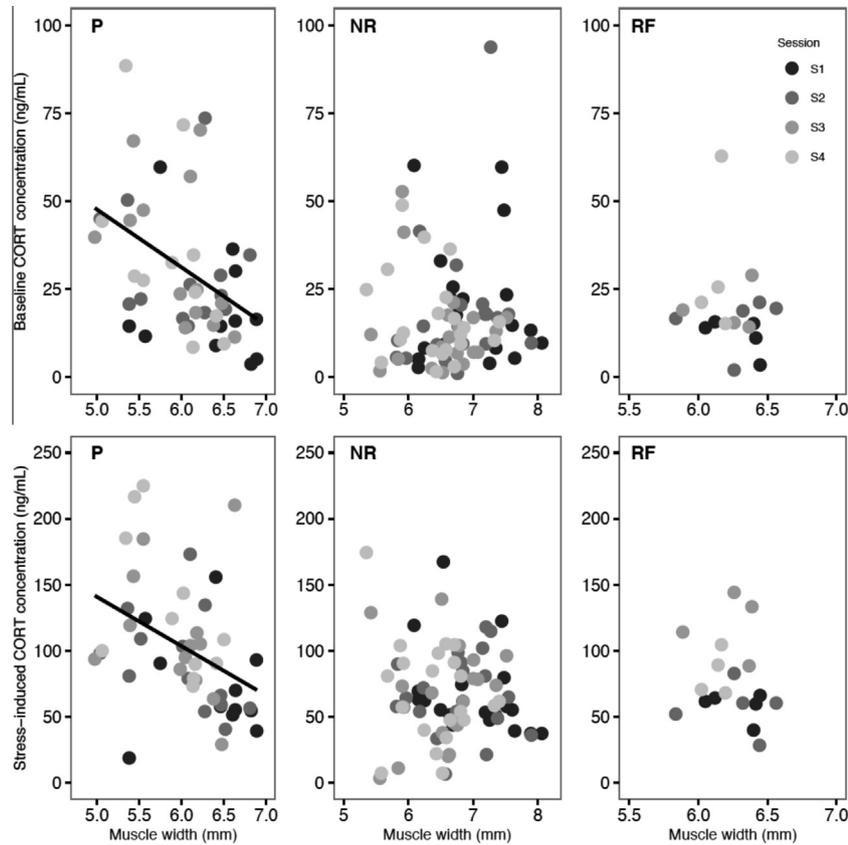


Fig. 3. Relationship between tail base width and [CORT] (baseline and stress-induced) in pregnant (P), non-reproductive females (NR), and females that failed reproduction (RF). All sessions were combined and each individual had 4 contributions (see text for statistics). Each session is represented by a different color. S1: late vitellogenesis, S2: early pregnancy, S3: mid-pregnancy, S4: late pregnancy.

increase only when individuals reach a low threshold in body condition (see Romero and Wikelski, 2001 for an example in a reptile species). Reproductive females had already mobilized their body reserves for vitellus synthesis (Lourdais et al., 2003; Van Dyke and Beaupre, 2011) at the start of the experiment. Thus, reproductive females were more constrained energetically than non-pregnant females at the onset of the experiment, as revealed by lowered muscular condition and performances. Non-pregnant females may not have reached this lower body condition threshold that is associated with a rise of baseline [CORT]. In support of this, we found that baseline [CORT] was negatively related to muscular condition in pregnant females but not in non-reproductive females suggesting a more pronounced stage of protein mobilization (Lourdais et al., 2004). Tail base width was closely related to traction force and pregnant females displayed lower traction force. A negative relationship was found between fecundity and traction force suggesting for a proximate link between reproductive effort and performance alteration.

To date, only a limited number of studies have examined how both baseline and stress-induced [CORT] change over gestation in ectotherms (Dauphin-Villemant and Xavier, 1986; Preest et al., 2005; Taylor et al., 2004). An increase in circulating [CORT] before parturition has been demonstrated in several snake species (Smith et al., 2012; Taylor et al., 2004), suggesting a specific role in preparing females for parturition (Taylor et al., 2004). Dauphin-Villemant and Xavier (1986) reported, both *in vivo* and *in vitro*, that adrenal gland activity was maximal in late gestation in the common lizard. The reported changes in baseline and stress-induced [CORT] in our study suggest that pregnancy imposes specific metabolic constraints. It is noteworthy that body temperature influences circulating [CORT] and may also drive the differences we observed

between reproductive statuses. Previous studies suggest a positive correlation between [CORT] and temperature in some reptile species (Telemeco and Addis, 2014; Preest and Cree, 2008; Cree et al., 2003) while another study found a negative relationship (Dupoué et al., 2013). Pregnant asp viper have higher thermal preferences compared to non-reproductive ones (Lorioux et al., 2013b). However, body temperature and basking effort are stable over pregnancy (see Lorioux et al., 2013b), whereas [CORT] (baseline and stress-induced) increase over gestation (this study) suggesting that CORT concentrations are not simple correlates of a thermoregulatory shift.

4.2. Influence of pregnancy on the adrenocortical stress response

According to the “inhibitory hypothesis” (Romero, 2002; Flechter et al., 2015) circulating [CORT] should be kept low during the reproductive season because of the potential negative effects on reproductive function. When exposed to a stressor, the secretion of glucocorticoids is often suppressed to allow the expression of reproductive behaviors (Wingfield and Sapolsky, 2003). Attenuated stress responses have been demonstrated in several non-avian reptiles species and can be associated to courtship in males (Cease et al., 2007), movement (Owen et al., 2014) or reproduction in females (Anderson et al., 2014; Jessop, 2001). Results obtained in mammals indicate that the stress response is often suppressed during late pregnancy (Atkinson and Waddell, 1995). HPA hypo-responsiveness to stressors is considered adaptive when investment in offspring is high, by minimizing offspring exposure to elevated glucocorticoid levels and female catabolism (Brunton et al., 2008; Field and Diego, 2008; Keller-Wood et al., 2014). Our results contradict this pattern, but are compatible with the

“enabling hypothesis” which posits that [CORT] is greater during reproduction than the post-reproductive season (Romero, 2002; Flechter et al., 2015) to support reproductive investment. Notably, increased [CORT] may prime individuals to mobilize energy to support the high energetic demands associated with reproduction. We reported a progressive increase in stress-induced [CORT] in pregnant females compared to non-reproductive ones and a decrease after parturition. We also found that the increase in stress-induced concentrations was proportional to baseline concentrations, supporting the idea that [CORT] is mainly a mediator of metabolic activities in our study species (Romero, 2002). Supporting this interpretation, Stahlschmidt et al. (2013) recently demonstrated in another capital breeder, the Children’s python, that reproductive females experience significant muscle loss, increased oxidative damage, reduced immune function, and increased circulating [CORT] compared to non-reproductive females.

We have previously demonstrated that pregnant vipers are behaviorally less responsive to stressors (a potential predator) and they are dedicated to behavioral thermoregulation during pregnancy (Lorioux et al., 2013a). Attenuated behavioral responses to stressors during reproduction have been found in various mammals (Neumann et al., 1998; Viérin and Bouissou, 2001) and also humans (Yazici et al., 2014). Our findings suggest that maternal behavioral shifts do not involve an HPA hypo-responsiveness in the asp viper. Addressing the impact of elevated circulating [CORT] on the progeny is an important aspect to address in the future. Deleterious effects have been demonstrated in mammals (Brunton et al., 2008; Field and Diego, 2008) and also in several lizard species (Cree et al., 2003; Warner et al., 2009; Hare and Cree, 2010). However, slight increases in maternal [CORT] has also been suggested to induce adaptive developmental responses (Meylan and Clobert, 2005).

4.3. Capital breeding and mitigation of reproductive failure

Capital breeding relies on the use of body reserves to support reproduction independently of current food intake (Bonnet et al., 1998). This strategy can be advantageous but is often associated with low reproductive frequency. Reproductive failure is predicted to be costly because it jeopardizes resources accumulated over a long period of time that were invested in reproductive activities and embryo development. Our results suggest that compensatory mechanisms exist as previously described (Lourdais et al., 2005b). Females that failed at pregnancy displayed stable tail base width and even regained muscular performance (traction force) likely by reabsorbing the yolk content in their reproductive tract. Both baseline and stress-induced [CORT] were also intermediate in this group, suggesting that these vipers were able to limit their protein catabolism. Yolk or embryo disappearance during pregnancy have been previously described in viviparous squamates (Blackburn et al., 2003; Bonnet et al., 2008). Our results suggest that females can recoup energy and nutrients allocated into the ova which may reduce the impact of failing at pregnancy.

5. Conclusions

Our study revealed that pregnancy was associated with a progressive increase in baseline and stress-induced [CORT] in a typical capital breeder. Moreover, we found that this increased CORT secretion was related to structural protein mobilization. Reduced food intake during pregnancy is facultative and largely results from modified behavior resulting in missed feeding opportunities. Therefore it would be interesting to address the impact of contrasted nutritional statuses during pregnancy on maternal [CORT] (French et al., 2007; Neuman-Lee et al., 2015). Further studies

are also required to clarify the proximate endocrine determinant of modified maternal thermoregulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2016.04.007>.

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