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Adrenocorticotrophic Hormone Contribute to Stress Response
Plasticity in Red-Sided Garter Snakes (*Thamnophis Sirtalis*
Parietalis)

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Seasonal and sex differences in responsiveness to adrenocorticotrophic hormone contribute to stress response plasticity in red-sided garter snakes (*Thamnophis sirtalis parietalis*)

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Abstract

Like many vertebrates, hormonal responses to stress vary seasonally in red-sided garter snakes (*Thamnophis sirtalis parietalis*). For example, males generally exhibit reduced glucocorticoid responses to a standard stressor during the spring mating season. We asked whether variation in adrenal sensitivity to adrenocorticotrophic hormone (ACTH) explains why glucocorticoid responses to capture stress vary with sex, season, and body condition in red-sided garter snakes. We measured glucocorticoids at 0, 1, and 4 hours after injection with ACTH (0.1 IU/g body mass) or vehicle in males and females during the spring mating season and fall pre-hibernation period. Because elevated glucocorticoids can influence sex steroids, we also examined androgen and estradiol responses to ACTH. ACTH treatment increased glucocorticoids in both sexes and seasons. Spring-collected males had a smaller integrated glucocorticoid response to ACTH than fall-collected males. The integrated glucocorticoid response to ACTH differed with sex during the spring, with males having a smaller glucocorticoid response than females. Although integrated glucocorticoid responses to ACTH did not vary with body condition, we observed an interaction among season, sex and body condition. In males, ACTH treatment did not alter androgens in either season, but androgens decreased during the sampling period. Similar to previous studies, plasma estradiol was low or undetectable during the spring and fall and therefore any effect of ACTH treatment on estradiol could not be determined. These data provide support for a mechanism that partly explains how the HPA axis integrates information about season, sex, and body condition: namely, variation in adrenal responsiveness to ACTH.

Keywords

ACTH; glucocorticoid; sex differences; sex steroids; season; body condition

List of symbols and abbreviations

Adrenocorticotrophic hormone (ACTH); Hypothalamic-pituitary-adrenal (HPA) axis; Analysis of variance (ANOVA)

Summary

The adrenal glucocorticoid response to ACTH varies with season and sex in garter snakes. Decreased responsiveness to ACTH partly explains reduced stress sensitivity in males during the spring breeding season.

Introduction

In light of the increasing impacts of climate change, habitat loss and fragmentation, endocrine disruption and loss of biodiversity on communities, it is critical that we understand the neuroendocrine mechanisms animals use to integrate information about their environment with their internal state to produce appropriate physiology and behavior. A cohesive understanding of these mechanisms is yet to emerge. The hypothalamus-pituitary-adrenal (HPA) axis is likely central to these mechanisms, because this hormone system enables an animal to integrate multiple signals (i.e., environmental conditions, life-history stage, reproductive history or status, and internal energy balance) and then respond appropriately by modulating circulating hormone concentrations.

In response to an acute perturbation or “stressor,” the HPA axis secretes glucocorticoid hormones, which coordinate changes in physiology and behavior that promote immediate survival by mobilizing energy stores and suppressing non-essential processes such as reproduction and digestion (Wingfield, 2008). Importantly, the magnitude of the stress-induced increase in glucocorticoids is often context dependent, varying with season (Astheimer et al., 1995; Klukowski, 2011; Lutterschmidt et al., 2009; Moore et al., 2001), sex (Cartledge and Jones, 2007; Pottinger et al., 2013), reproductive state (Cartledge and Jones, 2007; Lutterschmidt et al., 2009), and body condition (Dayger et al., 2013; Heath and Dufty, 1998). For example, male fence lizards (*Sceloporus undulatus*) generally respond to a standardized capture stress protocol by increasing plasma glucocorticoids. During the breeding season, however, the maximum glucocorticoid concentration attained is smaller compared to the nonbreeding season (Klukowski, 2011). Whether such variation in stress responses results from a change in the capacity of the adrenal glands to secrete glucocorticoids, upstream changes in the hypothalamus or pituitary, or a combination of both, is often unknown.

In addition to context-dependent variation in glucocorticoid synthesis, the responses to increased glucocorticoids, both physiological and behavioral, can also vary with context. For example, exogenous corticosterone treatment increases plasma glucocorticoid concentrations in both territorial orange-blue and non-territorial orange morphs of male tree lizards (*Urosaurus ornatus*). However, elevated corticosterone elicits a greater suppression of plasma testosterone in orange morphs compared to orange-blue morphs (Knapp and Moore, 1997). In another example, a low physiological dose of exogenous corticosterone suppresses mating behavior only in female red-sided garter snakes (*Thamnophis sirtalis parietalis*) in below-average body condition; mating behavior of females in above-average body condition

is not significantly affected (Dayger et al., 2013). Thus, there are contexts in which elevated glucocorticoid concentrations suppress neither sex steroid hormones nor mating behavior, but instead may facilitate reproduction by fueling energetically costly mating behavior (Lutterschmidt and Maine, 2014; Wingfield and Sapolsky, 2003). Indeed, many vertebrates exhibit elevated baseline glucocorticoids during energetically challenging life-history stages such as reproduction (reviewed in Landys et al., 2006; Moore and Jessop, 2003).

While the mechanisms mediating context-dependent variation in stress-induced glucocorticoids are poorly understood, a change in the sensitivity of the HPA axis is likely involved (Romero, 2002; Romero and Wingfield, 2001; Wingfield et al., 1994). One way to better understand the mechanisms underlying plasticity in stress responses is to administer an adrenocorticotropic hormone (ACTH) “challenge.” Administering a sufficiently high dose of pituitary ACTH induces the adrenal glands to secrete maximal levels of glucocorticoids. Comparing maximal glucocorticoid concentrations among contexts (e.g., life history stages, reproductive stages) can reveal how the sensitivity of the adrenal glands varies with those contexts. For example, snow buntings (*Plectrophenax nivalis*) respond to restraint stress with a larger glucocorticoid response during breeding compared to molting. The higher sensitivity to restraint stress during the breeding season is concomitant with increased sensitivity of the adrenal glands to ACTH (Romero et al., 1998). These and other studies demonstrate that variation in HPA axis sensitivity may mediate the effects of context on stress-induced changes in glucocorticoids and, by extension, physiology and behavior (reviewed in Wingfield, 2008).

We examined whether variation in responsiveness to ACTH could explain observed differences in hormonal responses to capture stress in a well-studied population of red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada. Following spring emergence from winter dormancy, red-sided garter snakes exhibit mating behavior for approximately 4 weeks. Female snakes generally leave the den post-copulation, but males remain near the den for several weeks, searching for mates and courting emerging females (Shine et al., 2001). Once snakes leave the den, they migrate up to 18 km to summer feeding areas (Gregory and Stewart, 1975). Neither sex feeds during winter dormancy or while at the den site, but because females migrate away within a few days, they likely have the opportunity to forage more quickly after emergence (Crews et al., 1987; Shine et al., 2001). In the fall, snakes migrate back to the den site before returning underground for winter dormancy.

Baseline glucocorticoids are generally elevated during the spring mating season

compared to other times of year, likely to support the energetic demands of mating behavior (Lutterschmidt and Maine, 2014; Moore et al., 2001; Whittier et al., 1987). Red-sided garter snakes also exhibit a temporally-dissociated reproductive pattern, whereby peak gonadal activity is temporally dissociated from mating behavior (Crews, 1984). In other words, mating behavior of red-sided garter snakes during the spring occurs when sex steroids tend to be low (reviewed in Lutterschmidt, 2012). Androgens in males and estrogens in females then rise during the summer when males commence spermatogenesis and females undergo vitellogenesis, ovulate and become gravid (Krohmer et al., 1987; Whittier et al., 1987). Estradiol rapidly declines after parturition in females (Whittier et al., 1987), but androgens in males remain significantly elevated in the fall compared to spring (Lutterschmidt and Mason, 2005; Lutterschmidt and Mason, 2009; Moore et al., 2001).

During the fall, capture stress elicits a significant increase in corticosterone (the primary glucocorticoid in reptiles) in male red-sided garter snakes, but not consistently during the spring (Lutterschmidt and Mason, 2005; Moore et al., 2000a; Moore et al., 2001). In addition, responses to capture stress vary with body condition. For example, male reptiles and amphibians in below-average body condition sometimes have higher baseline and stress-induced glucocorticoids (reviewed in Moore and Jessop, 2003). The relationship between glucocorticoids and body condition is important because the energetically demanding courtship and mating behavior in which male garter snakes engage can result in males losing an average of 10% of their body mass over the course of the mating season (O'Donnell et al., 2004). Thus, body condition may play a role in determining how long an individual male is capable of seeking reproductive opportunities (Shine and Mason, 2005). Similar to males, female responses to capture stress during the spring mating season also depend on body condition (Dayger et al., 2013). Females in below-average body condition significantly increased glucocorticoid concentrations in response to capture stress, while females in above-average body condition did not.

Taken together, these data suggest that the sensitivity of the HPA axis varies with season, sex and body condition in red-sided garter snakes. Here, we test the hypothesis that the observed variation in stress-induced glucocorticoid concentrations is related to the responsiveness of the adrenal glands to ACTH. Because elevated glucocorticoids, like those expected with ACTH challenge, are often associated with a decrease in sex steroid hormone concentrations (e.g., Duckworth et al., 2001; Hau et al., 2010; Leary and Harris, 2012; Leary et al., 2004; Moore et al., 1991; Moore et al., 2000a; Wingfield and Sapolsky, 2003), we also examined potential effects of ACTH-induced glucocorticoids on androgens in males and

estradiol in females. We predicted that differences in the sensitivity of the adrenal glands to ACTH would mirror previously-reported variation in responses to capture stress between seasons, sexes, and body conditions. Principally, we asked if the sensitivity of male snakes to ACTH is reduced during the spring mating season and if responses to ACTH, particularly during the spring mating season, vary with sex and/or body condition.

Materials and methods

The experiment described here was conducted in the field with free-ranging red-sided garter snakes (*Thamnophis sirtalis parietalis*) at a den site located in Inwood, Manitoba, Canada. Snakes were collected during the spring mating season from 15-18 May 2012 and before they descended underground for winter dormancy from 12-15 September 2012. This experiment was approved by the Manitoba Department of Conservation (scientific permit number WB14930) and Portland State University's Institutional Animal Care and Use Committee (approval number psu12.05.15.1).

Experimental Design and ACTH treatment

We collected 40 male and 43 female snakes during the spring and 39 males and 42 females during the fall. Females were confirmed to be unmated before including them in the experiment by checking for the absence of a mating plug. A blood sample (150 μ L) was collected from the caudal vein immediately upon capture ($t = 0$ h; mean sampling time \pm SE: 81 s \pm 4.8 s). Snakes were then weighed and randomly assigned to either ACTH or vehicle treatment. For ACTH treatment, an intraperitoneal injection of 0.1 IU ACTH/g body mass (product no. A6303, Sigma-Aldrich, St. Louis, MO, USA) was administered from a stock solution containing 20 IU/mL reptilian Ringer's solution. Injection volume was 0.5% of body mass. We chose this ACTH dose because it elicits a large increase in plasma glucocorticoids in lizards and birds (Klukowski, 2011; Phillips and Klukowski, 2008; Romero and Wingfield, 1999). Vehicle-treated snakes received an equivalent volume of reptilian Ringer's solution.

Following treatment, snakes were marked with a unique color pattern on the dorsal stripe using colored marker to identify individuals. Males and females were transferred to separate arenas (1 m x 1 m x 1 m) with hiding areas for the duration of the experiment; housing snakes in these arenas does not elicit a significant change in glucocorticoids (Moore and Mason, 2001). Less than 7 minutes elapsed from initial collection of the snake to its transfer to the holding arena. Additional blood samples were collected from each snake 1 hour and 4 hours after injection (150 μ L at each sampling time). At the conclusion of the experiment, snout-vent length was measured for each snake. Snakes were then released at the site of capture within 24 hours of collection; the colored marker patterns on the dorsal stripe enabled us to avoid recapturing a previously-sampled snake. To calculate body condition, we regressed body mass on snout-vent length to enable comparisons to previous studies (Cease et al., 2007; Dayger et al., 2013; Jessop et al., 2004; Moore et al., 2000b). We designated snakes with positive residuals as being in positive body condition and vice versa. Final

sample sizes within each treatment group were 21 male and 22 female snakes treated with ACTH during the spring, 19 male and 21 female snakes treated with vehicle during the spring, 20 male and 21 female snakes treated with ACTH during the fall, and 19 male and 21 female snakes treated with vehicle during the fall. Final sample sizes within body condition category for each analysis are listed in the figures.

Blood sampling and radioimmunoassay

Blood samples were collected using heparinized 25-g needles and 1-cc syringes. Blood samples were stored on ice before returning to the field station, where they were centrifuged to separate plasma from blood cells. The plasma was transferred to fresh tubes and stored at -4°C , transported on dry ice to Portland State University and stored at -80°C until analysis. Plasma hormone concentrations (corticosterone and estradiol for females; corticosterone and androgens for males) were determined for each sample by direct radioimmunoassay using the methods described and validated by Dayger et al. (2013) and Lutterschmidt and Mason (2005; 2009). Briefly, steroid hormones were extracted from 5-70 μL of plasma with anhydrous ethyl ether, and then evaporated under nitrogen gas in a 37°C bead bath. Hormone extracts were reconstituted in phosphate buffered saline. Samples were assayed in duplicate for each hormone, and each replicate aliquot was incubated with approximately 12,000 cpm [$1,2,6,7\text{-}^3\text{H}$] - corticosterone, [$2,4,6,7\text{-}^3\text{H}$] - estradiol, or [$1,2,6,7\text{-}^3\text{H}$] - testosterone (Product numbers: NET399250UC, NET317250UC, NET370250UC, respectively; Perkin-Elmer, Piscataway, NJ, USA) and 100 μL antiserum at 4°C for 18-24 hours (corticosterone antibody: 07120016 from MP Biomedicals, LLC, Solon, OH, USA; estradiol and testosterone antibody: 20R-ER012w and 20R-TR018w, respectively, from Fitzgerald Industries International, Acton, MA, USA). Unbound steroid was separated from bound steroid using dextran-coated charcoal and the radioactivity of the bound fraction was quantified using a liquid scintillation counter (Beckman Model No. 6500). The testosterone antibody we used significantly cross-reacts with 5- α -dihydrotestosterone (63% cross reactivity; Fitzgerald Industries International), and therefore we report data for androgens rather than testosterone concentrations.

Within each sex, samples were randomly distributed across assays. Samples were run in 8 assays for corticosterone, 4 assays for estradiol, and 6 assays for androgens, with mean intra-assay coefficients of variation of 11.1% for corticosterone, 11.8% for estradiol and 8.0% for androgens. The mean inter-assay coefficients of variation were 18.0% for corticosterone, 19.3% for estradiol, and 15.1% for androgens. Mean percent recovery was 78.2% for male

samples and 71.4% for female samples; concentrations were corrected for individual recovery variation. The average limit of detection was 29.3 pg/mL for corticosterone, 7.4 pg/mL for estradiol and 12.9 pg/mL for androgens.

Statistical analyses

We used SigmaPlot 12.0 software to perform all statistical analyses (Systat Software 2010, Systat systems, Inc., Point Richmond, CA, USA). All statistical comparisons were considered significant at $P \leq 0.05$. All data met the assumptions for parametric analysis (i.e., normality and equal variance) unless otherwise noted. In 3 of the 6 repeated measures analyses that we performed (i.e., fall corticosterone data from males and females and spring androgen data from males), data transformation could not correct for non-normality. Because non-parametric options for multifactorial repeated measures analysis of variance (ANOVA) do not exist, we proceeded with parametric analyses similar to Lutterschmidt and Mason (2008, 2009) because ANOVAs are robust against modest deviations from normality (Sheskin, 2007; Zar, 1999). For these analyses, we chose a transformation that most closely approximated normality based on a Shapiro-Wilk normality test. Some male samples had undetectable concentrations of corticosterone ($n = 1$ of 237 samples, 0.4%) and/or androgens ($n = 8$ of 237 samples, 3.4%) and were assigned the limit of detectability for analysis. Estradiol concentrations in most female samples (227 of 255 samples, 89%) were below the limits of detectability, so no statistical tests were performed on these data.

We first verified that ACTH treatment significantly increases plasma corticosterone concentrations over time using a two-way repeated measures ANOVA with treatment and sampling time as factors; each sex was analyzed separately within each season. We also examined if ACTH-induced changes in corticosterone alter androgen concentrations of male snakes using a two-way repeated measures ANOVA within each season with treatment and time as factors. We further investigated significant main effects of each ANOVA using a Student-Newman-Keuls multiple comparisons procedure.

To better understand the overall corticosterone response to ACTH challenge, we calculated an integrated corticosterone response for each ACTH-treated snake similar to the methods of Cockrem and Silverin (2002) and Rensel and Schoech (2011). For each snake, corticosterone concentration was plotted over time and the area under the curve was calculated using the trapezoid rule with Microsoft Excel (Microsoft Office 2010, Redmond, WA, USA). We corrected for variation in baseline corticosterone concentrations across individual snakes by multiplying the baseline corticosterone concentration by the entire 4

hour sampling period and subtracting this value from the total area under the curve. This procedure generated the corrected integrated corticosterone response (hereon referred to as the integrated corticosterone response). We then used these data to evaluate differences in the overall corticosterone response to ACTH treatment among snakes, as the integrated data incorporate, at least in part, temporal patterns in both the response to and recovery from ACTH treatment. Because transformation could not correct for deviations in normality, we performed a non-parametric three-way ANOVA using the Scheirer-Ray-Hare extension of a Kruskal-Wallis ANOVA on the integrated corticosterone response; season, sex and body condition were included as factors. There was a significant three-way interaction in this analysis, and therefore we reanalyzed the integrated corticosterone response data to further examine the potential effects of season and sex. Based on *a priori* knowledge, we focused our analyses on seasonal differences within males and potential sex differences within the spring. Within males, we reanalyzed the integrated corticosterone data with a non-parametric two-way ANOVA with season and body condition as factors. We compared the integrated corticosterone response of males and females within the spring by reanalyzing the data using a non-parametric two-way ANOVA with sex and body condition as factors.

Results

Effects of ACTH treatment on corticosterone

We first verified that ACTH treatment elicits an increase in plasma corticosterone concentrations in each season and sex separately. The main effects of sampling time were statistically significant in all analyses (Table 1). The main effect of treatment was statistically significant only in spring-collected males, but we observed a significant interaction between treatment and sampling time in all other groups (i.e., fall-collected males and in females collected during both the spring and fall). As expected, these results indicate that the effect of ACTH treatment on corticosterone depends on sampling time.

Compared to vehicle treatment, ACTH treatment significantly increased plasma corticosterone in males during the spring and in both sexes during the fall at the 1 h sampling time (Fig. 1A-D; from Student-Newman-Keuls multiple comparisons procedures). In males collected during fall and in females collected during both spring and fall, ACTH-induced corticosterone concentrations remained elevated at the 4 h sampling time (Figs. 1B - D). In contrast, plasma corticosterone of ACTH-treated, spring-collected males declined to below-baseline levels at the 4 h sampling time. Vehicle-treated males collected during spring did not change plasma corticosterone significantly from baseline throughout the entire sampling period (Fig. 1A). During the fall, vehicle-treated males increased plasma corticosterone above baseline at the 1 h and 4 h sampling times, although not to ACTH-induced levels (Fig. 1B). Vehicle-treated females during both the spring and fall did not significantly increase plasma corticosterone above baseline levels at any sampling time (Figs. 1C, D).

Effects of ACTH treatment on sex steroids

We asked if ACTH-induced increases in glucocorticoids alter plasma androgen concentrations in males. There was a significant main effect of sampling time but not hormone treatment on androgens during both spring and fall (Table 2). There were no significant interactions between treatment and sampling time (Table 2). In spring-collected males, a Student-Newman-Keuls multiple comparisons procedure revealed that plasma androgen concentrations decreased significantly at the 1 h sampling time (Fig. 2A). In fall-collected males, plasma androgen concentrations decreased significantly at the 4 h sampling time (Fig. 2B). Initial (0 h) androgen concentrations were significantly lower in spring compared to fall ($U = 260.00$, d.f. = 1, $P < 0.001$; from a Mann-Whitney U-test), consistent with previously published data.

Estradiol concentrations were low or undetectable in most female snakes at all

sampling times in both seasons (data not shown). We therefore did not perform any statistical tests on these data.

Integrated corticosterone response to ACTH treatment

To better evaluate how corticosterone responses to ACTH vary with season, sex and body condition, we calculated an integrated corticosterone response for each ACTH-treated snake and then compared these integrated responses using a non-parametric three-way ANOVA. Overall, there were no significant main effects of season ($H_{1,83} = 2.293$, $P = 0.130$), sex ($H_{1,83} = 2.977$, $P = 0.084$), or body condition ($H_{1,83} = 0.756$, $P = 0.385$). There were no significant interactions between season and sex ($H_{1,83} = 3.169$, $P = 0.075$), season and body condition ($H_{1,83} = 0.222$, $P = 0.637$) or sex and body condition ($H_{1,83} = 0.174$, $P = 0.677$). However, there was a significant three-way interaction among season, sex, and body condition ($H_{1,83} = 4.497$, $P = 0.034$). We noted a trend for ACTH treatment to elicit a larger integrated corticosterone response in males in positive body condition during the spring. Similarly, females in positive body condition collected during the fall also exhibited a larger response, although neither of these effects reached statistical significance (Fig. 3A).

Because of the significant interaction among season, sex, and body condition in our three-way ANOVA, we separated the data by sex and reanalyzed the male data to address one of our *a priori* questions: Does the sensitivity of male snakes to ACTH vary seasonally? A non-parametric two-way ANOVA with season and body condition as factors indicated that there was a significant main effect of season ($H_{1,37} = 5.689$, $P = 0.017$) but not body condition ($H_{1,37} = 0.137$, $P = 0.712$) on integrated corticosterone response to ACTH. The interaction between season and body condition ($H_{1,37} = 3.091$, $P = 0.079$) was not statistically significant. These results indicate that males have a significantly smaller integrated corticosterone response to ACTH during the spring mating season compared to the fall (Fig. 3B).

To address our second *a priori* question (i.e., Are there sex differences in the sensitivity to ACTH during the spring?), we separated the data by season and reanalyzed the spring data using a non-parametric two-way ANOVA with sex and body condition as factors. Within the spring, there was a significant main effect of sex ($H_{1,42} = 4.893$, $P = 0.027$) but not body condition ($H_{1,42} = 0.594$, $P = 0.441$) on integrated corticosterone response to ACTH. There was not a significant interaction between sex and body condition ($H_{1,42} = 1.003$, $P = 0.317$). Within the spring, males had a significantly smaller integrated corticosterone response to ACTH than females (Fig. 3B).

Discussion

We tested the hypothesis that observed variation in stress-induced glucocorticoid concentrations is related to the responsiveness of the adrenal glands to ACTH using an ACTH challenge. Overall, males collected during spring were less responsive to ACTH than males collected during fall or females in either season. These data explain, at least in part, why males are generally less sensitive to capture stress during the spring mating season. The corticosterone response to ACTH did not vary explicitly with body condition category, although we did observe a significant interaction among season, sex and body condition. Plasma androgens did not differ between vehicle- and ACTH-treated snakes. Taken together, our data elucidate a mechanism that explains how season, sex, and body condition affect glucocorticoid responses to capture stress: namely, variation in adrenal responsiveness to ACTH.

Seasonal differences in glucocorticoid responsiveness to ACTH challenge

Because of the direct connection between sensitivity to ACTH and responses to stress, we predicted that snakes would be least responsive to ACTH when they are also least responsive to capture stress. Indeed, male red-sided garter snakes are least responsive to ACTH (this study) as well as capture stress during the spring compared to fall (Lutterschmidt and Mason, 2005; Moore et al., 2001, but also see Lutterschmidt and Mason, 2010; Moore et al., 2000a). We also found that vehicle-treated males during the fall increased corticosterone concentrations within 1 hour of injection, though to a lesser degree than ACTH-treated males, indicating that males during the fall pre-dormancy period are comparatively more sensitive to handling stress than spring-collected males. Our data therefore support the hypothesis that decreased adrenal responsiveness to ACTH explains, at least in part, previous reports of suppressed stress responses in males during the mating season.

Seasonal variation in responses to ACTH occurs in fish, amphibians, reptiles, birds, and mammals (Astheimer et al., 1995; Capaldo et al., 2004; Cartledge and Jones, 2007; Girard et al., 1998; Gist, 1972; Ingram et al., 1999; Mashburn and Atkinson, 2008; Romero and Wingfield, 1998; van Lier et al., 2003). Studies in birds and reptiles further suggest that modulation of sensitivity to ACTH is associated with plasticity in stress responses (e.g., Romero and Wikelski, 2006; Romero and Wingfield, 1998; Romero and Wingfield, 1999; Romero et al., 1998). One study in Galápagos marine iguanas (*Amblyrhynchus cristatus*) examined diel variation in the sensitivity to exogenous ACTH and capture stress and found

that both treatments elicit larger corticosterone increases during the day versus the night (Romero and Wikelski, 2006). These data suggest that variation in ACTH responsiveness is a central mechanism used to modulate stress responses even on a daily basis.

Modulating responses to a stressor, whether by varying adrenal sensitivity to ACTH or by some other mechanism, may facilitate striking a balance between conflicting physiological processes. For example, elevated glucocorticoids can suppress non-essential processes like reproduction and digestion to promote immediate survival (reviewed in Landys et al., 2006; Wingfield and Sapolsky, 2003). However, the HPA axis may be adaptively regulated during certain time-sensitive life-history events to prevent elevated glucocorticoid concentrations from negatively impacting reproductive success (reviewed in Moore and Jessop, 2003; Romero, 2002; Wingfield and Sapolsky, 2003). In male red-sided garter snakes, elevated plasma corticosterone is associated with courtship behavior during the spring (Cease et al., 2007; Lutterschmidt and Maine, 2014), suggesting that energetically costly mating behavior relies on corticosterone to mobilize sufficient energy stores. When exogenous corticosterone is administered to males, however, courtship behavior is suppressed in a threshold-dependent manner (Lutterschmidt et al., 2004; Moore and Mason, 2001). Taken together, these data suggest that there is an optimal range of glucocorticoid concentrations during the mating season, below which insufficient energy is available to support courtship behavior, and above which glucocorticoids suppress reproduction (Lutterschmidt and Maine, 2014; Lutterschmidt et al., 2004; Moore and Mason, 2001). By remaining within the optimal range of corticosterone concentrations, males likely maximize reproductive opportunities. Overall, we interpret the seasonal difference in male responses to ACTH as a potentially adaptive modulation of the HPA axis to facilitate mating behavior and maximize reproductive success. Although we did not observe a seasonal difference in female responsiveness to ACTH, whether female responses to capture stress vary seasonally as they do in males is unknown. If the magnitude of a stress response primarily depends upon adrenal sensitivity to ACTH, then we posit that female glucocorticoid responses to capture stress will also be similar across seasons, as are female responses to ACTH. These data will be critical for gaining a more comprehensive understanding of the seasonal regulation of the HPA axis.

Sex differences in glucocorticoid responsiveness to ACTH challenge

We observed a significant sex difference in responsiveness to ACTH during the spring mating season, such that males had a smaller corticosterone response to ACTH compared to females. Because female glucocorticoid responses to ACTH are consistently

robust in both spring and fall, we suggest that the observed sex difference primarily reflects suppressed male responsiveness during the spring. Sex differences in the sensitivity of the HPA axis, particularly during the breeding season, have been reported previously (Ashley et al., 2011; Cartledge and Jones, 2007; Keenan et al., 2009; van Lier et al., 2003). For example, in male sheep (*Ovis aries*), ACTH elicits a smaller glucocorticoid response during the breeding season versus the non-breeding season, yet female sheep respond to ACTH equally in both seasons (van Lier et al., 2003).

Female red-sided garter snakes exhibit decreased receptivity to mating in response to elevated glucocorticoids, both from exogenous corticosterone administration and capture stress treatment (Dayger et al., 2013). Female snakes retain ACTH responsiveness during the spring even though elevated glucocorticoids can suppress receptivity, suggesting that female snakes may not be as limited by stress-induced changes in reproductive behavior as males. Because the operational sex ratio is highly skewed towards males during the mating season, females have relatively more opportunities to mate than males. In addition, female red-sided garter snakes can utilize stored sperm from previous copulations for fertilization (Friesen et al., 2013; Halpert et al., 1982), and therefore females are not strictly obligated to copulate during a given mating season in order to reproduce. Thus, stress-induced suppression of mating behavior may have little impact on the overall reproductive success of females. If correct, this would sharply contrast with the effects of highly elevated glucocorticoids on male reproductive success, potentially explaining why reduced adrenal sensitivity to ACTH during the spring has evolved only in males. Further studies investigating the effects of different doses of ACTH are needed to better elucidate the nature of the observed sex difference in the sensitivity to ACTH during the spring mating season as well as its significance to reproductive success.

The influence of body condition on glucocorticoid responsiveness to ACTH

Overall, corticosterone responses to ACTH did not vary significantly with body condition, although we did observe a significant three-way interaction among season, sex and body condition. The analyses presented here focus on using body condition as a categorical variable for better comparison to previous studies (Cease et al., 2007; Dayger et al., 2013; Jessop et al., 2004; Moore et al., 2000b). However, we also did not observe any significant relationships between corticosterone responses to ACTH and body condition using regression analyses (data not shown). Our results therefore suggest that the influence of body condition on stress responsiveness (as reported by Dayger et al., 2013; Moore et al., 2000b) is subtle,

and further research is needed to disentangle these effects from those of both season and sex. For example, there was a trend for ACTH-treated males in positive body condition during the spring to exhibit a larger corticosterone response than those in negative body condition; this trend may be related to the capacity to maintain the elevated corticosterone concentrations that support mating behavior (Cease et al., 2007; Lutterschmidt and Maine, 2014). In addition, fall-collected females in negative body condition tended to produce a smaller corticosterone response to ACTH than those in positive body condition. Because female red-sided garter snakes give birth every other year (Garstka et al., 1982; Gregory, 1977), females in negative body condition during the fall are most likely post-parturient. Similar to Lutterschmidt et al. (2009), the trend reported here may be related to modulation of HPA axis sensitivity in response to changing reproductive condition. The potential influence of body condition on stress response plasticity and the implications for reproductive outcomes in males and females requires further research in this and other taxa.

Effects of ACTH challenge on sex steroids

Elevated plasma glucocorticoid concentrations can elicit a decline in androgen concentrations (Moore et al., 2000a; Moore et al., 2000b; reviewed in Rivier and Rivest, 1991), but this is not always the case (Cease et al., 2007; Lutterschmidt and Mason, 2005; Lutterschmidt et al., 2004; Moore and Mason, 2001). We observed a significant increase in glucocorticoid concentrations in response to ACTH treatment during both the spring and fall, yet androgen concentrations were unaffected compared to vehicle-treated snakes. The absence of an effect of ACTH treatment on androgens during the spring is not surprising, as red-sided garter snakes are temporally-dissociated breeders that have relatively low androgen concentrations during mating (reviewed in Lutterschmidt, 2012). Indeed, we observed relatively lower androgen concentrations in males during the spring compared to fall, as has been previously reported (e.g., Lutterschmidt and Mason, 2005; Lutterschmidt and Mason, 2009; Moore et al., 2001). Similar to Lutterschmidt and Mason (2005), we also did not observe a significant decrease in androgens in response to ACTH-induced glucocorticoid concentrations during the fall. However, male androgen concentrations significantly declined over the sampling period during both the spring and fall, regardless of treatment group. These results suggest that the observed decline in androgens is due to some other component of capture and handling that is not directly corticosterone dependent. For example, activation of the sympathetic nervous system can directly alter hormone synthesis and release independently of corticosterone treatment (e.g., Lutterschmidt and Mason, 2010). It is

interesting to note that the time-course of these androgen changes varied between spring- and fall-collected males, suggesting that the mechanism mediating capture-stress induced changes in androgens, as yet undetermined, is also seasonally modulated.

While there have been several reports of elevated glucocorticoids influencing plasma estradiol concentrations in other female vertebrates (Clearwater and Pankhurst, 1997; Coddington and Cree, 1995; Elsey et al., 1991; Mahmoud et al., 1989; Shors et al., 1999; Woodley and Moore, 2002; Zerani et al., 1991), plasma estradiol concentrations are typically low or undetectable in unmated female red-sided garter snakes during the spring and in females collected during the fall pre-hibernation period (Garstka et al., 1985; Lutterschmidt and Mason, 2009; Uhrig et al., 2012; Whittier et al., 1987). Not surprising, plasma estradiol was undetectable in the majority of females in this experiment, and therefore any effects of ACTH treatment on estradiol concentrations could not be determined.

In summary, we observed seasonal and sex differences in the adrenal response to ACTH treatment in red-sided garter snakes. Here we present a mechanism explaining, at least in part, reduced stress sensitivity in male red-sided garter snakes during the spring breeding season (i.e., decreased responsiveness to ACTH). Females consistently exhibited a robust corticosterone response to ACTH that did not vary seasonally, suggesting that plasticity in adrenal sensitivity to ACTH is specific to males. Sex differences in the modulation of the HPA axis may therefore reflect differing pressures governing male and female fitness. Future research examining other mechanisms that alter HPA axis sensitivity, such as variation in negative feedback regulation of the HPA axis, are necessary to understand how the HPA axis facilitates the appropriate physiological and behavioral responses to a seasonally changing environment. Discovery of the processes that an animal uses to integrate internal and external cues will be crucial to understanding the fundamental mechanisms that impact reproductive success.

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Competing interests

The authors have no competing interests to declare.

Author contributions

Both authors conceived and designed the experiment, performed the experiment, analyzed the data, and wrote the paper.

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Tables

Table 1. Influence of adrenocorticotrophic hormone (ACTH) on plasma corticosterone in male and female red-sided garter snakes during the spring and fall.

Season	Sex	Treatment	Time	Interaction
Spring	♂	$F_{1,119} = 6.240$; $P = \mathbf{0.017}$	$F_{2,119} = 5.480$; $P = \mathbf{0.006}$	$F_{2,119} = 2.912$; $P = 0.060$
Spring	♀	$F_{1,128} = 0.536$; $P = 0.468$	$F_{2,128} = 10.756$; $P < \mathbf{0.001}$	$F_{2,128} = 3.461$; $P = \mathbf{0.036}$
Fall	♂	$F_{1,113} = 0.475$; $P = 0.495$	$F_{2,113} = 37.424$; $P < \mathbf{0.001}$	$F_{2,113} = 3.264$; $P = \mathbf{0.044}$
Fall	♀	$F_{1,125} = 3.462$; $P = 0.070$	$F_{2,125} = 17.450$; $P < \mathbf{0.001}$	$F_{2,125} = 8.056$; $P < \mathbf{0.001}$

All statistics are from two-way repeated measures ANOVAs with treatment and sampling time as factors.

Table 2. Influence of adrenocorticotrophic hormone (ACTH) on plasma androgens in male red-sided garter snakes during the spring and fall.

Season	Treatment	Time	Interaction
Spring	$F_{1,116} = 0.007$; $P = 0.936$	$F_{2,116} = 7.393$; $P = \mathbf{0.001}$	$F_{2,116} = 0.386$; $P = 0.681$
Fall	$F_{1,107} = 0.575$; $P = 0.454$	$F_{2,107} = 14.878$; $P < \mathbf{0.001}$	$F_{2,107} = 0.572$; $P = 0.567$

All statistics are from two-way repeated measures ANOVAs with treatment and sampling time as factors.

Figures

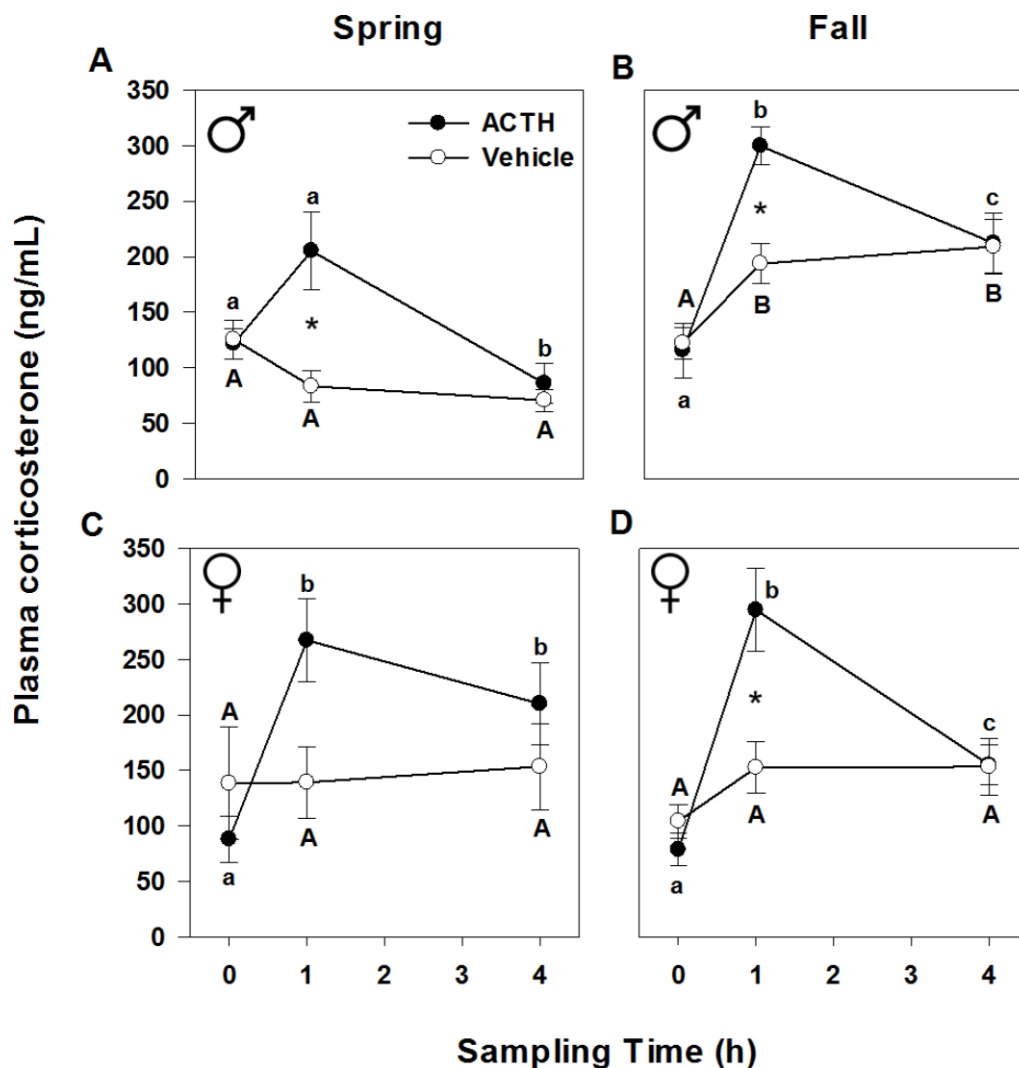


Figure 1. Effect of adrenocorticotrophic hormone (ACTH) challenge on plasma corticosterone (mean \pm SE) in male and female red-sided garter snakes (*Thamnophis sirtalis parietalis*) during the spring mating season (A and C, respectively) and fall pre-dormancy period (B and D, respectively). Letters indicate significant differences among sampling times within ACTH-treated (lowercase) and vehicle-treated (uppercase) snakes. Asterisks indicate that corticosterone concentrations of ACTH-treated snakes are significantly higher than vehicle-treated snakes within the 1 h sampling time. All statistics are from a two-way repeated measures ANOVA within each sex and season followed by a Student-Newman-Keuls multiple comparisons test. Note the relatively smaller increase in corticosterone in response to ACTH treatment in males during the spring mating season.

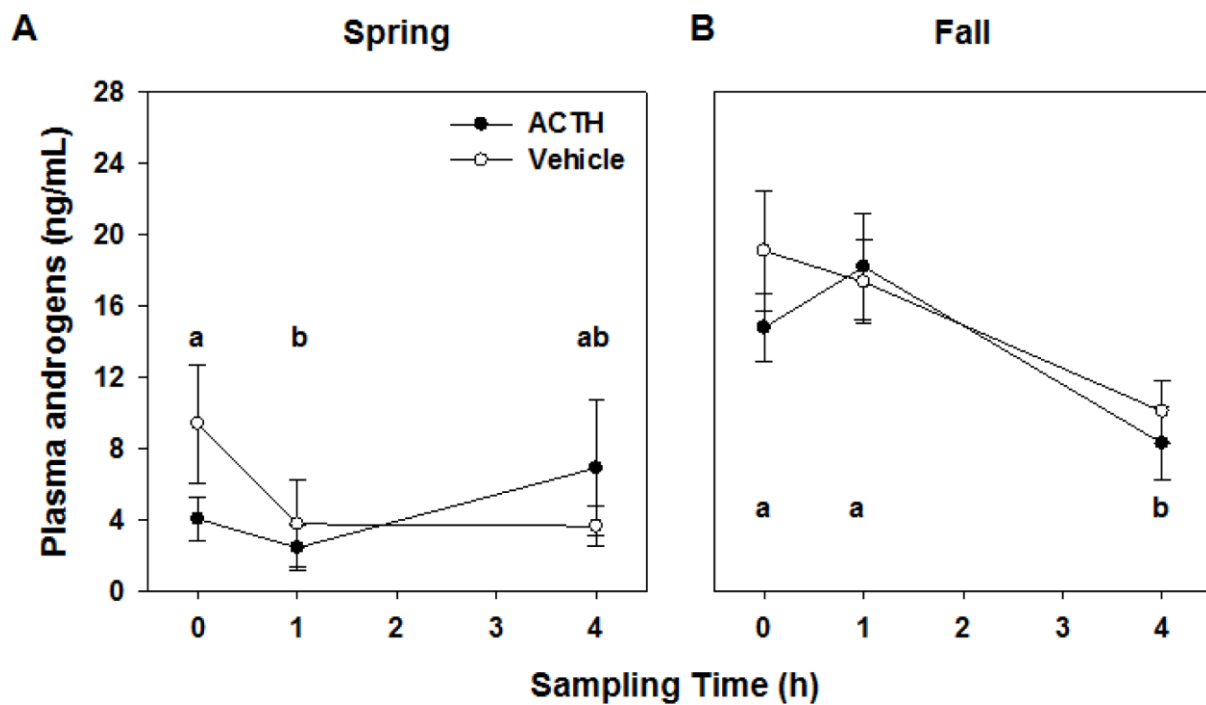


Figure 2. **Influence of ACTH-induced changes in corticosterone on plasma androgens (mean \pm SE) in male red-sided garter snakes (*Thamnophis sirtalis parietalis*) during the spring (A) and fall (B).** Letters indicate significant differences among sampling times; ACTH treatment did not alter androgens. All statistics are from a two-way repeated measures ANOVA within each season followed by a Student-Newman-Keuls multiple comparisons test. Baseline (0 h) androgen concentrations were significantly lower during the spring compared to the fall.

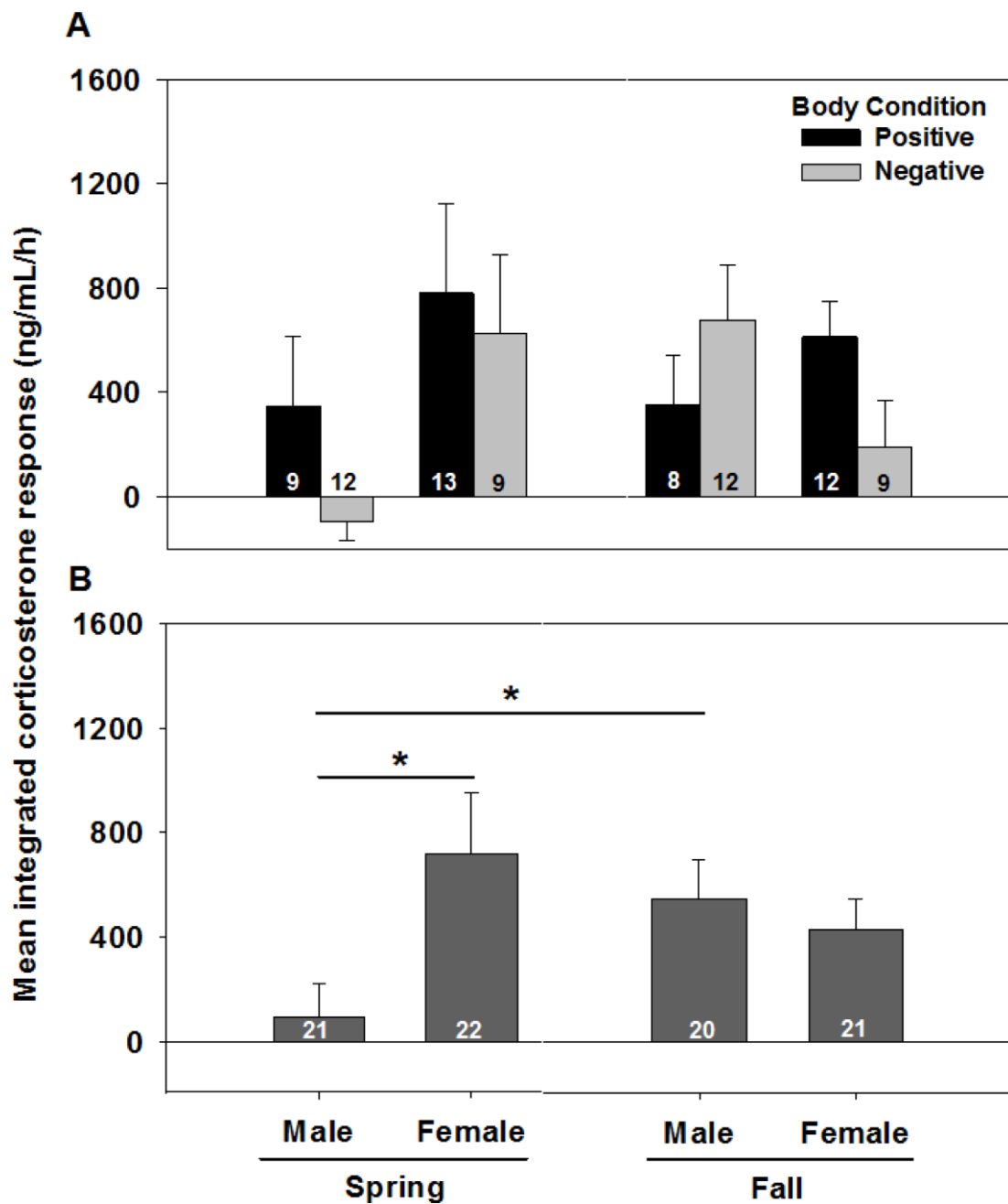


Figure 3. Mean integrated corticosterone response (+1 SE) to adrenocorticotrophic hormone (ACTH) challenge in male and female red-sided garter snakes (*Thamnophis sirtalis parietalis*) during the spring and fall (A). Body condition in panel A was calculated as the residual from a regression of body mass on snout-vent length. Because of a significant three-way interaction between season, sex and body condition, we reanalyzed the data to

focus on two *a priori* questions: Are there sex differences during the spring?, and Does male sensitivity to ACTH vary seasonally? These comparisons are shown in (B) with body condition category collapsed for clarity (the effects of body condition were not statistically significant in either comparison). Within males, the asterisk indicates a significant seasonal difference in integrated corticosterone response (from a non-parametric two-way ANOVA on male data with season and body condition as factors). Within spring, the asterisk indicates a significant sex difference in integrated corticosterone responses to ACTH (from a non-parametric two-way ANOVA on spring data with sex and body condition as factors). Numbers along the abscissa are sample sizes in each category.