Role of Melatonin in Temperature-Induced Activation of the Neuroendocrine Reproductive Axis in Garter Snakes

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Abstract

An animal's ability to respond optimally to changing environmental conditions is paramount to successfully reproducing and thus maximizing fitness. Studies on photoperiod-induced changes in neural thyroid hormone metabolism have conclusively linked environmental cues to the neuroendocrine reproductive axis of birds and mammals. Whether this conserved mechanism also transduces changes in environmental temperature, however, has not been fully addressed. We investigated whether the hormone melatonin mediates the effects of low-temperature dormancy on thyroid hormone metabolism within the hypothalamus of red-sided garter snakes (*Thamnophis sirtalis parietalis*). To address this question, we used immunohistochemistry to assess changes in thyroid-stimulating hormone (TSH) in the infundibulum of the pituitary and deiodinase 3 (Dio3) and gonadotropin-releasing hormone (GnRH) in the hypothalamus. We also asked if changes in TSH, Dio3, and/or GnRH immunoreactivity are associated with changes in male court-

ship behavior. In contrast to our predictions, 6 weeks of dormancy at 4°C significantly decreased the number of TSH-labeled cells in the infundibulum. It is possible that the observed decrease in TSH is related to the release of snakes from temperature refractoriness, but this idea needs further testing. Treatment of snakes with the melatonin precursor 5-hydroxytryptophan during dormancy at 4°C both reversed the temperature-induced change in TSH immunoreactivity and disrupted the temporal pattern of male courtship behavior. These results suggest that TSH cells within the infundibulum are both modulated by temperature and sensitive to changes in melatonin. As predicted, male snakes hibernated at an elevated temperature of 12°C for 6 weeks and treated with vehicle showed no change in TSH-, Dio3-, or GnRH-immunoreactive cell number. Treatment of snakes with the melatonin receptor antagonist luzindole was not sufficient in rescuing the effects of dormancy at 12°C on TSH immunoreactivity or courtship behavior. However, luzindole-treated snakes showed a significant increase in GnRH-immunoreactive cell number, suggesting that melatonin exerts an inhibitory effect on GnRH in garter snakes. In summary, our results provide critical insights into the mechanisms that mediate the effects of temperature on reproductive physiology and behavior.

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Introduction

Reproduction is energetically expensive, and animals cannot afford to expend resources at nonbeneficial times. Correctly timing life-history events requires that an animal properly adjust its physiology, behavior, and morphology to the environmental conditions in which survival and reproductive success are maximized. These adjustments are induced through the effects of environmental cues on the brain and associated changes in hormones and behavior. It is now well established, for example, that seasonal changes in photoperiod alter the metabolism of thyroid hormones within the brain, which in turn mediates seasonal changes in the neuroendocrine reproductive axis (reviewed by [Nakane and Yoshimura, 2019]). In spring-breeding birds and mammals, the effects of lengthening days manifest as an increase in thyrotropin or thyroid-stimulating hormone (TSH) specifically within the pars tuberalis, a region of the anterior pituitary that surrounds the hypophyseal stalk [Hanon et al., 2008; Nakao et al., 2008a; Unfried et al., 2009]. TSH acts retrogradely on the hypothalamus to increase deiodinase 2 (Dio2) expression, while decreasing deiodinase 3 (Dio3) expression within the tanycytes lining the third ventricle [Yamamura et al., 2006; Nakao et al., 2008a, b; Yasuo et al., 2009]. The Dio2 enzyme converts thyroxine (T_4) to bioactive triiodothyronine (T_3), whereas Dio3 inactivates T_3 via deiodination [Yasuo et al., 2005]. These reciprocal changes in deiodinase activity work in concert to favor the accumulation of T_3 within the hypothalamus [Yasuo et al., 2006]. Finally, the local accumulation of T_3 facilitates remodeling of the tanycytes and an increase in the expression, synthesis, and/or release of gonadotropin-releasing hormone (GnRH) [Yamamura et al., 2006; Perfito et al., 2012]. In this way, seasonal changes in photoperiod are relayed directly to GnRH neurons within the hypothalamus, which then modulate the activity of the anterior pituitary and gonads (i.e., the HPG axis) to modify reproductive physiology and behavior.

While many aspects of this neuroendocrine cascade are remarkably conserved across the vertebrates studied thus far [e.g., Wood and Loudon, 2014], the role of melatonin in mediating changes in TSH within the pars tuberalis differs between birds and mammals. The mammalian pars tuberalis has a high density of melatonin receptor type 1a [Weaver and Reppert, 1990; Dardente, 2007], and photoperiod-induced changes in melatonin directly modulate pars tuberalis thyrotropin [Hanon et al., 2008;

Ono et al., 2008; Unfried et al., 2009]. In contrast, the mechanisms transducing photoperiod information in birds are fundamentally different [Nakao et al., 2008a], as the avian pars tuberalis does not express melatonin receptors [Morgan and Williams, 1996]. Thus, while photoperiod-induced breeding in birds is also associated with increased thyrotropin in the pars tuberalis [Nakao et al., 2008a], these long-day changes are not directly regulated by melatonin signaling [Juss et al., 1993; Ball and Ketterson, 2008].

The role of this pathway in integrating other environmental cues has not been fully explored, and the lack of any studies in amphibians and reptiles, which are heavily influenced by seasonal changes in environmental temperature, is a notable gap in the comparative analysis of this pathway. A striking similarity between photoperiod- and temperature-activated reproduction is that both involve cues (long days and cold temperatures, respectively) that decrease melatonin signaling (Fig. 1; reviewed in [Lutterschmidt, 2012]). In the current study, we asked if low-temperature dormancy alters thyroid hormone metabolism within the hypothalamus. We used the well-studied red-sided garter snake (*Thamnophis sirtalis parietalis*) to address this question. Northern populations of red-sided garter snakes spend up to 8 months each year in underground hibernacula, with males first emerging in late April and females typically following suit 1–2 weeks later. The mating season is very attenuated, lasting only 4–5 weeks before snakes migrate away from the den to summer feeding grounds.

Seasonal changes in the expression of reproductive behavior are regulated exclusively by temperature in red-sided garter snakes [Whittier et al., 1987]. In addition, northern populations of red-sided garter snakes exhibit a temporally dissociated reproductive pattern, in which peak sex steroid hormone production and gametogenesis do not occur at the same time as mating behavior [Crews et al., 1984]. However, mounting evidence indicates that reproductive hormones may play a trans-seasonal role in facilitating changes in reproductive behavior. For example, prolonged low-temperature dormancy alters the number and size of GnRH-labeled neurons in the brain, plasma concentrations of sex steroid hormones, and male courtship behavior (see review in Lutterschmidt [2012]; [Lutterschmidt et al., 2022]). Previous studies also demonstrate that low-temperature dormancy alters plasma melatonin concentrations of snakes [Lutterschmidt, 2012]. Therefore, we asked if temperature-induced changes in the melatonin signal mediate subsequent changes in the neuro-

Fig. 1. a Schematic showing the effects of seasonal changes in photoperiod on neural thyroid hormone metabolism and gonadotropin-releasing hormone (GnRH) in spring-breeding mammals. Long days decrease the duration of elevated melatonin concentrations and this in turn increases the synthesis of thyroid-stimulating hormone (TSH) within cells of the pituitary pars tuberalis. TSH acts retrogradely on the hypothalamus to alter the expression of deiodinases (Dio); reciprocal changes in Dio2 and Dio3 allow triiodothyronine (T₃) to accumulate within the hypothalamus, resulting in increased expression of GnRH and an activation of the reproductive axis. **b** In garter snakes, low-temperature dormancy similarly decreases melatonin concentrations and increases both GnRH immunoreactivity and courtship behavior.

Based on these results and additional pilot data for TSH and Dio3, we predicted that (1) increasing melatonin synthesis with the precursor 5-hydroxytryptophan would block the effects of low-temperature dormancy (4°C) on TSH, Dio3, GnRH, and courtship behavior. Similarly, we predicted that (2) inhibiting melatonin signaling with the melatonin receptor antagonist luzindole would mimic exposure to 4°C and override the inhibitory effects of an elevated dormancy temperature (12°C). Data shown in bar graphs are the mean ± 1 SEM; sample sizes are shown along the x-axes. Significant changes in Dio3-labeled cell number ($p = 0.023$), GnRH-labeled cell number ($p = 0.026$), and courtship behavior [Garstka et al., 1982] following 8 weeks of dormancy at 4°C are indicated by asterisks.

endocrine reproductive axis (i.e., GnRH and thyroid hormone metabolism within the hypothalamus and/or TSH in the infundibulum of the pituitary gland; Fig. 1). We predicted that dormancy at 4°C would increase TSH, decrease Dio3, and increase GnRH immunoreactivity, and that elevated melatonin concentrations would reverse these effects. Similarly, we predicted that dormancy at an elevated temperature of 12°C would result in no changes in immunoreactive staining, but simultaneous inhibition of melatonin signaling via treatment with a melatonin receptor antagonist would increase TSH, decrease Dio3, and increase GnRH immunoreactivity. Such results would indicate that decreased melatonin concentrations in response to low-temperature dormancy are both necessary and sufficient to activate the reproductive axis.

Materials and Methods

Experimental protocols were approved by the Portland State University Animal Care and Use Committee 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 performed under the authority of Wildlife Scientific Permit WB18801 issued by the Manitoba Department of Sustainable Development.

Experimental Design

Snakes were collected from a den site in Inwood, MB, Canada, during the spring mating season and fall pre-hibernation period. Snakes were transported to the lab at Portland State University, where they were acclimatized to the photoperiod and temperature conditions shown in Table 1. Northern populations of red-sided garter snakes hibernate in underground dens for approximately 8 months each year. Both male and female snakes are refractory to warm temperatures, and previous studies indicate that significant

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

"Season"	Dates	Acclimatization conditions (photoperiod; thermoperiod)
Summer (feeding)	24 May to 15 Aug	16:8 h L:D; 23:18°C
Summer (feeding)	16 Aug to 03 Sept	14:10 h L:D; 20:16°C
Summer (feeding)	04 Sept to 19 Sept	13:11 h L:D; 20:12°C
Fall pre-hibernation	20 Sept to 03 Oct	12:12 h L:D; 16:12°C
Fall pre-hibernation	04 Oct to 17 Oct	11:13 h L:D; 14:12°C
Hibernation	18 Oct to 29 Nov	0:24 h L:D; either 4°C or 12°C
Emergence (mating)	30 Nov to 30 Dec	16:8 h L:D; 25:15°C

changes in hormones and behavior occur within 3–8 weeks of exposure to low-temperature dormancy [e.g., Garstka et al., 1982; Bona-Gallo and Licht, 1983; Lutterschmidt and Mason, 2009; Lutterschmidt et al., 2022].

The experimental design employed in this study closely replicates that of previous studies and simulates the environmental conditions experienced by red-sided garter snakes under natural field conditions [e.g., Lutterschmidt et al., 2006]. In independent studies, we first determined the effects of low-temperature dormancy at 4° on TSH-, Dio3-, and GnRH-labeled cells within the infundibulum and brain of untreated and unmanipulated garter snakes over time (Fig. 1b). Based on these data, we then asked if the effects of prolonged exposure to 4°C (i.e., significantly decreased Dio3 and increased GnRH) are mediated by the hormone melatonin. To address this question, animals were randomly assigned to one of five groups (Fig. 2): a pre-hibernation group that was used to establish a fall baseline level of immunoreactive cells prior to manipulating temperature and melatonin ($n = 12$), hibernation at 4°C for 6 weeks and treatment with vehicle ($n = 30$) or the melatonin precursor 5-hydroxytryptophan ($n = 30$), or hibernation at 12°C for 6 weeks and treatment with vehicle ($n = 30$) or the melatonin receptor antagonist luzindole ($n = 30$). Within each hibernation treatment group, snakes were further divided into two subgroups to determine the influence of treatment on immunoreactive cell number in the brain ($n = 12$) or courtship behavior ($n = 18$).

Hibernation was simulated by decreasing the ambient temperature in complete darkness (i.e., 0:24 h L:D photoperiod). The temperature regimes were chosen based upon previous laboratory studies in this species [e.g., Bona-Gallo and Licht, 1983; Krohmer and Crews, 1987; Whittier et al., 1987; Lutterschmidt and Mason, 2008, 2009] as well as recorded body temperatures of red-sided garter snakes during winter dormancy under natural field conditions [Lutterschmidt et al., 2006]. The elevated temperature treatment, consisting of a constant temperature of 12°C during dormancy, was chosen because this temperature is significantly higher than the minimal body temperatures of red-sided garter snakes recorded during winter dormancy in the field but low enough to prevent dramatic changes in body condition (in the absence of feeding) during these prolonged experiments [Lutterschmidt and Mason, 2009].

Hormone Treatments

Previous studies showed that low-temperature dormancy decreases plasma melatonin concentrations in garter snakes [Lutterschmidt and Mason, 2008, 2009]. In an effort 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, CA, USA), a precursor to melatonin synthesis. We chose to manipulate plasma levels of melatonin via a precursor because direct, prolonged manipulations of melatonin rarely mimic endogenous melatonin rhythms and often produce inconclusive results, both in garter snakes and in other species (e.g., see reviews in [Lutterschmidt et al., 2003; Lutterschmidt, 2012]. In contrast, experimentally elevated levels of a precursor allow more melatonin to be synthesized within the animal according to natural circadian rhythms in enzyme synthesis and activity, and Lutterschmidt and Mason [2010] demonstrated that treatment of snakes with 5-hydroxytryptophan significantly elevates plasma melatonin concentrations. As in this previous study, we treated snakes with 1.2 mg of 5-hydroxytryptophan diluted in 100 μ L of 10% ethanol in reptilian Ringer's solution. Control treatments consisted of the vehicle only (i.e., 100 μ L of 10% ethanol in reptilian Ringer's solution). Treatments were administered via intraperitoneal injection every 3 days for the entire hibernation period.

Similarly, we sought to inhibit melatonin signaling during hibernation at an elevated temperature to test the hypothesis that decreased melatonin is sufficient to activate the reproductive axis in response to low-temperature dormancy. We used the melatonin receptor antagonist luzindole to inhibit melatonin signaling; luzindole is a well-characterized and widely used nonselective antagonist for the melatonin type 1a and 1b receptors found in all vertebrates [Dubocovich, 1988; Dubocovich et al., 1990; Dubocovich et al., 2003; Boutin et al., 2005]. Animals hibernated at 12°C were treated with 2.0 mg of luzindole (N-Acetyl-2-benzyl-tryptamine, product number Q1885, Bachem Americas, Inc.) diluted in 50 μ L of 10% dimethyl sulfoxide (DMSO) in 80% ethanol. The dose approximated by these injections was 50 μ g/g body mass (males average 40 g) and is similar to doses used previously in both mammals and ectotherms [Dubocovich et al., 1990; Pinillos et al., 2001]. Control treatments consisted of the vehicle only (i.e., 50 μ L of 10% DMSO in 80% ethanol). Treatments were administered via intraperitoneal injection every 3 days for the entire hibernation period.

Despite several pilot studies conducted in our lab to validate the luzindole treatments and its effects on male courtship behavior, snakes in this experiment had an unexpected and negative response to prolonged, repeated injections of luzindole during dormancy. We observed increased mortality 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

Fig. 2. Experimental design for testing if melatonin is necessary and sufficient for mediating the effects of low-temperature dormancy on the neuroendocrine reproductive axis of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Animals were collected from the field and assigned to one of five groups: a pre-hibernation group to establish a fall baseline of immunoreactivity, hibernation at 4°C for 6 weeks and treatment with vehicle or the melatonin precursor 5-hydroxytryptophan (5HTP), or hibernation at 12°C

for 6 weeks and treatment with vehicle or the melatonin receptor antagonist luzindole. Within each hibernation treatment group, snakes were further divided into two subgroups to determine the influence of treatment on immunoreactive cell number in the brain ($n = 12$) or courtship behavior ($n = 18$). Spring emergence was simulated with spring-like environmental conditions (16:8 h L:D photoperiod and 25:20°C thermoperiod). Courtship behavior was assessed during weeks 6–10 of the experiment.

rate in the respective control group was 3.3%. Luzindole is not known to be cytotoxic in organismal studies, and, therefore, the cause of this increased mortality remains unknown. Although we had planned to retain snakes in hibernation for 8 weeks prior to measuring the effects of these treatments on neuropeptides 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. Importantly, our revised sampling time is still within the critical window of 3–8 weeks of low-temperature exposure when significant changes in behavior and hormones are observed [e.g., Garstka et al., 1982]. 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 μ L of 1% Brevital sodium 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 3 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- μ m sagittal sections that were thaw-mounted onto subbed slides (Superfrost Plus, ThermoFisher Scientific, Inc., Pittsburgh, PA, USA). Slides were stored at –20°C prior to immunohistochemical staining.

Immunohistochemistry – Primary Antibodies and Control Tests

We examined the effects of melatonin treatment on TSH within the infundibulum of the pituitary gland as well as thyroid hormone metabolism and GnRH in the hypothalamus using immunohistochemistry. To assess changes in hypothalamic thyroid hormone metabolism, we performed immunohistochemistry for the Dio3 peptide; Dio3 enzymatically inactivates T_3 by converting it to reverse T_3 and/or diiodothyronine (T_2). The expression of Dio3 mRNA is significantly decreased in the hypothalamus during photoperiod-induced activation of the reproductive axis [e.g., Yasuo et al., 2005], which in turn favors the accumulation of bioactive T_3 . For logistical reasons, we were not able to assess possible changes in the Dio2 peptide in this experiment; Dio2 enzymatically converts T_4 into T_3 .

TSH immunoreactivity was examined using a polyclonal anti-garter snake TSH antiserum generated in rabbit (PAC 12291-92). Similarly, Dio3 immunoreactivity was examined using a rabbit polyclonal, anti-garter snake Dio3 antiserum (PAC 59160). These antibodies were custom generated by Pacific Immunology, Inc., against the amino acid sequences for the β subunit of TSH (TSH β) and Dio3 predicted from the cDNA sequences we previously isolated from garter snake tissues (brain and pituitary). Primers used for PCR amplification and resulting cDNA sequences are shown in Table 2. GnRH immunoreactivity was examined using a commercially available, polyclonal rabbit anti-GnRH1 antiserum (PA1-122 ThermoFisher Scientific, Inc.).

To validate the specificity of each primary antibody in our assays, we first performed a series of immunohistochemistry controls using omission of the primary antibodies and pre-adsorption tests. Prior to immunohistochemistry, rabbit anti-TSH and rabbit

Table 2. Primers used for PCR and the resulting cDNA sequences isolated from the combined brain and pituitary tissues of red-sided garter snakes (*Thamnophis sirtalis parietalis*)

Gene	Forward and reverse primers, 5' to 3' (degenerate primers contain mixed bases)		Base pairs in isolated cDNA	% sequence identity (accession No.)
TSH β	F:	AAGACTGTGCAGCCAAGAGACTGA	402	96% with brown tree snake (AB575987.1)
	R:	ACATTTGCAACTCACTGCCACAGG		
Dio3	F:	GGSCAGAARYTGGAYTTYTTCAAG	267	86% with chicken (Y11273.1)
	R:	TGACCCAGCCGTCGGADGGGTG		
GnRH	F:	CTGAANGGNGTNGANATGGANGGNAG	1,221	96% with leopard gecko (DQ269480.1)
	R:	GNAANCCNTAGGACCACTGCTG		

The above cDNA sequences were used to predict amino acid sequences for the β subunit of thyroid-stimulating hormone (TSH β), deiodinase 3 (Dio3), and gonadotropin-releasing hormone (GnRH) peptides. These were in turn used to generate custom antibodies targeting garter snake peptides (TSH and Dio3) or corroborate results of immunoreactive staining with commercially available antiserum (GnRH). Because the mature GnRH peptide contains only 10 amino acids, forward and reverse primers were generated using the conserved synteny relationship between the genes encoding GnRH1 and potassium channel tetramerization domain containing 9 (KCTD9) as in Ikemoto and Park [2007]. The resulting amplified cDNA sequence in garter snake brain was 1,221 base pairs, which contains a 27-base pair sequence (CAGCACTGGTCTACGGGTACAAGGG) for which the % sequence identity is reported above.

anti-Dio3 antisera were diluted 1:1,000 in buffer (see next section) and incubated overnight at 4°C with 1 mg of TSH β or Dio3 peptide per mL of antibody solution, respectively. The peptides used for these pre-adsorption tests were synthesized by Pacific Immunology, Inc., and used as the inoculating antigens for antibody production. Pre-adsorption tests for the GnRH1 antiserum are described in Lutterschmidt et al. [2022] and the results corroborated by the cDNA sequence for GnRH isolated from red-sided garter snakes (Table 2). All tissues used in antibody omission and pre-adsorption tests were independent from the animals used in the hibernation experiments, and all tissues were processed in the same assay.

Immunohistochemistry Assays

For each peptide, one series of tissues was 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 45 min and outlined with a hydrophobic barrier (Liquid Blocker – Super Pap Pen; Electron Microscopy Sciences, Hatfield, PA, USA). Slides were then incubated for 5 min in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). We washed the slide-mounted tissues 3 times for 5 min 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 min and endogenous peroxidase activity quenched with 3% hydrogen peroxide (H₂O₂) in methanol for 30 min. Slides were washed 2 times for 5 min each with 0.1 M PBS followed by 1 time for 5 min in 0.3% Triton X solution in 0.1 M PBS (PBS-T). We incubated the slides for 60 min with a blocking solution of 10% horse serum (item H1270, Sigma-Aldrich Co.) and 10% avidin (item SP-2001, Vector Labs, Burlingame, CA, USA) to reduce nonspecific binding. Tissues were then incubated with primary antibody at a dilution of 1:1,000 in 10% horse serum and 10% biotin (item SP-2001, Vector

Labs) in PBS-T. Sections were coverslipped with parafilm and allowed to incubate with the primary antibody for 48 h at 4°C in a humid chamber.

The primary antibody signal was amplified via incubation with biotinylated horse anti-rabbit secondary antibody (item BA-1100, Vector Labs) diluted 1:400 in PBS-T for 60 min. Tissues were then incubated with avidin conjugated to horseradish peroxidase (Elite ABC peroxidase kit, item PK-6100, 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, UT, USA) diluted in 0.2% H₂O₂ in 0.05 M Tris-HCL buffer (pH 7.2). The reaction was terminated by immersing the slides in 3 washes of nanopure H₂O for 5 min each. Tissues were dehydrated using a series of graded ethanol washes (70, 85, 95, 100, and 100%) for 2 min each, cleared with Citrasolv (Fisher Scientific), and then sealed with Permount and glass coverslips.

Immunoreactive Cell Counting

Immunoreactive cell counting of stained tissues was performed using an Olympus BX40 microscope with a QIClick digital camera and QImaging software (QImaging; Surrey, BC, Canada). As in many neurobiology studies, we interpreted an increase in the number of immunoreactive cells as an indication that a greater number of cells within a given cell population were producing sufficient amounts of peptide to reach the threshold of detection for immunohistochemistry. While an increase in the number of immunoreactive cells suggests that more cells within the cell population are actively synthesizing and/or accumulating the target peptide, labeling with diaminobenzidine cannot be used to directly assess the concentration of peptide either across the cell population or within individual cells.

The locations of immunoreactive cells were mapped onto sagittal sections using anatomical brain sections adapted from Krohmer et al. [2010] and Maine et al [2014]. Animals were coded so that the observer was blind to the treatment group of individuals. For

Table 3. Ethogram of courtship behavior for male red-sided garter snakes (*Thamnophis sirtalis parietalis*)

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

Behaviors equal to and above 2 are exhibited only in a reproductive context. This ethogram using whole numbers was taken from Lutterschmidt et al. [2004]; for logistical reasons in recording scores of many snakes in multiple mating trials, it was modified from the half-point scale ranging from 0–3 used in Crews et al. [1984]. An additional modification was made by combining the behaviors of caudocephalic waves and tail wrestling with possible cloacal apposition into one score, because these behaviors typically occur in rapid succession or simultaneously.

each peptide of interest, immunoreactive cells were counted and quantified manually in all sections containing the hypothalamus and pituitary region under $\times 200$ magnification and again at $\times 400$ magnification. If the counts were not identical, the section was recounted under $\times 400$ magnification to verify cell counts. We followed counting methods and criteria described by Lutterschmidt and Wilczynski [2012]. The number of immunoreactive cells was quantified in one tissue series and then totaled for each individual. Missing and/or severely damaged sections were assigned the mean cell count of the previous and subsequent tissue section. If two or more consecutive sections were unusable, the animal was excluded from statistical analysis. Because each brain was divided into 4 different series of 25- μm sections, approximately 100 μm separated each section within a series, thereby eliminating the possibility that labeled cells were double counted and our cell counts were inflated.

Courtship Behavior

Following 6 weeks of hibernation in combination with hormone manipulations, animals were subjected to simulated spring emergence and housed under spring-like environmental conditions (Table 1). Using an ethogram of male courtship behavior (Table 3), we measured the courtship behavior of each male on days 1, 3, 7, 10, 14, and 21 after 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 5 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 un-

attractive and unreceptive after mating, we covered each female's cloaca with medical adhesive tape to prevent mating during the courtship trial [e.g., Lutterschmidt et al., 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 after emergence. At the conclusion of the behavior experiments, animals were maintained in the lab until they were returned to their site of capture and released.

Statistics

We used SigmaPlot 12.5 (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 varied significantly between treatment groups. For each peptide, we used a t test to compare the number of immunoreactive cells during the fall pre-hibernation period (i.e., baseline) to (1) the number of labeled cells following 6 weeks of dormancy with vehicle treatment and (2) the number of labeled cells following 6 weeks of dormancy with 5HTP or luzindole treatment. This allowed us to determine if there was a significant change in the immunoreactive cell number of animals after 6 weeks of dormancy with and without hormone manipulation.

To assess the effects of hibernation temperature and hormone treatments on behavior, 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. The within-subjects factor was days post-emergence 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.

Results

Omission of each primary antibody from the immunohistochemistry assay eliminated immunoreactive staining. Control tests with each primary antiserum pre-adsorbed with the respective peptide also eliminated all immunoreactive staining (Fig. 3; see also [Lutterschmidt et al., 2022]).

In addition to positive TSH β staining in the anterior pituitary gland, we observed a distinct population of TSH-immunoreactive (ir) cells located within the median eminence and infundibulum of the pituitary gland of all snakes (Fig. 3); we refer to this cell population collectively as the infundibulum from this point onward. Although few in number, a small cluster of TSH-ir cells was also consistently observed in the caudal aspect of the ventral hypothalamus, located adjacent to the most ventral regions of the third ventricle within the mediobasal hypothalamus. There is precedence for TSH β being localized outside of the pituitary [Joseph and Knigge, 1974]. More-

Fig. 3. Localization of thyroid-stimulating hormone (TSH) and deiodinase 3 (Dio3) immunoreactivity in the brain of male red-sided garter snakes (*Thamnophis sirtalis parietalis*). **a** Representative schematic showing the anatomical relationship between the hypothalamus and pituitary gland in garter snakes in the sagittal plane (adapted from Schreibman [1986]). The third ventricle is shaded in dark gray with hashed lines; both zones of the pars distalis and the pars intermedia are shaded in light gray. **b** Example photomicrograph showing the distribution of TSH-labeled cells in the median eminence, infundibulum, and anterior pituitary gland. Boxes delineate magnified spaces in the infundibulum (**c**) and anterior pituitary (**d**). **e, f** Example photomicrographs of TSH immunohisto-

chemistry in the infundibulum and anterior pituitary, respectively, following control tests with pre-adsorbed antiserum. **g–i** Images of the median eminence, infundibulum, and pituitary gland counter-stained with cresyl violet acetate. **j** Example photomicrograph showing the distribution of Dio3-labeled cells in the median eminence, infundibulum, and ventral hypothalamus; box delineates magnified space in **k**. **l** Example photomicrograph of Dio3 immunohistochemistry following pre-adsorption tests. The rostral brain is oriented to the left in all sections; all scale bars are 50 μ m. cPD, caudal pars distalis; Hyp, hypothalamus; Inf, infundibulum; ME, median eminence; PI, pars intermedia; PN, pars nervosa; rPD, rostral pars distalis; Sph, sphenoid bone; III, third ventricle.

over, positive immunoreactivity does not require that the peptide target has been synthesized within that cell [e.g., Schreibman, 1986]. Given the proximity of TSH β -ir cells to the third ventricle within the mediobasal hypothalamus, it is possible that these are the retrograde cells that

respond to (and possibly internalize) TSH β released from the infundibulum. Based on our hypothesis that TSH-positive cells in the infundibulum mediate the effects of melatonin on seasonal reproduction, as is the case for TSH cells within the pars tuberalis of mammals, we fo-

Table 4. Summary of results examining the influence of melatonin signaling on the number of cells immunoreactive (ir) for thyroid-stimulating hormone (TSH), deiodinase 3 (Dio3), or gonadotropin-releasing hormone (GnRH) in red-sided garter snakes (*Thamnophis sirtalis parietalis*)

Treatment group	TSH-ir cells		Dio3-ir cells		GnRH-ir cells	
	Mean ($\pm$ SEM)	<i>p</i> value	Mean ($\pm$ SEM)	<i>p</i> value	Mean ($\pm$ SEM)	<i>p</i> value
Pre-hibernation	134.7 $\pm$ 16.50	–	864.1 $\pm$ 61.15	–	50.6 $\pm$ 11.81	–
4°C + vehicle	83.7 $\pm$ 19.73	0.050	794.1 $\pm$ 95.59	0.268	45.4 $\pm$ 11.17	0.378
4°C + 5-HTP	132.3 $\pm$ 19.31	0.941	1,020.1 $\pm$ 92.83	0.084	72.7 $\pm$ 20.20	0.168
12°C + vehicle	162.1 $\pm$ 25.74	0.196	828.8 $\pm$ 103.22	0.382	50.0 $\pm$ 10.43	0.486
12°C + luzindole	162.1 $\pm$ 25.75	0.201	838.1 $\pm$ 59.81	0.388	108.7 $\pm$ 33.57	0.040

A pre-hibernation group was used to establish a fall baseline level of immunoreactive cells prior to manipulating temperature and melatonin. We predicted that 6 weeks of dormancy at 4°C would increase TSH-ir, decrease Dio3-ir, and increase GnRH-ir, and that treatment with the melatonin precursor 5-hydroxytryptophan (5-HTP) to elevate melatonin concentrations would reverse these effects. Similarly, we predicted that 6 weeks of dormancy at an elevated temperature of 12°C would result in no changes in immunoreactive staining, but simultaneous inhibition of melatonin signaling via treatment with the melatonin receptor antagonist luzindole would increase TSH-ir, decrease Dio3-ir, and increase GnRH-ir. All *p* values are from *t* tests comparing each treatment to the pre-hibernation baseline.

cused our analysis of TSH immunoreactivity in this experiment on cells localized within the infundibulum of the pituitary gland.

Immunoreactive Dio3 cells were observed within the median eminence, infundibulum of the pituitary gland, and ventral hypothalamus, from the rostral aspect of the median eminence through the caudal mediobasal hypothalamus (Fig. 3). Based on the hypothesis that changes in thyroid hormone metabolism specifically within the hypothalamus mediate the effects of temperature on the reproductive axis, we excluded cells in the infundibulum from our Dio3 cell counts, instead choosing to focus our analyses on changes in hypothalamic Dio3 immunoreactivity. Moreover, our preliminary analyses comparing changes in Dio3-ir cells between the hypothalamus and infundibulum indicated that seasonal changes in Dio3 were driven by changes specifically within the hypothalamus, with no significant changes observed within the infundibulum (not shown).

Finally, similar to Smith et al. [1997] and as reported in Lutterschmidt et al. [2022], we observed GnRH-ir cell bodies and/or fibers in the olfactory bulb, dorsal pallium of the rostral telencephalon, medial forebrain bundle, terminal nerve, lateral and medial septum, diagonal band of Broca, amygdala, medial and lateral preoptic area, supra-chiasmatic nuclei, hypothalamus in the caudal forebrain, and median eminence. We quantified GnRH-ir cell bodies in the terminal nerve ganglion, nucleus of the diagonal band of Broca, septum, and medial preoptic area.

Influence of Melatonin Signaling on Neuropeptide Immunoreactivity

Our immunohistochemistry results are summarized in Table 4. In contrast to our prediction, we observed a significant decrease in the number of TSH-labeled cells in the infundibulum of male snakes after 6 weeks of low-temperature dormancy at 4°C ($t = 2.091$, $df = 18$, $p = 0.050$; Fig. 4a). Treatment of snakes with the melatonin precursor 5-hydroxytryptophan eliminated the decrease in TSH-ir cells in response to 6 weeks of hibernation at 4°C ($t = 0.0746$, $df = 21$, $p = 0.941$; Fig. 4a). Neither Dio3-ir ($t = 0.628$, $df = 21$, $p = 0.269$; Fig. 4b) nor GnRH-ir ($t = 0.316$, $df = 15$, $p = 0.378$; Fig. 4c) cell number was altered by 6 weeks of low-temperature dormancy at 4°C. Treatment of snakes with 5-hydroxytryptophan during low-temperature dormancy did not significantly alter the number of cells labeled for Dio3 ($t = -1.426$, $df = 21$, $p = 0.084$; Fig. 4b) or GnRH ($t = -0.998$, $df = 14$, $p = 0.168$; Fig. 4c) within the hypothalamus.

There was no significant change in the number of TSH-labeled cells in the infundibulum of male snakes after 6 weeks of dormancy at an elevated temperature of 12°C ($t = -0.875$, $df = 19$, $p = 0.196$; Fig. 5a). Likewise, neither Dio3-ir ($t = 0.306$, $df = 20$, $p = 0.382$; Fig. 5b) nor GnRH-ir ($t = 0.035$, $df = 16$, $p = 0.486$; Fig. 5c) cell number in the hypothalamus was altered in response to 6 weeks of dormancy at 12°C. Treatment of snakes with the melatonin receptor antagonist luzindole did not alter the observed responses of TSH ($t = -0.860$, $df = 16$, $p = 0.201$;

Fig. 4. Effect of low-temperature winter dormancy on the number of cells immunoreactive (ir) for thyroid-stimulating hormone (TSH) (a) in the median eminence and infundibulum, deiodinase 3 (Dio3) (b) in the ventral hypothalamus, and gonadotropin-releasing hormone (GnRH) (c) in the preoptic area 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 fall, pre-hibernation baseline. Snakes were hibernated at an ecologically relevant temperature of 4°C for 6 weeks in complete darkness; snakes received intraperitoneal injections of vehicle or 5-hydroxytryptophan (5-HTP) every 3 days throughout the dormancy period. 5-HTP is a precursor for melatonin synthesis and is known to increase plasma melatonin concentrations in garter snakes [Lutterschmidt and Mason, 2010]. Data are the means + 1 SE; sample sizes are listed along the x-axes.

Fig. 5a) or Dio3 ($t = 0.290$, $df = 18$, $p = 0.388$; Fig. 5b) immunoreactivity to dormancy at 12°C. However, the number of GnRH-ir cells was significantly increased in the brain of luzindole-treated males ($t = -1.898$, $df = 13$, $p = 0.040$; Fig. 5c).

Influence of Melatonin Signaling on Courtship Behavior

For males hibernated at 4°C for 6 weeks, the main effects of 5-hydroxytryptophan versus vehicle treatment on mean courtship score were statistically nonsignificant ($F_{1,198} = 2.019$, $p = 0.165$, from a two-way repeated mea-

Fig. 5. Effect of elevated hibernation temperature (12°C) on the number of cells immunoreactive (ir) for thyroid-stimulating hormone (TSH) (**a**) in the median eminence and infundibulum, deiodinase 3 (Dio3) (**b**) in the ventral hypothalamus, and gonadotropin-releasing hormone (GnRH) (**c**) in the preoptic area 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 fall, pre-hibernation baseline. Snakes were hibernated at an elevated temperature of 12°C for 6 weeks in complete darkness; snakes received intraperitoneal injections of vehicle or luzindole, a melatonin receptor type 1a and 1b antagonist, every 3 days throughout the dormancy period. Data are the means $\pm$ 1 SE; sample sizes are listed along the x-axes.

sures ANOVA; Fig. 6a). However, mean courtship score varied significantly with days post-emergence ($F_{6,198} = 9.010$, $p < 0.001$; Fig. 6a). The interaction between treatment and days post-emergence was statistically nonsignificant ($F_{6,198} = 1.605$, $p = 0.147$).

To assess the effects of 5-hydroxytryptophan 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

Fig. 6. Influence of vehicle ($n = 17$) and 5-hydroxytryptophan (5-HTP, $n = 18$) treatment on the mean (a) and maximum (b) 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). Data are the means $\pm$ 1 SE.

courtship score were statistically nonsignificant ($F_{1,198} = 1.456$, $p = 0.236$), we observed a significant change in maximum courtship score over days post-emergence ($F_{6,198} = 5.989$, $p < 0.001$; Fig. 6b). In addition, the interaction between treatment and days post-emergence was statistically significant ($F_{6,198} = 2.898$, $p = 0.010$), indicating that the change in courtship intensity over time depended on treatment condition (Fig. 6b).

Fig. 7. Effect of treatment with vehicle ($n = 18$) and the melatonin receptor antagonist luzindole ($n = 9$) on the mean (a) and maximum (b) courtship scores achieved by male red-sided garter snakes (*Thamnophis sirtalis parietalis*) following emergence from 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 $\pm$ 1 SE.

For males hibernated at 12°C for 6 weeks, the main effects of luzindole versus vehicle treatment on mean courtship score were statistically nonsignificant ($F_{1,150} = 0.0166$, $p = 0.899$, from a two-way repeated-measures ANOVA; Fig. 7a). However, mean courtship score varied significantly with days post-emergence ($F_{6,150} = 8.048$, $p < 0.001$). The interaction between treatment and days post-emergence was statistically nonsignificant ($F_{6,150} = 0.702$, $p = 0.648$).

Similarly, while the main effects of luzindole treatment on courtship intensity (i.e., maximum courtship score) were statistically nonsignificant ($F_{1,150} = 0.059$, $p = 0.809$; Fig. 7b), we observed a significant change in maximum courtship score over days post-emergence ($F_{6,150} = 9.337$, $p < 0.001$; Fig. 7b). The interaction between treatment and days post-emergence was statistically nonsignificant ($F_{6,150} = 1.510$, $p = 0.179$).

Discussion

It is now well established that photoperiod-induced changes in thyroid hormone metabolism within the hypothalamus mediate seasonal changes in the neuroendocrine reproductive axis (reviewed by [Nakane and Yoshimura, 2019]). Given the role of GnRH neurons in integrating multiple inputs with reproductive effort [e.g., Godwin, 2010; Stevenson et al., 2012; Maruska, 2014], we asked if environmental temperature also modulates hypothalamic thyroid hormone metabolism. How temperature cues are integrated with photoperiod information is relevant to all animals, but the impacts of temperature on seasonal reproduction are particularly critical to ectotherms. In red-sided garter snakes, for example, exposure to low temperatures (4°C) for at least 3–8 weeks is required to increase the expression of reproductive behavior (reviewed in [Lutterschmidt, 2012]). In a previous study, we found that exposure to low-temperature dormancy for 8–16 weeks increased both the number and size (a proxy for cell activity) of GnRH-ir neurons in male snakes [Lutterschmidt et al., 2022]. In this study, 6 weeks of dormancy at 4°C decreased TSH-ir cell number in the infundibulum but did not significantly alter Dio3 or GnRH immunoreactivity. The apparent discrepancy in GnRH between these studies is likely due to the shorter time-course and/or repeated handling in the current study.

Because low-temperature dormancy increases GnRH-ir cell number in red-sided garter snakes, we predicted that TSH-ir cell number in the infundibulum would increase. In contrast to our prediction, we observed a significant decrease in the number of TSH-labeled cells. There are at least two possible explanations for these results. The first, related to a “release hypothesis,” is that temperature-induced increases in TSH release from cells results in a decrease in TSH peptide stored within those cells. This subsequently results in fewer cells reaching the threshold of detection in immunohistochemistry and fewer positively labeled cells. Alternatively, it is possible that the decrease in TSH-ir cell number is related to the “resetting” of the sensitivity of the

reproductive axis to temperature cues. For example, a decrease in GnRH release and synthesis has been reported in photorefractory birds (reviewed in [MacDougall-Shackleton et al., 2009]). Majumdar et al. [2015] additionally provide evidence that elevated Dio3 expression may sustain the photorefractory state in redheaded buntings. Red-sided garter snakes exhibit similar refractoriness to warm temperatures; that is, exposure to low-temperature conditions is necessary to elicit responsiveness to warm temperatures and reproductive behavior during the spring (reviewed in [Lutterschmidt, 2012]). The observed decrease in TSH-ir cell number during the relatively early stages of low-temperature dormancy may, therefore, be related to the release of snakes from temperature refractoriness. A longer time course of low-temperature dormancy is required to assess which, if either, of these hypotheses explains the observed changes in TSH-ir cell number.

Relative to photoperiod, very few studies have investigated if and how environmental temperature impacts the neuroendocrine pathway that regulates changes in hypothalamic thyroid hormone metabolism and subsequent reproductive activity. Despite seeing few temperature-induced changes in this study, our results align with those available thus far. More specifically, the sensitivity to and influence of temperature on pars tuberalis TSH and/or hypothalamic thyroid hormone metabolism varies among taxa. In common voles (*Microtus arvalis*), photoperiod and temperature interact to regulate Dio2/3 expression, with warm temperatures (21°C vs. 10°C) enhancing Dio2 expression and testosterone production during long days [Rosmalen et al., 2021]. However, pars tuberalis TSH expression was insensitive to temperature in voles. A similar enhancement in TSH, Dio2, and GnRH mRNA expression was observed in redheaded buntings (*Emberiza bruniceps*) housed under long-day conditions in combination with elevated temperatures (38°C vs. 22°C [Trivedi et al., 2019]). In the tropical spinefoot fish (*Siganus guttatus*), Dio2 expression in the mediobasal hypothalamus varied with the duration and phase of the photoperiod and decreased in response to melatonin treatment, but it was not significantly altered by water temperature [Wambiji et al., 2011]. Similar to known variation in the responses of both plants and animals to changing day length (e.g., differences in spring vs. fall breeders), how environmental temperature supplements other cues such as photoperiod, social stimuli, and food availability [e.g., Wambiji et al., 2011; Perfito et al., 2015] to regulate neural thyroid hormone metabolism likely varies with the ecology and life history of each species.

Interestingly, comparative histological analyses reveal that in some groups of reptiles, including snakes, the pituitary pars tuberalis is reduced or absent altogether [Schreibman, 1986; Chester-Jones et al., 1987]. Despite this fact, we observed immunoreactive staining for TSH within the median eminence and infundibulum and in close proximity to where one might expect to find a pars tuberalis if it existed. We do not suggest from our immunohistochemical and histological analyses that garter snakes possess a distinct or even partial pars tuberalis. However, our results do indicate that a population of cells capable of producing TSH and responding to both temperature and treatment with 5-hydroxytryptophan resides within the pituitary stalk. Whether these cells exhibit other attributes of pars tuberalis cells is unknown, and it would be valuable to compare these results to other reptiles that do possess a distinct pars tuberalis (e.g., turtles and crocodiles). Further studies are also needed to identify the phenotype(s) of TSH-ir and Dio3-ir cells (e.g., ependymal versus other glial cells or neurons) and confirm the cellular location of staining (e.g., cytoplasm vs. nucleus). Nevertheless, our findings in garter snakes are supported by those of Nakane et al. [2013], who used masu salmon (*Oncorhynchus masou masou*) to demonstrate that fish, which also do not possess an anatomically distinct pars tuberalis, still exhibit the same characteristics of the photoperiodic regulatory center observed in birds and mammals. Specifically, in fish, this center is localized to the coronet cells of the saccus vasculosus, which both express TSH β and deiodinases as well as respond to photoperiod information (but see also [Irachi et al., 2021]). Together, these results underscore the evolutionarily conserved nature of this pathway irrespective of whether a distinct pars tuberalis is present.

One question we sought to address in this research is whether the regulation of neural thyroid hormone metabolism in reptiles is more similar to birds or mammals. Although our data in garter snakes cannot provide a thorough test of this hypothesis (because we cannot assume that they represent the pattern found in other reptiles, including the ancestral reptiles that gave rise to birds), they do suggest that temperature-induced changes in TSH are sensitive to melatonin, as is the case in mammals. Male snakes treated with vehicle showed a significant decrease in TSH immunoreactivity within the infundibulum after 6 weeks of dormancy at 4°C; this effect was reversed in males treated with the melatonin precursor 5-hydroxytryptophan. Although not statistically significant, treatment with 5-hydroxytryptophan at 4°C also increased the number of Dio3-ir cells within the hypothala-

mus ($p = 0.084$); increased levels of Dio3 result in degradation of thyroid hormones and are associated with reproductive quiescence [Nakane and Yoshimura, 2019]. Previous studies in garter snakes demonstrated that intraperitoneal injection of 5-hydroxytryptophan increases melatonin synthesis and circulating plasma melatonin concentrations [Lutterschmidt and Mason, 2010]. Together, these results indicate that TSH-ir cell number within the infundibulum is sensitive to low-temperature dormancy, and they additionally suggest that temperature-induced changes in TSH are mediated by decreased melatonin concentrations. Evidence that type 1a melatonin receptors are co-expressed within TSH-labeled cells in the infundibulum would support this conclusion.

As predicted, male snakes undergoing dormancy at an elevated temperature of 12°C for 6 weeks and treated with vehicle showed no change in TSH-ir, Dio3-ir, or GnRH-ir cell number. Inhibition of melatonin signaling via treatment with the melatonin receptor antagonist luzindole did not alter TSH- or Dio3-ir cell number. These results suggest that at elevated dormancy temperatures, inhibition of melatonin signaling is not sufficient in itself to mimic the effects of low-temperature dormancy. However, snakes treated with luzindole at 12°C showed a significant increase in GnRH-ir cell number, suggesting that melatonin exerts an inhibitory effect on GnRH in garter snakes. However, these findings should be confirmed with additional studies, considering the increased mortality rate we observed among luzindole-treated snakes. Luzindole is a competitive melatonin receptor antagonist that has been shown to inhibit the effects of melatonin across a variety of species, including red-sided garter snakes [e.g., Dubocovich, 1988; Dubocovich et al., 1990; upubl. data]. While there is no indication in the literature or from our pilot studies that luzindole is cytotoxic, negative side effects of chronic exposure to luzindole at 12°C were apparent in this study. The negative impacts do not appear to result from a global increase in cellular processes, because luzindole treatment did not affect all of the variables included in this study.

Finally, we asked if observed changes in TSH, Dio3, and/or GnRH immunoreactivity are associated with changes in male courtship behavior. Several previous studies have documented the inhibitory effects of elevated melatonin, both endogenous and exogenous, on male courtship behavior (reviewed in [Lutterschmidt, 2012]). Further, male red-sided garter snakes hibernated at an elevated temperature (10°C) had significantly higher plasma melatonin concentrations and also showed a significant delay in the onset of maximum courtship behav-

ior [Lutterschmidt and Mason, 2009]. In the current study, both the mean and maximum courtship scores (measures of courtship endurance and intensity, respectively) of snakes maintained at 4°C and treated with 5-hydroxytryptophan declined significantly sooner after emergence compared to vehicle-treated snakes. These results suggest that the disruptive effects of elevated hibernation temperatures on courtship behavior are mediated through increased plasma melatonin concentrations, and these behavioral changes are concomitant with altered patterns of TSH immunoreactivity in the infundibulum. Of course, direct manipulations of TSH β are needed to discern if and how it modulates seasonal changes in reproductive behavior and/or physiology. 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. Thus, we suggest that decreases in 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. While the observed impacts on reproductive behavior may appear relatively minor, it should be noted that the mating season in northern populations of red-sided garter snakes is extremely attenuated, and individual snakes can typically only sustain mate searching and courtship behavior for 5–14 days in this intense, scramble competition mating system [e.g., Lutterschmidt and Mason, 2008, 2009; this study]. Thus, any reduction or temporal shift in courtship behavior, however small, could have a disproportionately large impact on mating opportunities and reproductive fitness.

In summary, we found that 6 weeks of low-temperature dormancy at 4°C significantly decreased TSH-ir cell number in the infundibulum of the pituitary gland. Treatment of snakes with the melatonin precursor 5-hydroxytryptophan during dormancy at 4°C both eliminated the temperature-induced change in TSH immunoreactivity and disrupted the temporal pattern of male courtship behavior. Treatment of snakes with the melatonin receptor antagonist luzindole was not sufficient in rescuing the effects of dormancy at an elevated temperature (12°C) on TSH immunoreactivity or courtship behavior. However, treatment with luzindole significantly increased GnRH-ir cell number within the hypothalamus of male snakes, providing evidence for an inhibitory effect of melatonin on GnRH peptide synthesis and/or release in vivo. Our results provide further evidence that the neuroendocrine pathway regulating photoperiod-induced changes in neural thyroid hormone metabolism is (1) modulated by en-

vironmental temperature; (2) sensitive to changes in the hormone melatonin in a reptile; and (3) conserved among vertebrates, even in the absence of a well-developed pituitary pars tuberalis. Together, these results provide critical insights into the mechanisms that mediate the effects of temperature on reproductive physiology and behavior, information that is increasingly more imperative for understanding the impacts of our changing climate.

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Statement of Ethics

Experimental protocols were approved by the Portland State University Animal Care and Use Committee (approval number 59) 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 performed under the authority of Wildlife Scientific Permit WB18801 issued by the Manitoba Department of Sustainable Development.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

D.I.L. and T.J.W. designed and conducted the experiments. T.J.W. and S.M. processed tissues and performed the immunohistochemistry assays. S.M., H.A., and N.D.P. quantified the immunoreactive cell data. D.I.L. and T.J.W. analyzed and interpreted the data and wrote the paper.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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