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Temperature-induced Activation of the Reproductive Axis through Melatonin-mediated Changes in Thyrotropin

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by

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Abstract

An animal's ability to synchronize life-history events or stages with optimal environmental conditions is paramount to successfully reproducing and maximizing fitness. Additional events including migration, foraging, rearing of young, and emergence from hibernation are all examples of processes under environmental control in one species or another. An exciting new mechanism involving neural thyroid hormone metabolism has been elucidated that links environmental control to the neuroendocrine reproductive axis. In birds and mammals with seasonal breeding cycles, this neuroendocrine pathway is activated by photoperiod-induced changes in thyroid stimulating hormone (TSH, also known as thyrotropin) production within the pars tuberalis region of the pituitary. Thyrotropin then alters neural thyroid hormone metabolism in the hypothalamus to stimulate the release of gonadatropin-releasing hormone (GnRH). Melatonin, the primary hormone produced by the pineal gland, has long been known as the primary transducer of photoperiod and thermoperiod in seasonally breeding animals. It is possible that this mechanism is conserved across all seasonally breeding vertebrates and that the mechanism itself is directly linked to reproductive physiological changes. Through melatonin manipulation we experimented to see if melatonin directly mediates the effects of thermoperiod on hypothalamic thyroid hormone metabolism the same way it mediates the effects of photoperiod in seasonal breeding vertebrates. In this study we used the red-sided garter snake (*Thamnophis sirtalis parietalis*), an ectothermic vertebrate that is known to be a temperature-activated

seasonal breeder, to investigate changes in thyrotropin stimulating hormone (TSH) and courtship behavior at different hibernation temperatures (4°C or 12°C) and with different melatonin treatments: the melatonin precursor 5-hydroxytryptophan (5-HTP) or the melatonin receptor antagonist luzindole. Males that hibernated at the ecologically relevant temperature of 4°C exhibited a significant decrease in TSH immunoreactivity within the median eminence area of the hypothalamus, an effect that was reversed in males treated with the melatonin precursor 5-HTP. Additionally, males hibernated at 4°C and treated with 5-HTP had significantly lower courtship intensity. Males hibernated at a warmer 12°C temperature, with or without luzindole treatment, did not show any difference in TSH immunoreactivity or mating behavior. These results suggest that while changes in melatonin may be necessary for transducing the effects of low-temperature exposure on the reproductive axis, melatonin is not sufficient in overriding the influence of elevated hibernation temperatures. Together, these results indicate that more research is needed if we are to understand the mechanisms by which increases in environmental temperature will impact physiology and behavior.

Dedication

This thesis and the research behind it is dedicated to Anatole Farci. Thank you for taking me into your house and treating me like family during my time at Portland State University. Graduate school would have been significantly more difficult without your generosity and kindness. You were a mentor and a role model to me; I hope one day to be able to help someone the same way you helped me.

"Every man's life ends the same way. It is only the details of how he lived and how he died that distinguish one man from another.

- Ernest Hemingway

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Chapter 1: Introduction

Life History Stages and Biological Rhythms

Animals constantly transition through periods or stages of their life that correspond with certain—and usually distinct—changes in physiology and behavior. These stages are the culminative product of the communication between endogenous and exogenous biological environments, ultimately dictating the way in which environmental cues are translated into important behaviors and physiological conditions (Licht 1972; Whittier et al. 1987; Ball Gregory F and Ketterson Ellen D 2008; Crespi et al. 2013). This means the internal biology of an animal is constantly adjusting to changes in environmental conditions. The relationship is a dynamic process, and it is perpetually being regulated and modulated by both intrinsic (i.e., hormonal) and extrinsic (i.e., environmental and social) signals through complex signaling pathways and feedback loops (Lutterschmidt 2012). This allows life-history events to be optimally synchronized with the environment, which is in turn critical to survival and reproductive success.

Reproduction is energetically expensive, and animals cannot afford to expend resources at nonbeneficial times. As such, many use environmental cues such as photoperiod and temperature to time important stages in their life. In the green anole (*Anolis carolinensis*), for example, males have annual cycles of testis activity, with long days during the spring and summer stimulating testis growth while the shorter, colder days of fall initiate regression of the testes (Licht 1971). Many mammal and bird species use seasonal changes in photoperiod to regulate reproductive cycles; small mammals and

birds with short gestation and egg incubation periods, respectively, key in on long days (spring and summer) to mate and raise their young during that one season. Larger mammals such as goats and sheep have gestation periods that reach six months, and they in turn coordinate mating behaviors with short days (fall and winter) (Reviewed by Shinomiya et al. 2014). This allows the birthing and raising of offspring to coincide with the warmer periods of spring when resources are in abundance. The robustness of these reproductive control mechanisms allows many different types that can vary from species to species while retaining the same ultimate function, the ability to optimally time reproductive activities.

The importance of optimal timekeeping is so great that the cycles of seasonal reproduction and the extent of an animal's plasticity in regards to these cycles are under heavy selection pressure (Nilsson 1994). With the high level of nuance behind these systems, it is not surprising that a "disconnection" between environmental conditions and the internal biological state of an animal can have serious consequences. Mistiming of life-history events such that they occur at suboptimal conditions can have repercussions ranging from diminished mating success to death (McClintock 1981). Correct timing of life-history events requires that an animal properly adjusts physiologically, behaviorally, and/or morphologically to the environmental conditions in which survival and reproductive success are maximized (Ho, Lance, and Megaloudis 1987; Lutterschmidt et al. 2002; Sharp 2005; Lutterschmidt and Mason 2009, 2010; McCormick, Farrell, and Brauner 2013; McCormick et al. 2019).

For example, in Atlantic salmon (*Salmo salar*), elevated water temperatures cause delayed or truncated spawning seasons (Pankhurst and Munday 2011). This abrupt

change could have downstream effects on future salmon populations due to a reduction in spawning success. In another study, Parmesan and Yohe (2003) quantitatively assessed 677 species from the literature, including mammals, reptiles, amphibians, insects, marine invertebrates, and some plant species, and found that 71% have undergone some temporal shift in life-history events in the last 16-132 years. Eighty-seven percent of those species trended in the direction expected from climate change, many showing spring events advancing or occurring earlier than they had historically. In a final example, a study on pelagic ecosystems found that climate change has altered the highly complex food-web structure between many trophic levels. This in turn has had downstream effects which has disrupted the entire relationship between primary, secondary, and tertiary producers (Edwards and Richardson 2004). As a scientific community, we are only just beginning to understand the true extent of climate shifts, why some species will be unable to adapt, and why other species may benefit. If changes in seasonal weather profiles keep accelerating at current rates there is the possibility of entire ecosystems collapsing (Edwards and Richardson 2004; Riddell et al. 2021). The need for continued work elucidating how animals respond to a changing environment has never been greater.

Environmental Control of Seasonal Rhythms

The transitions between life history stages introduced above are often induced through environmental cues (i.e., photoperiod and temperature) that change in a cyclical pattern at specific times of year with a high degree of predictability. These rhythms or cycles can be annual, seasonal, or under even shorter time constraints such as the well-known circadian cycle. Each phase of a cycle (i.e., sleep vs wake or breeding period vs

non-breeding period) demonstrates a unique set of physiological and behavioral processes, because the specific needs of each event are often distinct from each other. A good example of this is the yearly spring mating season that every deer species in the family *Cervidae* exhibits. Strict neuroendocrine control keeps deer from exhibiting mating behaviors at undesirable times, or from abandoning their young in lieu of other behaviors (Pereira, Duarte, and Negrão 2005; Lincoln 1992). The sreason for this strict behavioral adherence lies in hormonally controlled circuits that are "primed or triggered" through optimal biotic and abiotic conditions or cues.

Some of the most well-known cues used to time life-history stages include photoperiod, thermoperiod, resource availability and social stimuli (Paul et al. 2007). There is usually a primary cue that serves as the *zeitgeber* which is most responsible for entraining the animal's internal state; supplementary cues are then often used to "fine-tune" these states (Ball G and Ketterson 2008; Goldman 2001; E. Gwinner 1990; Krohmer and Lutterschmidt 2016). Photoperiod has received the most attention, as it is undeniably the most reliable environmental cue underpinning the environmental control of an animal's seasonal behavior and physiology. One of the first papers to explore this idea was by Rowan (1930) in which he hypothesized that day length was a cue for migration in dark-eyed juncos (*Junco hyemalis*). This proved to be fruitful, as there has been a wealth of studies on photoperiodic control of various life-history stages since then (Ball and Ketterson 2008; Dawson and Sharp 2007; Gwinner 1990).

Photoperiodic control of seasonal rhythms has been documented in all vertebrate classes (Bromage et al. 1984; Young 1983). Research on other cues such as temperature is much sparser, even though recent advances have continued to shine light on their

importance. For example, most ectothermic vertebrates rely on temperature to regulate many of the processes behind their biological rhythms (Ho, Lance, and Megaloudis 1987; Licht 1972; Lutterschmidt and Mason 2009; Tilden and Hutchison 1993). Well-studied reptilian model systems in particular offer the ability to study the effects of temperature in depth, which may in turn lead to a better understanding of how environmental cues work in concert to "guide" the endogenous state of organisms through different seasonal rhythms.

Endogenous Mechanisms

The way in which environmental signals modulate behavior is complex and use a wide spectrum of chemical "messenger" molecules that act at all levels of biological communication. Some of the most common examples of these chemical messengers are hormones and neurohormones—commonly peptides or steroids that are released into the bloodstream that induce changes on target cells/tissues. Many of the endogenous hormonal mechanisms/pathways are directly linked to environmental stimuli and act to transduce these cues (i.e., photoperiod and thermoperiod) into hormonal signals that affect animal physiology and behavior.

One of the most widely studied transducers of environmental information is melatonin or *N*-acetyl-5-methoxytryptamine, which is the primary hormone produced by the pineal gland. It is the product of a biosynthetic pathway that begins with the amino acid tryptophan that is enzymatically converted into serotonin and then lastly melatonin (Hardeland, Pandi-Perumal, and Cardinali 2006; R. W. Krohmer and Lutterschmidt

2011). All animals, diurnal and nocturnal alike, exhibit an increasing melatonin synthesis pattern in the hours of darkness that then returns to a baseline level in the daylight hours (Dawson and Sharp 2007; Hut and Beersma 2011; Lutterschmidt and Mason 2008, 2009). This 24-hour melatonin cycle becomes a consistent pattern that can be used to interpret time of day and seasonal changes in light availability. Temperature also modulates melatonin levels in diamondback watersnakes (*Nerodia* rhombifer) and red-sided garter snakes (*Thamnophis sirtalis parietalis*); low temperatures decreased the nightly amplitude of melatonin concentrations but had no effect on the phase/duration of melatonin synthesis.(Axelrod 1974; Lutterschmidt, LeMaster, and Mason 2004; Lutterschmidt and Mason 2009; Tilden and Hutchison 1993). Thus, a complete timekeeping mechanism through melatonin signaling is responsible, at least in part, for the photothermal entrainment of the circadian rhythm (García□Allegue, Madrid, and Sánchez□Vázquez 2001; Lutterschmidt and Mason 2009; Underwood and Calaban 1987).

Removal of the pineal gland, the source of melatonin, causes physiological and behavioral changes across multiple species that indicate a role for melatonin in regulating seasonal cycles. For example, pinealectomy abolished seasonal circadian rhythms of melatonin and daily activity patterns in ruin lizards (*Podarcis sicula*) (Foa, Janik, and Minutini 1992; Innocenti et al. 1996; McMillan 1972). In red-sided garter snakes pinealectomy in the fall prior to hibernation inhibited male courtship behavior upon spring emergence (Foa, Janik, and Minutini 1992; Innocenti et al. 1996; McMillan 1972; Nelson et al. 1987). In pinealectomized White-throated Sparrows (*Zonotrichia albicollis*), House sparrows (*Passser domesticus*) and European starlings (*Sturnus vulgaris*),

migration and activity patterns became arrhythmic post-surgery, but in house sparrows and starlings the effect could be reversed through daily injections of melatonin (E. Gwinner 1990; Eberhard Gwinner and Benzinger 1978; Lu and Cassone 1993; McMillan 1972).

There is a delicate balance that must be maintained in hormonal signaling pathways and this is evident by the extensive controls or feedback systems that act at many different levels of hormonal cascades. Examples of these cascades are the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-gonadal (HPG) axis, where three different organs tightly control different parts of a shared hormone pathway. The HPG axis in particular serves as the prime regulatory pathway in reproduction (Figure 1): gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland, which in turn stimulates the gonads to release sex steroid hormones as well as facilitate other reproductive functions (Hiller-Sturmhöfel and Bartke 1998). At each level of the axis, feedback loops tightly control the pathway's activity, and sex steroid hormones generally have an inhibitory effect on hormone release from the hypothalamus and the pituitary. There are also shorter feedback loop interactions that occur between the pituitary and the hypothalamus. The sensitivity of these feedback loops are plastic and can be affected by the physiological states of other systems, creating a dynamic communication system that is able to fluctuate by various increments in an effort to maintain homeostasis (Hiller-Sturmhöfel and Bartke 1998). This neuroendocrine system facilitates all stages of reproduction; maturation, activation and deactivation of sexual function, along with behavioral responses.

Melatonin's influence on the HPG axis has been studied extensively, and it is well known that photoperiodic changes in melatonin are the catalyst for seasonal changes in reproductive states. For example, male green anoles (*Anolis carolinensis*) showed a difference in the seasonal response to melatonin manipulation. In the summer, neither treatment with melatonin nor pinealectomy influenced gonadal measurements, while in the winter pinealectomized males showed increased gonadal growth and the effect was reversed with melatonin treatment (Underwood 1981; Underwood and Calaban 1987). While it is well known that GnRH is sensitive to day length in many species, to date no studies have shown that melatonin directly acts on GnRH, as melatonin receptors have never been found on GnRH neurons or gonadotrophs located in the pituitary gland (Malpaux, Thiéry, and Chemineau 1999). Thus, the exact mechanism controlling the effects of environmental cues on the HPG axis remains unclear.

Questions over the coordination of this system remain and research into a photoperiod-sensitive neural thyroid hormone mechanism in mammals and birds has begun to bridge the gap in our understanding of this system. Japanese quail (*Coturnix japonica*) and Djungarian hamsters (*Phodopus sungorus*), both spring breeders as is common in birds and small mammals, both exhibited increases in the metabolism of hypothalamic thyroid hormones when exposed to long day conditions (Figure 2). These signals have in turn been found to stimulate GnRH expression within the hypothalamus, thus activating the reproductive axis (Nakao et al. 2008; Watanabe et al. 2004). Both mammalian and avian species show accumulation of active thyroid hormone within the hypothalamus when exposed to long-day conditions. The changes in mammals are believed to be initiated through melatonin on melatonin-1a receptors located on the

pituitary pars tuberalis that influence changes in thyroid stimulating hormone (TSH) and ultimately GnRH. Interestingly, no melatonin receptors have been found on the avian pituitary, suggesting that melatonin does not directly transduce photoperiod signals to the pars tuberalis in these animals (Dawson and Sharp 2007).

Recent data from our lab suggest a similar pathway exists in reptiles (Figure 3). That is, temperature is the primary cue stimulating reproduction and it is transduced through changes in melatonin synthesis (Lutterschmidt and Mason 2008, 2009). In this thesis, we asked whether melatonin directly transduces temperature cues downstream to the same neural thyroid hormone cascade utilized in many photoperiod-sensitive breeders. To address this question, we manipulated the melatonin signal through the use of a biochemical precursor and a melatonin receptor antagonist to disrupt the signal at different levels.

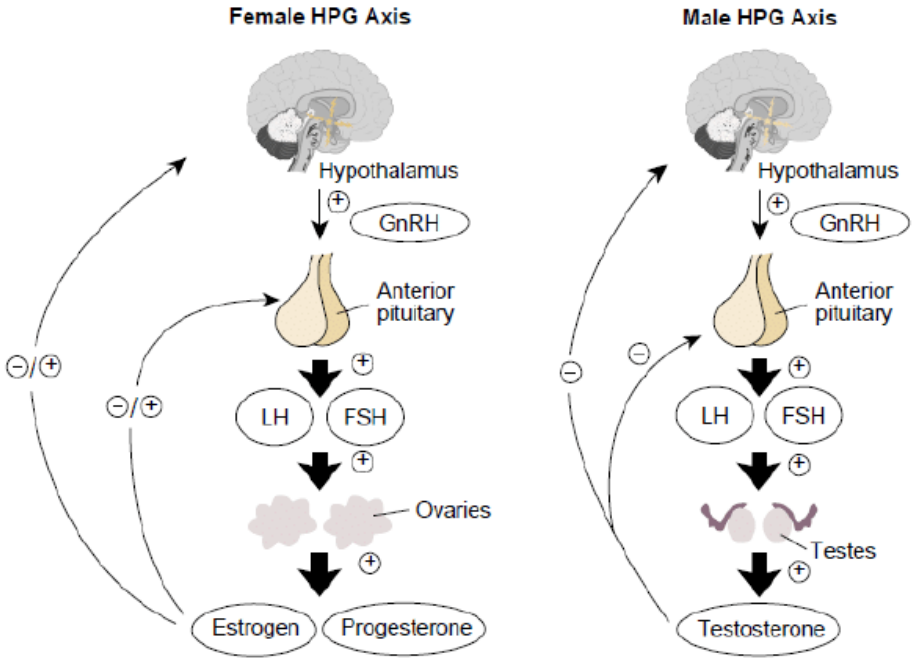

Fig. 1. Schematic overview of the male and female Hypothalamic-Pituitary-Gonadal (HPG) Axis. GnRH is released from the hypothalamus and acts upon the anterior pituitary gland. GnRH stimulation upregulates the secretion of luteinizing and follicle stimulating hormones which in

turn stimulate the synthesis of sex steroid hormones (e.g., estrogen and testosterone) from the gonads), ultimately controlling reproductive function (Hiller-Sturmhöfel and Bartke 1998).

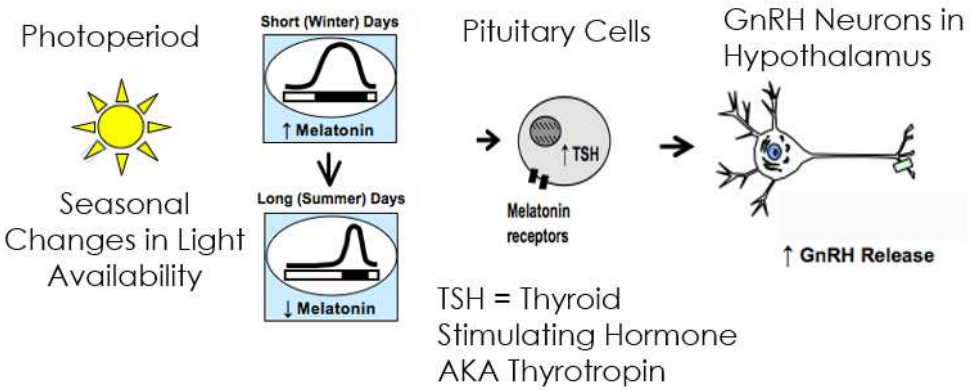

Fig. 2. The neuroendocrine pathway regulating photoperiod-induced reproduction. Changes in light availability are directly measured through the phase and duration of melatonin secretion. Long summer days decrease melatonin concentrations and with it the inhibitory effect of melatonin on reproductive physiological functions. This decrease in melatonin upregulates TSH synthesis in the pituitary pars tuberalis and ultimately upregulates GnRH and reproductive behavior.

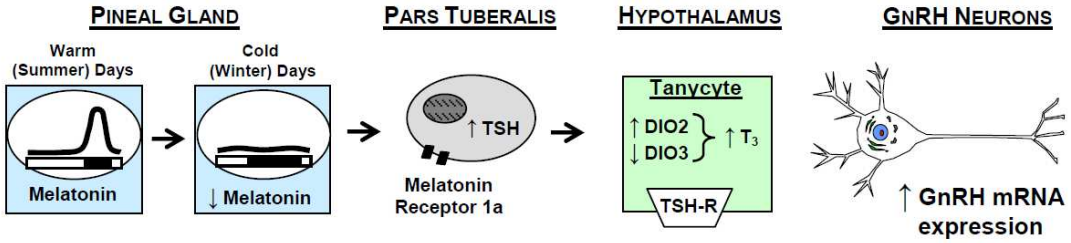

Fig. 3. Hypothesized neuroendocrine pathway regulating temperature-induced reproduction. Similar to the photoperiod-induced pathway shown in Fig. 2, cold winter days result in a decrease in the amplitude of melatonin synthesis. We hypothesize that this decrease in melatonin upregulates TSH synthesis in the pituitary, alters thyroid hormone metabolism in the hypothalamus, and ultimately upregulates GnRH and reproductive behavior.

Chapter 2: Materials and Methods

Study System

We used the well-studied red-sided garter snake system to address our research question: "Does melatonin mediate the influence of hibernation temperature on the synthesis of TSH in the hypothalamus and pituitary and/or reproductive behavior?" This study system is ideal for addressing these questions because, among other reasons, obtaining large sample sizes is feasible. Garter snakes are arguably the most abundant reptile in North America and are the most studied reptile with respect to reproductive behavior and neuroendocrinology (Lutterschmidt 2012). The majority of our knowledge comes from one large population found in south-central Manitoba, Canada, that is extremely resilient to capture stress (Krohmer and Lutterschmidt 2011; Lutterschmidt 2012). At this extreme northern latitude location where winter temperatures are extremely low and the rocky prairie terrain offers few sources of cover from the elements, snakes congregate together during the freezing months in communal underground dens that can contain more than 30,000 snakes (Shine et al. 2004)! Snakes spend up to 8 months each year in underground hibernacula with males first emerging in late April and females typically following by one or two weeks. Thousands of snakes aggregate near the dens and immediately begin breeding in large mating balls that consist of up to 100 males courting a single female. These conditions allow for large sample sizes to be collected with ease and have also allowed for lab conditions to be developed that accurately mimic field environments. Red-sided garter snakes have long been thought to have a reproductive pattern that was completely disassociated from control by sex steroid

hormones due to regressed gonads and a low concentration of sex steroids during the mating season. While this view is now rejected, with sex steroids playing a critical role in reproduction while remaining temporally disassociated from mating behavior, this disassociation allows for neuroendocrine mechanisms to be studied without the confounding effects of sex steroids on behavior. Finally, and most importantly, seasonal reproduction in red-sided garter snakes is regulated exclusively by temperature (Whittier et al. 1987), which creates an ideal condition to answer questions regarding a temperature sensitive pathway possibly under melatonin control.

Experimental Design

Snakes were collected from the den site in Inwood, Manitoba, Canada, during spring and fall and were transported back to the lab at PSU where they were acclimatized to the photoperiod and temperature conditions as listed in Table 1. Prior to hibernation animals were randomly assigned to one of three groups; a pre-hibernation group that was used to establish a baseline pre-hibernation level of TSH-immunoreactive cells (TSH-ir) prior to manipulating temperature and melatonin (n = 12), hibernation at 4°C and treatment with vehicle or 5-HTP (n = 60), or hibernation at 12°C and treatment with vehicle or luzindole (n = 60). Within the two hibernation temperature groups, snakes were further divided into two subgroups to determine the influence of treatment on TSH-ir cell number in the brain (n = 12 per treatment group for a total of 24 animals) and courtship behavior (n = 18 per treatment group for a total of 36 animals).

Table 1. Acclimatization regimes for investigating the influence of melatonin manipulation on reproductive physiology and behavior in red-sided garter snakes *(Thamnophis sirtalis parietalis*) hibernated at different temperatures.

Acclimatization Period		**Acclimatization Conditions (Photoperiod; Thermoperiod)**	
		Cold Temperature 4°C Hibernation	Warm Temperature 12°C Hibernation
Summer Feeding	24 May - 2 July	16L:8D; 23:18°C	16L:8D; 23:18°C
Summer Feeding	16 Aug - 3 Sep	14L:10D; 20:16°C	14L:10D; 20:16°C
Summer Feeding	4 Sep - 19 Sep	13L:11D; 20:12°C	13L:11D; 20:12°C
Pre-Hibernation	20 Sep - 3 Oct	12L:12D; 16:12°C	12L:12D; 16:12°C
Pre-Hibernation	4 Oct - 17 Oct	11L:13D; 14:12°C	11L:13D; 14:12°C
Hibernation	18 Oct - 29 Nov	0L:24D; 4°C	0L:24D; 12°C
Emergence	30 Nov +	16L:8D; 25:15°C	16L:8D; 25:15°C

Hormone Treatments

Previous studies have shown that low temperature winter dormancy decreases plasma melatonin concentrations in garter snakes e.g., (Lutterschmidt and Mason 2008, 2009). In an attempt to reverse this effect and elevate melatonin synthesis during hibernation, we injected animals hibernated at 4°C with 5-Hydroxytryptophan (product number F4150, Bachem Americas, Inc., Torrance, California, USA), a precursor to melatonin synthesis. Similar to Lutterschmidt and Mason (2010) snakes were treated with 1.2 mg of 5-hydroxytryptophan diluted in 100 µl of 10% ETOH in reptilian Ringers solution. Control treatments consisted of the vehicle only (i.e., 100 µl of 10% ethanol in reptilian Ringers solution). Treatments were administered via intraperitoneal injection every 3 days for the entire 6-week hibernation period.

Similarly, we attempted to inhibit melatonin signaling during hibernation at an elevated temperature, with the goal of testing the hypothesis that decreased melatonin signaling is sufficient to increase thyrotropin immunoreactivity and courtship behavior to the levels observed in response to low temperature dormancy. We used the melatonin receptor antagonist luzindole to inhibit melatonin signaling; luzindole is a well-characterized and widely known non-selective antagonist for vertebrate melatonin type 1a and 1b receptors (M. L. Dubocovich 1988; Margarita L. Dubocovich, Mogilnicka, and Areso 1990). Animals hibernated at 12°C were treated with 2.0 mg of luzindole (N-Acetyl-2benzyl-tryptamine, product number Q1885, Bachem Americas, Inc.) diluted in 50 µl of 10% DMSO in 80% ethanol. Control treatments consisted of the vehicle only (i.e., 50 µl of 10% DMSO in 80% ethanol). Treatments were administered via intraperitoneal injection every 3 days for the entire 6-week hibernation period.

Unfortunately, and despite several pilot studies conducted in our lab to validate the luzindole treatments, snakes in this experiment had an unexpected and negative response to prolonged, repeated injections of luzindole during winter dormancy. We observed a higher than normal mortality rate after approximately 5 weeks of luzindole treatments. The mortality rate typically observed during hibernation of garter snakes for 16-24 weeks in our lab is less than 1%. Prior to week 5, the mortality rate of all snakes was also < 1%. During week 5, however, the mortality rate in the luzindole treatment group increased to 30% while the mortality rate in the respective control group was 3.3%. Luzindole is not known to be cytotoxic in organismal studies, and therefore the cause of the increased mortality remains unknown. Although we had planned to retain snakes in hibernation for 8 weeks prior to measuring the treatment effects on TSH and courtship

behavior, once we observed the increased mortality rate during week 5 we immediately ceased all treatments and ended the experiment after 6 weeks of hibernation. We redistributed the remaining luzindole-treated animals to optimize sample sizes for brain immunohistochemistry versus behavior. Final sample sizes for all treatment groups are shown in the figures.

Tissue Processing and Collection

Snakes were euthanized via injection with 250 µl of sodium Brevital near the heart and decapitated. Brains were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 16 h at 4°C and then rinsed and stored in 0.1 M phosphate buffer (pH 7.2). Brains were dissected with the ventral skull left intact and then decalcified by incubating tissues in 10% Ethylenediaminetetraacetic acid (EDTA) diluted in 0.1 M phosphate-buffer (pH 7.2) solution for three days; tissues were transferred to fresh solution each day. The brain and decalcified ventral skull were then cryoprotected in 30% sucrose in 0.1 M phosphate buffer. Brains with ventral skull still intact were sectioned on a cryostat (Leica 3050S) into four series of 25-µm sagittal sections that were thaw-mounted onto subbed slides (Superfrost Plus, Thermo Fisher Scientific, Inc., Pittsburgh, PA., USA). Slides were stored at -20°C prior to immunohistochemical staining.

Immunohistochemistry

We examined potential differences in TSH within the hypothalamus and pituitary gland among treatment groups using immunohistochemistry . All slides were processed in a single assay using the methods detailed in Lutterschmidt and Maine (2014). Slides that contained the hypothalamic region and the pituitary were defrosted and dried on a slide warmer at 50 °C for 60 minutes and outlined with a hydrophobic barrier (Liquid Blocker – Super Pap Pen; Electron Microscopy Sciences, Hatfield, Pa., USA). Slides were then incubated for 5 minutes in a 4% paraformaldehyde solution in 0.1 M phosphate-buffered saline (PBS; pH 7.4). We washed the slide-mounted tissues 3 times for 5 minutes each with 0.1 M PBS; this was completed after every step of the assay to reduce potential background staining. The paraformaldehyde fixative was neutralized with a 0.1% sodium borohydride solution (pH 8.5) for 20 minutes and endogenous peroxidase activity quenched with 3% hydrogen peroxide (H_2O_2) in methanol for 30 minutes. This last step was to reduce Horseradish Peroxidase (HRP) substrates which can lead to background staining later in the assay when the tissues undergo a chromogenic reaction. Slides were washed twice for 5 min each with 0.1 M PBS followed by a single 5 min wash in 0.3% Triton X solution in 0.1 M PBS (PBS-T). We incubated the slides for 60 minutes with a blocking solution of 10% horse serum (item H1270, Sigma-Aldrich Co.) and 10% Avidin (item SP-2001, Vector Labs, Burlingame, CA 94010, USA) to reduce nonspecific binding. TSH immunoreactivity was examined using an anti-garter snake TSH antiserum made in a rabbit. This antibody was custom generated by Pacific Immunology, Inc. against the predicted amino acid sequence for TSHβ using the cDNA sequence we previously isolated from garter snake brain. Tissues were incubated with the TSH

antibody at a dilution of 1:1000 in 10% horse serum and 10% biotin (item SP-2001, Vector Labs) solution in PBS-T. Sections were coverslipped with parafilm and allowed to incubate with the primary TSH antibody for 48 hours at 4°C in a humid chamber. The primary antibody signal was amplified with a 60 min incubation with a biotinylated horse-anti rabbit secondary antibody (Vector Labs/BA-1100) diluted 1:400 in PBS-T. Tissues were then incubated with avidin conjugated to horseradish peroxidase (Elite ABC peroxidase kit; Vector Labs). Primary antibody binding was visualized with a chromogenic reaction carried out with a 0.25 mg/mL solution of diaminobenzidine (item 0430-5G; BioExpress, Kaysville, Utah, USA) diluted in 0.2% H_2O_2 in 0.05 M Tris-HCL buffer (pH 7.2). The reaction was terminated by immersion of the slides in 3 washes of nanopure H_2O for 5 min each. Tissues were dehydrated using a series of graded ethanol washes (70, 85, 95, 100%) for 2 min each and then cleared with Citrasolv (Fisher Scientific) and covered with Permount and coverslips.

To validate the specificity of the primary antibody for TSH in our assay, we first performed a series of immunohistochemistry controls using preadsorption tests. Prior to immunohistochemistry, rabbit anti-TSH antiserum diluted 1:1000 in 10% horse serum and 10% biotin solution in PBS-T was incubated overnight at 4°C with 1 mg of TSH Peptide used as the inoculating antigen (Pacific Immunology) per mL of antibody solution. We used two series of tissue from three individual males; one series was incubated with the preadsorbed antibody and the other series was incubated with the regular, non-adsorbed antibody. Both series were tissue from the same animals. All tissue used in the preadsorption tests were independent from the animals used in the hibernation experiments, and all tissue was processed in the same assay.

Immunoreactive Cell Counting

Immunoreactive cell counting of stained tissue was performed using an Olympus BX40 microscope with a QIClick digital camera and QImaging software (QImaging; Surrey, B.C., Canada). The locations of TSH immunoreactive (TSH-ir) cells were mapped onto sagittal sections using anatomical brain sections adapted from Krohmer et al. [2010]. We observed one distinct TSH-ir cell population located in the median eminence region of the hypothalamus and infundibulum of the pituitary gland; staining in the anterior pituitary region was also observed as expected. Animals were coded so that the observer was blind to the treatment group of individuals. Immunoreactive cells were counted and quantified manually in sections through the entirety of the hypothalamic/pituitary region under x200 magnification and again at x400 magnification. If the counts were not identical the section was recounted under x400 magnification as a verification and quality control measure. We followed counting methods and criteria described by (Lutterschmidt and Wilczynski 2012). The number of TSH-ir cells was quantified in one tissue series and then totaled for each individual. Missing and/or severely damaged sections were designated as the mean cell count of TSH-ir cells in the previous and subsequent tissue section. If two or more consecutive sections were unusable the animal was excluded from the statistical analysis. Because tissue was divided into 4 different 25-µm sections, approximately 100µm separated each section within a series, thereby eliminating the possibility that counts of TSH-ir cells were were inflated due to double counting.

Courtship behavior

Following hibernation and treatment for 6 weeks, animals were subjected to simulated spring emergence and housed under spring-like environmental conditions (Table 1). Using an ethogram of male courtship behavior (Table 2), we measured the courtship behavior of each male on days 1, 3, 7, 10, 14, and 21 post-emergence. During each courtship trial, we scored the behavior of each male 10, 30 and 60 min following the introduction of a sexually attractive stimulus female into each arena. Males were tested in groups of 8 to simulate natural mating conditions, where mating balls rarely contain fewer than five males courting a single female (Joy and Crews 1985). Males from each treatment group were equally distributed across all behavior arenas. Because female red-sided garter snakes become unattractive and unreceptive after mating, we covered each female's cloaca with medical adhesive tape to prevent mating during the courtship trial e.g.,(Lutterschmidt, LeMaster, and Mason 2004). Thus, the highest courtship score each male could achieve was 4.0. We used these data to calculate a mean and maximum courtship score for each male on each day post-emergence.

Table 2. Ethogram of courtship behavior for the male red-sided garter snake *(Thamnophis sirtalis parietalis)*. Behaviors equal to and above 3 are exhibited in a reproductive context only (modified from Lutterschmidt 2006, 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

Statistics

We used SigmaPlot 12.0 (Systat Software 2010, Systat systems, Inc., Port Richmond, CA, USA) to perform all statistical analyses. Statistical comparisons were considered significant at $p \leq 0.05$. Except where noted, all data met the assumptions of normality and equal variance required for parametric tests. We first used a one-way ANOVA within each temperature group to confirm that neither body mass nor snout-vent length differed significantly between treatment groups. T-tests were used to determine if there was a significant difference in TSH-immunoreactive cell number of animals between week 0 and week 6 with and without hormone manipulation. We used a two-way repeated measures analysis of variance (ANOVA) followed by a Holm-Sidak multiple comparisons test to detect possible differences in both average and maximum courtship scores between treatment groups; days post-emergence was used as the within-subjects factor and each day was compared against the first day (day 1) of emergence in the multiple comparisons test. Separate analyses were performed for each hibernation temperature, and all pairwise comparisons were not performed to maintain statistical power.

Chapter 3: Results

Experiment 1: Influence of Melatonin Signaling on Thyrotropin Immunoreactivity

Prior to hibernation (i.e., time 0), males had a mean of 134.73 (± 16.497 SE) TSH-labeled cells in the brain. Following 6 weeks of low temperature dormancy at 4°C, in combination with intraperitoneal vehicle treatment, we detected a significant decrease in the number of TSH-labeled cells in the brains of male snakes (83.67 ± 17.940 SE; t = 2.091, df = 18, P = 0.050; Figure 4). By contrast, treatment of snakes with the melatonin precursor 5-HTP eliminated the decrease in TSH-labeled cells in response to 6 weeks of hibernation at 4°C (t = 0.0746, df = 21, P = 0.941; Figure 4). The mean number of TSH-immunoreactive cells in the brain of 5-HTP-treated males was 132.333 (± 26.72 SE).

There was no significant change in the number of TSH-labeled cells in the brain of male snakes after 6 weeks of high temperature dormancy at 12°C (t = -0.875, df = 19, P = 0.393; Figure 5). Following 6 weeks of high temperature dormancy at 12°C, in combination with intraperitoneal vehicle treatment, males had a mean of 162.100 (± 27.398 SE) TSH-labeled cells in the brain, which did not differ from pre-hibernation values (see above). Treatment of snakes with the melatonin receptor antagonist luzindole elicited no significant changes in the number of TSH-labeled cells in males that underwent 6 weeks of hibernation at 12°C (t = -0.860, df = 16, P = 0.402; Figure 5). The mean number of TSH-immunoreactive cells in the brain of luzindole-treated males was 162.143 (± 30.711 SE).

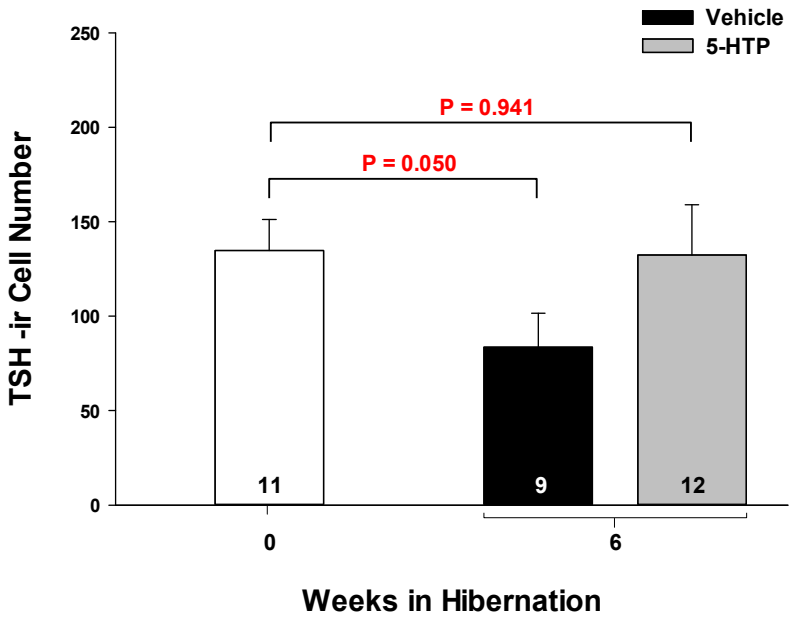

Figure 4. Effect of low temperature winter dormancy on the number of cells labeled for thyroid stimulating hormone (TSH) in the region of the median eminence of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Samples collected at time 0 occurred prior to snakes being subjected to any temperature or hormone manipulation and serve as a pre-hibernation baseline. The number of TSH-immunoreactive (ir) cells decreased significantly after snakes were hibernated at 4°C for 6 weeks and treated with control (vehicle) solution. Treatment of snakes with the melatonin precursor 5-hydroxytryptophan (5-HTP), which is known to increase plasma melatonin

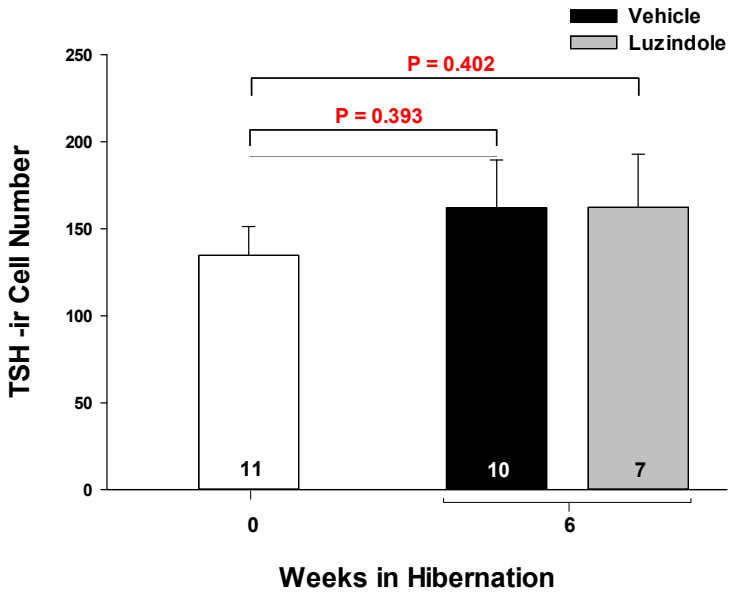

Figure 5. Effect of elevated hibernation temperature (12°C) on the number of cells labeled for thyroid stimulating hormone (TSH) in the region of the median eminence of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Samples collected at time 0 occurred prior to snakes being subjected to any temperature or hormone manipulation and serve as a pre-hibernation baseline. The number of TSH-immunoreactive (ir) cells did not change significantly in response to hibernation at 12°C for 6 weeks and treatment with control (vehicle) solution. Treatment of snakes with the melatonin receptor antagonist luzindole did not influence TSH-ir cell number. Data are the

Experiment 2: Influence of Melatonin Signaling on Courtship Behavior

For males hibernated at 4°C for 6 weeks, the main effects of 5-HTP versus vehicle treatment on mean courtship score were not significant ($F_{1,198} = 2.019$, P = 0.165, two-way repeated measures ANOVA; Figure 6A). However, mean courtship score varied significantly with days post-emergence ($F_{6,198} = 9.010$, P = <0.001; Figure 6A). The interaction between treatment and days post-emergence was not significant ($F_{6,198} = 1.605$, P = 0.147).

To assess the effects of 5-HTP treatment on courtship intensity, we investigated possible differences in the maximum courtship score achieved by males between

treatments and across days post-emergence. While the main effects of treatment on maximum courtship score were not significant ($F_{1.,198} = 1.456$, $P = 0.236$), we observed a significant change in maximum courtship score over days post-emergence ($F_{6,198} = 5.989$, $P = <0.001$; Figure 6B). In addition, the interaction between treatment and days post-emergence was significant ($F_{6,198} = 2.898$, $P = 0.010$), indicating that the change in courtship intensity over time depended on treatment condition (Figure 6B).

For males hibernated at 12°C for 6 weeks, the main effects of luzindole versus vehicle treatment on mean courtship score were not significant ($F_{1,150} = 0.0166$, $P = 0.899$, two-way repeated measures ANOVA; Figure 7A). However, mean courtship score varied with days post-emergence ($F_{6,150} = 8.048$, $P = <0.001$; Figure 7A). The interaction between treatment and days post-emergence was not significant ($F_{6,150} = 0.702$, $P = 0.648$).

Similarly, while the main effects of luzindole treatment on courtship intensity (i.e., maximum courtship score) were not significant ($F_{1,150} = 0.059$, $P = 0.809$; Figure 7B), we observed a significant change in maximum courtship score over days post-emergence ($F_{6,150} = 9.337$, $P = <0.001$; Figure 7B). The interaction between treatment and days post-emergence was not significant ($F_{6,150} = 1.510$, $P = 0.179$).

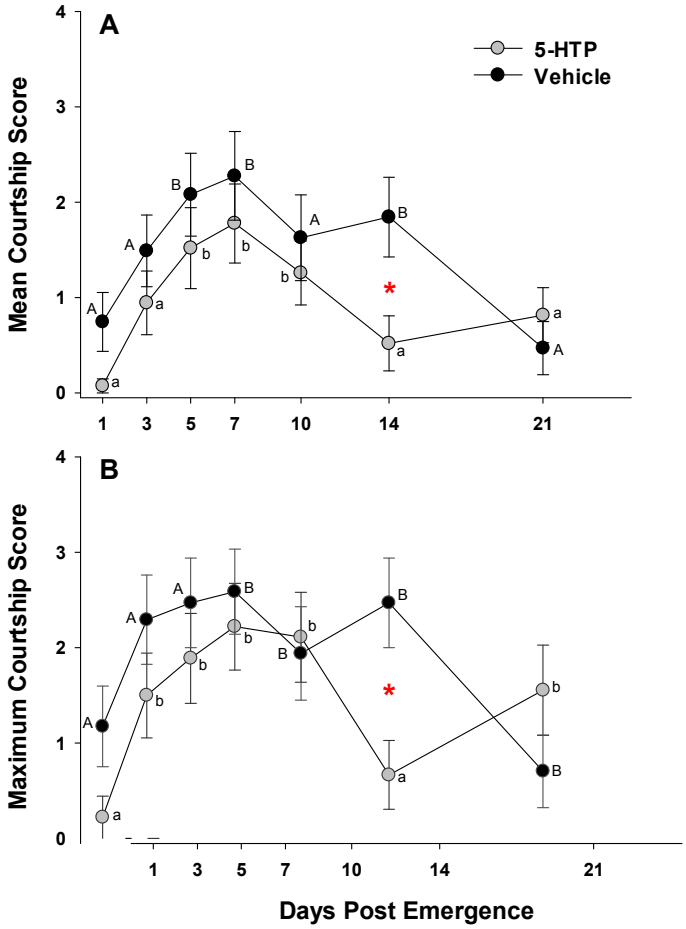

Figure 6. Effect of vehicle (n = 17) and 5-hydroxytryptophan (5-HTP, n = 18) treatment on the (A) mean and (B) maximum courtship scores achieved by male red-sided garter snakes (*Thamnophis sirtalis parietalis*) following emergence from simulated winter dormancy at 4°C for 6 weeks. Treatment of snakes with the melatonin precursor 5-HTP is known to increase plasma melatonin concentrations in this species. Capital letters indicate significant differences between days post-emergence within the vehicle treatment group; lower case letters indicate significant differences between days within the 5-HTP group. For both analyses, multiple comparisons tests were performed by comparing each day post-emergence to day 1 (i.e., all pairwise comparisons were not performed to maintain statistical power). The asterisks indicate a significant difference between treatment groups on day 14 post-emergence. Data are the means ± 1 SE.

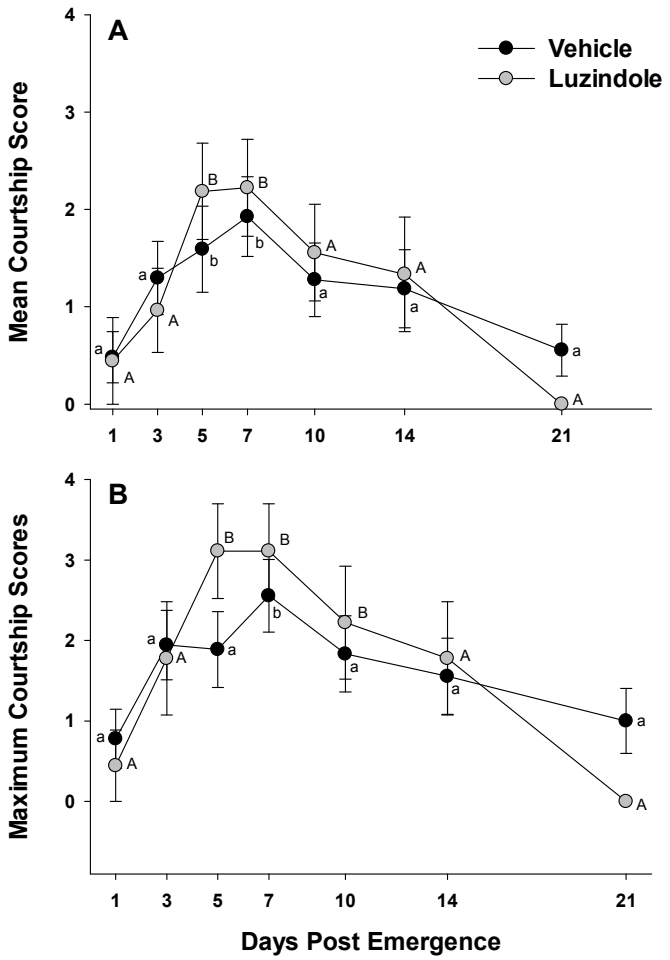

Figure 7. Effect of treatment with vehicle (n = 18) or the melatonin receptor antagonist luzindole (n = 9) on the (A) mean and (B) maximum courtship scores achieved by male red-sided garter snakes (*Thamnophis sirtalis parietalis*) following emergence from simulated winter dormancy at 12°C for 6 weeks. Capital letters indicate significant differences between days post-emergence within the vehicle treatment group; lower case letters indicate significant differences between days within the luzindole group. For both analyses, multiple comparisons tests were performed by comparing each day post-emergence to day 1 (i.e., all pairwise comparisons were not performed to maintain statistical power). Data are the means ± 1 SE.

Chapter 4: Discussion and Conclusions

This is the first study to address whether low temperature winter dormancy, which activates seasonal cycles of reproductive behavior in many ectotherms, alters the metabolism of thyroid hormones within the hypothalamus of the brain. Similar to the effects of long days in spring on breeding birds and mammals, our data indicate that low temperature winter dormancy also influences neural thyroid hormone metabolism. Furthermore, our data suggest that these changes are sensitive to the hormone melatonin.

In our study male snakes that received vehicle injections showed a significant decrease in TSH immunoreactivity within the median eminence after 6 weeks of hibernation at 4°C; this effect was lost in males that were instead given intraperitoneal (IP) injections of the melatonin precursor 5-HTP. Previous research has shown that increasing 5-HTP leads to an increase in melatonin synthesis and circulating melatonin concentrations (Lutterschmidt and Mason 2010). These results indicate that TSH-immunoreactive cell number within the median eminence is sensitive to low temperature winter dormancy, and additionally suggest that temperature induced changes in TSH are mediated by decreased melatonin concentrations. Conversely, male snakes hibernated at an elevated 12°C for six weeks while receiving vehicle or luzindole injections showed no change in TSH-immunoreactive cell number. Luzindole is a competitive melatonin receptor antagonist that has been shown to inhibit the effects of melatonin across a variety of species (Dubocovich 1988; Dubocovich et al. 1990).

Together, these results suggest that at elevated hibernation temperatures, blocking melatonin signaling is not sufficient in itself to mimic the effects of low-temperature winter dormancy. Red-sided garter snakes must undergo a period of low-temperature exposure before they initiate courtship behavior et al. 1982), and it is likely that blocking melatonin signaling with luzindole is not sufficient to mimic the exposure to low temperature winter dormancy. It is also possible that melatonin levels are being significantly affected but the pathway remains refractory to these changes without a "reset" of the system induced by low temperatures. This hypothesis is supported as we see this same phenomenon in hamsters; male and female hamsters with intact pineal glands showed no regression of their reproductive systems if kept in constant darkness at the start of the spring breeding season (Reiter 1973). The effect on TSH immunoreactivity may, in general, be masked or confounded by other factors that are present at this elevated hibernation temperature. Lastly, there is the possibility that luzindole did not serve as a suitable melatonin antagonist in this study. Thirty percent of the animals treated with luzindole in this experiment died, suggesting that chronic exposure to luzindole at 12°C is toxic. Incorrect dose and delivery methods are unlikely as the dose was on the low-end and in line with previous studies in this and other species (Dubocovich et al. 1990; Pinillos et al. 2001).

The second part of this study examined the effects of melatonin manipulation on courtship behavior in male red-sided garter snakes hibernated at low (4°C) or elevated (12°C) temperatures. Specifically, we examined changes in the mean and maximum courtship scores, which are measures of courtship endurance and intensity, respectively. Both are important because they give a more complete look into understanding sex

drive—the motivating force responsible for passing genes to one's progeny. We observed that both the mean and maximum courtship scores of snakes hibernated at 4°C and treated with vehicle stayed elevated after emergence significantly longer than animals that had been given 5-HTP injections. In contrast, males that were hibernated at 12°C and treated with vehicle or luzindole showed no significant differences in mean or maximum courtship scores upon emergence. We know from past research that red-sided garter snakes needs a period of cold during hibernation to show maximum courtship upon emergence (Garstka, Camazine, and Crews 1982, 1982). Thus, the fact that blocking melatonin did little to courtship behavior suggests that the process is more complex and cold temperatures are likely affecting other physiological systems responsible for mating behavior. Alternatively, it is possible that decreasing melatonin concentrations, which are observed in response to low temperature dormancy, are not sufficient to override the effects of elevated winter dormancy temperatures on reproductive behavior.

Male reproductive behavior in this model system is very stereotyped and has been consistently incorporated into past research; a number of these studies have documented the inhibitory effects of elevated melatonin on male courtship (Lutterschmidt et al. 2004; Lutterschmidt and Mason 2009; Whittier et al. 1987b; Whittier and Crews 1987). Specifically, Lutterschmidt and Mason (2009) observed that male red-sided garter snakes hibernated at elevated temperatures had significantly higher diel melatonin concentrations during hibernation and these elevated levels persisted through spring emergence. They also showed a significant delay in the onset of courtship behavior in males that were hibernated at elevated temperatures. Our results corroborate these

findings and provide further insight into the mechanisms that mediate the effects of temperature on reproduction.

We observed that treatment with 5-HTP significantly decreased or delayed appetitive courtship behaviors in male red-sided garter snakes hibernated at 4°C. Courtship intensity showed a more pronounced change with melatonin manipulation overall. This is interesting because up to this point studies of courtship behavior in garter snakes have primarily focused on mean mating behavior scores. By expanding the way in which we look at these behaviors we gained the insight that changes in melatonin (and its downstream effects) may act upon certain aspects of courtship behavior preferentially. Both groups of male red-sided garter snakes hibernated at 4°C reached their highest mean courtship scores by day 7 post-emergence; similarly, courtship intensity in both the 5-HTP- and vehicle-treated snakes plateaued at days 7-10 post-emergence. However, on day 14 post-emergence, both mean and maximum courtship scores of vehicle- treated males were significantly higher than males treated with 5-HTP. This is important because most females only mate once before dispersing from the den within 24 hours of emerging (Whittier et al. 1985). Because of the very brief and intense mating behavior of red-sided garter snakes after emergence, a delay of even a few days could have large repercussions on reproductive fitness.

Male garter snakes hibernated at 12°C were treated with either vehicle or the melatonin receptor antagonist luzindole to test the hypothesis that decreasing melatonin signaling would rescue courtship behavior. This hypothesis was based on the results of Lutterschmidt and Mason (2009), in which males hibernated at elevated temperatures had

elevated melatonin levels and a delayed onset of courtship behavior. Overall our results suggest that decreasing melatonin signaling is not sufficient in itself to override the effects of elevated winter dormancy temperatures on reproductive behavior. The fact that blocking melatonin did little may suggest that the process is more complex and cold temperatures are likely affecting other physiological systems responsible for mating behavior.

Conclusion

One question we aimed to answer was whether low-temperature changes in TSH immunoreactivity in the brain—specifically the median eminence of the hypothalamus—was connected to a temperature mediated melatonin signaling pathway, a pathway that may ultimately direct sexual behavior. We found that there is a link between melatonin and TSH immunoreactivity in the brain of male red-sided garter snakes hibernated at 4°C. Our results suggest that temperature-induced changes in TSH within the median eminence may be mediated, at least in part, by temperature-induced changes in melatonin signaling. We also found that changes in melatonin affect appetitive reproductive behaviors of male garter snakes hibernated at 4°C. The results of this study are important because they demonstrate that the neuroendocrine factors regulating the reproductive axis and reproductive behavior are sensitive to hibernation temperature and are mediated by a conserved cascade of hormonal control.

In conclusion, an animal's ability to synchronize life-history events or stages with optimal environmental conditions is paramount to successfully reproducing and thus maximizing its fitness. These life-stages are intrinsically tied to particular environmental

conditions that allow them to be optimally timed. Through a wide and continued study of comparative physiology we can better understand what outcomes are probable and gain a deeper understanding of ecological changes on a grander scale. This will allow specificity of research to intimately tailor itself to the needs of the changing planet.

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