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Endocrine mechanisms mediating temperature-induced reproductive behavior in red-sided garter snakes (*Thamnophis sirtalis parietalis*)

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SUMMARY

We investigated the mechanisms by which temperature induces seasonal reproductive behavior in red-sided garter snakes (*Thamnophis sirtalis parietalis*). Specifically, we addressed whether elevated temperatures during winter dormancy influence (1) diel melatonin and corticosterone rhythms; (2) sex steroid hormone and corticosterone profiles; and (3) the expression of reproductive behavior following emergence. Elevated hibernation temperatures (i.e. 10°C *versus* 5°C) significantly increased overall melatonin and decreased corticosterone concentrations of snakes. The temperature-induced differences in melatonin rhythms between the 5°C and 10°C treatment groups persisted even after both groups were again acclimated to 10°C, indicating that cold temperature exposure has a lasting influence on melatonin rhythms. Elevated hibernation temperatures also significantly altered androgen and corticosterone profiles of snakes, providing a potential mechanism to explain reported annual variation in steroid hormones. Although previous studies indicate that male red-sided garter snakes exhibit a dissociated reproductive strategy, we demonstrate the presence of intersexual variation in sex steroid hormone profiles, as estradiol concentrations of female snakes increased significantly prior to spring mating activity. Importantly, the percentage change in body mass did not differ significantly between snakes in the hibernation treatments, indicating that the observed changes in hormone profiles are indeed temperature induced and not simply an indirect result of significant changes in the energy balance of snakes. Finally, in males maintained at 10°C during winter dormancy the onset of courtship behavior following emergence was delayed. Our results suggest that environmental temperatures induce reproductive behavior, in part, *via* changes in diel melatonin and/or corticosterone rhythms in this seasonally breeding reptile.

Key words: melatonin, corticosterone, diel rhythm, androgen, estradiol, courtship behavior, hibernation, winter dormancy, reptile.

INTRODUCTION

Although hibernation and daily torpor are expressed primarily among birds and mammals, many other taxonomic groups from diverse environments undergo periods of prolonged winter dormancy. In ectothermic vertebrates, relatively little is known about the environmental and hormonal mechanisms regulating entrance into or emergence from winter dormancy. Most intriguing is that in species where reproduction occurs immediately following spring emergence, the concomitant changes in neurophysiology and behavior that accompany reproduction are likely to occur during winter dormancy. Thus, significant changes in reproductive physiology and behavior may occur during this 'dormancy' period, and the seasonal control of reproduction is therefore probably linked to the environmental and hormonal mechanisms that control the timing of winter dormancy.

In ectothermic vertebrates, increases in ambient and ground temperatures during spring are thought to play a role in initiating emergence from winter dormancy and subsequent reproductive behavior (e.g. Hawley and Aleksiuk, 1975; Hawley and Aleksiuk, 1976; Jacob and Painter, 1980; Crews and Garstka, 1982; Licht, 1984; Whittier et al., 1987a; Macartney et al., 1989; Grobman, 1990; Crawford, 1991). For example, Etheridge et al. (Etheridge et al., 1983) demonstrated experimentally that increasing ambient temperatures stimulate emergence of the six-lined racerunner (*Cnemidophorus sexlineatus*) from winter dormancy. However, some species (especially those inhabiting extreme northern latitudes) can occupy underground dens at depths where ground temperatures do not change significantly before spring emergence. Red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada emerge at body temperatures as low as 0.5°C (Lutterschmidt et al., 2006), suggesting that increases in ground temperatures may not be the only thermal cue utilized by ectotherms (Macartney et al., 1989; Lutterschmidt et al., 2006). Indeed, emergence of red-sided garter snakes from hibernation may be regulated, at least in part, by an endogenous circannual cycle (Lutterschmidt et al., 2006).

A potential hormonal mechanism mediating the chronobiology of spring emergence and seasonal reproduction in ectotherms is the pineal gland and its major secretory product, melatonin. Circadian melatonin rhythms function in the endocrine transduction of environmental stimuli in vertebrates (Axelrod, 1974). Photoperiod influences the phase of the melatonin cycle, but environmental temperature modulates its amplitude. In diamondback water snakes (*Nerodia rhombifer*), extreme cold and warm temperatures decrease the amplitude of the melatonin cycle (Tilden and Hutchison, 1993). Thus, photoperiod and temperature interact to influence circadian melatonin rhythms. This relationship has also been observed in the European sea bass (*Dicentrarchus labrax*), the mudpuppy (*Necturus maculosus*), the three-toed box turtle (*Terrapene carolina triunguis*), the marbled gecko (*Christinus marmoratus*), and the green anole (*Anolis carolinensis*) (Vivien-Roels et al., 1988; Underwood, 1985a; Rawding and Hutchison, 1992; Tilden and Hutchison, 1993; Moyer et al., 1995; García-Allegue et al., 2001). Thus, circadian melatonin rhythms may transduce low temperature exposure during winter

dormancy as well as changing temperature profiles during spring emergence.

Melatonin is poised to play an important role in orchestrating temperature-induced activation of reproductive function because it in turn influences many different physiological and behavioral processes. For example, changes in the duration of the melatonin signal reflect annual changes in day length and are implicated in the timing of seasonal reproduction in some species (Bittman et al., 1983; Carter and Goldman, 1983). Although extensive experiments have been conducted in both birds and mammals, there are fewer (and inconclusive) studies investigating the relationship between melatonin and seasonal reproduction in other vertebrates (reviewed by Turek and Van Cauter, 1994; Mayer et al., 1997). However, pinealectomy of male green anoles (*A. carolinensis*) stimulates testicular growth and spermatogenesis (Underwood, 1985b); melatonin treatment abolishes the effects of pinealectomy on reproduction in female anoles (Levey, 1973). In male red-sided garter snakes (*Thamnophis sirtalis parietalis*), pinealectomy prior to hibernation abolishes courtship behavior upon spring emergence (Nelson et al., 1987; Crews et al., 1988; Mendonça et al., 1996a). These results suggest the pineal gland is necessary for both the transduction of environmental stimuli during winter dormancy and the induction of seasonal reproductive behavior following spring emergence.

To better understand the hormonal mechanisms controlling seasonal reproduction in ectotherms, we examined the influence of environmental temperatures during hibernation on seasonal reproductive physiology and behavior in a well-studied population of red-sided garter snakes (*T. sirtalis parietalis*) in Manitoba, Canada. These extreme-latitude populations of snakes undergo a period of continuous winter dormancy for approximately 8 months each year. Immediately following spring emergence, an attenuated mating season lasting 4–5 weeks is initiated (e.g. Crews and Garstka, 1982). In this dissociated breeder, reproductive behavior does not coincide with peak gonadal activity (Crews, 1984; Crews, 1991; Crews et al., 1984). Rather, mating occurs 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., 1987b). A secretory product of the hypothalamus–pituitary–adrenal axis, glucocorticoids modify metabolism and regulate energy balance, especially in response to homeostatic challenges. Because these snakes are aphagic during winter dormancy and the mating season, elevated glucocorticoid levels may play an important role in mobilizing energy stores during spring emergence and mating. Such seasonal elevations in glucocorticoids are often observed in vertebrates in which reproductive opportunities are both limited and energetically costly (e.g. Silverin and Wingfield, 1998; Wingfield et al., 1998) (reviewed by Moore and Jessop, 2003).

In red-sided garter snakes, mating behavior is triggered by cold temperature exposure. Snakes are refractory to warm temperatures and require a period of low temperature conditions for more than 4 weeks to initiate sexual behavior (Camazine et al., 1980; Garstka et al., 1982; Bona-Gallo and Licht, 1983). Reproductive behavior of red-sided garter snakes is also independent of changes in photoperiodic conditions (Nelson et al., 1987; Whittier et al., 1987a). Thus, this model system provides an excellent opportunity to examine the hormonal mechanisms by which environmental temperatures activate seasonal reproductive physiology and behavior. Specifically, we investigated whether increased environmental temperatures during winter dormancy influence (1) 24-h melatonin and glucocorticoid rhythms; (2) sex steroid hormone and glucocorticoid profiles; and (3) the expression of reproductive behavior following emergence from winter dormancy. Importantly, both melatonin and corticosterone [the primary glucocorticoid in reptiles (Idler, 1972)] play a role in regulating the seasonal biology of red-sided garter snakes (e.g. Mendonça et al., 1996a; Mendonça et al., 1996b; Moore and Mason, 2001; Moore et al., 2000; Moore et al., 2001; Lutterschmidt et al., 2004; Lutterschmidt and Mason, 2005; Cease et al., 2007). Furthermore, interactions between melatonin, glucocorticoids and the hypothalamus–pituitary–adrenal axis are also well established (e.g. Maestroni et al., 1989; Brotto et al., 2001; Otsuka et al., 2001; Barriga et al., 2002).

MATERIALS AND METHODS

Animals, captive care and acclimatization conditions

These experiments were conducted with red-sided garter snakes (*Thamnophis sirtalis parietalis* Say in James 1823) collected from the Interlake region of Manitoba, Canada. A total of 132 snakes were collected from the den site in the fall after they had migrated from summer feeding grounds to the hibernaculum in preparation for winter dormancy. To identify individual snakes throughout these experiments, we scale-clipped each snake on the ventral surface with a unique number. Snakes were then transported to the laboratory at Oregon State University where they were housed in 10-gallon (~45l) aquaria within microprocessor-controlled environmental chambers. All aspects of these experiments (captive care, blood sampling, courtship trials, etc.) were performed within the environmental chambers. Water was provided *ad libitum*, but food was not offered as snakes do not forage during the winter dormancy period. Snout-vent length and body mass of snakes were measured regularly during all experiments to monitor changes in body condition.

Photoperiod and temperature regimes were adjusted throughout these experiments as shown in Table 1. Daily photoperiod and temperature cycles began at 06:00 h. Hibernation was induced by decreasing ambient temperatures; an absence of photoperiod cues during hibernation simulated underground hibernacula. The cold temperature hibernation regime was chosen based upon previous

Table 1. Acclimatization regimes for investigating the influence of hibernation temperatures on reproductive physiology and behavior in red-sided garter snakes, *Thamnophis sirtalis parietalis*

Acclimatization period		Acclimatization conditions (photoperiod; thermoperiod)	
		Cold temperature hibernation	Warm temperature hibernation
Activity	Weeks	Hours; °C	Hours; °C
Pre-hibernation	–4 to 0	11:13 L:D; 18:10	11:13 L:D; 18:10
Hibernation	1 to 6	0:24 L:D; 10:10	0:24 L:D; 10:10
Hibernation	7 to 20	0:24 L:D; 5:5	0:24 L:D; 10:10
Hibernation	21 to 26	0:24 L:D; 10:10	0:24 L:D; 10:10
Emergence	27+	16:8 L:D; 25:15	16:8 L:D; 25:15

laboratory studies of this species (e.g. Bona-Gallo and Licht, 1983; Krohmer and Crews, 1987; Whittier et al., 1987a) as well as recorded body temperatures of red-sided garter snakes during winter dormancy under natural field conditions (Lutterschmidt et al., 2006). The warm temperature hibernation regime, consisting of a constant temperature of 10°C during winter dormancy, was chosen because (1) this temperature is significantly higher than the body temperatures of red-sided garter snakes recorded during winter dormancy in the field [i.e. 1–3°C minimum (Lutterschmidt et al., 2006)] and (2) this temperature is low enough to prevent dramatic changes in body condition (in the absence of feeding) during these prolonged experiments. All experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol number: 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 Permit WSP 04004.

Experimental design

Diel melatonin and corticosterone rhythms

To investigate whether hibernation temperatures modulate 24-h melatonin and corticosterone rhythms, we randomly assigned 84 male red-sided garter snakes to one of two treatment groups (N=42 in each): cold temperature hibernation or warm temperature hibernation. Once every 4–8 weeks during winter dormancy, we measured diel melatonin and corticosterone rhythms following acclimatization to each set of environmental conditions (Table 1). Snakes were allowed a minimum of 2 weeks for acclimatization. For each treatment group, we measured diel hormone cycles by collecting blood samples from a randomly selected subset of snakes (N=7 at each sampling time selected from a total of 42 snakes) every 4 h for one 24-h period; no snake was bled more than once during a 24-h sampling period. Within a 24-h cycle, each of the six sampling periods (t=08:00, 12:00, 16:00, 20:00; 00:00, and 04:00 h) was completed within approximately 60 min and was centered on the circadian sampling time. All scotophasic blood samples were collected under dim red light, as this wavelength of light does not inhibit melatonin production (e.g. Benshoff et al., 1987; Oliveira et al., 2007).

Sex steroid hormone and corticosterone profiles

We collected blood samples from 20 male and 20 female red-sided garter snakes immediately upon capture in the field (8–9 September) to examine steroid hormone concentrations during the fall (autumn) pre-hibernation period under natural field conditions. To determine if elevated hibernation temperatures influence patterns of steroid hormones during winter dormancy, we randomly assigned 48 red-sided garter snakes to either a cold temperature hibernation or warm temperature hibernation treatment group (N=12 males and 12 females in each). Using a repeated measures design, we collected blood samples from these snakes once every 4–8 weeks to examine changes in steroid hormones during winter dormancy. All blood samples in this experiment were collected between 12:00 and 15:00 h to avoid diel variation in hormone concentrations. Scotophasic blood samples were collected under dim red light as described previously.

Reproductive behavior

After snakes were transferred to spring-like environmental conditions (16 h:8 h L:D; 25:15°C), we measured male courtship behavior to determine if elevated hibernation temperatures influence the expression of reproductive behavior. For these measures of courtship behavior, we used the male garter snakes that were used to determine diel melatonin and corticosterone rhythms during hibernation (N=84 snakes). Courtship behavior was assessed every 3 days for approximately 3 weeks and again at 48 days post-emergence. All courtship trials were conducted between 12:00 and 16:00 h.

Courtship trials were performed in 10-gallon aquaria with eight males simultaneously introduced to an unmated, attractive female. Males were randomly selected from both treatment groups and introduced in groups of eight to simulate natural mating conditions, where the presence of a mating ball facilitates male courtship behavior (Joy and Crews, 1985). Mating balls rarely contain fewer than five males courting a single female because males are attracted to females by both the presence of pheromonal cues expressed on the female's dorsal surface as well as the presence of a mating ball (Joy and Crews, 1985).

Using an ethogram of male courtship behavior (Table 2), we recorded the courtship score of each male 5 and 30 min after introduction into the arena; the observer was blind to the treatment group of each male. Because we had a limited number of female snakes for testing male courtship behavior, and because female attractivity declines significantly following mating (Garstka et al., 1982), we placed a small piece of medical adhesive tape around the cloaca of each stimulus female to prevent mating. The tape does not influence male or female reproductive behavior (LeMaster and Mason, 2002; Lutterschmidt et al., 2004) and was removed from female snakes immediately following each courtship trial. Each male was therefore assigned a courtship score of 0 (no reproductive behavior) to 4 (male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves). Behavioral scores of 3.0 and greater are exhibited only in a reproductive context.

Blood sampling and radioimmunoassay

Blood samples (approximately 300 µl) were obtained from the caudal vein as quickly as possible (86.3±1.5 s; mean ± s.e.m.) after capture, using heparinized 1 cm^3 syringes and 25 gauge needles. Samples were stored on ice until centrifuged and the plasma separated. Plasma samples were then stored at –70°C until analyzed for melatonin

Table 2. Ethogram of courtship behavior for the male red-sided garter snake, *Thamnophis 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 Moore et al. (Moore et al., 2000) and Crews et al. (Crews et al., 1984)].

and/or steroid hormone concentrations following radioimmunoassay procedures described by Tilden and Hutchison (Tilden and Hutchison, 1993), Lutterschmidt et al. (Lutterschmidt et al., 2004), and Lutterschmidt and Mason (Lutterschmidt and Mason, 2008).

Briefly, plasma samples were analyzed in duplicate for each hormone. Plasma volumes were typically 100 µl for melatonin and 4–120 µl for steroid hormone samples. Melatonin and steroid 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°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 c.p.m. tritiated melatonin (*O*-methyl-[^{3}H]melatonin; Amersham Biosciences, Piscataway, NJ, USA) or 12,000 c.p.m. tritiated steroid (1,2,6,7-[^{3}H]corticosterone, 1,2,6,7-[^{3}H]testosterone, or 2,4,6,7,16,17-[^{3}H]estradiol, Amersham Biosciences, Piscataway, NJ, USA). Samples, standards and maximum binding tubes were also incubated with 100 µl antiserum at 4°C for 18–24 h (melatonin antibody from Stockgrand LTD, Surrey, UK; corticosterone antibody B3-163 from Esoterix Endocrinology, Calabasas Hills, CA, USA; testosterone antibody T-3003 and estradiol antibody E-6006 from Wein Laboratories, Succasunna, NJ, USA). 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.

Diel melatonin and corticosterone rhythm samples from both treatment groups, all sampling months, and all sampling times (*N*=413 samples) were randomly distributed across seven melatonin and four corticosterone hormone assays. Owing to limited plasma volumes, corticosterone rhythms were not analyzed during weeks 5 or 10 in hibernation. Steroid hormone profile samples from both treatment groups and all sampling months (*N*=280 samples) were randomly distributed across six steroid hormone assays. Hormone concentrations were corrected for individual recovery variation. Mean extraction efficiency was 100.0% for melatonin and 97.5% for corticosterone; mean extraction efficiencies for testosterone and estradiol were 97.3 and 92.2%, respectively. Mean intra- and inter-assay coefficients of variation were 4.6 and 5.0% for melatonin, 11.1 and 15.5% for corticosterone, 6.3 and 11.6% for testosterone, and 12.4 and 15.3% for estradiol. Limits of detectability were approximately 2 pg for melatonin, 8 pg for corticosterone, 4 pg for testosterone, and 1 pg for estradiol. In some instances (*N*=13 of 140 female steroid samples), estradiol concentrations were below the limits of detectability. To retain these samples in our statistical analyses, and because of the high sensitivity of our estradiol assay, we assigned each undetectable plasma sample the limit of detectability (i.e. 0.001 ng ml^{-1}).

We validated this melatonin radioimmunoassay for use in red-sided garter snakes by demonstrating parallelism between serially diluted snake plasma and serially diluted melatonin standards (Lutterschmidt and Mason, 2008). Further, quantitative recovery tests following addition of melatonin to charcoal-stripped plasma also indicate there are no factors in snake plasma that interfere with this competitive binding assay (Lutterschmidt and Mason, 2008). The methods used for direct radioimmunoassay of corticosterone and testosterone have been previously validated for male red-sided garter snakes (Lutterschmidt et al., 2004; Lutterschmidt and Mason, 2005). To further test whether chromatography of steroid hormones extracted from female snake plasma is necessary, we simultaneously analyzed a subset of plasma samples (*N*=30) for corticosterone and estradiol concentrations using both radioimmunoassay with partition chromatography (e.g. Moore et al., 2000) and radioimmunoassay without partition chromatography (i.e. direct radioimmunoassay). Similar to Lutterschmidt and Mason (Lutterschmidt and Mason, 2005), we included fall, winter and spring plasma samples (*N*=10, 9 and 11, respectively) in these tests to account for seasonal variation in plasma lipid concentrations, and hence different levels of nonspecific binding of steroids. As in male red-sided garter snakes, we observed excellent correlation between the steroid concentrations of female plasma samples assayed by both direct radioimmunoassay and radioimmunoassay with partition chromatography (data not shown; R^2=0.958, P<0.001 for estradiol; R^2=0.938, P<0.001 for corticosterone, from a regression). Thus, we elected to analyze all male and female plasma samples for steroid hormones using direct radioimmunoassay methods. Our direct assay measures both plasma testosterone and 5-α-dihydrotestosterone concentrations because our testosterone antibody (Wein Laboratories, Inc., Succasunna, NJ, USA) cross-reacts significantly with 5-α-dihydrotestosterone (63.2% cross-reactivity). For these reasons, we present here data for androgen concentrations.

Statistical analyses

We first examined whether increased hibernation temperatures significantly affected the body condition of snakes using a *t*-test. To investigate the influence of increased hibernation temperatures on diel melatonin and corticosterone rhythms, we used a two-way analysis of variance (ANOVA) within each sampling month for each hormone cycle. Treatment (i.e. cold versus warm hibernation regime) and sampling time within the 24-h cycle were included in these analyses as between-subjects factors. Before analysis, data were natural log-transformed where necessary to correct for non-normality and/or unequal variance. Significant main effects detected by the two-way ANOVA were followed by a Tukey's multiple comparisons procedure.

We investigated the influence of hibernation temperatures on steroid hormone profiles during winter dormancy using a two-way repeated-measures ANOVA. Changes in hormone concentrations were analyzed separately for male and female snakes. The steroid hormone data presented here represent a mixed design ANOVA, in which hibernation temperature is a between-subjects factor and sampling month is a within-subjects or repeated factor (i.e. the same subjects serve under all five levels of the factor: weeks 8, 13, 21, 28 and 30) (e.g. Field, 2005; Sheskin, 2007). However, assumptions of normality and equal variance required for parametric analysis of these data were violated, and data transformation could not correct these distributional characteristics. Because acceptable nonparametric procedures for a factorial mixed design ANOVA are not available, we analyzed these data using a parametric two-way mixed design ANOVA on the natural-log transformed hormone concentrations. Out of necessity, we assumed that ANOVA is robust against moderate departures from normality and equal variance (e.g. Zar, 1999; Sheskin, 2007).

The above analyses were performed to determine the main effects of hibernation temperature and time (as well as interactions between these factors) on steroid hormone profiles. Because of the inability to meet the assumptions necessary for parametric multifactor analysis, we chose to examine how hormone concentrations change over time within each hibernation temperature group. For these analyses, we used a one-way repeated-measures ANOVA with sampling time as the within-subjects factor (5 levels: 8, 13, 21, 28

and 30 weeks). Prior to analysis, data were natural log-transformed where necessary to correct for non-normality and/or unequal variance. If data transformation could not correct these distributional characteristics, we used a nonparametric Friedman's repeated-measures ANOVA to investigate changes in steroid hormone profiles over time. A Friedman's ANOVA is a rank-based test in which the observations are ranked within each block prior to analysis (e.g. Zar, 1999; Sheskin, 2007). For these data, hormone concentrations within each snake (i.e. the block) were therefore ranked across all levels of sampling time. Significant main effects detected by the repeated-measures ANOVAs were followed by a Tukey's multiple comparisons procedure.

Lastly, we used a two-way repeated-measures ANOVA to examine the influence of increased hibernation temperatures on the expression of courtship behavior following emergence. This analysis was performed on the highest courtship score achieved by each male during a courtship trial. All snakes, including those having a courtship score of 0, were included in the analysis of courtship behavior. Because the same males were tested on each day of the courtship trials, time (i.e. days post-emergence) was included as the repeated or within-subjects factor, while treatment condition was included in the analysis as a between-subjects factor. Significant main effects detected by the ANOVA were followed by a Student–Newman–Keuls multiple comparisons test. We used this multiple comparisons test, which employs step-down logic, because of *a priori* knowledge that courtship behavior would exhibit a step-wise decrease over time (e.g. Zar, 1984; Toothaker, 1993). We used SigmaStat® 3.11 [Systat Software (2005), Systat Systems, Inc., Point Richmond, CA, USA] for statistical analyses. All statistical comparisons were considered significant at $P \leq 0.05$.

RESULTS

Diel melatonin and corticosterone rhythms

Our elevated hibernation temperature of 10°C did not significantly increase the percentage body mass loss of snakes in the warm temperature hibernation treatment (t=–1.645, d.f.=77, P=0.104, from a t-test; Fig. 1). As expected, there were no significant differences between the diel melatonin rhythms of snakes in the cold and warm temperature hibernation treatments prior to temperature manipulation (i.e. during week 5 in hibernation; $F_{1,83}$=2.639, P=0.109, from a two-way ANOVA; Fig. 2A). During this week-5 sampling period, the melatonin concentrations of snakes varied significantly over the 24-h sampling period ($F_{5,83}$=2.613, P=0.031, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 2A). This statistically significant diel melatonin rhythm is expected, as melatonin secretion in snakes is thought to be governed by an endogenous circadian rhythm.

During week 10 in hibernation, snakes acclimated to 5°C in the cold hibernation treatment had significantly lower melatonin concentrations than snakes acclimated to 10°C in the warm hibernation treatment ($F_{1,83}$=4.895, P=0.030, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 2B). Diel melatonin rhythms were not statistically significant in either treatment group ($F_{5,83}$=2.016, P=0.087, from a two-way ANOVA; Fig. 2B). The differences between the overall melatonin concentrations of snakes in the cold and warm temperature hibernation treatments were also observed during week 18 in hibernation ($F_{1,83}$=4.181, P=0.045, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 3A). Again, 24-h melatonin rhythms were not statistically significant in either treatment group ($F_{5,83}$=1.323, P=0.264, from a two-way ANOVA; Fig. 3A).

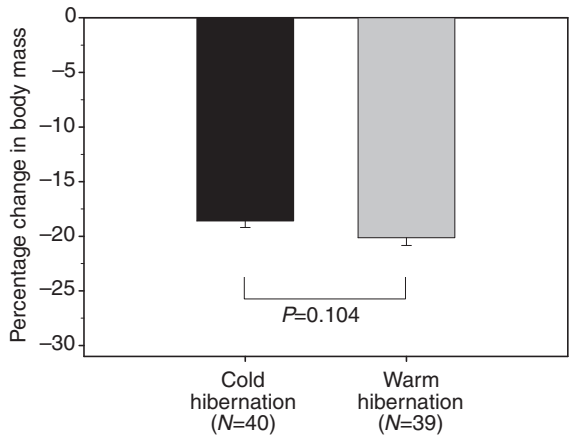


Fig. 1. Mean percentage change in body mass (+s.e.m.) of snakes in the cold *versus* warm temperature hibernation treatments (P-value from a t-test).

When snakes in the cold temperature hibernation treatment were again returned to 10°C during weeks 21–26 in hibernation, significant differences in melatonin between the two hibernation temperature treatments persisted ($F_{1,81}$=4.476, P=0.038, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 3B). Diel melatonin rhythms during week 23 in hibernation were not statistically significant ($F_{5,81}$=2.137, P=0.071, from a two-way ANOVA; Fig. 3B). After both hibernation temperature treatment groups were acclimated to spring-like environmental conditions (i.e. 16 h:8 h L:D; 25:15°C), diel melatonin rhythms did not differ significantly between treatment groups ($F_{1,78}$=1.009, P=0.319, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 3C). A statistically significant 24-h cycle, with higher levels occurring during scotophase, was observed in melatonin concentrations at day 11 post-emergence ($F_{5,78}$=3.703, P=0.005, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 3C). There were no statistically significant interactions between hibernation treatment and 24-h sampling time in any of these analyses of melatonin rhythms.

Hibernation temperature significantly influenced diel corticosterone rhythms of snakes during week 18 in hibernation ($F_{1,81}$=6.757, P=0.011, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 3D). Overall, corticosterone concentrations of snakes acclimated to 5°C were significantly higher than those of snakes acclimated to 10°C (Fig. 3D). A statistically significant diel rhythm in corticosterone levels, with two distinct peaks, was observed in the cold temperature hibernation treatment ($F_{5,81}$=2.474, P=0.040, from a two-way ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 3D). The observed differences in 24-h corticosterone rhythms between snakes in the cold and warm temperature hibernation treatment groups were abolished when snakes were acclimated to identical acclimatization conditions during week 23 in hibernation ($F_{1,79}$=0.173, P=0.679, from a two-way ANOVA; Fig. 3E) and day 11 post-emergence ($F_{1,75}$=0.026, P=0.873, from a two-way ANOVA; Fig. 3F). During week 23 in hibernation, diel corticosterone rhythms were not statistically significant in either hibernation temperature group ($F_{5,79}$=0.050, P=0.998, from a two-way ANOVA), but the corticosterone rhythms of snakes in the two treatment groups appeared to be out

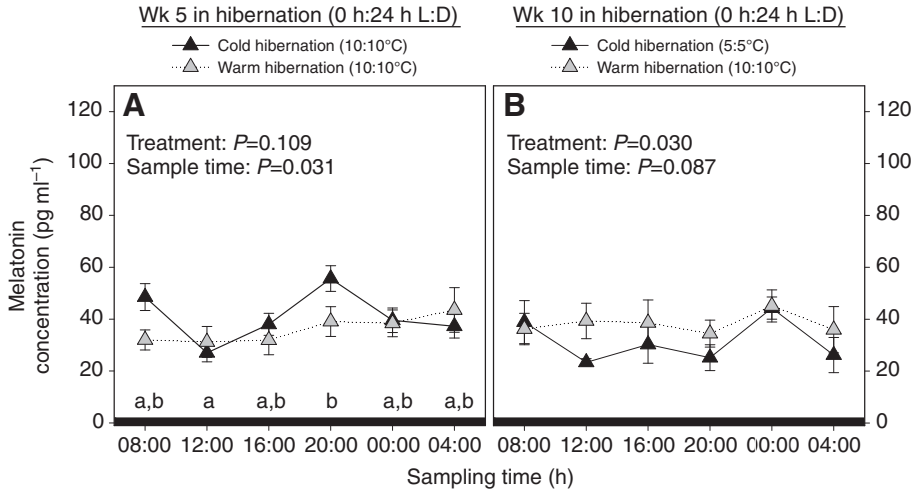
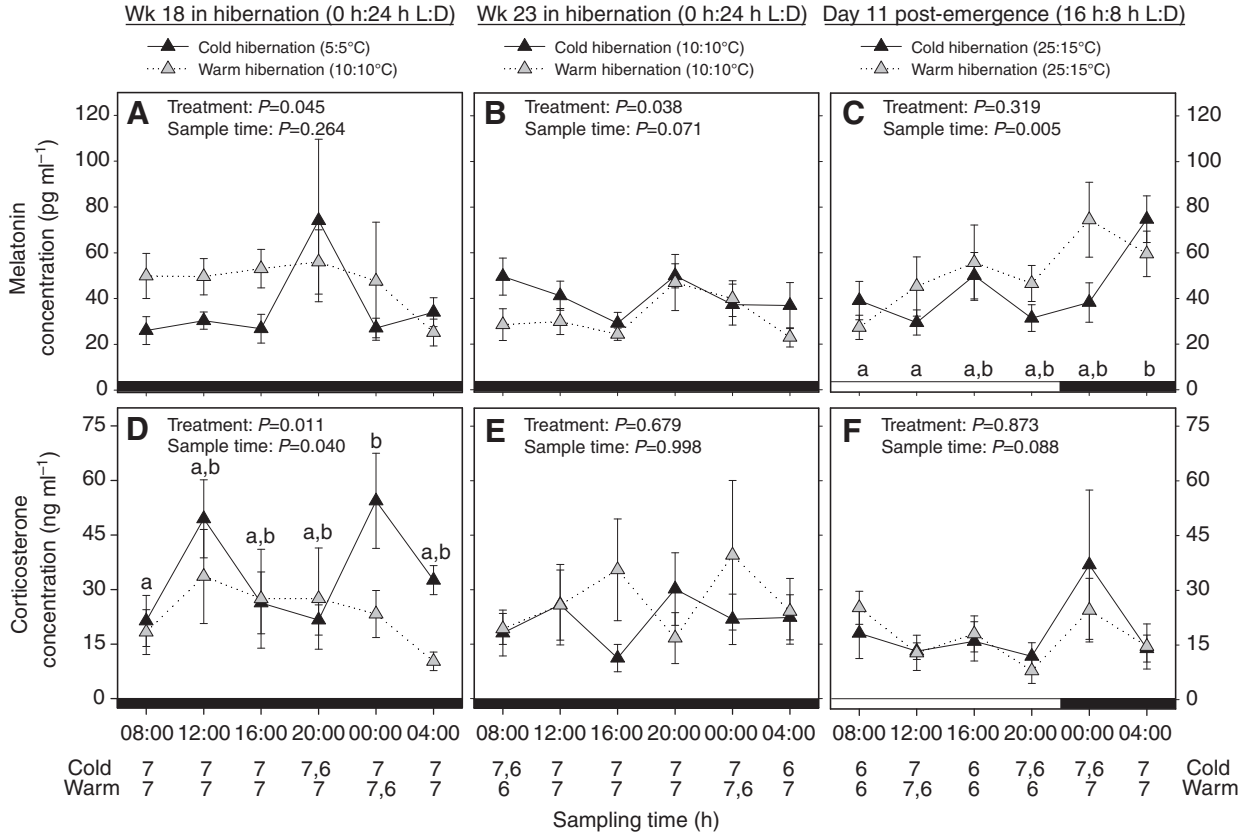


Fig. 2. Influence of elevated hibernation temperatures on diel melatonin rhythms of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) during (A) week 5 and (B) week 10 in hibernation. Temperature acclimation conditions are listed in parentheses above each panel. Black bars above each abscissa indicate the period of scotophase. Each data point is the mean melatonin concentration ±1 s.e.m. of seven snakes randomly selected from a total of 42 animals in each treatment group. Main effects of temperature treatment and sampling time are listed in the top left corner of each panel (statistical values from two-way ANOVAs). Note that melatonin rhythms of snakes in the two treatment groups do not differ significantly before temperature manipulation (A). Letters above the abscissa indicate statistically significant variation in melatonin concentrations during the 24-h sampling period (results from a Tukey's multiple comparisons test).

of phase with one another (Fig. 3E). During post-emergence, snakes in both the cold and warm temperature hibernation treatments had higher corticosterone concentrations during the scotophase, although these diel corticosterone rhythms were not statistically significant ($F_{5,75}$=2.014, P=0.088, from a two-way ANOVA; Fig. 3F). There were no statistically significant interactions between treatment group and sampling time in any of the analyses of corticosterone rhythms.

Sex steroid hormone and corticosterone profiles

Elevated hibernation temperatures significantly altered androgen concentrations of male red-sided garter snakes during winter



Tukey's multiple comparisons test).

Fig. 3. Influence of elevated hibernation temperatures on diel (A-C) melatonin and (D-F) corticosterone rhythms of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) during weeks 18 and 23 in hibernation and day 11 post-emergence. Temperature acclimation conditions are listed in parentheses above panels A, B and C. Black bars above each abscissa indicate the period of scotophase. Each data point is the mean hormone concentration ±1 s.e.m. of a randomly selected subset of snakes. Sample sizes at each sampling time are shown below the *x*-axis for each treatment group; when melatonin and corticosterone sample sizes differ, the sample sizes for melatonin are given first. Main effects of temperature treatment and sampling time are listed in the top left corner of each panel (statistical values from two-way ANOVAs). Letters appearing above the abscissa in C indicate significant differences among sampling times when treatment groups do not differ significantly. Differences among sampling times within the cold temperature hibernation treatment (black symbols) are indicated by lowercase letters near the error bars in D (results from Tukey's multiple comparisons tests).

dormancy ($F_{1,119}$=18.410, P<0.001, from a two-way repeated-measures ANOVA; Fig. 4A). In addition, androgen concentrations changed significantly over time ($F_{4,119}$=47.817, P<0.001, from a two-way repeated-measures ANOVA; Fig. 4A). As expected, the effect of hibernation temperatures on androgen concentrations depended on how long the snakes were in winter dormancy [i.e. there was a statistically significant interaction between hibernation temperature treatment and sampling month ($F_{14,119}$=11.475, P<0.001, from a two-way repeated-measures ANOVA)] (Fig. 4A). Within the cold temperature hibernation treatment, androgen concentrations of male snakes declined significantly following emergence from winter dormancy (χ_r^2=20.200, d.f.=4, P<0.001, from a nonparametric Friedman's repeated-measures ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 4A).

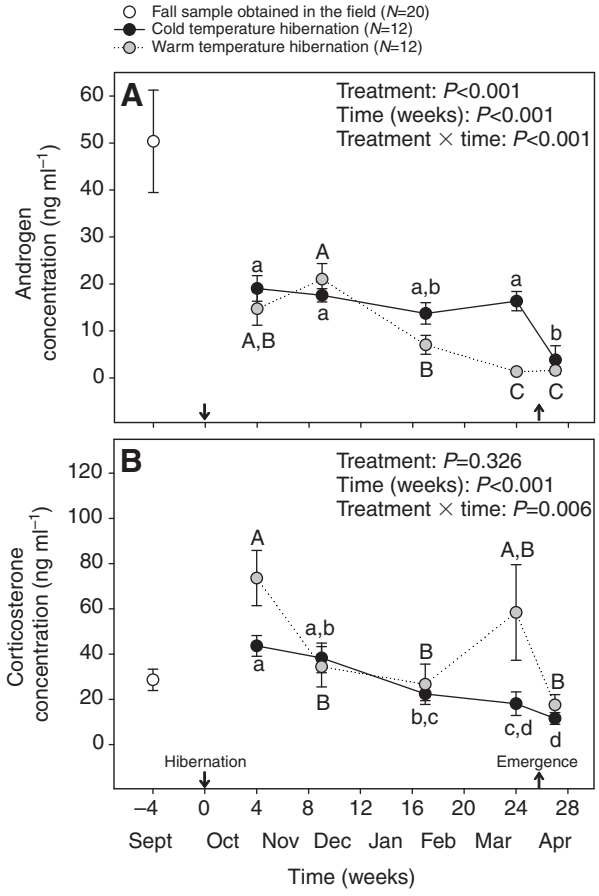


Fig. 4. Influence of elevated hibernation temperatures on (A) androgen and (B) corticosterone concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) during winter dormancy. Fall pre-hibernation steroid concentrations were determined from a randomly selected subset of males (N=20) sampled immediately upon capture in the field. Each subsequent data point is the mean hormone concentration ±1 s.e.m. of snakes in the cold (N=12) and warm (N=12) hibernation treatment groups. Main effects of temperature treatment and sampling month are listed in the top right corner of each panel (statistical values from two-way repeated-measures ANOVAs). Within the cold temperature hibernation treatment (black symbols), differences among sampling periods are indicated by lowercase letters near the error bars, whereas those within the warm temperature hibernation treatment (gray symbols) are indicated by capital letters (results from Tukey's multiple comparisons tests). Induction of hibernation and spring emergence are indicated by arrows along the abscissae.

By contrast, androgen concentrations of snakes in the warm temperature hibernation treatment decreased significantly and steadily throughout hibernation (F=29.011, d.f.=4, P<0.001, from a one-way repeated-measures ANOVA followed by a Tukey's multiple comparisons test), reaching basal levels prior to emergence from winter dormancy (Fig. 4A).

Overall, hibernation temperature did not significantly influence corticosterone concentrations of male snakes ($F_{1,119}$=1.009, P=0.326, from a two-way repeated-measures ANOVA; Fig. 4B). However, we observed a statistically significant interaction between temperature treatment and sampling month ($F_{4,119}$=3.863, P=0.006, from a two-way repeated-measures ANOVA; Fig. 4B). Corticosterone also varied significantly with time ($F_{4,119}$=21.124, P<0.001, from a two-way repeated-measures ANOVA; Fig. 4B). Within the cold temperature hibernation treatment, corticosterone concentrations of male snakes declined significantly during winter dormancy (F=16.196, d.f.=4, P<0.001, from a one-way repeated-measures ANOVA followed by a Tukey's multiple comparisons test; Fig. 4B). By contrast, corticosterone concentrations of snakes in the warm temperature hibernation treatment changed significantly over time but not in a consistent pattern (χ_r^2=28.067, d.f.=4, P<0.001, from a nonparametric Friedman's repeated-measures ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 4B).

In female snakes, elevated hibernation temperatures did not significantly influence estradiol concentrations ($F_{1,119}$=0.019, P=0.891, from a two-way repeated-measures ANOVA; Fig. 5A). In addition, there were no statistically significant interactions between temperature treatment and sampling month ($F_{4,119}$=0.558, P=0.694, from a two-way repeated-measures ANOVA; Fig. 5A). Thus, we collapsed the temperature treatment groups and reanalyzed these data using a one-way repeated-measures ANOVA to determine how estradiol concentrations changed over time. These results indicate that estradiol concentrations increased significantly during winter dormancy and again just before or during spring emergence (χ_r^2=44.064, d.f.=4, P<0.001, from a nonparametric Friedman's repeated-measures ANOVA followed by a Tukey's multiple comparisons procedure; Fig. 5A).

Similar to the corticosterone results in male garter snakes, elevated hibernation temperatures did not significantly influence corticosterone concentrations of female snakes ($F_{1,119}$=0.028, P=0.868, from a two-way repeated-measures ANOVA; Fig. 5B). However, we observed a statistically significant interaction between temperature treatment and sampling month ($F_{4,119}$=3.004, P=0.023, from a two-way repeated-measures ANOVA; Fig. 5B). Corticosterone also varied significantly with sampling month ($F_{4,119}$=28.673, P<0.001, from a two-way repeated-measures ANOVA; Fig. 5B). Within the cold temperature hibernation treatment, corticosterone concentrations of female snakes decreased significantly before emergence from winter dormancy (F=10.801, d.f.=4, P<0.001, from a one-way repeated-measures ANOVA followed by a Tukey's multiple comparisons test; Fig. 5B). Within the warm temperature hibernation treatment, corticosterone concentrations also decreased significantly over time (χ_r^2=31.467, d.f.=4, P<0.001, from a nonparametric Friedman's repeated-measures ANOVA followed by a Tukey's multiple comparisons procedure), reaching the lowest values just before emergence from winter dormancy (Fig. 5B).

Reproductive behavior

Elevated hibernation temperatures did not significantly influence the highest courtship scores achieved by male red-sided garter snakes

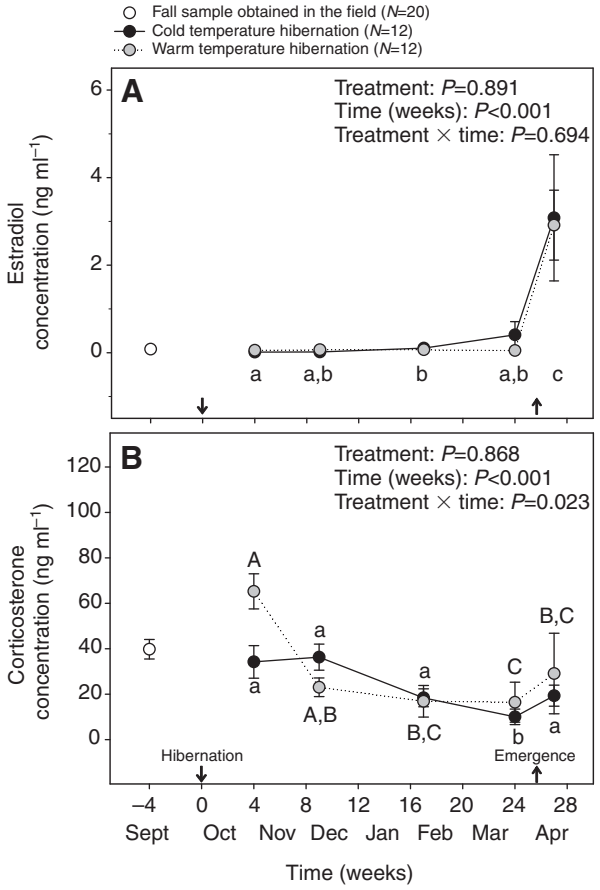


Fig. 5. Influence of elevated hibernation temperatures on (A) estradiol and (B) corticosterone concentrations of female red-sided garter snakes (*Thamnophis sirtalis parietalis*) during winter dormancy. Fall pre-hibernation steroid concentrations were determined from a randomly selected subset of females (N=20) sampled immediately upon capture in the field. Each subsequent data point is the mean ±1 s.e.m. of snakes in the cold (N=12) and warm (N=12) hibernation treatment groups. Main effects of temperature treatment and sampling month are listed in the top right corner of each panel (statistical values from two-way repeated-measures ANOVAs). Letters above the abscissa indicate differences among sampling periods when temperature treatments do not differ significantly. Lowercase letters near the error bars indicate differences among sampling times within the cold temperature hibernation treatment (black symbols); differences among sampling periods within the warm temperature hibernation treatment (gray symbols) are indicated by uppercase letters (results from Tukey's multiple comparisons tests). Induction of hibernation and spring emergence are indicated by arrows along the abscissae.

($F_{1,631}$=1.178, P=0.281, from a two-way repeated-measures ANOVA; Fig. 6). The expression of courtship behavior changed significantly over time during the course of emergence ($F_{7,631}$=20.790, P<0.001, from a two-way repeated-measures ANOVA followed by a Student–Newman–Keuls multiple comparisons procedure; Fig. 6). Although the main effects of hibernation temperature were not statistically significant, the effect of hibernation temperature depended on the day courtship behavior was measured post-emergence ($F_{7,631}$=2.156, P=0.037, from a two-way repeated-measures ANOVA). This significant interaction between hibernation temperature treatment and time is evident in Fig. 6.

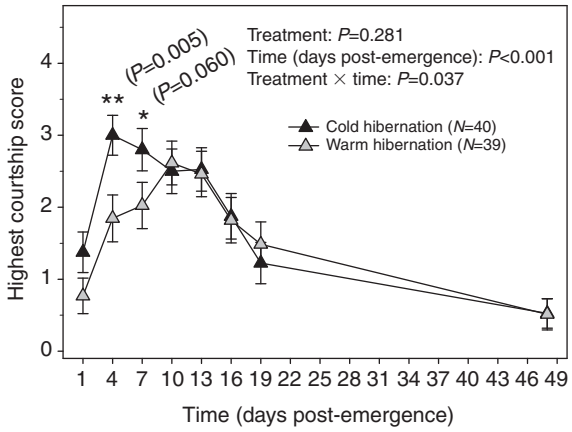


Fig. 6. Influence of elevated hibernation temperatures on the expression of courtship behavior following winter dormancy in male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Each data point is the mean highest courtship score ±1 s.e.m. Main effects of temperature treatment and days post-emergence are listed in the top right corner (from a two-way repeated-measures ANOVA). Differences between the highest courtship scores of snakes in the cold and warm temperature hibernation treatments within days post-emergence are indicated by asterisks (results from a Student–Newman–Keuls multiple comparisons test).

DISCUSSION

We investigated the endocrine mechanisms by which temperature induces seasonal reproductive behavior in red-sided garter snakes (*T. sirtalis parietalis*). Our results demonstrate that environmental temperature, in the absence of changing photoperiodic cues, is sufficient in modulating diel melatonin and corticosterone rhythms as well as androgen concentrations of red-sided garter snakes during winter dormancy. Importantly, the percentage body mass loss of snakes did not differ significantly between our hibernation temperature treatments, suggesting that the observed changes in hormone profiles were indeed temperature-induced and not simply an indirect result of significant changes in the energy balance of snakes in the warm temperature hibernation group. These experiments also support previous findings that male snakes show a dissociated reproductive strategy, in which mating behavior does not coincide with peak steroidogenesis. However, the observation that estradiol concentrations increase significantly before spring emergence suggests that there is intersexual variation in seasonal sex steroid hormone profiles. Following emergence from winter dormancy, we observed robust courtship behavior in both the cold and warm temperature hibernation groups, but male snakes maintained at 10°C during winter dormancy were delayed in the onset of courtship behavior. Collectively, these results suggest that exposure to cold temperature may induce reproductive behavior in red-sided garter snakes through changes in melatonin and/or corticosterone rhythms.

Diel melatonin and corticosterone rhythms

Elevated hibernation temperatures (i.e. 10°C) significantly increased overall melatonin concentrations (Fig. 2B, Fig. 3A) and decreased corticosterone concentrations in male snakes (Fig. 3D). Our results are similar to those of Tilden and Hutchison (Tilden and Hutchison, 1993), who showed that low environmental temperatures decrease the amplitude of the melatonin cycle in diamondback water snakes (*Nerodia rhombifer*). These results demonstrate that temperature during winter dormancy, in the absence of changing photoperiodic

cues, is sufficient to modulate diel melatonin and corticosterone rhythms.

Intriguingly, the amplitude of the melatonin cycle appeared to increase during prolonged exposure to low temperature conditions. Peak melatonin concentrations of snakes were higher after exposure to 5°C for 12 weeks (Fig. 3A) as compared to those of snakes following exposure to 5°C for 4 weeks (Fig. 2B). This trend was also observed in diel melatonin cycles during a preliminary hibernation study, in which a melatonin cycle was observed only in red-sided garter snakes acclimated to low temperature conditions (5°C *versus* 15°C; D.I.L., unpublished data). Previous studies in red-sided garter snakes have shown that the period of low temperature exposure must be at least 4 weeks in duration to elicit courtship behavior after return to high temperature conditions (Bona-Gallo and Licht, 1983). The duration of exposure to cold temperature may be transduced by changes in the amplitude of the melatonin rhythm. This hypothesis is supported by the observation that the cold temperature-induced differences in melatonin rhythms persisted even after snakes in the cold temperature hibernation treatment were returned to 10°C (Fig. 3B). However, it must be noted that diel melatonin cycles were not statistically significant during either of the cold-temperature sampling periods (week 10 and week 18). The lack of a statistically significant melatonin rhythm, especially during week 18 in hibernation (Fig. 3A), is probably a result of the large variation observed in hormone concentrations. This variation may, in turn, reflect asynchronous freerunning hormone rhythms among individuals within each treatment group. Future studies using larger sample sizes within the 24-h sampling period, or perhaps using repeated sampling techniques within individuals, are necessary to determine whether the duration of cold-temperature exposure is indeed coded by changes in the amplitude of the melatonin rhythm.

Alternatively, low temperature exposure during winter dormancy may be transduced by changes in corticosterone rhythms. Because of the role of corticosterone in energy balance, we hypothesized that increased hibernation temperatures would increase corticosterone concentrations of snakes. Surprisingly, a significant diel corticosterone rhythm, with overall higher concentrations, was observed only when snakes were acclimated to 5°C during winter dormancy (Fig. 3D). Whether the two distinct peaks in corticosterone concentrations observed during this sampling period are functionally significant in transducing temperature cues requires further investigation.

In contrast to the observed temperature-induced changes in melatonin rhythms, the effects of low temperature exposure on corticosterone rhythms were transient: neither the temperature-induced differences in corticosterone rhythms nor a significant diel cycle persisted when the acclimation temperature of the cold hibernation treatment group was increased to 10°C (Fig. 3E). During this time, when snakes in both temperature hibernation treatments were acclimated to 10°C, corticosterone rhythms of snakes in these treatment groups were out of phase with one another (Fig. 3E). It is possible that the differences between these corticosterone rhythms contributed to the delayed onset of courtship behavior observed in the warm temperature hibernation group following winter dormancy. Additional experiments focusing on manipulating corticosterone concentrations would help to discern the role corticosterone rhythms play in the induction of seasonal reproduction.

During spring emergence, we did not observe any differences in the melatonin and corticosterone rhythms of snakes between the cold and warm temperature hibernation treatments (Fig. 3C,F). This is not surprising, however, as diel hormone cycles of snakes were measured on day 11 post-emergence. As evident in Fig. 6, the courtship behavior of males in the cold and warm temperature hibernation groups was indistinguishable after day 10 post-emergence. As discussed below, these results suggest that an entrainable mechanism is involved in the transduction of temperature cues, as acclimatization to spring-like environmental conditions increased the courtship behavior of snakes in the warm temperature hibernation treatment within 10 days post-emergence.

Sex steroid hormone and corticosterone profiles

Male red-sided garter snakes (*T. sirtalis parietalis*) are one of the most studied reptilian models of dissociated reproduction (reviewed by Woolley et al., 2004). Reproductive behavior of red-sided garter snakes does not coincide with peak gonadal activity (Crews 1984; Crews et al., 1984). Rather, mating occurs 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., 1987b). In addition, castration prior to hibernation or following spring emergence does not eliminate courtship behavior (Garstka et al., 1982; Crews et al., 1984), and treatment of male snakes with androgens does not induce reproductive behavior (Garstka et al., 1982; Crews et al., 1984).

Our results are consistent with previous findings that male red-sided garter snakes exhibit a dissociated reproductive strategy in which mating behavior does not coincide with maximal androgen synthesis (Fig. 4A). We demonstrate that androgen concentrations are elevated when male snakes return to the hibernaculum in preparation for winter dormancy. Androgen concentrations gradually decline during winter dormancy, reaching basal levels during the period of spring emergence. These experiments indicate that androgen concentrations decline during winter dormancy as a result of metabolic clearance, as androgens reached basal levels significantly earlier when snakes were maintained at higher (10°C) hibernation temperatures (Fig. 4A).

In contrast to the numerous studies in male red-sided garter snakes, we provide evidence of intersexual variation in seasonal sex steroid hormone profiles. Estradiol concentrations of female snakes were very low throughout the fall pre-hibernation period and winter dormancy. During the latter portion of winter dormancy, estradiol concentrations increased slightly, and following emergence we observed a highly significant increase in sex steroid hormone levels (Fig. 5A). This increase in estradiol concentrations was independent of hibernation temperature. Although these data suggest that increased estradiol concentrations occur during winter dormancy in the absence of changing environmental cues, further research is necessary to determine sex steroid hormone concentrations immediately preceding and following spring emergence. Additional studies are also necessary to determine if this observed pre-mating estradiol surge induces female receptivity during the mating season (e.g. Mendonça and Crews, 1996; Whittier et al., 1987b). Such studies would help clarify whether red-sided garter snakes indeed exhibit intersexual variation in the control of seasonal reproductive physiology and behavior.

In both male and female red-sided garter snakes, corticosterone concentrations significantly declined during winter dormancy when snakes were exposed to low temperature conditions (Fig. 4B, Fig. 5B). Within the cold temperature hibernation treatment, corticosterone levels significantly decreased following spring emergence in male red-sided garter snakes but significantly increased during emergence in female red-sided garter snakes. This increase in corticosterone in female snakes coincided with

increased estradiol synthesis (Fig. 5A). Although we did not observe a significant overall effect of hibernation temperature on corticosterone, the effect of hibernation temperature interacted significantly with sampling month, making it difficult to interpret how corticosterone profiles are influenced by elevated hibernation temperatures. Furthermore, the ambiguous pattern of corticosterone in the warm temperature hibernation groups, particularly for male snakes, may reflect changing diel hormone rhythms, as discussed previously.

Importantly, these experiments provide a potential mechanism to explain observed variation in steroid hormone concentrations of snakes among years, sampling times and snake populations (e.g. Woolley et al., 2004). For example, initial studies of male red-sided garter snakes demonstrated that androgen concentrations were basal during winter dormancy and spring emergence (Camazine et al., 1980; Crews, 1984). Subsequent studies showed that androgen concentrations were elevated upon spring emergence and rapidly declined over the mating season (Krohmer and Crews, 1987; Krohmer et al., 1987; Moore et al., 2000). Similar annual variation has also been reported in corticosterone concentrations (e.g. Moore et al., 2000; Moore et al., 2001; Lutterschmidt and Mason, 2005). If androgen concentrations of snakes indeed decline during winter dormancy and spring emergence *via* metabolic clearance, then androgen concentrations will vary with how much time elapses between the summer/fall peak in androgen synthesis and entry into hibernation. For example, during fall 2003, we recorded basking activity in snakes late into October (Lutterschmidt et al., 2006). In such years, when environmental conditions permit delayed entry into winter dormancy, androgen and corticosterone concentrations would be expected to be lower throughout winter dormancy and spring emergence.

Reproductive behavior

Following acclimatization to spring-like environmental conditions, we observed robust courtship behavior in both temperature treatment groups, with most male snakes exhibiting reproductive behavior for approximately 2 weeks following winter dormancy (Fig. 6). Although elevated hibernation temperatures did not influence the overall expression of reproductive behavior, males maintained at 10°C during winter dormancy showed a significant delay in the onset of courtship behavior. For male red-sided garter snakes in this extreme-latitude population, such a delay in courtship behavior (i.e. 10 days to reach maximal courtship behavior during an individual mating season lasting approximately 14 days) could have disproportionately large consequences on reproductive fitness.

The effects of elevated hibernation temperatures on courtship behavior did not persist throughout the entire mating period, suggesting that the hibernation temperatures tested in this experiment do not influence reproductive behavior by an all-or-none mechanism. Rather, plastic mechanisms are implicated in the transduction of temperature cues, as acclimatization to spring-like environmental conditions increased courtship behavior of snakes in the warm temperature hibernation treatment within 10 days of emergence (Fig. 6).

It is unlikely that this observed delay in the activation of courtship behavior is related to differences in androgen concentrations between the hibernation temperature treatment groups, particularly because androgen concentrations of snakes in the warm temperature hibernation group did not change significantly during the period of spring emergence. By contrast, differences in melatonin and corticosterone rhythms between these treatment groups may have contributed to the delayed courtship behavior, as both melatonin (Lutterschmidt et al., 2004) and corticosterone (Moore and Mason, 2001; Lutterschmidt et al., 2004) inhibit courtship behavior of male red-sided garter snakes. This hypothesis is supported by the fact that the differences in diel hormone cycles observed between the cold and warm temperature treatments (Fig. 2B, Fig. 3A,B,D) were eliminated following acclimatization to spring-like environmental conditions (Fig. 3C,F). However, further studies are necessary to determine how a larger range of hibernation temperatures (e.g. 3–15°C) influence both diel hormone rhythms and courtship behavior during the mating season. Future research, manipulating both melatonin and corticosterone levels within physiological limits during winter dormancy, is also needed to evaluate the roles of these hormones in the chronobiology of seasonal reproduction. Such studies would help elucidate the hormonal mechanisms mediating temperature-induced reproductive physiology and behavior in ectothermic vertebrates.

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