AN ABSTRACT OF THE DISSERTATION OF

Deborah I. Lutterschmidt for the degree of Doctor of Philosophy in Zoology presented on April 20, 2006.

Title: Chronobiology of Garter Snakes: Environmental and Hormonal Mechanisms Mediating Hibernation and Reproduction.

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Most vertebrates exhibit seasonality in many life history traits. Such seasonal rhythms are temporally organized via the transduction of environmental cues (e.g., photoperiod, temperature) into appropriate endocrine signals. However, among ectothermic vertebrates that undergo continuous winter dormancy, temperature is the only environmental cue available for synchronizing seasonal rhythms. Most intriguing is that in species where reproduction occurs immediately following spring emergence, the associated changes in neurophysiology and behavior that accompany reproduction likely occur during winter dormancy. The purpose of this dissertation research was to explore the mechanisms by which temperature cues affect the chronobiology and seasonal reproduction of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Because of their roles in circadian organization and energy balance, melatonin and corticosterone are likely hormonal components of these time-keeping systems. I first characterized the interactions between melatonin and corticosterone to better understand the hormonal

mechanisms facilitating temperature-induced reproduction. Melatonin and corticosterone additively inhibit reproductive behavior during the spring mating season. Experimental manipulations with a serotonin receptor antagonist suggest the mechanism underlying these effects involves a serotonin-regulated system. Although melatonin does not influence corticosterone responses to capture stress, capture stress significantly increases melatonin concentrations. To investigate the functional significance of these interactions in regulating temperature-induced reproduction, I measured body temperatures of snakes as well as circadian melatonin and corticosterone cycles during winter dormancy and spring emergence using a combination of field and laboratory experiments. Surprisingly, an increase in body temperature is not necessary for emergence from winter dormancy. Rather, critically low temperatures may serve as a *zeitgeber* entraining an endogenous circannual cycle that regulates emergence. Decreased environmental temperatures, in the absence of changing photoperiod cues, modulate circadian melatonin and corticosterone rhythms during hibernation. Such temperature-induced changes in hormone rhythms may facilitate seasonal reproductive behavior following spring emergence. Furthermore, a phase-shift in corticosterone rhythms during the mating season may regulate the seasonal transition between reproductive and non-reproductive states in red-sided garter snakes. Such studies investigating the environmental and hormonal mechanisms underlying time-keeping systems may provide valuable insight into the potential impact of environmental perturbations (e.g., climate change) on seasonal rhythms in physiology and behavior.

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Chronobiology of Garter Snakes:

Environmental and Hormonal Mechanisms Mediating Hibernation and Reproduction

by

Deborah I. Lutterschmidt

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APPROVED:

Major Professor, representing Zoology

Chair of the Department of Zoology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Deborah I. Lutterschmidt, Author

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Contribution of Authors

Michael P. LeMaster assisted in the data collection of chapter three and contributed to the experimental design, data collection, and interpretation of chapter five. Robert T. Mason has served as my graduate advisor in the Department of Zoology at Oregon State University.

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DEDICATION

This dissertation is dedicated to the following people in honor of the roles they have played in my life:

In memory of Isacc Skaff and Arthur T. Sevem,

for believing in me,

and

William I. Lutterschmidt, who quite literally changed the course of my life when he introduced me to the wonders of research.

CHRONOBIOLOGY OF GARTER SNAKES: ENVIRONMENTAL AND HORMONAL MECHANISMS MEDIATING HIBERNATION AND REPRODUCTION

CHAPTER 1 – INTRODUCTION

Almost all vertebrates exhibit seasonality in many behavioral and physiological processes, including activity, feeding, immune function, molt of skin, fur, and plumage, migration, and reproduction. One of the most reliable environmental cues thought to function in "time-keeping" in vertebrates is photoperiod. Unlike many environmental signals (e.g., temperature and humidity) that can vary quite dramatically both within seasons and among years, changes in photoperiod length accurately and reliably reflect changing environmental seasons. Changes in environmental cues are in turn transduced via hormonal signals whose amplitude, duration, and phase reflect changing photoperiod and temperature regimes.

To optimize survival and reproduction, vertebrates must track changing environmental seasons so that physiological and behavioral processes may be synchronized with optimal environmental conditions. Thus, it is important to understand how environmental cues are transduced into hormonal signals and how these hormonal signals in turn function to integrate an animal's physiology and behavior with its environment. The pineal gland and its major secretory product, melatonin, are thought to be the primary neuroendocrine transducers of environmental stimuli in vertebrates.

Anatomy of the Pineal Complex

The pineal complex of vertebrates is composed of the pineal gland or epiphysis and, in some taxonomic groups, a parapineal organ. Although a well-developed parapineal organ has been retained only in clyclostomes, tuatara, and lizards, almost all vertebrate groups possess a pineal gland. Interesting exceptions to this rule include the reported absence of a pineal gland in crocodilians and the nine-banded armadillo (*Dasypus novemcinctus*) (Harlow *et al*. 1981; Norris 1997; Tosini 1997).

Three different types of pinealocytes are found throughout the evolution of the vertebrate pineal complex: photoreceptor cells, modified or rudimentary photoreceptor cells, and true pinealocytes (Filadelfi and Castrucci 1996; Norman and Litwack 1997). The most highly differentiated sensory components of the pineal complex are found in the pineal vesicle of some cyclostomes and teleosts, the frontal organ or stirnorgan in some amphibians, and the parietal eye of tuatara and lizards (Norman and Litwack 1997; Tosini 1997). The parapineal organs of these vertebrates contain photoreceptors morphologically similar to those found in the vertebrate retina. External processes from these photoreceptors extend into the lumen of the pineal gland and are thought to be responsible for the transduction of photic stimuli (Filadelfi and Castrucci 1996; Korf and Oksche 1986; Maksimovich 2002). However, evidence suggests the role of the parapineal organ in modulating pineal gland function may be insignificant compared to that of the lateral eyes (Bethea and Walker 1978; Tosini 1997; Tosini and Avery 1994, 1996).

In other reptiles (turtles and snakes) and in birds, the parapineal organ has been lost and the pineal gland consists primarily of modified photoreceptor cells with properties intermediate between sensory receptors and secretory cells. The pineal gland of mammals lacks direct photosensitivity and consists of true secretory pinealocytes (Norman and Litwack 1997). The reduction in pineal photosensitivity seen in some reptiles, birds, and mammals is concurrent with the development of more pronounced sympathetic noradrenergic innervation of the pineal gland. This sympathetic innervation is most pronounced in the mammalian pineal gland, such that light cues detected by the retinas are relayed to the suprachiasmatic nucleus (SCN) via the retinohypothalamic pathway. The SCN in turn alters the activity of postganglionic sympathetic nerve fibers innervating the pinealocytes of the pineal gland (Norman and Litwack 1997; Norris 1997).

Biochemistry and Modulation of Melatonin Secretion

The major secretory product of the pineal gland and parapineal organ is *N*-acetyl-5-methoxytryptamine, or melatonin. Although detectable levels of melatonin are present in the plasma during daylight, large increases in melatonin are observed during darkness (e.g., García-Allegue *et al.* 2001; Moyer *et al.* 1995; Underwood 1985*a*; Vivien-Roels *et al.* 1988). Nocturnal increases in plasma melatonin concentrations result primarily from increased synthesis and secretion of melatonin from the pineal gland. Pinealectomy abolishes circadian rhythms of plasma melatonin in desert iguanas (*Dipsosaurus dorsalis*) and ruin lizards (*Podarcis sicula*) (Foà *et al*. 1992; Janik and Menaker 1990). However,

extrapineal melatonin synthesis occurs at several sites in the body, including the harderian gland (or Harder's lacrimal gland), retina, and intestine (Gern and Karn 1983; Gern and Ralph 1979; Hadley 1996; Norris 1997; Ralph 1980). Thus, detectable levels of circulating melatonin are often still present even after pinealectomy. For example, pinealectomy of neotenic tiger salamanders (*Ambystoma trigrinum*) provoked little change in plasma melatonin levels during photophase and reduced melatonin levels during scotophase by only 55% (Gern and Norris 1979). Similarly, photophasic and scotophasic plasma melatonin levels did not differ significantly between pinealectomized and sham-operated red-sided garter snakes (*Thamnophis sirtalis parietalis*) (Mendonça *et al*. 1996*a*). Thus, it must be noted that extrapineal melatonin synthesis may significantly contribute to baseline levels of plasma melatonin in some species, with the pineal gland contributing to this baseline level in an additive manner during scotophase (Gern and Norris 1979).

In most nonmammalian vertebrates, the circadian melatonin cycle is an endogenously-generated rhythm controlled by circadian oscillators or "pineal clocks" located within the pineal gland (Binkley *et al*. 1978; Bolliet *et al*. 1996; Cahill 1996; Cassone and Natesan 1997; Deguchi 1979; Falcon *et al*. 1989; Gern and Greenhouse 1988; Iigo *et al*. 1991; Menaker and Wisner 1983; Okimoto and Stetson 1997, 1999a, 1999b; Tosini and Menaker 1998; Zachmann *et al*. 1992). Mammalian pinealocytes have not retained the endogenous oscillatory capacity observed in other vertebrates. Rather, melatonin production is controlled remotely by circadian oscillators located within the hypothalamic SCN (Cassone 1990; reviewed in Cassone and Natesan 1997). Although

melatonin secretion may occur rhythmically even in the absence of photic cues, the melatonin cycles of all vertebrates are entrained by daily photoperiodic conditions. This photoperiodic entrainment results in a circadian cycle of melatonin secretion that has been observed across many taxa (e.g., García-Allegue *et al.* 2001; Moyer *et al.* 1995; Underwood 1985*a*; Vivien-Roels and Pévet 1993; Vivien-Roels *et al.* 1988;).

The initial substrate for indoleamine synthesis in the pinealocyte is the amino acid tryptophan, which is first converted into 5-hydroxytryptamine (i.e., serotonin) before its final conversion to melatonin (Figure 1.1). Melatonin is then secreted either directly into the blood or into the cerebrospinal fluid before entering the bloodstream (Norman and Litwack 1997). The enzymes involved in the synthesis of melatonin are also regulated by photic cues. For example, circadian regulation of enzymatic activity and/or transcription occurs for tryptophan hydroxylase, *N*-acetyltransferase, and hydroxyindole-*O*-methyltransferase (Cassone and Natesan 1997; Falcón *et al.* 1987, 1989; Morton and Forbes 1988). The biochemistry of extrapineal melatonin synthesis is similar to that of pineal melatonin synthesis (Figure 1.1). In addition, diel rhythms in ocular *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase activity have been observed (reviewed by Gern and Karn 1983).

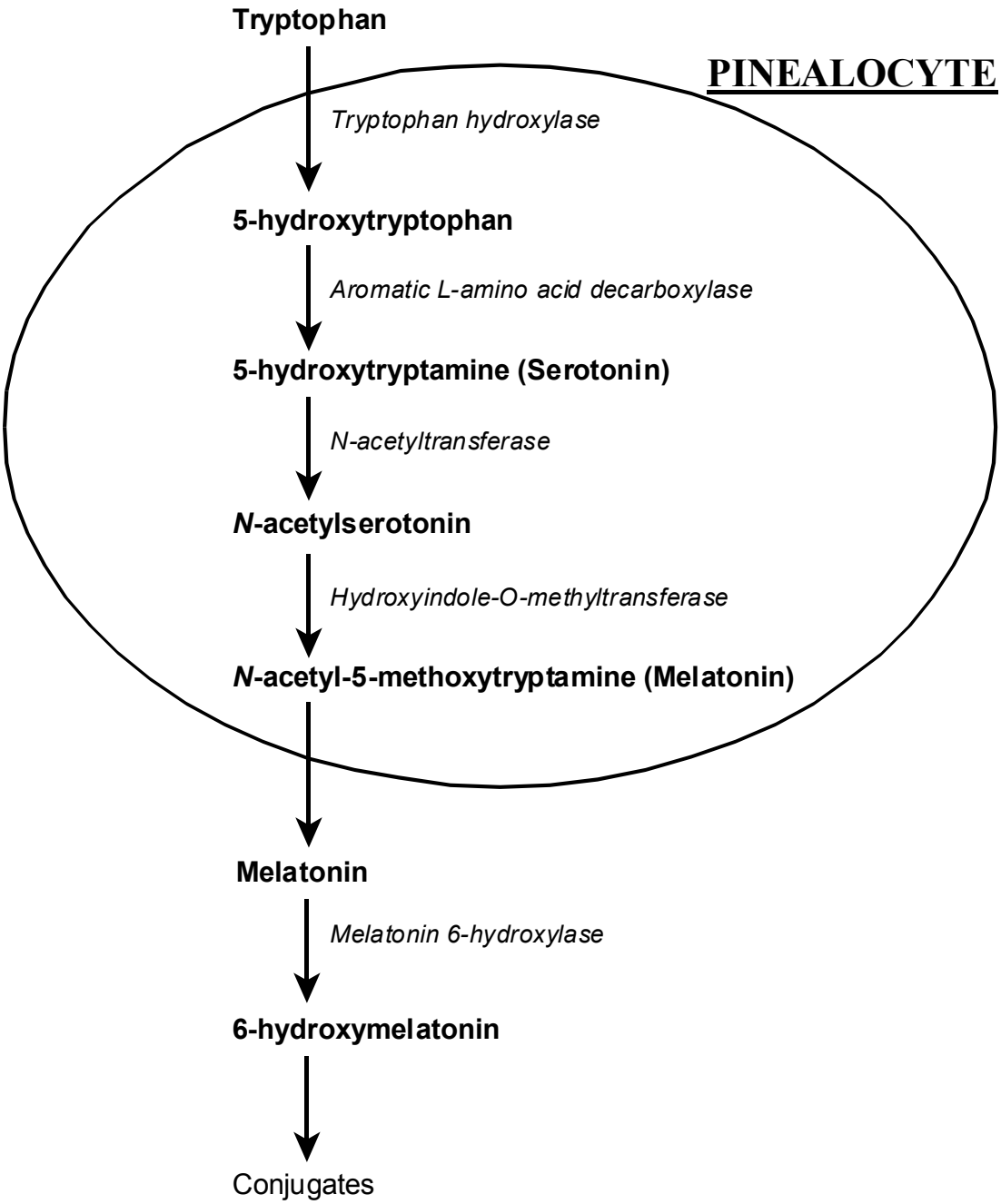

Figure 1.1. Simplified biosynthetic pathway for the production of melatonin by the pinealocyte. Enzymes involved in the metabolism of melatonin are shown to the right of the arrows in italics. The area within the pinealocyte includes reactions for the anabolism of melatonin while the enzyme melatonin 6-hydroxylase is involved in catabolism of melatonin by the liver. Cofactors are not shown; "conjugates" include at least three metabolites of 6-hydroxymelatonin identified from mammalian liver microsome fractions (modified from Lutterschmidt *et al*. 2003).

The cycle of melatonin synthesis and secretion can itself be modulated by external factors, including magnetic fields, photoperiod, light intensity, and temperature (Birks and Ewing 1986; Falcón *et al.* 1994; Firth and Kennaway 1987, 1989; Firth *et al.* 1989; García-Allegue *et al.* 2001; Gern and Norris 1979; Hyde and Underwood 1993; Lerchl *et al.* 1998; Rawding and Hutchison 1992; Reiter 1992, 1993; Tilden and Hutchison 1993; Underwood and Calaban 1987; Underwood and Hyde 1989; Wilson *et al.* 1986). Photoperiod and temperature interact to influence plasma concentrations of melatonin. This relationship has 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*), the green anole (*Anolis carolinensis*), and the diamondback water snake (*Nerodia rhombifer*) (García-Allegue *et al.* 2001; Moyer *et al.* 1995; Rawding and Hutchison 1992; Tilden and Hutchison 1993; Underwood 1985*a*; Vivien-Roels *et al.* 1988). While photoperiod influences the phase of the melatonin cycle, environmental temperature appears to modulate its amplitude (García-Allegue *et al.* 2001; Tilden and Hutchison 1993). In diamondback water snakes (*N. rhombifer*) very cold and very warm temperatures decrease the amplitude of the melatonin cycle (Tilden and Hutchison 1993). Furthermore, changes in the duration of the melatonin signal reflect annual changes in the photophase: scotophase ratio and are implicated in the timing of seasonal reproductive cycles in some species (Bittman *et al*. 1983; Carter and Goldman 1983). Thus, interactions among the amplitude, duration, and phase of the melatonin cycle determine the effects of this hormone on an animal's physiology and behavior. Melatonin's ability to transduce environmental information

into appropriate endocrine signals (Axelrod 1974) plays an important role in synchronizing an animal's physiology and behavior with the environmental conditions of its unique habitat.

Functions of the Pineal Gland and Melatonin

Because of its circadian and circannual cycle of synthesis and secretion, melatonin is often implicated in modulating and synchronizing many physiological and behavioral rhythms (e.g., Kavaliers 1981; Kavaliers *et al*. 1980; Pévet *et al*. 2002; Prasad *et al*. 2001; Ralph 1978; Ralph *et al*. 1979a, 1979b; Tosini 1997; Tosini *et al*. 2001; Turek *et al*. 1976; Underwood 1989; Underwood and Harless 1985). For example, melatonin regulates circadian activity rhythms, melanophore aggregation, thyroid function and activity, immune function, thermoregulation, aggression, and free radical scavenging (Cagnoli *et al.* 1995; Dubbels *et al.* 1995; Hadley 1996; Hyde and Underwood 2000; Jasnow *et al*. 2002; Krotewicz and Lewinski 1994; Krotewicz *et al.* 1992; Lutterschmidt *et al*. 1997, 1998; Maestroni *et al*. 1989; Persengiev *et al*. 1991*a*; Reiter 1996; Reiter *et al.* 1995; Wright *et al.* 1991, 1996). Melatonin can also modulate an animal's sensitivity to temperature extremes by reducing thermal tolerance (Erskine and Hutchison 1982). Of the many actions attributed to the pineal gland and melatonin, their role in modulating physiological stress responses and reproductive function are of particular importance here; these specific actions of the pineal gland and melatonin are discussed below.

Physiological and Behavioral Stress Responses

Interactions between melatonin and other hormones are important in synchronizing and modulating physiological and behavioral parameters necessary for daily activity. For example, melatonin is a potent inhibitor of the hypothalamus-pituitary-adrenal (HPA) axis (Reiter 1991; Wang *et al*. 1999), which mediates an animal's physiological and behavioral responses to stressors. A stressor refers to any perturbation that disrupts the predictability of an animal's internal or external environment (Hiebert *et al*. 2000). Responses to such stressors are mediated by an increase in the secretion of adrenal glucocorticoid "stress hormones" (Wingfield and Ramenofsky 1999). Unpredicted challenges to energy homeostasis, such as unusually low environmental temperatures, predation attempts, food shortage, and starvation, stimulate the HPA axis and result in increased plasma glucocorticoids (Harvey *et al*. 1984; Schwabl *et al*. 1985; Wingfield 1988). Glucocorticoids in turn modulate a variety of physiological and behavioral processes that promote survival while suppressing behaviors, such as reproduction, that are not crucial to immediate survival (e.g., Pottinger 1999; Sapolsky 1992; Wingfield 1988; Wingfield and Silverin 1986). Such acute physiological stress responses are normally adaptive responses used to modify metabolism and mobilize energy stores.

A frequently studied action of elevated glucocorticoids is its inhibition of reproductive function (e.g., Carragher and Rees 1994; Moore *et al*. 1991; Rivier and Rivest 1991). Negative interactions between the HPA and the hypothalamic-pituitary-gonadal axes suggest that activation of one system is accompanied by an inactivation of

the other. Decreased plasma testosterone accompanies increased glucocorticoids in male tree lizards (*Urosaurus ornatus*) and male red-sided garter snakes (*Thamnophis sirtalis parietalis*) (Moore *et al*. 2000*a*; Moore *et al*. 1991). Thus, physiological stress responses, marked by an increase in plasma glucocorticoid concentrations, generally result in decreased production of sex steroid hormones and lead to an overall suppression of reproductive behavior in many species (Carragher and Rees 1994; Coddington and Cree 1995; Moore *et al*. 1991; Moore *et al*. 2000*a*; Rivier and Rivest 1991).

Although interactions between the pineal gland and HPA axis are well established (Barriga *et al*. 2002; Demisch *et al*. 1998; Jessop *et al*. 2002; Khan *et al*. 1990; Kirby *et al*. 1999; Maestroni *et al*. 1986, 1989; Marinova *et al*. 1991; Otsuka *et al*. 2001; Persengiev *et al*. 1991*b*; Rodríguez *et al*. 2001*a*, 2001*b*; Rogers *et al*. 1997; Vaughan *et al*. 1972, 1984), the nature of this relationship is poorly understood. For example, both stimulatory (Al-Dujaili *et al*. 1982; Haus *et al*. 1996; Persengiev *et al*. 1989; Touitou *et al*. 1989) and inhibitory (Heiman and Porter 1980; Ng 1987; Nussdorfer *et al*. 1990) effects of melatonin on the secretory activity of the adrenal cortex have been described. However, all of these studies examined the effects of melatonin on cultured adrenocortical cells (Appa-Rao *et al*. 2001). In an experiment using *in vivo* physiological conditions, melatonin exerted a direct antisecretory effect on the adrenal gland (Appa-Rao *et al*. 2001). Chronic melatonin treatment also considerably alters the affinity of glucocorticoid receptors in the brain and pituitary (Marinova *et al*. 1991). Furthermore, melatonin treatment of both adult and juvenile rats prevents many of the injurious effects induced by chronically elevated glucocorticoids, such as the reduction in growth, atrophy

of the thymus and adrenal glands, and elevation of blood glucose, free fatty acids, triglycerides, and total cholesterol (Aoyama *et al*. 1986, 1987). Daily melatonin treatment in mice prevents several chronic stress-induced disturbances, including a reduction in preference for sucrose solution and a reduction in spontaneous locomotor activity (Kopp *et al*. 1999*a*). In male rats, acute melatonin treatment attenuates the effects of glucocorticoids on sexual behavior (Gorzalka *et al*. 1999). More recently, Brotto *et al*. (2001) also demonstrated that melatonin treatment significantly reduces the inhibitory effects of acute and chronic stress on sexual behavior in male rats. These are the only accounts describing melatonin's ability to modulate the effects of glucocorticoids on reproductive function in any vertebrate. Whether the effects of melatonin on physiological stress responses result from direct antisecretory actions on the adrenal cortex, antagonism of glucocorticoid actions at the target tissues, or a combination of both mechanisms remains to be determined.

Increases in melatonin secretion following stressful events have been attributed to its protective actions against the deleterious effects of glucocorticoids. However, increases in melatonin following stressful events occur only in some species (Carr *et al*. 1981; Champney *et al*. 1985; Lynch *et al*. 1973; Persengiev *et al*. 1991*b*; Rodríguez *et al*. 2001*a*; Tannenbaum *et al*. 1987; Thientz *et al*. 1984). Furthermore, diel differences occur in the response of the pineal gland to stress (Joshi *et al*. 1986; Monteleone *et al*. 1990; Troiani *et al*. 1988). Immobilization stress significantly increases both photophasic and scotophasic glucocorticoid concentrations in ring doves (*Streptopelia risoria*) while increasing photophasic melatonin and decreasing scotophasic melatonin levels (Barriga *et*

al. 2002; Rodríguez *et al*. 2001*a*). Thus, physiological stress responses appear to abolish the cyclicity of melatonin synthesis (Persengiev and Kanchev 1991). Increased photophasic melatonin synthesis following a stress response is thought to be an adaptation for combating oxidative damage. Indeed, Sainz *et al*. (1995) found that melatonin alleviates glucocorticoid-induced apoptosis of thymocytes via an antioxidant mechanism. In contrast, the decrease in nocturnal melatonin levels following a stress response is hypothesized to be the result of a rapid decline in the concentration of tryptophan, the amino acid precursor for melatonin synthesis (Clark and Russo 1997). These observations may explain some of the disparate results found in the response of the pineal gland to stress as well as why increases in melatonin following a stressful event have only been observed in some species (i.e., some experimental protocols). However, in humans and some other mammalian species, physical stress administered during scotophase still attenuates the nocturnal peak in melatonin secretion (Champney *et al*. 1985; Joshi *et al*. 1986; Monteleone *et al*. 1990, 1992; Tannenbaum *et al*. 1987; Troiani *et al*. 1988). Thus, diel variation in the response of the pineal gland to elevated glucocorticoids is supported.

The inhibitory effect of stress on circadian melatonin secretion may result from direct effects of glucocorticoids on the pineal gland. Administration of dexamethasone, a synthetic glucocorticoid, to humans reduces plasma melatonin levels (Beck-Friis *et al*. 1983), while metyrapone, a glucocorticoid synthesis-inhibitor, increases urinary excretion of melatonin (Brismar *et al*. 1985). The hypothesis that adrenal glucocorticoids inhibit pineal melatonin secretion is supported by the finding that glucocorticoids decrease

norepinephrine-induced melatonin synthesis by cultured pineal glands (unpublished data by Fevre-Montange and Abu-Samra, cited in Monteleone *et al*. 1992).

The mechanisms responsible for the complex interactions between melatonin and glucocorticoids during and following physiological stress responses have not been elucidated. Because both the adrenal cortex and the pineal gland are innervated by sympathetic nerve fibers, the immediate increases in glucocorticoid and melatonin secretion following a stressful event may result from the increase in sympathetic nervous system activity necessary for initiation of the stress response. However, potential roles of catecholamines or other factors released during the physiological stress response cannot be dismissed (Monteleone *et al*. 1992). The initial increase in melatonin during a stress response most likely helps to protect the organism from some of the deleterious effects (e.g., oxidative damage) of acute glucocorticoid elevation. Melatonin's observed antisecretory effects on the adrenal cortex may assist in regulating glucocorticoid responses to acute stress. In contrast, inhibition of melatonin secretion by glucocorticoids likely helps in facilitating the "fight or flight" response to stressors. Because melatonin has analgesic, anxiolytic, and sedative effects (Albertson *et al*. 1981; Datta and King 1977, 1979; Golombek *et al*. 1993; Kopp *et al*. 1999*b*; Kumar *et al*. 1982; Lakin *et al*. 1981; Lowenstein *et al*. 1984), the reduction in melatonin synthesis during the latter portion of a physiological stress response may function in maintaining the organism's preparedness for adapting to unpredicted stressors. These hypotheses may account for some of the disparate results regarding the response of the pineal gland to stress and may

also explain some of the complex interactions observed among melatonin, glucocorticoids, and an organism's physiological and behavioral responses to stress.

Seasonal Reproduction

One of the best-studied functions of the pineal gland and melatonin is their role in synchronizing seasonal reproductive cycles with optimal environmental conditions. Although extensive experiments have been done in both birds and mammals, there are fewer studies investigating the relationship between the pineal complex and reproductive function in other vertebrates (reviewed in Mayer *et al*. 1997 and Turek and Van Cauter 1994). An extensively studied model organism used for investigating the photoperiodicity of reproduction in ectothermic vertebrates is the green anole lizard, *Anolis carolinensis* (Mayer *et al*. 1997). There is clear evidence for an inhibitory role of the pineal gland and melatonin in the control of reproduction in this species (Levey 1973; Mayer *et al*. 1997; Underwood 1981, 1985*b*). For example, pinealectomy of male anoles stimulates testicular growth and spermatogenesis (Underwood 1985*b*), while pinealectomy of female anoles stimulates follicular development and increases ovarian and oviduct weights (Levey 1973). Melatonin treatment abolishes the effects of pinealectomy on reproduction in female anoles (Levey 1973). In other experiments, treatment of pinealectomized and intact anoles with melatonin had either no effect or inhibited reproductive parameters depending upon the photoperiod and season tested (Underwood 1981, 1985*b*).

Antigonadotropic effects of melatonin and the pineal gland also occur in other reptiles, including the Indian garden lizard (*Calotes versicolor*) and the zebra-tailed lizard (*Callisaurus draconoides*) (Haldar and Thapliyal 1977; Haldar-Misra and Thapliyal 1981*a*, 1981*b*; Mayer *et al*. 1997; Packard and Packard 1977; Thapliyal and Misra nee Haldar 1979; Vivien-Roels and Pévet 1983). In red-sided garter snakes (*Thamnophis sirtalis parietalis*), pinealectomy inhibited male courtship behavior but did not influence plasma androgen levels (Crews *et al*. 1988; Mendonça *et al*. 1996*a*); treatment with melatonin had no influence on the effects of pinealectomy on courtship behavior (Mendonça *et al*. 1996*a*). In contrast, melatonin treatment (subcutaneous implants) decreased the gonadosomatic index (gonad mass expressed as a percentage of body mass) and testosterone concentrations in male Hermann's tortoises (*Testudo hermanni*) (Vivien-Roels and Arendt 1983; Vivien-Roels and Pévet 1983).

Although available data are inconclusive, several studies suggest the pineal gland and melatonin also play a role in regulating reproductive physiology and behavior in amphibians and several teleosts, including killifish (*Fundulus similes*), goldfish (*Carrassius auratus*), cyprinid golden shiners (*Notemigonus chrysoleucas*), and walking catfish (*Clarias batrachus*) (Fenwick 1970; Mayer *et al*. 1997; Nayak and Singh 1988; de Vlaming 1975; de Vlaming and Vodicnik 1977, 1978; de Vlaming *et al*. 1974). For example, in female Palearctic water frogs (*Rana perezi*), pinealectomy increased concentrations of estradiol and ovarian lipids but did not influence ovary or oviduct masses (Alonso-Gómez *et al*. 1990.). Melatonin treatment decreased gonadosomatic

indices and inhibited reproduction in both male and female green tree frogs (*Hyla cinerea*) (de Vlaming *et al*. 1974).

Melatonin's effects on reproduction are thought to be mediated via the hypothalamus and pituitary gland (e.g., Norris 1997). Indeed, melatonin receptors have been localized in the hypothalamus, including the SCN in birds and mammals, and in the pituitary pars tuberalis, although the specific receptor distribution varies among taxonomic groups and, in some cases, species (Cassone and Brooks 1991; Davies *et al*. 1994; Ekström and Vanecek 1992; Martinoli *et al*. 1991; Mayer *et al*. 1997; Reppert *et al*. 1995; Rivkees *et al*. 1989). Several studies have also demonstrated a direct effect of melatonin at the gonadal level. For example, melatonin treatment inhibits *in vitro* ovulation of oocytes in Northern leopard frogs (*Rana pipiens*) and toads (*Bufo arenarum*) (de Atenor *et al*. 1994; O'Connor 1969). In ducks, incubation of homogenated testes with melatonin reduced the production of androgens (Cardinali and Rosner 1971). In contrast, melatonin had no effect on testicular androgen production in edible frogs (*Rana esculenta*) or water frogs (*R. perezi*) (Gancedo *et al*. 1991; Pierantoni *et al*. 1986). Melatonin also did not affect *in vitro* oocyte maturation in either *R. perezi* or painted frogs (*Discoglossus pictus*) (Gancedo *et al*. 1991). However, binding sites specific for melatonin have been found in both bird gonads and in oocytes of clawed frogs (*Xenopus* sp.) (Ayre *et al*. 1992, 1994; Fischer *et al*. 1996). Thus, a direct role of melatonin in regulating gonadal physiology is suggested.

Melatonin's antigonadal effects, in combination with its seasonal rhythm of secretion, are thought to modulate and synchronize reproductive behavior (Crews *et al.*

1988; Mendonça *et al.* 1995; Rismiller and Heldmaier 1987; Underwood 1981, 1985*b*). However, the effects of pinealectomy and/or melatonin treatment on reproductive parameters vary among species, experimental protocols, and seasons, making it difficult to draw conclusions about the role of the pineal gland in reproduction (Chanda and Biswas 1982, 1992; Garg 1988; Haldar and Pandey 1988; Haldar and Thapliyal 1977; Hontela and Peter 1980; Nayak and Singh 1988; Thapliyal and Misra nee Haldar 1979; Underwood 1981, 1985*b*; de Vlaming 1975; de Vlaming and Vodicnik 1977; Vodicnik *et al*. 1978). For example, the effects of pinealectomy on testicular size in the Indian chequered water snake (*Natrix piscator*) are influenced by humidity; pinealectomy decreased testicular size in high humidity (which normally stimulates testicular growth) but increased testicular size in both low and moderate humidity (Haldar and Pandey 1989*a*, 1989*b*). In addition, treatment of Indian chequered water snakes with either melatonin or 5-methoxytryptamine suppressed testicular function of active testes, but did not influence inactive testes (Haldar and Pandey 1988). The effects of pinealectomy in black-spined toads (*Bufo melanostictus*) also vary among this species' seasonal activity patterns. For example, pinealectomy of toads during the hibernation phase stimulated testicular maturation, while pinealectomy during the breeding season did not influence testicular function (Chanda and Biswas 1984).

Similar differences in the effects of pinealectomy and melatonin treatment have been observed in other physiological and behavioral parameters. For example, thermoregulatory responses of collared lizards (*Crotophytus collaris*) to melatonin treatment varied depending on the phase of the photoperiod. Injection of melatonin (1 μg

g^{-1} M_b) for 2 consecutive days increased mean preferred body temperature (T_b) of lizards during the photophase, but decreased mean preferred T_b of *C. collaris* during scotophase (Cothran and Hutchison 1979). Similarly, Tosini and Menaker (1996) demonstrated that pinealectomized green iguanas (*Iguana iguana*) selected significantly lower median photophasic T_b and significantly higher mean scotophasic T_b.

Such results indicate that the pineal gland and its secretory product function primarily in synchronizing physiological and behavioral parameters with changing environmental stimuli, rather than strictly inhibiting any one particular parameter. Pinealectomy therefore merely abolishes the controlling influences of environmental stimuli, which in turn desynchronizes an animal's physiology and behavior with environmental cues. Melatonin treatment would be expected to restore pineal function only if melatonin administration closely mimics the natural melatonin cycle normally determined by interacting environmental factors. Thus, pinealectomy and melatonin treatment exhibit both positive and negative influences on reproduction depending on when experiments are conducted in relation to the species' unique natural history traits.

Hypotheses for Variation in Pineal Complex and Melatonin Function

Despite much research, the role of the pineal gland and melatonin in modulating the behavior and physiology of ectotherms remains unclear. Furthermore, the available data do not clearly elucidate melatonin's regulatory actions in ectotherms, primarily because responses to melatonin are very disparate among different species and different experimental protocols. Several hypotheses have been postulated in an attempt to explain

and account for reported anatomical and physiological differences in melatonin and pineal complex function among vertebrates. For example, Ralph (1975) suggested that nocturnality may be correlated with pineal gland size and/or function. Some nocturnal vertebrates may not possess a well-developed pineal gland or may not synthesize concentrations of pineal melatonin comparable to those of diurnal vertebrates having fully functional pineal glands. For example, under natural environmental conditions during autumn, nocturnal tuatara (*Sphenodon punctatus*) had a much lower amplitude in the melatonin cycle than diurnal shingle-back skinks (*Tiliqua rugosa*) (Firth *et al*. 1989). However, laboratory experiments demonstrated that both species were capable of attaining comparable amplitudes in melatonin synthesis (Firth *et al*. 1989).

Concentrations of melatonin comparable to those observed in other vertebrates also occur in the nocturnal mudpuppy (*N. maculosus*) and the nocturnal diamond-back water snake (*N. rhombifer*) (Rawding and Hutchison 1992; Tilden and Hutchison 1993). In addition, pronounced cycles of melatonin synthesis, with highest concentrations occurring during scotophase, occur in these nocturnal species. Furthermore, although it has been reported that owls do not have a pineal gland, nocturnal Indian spotted owlets (*Athene brama*) possess a large pineal gland with defined secretory activity (Haldar and Guchhait 2000).

Alternatively, it has been hypothesized that the presence and/or size of the pineal complex is correlated with latitudinal distribution. The pineal complex may play a more important role in thermoregulatory and reproductive adaptations at higher latitudes, where seasons tend to vary more dramatically (Ralph 1975). Gundy *et al*. (1975) showed

that families of lizards in which most genera lack parietal eyes have geographic ranges restricted to low latitudes. Likewise, families of lizards in which most genera possess parietal eyes have ranges that extend to higher latitudes (Gundy *et al*. 1975). Similar geographic trends were observed for a few species that have either greatly enlarged or reportedly absent pineal glands (Ralph 1975). However, some taxonomic groups do not conform to this hypothesis (Ralph 1975). For example, the presence of the anuran frontal organ provides little insight into an animal's geographic distribution. Thus, data to support this hypothesis are lacking.

Although little evidence is available to support a correlation between either nocturnality or latitudinal distribution and pineal gland anatomy, it is likely that pineal gland and melatonin physiology vary among species. For example, melatonin treatments that significantly decreased mean preferred T_b in the diurnal bullsnake (*Pituophis melanoleucus*) failed to modulate thermoregulatory behavior in the nocturnal African house snake (*Lamprophis fuliginosus*). However, chronic (i.e., repeated injections) melatonin treatment affected the phase of snakes' T_b rhythm, most likely by "re-entraining" the circadian clock (Lutterschmidt *et al.* 2002). Thus, although melatonin treatment did not significantly influence mean preferred T_b of nocturnal African house snakes, melatonin may retain its *zeitgeber* role in this species. These results suggest melatonin may serve different roles in species with different natural histories.

Melatonin likely plays an important role in synchronizing physiological and behavioral processes with environmental cues in most vertebrates, but may only directly influence particular behaviors or physiologies in some species. Melatonin's function as a

zeitgeber is believed to have evolved secondarily to its function as a local hormone regulating the distribution of melanosomes in the retina (Gern *et al*. 1987). Given the vast evolutionary changes observed in the functional anatomy of the pineal complex, it is reasonable to hypothesize that the actions of melatonin in the timing of circadian and circannual physiologies and behaviors may be highly species-specific and therefore may vary substantially among vertebrates.

The Garter Snake Model

The common garter snake, *Thamnophis sirtalis*, provides a unique system for investigating the roles environmental stimuli and hormones play in regulating hibernation and reproduction. Garter snakes are arguably the most abundant reptiles in North America and have an extensive range. Furthermore, the reproductive behavior and endocrinology of garter snakes are the most investigated of any reptile, providing an invaluable framework for continued research (e.g., Aleksiuk and Gregory 1974; Camazine *et al*. 1980; Crews 1979, 1983, 1984, 1991; Crews *et al*. 1984; Krohmer *et al*. 1987; Lerner and Mason 2001; Mason 1992; Mason and Crews 1985; Mason *et al*. 1989; Moore *et al*. 2000*a*, 2000*b*; Whittier and Mason 1996; Whittier and Tokarz 1992; Whittier *et al*. 1987*a*, 1987*b*).

Much of our knowledge regarding reproduction in garter snakes is derived from studies of one species, the red-sided garter snake (*T. sirtalis parietalis*). Red-sided garter snakes are the most northerly living reptile in North America and are found in extremely large numbers throughout south central Manitoba, Canada. These populations of snakes

hibernate for approximately 8 months each year in underground hibernacula, void of any photoperiod cues. Beginning in early May, red-sided garter snakes emerge from hibernacula and remain within the vicinity of the dens during the attenuated mating season (4-5 weeks). Mating occurs in large mating balls of up to 100 males courting a single female (Gregory 1974). Dispersal of the snakes to the feeding grounds from the hibernacula occurs in late May through early June. Snakes spend the spring and summer at the feeding grounds before returning to the hibernacula in late August through September (Crews and Garstka 1982; Crews *et al*. 1984; Whittier *et al*. 1987*a*).

The reproductive behavior of red-sided garter snakes does not depend upon the activational effects of sex steroid hormones (e.g., estradiol 17β, testosterone, and progesterone) (Crews 1984, 1991; Whittier and Tokarz 1992). This steroid-independent induction of reproductive behavior is thought to be an adaptation to the environmental constraints encountered by this species. Such dissociated reproductive patterns are commonly observed in animals inhabiting environments that provide predictable but brief opportunities for reproduction (Crews and Gans 1992). In red-sided garter snakes, a well studied dissociated breeder, increases in gonadal activity (i.e., gametogenesis and steroidogenesis) do not coincide with mating. Rather, mating occurs when gonads are regressed and circulating levels of sex steroid hormones are low (Crews 1984; Crews and Garstka 1982). Gonadal recrudescence and activity peak during summer, when females ovulate and fertilize eggs and males produce sperm to be used the following spring (Crews 1979, 1984, 1991; Halpert *et al*. 1982; Krohmer *et al*. 1987; Whittier and Crews 1986*a*; Whittier *et al*. 1987*b*).

Spring mating in female garter snakes results in an estradiol surge, after which females become unreceptive and, within 24 hours, unattractive (Devine 1977; Ross and Crews 1977, 1978; Whittier *et al*. 1985, 1987*b*). This estradiol surge may facilitate vitellogenin production by the liver and induce ovarian development (Garstka e*t al*. 1982). However, normal ovarian development has been observed in the absence of a postmating estradiol surge (Mendonça and Crews 1989; Whittier and Crews 1986*b*; Whittier *et al*. 1987*b*). In addition, it is likely that a neural reflex is involved, as spinal transection or treatment of females with an anesthetic prior to mating abolishes the postmating estradiol surge and subsequent ovarian development (Halpert *et al*. 1982; Mendonça and Crews 1990, 1996, 2001; Whittier and Crews 1986*a*; Whittier *et al*. 1985, 1987*b*).

The dramatic postmating decline in female receptivity and the termination of receptive behavior may be mediated by prostaglandins, as elevated levels of prostaglandin $F_{2\alpha}$ have been observed in response to mating (Whittier and Crews 1989). Furthermore, injection of prostaglandin $F_{2\alpha}$ significantly inhibits sexual receptivity in unmated, newly emerged females (Whittier and Crews 1986*b*). One possible source of prostaglandins is the seminal fluids that are transferred to female snakes during mating (Whittier and Tokarz 1992). Some garter snakes (*T. butleri*, *T. radix*, *T. sauritu*s, *and T. sirtalis*) deposit a gelatinous copulatory plug in the cloaca of females during mating; changes in female receptivity following mating have been attributed to these copulatory plugs (Devine 1975; Ross and Crews 1977, 1978). However, approximately 30% of all females have been observed to mate 2 or more times, even when a copulatory plug is

present from a previous mating. Thus, copulatory plugs appear to have limited effectiveness in blocking subsequent matings (Devine 1975, 1977; Ross and Crews 1977; Shine *et al*. 2000*b*).

As in female red-sided garter snakes, mating in male garter snakes occurs when gonads are regressed and levels of circulating testosterone are low (Crews 1976, 1983, 1984, 1991; Crews and Garstka 1982). Following spring mating, males demonstrate testicular recrudescence and androgen synthesis. Male red-sided garter snakes undergo spermatogenesis in late summer and store their sperm through hibernation (Crews 1979, 1984, 1991; Krohmer *et al*. 1987; Moore and Lindzey 1992). Thus, mating behavior in males is also independent of steroids. For example, neither castration nor androgen treatment affects the expression of mating behavior in adult males (Camazine *et al*. 1980; Crews 1985, 1991; Crews *et al*. 1984).

Mating behavior of red-sided garter snakes appears to be triggered by increases in environmental temperatures following winter dormancy (Bona-Gallo and Licht 1983; Garstka *et al*. 1982; Krohmer and Crews 1987; Ross and Crews 1978; Whittier *et al*. 1987*a*). Both male and female red-sided garter snakes require a period of low temperature conditions to initiate sexual behavior upon re-exposure to warm temperatures (Bona-Gallo and Licht 1983; Camazine *et al*. 1980;). Several studies have also demonstrated that photoperiod prior to and during hibernation has no influence on the initiation and timing of reproductive behavior upon emergence (e.g., Nelson *et al.*1987; Whittier *et al.*1987*a*). Given the unique life history of the red-sided garter snake, temperature is likely the most important proximate environmental cue governing the

initiation of emergence and subsequent mating behavior. Furthermore, environmental induction of mating may be mediated by the pineal gland. For example, male snakes pinealectomized prior to hibernation fail to court female snakes upon emergence (Crews *et al*. 1988; Mendonça *et al*. 1996*a*, 1996*b*; Nelson *et al*. 1987). In contrast, pinealectomy following spring emergence has no effect on the expression of courtship behavior in male red-sided garter snakes (Mendonça *et al*. 1996*a*; Nelson *et al*. 1987). These results indicate the pineal gland is necessary for transducing environmental stimuli during winter dormancy, of which temperature is the most important (and perhaps the only) cue. Thus, this system is unique in that in all other vertebrates studied, interactions among both photoperiod and temperature determine the physiological functions of the pineal gland and its major secretory product, melatonin.

The discipline of comparative physiology offers an excellent mechanism of delineating the factors playing an important role in a species' unique physiological and behavioral adaptations. The red-spotted garter snake (*T. sirtalis concinnus*) found throughout the Willamette Valley of western Oregon provides a valuable platform for investigating the importance of the pineal gland and melatonin in regulating seasonal biology. For example, the pineal complex may play a more important role in activity and reproductive adaptations at higher latitudes, where seasons tend to vary more dramatically (Ralph 1975). Because *T. sirtalis parietalis* and *T. sirtalis concinnus* inhabit very different environments, studying these two populations may provide insight into the adaptations of garter snakes to their unique environments. Unlike red-sided garter snakes, the red-spotted garter snake (*T. sirtalis concinnus*) of the Willamette Valley in

western Oregon has an extended breeding season lasting 10-12 weeks following spring emergence (Moore *et al*. 2000*b*). Although the red-spotted garter snake does exhibit a period of winter dormancy, they can be active during 10-12 months of the year given appropriate environmental conditions (Moore *et al*. 2002*b*). Investigating possible differences in the function of melatonin among these different populations of garter snakes may help elucidate whether pineal gland function varies with geographic distribution. Furthermore, a comparative approach may provide insight into the species differences and complex interactions observed among the pineal gland and melatonin, the transduction of environmental stimuli, and reproductive physiology and behavior.

Synopsis of Dissertation Research

Seasonal rhythms in physiology and behavior are temporally organized via the transduction of environmental cues (e.g., photoperiod, temperature) into appropriate endocrine signals. The role of the pineal gland and melatonin in transducing environmental stimuli and regulating the behavior and physiology of ectotherms, however, remains unclear. Furthermore, among ectothermic vertebrates that undergo continuous winter dormancy, temperature is the only environmental cue available for synchronizing seasonal rhythms. Most intriguing is that in species where reproduction occurs immediately following spring emergence, the associated changes in neurophysiology and behavior that accompany reproduction must occur during winter dormancy. The purpose of this dissertation research was to explore the mechanisms by

which temperature cues affect the chronobiology and seasonal reproduction of red-sided garter snakes (*T. sirtalis parietalis*).

The garter snake model system utilized during these studies provides a unique framework for investigating these questions. In most other vertebrates, photoperiod is the primary environmental cue used in synchronizing circadian systems. However, during winter dormancy, the time-keeping mechanisms of red-sided garter snakes (*T. sirtalis parietalis*), and most importantly the control of seasonal reproduction, rely exclusively on changes in seasonal temperatures. Thus, the red-sided garter snake is an excellent model for studying the role of temperature in synchronizing seasonal rhythms.

Because of their roles in circadian organization and energy balance, melatonin and glucocorticoids are likely hormonal components of the mechanisms regulating temperature-induced reproduction. To better understand how melatonin and corticosterone, the primary glucocorticoid in reptiles (Idler 1972), might facilitate temperature-induced reproduction, I first characterized the reciprocal interactions between these two hormonal systems. No studies have investigated the influence of melatonin on the hormonal and behavioral responses to physiological stress in an ectotherm. Furthermore, no investigations examining melatonin's ability to antagonize glucocorticoid actions at the level of the target tissues have been conducted in nonmammalian vertebrates. The first three research chapters of this dissertation explore the effects of melatonin on behavioral and hormonal responses to exogenous corticosterone, the effects of melatonin on hormonal responses to capture stress in two

different populations of garter snakes, and the influence of capture stress and exogenous corticosterone on circadian melatonin cycles.

If interactions between melatonin, glucocorticoids, and physiological stress responses are conserved in this model system, then it is important to understand how these interactions might regulate physiology and behavior during both winter dormancy and spring emergence. For example, are melatonin levels sufficiently elevated following spring emergence to play a modulatory role in stress responses? Does the pineal gland function in signaling spring emergence and subsequent mating behavior via changes in the amplitude and/or duration of circadian melatonin rhythms? And given the lack of photoperiod cues to snakes hibernating in underground dens, is temperature a sufficient cue in driving such changes in circadian melatonin secretion?

To better understand the environmental mechanisms mediating spring emergence and temperature-induced reproductive behavior, I first measured body temperatures of red-sided garter snakes (*T. sirtalis parietalis*) during winter dormancy under natural field conditions (chapter five). Chapter six of this dissertation focuses on the hormonal mechanisms mediating temperature-induced reproductive behavior in red-sided garter snakes. In summary, this dissertation research focuses on the environmental and hormonal mechanisms underlying time-keeping systems and the control of seasonal reproduction. Specifically, I will determine if (1) a physiological coupling exists between melatonin and corticosterone rhythms in this ectothermic model and (2) whether such interactions between melatonin and corticosterone contribute to the hormonal time-keeping mechanisms regulating circannual rhythms in physiology and behavior.

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CHAPTER 2

EFFECTS OF MELATONIN ON THE BEHAVIORAL AND HORMONAL RESPONSES OF RED-SIDED GARTER SNAKES (*THAMNOPHIS SIRTALIS PARIETALIS*) TO EXOGENOUS CORTICOSTERONE

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Abstract

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

additive inhibitory effects on courtship behavior. *Key Words:* melatonin, corticosterone, ketanserin, serotonin, courtship, mating, reptile, garter snake, *Thamnophis sirtalis parietalis*.

Introduction

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

Interactions between melatonin and other hormones are also important in synchronizing and modulating physiological and behavioral parameters necessary for daily activity. For example, melatonin inhibits the hypothalamic-pituitary-adrenal axis (Reiter 1991; Wang *et al*. 1999), which mediates an animal's physiological and

behavioral responses to stressors. Unpredicted challenges to energy homeostasis, such as unusually low environmental temperatures or food shortage, stimulate the hypothalamic-pituitary-adrenal axis and result in increased plasma glucocorticoids (Harvey *et al*. 1984; Schwabl *et al*. 1985; Wingfield 1988). Glucocorticoids in turn modulate a variety of physiological and behavioral processes that promote survival while suppressing behaviors that are not crucial to immediate survival (e.g., Pottinger 1999; Sapolsky 1992; Wingfield 1988). For example, physiological stress responses, marked by an increase in plasma glucocorticoid concentrations, generally result in decreased plasma concentrations of sex steroid hormones and an overall suppression of reproductive behavior in many species (Carragher and Rees 1994; Coddington and Cree 1995; Moore *et al*. 2000; Moore *et al*. 1991; Rivier and Rivest 1991). Such acute physiological stress responses are normally adaptive responses used to modify metabolism and mobilize energy stores during a stressful event.

Although interactions between the pineal gland and hypothalamic-pituitary-adrenal axis are well established (Barriga *et al*. 2002; Demisch *et al*. 1998; Khan *et al*. 1990; Kirby *et al*. 1999; Maestroni *et al*. 1986, 1989; Otsuka *et al*. 2001; Vaughan *et al*. 1972), the nature of this relationship is poorly understood. For example, both stimulatory (Al-Dujaili *et al*. 1982; Haus *et al*. 1996; Persengiev *et al*. 1989; Touitou *et al*. 1989) and inhibitory (Heiman and Porter 1980; Ng 1987; Nussdorfer *et al*. 1990) effects of melatonin on the secretory activity of the adrenal cortex have been described. However, all of these studies examined the effects of melatonin on cultured adrenocortical cells. In an experiment using *in vivo* physiological conditions, melatonin exerted a direct

antisecretagogue effect on the adrenal gland (Appa-Rao *et al*. 2001). Chronic melatonin treatment considerably altered the affinity of glucocorticoid receptors in the brain and pituitary (Marinova *et al*. 1991). Furthermore, melatonin treatment of both adult and juvenile rats prevented many of the injurious effects induced by chronically elevated glucocorticoids, such as the reduction in growth, atrophy of the thymus and adrenal glands, and elevation of blood glucose, free fatty acids, triglycerides, and total cholesterol (Aoyama *et al*. 1986, 1987). Daily melatonin treatment in stressed mice prevented several chronic stress-induced disturbances, including a reduction in preference for sucrose solution and a reduction in spontaneous locomotor activity (Kopp *et al*. 1999). In male rats, melatonin treatment significantly reduced the inhibitory effects of acute and chronic stress on sexual behavior (Brotto *et al*. 2001). Gorzalka *et al*. (1999) demonstrated that acute melatonin treatment also attenuated the effects of glucocorticoids on sexual behavior and wet-dog shakes in male rats. Such effects of melatonin are thought to be mediated by melatonin's properties as a serotonergic type 2A receptor antagonist (Eison *et al*. 1995; Gorzalka *et al*. 1999).

We investigated the influence of melatonin on the behavioral and hormonal responses to exogenous glucocorticoids in a non-mammalian model. Male red-sided garter snakes (*Thamnophis sirtalis parietalis*) provide an excellent model for investigating these questions because they are known to exhibit significantly reduced courtship behavior in response to exogenous glucocorticoid treatment (Moore and Mason 2001). In this study, we investigated differences in courtship behavior and plasma testosterone + 5-α-dihydrotestosterone concentrations among snakes treated with

melatonin and/or corticosterone [the primary glucocorticoid in snakes (Idler 1972)]. We asked the following questions: (1) Does melatonin modulate behavioral and hormonal responses of male red-sided garter snakes to exogenous corticosterone? and (2) Does melatonin's influence on behavioral and hormonal responses to exogenous corticosterone result from antagonism of serotonin receptors?

Materials and Methods

Experiments were conducted in the field with free-living red-sided garter snakes (*T. sirtalis parietalis*) in Inwood, Manitoba, Canada. Studies were conducted between 1030 and 1430 h on 14-17 May 2003, during the month following emergence from hibernacula when snakes are mating and plasma testosterone concentrations are declining (Krohmer *et al*. 1987). All experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol number: LAR-2661) and were in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals". This research was approved by the Manitoba Wildlife Animal Care Committee (protocol number: 2002-06) and was performed under the authority of Manitoba Wildlife Scientific Permit No. WSP 03009.

Reagents

Melatonin and corticosterone were purchased from Sigma Chemical Company (St. Louis, MO). Ketanserin, a serotonergic type 2A receptor antagonist, was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). We chose this antagonist because (1)

serotonergic type 2A receptors reliably modulate sexual behavior (Gorzalka *et al*. 1990), (2) corticosterone regulates the density of serotonergic type 2A receptors as well as serotonergic type 2A receptor-mediated behaviors (Berendsen *et al.* 1996; Fernandes *et al.* 1997; Gorzalka and Hanson 1998; Takao *et al.* 1997), and (3) ketanserin itself exerts no significant effects on sexual behavior (Watson and Gorzalka 1991). All pretreatments and treatments were administered via intraperitoneal injection with an injection volume of 0.1 ml. Injection volumes of vehicle (5% ethanol in reptile Ringer's solution) were also 0.1 ml.

Melatonin solutions for the low and high melatonin doses were prepared by first dissolving 6 mg or 60 mg, respectively, in 1 ml of 100% ethanol. Stock solutions were diluted to 20 ml with reptile Ringer's solution, producing melatonin concentrations for the low and high doses of 0.3 mg ml^{-1} and 3.0 mg ml^{-1}, respectively. Thus, the melatonin pretreatment doses were 0.03 and 0.3 mg per snake (i.e., 0.03 and 0.3 mg per 0.1 ml). For an average male snake weighing 0.03 kg, the high melatonin dose was 10 mg kg^{-1} body mass, which is similar to the doses used to test the effects of melatonin on thermoregulation in ectotherms (Erskine and Hutchison 1981; Skinner 1991).

Ketanserin solution was prepared by first dissolving 9 mg in 1 ml of 100% ethanol and then diluting to 20 ml with reptile Ringer's solution. This produced a ketanserin concentration of 0.45 mg ml^{-1} and a ketanserin dose of 0.045 mg per snake. For an average male snake weighing 0.03 kg, this ketanserin dose was 1.5 mg kg^{-1} body mass. We chose this dose based on previous research done by Wilson and Pulido (2000),

who used a 1.5 mg kg^{-1} body mass dose to test the effects of this serotonin receptor antagonist on the transport response of rats.

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

Experimental Design

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

arenas for mating trials (n = 32 in each corticosterone treatment subgroup) or blood sampling (n = 10 in each corticosterone treatment subgroup).

Behavioral Responses to Corticosterone

Mating trials were conducted in nylon cloth arenas measuring 1 × 1 × 1 m with 12 males (i.e., one male randomly selected from each combination of the 4 pretreatment and 3 corticosterone treatment conditions) simultaneously introduced to an unmated, attractive female. Males were introduced in groups of 12 to simulate natural mating conditions, where the presence of a mating ball facilitates male courtship behavior (Joy and Crews 1985). Because males are attracted to females by both the presence of pheromonal cues expressed on the dorsal surface of females as well as the presence of a mating ball, mating balls rarely contain fewer than five males courting a single female (Joy and Crews 1985). Using an ethogram of male courtship behavior (Table 2.1), we recorded the mating score of each male every 10 min for a period of 1 h after introduction into the arena; the observer was blind to the treatment group of each male. Adhesive tape was placed across the female's cloaca to prevent mating during the trials, as mating significantly reduces further male courtship behavior (Garstka *et al*. 1982). The tape does not alter male or female reproductive behavior (LeMaster and Mason 2002) and was immediately removed following each trial. Each male was therefore assigned a mating score of 0 (no reproductive behavior) through 4 (male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves) every 10 min for 1 h. Behavioral scores of 3.0 and greater are exhibited only in a reproductive

Table 2.1. Ethogram of courtship behavior for the male red-sided garter snake (*Thamnophis sirtalis parietalis*). Behaviors 3.0 and greater are exhibited only in a reproductive context (modified from Moore *et al*. 2000 and Crews *et al*. 1984).

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

context (Table 2.1). We collected a total of 6 courtship scores for each snake during the 1-h trial period; these courtship scores were used to calculate a mean courtship score for each snake.

Hormonal Responses to Corticosterone

To measure testosterone + 5-α-dihydrotestosterone concentrations, we collected blood samples from a subset (n = 10) of males in each corticosterone subgroup that were not used in the mating trials. All blood samples were collected approximately 1 h following completion of the mating trials (i.e., approximately 3 h following pretreatment with melatonin or ketanserin). Moore and Mason (2001) demonstrated that 4 h following intraperitoneal injection with 50 μg corticosterone (in 0.1 ml 1% ethanol in reptile

Ringer's solution), male red-sided garter snakes tended to have higher, but physiologically relevant, plasma corticosterone levels (approximately 90 ng ml^{-1}) than snakes receiving only reptile Ringer's solution (approximately 55 ng ml^{-1}). Similarly, the half-life of melatonin after injection into endotherms can be 1 h or less (Rollag and Stetson 1982). Although the half-life of melatonin in whole-animal ectotherms has not been measured, it is likely to be much longer because metabolic rate is as much as 10 times lower, depending on body temperature (Filadelfi and Castrucci 1996). We are confident that plasma corticosterone and melatonin levels, in response to hormone treatments, remained elevated throughout the duration of our experiments.

Blood samples were obtained from the caudal vein within 1 min using heparinized 1-cm^3 syringes and 25-g needles. Samples were stored on ice until return to the field station, where they were centrifuged and the plasma separated. Plasma samples were stored at – 4ºC until return to Oregon State University, where they were stored at - 70ºC until analyzed for androgen concentrations following radioimmunoassay procedures modified from Moore *et al*. (2000). To test whether chromatography of steroid hormones extracted from snake plasma is necessary, we simultaneously analyzed a subset of plasma samples (n = 40) for testosterone concentrations using both radioimmunoassay with partition chromatography (Moore *et al*. 2000) and radioimmunoassay without partition chromatography (i.e., direct radioimmunoassay). The methods used for direct radioimmunoassay were similar to those described by Jessop *et al*. (1999a, 1999b, 2000, 2004) and Whittier *et al*. (1997).

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

Statistical Analyses

To investigate behavioral responses of snakes to corticosterone, we collected a total of 6 courtship scores for each snake during the 1-h trial period. These courtship scores were used to calculate a mean courtship score for each snake due to the statistical

non-independence of repeated measurements. Using a mean courtship score also helps account for possible metabolism (and therefore changing effects) of hormone treatments during the mating trials. We rank-transformed the mean courtship scores of snakes to adjust for the non-normality of these data. Differences in mean courtship scores among treatment groups were then investigated using a two-way analysis of variance (ANOVA; with pretreatment and treatment as the between-subjects factors) on rank-transformed data followed by a Student-Newman-Keuls multiple comparisons test. We used this multiple comparisons test, which employs step-down logic [i.e., first testing larger pairwise differences in ordered means and then proceeding to smaller differences (Toothaker 1993; Zar 1984)] because of *a priori* knowledge that corticosterone would have a step-wise effect (i.e., a dose-response) on courtship behavior.

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

Results

Behavioral Responses to Corticosterone

Pretreatment with both melatonin and ketanserin significantly reduced average courtship scores of male garter snakes (Figure 2.1; $F_{(3,372)} = 8.62$, $P < 0.001$ from a two-way ANOVA). There were no statistically significant differences in the courtship scores of snakes among melatonin and ketanserin pretreatment groups (Figure 2.1). Within each pretreatment group, corticosterone significantly decreased the average courtship scores of male red-sided garter snakes (Figure 2.1; $F_{(2,372)} = 64.58$, $P < 0.001$ from a two-way ANOVA). Results from the Student-Newman-Keuls multiple comparisons procedure for comparisons of pretreatment condition within the corticosterone treatment subgroups are shown in Table 2.2. There were no statistically significant interactions between pretreatment and treatment conditions.

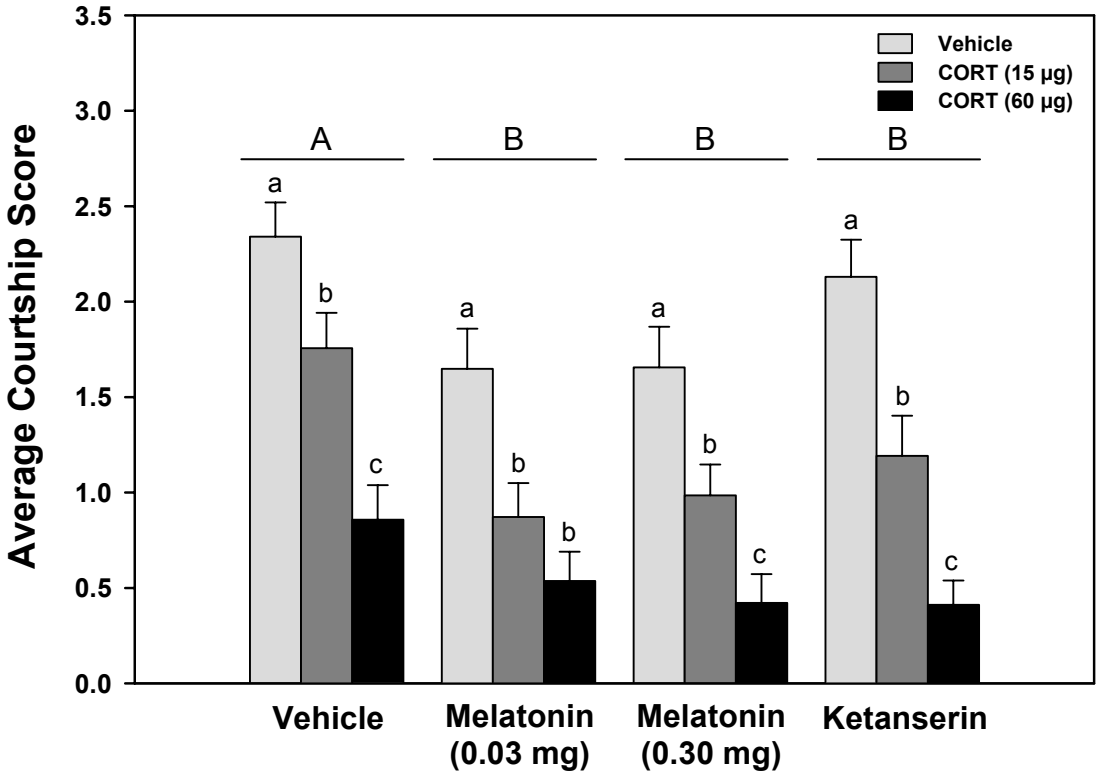

Figure 2.1. Average courtship scores of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) following pretreatment with vehicle, melatonin (0.03 or 0.30 mg), or ketanserin, a serotonergic type 2A receptor antagonist. Pretreatment conditions are indicated along the abscissa. Thirty minutes after pretreatment, snakes received treatment with vehicle or corticosterone (CORT; 15 or 60 µg). Standard errors (+ 1) are shown by the vertical lines; $n = 32$ in each treatment. Statistically significant differences among pretreatment groups are indicated by letters above the horizontal lines. Within each pretreatment group, statistically significant differences among vehicle and corticosterone treatments are indicated by letters above each standard error bar.

Table 2.2. Results from a two-way analysis of variance on mean courtship scores of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) followed by a Student-Newman-Keuls multiple comparisons procedure. Data were rank-transformed to correct for non-normality. Results shown below are for comparisons of pretreatment conditions within the corticosterone treatment groups; statistically significant differences are highlighted in bold type.

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

Hormonal Responses to Corticosterone

We obtained excellent correlation between the testosterone concentrations of a subset of plasma samples (n = 40) assayed by both direct radioimmunoassay and radioimmunoassay with partition chromatography (Figure 2.2; $R^2 = 0.97$, $P < 0.001$ from a regression). Thus, we elected to analyze all plasma samples using direct radioimmunoassay methods. Because our testosterone antibody (Wien Laboratories, Inc.,

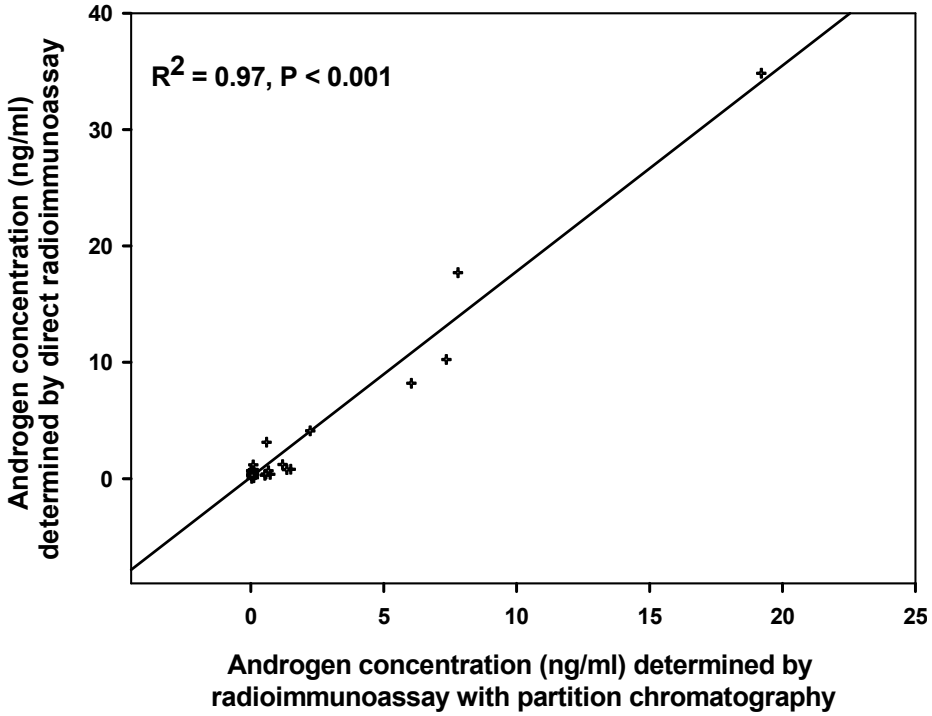

Figure 2.2. Regression of androgen (testosterone + 5-α-dihydrotestosterone) concentrations (ng ml^{-1}) determined by direct radioimmunoassay on androgen (testosterone) concentrations (ng ml^{-1}) determined by radioimmunoassay with partition chromatography using celite microcolumns. Each data point represents the androgen concentration of one plasma sample as determined by direct radioimmunoassay versus radioimmunoassay with partition chromatography (n = 40 plasma samples).

Succasunna, NJ) cross-reacts significantly with 5-α-dihydrotestosterone, our direct assay measures both plasma testosterone and 5-α-dihydrotestosterone concentrations. Thus, our direct assay-testosterone concentrations tend to be higher than those measured by radioimmunoassay with partition chromatography (which separates testosterone from 5-α-dihydrotestosterone). For these reasons, we present here data for both testosterone and 5-α-dihydrotestosterone concentrations.

A two-way ANOVA shows that pretreatment with melatonin and ketanserin did not significantly influence testosterone + 5-α-dihydrotestosterone concentrations of snakes (Figure 2.3). Within each pretreatment group, corticosterone (15 and 60 μg) did not significantly influence testosterone + 5-α-dihydrotestosterone concentrations of male red-sided garter snakes (Figure 2.3). There were no statistically significant interactions between pretreatment and treatment conditions.

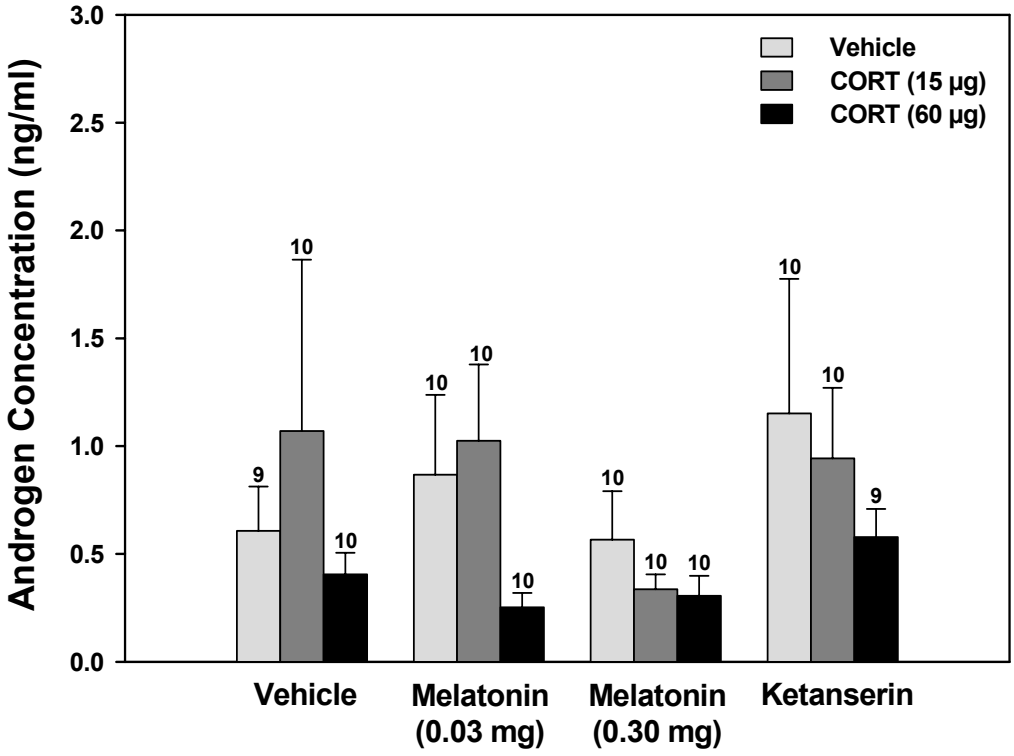

Figure 2.3. Mean plasma androgen (testosterone + 5-α-dihydrotestosterone) concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) following pretreatment with vehicle, melatonin (0.03 or 0.30 mg), or ketanserin, a serotonergic type 2A receptor antagonist. Pretreatment conditions are indicated along the abscissa. Thirty minutes after pretreatment, snakes received treatment with vehicle or corticosterone (CORT; 15 or 60 μg). Standard errors (+ 1) are shown by the vertical lines; sample sizes are listed above the standard error bars.

Discussion

Our results provide evidence that both melatonin and corticosterone inhibit courtship behavior in male red-sided garter snakes (*T. sirtalis parietalis*) (Figure 2.1). These data also demonstrate that the effects of melatonin and corticosterone on courtship behavior are independent of their effects on the hypothalamic-pituitary-gonadal axis, as neither hormone influenced plasma testosterone + 5-α-dihydrotestosterone concentrations (Figure 2.3). Furthermore, we demonstrate that melatonin and corticosterone, at the 15 μg dose, have significant additive inhibitory effects on male reproductive behavior (Figure 2.1, Table 2.2). Our experiments with the serotonergic type 2A receptor antagonist ketanserin suggest that a serotonin-regulated system is involved in the inhibition of courtship behavior by melatonin and corticosterone (Figure 2.1, Table 2.2). These data also support the hypothesis that melatonin acts as a serotonin receptor antagonist.

These experiments support a direct modulatory role of melatonin on reproductive behavior in an ectothermic model. Pretreatment with either dose of melatonin followed by vehicle significantly reduced courtship of male snakes as compared to snakes receiving vehicle pretreatment followed by treatment with vehicle (Table 2.2). This modulatory role, in combination with melatonin's circadian and circannual rhythm of secretion, may be important in synchronizing seasonal reproductive function with optimal environmental conditions in this species.

Although extensive experiments have been done in both birds and mammals, there are fewer studies investigating the relationship between the pineal complex,

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

Previous studies in red-sided garter snakes (*T. sirtalis parietalis*) showed that pinealectomy prior to winter hibernation inhibits male courtship behavior upon spring emergence (Crews *et al*. 1988; Mendonça *et al*. 1996*a*; Nelson *et al*. 1987). In contrast, pinealectomy following spring emergence has no effect on the expression of male

courtship behavior (Nelson *et al*. 1987; Mendonça *et al*. 1996*a*). These results indicate the pineal gland is necessary for transducing environmental stimuli (and synchronizing reproduction) during winter dormancy, but once reproductive behavior is induced, pinealectomy is no longer effective in modulating reproduction. Treatment of pinealectomized snakes with melatonin did not influence the effects of pinealectomy on courtship behavior (Mendonça *et al*. 1996*a*). This suggests that the physical presence of the pineal gland, and not melatonin per se, is necessary for synchronizing reproductive behavior with spring emergence. It is possible that a neural component of the pineal gland, in combination with melatonin secretion, is necessary for initiating reproductive behavior of red-sided garter snakes following winter dormancy. Our results demonstrate that melatonin modulates reproductive behavior of male red-sided garter snakes during the spring mating season. Thus, although pinealectomy following spring emergence does not influence reproductive behavior, male snakes are sensitive to melatonin during the mating season. Circadian melatonin cycles following spring emergence most likely play a role in synchronizing reproductive behavior (and activity) with the appropriate time of day. Indeed, disrupted circadian melatonin cycles, with peak melatonin secretion occurring during the photophase, were observed in red-sided garter snakes that failed to exhibit courtship behavior during the spring breeding season (Mendonça *et al*. 1996*b*).

The hypothalamic-pituitary-adrenal axis is also important in regulating reproductive function in red-sided garter snakes. In this species, mating occurs upon emergence from winter dormancy while plasma sex steroid concentrations are basal, gonads are regressed, and glucocorticoid levels are high (Aleksiuk and Gregory 1974;

Crews 1984; Crews and Garstka 1982; Crews *et al*. 1984; Whittier *et al*. 1987). Because these snakes are aphagic during the mating season, elevated corticosterone levels likely play an important role in mobilizing energy stores during spring emergence and mating. When reproductive opportunities are limited, however, it is not uncommon to observe an absence of glucocorticoid-induced reproductive suppression. For example, courtship behavior of male garter snakes during the spring breeding season is not inhibited following capture and handling stress (which significantly increases plasma corticosterone concentrations and significantly decreases plasma testosterone concentrations; Moore *et al*. 2000). However, treatment of male red-sided garter snakes with exogenous corticosterone suppresses courtship behavior in a threshold-dependent manner (Moore and Mason 2001). Thus, male garter snakes retain sensitivity behaviorally to elevated corticosterone during the breeding season, even though they have uncoupled hormonal responses to capture stress from behavioral responses to capture stress (Moore and Mason 2001).

Similar to Moore and Mason (2001), we demonstrate that exogenous corticosterone significantly suppresses courtship behavior of male red-sided garter snakes but does not influence plasma androgen concentrations. Although the testosterone + 5-α-dihydrotestosterone concentrations we measured are much lower than those reported by Moore and Mason (2001), this is likely due to the unusually warm mating season during May 2003. Testosterone concentrations normally decline during the mating season (Krohmer *et al*. 1987), and therefore the warmer temperatures most likely increased the metabolic clearance of testosterone. If we had conducted these experiments earlier in the

season we would most likely have observed testosterone concentrations in the range of those reported in previous studies.

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

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

We propose that a serotonin system is involved in the modulation of male courtship behavior by melatonin and corticosterone in red-sided garter snakes. As expected, pretreatment with ketanserin, a serotonergic type 2A receptor antagonist, followed by vehicle did not significantly influence courtship behavior (Watson and Gorzalka 1991). However, the combination of ketanserin pretreatment followed by

treatment with the low corticosterone dose significantly reduced courtship behavior of male snakes (as compared to the courtship scores of snakes receiving vehicle pretreatment followed by treatment with the low corticosterone dose; see Table 2.2). These results suggest that corticosterone inhibits reproductive behavior of red-sided garter snakes by modulating a serotonin-regulated system; these inhibitory effects of corticosterone are significantly augmented with ketanserin.

There is much precedence for interactions between serotonin and corticosterone in modulating physiology and behavior (e.g., Chaoloff 1993; Gorzalka *et al*. 1998; Kawahara *et al*. 1993; Mendelson and McEwen 1992; Popava and Lobacheva 1982; Stutzmann *et al*. 1998). For example, corticosterone increases the density of central serotonergic type 2A receptors and facilitates serotonergic type 2A receptor-mediated behaviors (Berendsen *et al.* 1996; Fernandes *et al.* 1997; Gorzalka and Hanson 1998; Takao *et al.* 1997). Corticosterone has little or no effect on modulating serotonin metabolism (Chaouloff 1993). Thus, the effects of corticosterone on serotonergic type 2A receptor-mediated behaviors, such as an increase in wet-dog shakes, are likely due to a specific receptor-mediated mechanism, rather than simply a modulation of serotonin metabolism (Gorzalka *et al*. 1999). These actions of corticosterone, when combined with a serotonin antagonist, could explain the additive inhibitory effects of corticosterone and ketanserin we observed on reproductive behavior. This hypothesis is supported by the lack of an additional inhibitory effect of ketanserin pretreatment when combined with the high corticosterone treatment dose (Table 2.2), as the high corticosterone dose may have saturated the system.

Similar to our observations of additive inhibitory effects of ketanserin and corticosterone, melatonin and corticosterone, at a dose of 15 μg, also had additive inhibitory effects on reproductive behavior. Snakes receiving pretreatment with both the low and high melatonin doses followed by treatment with the low corticosterone dose exhibited significantly reduced courtship behavior (as compared to those snakes receiving pretreatment with vehicle followed by treatment with the low corticosterone dose; Table 2.2). We observed no significant differences in the courtship behavior of male snakes receiving pretreatment with ketanserin or either melatonin dose among any of the corticosterone treatments (Table 2.2). These results suggest that both melatonin and ketanserin reduce courtship behavior via modulation of a serotonin system, thus supporting the hypothesis that melatonin may act as a serotonin receptor antagonist (Gorzalka *et al*. 1999). However, pretreatment with both melatonin doses, but not ketanserin, followed by vehicle significantly reduced courtship behavior of male snakes. Thus, melatonin may inhibit courtship behavior via a mechanism other than antagonism of serotonin receptors. It is also possible, however, that melatonin is a more potent serotonin receptor antagonist than ketanserin. Future studies examining dose response curves for ketanserin would provide insight into whether the effects of melatonin on courtship behavior reported here result primarily from its antagonism of serotonergic type 2A receptors or some other mechanism. Further research is also needed to determine if the actions of corticosterone on reproductive behavior do indeed involve modulation of a serotonin-regulated system. Furthermore, additional research is necessary to discern

whether melatonin and corticosterone are converging on the same pathway or if their effects on different pathways are having additive inhibitory effects on courtship behavior.

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CHAPTER 3

A SEROTONIN RECEPTOR ANTAGONIST, BUT NOT MELATONIN, MODULATES HORMONAL RESPONSES TO CAPTURE STRESS IN TWO POPULATIONS OF GARTER SNAKES (*THAMNOPHIS SIRTALIS PARIETALIS* AND *THAMNOPHIS SIRTALIS CONCINNUS*)

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Abstract

Hormonal and behavioral responses to a stressor depend on many factors, including the influence of other hormones. We examined the role of melatonin in modulating hormonal responses to capture stress in two populations of male garter snakes, *Thamnophis sirtalis*. Studies of red-sided (*T. sirtalis parietalis*) and red-spotted (*T. sirtalis concinnus*) garter snakes were conducted in the field with free-living snakes. Populations of red-sided garter snakes in south-central Manitoba, Canada undergo a period of winter dormancy for approximately 8 months each year followed by an attenuated mating season (4-5 weeks) in early spring. In contrast, the mid-latitude red-spotted garter snake in western Oregon, USA has an extended breeding season and can be active during 10-12 months of the year given appropriate environmental conditions. We chose to study these two populations of garter snakes to investigate possible variation in melatonin function among snakes with different suites of environmental adaptations. To better address these questions, we also examined the effects of 5-hydroxytryptophan (a precursor of melatonin synthesis) and ketanserin (a serotonergic type 2A receptor antagonist) on hormonal responses to capture stress. We observed a trend of increased corticosterone and decreased androgen concentrations in northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) subjected to 4 h of capture stress during the spring. However, these differences were not statistically significant. During the fall, red-sided garter snakes showed no change in corticosterone or androgen concentrations in response to the capture stress treatments. We speculate that northern-latitude red-sided garter snakes suppress hormonal responses to capture stress during preparation for winter

dormancy. Treatment with melatonin, 5-hydroxytryptophan, or ketanserin did not significantly influence corticosterone or androgen concentrations of northern-latitude red-sided garter snakes during the spring or fall. Mid-latitude red-spotted garter snakes (*T. sirtalis concinnus*) from Oregon showed a statistically significant increase in corticosterone concentrations in response to 4 h of capture stress; treatment with melatonin, 5-hydroxytryptophan, or ketanserin prior to capture stress had no significant influence on plasma corticosterone concentrations. Androgen concentrations of mid-latitude red-spotted garter snakes in response to capture stress were significantly lower than those of non-stressed control snakes. Neither melatonin nor 5-hydroxytryptophan influenced the change in androgen concentrations during capture stress. However, androgen concentrations of snakes treated with ketanserin prior to 4 h of capture stress did not differ significantly from those of non-stressed control snakes. These studies suggest that melatonin does not modulate hormonal responses to capture stress in this ectothermic model. Our results also suggest that a serotonin-regulated system may play a role in modulating the activity of the hypothalamic-pituitary-gonadal axis during physiological stress responses.

Introduction

Hormonal responses to stressors are protective events that help vertebrates respond to external noxious stimuli. A stressor can be any perturbation that disrupts the predictability of an animal's environment. Examples of stressors include predation events and unpredicted challenges to energy homeostasis, such as unusually low

environmental temperatures, food shortage, and starvation. Responses to stressors are mediated by an increase in the activity of the hypothalamic-pituitary-adrenal axis and are marked by increased glucocorticoid secretion (Harvey *et al*. 1984; Schwabl *et al*. 1985; Wingfield 1988). Glucocorticoids in turn modulate a variety of physiological and behavioral processes that promote survival while suppressing behaviors, such as reproduction, that are not crucial to immediate survival (e.g., Pottinger 1999; Sapolsky 1992; Wingfield 1988). Such acute physiological stress responses are normally adaptive responses used to modify metabolism and mobilize energy stores.

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

Hormonal and behavioral responses to a stressor depend on many factors, including the environment inhabited, the season, and the influence of other hormones. Melatonin, for example, the major secretory product of the pineal gland, inhibits the hypothalamic-pituitary-adrenal axis (Reiter 1991; Wang *et al*. 1999). Interactions between the pineal gland and hypothalamic-pituitary-adrenal axis are well established (e.g., Khan *et al*. 1990; Kirby *et al*. 1999; Maestroni *et al*. 1989), although the nature of

this relationship is poorly understood. Both stimulatory (Al-Dujaili *et al*. 1982; Haus *et al*. 1996; Persengiev *et al*. 1989; Touitou *et al*. 1989) and inhibitory (Heiman and Porter 1980; Ng 1987; Nussdorfer *et al*. 1990) effects of melatonin on the secretory activity of the adrenal cortex have been described. However, all of these studies examined the effects of melatonin on cultured adrenocortical cells (Appa-Rao *et al*. 2001). In an experiment using *in vivo* physiological conditions, melatonin exerted a direct antisecretory effect on the adrenal gland (Appa-Rao *et al*. 2001). In addition, chronic melatonin treatment alters the affinity of glucocorticoid receptors in the brain and pituitary (Marinova *et al*. 1991).

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

In mammals, the effects of melatonin on physiological stress responses appear to involve both direct antisecretory actions on the adrenal cortex and antagonism of glucocorticoid actions at the target tissues. However, the role of melatonin in modulating behavioral and hormonal responses to stress and exogenous glucocorticoids has been largely uninvestigated in nonmammalian species. Lutterschmidt *et al*. (2004) demonstrated that melatonin does not influence responses of red-sided garter snakes (*T.*

sirtalis parietalis) to exogenous corticosterone [the primary glucocorticoid in snakes (Idler 1972)]. In contrast, melatonin and corticosterone have significant additive inhibitory effects on reproductive behavior (Lutterschmidt *et al*. 2004). These results indicate that melatonin does not antagonize glucocorticoid actions in red-sided garter snakes. In the present studies, we investigated whether melatonin has antisecretory effects on the adrenal cortex by examining the influence of melatonin on hormonal responses to capture stress in two populations of male garter snakes, *Thamnophis sirtalis*.

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

concentrations (Moore *et al*. 2001; see Discussion for a more detailed description of these studies).

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

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

responses might be due to antagonism of serotonin receptors, we examined the influence of ketanserin, a serotonergic type 2A receptor antagonist, on hormonal responses to capture stress.

Materials and Methods

These experiments were conducted in the field with free-living garter snakes (*Thamnophis sirtalis*) in the Interlake region of Manitoba, Canada (50° 37' N, 97° 32' W) and the Willamette Valley of western Oregon, USA (44° 30' N, 123° 17' W). Studies of northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) during the spring were conducted between 1000 and 1630 h on 19-21 May 2003, during the month following emergence from hibernacula when snakes are mating and plasma testosterone concentrations are declining (Krohmer *et al*. 1987). Fall experiments in northern-latitude *T. sirtalis parietalis* were conducted between 1200 and 1800 h on 11-13 September 2003, when snakes are returning to the den site to over-winter. Both spring and fall experiments were conducted with northern-latitude red-sided garter snakes collected from a den located in Inwood, Manitoba, Canada. Studies of the mid-latitude red-spotted garter snake were conducted during the breeding season at E.E. Wilson Wildlife Area (approximately 15 km north of Corvallis, Oregon, USA) between 1030 and 2000 h on 23 March-12 April 2004. All experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol number: LAR-2661) and were in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals”. This research was approved by the Manitoba Wildlife Animal

Care Committee (protocol number: 2002-06) and was performed under the authority of Manitoba Wildlife Scientific Permit No. WSP 03009.

Reagents

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

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

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

5-Hydroxytryptophan solution was prepared by dissolving 30 mg of 5-hydroxytryptophan in 5 ml of 5% ethanol in reptile Ringer's solution, producing a 5-hydroxytryptophan concentration of 6 mg ml^{-1}. Thus, the 5-hydroxytryptophan treatment dose was 0.6 mg per snake. For an average male snake weighing 0.03 kg, this dose is 20

mg kg^{-1} body mass, which is identical to the dose used by Rawding and Hutchison (1993) for testing the effects of 5-hydroxytryptophan on melatonin synthesis in mudpuppies (*Necturus maculosus*).

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

Experimental Design

Male garter snakes collected in the field were randomly assigned to one of 6 capture stress treatments (10-12 in each group): no injection, vehicle (5% ethanol in reptile Ringer's solution), low melatonin dose (0.03 mg), high melatonin dose (0.3 mg), 5-hyroxytryptophan (0.6 mg), or ketanserin (0.045 mg). Following treatment injections, snakes were housed in circular outdoor arenas (48 cm diameter) and allowed to absorb the treatment drugs for 30 min. Snakes were then immediately isolated individually in small, opaque cloth bags (approximately 20 × 20 cm) for 4 h. This capture-stress protocol is identical to that of Moore *et al*. (2000*a*), who used capture and isolation of

snakes in these cloth bags to induce physiological stress responses. We collected blood samples from snakes at the end of each 4-h capture stress treatment. For the non-stressed control groups, blood samples were collected at approximately the same time as the capture stress-treated snakes from snakes captured in the field.

Blood Sampling and Radioimmunoassay

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

To test whether chromatography of steroid hormones extracted from snake plasma is necessary, we simultaneously analyzed a subset of plasma samples (n = 30 for northern-latitude *T. sirtalis parietalis*; n = 18 for mid-latitude *T. sirtalis concinnus*) for corticosterone and testosterone concentrations using both radioimmunoassay with partition chromatography (Moore *et al*. 2000*a*) and radioimmunoassay without partition chromatography (i.e., direct radioimmunoassay). For the northern-latitude red-sided

garter snake, we used a subset of plasma samples representing both spring and fall seasons (n = 15 spring and 15 fall samples) to test the necessity of steroid hormone chromatography. We included both spring and fall samples in our evaluations to account for variation in plasma lipid concentrations, and hence different levels of nonspecific binding of steroids, typically observed at different times of the year. The subset of plasma samples used to evaluate the necessity of chromatography of samples from mid-latitude red-spotted garter snakes represented the spring season only, as these snakes were only sampled during the spring. The methods used for direct radioimmunoassay are described in Lutterschmidt *et al*. (2004) and are similar to those of Jessop *et al*. (1999, 2000).

For individual sample recovery determination, duplicate aliquots (20-70 µl) of each plasma sample were incubated 12-24 h with 2000 cpm of tritiated steroid (Amersham Biosciences, Piscataway, NJ). Steroids were extracted from each plasma sample twice with 2 ml anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas in a warm (37°C) water bath. Hormone extracts were then either reconstituted in phosphate-buffered saline for direct assay or reconstituted in 10% ethyl acetate in isooctane and chromatographed through individual celite microcolumns. Steroid fractions and neutral lipids were eluted using increasing proportions of ethyl acetate in isooctane. The purified eluates were dried under nitrogen gas and reconstituted in phosphate-buffered saline for assay.

Individual sample recoveries were determined from a 50-µl aliquot of each extracted and reconstituted sample. For each steroid hormone being assayed, the

remaining sample was allocated to two duplicate culture tubes for assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were then incubated with 100 µl tritiated steroid (1,2,6,7-^{3}H testosterone or 1,2,6,7-^{3}H corticosterone, Amersham Biosciences, Piscataway, NJ) and 100 µl antiserum (testosterone antibody T3003 from Wien Laboratories, Inc., Succasunna, NJ; corticosterone antibody B3-163 from Esoterix Endocrinology, Calabasas Hills, CA) at 4ºC for 12-24 h. Unbound steroid was separated from bound hormone using dextran-coated charcoal. The bound steroid was decanted into scintillation vials and incubated in toluene-based scintillation fluid for 12 h. The radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter.

All samples were corrected for individual recovery variation. Mean extraction efficiency was 101.8% (98.7% for corticosterone and 104.8% for testosterone), as determined by the recovery of tritiated steroid added to samples prior to extraction with ethyl ether. Extraction efficiency in direct radioimmunoassay is typically much greater (> 95%) than mean extraction efficiency following column chromatography. Because we have recently checked the calibration of our pipettes, the extraction efficiency reported here likely represents random variation around this greater mean extraction efficiency. All spring and fall samples (i.e., treatment groups) from northern-latitude *T. sirtalis parietalis* were randomly distributed across 4 steroid assays. Mean intra-assay variation was 13.5% for testosterone and 12.2% for corticosterone; inter-assay variation was 18.1% for testosterone and 13.7% for corticosterone. All samples (i.e., treatment groups) from mid-latitude *T. sirtalis concinnus* were randomly distributed across 2 steroid assays.

Mean intra-assay variation of samples from *T. sirtalis concinnus* was 7.8% for testosterone and 13.9% for corticosterone; inter-assay variation was 9.3% for testosterone and 13.3% for corticosterone.

Statistical Analyses

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

Results

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

Results from a MANOVA indicate no statistically significant differences in corticosterone or androgen concentrations among the treatment groups of northern-latitude red-sided garter snakes (Figure 3.1 A-D). There were no significant effects of season on corticosterone concentrations. As expected, androgen concentrations of northern-latitude red-sided garter snakes were significantly higher during the fall ($P \leq$

0.001 from a MANOVA). There were no statistically significant interactions between season and treatment.

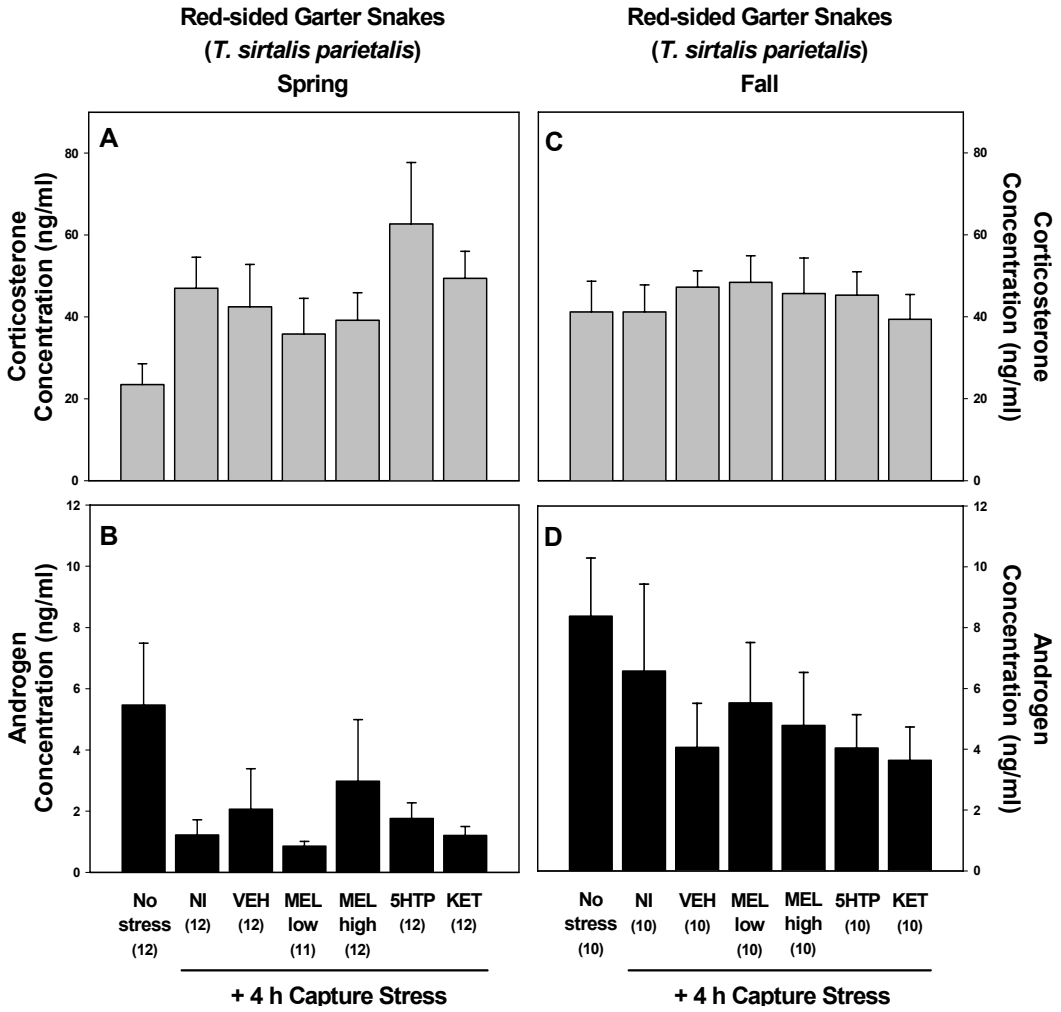

Figure 3.1. Hormonal responses of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) to capture stress during the spring and fall. Male garter snakes collected in the field in south-central Manitoba, Canada received the following treatments (indicated along the abscissa) prior to 4 h of capture stress: no injection (NI), vehicle (5% ethanol in reptile Ringer's solution; VEH), low melatonin dose (0.03 mg; MEL low), high melatonin dose (0.3 mg; MEL high), 5-hydroxytryptophan (0.6 mg; 5HTP), or ketanserin (0.045 mg; KET). A control group of snakes (No stress) received no capture stress treatment. Panels (A) and (C) show mean corticosterone concentrations of snakes during the spring and fall, respectively. Panels (B) and (D) show mean androgen concentrations during the spring and fall, respectively. Sample sizes are indicated in parentheses below the treatment groups. Standard errors (+ 1) are shown by the vertical lines.

Mid-latitude red-spotted garter snakes showed a statistically significant increase in corticosterone in response to 4 h of capture stress (Figure 3.2A; $F = 3.425$, $df = 6$, $P = 0.005$, from a MANOVA). Results from a Student-Newman-Keuls multiple comparisons procedure indicate that treatment with melatonin, 5-hydroxytryptophan, or ketanserin had no significant influence on the corticosterone concentrations of red-spotted garter snakes in response to capture stress (Figure 3.2A). Androgen concentrations of mid-latitude red-spotted garter snakes subjected to 4 h of capture stress were significantly lower than those of non-stressed control snakes (Figure 3.2B; $F = 2.906$, $df = 6$, $P = 0.014$, from a MANOVA). Treatment of snakes with either melatonin or 5-hydroxytryptophan did not influence the androgen concentrations of mid-latitude red-spotted garter snakes in response to capture stress (Figure 3.2B; results from a Student-Newman-Keuls multiple comparisons procedure). Androgen concentrations of snakes treated with ketanserin prior to 4 h of capture stress did not differ significantly from the androgen concentrations of non-stressed control snakes (Figure 3.2B).

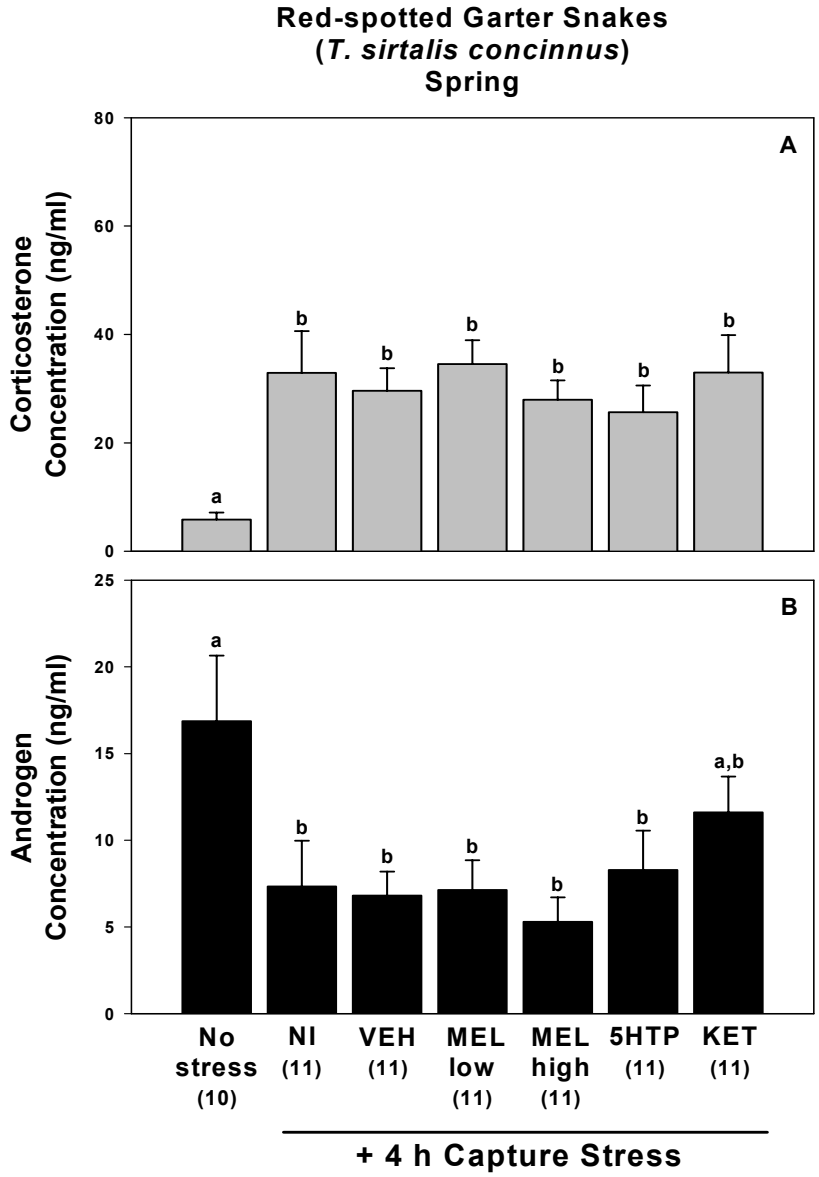

Figure 3.2. Hormonal responses of male red-spotted garter snakes (*Thamnophis sirtalis concinnus*) to capture stress during the spring mating season. Male garter snakes collected in the field in western Oregon, USA, received the following treatments (indicated along the abscissa) prior to 4 h of capture stress: no injection (NI), vehicle (5% ethanol in reptile Ringer's solution; VEH), low melatonin dose (0.03 mg; MEL low), high melatonin dose (0.3 mg; MEL high), 5-hydroxytryptophan (0.6 mg; 5HTP), or ketanserin (0.045 mg; KET). A control group of snakes (No stress) received no capture stress treatment. Panel (A) shows mean plasma corticosterone concentrations of snakes; panel (B) shows mean plasma androgen concentrations. Sample sizes are indicated in parentheses below the treatment groups. Statistically significant differences among treatment groups are indicated by letters above each standard error (+ 1) bar.

Discussion

Our results indicate that northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) respond to 4 h of capture stress during the spring and fall seasons with no significant changes in either corticosterone or androgen concentrations. Although we observed a trend of increased corticosterone and decreased androgen concentrations in response to capture stress during the spring mating season (Figures 3.1A, 3.1B), our multivariate analyses indicated that these differences were not statistically significant. This lack of statistical difference most likely results from the large variation in hormone concentrations both within and among groups. Indeed, a t-test indicates that northern-latitude red-sided garter snakes receiving no injection + 4 h of capture stress during the spring had significantly higher corticosterone levels than non-stressed control snakes ($P = 0.018$; Figure 3.1A). A t-test between the androgen concentrations of non-stressed control snakes and non-injected capture stress-treated snakes does not resolve the large variation observed in androgen concentrations, as it indicates no statistically significant difference between these groups of northern-latitude red-sided garter snakes (Figure 3.1B). Similar to Moore *et al*. (2001), we demonstrate that mid-latitude red-spotted garter snakes (*T. sirtalis concinnus*) respond to 4 h of capture stress with a significant increase in plasma corticosterone and a significant decrease in androgen concentrations.

Seasonal Variation in Hormonal Responses to Capture Stress

The trends we observed in northern-latitude red-sided garter snakes are similar to those reported by Moore *et al*. (2000*a*), who demonstrated a significant increase in

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

In these populations of northern-latitude red-sided garter snakes, a significant negative relationship exists between basal corticosterone concentrations and body condition (Moore *et al*. 2001). Because snakes actively forage for only 3-4 months before undergoing 8 months of winter dormancy, seasonal changes in body condition are pronounced. For example, during the summer and fall, northern-latitude red-sided garter snakes have positive body condition (determined as an individual's residual from a regression of body mass on snout-to-vent length for all snakes across seasons), with the greatest positive body condition occurring during the summer feeding months (Moore *et al*. 2001). In contrast, snakes captured immediately following emergence from the hibernaculum had a negative body condition. Body condition of snakes during late spring (i.e., late in the mating season) was more than 6-fold lower than body condition of snakes during early spring (Moore *et al*. 2001), likely due to sustained mating activity.

Snakes are aphagic during the mating season, and therefore sustaining mating activity is extremely costly; snakes can lose up to 1-2 % of their body mass per day (Mason, unpublished data).

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

During the fall, northern-latitude red-sided garter snakes showed no change in corticosterone or androgen concentrations in response to capture stress (Figure 3.1C and 3.1D). These results are similar to the findings of Moore *et al*. (2001), who reported no significant change in corticosterone concentrations and a significant decrease in testosterone levels following 4 h of capture stress during the fall. In both this study and in Moore *et al*. (2001), experiments were conducted at the den site with snakes that had returned to the den in preparation for winter dormancy. We hypothesize that northern-

latitude red-sided garter snakes suppress at least hormonal responses to capture stress during the fall pre-hibernation period. Although further investigation is necessary, suppression of corticosterone responses to capture stress prior to entering winter dormancy may be adaptive. Because fat stores are necessary to sustain snakes during winter dormancy, suppression of hormonal stress responses prior to entering winter dormancy would conserve these much-needed fat stores. Additional studies of hormonal stress responses in snakes both pre- and post-migration would provide insight into whether stress responses are modulated by the mechanisms regulating fall migration to the dens and preparation for winter dormancy.

Melatonin and Hormonal Responses to Capture Stress

We investigated whether melatonin and 5-hyroxytyrptophan, a precursor of melatonin synthesis, modulate the responses of garter snakes to capture stress. Although there is evidence that melatonin inhibits the secretion of glucocorticoids in mammals *in vivo* (Appa-Rao *et al*. 2001), our results suggest that melatonin does not play a role in modulating hormonal stress responses in this ectothermic model. However, we measured corticosterone and androgen concentrations of snakes at only one time point (i.e., 4 h) following capture stress. Thus, it is possible that melatonin does indeed modulate stress responses in these snakes but we were unable to observe these effects due to our sampling regime. For example, melatonin may reduce initial hormonal responses to acute stressors but may not affect responses to stressors of longer (i.e., 4 h) duration. During the spring, levels of corticosterone in both northern-latitude red-sided garter

snakes and mid-latitude red-spotted garter snakes subjected to 1 h of capture stress are similar to those in response to 4 h of capture stress (Moore *et al*. 2001). Thus, it is not likely that corticosterone concentrations had significantly declined over our 4-h capture stress period. In addition, we are confident that plasma melatonin levels, in response to hormone treatments, remained elevated throughout the duration of our experiments. The half-life of melatonin after injection into endotherms can be 1 h or less (Rollag and Stetson 1982); the half-life of melatonin in whole-animal ectotherms is likely to be much longer because metabolic rate is as much as 10 times lower, depending on body temperature (Filadelfi and Castrucci 1996). Future studies investigating the time course of melatonin's influence on hormonal responses to capture stress are necessary to discern whether melatonin does indeed regulate stress responses.

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

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

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

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

Northern-latitude red-sided garter snakes spend 8 months each year in winter dormancy in underground hibernacula. Throughout much of the year, these snakes are therefore not exposed to the photoperiod cues that regulate melatonin cycles. However, circadian melatonin cycles have been observed in red-sided garter snakes during the spring (e.g., Mendonça *et al*. 1996*b*). In addition, melatonin modulates reproductive behavior of northern-latitude red-sided garter snakes during the spring mating season (Lutterschmidt *et al*. 2004). Thus, melatonin appears to play a functional role in regulating the seasonal biology of red-sided garter snakes. Given the unique life history of the northern-latitude red-sided garter snake, temperature is likely the most important proximate environmental cue regulating circadian melatonin cycles and seasonal reproductive behavior. For example, photoperiod prior to and during hibernation has no influence on the initiation and timing of reproductive behavior of northern-latitude red-sided garter snakes upon emergence (e.g., Nelson *et al.*1987; Whittier *et al.*1987*b*). Red-

sided garter snakes also require a period of low temperature conditions to initiate sexual behavior upon re-exposure to warm temperatures (Camazine *et al*. 1980; Bona-Gallo and Licht 1983). Our laboratory is currently examining how temperature, in the absence of photoperiod cues, may function as a *zeitgeber* in entraining circadian melatonin cycles and regulating seasonal reproduction. We are particularly interested in investigating possible differences in both the production and function of melatonin among populations having very different seasonal biologies.

Serotonin and Hormonal Responses to Capture Stress

To test whether a serotonin-regulated system may be involved in inducing responses to capture stress, we examined the influence of ketanserin, a serotonergic type 2A receptor antagonist, on hormonal responses to capture stress. Ketanserin had no effect on the corticosterone responses of northern-latitude red-sided garter snakes or mid-latitude red-spotted garter snakes to 4 h of capture stress. However, treatment of mid-latitude red-spotted garter snakes with ketanserin prior to capture stress reduced the decline in androgen concentrations (Figure 3.2B). These results suggest that a serotonin-regulated system is involved in mediating the effects of capture stress on the hypothalamic-pituitary-gonadal axis.

The modulation of glucocorticoid actions by melatonin are thought to be mediated by its properties as a serotonergic type 2A receptor antagonist (Eison *et al*. 1995; Gorzalka *et al*. 1999). In these experiments, ketanserin, but not melatonin, influenced androgen concentrations of northern-latitude red-spotted garter snakes (*T. sirtalis*

concinnus) in response to capture stress. In addition, although serotonin influences testosterone secretion in rat testis (Csaba 1998; Pieścikowska *et al*. 1999), ketanserin (0.045 mg) does not significantly influence plasma androgen concentrations of northern-latitude red-sided garter snakes (Lutterschmidt *et al*. 2004). Thus, the effects of ketanserin on androgen concentrations during stress responses are not a direct effect of ketanserin itself, but rather may be mediated via a serotonin-regulated pathway that is activated during the stress response.

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

in modulating the activity of the hypothalamic-pituitary-gonadal axis during physiological stress responses.

There is much precedence for interactions between serotonin and corticosterone in modulating physiology and behavior (e.g., Gorzalka *et al*. 1998; Mendelson and McEwen 1992; Stutzmann *et al*. 1998). For example, corticosterone increases the density of central serotonergic type 2A receptors and facilitates serotonergic type 2A receptor-mediated behaviors (Berendsen *et al.* 1996; Fernandes *et al.* 1997; Gorzalka and Hanson 1998; Takao *et al.* 1997). In addition, Lutterschmidt *et al*. (2004) suggest that a serotonin-regulated system is involved in mediating the inhibitory effects of melatonin and corticosterone on courtship behavior in northern-latitude red-sided garter snakes (*T. sirtalis parietalis*). In mammals, corticosterone has little or no effect on modulating serotonin metabolism (Chaouloff 1993). Thus, the effects of corticosterone on serotonergic type 2A receptor-mediated behaviors, such as an increase in wet-dog shakes in rats, are likely due to a specific receptor-mediated mechanism, rather than simply a modulation of serotonin metabolism (Gorzalka *et al*. 1999). Further studies are necessary to determine whether serotonin does indeed play a role in orchestrating changes in physiology and behavior during stress responses.

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CHAPTER 4

CIRCADIAN MELATONIN AND CORTICOSTERONE RHYTHMS DURING SPRING EMERGENCE IN RED-SIDED GARTER SNAKES (*THAMNOPHIS SIRTALIS PARIETALIS*): EFFECTS OF STRESS AND EXOGENOUS CORTICOSTERONE

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Abstract

Circadian and circannual rhythms in physiology and behavior are temporally organized via hormonal signals that reflect changing environmental cues (e.g., photoperiod and temperature). Interactions between endocrine signals are in turn important in integrating multiple physiological and behavioral rhythms. In the present study, we sought to examine interactions between melatonin, the hypothalamus-pituitary-adrenal (HPA) axis, and corticosterone in a well-studied population of red-sided garter snakes (*Thamnophis sirtalis parietalis*). We demonstrate that capture stress, but not exogenous corticosterone, significantly increases melatonin concentrations of snakes. Pretreatment of snakes with both capture stress and exogenous corticosterone abolishes the increase in scotophasic melatonin synthesis induced by 5-hydroxytryptophan treatment. These experiments indicate the different phases of an acute physiological stress response (i.e., activation of the HPA axis, increased glucocorticoid secretion) have distinct and temporally different effects on pineal melatonin synthesis. To investigate whether interactions between melatonin and corticosterone play a functional role in regulating the seasonal biology of red-sided garter snakes, we measured circadian melatonin and corticosterone rhythms during spring emergence from prolonged winter dormancy. When red-sided garter snakes first emerge from underground hibernacula following 8 months of winter dormancy, melatonin cycles are already synchronized with photoperiodic conditions (i.e., the highest melatonin concentrations occur during scotophase). These findings suggest the *zeitgeber* entraining circadian melatonin rhythms occurs prior to, or during, spring emergence. A significant corticosterone

rhythm was also observed upon emergence with peak levels occurring during scotophase; corticosterone rhythms phase-shifted approximately 180º following emergence from winter dormancy. This phase-shift in the corticosterone rhythm may play a role in regulating the seasonal transition between reproduction and dispersal from the den site in red-sided garter snakes. Collectively, our experiments demonstrate that a physiological coupling between melatonin, glucocorticoids, and the HPA axis is conserved in this ectothermic model. We suggest that such a physiological coupling plays a functional role in regulating seasonal rhythms.

Introduction

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

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

Interactions between melatonin, glucocorticoids, and the HPA axis are well established (e.g., Barriga *et al*. 2002; Maestroni *et al*. 1989; Otsuka *et al*. 2001). For example, treatment of adult and juvenile rats with melatonin prevents some of the injurious effects induced by chronically-elevated glucocorticoids, such as the reduction in growth and atrophy of the thymus and adrenal glands (Aoyama *et al*. 1986, 1987). In male rats, melatonin also significantly reduces the inhibitory effects of acute and chronic stress on sexual behavior (Brotto *et al*. 2001).

Activation of the HPA axis during physiological stress responses in turn modulates melatonin cycles. Social stress modulates melatonin cycles in rainbow trout, *Oncorhynchus mykiss* (Larson *et al*. 2004) and tree shrews, *Tupaia belangeri* (Fuchs and

Schumacher 1990). In ring doves (*Streptopelia risoria*), immobilization stress significantly increases melatonin levels during photophase but decreases melatonin levels during scotophase (Barriga *et al*. 2002; Rodríguez *et al*. 2001). Physiological stress responses therefore appear to abolish the cyclicity of melatonin synthesis (Persengiev and Kanchev 1991). Increased melatonin synthesis following a stress response is thought to aid in combating oxidative damage induced by elevated glucocorticoid concentrations. Indeed, Sainz *et al*. (1995) demonstrated that melatonin alleviates glucocorticoid-induced apoptosis of thymocytes via an antioxidant mechanism. The observed decrease in scotophasic melatonin levels following a stress response is hypothesized to be the result of a rapid decline in the concentration of tryptophan, the amino acid precursor for melatonin synthesis (Clark and Russo 1997). The effects of stress on circadian melatonin secretion may result from the direct actions of glucocorticoids on the pineal gland (e.g., Beck-Friis *et al*. 1983; Brismar *et al*. 1985; Ferreira *et al*. 2005).

Despite much research, the physiological coupling between melatonin, glucocorticoids, and the HPA axis remains unclear. Furthermore, whether or not a possible coupling between melatonin and glucocorticoids is evolutionarily conserved or more recently derived among birds and mammals is poorly understood, primarily because studies addressing these questions in ectothermic vertebrates are lacking. In the present study, we sought to examine interactions between melatonin and the HPA axis in a well-studied population of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Red-sided garter snakes are the most northerly living reptile in North America and are found in extremely high numbers throughout south central Manitoba, Canada. These extreme-

latitude populations hibernate for approximately 8 months each year in underground hibernacula, void of any photoperiod cues. Snakes emerge in the spring and immediately enter an intense mating season for approximately 4-5 weeks. During this time, gonads are regressed, sex steroid levels are basal, and glucocorticoid levels are elevated (Crews *et al*. 1984; Krohmer *et al*. 1987). Female red-sided garter snakes generally leave the den site immediately following emergence and mating, while male snakes remain within the vicinity of the den and typically exhibit courtship behavior for several weeks (Shine *et al*. 2001). Snakes are aphagic while actively courting at the den site (O'Donnell *et al*. 2004) and must disperse as far as 20 km to summer feeding grounds to forage (Gregory 1977). Because snakes enter this energetically costly courtship and dispersal period subsequent to an eight-month dormancy period, elevated glucocorticoid levels likely facilitate reproduction and daily activity by mobilizing much-needed energy stores (e.g., Moore and Jessop 2003).

Both melatonin and glucocorticoids play important roles in regulating the seasonal biology of red-sided garter snakes (e.g., Lutterschmidt *et al*. 2004; Mendonça *et al*. 1996; Moore *et al*. 2000). This model system therefore provides an excellent opportunity for investigating possible physiological coupling between melatonin and the HPA axis. We previously demonstrated that melatonin does not possess "anti-stress" actions in red-sided garter snakes, as melatonin neither antagonizes glucocorticoid actions on reproductive behavior (Lutterschmidt *et al*. 2004) nor influences glucocorticoid responses to capture stress (Lutterschmidt and Mason 2005). The aim of the present study was to determine if physiological stress responses alter circadian

melatonin rhythms in this population of garter snakes and, if so, whether the effects of stress on melatonin cycles results from increased glucocorticoid secretion or the stress response itself (i.e., increased activity of the sympathetic nervous system). In addition, we investigated whether stress-induced melatonin rhythms could be restored by treatment with 5-hydroxytryptophan, a precursor of melatonin synthesis. Lastly, we examined whether a physiological coupling between melatonin and glucocorticoid rhythms might play a role in regulating the seasonal biology of red-sided garter snakes during the spring mating season.

Materials and Methods

Experiments were conducted in the field with free-ranging red-sided garter snakes (*Thamnophis sirtalis parietalis*) in the Interlake region of Manitoba, Canada (50° 37' N, 97° 32' W). Studies were conducted during the spring mating seasons of 2003-2005 during the month following emergence from winter hibernacula. All animals were returned to the site of capture upon conclusion of the experiments. Experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol numbers: 2661, 3120) and were in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This research was approved by the Manitoba Wildlife Animal Care Committee (protocol number: 2002-06) and was performed under the authority of Manitoba Wildlife Scientific Permits WSP 03009 and 04004.

Hormone Treatments

Corticosterone, the primary glucocorticoid in snakes (Idler 1972), and 5-hydroxytryptophan, a precursor of melatonin synthesis, were purchased from Sigma (St. Louis, MO). All treatments were administered via intraperitoneal injection with an injection volume of 0.1 ml. Injection volumes of vehicle (5% ethanol in reptile Ringer's solution) were also 0.1 ml. All treatment solutions were prepared fresh daily.

Corticosterone solutions were prepared by first dissolving 3 or 12 mg corticosterone in 1 ml of 100% ethanol. Stock solutions were then diluted to 20 ml with reptile Ringer's solution, producing corticosterone concentrations for the low and high doses of 150 and 600 μg ml^{-1}, respectively. Thus, the corticosterone treatment doses were 15 or 60 μg per snake (i.e., 15 or 60 μg per 0.1 ml). These corticosterone doses are identical to those used by Lutterschmidt *et al*. (2004) to test the effects of melatonin on the behavioral responses of red-sided garter snakes to exogenous corticosterone. As demonstrated behaviorally by Moore and Mason (2001) and Lutterschmidt *et al*. (2004), these corticosterone treatment doses produce physiologically relevant increases in circulating corticosterone.

5-Hydroxytryptophan solutions were prepared by dissolving 30 or 60 mg 5-hydroxytryptophan in 5 ml of 5% ethanol in reptile Ringer's solution. This produced 5-hydroxytryptophan concentrations for the low and high doses of 6 and 12 mg ml^{-1}, respectively. Thus, the 5-hydroxytryptophan treatment doses were 0.6 or 1.2 mg per snake (i.e., per 0.1 ml). For an average male snake weighing 0.03 kg, this dose is 20 mg kg^{-1} body mass, which is identical to the dose used by Rawding and Hutchison (1993) for

testing the effects of 5-hydroxytryptophan on melatonin synthesis in mudpuppies (*Necturus maculosus*). During the scotophase, treatment of mudpuppies with 20 mg kg^{-1} 5-hydroxytryptophan significantly elevates melatonin synthesis for more than 4 h (Rawding and Hutchison 1993).

Experimental Design

Experiment 1: Effect of capture stress on melatonin rhythms. Twenty-four male red-sided garter snakes were collected from the den site and subjected to a capture stress protocol identical to that of Moore *et al*. (2000) and Lutterschmidt and Mason (2005). Upon capture, snakes were immediately isolated individually in small, opaque cloth bags (approximately 20 × 20 cm) for 4 h to induce physiological stress responses. Following capture stress treatment from 1000-1400 h, we collected blood samples from a subset of snakes (n = 12) to determine the effects of stress on photophasic melatonin levels. The remainder of the capture stress-treated snakes (n = 12) were removed from the cloth bags and housed in a circular outdoor arena (48 cm diameter) until blood samples were collected at 0000 h to determine the effects of capture stress on scotophasic melatonin levels. For the non-stress control groups, photophasic blood samples were collected at approximately the same time as the capture stress-treated snakes from snakes captured in the field. To determine scotophasic melatonin levels of untreated male snakes, 12 additional males were collected from the den site at 1400 h and housed in an outdoor arena until blood samples were collected at 0000 h.

Experiment 2: Effect of exogenous corticosterone on melatonin rhythms. To investigate whether exogenous corticosterone treatment mimics the effects of capture stress on melatonin cycles, we collected male red-sided garter snakes from the den site and randomly assigned them to one of 3 treatment groups (n = 48 in each group): vehicle (5% ethanol in reptile Ringer's solution), low corticosterone dose (15 μg), or high corticosterone dose (60 μg). Following intraperitoneal treatment injections at 1200 h, snakes were housed in outdoor arenas (48 cm diameter) and allowed to absorb the treatments. Photophasic blood samples were collected at 1400 h from a subset of snakes (n = 12) in each treatment group. The remaining snakes were kept in the outdoor arenas until blood samples were collected during the scotophase at 2200, 0000, and 0200 h (n = 12 for each sampling period in each treatment group).

Experiment 3: Effect of 5-hydroxytrptophan on melatonin rhythms. To verify that treatment with 5-hydroxytryptophan increases scotophasic melatonin production, we collected 108 male snakes from the den site at 1200 h. Snakes were pretreated with an intraperitoneal injection of 5% ethanol in reptile Ringer's solution (i.e., vehicle) and placed in outdoor arenas (48 cm diameter). We pretreated snakes with vehicle to enable appropriate evaluation of the effects of corticosterone pretreatment as described below. At 2000 h, snakes were randomly assigned to one of 3 treatment groups (n = 36 in each group): vehicle (5% ethanol in reptile Ringer's solution), low 5-hydroxytrptophan dose (0.6 mg), or high 5-hydroxytrptophan dose (1.2 mg). Following intraperitoneal treatment injections, snakes were returned to the outdoor arenas and allowed to absorb the

treatment drugs. Blood samples were then collected during the scotophase at 2200, 0000, and 0200 h (n = 12 for each sampling period in each treatment group).

To determine if 5-hydroxytryptophan restores stress-induced scotophasic melatonin concentrations to non-stress levels, we collected 36 male red-sided garter snakes from the den site and subjected them to 4 h of capture stress from 1000 to 1400 h. Following capture-stress treatment, snakes were removed from the cloth bags and housed in outdoor arenas. At 2000 h, snakes received one of the following treatments (n = 12 in each): vehicle (5% ethanol in reptile Ringer's solution), low 5-hydroxytrptophan dose (0.6 mg), or high 5-hydroxytrptophan dose (1.2 mg). Following intraperitoneal treatment injections, snakes were returned to the outdoor arenas and allowed to absorb the treatment drugs. Blood samples were then collected during the scotophase at 0000 h (n = 12 for each treatment group).

To examine if corticosterone alters the effect of 5-hydroxytryptophan on scotophasic melatonin levels, we collected 108 male snakes from the den site at 1200 h. Snakes were pretreated with an intraperitoneal injection of 60 μg corticosterone and were then placed in outdoor arenas. At 2000 h, snakes were randomly assigned to one of 3 treatment groups (n = 36 in each group): vehicle (5% ethanol in reptile Ringer's solution), low 5-hydroxytrptophan dose (0.6 mg), or high 5-hydroxytrptophan dose (1.2 mg). Following treatment, snakes were returned to the outdoor arenas and allowed to absorb the treatment drugs. Blood samples were then collected during the scotophase at 2200, 0000, and 0200 h (n = 12 for each sampling period in each treatment group).

Experiment 4: Role of melatonin and corticosterone rhythms in spring emergence. We examined melatonin and corticosterone rhythms during the spring mating season to investigate whether interactions between these two hormones might play a role in regulating the seasonal emergence of red-sided garter snakes. Female snakes were used in this study because female red-sided garter snakes generally leave the den site immediately following emergence (Shine *et al*. 2001). In contrast, male snakes remain near the den for several weeks (Shine *et al*. 2001) and will typically use the more shallow depths of the den as a nocturnal retreat site, "re-emerging" each day. Thus, we could be confident that an emerging female snake was indeed emerging from the hibernaculum for the first time and had not been exposed previously to photoperiod cues.

During each of the spring mating seasons of 2003-2005, we collected blood samples from female red-sided garter snakes immediately following emergence from the hibernaculum. Blood samples were collected from a subset of snakes ($n \geq 5$ for each sample) every 4 h for a 24-h period; no snake was bled more than once during a 24-h sample. The 1200 h and 1600 h samples were obtained from female snakes collected immediately upon emergence in the field. For all subsequent samples, female red-sided garter snakes were collected following emergence during the afternoon (1200-1700 h) and were housed in outdoor nylon cloth arenas ($1 \times 1 \times 1$ m) until sampled at 2000, 0000, 0400, or 0800 h. This protocol was necessary to obtain blood samples at these sampling times because (1) snakes are inaccessible prior to emergence from the hibernaculum and (2) snakes emerge from the hibernaculum only during the day.

To determine if melatonin and corticosterone rhythms change over the mating season, we collected 56 female snakes following emergence on 3 May 2005. Snakes were housed in outdoor nylon cloth arenas (1 × 1 × 1 m) for approximately 3 weeks to allow acclimatization to ambient photoperiod and temperature rhythms. Hide boxes placed inside the arenas provided retreat sites for the snakes; water was provided ad libitum. On 25-26 May, blood samples were collected from a subset of snakes (n = 8-10 for each sample) every 4 h for a 24-h period beginning at 0800 h; no snake was bled more than once during the 24-h period.

Blood Sampling and Radioimmunoassay

Blood samples (300 μl) were obtained from the caudal vein as quickly as possible (mean ± 1 standard error: 78.1 ± 2.4 seconds) using heparinized 1-cm^3 syringes and 25-g needles. Scotophasic blood samples were collected under dim red light. Samples were stored on ice until return to the field station, where they were centrifuged and the plasma separated. Plasma samples were stored at – 4ºC until return to Oregon State University, where they were transferred to – 70ºC until analyzed for corticosterone and/or melatonin concentrations following radioimmunoassay procedures described by Lutterschmidt *et al*. (2004) and Tilden and Hutchison (1993).

For determination of corticosterone concentrations, an aliquot (6-20 μl) of each plasma sample was incubated overnight with 2000 cpm tritiated steroid (1,2,6,7-^{3}H corticosterone, Amersham Biosciences, Piscataway, NJ) to determine extraction efficiency. Steroids were extracted from each plasma sample with 3 ml anhydrous ethyl

ether. The ether phase was removed and dried under nitrogen gas in a warm water bath and the hormone extracts were then reconstituted in 500 µl phosphate-buffered saline for direct assay. The methods used for direct radioimmunoassay of steroids from male red-sided garter snakes were described and validated in Lutterschmidt *et al*. (2004) and Lutterschmidt and Mason (2005). Individual sample recoveries were determined from a 50-µl aliquot of each reconstituted sample. The remaining sample was allocated to two duplicate culture tubes for assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were then incubated with 100 µl each corticosterone antiserum (B3-163 from Esoterix Endocrinology, Calabasas Hills, CA) and tritiated corticosterone (approximately 10 000 cpm) at 4ºC for 12-24 h. Unbound steroid was separated from bound hormone using dextran-coated charcoal. The bound steroid was decanted into scintillation vials and incubated in toluene-based scintillation fluid for 12 h. The radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter.

Melatonin concentrations were determined from duplicate aliquots (100 µl) of each plasma sample. To determine extraction efficiency, 12-24 replicates of garter snake plasma (100 µl each) were incubated overnight with 2000 cpm tritiated melatonin (*O*-methyl-^{3}H melatonin, Amersham Biosciences, Piscataway, NJ). Tests for sample recovery were run simultaneously with each melatonin assay. Melatonin was extracted from each plasma sample twice with a total of 4.5 ml HPLC-grade chloroform. A portion (4.0 ml) of the chloroform phase was removed and dried under nitrogen gas in a warm water bath and the hormone extracts were then reconstituted in 200 µl tricine-buffered

saline (pH 8.0) for assay. Recovery samples were reconstituted in 400 µl tricine-buffered saline and incubated overnight at 4ºC. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were then incubated with 100 µl melatonin antiserum (Stockgrand LTD, Surrey, UK), 50 µl sheep serum (Sigma, St. Louis, MO), and 50 µl (approximately 6000 cpm) tritiated melatonin at 4ºC for 18-26 h. Unbound melatonin was separated from bound hormone using dextran-coated charcoal. The bound hormone was decanted into scintillation vials and incubated in toluene-based scintillation fluid for 12 h. The radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter. To validate the use of this melatonin radioimmunoassay with plasma from red-sided garter snakes, we tested serially-diluted snake plasma for parallelism with the standard curve (Figure 4.1A). We also performed quantitative recovery tests following addition of melatonin to charcoal-stripped snake plasma to demonstrate that there are no factors in snake plasma that interfere with this competitive binding assay (Figure 4.1B).

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

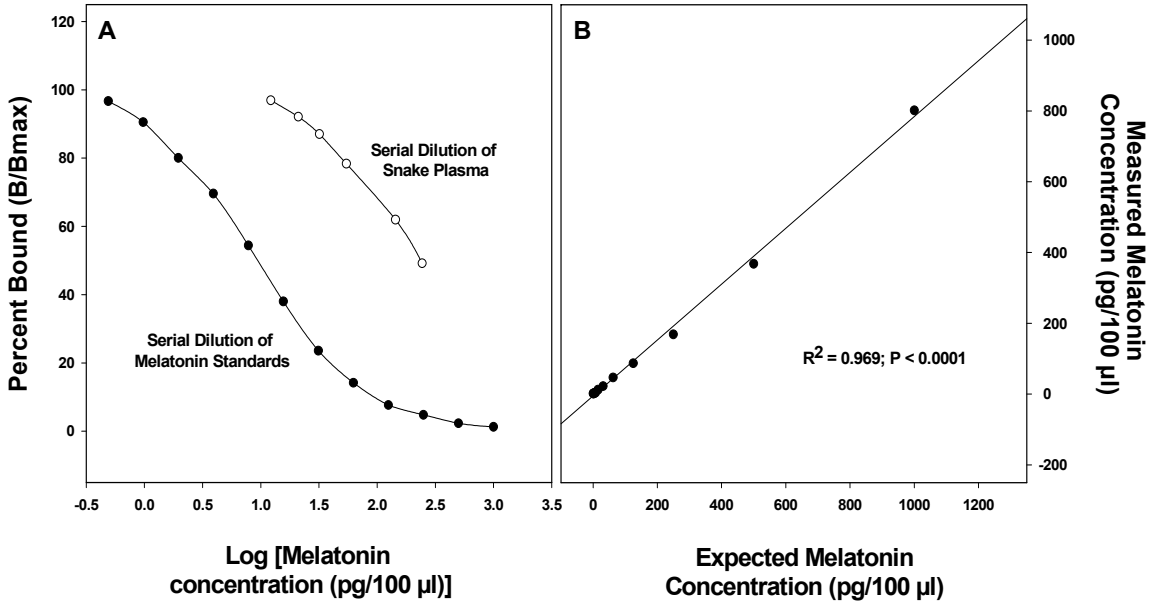

Figure 4.1. Validation of the radioimmunoassay for determining melatonin concentrations in plasma of red-sided garter snakes (*Thamnophis sirtalis parietalis*). (A) Parallelism between serially-diluted melatonin standards (closed symbols) and serially-diluted snake plasma (open symbols). A value of 1 was added to each data point in the serial dilutions of snake plasma to offset the curves. (B) Quantitative recovery of melatonin standard added to charcoal-stripped snake plasma. Both tests demonstrate that there are no factors in snake plasma that interfere with this competitive binding assay.

Statistical Analyses

We used a t-test to verify that capture stress increased corticosterone concentrations of male red-sided garter snakes (Experiment 1). To examine if capture stress modulates melatonin rhythms, we used a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons procedure. Both treatment and time were included in the analysis as between-subjects factors.

To verify that exogenous corticosterone treatment increased corticosterone concentrations of male snakes (Experiment 2), we used a two-way ANOVA followed by

Tukey's multiple comparisons procedure. Both treatment and time were included in the analysis as between-subjects factors. To examine if exogenous corticosterone modulates melatonin rhythms, we used a two-way ANOVA with treatment and time as between-subjects factors.

We used a two-way ANOVA to examine if 5-hydroxytryptophan increases melatonin concentrations of red-sided garter snakes during the scotophase (Experiment 3). Because melatonin responses to 5-hydroxytryptophan treatment did not vary significantly with sampling time, we pooled the 2200, 0000, and 0200 h sampling times and performed an ANOVA followed by a Student-Newman-Keuls multiple comparisons test. We used an ANOVA to investigate whether pretreatment with capture stress alters the effects of 5-hydroxytrptophan on melatonin concentrations. To examine if pretreatment with exogenous corticosterone influences melatonin responses to 5-hydroxytrptophan, we used a two-way ANOVA with treatment and time included in the analysis as between-subjects factors.

Lastly, we used ANOVA's to describe melatonin and corticosterone rhythms upon emergence from winter dormancy (Experiment 4). Because the same trends were observed during each of the 3 mating seasons sampled (2003-2005), we chose to pool the melatonin and corticosterone data across years. Melatonin rhythms were examined using an ANOVA on ranks (due to the non-normal distribution of data) followed by Dunn's multiple comparisons procedure. Corticosterone rhythms were examined using an ANOVA followed by a Tukey's multiple comparisons test. Post-emergence melatonin

and corticosterone rhythms were examined using an ANOVA and an ANOVA followed by a Tukey's multiple comparisons test, respectively.

We used SigmaStat® 2.03 (SPSS 1997) for all statistical analyses. Prior to analysis, data were natural log- or rank-transformed where necessary to correct for non-normality and unequal variance. All statistical comparisons were considered significant at $P \leq 0.05$.

Results

Experiment 1: Effect of Capture Stress on Melatonin Rhythms.

Treatment with 4 h of capture stress during the photophase significantly increased corticosterone concentrations of male snakes (Figure 4.2A; $t = -3.524$, $df = 22$, $P = 0.002$, from a t-test). Melatonin rhythms of male snakes were significantly altered following capture stress (Figure 4.2B). As expected, the effects of capture stress varied significantly with sampling time ($F_{(1,41)} = 6.635$, $P = 0.014$, from a two-way ANOVA). Capture stress significantly increased photophasic melatonin concentrations ($q = 3.249$, $P = 0.027$, from a Tukey's multiple comparisons test) but did not significantly influence scotophasic melatonin concentrations (Figure 4.2B).

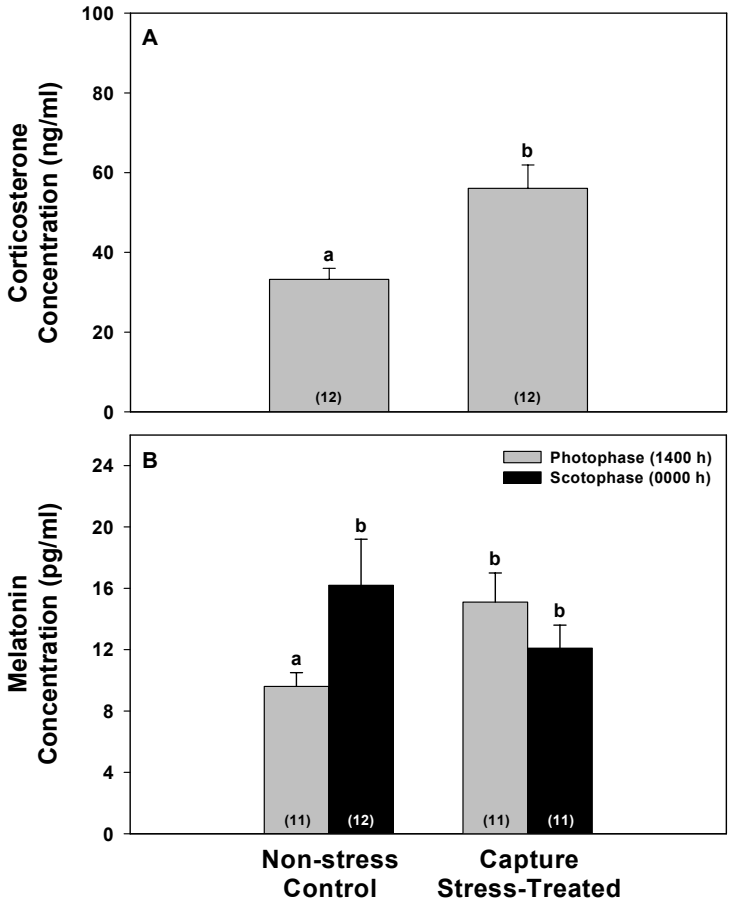

Figure 4.2. Effect of capture stress on (A) corticosterone and (B) melatonin concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Snakes were subjected to capture stress during the photophase from 1000-1400 h. Gray and black bars represent hormone concentrations during the photophase (1400 h) and scotophase (0000 h), respectively. Sample sizes are indicated in parentheses above the abscissa. Statistically significant differences are indicated by letters above each standard error (+ 1) bar.

Experiment 2: Effect of Exogenous Corticosterone on Melatonin Rhythms.

Circulating corticosterone concentrations were significantly elevated following treatment with exogenous corticosterone at 1200 h (Figure 4.3A; $F_{(2,63)} = 15.925$, $P < 0.001$, from a two-way ANOVA). Twelve hours following treatment with exogenous

corticosterone (i.e., at 0000 h), corticosterone levels were not significantly different from those of vehicle-treated snakes (Figure 4.3A). Treatment with exogenous corticosterone did not significantly influence either photophasic or scotophasic melatonin concentrations of snakes (Figure 4.3B).

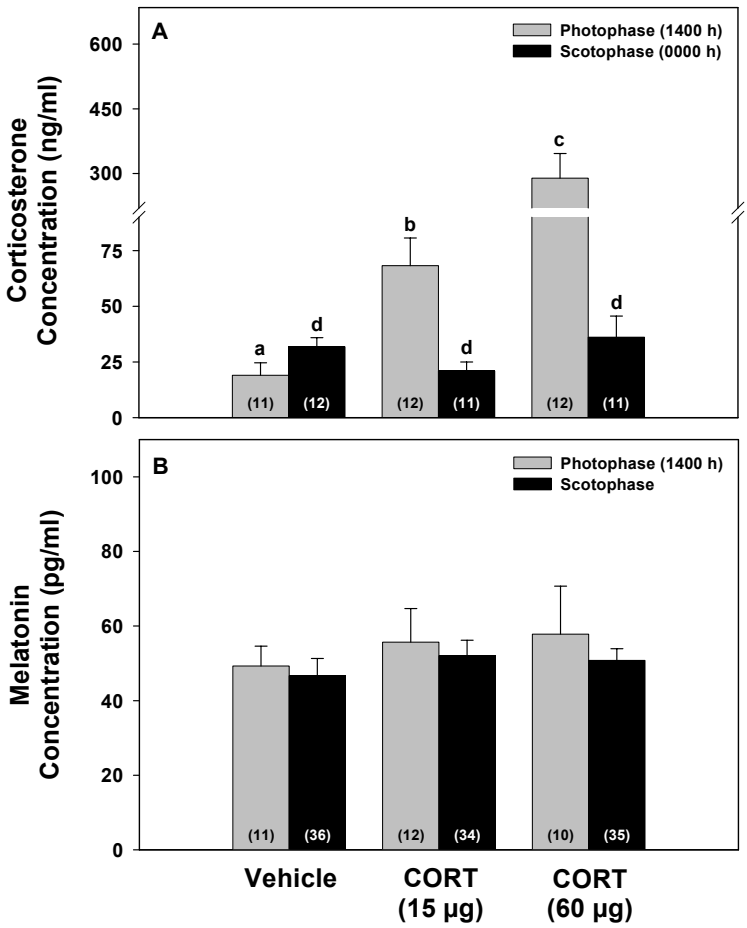

Figure 4.3. Effect of exogenous corticosterone (15 and 60 µg) on (A) corticosterone and (B) melatonin concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Snakes received corticosterone treatments at 1200 h. Gray and black bars represent hormone concentrations during the photophase and scotophase, respectively. Scotophasic melatonin samples (i.e., at 2200, 0000, and 0200 h) are shown pooled for ease of visual interpretation. Sample sizes are indicated in parentheses above the abscissa. Statistically significant differences are indicated by letters above each standard error (+ 1) bar.

Experiment 3: Effect of 5-Hydroxytrptophan on Melatonin Rhythms.

Treatment with 5-hydroxytryptophan at 2000 h significantly increased scotophasic melatonin concentrations of male snakes pretreated with vehicle (Figure 4.4A; $F = 6.208$, $df = 2$, $P = 0.003$, from a one-way ANOVA). Melatonin concentrations of snakes subjected to 4 h of capture stress (from 1000 to 1400 h) prior to receiving treatment with 5-hydroxytryptophan at 2000 h did not differ significantly from those of vehicle-treated snakes (Figure 4.4B). Likewise, 5-hydroxytryptophan did not significantly influence scotophasic melatonin concentrations of snakes when preceded by pretreatment with exogenous corticosterone at 1200 h (Figure 4.4C).

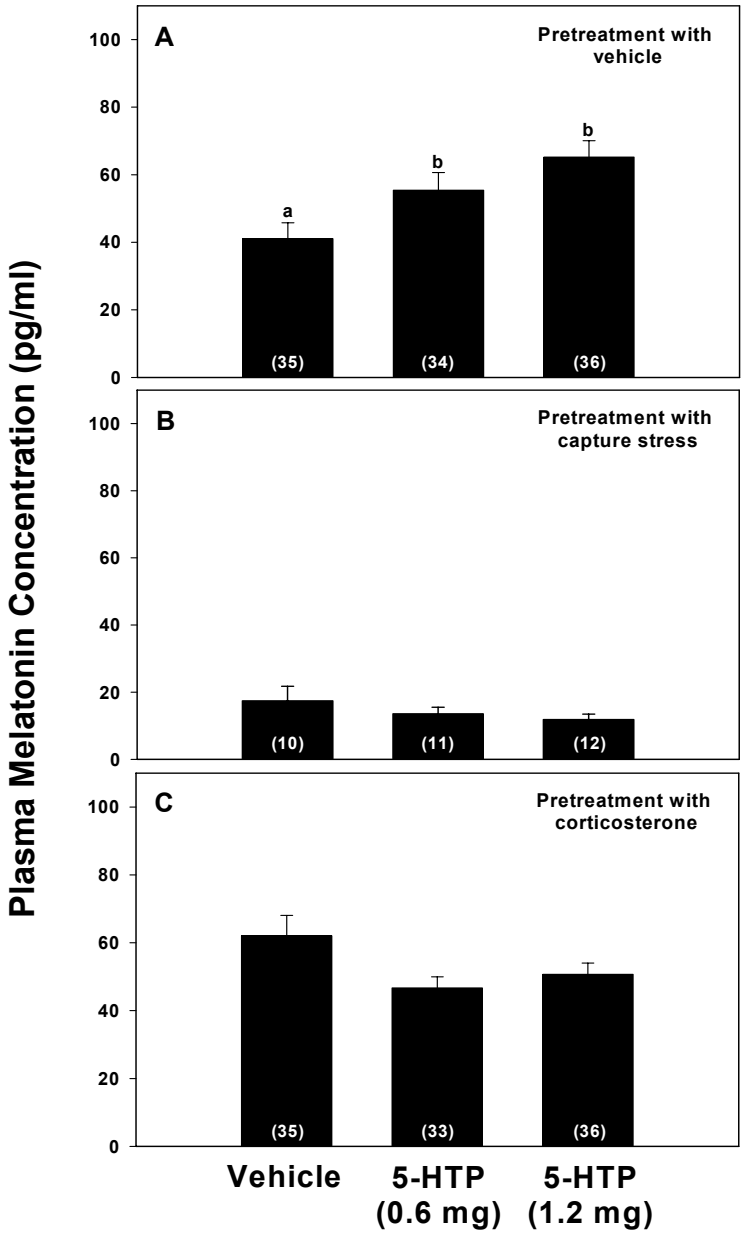

Figure 4.4. Effect of 5-hydroxytryptophan (0.6 and 1.2 mg) on scotophasic melatonin concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). During the photophase, snakes were pretreated with (A) vehicle at 1200 h, (B) 4 h of capture stress from 1000 to 1400 h, or (C) 60 μg corticosterone at 1200 h. Snakes received 5-hydroxytryptophan treatment at 2000 h. In (A) and (C), scotophasic melatonin samples (i.e., at 2200, 0000, and 0200 h) are shown pooled; melatonin concentrations in (B) were measured at 0000 h. Sample sizes are indicated in parentheses above the abscissa. Statistically significant differences are indicated by letters above each standard error (+ 1) bar.

Experiment 4: Role of Melatonin and Corticosterone Rhythms in Spring Emergence.

Upon emergence from hibernacula, female red-sided garter snakes exhibited a significant rhythm in both melatonin ($H = 20.313$, $df = 5$, $P = 0.001$, from a one-way ANOVA on ranks) and corticosterone ($F = 4.455$, $df = 5$, $P < 0.001$, from a one-way ANOVA) concentrations (Figure 4.5A). The melatonin and corticosterone rhythms appear to be in phase with one another, with both hormones having the highest levels during scotophase (Figure 4.5A). After a 3-wk acclimatization period in the field, post-emergence melatonin and corticosterone rhythms were approximately 180° out of phase with one another (Figure 4.5B). Although we did not observe a significant post-emergence melatonin rhythm, post-emergence corticosterone concentrations changed significantly throughout the 24-h sampling period, with the highest hormone concentrations occurring during photophase (Figure 4.5B; $F = 4.120$, $df = 5$, $P = 0.003$, from a one-way ANOVA).

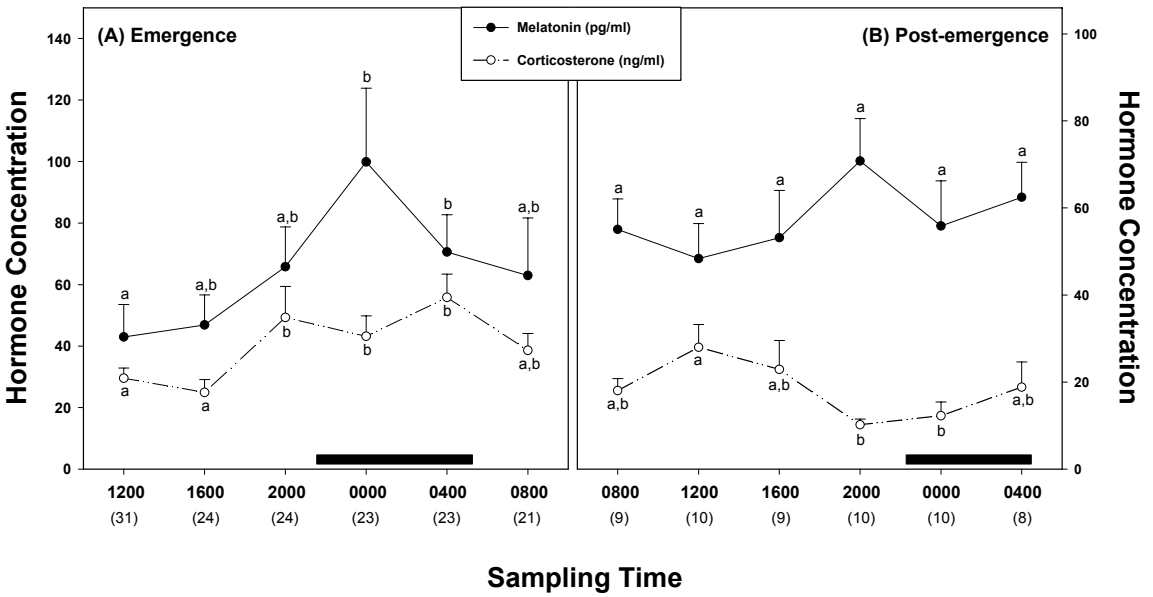

Figure 4.5. Melatonin and corticosterone rhythms of female red-sided garter snakes, *Thamnophis sirtalis parietalis*, (A) on the day of emergence from winter dormancy during the mating seasons of May 2003-2005 and (B) after a 3-wk acclimatization period in the field during May 2005. Closed and open circles represent melatonin and corticosterone concentrations, respectively. Black bars above each abscissa indicate the period of scotophase. Sample sizes are indicated in parentheses below the abscissa. Statistically significant differences in melatonin and corticosterone rhythms are indicated by letters above and below, respectively, the standard error (+ 1) bars.

Discussion

Our results demonstrate that physiological stress responses significantly modulate circadian melatonin rhythms in red-sided garter snakes, *T. sirtalis parietalis*. While activation of the HPA axis increases plasma melatonin concentrations, elevated corticosterone levels following a physiological stress response can inhibit the production of melatonin by the pineal gland. Our findings are unique because they clearly demonstrate that the different phases of an acute physiological stress response (i.e., activation of the HPA axis, increased glucocorticoid secretion) have distinct and

temporally different effects on pineal melatonin synthesis. We provide the first field study to describe circadian melatonin and corticosterone rhythms at spring emergence from prolonged winter dormancy in any animal. When red-sided garter snakes (*T. sirtalis parietalis*) first emerge from underground hibernacula following 8 months of winter dormancy, melatonin cycles are already synchronized with photoperiodic conditions, suggesting that the *zeitgeber* entraining circadian melatonin rhythms occurs prior to, or during, emergence. We also demonstrate that corticosterone rhythms phase-shift approximately 180º following emergence from winter dormancy. This change in the phase of the corticosterone rhythm may play a role in regulating the seasonal transition between reproduction and dispersal from the den site in red-sided garter snakes. Collectively, these experiments demonstrate that a physiological coupling between melatonin, glucocorticoids, and the HPA axis is conserved in this ectothermic model. We suggest that such a physiological coupling plays a functional role in regulating circadian and circannual rhythms in physiology and behavior.

Melatonin Rhythms and Physiological Stress Responses

A physiological coupling between stress responses and melatonin rhythms has been reported previously. Chronic sleep deprivation significantly elevates photophasic melatonin concentrations and abolishes the circadian pattern of melatonin secretion in male rats (Persengiev and Kanchev 1991). Physical exercise increases plasma melatonin concentrations in humans (Carr *et al*. 1981), and a limited number of studies indicate that social stress also influences pineal function and melatonin synthesis. For example,

chronic social confrontation significantly increases urinary excretion of the melatonin metabolite 6-sulfatoxymelatonin in subordinate tree shrews, *Tupaia belangeri* (Fuchs and Schumacher 1990). Subordinate rainbow trout (*Oncorhynchus mykiss*) have significantly higher scotophasic melatonin levels than dominant individuals or control fish (Larson *et al*. 2004). In such species that demonstrate strong dominance hierarchies, adjustment of diel activity patterns in subordinate individuals may aid in limiting interactions with dominant competitors (Chen *et al*. 2002). These results suggest that different types of stressors (i.e., social stress versus physical stress) may influence the pineal gland and circadian melatonin rhythms differently.

Melatonin's role as an antioxidant suggests that increased melatonin synthesis following a physiological stress response may be adaptive in combating oxidative damage induced by elevated glucocorticoid concentrations (e.g., Reiter 1996; Reiter *et al*. 1995). Sainz *et al*. (1995) demonstrated that melatonin alleviates glucocorticoid-induced apoptosis of thymocytes via an antioxidant mechanism. Similar to our results, immobilization stress increased photophasic melatonin concentrations but decreased nocturnal melatonin levels in ring doves, *Streptopelia risoria* (Barriga *et al*. 2002; Rodríguez *et al*. 2001). Our experiments with red-sided garter snakes (*T. sirtalis parietalis*) demonstrate that increased plasma melatonin levels following a stress response result directly from the stress response itself, rather than an increase in glucocorticoid secretion (Figures 4.2B, 4.3B). These results corroborate those of Ferreira *et al*. (2005), who demonstrated that corticosterone alone cannot induce melatonin synthesis in cultured pineal glands. Because both the adrenal cortex and the pineal gland

are innervated by sympathetic nerve fibers, the immediate increase in glucocorticoid and melatonin secretion during an acute stress response may result from increased sympathetic nervous system activity necessary for initiation of the stress response (e.g., Lynch *et al*. 1973, 1977; Parfitt and Klein 1976). However, diel differences occur in the response of the pineal gland to stress (Monteleone *et al*. 1990; Troiani *et al*. 1988). In rats, water-immersion restraint stress administered during the scotophase still attenuates the nocturnal peak in melatonin secretion (Otsuka *et al*. 2001). Thus, potential interactions between the pineal gland and catecholamines or other factors released during the physiological stress response must also be considered.

While the initial increase in melatonin levels following a stress response results from activation of the HPA axis, the persistent effects of stress on scotophasic melatonin levels may result from elevated glucocorticoid secretion (Figures 4.2B, 4.4C). Scotophasic melatonin levels of male snakes treated with 4 h of capture stress (from 1000 to 1400 h) tended to be lower than those of non-stress control snakes (Figure 4.2B). Although these differences were not statistically significant, similar results describing decreased scotophasic melatonin synthesis following a stress response have been reported (e.g., Barriga *et al*. 2002; Otsuka *et al*. 2001; Rodríguez *et al*. 2001). Treatment of snakes with 5-hydroxytryptophan did not restore stress-induced decreases in scotophasic melatonin concentrations (Figure 4.4B). Thus, our results do not support the hypothesis that decreased scotophasic melatonin levels following a stress response result from a rapid decline in the concentration of tryptophan, the amino acid precursor for melatonin synthesis (Clark and Russo 1997). Rather, we demonstrate that acutely-elevated

corticosterone concentrations following a physiological stress response may act directly on the pineal gland to modulate melatonin synthesis (Figure 4.4C).

Although corticosterone itself does not influence melatonin rhythms of red-sided garter snakes (Figure 4.3B), we show that corticosterone can inhibit the synthesis of melatonin from 5-hydroxytryptophan (Figure 4.4C). These results suggest the persistent effects of stress (at least 10 h following capture stress in these experiments) on scotophasic melatonin levels result from some concomitant change in pineal physiology that occurs following a stress response. This mechanism may involve the enzymes responsible for synthesizing melatonin from 5-hydroxytryptophan, including *L*-aromatic amino acid decarboxylase, *N*-acetyltransferase, or hydroxyindole-O-methyltransferase. For example, the adrenal gland mediates decreases in *N*-acetyltransferase activity, the enzyme responsible for converting serotonin into *N*-acetylserotonin in the biosynthetic pathway for melatonin synthesis (Joshi *et al*. 1986). Such an interaction between stress responses and pineal physiology might facilitate the inhibition of melatonin synthesis by corticosterone following acute stress responses.

Differences between acute and chronic physiological stress responses likely contribute to the disparate results regarding a possible coupling between melatonin and glucocorticoids. For example, corticosterone potentiates the synthesis of *N*-acetylserotonin and melatonin when cultured pineal glands are incubated with corticosterone for 48 h, but not 1 h, prior to stimulation with noradrenaline (Ferreira *et al*. 2005). Likewise, adrenal hormones positively influence pineal melatonin secretion of mice experiencing chronic inflammation (Lopes *et al*. 2001). Such differences between

the effects of acute and chronic stress responses suggest that different mechanisms (i.e., both genomic and rapid nongenomic pathways) are in place for adrenal modulation of pineal melatonin synthesis. The pineal gland of rats expresses functional glucocorticoid receptors at a density similar to that found in the liver, a tissue known to respond to glucocorticoids (Ferreira *et al*. 2005). Mifepristone, a glucocorticoid receptor antagonist, inhibits the potentiation of noradrenaline-induced *N*-acetylserotonin synthesis by corticosterone (Ferreira *et al*. 2005). In addition, Ferreira *et al*. (2005) demonstrated that corticosterone enhances noradrenaline-induced pineal melatonin synthesis via inhibition of the nuclear factor kappa B pathway, a transcription factor that regulates the expression of genes involved in immune responses. Further research is necessary to determine if both genomic and/or non-genomic glucocorticoid pathways are activated in the pineal gland during a physiological stress response. Furthermore, information regarding mechanistic differences between acute versus chronic physiological stress responses, as well possible differences among different types of stressors (e.g., social stress, capture/restraint stress, physical stress), would provide much insight into the disparate results regarding a physiological coupling between melatonin, glucocorticoids, and the HPA axis.

Role of Melatonin and Corticosterone Rhythms in Spring Emergence

Many ectotherms inhabiting north-temperate climates undergo periods of prolonged winter dormancy prior to spring breeding. Animals that occupy underground hibernacula during winter dormancy are not exposed, or receive little exposure, to

changing photoperiodic conditions (e.g., Whittier *et al*. 1987). Thus, photoperiod is likely not a critical factor in synchronizing spring emergence from over-wintering locations. For example, photoperiod prior to and during winter dormancy has no influence on the initiation and timing of reproductive behavior of red-sided garter snakes (*T. sirtalis parietalis*) upon spring emergence (Whittier *et al*.1987). Rather, temperature appears to be the most important environmental cue for synchronizing seasonal reproduction in reptiles (Duvall *et al*. 1982; Licht 1972).

The red-sided garter snake is an excellent model for studying the contribution of temperature as an important environmental cue synchronizing seasonal rhythms. In addition, environmental induction of mating in red-sided garter snakes is mediated by the pineal gland. Male snakes pinealectomized prior to hibernation fail to exhibit courtship behavior upon emergence (Mendonça *et al*. 1996; Nelson *et al*. 1987). These results indicate the pineal gland is necessary for transducing environmental stimuli during winter dormancy, of which temperature is the most important (and perhaps the only) cue. Melatonin also modulates reproductive behavior of red-sided garter snakes during the spring mating season (Lutterschmidt *et al*. 2004). Thus, melatonin plays a functional role in regulating seasonal reproduction in this species.

To better understand the environmental cues and hormonal mechanisms regulating seasonal emergence and reproductive behavior, we measured changes in melatonin and glucocorticoid rhythms following emergence from winter dormancy. When red-sided garter snakes (*T. sirtalis parietalis*) first emerge from underground hibernacula following 8 months of winter dormancy, melatonin cycles are already

synchronized with photoperiodic conditions (i.e., the highest melatonin concentrations occur during the scotophase; Figure 4.5A). We also observed a significant corticosterone rhythm with peak corticosterone levels occurring during the scotophase (Figure 4.5A). Such melatonin and corticosterone rhythms were observed during each of 3 different mating seasons. These findings suggest that the *zeitgeber* entraining circadian melatonin rhythms occurs prior to, or during, spring emergence from hibernacula.

Previous work in our laboratory has shown that snakes encounter slight increases in ground temperature (approximately 3°C) as they migrate vertically towards the surface of the hibernaculum (Lutterschmidt *et al*. 2006). It is possible that such an increase in ground temperature (and hence snake body temperature) prior to emergence from the hibernaculum is the *zeitgeber* entraining melatonin rhythms. However, some snakes emerging from winter dormancy early in the mating season (late April to early May) do not experience an increase in body temperature prior to emergence, likely because ground temperatures do not increase significantly during this time. For example, some snakes emerge at body temperatures of only 0.5°C (Lutterschmidt *et al*. 2006; Macartney *et al*. 1989). Thus, an increase in body temperature does not appear to be necessary to elicit spring emergence from winter dormancy. We are currently investigating how temperature cues, in the absence of photoperiod stimuli, interact with circadian and circannual hormone cycles to regulate spring emergence and reproductive behavior of red-sided garter snakes (*T. sirtalis parietalis*).

Following emergence from winter dormancy, we observed a phase shift of approximately 180º in the corticosterone rhythm of snakes. Although we did not observe

a significant melatonin cycle post-emergence, melatonin levels did increase just prior to the onset of darkness (Figure 4.5B). The lack of a statistically significant post-emergence melatonin cycle is likely due to the smaller sample sizes at each sampling period. Circadian melatonin rhythms may function in synchronizing the corticosterone rhythm with photophase following emergence, as interactions between melatonin and the adrenal gland are well established (e.g., Barriga *et al*. 2002; Maestroni *et al*. 1989; Otsuka *et al*. 2001). For example, melatonin exerts a direct antisecretory effect on the adrenal gland *in vivo* (Appa-Rao *et al*. 2001). Melatonin treatment also alters the affinity of glucocorticoid receptors in the brain and pituitary (Marinova *et al*. 1991).

We speculate that temporal shifts in the corticosterone rhythm play a role in regulating the seasonal transition between reproduction and dispersal from the den site in red-sided garter snakes. Although female snakes disperse from the den immediately following emergence, male snakes remain within the vicinity of the den and typically exhibit courtship behavior for several weeks (Shine *et al*. 2001). Snakes are aphagic while actively courting at the den site (O'Donnell *et al*. 2004) and must disperse as far as 20 km to summer feeding grounds to forage (Gregory 1977). Because snakes enter this energetically costly courtship and dispersal period subsequent to an eight-month dormancy period, elevated glucocorticoid levels likely facilitate reproduction and daily activity by mobilizing much-needed energy stores (e.g., Moore and Jessop 2003). However, elevated corticosterone levels will inhibit courtship behavior in the diurnally-active red-sided garter snake (Lutterschmidt *et al*. 2004; Moore and Mason 2001). Thus, elevated scotophasic corticosterone levels may enable the mobilization of energy stores

needed to maintain reproductive activity while avoiding the reproductive costs of corticosterone-induced inhibition of courtship behavior during the photophase. Although we measured melatonin and corticosterone rhythms at emergence in female red-sided garter snakes, our results indicate that male snakes actively courting at the den site (potentially as long as 3 weeks post-emergence) also have higher corticosterone levels during scotophase (Figure 4.3A, vehicle-treated snakes). Thus, actively courting male snakes at the den site may exhibit a corticosterone cycle similar to that observed in newly-emerged female red-sided garter snakes.

There is precedence for temporal shifts in endocrine cycles in response to changes in daily activity patterns. For example, nocturnal activity in free-ranging green sea turtles (*Chelonia mydas*), including nesting, mate searching, and feeding/swimming behaviors, abolishes diel variation in both melatonin and corticosterone cycles (Jessop *et al*. 2002). Further research is necessary to evaluate whether the transition from courtship behavior to dispersal in red-sided garter snakes may be initiated by a temporal shift in the corticosterone rhythm during the mating season. Future studies are also needed to evaluate the influence of melatonin on the synchronization of corticosterone rhythms, as well as possible sex differences in the temporal scale required for synchronization. Such studies would help elucidate the functional importance of a physiological coupling between melatonin, glucocorticoids, and the HPA axis in regulating the seasonal biology of this and other species.

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CHAPTER 5

MINIMAL OVER-WINTERING TEMPERATURES OF RED-SIDED GARTER SNAKES (*THAMNOPHIS SIRTALIS PARIETALIS*): A POSSIBLE CUE FOR EMERGENCE?

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Abstract

Red-sided garter snakes (*Thamnophis sirtalis parietalis* (Linnaeus, 1758)) in Manitoba, Canada undergo 8 months of continuous winter dormancy prior to spring emergence. As in other ectothermic species, increases in ground temperature may be the cue for emergence from winter dormancy in these populations. To test this hypothesis, we measured body temperatures during winter dormancy by surgically implanting small temperature-loggers into 32 female red-sided garter snakes before they entered their native hibernaculum. The following spring, we recaptured 7 of the snakes implanted with temperature-loggers. Body temperature declined gradually from mid-September (mean = 14.7°C ± 0.24 SE) to early April (mean = 1.1°C ± 0.16 SE) during winter dormancy, reaching minimal values approximately 1 month prior to spring emergence. Body temperatures of emerging snakes ranged from 0.5°C during early spring to 6.3°C during late spring (mean = 3.4°C ± 0.84 SE). These results do not support the hypothesis that an increase in ground temperature (and hence body temperature) is necessary for emergence from winter dormancy. We suggest that critically low temperatures (i.e., 0.5 - 1°C) are a *zeitgeber* entraining an endogenous circannual cycle that regulates snake emergence. These results offer new insight into the mechanisms regulating seasonal emergence from winter dormancy.

Introduction

Most vertebrates exhibit some seasonality in many behavioral and physiological processes. One of the most reliable environmental cues thought to function in regulating

seasonality in vertebrates is photoperiod. Unlike other environmental signals (e.g., temperature and humidity) that can vary quite dramatically both within seasons and among years, changes in photoperiod length accurately and reliably reflect changing environmental seasons. Many ectotherms inhabiting north-temperate climates, however, undergo periods of prolonged winter dormancy prior to spring breeding. Animals that occupy underground hibernacula during winter dormancy are not exposed, or receive little exposure, to changing photoperiodic conditions (e.g., Grobman 1990; Whittier *et al*. 1987). Thus, photoperiod is likely not a critical factor in synchronizing spring emergence from over-wintering locations. For example, photoperiod prior to and during winter dormancy has no influence on the initiation and timing of reproductive behavior of red-sided garter snakes (*Thamnophis sirtalis parietalis* (Say in James, 1823)) upon spring emergence (Nelson *et al*.1987; Whittier *et al*.1987). Rather, temperature appears to be the most important environmental cue for synchronizing reproduction in reptiles (Duvall *et al*. 1982; Licht 1972, 1984; Whittier *et al*. 1987).

In some ectothermic species, 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., Crawford 1991; Crews and Garstka 1982; Hawley and Aleksiuk 1975, 1976; Jacob and Painter 1980; Licht 1984; Macartney *et al*. 1989; Whittier *et al*. 1987). For example, emergence from winter dormancy in the box turtles *Terrapene carolina* and *T. ornata* occurs after subsurface ground temperatures increase for several consecutive days (Grobman 1990). Emergence of northern Pacific rattlesnakes (*Crotalus oreganus oreganus*) also occurs as hibernaculum temperatures

increase (Macartney *et al*. 1989). Etheridge *et al*. (1983) demonstrated experimentally that increasing ambient temperatures stimulate emergence of the six-lined racerunner (*Cnemidophorus sexlineatus*) from winter dormancy. However, some ectothermic species (especially those inhabiting extreme northern latitudes) can occupy underground dens at depths where ground temperatures do not change significantly prior to spring emergence (e.g., Macartney *et al*. 1989), suggesting that increases in ground temperatures may not be the only thermal cue utilized by reptiles.

To better understand the environmental cues regulating spring emergence and reproduction in reptiles, we measured body temperatures of red-sided garter snakes (*T. sirtalis parietalis*) during winter dormancy under natural field conditions. Red-sided garter snakes are the most northerly living reptile in North America and are found in extremely large numbers throughout south central Manitoba, Canada. These northern-latitude populations of snakes undergo a period of continuous winter dormancy for approximately 8 months each year. Following spring emergence, an attenuated mating season lasting approximately 4-5 weeks is initiated (e.g., Crews and Garstka 1982). In this well-studied dissociated breeder, mating behavior is triggered by increases in environmental temperatures following winter dormancy (Ross and Crews 1978; Garstka *et al*. 1982; Bona-Gallo and Licht 1983; Krohmer and Crews 1987; Whittier *et al*. 1987).

Given the unique life history traits of these snake populations, temperature is likely the most important proximate environmental cue governing the initiation of emergence from winter dormancy. Previous studies investigating the role of temperature in regulating spring emergence in red-sided garter snakes were conducted in the

laboratory (e.g., Bona-Gallo and Licht 1983; Krohmer and Crews 1987; Whittier *et al*. 1987). Furthermore, these studies focused on the role of temperature in initiating reproductive behavior, as it is a more conspicuous endpoint to measure. There are limited data regarding over-wintering temperatures of ectotherms under natural field conditions, especially in northern latitudes. In addition, few field studies have focused on the thermal cues regulating spring emergence. We examined the efficacy of temperature as an important cue for synchronizing spring emergence under natural field conditions. Specifically, we sought to determine if hibernaculum temperatures (and hence snake body temperatures) increase significantly prior to emergence from winter dormancy. Because the initiation of spring emergence of red-sided garter snakes is independent of changes in photoperiodic conditions (Nelson *et al*.1987; Whittier *et al*.1987), this model system provides an excellent opportunity to examine the role of temperature as the primary environmental cue synchronizing emergence from prolonged winter-dormancy.

Materials and Methods

Experiments were conducted in the field with free-ranging red-sided garter snakes (*T. sirtalis parietalis*) in the Interlake region of Manitoba, Canada (50° 30' N, 97° 30' W). All experimental protocols were approved by the Oregon State University Animal Care and Use Committee (protocol number: 2661) and were in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals". This research was approved by the Manitoba Wildlife Animal Care Committee (protocol number:

2002-06) and was performed under the authority of Manitoba Wildlife Scientific Permit No. WSP 03009.

Similar to Grayson and Dorcas (2004) and Angilletta and Krochmal (2003), we measured body temperatures of red-sided garter snakes during winter dormancy by surgically implanting snakes with small temperature-loggers (Thermochron iButton, Dallas Semiconductor, Dallas, TX, USA). Dataloggers were programmed to sample temperature once every 3 h using the 32-Bit iButton-TMEX Runtime Environment software (Dallas Semiconductor, Dallas, TX, USA). This sampling rate was used to ensure continuous recordings of body temperatures throughout the 8-month dormancy period (i.e., for approximately 256 days). The sampling times of all dataloggers were synchronized so that body temperatures of individual snakes were recorded at the same time of day.

Thirty-two female red-sided garter snakes having a snout-vent length $\geq$ 62 cm (mean = 71.2 cm $\pm$ 0.78 SE) were collected in the fall (9-15 September 2003) after snakes returned to the den site to over-winter. Female snakes were used in this study because of their much larger body size. Snakes were anesthetized with sodium brevital (0.003 ml 0.5% brevital per g body mass) and a temperature-logger was surgically implanted into the peritoneal cavity. Dataloggers (17.4 mm diameter $\times$ 5.7 mm height, 3.1 g) were 2.2% of the mean body mass of snakes (range = 2.8% of the smallest snake to 1.4% of the largest snake) and produced a slight, noticeable bulge in the mid-body area containing the temperature-logger. To aid in recapture of female snakes during the spring when they are emerging among thousands of garter snakes, we individually scale-clipped each snake

with a unique number and a silver sequin was secured to the parietal scales with glue. Snakes were allowed to recover from surgery for 1-5 days before being released at the site of capture, where they were allowed to hibernate under natural conditions.

The following spring, we recaptured 7 of the snakes implanted with temperature-loggers. We attribute this recapture rate to the difficulty of locating snakes among the extremely large numbers of snakes (~35 000; Shine *et al*. 2006) at this den site. Mortality may have contributed to reducing the number of females recaptured, but mortality rates of red-sided garter snakes during winter dormancy in the field are unknown. All females were captured immediately following emergence from the hibernaculum and the specific time and date of capture were recorded for each snake. The temperature-loggers were surgically removed and the females released at the site of capture following recovery; data were downloaded for analysis. Only body temperature data for snakes prior to complete emergence were used in the analyses of winter dormancy temperatures.

Ground temperatures were measured during the period of winter dormancy at 6 different soil depths (0, 0.3, 0.6, 0.9, 1.2, and 1.5 m) using Thermochron iButton temperature-loggers (Dallas Semiconductor, Dallas, TX, USA). Dataloggers were programmed to sample temperature once every 3 h and were synchronized with the dataloggers recording body temperatures of female snakes. To protect the dataloggers from ground water during the observation period, we sealed each iButton in a small balloon (not inflated) before placement in the ground. Due to the rocky terrain at the den site and because the den site is located on public property, ground temperatures were measured at the field station approximately 20 km north of the den (50° 37' N, 97° 32'

W). To aid in the retrieval of temperature-loggers in the spring, we first dug a hole 1.5 m deep by inserting a metal cylinder into the ground. Temperature loggers were then placed into the ground at 0.3 m intervals. To mimic the rocky terrain at the den site, we used gravel to fill the spaces between dataloggers and placed rocks over the site. Mean high and low ambient temperatures during September 2003 through May 2004 were obtained for a nearby area (Lundar, Manitoba; 50° 43' N, 97° 51' W) from Environment Canada.

Results

Body temperatures of female red-sided garter snakes declined gradually from mid-September (mean = 14.7°C ± 0.24 SE) to early April (mean = 1.1°C ± 0.16 SE) during the 8-month dormancy period (Figure 5.1). Body temperatures did not reach minimal values until April, approximately 1 month prior to the beginning of spring emergence. Mean body temperature of snakes 1 week prior to emergence was 2.6°C ± 0.39 SE. Mean body temperature of snakes 1 day prior to emergence was 3.4°C ± 0.84 SE. Because female garter snakes emerge over the entire 4-wk mating season, mean body temperature one-day prior to emergence ranged from 0.5°C when snakes emerged during early spring to 6.3°C when snakes emerged during late spring. Pre-hibernation body mass of snakes was significantly higher than post-hibernation body mass ($P < 0.001$ from a paired t-test; data not shown). The mean percent body mass loss of female snakes during winter dormancy was 10.4% ± 1.6 SE.

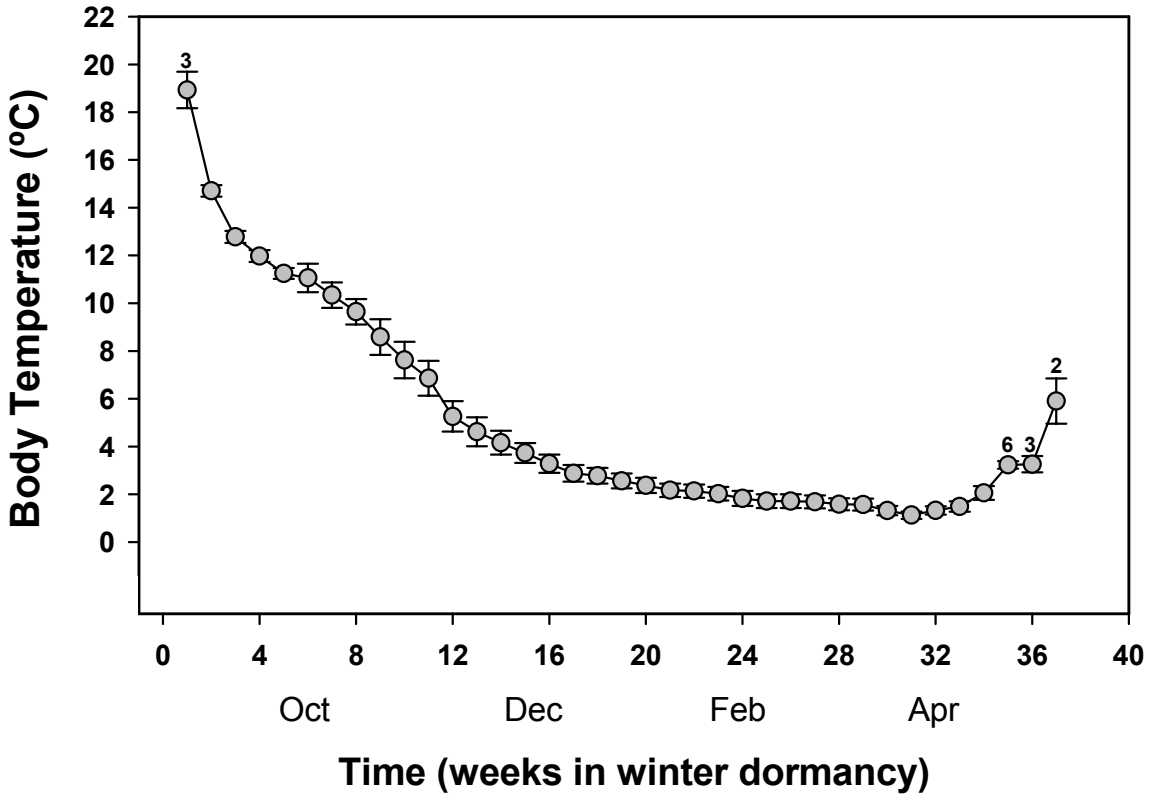

Figure 5.1. Body temperatures of female red-sided garter snakes (*Thamnophis sirtalis parietalis*) during winter dormancy in dens under natural field conditions in Manitoba, Canada. Unless noted otherwise by sample sizes above the standard error bars, each data point is a weekly mean of 7 snakes ± 1 standard error.

Mean high ambient temperatures were below 0°C from late October through late March (Figure 5.2). Although the underground hibernaculum protected snakes from ambient temperatures, snakes could only escape freezing temperatures at a depth of 1.2 m or greater (Figure 5.3). Ground water was observed at soil depths of 1.2 and 1.5 m during retrieval of the ground temperature dataloggers. During the period of spring emergence (i.e., from 29 April to 22 May, weeks 34-37 of winter dormancy), ground temperatures at depths of 1.2 and 1.5 m increased by only 3.0 and 2.5°C, respectively (Figure 5.3).

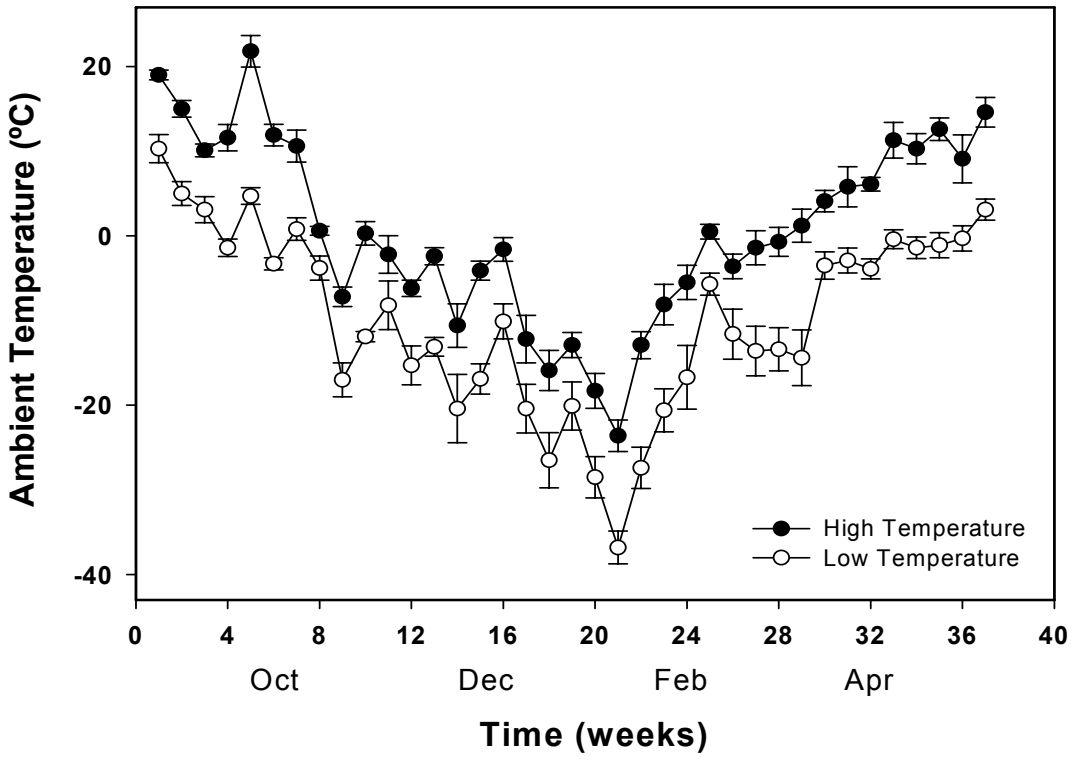

Figure 5.2. Mean high and low ambient temperatures from September 2003 to May 2004 (i.e., during the dormancy period of red-sided garter snakes, *Thamnophis sirtalis parietalis*). Data were obtained for a nearby area from Environment Canada. Each data point is a weekly mean ± 1 standard error.

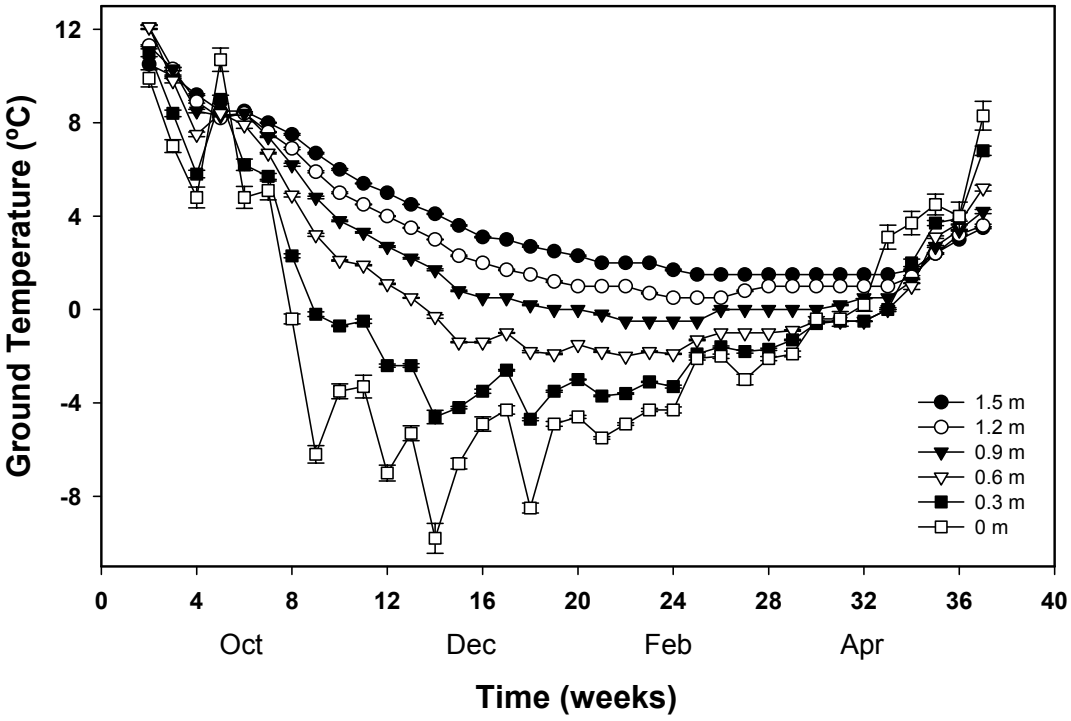

Figure 5.3. Ground temperatures during the dormancy period of red-sided garter snakes (*Thamnophis sirtalis parietalis*) at 6 different soil depths (0, 0.3, 0.6, 0.9, 1.2, and 1.5 m). Temperatures were measured at a site near the snake hibernaculum. Each data point is a weekly mean ± 1 standard error.

Discussion

Our results indicate that red-sided garter snakes (*T. sirtalis parietalis*) near the northern limit of this species' range in Manitoba, Canada have a mean minimum body temperature of 1.1°C ± 0.16 SE during winter dormancy. This body temperature is much lower than that estimated previously for these populations of snakes during hibernation (i.e., 3-6°C; Whittier *et al*. 1987). The range of body temperatures we observed during winter dormancy is similar to that reported by Macartney *et al*. (1989), who measured body temperatures of red-sided garter snakes in a communal den in northern Alberta,

Canada. However, due to the failure of the radiotelemetry equipment, body temperatures of only 1 red-sided garter snake could be monitored (Macartney *et al*. 1989). The mean body temperature of this snake during hibernation was 3.9°C ± 0.34 SE (n = 16 observations during hibernation); body temperature ranged from 1.8 to 6.5°C (Macartney *et al*. 1989).

Body temperatures of snakes remained above 0°C throughout winter dormancy and were similar to ground temperatures observed at a depth of 1.5 m from November through late April (weeks 12-33; Figure 5.4). These results support previous findings

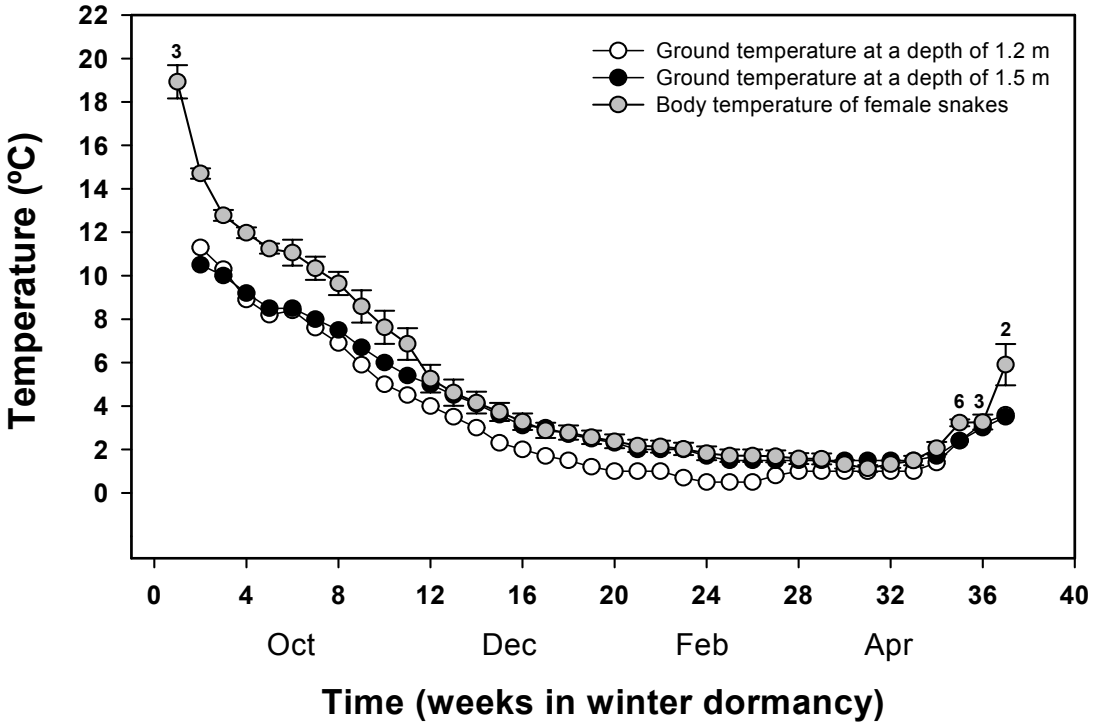

Figure 5.4. Body temperatures of red-sided garter snakes (*Thamnophis sirtalis parietalis*) during winter dormancy shown with ground temperatures at depths of 1.2 and 1.5 m. Unless noted otherwise by sample sizes above the standard error bars, each data point for body temperature is a weekly mean of 7 snakes ± 1 standard error; ground temperatures are a weekly mean ± 1 standard error.

that garter snakes cannot endure prolonged freezing stress (reviewed in Storey and Storey 1992) and instead seek thermally-buffered hibernaculum sites.It is evident that ambient temperatures directly influence hibernaculum temperatures during the dormancy period, especially at shallower hibernaculum depths (Figures 5.2, 5.3). Ground temperature measurements indicate that snakes must have moved to a depth of at least 1.2 m below the den surface to escape freezing temperatures (Figure 5.3). At depths of 1.2 and 1.5 m, ground temperatures decreased gradually but were fairly stable during the period of winter dormancy; ground temperatures at these soil depths increased no more than 3°C during the period of spring emergence (weeks 34-37; Figure 5.3).

An increase in hibernaculum temperatures could potentially be a cue for emergence, but the underlying mechanism must be sensitive to very small changes in temperature (i.e., 2.5 - 3.0°C) over a period of less than 1 month. Similar patterns of spring emergence have been observed in the box turtles *T. carolina* and *T. ornata* (Grobman 1990). Spring emergence (and subsequent reproductive behavior) of red-sided garter snakes may be controlled by an endogenous circannual rhythm that is entrained by the slight increase in ground temperatures observed during this study (e.g., Licht 1972; Gregory 1982).

In contrast, some snakes were observed to emerge from the hibernaculum without a significant increase in body temperature. These results therefore do not support the hypothesis that an increase in ground temperature (and therefore body temperature) is a necessary cue for emergence from winter dormancy. For example, during early to mid-May, 2 snakes emerged at a body temperature of only 0.5°C. These observations are

similar to those reported by Macartney *et al*. (1989), who also observed cloacal temperatures as low as 0.5°C in emerging garter snakes. In late May, however, body temperatures of emerging snakes were as high as 6.3°C. This higher body temperature at emergence is likely attributable to the higher ground temperatures experienced at the hibernaculum surface as snakes emerged later in the season (Figure 5.3). During the period of spring emergence (May, weeks 34-37), ground temperatures at depths of 1.2 and 1.5 m did not increase above 4°C. Thus, snakes (i.e., ectotherms) with body temperatures higher than 4°C prior to complete emergence must have been occupying shallower locations within the hibernaculum, and therefore were likely already in the process of emergence. (Although ground temperatures were recorded at a site approximately 20 km north of the den, we presume that ground temperatures do not differ greatly between these sites.) We currently have no way of estimating the time required for a snake to make its journey from the location of winter dormancy within the hibernaculum to the surface of the den. We hypothesize that the time required for complete emergence is highly variable among snakes and depends upon the position of each snake within the hibernaculum, as entrances into the den as well as the den itself are composed of narrow, rocky tunnels. Indeed, early versus late emergence from hibernacula has been correlated with snake depth in other studies (e.g., Carpenter 1953). Thus, increases in body temperature prior to emergence (especially increases above ground temperatures at depths of 1.2 and 1.5 m) most likely reflect the vertical migration of snakes to the surface of the hibernaculum.

We speculate that critically low temperatures (i.e., 0.5 - 1°C) may play a role in initiating snake emergence. For example, mean snake body temperatures were similar to those temperatures observed at a depth of 1.5 m from November through late April (from weeks 12-33; Figure 5.4). However, the mean body temperature of snakes increased more rapidly than ground temperatures at this depth (Figure 5.4), suggesting that snakes were vertically migrating to the surface of the hibernaculum during weeks 34-37 of winter dormancy. However, it again must be noted that ground temperatures were recorded at a site away from the hibernaculum. These observations suggest that when snakes reach a critical minimum temperature, they may be stimulated to change their vertical position in the den. Such critically low temperatures (and/or the duration of exposure to low temperatures) may act as a *zeitgeber* entraining an endogenous circannual cycle that governs spring emergence from winter dormancy.

There is precedence for vertical migration within hibernacula during winter dormancy in other ectothermic species. Sexton and Marion (1981) demonstrated that emergence of prairie rattlesnakes (*Crotalus viridis*) from winter hibernacula is regulated by a reversing thermal gradient within natural dens. In northern latitudes, where ambient temperatures can be below freezing during much of the winter, hibernating ectotherms select the warmest portion of the naturally-occurring thermal gradient in the hibernaculum (Sexton and Marion 1981). Thus, in the early stages of winter dormancy, the cooling of the hibernaculum surface stimulates animals to migrate farther into the den. Vertical migration to the surface of the den occurs during the spring, when surface

temperatures warm more quickly then the lower portions of the hibernaculum (e.g., Etheridge *et al*. 1983; Grobman 1990; Sexton and Marion 1981).

It is unlikely that the vertical migration of red-sided garter snakes within the den results from active behavioral thermoregulation, as snakes can emerge from winter dormancy at body temperatures of only 0.5°C (Macartney *et al*. 1989; this study). Rather, vertical migration within and emergence from hibernacula may be regulated by a circannual cycle that is influenced by low temperatures. Because of the extreme environmental constraints on survival and reproduction in these northern populations of garter snakes, it is likely that a very sensitive mechanism regulating spring emergence has evolved in these populations.

Further research is necessary to determine the temperature threshold as well as the role of other environmental cues (e.g., humidity) in initiating vertical migration within and emergence from hibernacula. Studies of the spatial and temporal distribution of snakes in hibernacula, perhaps via artificial dens, would be particularly informative about the role of minimal over-wintering temperatures in spring emergence. We are currently investigating how temperature cues interact with circadian and circannual hormone cycles to regulate spring emergence and reproductive behavior. Because male and female red-sided garter snakes demonstrate differential timing of emergence from winter dormancy, future studies examining possible sex differences in the mechanisms regulating spring emergence are needed. Such studies would provide much insight into the circannual rhythms and environmental cues regulating seasonality in ectothermic vertebrates.

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CHAPTER 6

MECHANISMS GOVERNING TEMPERATURE-INDUCED REPRODUCTIVE BEHAVIOR IN RED-SIDED GARTER SNAKES (*THAMNOPHIS SIRTALIS PARIETALIS*)

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Abstract

We investigated the mechanisms by which temperature induces seasonal reproductive behavior in red-sided garter snakes (*T. sirtalis parietalis*). Specifically, we addressed whether elevated melatonin levels and/or increased environmental temperatures during winter dormancy influence (1) patterns of sex steroid hormone and corticosterone secretion; (2) the expression of reproductive behavior following emergence; and (3) circadian melatonin and corticosterone rhythms during winter dormancy. Our results demonstrate that elevated temperatures during hibernation, but not exogenous melatonin treatment, significantly influence androgen concentrations of male red-sided garter snakes during winter dormancy. Neither exogenous melatonin nor elevated hibernation temperatures significantly influenced estradiol concentrations of snakes. Following winter dormancy, we observed robust courtship behavior in all treatment groups. Neither exogenous melatonin nor elevated hibernation temperatures significantly influenced the expression of reproductive behavior. However, males maintained at 10°C during winter dormancy exhibited delayed onset of courtship behavior and a higher percent change in body mass. Snakes treated with exogenous melatonin also demonstrated a significantly larger change in body mass than control-treated snakes during these experiments. Elevated hibernation temperatures (i.e., 10°C) significantly increased melatonin concentrations and significantly decreased corticosterone concentrations of male snakes. Melatonin and corticosterone rhythms were observed in snakes maintained at 5°C (but not at 10°C) during hibernation, although melatonin rhythms were not statistically significant. The small proportion of male snakes

that did not exhibit courtship behavior appeared to have higher corticosterone concentrations during photophase. Because the sample size of non-courting snakes was small and inconsistent among sampling times within the 24-h cycle, we investigated circadian melatonin and corticosterone rhythms of snakes from a preliminary study performed during the winter dormancy period of 2002-2003. During this experiment, we observed a very low incidence of courtship behavior among male snakes. Circadian melatonin rhythms were absent, and overall concentrations were lower, in snakes observed during 2003. In addition, corticosterone concentrations of snakes were higher during photophase, although corticosterone rhythms were not statistically significant. These experiments support previous findings that male red-sided garter snakes exhibit a dissociated reproductive strategy and offer a potential mechanism explaining observed annual variation in steroid levels. However, the observation that estradiol concentrations increase significantly during spring emergence suggests the presence of inter-sexual differences in reproductive strategy. Our results suggest that environmental temperatures induce reproductive behavior of red-sided garter snakes via changes in melatonin and/or corticosterone rhythms. We also demonstrate that the phase of corticosterone rhythms during the spring mating season may play a role in regulating reproductive behavior in this seasonally breeding reptile.

Introduction

Hibernation is a type of winter dormancy accompanied by prolonged hypometabolism and decreases in body temperature to within 1-3°C of ambient

temperature. Among most true hibernators, this dormant or torpid period lasts for days or weeks at a time and is punctuated by periodic arousal from hibernation and a return of body temperature to euthermic conditions (Lyman *et al*. 1982). Many small birds and mammals undergo daily periods of torpor rather than prolonged hibernation. Nevertheless, hibernation and daily torpor are analogous phenomena that are likely regulated by similar mechanisms (Wilz and Heldmaier 2000). Much research indicates that daily bouts of torpor are temporally organized by the circadian system, specifically the suprachiasmatic nucleus (reviewed in Ruby 2003). Exposure to both photoperiod cues (albeit limited) and fluctuations in body temperature resulting from torpor itself may function in synchronizing circadian pacemakers during hibernation (Ruby 2003).

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 such ectothermic vertebrates, body temperature is dependent on ground temperatures within the hibernacula, and ground temperatures are typically stable throughout winter dormancy (e.g., Lutterschmidt *et al*. 2006). Thus, daily body temperature fluctuations like those observed in species exhibiting daily torpor are not a potential cue for synchronizing the circadian system during winter dormancy. Furthermore, animals that occupy underground hibernacula during continuous winter dormancy are not exposed to changing photoperiodic conditions (e.g., Grobman 1990; Whittier *et al*. 1987*a*). Most intriguing is that in species where reproduction occurs immediately following spring emergence, the associated changes in neurophysiology and behavior that accompany reproduction must occur during winter dormancy. Thus,

significant changes in reproductive physiology and behavior occur during this "dormancy" period, and the seasonal control of reproduction is therefore likely linked to the environmental and hormonal mechanisms controlling winter dormancy.

Relatively little is known about the mechanisms regulating seasonal reproduction in ectotherms. In some species, 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., Crawford 1991; Crews and Garstka 1982; Grobman 1990; Hawley and Aleksiuk 1975, 1976; Jacob and Painter 1980; Licht 1984; Macartney *et al*. 1989; Whittier *et al*. 1987*a*). For example, 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 prior to spring emergence. Red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada emerge at body temperatures as low as 0.5°C, suggesting that increases in ground temperatures may not be the only thermal cue utilized by ectotherms (Lutterschmidt *et al*. 2006; Macartney *et al*. 1989).

A potential hormonal mechanism regulating seasonal reproduction in ectotherms is the pineal gland and its major secretory product, melatonin. Circadian melatonin rhythms function in the neuroendocrine transduction of environmental stimuli in vertebrates (Axelrod 1974). While photoperiodic entrainment influences the phase of the melatonin cycle, environmental temperature modulates its amplitude. In diamondback

water snakes (*Nerodia rhombifer*), very cold and very 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*) (García-Allegue *et al.* 2001; Moyer *et al.* 1995; Rawding and Hutchison 1992; Tilden and Hutchison 1993; Underwood 1985*a*; Vivien-Roels *et al.* 1988).

Melatonin's influence on multiple systems in turn plays an important role in integrating an animal's physiology and behavior with its environment. For example, changes in the duration of the melatonin signal 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 in Turek and Van Cauter 1994 and Mayer *et al*. 1997). Pinealectomy of male anoles stimulates testicular growth and spermatogenesis (Underwood 1985*b*); melatonin treatment abolishes the effects of pinealectomy on reproduction in female anoles (Levey 1973). In male red-sided garter snakes (*T. sirtalis parietalis*), pinealectomy prior to hibernation abolishes courtship behavior upon spring emergence (Crews *et al*. 1988; Mendonça *et al*. 1996*a*; Nelson *et al*. 1987). In contrast, pinealectomy following spring emergence has no effect on courtship behavior (Nelson *et*

al. 1987; Mendonça *et al*. 1996*a*). These results suggest the pineal gland is necessary for transducing environmental stimuli during winter dormancy in red-sided garter snakes.

Interactions between melatonin and other endocrine signals are also important in integrating multiple physiological and behavioral rhythms. For example, glucocorticoids, a secretory product of the hypothalamus-pituitary-adrenal (HPA) axis that modifies metabolism and regulates energy balance, is also a hormonal pacemaker of physiological and behavioral processes. Interactions between melatonin, glucocorticoids, and the HPA axis are well established (e.g., Barriga *et al*. 2002; Maestroni *et al*. 1989; Otsuka *et al*. 2001). Activation of the HPA axis during capture stress significantly increases photophasic melatonin levels in red-sided garter snakes, *T. sirtalis parietalis* (Lutterschmidt, unpublished data). Such a physiological coupling between melatonin and glucocorticoid cycles may play a role in regulating seasonal rhythms in physiology and behavior.

To better understand the environmental cues and hormonal mechanisms controlling seasonal reproduction in ectotherms, we examined interactions among environmental temperatures, melatonin, and glucocorticoid rhythms 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 of red-sided garter snakes does not depend upon the activational effects of sex steroid hormones (Crews 1984, 1991; Crews

et al. 1984; Whittier and Tokarz 1992). Rather, mating behavior is triggered by increased environmental temperatures following winter dormancy (Ross and Crews 1978; Garstka *et al*. 1982; Bona-Gallo and Licht 1983; Krohmer and Crews 1987; Whittier *et al*. 1987*a*). Both male and female snakes are refractory to warm temperatures; snakes require a period of low temperature conditions (> 4 wk) to initiate sexual behavior (Camazine *et al*. 1980; Bona-Gallo and Licht 1983; Garstka *et al*. 1982). Such steroid-independent reproductive patterns are typically observed in animals inhabiting environments that provide predictable but brief opportunities for reproduction (Crews and Gans 1992).

Because spring emergence in red-sided garter snakes is independent of changes in photoperiodic conditions (Nelson *et al.*1987; Whittier *et al.*1987*a*), this model system provides an excellent opportunity to examine the role of temperature as the primary environmental cue synchronizing seasonal reproduction. In addition, both melatonin and glucocorticoids play important roles in regulating the seasonal biology of red-sided garter snakes (e.g., Lutterschmidt and Mason 2005, Lutterschmidt *et al*. 2004; Mendonça *et al*. 1996*a*, 1996*b*; Moore and Mason 2001; Moore *et al*. 2000*a*, 2001). Thus, this model system provides a valuable framework for investigating interactions among environmental temperatures, melatonin, and corticosterone [the primary glucocorticoid in reptiles (Idler 1972)]. We sought to investigate the mechanisms by which temperature induces seasonal reproductive behavior in red-sided garter snakes. Specifically, we asked whether elevated melatonin levels and/or increased environmental temperatures during winter dormancy influence (1) patterns of sex steroid hormone and corticosterone

secretion; (2) the expression of reproductive behavior following emergence; and (3) circadian melatonin and corticosterone rhythms during winter dormancy.

Materials and Methods

Animals, Captive Care, and Acclimatization Conditions

These experiments were conducted with red-sided garter snakes (*Thamnophis sirtalis parietalis*) collected 8-15 Sept 2004 from the Interlake region of Manitoba, Canada (50° 37' N, 97° 32' W). 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 aquaria within microprocessor-controlled 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.

Hibernation was induced by decreasing ambient temperatures; an absence of photoperiod cues during hibernation simulated underground hibernacula. Throughout these experiments, photoperiod and temperature regimes were adjusted as described in Table 6.1. The cold temperature hibernation regime was chosen based upon previous laboratory studies of this species (e.g., Bona-Gallo and Licht 1983; Krohmer and Crews 1987; Whittier *et al*. 1987*a*) as well as recorded body temperatures of red-sided garter

Table 6.1. Acclimatization regimes for investigating the influence of hibernation temperatures on reproductive physiology and behavior in red-sided garter snakes (*Thamnophis sirtalis parietalis*). Snakes in the melatonin and control implant treatment groups were housed under the cold temperature hibernation regime.

Acclimatization Period		**Acclimatization Conditions (Photoperiod; Thermoperiod)**	
		Cold Temperature Hibernation	Warm Temperature Hibernation
Pre-hibernation	Wks -4 - 0	11L:13D; 18:10ºC	11L:13D; 18:10ºC
Hibernation	Wks 1 - 6	0L:24D; 10:10ºC	0L:24D; 10:10ºC
Hibernation	Wks 7 - 20	0L:24D; 5:5ºC	0L:24D; 10:10ºC
Hibernation	Wks 21 - 26	0L:24D; 10:10ºC	0L:24D; 10:10ºC
Emergence	Wks 27 +	16L:8D; 25:15ºC	16L:8D; 25:15ºC

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 sufficiently 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 numbers: 2661, 3120) and were in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This research was approved by the Manitoba Wildlife Animal Care Committee (protocol number:

2002-06) and was performed under the authority of Manitoba Wildlife Scientific Permits WSP 03009 and 04004.

Hormone Treatments

To investigate the influence of elevated melatonin concentrations on courtship behavior, circadian corticosterone rhythms, and sex-steroid hormones, we treated snakes with exogenous melatonin during winter dormancy. To significantly elevate melatonin levels throughout these experiments, we used melatonin-filled silastic-capsule implants similar to Mendonça *et al*. (1996*a*). Small pieces of silastic tubing (0.76 mm i.d. × 1.65 mm o.d. × 10 mm length) were packed with crystalline melatonin (purchased from Sigma, St. Louis, MO, USA). Control implants were identical in size to the melatonin implants but were not filled with melatonin. Both ends of each silastic capsule were then sealed with Silastic Brand® medical adhesive (Dow Corning, Midland, MI, USA). Snakes were anesthetized with sodium brevital (0.0025 and 0.003 ml 0.5% brevital per g body mass for males and females, respectively) and a silastic capsule was surgically implanted into the peritoneal cavity. Implants were less than 0.05% of the mean body mass of snakes. Snakes were allowed to recover from surgery for 1-2 days before being returned to the environmental chambers. Snakes were then housed in pre-hibernation environmental conditions (Table 6.1) for 3 wk prior to the onset of hibernation on 11 Oct 2004.

Experimental Design

Patterns of Sex Steroid Hormone and Corticosterone Secretion. We collected blood samples from 20 male and 20 female snakes immediately upon capture in the field (8-9 Sept 2004) to examine steroid hormone concentrations during the fall pre-hibernation period under natural field conditions. To determine if elevated melatonin concentrations and increased hibernation temperatures influence patterns of steroid hormones during winter dormancy, we randomly assigned 48 male and 48 female red-sided garter snakes to one of the following 4 treatments (n = 12 males and 12 females in each): melatonin implant treatment, control (blank) implant treatment, cold temperature hibernation, or warm temperature hibernation. Both the melatonin and control implant treatment groups were housed under the cold temperature hibernation regime (Table 6.1). Once every 4-8 weeks, we collected blood samples from snakes to examine patterns of steroid hormones during winter dormancy. All blood samples in this experiment were collected between 1200 and 1500 h.

Reproductive Behavior. To investigate the influence of elevated melatonin concentrations and increased hibernation temperatures on reproductive behavior, we randomly assigned an additional 168 male red-sided garter snakes to one of the following 4 treatments (n = 42 in each): melatonin implant, control (blank) implant, cold temperature hibernation, or warm temperature hibernation. Both the melatonin and control implant treatment groups were housed under the cold temperature hibernation regime (Table 6.1). Following emergence from winter dormancy, courtship behavior was

assessed for each male every 3 days for approximately 3 wks and again at 48 days post-emergence. All courtship trials were conducted between 1200 and 1600 h.

Courtship trials were performed in 10-gallon aquaria with 8 males (i.e., 2 males randomly selected from each of the 4 treatment groups) simultaneously introduced to an unmated, attractive female. Males were introduced in groups of 8 to simulate natural mating conditions, where the presence of a mating ball facilitates male courtship behavior (Joy and Crews 1985). Because males are attracted to females by both the presence of pheromonal cues expressed on the dorsal surface of females as well as the presence of a mating ball, mating balls rarely contain fewer than five males courting a single female (Joy and Crews 1985).

Using an ethogram of male courtship behavior (Table 6.2), 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. Adhesive tape was placed across the female's cloaca to prevent mating during the trials, as mating significantly reduces further male courtship behavior (Garstka *et al*. 1982). The tape does not alter male or female reproductive behavior and was immediately removed following each trial (LeMaster and Mason 2002). Each male was therefore assigned a courtship score of 0 (no reproductive behavior) through 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 (Table 6.2).

Table 6.2. Ethogram of courtship behavior for the male red-sided garter snake (*T. sirtalis parietalis*). Behaviors 3.0 and greater are exhibited only in a reproductive context (modified from Moore *et al*. 2000 and Crews *et al*. 1984).

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

Circadian Melatonin and Corticosterone Rhythms. The aim of this experiment was to investigate whether elevated melatonin concentrations and increased hibernation temperatures influence reproductive behavior via modulation of circadian melatonin and corticosterone rhythms. During winter dormancy, we measured circadian hormone cycles of snakes in each of the 4 treatment groups described above (n = 42 in each); the same male snakes used to assess courtship behavior (n = 168 total) were used in this experiment. Once every 4-8 weeks during winter dormancy, we measured circadian melatonin and corticosterone rhythms in each treatment group by collecting blood samples from a randomly-selected subset of snakes (n = 7 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.

Courtship Behavior and Circadian Hormone Rhythms in 2002-2003. During the winter dormancy period of 2002-2003, we performed preliminary experiments similar to those described above. For these preliminary experiments, male and female red-sided garter snakes were collected during the spring mating season of May 2002 and again in fall 2002. Snakes collected during the spring were transported to the laboratory and housed under summer-like environmental conditions (i.e., 16L:8D photoperiod; 24:18°C thermoperiod). Snakes were fed twice weekly with a combination of fish and worms fortified with vitamins; water was provided *ad libitum*.

From late December 2002 through March 2003, snakes were maintained in hibernation under a constant photoperiod of 0L:24D and temperature of 5°C. Environmental conditions were then adjusted to a photoperiod of 16L:8D and temperature of 20°C to simulate spring emergence. We measured courtship behavior of male snakes (n = 15) every 5-10 days during spring emergence. Courtship trials were performed in 10-g aquaria with 5 males simultaneously introduced to an unmated, attractive female. Using an ethogram of male courtship behavior (Table 6.2), we recorded the courtship score of each male every 10 min for a period of 1 h after introduction into the arena; the observer was blind to the treatment group of each male. All courtship trials were conducted between 1200 and 1800 h. Three weeks following emergence, we measured circadian melatonin and corticosterone rhythms by collecting blood samples from a randomly-selected subset of snakes (n = 4-5 at each sampling time) every 4 h for one 24-h period. No snake was bled more than once during the 24-h sampling period.

Blood Sampling and Radioimmunoassay

Blood samples (300 µl) were obtained from the caudal vein within 3 min using heparinized 1-cm^3 syringes and 25-g needles. Scotophasic blood samples were collected under dim red light. Samples were stored on ice until centrifuged and the plasma separated. Plasma samples were then stored at – 70ºC until analyzed for melatonin and /or steroid hormone concentrations following radioimmunoassay procedures described by Lutterschmidt *et al*. (2004).

Briefly, melatonin concentrations were determined from duplicate aliquots (20 µl for melatonin implant samples; 100 µl for all others) of each plasma sample. To determine extraction efficiency, 20-30 replicates of garter snake plasma were incubated overnight with 2000 cpm tritiated melatonin (*O*-methyl-^{3}H melatonin, Amersham Biosciences, Piscataway, NJ). Tests for sample recovery were run simultaneously with each melatonin assay. Melatonin was extracted from each plasma sample twice with a total of 4.5 ml HPLC-grade chloroform. A portion (4.0 ml) of the chloroform phase was removed and dried under nitrogen gas in a warm water bath and the hormone extracts were then reconstituted in 200 µl tricine-buffered saline (pH 8.0) for assay. Recovery samples were reconstituted in 400 µl tricine-buffered saline and incubated overnight at 4ºC. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were then incubated with 100 µl melatonin antiserum (Stockgrand LTD, Surrey, UK), 50 µl sheep serum (Sigma, St. Louis, MO), and 50 µl (approximately 6000 cpm) tritiated melatonin at 4ºC for 18-26 h. Unbound melatonin was separated from bound hormone using dextran-coated charcoal.

The bound hormone was decanted into scintillation vials and incubated in toluene-based scintillation fluid for 12 h. The radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter. We previously validated this melatonin radioimmunoassay for use with plasma from red-sided garter snakes by demonstrating parallelism between serially-diluted snake plasma and serially-diluted melatonin standards. In addition, quantitative recovery tests following addition of melatonin to charcoal-stripped snake plasma also indicate there are no factors in snake plasma that interfere with this competitive binding assay.

Steroid hormone concentrations were determined using radioimmunoassay procedures described by Lutterschmidt *et al*. (2004) and Lutterschmidt and Mason (2005). Briefly, an aliquot (4-120 µl) of each plasma sample was incubated overnight with 2000 cpm tritiated steroid (1,2,6,7-^{3}H testosterone, 2,4,6,7,16,17-^{3}H Oestradiol, or 1,2,6,7-^{3}H corticosterone, Amersham Biosciences, Piscataway, NJ) to determine extraction efficiency. Steroids were extracted from each plasma sample with 3 ml anhydrous ethyl ether. The ether phase was removed and dried under nitrogen gas in a warm water bath and the hormone extracts were then reconstituted in phosphate-buffered saline for direct assay. Individual sample recoveries were determined from a 50-µl aliquot of each reconstituted sample. For each hormone being assayed, the remaining sample was allocated to two duplicate culture tubes for assay. Serial dilutions of the standard curve (performed in triplicate), 0% bound (or non-specific binding), 100% bound, and all samples were then incubated with 100 µl each antiserum (testosterone antibody T-3003 and estradiol antibody E-6006 from Wien Laboratories, Inc.,

Succasunna, NJ; corticosterone antibody B3-163 from Esoterix Endocrinology, Calabasas Hills, CA) and tritiated steroid (approximately 10 000 cpm) at 4ºC for 12-24 h. Because the testosterone antibody cross-reacts significantly with 5-α-dihydrotestosterone (63.2% cross-reactivity), our direct testosterone assay measures both plasma testosterone and 5-α-dihydrotestosterone concentrations. For these reasons, we present data for total androgen concentrations in male snakes. Unbound steroid was separated from bound hormone using dextran-coated charcoal. The bound steroid was decanted into scintillation vials and incubated in toluene-based scintillation fluid for 12 h. The radioactivity of each sample was quantified in a Beckman LS 1800 scintillation counter.

All samples were randomly distributed across hormone assays. Hormone concentrations were corrected for individual recovery variation. Mean extraction efficiency for melatonin was 100.0%; mean extraction efficiencies for testosterone, estradiol, and corticosterone were 97.3, 92.2, and 97.5%, respectively. Mean intra- and inter-assay coefficients of variation were 4.6 and 5.0% for melatonin, 6.3 and 11.6% for testosterone, 12.4 and 15.3% for estradiol, and 11.1 and 15.5% for corticosterone.

Statistical Analyses

We investigated the influence of exogenous melatonin and hibernation temperatures on patterns of steroid hormone production during winter dormancy using a two-way repeated measures ANOVA on androgen and corticosterone concentrations within male snakes and estradiol and corticosterone concentrations within female snakes.

Time (i.e., sampling month) was included as the repeated or within-subjects factor, while treatment condition was included in the analyses as a between-subjects factor.

We used a two-way repeated measures ANOVA to examine the influence of exogenous melatonin treatment on courtship behavior following winter dormancy. Likewise, we investigated the influence of hibernation temperatures on the expression of courtship behavior following emergence using a two-way repeated measures ANOVA. These analyses were performed on the highest courtship score achieved by each male during a courtship trial. 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 analyses as a between-subjects factor. A one-way ANOVA was used to ensure there were no differences in body composition index among treatment groups at the beginning of these experiments. Body composition index is determined as an individual's residual from a regression of body mass on snout-to-vent length for all snakes (e.g., Lutterschmidt and Mason 2005; Moore *et al*. 2000*b*). Possible differences among treatment groups in percent change in body mass over the course of the experiment were investigated using a one-way ANOVA.

To investigate the influence of exogenous melatonin treatment on circadian melatonin and corticosterone rhythms of snakes, we performed a two-way analysis of variance (ANOVA) within each sampling month on each hormone cycle. Treatment (i.e., melatonin versus control implant) and sampling time within the 24-h cycle were included in these analyses as between-subjects factors. To investigate the influence of increased hibernation temperatures on circadian melatonin and corticosterone rhythms, we

performed a two-way ANOVA within each sampling month on each hormone cycle. Treatment (i.e., cold versus warm hibernation regime) and sampling time within the 24-h cycle were included as between-subjects factors.

To examine possible differences in melatonin and corticosterone cycles among courting and non-courting snakes, we further evaluated reproductive behavior of snakes recorded the day before (i.e., on day 10 post-emergence) circadian hormone cycles were measured. Snakes in the cold and warm hibernation temperature treatments achieving a courtship score ≥ 3 were categorized as courting, while snakes achieving a courtship score ≤ 2 were categorized as non-courting snakes. We then reanalyzed the melatonin and corticosterone hormone rhythms measured post-emergence using a two-way ANOVA on each hormone with category (i.e., courting versus non-courting) and sampling time within the 24-h cycle included as between-subjects factors.

Lastly, we used a z-test to compare the proportion of snakes exhibiting courtship behavior (courtship score ≥ 3) between the experiments conducted in 2003 versus 2005 (cold and warm hibernation temperature treatments pooled). A t-test was used to compare differences in body composition index of male snakes between years. To examine possible differences in melatonin and corticosterone rhythms between different years, we performed a two-way ANOVA on each hormone cycle with year (i.e., 2003 versus 2005, cold and warm temperature treatments pooled) and sampling time within the 24-h cycle included as between-subjects factors.

We used SigmaStat® 3.11 (Systat 2004) for all statistical analyses. Data were ln- or rank-transformed where necessary to correct for non-normality and unequal variance prior to analysis. All statistical comparisons were considered significant at $P \leq 0.05$.

Results

Patterns of Sex Steroid Hormone and Corticosterone Secretion.

Treatment with exogenous melatonin did not significantly influence either androgen or corticosterone concentrations of male snakes during winter dormancy (from a two-way repeated measures ANOVA for each hormone). Because there were no statistically significant differences among androgen or corticosterone concentrations of snakes in the cold temperature hibernation regime (i.e., among melatonin implant, control implant, and cold temperature hibernation treatments), we chose to pool these groups into one treatment parameter to evaluate the effects of hibernation temperatures on steroid hormone patterns. Increased hibernation temperature significantly decreased androgen concentrations of snakes during winter dormancy (Figure 6.1A; $F_{(1,46)} = 11.041$, $P = 0.002$ from a two-way repeated measures ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure); hibernation temperature did not significantly influence corticosterone concentrations of male snakes (Figure 6.1B; from a two-way repeated measures ANOVA). Both androgen and corticosterone concentrations changed significantly with time during the course of winter dormancy (Figure 6.1A; $F_{(4,43)} = 52.429$, $P < 0.001$ and Figure 6.1B; $F_{(4,43)} = 36.902$, $P < 0.001$, respectively, from a two-way repeated measures ANOVA followed by a Student-Newman-Keuls multiple

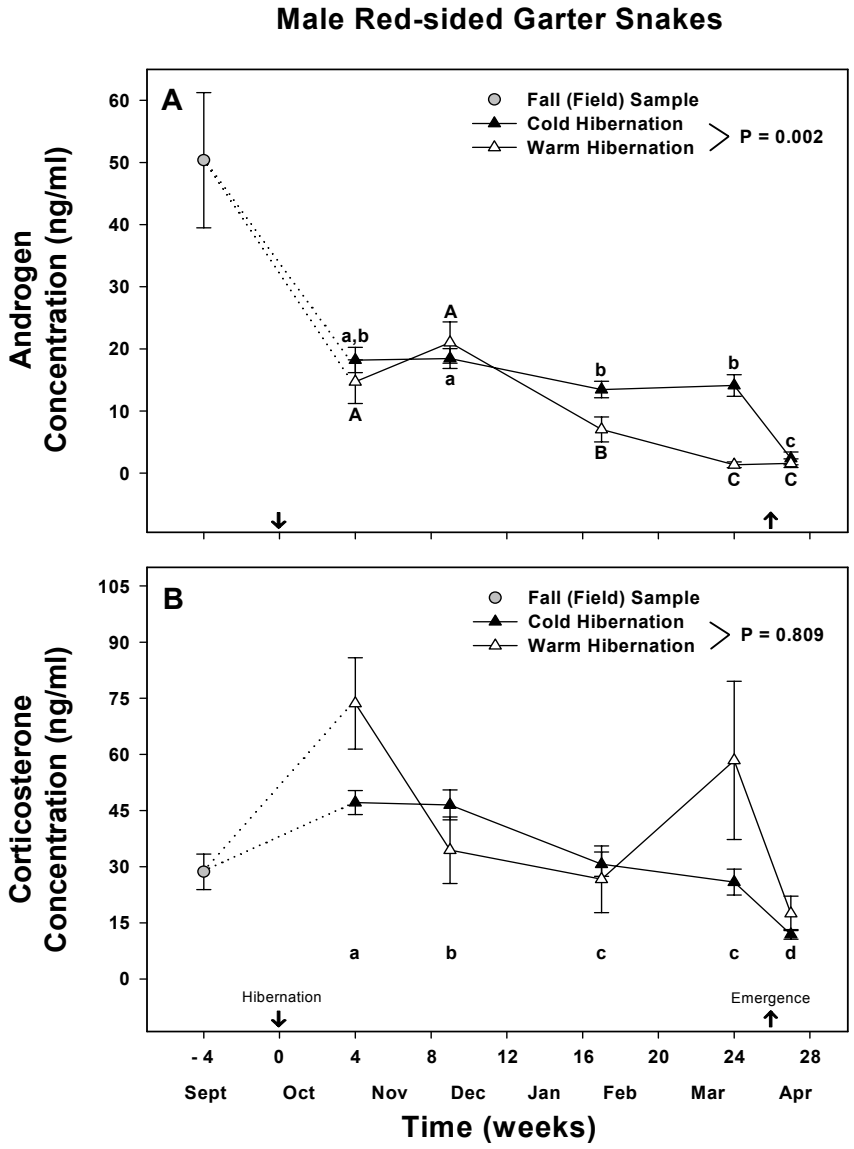

Figure 6.1. Influence of elevated hibernation temperatures on (A) androgen concentrations and (B) corticosterone concentrations of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) during winter dormancy. Fall pre-hibernation steroid concentrations were determined from a randomly-selected subset of males (n = 20) sampled immediately upon capture in the field. Each subsequent data point is the mean ± 1 standard error of snakes in the cold hibernation (n = 36) and warm hibernation (n = 12) treatment groups. Differences among sampling periods within the cold temperature hibernation treatment (closed symbols) are indicated by lower-case letters, while those within the warm temperature hibernation treatment (open symbols) are indicated by capital letters. Letters appearing above the abscissa indicate differences among sampling periods when treatment conditions do not differ significantly. Induction of hibernation and spring emergence are indicated by arrows along the abscissa.

comparisons procedure for each hormone). As expected, the effect of hibernation temperatures on androgen and corticosterone concentrations significantly 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 for both androgen ($F_{(1 \times 4)} = 8.782$, $P < 0.001$) and corticosterone ($F_{(1 \times 4)} = 6.236$, $P < 0.001$).

Neither estradiol nor corticosterone concentrations of female snakes were significantly influenced by treatment with exogenous melatonin (from a two-way repeated measures ANOVA for each hormone). Because there were no statistically significant differences among estradiol or corticosterone concentrations of snakes in the cold temperature hibernation regime (i.e., among melatonin implant, control implant, and cold temperature hibernation treatments), we pooled these treatment groups prior to investigating the influence of hibernation temperatures on steroid hormone patterns. Increased hibernation temperature did not significantly influence either estradiol or corticosterone concentrations of female snakes (Figures 6.2A and 6.2B, respectively; from a two-way repeated measures ANOVA). Both estradiol and corticosterone concentrations changed significantly with time during the course of winter dormancy (Figure 6.2A; $F_{(4,43)} = 43.876$, $P < 0.001$ and Figure 6.2B; $F_{(4,43)} = 57.313$, $P < 0.001$, respectively, from a two-way repeated measures ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure for each hormone). As expected, the effect of hibernation temperatures on estradiol and corticosterone concentrations significantly 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 for both estradiol ($F_{(1 \times 4)} = 4.301$, $P = 0.002$) and corticosterone ($F_{(1 \times 4)} = 5.972$, $P < 0.001$).

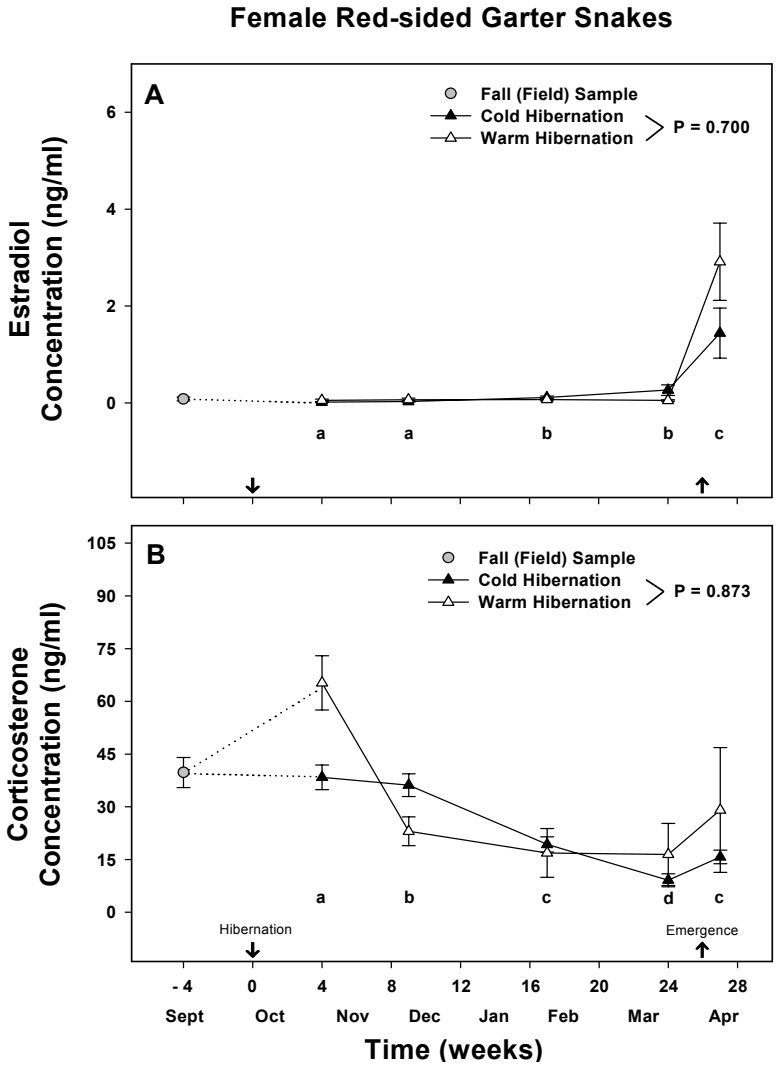

Figure 6.2. Influence of elevated hibernation temperatures on (A) estradiol concentrations and (B) corticosterone concentrations of female red-sided garter snakes (*T. sirtalis parietalis*) during winter dormancy. Fall pre-hibernation steroid concentrations were determined from a randomly-selected subset of females (n = 20) sampled immediately upon capture in the field. Each subsequent data point is the mean ± 1 standard error of snakes in the cold hibernation (n = 36) and warm hibernation (n = 12) treatment groups. Letters above the abscissa indicate statistically significant differences among sampling periods. Induction of hibernation and spring emergence are indicated by arrows along the abscissa.

Reproductive Behavior and Body Condition.

Exogenous melatonin treatment did not significantly influence the highest courtship scores achieved by male red-sided garter snakes (Figure 6.3A; from a two-way repeated measures ANOVA). The expression of courtship behavior significantly changed with time during the course of emergence (Figure 6.3A; $F_{(7,74)} = 27.324$, $P < 0.001$ from a two-way repeated measures ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). There were no significant interactions between treatment group and time (i.e., days post-emergence).

Increased hibernation temperature did not significantly influence the highest courtship scores achieved by male red-sided garter snakes (Figure 6.3B; from a two-way repeated measures ANOVA). The expression of courtship behavior significantly changed with time during the course of emergence (Figure 6.3B; $F_{(7,71)} = 20.790$, $P < 0.001$ from a two-way repeated measures ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). Although the different hibernation temperature treatments overall did not significantly influence courtship behavior, the effect of hibernation temperature significantly depended on the day courtship behavior was measured post-emergence ($F_{(1 \times 7)} = 2.156$, $P = 0.037$ from a two-way repeated measures ANOVA). This significant interaction between hibernation temperature treatment and time (days post-emergence) is evident in Figure 6.3B.

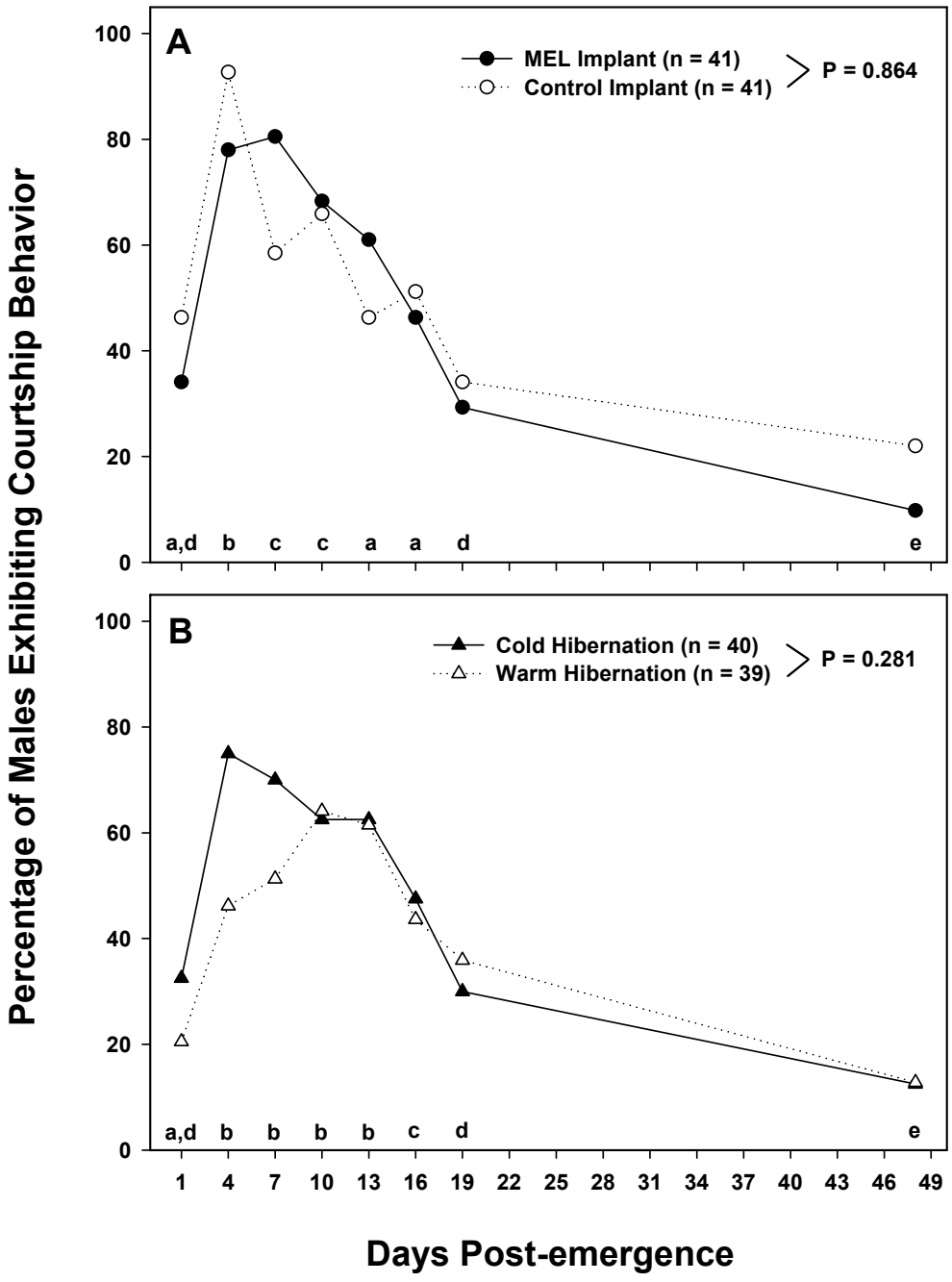

Figure 6.3. Influence of (A) exogenous melatonin and (B) elevated hibernation temperatures on the expression of courtship behavior following winter dormancy in male red-sided garter snakes (*T. sirtalis parietalis*). Statistically significant differences among days post-emergence are indicated by letters above the abscissa (from a two-way ANOVA on highest courtship scores). For ease of visual interpretation, data are presented as percentage of males exhibiting courtship behavior during spring emergence.

At the beginning of these experiments, body composition indices of male red-sided garter snakes did not differ significantly among treatment groups (from a one-way ANOVA). However, the percent body mass lost during the experiment differed significantly among treatments (Figure 6.4; H = 15.912, df = 3, P = 0.001 from a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's multiple comparisons procedure). Male snakes treated with exogenous melatonin lost significantly more body mass than snakes in the control implant treatment group (Figure 6.4). Percent body mass lost did not differ significantly among snakes in the cold and warm temperature hibernation regimes (Figure 6.4).

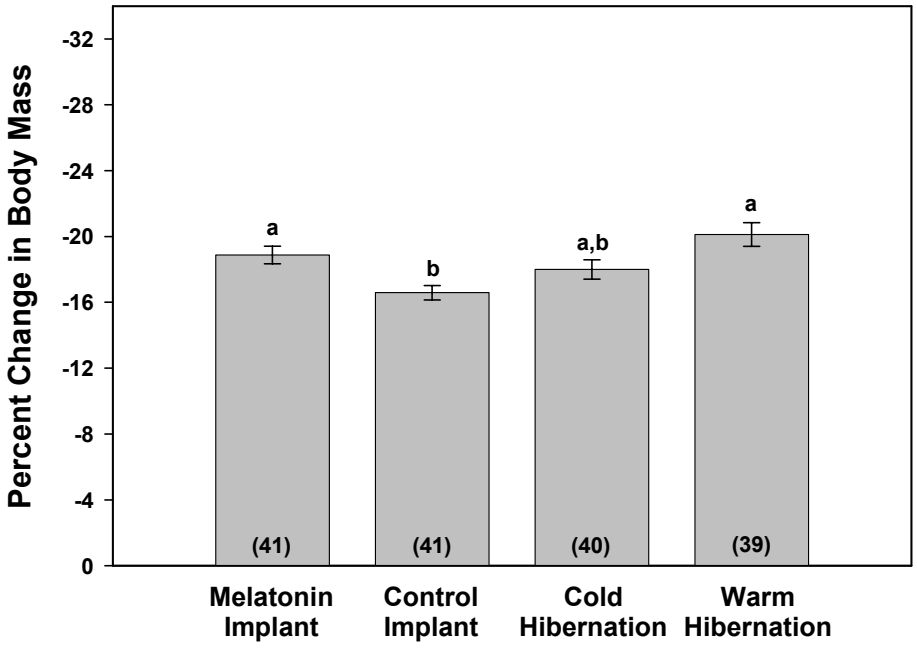

Figure 6.4. Influence of exogenous melatonin and elevated hibernation temperatures on the percent change in body mass of male red-sided garter snakes (*T. sirtalis parietalis*) during winter dormancy. Sample sizes are indicated in parentheses below the treatment group; standard errors (± 1) are shown by the vertical lines. Statistically significant differences are indicated by letters below each standard error bar.

Circadian Melatonin and Corticosterone Rhythms.

Treatment of snakes with melatonin-filled silastic-tubing implants significantly elevated melatonin concentrations during these experiments (wk 18 in hibernation, data not shown: $F_{(1,83)} = 591.996$, $P < 0.001$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure; day 11 post-emergence, Figure 6.5A: $F_{(1,81)} = 433.056$, $P < 0.001$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). Melatonin concentrations of snakes did not vary significantly over the 24-h sampling period during either sampling month [i.e., during wk 18 in hibernation (data not shown) nor day 11 post-emergence (Figure 6.5A)]; there were no significant interactions between treatment and sampling time within the 24-h cycle (from a two-way ANOVA within each sampling month). Exogenous melatonin treatment did not significantly influence corticosterone rhythms of snakes at day 11 post-emergence (Figure 6.5B; from a two-way ANOVA). A statistically significant 24-h cycle was observed in corticosterone concentrations at day 11 post-emergence (Figure 6.5B; $F_{(5,76)} = 2.936$, $P = 0.018$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). There were no significant interactions between treatment and sampling time within the 24-h cycle.

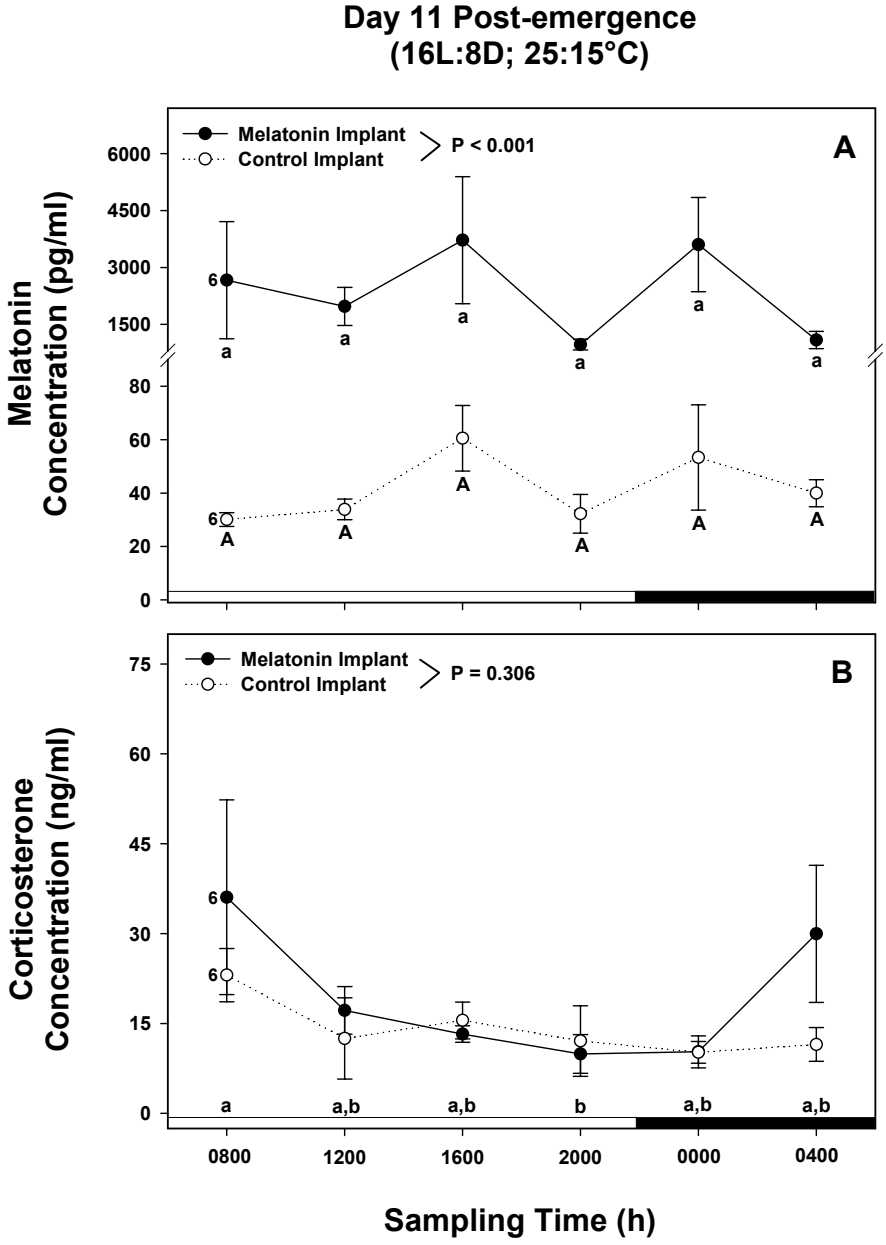

Figure 6.5. Influence of exogenous melatonin on circadian (A) melatonin and (B) corticosterone rhythms of male red-sided garter snakes (*T. sirtalis parietalis*) during the period of spring emergence. Black bars above each abscissa indicate the period of scotophase. Unless otherwise noted, each data point is the mean hormone concentration of 7 snakes ± 1 standard error. Differences among sampling times within the melatonin implant treatment (closed symbols) are indicated by lower-case letters, while those within the control implant treatment (open symbols) are indicated by capital letters. Letters appearing above the abscissa indicate statistically significant differences among sampling times when treatment conditions do not differ significantly.

As expected, there were no significant differences between the melatonin cycles of snakes in the cold and warm temperature hibernation treatments during wk 5 in hibernation, as these treatment groups were initially acclimated to the same temperature of 10°C (Figure 6.6A; from a two-way ANOVA). A significant melatonin rhythm was observed in snakes during wk 5 in hibernation (Figure 6.6A; $F_{(5,78)} = 2.613$, $P = 0.031$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). There were no significant interactions between treatment and sampling time within the 24-h cycle.

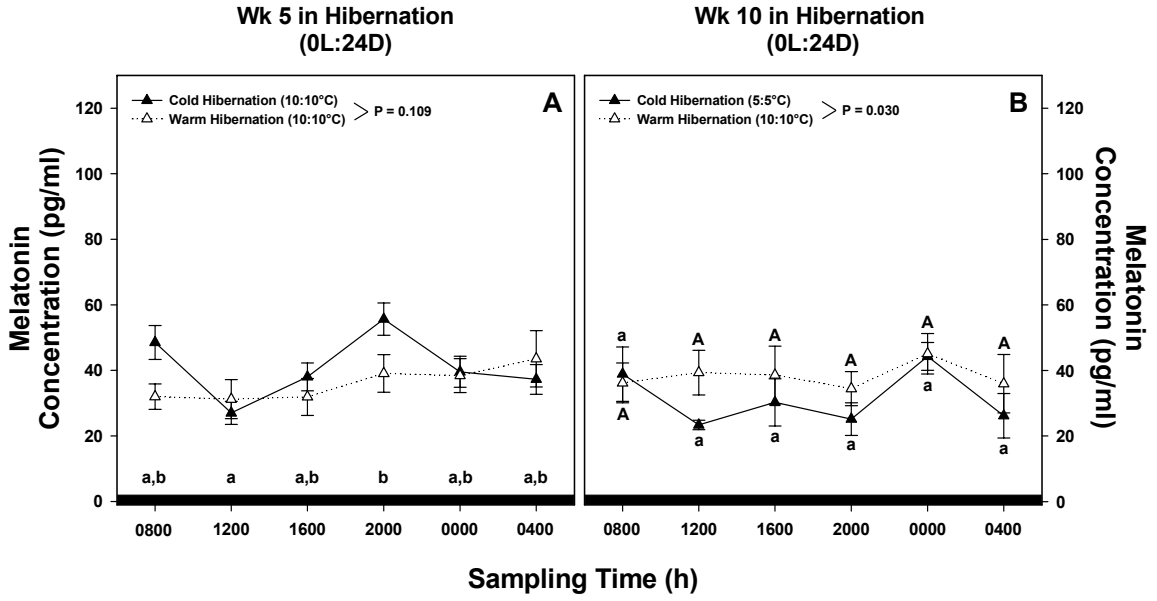

Figure 6.6. Influence of elevated hibernation temperatures on circadian melatonin rhythms of male red-sided garter snakes (*T. sirtalis parietalis*) during (A) wk 5 and (B) wk 10 in hibernation. Temperature acclimation conditions are listed in parentheses within the legend. Black bars above each abscissa indicate the period of scotophase. Each data point is the mean of 7 snakes ± 1 standard error. Differences among sampling times within the cold temperature hibernation treatment (closed symbols) are indicated by lower-case letters, while those within the warm temperature hibernation treatment (open symbols) are indicated by capital letters. Letters appearing above the abscissa indicate statistically significant differences among sampling times when treatment conditions do not differ significantly.

During wk 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 (Figure 6.6B; $F_{(1,83)} = 4.895$, $P = 0.030$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). Melatonin rhythms were not statistically significant in either treatment group (Figure 6.6B; from a two-way ANOVA). There were no significant interactions between treatment and sampling time within the 24-h cycle. These differences between the melatonin concentrations of snakes in the cold and warm temperature hibernation treatments were also observed during wk 18 in hibernation (Figure 6.7A; $F_{(1,83)} = 4.181$, $P = 0.045$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). Again, melatonin rhythms were not statistically significant in either treatment group (Figure 6.7A; from a two-way ANOVA), and there were no significant interactions between treatment and sampling time within the 24-h cycle.

When snakes in the cold temperature hibernation treatment were again acclimated to 10°C during wks 21-26 in hibernation (i.e., the same temperature as in the warm temperature hibernation treatment), significant differences in the melatonin concentrations of snakes between the two hibernation temperature treatments persisted (Figure 6.7B; $F_{(1,81)} = 4.068$, $P = 0.048$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). Melatonin rhythms during wk 23 in hibernation were not statistically significant in either treatment group (Figure 6.7B; from a two-way ANOVA), and there were no significant interactions between treatment and sampling time within the 24-h cycle. After both hibernation temperature treatment

Figure 6.7. Influence of elevated hibernation temperatures on circadian melatonin and corticosterone rhythms of male red-sided garter snakes (*T. sirtalis parietalis*) during (A and D, respectively) wk 18 in hibernation, (B and E) wk 23 in hibernation, and (C and F) day 11 post-emergence. Temperature acclimation conditions are listed in parentheses within the legend. Black bars above each abscissa indicate the period of scotophase. Unless otherwise noted, each data point is the mean hormone concentration of 7 snakes ± 1 standard error. Differences among sampling times within the cold temperature hibernation treatment (closed symbols) are indicated by lower-case letters, while those within the warm temperature hibernation treatment (open symbols) are indicated by capital letters. Letters appearing above the abscissa indicate differences among sampling times when treatment conditions do not differ significantly.

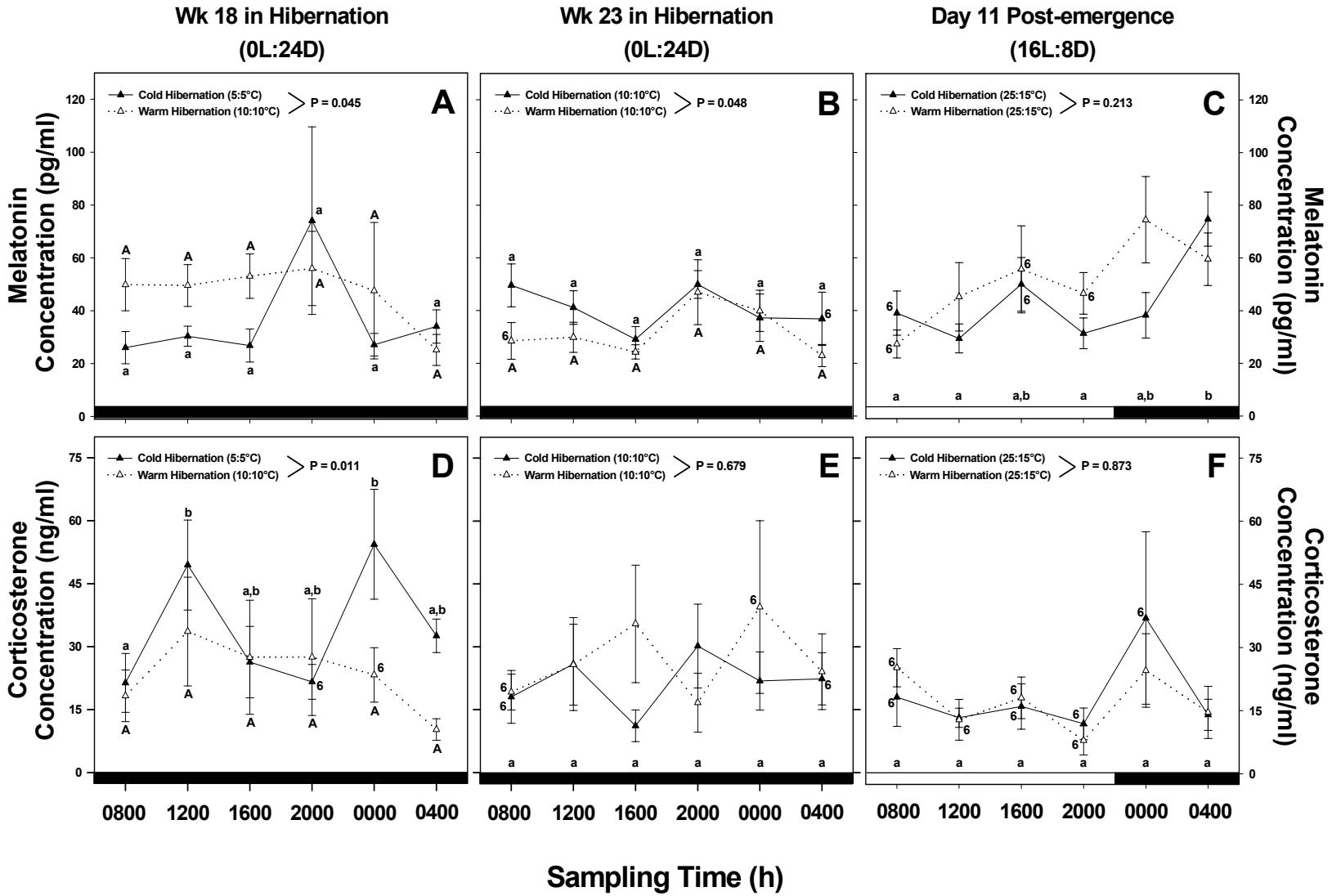
Wk 18 in Hibernation
(0L:24D)
Wk 23 in Hibernation
(0L:24D)
Day 11 Post-emergence
(16L:8D)
Cold Hibernation (5:5°C)
Warm Hibernation (10:10°C)
P = 0.045
A
Cold Hibernation (10:10°C)
Warm Hibernation (10:10°C)
P = 0.048
B
Cold Hibernation (25:15°C)
Warm Hibernation (25:15°C)
P = 0.213
C
Melatonin Concentration (pg/ml)
Cold Hibernation (5:5°C)
Warm Hibernation (10:10°C)
P = 0.011
D
Cold Hibernation (10:10°C)
Warm Hibernation (10:10°C)
P = 0.679
E
Cold Hibernation (25:15°C)
Warm Hibernation (25:15°C)
P = 0.873
F
Corticosterone Concentration (ng/ml)
0800
1200
1600
2000
0000
0400
Sampling Time (h)

Figure 6.7.

groups were acclimated to spring-like environmental conditions (i.e., 16L:8D; 25:15°C), melatonin rhythms did not differ significantly between treatment groups (Figure 6.7C; from a two-way ANOVA). A statistically significant 24-h cycle, with higher levels occurring during scotophase, was observed in melatonin concentrations at day 11 post-emergence (Figure 6.7C; $F_{(5,73)} = 3.192$, $P = 0.012$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). There were no statistically significant interactions between treatment and sampling time within the 24-h cycle.

Hibernation temperature significantly affected corticosterone rhythms of snakes during wk 18 in hibernation (Figure 6.7D; $F_{(1,81)} = 6.757$, $P = 0.011$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure); corticosterone concentrations of snakes acclimated to 5°C were significantly higher than those of snakes acclimated to 10°C. A statistically significant rhythm in corticosterone levels, with two distinct peaks, was observed in the cold temperature hibernation treatment (Figure 6.7D; $F_{(5,77)} = 2.474$, $P = 0.040$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). The observed differences in corticosterone rhythms between snakes in the cold and warm temperature hibernation treatment groups did not persist when snakes were acclimated to identical acclimatization conditions (Figures 6.7E, 6.7F; from a two-way ANOVA within each sampling period). During week 23 in hibernation, corticosterone rhythms were not statistically significant in either hibernation temperature group, but the corticosterone rhythms of snakes in the two treatment groups appeared to be out of phase with one another (Figure 6.7E). During post-emergence, snakes in both the cold and warm temperature hibernation treatments

exhibited higher corticosterone levels during the scotophase (Figure 6.7F). However, these corticosterone rhythms were not statistically significant (from a two-way ANOVA). There were no statistically significant interactions between treatment group and sampling time in any of the analyses of corticosterone rhythms.

During post-emergence, neither melatonin nor corticosterone rhythms differed significantly between courting and non-courting male red-sided garter snakes (Figures 6.8A, 6.8B; from a two-way ANOVA for each hormone). A statistically significant cycle, with higher levels during scotophase, was observed in melatonin concentrations of snakes (Figure 6.8A; $F_{(5,73)} = 3.579$, $P = 0.006$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure). Corticosterone concentrations did not differ significantly over the 24-h sampling period (Figure 6.8B; from a two-way ANOVA). There were no statistically significant interactions between courtship category and sampling time for either melatonin or corticosterone rhythms. Although Figure 6.8B suggests that non-courting males may have higher corticosterone concentrations during the photophase, only one of the snakes sampled at 1200 h was categorized as a non-courting male.

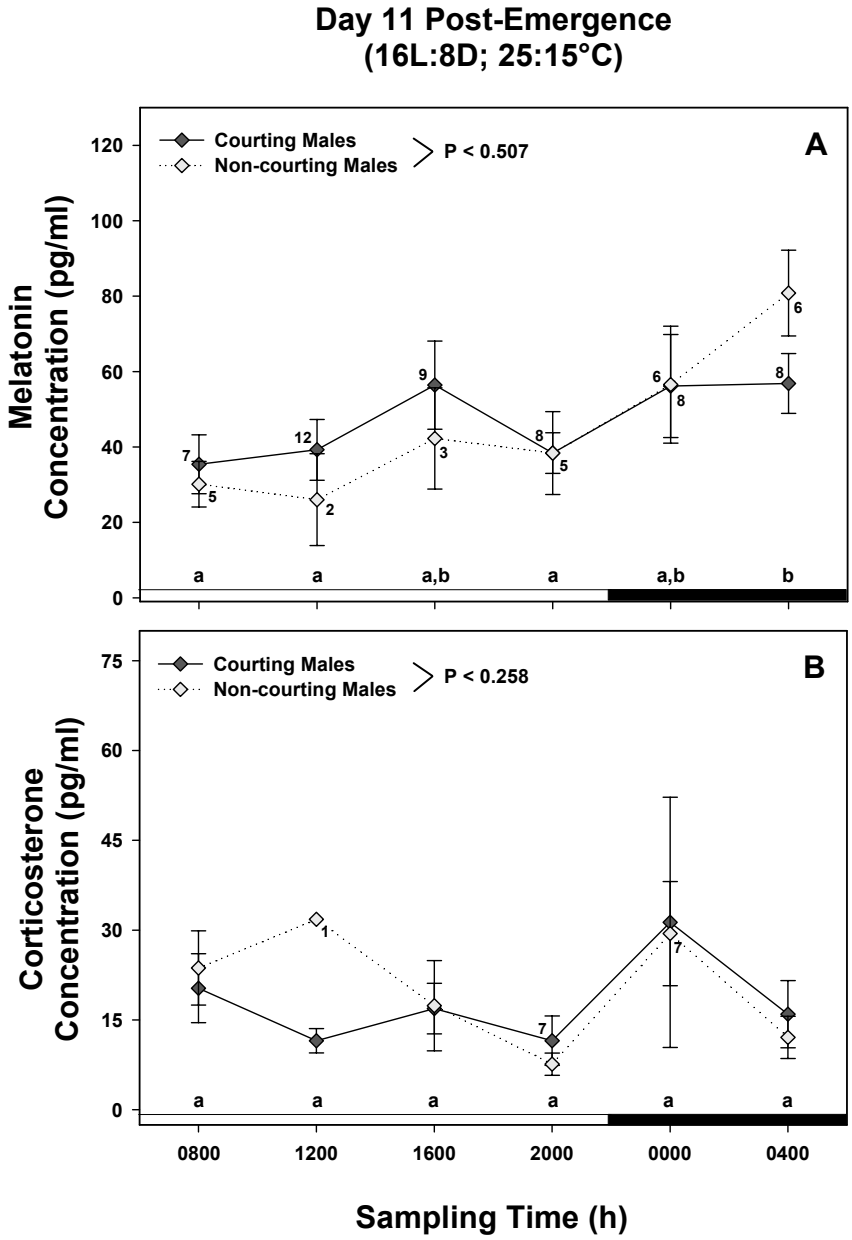

Figure 6.8. Circadian (A) melatonin and (B) corticosterone rhythms of courting versus non-courting male red-sided garter snakes (*T. sirtalis parietalis*) during day 11 post-emergence. Males achieving a courtship score ≥ 3 were categorized as courting snakes (dark gray symbols); scores of non-courting snakes were ≤ 2 (light gray symbols). Black bars above each abscissa indicate the period of scotophase. Each data point is the mean hormone concentration ± 1 standard error; sample sizes for melatonin rhythms are indicated by numbers next to each data point. Unless otherwise noted, sample sizes for corticosterone rhythms are the same as those listed in (A). Letters appearing above the abscissa indicate statistically significant differences among sampling times.

Courtship Behavior and Circadian Hormone Rhythms in 2002-2003.

We observed a statistically significant difference in the proportion of male snakes exhibiting courtship behavior between the 2 different years these experiments were conducted (Figure 6.9A; $z = 3.285$, $P = 0.001$ from a z-test). In 2003, only 13.3% of male snakes (i.e., 2 of 15 males) exhibited courtship behavior during any of the post-emergence sampling periods, whereas 63.3% of males (i.e., 50 of 79 males in the cold and warm temperature hibernation treatments) exhibited a courtship score ≥ 3 at day 10 post-emergence. Body composition index of male snakes did not differ significantly between years (from a Mann-Whitney rank sum test; data not shown).

We investigated whether the circadian melatonin and corticosterone rhythms of snakes during post-emergence might explain the lack of courtship behavior following winter dormancy in 2002-2003. We compared the melatonin and corticosterone rhythms of snakes observed during post-emergence 2005 (cold and warm temperature hibernation treatments pooled) to those of snakes observed during post-emergence 2003. Although the experiments in 2002-2003 included both male and female snakes in the cold temperature hibernation treatment, neither melatonin nor corticosterone concentrations varied significantly with snake sex (from a two-way ANOVA on each hormone with sex and sampling time as between-subjects factors). Thus, we excluded sex from all subsequent analyses.

Melatonin rhythms were statistically significantly different between the two years these experiments were conducted (Figure 6.9B; $F_{(1,103)} = 5.374$, $P = 0.023$ from a two-way ANOVA followed by a Student-Newman-Keuls multiple comparisons procedure).

Melatonin rhythms differed significantly over the 24-h sampling period only during post-emergence 2005 (Figure 6.9B). Corticosterone rhythms of snakes did not differ significantly between years (Figure 6.9C; from a two-way ANOVA). Although the corticosterone rhythms observed during post-emergence 2005 and 2003 are 180° out of phase with one another, the corticosterone rhythms were not significant in either year (Figure 6.9C). There were no significant interactions between year and sampling time within the 24-h cycle for either hormone.

Figure 6.9. Differences in (A) the proportion of males exhibiting courtship behavior, (B) circadian melatonin rhythms, and (C) circadian corticosterone rhythms of red-sided garter snakes (*T. sirtalis parietalis*) among the two different years these experiments were conducted. The cold and warm temperature hibernation treatments during the winter dormancy period of 2004 - 2005 were pooled for analysis. Black bars above each abscissa indicate the period of scotophase. Sample sizes in (A) are listed in parentheses above the abscissa. Each data point in (B) and (C) is the mean hormone concentration ± 1 standard error; sample sizes for melatonin rhythms are indicated by numbers next to each data point. Unless otherwise noted, sample sizes for corticosterone rhythms are the same as those listed in (B). Differences among sampling times within the 2005 sampling period (black symbols) are indicated by lower-case letters, while those within the 2003 sampling period (gray symbols) are indicated by capital letters. Letters appearing above the abscissa indicate differences among sampling times when hormone rhythms do not differ significantly between years.

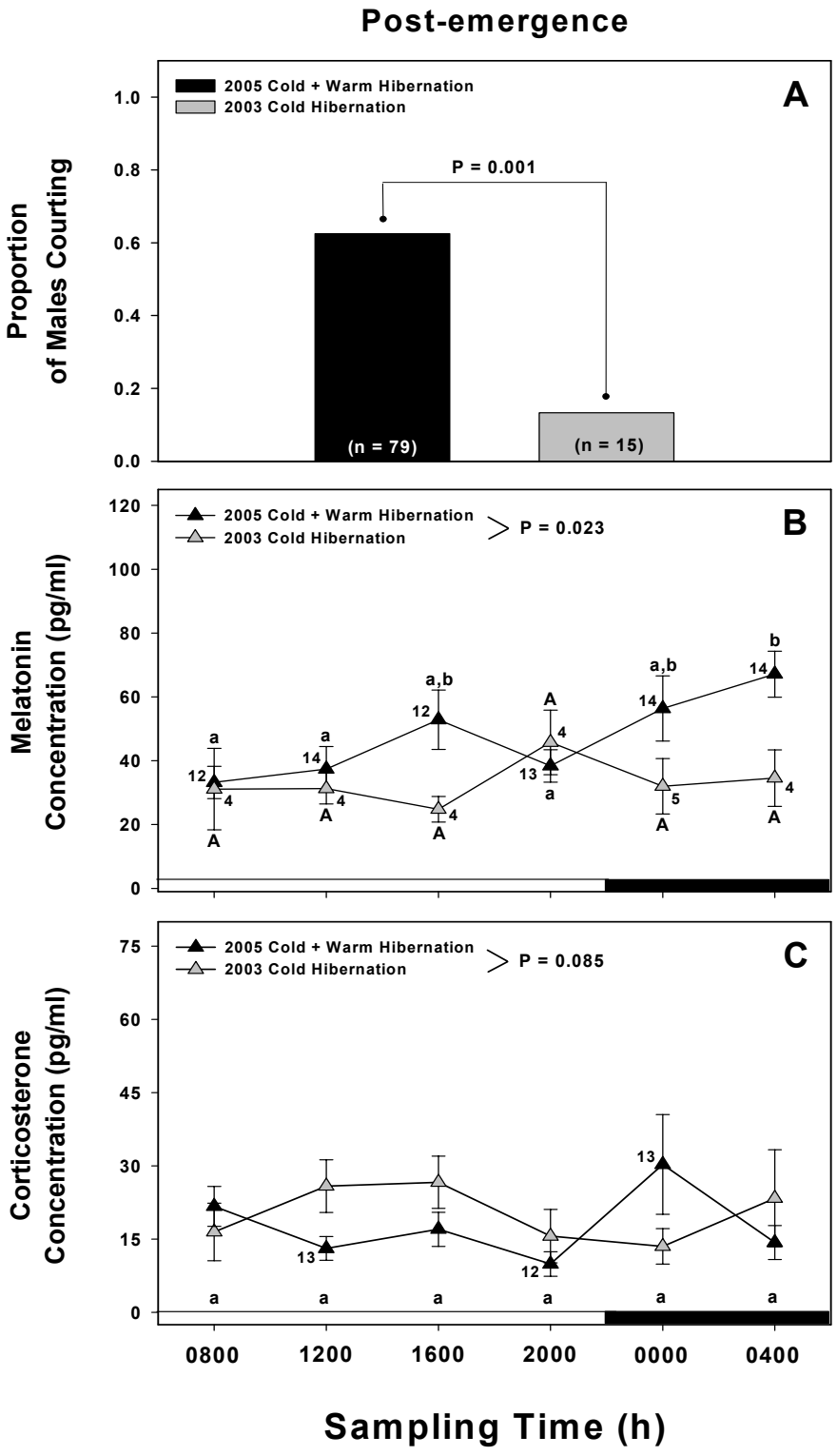
Post-emergence
2005 Cold + Warm Hibernation
2003 Cold Hibernation
A
P = 0.001
Proportion of Males Courting
1.0
0.8
0.6
0.4
0.2
0.0
(n = 79)
(n = 15)
B
P = 0.023
Melatonin Concentration (pg/ml)
120
100
80
60
40
20
0
C
P = 0.085
Corticosterone Concentration (pg/ml)
75
60
45
30
15
0
0800
1200
1600
2000
0000
0400
Sampling Time (h)

Figure 6.9.

Discussion

We investigated the mechanisms by which temperature induces seasonal reproductive behavior in red-sided garter snakes (*T. sirtalis parietalis*). Specifically, we addressed whether elevated melatonin levels and/or increased environmental temperatures during winter dormancy influence (1) patterns of sex steroid hormone and corticosterone secretion; (2) the expression of reproductive behavior following emergence; and (3) circadian melatonin and corticosterone rhythms during winter dormancy. These experiments support previous findings that male red-sided garter snakes exhibit a dissociated reproductive strategy. The observation that estradiol concentrations increase significantly during spring emergence, however, suggests the presence of inter-sexual differences in reproductive strategy. Following winter dormancy, we observed robust courtship behavior in all treatment groups, but male snakes maintained at 10°C during winter dormancy exhibited delayed onset of courtship behavior. Decreased environmental temperatures may induce reproductive behavior of red-sided garter snakes via changes in melatonin and/or corticosterone rhythms. Our results demonstrate that environmental temperature, in the absence of changing photoperiodic cues, is a sufficient *zeitgeber* in modulating circadian melatonin and corticosterone rhythms of red-sided garter snakes during winter dormancy. Furthermore, we suggest that the phase of corticosterone rhythms during the spring mating season plays a role in regulating reproductive behavior in this seasonally breeding reptile.

Patterns of Sex Steroid Hormone and Corticosterone Secretion.

Melatonin's capacity to transduce environmental stimuli into endocrine signals is thought to play an important role in synchronizing reproductive physiology and behavior with appropriate environmental conditions in seasonal breeders. Studies investigating the relationship between melatonin and seasonal reproduction in ectothermic vertebrates have produced inconclusive results (reviewed in Mayer *et al*. 1997). The effects of pinealectomy and/or melatonin treatment on reproductive parameters vary among species, experimental protocols, and seasons, making it difficult to draw conclusions about the role of the pineal gland in reproduction. For example, the effects of pinealectomy on testicular size in the Indian chequered water snake (*Natrix piscator*) are influenced by humidity; pinealectomy decreased testicular size in high humidity (which normally stimulates testicular growth) but increased testicular size in both low and moderate humidity (Haldar and Pandey 1989*a*, 1989*b*). In addition, treatment of Indian chequered water snakes with either melatonin or 5-methoxytryptamine suppressed testicular function of active testes, but did not influence inactive testes (Haldar and Pandey 1988). The effects of pinealectomy in black-spined toads (*Bufo melanostictus*) also vary among this species' seasonal activity patterns. Pinealectomy of toads during the hibernation phase stimulated testicular maturation, while pinealectomy during the breeding season did not influence testicular function (Chanda and Biswas 1984).

Similar to previous studies, melatonin treatment did not significantly influence steroid hormone concentrations in either male or female red-sided garter snakes (*T. sirtalis parietalis*). Crews *et al*. (1988) and Mendonça *et al*. (1996*a*) demonstrated that

pinealectomy inhibits male courtship behavior but does not influence plasma androgen levels in red-sided garter snakes. Treatment with melatonin did not influence the effects of pinealectomy on reproductive behavior (Mendonça *et al*. 1996*a*). In light of the fact that reproductive behavior in red-sided garter snakes is independent of sex steroid hormones, these results are not surprising.

Male red-sided garter snakes (*T. sirtalis parietalis*) are one of the most studied reptilian models of dissociated reproduction [reviewed in Woolley *et al*. (2004)]. Reproductive behavior of red-sided garter snakes does not depend upon the activational effects of sex steroid hormones, as castration prior to hibernation or following spring emergence does not eliminate courtship behavior (Crews *et al*. 1984; Garstka *et al* 1982). In addition, treatment of male snakes with androgens does not induce reproductive behavior (Crews *et al*. 1984; Garstka *et al* 1982). Increases in gonadal activity (i.e., gametogenesis and steroidogenesis) occur during the summer feeding period and do not coincide with spring mating. Rather, mating occurs when gonads are regressed and circulating levels of sex steroid hormones are low (Crews and Garstka 1982; Crews 1984). This steroid-independent induction of reproductive behavior is hypothesized to be an adaptation to the environmental constraints on reproduction in this species. Such dissociated reproductive patterns are generally observed in animals inhabiting environments that provide predictable but brief opportunities for reproduction (e.g., Crews 1987; Crews and Gans 1992).

Our results are consistent with previous findings that male red-sided garter snakes exhibit a dissociated reproductive strategy, in which mating does not coincide with

increased androgen synthesis (Figure 6.1A). We demonstrate that androgen concentrations are elevated when male snakes return to the hibernaculum in preparation for winter dormancy. Androgen concentrations gradually declined during winter dormancy, reaching basal levels during the period of spring emergence. These experiments suggest that androgen concentrations decline during winter dormancy via metabolic clearance, as androgen concentrations reached basal levels significantly earlier when snakes were maintained at higher (10°C) hibernation temperatures (Figure 6.1A). A higher metabolic rate of snakes in the warm temperature hibernation treatment is supported by the finding that these snakes demonstrated a larger (although not significant) percent change in body mass than snakes maintained at 5°C during winter dormancy (Figure 6.4).

In contrast to the numerous studies in male red-sided garter snakes, we provide evidence that female red-sided garter snakes may exhibit an associated reproductive strategy. Estradiol concentrations of female snakes were very low throughout the fall pre-hibernation period as well as 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 (Figure 6.2A). This increase in estradiol concentrations was independent of hibernation temperature. Although these experiments indicate 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. Such experiments would help elucidate whether spring

emergence results in increased estradiol synthesis or if increased estradiol synthesis facilitates spring emergence.

Similar to the pattern observed in androgen concentrations, corticosterone concentrations also significantly declined during winter dormancy in both male and female red-sided garter snakes. However, we did not observe a significant effect of hibernation temperature on corticosterone levels (Figures 6.1B, 6.2B). 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 concentrations in female snakes coincided with increased estradiol synthesis (Figure 6.2). These observations support previous findings that elevated corticosterone concentrations do not negatively influence sex steroid hormones in this species (Lutterschmidt *et al*. 2004; Moore and Mason 2001). The ambiguous pattern in corticosterone concentrations during this portion of the experiment is likely attributable to the changing circadian hormone rhythms we observed during winter dormancy.

These experiments provide a potential mechanism to explain observed variation in steroid concentrations of snakes among years, sampling times, and snake populations (e.g., Woolley *et al*. 2004). For example, initial studies in male red-sided garter snakes demonstrated that androgen concentrations were basal during winter dormancy and spring emergence (Crews 1984; Camazine *et al*. 1980). In contrast, 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*a*). Similar annual variation has also been reported in corticosterone concentrations (e.g., Lutterschmidt and Mason 2005; Moore *et al*. 2000*a*, 2001). 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 concentrations would be expected to be lower throughout winter dormancy and spring emergence.

In female red-sided garter snakes, spring mating results in an estradiol surge, after which females become unreceptive and, within 24 hours, unattractive (Devine 1977; Ross and Crews 1977, 1978; Whittier *et al*. 1985, 1987*b*). It is likely that a neural reflex is involved, as spinal transection or treatment of females with an anesthetic prior to mating abolishes the post-mating estradiol surge and subsequent ovarian development (Halpert *et al*. 1982; Whittier *et al*. 1985, 1987*b*; Whittier and Crews 1986*a*; Mendonça and Crews 1990, 1996, 2001). This estradiol surge may facilitate vitellogenin production by the liver and induce ovarian development (Garstka e*t al*. 1982). However, normal ovarian development has been observed in the absence of a post-mating estradiol surge (Whittier and Crews 1986*b*; Whittier *et al*. 1987*b*; Mendonça and Crews 1989). Further studies are necessary to determine the degree to which the pre-mating increase in estradiol concentrations observed during this study contributes to the post-mating estradiol surge. Additional studies are also necessary to determine if a pre-mating

estradiol surge during spring emergence induces female receptivity during the mating season. Such studies would help clarify whether red-sided garter snakes indeed exhibit inter-sexual variation in the control of seasonal reproductive physiology and behavior.

Reproductive Behavior and Body Condition.

Many ectotherms inhabiting north-temperate climates undergo periods of prolonged winter dormancy prior to spring breeding. Animals that occupy underground hibernacula during winter dormancy are not exposed, or receive little exposure, to changing photoperiodic conditions (e.g., Whittier *et al*. 1987*a*). Thus, photoperiod is likely not a critical factor in synchronizing spring emergence from over-wintering locations. Indeed, temperature appears to be the most important environmental cue for synchronizing the seasonal biology of reptiles (Duvall *et al*. 1982; Licht 1972).

The red-sided garter snake is an excellent model for studying the role of temperature as an important environmental cue synchronizing seasonal rhythms. Photoperiod prior to and during winter dormancy has no influence on the initiation and timing of reproductive behavior of red-sided garter snakes (*T. sirtalis parietalis*) upon spring emergence (Whittier *et al*.1987*a*). Rather, mating behavior is induced by increases in environmental temperatures following winter dormancy (Ross and Crews 1978; Garstka *et al*. 1982; Bona-Gallo and Licht 1983; Krohmer and Crews 1987; Whittier *et al*. 1987*a*). Both male and female red-sided garter snakes are refractory to warm temperatures; snakes require a period of low temperature conditions to initiate sexual behavior upon re-exposure to warm temperatures (Camazine *et al*. 1980; Bona-

Gallo and Licht 1983). Furthermore, environmental induction of mating appears to be mediated by the pineal gland. Male snakes pinealectomized prior to hibernation fail to court female snakes upon emergence (Nelson *et al*. 1987; Crews *et al*. 1988; Mendonça *et al*. 1996*a*). In contrast, pinealectomy following spring emergence has no effect on the expression of courtship behavior in male red-sided garter snakes (Nelson *et al*. 1987; Mendonça *et al*. 1996*a*). These results indicate the pineal gland is necessary for transducing environmental stimuli during winter dormancy, of which temperature is the most important (and perhaps the only) cue.

To investigate the mechanisms by which temperature induces seasonal reproduction, we examined the influence of elevated hibernation temperatures on reproductive behavior in male red-sided garter snakes. We also investigated the influence of exogenous melatonin treatment on reproductive behavior to determine if the mechanisms governing temperature-induced reproduction involve modulation of melatonin concentrations. Lutterschmidt *et al*. (2004) demonstrated that melatonin significantly inhibits courtship behavior of male red-sided garter snakes during the spring mating season. In contrast, we did not observe a significant influence of exogenous melatonin treatment on the expression of male courtship behavior following winter dormancy. However, experimental differences in hormone manipulation between these studies must be considered. We verified that melatonin-filled silastic capsule implants increased melatonin concentrations of snakes by measuring circadian hormone cycles (Figure 6.5A). The supra-physiological melatonin levels (e.g., approximately 3 ng/ml) resulting from these hormone implants may explain why an effect of melatonin on

courtship behavior was not observed during this study, as such high melatonin levels may have caused down-regulation of melatonin receptors in the neural areas controlling reproductive behavior (e.g., Krohmer and Crews 1987). Further studies using alternative techniques to increase melatonin levels within physiological concentrations are needed to appropriately evaluate the effects of melatonin rhythms during winter dormancy on reproductive behavior.

Although elevated hibernation temperatures did not significantly influence the overall expression of reproductive behavior, males maintained at 10°C during winter dormancy exhibited delayed onset of courtship behavior (Figure 6.3B). Because the effects of elevated hibernation temperatures on courtship behavior did not persist throughout the entire mating period, these results suggest that temperature does not influence reproductive behavior via a threshold-dependent mechanism. Rather, plastic mechanisms are implicated in the transduction of temperature cues, as acclimatization to spring-like environmental conditions increased the expression of courtship behavior of snakes in the warm temperature hibernation treatment within 10 days of post-emergence (Figure 6.3B). Further studies are necessary, however, to determine how higher hibernation temperatures (e.g., 15 and 20°C) influence courtship behavior during the mating season.

Following acclimatization to spring-like environmental conditions, we observed robust courtship behavior in all treatment groups (Figures 6.3A, 6.3B). Most male snakes exhibited reproductive behavior for approximately 2 wks following winter dormancy. These results suggest that individual snakes migrate to summer feeding grounds within 2-

3 wks following spring emergence and do not engage in mating behavior during the entire abbreviated 4-5 wk mating season. Thus, male snakes observed at den sites during the spring mating season likely represent "populations" of males that have recently emerged from winter dormancy (e.g., Shine *et al*. 2001, 2006).

As expected, snakes maintained at elevated hibernation temperatures of 10°C lost a larger percentage of body mass than snakes in the cold temperature (i.e., 5°C) hibernation treatment, although this difference was not statistically significant (Figure 6.4). Snakes treated with exogenous melatonin demonstrated a statistically significant change in body mass as compared to control-treated snakes (Figure 6.4). As noted in the following section, snakes maintained at 10°C also had significantly higher melatonin concentrations than snakes acclimated to 5°C (Figures 6.6B, 6.7A). The difference in percent change in body mass among treatment groups is not likely a result of corticosterone actions on metabolism, as corticosterone concentrations did not differ between melatonin and control-treated snakes (Figure 6.5B). In addition, snakes acclimated to 10°C had significantly lower corticosterone concentrations than snakes acclimated to 5°C (Figure 6.7D). These results therefore suggest that melatonin positively influences metabolism during winter dormancy in red-sided garter snakes. A similar role of melatonin has been implicated in arousal from torpor. For example, a brief surge in plasma melatonin concentrations accompanies arousal from torpor despite the presence of light (Larkin *et al*. 2003). Although pineal melatonin is very low during torpor, melatonin levels rapidly increase in golden (*Mesocricetus auratus*) and Turkish (*Mesocricetus brandti*) hamsters following arousal (Vanecek *et al*. 1984, 1985; Darrow *et*

al. 1986). In Siberian hamsters (*Phodopus sungorus*), melatonin production is stimulated by the pronounced sympathetic nervous system activity that accompanies the arousal process (Larkin *et al*. 2003). Although further research is necessary, these results suggest that low melatonin levels are characteristic of deep hibernation and torpor, while increased melatonin levels may facilitate a state of arousal in hibernating animals.

Circadian Melatonin and Corticosterone Rhythms.

To further investigate the mechanisms by which temperature induces seasonal reproductive behavior, we examined circadian melatonin and corticosterone rhythms during winter dormancy in snakes maintained at different hibernation temperatures. Elevated hibernation temperatures (i.e., 10°C) significantly increased melatonin concentrations (Figures 6.6B, 6.7A) and significantly decreased corticosterone concentrations of male snakes (Figure 6.7D). Our results are similar to those of Tilden and Hutchison (1993), who showed that low environmental temperatures decrease the amplitude of the melatonin cycle in diamondback water snakes (*N. rhombifer*). Interactions between photoperiod and temperature in regulating circadian melatonin rhythms also occur in several other species (e.g., García-Allegue *et al.* 2001; Moyer *et al.* 1995; Rawding and Hutchison 1992; Underwood 1985*a*; Vivien-Roels *et al.* 1988). Our results demonstrate that temperature during winter dormancy, in the absence of changing photoperiodic cues, is a sufficient *zeitgeber* in modulating circadian melatonin rhythms.

Melatonin and corticosterone rhythms during winter dormancy were observed only in snakes maintained at 5°C, although melatonin rhythms were not statistically

significant (Figures 6.6B, 6.7A, 6.7D). This trend was also observed in melatonin cycles during preliminary studies conducted in the laboratory. During these preliminary experiments, melatonin concentrations of snakes acclimated to 5°C during winter dormancy were significantly lower than those of snakes acclimated to 15 °C; a melatonin cycle was observed only in snakes acclimated to low temperature conditions (Lutterschmidt, unpublished data). Intriguingly, the amplitude of this 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 wks (Figure 6.7A) as compared to those of snakes following 4 wks of cold temperature exposure (Figure 6.6B). Previous studies in red-sided garter snakes have shown that the period of low temperature exposure must be at least 4 wks in duration to elicit courtship behavior following return to high temperature conditions (Garstka *et al*. 1982). Furthermore, the proportion of males exhibiting courtship behavior increases as the length of low temperature exposure increases (Garstka *et al*. 1982). We speculate that the duration of cold temperature exposure may be transduced by such changes in the amplitude of the melatonin rhythm. It must be noted, however, that circadian melatonin cycles were not statistically significant during either of the cold-temperature sampling periods. The lack of a statistically significant melatonin rhythm, especially during wk 18 in hibernation (Figure 6.7A), likely results from the large variation observed in hormone concentrations among snakes. Future studies using larger sample sizes within the 24-h sampling period are necessary to determine whether the duration of cold-temperature exposure may be coded by changes in the amplitude of the melatonin rhythm.

Alternatively, exposure to cold temperatures during winter dormancy may be transduced by changes in the corticosterone rhythm. Because of corticosterone's role in energy balance, we hypothesized that increased hibernation temperatures would increase corticosterone concentrations of snakes. Surprisingly, a significant corticosterone rhythm, with overall higher concentrations, was observed only when snakes were acclimated to 5°C during winter dormancy (Figure 6.7D). Whether the two distinct peaks in corticosterone concentrations observed during this sampling period are functionally significant in transducing temperature cues requires further investigation. A significant corticosterone cycle did not persist when the acclimation temperature of the cold hibernation treatment group was increased to 10°C (Figure 6.7E). During this time, when snakes in both temperature hibernation treatments were acclimated to 10°C, corticosterone rhythms of snakes in the two different treatment groups were out of phase with one another (Figure 6.7E). Differences among these corticosterone rhythms may have contributed to the delayed onset of courtship behavior observed in the warm temperature hibernation group following winter dormancy (Figure 6.3B).

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 (Figures 6.7C, 6.7F). This is not surprising, however, as circadian hormone cycles of snakes were measured on day 11 post-emergence. As shown in Figure 6.3B, the proportion of males exhibiting courtship behavior between the cold and warm temperature hibernation treatments was indistinguishable after day 10 post-emergence. Thus, to further investigate whether the delayed onset of courtship behavior in the warm

temperature hibernation group might have resulted from hormonal mechanisms, we examined possible differences in melatonin and corticosterone rhythms between courting and non-courting snakes.

Following winter dormancy, both courting and non-courting male red-sided garter snakes exhibited a significant melatonin rhythm with the highest melatonin concentrations occurring during scotophase (Figure 6.8A). These results contrast with those of Mendonça *et al*. (1996*b*), who observed disrupted circadian melatonin cycles, with peak melatonin secretion during the photophase, in red-sided garter snakes that failed to exhibit courtship behavior during the spring breeding season. However, corticosterone rhythms were not reported in this study, making it difficult to determine whether disrupted melatonin cycles are directly or indirectly related to the observed lack of courtship behavior (Mendonça *et al*. 1996*b*). For example, previous studies in our laboratory have shown that circadian corticosterone cycles may be more important in synchronizing seasonal reproductive behavior in red-sided garter snakes (Lutterschmidt, unpublished data). We observed a phase shift of approximately 180º in the corticosterone rhythm of snakes following emergence from winter dormancy in the field. During the spring mating season, a significant corticosterone rhythm, with peak concentrations occurring during the photophase, was observed in the absence of a significant melatonin cycle (Lutterschmidt, unpublished data). Such temporal shifts in corticosterone rhythms may regulate the seasonal transition between reproductive and non-reproductive states in red-sided garter snakes.

The small proportion of male snakes that did not exhibit courtship behavior during this experiment appeared to have higher corticosterone concentrations during photophase (Figure 6.8B). Because the sample size of non-courting snakes was small and inconsistent among sampling times within the 24-h cycle, we investigated circadian melatonin and corticosterone rhythms of snakes from a preliminary study performed during the winter dormancy period of 2002-2003. During this experiment, we observed a very low incidence of courtship behavior among male snakes following hibernation at 5°C (Figure 6.9A). The reasons for this lack of courtship behavior are unknown, but we hypothesize that spring collection of animals and delayed induction of hibernation (i.e., in late December) contributed to the lack of reproductive behavior. Circadian melatonin rhythms were absent, and overall concentrations were lower, in snakes observed during emergence 2003 (Figure 6.9B). In addition, corticosterone concentrations of snakes in this experiment tended to be higher during photophase, although corticosterone rhythms were not statistically significant (Figure 6.9C).

Similar to Mendonça *et al*. (1996*b*), the lack of a significant melatonin cycle in red-sided garter snakes may inhibit courtship behavior during the spring mating season (Figures 6.9A, 6.9B). In addition, this experiment supports previous findings that the phase of circadian corticosterone rhythms during the spring mating season may play a role in regulating courtship behavior (Figure 6.9C; Lutterschmidt, unpublished data). There is precedence for temporal shifts in endocrine cycles in response to changes in daily activity patterns. For example, nocturnal activity in free-ranging green sea turtles

(*Chelonia mydas*), including nesting, mate searching, and feeding/swimming behaviors, abolishes diel variation in both melatonin and corticosterone cycles (Jessop *et al*. 2002).

Further research is necessary to determine the hormonal mechanisms governing temperature-induced reproductive physiology and behavior in ectothermic vertebrates. Although circadian melatonin and corticosterone rhythms are likely candidates for these hormonal mechanisms, the role of other temperature-sensitive endocrine signals (e.g., prolactin, thyroxine, arginine vasotocin) must also be considered. Additional studies are needed to evaluate the roles of melatonin and corticosterone in transducing temperature cues and synchronizing seasonal reproductive behavior. For example, circadian melatonin rhythms may function in synchronizing the corticosterone rhythm with photophase following emergence, as interactions between melatonin and the adrenal gland are well established (e.g., Barriga *et al*. 2002; Maestroni *et al*. 1989; Otsuka *et al*. 2001). Similar experiments manipulating corticosterone rhythms would prove useful in evaluating independently the importance of corticosterone in regulating seasonal reproductive behavior. Melatonin and corticosterone rhythms are likely physiologically coupled via reciprocal interactions, and such a physiological coupling likely contributes to regulating circadian and circannual rhythms in physiology and behavior.

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CHAPTER 7 – CONCLUSIONS

One of the most important factors governing survival of species is the ability to track changing environmental cues so that life history processes are synchronized with optimal environmental conditions. Almost all vertebrates exhibit seasonality in some processes, including molt of skin, fur, and plumage, feeding, immune function, activity, migration, and especially reproduction. Circadian and circannual rhythms in physiology and behavior are temporally organized via changing environmental cues. The most reliable environmental cue thought to function in vertebrate time-keeping is photoperiod. Unlike other environmental signals that can vary quite dramatically both within seasons and among years (e.g., temperature and precipitation), changes in photoperiod reliably reflect changing environmental seasons. These environmental cues are transduced into hormonal signals that in turn regulate physiology and behavior. Thus, the hormonal mechanisms underlying time-keeping systems provide a direct link between an organism's environment and its physiology and behavior.

A particularly intriguing challenge to time-keeping mechanisms is hibernation and daily torpor. Such prolonged periods of inactivity, hypometabolism, and decreased body temperatures present unique challenges to maintaining the synchrony of the circadian system (Lyman *et al*. 1982; Ruby 2003). Much research indicates that daily bouts of torpor are temporally organized by the circadian system, specifically the suprachiasmatic nucleus (reviewed in Ruby 2003). Exposure to both photoperiod cues (albeit limited) and fluctuations in body temperature resulting from periodic arousal from

torpor may function in synchronizing circadian pacemakers during hibernation (Ruby 2003).

Although true 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 such ectothermic vertebrates, body temperature is dependent on ground temperatures within the hibernaculum. Furthermore, animals that occupy underground hibernacula during continuous winter dormancy are not exposed to changing photoperiodic conditions (e.g., Grobman 1990; Whittier *et al*. 1987). Thus, photoperiod is likely not a critical factor in synchronizing spring emergence from over-wintering locations. Indeed, temperature appears to be the most important environmental cue for synchronizing the seasonal biology of ectothermic vertebrates (e.g., Duvall *et al*. 1982; Licht 1972). However, relatively little is known about how possible changes in temperature cues during winter dormancy are transduced into endocrine signals. Most intriguing is that in species where reproduction occurs immediately following spring emergence, the associated changes in neurophysiology and behavior that accompany reproduction must occur during winter dormancy. Thus, significant changes in reproductive physiology and behavior occur during this "dormancy" period, and the seasonal control of reproduction is therefore likely linked to the environmental and hormonal mechanisms controlling winter dormancy.

The research described here uses a multidisciplinary approach in a comparative framework to investigate the role of chronobiology in a species' adaptation to its environment. Specifically, these studies focus directly on the mechanisms by which

temperature cues affect the chronobiology and seasonal reproduction of a nonmammalian vertebrate. The garter snake model system utilized during these studies provides a unique framework for investigating these questions. In most other vertebrates, photoperiod is the primary environmental cue used in synchronizing circadian systems. However, during winter dormancy, the time-keeping mechanisms of red-sided garter snakes (*Thamnophis sirtalis parietalis*), and most importantly the control of seasonal reproduction, rely exclusively on changes in seasonal temperatures. Photoperiod prior to and during winter dormancy has no influence on the initiation and timing of reproductive behavior in red-sided garter snakes (*T. sirtalis parietalis*) (Whittier *et al*.1987). Rather, mating behavior is induced by increases in environmental temperatures following winter dormancy (Ross and Crews 1978; Garstka *et al*. 1982; Bona-Gallo and Licht 1983; Krohmer and Crews 1987; Whittier *et al*. 1987). Both male and female red-sided garter snakes are refractory to warm temperatures; snakes require a period of low temperature conditions to initiate sexual behavior upon return to warm temperatures (Camazine *et al*. 1980; Bona-Gallo and Licht 1983). Thus, the red-sided garter snake is an excellent model for studying the role of temperature in synchronizing seasonal rhythms.

Because of their roles in circadian organization and energy balance, melatonin and glucocorticoids are likely hormonal components of the mechanisms regulating temperature-induced reproduction. To better understand how melatonin and corticosterone, the primary glucocorticoid in reptiles, might facilitate temperature-induced reproduction, I first characterized the reciprocal interactions between these two hormonal systems. The first three chapters of this dissertation explored the effects of

melatonin on hormonal responses to capture stress, the effects of melatonin on behavioral and hormonal responses to exogenous corticosterone, and the influence of capture stress on circadian melatonin cycles. After characterizing these interactions, I then examined how environmental temperatures during winter dormancy influence circadian melatonin and corticosterone rhythms using both field and laboratory studies.

Chapter two examined the role of melatonin in modulating hormonal responses to capture stress in two populations of male garter snakes, *Thamnophis sirtalis*. I chose to study these two populations to investigate possible variation in melatonin function among snakes with different suites of environmental adaptations. For example, populations of red-sided garter snakes (*T. sirtalis parietalis*) in south-central Manitoba, Canada undergo a period of winter dormancy for approximately 8 months each year followed by an attenuated mating season lasting 4-5 weeks in early spring. In contrast, the mid-latitude red-spotted garter snake (*T. sirtalis concinnus*) in western Oregon, USA has an extended breeding season of several months and can be active during 10-12 months of the year given appropriate environmental conditions. To better address the influence of melatonin on the secretory activity of the adrenal gland, I also examined the effects of 5-hydroxytryptophan (a precursor of melatonin synthesis) and ketanserin (a serotonergic type 2A receptor antagonist) on hormonal responses to capture stress. I observed a trend of increased corticosterone and decreased androgen concentrations in northern-latitude red-sided garter snakes (*T. sirtalis parietalis*) following 4 h of capture stress during the spring. During the fall, red-sided garter snakes showed no change in corticosterone or androgen concentrations in response to the capture stress treatments, suggesting that

northern-latitude red-sided garter snakes suppress hormonal responses to capture stress during preparation for winter dormancy. Treatment with melatonin, 5-hydroxytryptophan, or ketanserin did not significantly influence corticosterone or androgen concentrations of northern-latitude red-sided garter snakes during the spring or fall. Mid-latitude red-spotted garter snakes (*T. sirtalis concinnus*) from Oregon showed a statistically significant increase in corticosterone concentrations in response to 4 h of capture stress; treatment with melatonin, 5-hydroxytryptophan, or ketanserin prior to capture stress had no significant influence on plasma corticosterone concentrations. Androgen concentrations of mid-latitude red-spotted garter snakes in response to capture stress were significantly lower than those of non-stress control snakes. Neither melatonin nor 5-hydroxytryptophan influenced the change in androgen concentrations during capture stress. However, androgen concentrations of snakes treated with ketanserin prior to 4 h of capture stress did not differ significantly from those of non-stressed control snakes.

In chapter three, I investigated possible interactions between melatonin and corticosterone in modulating the reproductive behavior of male red-sided garter snakes (*T. sirtalis parietalis*) following spring emergence. I also examined whether melatonin's modulatory actions could be explained by its potential properties as a serotonin receptor antagonist. Exogenous corticosterone significantly reduced courtship behavior of male snakes in a dose-dependent manner. Melatonin also significantly reduced courtship behavior of male garter snakes. Pretreatment with melatonin prior to administering corticosterone treatments further suppressed courtship behavior. Snakes receiving

ketanserin, a serotonergic type 2A receptor antagonist, followed by corticosterone also showed reduced courtship behavior; this serotonin receptor antagonist itself did not significantly influence reproductive behavior. Neither melatonin nor corticosterone treatments significantly influenced androgen concentrations of male garter snakes, supporting a direct effect of melatonin and corticosterone on courtship behavior that is independent of any effect on sex steroid concentrations.

The results from these two studies suggest that melatonin does not inhibit corticosterone secretion in this ectothermic model. However, both melatonin and corticosterone inhibit reproductive behavior. The additive inhibitory effects of melatonin and corticosterone on courtship behavior suggest that these hormones might modulate reproduction via a common mechanism. Results from the experimental manipulations with the serotonin receptor antagonist ketanserin suggest that this mechanism involves a serotonin-regulated system. The hypothesis that melatonin functions as a serotonin receptor antagonist is supported, although the serotonergic properties of melatonin cannot completely explain its effects on courtship behavior. In addition, these studies suggest that a serotonin-regulated system may play a role in modulating the activity of the hypothalamus-pituitary-gonadal axis during physiological stress responses. Additional studies are necessary to investigate the role of serotonin in regulating hormonal and behavioral responses to stress in this species. Further research is also necessary to discern whether the actions of melatonin and corticosterone on reproductive behavior are converging on the same regulatory pathway or if their effects on different pathways are having additive inhibitory effects on reproduction.

To examine the reciprocal interactions between melatonin and corticosterone, I investigated the influence of hormonal stress responses on circadian melatonin rhythms in chapter four. Capture stress, but not exogenous corticosterone, significantly increases melatonin concentrations of red-sided garter snakes. Pretreatment of snakes with both capture stress and exogenous corticosterone abolishes the increase in scotophasic melatonin synthesis induced by 5-hydroxytryptophan treatment. These experiments indicate the different phases of an acute physiological stress response (i.e., activation of the hypothalamus-pituitary-adrenal axis, increased glucocorticoid secretion) have distinct and temporally different effects on pineal melatonin synthesis.

To investigate whether these interactions between melatonin and corticosterone play a functional role in regulating the seasonal biology of red-sided garter snakes, I measured circadian melatonin and corticosterone rhythms during spring emergence from prolonged winter dormancy. When red-sided garter snakes first emerge from underground hibernacula following 8 months of winter dormancy, melatonin cycles are already synchronized with photoperiodic conditions (i.e., the highest melatonin concentrations occur during scotophase). These findings suggest the *zeitgeber* entraining circadian melatonin rhythms occurs prior to, or during, spring emergence. A significant corticosterone rhythm was also observed upon emergence with peak levels occurring during scotophase; corticosterone rhythms phase-shifted approximately 180º following emergence from winter dormancy. This phase-shift in the corticosterone rhythm may play a role in regulating the seasonal transition between reproduction and migration from the den site in red-sided garter snakes.

Collectively, these first three chapters demonstrate that a physiological coupling between melatonin, glucocorticoids, and the hypothalamus-pituitary-adrenal axis is conserved in this ectothermic model. This physiological coupling likely functions in regulating seasonal rhythms in physiology and behavior. However, controlled laboratory experiments were necessary to determine if the interactions between melatonin and corticosterone have a functional role in regulating seasonal rhythms. Specifically, I was interested in determining if melatonin and corticosterone cycles play a role in transducing environmental temperatures during winter dormancy.

To appropriately evaluate the influence of hibernation temperatures on circadian melatonin and corticosterone rhythms, it was first necessary to determine what range of temperatures snakes are exposed to during winter dormancy in natural hibernacula. The observation that the *zeitgeber* entraining circadian melatonin rhythms occurs prior to (or during) spring emergence contributed to the importance of measuring body temperatures of red-sided garter snakes under natural field conditions. In chapter five I tested the hypothesis that increased ground temperature is the cue for emergence from hibernation. I measured body temperatures of snakes during winter dormancy by surgically implanting small temperature-loggers into female red-sided garter snakes before they entered their native hibernaculum. The following spring, I recaptured 7 of the snakes implanted with temperature-loggers. Body temperature declined gradually during winter dormancy from 15°C in mid-September to 1°C in early April, reaching minimal values approximately one month prior to spring emergence. Body temperatures of emerging snakes ranged from 0.5°C during early spring to 6.3°C during late spring. These results

do not support the hypothesis that an increase in ground temperature (and hence body temperature) is necessary for emergence from winter dormancy. Rather, critically low temperatures (i.e., 0.5 - 1°C) may be a *zeitgeber* entraining an endogenous circannual cycle that regulates snake emergence.

Finally, in chapter six I investigated the mechanisms by which environmental temperatures during winter dormancy induce seasonal reproductive behavior in red-sided garter snakes (*T. sirtalis parietalis*). Specifically, I addressed whether elevated melatonin levels and/or increased environmental temperatures during winter dormancy influence (1) patterns of sex steroid hormone and corticosterone secretion; (2) the expression of reproductive behavior following emergence; and (3) circadian melatonin and corticosterone rhythms during winter dormancy. These experiments support previous findings that male red-sided garter snakes exhibit a dissociated reproductive strategy. The observation that estradiol concentrations increase significantly during spring emergence, however, suggests the presence of inter-sexual differences in reproductive strategy. Following winter dormancy, I observed robust courtship behavior in all treatment groups, but male snakes maintained at 10°C during winter dormancy exhibited delayed onset of courtship behavior. Decreased environmental temperatures may induce reproductive behavior of red-sided garter snakes via changes in melatonin and/or corticosterone rhythms. These experiments demonstrate that environmental temperature, in the absence of changing photoperiodic cues, is a sufficient *zeitgeber* in modulating circadian melatonin and corticosterone rhythms of red-sided garter snakes during winter dormancy. Furthermore, these laboratory studies support previous field observations that

the phase of corticosterone rhythms during the spring mating season plays a role in regulating seasonal reproductive behavior.

Over 30 years of intense laboratory and field studies have provided much insight into the reproductive behavior and physiology, neuroanatomy, behavioral neuroendocrinology, chemical ecology, evolutionary physiology, behavioral ecology, population ecology, and genetics of the red-sided garter snake (*T. sirtalis parietalis*). The studies presented here also advance *T. sirtalis* as a model system for chronobiology. This dissertation research provides further insight into the environmental and hormonal mechanisms controlling winter dormancy and seasonal reproductive behavior in ectothermic vertebrates. These results fit within the larger framework of this species' annual life history cycle (Figure 7.1). For example, male and female red-sided garter snakes have significantly different levels of sex steroid hormones during the fall pre-hibernation period, suggesting inter-sexual variation in reproductive strategy. I provide evidence that exposure to low-temperature conditions during winter dormancy may be transduced by changing circadian melatonin and corticosterone rhythms. The experiments presented in chapter three establish a functional link between melatonin and spring mating behavior in red-sided garter snakes. Furthermore, these studies suggest that the phase of corticosterone rhythms during the spring mating season regulates the seasonal transition from reproductive to non-reproductive states, as manifested by migration from the den site to summer feeding grounds.

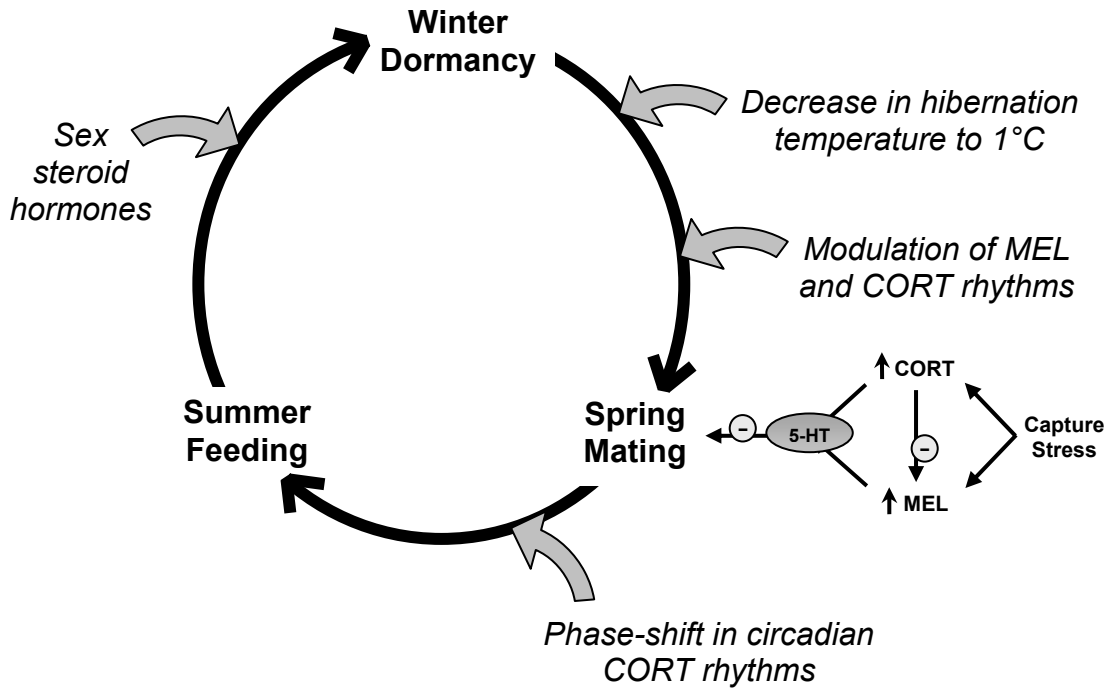

Figure 7.1. A revised model describing how the results of this dissertation research fit within the larger framework of the annual life history cycle of red-sided garter snakes (*Thamnophis sirtalis parietalis*). During the spring mating season, hormonal stress responses increase both corticosterone (CORT) and melatonin (MEL) levels. Melatonin and corticosterone in turn inhibit reproductive behavior, likely via modulation of a serotonin (5-hydroxytryptamine, 5-HT) system.

Overall, the studies conducted here demonstrate that a physiological coupling between melatonin and glucocorticoids is conserved in this ectothermic model. Furthermore, this physiological coupling plays a significant role in transducing environmental cues, particularly temperature stimuli, during winter dormancy. Both melatonin and corticosterone directly influence reproductive behavior, and interactions between these endocrine systems therefore contribute to the regulation of this species' seasonal biology. Although much remains to be explored, circadian melatonin and corticosterone rhythms during winter dormancy may facilitate the necessary changes in

neurophysiology and neuroanatomy required to elicit reproductive behavior upon spring emergence. For example, red-sided garter snakes require at least 4 wks of low-temperature exposure to elicit reproductive behavior upon return to warm temperatures. Although this refractoriness has been well characterized, we know very little about how the duration of cold temperature exposure is transduced. My results suggest that changes in the amplitude of the melatonin cycle during prolonged exposure to low temperatures may serve as a potential hormonal mechanism in this time-keeping system. However, future studies using larger sample sizes within the circadian sampling period are necessary to determine whether the duration of cold-temperature exposure is indeed coded by changes in the amplitude of the melatonin rhythm.

Such seasonal neuroplasticity has been described in the song control system of several birds (Bentley *et al*. 1999; Jansen *et al*. 2005; Thompson and Brenowitz 2005; Tramontin *et al*. 1998). Furthermore, evidence suggests that melatonin plays a direct functional role in the neural control of the avian song system (Jansen *et al*. 2005). Very little is known about the control mechanisms regulating seasonal reproduction in ectotherms, but it is likely that the hormonal time-keeping mechanisms described here facilitate seasonal changes in the anatomy and physiology of neural circuits controlling reproductive behavior.

Further research is necessary to examine the hormonal mechanisms governing temperature-induced reproductive physiology and behavior in ectothermic vertebrates. Although circadian melatonin and corticosterone rhythms are likely candidates for these hormonal mechanisms, the role of other temperature-sensitive endocrine signals (e.g.,

prolactin, thyroxine, arginine vasotocin) must also be considered. Relatively little is known about the function of arginine vasotocin, for example, in regulating courtship behavior in reptiles. However, because temperature influences the expression of arginine vasotocin in some reptiles, it is possible that it is involved in regulating temperature-induced reproduction (reviewed in Woolley *et al*. 2004). Additional studies are needed to evaluate the roles of such hormones in transducing temperature cues and synchronizing seasonal reproductive behavior. For example, circadian melatonin rhythms may function in synchronizing the corticosterone rhythm with photophase following emergence, as interactions between melatonin and the adrenal gland are well established (e.g., Barriga *et al*. 2002; Maestroni *et al*. 1989; Otsuka *et al*. 2001). Experiments using alternative techniques to increase melatonin levels within physiological concentrations during winter dormancy are necessary to evaluate the role of circadian melatonin rhythms on reproductive behavior. Similar experiments manipulating corticosterone rhythms would prove useful in evaluating independently the importance of corticosterone in regulating seasonal reproductive behavior. However, melatonin and corticosterone rhythms are physiologically coupled via reciprocal interactions, and such a physiological coupling likely contributes to the regulation of circadian and circannual rhythms in physiology and behavior.

In a much broader context, studies of the mechanisms underlying the effects of environmental stimuli on time-keeping systems and seasonal rhythms in physiology and behavior are extremely valuable. Knowledge of these mechanisms will provide insights regarding the potential impact of environmental perturbations on the stability of

populations. Global climate change is one of the most significant environmental perturbations threatening numerous species (e.g., McCarty 2001). It is hypothesized that global climate change will ultimately affect the predictability of overall weather patterns, including global water cycles, cloud cover, and average temperatures (e.g., IPCC 2001). Regardless of its cause, we have already begun to see evidence of global climate change; it has had a consistent and marked influence on many species ranging from mollusks to mammals and from grasses to trees (Root *et al*. 2003).

Disrupted breeding cycles and consistently earlier breeding are occurring in birds and amphibians and have been associated with global climate change (Forchhammer *et al*. 1998; Brown *et al*. 1999; Dunn and Winkler 1999; Moss *et al*. 2001). Root *et al*. (2003) demonstrated that in more than 80% of the species showing temperature-related shifts in seasonal rhythms, the changes are consistent with and predicted by the species' physiological constraints. While it is speculated that climate change will drastically affect many species (e.g., Sorenson *et al*. 1998; Stevenson and Bryant 2000), we know exceedingly little about the physiological mechanisms that will mediate these changes. Because the predictability of environments ultimately will be altered, the most likely mechanism mediating disruption of seasonal biology is the disruption of time-keeping mechanisms.

Disruptions in the ability to synchronize appropriate physiological and behavioral responses with the environment could be disastrous for animal populations. The necessity for research in this area is made obvious by recent reports of reduced survival and reproductive fitness where environmental perturbations (e.g., endocrine disruptors and

global climate change) are prevalent (Wingfield 1988; Blaustein and Wake 1995; Forchhammer *et al*. 1998; Moss *et al*. 2001; Root *et al*. 2003). Understanding the sensitivity of time-keeping systems to environmental perturbations will enable us to more accurately estimate the impact of such perturbations on the long-term survival and persistence of populations. For example, if time-keeping systems are ecologically and/or phylogenetically constrained, then changes in climate predictability could pose a serious threat to seasonal breeders.

The unique life history of the red-sided garter snake (*T. sirtalis parietalis*) makes it a remarkable model for studying the potential impacts of environmental perturbations on time-keeping mechanisms and reproduction. Because of the extreme environmental constraints on spring emergence and reproduction in this species, even small changes in climate predictability or exposure to endocrine disruptors may have disproportionately large consequences on reproductive fitness and survival. Studies investigating the disruption of time-keeping mechanisms in common garter snakes may therefore elucidate a necessary "canary in the coal mine" for modeling the effects of environmental perturbations in other species.

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