



## Temporally distinct effects of stress and corticosterone on diel melatonin rhythms of red-sided garter snakes (*Thamnophis sirtalis*)

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### ABSTRACT

Circadian and circannual rhythms in physiology and behavior are temporally organized via hormonal signals that reflect changing environmental cues. Interactions between endocrine signals are in turn important for integrating multiple physiological and behavioral rhythms. In the present study, we examined interactions between melatonin, the hypothalamus–pituitary–adrenal (HPA) axis, and corticosterone in a well-studied population of red-sided garter snakes (*Thamnophis sirtalis parietalis*). We demonstrate that 4 h of capture stress significantly increased photophasic melatonin and decreased scotophasic melatonin concentrations of male snakes. Treatment with exogenous corticosterone (15 and 60  $\mu\text{g}$ ) did not mimic the effects of stress on diel melatonin rhythms. To determine if capture stress decreases scotophasic melatonin by depleting the precursors necessary for melatonin synthesis, we used a paradigm in which snakes were treated with the melatonin precursor 5-hydroxytryptophan (0.6 and 1.2 mg) to elevate melatonin concentrations. Pretreatment of snakes with both capture stress and exogenous corticosterone blocked the effect of 5-hydroxytryptophan on scotophasic melatonin. Thus, although corticosterone itself does not influence melatonin rhythms of snakes, corticosterone can inhibit the synthesis of melatonin from 5-hydroxytryptophan. These experiments suggest that the initial versus later phases of an acute physiological stress response have temporally distinct effects on melatonin synthesis: activation of the sympathoadrenal system increases melatonin, while increased glucocorticoids can inhibit melatonin synthesis. Collectively, we demonstrate that a physiological coupling between melatonin, glucocorticoids, and the sympathoadrenal system is conserved in this ectothermic model and propose that such interactions may mediate stress-induced changes in physiology and behavior.

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### 1. Introduction

Many vertebrates exhibit both circadian and circannual rhythms in physiology and behavior. Such biological rhythms are temporally organized via hormonal signals that reflect daily- and seasonally-changing environmental cues (e.g., photoperiod and temperature). The pineal gland and its major secretory product, melatonin, are the primary neuroendocrine transducers of environmental stimuli in vertebrates (Axelrod, 1974). Melatonin, in turn, regulates many physiological and behavioral processes, including reproduction, activity, aggression, immune function, thermoregulation, and free radical scavenging (e.g., Underwood, 1981, 1985; Maestroni et al., 1989; Reiter et al., 1995; Hyde and Underwood, 2000; Jasnaw et al., 2002; Lutterschmidt et al., 2003, 2004). Thus, melatonin's ability to transduce environmental information into appropriate endocrine signals plays an important role in integrat-

ing an animal's physiology and behavior with optimal environmental conditions.

Interactions between melatonin and other endocrine signals are important in integrating multiple physiological and behavioral rhythms. For example, melatonin and glucocorticoids, a secretory product of the hypothalamus–pituitary–adrenal (HPA) axis, are hormonal pacemakers of different physiological and behavioral processes. The HPA axis mediates hormonal responses to noxious stimuli, or stressors (e.g., food shortage, extreme environmental temperatures and predators), which are marked by increased secretion of adrenal glucocorticoids (e.g., Harvey et al., 1984; Wingfield et al., 1998). Elevated glucocorticoids in turn modulate a variety of physiological and behavioral processes to promote immediate survival (e.g., Wingfield, 1988; Sapolsky, 1992; Pottinger, 1999). Thus, acute physiological stress responses are normally adaptive responses used to modify metabolism and mobilize energy stores.

Reciprocal interactions between melatonin, glucocorticoids, and the HPA axis are well established (e.g., Maestroni et al., 1989; Otsuka et al., 2001; Barriga et al., 2002). In male rats, melatonin significantly reduces the inhibitory effects of acute and chronic

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stress on sexual behavior (Brotto et al., 2001). Activation of the HPA axis during physiological stress responses also modulates melatonin rhythms. For example, social stress alters melatonin cycles in rainbow trout, *Oncorhynchus mykiss* (Larson et al., 2004) and tree shrews, *Tupaia belangeri* (Fuchs and Schumacher, 1990). In ring doves (*Streptopelia risoria*), immobilization stress significantly increases melatonin levels during photophase (light phase of the photoperiod) but decreases melatonin during scotophase (dark phase of the photoperiod; Rodríguez et al., 2001; Barriga et al., 2002). Physiological stress responses therefore appear to alter the cyclicity of melatonin synthesis (Persengiev and Kanchev, 1991).

Increases in melatonin following a stress response are thought to aid in combating oxidative damage induced by elevated glucocorticoid concentrations. Sainz et al. (1995) demonstrated that melatonin alleviates glucocorticoid-induced apoptosis of thymocytes via an antioxidant mechanism. In contrast, decreased scotophasic melatonin following a stress response is hypothesized to result from a rapid decline in tryptophan, the amino acid precursor for melatonin synthesis (Clark and Russo, 1997). Alternatively, the effects of stress on circadian melatonin rhythms may result from the direct actions of glucocorticoids on the pineal gland (e.g., Beck-Friis et al., 1983; Brismar et al., 1985; Ferreira et al., 2005).

Despite much research, the physiological relationship between melatonin, glucocorticoids, and the HPA axis remains unclear. Furthermore, whether or not a possible coupling between melatonin and glucocorticoids is evolutionarily conserved or more recently derived among birds and mammals is poorly understood, primarily because studies addressing these questions in ectothermic vertebrates are lacking. In the present study, we sought to examine interactions between melatonin and the HPA axis in a well-studied population of red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada. These extreme-latitude populations exhibit a temporal dissociation between mating behavior and maximal gonadal activity (e.g., Crews et al., 1984). Following 8 months of continuous winter dormancy, snakes emerge in the spring and immediately enter an intense mating season lasting approximately 4–5 weeks. During this time, gonads are regressed, sex steroid levels are declining, and glucocorticoid levels are elevated (Crews et al., 1984; Krohmer et al., 1987; Moore et al., 2000; Lutterschmidt and Mason, 2005, 2009; Cease et al., 2007). Snakes are aphagic while actively courting at the den site (O'Donnell et al., 2004) and must migrate as far as 20 km to summer feeding grounds to forage (Gregory, 1977). Thus, elevated glucocorticoid levels facilitate reproduction and daily activity by mobilizing much-needed energy stores (e.g., Moore and Jessop, 2003).

This ectothermic model system provides an excellent opportunity for investigating interactions between melatonin and the HPA axis. Both melatonin and glucocorticoids play important roles in regulating the seasonal biology of red-sided garter snakes (e.g., Nelson et al. 1987; Mendonça et al., 1996; Moore et al., 2000; Lutterschmidt et al., 2004; Lutterschmidt and Mason, 2009). Specifically, acutely elevated melatonin and glucocorticoids significantly inhibit male reproductive behavior (Moore and Mason, 2001; Lutterschmidt et al., 2004). We previously demonstrated that melatonin does not possess “anti-stress” actions in red-sided garter snakes, as melatonin neither antagonizes glucocorticoid actions on reproductive behavior (Lutterschmidt et al., 2004) nor influences glucocorticoid responses to capture stress (Lutterschmidt and Mason, 2005). The aim of the present study was to determine if physiological stress responses alter melatonin rhythms in this population of garter snakes and, if so, whether the effects of stress on melatonin results from increased glucocorticoid secretion. In addition, we investigated whether stress-induced melatonin rhythms could be restored by treatment with 5-hydroxytryptophan, a precursor of melatonin synthesis. Because both melatonin and glucocorticoids regulate reproductive behavior of red-sided garter snakes, understanding how

these endocrine signals interact will provide insight into the underlying reproductive regulatory mechanisms.

## 2. Methods

The following experiments were conducted in the field with free-ranging red-sided garter snakes (*Thamnophis sirtalis parietalis*) in the Interlake region of Manitoba, Canada. Studies were conducted during the spring mating season when the photoperiod is approximately 16 h:8 h LD. All animals were returned to the site of capture upon conclusion of the experiments. Experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol numbers: 2661, 3120) and were in compliance with guidelines established by 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 Permits WSP 03009 and 04004.

### 2.1. Hormone treatments

Corticosterone, the primary glucocorticoid in snakes (Idler, 1972), and 5-hydroxytryptophan, a precursor of melatonin synthesis, were purchased from Sigma (St. Louis, MO). 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.

Corticosterone solutions were prepared by first dissolving 3 or 12 mg 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 15 and 60  $\mu\text{g}$  per 0.1 ml (i.e., per snake). These corticosterone doses are identical to those used by Lutterschmidt et al. (2004) to test the effects of melatonin on the behavioral responses of red-sided garter snakes to exogenous corticosterone. As demonstrated behaviorally by Moore and Mason (2001) and Lutterschmidt et al. (2004), these corticosterone treatment doses produce physiologically relevant increases in circulating corticosterone.

5-Hydroxytryptophan solutions were prepared by dissolving 30 or 60 mg 5-hydroxytryptophan in 5 ml of 5% ethanol in reptile Ringer's solution. This produced 5-hydroxytryptophan concentrations for the low and high doses of 0.6 and 1.2 mg per 0.1 ml, respectively. For an average male snake weighing 0.03 kg, the low dose is 20 mg  $\text{kg}^{-1}$  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 (*Necturus maculosus*). During the scotophase, treatment of mudpuppies with 20 mg  $\text{kg}^{-1}$  5-hydroxytryptophan significantly elevates melatonin synthesis for more than 4 h (Rawding and Hutchison, 1993).

### 2.2. Experimental design

#### 2.2.1. Experiment 1: effect of capture stress on melatonin rhythms

Twenty-four male red-sided garter snakes were collected from the den site and subjected to a capture stress protocol identical to that of Moore et al. (2000) and Lutterschmidt and Mason (2005). Upon capture, snakes were immediately isolated individually in small, opaque cloth bags (approximately 20  $\times$  20 cm) for 4 h to induce physiological stress responses. Following capture-stress treatment from 1000 to 1400 h, we collected blood samples from a subset of snakes ( $n = 12$ ) to determine the effects of stress on photophasic melatonin levels. The remaining 12 capture stress-

treated snakes were removed from the cloth bags and housed in a circular outdoor arena (48 cm diameter) until blood samples were collected at 0000 h to determine the effects of capture stress on scotophasic melatonin. For the non-stress control group, photophasic blood samples were collected from 12 snakes immediately upon capture from the den site and were temporally matched to those of the capture stress-treated snakes. To determine scotophasic melatonin levels of untreated, non-stress control snakes, an additional 12 male snakes were collected from the den site at 1400 h and housed in a circular outdoor arena (48 cm diameter) until blood samples were collected at 0000 h. We housed non-stress control snakes in outdoor arenas until scotophasic sampling because (1) this is the most appropriate control treatment, as snakes treated with capture stress from 1000 to 1400 h were also housed in outdoor arenas until scotophasic sampling at 0000 h and (2) housing snakes in arenas during the spring mating season does not significantly influence plasma corticosterone concentrations (Moore and Mason, 2001). Photophasic samples collected at 1400 h were analyzed for corticosterone and melatonin; scotophasic samples collected at 0000 h were analyzed for melatonin only due to a priori knowledge that stress-induced corticosterone concentrations would return to baseline levels before the scotophasic sampling period (i.e., within 10 h).

### 2.2.2. Experiment 2: effect of exogenous corticosterone on melatonin rhythms

To investigate whether exogenous corticosterone treatment mimics the effects of capture stress on melatonin cycles, we collected 144 male red-sided garter snakes from the den site and randomly assigned them to one of three treatment groups ( $n = 48$  in each): vehicle (5% ethanol in reptile Ringer's solution), low corticosterone dose (15  $\mu\text{g}$ ), or high corticosterone dose (60  $\mu\text{g}$ ). Following intraperitoneal treatment injections at 1200 h, snakes were housed in outdoor arenas (48 cm diameter) and allowed to absorb the treatments. Photophasic blood samples were collected at 1400 h from a subset of snakes ( $n = 12$ ) in each treatment group. The remaining snakes were kept in the outdoor arenas until blood samples were collected during the scotophase at 2200, 0000, and 0200 h ( $n = 12$  for each treatment group at each sampling time). We measured corticosterone in both photophase (1400 h) and scotophase (0000 h) samples to verify that exogenous corticosterone treatment significantly elevated plasma corticosterone. Melatonin was measured in both photophasic (1400 h) and scotophasic (2200, 0000, and 0200 h) samples.

### 2.2.3. Experiment 3: effect of stress and exogenous corticosterone on 5-hydroxytryptophan-induced melatonin rhythms

This experiment was conducted to test the hypothesis that stress decreases scotophasic melatonin concentrations simply because melatonin precursors are depleted during the stress response. Male red-sided garter snakes were collected from the den site at 1200 h and pretreated with an intraperitoneal injection of either vehicle (5% ethanol in reptile Ringer's solution) or 60  $\mu\text{g}$  corticosterone ( $n = 108$  in each group). Snakes were then placed in outdoor arenas (48 cm diameter). To determine if capture stress influences the response to 5-hydroxytryptophan, we collected an additional 36 male snakes from the den site and subjected them to 4 h of capture stress from 1000 to 1400 h. Following capture-stress treatment, snakes were removed from the cloth bags and housed in outdoor arenas.

At 2000 h, snakes from each pretreatment group (i.e., vehicle, capture stress, corticosterone) were randomly assigned to one of the three following treatment groups: vehicle (5% ethanol in reptile Ringer's solution), low 5-hydroxytryptophan dose (0.6 mg), or high 5-hydroxytryptophan dose (1.2 mg). Following treatment, snakes were returned to the outdoor arenas and allowed to absorb

the treatment drugs. Blood samples were then collected during the scotophase at 2200, 0000, and 0200 h ( $n = 12$  for each sampling period in each treatment group). Due to a limited number of bags used to induce physiological stress responses, scotophasic melatonin samples were collected from the capture stress-treated group at 0000 h only. All scotophase samples were analyzed for melatonin concentrations.

### 2.3. Blood sampling and radioimmunoassay

Blood samples (300  $\mu\text{l}$ ) were obtained from the caudal vein as quickly as possible (mean  $\pm 1$  standard error:  $78.1 \pm 2.4$  s) using heparinized 1-cm<sup>3</sup> syringes and 25-g needles. All scotophasic blood samples were collected under dim red light, as this wavelength of light does not inhibit melatonin production (e.g., Benschhoff et al., 1987; Oliveira et al., 2007). Samples were stored on ice until return to the field station, where they were centrifuged and the plasma separated. Plasma samples were stored at  $-4^\circ\text{C}$  until return to Oregon State University, where they were transferred to  $-70^\circ\text{C}$  until analyzed for corticosterone and/or melatonin concentrations following radioimmunoassay procedures described and validated for red-sided garter snakes by Lutterschmidt et al. (2004) and Lutterschmidt and Mason (2008, 2009).

Briefly, plasma samples were analyzed in duplicate for each hormone. Plasma volumes were typically 100  $\mu\text{l}$  for melatonin and 6–20  $\mu\text{l}$  for steroid hormone samples. Melatonin and corticosterone hormones were extracted from each plasma sample with HPLC-grade chloroform or anhydrous ethyl ether, respectively. The solvent phase was removed and dried under nitrogen gas in a warm ( $37^\circ\text{C}$ ) water bath. Hormone extracts were then reconstituted in either tricine-buffered saline for melatonin assay or phosphate-buffered saline for steroid hormone assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were incubated with 6000 cpm tritiated melatonin (*O*-methyl-[<sup>3</sup>H]melatonin, Amersham Biosciences, Piscataway, NJ) or 12,000 cpm tritiated steroid (1,2,6,7-[<sup>3</sup>H]corticosterone, Amersham Biosciences, Piscataway, NJ). Samples and maximum binding tubes also received 100  $\mu\text{l}$  antiserum (melatonin antibody from Stockgrand Ltd., Surrey, UK; corticosterone antibody B3-163 from Esoterix Endocrinology, Calabasas Hills, CA), and all tubes were incubated at  $4^\circ\text{C}$  for 18–24 h. Unbound hormone was separated from bound hormone using dextran-coated charcoal. The bound hormone 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.

Within each experiment, hormone samples were randomly distributed across hormone assays. Hormone concentrations were corrected for individual recovery variation. Mean extraction efficiency was 99.3% for melatonin and 93.4% for corticosterone. Mean intra-assay variation was 9.4 and 12.7% for melatonin and corticosterone, respectively. Inter-assay variation was 15.6% for melatonin and 17.8% for corticosterone.

### 2.4. Statistical analyses

We used SigmaStat<sup>®</sup> 3.11 (1999) (Systat Software, Inc.) for all statistical analyses. Prior to analysis, data were natural log-transformed where necessary to correct for non-normality and/or unequal variance. Significant main effects detected by analysis of variance (ANOVA) were followed by a Tukey's multiple comparisons procedure. All statistical comparisons were considered significant at  $P \leq 0.05$ .

We used a *t*-test to verify that capture stress increased corticosterone concentrations of male red-sided garter snakes (Experiment 1). To examine if capture stress modulates melatonin

rhythms, we used a two-way ANOVA with treatment and sampling time as between-subjects factors. To examine the effects of exogenous corticosterone treatment on corticosterone and melatonin concentrations of male snakes (Experiment 2), we used a two-way ANOVA for each hormone with treatment and sampling time as between-subjects factors.

Lastly, to determine the influence of 5-hydroxytryptophan on melatonin rhythms within each pretreatment condition (Experiment 3), we used a two-way ANOVA with treatment and sampling time as between-subjects factors. Because melatonin responses to 5-hydroxytryptophan did not vary significantly with sampling time in either the vehicle or corticosterone pretreatment groups, we collapsed the 2200, 0000, and 0200 h sampling times. We then performed a two-way ANOVA with pretreatment (3 levels: vehicle, capture stress, exogenous corticosterone) and treatment (3 levels: vehicle, 0.6 mg or 1.2 mg 5-hydroxytryptophan) as the between-subjects factors. Due to violation of the equal variance assumption necessary for parametric analysis, we used the nonparametric Scheirer–Ray–Hare extension of the Kruskal–Wallis analysis (e.g., Sokal and Rohlf, 1995) followed by a nonparametric multiple comparisons test (Zar, 1999) similar to Lutterschmidt and Mason (2008).

### 3. Results

#### 3.1. Experiment 1: capture stress alters melatonin rhythms

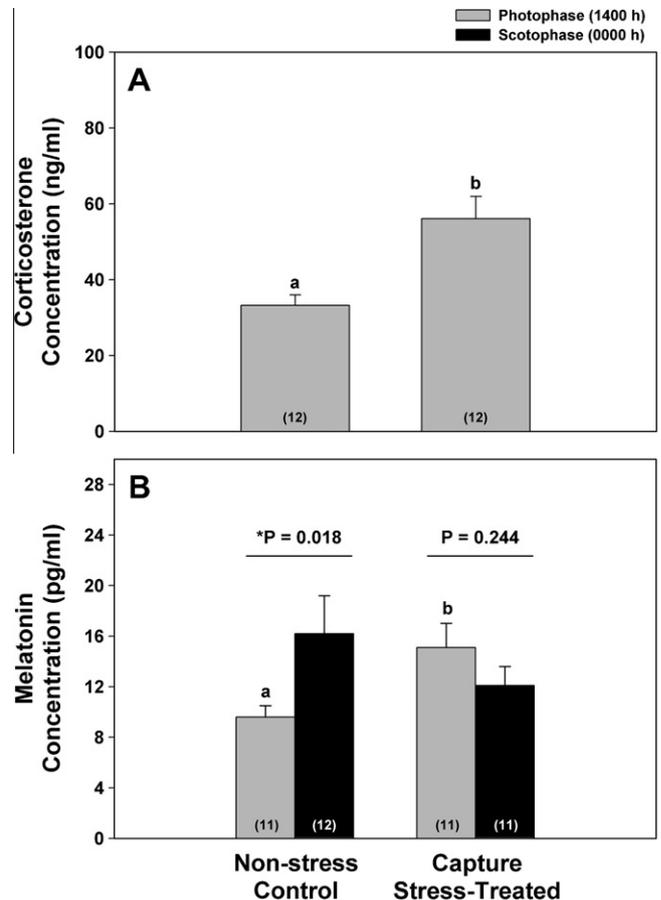
Treatment with 4 h of capture stress during the photophase significantly increased corticosterone concentrations of male snakes (Fig. 1A,  $t = -3.524$ ,  $df = 22$ ,  $P = 0.002$ , from a  $t$ -test). The effects of capture-stress treatment on melatonin varied significantly with sampling time (interaction term:  $F = 6.635$ ,  $df = 1$ ,  $P = 0.014$ , from a two-way ANOVA). Capture stress significantly increased photophasic melatonin concentrations (Fig. 1B,  $q = 3.249$ ,  $P = 0.027$ ) but did not significantly influence scotophasic melatonin concentrations ( $q = 1.888$ ,  $P = 0.189$ , from a Tukey's multiple comparisons test).

#### 3.2. Experiment 2: exogenous corticosterone does not influence melatonin rhythms

Exogenous corticosterone treatment significantly increased plasma corticosterone concentrations ( $F = 15.925$ ,  $df = 2$ ,  $P < 0.001$ , from a two-way ANOVA). Further, plasma corticosterone varied significantly with time ( $F = 23.254$ ,  $df = 1$ ,  $P < 0.001$ ), and a significant interaction between treatment and sampling time existed ( $F = 21.243$ ,  $df = 2$ ,  $P < 0.001$ , from a two-way ANOVA). Within the photophase sampling time, circulating corticosterone concentrations were significantly elevated following treatment at 1200 h (Fig. 2A, from a Tukey's multiple comparisons test). Twelve hours following exogenous corticosterone treatment (i.e., at 0000 h), corticosterone levels were not significantly different from those of vehicle-treated snakes (Fig. 2A). Note that within the vehicle treatment, scotophasic corticosterone concentrations were significantly higher than those during photophase ( $q = 3.112$ ,  $P = 0.032$ , from a Tukey's multiple comparisons test). Treatment with exogenous corticosterone did not significantly influence either photophasic or scotophasic melatonin concentrations of snakes (Fig. 2B, from a two-way ANOVA).

#### 3.3. Experiment 3: both capture stress and exogenous corticosterone block the effect of 5-hydroxytryptophan on melatonin

Pretreatment condition significantly influenced responses to 5-hydroxytryptophan ( $H_{2,245} = 73.075$ ,  $P < 0.001$ , from a Scheirer–Ray–Hare extension of the Kruskal–Wallis analysis). In control snakes (i.e., pretreatment with corticosterone vehicle), 5-hydroxy-

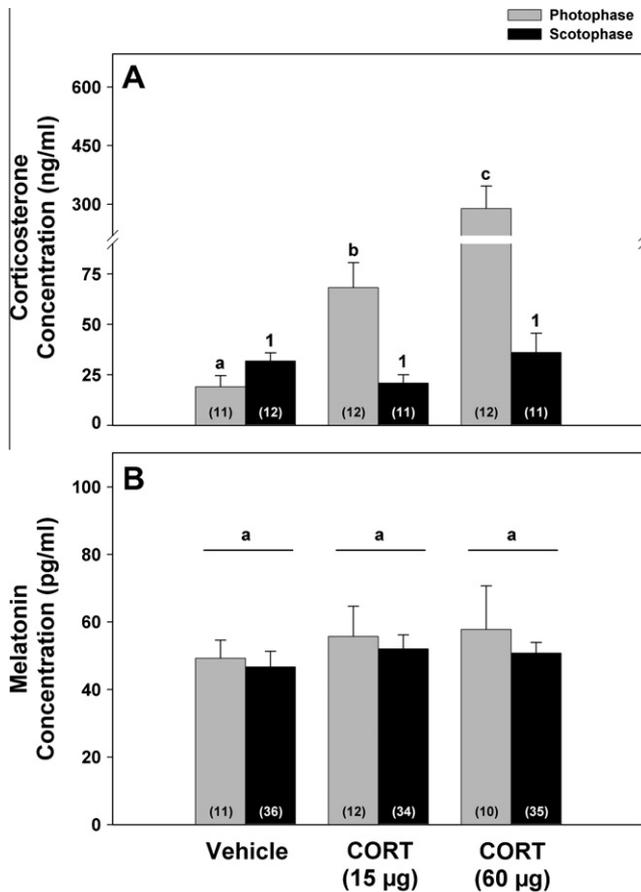


**Fig. 1.** Effect of capture stress on (A) corticosterone and (B) melatonin concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Snakes were subjected to capture stress during the photophase from 1000 to 1400 h. Gray and black bars represent hormone concentrations during the photophase (1400 h) and scotophase (0000 h), respectively. Sample sizes are indicated in parentheses above the abscissa; standard errors (+) are shown by the vertical lines. Statistically significant differences in hormone concentrations within the photophase sampling time are indicated by letters above the standard error bars. Statistical differences between photophasic and scotophasic melatonin within the non-stress control and capture-stress treatment groups are indicated above the horizontal lines in (B).

tryptophan significantly increased scotophasic melatonin concentrations (Fig. 3A, from a nonparametric multiple comparisons test). Pretreatment with both capture stress and exogenous corticosterone blocked this effect. 5-hydroxytryptophan did not significantly influence melatonin concentrations of snakes that were pretreated with 4 h of capture stress from 1000 to 1400 h (Fig. 3B, from a nonparametric multiple comparisons test). Likewise, 5-hydroxytryptophan did not increase scotophasic melatonin concentrations when preceded by pretreatment with exogenous corticosterone (Fig. 3C, from a nonparametric multiple comparisons test). Finally, melatonin concentrations of snakes receiving 5-hydroxytryptophan vehicle differed significantly among pretreatment conditions. Capture stress + 5-hydroxytryptophan vehicle significantly decreased scotophasic melatonin concentrations (Fig. 3B), whereas corticosterone + 5-hydroxytryptophan vehicle had no effect on scotophasic melatonin (Fig. 3C, from a nonparametric multiple comparisons test). There were no significant interactions between pretreatment and treatment conditions ( $H_{4,245} = 8.624$ ,  $P = 0.071$ ).

### 4. Discussion

Our results demonstrate that physiological stress responses significantly modulate diel melatonin rhythms in red-sided garter snakes, *T. sirtalis parietalis*. While the initial phase of an acute stress

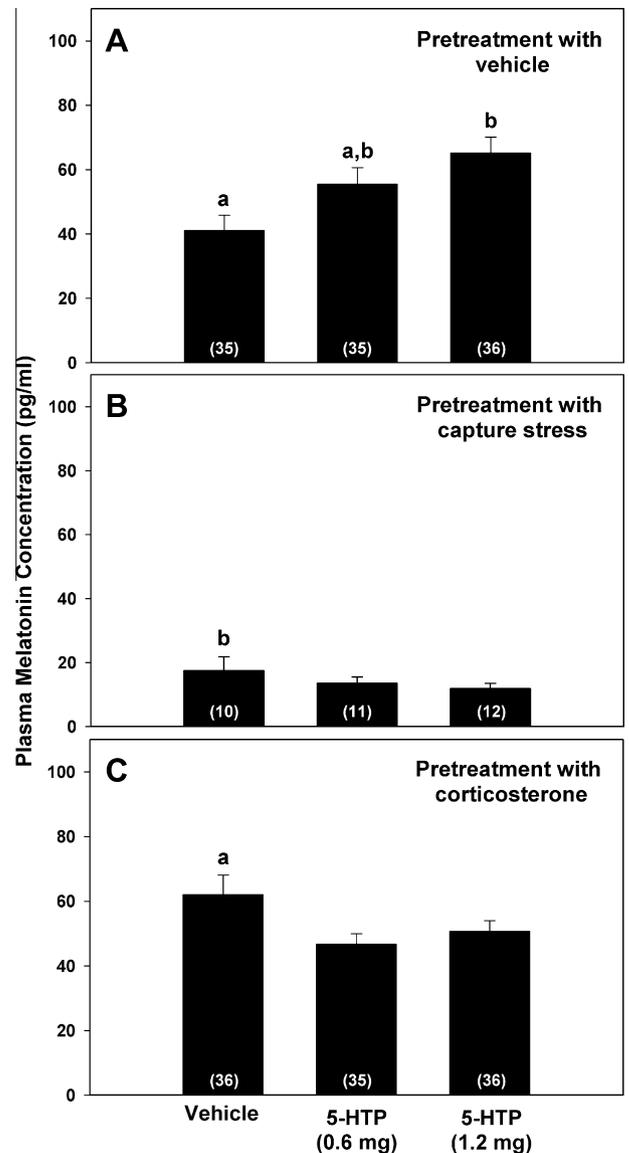


**Fig. 2.** Effect of exogenous corticosterone (CORT; 15 and 60 µg) on plasma (A) corticosterone and (B) melatonin concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Snakes received corticosterone treatments at 1200 h. Gray and black bars represent hormone concentrations during the photophase and scotophase, respectively. Scotophasic melatonin samples (i.e., at 2200, 0000, and 0200 h) are shown collapsed. Sample sizes are indicated in parentheses above the abscissa; standard errors (+1) are shown by the vertical lines. In (A), statistically significant differences in corticosterone concentrations within the photophase sampling time are indicated by letters above the standard error bars; differences within scotophase are shown by numbers.

response increases plasma melatonin concentrations, elevated corticosterone levels can inhibit the production of melatonin from a melatonin precursor. Our findings are unique because they suggest that the different phases of an acute physiological stress response (e.g., initial activation of the sympathoadrenal system followed by increased glucocorticoid secretion) have distinct and temporally different effects on melatonin synthesis. We speculate that interactions between melatonin, glucocorticoids, and the sympathoadrenal system play a role in mediating stress-induced changes in physiology and behavior.

#### 4.1. Effects of stress and corticosterone on photophasic melatonin

A physiological coupling between stress responses and melatonin rhythms has been reported previously. For example, immobilization stress increases photophasic melatonin concentrations in ring doves, *S. risoria* (Rodríguez et al., 2001; Barriga et al., 2002). Melatonin's role as an antioxidant suggests that increased melatonin synthesis following a physiological stress response may be adaptive in combating oxidative damage induced by elevated glucocorticoids (e.g., Reiter et al., 1995; Reiter, 1996). Indeed, Sainz et al. (1995) demonstrated that melatonin alleviates glucocorti-



**Fig. 3.** Effect of 5-hydroxytryptophan (5-HTP; 0.6 and 1.2 mg) on scotophasic melatonin concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). During the photophase, snakes were pretreated with (A) corticosterone vehicle at 1200 h, (B) 4 h of capture stress from 1000 to 1400 h, or (C) 60 µg corticosterone at 1200 h. Snakes received 5-hydroxytryptophan treatment at 2000 h. In (A) and (C), scotophasic melatonin samples (i.e., at 2200, 0000, and 0200 h) are shown collapsed; melatonin concentrations in (B) were measured at 0000 h. Sample sizes are indicated in parentheses above the abscissa; standard errors (+1) are shown by the vertical lines. Statistically significant differences are indicated by letters above the standard error bars.

coid-induced apoptosis of thymocytes via an antioxidant mechanism.

Our experiments with red-sided garter snakes (*T. sirtalis parietalis*) indicate that increased plasma melatonin levels following a stress response result directly from the stress response itself, rather than an increase in glucocorticoid secretion (Figs. 1B and 2B). However, it should be noted that photophasic melatonin concentrations were elevated in snakes treated with vehicle in Experiment 2 (Fig. 2B). This elevation in melatonin may be due to the stress associated with capture and intraperitoneal injection of snakes. Alternatively, elevated photophasic melatonin may be related to individual variation in melatonin rhythms (e.g., Mendonça et al., 1996). Nevertheless, corticosterone treatment had no additional influence on photophasic melatonin concentrations as

compared to vehicle-treated snakes (Fig. 2), suggesting that corticosterone itself does not modulate melatonin.

Our results corroborate those of Ferreira et al. (2005), who demonstrated that corticosterone alone cannot induce melatonin synthesis in cultured pineal glands. Because the pineal gland is innervated by sympathetic nerve fibers, the immediate increase in melatonin secretion during an acute stress response may result from increased sympathetic nervous system activity (e.g., Lynch et al., 1973, 1977; Parfitt and Klein, 1976). For example, changes in sympathetic tone influence pineal melatonin synthesis. Lynch et al. (1973) demonstrated that immobilization stress in rats significantly increases pineal melatonin content as well as *N*-acetyltransferase activity, the enzyme responsible for converting serotonin into *N*-acetylserotonin in the biosynthetic pathway for melatonin synthesis. This effect can be blocked by treatment with a  $\beta$ -adrenergic receptor antagonist, indicating that the effect of stress on melatonin is due to the actions of catecholamines (likely norepinephrine) on pineal  $\beta$ -adrenergic receptors (Lynch et al., 1973).

#### 4.2. Effects of stress and corticosterone on scotophasic melatonin

Scotophasic melatonin concentrations of male snakes treated with 4 h of capture stress (from 1000 to 1400 h) were lower than those of non-stress control snakes. Although this effect was not significant in Experiment 1 (Fig. 1B), capture-stress treatment did significantly decrease scotophasic melatonin concentrations in Experiment 3 (Fig. 3B). Treatment with 5-hydroxytryptophan did not restore stress-induced decreases in scotophasic melatonin (Fig. 3B). Our results therefore do not support the hypothesis that decreased scotophasic melatonin levels following a stress response result from a rapid decline in the concentration of tryptophan, the amino acid precursor for melatonin synthesis (Clark and Russo, 1997).

Although corticosterone itself does not influence melatonin rhythms of red-sided garter snakes (Fig. 2B), we show that corticosterone can inhibit the synthesis of melatonin from 5-hydroxytryptophan (Fig. 3C). These results suggest the persistent effects of stress (at least 10 h following capture stress in these experiments) on scotophasic melatonin result from a combination of sympathoadrenal activation and increased glucocorticoid secretion that, together, induce changes in pineal physiology. This mechanism may involve the enzymes responsible for synthesizing melatonin from 5-hydroxytryptophan, including *L*-aromatic amino acid decarboxylase, *N*-acetyltransferase, or hydroxyindole-*O*-methyltransferase. For example, adrenalectomy blocks stress-induced decreases in pineal *N*-acetyltransferase activity in rats, although whether this effect results from removal of the adrenal medulla or adrenal cortex is unknown (Joshi et al., 1986).

## 5. Conclusions

Further research is necessary to understand the mechanisms by which stress responses influence diel melatonin rhythms. For example, additional experiments using a glucocorticoid synthesis inhibitor such as metyrapone would aid in teasing apart the effects of increased sympathetic activity from those of elevated glucocorticoid synthesis. Although not addressed in the present study, additional experiments are also needed to understand why diel differences occur in the response of the pineal gland to stress (Troiani et al., 1988; Monteleone et al., 1990). For example, water-immersion restraint stress administered to rats during the scotophase still attenuates the nocturnal peak in melatonin secretion (Otsuka et al., 2001). Thus, potential interactions between the pineal gland and catecholamines or other factors released during

the physiological stress response (e.g., endogenous opioids) must be considered. Possible differences between chronic versus acute stress responses also warrant future attention. Chronic immobilization stress in rats significantly increases plasma melatonin and decreases the expression of sympathetic markers in the pineal gland (i.e., tyrosine hydroxylase, p75 neurotrophin receptor and  $\alpha$ -tubulin; Dagnino-Subiabre et al., 2006). The authors suggest that chronic stress impairs sympathetic inputs to the pineal gland, leading to disrupted melatonin signaling and possible environmental maladaptation (Dagnino-Subiabre et al., 2006).

Collectively, these experiments demonstrate that a physiological coupling between melatonin, glucocorticoids, and the sympathoadrenal axis is conserved in this ectothermic model. We suggest that interactions between these neuroendocrine pathways play a role in integrating multiple physiological and behavioral rhythms. For example, Mendonça et al. (1996) reported that male red-sided garter snakes (*T. sirtalis parietalis*) that fail to exhibit courtship behavior during the spring mating season have disrupted melatonin cycles, with highest concentrations occurring during the photophase. Preliminary evidence also suggests that temporal shifts in the corticosterone rhythm of *T. sirtalis parietalis* are associated with the seasonal transition between reproductive and non-reproductive behaviors (Fig. 2A of this study; Lutterschmidt, unpublished data). Interactions between melatonin rhythms and stress responses, like those observed during the present study, may contribute to the mechanisms underlying stress- and/or glucocorticoid-induced changes in activity, reproductive behavior, foraging, and social behavior. Future studies are needed to examine the impact of stress-induced changes in melatonin rhythms on activity patterns and reproductive behavior, particularly with regard to the timecourse of these effects. Such studies would help elucidate whether the observed interactions among melatonin, glucocorticoids, and the sympathoadrenal system are functionally significant to regulating physiology and behavior.

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