Geographic Variation in Timekeeping Systems among Three Populations of Garter Snakes (*Thamnophis sirtalis*) in a Common Garden

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ABSTRACT

Transduction of environmental cues into endocrine signals that synchronize physiology and behavior with optimal environmental conditions is central to an animal's timekeeping system. Using a common garden approach, we investigated possible geographic variation in timekeeping systems by comparing 24-h melatonin and corticosterone rhythms and reproductive behavior among three populations of garter snakes with very different life histories: red-sided garter snakes (*Thamnophis sirtalis parietalis*) from Manitoba, Canada; red-spotted garter snakes (*Thamnophis sirtalis concinnus*) from western Oregon; and eastern garter snakes (*Thamnophis sirtalis sirtalis*) from southern Florida. Melatonin and corticosterone cycles differed significantly among the three snake populations in a majority of the sampling periods. Population differences were observed across a wide range of acclimatization conditions and were themselves plastic (i.e., one snake population was not consistently different from the others). Changes in courtship behavior during emergence also varied significantly among populations. Our data support the hypothesis that endogenous timekeeping systems have evolved in the presence of unique environmental conditions. Further research is necessary to determine whether this geographic variation results from inherent genetic differences or whether it is a product of development. These studies provide insight into the evolution of timekeeping systems and may aid in understanding the potential effects of environmental perturbations on seasonal physiology and behavior.

Introduction

A crucial factor governing individual survival and fitness is the ability to track changing environmental cues so that physiological and behavioral processes are synchronized with optimal environmental conditions. Most vertebrates exhibit seasonality and/or annual cyclicity in a variety of processes, including molting, feeding, immune function, activity, migration, hibernation, and especially reproduction (e.g., Goldman et al. 2004). Such seasonal cycles in physiology and behavior generally are thought to be a product of endogenous rhythmicity and responses to annual changes in day length (e.g., Lincoln et al. 2003).

Photoperiod is transduced into an endocrine signal by the pineal gland, which produces the hormone melatonin in the absence of inhibitory light stimuli (e.g., Axelrod 1974). The 24-h melatonin cycle therefore provides an endocrine index of time of day (via the phase of increased melatonin secretion) as well as time of year (via the duration of increased melatonin concentrations) to all tissues (e.g., Axelrod 1974; Tamarkin et al. 1985). Additionally, environmental temperature modulates the amplitude of the melatonin cycle (Firth and Kennaway 1987; Tilden and Hutchison 1993; García-Allegue et al. 2001). Together, the duration, phase, and amplitude of the melatonin signal play an important role in coding environmental conditions.

The endocrine melatonin signal forms the interface between the environment and the internal milieu: environmental cues influence the melatonin cycle, and the melatonin cycle in turn regulates physiology and behavior. Melatonin signaling therefore constitutes, at least in part, an animal's timekeeping system (e.g., Lincoln et al. 2003). Among the many actions reported for melatonin in both mammalian and nonmammalian vertebrates are regulation of activity rhythms, reproductive physiology and behavior, thyroid activity, immune function, thermoregulation, aggression, and free radical scavenging (e.g., Bittman et al. 1983; Carter and Goldman 1983; Crews et al. 1988; Maestroni et al. 1989; Krotewicz et al. 1992; Cagnoli et al. 1995; Dubbels et al. 1995; Mendonça et al. 1996*a*, 1996*b*; Reiter 1996; Wright et al. 1996; Lutterschmidt et al. 1997, 1998, 2002, 2004; Hyde and Underwood 2000; Jasnow et al. 2002). Melatonin signaling also modulates clock gene synthesis in calendar cells in the pituitary gland to entrain endogenous circannual rhythms to environmental cues (reviewed in Lincoln et al. 2003). It is worth noting here that the influence of melatonin signaling on physiology and behavior varies both tax-

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onomically as well as among animals with different life-history characteristics (e.g., Lutterschmidt et al. 2002, 2003; Goldman et al. 2004). Nevertheless, melatonin signaling functions in synchronizing extrinsic environmental cues with at least some aspects of intrinsic physiology and behavior in most organisms.

As part of this synchronizing interface, melatonin significantly modulates other endocrine pathways that regulate physiology and behavior. For example, interactions between the pineal gland and hypothalamus-pituitary-adrenal (HPA) axis are well established (e.g., Maestroni et al. 1989; Khan et al. 1990; Kirby et al. 1999). The HPA axis mediates responses to stressors such as predation events or limited food availability by increasing glucocorticoid secretion (Harvey et al. 1984; Schwabl et al. 1985; Wingfield 1988). Glucocorticoids in turn promote survival by modifying metabolism and mobilizing energy stores (e.g., Wingfield 1988; Sapolsky 1992; Pottinger 1999).

Together, the HPA axis and glucocorticoid tone play an important role in integrating information about energy homeostasis with seasonal changes in physiology and behavior (e.g., Wilson and Wingfield 1992, 1994; Schramm et al. 1999; Jessop et al. 2002; reviewed in Moore and Jessop 2003; Lutterschmidt 2006). For example, seasonal elevation in glucocorticoid levels is adaptive during periods of high energy demand and has been described in many vertebrates (e.g., Zerani and Gobbetti 1993; Wilson and Wingfield 1994; O'Reilly and Wingfield 1995; Schramm et al. 1999; Landys-Ciannelli et al. 2002; Reneerkens et al. 2002; Moore and Jessop 2003; Leary et al. 2004). A physiological coupling between the HPA axis and melatonin signaling functions in integrating multiple diel and seasonal rhythms in physiology and behavior (e.g., Jessop et al. 2002; Lutterschmidt et al. 2004; Lutterschmidt 2006). One question that requires further investigation is how diel melatonin and glucocorticoid rhythms vary among different environments. For example, does the neuroendocrine pathway transduce environmental cues similarly in all species regardless of phylogeny and evolutionary history? Do glucocorticoid rhythms contribute to seasonal biology to a greater extent in extreme environments?

There is precedence for these questions in observations of geographic variation in the molecular genetics of circadian clock cells in both *Drosophila* and *Arabidopsis* (Michael et al. 2003; Khare et al. 2005). Furthermore, Firth et al. (1989) demonstrated significant differences in melatonin rhythmicity between cold-temperature-adapted tuatara (*Sphenodon punctatus*) and desert-adapted sleepy lizards (*Tiliqua rugosa*). However, whether the neuroendocrine interface between the environment and an organism's physiology and behavior is genetically and/or phylogenetically constrained is not clear. Studies addressing how melatonin cycles respond to extreme environmental conditions, such as those found in the Artic, have been conducted (e.g., Reierth et al. 1999; Hau et al. 2002). However, comparisons across species and geographic regions cannot be made without phylogenetic controls for such comparisons (e.g., Felsenstein 1985).

The common garter snake (*Thamnophis sirtalis*) model provides a powerful framework for investigating these questions. This diurnal species has the most extensive range of any ectotherm in North America (from central Canada southward to the Gulf of Mexico and southern Florida and from the Atlantic to the Pacific coasts) and inhabits a great diversity of environments. These characteristics are invaluable for investigating evolutionary differences in timekeeping systems among geographically distinct populations. Furthermore, both laboratory and field studies have demonstrated functional roles for melatonin and corticosterone, the primary glucocorticoid in snakes (Idler 1972), in regulating the seasonal biology of this ectothermic model (e.g., Nelson et al. 1987; Crews et al. 1988; Mendonça et al. 1996*a*, 1996*b*; Moore et al. 2000*a*, 2000*b*, 2001; Moore and Mason 2001; Lutterschmidt et al. 2004).

We investigated whether variation in melatonin and corticosterone rhythms among snake populations is environmentally induced or whether this variation reflects evolutionary differences in timekeeping systems. We hypothesized that physiological timekeeping systems evolved to ensure that individuals are temporally well adapted to a particular environment. To test this hypothesis, we compared melatonin and corticosterone rhythms among three populations of garter snakes (*T. sirtalis*) from Manitoba, Canada, and from Oregon and Florida. These snake populations have very distinct latitudinal and longitudinal distributions and are closely related phylogenetically (e.g., de Queiroz et al. 2002). Because these populations of *T. sirtalis* have very different life histories and seasonal biologies, they provide an excellent opportunity to investigate possible geographic variation in timekeeping systems. Using a common garden approach, we posed the following specific questions. (1) Are environmental cues transduced into diel melatonin and corticosterone rhythms similarly among snake populations? (2) Does reproductive behavior vary among populations, even when snakes are acclimatized to identical environmental conditions?

Study Populations

Red-sided garter snakes (*Thamnophis sirtalis parietalis*) are the most northerly living reptile in North America and are found in extremely high numbers throughout south central Manitoba, Canada. These northern-latitude populations of snakes undergo a period of winter dormancy for approximately 8 mo each year. Following spring emergence, red-sided garter snakes remain within the vicinity of the dens during the attenuated mating season (4–5 wk; Crews and Garstka 1982). In this dissociated reproductive system, reproductive behavior 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. 1987). Because these snakes are aphagic during winter dormancy and the mating season, elevated corticosterone levels probably play an im-

portant role in mobilizing energy stores during spring emergence and mating. Seasonal elevations in glucocorticoids are often observed in vertebrates whose reproductive opportunities are both limited and energetically costly (e.g., Silverin and Wingfield 1998; Wingfield et al. 1998; reviewed in Moore and Jessop 2003). Further, dissociated reproductive patterns are thought to be an adaptation to environments that provide predictable but brief opportunities for reproduction (Crews and Gans 1992).

In contrast, the midlatitude red-spotted garter snake (*T. sirtalis concinnus*) of western Oregon has an extended breeding season that lasts 10–12 wk from March through May (Moore et al. 2000*b*, 2001). Although red-spotted garter snakes do exhibit periods of winter dormancy, they can be active during 10–12 mo of the year, given appropriate environmental conditions (Moore et al. 2000*b*, 2001). Moore et al. (2000*b*) reported elevated testosterone levels at the beginning of the mating season (late February–April) in red-spotted garter snakes (*T. sirtalis concinnus*). The presence of measurable testosterone concentrations during the mating season indicates that the testes are active and steroidogenic (Moore et al. 2000*b*; Lutterschmidt and Mason 2005). Similarly, eastern garter snakes (*T. sirtalis sirtalis*) in the southern subtropical regions of Florida may be active during most of the year, since environmental conditions are extremely mild during winter months (e.g., Tennant 1997). Like the red-spotted garter snake (*T. sirtalis concinnus*), eastern garter snakes (*T. sirtalis sirtalis*) also demonstrate elevated plasma and testicular testosterone concentrations on emergence from hibernation (Weil 1985). However, it should be noted that these data are from northern populations of eastern garter snakes in Wisconsin (Weil 1985). Although extensive studies of the reproductive endocrinology of midlatitude red-spotted (*T. sirtalis concinnus*) and southern-latitude eastern (*T. sirtalis sirtalis*) garter snakes have not been conducted, the data of Moore et al. (2000*b*) and Weil (1985) suggest that reproductive behavior and peak gonadal steroidogenesis coincide during the spring mating season in both populations, a phenomenon characteristic of associated reproductive patterns (e.g., Woolley et al. 2004).

Material and Methods

Using a common garden approach, we investigated whether variation in diel hormone cycles and courtship behavior is environmentally induced or whether this variation reflects evolutionary differences in timekeeping systems. These experiments were conducted with male and female common garter snakes (*Thamnophis sirtalis*) collected from three different populations: red-sided garter snakes (*T. sirtalis parietalis*) were collected from a den located in the Interlake region of Manitoba, Canada (50°37′N, 97°32′W); red-spotted garter snakes (*T. sirtalis concinnus*) were collected from the E. E. Wilson Wildlife Area in the Willamette Valley of western Oregon (44°30′N, 123°17′W); and eastern garter snakes (*T. sirtalis sirtalis*) were collected from Broward (26°07′N, 80°15′W), Lee (26°58′N, 81°87′W), and Palm Beach (26°68′N, 80°12′W) counties in south Florida. These snake populations represent three very distinct latitudinal and longitudinal distributions and suites of environmental adaptations, especially with respect to overwintering temperatures.

Red-sided (*T. sirtalis parietalis*) and red-spotted (*T. sirtalis concinnus*) garter snakes were collected in the field from March through September 2003 and were transported to the laboratory at Oregon State University. Wild-caught eastern garter snakes were purchased from Glades Herp (formerly of Fort Myers, FL; current location is Bushnell, FL) and Strictly Reptiles (Hollywood, FL) from March through July 2003. Eastern garter snakes were purchased and shipped to Oregon State University on a weekly basis shortly after collection; snakes were housed in the suppliers' vivaria typically less than 1 wk. At Oregon State University, groups of two to four same-sex conspecific snakes were housed in 10-gal aquaria within microprocessor-controlled environmental chambers. All aspects of these experiments (captive care, blood sampling, and courtship trials) were performed within the environmental chambers.

To identify individual snakes throughout these experiments, we scale clipped each snake on the ventral surface with a unique number. During their normal active foraging season, snakes were housed under summerlike environmental conditions (i.e., 14L : 10D photoperiod; 24° : 18°C thermoperiod) and were fed twice weekly with vitamin-fortified fish and earthworms. Water was provided ad lib. at all times, but food was not offered during hibernation because 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 body condition. Throughout these experiments, photoperiod and temperature regimes were adjusted as described in Table 1.

All experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol 2661) 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 2002-06) and was performed under the authority of the Manitoba Wildlife Scientific Permit WSP 03009.

Melatonin and Corticosterone Cycles

In October 2003, we randomly assigned snakes from each population to one of two acclimatization regimes: 11L : 13D, 15° : 10°C (hereafter referred to as the cold acclimatization regime) or 11L : 13D, 25° : 20°C (hereafter referred to as the warm acclimatization regime; Table 1). Daily photoperiod and temperature cycles began at 0600 hours. This experimental design allowed us to examine the direct effects of acclimatization regimes on melatonin and corticosterone cycles within and among populations. An advantage of this design is that all snake populations have spent equal amounts of time in winter dor-

Table 1: Acclimatization regimes for investigating geographic variation in hormone cycles and reproductive behavior in common garter snakes (*Thamnophis sirtalis*)

Acclimatization Period	Sampling Month	Acclimatization Conditions (Photoperiod, Thermoperiod)
Summer (March 10–October 25)	…	14L : 10D, 24° : 18°C
Prehibernation (October 26–December 1)	November	11L : 13D, 15° : 10°C (cold regime)
		11L : 13D, 25° : 20°C (warm regime)
Hibernation (December 2–29)	December	0L : 24D, 15° : 10°C
Hibernation (December 30–February 29)	February	0L : 24D, 10° : 10°C
Spring emergence (March 1–April 20)	April	16L : 8D, 25° : 20°C

mancy, and therefore time does not confound these comparisons.

Approximately once every 4–6 wk from October 2003 to April 2004, we measured melatonin and corticosterone rhythms following acclimatization to each set of environmental conditions (Table 1). Snakes were allowed no less than 3 wk for acclimatization. For each population in each acclimatization regime, we measured diel hormone cycles by collecting blood samples from a randomly selected subset of snakes ($n =$ 5–10 at each sampling time) every 4 h for one 24-h period; no snake was bled more than once during a 24-h sampling period. Scotophasic blood samples were collected under dim red light, since this wavelength of light does not inhibit melatonin production (e.g., Benshoff et al. 1987; Oliveira et al. 2007). Each of the six sampling periods within a 24-h cycle ($t =$ 0800, 1200, 1600, 2000, 0000, and 0400 hours) were completed within approximately 60 min and were centered on the circadian sampling time.

After the initial samples were collected in November, we collapsed the cold and warm acclimatization groups to increase sample sizes within each population and sampling time. Snakes were then subjected to a winter dormancy period that simulated a mild temperate climate (Table 1). Snakes were hibernated in complete darkness to mimic the natural conditions of underground hibernation sites. We chose acclimatization temperatures that were intermediate between the two extreme populations of red-sided garter snakes in Manitoba, Canada, and eastern garter snakes in south Florida (e.g., Lutterschmidt et al. 2006). These intermediate temperatures were chosen to maximize survival of animals throughout this prolonged experiment.

Courtship Behavior

After snakes were transferred to springlike environmental conditions (16L : 8D, 25° : 20°C), we measured courtship behavior of males to determine whether population differences in hormone cycles were correlated with differences in the expression of reproductive behavior. Similar to the study by Lutterschmidt et al. (2004), we used an ethogram of male courtship behavior (Table 2) to score reproductive behaviors on a scale of 0 (no courtship behavior) to 5 (mating). Individual female snakes were introduced into groups of three to four conspecific males to simulate natural mating conditions, where the presence of a mating ball facilitates male courtship behavior (Joy and Crews 1985). We measured the highest courtship score achieved by individual male snakes during each 30-min courtship trial. 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. Thus, the highest achievable mating score for male snakes was 4 (Table 2). Courtship behavior of each male was assessed every third day for 1 wk following emergence from winter dormancy and once every 7 d for 3 wk thereafter. All courtship trials were conducted between 1000 and 1400 hours.

Blood Sampling and Radioimmunoassay

Blood samples were obtained from the caudal vein as quickly after capture as possible (mean = 112.3 s, SE = 3.0) using heparinized 1-cm^3 syringes and 25-g needles. The sensitivity of the HPA axis to stress is modulated seasonally in garter snakes. For example, during the spring reproductive season, red-spotted (*T. sirtalis concinnus*) and red-sided (*T. sirtalis parietalis*) garter snakes require 4 h or longer to mount a significant response

Table 2: Ethogram of courtship behavior for male garter snakes (*Thamnophis sirtalis*)

Description of Behavior	Courtship Score
No reproductive behavior	0
Male investigates female, increased tongue-flick rate	1
Male chin rubs female with rapid tongue flicks	2
Male aligns body with female	3
Male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves	4
Male copulates with female	5

Note. Behaviors 3 and greater are exhibited only in a reproductive context (modified from Crews et al. 1984; Moore et al. 2000*a*).

to capture stress (Moore et al. 2001). In contrast, significant stress-induced increases in corticosterone occur within 1 h during the summer (red-spotted and red-sided garter snakes) and fall (red-spotted garter snakes; Moore et al. 2001). Although the rate at which garter snakes respond to capture and handling stress varies with environmental temperature as well as seasonal adrenocortical modulation, our mean sampling time is well within the acceptable limits for investigating baseline hormone concentrations without inducing a physiological stress response (e.g., Moore et al. 2000*a*, 2000*b*, 2001; Lutterschmidt and Mason 2005; Cease et al. 2007).

Blood samples were stored on ice until centrifuged (within 90 min) at 2,500 rpm and 4°C and the plasma separated. Plasma samples were stored at −70°C until analyzed for melatonin and corticosterone concentrations using radioimmunoassay procedures modified from Tilden and Hutchison (1993) and Lutterschmidt and Mason (2005). Briefly, plasma samples were analyzed in duplicate for each hormone. Plasma volumes were typically 100 μL for melatonin and 10–14 μL for corticosterone. Melatonin and corticosterone were extracted from each plasma sample twice with high-performance liquid chromatography–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 buffered saline for assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or nonspecific binding), 100% bound, and all samples were incubated with 6,000 cpm tritiated melatonin (*O*-methyl-^{3}H melatonin; Amersham Biosciences, Piscataway, NJ) or 12,000 cpm tritiated corticosterone (1,2,6,7-^{3}H corticosterone; Amersham Biosciences). All samples (except nonspecific binding tubes) were also incubated with 100 μL antiserum at 4°C for 18–24 h (melatonin antibody from Stockgrand, Surrey; corticosterone antibody B3-163 from Esoterix Endocrinology, Calabasas Hills, CA). Melatonin samples were additionally incubated with 50 μL diluted normal sheep serum (Sigma, St. Louis, MO) to decrease nonspecific binding (e.g., Tilden and Hutchison 1993; Mendonça et al. 1996*a*). Unbound hormone was separated from the bound fraction 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 (Beckman Coulter; Fullerton, CA).

We validated this melatonin radioimmunoassay for use in each garter snake population by demonstrating parallelism between serially diluted snake plasma and serially diluted melatonin standards (data not shown). Further, quantitative recovery tests following the addition of melatonin to charcoal-stripped plasma from each population also indicated that there are no factors in snake plasma that interfere with this competitive binding assay (red-sided garter snakes: $r^2 = 0.917$, $P < 0.0001$; red-spotted garter snakes: $r^2 = 0.779$, $P = 0.0001$; eastern garter snakes: $r^2 = 0.973$, $P < 0.0001$; from regressions of measured melatonin concentrations on expected melatonin concentrations). The methods used for direct radioimmunoassay of steroid hormones have been previously validated for male and female red-sided (*T. sirtalis parietalis*) and red-spotted (*T. sirtalis concinnus*) garter snakes (e.g., Lutterschmidt et al. 2004; Lutterschmidt and Mason 2005; D. I. Lutterschmidt, unpublished data). Limited sample sizes and plasma volumes from eastern garter snakes (*T. sirtalis sirtalis*) prevented us from conducting similar comparisons between samples processed with column chromatography and samples assayed by direct steroid radioimmunoassay. However, it is unlikely that the highly significant relationship between hormone concentrations obtained by direct radioimmunoassay and radioimmunoassay with column chromatography observed for red-sided and red-spotted garter snakes across different seasons would not also hold true for eastern garter snakes.

All samples were corrected for individual recovery variation. Mean extraction efficiency was 93.9% for melatonin and 92.0% for corticosterone. Plasma samples from all populations, all sampling months, and all sampling times ($n = 702$) were randomly distributed across six melatonin and nine steroid hormone assays. Mean intraassay variation was 11.6% for melatonin and 13.1% for corticosterone; interassay variation was 23.8% for melatonin and 20.7% for corticosterone. This relatively higher interassay variation results from the large number of radioimmunoassays required to analyze all plasma samples. Limits of detectability were approximately 5 pg/mL for melatonin and 16 pg/mL for corticosterone. In some instances ($n = 0$ of 701 melatonin samples; $n = 11$ of 702 corticosterone samples), hormone concentrations were below the limits of detectability. To retain these corticosterone samples in our statistical analyses and because of the high sensitivity of our corticosterone assay, we assigned each undetectable plasma sample the limit of detectability observed for that assay (i.e., 0.01–0.02 ng/mL).

Statistical Analyses

To examine differences in hormone cycles among snake populations, we first determined whether the data within each sampling month were normally distributed and whether they exhibited equal variance among groups. All hormone data in these experiments demonstrated homogeneity in variance. However, assumptions of normality required for parametric analysis were consistently violated, and data transformation could not correct nonnormality. Although parametric ANOVA and multiple comparisons tests are generally regarded to be robust against violations of normality (e.g., Toothaker 1993; Sheskin 2007), this is true only when distributions differ moderately from normality. Because these data grossly violate the assumption of normality required for reliable results from parametric analysis, we performed nonparametric analyses described by Groggel and Skillings (1986), Sokal and Rohlf (1995), and Zar (1999).

The hormone data presented here are replicated, and all factors are fixed treatment effects: snake population (three levels), circadian sampling time (six levels), and sex (two levels). Thus, we employed the Scheirer-Ray-Hare extension of the

Kruskal-Wallis analysis (e.g., Sokal and Rohlf 1995) to examine differences in hormone cycles among snake populations. The methodology underlying this nonparametric rank-based test for two-way ANOVA designs "can be extended to multiway ANOVAs in a straightforward manner" (Sokal and Rohlf 1995, p. 447). As detailed by Sokal and Rohlf (1995), we also tested for interactions between the fixed factors. Rank-based tests are more conservative than parametric analyses (Toothaker 1993), and some concern has been expressed regarding the accuracy of rank-transform methods (Sawilowsky et al. 1989). However, the extreme violation of normality in these hormone data combined with unequal sample sizes across snake populations produce spurious and unreliable results in parametric analysis. Thus, the rank-based Scheirer-Ray-Hare extension of the Kruskal-Wallis analysis is most appropriate for investigating differences in hormone cycles among snake populations.

Within each acclimatization regime (i.e., month), all observations were ranked as a single array, and a three-way ANOVA was used to investigate possible differences in diel hormone cycles among snake populations. Melatonin and corticosterone cycles were analyzed individually with population, sampling time, and sex as between-subjects factors. When sex did not significantly influence hormone cycles and did not significantly interact with other factors, we collapsed the male and female groups to increase sample size and reanalyzed the data using a two-way ANOVA on rank-transformed data, with population and sampling time as between-subjects factors.

To examine the direct effects of acclimatization regime on melatonin rhythms, we compared hormone profiles of snakes in the cold and warm acclimatization regimes sampled during November via a three-way ANOVA on rank-transformed data (Groggel and Skillings 1986; Sokal and Rohlf 1995). We excluded sex as a factor in this analysis because melatonin cycles did not vary significantly between male and female snakes during this sampling period. Thus, snake population, acclimatization regime, and sampling time were the between-subjects factors. To examine the direct effects of acclimatization regime on corticosterone rhythms during the November sampling period, we performed a preliminary four-way ANOVA on ranks, with acclimatization regime, population, sampling time, and sex as between-subjects factors. However, sex did not significantly influence corticosterone cycles of snakes and did not significantly interact with the other main factors. Thus, we collapsed the male and female groups and reanalyzed these data using a three-way ANOVA on rank-transformed data, with snake population, acclimatization regime, and sampling time as between-subjects factors.

When the Scheirer-Ray-Hare extension of the Kruskal-Wallis analysis (Sokal and Rohlf 1995) rejected the null hypothesis of no differences in hormone cycles among snake populations, we followed the analysis with a multiple comparisons test to determine which snake populations differed significantly. We followed the procedures developed by Dunn (1964) and outlined by Zar (1999) for nonparametric multiple comparisons tests with unequal sample sizes. Because our primary question of interest was whether hormone cycles vary among snake populations, we limited our multiple comparisons tests to those necessary for examining population differences only. We included sampling time and sex as fixed treatment effects in our statistical model to account for a priori knowledge that hormone concentrations would vary significantly with both the circadian cycle and sex. However, we did not perform multiple comparisons tests for these factors because these comparisons are not the primary focus of this study. Furthermore, multiple comparisons tests for sampling time and sex would not produce reliable results for examining the effects of these factors on hormone rhythms in this experimental design. The extremely large number of possible comparisons for sampling time (six levels) and sex (two levels) within snake population (three levels) would either grossly inflate the familywise Type I error rate (i.e., the likelihood of making at least one Type I error, or incorrectly rejecting the null hypothesis when it is in fact true) or, if the familywise error rate is strictly controlled, greatly reduce the statistical power of the test (i.e., increase the probability of making Type II errors, or incorrectly accepting the null hypothesis when it is in fact false; Field 2005; Sheskin 2007).

Finally, we examined differences in the expression of courtship behavior (i.e., the highest courtship score achieved by each male) among snake populations. All snakes, including those having a courtship score of 0, were included in analyses of courtship behavior. The courtship data presented here represent a mixed design ANOVA in which snake population is a between-subjects factor and days postemergence is a within-subjects or repeated factor (i.e., the same subjects serve under all six levels of the factor: 1, 4, 7, 14, 21, and 28 d postemergence; Field 2005; Sheskin 2007). We included days postemergence in the statistical model to account for a priori knowledge that courtship behavior would change significantly over the emergence period. These courtship data demonstrate both unequal variance as well as nonnormality, 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 raw, untransformed courtship scores. 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 analysis was performed to determine the main effects of snake population and days postemergence on courtship behavior. Within each snake population, we investigated how courtship behavior changed over the 28-d emergence period using a nonparametric Friedman's repeated-measures ANOVA with days postemergence as the within-subjects factor (six levels: 1, 4, 7, 14, 21, and 28 d). A Friedman's ANOVA is a rank-based test in which the observations are ranked within each block before analysis (e.g., Zar 1999; Sheskin 2007). For these data, courtship scores within each snake (i.e., the block) were therefore ranked across all levels of days postemergence. Significant results of the Friedman's test were followed by a

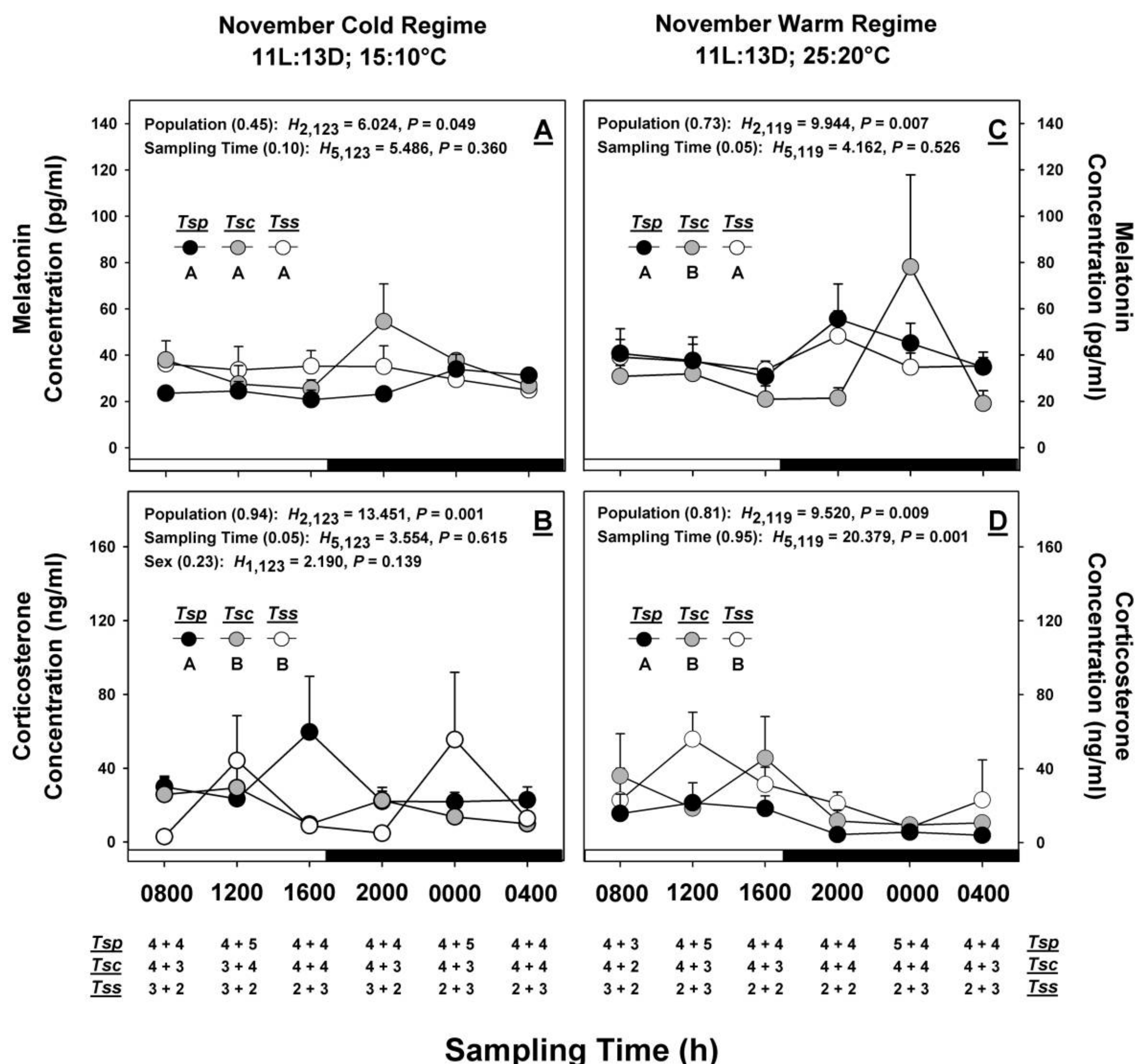

Figure 1. Diel hormone rhythms in red-sided garter snakes (*Thamnophis sirtalis parietalis*; *Tsp*) from Manitoba, Canada; red-spotted garter snakes (*T. sirtalis concinnus*; *Tsc*) from western Oregon; and eastern garter snakes (*T. sirtalis sirtalis*; *Tss*) from southern Florida. *A*, *C*, Plasma melatonin concentrations (pg/mL) measured every 4 h for one 24-h period. *B*, *D*, Plasma corticosterone concentrations (ng/mL) for the same snakes. Each data point is the mean hormone concentration + SE. Sample sizes at each sampling time are shown below the *X*-axis for each snake population (males + females). Main effects of snake population and sampling time are listed in the top left corner (statistical values from two-way ANOVAs on ranks with males and females collapsed). Statistical values in *B* are from a three-way ANOVA with sex included as a between-subjects factor, because a significant population × sex interaction was observed in corticosterone cycles during this sampling period. Post hoc statistical power (i.e., the probability of not making a Type II error) is shown in parentheses behind each factor. Statistically significant differences among snake populations are indicated by letters below the key (from nonparametric multiple comparisons tests). Acclimatization conditions are listed above *A* and *C*; shaded bars along the *X*-axis indicate scotophase.

Bonferroni-corrected Wilcoxon signed-ranks test with day 1 postemergence as the control group (Field 2005).

We used SigmaStat 3.11 (Systat 2005) and SPSS 15.0 (SPSS 2006) for statistical analyses. Critical values for the Scheirer-Ray-Hare extension of the Kruskal-Wallis test and nonparametric multiple comparisons tests for unequal sample sizes were calculated as outlined by Sokal and Rohlf (1995) and Zar (1999). All statistical comparisons were considered significant at $P \geq 0.05$.

Results

Melatonin and Corticosterone Cycles

The main effects of population and sampling time on hormone cycles are shown in Figures 1 and 2 (statistical values from Scheirer-Ray-Hare extension of the Kruskal-Wallis analysis). Main effects of sex from three-way ANOVAs on ranks are indicated where significant sex differences or significant sex × population interactions were observed (melatonin cycles

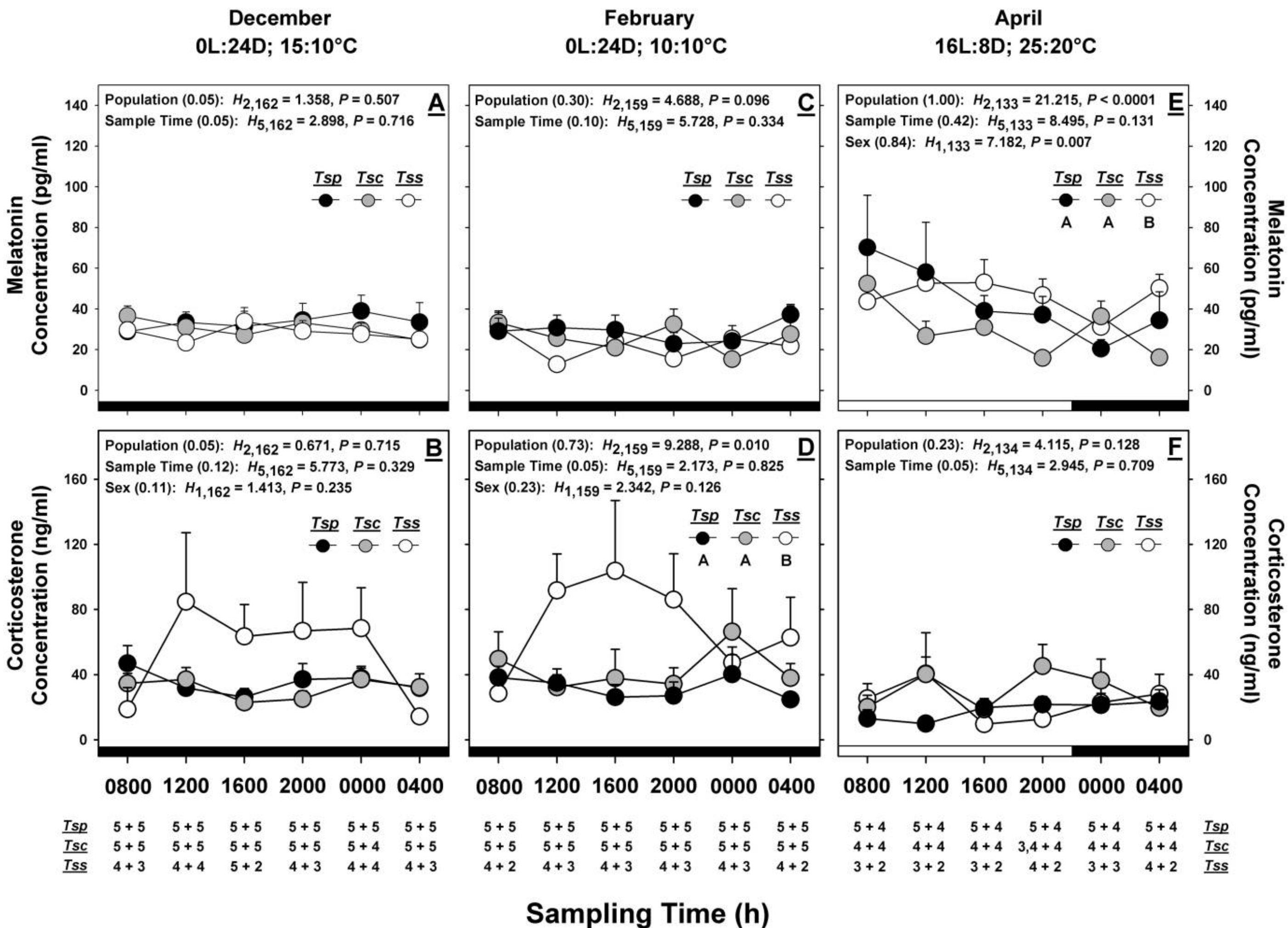

Figure 2. Diel hormone rhythms in red-sided garter snakes (*T. sirtalis parietalis*; *Tsp*) from Manitoba, Canada; red-spotted garter snakes (*T. sirtalis concinnus*; *Tsc*) from western Oregon; and eastern garter snakes (*T. sirtalis sirtalis*; *Tss*) from southern Florida. *A*, *C*, *E*, Plasma melatonin concentrations (pg/mL) measured every 4 h for one 24-h period. *B*, *D*, *F*, Plasma corticosterone concentrations (ng/mL) for the same snakes. Each data point is the mean hormone concentration + SE. Sample sizes at each sampling time are shown below the *X*-axis for each snake population (males + females; when melatonin and corticosterone sample sizes differ, the sample sizes are given as melatonin, corticosterone). Main effects of snake population, sampling time, and sex are listed in the top left corner (statistical values from three-way ANOVAs on ranks). When sex did not significantly influence hormone concentrations and did not significantly interact with the other factors, the statistical values listed are from two-way ANOVAs on ranks with males and females collapsed. Post hoc statistical power (i.e., the probability of not making a Type II error) is shown in parentheses behind each factor. Statistically significant differences among snake populations are indicated by letters below the key (from nonparametric multiple comparisons tests). Acclimatization conditions are listed above *A*, *C*, and *E*; shaded bars along the *X*-axis indicate scotophase.

Table 3: Results from a three-way ANOVA on ranks for investigating geographic variation in melatonin rhythms among populations of common garter snakes (*Thamnophis sirtalis*) in the cold versus warm acclimatization regimes sampled during November

Source of Variation	Power	df	SS	MS	H	P
Population	.40	2	17,905.338	8,952.669	3.626	.163
Acclimatization regime	.40	1	13,368.050	13,368.050	2.707	.099
Sampling time	.59	5	39,667.522	7,933.504	8.033	.154
Population × acclimatization regime	.93	2	63,996.702	31,998.351	12.960	.002
Population × sampling time	.68	10	61,211.719	6,121.172	12.396	.259
Acclimatization regime × sampling time	.11	5	5,703.040	1,140.608	1.155	.949
Population × sampling time × acclimatization regime	.58	10	50,932.534	5,093.253	10.314	.414
Residual		208	947,150.277	4,553.607		
Total		243	1,199,935.182	4,938.005		

Note. Post hoc power analyses were computed using $\alpha = 0.05$.

during April; corticosterone cycles during November [cold acclimatization regime], December, and February). Post hoc power analyses (calculated with $\alpha = 0.05$) are also shown in Figures 1 and 2 for each main factor of population, sampling time, and sex. These power statistics represent the probability of not making a Type II error (i.e., the probability of not making the conservative error of accepting the null hypothesis when it is indeed false).

Melatonin and corticosterone cycles differed significantly among the three snake populations in a majority of the sampling periods during this experiment. Nonparametric multiple comparisons procedures for unequal sample sizes indicate that the observed population differences in hormone cycles were not static and changed over the course of the experiment (Figs. 1, 2).

Sampling time did not significantly influence melatonin concentrations during any of the sampling periods. Corticosterone concentrations varied significantly with sampling time only in the warm acclimatization regime sampled during November ($H_{5,119} = 20.379$, $P = 0.001$; Scheirer-Ray-Hare extension of the Kruskal-Wallis analysis; Fig. 1*D*). All interaction terms for sampling time were not statistically significant.

A significant effect of sex on melatonin cycles was observed during the April sampling period ($H_{1,133} = 7.182$, $P = 0.007$; three-way ANOVA on ranks; Fig. 2*E*). There were no statistically significant main effects of sex on corticosterone cycles during any of the sampling periods (Figs. 1, 2). However, we observed a significant interaction between snake population and sex in corticosterone cycles sampled in the cold acclimatization regime during November ($H_{2,123} = 6.353$, $P = 0.042$; three-way ANOVA on ranks). Additional significant population × sex interactions were observed in corticosterone cycles sampled during December ($H_{2,162} = 7.509$, $P = 0.023$; three-way ANOVA on ranks) and February ($H_{2,159} = 9.476$, $P = 0.009$; three-way ANOVA on ranks). All other interaction terms for sex were not statistically significant.

To investigate the direct effects of acclimatization regime on diel melatonin and corticosterone rhythms, we compared hormone profiles of snakes in the cold and warm regimes sampled during November. Neither acclimatization regime nor population alone significantly influenced melatonin (Table 3) or corticosterone (Table 4) cycles. However, we found highly significant interactions between snake population and acclimatization regime in both melatonin cycles ($H_{2,243} = 12.960$, $P = 0.002$; three-way ANOVA on ranks; Table 3) and corticosterone cycles ($H_{2,243} = 25.533$, $P < 0.001$; three-way ANOVA on ranks; Table 4). These results indicate that the effect of acclimatization regime on hormone cycles depends on the snake population.

Courtship Behavior

Courtship behavior of male snakes differed significantly among the three populations ($F_{2,443} = 16.294$, $P < 0.001$; mixed-model ANOVA with one repeated factor; Fig. 3). Courtship scores of male snakes changed significantly during the spring emergence period ($F_{5,443} = 6.156$, $P < 0.001$), and this change in courtship behavior depended on snake population (i.e., there was a statistically significant interaction between days postemergence and snake population: $F_{10,443} = 2.596$, $P = 0.005$; mixed-model ANOVA with one repeated factor; Fig. 3).

Within populations, courtship behavior of red-sided garter snakes (*Thamnophis sirtalis parietalis*) increased significantly during days 4–7 postemergence and then significantly declined to baseline levels (Friedman's rank-sum test statistic [χ_r^2] = 37.758, df = 5, $P < 0.0001$; nonparametric Friedman's repeated-measures ANOVA followed by a Bonferroni-corrected Wilcoxon signed-ranks test with day 1 postemergence as the control group; Fig. 3). In contrast, courtship behavior did not change significantly over the emergence period in either red-spotted garter snakes, *T. sirtalis concinnus* ($\chi_r^2 = 0.000$, df = 5, $P = 1.000$; nonparametric Friedman's repeated-measures ANOVA; Fig. 3), or eastern garter snakes, *T. sirtalis sirtalis* ($\chi_r^2 = 7.967$, df = 5, $P = 0.158$; nonparametric Friedman's repeated-measures ANOVA; Fig. 3).

Table 4: Results from a three-way ANOVA on ranks for investigating geographic variation in corticosterone rhythms among populations of common garter snakes (*Thamnophis sirtalis*) in the cold versus warm acclimatization regimes sampled during November

Source of Variation	Power	df	SS	MS	*H*	*P*
Population	.07	2	867.556	433.778	.174	.917
Acclimatization regime	.46	1	13,857.435	13,857.435	2.785	.095
Sampling time	.97	5	89,151.454	17,830.291	17.920	.003
Population × acclimatization regime	1.00	2	117,079.864	58,539.932	25.533	<.001
Population × sampling time	.71	10	57,409.652	5,740.965	11.540	.317
Acclimatization regime × sampling time	.75	5	47,131.175	9,426.235	9.474	.092
Population × sampling time × acclimatization regime	.62	10	48,237.079	4,823.708	9.696	.468
Residual		208	835,200.104	4,015.385		
Total		243	1,208,934.319	4,975.038		

Note. Post hoc power analyses were computed using $\alpha = 0.05$.

Discussion

Our results demonstrate significant geographic variation among snake populations in both diel melatonin and corticosterone rhythms and patterns of courtship behavior. These data support the hypothesis that timekeeping systems have evolved in the presence of unique environmental conditions and are not plastic among geographic regions. However, traditional common garden rearing experiments are needed to determine whether this observed geographic variation in timekeeping systems results from inherent genetic differences among these populations or different environmental conditions during key developmental stages. Such studies would aid in understanding the evolution of circadian and circannual timekeeping mechanisms.

Melatonin and Corticosterone Cycles

While we know that environmental cues modify melatonin (and perhaps corticosterone) cycles, we were interested in examining whether the response of the pineal gland and HPA axis to environmental cues varies among geographic regions. We found that melatonin and corticosterone cycles differed significantly among the three snake populations in a majority of the sampling periods during this experiment. These population differences were observed across a wide range of acclimatization conditions, suggesting that the underlying basis for this observed geographic variation is complex. Indeed, we observed significant interactions between snake population and the other between-subjects factors (i.e., sex), indicating that the effects of these factors depended on the snake population being examined.

The population differences we observed in melatonin and corticosterone rhythms were themselves plastic. Under some acclimatization conditions, the northern-latitude red-sided garter snake (*Thamnophis sirtalis parietalis*) responded differently than the two more southern populations (Fig. 1*B*, 1*D*). In other instances, either the midlatitude red-spotted garter snake (*T. sirtalis concinnus*; Fig. 1*C*) or the southern-latitude eastern garter snake (*T. sirtalis sirtalis*; Fig. 2*D*, 2*E*) had significantly different responses to the acclimatization conditions. No obvious relationship is apparent between these population differences and the environmental conditions. For example, we hypothesized that northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) would respond differently when acclimatized to warmer temperatures during their normal winter dormancy period (i.e., 25° : 20°C in November; Lutterschmidt et al. 2006). Rather, midlatitude red-spotted garter snakes (*T. sirtalis concinnus*) showed melatonin rhythms that were significantly different from those of the other two populations in response to this acclimatization regime (Fig. 1*C*). Similarly, it was the southern-latitude eastern garter snake (*T. sirtalis sirtalis*) that had significantly higher corticosterone concentrations in response to the mild winter dormancy thermoperiod of 10° : 10°C in February (Fig. 2*D*). Further study is necessary to understand why the observed geographic differences in melatonin and corticosterone cycles vary across the different acclimatization regimes.

When comparing the cold and warm acclimatization regimes sampled during November, we found that neither acclimatization regime nor population alone significantly affected hormone cycles. However, we found highly significant interactions between snake population and acclimatization regime in both melatonin (Table 3) and corticosterone (Table 4) cycles. These results indicate that the effect of acclimatization regime on hormone cycles varies among the three snake populations, further supporting geographic variation and perhaps evolutionary differences in timekeeping systems.

Surprisingly, statistically significant melatonin cycles were not observed during any of the sampling months in this experiment. Although necessary for these experiments in the relatively small-bodied garter snake, our methodology of collecting hormone samples from a subset of snakes at each sampling time may have obscured hormone rhythmicity within individuals. Further studies using repeated sampling techniques within individuals are necessary to accurately determine how environmental conditions influence hormone cycles, especially under free-running conditions. These logistical issues combined

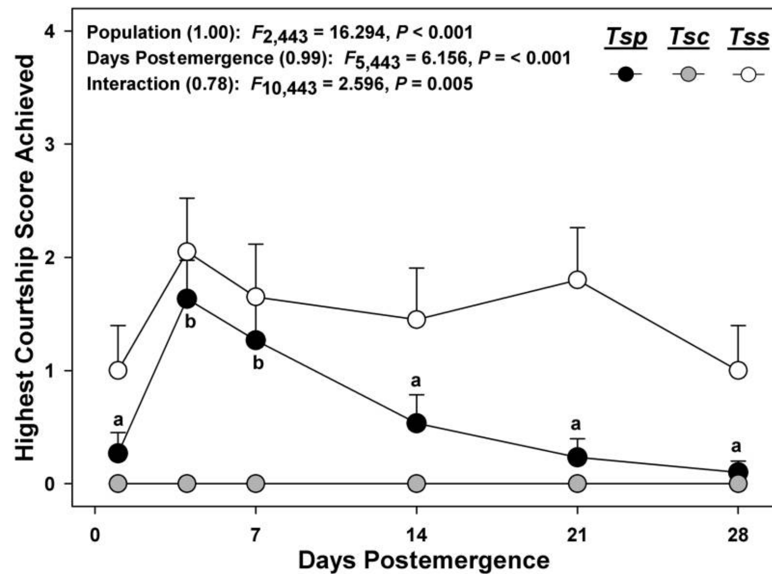

Figure 3. Courtship scores of male garter snakes following winter dormancy in the laboratory. Reproductive behavior of red-sided garter snakes (*Thamnophis sirtalis parietalis*; *Tsp*; $n = 30$) from Manitoba, Canada; red-spotted garter snakes (*T. sirtalis concinnus*; *Tsc*; $n = 24$) from western Oregon; and eastern garter snakes (*T. sirtalis sirtalis*; *Tss*; $n = 20$) from southern Florida was scored using an ethogram of male courtship behavior. Each data point is the mean highest courtship score achieved by male snakes + SE. Main effects of snake population and days postemergence on courtship behavior are shown in the top left corner (statistical values from a mixed-model ANOVA). Post hoc statistical power (i.e., the probability of not making a Type II error) is shown in parentheses behind each factor. Within red-sided garter snakes (*T. sirtalis parietalis*), statistically significant differences in courtship behavior over days postemergence are indicated by lowercase letters (from a nonparametric Friedman's repeated-measures ANOVA followed by a Bonferroni-corrected Wilcoxon signed-rank test with day 1 postemergence as the control group).

with the large variation in hormone concentrations and the observed sex differences indicate that larger sample sizes are necessary to observe significant rhythmicity in hormone production. Indeed, the power associated with most of our statistical tests for diel sampling time is quite low (Figs. 1, 2). Thus, we have a high probability of accepting the null hypothesis when it is actually false (i.e., of making a Type II error). Although inadequate sample sizes and large variation in hormone concentrations obscure significant rhythmicity in hormone cycles, these data do demonstrate significant population differences in 24-h melatonin and corticosterone cycles.

Because moderately elevated levels of glucocorticoids play an important role in facilitating seasonal reproductive activity (reviewed in Moore and Jessop 2003), the significant interactions observed between snake population and sex in corticosterone rhythms are not surprising. Indeed, these significant interactions corroborate known differences in the timing of seasonal reproductive events both among and within populations (e.g., spring emergence of red-spotted garter snakes in February–March vs. spring emergence of red-sided garter snakes in April–May; spring courtship behavior in male snakes vs. summer gestation in female snakes). We also observed a significant effect of sex on melatonin cycles during the spring emergence period (Fig. 2*E*). Male snakes exhibited diel changes in melatonin secretion, while female snakes did not. In fact, the observed population differences in melatonin cycles during the April sampling period appeared to result from differences in melatonin cycles among male snakes; melatonin cycles of female snakes did not vary among populations. Future studies are necessary to determine whether these sex differences in melatonin cycles are related to the expression of male courtship behavior during spring mating (e.g., Mendonça et al. 1996*a*). Further research with these populations would also help elucidate whether the observed geographic variation in melatonin and corticosterone cycles is the foundation for differences in the natural history of these populations, particularly with respect to the timing and duration of spring reproductive behavior.

Courtship Behavior

The observed population differences in hormone cycles also extended to the expression of courtship behavior in male snakes following winter dormancy (Fig. 3). However, there was a significant interaction between population and time postemergence, making it difficult to interpret the observed geographic variation in patterns of courtship behavior. Thus, we discuss here how courtship behavior changes over time within levels of the factor population. For example, male red-sided garter snakes (*T. sirtalis parietalis*) showed a significant increase in courtship behavior during the first few days following exposure to springlike environmental conditions. This was followed by a rapid decline in courtship behavior to baseline levels by 14

d postemergence (Fig. 3). These data reflect the natural history of red-sided garter snakes during the abbreviated spring mating season in Manitoba, Canada (e.g., Crews 1984; Crews et al. 1984). Similarly, the expression of courtship behavior in male eastern garter snakes (*T. sirtalis sirtalis*) reflects the extended mating season exhibited by this southern-latitude population (e.g., Tennant 1997). Male eastern garter snakes exhibited reproductive behavior on emergence as well as over the entire 4 wk that we measured courtship behavior (Fig. 3).

In contrast, red-spotted garter snakes (*T. sirtalis concinnus*) exhibited no courtship behavior over the 4-wk observation period. This lack of reproductive behavior may reflect the extremely low androgen concentrations of male red-spotted garter snakes observed during the spring emergence period in this study (data not shown; mean = 0.1 ng/mL [SE = 0.02, n = 8] vs. 4.1 ng/mL [SE = 1.92, n = 8] for eastern garter snakes and 0.8 ng/mL [SE = 0.32, n = 8] for red-sided garter snakes). The androgen concentrations observed in red-spotted garter snakes (*T. sirtalis concinnus*) are lower than those expected for an associated reproductive pattern. For example, the elevated androgen concentrations observed during the period of reproductive behavior in male eastern garter snakes (*T. sirtalis sirtalis*) support the hypothesis that eastern garter snakes exhibit associated reproduction (Fig. 3). In contrast, the androgen concentrations measured in red-spotted garter snakes are much lower than those of eastern garter snakes in this study as well as those reported for red-spotted garter snakes sampled in the field during the spring mating season (Moore et al. 2000*b*; Lutterschmidt and Mason 2005). Together, these results may explain the lack of courtship behavior observed in red-spotted garter snakes, although an additional possibility is that the stimulus females were simply not sexually attractive. Although more secretive than red-sided garter snakes (*T. sirtalis parietalis*), the courtship behavior of red-spotted garter snakes (*T. sirtalis concinnus*) is identical to that described in Table 2 (D. I. Lutterschmidt, personal observation). Thus, we are confident that our ethogram of male courtship behavior is appropriate for all three snake populations studied in these experiments.

In red-sided (*T. sirtalis parietalis*) and eastern (*T. sirtalis sirtalis*) garter snakes, the highest proportions of male snakes exhibiting mating behavior (i.e., having courtship scores ≥3; Table 2) were 46.7 and 50.0, respectively. These proportions are lower than those observed in the field and explain the relatively lower mean courtship scores observed in this study (0.5–2.0; Fig. 3) compared with mean courtship scores measured in the field (e.g., mean highest courtship score of 3.6 [SE = 0.12, n = 32] for red-sided garter snakes using similar behavioral methods; Lutterschmidt et al. 2004). The lower proportion of males exhibiting courtship behavior may be an artifact of housing snakes at mild, intermediate temperatures during winter dormancy. This may be especially true for red-sided garter snakes, because exposure to low temperatures (i.e., 4°C) is required to elicit robust courtship behavior on spring emergence (Camazine et al. 1980; Bona-Gallo and Licht 1983). Nevertheless, our observations of population differences in courtship behavior under a common garden regime demonstrate geographic variation in the endogenous circannual rhythms regulating seasonal reproduction.

Conclusions and Significance

Our results suggest that endogenous timekeeping systems have evolved in the presence of unique suites of environmental conditions. Additional studies are necessary to determine whether the observed geographic variations in hormone rhythms and courtship behavior result from inherent genetic differences among these populations or whether they are a product of different environmental conditions during key developmental stages. If timekeeping systems are indeed evolutionarily constrained, then rapid changes in climate predictability, such as those associated with global climate change (e.g., IPCC 2001), could have a disproportionately large influence on reproductive fitness. For example, disrupted breeding cycles and consistently earlier breeding are occurring in birds and amphibians and have been associated with global climate change (e.g., Forchhammer et al. 1998; Brown et al. 1999; Dunn and Winkler 1999; Moss et al. 2001). Further, Root et al. (2003) demonstrated that in more than 80% of the species showing temperature-related shifts, the changes are consistent with and predicted by the species' physiological constraints.

While our results suggest that animal populations will respond uniquely to changing environmental cues (and environmental perturbations), additional studies are necessary to determine the degree of plasticity in timekeeping systems within populations. For example, high phenotypic plasticity in one or more traits may be adaptive in highly variable environments, which may allow some populations to adjust more readily, and perhaps favorably, to environmental perturbations (e.g., Visser 2008). Comparative phylogenetic analyses of the biochemistry underlying the transduction of environmental cues would also provide insight into the evolution of circadian and circannual rhythmicity. The geographic variation in melatonin rhythms reported here, for instance, might be due to different isoforms of the enzymes regulating melatonin synthesis (e.g., *N*-acetyltransferase, hydroxyindole-*O*-methyltransferase). Such data regarding plasticity in timekeeping systems both within and among animal populations are necessary for understanding the potential effects of environmental perturbations on seasonal rhythms in physiology and behavior.

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