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Effects of melatonin on the behavioral and hormonal responses of red-sided garter snakes (*Thamnophis sirtalis parietalis*) to exogenous corticosterone

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Abstract

We investigated possible interactions between melatonin and corticosterone in modulating the reproductive behavior of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) following spring emergence. We also examined whether melatonin's modulatory actions could be explained by its potential properties as a serotonin receptor antagonist. Exogenous corticosterone significantly reduced courtship behavior of male snakes in a dose-dependent manner. Melatonin also significantly reduced courtship behavior of male garter snakes. Pretreatment with melatonin before administering corticosterone treatments further suppressed courtship behavior of red-sided garter snakes. These results indicate additive inhibitory effects of melatonin and corticosterone in modulating reproductive behavior. Snakes receiving ketanserin, a serotonergic type 2A receptor antagonist, followed by corticosterone also showed reduced courtship behavior; this serotonin receptor antagonist followed by treatment with vehicle did not significantly influence courtship behavior of male snakes. Neither melatonin nor corticosterone treatments significantly influenced testosterone + 5-α-dihydrotestosterone concentrations of male garter snakes, supporting a direct effect of melatonin and corticosterone on courtship behavior that is independent of any effect on androgen concentrations. We propose that a serotonin system is involved in the modulation of male courtship behavior by melatonin and corticosterone. In addition, our data support the hypothesis that melatonin may function as a serotonin receptor antagonist. Further research is necessary to discern whether the actions of melatonin and corticosterone are converging on the same pathway or if their effects on different pathways are having additive inhibitory effects on courtship behavior.
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Introduction

The pineal gland and its major secretory product melatonin are the primary neuroendocrine transducers of environmental stimuli in vertebrates (Axelrod, 1974). Environmental factors such as photoperiod and temperature interact to modulate the cycle of melatonin synthesis and secretion (e.g., Firth and Kennaway, 1987, 1989; García-Allegue et al., 2001; Gern and Norris, 1979; Tilden and Hutchison, 1993; Underwood and Calaban, 1987; Vivien-Roels et al., 1988). Melatonin cycles, in turn, regulate many physiological and behavioral rhythms, including reproduction, activity, aggression, immune function, thermoregulation, and free radical scavenging (e.g., Cagnoli et al., 1995; Hyde and Underwood, 2000; Jasnow et al., 2002; Lutterschmidt et al., 2003; Maestroni et al., 1989; Reiter, 1996, Reiter et al., 1995; Underwood, 1981, 1985). Melatonin's ability to transduce environmental information into appropriate endocrine signals plays an important role in synchronizing an animal's physiology and behavior with optimal environmental conditions.

Interactions between melatonin and other hormones are also important in synchronizing and modulating physiologi-

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cal and behavioral parameters necessary for daily activity. For example, melatonin inhibits the hypothalamic–pituitary–adrenal axis (Reiter, 1991; Wang et al., 1999), which mediates an animal's physiological and behavioral responses to stressors. Unpredicted challenges to energy homeostasis, such as unusually low environmental temperatures or food shortage, stimulate the hypothalamic–pituitary–adrenal axis and result in increased plasma glucocorticoids (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 that are not crucial to immediate survival (e.g., Pottinger, 1999; Sapolsky, 1992; Wingfield, 1988). For example, physiological stress responses, marked by an increase in plasma glucocorticoid concentrations, generally result in decreased plasma concentrations of sex steroid hormones and an overall suppression of reproductive behavior in many species (Carragher and Rees, 1994; Coddington and Cree, 1995; Moore et al., 1991, 2000; Rivier and Rivest, 1991). Such acute physiological stress responses are normally adaptive responses used to modify metabolism and mobilize energy stores during a stressful event.

Although interactions between the pineal gland and hypothalamic–pituitary–adrenal axis are well established (Barriga et al., 2002; Demisch et al., 1998; Khan et al., 1990; Kirby et al., 1999; Maestroni et al., 1986, 1989; Otsuka et al., 2001; Vaughan et al., 1972), the nature of this relationship is poorly understood. For example, 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. In an experiment using in vivo physiological conditions, melatonin exerted a direct antisecretagogue effect on the adrenal gland (Appa-Rao et al., 2001). Chronic melatonin treatment considerably altered the affinity of glucocorticoid receptors in the brain and pituitary (Marinova et al., 1991). Furthermore, melatonin treatment of both adult and juvenile rats prevented many of the injurious effects induced by chronically elevated glucocorticoids, such as the reduction in growth, atrophy of the thymus and adrenal glands, and elevation of blood glucose, free fatty acids, triglycerides, and total cholesterol (Aoyama et al., 1986, 1987). Daily melatonin treatment in stressed mice prevented several chronic stress-induced disturbances, including a reduction in preference for sucrose solution and a reduction in spontaneous locomotor activity (Kopp et al., 1999). In male rats, melatonin treatment significantly reduced 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 attenuated the effects of glucocorticoids on sexual behavior and wet-dog shakes in male rats. Such 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).

We investigated the influence of melatonin on the behavioral and hormonal responses to exogenous glucocorticoids in a nonmammalian model. Male red-sided garter snakes (*Thamnophis sirtalis parietalis*) provide an excellent model for investigating these questions because they are known to exhibit significantly reduced courtship behavior in response to exogenous glucocorticoid treatment (Moore and Mason, 2001). In this study, we investigated differences in courtship behavior and plasma testosterone + 5-α-dihydrotestosterone concentrations among snakes treated with melatonin or corticosterone [the primary glucocorticoid in snakes (Idler, 1972)]. We asked the following questions: (1) does melatonin modulate behavioral and hormonal responses of male red-sided garter snakes to exogenous corticosterone? and (2) does melatonin's influence on behavioral and hormonal responses to exogenous corticosterone result from antagonism of serotonin receptors?

Materials and methods

Experiments were conducted in the field with free-living red-sided garter snakes (*T. sirtalis parietalis*) in Inwood, Manitoba, Canada. Studies were conducted between 1030 and 1430 h on 14–17 May 2003, during the month following emergence from hibernacula when snakes are mating and plasma testosterone concentrations are declining (Krohmer et al., 1987). All experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol number: 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 number: 2002-06) and was performed under the authority of Manitoba Wildlife Scientific Permit No. WSP 03009.

Reagents

Melatonin and corticosterone were purchased from Sigma (St. Louis, MO). Ketanserin, a serotonergic type 2A receptor antagonist, was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). We chose this antagonist because (1) serotonergic type 2A receptors reliably modulate sexual behavior (Gorzalka et al., 1990), (2) corticosterone regulates the density of serotonergic type 2A 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), and (3) ketanserin itself exerts no significant effects on sexual behavior (Watson and Gorzalka, 1991). All pretreatments and 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.

Melatonin solutions for the low and high melatonin doses were prepared by first dissolving 6 or 60 mg, respectively, in 1 ml of 100% ethanol. Stock solutions were diluted to 20 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 pretreatment 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).

Ketanserin solution was prepared by first dissolving 9 mg in 1 ml of 100% ethanol and then diluting to 20 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 transport response of rats.

Corticosterone solutions were prepared by first dissolving 3 or 12 mg of corticosterone in 1 ml of 100% ethanol. Stock solutions were then diluted to 20 ml with reptile Ringer's solution, producing corticosterone concentrations for the low and high doses of 150 and 600 μg ml^{-1}, respectively. Thus, the corticosterone treatment doses were 15 and 60 μg per snake. These corticosterone doses are similar to those used by Moore and Mason (2001) to test the behavioral responses of red-sided garter snakes to exogenous corticosterone.

Experimental design

We used a 4 × 3 factorial design to investigate whether melatonin modulates the behavioral and hormonal responses of male garter snakes to exogenous corticosterone. Courting male red-sided garter snakes were collected from the den site and randomly assigned to one of four pretreatment groups (n = 126 in each): vehicle, low melatonin dose (0.03 mg), high melatonin dose (0.3 mg), or ketanserin (0.045 mg). Following pretreatment, snakes were housed in outdoor arenas and allowed to absorb the pretreatment drugs for approximately 30 min. Within each pretreatment group, snakes were then randomly assigned to one of three corticosterone treatment subgroups (n = 42 in each): 0 (i.e., vehicle), 15, or 60 μg corticosterone. As snakes received the corticosterone treatments, a small treatment-unique marking was made on each snake's anterior dorsal stripe using magic markers. Snakes were then immediately placed in arenas for mating trials (n = 32 in each corticosterone treatment subgroup) or blood sampling (n = 10 in each corticosterone treatment subgroup).

Behavioral responses to corticosterone

Mating trials were conducted in nylon cloth arenas measuring 1 × 1 × 1 m with 12 males (i.e., one male randomly selected from each combination of the four pretreatment and three corticosterone treatment conditions) simultaneously introduced to an unmated, attractive female. Males were introduced in groups of 12 to simulate natural mating conditions, where the presence of a mating ball facilitates male courtship behavior (Joy and Crews, 1985). Because males are attracted to females by both the presence of pheromonal cues expressed on the dorsal surface of females as well as the presence of a mating ball, mating balls rarely contain fewer than five males courting a single female (Joy and Crews, 1985). Using an ethogram of male courtship behavior (Table 1), we recorded the mating score of each male every 10 min for a period of 1 h after introduction into the arena; the observer was blind to the treatment group of each male. Adhesive tape was placed across the female's cloaca to prevent mating during the trials, as mating significantly reduces further male courtship behavior (Garstka et al., 1982). The tape does not alter male or female reproductive behavior and was immediately removed following each trial (LeMaster and Mason, 2002). Each male was therefore assigned a mating score of 0 (no reproductive behavior) through 4 (male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves) every 10 min for 1 h. Behavioral scores of 3.0 and greater are exhibited only in a reproductive context (Table 1). We collected a total of six courtship scores for each snake during the 1-h trial period; these courtship scores were used to calculate a mean courtship score for each snake.

Hormonal responses to corticosterone

To measure testosterone + 5-α-dihydrotestosterone concentrations, we collected blood samples from a subset (n = 10) of males in each corticosterone subgroup that

Table 1
Ethogram of courtship behavior for the male red-sided garter snake *T. sirtalis parietalis*

Courtship score	Description of behavior
0.0	No reproductive behavior
1.0	Male investigates female, increased tongue-flick rate
2.0	Male chin-rubs female with rapid tongue-flicks
3.0	Male aligns body with female
4.0	Male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves
5.0	Male copulates with female

Behaviors 3.0 and greater are exhibited only in a reproductive context (modified from Crews et al., 1984; Moore et al., 2000).

were not used in the mating trials. All blood samples were collected approximately 1 h following completion of the mating trials (i.e., approximately 3 h following pretreatment with melatonin or ketanserin). Moore and Mason (2001) demonstrated that 4 h following intraperitoneal injection with 50 μg corticosterone (in 0.1 ml 1% ethanol in reptile Ringer's solution), male red-sided garter snakes tended to have higher, but physiologically relevant, plasma corticosterone levels (approximately 90 ng ml^{-1}) than snakes receiving only reptile Ringer's solution (approximately 55 ng ml^{-1}). Similarly, the half-life of melatonin after injection into endotherms can be 1 h or less (Rollag and Stetson, 1982). Although the half-life of melatonin in whole-animal ectotherms has not been measured, it is likely to be much longer because metabolic rate is as much as 10 times lower, depending on body temperature (Filadelfi and Castrucci, 1996). We are confident that plasma corticosterone and melatonin levels, in response to hormone treatments, remained elevated throughout the duration of our experiments.

Blood samples were obtained from the caudal vein within 1 min using heparinized 1-cm^3 syringes and 25-g needles. Samples 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, where they were stored at −70°C until analyzed for androgen concentrations following radioimmunoassay procedures modified from Moore et al. (2000). To test whether chromatography of steroid hormones extracted from snake plasma is necessary, we simultaneously analyzed a subset of plasma samples (n = 40) for testosterone concentrations using both radioimmunoassay with partition chromatography (Moore et al., 2000) and radioimmunoassay without partition chromatography (i.e., direct radioimmunoassay). The methods used for direct radioimmunoassay were similar to those described by Jessop et al. (1999a,b, 2000, 2004) and Whittier et al. (1997).

Briefly, we extracted steroids from 100-μl aliquots of plasma twice with anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas. Hormone extracts were then either reconstituted in phosphate-buffered saline for direct assay or reconstituted in 10% ethyl acetate in isooctane and chromatographed on celite microcolumns. Extracted and reconstituted samples were then incubated with tritiated testosterone (1,2,6,7-^{3}H testosterone, Amersham Biosciences, Piscataway, NJ) and testosterone antiserum (Wein Laboratories, Inc., Succasunna, NJ) at 4°C for 12–24 h. Cross-reactivity of this antiserum with 5-α-dihydrotestosterone is 63.2%; cross-reactivity with progesterone and estradiol is <0.3% and 2.3%, respectively. Unbound steroid was separated from bound hormone using dextran-coated charcoal, and the radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter. Samples were assayed in duplicate and corrected for individual recovery variation. Mean extraction efficiency for testosterone was 94.1%, as determined by the recovery of tritiated testosterone added to samples before extraction with ethyl ether. All samples (i.e., treatment groups) were randomly distributed across the steroid assays. Mean intra-assay variation was 14.6% and inter-assay variation was 19.7%. The limits of detection were 0.49 pg per 100 μl.

Statistical analyses

To investigate behavioral responses of snakes to corticosterone, we collected a total of six courtship scores for each snake during the 1-h trial period. These courtship scores were used to calculate a mean courtship score for each snake due to the statistical nonindependence of repeated measurements. Using a mean courtship score also helps account for possible metabolism (and therefore changing effects) of hormone treatments during the mating trials. We rank-transformed the mean courtship scores of snakes to adjust for the nonnormality of these data. Differences in mean courtship scores among treatment groups were then investigated using a two-way analysis of variance (ANOVA; with pretreatment and treatment as the between-subjects factors) on rank-transformed data followed by a Student–Newman–Keuls multiple comparisons test. We used this multiple comparisons test, which employs step-down logic (i.e., first testing larger pairwise differences in ordered means and then proceeding to smaller differences; Toothaker, 1993; Zar, 1984) because of a priori knowledge that corticosterone would have a step-wise effect (i.e., a dose-response) on courtship behavior.

We used a regression analysis to examine the correlation between androgen concentrations of samples determined by radioimmunoassay with and without partition chromatography. Hormone concentrations were rank-transformed to account for nonnormality. Differences in androgen concentrations among treatment groups were then analyzed using a two-way ANOVA (with pretreatment and treatment as the between-subjects factors) on rank-transformed data. We used SigmaStat® 2.03 (SPSS, 1997) for all statistical analyses. All statistical comparisons were considered significant at $P \leq 0.05$.

Results

Behavioral responses to corticosterone

Pretreatment with both melatonin and ketanserin significantly reduced average courtship scores of male garter snakes (Fig. 1; $F(3,372) = 8.62$; $P < 0.001$ from a two-way ANOVA). There were no statistically significant differences in the courtship scores of snakes among melatonin and ketanserin pretreatment groups (Fig. 1). Within each pretreatment group, corticosterone significantly decreased the average courtship scores of male red-sided garter snakes (Fig. 1; $F(2,372) = 64.58$; $P < 0.001$

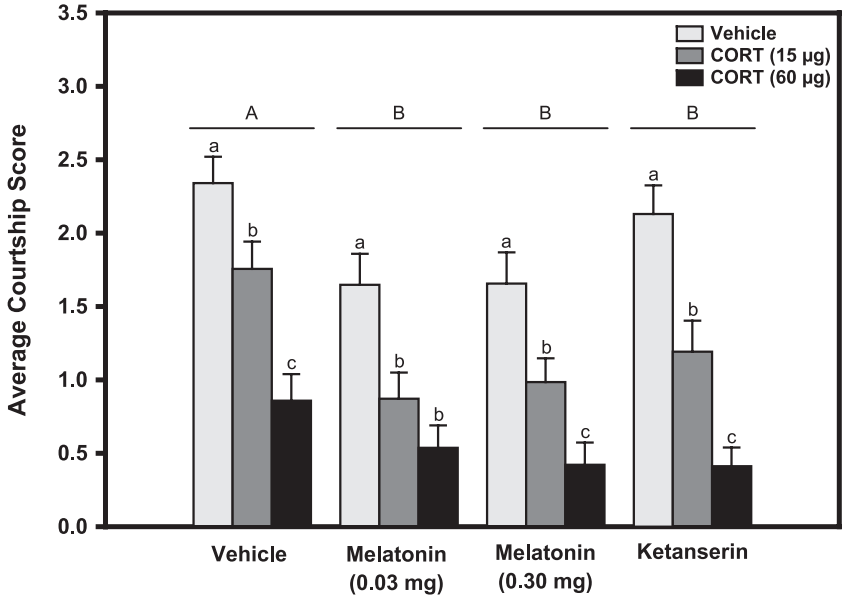

Fig. 1. Average courtship scores of male red-sided garter snakes (*T. sirtalis parietalis*) following pretreatment with vehicle, melatonin (0.03 or 0.30 mg), or ketanserin, a serotonergic type 2A receptor antagonist. Pretreatment conditions are indicated along the abscissa. Thirty minutes after pretreatment, snakes received treatment with vehicle or corticosterone (CORT; 15 or 60 μg). Standard errors (±1) are shown by the vertical lines; $n = 32$ in each treatment. Statistically significant differences among pretreatment groups are indicated by letters above the horizontal lines. Within each pretreatment group, statistically significant differences among vehicle and corticosterone treatments are indicated by letters above each standard error bar.

from a two-way ANOVA). Results from the Student–Newman–Keuls multiple comparisons procedure for comparisons of pretreatment condition within the corticosterone treatment subgroups are shown in Table 2. There were no statistically significant interactions between pretreatment and treatment conditions.

Hormonal responses to corticosterone

We obtained excellent correlation between the testosterone concentrations of a subset of plasma samples ($n = 40$) assayed by both direct radioimmunoassay and radioimmunoassay with partition chromatography (Fig. 2; $R^2 = 0.97$, $P < 0.001$ from a regression). Thus, we elected to analyze all plasma samples using direct radioimmunoassay methods. Because our testosterone antibody (Wein Laboratories, Inc.) cross-reacts significantly with 5-α-dihydrotestosterone, our direct assay measures both plasma testosterone and 5-α-dihydrotestosterone concentrations. Thus, our direct assay-testosterone concentrations tend to be higher than those measured by radioimmunoassay with partition chromatography (which separates testosterone from 5-α-dihydrotestosterone). For these reasons, we present here data for both testosterone and 5-α-dihydrotestosterone concentrations.

A two-way ANOVA shows that pretreatment with melatonin and ketanserin did not significantly influence testosterone + 5-α-dihydrotestosterone concentrations of snakes (Fig. 3). Within each pretreatment group, corticosterone (15 and 60 μg) did not significantly influence testosterone + 5-α-dihydrotestosterone concentrations of male red-sided garter snakes (Fig. 3). There were no

Table 2
Results from a two-way analysis of variance on mean courtship scores of male red-sided garter snakes followed by a Student–Newman–Keuls multiple comparisons procedure

Comparison of pretreatment conditions	*P* values from a Student–Newman–Keuls multiple comparisons procedure		
	Within vehicle treatment	Within corticosterone (15 μg) treatment	Within corticosterone (60 μg) treatment
Vehicle vs. Melatonin (0.03 mg)	0.035	0.001	0.197
Vehicle vs. Melatonin (0.30 mg)	0.038	0.010	0.137
Vehicle vs. Ketanserin	0.431	0.019	0.138
Melatonin (0.03 mg) vs. Melatonin (0.30 mg)	0.845	0.421	0.665
Melatonin (0.03 mg) vs. Ketanserin	0.091	0.343	0.541
Melatonin (0.30 mg) vs. Ketanserin	0.143	0.555	0.803

Data were rank-transformed to correct for nonnormality. Results shown above are for comparisons of pretreatment conditions within the corticosterone treatment groups.

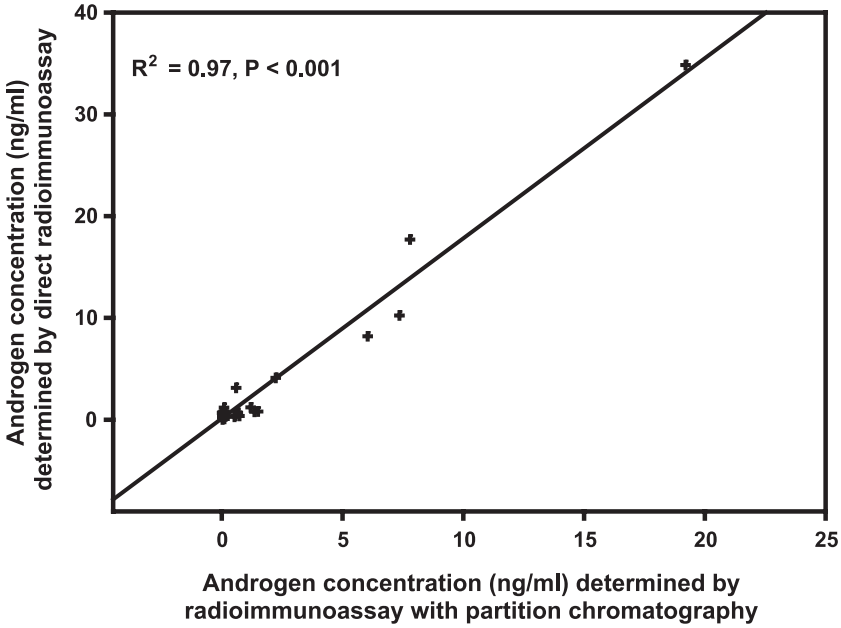

Fig. 2. Regression of androgen (testosterone + 5-α-dihydrotestosterone) concentrations (ng ml^{-1}) determined by direct radioimmunoassay on androgen (testosterone) concentrations (ng ml^{-1}) determined by radioimmunoassay with partition chromatography using celite microcolumns. Each data point represents the androgen concentration of one plasma sample as determined by direct radioimmunoassay vs. radioimmunoassay with partition chromatography (n = 40 plasma samples).

statistically significant interactions between pretreatment and treatment conditions.

Discussion

Our results provide evidence that both melatonin and corticosterone inhibit courtship behavior in male red-sided garter snakes (*T. sirtalis parietalis*) (Fig. 1). These data also demonstrate that the effects of melatonin and corticosterone on courtship behavior are independent of their effects on the hypothalamic–pituitary–gonadal axis, as neither hormone influenced plasma testosterone + 5-α-dihydrotestosterone concentrations (Fig. 3). Furthermore, we demonstrate that melatonin and corticosterone, at the 15-μg dose, have significant additive inhibitory effects on male reproductive

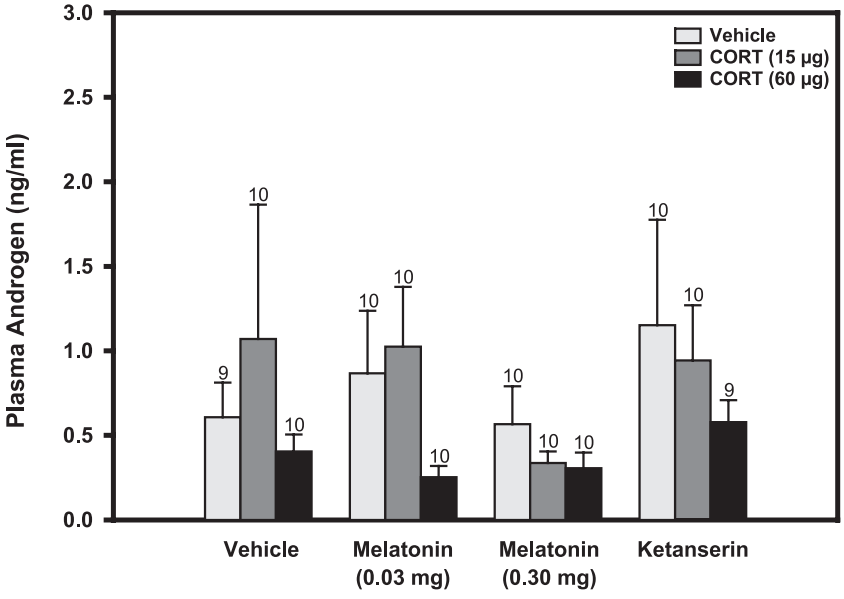

Fig. 3. Mean plasma androgen (testosterone + 5-α-dihydrotestosterone) concentrations of male red-sided garter snakes (*T. sirtalis parietalis*) following pretreatment with vehicle, melatonin (0.03 or 0.30 mg), or ketanserin, a serotonergic type 2A receptor antagonist. Pretreatment conditions are indicated along the abscissa. Thirty minutes after pretreatment, snakes received treatment with vehicle or corticosterone (CORT; 15 or 60 μg). Standard errors (±1) are shown by the vertical lines; sample sizes are listed above the standard error bars.

behavior (Fig. 1, Table 2). Our experiments with the serotonergic type 2A receptor antagonist ketanserin suggest that a serotonin-regulated system is involved in the inhibition of courtship behavior by melatonin and corticosterone (Fig. 1, Table 2). These data also support the hypothesis that melatonin acts as a serotonin receptor antagonist.

These experiments support a direct modulatory role of melatonin on reproductive behavior in an ectothermic model. Pretreatment with either dose of melatonin followed by vehicle significantly reduced courtship of male snakes as compared to snakes receiving vehicle pretreatment followed by treatment with vehicle (Table 2). This modulatory role, in combination with melatonin's circadian and circannual rhythm of secretion, may be important in synchronizing seasonal reproductive function with optimal environmental conditions in this species.

Although extensive experiments have been done in both birds and mammals, there are fewer studies investigating the relationship between the pineal complex, melatonin, and reproductive function in other vertebrates (reviewed in Mayer et al., 1997; Turek and Van Cauter, 1994). In some ectothermic vertebrates, the effects of pinealectomy and melatonin treatment on reproductive parameters vary among experimental protocols and seasons, making it difficult to draw conclusions about the role of the pineal gland in reproduction (e.g., Chanda and Biswas, 1982, 1992; De Vlaming, 1975; Haldar and Thapliyal, 1977; Hontela and Peter, 1980; Nayak and Singh, 1988; Thapliyal and Misra nee Haldar, 1979; Underwood, 1981, 1985; Vodicnik et al., 1978). For example, the effects of pinealectomy on testicular size in the Indian chequered water snake (*Natrix piscator*) are influenced by humidity; pinealectomy decreased testicular size in high humidity (which normally stimulates testicular growth) but increased testicular size in both low and moderate humidity (Haldar and Pandey, 1989a,b). In addition, treatment of Indian chequered water snakes with either melatonin or 5-methoxytryptamine suppressed testicular function of active testes, but did not influence inactive testes (Haldar and Pandey, 1988). The effects of pinealectomy in black-spined toads (*Bufo melanostictus*) also vary among this species' seasonal activity patterns. Pinealectomy of toads during the hibernation phase stimulated testicular maturation, while pinealectomy during the breeding season did not influence testicular function (Chanda and Biswas, 1984).

Previous studies in red-sided garter snakes (*T. sirtalis parietalis*) showed that pinealectomy before 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. Treatment of pinealectomized snakes with melatonin did not influence the effects of pinealectomy on courtship behavior (Mendonça et al., 1996a). This suggests that the physical presence of the pineal gland, and not melatonin per se, is necessary for synchronizing reproductive behavior with spring emergence. It is possible that a neural component of the pineal gland, in combination with melatonin secretion, is necessary for initiating reproductive behavior of red-sided garter snakes following winter dormancy. Our results demonstrate that melatonin modulates reproductive behavior of male red-sided garter snakes during the spring mating season. Thus, although pinealectomy following spring emergence does not influence reproductive behavior, male snakes are sensitive to melatonin during the mating season. Circadian melatonin cycles following spring emergence most likely play a role in synchronizing reproductive behavior (and activity) with the appropriate time of day. Indeed, disrupted circadian melatonin cycles, with peak melatonin secretion occurring during the photophase, were observed in red-sided garter snakes that failed to exhibit courtship behavior during the spring breeding season (Mendonça et al., 1996b).

The hypothalamic–pituitary–adrenal axis is also important in regulating reproductive function in red-sided garter snakes. In this species, mating occurs upon emergence from winter dormancy while plasma sex steroid concentrations are basal, gonads are regressed, and glucocorticoid levels are high (Aleksiuk and Gregory, 1974; Crews, 1984; Crews and Garstka, 1982; Crews et al., 1984; Whittier et al., 1987). 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. When reproductive opportunities are limited, however, it is not uncommon to observe an absence of glucocorticoid-induced reproductive suppression. For example, courtship behavior of male garter snakes during the spring breeding season is not inhibited following capture and handling stress (which significantly increases plasma corticosterone concentrations and significantly decreases plasma testosterone concentrations; Moore et al., 2000). However, treatment of male red-sided garter snakes with exogenous corticosterone suppresses courtship behavior in a threshold-dependent manner (Moore and Mason, 2001). Thus, male garter snakes retain sensitivity behaviorally to elevated corticosterone during the breeding season, even though they have uncoupled hormonal responses to capture stress from behavioral responses to capture stress (Moore and Mason, 2001).

Similar to Moore and Mason (2001), we demonstrate that exogenous corticosterone significantly suppresses courtship behavior of male red-sided garter snakes but does not influence plasma androgen concentrations. Although the testosterone + 5-α-dihydrotestosterone concentrations we measured are much lower than those reported by Moore and Mason (2001), this is likely due to the unusually warm

mating season during May 2003. Testosterone concentrations normally decline during the mating season (Krohmer et al., 1987), and therefore the warmer temperatures most likely increased the metabolic clearance of testosterone. If we had conducted these experiments earlier in the season we would most likely have observed testosterone concentrations in the range of those reported in previous studies.

Because the reproductive behavior of red-sided garter snakes does not depend upon the activational effects of sex steroid hormones (Crews, 1984, 1991; Crews et al., 1984), it is not surprising that the observed effects of corticosterone and melatonin on reproductive behavior are independent of any effect on androgen concentrations. Although ketanserin has been reported to inhibit testosterone secretion from Leydig cells of rats (Csaba et al., 1998; Pieścikowska et al., 1999), our results demonstrate no effect of ketanserin, at a dose of 0.045 mg, on plasma testosterone + 5-α-dihydrotestosterone concentrations.

Direct and rapid sex steroid-independent effects of corticosterone on reproductive behavior have been reported in other species. In song sparrows (*Melospiza melodia*), treatment with exogenous corticosterone reduces territorial behavior but does not significantly influence plasma testosterone concentrations (Wingfield and Silverin, 1986). Corticosterone suppresses mating behavior in male rough-skinned newts (*Taricha granulosa*) by binding to a membrane-bound corticosterone receptor on neuronal membranes (Orchinik et al., 1991).

We propose that a serotonin system is involved in the modulation of male courtship behavior by melatonin and corticosterone in red-sided garter snakes. As expected, pretreatment with ketanserin, a serotonergic type 2A receptor antagonist, followed by vehicle did not significantly influence courtship behavior (Watson and Gorzalka, 1991). However, the combination of ketanserin pretreatment followed by treatment with the low corticosterone dose significantly reduced courtship behavior of male snakes (as compared to the courtship scores of snakes receiving vehicle pretreatment followed by treatment with the low corticosterone dose; see Table 2). These results suggest that corticosterone inhibits reproductive behavior of red-sided garter snakes by modulating a serotonin-regulated system; these inhibitory effects of corticosterone are significantly augmented with ketanserin.

There is much precedence for interactions between serotonin and corticosterone in modulating physiology and behavior (e.g., Chaouloff, 1993; Gorzalka et al., 1998; Kawahara et al., 1993; Mendelson and McEwen, 1992; Popova and Lobacheva, 1982; 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). 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, are likely due to a specific receptor-mediated mechanism, rather than simply a modulation of serotonin metabolism (Gorzalka et al., 1999). These actions of corticosterone, when combined with a serotonin antagonist, could explain the additive inhibitory effects of corticosterone and ketanserin we observed on reproductive behavior. This hypothesis is supported by the lack of an additional inhibitory effect of ketanserin pretreatment when combined with the high corticosterone treatment dose (Table 2), as the high corticosterone dose may have saturated the system.

Similar to our observations of additive inhibitory effects of ketanserin and corticosterone, melatonin and corticosterone, at a dose of 15 μg, also had additive inhibitory effects on reproductive behavior. Snakes receiving pretreatment with both the low and high melatonin doses followed by treatment with the low corticosterone dose exhibited significantly reduced courtship behavior (as compared to those snakes receiving pretreatment with vehicle followed by treatment with the low corticosterone dose; Table 2). We observed no significant differences in the courtship behavior of male snakes receiving pretreatment with ketanserin or either melatonin dose among any of the corticosterone treatments (Table 2). These results suggest that both melatonin and ketanserin reduce courtship behavior via modulation of a serotonin system, thus supporting the hypothesis that melatonin may act as a serotonin receptor antagonist (Gorzalka et al., 1999). However, pretreatment with both melatonin doses, but not ketanserin, followed by vehicle significantly reduced courtship behavior of male snakes. Thus, melatonin may inhibit courtship behavior via a mechanism other than antagonism of serotonin receptors. It is also possible, however, that melatonin is a more potent serotonin receptor antagonist than ketanserin. Future studies examining dose response curves for ketanserin would provide insight into whether the effects of melatonin on courtship behavior reported here result primarily from its antagonism of serotonergic type 2A receptors or some other mechanism. Further research is also needed to determine if the actions of corticosterone on reproductive behavior do indeed involve modulation of a serotonin-regulated system. Furthermore, additional research is necessary to discern whether melatonin and corticosterone are converging on the same pathway or if their effects on different pathways are having additive inhibitory effects on courtship behavior.

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