

Behavioral and hormonal responses to corticosterone in the male red-sided garter snake, *Thamnophis sirtalis parietalis*

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Abstract

Stress and glucocorticoids are generally thought to suppress reproductive function at multiple levels. We tested the hypotheses that exogenous corticosterone would suppress sexual behavior in a dose-dependent manner, as well as drive a decrease in plasma testosterone levels in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. We examined this by challenging individual males with intraperitoneal injections of exogenous corticosterone, and subsequently exposing them to sexually attractive females or taking a blood sample. Previous work has demonstrated a hormonal but no behavioral response to stress in this species. In this study, increasing concentrations of exogenous corticosterone rapidly suppressed mating behavior in a threshold manner. However, exogenous corticosterone had no effect on plasma levels of testosterone. Thus, these data suggest that the mechanism is in place for corticosterone to suppress mating behavior in this species and that these effects do not occur because of an indirect effect on plasma levels of testosterone but rather are the direct effect of the hormone itself. In addition, the negative relationship observed previously between plasma levels of corticosterone and testosterone in this species was probably not the direct result of corticosterone acting on the hypothalamic–pituitary–gonadal (HPG) axis. Rather, our results seem to indicate that the negative associations between the hypothalamic–pituitary–adrenal axis (HPA) and the HPG axis occur at other levels of these neuroendocrine pathways. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

Stress can have dramatic negative effects on reproduction [10]. These primarily occur through the actions of glucocorticoid stress hormones from the adrenal cortex through activation of the hypothalamic–pituitary–adrenal (HPA) axis. For example, corticosterone has been shown to directly suppress mating behavior, in a dose-dependent manner, in the male rough-skinned newt, *Taricha granulosa* [22]. In addition, these hormones have been shown to directly inhibit testicular function in a variety of species [6,28,31]. Thus, stress hormones may suppress reproduction at multiple levels.

Reptiles exposed to stressful conditions respond with increases in plasma levels of corticosterone (reviewed in Refs. [11,19,36]), the primary glucocorticoid in reptiles

[14]. However, relatively few studies in reptiles, especially squamates, have progressed past the effects of stress on plasma levels of corticosterone. Investigations of how activation of the HPA axis affects reproductive behavior and plasma levels of other hormones, such as sex steroids, have been conducted on only a few reptile species. Capture stress has been shown to result in increases in plasma corticosterone and decreases in plasma testosterone in the male alligator, *Alligator mississippiensis* [20] and in the male tuatara, *Sphenodon punctatus* [2,36]. Male *A. mississippiensis* also responds to an injection of adrenocorticotrophic hormone (ACTH) with increased plasma levels of corticosterone but no change in plasma testosterone [21]. Male tree lizards, *Urosaurus ornatus*, respond to acute stress with increases in plasma corticosterone and decreases in plasma testosterone [26]. Implants of exogenous corticosterone cause a decrease in plasma levels of testosterone in this species [17], as well as in the male side-blotched lizard, *Uta stansburiana* [7]. In addition, corticosterone implants cause a decrease in activity and aggression in male *Uta stansburiana*, even if plasma levels of testosterone were

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experimentally elevated [7,8]. However, exogenous corticosterone has no effect on courtship or copulatory behavior in males of this species [7]. Thus, stress and corticosterone generally seem to suppress territorial behaviors, but may or may not affect plasma levels of sex steroids and sexual behavior in reptiles.

Our study examines the behavioral and hormonal responses to exogenous corticosterone in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. Previously, Moore et al. [24] documented 4 h of capture stress as having no effect on male mating behavior, but did result in an increase in plasma levels of corticosterone and a decrease in plasma levels of testosterone. There was a strong negative relationship between the two steroids, suggesting that plasma corticosterone acted directly to suppress plasma levels of testosterone. In the first experiment, we hypothesized that exogenous administration of corticosterone would suppress mating behavior in a dose-dependent manner. In the second experiment, we hypothesized that if the stress-induced increase in corticosterone drives the decrease in testosterone, then exogenous administration of corticosterone should result in decreased plasma levels of testosterone.

2. Methods

All studies were conducted in the field with free-living male red-sided garter snakes, *Tha. sirtalis parietalis*, at the Narcisse Wildlife Management Area in the Interlake region of Manitoba, Canada. These investigations occurred during the month following spring emergence when testosterone levels are declining and animals are mating [18]. All animals were held in captivity for less than 24 h and then returned to the site of capture.

2.1. Effect of corticosterone on mating behavior

This experiment was performed between May 9 and 25, 1997 to investigate the direct effects of corticosterone on mating behavior. Male *Tha. sirtalis parietalis* were captured and randomly assigned to one of six different treatment groups. To define a dose-dependent behavioral response to corticosterone, individuals in the first four groups received an intraperitoneal injection of 10 μg ($N=27$), 25 μg ($N=24$), 50 μg ($N=24$), or 100 μg ($N=27$) of corticosterone. These doses were chosen based on previous studies of *T. granulosa* that determined a dose-dependent behavioral response to exogenous corticosterone [22]. Average body mass for male *Tha. sirtalis parietalis* in this study was 33 g and did not differ between the groups. Corticosterone was first dissolved in 0.10-ml ethanol and subsequently in 10-ml reptilian ringers. Dilutions of this corticosterone solution were done to achieve the desired concentrations. The volume of all injections was 0.10 ml. The first set of controls (reptilian ringers) received an intraperitoneal injection

of reptilian ringers with ethanol in a similar concentration to the experimental animals ($N=53$). The second set received no injection ($N=53$). After treatment, all males had an individual number written on their heads and were then allowed to settle in nylon cloth arenas ($1 \times 1 \times 1$ m) for 30 min. At the end of the adjustment time, a sexually attractive unmated female (removed from a mating ball in which she was being courted) was introduced into the arena. Behaviors exhibited in the arenas were similar to those exhibited in the field and the use of arenas allowed us to follow individuals without losing sight of them. Each arena included eight males, two of each control group and two each of two different dosage treatments. The females did not appear to respond differently to the males and there is no male–male combat in garter snakes to confound the results [32]. The observer of each behavioral trial was blind to the treatment of the individuals and males were observed for 2 h after introduction. Individuals were scored as exhibiting mating behavior if they courted the female [24]. The proportion of males exhibiting mating behavior in each group was compared by chi-square analysis with the null hypothesis being no difference in the proportion of males exhibiting courtship between the groups.

2.2. Effect of corticosterone on plasma hormones

This experiment was performed between April 29 and May 2, 1998 to investigate the direct effects of corticosterone on plasma levels of testosterone. Male *Tha. sirtalis parietalis* (average mass 37 g) were captured and randomly assigned to one of three treatment groups. The first group, corticosterone-injected ($N=12$), received an intraperitoneal injection of 50 μg of corticosterone. We chose this dosage as it was sufficient to elicit a consistent behavioral response. The second group, ringers-injected ($N=12$), received an intraperitoneal injection of reptilian ringers. Both of these solutions were prepared as outlined above. Injection volume for each group was 0.10 ml. The third group, arena controls ($N=12$), received no injection and controlled for the effect of introducing the animals to the arenas. Males were introduced into arenas ($1 \times 1 \times 1$ m) in four groups of nine, three from each of the three treatment groups. Two females, previously determined to be sexually attractive (removed from mating balls in which they were being courted), were introduced into the arenas at the same time to resemble a natural situation of groups of males courting females [15]. All individuals were bled after being in the arena for 4 h, as this was the time span when capture-stressed males exhibited a significant decrease in testosterone [24]. Blood samples from 10 of the 12 animals in each group were randomly selected to be analyzed for hormone levels. A fourth group of males, controls ($N=10$), was captured randomly from the field, at the same time of day, and a blood sample was immediately obtained.

Plasma levels of testosterone were measured by radioimmunoassay following the procedures of Moore et al. [25].

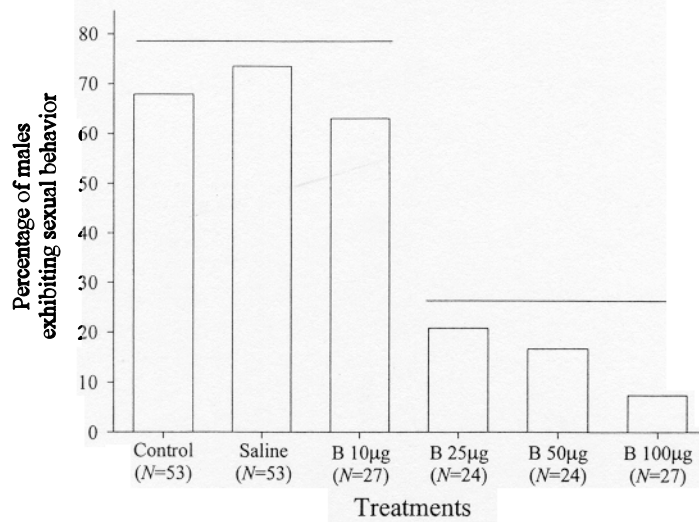


Fig. 1. Percentage of male red-sided garter snakes, *Tha. sirtalis parietalis*, exhibiting sexual behavior in the six different treatment groups. Groups underneath the solid lines are not significantly different from one another.

Plasma levels of corticosterone were measured by direct radioimmunoassay following the procedures of Ball and Wingfield [1]. All samples were assayed in duplicate, in a single assay, and corrected for individual recovery variation. Intra-assay variation was less than 10% for both testosterone and corticosterone. Plasma levels of testosterone were compared between the four study groups by one-way ANOVA on ranks. Plasma levels of corticosterone were log-transformed and compared by one-way ANOVA.

3. Results

3.1. Effect of corticosterone on mating behavior

Exogenous corticosterone suppressed mating behavior in a threshold manner. There was a significant difference

between the proportion of animals exhibiting mating behavior between the six treatment groups ($\chi^2=49.540$, $df=5$, $P<.001$). There was no difference in the proportion of animals displaying mating behavior between control, saline, and 10 µg corticosterone-injected animals ($\chi^2=0.433$, $df=2$, $P=.805$), so we combined these groups. We also saw no difference between males treated with 25, 50, and 100 µg of corticosterone ($\chi^2=1.943$, $df=2$, $P=.379$), so we combined these groups. When we compared the two combined groups, we found a significant difference between them (Fig. 1; $\chi^2=46.177$, $df=1$, $P<.001$). Male *Tha. sirtalis parietalis*, treated with the three highest dosages of corticosterone, suppressed mating behavior relative to the lowest corticosterone dosage and controls.

3.2. Effect of corticosterone on plasma hormones

We found no effect of exogenous corticosterone on plasma levels of testosterone. There was no difference in plasma levels of testosterone between the four treatment groups (Fig. 2, ANOVA on ranks, $H=1.15$, $P=.77$). There was also no overall difference in log-transformed plasma levels of corticosterone between the four treatment groups (Fig. 2, ANOVA, $F=1.10$, $P=.36$). However, there was a trend for the corticosterone-injected individuals to have higher plasma levels of corticosterone than the saline-injected animals (t test, $t=1.84$, $P=.08$).

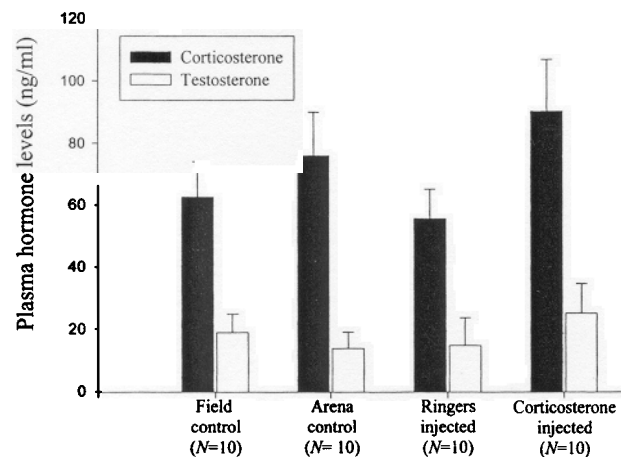


Fig. 2. Plasma levels of testosterone (mean \pm S.E.) and corticosterone (mean \pm S.E.) in four different treatment groups of male red-sided garter snakes, *Tha. sirtalis parietalis*. There are no significant differences between the groups.

4. Discussion

Exogenous administration of corticosterone suppressed mating behavior in male *Tha. sirtalis parietalis* in a threshold manner. Individuals treated with 25 µg or more of corticosterone significantly suppressed mating behavior

compared to controls and 10 µg corticosterone-treated individuals. We are confident that we would have seen a more traditional dose-dependent response if we had chosen additional dosages between 10 and 25 µg. Previous studies have documented that this species responds to 4 h of capture stress with an increase in plasma levels of corticosterone but no suppression of mating behavior [24]. Thus, it appears that the higher corticosterone treatments used in this study raised levels of the hormone above a threshold level at which point they had behavioral effects. This seems to reject an alternative hypothesis that this animal lacks receptors in the appropriate brain areas to exhibit suppression of mating behavior in response to stress or corticosterone. These studies suggest that, under normal circumstances, the mechanism exists for corticosterone to suppress mating behavior but that endogenous levels are kept below a threshold level or are isolated from target tissues. This could occur if, for example, plasma levels of corticosterone-binding globulin (CBG) were high enough to prevent any endogenous corticosterone from being free and biologically active [12,13,33,34]. Exogenous corticosterone would elevate plasma levels above the binding capacity of CBG resulting in free steroid, which could find its way to target tissues and exert behavioral effects.

We believe that the behavioral effects of corticosterone that we document here are not the result of a pharmacological dose of the hormone. The fact that we saw no behavioral effect with our lowest dose and found a threshold above this supports our contention that our dosages were in the physiological range. Dose-dependent behavioral responses with similar doses of corticosterone have been reported in other vertebrate classes (e.g., Refs. [22,29]). In addition, the 50-µg corticosterone dosage is the same as used in the second part of this study, which resulted in a physiologically relevant plasma level of the hormone, albeit at a later time. Finally, there did not appear to be any other behavioral effects (e.g., increased or decreased locomotion) of the corticosterone treatment.

There is precedence for the direct behavioral effects of corticosterone we document here. Corticosterone suppresses mating behavior in male *T. granulosa* [22], similar to what we observed in male *Tha. sirtalis parietalis*. In *T. granulosa*, this results from corticosterone binding a neuronal membrane-bound corticosteroid receptor rather than through traditional genomic action of the steroid [27]. It is not surprising that the behavioral effects of exogenous corticosterone in *Tha. sirtalis parietalis* occur independently of plasma testosterone. Previous studies have shown that the testes are not necessary for mating behavior to be exhibited by males of this species [3–5]. Although we cannot rule out more traditional genomic effects of corticosterone, our data (direct and rapid effect within 30 min) are consistent with the action of a membrane-bound corticosteroid receptor.

In our second experiment, we found that exogenous administration of corticosterone had no effect on plasma

levels of testosterone after 4 h. This was surprising as a previous study of this population of snakes documented an increase in plasma corticosterone and a decrease in plasma testosterone in response to 4 h of capture stress with a negative relationship between the two hormones [24]. The negative relationship between plasma levels of corticosterone and testosterone is usually evidence of a direct negative effect of corticosterone on testosterone production and has been documented in other reptiles [9,16]. Our injections of corticosterone did not appear to raise plasma levels of this hormone, however, there was a trend in that direction. Other studies have documented intraperitoneal injections of corticosterone as resulting in a transient elevation of plasma corticosterone and depression of plasma testosterone at 1 and 3 h that is not evident at 6 h after treatment [29]. Confirming that corticosterone was effective in this experiment, we did not notice any courtship behavior in the corticosterone-treated animals but did in the controls and saline-treated. Thus, it is possible that in the current study, plasma levels of corticosterone and testosterone changed and then recovered over the period of the experiment. Another possibility to explain the lack of a decline in testosterone is that a sudden spike in corticosterone is physiologically perceived differently than 4 h of capture stress by the HPG axis. It is possible then that a more chronic elevation of plasma corticosterone would drive a decrease in plasma testosterone.

Investigations of other reptiles, particularly lizards, suggest that exogenous corticosterone does not have a consistent negative effect on endogenous testosterone, regardless of the direct relationship between plasma levels of the two hormones [7,17]. Corticosterone implants significantly decreased plasma levels of testosterone but have no effect on male courtship and copulatory behaviors in *Uta stansburiana* [7]. Corticosterone significantly decreased aggressive behavior, as well as plasma testosterone and spermatogenic activity in the male brown anole, *Anolis sagrei* [35]. However, whether there is a direct relationship between plasma levels of the two hormones is not addressed in these studies. Different male morphs (associated with alternative reproductive tactics) of *U. ornatus* display decreases in plasma testosterone in response to dermal corticosterone patches [17]. However, only the nonterritorial morph displays a significant negative relationship between endogenous levels of the hormones [17]. This is hypothesized to allow the territorial morph to maintain aggressiveness despite the stress of intrasexual agonistic encounters. In contrast, male *A. mississippiensis* treated with ACTH displayed an increase in plasma corticosterone but no change in plasma testosterone [21]. Thus, it appears that, at least within reptiles, the direct effects of corticosterone on plasma levels of testosterone are not consistent. This may be associated with the need to maintain elevated plasma testosterone levels, despite being stressed, during certain periods, such as the breeding season or during gametogenesis and not during other times.

Studies in birds support a behavioral action of corticosterone separate from its effects on other hormones. Free-living populations of white-crowned sparrows, *Zonotrichia leucophrys*, and song sparrows, *Melospiza melodia*, respond to storms during the breeding season with an increase in plasma corticosterone levels but no change in plasma testosterone levels [37–39]. Similarly, treatment of song sparrows with exogenous corticosterone caused a slight decrease in plasma levels of testosterone, but still well within the normal levels witnessed during the breeding season [40]. These same experimental animals displayed significantly reduced territorial behavior, relative to controls. Thus, the authors conclude that song sparrows display a significant behavioral response to exogenous corticosterone independent of its actions on the HPG axis [40]. This is similar to what we report with corticosterone suppressing a behavior independent of its effect on the HPG axis.

The lack of an effect of exogenous corticosterone on plasma levels of testosterone does not necessarily mean that the HPA and HPG axes are not reciprocally related in this species. For example, corticosterone acts at the level of the hypothalamus to suppress the release of gonadotropin-releasing hormone (GnRH) [23]. Our data suggest that endogenous corticosterone levels are not responsible for driving the acute decrease in testosterone, leaving other parts of the HPA axis as more likely being responsible. There is evidence for direct negative effects, such as paracrine actions of corticotropin-releasing factor on GnRH producing neurons, between higher levels of the HPA and HPG axes [30]. In addition, the release of ACTH from the anterior pituitary is accompanied by the co-release of β -endorphin that can act to suppress the HPG axis [30].

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