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ScienceDirect
General and Comparative Endocrinology 150 (2007) 132–139
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Does adrenal responsiveness vary with sex and reproductive status in *Egernia whitii*, a viviparous skink?

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Received 21 February 2006; revised 23 May 2006; accepted 31 July 2006
Available online 18 September 2006

Abstract

In mammals, oestrogens generally stimulate adrenal function whilst androgens are inhibitory, and gestating females down-regulate their acute response to stressors in order to protect current reproductive investment. This study aimed to determine if adrenocortical function is similarly modulated by sex and reproductive status in the viviparous lizard, *Egernia whitii*. We compared the adrenocortical response to acute capture stress in female *E. whitii* during active (post-ovulatory and gestating) and quiescent (post-partum) phases of their reproductive cycle. We also compared the responses of reproductively quiescent males and females to acute stress and ACTH challenge to determine if there are sex-related differences in HPA axis activity when the influence of reproductive hormones is minimal. The females' responses to acute capture stress varied significantly with reproductive stage, and quiescent females displayed the strongest immediate response, with a rapid and sustained increase in plasma corticosterone (CORT) concentrations. Post-ovulatory females showed the most conservative adrenocortical response and while gestating females showed a large immediate response, this was not as prolonged as in quiescent females. Reproductively quiescent males and females exhibited similar responses to acute stress, and also responded similarly to ACTH injection, with plasma CORT reaching maximal concentrations of 52.1 and 59.4 ng/mL, respectively. Reproductively quiescent females treated with oestrogen exhibited greater responsiveness to ACTH than control females, although basal plasma CORT concentrations were unaltered: these results suggest that the attenuation of the acute stress response observed in reproductively active females of *E. whitii* may be regulated upstream of ACTH secretion. Our results demonstrate that the activity of the HPA axis is modulated by reproductive status in this viviparous reptile, and that gestating females are able to buffer their embryos from the potentially adverse effects of elevated plasma corticosteroids.

Keywords: ACTH; Capture; Corticosterone; Oestrogen; Reptile; Stress; Viviparity

1. Introduction

In vertebrates, the hypothalamo–pituitary–adrenal (HPA) axis is central to promoting survival by allowing the animal to respond behaviourally and physiologically to environmental stressors. Such responses may, however, include suppression of reproductive activity via inhibition of the hypothalamo–pituitary–gonadal (HPG) axis: current survival is then traded off against future reproductive success (Klose et al., 2006; Wingfield, 2003). Modulation of adrenocortical activity during particular stages of life history may therefore be an important factor in determining an animal's overall fitness (Moore and Jessop, 2003). For example, in arctic (Astheimer et al., 1995; Wingfield et al., 1995) and in desert (Wingfield et al., 1992) birds with limited time for breeding, the stress response is suppressed during the reproductive phase and breeding appears to be prioritised, albeit at the risk of increased mortality due to decreased responses to immediate stressors.

The anti-gonadal effects of stress in reptiles have been well documented (e.g. Lance and Elsey, 1986; Greenberg and Wingfield, 1987; Mahmoud et al., 1989; Elsey et al., 1991; Moore et al., 1991), but reciprocal influences of

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doi:10.1016/j.ygcen.2006.07.021

reproductive status on the function of the HPA axis have received much less attention. Marine turtles have been the most comprehensively studied group, with the consistent finding that females exhibit a depressed adrenocortical response to stress when breeding (*Lepidochelys mydas*: Valverde et al., 1999; *Chelonia mydas* and *Eretmochelys imbricata*: Jessop, 2001). This appears to represent a mechanism to ensure that the large energetic reserves accumulated by these capital (oviparous) breeders are not wasted through CORT-mediated stress responses (Jessop, 2001). Similarly in female tree lizards, *Urosaurus ornatus*, vitellogenic females show a large increase in plasma CORT concentrations in response to an acute stressor, but gravid females do not (Woodley and Moore, 2002). In the garter snake *Thamnophis sirtalis*, breeding males from populations with shorter breeding seasons exhibit reduced adrenocortical responses to stress compared with males from latitudes allowing more extended breeding (Moore et al., 2001), reflecting the greater potential of stressors to affect reproductive success in males with shorter breeding seasons.

In mammals, both basal and stress induced concentrations of glucocorticoids are greater in females than in males (Gaskin and Kitay, 1970; Handa et al., 1994a). Sex differences in stress responses of reptiles remain largely unknown but there is some evidence to suggest that reproductive steroids may influence stress responses, with females exhibiting greater adrenocortical responses to stressors than males. For example, Grassman and Hess (1992a) examined the effect of gonadectomy on the acute stress responses of male and female lizards of the species *Cnemidophorus sexlineatus*: intact females subject to the acute stress of hand-chasing in a pail exhibit higher plasma CORT concentrations than males exposed to the same stressor, while castrated males show more dramatic increases in plasma CORT concentrations following acute stress than intact males. In marine green and hawksbill turtles, differences in the stress responses of females and males of differing reproductive states are evident within the first few hours (Jessop, 2001). It remains unclear, however, whether there are differences in the activation of the HPA axis independent of gonadal hormone effects.

Adrenal function may also vary between reproductive stages. In mammals, there is strong evidence that gestating females down-regulate their acute stress responses to protect their current reproductive investment (Neumann et al., 1998), and the negative effects of high maternal corticosteroids on the development of the foetus have been repeatedly demonstrated (e.g. Hansen et al., 1999; Weinstock, 2001). Do viviparous female reptiles similarly buffer their developing embryos against the potentially detrimental effects of high levels of circulating corticosteroids? The available evidence seems contradictory. Pregnant females of the viviparous gecko *Hoplodactylus maculatus* exhibit no suppression of the stress response to capture compared with vitellogenic females although both groups do exhibit greater stress responses than males (Cree et al., 2003), and there is no observable effect on offspring quality in females of this species challenged with ACTH during gestation (Preest et al., 2005). Gestating and post-partum females of the viviparous skink *Egernia whitii* show very similar adrenocortical responses to chronic captivity with corticosterone concentrations returning to baseline levels one week following capture (Cartledge et al., 2005). However, the acute stress response has not been compared between reproductive states in this species and it may be more adaptive to modulate acute stress responses than chronic ones. Once a stressor becomes chronic in nature the reproductive benefits of curtailing the adrenocortical response are likely to be outweighed by the potential threat to fitness or mortality due to the chronic stressor.

Thus although there appears to be considerable interspecies variation in how, and if, stress responses are modulated with reproductive status, there is evidence of reciprocal relationships between the adrenal and gonadal axes in reptiles. Such results may reflect variations in circulating concentrations of gonadal steroids. Generally, oestrogens are stimulatory to adrenal function and androgens are inhibitory (see Handa et al., 1994a for review). For example, in female rats, increased basal CORT concentrations, as well as increased ACTH and CORT responses to stressors, are correlated with higher oestradiol concentrations (Kitay, 1963; Viau and Meaney, 1991; Burgess and Handa, 1992), but there is only indirect evidence that oestrogens may stimulate adrenal activity in reptiles. In female *Uta stansburiana* lizards, peak CORT concentrations are correlated with increased gonadal mass (Wilson and Wingfield, 1992), and in females of the viviparous *Lacerta vivipara*, plasma CORT varies significantly with reproductive status, while no significant variation is observed for males (Dauphin-Villemant et al., 1990).

Building on previous work on adrenal function in the viviparous skink *E. whitii* (Jones and Bell, 2004; Cartledge et al., 2005), we aim to establish whether reproductive status modulates adrenocortical activity in females of this species, and to investigate the endocrine mechanisms that may potentiate differential secretion of CORT between reproductive stages. We therefore compare the changes in plasma CORT concentration in response to the acute stress of capture in females of *E. whitii* at three reproductive stages: post-ovulatory, gestating and postpartum. We hypothesise that, due to their current reproductive investment, both post-ovulatory and gestating (carrying well-developed embryos) females will down-regulate their acute stress response compared with postpartum females, and that gestating females will show the most marked reduction. We extend the comparison to reproductively quiescent males collected at the same time as the postpartum females (Bell, 1997). A comparison of stress responses in males and females when both are reproductively quiescent is included to determine if there are integral differences in the stress responses of the sexes even when the influence of reproductive steroids should be minimal.

To investigate whether the adrenal response to acute capture stress reflects the maximal secretory capacity of the adrenal glands in this species, we assess the adrenocortical response to exogenous ACTH in reproductively quiescent females and males; however, post-ovulatory and gestating females are not tested due to concerns that ACTH stimulation may result in extraordinarily high CORT levels, capable of disrupting development of embryos. The influence of elevated plasma steroids on adrenocortical function in female *E. whitii* is investigated by treating reproductively quiescent females with oestrogen, and measuring plasma concentrations of CORT before and after injection of ACTH. We chose to use oestradiol-17β because this steroid is at the end of the steroidogenic pathway, and because the mammalian literature (see Handa et al., 1994a) provides direct evidence that oestradiol-17β interacts with the HPA axis. We hypothesise that oestrogen-treated females will exhibit increased basal plasma CORT, and an increased response to exogenous ACTH.

2. Methods

2.1. Animals

Sexually mature (>65 mm snout-vent length) female and male *Egernia whitii* were collected from a single site at Orford, Tasmania, Australia (42°34′S, 147°52′E) during their active season, the austral spring/summer, October through March (1999–2000). Females were collected at times of year corresponding to the following stages of their reproductive cycle: (1) post-ovulation (early-November) (2) gestation (late-January) and (3) post-partum (early-March): post-partum females were considered to be reproductively quiescent and the term "gestation" herein refers to females carrying well formed embryos in the third trimester (Hickman, 1960; Milton, 1987; Chapple, 2005). Males were collected in late March, when they are reproductively quiescent (Milton, 1987). Males were preliminarily identified by their wider heads and sex was then confirmed by the presence of eversible hemipenes (Chapple, 2005). Females were palpated to confirm their reproductive status similar to Chapple (2005) and Chapple and Keogh (2005). Post-partum females were also quite obvious by their reduced body weight and flaccid appearance following birth, and follicles were easily detected in the abdomens of post-ovulatory females.

Animals were captured by "fishing" with mealworms and depositing the animal in a bucket. Blood samples of approximately 100 μL were collected by gently inserting a heparinised capillary tube into the sub-orbital sinus and allowing the blood to drip out into a small vial. Blood samples were placed upon ice until return to the laboratory where plasma was separated from cells by centrifugation and stored at −20 °C until assay for CORT by radioimmunoassay (RIA) (see below).

2.2. Acute capture stress

This experiment was conducted in the field. Twenty females were caught at each reproductive stage and 20 reproductively quiescent males were also captured. Blood samples were taken immediately after capture for five animals of each group. If the blood sample was not completed within 5 min of capture, that lizard was not included in the experiment. The remaining animals were held individually in small cloth bags in a shady position for 10, 60, or 240 min (five animals per group) prior to the blood sample being taken. To avoid the influence of possible daily cyclic variation in plasma CORT concentrations, catching of lizards was timed so that all post-confinement blood sampling occurred within the same two-hour period (14:00–16:00). To avoid recapture of the same individual, no animals were released until all blood samples had been taken.

2.3. The ACTH stimulation test

The ACTH stimulation test was conducted in the field in March, using reproductively quiescent (post-partum) females and males. The aim of this protocol was to assess the maximum secretory capacity of the adrenal gland. Blood samples were taken from 10 female and 10 male *E. whitii* immediately upon capture. Lizards were alternately assigned to ACTH or saline injection as caught. Five lizards of each sex were injected intramuscularly (forelimb) with 2.5 IU ACTH in 25 μL (Syncanthen Depot, Novartis), while five control lizards of each sex were injected with 25 μL buffered lizard Ringer's solution. All lizards were then held in individual cloth bags for a period of four hours. This period was chosen because this species' adrenocortical response to acute stress has been shown to plateau at four hours (Jones and Bell, 2004; this study). At the conclusion of the four-hour period, a second blood sample was taken, and lizards were released at their point of capture. All blood samples were placed on ice until return to laboratory where samples were centrifuged and plasma separated and stored for CORT assay as detailed below.

2.4. The influence of exogenous oestrogen on adrenal function

Fifteen post-partum females were caught in March. A blood sample was collected immediately after capture, and the animals were palpated to ensure they were not still gestating (no gestating females were caught). They were placed into individual cloth bags for transportation back to the laboratory where they were housed individually in plastic containers (30 × 20 × 10 cm). Each cage contained a basking site located under a 25 W light globe on a 13 h ON:11 h OFF cycle. Lizards were provided with a retreat, and the cage substrate consisted of paper pellets to a depth of approximately 2 cm. Lizards were fed three times per week on a diet consisting predominantly of cat food supplemented by tenebrionid larvae (mealworms) and water was available *ad lib*. The room air temperature was maintained at 20 °C and fluorescent overhead lighting was set to 13 L:11 D, mimicking the natural photoperiod. All females were held under these conditions in the laboratory for one week prior to the commencement of the experiment, to allow recovery from the increased adrenal activity associated with the stress of capture and housing. Previous work indicates that, in this species, one week is sufficient for CORT levels to return to baseline (Cartledge et al., 2005).

After one week of confinement, a blood sample was collected and females were randomly assigned to one of three treatment groups:

(i) Saline + ACTH ($n = 5$). Females in this treatment group were administered 50 μL of 0.7% saline intraperitoneally on three consecutive days. On the fourth day females were administered the ACTH stimulation test as described in the previous section.
(ii) Oestrogen + ACTH ($n = 5$). Females in this treatment group received an intraperitoneal injection of 200 ng oestrogen in 50 μL 0.7% saline on three consecutive days. On the fourth day these animals were administered the ACTH stimulation test.
(iii) Oestrogen + saline ($n = 4$). To control for the likely increase in CORT concentration due to the stress of injection (as separate from the stimulatory effects of ACTH injection), females in this treatment group received an intraperitoneal injection of 200 ng oestrogen in 50 μL of 0.7% saline on three consecutive days. On the fourth day females received an intramuscular injection of 25 μL saline instead of ACTH, and four hours later a second blood sample was taken.

These treatment groups allowed a comparison of plasma CORT concentrations in low oestrogen (saline injected) against high oestrogen (oestrogen injected) females; and a comparison of the CORT responses to ACTH in both high oestrogen and low oestrogen females. In the skink *Tiliqua nigrolutea*,an equivalent dose and injection regime of oestrogen resulted in elevated plasma oestrogen concentrations after four days of treatment (Edwards, 1999). Females treated with oestrogen were held in a separate room to saline treated controls to minimise the possibility of

cross contamination or secondary pheromonal effects due to the oestrogen treatment.

2.5. Corticosterone radioimmunoassay

The radioimmunoassay is described by Jones and Bell (2004). Briefly, CORT was extracted from plasma aliquots (25–50 μL) with 500 μL A.R. grade absolute ethanol; duplicate aliquots of 200 μL of ethanol extract were assayed. Duplicate standards (0, 25, 50, 100, 200, 400 and 800 pg authentic CORT) and sample extracts were dried down, and 100 μL of antiserum (Endocrine Services) and 100 μL of tritiated CORT in phosphate buffered saline were added to each tube. Tubes were incubated at 4 °C overnight, and the free and bound fractions separated with dextran-coated charcoal.

Validation of this assay for this species was carried out by Jones and Bell (2004). The extraction efficiency was 100% and the amount of hormone that can be determined as statistically different from the zero tubes was found to be 0.5 ng/mL plasma. The intra-assay coefficient of variation was 11.2%, and the inter-assay variation was 3.4% ($n = 5$). Serial dilutions of lizard plasma ran parallel to the standard curve tested over 3–17 ng/mL plasma (Jones and Bell, 2004).

2.6. Statistical analyses

The effects of reproductive stage (females: post-ovulatory, gestating, post-partum) and time held captive upon plasma CORT concentration during the acute stress protocol were tested using a factorial design in which females of all reproductive stages were held captive for all periods of restraint. This design permitted two-way analysis of variance (ANOVA) within each reproductive state and between reproductive states for each period of restraint. Variances of mean plasma CORT concentrations were homogeneous ($F_{11,44} = 0.410$, $p > 0.5$). Following the ANOVA, a plot of Studentised residuals against predicted values was examined to check the normality of the residuals. When the ANOVA indicated significant effects, post-hoc pair-wise comparisons by Tukey's multiple range tests were conducted to investigate which individual groups differed significantly.

Similarly, the responses of reproductively quiescent males were compared to post-partum females using two-way ANOVA with the factors being sex and time held captive. Post–hoc pair-wise comparisons were made within each sex using Student–Neuman–Keuls (SNK) tests. At each time, mean plasma CORT concentrations were compared between males and females using *t*-tests.

When testing the effect of ACTH injection, the dependent variable analysed was the change in plasma CORT concentrations pre- and post-injection of ACTH. Two-way ANOVA was employed to compare plasma CORT concentrations as a function of the within-groups factor, injection type (experimental or control) and between-groups factor, sex. When the ANOVA indicated significant effects, post–hoc pairwise comparisons (SNK tests) were conducted to reveal the points of significant difference between groups.

In the oestrogen injection experiment, repeated-measures analysis of variance was employed to make comparisons within each treatment group (saline + ACTH, oestrogen + ACTH, oestrogen + saline) of CORT concentrations at time zero, one week, following priming and following ACTH or saline injection. SNK was used for post-hoc comparisons. To compare between treatment groups at each of these times, separate one-way ANOVA were employed.

Statistical analyses were conducted using the softwares StatistiXL v1.4 (www.statistixl.com) and SAS. In all statistical tests, $p < 0.05$ was considered significant.

3. Results

3.1. Acute stress response

The initial mean plasma CORT concentration did not differ between reproductive stages ($F_{2,12} = 1.17$, $p > 0.3$). However, two-way ANOVA indicated that the profile of the acute stress response over the 240 min period differed between the reproductive stages (reproductive stage × time restrained interaction, $F_{6,60} = 2.72$, $p < 0.03$) (Fig. 1). Post-ovulatory and gestating females exhibited significant elevations in plasma CORT concentration following both 10 min and 1 h of confinement (post-ovulatory: $F_{3,14} = 9.17$, $p < 0.01$, gestating: $F_{3,15} = 4.92$, $p < 0.02$); however, at 4 h mean plasma CORT concentrations were no longer significantly different from initial values in either reproductive state. The mean rise in plasma CORT concentration following the first 10 min of restraint was significantly greater in post-partum than either post-ovulatory or gestating females ($F_{2,12} = 8.04$, $p < 0.007$). For post-partum females the mean plasma CORT concentrations at 1 h and 4 h were in fact not significantly below that of 10 min. Reproductively quiescent males exhibited plasma CORT concentrations consistently higher than those of post-partum females, but the difference was significant only at 10 min ($F_{1,8} = 8.89$, $p < 0.02$). Concentrations of plasma CORT varied significantly through time for both sexes ($F_{3,16} = 12.489$, $p < 0.001$). Reproductively quiescent males exhibited significantly higher plasma CORT concentrations than at capture at 10 min ($p < 0.01$), 1 h ($p < 0.01$), and 4 h ($p < 0.01$) as compared with time zero.

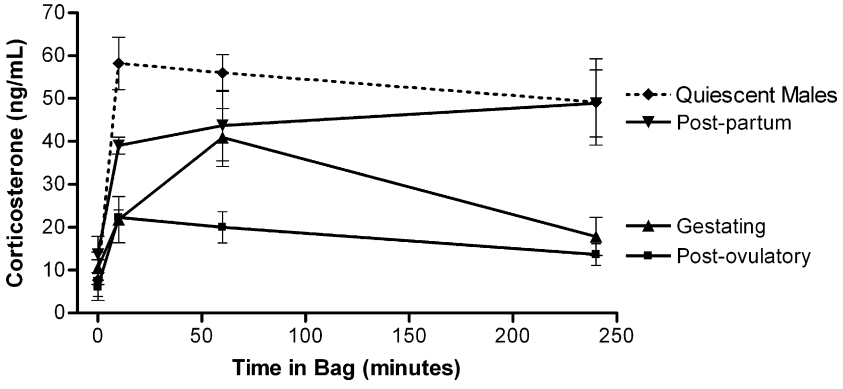

Fig. 1. Profile of plasma corticosterone concentration (ng/mL) over 4 h of acute capture stress in *Egernia whitii*. This investigation was carried out in females of three reproductive states: post-ovulatory, gestating and post-partum and in reproductively quiescent males. Values are mean ± SE (20 females/males per reproductive state, i.e. $n = 5$ per experimental group).

Table 1
Plasma CORT concentration changes in male and female *Egernia whitii* in response to ACTH (2.5 IU) versus saline injection

	Treatment	Plasma CORT concentration (ng/mL ± SE)		
		Initial	Post-injection	Difference
Males	Saline control	10.4 ± 2.18	29.5 ± 8.06	19.1 ± 9.36
	ACTH (2.5 IU)	12.2 ± 3.40	52.1 ± 12.6	39.9 ± 6.72
Females	Saline control	9.5 ± 4.41	31.1 ± 11.07	21.6 ± 5.90
	ACTH (2.5 IU)	2.2 ± 1.78	59.4 ± 17.14	57.2 ± 9.13*

Plasma CORT concentrations (ng/mL ± SE) pre- and post-injection are presented along with the difference. Five lizards were included in each treatment group.
* $p < 0.05$, significantly different from female controls.

3.2. Adrenocorticotropic hormone (ACTH) stimulation test

Both reproductively quiescent male and female *E. whitii* responded to saline injection with a rise in plasma CORT (Table 1). ACTH injection also induced a rise in plasma CORT concentration in reproductively quiescent males, but this was not significantly different from the response to saline injection ($F_{1,9} = 3.2$, $p > 0.1$). In contrast, the rise in plasma CORT levels exhibited by reproductively active females in response to ACTH injection was significantly greater than in saline-injected controls ($F_{1,8} = 10.8$, $p < 0.05$).

3.3. The influence of exogenous oestrogen on adrenal function

Mean plasma CORT concentrations in females at the four stages of the oestrogen experiment: immediately following capture, following one week of confinement, following four days of priming with oestrogen or saline, and then lastly on the 12th day either administration of the ACTH stimulation test or otherwise a control saline injection are shown in Fig. 2. One-way ANOVA revealed that mean plasma CORT concentrations did not vary significantly between females at time zero ($F_{2,12} = 0.84$, $p > 0.4$) or one week ($F_{2,12} = 3.28$, $p > 0.07$), or between females primed with oestrogen or saline ($F_{2,12} = 3.39$, $p > 0.07$).

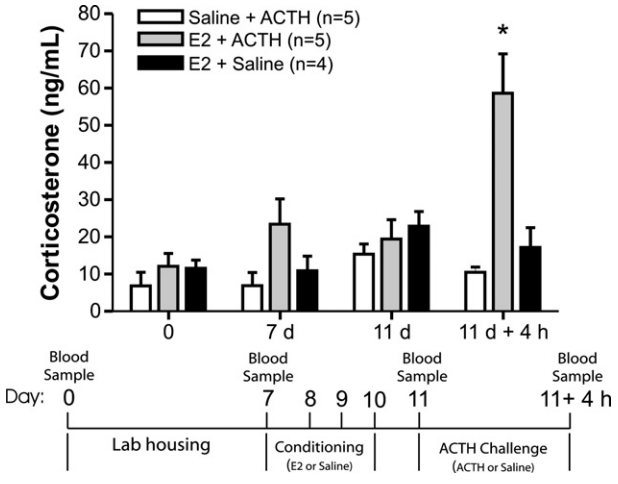

Fig. 2. Plasma corticosterone concentrations of post-partum females upon capture in the field, after 7 d confinement, and following priming with either oestrogen (200 ng/lizard/day) or saline (0.7%) depending on treatment group. Females were primed with either saline (injection control) or oestrogen and then given either the ACTH stimulation test or a saline injection (experimental control). Values are mean ± SE. *Indicates significant difference from 11 d (pre-injection) concentration.

Comparisons within treatment groups over time revealed no significant variation in CORT concentration over the course of the experiment for females in the oestrogen + saline treatment group ($F_{3,12} = 1.957$, $p > 0.17$) or the saline + ACTH treatment group ($F_{3,16} = 1.819$, $p > 0.1$). However, females in the oestrogen + ACTH treatment did display significant variation in CORT between times ($F_{3,16} = 8.712$, $p < 0.01$) with concentrations post-ACTH injection higher than pre-injection ($p < 0.01$).

4. Discussion

In this study we compared the acute adrenal stress response in female *E. whitii* at three reproductive stages: post-ovulation, gestation, and post-partum, to test the hypothesis that, in this viviparous lizard, gestating females will exhibit a reduced adrenocortical response compared with post-ovulatory and post-partum females. Basal plasma CORT concentrations did not differ significantly between post-ovulatory, gestating and post-partum females in *E. whitii*. CORT is a hormone with catabolic effects associated with increased energy requirements (Greenberg and Wingfield, 1987; Bentley, 1998). The lack of variation in basal circulating CORT concentrations in female *E. whitii* at different reproductive stages indicates that females are exhibiting tight regulation over CORT levels even in the presence of differing energetic demands.

However, studies investigating the natural fluctuations in plasma corticosterone through the annual cycle in female reptiles have generally failed to demonstrate a consistent pattern of variation with reproductive condition. For example, in the oviparous lizard *U. stansburiana* plasma CORT levels are positively correlated with the relative masses of reproductive tissue (Wilson and Wingfield, 1992, 1994), whereas in the parthenogenetic lizard *Cnemidophorus uniparens* plasma CORT concentrations decline during vitellogenesis and ovulation (Grassman and Crews, 1990). The oviparous lizard *C. sexlineatus* shows no significant variation in plasma corticosterone over the reproductive season (Grassman and Hess, 1992b). Similarly, females of the viviparous common gecko *H. maculatus* show no significant difference in plasma CORT concentrations among

four stages of reproduction (mid/late vitellogenic, mid-pregnant, late pregnant prior to winter dormancy and spent) (Girling and Cree, 1995). Thus, the way in which plasma CORT concentration varies with reproductive condition of females shows considerable variation both within and between viviparous and oviparous lizard species.

Reproductively quiescent (post-partum) females exhibited the most marked immediate response, and the most prolonged response, to acute capture stress. Post-ovulatory females displayed the most conservative adrenocortical response to acute capture, with plasma CORT concentrations lower at all times than in the other two groups of females. In the gestating females, mean CORT concentrations were high (41 ng/mL) at 1 h, similar to those in post-partum females. However, both groups of reproductive females (gestating and post-ovulatory) displayed a recovery by 4 h post-capture, with plasma CORT concentrations at this time no longer significantly different from initial levels: this was particularly marked for gestating females. Therefore, although the gestating females did exhibit initial adrenal responsiveness, their response was attenuated, suggesting the presence of a mechanism to reduce the duration of the acute stress response. This may represent an adaptation to mitigate negative impacts of chronically elevated levels of CORT on gestation: a suite of papers demonstrate that in *L. vivipara* treating gestating females with exogenous CORT does influence both offspring phenotype and dispersal behaviour (e.g. Meylan et al., 2002; Belliure et al., 2004; Meylan and Clobert, 2005). Painter et al. (2002) suggest that placental buffering means that maternal steroids have little influence on foetal steroid levels in the viviparous lizard *Sceloporus jarrovi*. The presence of mechanisms for placental buffering of foetal steroid concentrations may, therefore, in part explain why gestating females of *E. whitii* exhibited a moderate, though attenuated, stress response, while post-ovulatory females, in which the chorioallantoic placentae have not yet developed, exhibited a more marked suppression of the acute stress response. In contrast, in the viviparous gecko *H. maculatus*, the adrenocortical response to acute capture in gestating females is as great as that in vitellogenic females (Cree et al., 2003) and more recent work on gestating females of that species indicates that high CORT concentrations do not negatively affect the development of the young following birth (Preest et al., 2005). The authors propose that the capture-induced CORT levels are lower and of shorter duration than necessary to have deleterious effects on the embryos and also suggest that the extended period of gestation in this species may require reduced sensitivity to elevated corticosterone (Preest et al., 2005). Gravid females of the oviparous *U. ornatus* show no change in plasma CORT concentrations in response to an acute stressor, while vitellogenic females respond with a large increase (Woodley and Moore, 2002). An in vitro study of adrenal CORT production in the presence or absence of ACTH showed no difference between reproductive stages in the viviparous lizard *L. vivipara* (Dauphin-Villemant et al., 1990). These results demonstrate that further work is needed on modulation of the adrenocortical response with reproductive stage in females of oviparous and viviparous reptiles.

In comparison with females at all three reproductive stages, reproductively quiescent males of *E. whitii* exhibited the greatest increase in plasma CORT in response to the acute stressor. Furthermore, CORT concentrations then remained high over the 4 h period, a pattern similar to that in the post-partum females. This was unexpected as sex differences in adrenal stress responses have been clearly demonstrated in mammals, with the majority of work indicating that oestradiol stimulates CORT production while testosterone ameliorates the acute stress response (Handa et al., 1994a,b; Viau, 2002; Lund et al., 2004). Whilst very little is known for reptiles, subordinate male lizards of the species *Anolis carolinenesis*, having low plasma testosterone, exhibit a stronger adrenal stress response than dominant males (Greenberg et al., 1984). As our males were captured during their period of reproductive quiescence, plasma testosterone concentrations were low (Bell, 1997), which may explain why their adrenocortical response was greater than females.

Reproductively quiescent males and females responded similarly to exogenous ACTH. Plasma CORT concentrations increased to 52 and 59 ng/mL, respectively, but plasma CORT also increased in the saline- injected animals, reaching levels of around 30 ng/mL in both sexes, suggesting that the injection induced an acute stress response. Only in females was the CORT increase caused by ACTH injection significantly greater than that induced by saline injection alone, partly reflecting the considerable variation in response between individual males. Carsia and John-Alder (2003) found that isolated adrenal cells from males showed reduced sensitivity compared to females, suggesting a mechanism for differences in acute stress responses. In *E. whitii*, the plasma CORT concentrations induced by ACTH injection were comparable with the peak levels measured during the acute stress protocol. Given the supra-physiological dose of ACTH employed, this may indicate that this is the maximal concentration of plasma CORT that can be achieved in this species. Similarly, Preest et al. (2005) found that exogenous ACTH induced CORT concentrations (62 ng/mL) similar to the maximum (69 ng/mL) measured in pregnant *H. maculatus* following capture and confinement (Girling and Cree, 1995).

To examine the influence of oestrogen upon basal levels of plasma CORT and the response to ACTH, we treated captive females with oestrogen or saline for four days, followed by challenge with an injection of ACTH or saline. Plasma CORT concentrations did not vary between times or between treatments prior to the ACTH challenge, indicating that exogenous oestrogen did not influence basal CORT concentrations in this species. This result contrasts with that of Callard and Callard (1978), who found that oestrogen injection results in an increase in circulating concentrations of CORT in female *Dipsosaurus dorsalis* in both the reproductively active and inactive phases of their cycle.

Similarly, in mammals, exogenous oestrogen increases circulating concentrations of both cortisol and corticosteroid-binding proteins (Coe et al., 1986). However, ovariectomized lizards *C. uniparens*, in which plasma oestrogen concentrations were significantly decreased, show elevated levels of CORT in the plasma (Grassman and Crews, 1990), while ovariectomized *C. sexlineatus* exhibit plasma CORT concentrations that are not statistically different from those of intact females (Grassman and Hess, 1992a). Such disparate results suggest that adrenal–gonadal relationships in female reptiles are complex, reflecting the numerous interactions between CORT, reproductive hormones and metabolic processes (Guillette et al., 1995), and may display a high degree of inter-species variation.

In this study, female *E. whitii* treated with oestrogen responded to ACTH with a marked increase in plasma CORT (similar to females subject to the ACTH stimulation test directly following capture in the field). In contrast, females receiving the control saline injections in place of oestrogen injection showed no such large CORT response to ACTH injection. This may reflect adrenal exhaustion if these post-partum females were responding to the acute stress of each saline injection with a large and extended secretion of CORT, causing exhaustion of adrenal reserves of CORT over the four days of injection: there is little storage of corticosteroids in the adrenals of the lizard *S. undulatus* (Carsia and John-Alder, 2003). However, if the adrenals were becoming exhausted, we would expect to find undetectable levels of CORT in these saline-injected females at day four but instead levels were not significantly different from time zero. Alternatively, saline-injected females may have become desinsitised to ACTH during the four days injection period whilst sensitivity was maintained in oestrogen-treated females. In mammals, oestrogen can increase cortisol production by the adrenals (Kitay, 1963), and the response to ACTH is most pronounced when estrogen levels are highest, such as at pro-oestrous (Viau and Meaney, 1991). Indeed, in viviparous reptiles, oestrogen is highest during late vitellogenesis, and drops markedly during early gestation (e.g. Jones and Swain, 1996; Edwards and Jones, 2001), perhaps suggesting a mechanism for modulation of the stress response between reproductive phases.

In conclusion, our results support our hypothesis that, in comparison with reproductively quiescent females and males, pregnant females of the viviparous *E. whitii* down-regulate their acute stress response, presumably to protect their current reproductive investment, with post-ovulatory females showing the most reduced response. Circulating oestrogen concentrations also appear to modulate the response to exogenous ACTH, although our results are somewhat equivocal. The variation in results from the limited number of similar studies in reptiles shows that further work is required to tease out the mechanisms through which females of viviparous reptilian species may protect their developing embryos from the potentially damaging effects of elevated corticosteroids.

Acknowledgments

Dr. Ashley Edwards, Dr. Jane Girling, Natalia Atkins and Dr. Erik Waptstra kindly assisted with animal capture. We thank Dr. Stewart Ford for his helpful comments on the manuscript. All procedures were approved by the University of Tasmania Animal Ethics Committee (Approval no. 99064).

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