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General and Comparative Endocrinology 141 (2005) 259–270
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

A serotonin receptor antagonist, but not melatonin, modulates hormonal responses to capture stress in two populations of garter snakes (*Thamnophis sirtalis parietalis* and *Thamnophis sirtalis concinnus*)

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Received 23 October 2004; revised 13 January 2005; accepted 18 January 2005

Abstract

Hormonal and behavioral responses to a stressor depend on many factors, including the influence of other hormones. We examined the role of melatonin in modulating hormonal responses to capture stress in two populations of male garter snakes, *Thamnophis sirtalis*. Studies of red-sided (*T. sirtalis parietalis*) and red-spotted (*T. sirtalis concinnus*) garter snakes were conducted in the field with free-living snakes. Populations of red-sided garter snakes in south-central Manitoba, Canada undergo a period of winter dormancy for approximately 8 months each year followed by an attenuated mating season (4–5 weeks) in early spring. In contrast, the mid-latitude red-spotted garter snake in western Oregon, USA has an extended breeding season and can be active during 10–12 months of the year given appropriate environmental conditions. We chose to study these two populations of garter snakes to investigate possible variation in melatonin function among snakes with different suites of environmental adaptations. To better address these questions, we also examined the effects of 5-hydroxytryptophan (a precursor of melatonin synthesis) and ketanserin (a serotonergic type 2A receptor antagonist) on hormonal responses to capture stress. We observed a trend of increased corticosterone and decreased androgen concentrations in northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) subjected to 4 h of capture stress during the spring. However, these differences were not statistically significant. During the fall, red-sided garter snakes showed no change in corticosterone or androgen concentrations in response to the capture stress treatments. We speculate that northern-latitude red-sided garter snakes suppress hormonal responses to capture stress during preparation for winter dormancy. Treatment with melatonin, 5-hydroxytryptophan, or ketanserin did not significantly influence corticosterone or androgen concentrations of northern-latitude red-sided garter snakes during the spring or fall. Mid-latitude red-spotted garter snakes (*T. sirtalis concinnus*) from Oregon showed a statistically significant increase in corticosterone concentrations in response to 4 h of capture stress; treatment with melatonin, 5-hydroxytryptophan, or ketanserin prior to capture stress had no significant influence on plasma corticosterone concentrations. Androgen concentrations of mid-latitude red-spotted garter snakes in response to capture stress were significantly lower than those of non-stressed control snakes. Neither melatonin nor 5-hydroxytryptophan influenced the change in androgen concentrations during capture stress. However, androgen concentrations of snakes treated with ketanserin prior to 4 h of capture stress did not differ significantly from those of non-stressed control snakes. These studies suggest that melatonin does not modulate hormonal responses to capture stress in this ectothermic model. Our results also suggest that a serotonin-regulated system may play a role in modulating the activity of the hypothalamic–pituitary–gonadal axis during physiological stress responses.

Keywords: Melatonin; 5-Hydroxytryptophan; Ketanserin; Serotonin; Stress; Corticosterone; Androgen; Reptile; Garter snake; *Thamnophis sirtalis*

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doi:10.1016/j.ygcen.2005.01.012

1. Introduction

Hormonal responses to stressors are protective events that help vertebrates respond to external noxious stimuli. A stressor can be any perturbation that disrupts the predictability of an animal's environment. Examples of stressors include predation events and unpredicted challenges to energy homeostasis, such as unusually low environmental temperatures, food shortage, and starvation. Responses to stressors are mediated by an increase in the activity of the hypothalamic–pituitary–adrenal axis and are marked by increased glucocorticoid secretion (Harvey et al., 1984; Schwabl et al., 1985; Wingfield, 1988). Glucocorticoids in turn modulate a variety of physiological and behavioral processes that promote survival while suppressing behaviors, such as reproduction, that are not crucial to immediate survival (e.g., Pottinger, 1999; Sapolsky, 1992; Wingfield, 1988). Such acute physiological stress responses are normally adaptive responses used to modify metabolism and mobilize energy stores.

Negative interactions between the hypothalamic–pituitary–adrenal axis and the hypothalamic–pituitary–gonadal axis suggest that activation of one system is accompanied by decreased activity in the other. Decreased plasma testosterone accompanies increased glucocorticoids in male tree lizards (*Urosaurus ornatus*) and male red-sided garter snakes (*Thamnophis sirtalis parietalis*) (Moore et al., 1991, 2000a). Thus, physiological stress responses, marked by an increase in plasma glucocorticoid concentrations, result in decreased sex steroid hormones and suppression of reproductive behavior in many species (e.g., Rivier and Rivest, 1991; Wingfield, 1988).

Hormonal and behavioral responses to a stressor depend on many factors, including the environment inhabited, the season, and the influence of other hormones. Melatonin, for example, the major secretory product of the pineal gland, inhibits the hypothalamic–pituitary–adrenal axis (Reiter, 1991; Wang et al., 1999). Interactions between the pineal gland and hypothalamic–pituitary–adrenal axis are well established (e.g., Khan et al., 1990; Kirby et al., 1999; Maestroni et al., 1989), although the nature of this relationship is poorly understood. Both stimulatory (Al-Dujaili et al., 1982; Haus et al., 1996; Persengiev et al., 1989; Touitou et al., 1989) and inhibitory (Heiman and Porter, 1980; Ng, 1987; Nussdorfer et al., 1990) effects of melatonin on the secretory activity of the adrenal cortex have been described. However, all of these studies examined the effects of melatonin on cultured adrenocortical cells (Appa-Rao et al., 2001). In an experiment using in vivo physiological conditions, melatonin exerted a direct antisecretory effect on the adrenal gland (Appa-Rao et al., 2001). In addition, chronic melatonin treatment alters the affinity of glucocorticoid receptors in the brain and pituitary (Marinova et al., 1991).

Behavioral responses to stress and exogenous glucocorticoids are also modulated by melatonin. In male rats, melatonin treatment significantly reduces the inhibitory effects of acute and chronic stress on sexual behavior (Brotto et al., 2001). Gorzalka et al. (1999) demonstrated that acute melatonin treatment also attenuates the effects of glucocorticoids on sexual behavior and wet-dog shakes in male rats. These effects of melatonin are thought to be mediated by melatonin's properties as a serotonergic type 2A receptor antagonist (Eison et al., 1995; Gorzalka et al., 1999).

In mammals, the effects of melatonin on physiological stress responses appear to involve both direct antisecretory actions on the adrenal cortex and antagonism of glucocorticoid actions at the target tissues. However, the role of melatonin in modulating behavioral and hormonal responses to stress and exogenous glucocorticoids has been largely uninvestigated in nonmammalian species. Lutterschmidt et al. (2004) demonstrated that melatonin does not influence responses of red-sided garter snakes (*T. sirtalis parietalis*) to exogenous corticosterone [the primary glucocorticoid in snakes (Idler, 1972)]. In contrast, melatonin and corticosterone have significant additive inhibitory effects on reproductive behavior (Lutterschmidt et al., 2004). These results indicate that melatonin does not antagonize glucocorticoid actions in red-sided garter snakes. In the present studies, we investigated whether melatonin has antisecretory effects on the adrenal cortex by examining the influence of melatonin on hormonal responses to capture stress in two populations of male garter snakes, *T. sirtalis*.

Red-sided garter snakes (*T. sirtalis parietalis*) are the most northerly living reptile in North America and are found in extremely high numbers throughout south central Manitoba, Canada. These northern-latitude populations of snakes undergo a period of winter dormancy for approximately 8 months each year. Following spring emergence, red-sided garter snakes remain within the vicinity of the dens for the duration of the attenuated mating season (4–5 weeks) (e.g., Crews and Garstka, 1982). Moore et al. (2000a) demonstrated that during the spring mating season, red-sided garter snakes respond to 4 h of capture stress with an increase in corticosterone and a decrease in testosterone concentrations. However, later work indicated that male red-sided garter snakes display no change in either plasma corticosterone or testosterone during the spring mating season, suggesting the presence of annual variation in stress responses (Moore et al., 2001). During the fall, when mating has also been observed (Krohmer et al., 1987; Mendonça and Crews, 1989), male red-sided garter snakes respond to 4 h of capture stress with no significant increase in corticosterone but a significant decrease in testosterone concentrations (Moore et al., 2001; see Section 4 for a more detailed description of these studies).

In contrast, the mid-latitude red-spotted garter snake (*T. sirtalis concinnus*) of western Oregon has an extended breeding season that lasts 10–12 weeks from March to May. Although red-spotted garter snakes do exhibit periods of winter dormancy, they can be active during 10–12 months of the year given appropriate environmental conditions (Moore et al., 2000b). During the spring, summer, and fall, red-spotted garter snakes respond similarly to 4 h of capture stress with significantly increased corticosterone and significantly decreased testosterone concentrations (Moore et al., 2001). During the summer, however, when gametogenesis is occurring, mid-latitude red-spotted garter snakes show no decline in plasma testosterone in response to capture stress, despite a concomitant increase in corticosterone (Moore et al., 2001).

Because these two populations of *T. sirtalis* have very different life history characteristics, they provide an excellent opportunity to investigate possible population differences in the regulation of hormonal stress responses. We were specifically interested in examining the following questions: (1) Does melatonin influence hormonal responses to capture stress?; (2) Is there seasonal variation in the effects of melatonin on hormonal stress responses?; and (3) Are there population differences in the influence of melatonin on hormonal stress responses? To better address these questions, we also investigated whether 5-hydroxytryptophan, a precursor of melatonin synthesis, modifies responses to capture stress. Lastly, to test whether the effects of melatonin on stress responses might be due to antagonism of serotonin receptors, we examined the influence of ketanserin, a serotonergic type 2A receptor antagonist, on hormonal responses to capture stress.

2. Materials and methods

These experiments were conducted in the field with free-living garter snakes (*T. sirtalis*) in the Interlake region of Manitoba, Canada (50°37′N, 97°32′W) and the Willamette Valley of western Oregon, USA (44°30′N, 123°17′W). Studies of northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) during the spring were conducted between 10:00 and 16:30 h on 19–21 May 2003, during the month following emergence from hibernacula when snakes are mating and plasma testosterone concentrations are declining (Krohmer et al., 1987). Fall experiments in northern-latitude *T. sirtalis parietalis* were conducted between 12:00 and 18:00 h on 11–13 September 2003, when snakes are returning to the den site to over-winter. Both spring and fall experiments were conducted with northern-latitude red-sided garter snakes collected from a den located in Inwood, Manitoba, Canada. Studies of the mid-latitude red-spotted garter snake were conducted during the breeding season at E.E. Wilson Wildlife Area (approximately 15 km north of Corvallis, Oregon, USA) between 10:30 and 20:00 h on 23 March–12 April 2004. All experimental protocols were approved by the Oregon State University Animal Care and Use Committee (Protocol No.: LAR-2661) and were in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals." This research was approved by the Manitoba Wildlife Animal Care Committee (Protocol No.: 2002–06) and was performed under the authority of Manitoba Wildlife Scientific Permit No. WSP 03009.

2.1. Reagents

Melatonin and 5-hydroxytryptophan, a precursor of melatonin synthesis, were purchased from Sigma Chemical (St. Louis, MO). We were specifically interested in examining differences between the effects of melatonin and this melatonin precursor on hormonal stress responses. During the scotophase (i.e., the dark phase of the photoperiod), treatment of mudpuppies (*Necturus maculosus*) with 5-hydroxytryptophan (20 mg kg^{-1} body mass) significantly elevates melatonin synthesis for greater than 4 h following injection (Rawding and Hutchison, 1993). However, we administered treatment injections during the photophase (i.e., the light phase of the photoperiod), when the activity of enzymes responsible for melatonin synthesis is reduced. For example, circadian regulation of enzymatic activity and/or transcription occur for tryptophan hydroxylase, *N*-acetyltransferase, and hydroxyindole-*O*-methyltransferase (e.g., Cassone and Natesan, 1997; Falcón et al., 1987, 1989; Morton and Forbes, 1988). Because melatonin synthesis typically occurs during the scotophase, administering treatments during the photophase ensures that a majority of the melatonin precursor will not be synthesized into melatonin. Thus, we expected that most of our 5-hydroxytryptophan treatment would remain in the circulation. We used this protocol to test the direct effects of 5-hydroxytryptophan on hormonal stress responses.

Ketanserin, a serotonergic type 2A receptor antagonist, was purchased from ICN Biomedicals (Costa Mesa, CA). We chose this antagonist because: (1) the effects of melatonin on stress responses are thought to be mediated by melatonin's properties as a serotonergic type 2A receptor antagonist (Eison et al., 1995; Gorzalka et al., 1999) and (2) corticosterone regulates the density of serotonergic type 2 A receptors as well as serotonergic type 2A receptor-mediated behaviors (Berendsen et al., 1996; Fernandes et al., 1997; Gorzalka and Hanson, 1998; Takao et al., 1997). All treatments were administered via intraperitoneal injection with an injection volume of 0.1 ml. Injection volumes of vehicle (5% ethanol in reptile Ringer's solution) were also 0.1 ml. All treatment solutions were prepared fresh daily.

Melatonin solutions for the low and high melatonin doses were prepared by first dissolving 1.5 or 15 mg, respectively, in 0.25 ml of 100% ethanol. Stock solutions were diluted to 5 ml with reptile Ringer's solution, producing melatonin concentrations for the low and high doses of 0.3 and 3.0 mg ml^{-1}, respectively. Thus, the melatonin doses were 0.03 and 0.3 mg per snake (i.e., 0.03 and 0.3 mg per 0.1 ml). For an average male snake weighing 0.03 kg, the high melatonin dose was 10 mg kg^{-1} body mass, which is similar to the doses used to test the effects of melatonin on thermoregulation in ectotherms (Erskine and Hutchison, 1981; Skinner, 1991).

5-Hydroxytryptophan solution was prepared by dissolving 30 mg of 5-hydroxytryptophan in 5 ml of 5% ethanol in reptile Ringer's solution, producing a 5-hydroxytryptophan concentration of 6 mg ml^{-1}. Thus, the 5-hydroxytryptophan treatment dose was 0.6 mg per snake. For an average male snake weighing 0.03 kg, this dose is 20 mg kg^{-1} body mass, which is identical to the dose used by Rawding and Hutchison (1993) for testing the effects of 5-hydroxytryptophan on melatonin synthesis in mudpuppies (*N. maculosus*).

Ketanserin solution was prepared by first dissolving 2.25 mg in 0.25 ml of 100% ethanol and then diluting to 5 ml with reptile Ringer's solution. This produced a ketanserin concentration of 0.45 mg ml^{-1} and a ketanserin dose of 0.045 mg per snake. For an average male snake weighing 0.03 kg, this ketanserin dose was 1.5 mg kg^{-1} body mass. We chose this dose based on previous research done by Wilson and Pulido (2000), who used a 1.5 mg kg^{-1} dose to test the effects of this serotonin receptor antagonist on the behavioral transport response of rats. In rat pups, the transport response is characterized by adduction of the limbs and tail to aid in transport by the mother and is known to be modulated by serotonin.

2.2. Experimental design

Male garter snakes collected in the field were randomly assigned to one of six capture stress treatments ($\geqslant 10$ in each group): no injection, vehicle (5% ethanol in reptile Ringer's solution), low melatonin dose (0.03 mg), high melatonin dose (0.3 mg), 5-hyroxytryptophan (0.6 mg), or ketanserin (0.045 mg). Following treatment injections, snakes were housed in circular outdoor arenas (48 cm diameter) and allowed to absorb the treatment drugs for 30 min. Snakes were then immediately isolated individually in small, opaque cloth bags (approximately 20×20 cm) for 4 h. This capture-stress protocol is identical to that of Moore et al. (2000a), who used capture and isolation of snakes in these cloth bags to induce physiological stress responses. We collected blood samples from snakes at the end of each 4-h capture stress treatment. For the non-stressed control groups, blood samples were collected at approximately the same time as the capture stress-treated snakes from snakes captured in the field.

2.3. Blood sampling and radioimmunoassay

Blood samples were obtained from the caudal vein as quickly as possible (mean ± 1 SE: 70.1 ± 3.3 s) using heparinized 1-cm^3 syringes and 25-g needles. Samples from red-sided garter snakes obtained in Canada were stored on ice until return to the field station, where they were centrifuged and the plasma separated. Plasma samples were stored at −4 °C until return to Oregon State University. Samples obtained from red-spotted garter snakes in Oregon were stored on ice until return to the laboratory at Oregon State University, where they were centrifuged and the plasma separated. All plasma samples were then stored at −70 °C until analyzed for corticosterone and testosterone concentrations following radioimmunoassay procedures modified from Moore et al. (2000a).

To test whether chromatography of steroid hormones extracted from snake plasma is necessary, we simultaneously analyzed a subset of plasma samples ($n = 30$ for northern-latitude *T. sirtalis parietalis*; $n = 18$ for mid-latitude *T. sirtalis concinnus*) for corticosterone and testosterone concentrations using both radioimmunoassay with partition chromatography (Moore et al., 2000a) and radioimmunoassay without partition chromatography (i.e., direct radioimmunoassay). For the northern-latitude red-sided garter snake, we used a subset of plasma samples representing both spring and fall seasons ($n = 15$ spring and 15 fall samples) to test the necessity of steroid hormone chromatography. We included both spring and fall samples in our evaluations to account for variation in plasma lipid concentrations, and hence different levels of nonspecific binding of steroids, typically observed at different times of the year. The subset of plasma samples used to evaluate the necessity of chromatography of samples from mid-latitude red-spotted garter snakes represented the spring season only, as these snakes were only sampled during the spring. The methods used for direct radioimmunoassay are described in Lutterschmidt et al. (2004) and are similar to those of Jessop et al. (1999, 2000).

For individual sample recovery determination, duplicate aliquots (20–70 μl) of each plasma sample were incubated 12–24 h with 2000 cpm of tritiated steroid (Amersham Biosciences, Piscataway, NJ). Steroids were extracted from each plasma sample twice with anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas in a warm (37 °C) water bath. Hormone extracts were then either reconstituted in phosphate-buffered saline for direct assay or reconstituted in 10% ethyl acetate in isooctane and chromatographed through individual celite microcolumns. Steroid fractions and neutral lipids were eluted using increasing

proportions of ethyl acetate in isooctane. The purified eluates were dried under nitrogen gas and reconstituted in phosphate-buffered saline for assay.

Individual sample recoveries were determined from a 50-μl aliquot of each extracted and reconstituted sample. For each steroid hormone being assayed, 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-^{3}H testosterone or 1,2,6,7-^{3}H corticosterone, Amersham Biosciences, Piscataway, NJ) and 100 μl antiserum (testosterone antibody T3003 from Wein Laboratories, Succasunna, NJ; corticosterone antibody B3-163 from Esoterix Endocrinology, Calabasas Hills, CA) at 4 °C for 12–24 h. 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 101.8% (98.7% for corticosterone and 104.8% for testosterone), as determined by the recovery of tritiated steroid added to samples prior to extraction with ethyl ether. Extraction efficiency in direct radioimmunoassay is typically much greater (>95%) than mean extraction efficiency following column chromatography. Because we have recently checked the calibration of our pipettes, the extraction efficiency reported here likely represents random variation around this greater mean extraction efficiency. All spring and fall samples (i.e., treatment groups) from northern-latitude *T. sirtalis parietalis* were randomly distributed across four steroid assays. Mean intra-assay variation was 13.5% for testosterone and 12.2% for corticosterone; inter-assay variation was 18.1% for testosterone and 13.7% for corticosterone. All samples (i.e., treatment groups) from mid-latitude *T. sirtalis concinnus* were randomly distributed across two steroid assays. Mean intra-assay variation of samples from *T. sirtalis concinnus* was 7.8% for testosterone and 13.9% for corticosterone; inter-assay variation was 9.3% for testosterone and 13.3% for corticosterone.

2.4. Statistical analyses

We used regression analyses to examine the correlation between steroid concentrations of samples determined by radioimmunoassay with and without partition chromatography. Within each population, we used a multivariate analysis of variance (MANOVA) followed by a Student–Newman–Keuls multiple comparisons procedure to investigate possible differences in hormone concentrations among treatment groups. We used this multivariate approach to simultaneously analyze both corticosterone and androgen responses to the capture stress treatments. For analysis of samples from northern-latitude red-sided garter snakes (*T. sirtalis parietalis*), treatment and season were included in the MANOVA as fixed factors. Because studies of mid-latitude red-spotted garter snakes (*T. sirtalis concinnus*) were conducted only during the spring, only treatment was included in the MANOVA as a fixed factor. Prior to analysis, spring and fall androgen concentrations of northern-latitude red-sided garter snakes were natural-log-transformed to correct for non-normality. We used SigmaStat 2.03 (SPSS, 1997) and SPSS 13.0 (SPSS, 2004) for all statistical analyses. All statistical comparisons were considered significant at $P \leqslant 0.05$.

3. Results

We obtained excellent correlation between the steroid concentrations of a subset of plasma samples ($n = 15$ spring samples + 15 fall samples) collected from northern-latitude *T. sirtalis parietalis* and assayed by both direct radioimmunoassay and radioimmunoassay with partition chromatography ($R^2 = 0.95$, $P < 0.001$ for corticosterone; $R^2 = 0.99$, $P < 0.001$ for testosterone, from a regression). Likewise, we obtained excellent correlation between the steroid concentrations of a subset of plasma samples ($n = 18$) collected from mid-latitude *T. sirtalis concinnus* during the spring breeding season and assayed by both direct radioimmunoassay and radioimmunoassay with partition chromatography ($R^2 = 0.90$, $P < 0.001$ for corticosterone; $R^2 = 0.93$, $P < 0.001$ for testosterone, from a regression). Thus, we elected to analyze all plasma samples using direct radioimmunoassay methods. Because our testosterone antibody (Wein Laboratories, Succasunna, NJ) cross-reacts significantly with 5-α-dihydrotestosterone (63.2% cross-reactivity), our direct assay measures both plasma testosterone and 5-α-dihydrotestosterone concentrations. For these reasons, we present here data for total androgen concentrations.

Results from a MANOVA indicate no statistically significant differences in corticosterone or androgen concentrations among the treatment groups of northern-latitude red-sided garter snakes (Figs. 1A–D). There were no significant effects of season on corticosterone concentrations. As expected, androgen concentrations of northern-latitude red-sided garter snakes were significantly higher during the fall ($P \leqslant 0.001$ from a MANOVA). There were no statistically significant interactions between season and treatment.

Mid-latitude red-spotted garter snakes showed a statistically significant increase in corticosterone in response to 4 h of capture stress ($F = 3.425$; df = 6; $P = 0.005$, from a MANOVA). Results from a Student–Newman–Keuls multiple comparisons procedure indicate that treatment with melatonin, 5-hydroxytryptophan, or ketanserin had

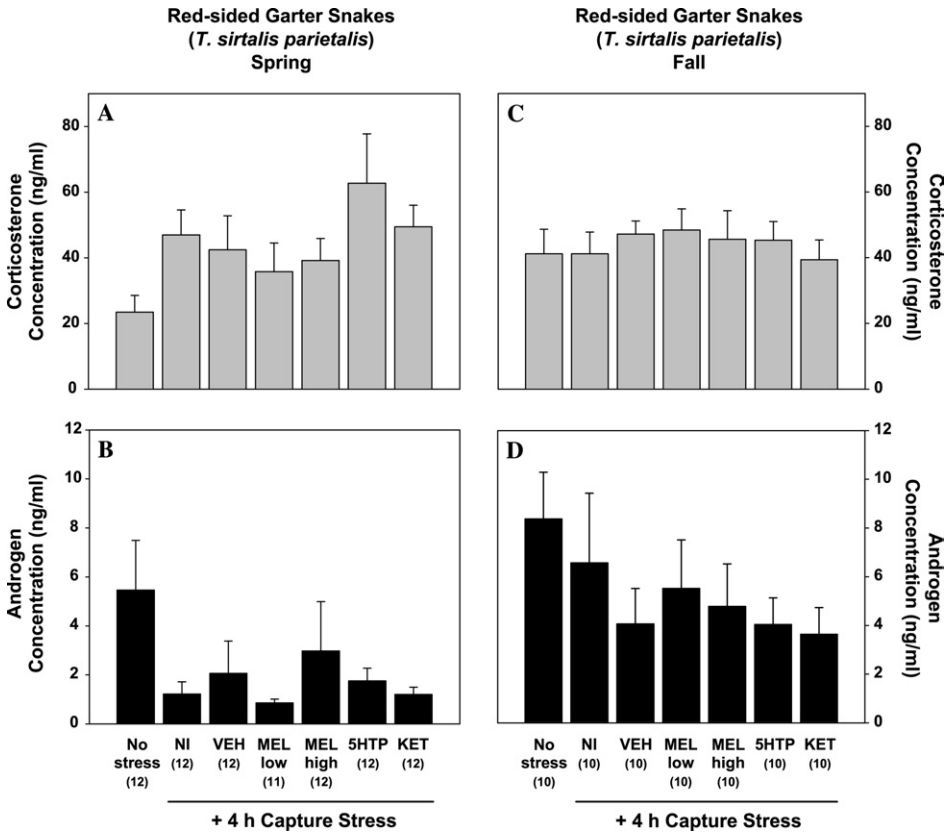

Fig. 1. Hormonal responses of male red-sided garter snakes (*T. sirtalis parietalis*) to capture stress during the spring and fall seasons. Male garter snakes collected in the field in south-central Manitoba, Canada received the following treatments (indicated along the abscissa) prior to 4 h of capture stress: no injection (NI), vehicle (5% ethanol in reptile Ringer's solution; VEH), low melatonin dose (0.03 mg; MEL low), high melatonin dose (0.3 mg; MEL high), 5-hydroxytryptophan (0.6 mg; 5HTP), or ketanserin (0.045 mg; KET). A control group of snakes (No stress) received no capture stress treatment. (A) and (C) mean plasma corticosterone concentrations of snakes during the spring and fall, respectively. (B) and (D) mean plasma androgen concentrations during the spring and fall, respectively. Sample sizes are indicated in parentheses below the treatment groups. Standard errors (±1) are shown by the vertical lines.

no significant influence on the corticosterone concentrations of red-spotted garter snakes in response to capture stress (Fig. 2A). Androgen concentrations of mid-latitude red-spotted garter snakes subjected to 4 h of capture stress were significantly lower than those of non-stressed control snakes ($F = 2.906$; df = 6; $P = 0.014$, from a MANOVA). Treatment of snakes with either melatonin or 5-hydroxytryptophan did not influence the androgen concentrations of mid-latitude red-spotted garter snakes in response to capture stress (Fig. 2B; results from a Student–Newman–Keuls multiple comparisons procedure). Androgen concentrations of snakes treated with ketanserin prior to 4 h of capture stress did not differ significantly from the androgen concentrations of non-stressed control snakes (Fig. 2B).

4. Discussion

Our results indicate that northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) respond to 4 h of capture stress during the spring and fall seasons with no significant changes in either corticosterone or androgen concentrations. Although we observed a trend of increased corticosterone and decreased androgen concentrations in response to capture stress during the spring mating season (Figs. 1A and B), our multivariate analyses indicated that these differences were not statistically significant. This lack of a statistical difference most likely results from the large variation in hormone concentrations both within and among groups. Indeed, a *t* test indicates that northern-latitude red-sided garter snakes receiving no injection + 4 h of capture stress during the spring had significantly higher corticosterone levels than non-stressed control snakes ($P = 0.018$; Fig. 1A). A *t* test between the androgen concentrations of non-stressed control snakes and non-injected capture stress-treated snakes does not resolve the large variation observed in androgen concentrations, as it indicates no statistically significant difference between these groups of northern-latitude red-sided garter snakes (Fig. 1B). Similar to Moore et al. (2001), we demonstrate that mid-latitude red-spotted garter snakes (*T. sirtalis concinnus*) respond to 4 h of capture stress with a significant

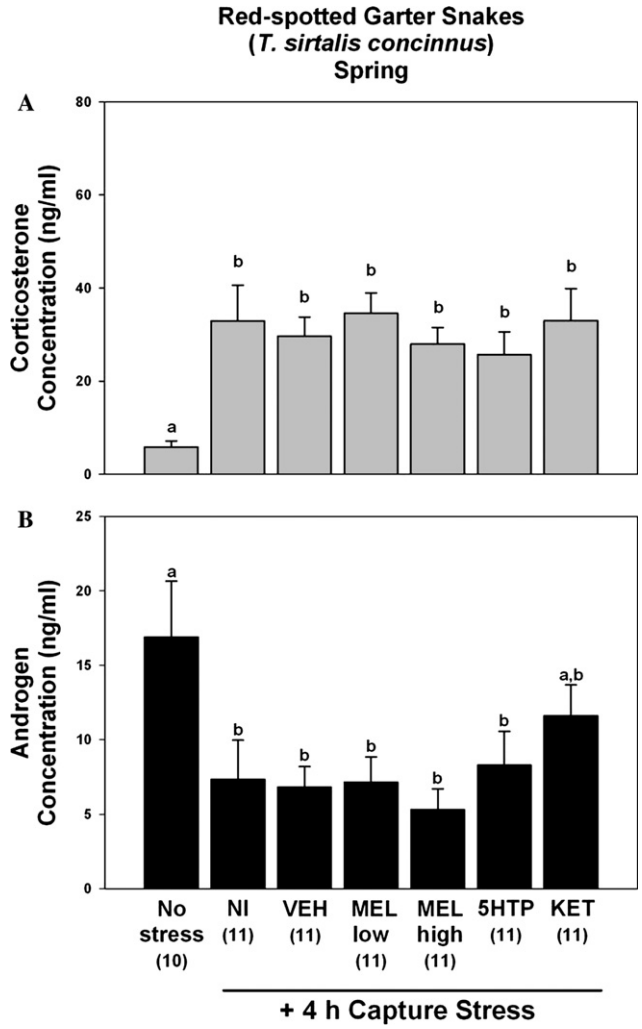

Fig. 2. Hormonal responses of male red-spotted garter snakes (*T. sirtalis concinnus*) to capture stress during the spring mating season. Male garter snakes collected in the field in western Oregon, USA, received the following treatments (indicated along the abscissa) prior to 4 h of capture stress: no injection (NI), vehicle (5% ethanol in reptile Ringer's solution; VEH), low melatonin dose (0.03 mg; MEL low), high melatonin dose (0.3 mg; MEL high), 5-hydroxytryptophan (0.6 mg; 5HTP), or ketanserin (0.045 mg; KET). A control group of snakes (no stress) received no capture stress treatment. (A) Mean plasma corticosterone concentrations of snakes; (B) mean plasma androgen concentrations. Sample sizes are indicated in parentheses below the treatment groups. Standard errors (±1) are shown by the vertical lines. Statistically significant differences among treatment groups are indicated by letters above each standard error bar.

increase in plasma corticosterone and a significant decrease in androgen concentrations.

4.1. Seasonal variation in hormonal responses to capture stress

The trends we observed in northern-latitude red-sided garter snakes are similar to those reported by Moore et al. (2000a), who demonstrated a significant increase in plasma corticosterone and a significant decrease in plasma testosterone concentrations of red-sided garter snakes in response to 4 h of capture stress during the spring mating season. In contrast, Moore et al. (2001) reported no significant change in corticosterone or testosterone concentrations of northern-latitude red-sided garter snakes during the spring. The basal corticosterone concentrations of snakes during this study (23.5 ng ml^{-1} plasma) were much lower than the basal corticosterone concentrations reported for red-sided garter snakes during the spring in both Moore et al. (2000a; 62 ng ml^{-1}) and Moore et al. (2001; 129 ng ml^{-1}). The lack of an effect of capture stress on corticosterone levels reported in Moore et al. (2001) was attributed to the already elevated corticosterone concentrations of snakes during that study.

In these populations of northern-latitude red-sided garter snakes, a significant negative relationship exists between basal corticosterone concentrations and body condition (Moore et al., 2001). Because snakes actively forage for only 3–4 months before undergoing 8 months of winter dormancy, seasonal changes in body condition are pronounced. For example, during the summer and fall, northern-latitude red-sided garter snakes have positive body condition (determined as an individual's residual from a regression of body mass on snout-to-vent length for all snakes across seasons), with the greatest positive body condition occurring during the summer feeding months (Moore et al., 2001). In contrast, snakes captured immediately following emergence from the hibernaculum had a negative body condition. Body condition of snakes during late spring (i.e., late in the mating season) was more than sixfold lower than body condition of snakes during early spring (Moore et al., 2001), likely due to sustained mating activity. Snakes are aphagic during the mating season, and therefore sustaining mating activity is extremely costly; snakes can lose up to 1–2% of their body mass per day (Mason, unpublished data).

The northern-latitude red-sided garter snakes sampled during this study also had a negative body condition during the late spring. However, body condition of snakes during this study was much greater (mean residual from a regression of body mass on snout-to-vent length = −0.05) than the body condition of snakes reported in Moore et al. (2001) (mean residual ≈−6.0). During the spring mating season, basal corticosterone concentrations are related to body condition and therefore likely reflect the climatic conditions and food availability of the previous summer feeding season as well as the length of winter dormancy. These observations reflect the unique life history of red-sided garter snakes living at the extreme northern limit of their range, as neither seasonal changes in body condition nor a correlation between basal corticosterone levels and body condition have been observed in the red-spotted garter snake, which experiences the much milder environmental conditions of western Oregon (Moore et al., 2001).

During the fall, northern-latitude red-sided garter snakes showed no change in corticosterone or androgen concentrations in response to capture stress (Figs. 1C and D). These results are similar to the findings of Moore et al. (2001), who reported no significant change in corticosterone concentrations and a significant decrease in testosterone levels following 4 h of capture stress during the fall. In both this study and in Moore et al. (2001), experiments were conducted at the den site with snakes that had returned to the den in preparation for winter dormancy. We hypothesize that northern-latitude red-sided garter snakes suppress at least hormonal responses to capture stress during the fall pre-hibernation period. Although further investigation is necessary, suppression of corticosterone responses to capture stress prior to entering winter dormancy may be adaptive. Because fat stores are necessary to sustain snakes during winter dormancy, suppression of hormonal stress responses prior to entering winter dormancy would conserve these much-needed fat stores. Additional studies of hormonal stress responses in snakes both pre- and post-migration would provide insight into whether stress responses are modulated by the mechanisms regulating fall migration to the dens and preparation for winter dormancy.

4.2. Melatonin and hormonal responses to capture stress

We investigated whether melatonin and 5-hyroxytyrptophan, a precursor of melatonin synthesis, modulate the responses of garter snakes to capture stress. Although there is evidence that melatonin inhibits the secretion of glucocorticoids in mammals in vivo (Appa-Rao et al., 2001), our results suggest that melatonin does not play a role in modulating hormonal stress responses in this ectothermic model. However, we measured corticosterone and androgen concentrations of snakes at only one time point (i.e., 4 h) following capture stress. Thus, it is possible that melatonin does indeed modulate stress responses in these snakes but we were unable to observe these effects due to our sampling regime. For example, melatonin may reduce initial hormonal responses to acute stressors but may not affect responses to stressors of longer (i.e., 4 h) duration. During the spring, levels of corticosterone in both northern-latitude red-sided garter snakes and mid-latitude red-spotted garter snakes subjected to 1 h of capture stress are similar to those in response to 4 h of capture stress (Moore et al., 2001). Thus, it is not likely that corticosterone concentrations had significantly declined over our 4-h capture stress period. In addition, we are confident that plasma melatonin levels, in response to hormone treatments, remained elevated throughout the duration of our experiments. The half-life of melatonin after injection into endotherms can be 1 h or less (Rollag and Stetson, 1982); the half-life of melatonin in whole-animal ectotherms is likely to be much longer because metabolic rate is as much as 10 times lower, depending on body temperature (Filadelfi and Castrucci, 1996). Future studies investigating the time course of melatonin's influence on hormonal responses to capture stress are necessary to discern whether melatonin does indeed regulate stress responses.

In northern-latitude red-sided garter snakes, mating occurs upon emergence from winter dormancy while plasma sex steroid concentrations are declining, gonads are regressed, and glucocorticoid levels are high (Aleksiuk and Gregory, 1974; Crews, 1984; Crews et al., 1984; Krohmer et al., 1987; Whittier et al., 1987a). Because these snakes are aphagic during the mating season, elevated corticosterone levels likely play an important role in mobilizing energy stores during spring emergence and mating. Indeed, red-sided garter snakes do not respond to capture stress during the spring mating season with a decrease in mating behavior (Moore et al., 2000a). This phenomenon is often observed in vertebrates whose reproductive opportunities are limited (e.g., Silverin and Wingfield, 1998; Wingfield et al., 1998).

Because stress responses and elevated corticosterone levels play an important role in mobilizing energy stores during spring emergence and mating, it is not surprising that melatonin does not appear to modulate responses of garter snakes to capture stress. Rather, melatonin likely plays a role in synchronizing reproductive behavior following winter dormancy (Crews et al., 1988; Lutterschmidt et al., 2004; Mendonça et al., 1996a,b; Nelson et al., 1987). Pinealectomy of northern-latitude red-sided garter snakes prior to winter hibernation inhibits male courtship behavior upon spring emergence (Crews et al., 1988; Mendonça et al., 1996a; Nelson et al., 1987). In contrast, pinealectomy following spring emergence has no effect on the expression of male courtship behavior (Mendonça et al., 1996a; Nelson et al., 1987). These results indicate the pineal gland is necessary for transducing environmental stimuli (and synchronizing reproduction) during winter dormancy, but once reproductive behavior is induced, pinealectomy is no longer effective in modulating reproduction. However, melatonin modulates reproductive behavior of male red-sided garter snakes (*T. sirtalis parietalis*) during the spring mating season (Lutterschmidt et al., 2004). Thus, although pinealectomy following spring emergence does not influence reproductive behavior, male snakes are sensitive to melatonin during the mating season.

It is possible that the sensitivity of northern-latitude red-sided garter snakes to melatonin during the spring, in the absence of a behavioral response to pinealectomy, could be related to an extrapineal source of melatonin. Extrapineal melatonin synthesis occurs at several sites in the body, including the harderian gland, retina, and intestine (e.g., Gern and Ralph, 1979; Norris, 1997; Ralph, 1980). Thus, detectable levels of circulating

melatonin are often still present even after pinealectomy. For example, pinealectomy of neotenic tiger salamanders (*Ambystoma trigrinum*) provoked little change in plasma melatonin levels during photophase and reduced melatonin levels during scotophase by only 55% (Gern and Norris, 1979). Similarly, photophasic and scotophasic plasma melatonin levels did not differ significantly between pinealectomized and sham-operated red-sided garter snakes (*T. sirtalis parietalis*) (Mendonça et al., 1996a). Extrapineal melatonin synthesis may contribute significantly to baseline levels of plasma melatonin in some species, with the pineal gland contributing to this baseline level in an additive manner during scotophase (Gern and Norris, 1979).

Northern-latitude red-sided garter snakes spend 8 months each year in winter dormancy in underground hibernacula. Throughout much of the year, these snakes are therefore not exposed to the photoperiod cues that regulate melatonin cycles. However, circadian melatonin cycles have been observed in red-sided garter snakes during the spring (e.g., Mendonça et al., 1996b). In addition, melatonin modulates reproductive behavior of northern-latitude red-sided garter snakes during the spring mating season (Lutterschmidt et al., 2004). Thus, melatonin appears to play a functional role in regulating the seasonal biology of red-sided garter snakes. Given the unique life history of the northern-latitude red-sided garter snake, temperature is likely the most important proximate environmental cue regulating circadian melatonin cycles and seasonal reproductive behavior. For example, photoperiod prior to and during hibernation has no influence on the initiation and timing of reproductive behavior of northern-latitude red-sided garter snakes upon emergence (e.g., Nelson et al., 1987; Whittier et al., 1987b). Red-sided garter snakes also require a period of low temperature conditions to initiate sexual behavior upon re-exposure to warm temperatures (Bona-Gallo and Licht, 1983; Camazine et al., 1980). Our laboratory is currently examining how temperature, in the absence of photoperiod cues, may function as a zeitgeber in entraining circadian melatonin cycles and regulating seasonal reproduction. We are particularly interested in investigating possible differences in both the production and function of melatonin among populations having very different seasonal biologies.

4.3. Serotonin and hormonal responses to capture stress

To test whether a serotonin-regulated system may be involved in inducing responses to capture stress, we examined the influence of ketanserin, a serotonergic type 2A receptor antagonist, on hormonal responses to capture stress. Ketanserin had no effect on the corticosterone responses of northern-latitude red-sided garter snakes or mid-latitude red-spotted garter snakes to 4 h of capture stress. However, treatment of mid-latitude red-spotted garter snakes with ketanserin prior to capture stress reduced the decline in androgen concentrations (Fig. 2B). These results suggest that a serotonin-regulated system is involved in mediating the effects of capture stress on the hypothalamic–pituitary–gonadal axis.

The modulation of glucocorticoid actions by melatonin are thought to be mediated by its properties as a serotonergic type 2A receptor antagonist (Eison et al., 1995; Gorzalka et al., 1999). In these experiments, ketanserin, but not melatonin, influenced androgen concentrations of northern-latitude red-spotted garter snakes (*T. sirtalis concinnus*) in response to capture stress. In addition, although serotonin influences testosterone secretion in rat testis (Csaba et al., 1998; Pieścikowska et al., 1999), ketanserin (0.045 mg) does not significantly influence plasma androgen concentrations of northern-latitude red-sided garter snakes (Lutterschmidt et al., 2004). Thus, the effects of ketanserin on androgen concentrations during stress responses are not a direct effect of ketanserin itself, but rather may be mediated via a serotonin-regulated pathway that is activated during the stress response.

Previous studies in red-sided garter snakes (*T. sirtalis parietalis*) from Manitoba, Canada have shown that capture and handling stress significantly increases plasma corticosterone concentrations and significantly decreases plasma testosterone concentrations (Moore et al., 2000a). While treatment with exogenous corticosterone significantly suppresses courtship behavior of male red-sided garter snakes, it does not influence plasma androgen concentrations (Lutterschmidt et al., 2004; Moore and Mason, 2001). Thus, the effects of corticosterone on courtship behavior of red-sided garter snakes are independent of its effects on androgen concentrations. Furthermore, the significant decline in plasma androgen concentrations in response to capture stress does not result directly from the elevation of corticosterone levels but rather some other aspect of the stress response (Moore and Mason, 2001). There are many factors that play a role in mediating the stress response, including elevated catecholamine secretion when the hypothalamic–pituitary–adrenal axis is activated. Our studies in mid-latitude red-spotted garter snakes (*T. sirtalis concinnus*) suggest that a serotonin-regulated system plays a role in modulating the activity of the hypothalamic–pituitary–gonadal axis during physiological stress responses.

There is much precedence for interactions between serotonin and corticosterone in modulating physiology and behavior (e.g., Gorzalka et al., 1998; Mendelson and McEwen, 1992; Stutzmann et al., 1998). For example, corticosterone increases the density of central serotonergic type 2A receptors and facilitates serotonergic type 2A receptor-mediated behaviors (Berendsen et al., 1996; Fernandes et al., 1997; Gorzalka and Hanson, 1998; Takao et al., 1997). In addition, Lutterschmidt et al. (2004) suggest that a serotonin-regulated system is

involved in mediating the inhibitory effects of melatonin and corticosterone on courtship behavior in northern-latitude red-sided garter snakes (*T. sirtalis parietalis*). In mammals, corticosterone has little or no effect on modulating serotonin metabolism (Chaouloff, 1993). Thus, the effects of corticosterone on serotonergic type 2A receptor-mediated behaviors, such as an increase in wet-dog shakes in rats, are likely due to a specific receptor-mediated mechanism, rather than simply a modulation of serotonin metabolism (Gorzalka et al., 1999). Further studies are necessary to determine whether serotonin does indeed play a role in orchestrating changes in physiology and behavior during stress responses.

Acknowledgments

We thank the Manitoba Department of Conservation and Dave Roberts for technical assistance in Manitoba, Canada and Dave Budeau for technical assistance at E.E. Wilson Wildlife Area, Oregon, USA. We thank Al and Gerry Johnson for support and encouragement and Suzanne Estes, Michael LeMaster, and Ruth Nesbitt for field assistance in Manitoba. We especially thank Arianne Cease for field assistance at E.E. Wilson Wildlife Area. This work was supported in part by a Pennsylvania Academy of Science Graduate Student Research Grant, Zoology Research Funds from Oregon State University, and a P.E.O. Scholar Award to D.I.L.

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