

Seasonal variation in hormonal responses of timber rattlesnakes (*Crotalus horridus*) to reproductive and environmental stressors

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Abstract Data addressing adrenocortical modulation across taxonomic groups are limited, especially with regard to how female reproductive condition influences the sensitivity of the hypothalamus–pituitary–adrenal axis. We investigated seasonal and reproductive variation in basal and stress-induced hormone profiles in a population of free-ranging timber rattlesnakes (*Crotalus horridus*) in north-central Pennsylvania during spring (i.e., May), summer (i.e., July), and early fall (i.e., September). Baseline corticosterone concentrations varied seasonally and were significantly lower during the summer sampling period in July. We observed a significant negative relationship between baseline corticosterone and testosterone in male snakes, while baseline corticosterone and estradiol tended to be positively correlated in females. Treatment of snakes with 1 h of capture stress significantly increased corticosterone across all seasons. However, there was a significant interaction between corticosterone responses to capture stress and season, suggesting that adrenocortical function is

modulated seasonally. Because elevated corticosterone may be associated with reproduction, we asked whether hormonal stress responses vary with female reproductive condition. Although sample sizes are low, reproductive snakes had significantly higher baseline and stress-induced corticosterone concentrations than non-reproductive or post-parturient females. Further, despite similar baseline corticosterone concentrations between non-reproductive and post-parturient rattlesnakes, post-parturient females responded to capture stress with a significantly higher increase in corticosterone. Collectively, these data suggest that the sensitivity of the hypothalamus–pituitary–adrenal axis varies both seasonally and with changing reproductive states.

Keywords Reproduction · Capture stress · Corticosterone · Testosterone · Estradiol

Introduction

Seasonal reproduction in animals is accompanied by both environmental and energetic challenges. Appropriate timing of gamete production, mating, gestation, and birth are crucial to reproductive fitness, and both males and females must have adequate energy stores to sustain these events. The hypothalamus–pituitary–adrenal (HPA) axis is an endocrine mechanism that functions in energy homeostasis and plays a particularly important role in seasonal reproduction (reviewed in Wingfield et al. 1998; McEwen and Wingfield 2003; Moore and Jessop 2003). The HPA axis aids in maintaining homeostasis in response to stressors via increased glucocorticoid secretion (Harvey et al. 1984; Schwabl et al. 1985; Wingfield 1988). Glucocorticoids in turn modify physiology and behavior to promote survival and suppress those processes not critical to immediate

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survival (e.g., Wingfield 1988; Sapolsky 1992; Pottinger 1999). For example, physiological stress responses generally result in decreased plasma concentrations of sex steroid hormones and/or suppression of reproductive behavior in many species (Wingfield 1988; Moore et al. 1991; Rivier and Rivest 1991; Carragher and Rees 1994; Coddington and Cree 1995; Moore et al. 2000a). Such acute physiological stress responses are normally adaptive responses used to modify metabolism and mobilize energy stores. Thus, activation of the HPA axis can potentially result in animals foregoing reproduction and other non-critical processes in favor of conserving energy for survival during stressful events.

Challenges to homeostasis are most commonly thought of as external noxious stimuli such as injury and predation events, but they also include natural physiological states such as the energetically demanding processes of reproduction (e.g., Moore and Jessop 2003; Cease et al. 2007). Such predictable and demanding life-history stages generally represent different physiological states (or set-points) in an animal. The process of allostasis allows many internal variables to be adjusted among these different set-points such that a particular physiological state (e.g., reproduction, migration) is maintained (e.g., McEwen and Wingfield 2003; Landys et al. 2006). For many animals, seasonal changes in physiology represent a modulation of their allostatic load. For example, seasonal elevation in glucocorticoid levels is adaptive during periods of high energy demand. A correlation between periods of high activity and elevated baseline glucocorticoid levels has been described in many vertebrates, especially with relation to avian migratory flight (e.g., O'Reilly and Wingfield 1995; Holberton 1999; Piersma et al. 2000; Reneerkens et al. 2002; Landys-Ciannelli et al. 2002) and male anuran vocalization (Emerson and Hess 2001; Leary et al. 2004). Further, glucocorticoids are elevated during the breeding season in several amphibians and reptiles (Wilson and Wingfield 1992, 1994; Zerani and Gobbetti 1993; Schramm et al. 1999; Moore et al. 2001; also see reviews by Romero 2002; Moore and Jessop 2003).

Despite much research utilizing physiological stress responses as a paradigm for understanding how animals cope with environmental and energetic stressors, data addressing seasonal modulation of adrenocortical function across taxonomic groups are limited. There is also a lack of research investigating physiological stress responses in female vertebrates, especially with regard to how varying reproductive condition influences the sensitivity of the HPA axis (e.g., Woodley and Moore 2002). Further, studies investigating the female aspects of stress biology are often conducted in different years than the complement studies addressing similar questions in males (e.g., Whittier et al. 1987; Moore et al. 2000a), making intersexual comparisons

difficult. The need for such data is emphasized by the knowledge that both baseline and stress-induced glucocorticoid production varies both seasonally and annually (e.g., Moore and Jessop 2003; Lutterschmidt and Mason 2005).

In the present study, we investigated seasonal variation in baseline corticosterone concentrations (the primary glucocorticoid in reptiles; Idler 1972), sex steroid hormones, and the adrenocortical stress response in a population of timber rattlesnakes (*Crotalus horridus*). Although there is extensive information on the seasonal reproductive cycles of rattlesnakes from behavioral, morphological, and histological studies (e.g., Brown 1991; Martin 1992b, 1993, 1996; Graves and Duvall 1993; Aldridge and Brown 1995; Martin 1996; Goldberg 1999; Goldberg and Rosen 2000; Mero and Aubertin 2005; Taylor and Denardo 2005), there are fewer data on the endogenous annual hormone cycles of free-ranging populations (Taylor et al. 2004; Schuett et al. 2006). Most importantly, there are no studies of reproductive endocrinology or stress biology in timber rattlesnakes (*C. horridus*), a species of concern in much of its northern distributional range (Brown 1993). To obtain an endocrinological framework for future research, we examined the following questions in timber rattlesnakes: (1) do baseline and stress-induced hormone profiles vary among spring (May), summer (July), and fall (September) seasons?; (2) is there a relationship between sex steroid and corticosterone hormone profiles?; (3) does body condition influence hormone profiles?; and (4) does female reproductive condition influence baseline and stress-induced hormone profiles?

Materials and methods

Study site and animals

This study was conducted on a population of timber rattlesnakes (*C. horridus*) within the Tiadaghton State Forest (Lycoming County) of north-central Pennsylvania. Timber rattlesnakes are large-bodied, long-lived pit vipers (Viperidae) that generally demonstrate low fecundity and triennial reproduction within their northern distribution (Brown 1991; Martin 1993; Clark et al. 2003). The ecology, behavior, and some aspects of the physiology of timber rattlesnakes have been extensively studied (e.g., Reinert 1984; Reinert et al. 1984; Reinert and Rupert 1999; Wills and Beaupre 2000; Beaupre 2001; Zaidan and Beaupre 2003; Clark 2004), making them an excellent experimental model. Secondly, conservation concerns and the vulnerable status of *C. horridus* create urgency for understanding how timber rattlesnakes respond hormonally to reproductive and environmental stressors. All experimental protocols were supported and conducted through The College of New

Jersey's Institutional Animal Care and Use Committee (Animal Care and Use Procedure #0406-008) and were in accordance with the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals". The collection of specimens was performed under the authority of a Pennsylvania Fish and Boat Commission Scientific Collector's Permit No. 042-1 (to HKR).

Experimental design

We investigated seasonal variation in the effects of capture stress on corticosterone, testosterone, and estradiol as part of a larger investigation evaluating the influences of timbering practices on the ecology, movement patterns, and mortality of rattlesnakes. A total of 307 rattlesnakes were collected, measured, and PIT-tagged for population census. A randomly-selected subset ($n = 67$) of this population was surgically implanted with radio-transmitters (Reinert and Cundall 1982) and monitored with radio-telemetry between spring 2002 and fall 2006. From this pool of 67 telemetered rattlesnakes, we collected blood samples from a total of 26 individuals on 5–7 May (i.e., spring; $n = 9$), 15–16 July (i.e., summer; $n = 8$), and 17–18 September 2004 (i.e., early fall; $n = 9$). Free-ranging rattlesnakes were selected arbitrarily for bleeding based upon accessibility.

Once an individual was located with radio-telemetry, the rattlesnake was coerced to crawl into a clear plastic tube and then secured at midbody for safe handling. Immediately upon capture a blood sample was obtained from the basal rattle as described by Reinert and Bushar (1991). The procedure of collecting, tubing, and obtaining a 300–500 μ l blood sample from the snake's basal rattle was completed in approximately 2 min (mean = 124.1 s \pm 10.6 SE) to establish an initial hormone baseline in which samples represent pre-stress steroid concentrations (e.g., see reviews by Sapolsky et al. 2000; Wingfield and Romero 2001; Romero 2002 and references cited therein). To investigate seasonal variation in the influence of hormonal stress responses on corticosterone and sex steroid hormone concentrations, we subjected both male and female rattlesnakes to a capture-stress protocol similar to Moore et al. (2001). Rattlesnakes were placed individually in opaque cloth snake bags (Fuhrman Diversified Inc.) and kept individually in a white 23-l bucket for 1 h. Immediately following the 1 h capture stress treatment, each rattlesnake was again tubed and a second blood sample was collected to examine hormonal responses to capture stress.

Individual snakes sampled in this study were subjected to the capture-stress protocol one time either during spring (May), summer (July), or fall (September) sampling periods. Thus, the 26 snakes sampled during this study represent 26 independent pairs of pre- and post-stress hormone samples that were used to statistically investigate seasonal

variation in hormonal stress responses. Morphometric measures were collected for analysis of body condition before rattlesnakes were released at their site of capture. Body condition was calculated as the residual from a regression of body mass on snout-to-vent length for 176 snakes (which included the 67 radiotelemetered individuals as well as the 26 snakes from which blood samples were collected) across both sexes and all seasons similar to Moore et al. (2001) and Cease et al. (2007). We used this large population sample of 176 snakes in our regression analysis to ensure that the relationship between body mass and snout-to-vent length was robust, representative of the population, and not heavily influenced by any one particular group of snakes and/or season. We then compared the resulting individual residuals to determine if body condition varies with sex, season, and hormone concentrations.

An advantage to conducting this experiment as part of a radiotelemetry study is that blood samples were collected during a narrow 2-day window of field monitoring and can be used to represent discrete spring (May), summer (July), and fall samples (September), respectively. Furthermore, because this experiment was part of a long-term telemetry study, frequent observation of free-ranging individuals over multiple activity seasons allowed us to monitor and characterize female reproductive condition. This is particularly critical for examining if the sensitivity of the HPA axis varies among female reproductive condition, as female timber rattlesnakes within this population demonstrate a triennial reproductive pattern. The typical triennial reproductive cycle is characterized by (1) females that are non-reproductive but receptive in the first activity season, with mating of receptive females occurring in mid to late summer; (2) females that are reproductive and gravid in the second activity season, with females giving birth to a brood of young prior to entering hibernation; and (3) females that are post-parturient and unreceptive to mating in the third activity season. Thus, we categorized female reproductive condition as non-reproductive, reproductive, or post-parturient. Non-reproductive females were those females that were neither gravid nor post-parturient during the active season. Reproductive females were those females that were gravid during the active season and gave birth to a brood of young prior to entering hibernation. Finally, post-parturient females were those females having an activity season after giving birth to a brood of young during the previous active season. In contrast to female reproductive patterns, mating behavior of male timber rattlesnakes occurs annually during the summer and coincides with spermatogenesis. All individual snakes used in this experiment were radio-tracked for a minimum of 3 months prior to measuring hormone concentrations.

Blood sampling and radioimmunoassay

Blood samples were collected from the basal rattle using heparinized 1-cm³ syringes and 22-g needles. Blood samples were stored on ice in the field until return to the field station, where they were centrifuged and the plasma separated. Both plasma and red blood cells were immediately frozen at -15°C for hormone assays and further genetic analyses, respectively. Plasma samples were then stored at -70°C until analyzed for corticosterone and sex steroid hormone concentrations following radioimmunoassay procedures similar to Moore et al. (2000a) and Lutterschmidt et al. (2004).

For individual sample recovery determination, 50 μl aliquots of each plasma sample were incubated 12–24 h with 2,000 cpm of both tritiated corticosterone and the appropriate sex steroid hormone (Amersham Biosciences, Piscataway, NJ). Steroids were extracted from each plasma sample twice with 2 ml anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas in a warm (37°C) water bath. Hormone extracts were then reconstituted in 10% ethyl acetate in isooctane and chromatographed through individual celite microcolumns. Neutral lipids and steroid fractions were eluted using increasing proportions of ethyl acetate in isooctane. The purified eluates were dried under nitrogen gas and reconstituted in 500 μl phosphate-buffered saline for assay.

Individual sample recoveries were determined from a 50 μl aliquot of each extracted and reconstituted sample. The remaining sample was allocated to two duplicate culture tubes for assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were then incubated with 100 μl tritiated steroid (1,2,6,7-³H corticosterone, 1,2,6,7-³H testosterone, or 2,4,6,7,16,17-³H Oestradiol, Amersham Biosciences, Piscataway, NJ). Samples, maximum binding tubes, and the standard curve dilutions were also incubated with 100 μl antiserum at 4°C for 12–24 h (corticosterone antibody B3-163 from Esoterix Endocrinology, Calabasas Hills, CA; testosterone antibody T-3003 and estradiol antibody E-6006 from Wein Laboratories, Inc., Succasunna, NJ). Unbound steroid was separated from bound hormone using dextran-coated charcoal. The bound steroid was decanted into scintillation vials and incubated in toluene-based scintillation fluid for 12 h. The radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter.

All samples were corrected for individual recovery variation. Mean extraction efficiency was 92.0% for testosterone (fourth fraction using partition chromatography), 71.3% for estradiol (fifth fraction), and 57.4% for corticosterone (sixth fraction). Samples were analyzed within a single assay with an intra-assay coefficient of variation of

14.0% for testosterone, 7.9% for estradiol, and 15.4% for corticosterone.

Statistical analysis

Seasonal variation in baseline (i.e., pre-stress) hormone concentrations was first investigated using analysis of variance (ANOVA). We used repeated measures ANOVAs followed by Tukey's multiple comparisons procedures to investigate the effects of season, sex, and reproductive condition (i.e., the between-subjects factors) on pre- versus post-stress hormone profiles (i.e., the within-subjects or repeated factor). To ensure that body size did not differ among snakes sampled during the three different seasons, we compared the body mass and snout-to-vent length of male and female snakes using one-way ANOVAs. Because body condition is known to be much more closely associated with changes in hormone profiles (e.g., Husak and Moore 2008), we examined differences in body condition (calculated as the residuals from a regression of body mass on snout-to-vent length) between sexes and among seasons using a two-way ANOVA followed by a Tukey's multiple comparisons procedure. We used a Model-II principal axis regression (Sokal and Rohlf 1995) to investigate the relationship between sex steroid and corticosterone hormone profiles as well as the relationship between body condition and steroid hormone profiles. A Model-II principal axis regression was required for these analyses because the x -axis variable (i.e., corticosterone concentrations) is a measured variable, rather than a pre-set or determined x -axis variable (Sokal and Rohlf 1995). All assumptions for parametric comparisons were met by transforming data where necessary. Results were considered significant at $P < 0.05$. We used SigmaStat 3.11 (Systat 2005) and SPSS 15.0 (SPSS 2006) for all statistical analyses.

Results

Question 1: Do baseline and stress-induced hormone profiles vary seasonally?

The results from a two-way ANOVA indicate that baseline corticosterone concentrations did not vary significantly between male and female snakes (main effects of sex). Thus, within each season, we collapsed sex and reanalyzed these data using a one-way ANOVA to examine possible seasonal variation in pre-stress corticosterone concentrations. Baseline corticosterone concentrations varied significantly with season, with lower concentrations observed during the summer (July) sampling period (Fig. 1a; $F = 5.182$, $df = 2$, $P = 0.014$, from a one-way ANOVA followed by a Tukey's multiple comparisons test). Neither

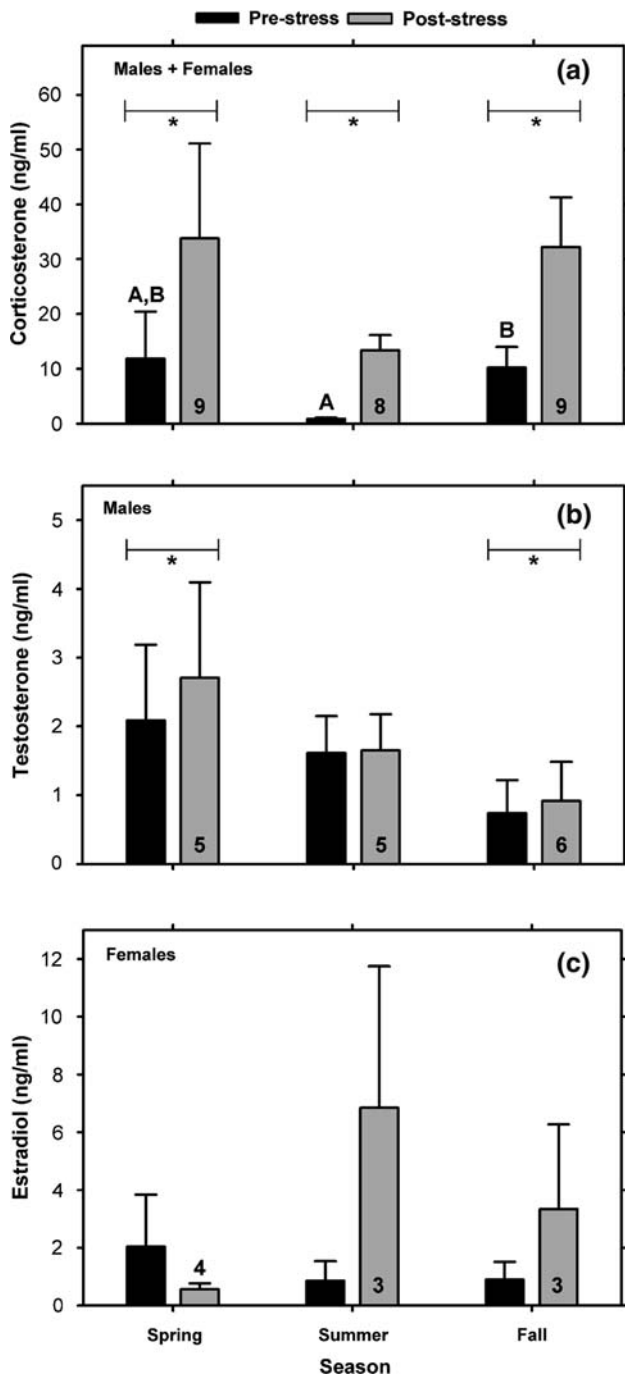


Fig. 1 Seasonal variation in hormonal responses of timber rattlesnakes (*Crotalus horridus*) to 1 h of capture stress treatment. **a** Mean plasma corticosterone concentrations are shown for male and female snakes combined as there were no sex differences in either baseline or stress-induced corticosterone concentrations; **b** mean plasma testosterone concentrations of male rattlesnakes; and **c** mean plasma estradiol concentrations of female snakes. Standard errors (+1) are shown by the vertical lines. Sample sizes within each season are listed above the x-axes. Capital letters above the standard error bars indicate significant differences in pre-stress (i.e. baseline) hormone concentrations across seasons (from a one-way ANOVA). Statistically significant effects of capture stress treatment on hormone concentrations within each season are indicated by an asterisk below the horizontal brackets. Significant seasonal differences in corticosterone responses to capture stress are not shown due to a statistically significant interaction between pre- versus post-stress sampling time and season (from a three-way repeated measures ANOVA)

($F_{1,20} = 1.875, P = 0.186$). We observed a statistically significant effect of season on corticosterone responses to capture stress ($F_{2,20} = 4.406, P = 0.026$). Further, there was a statistically significant interaction between pre- versus post-stress sampling time and season ($F_{2,20} = 5.945, P = 0.009$). All other interaction terms were not statistically significant.

Capture stress treatment also significantly increased testosterone concentrations of male snakes (Fig. 1b; $F_{1,31} = 9.127, P = 0.010$ from a two-way repeated measures ANOVA followed by a Tukey’s multiple comparisons test). Season did not significantly influence testosterone responses to capture stress (Fig. 1b; $F_{2,31} = 0.905, P = 0.429$, main effects of season from a two-way repeated measures ANOVA). Despite the lack of a main effect of season on testosterone responses to capture stress, the results of a Tukey’s multiple comparisons procedure indicate a significant difference between pre- and post-stress testosterone levels within the spring (May) and fall (September) seasons only. In contrast, neither capture stress ($F_{1,19} = 1.778, P = 0.224$) nor season ($F_{2,19} = 0.580, P = 0.585$) significantly influenced plasma estradiol concentrations of female snakes (Fig. 1c; from a two-way repeated measures ANOVA). There were no statistically significant interactions between pre- versus post-stress sampling time and season for either male or female snakes.

baseline testosterone (Fig. 1b; $F = 0.923, df = 2, P = 0.422$) nor estradiol (Fig. 1c; $F = 0.253, df = 2, P = 0.783$) concentrations of male or female snakes, respectively, varied significantly with season (results from a one-way ANOVA for each hormone).

Snakes responded to capture stress with a statistically significant increase in plasma corticosterone across all seasons (Fig. 1a; $F_{1,20} = 150.165, P < 0.001$, from a three-way repeated measures ANOVA). Corticosterone responses to capture stress did not vary between male and female snakes

Question 2: Is there a relationship between sex steroid and corticosterone hormone profiles?

We found a significant negative relationship between baseline (i.e., pre-stress) corticosterone concentrations and baseline testosterone concentrations in male snakes (Fig. 2a; $F_{1,14} = 7.809, P = 0.014; r^2 = 0.358$ from a Model-II reduced major axis regression). Baseline corticosterone concentrations were not significantly related to baseline estradiol concentrations in female snakes (Fig. 2b). In male

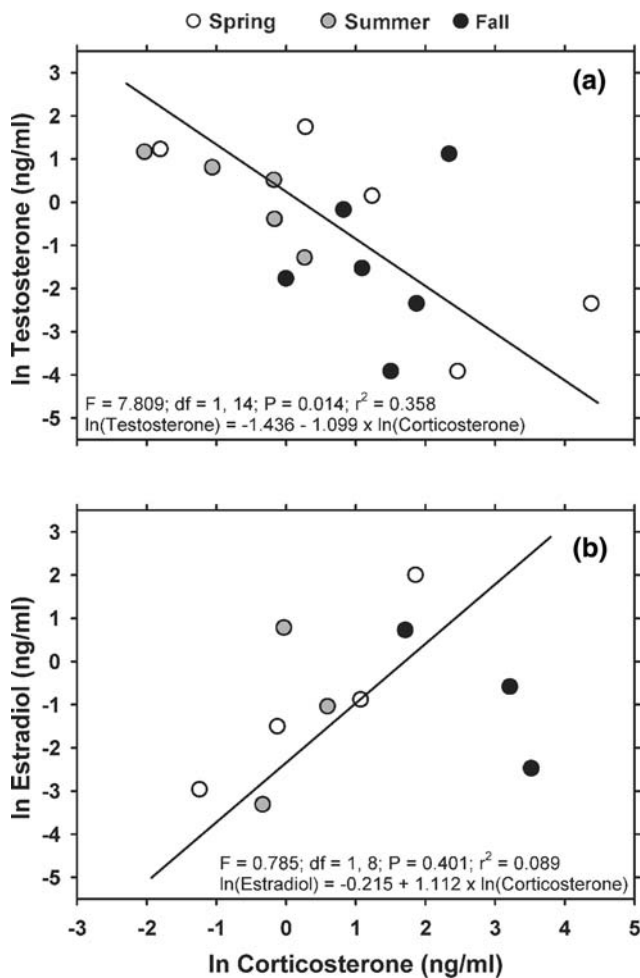


Fig. 2 Natural log–log Model-II principal axis regression showing the relationship between baseline corticosterone and baseline sex steroid hormone concentrations in **a** male and **b** female timber rattlesnakes (*Crotalus horridus*). The respective model-II regression lines indicate a significant negative relationship for males and a nonsignificant positive relationship for females. Open, grey, and black circles indicate spring, summer, and fall hormone samples, respectively

snakes, neither baseline corticosterone nor capture stress-induced changes in corticosterone were related to the observed change in testosterone concentrations following capture stress treatment (data not shown). Likewise, the effect of capture stress on estradiol concentrations was not related to either baseline corticosterone or the corticosterone response to capture stress (data not shown).

Question 3: Does body condition influence hormone profiles?

Neither body mass nor snout-to-vent length differed significantly among our seasonal samples of male or female timber rattlesnakes. Body condition of snakes (calculated as the residuals from a regression of body mass on snout-to-vent length for all snakes across seasons) also did not differ

significantly among seasons (data not shown; $F_{2,25} = 2.276$, $P = 0.129$ from a two-way ANOVA). However, body condition of female snakes was significantly lower than that of males across all seasons (data not shown; $F_{1,25} = 12.889$, $P = 0.002$, main effect of sex from a two-way ANOVA followed by a Tukey's multiple comparisons test). There were no statistically significant interactions between sex and season on body condition. Body condition of male and female snakes was not significantly related to either baseline (pre-stress) or capture stress-induced changes in plasma corticosterone, testosterone, or estradiol concentrations (data not shown).

Question 4: Does female reproductive condition influence baseline and stress-induced hormone profiles?

Capture stress significantly increased corticosterone concentrations of female snakes across all reproductive conditions (Fig. 3a; $F_{1,19} = 115.240$, $P < 0.001$; from a two-way repeated measures ANOVA followed by a Tukey's multiple comparisons test). Corticosterone responses to capture stress varied significantly with reproductive condition ($F_{2,19} = 18.440$, $P = 0.002$). There were no significant interactions between reproductive condition and pre- versus post-stress corticosterone concentrations (Fig. 3a; $F_{2,19} = 2.500$, $P = 0.152$ from a two-way repeated measures ANOVA). Neither capture stress treatment ($F_{1,19} = 3.583$, $P = 0.100$) nor reproductive condition ($F_{2,19} = 2.191$, $P = 0.182$) significantly influenced estradiol concentrations of female snakes (Fig. 3b; from a two-way repeated measures ANOVA). There were no significant interactions between reproductive condition and pre- versus post-stress estradiol concentrations ($F_{2,19} = 1.273$, $P = 0.338$ from a two-way repeated measures ANOVA).

Discussion

These experiments provide data on the physiological responses to environmental and reproductive stressors in a free-ranging population of timber rattlesnakes (*C. horridus*). Male and female snakes demonstrated significant variation in baseline corticosterone levels among spring (May), summer (July), and fall (September) seasons. During summer activity, corticosterone concentrations were lower than those observed during both the spring and fall sampling periods, while testosterone concentrations of male snakes tended to be higher during spring and summer activity. In addition, we observed a significant negative relationship between baseline corticosterone and testosterone concentrations in male timber rattlesnakes (*C. horridus*). Although Taylor et al. (2004) reported that corticosterone levels are not significantly related to testosterone concentrations in

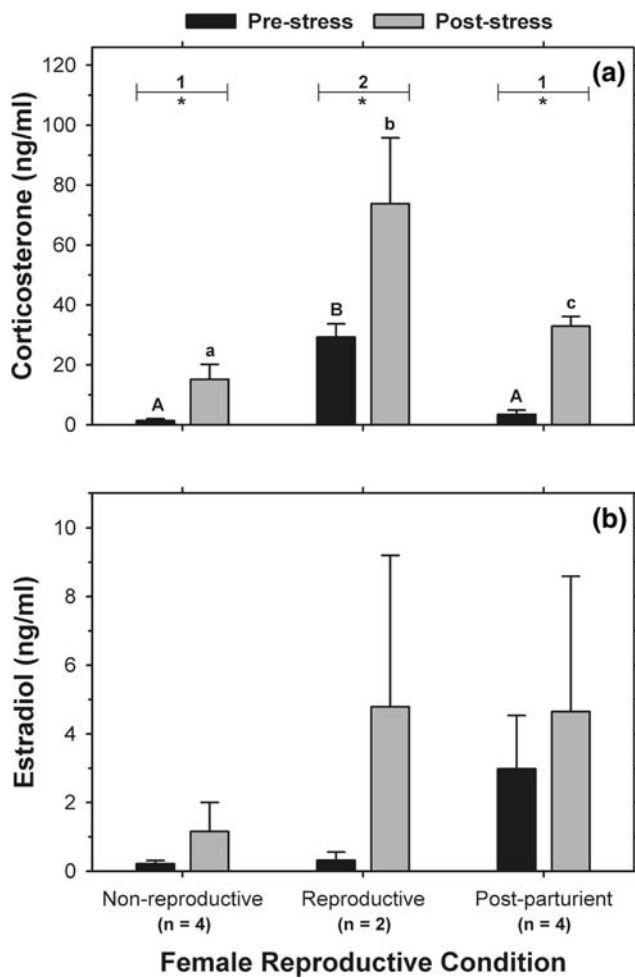


Fig. 3 **a** Corticosterone and **b** estradiol responses of female timber rattlesnakes (*Crotalus horridus*) to 1 h of capture stress treatment with respect to reproductive condition (i.e., non-reproductive, reproductive, or post-parturient). Sample sizes are listed in parentheses below the x-axis. Standard errors (+1) are shown by the vertical lines. Overall differences in hormonal responses to capture stress among reproductive conditions are indicated by numbers above the horizontal brackets. Statistically significant effects of capture stress on hormone concentrations within each reproductive condition are indicated by an asterisk below the horizontal brackets. Capital letters above the standard error bars indicate significant differences in pre-stress (i.e. baseline) hormone concentrations among reproductive conditions. Lower-case letters above the standard error bars indicate significant differences in post-stress hormone concentrations among reproductive conditions

male western diamond-backed rattlesnakes (*C. atrox*), the relationship between the HPA axis and reproduction is highly context-dependent (Moore and Jessop 2003), and possible differences in the natural history and biogeography of these two species must be considered. For example, the relationship between corticosterone and testosterone differs between two populations of common garter snakes (*Thamnophis sirtalis*): male red-sided garter snakes (*T. s. parietalis*) in Manitoba, Canada exhibit a significant negative relationship between stress-induced levels of corticosterone

and testosterone (Moore et al. 2000a), while male red-spotted garter snakes (*T. s. concinnus*) in western Oregon exhibit a significant positive relationship between baseline corticosterone and testosterone concentrations (Moore et al. 2000b). In male timber rattlesnakes, we observed a significant negative relationship between baseline, but not stress-induced concentrations of corticosterone and testosterone. Although additional studies with larger sample sizes would be beneficial, our data suggest the presence of a reciprocal relationship between the HPA and hypothalamus–pituitary–gonad (HPG) axes under non-stress conditions in male timber rattlesnakes (e.g., Greenberg and Wingfield 1987; Wingfield 1988).

In contrast, female snakes demonstrated a positive, although nonsignificant relationship between baseline corticosterone and estradiol concentrations, suggesting that the relationship between reproduction and the HPA axis in female timber rattlesnakes may differ from that of males. To better understand these possible sex differences, we examined how corticosterone and estradiol concentrations vary with female reproductive condition. Although sample sizes are low, baseline corticosterone concentrations were significantly higher in reproductive female snakes as compared to both non-reproductive and post-parturient females. Differences in estradiol concentrations among female snakes were not statistically significant due to the large variation in hormone concentrations combined with limited sample sizes.

Our results are similar to those reported by Woodley and Moore (2002), in which gravid female tree lizards (*Urosaurus ornatus*) had higher baseline corticosterone levels than females that were vitellogenic. Similarly, reproductive female western diamond-backed rattlesnakes (*C. atrox*) in the Sonoran Desert of Arizona also have significantly higher baseline corticosterone concentrations at the end of gestation than non-reproductive females (Taylor et al. 2004). If corticosterone is important for sustaining energetically demanding processes associated with reproduction (e.g., Moore and Jessop 2003), then a lack of a negative relationship between the HPA and HPG axes would be expected. Rather, a positive relationship between corticosterone and estradiol concentrations, like the trend observed in this study, might be more physiologically relevant. The demands of reproduction on female snakes are corroborated by our observation that female body condition was significantly lower than that of male snakes across all seasons (data not shown; $F_{1,25} = 12.889$, $P = 0.002$, main effects of sex from a two-way ANOVA followed by Tukey’s multiple comparisons test). As discussed by Husak and Moore (2008), decreased food availability and poor body condition have been linked with elevated stress hormone levels in a variety of animals. Because reproductive female rattlesnakes may experience decreased food availability while

brooding young, and post-parturient females likely experience reduced lipid stores, a lower body condition among female rattlesnakes may be expected when compared to males.

These data on seasonal cycles in baseline hormone concentrations complement current knowledge of reproductive physiology and behavior in timber rattlesnakes (e.g., Brown 1991; Martin 1993; Clark et al. 2003). For example, vitellogenic female timber rattlesnakes emerge from hibernation and immediately select and remain in favorable microhabitats to behaviorally regulate body temperature during gestation (Reinert 1984), thus ensuring complete embryonic development of young prior to giving birth in late August to mid September (Brown 1991; Martin 1993). The significantly elevated baseline corticosterone concentrations of reproductive snakes may reflect the increased energetic demands of pregnancy. Although neither estradiol nor testosterone concentrations varied seasonally in this study, the lack of a seasonal cycle likely reflects limited sample sizes, varying reproductive condition among female snakes, and the inability to specifically measure sex steroid hormones when females are vitellogenic. Baseline corticosterone concentrations were also higher during the fall and spring sampling periods. These seasonal elevations in plasma corticosterone may be associated with preparation for winter dormancy during the fall (e.g., Lutterschmidt and Mason 2005) and the energetically costly events associated with prolonged winter dormancy and spring emergence (e.g., Moore et al. 2000a; Cease et al. 2007). Alternatively, the lower baseline corticosterone concentrations observed during the summer sampling period may reflect a decrease in the activity and/or sensitivity of the HPA axis in conjunction with optimizing reproductive activity. Traditional HPA axis challenge experiments are necessary to distinguish between these possible explanations.

Similar to our data, Moore et al. (2001) described a consistent increase in corticosterone in response to capture stress across seasons in male red-spotted garter snakes (*T. s. concinnus*), a population of snakes with an extended spring mating season. In the present study, the corticosterone responses to capture stress depended on season, thus suggesting that adrenocortical function is modulated seasonally in timber rattlesnakes. For example, during the summer sampling period, stress-induced corticosterone levels appear to be much lower than those observed during both the spring and fall. The lower magnitude of corticosterone responses to stress could result from the lower baseline corticosterone levels, a decrease in the responsiveness of the HPA axis, or a combination of both mechanisms. Similarly, the corticosterone responses of reproductive snakes differed significantly from those of both non-reproductive and post-parturient snakes. Again,

whether the larger magnitude in the stress-induced change in corticosterone of reproductive females reflects the higher baseline corticosterone concentrations or an effect of reproductive condition on the sensitivity of the HPA axis requires additional study. Although the sample sizes for female reproductive condition are low, particularly for the reproductive group, our statistical analyses indicate that corticosterone responses to capture stress vary significantly with reproductive condition. Further investigations with larger sample sizes (and hence increased statistical power) are needed to confirm the findings of this study. Nevertheless, the observed differences in the magnitude of corticosterone responses to capture stress among seasons and reproductive conditions suggest that hormonal stress responses are dynamic and do not simply reflect a maximal production of corticosterone.

In contrast to our findings, Woodley and Moore (2002) demonstrated that gravid female tree lizards (*U. ornatus*) do not show a significant increase in corticosterone following 10 min of handling stress. However, methodological differences in the length of the handling stress treatment, in addition to taxonomic divergence and ecological differences between these reptiles make it difficult to compare these studies. Future studies examining responses to capture stress over varying time courses would aid in understanding whether female reproductive condition modulates adrenocortical function, as has been reported in female green turtles, *Chelonia mydas* (Jessop 2001) and female tree lizards, *U. ornatus* (Woodley and Moore 2002). Because gravid female tree lizards (Woodley and Moore 2002) and reproductive female timber rattlesnakes (this study) both had higher baseline corticosterone concentrations than non-gravid and non-reproductive females, it is possible that reproductive condition influences the responsiveness of the HPA axis. Further, although baseline corticosterone concentrations do not differ between non-reproductive and post-parturient female rattlesnakes, stress-induced corticosterone concentrations are significantly higher in post-parturient snakes (Fig. 3a). These observations support the hypothesis that reproductive condition modulates the sensitivity of the HPA axis, independent of initial, baseline hormone concentrations.

Unlike previous reports in other reptiles (e.g., Moore et al. 1991; 2000a), capture stress treatment did not decrease sex steroid hormones of male and female timber rattlesnakes. Instead, capture stress treatment produced a small, but statistically significant increase in testosterone in male snakes during the spring and fall sampling periods. The functional significance of this stress-induced increase in testosterone in male timber rattlesnakes is currently not understood. However, there is precedence for increases in sex steroid hormones following exposure to environmental and social stressors in other ectothermic vertebrates

(reviewed in Moore and Jessop 2003). For example, social encounters with conspecific males during the breeding season result in increased plasma corticosterone as well as plasma testosterone concentrations in male eastern fence lizards, *Sceloporus undulatus* (Smith and John-Alder 1999). In male marine toads (*Bufo marinus*), amplexus behavior increases both corticosterone and androgen concentrations (Orchinik et al. 1988). Future studies examining possible context dependence in the stress response in timber rattlesnakes may prove helpful in understanding why male timber rattlesnakes demonstrate stress-induced increases in testosterone. Whether the relationship between baseline corticosterone and sex steroid hormones in timber rattlesnakes is indeed sexually dimorphic also requires further investigation. Such studies would provide insight into the variation observed in the relationship between adrenocortical function and reproductive hormones across taxonomic groups.

This study provides much needed information regarding the physiological stress responses of timber rattlesnakes across seasons and changing reproductive states. These data are particularly important considering that timber rattlesnake populations have declined across much of the historic range due to habitat loss, over collecting, and wanton killing (Martin 1992a; Brown 1993). The alarming rate of habitat alteration and loss in the northeastern United States has exacerbated such population declines and this species is now a species of special concern in Pennsylvania (Anonymous 2005). The results presented here provide an initial and necessary framework for using changes in hormone profiles as a paradigm for understanding the impacts of more pervasive ecological perturbations. For example, we are conducting preliminary studies to investigate if the activity and sensitivity of the HPA axis are influenced by the amount of time rattlesnakes are associated with habitats affected by timber harvesting activities. Although the disturbances associated with timber harvesting may influence the HPA axis differently than the capture and handling stress paradigm reported here, such data may aid conservation efforts for the protection of threatened and endangered animal populations.

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