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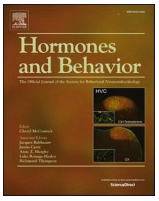

Neural thyroid hormone metabolism integrates seasonal changes in environmental temperature with the neuroendocrine reproductive axis

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ABSTRACT

We asked if environmental temperature alters thyroid hormone metabolism within the hypothalamus, thereby providing a neuroendocrine mechanism by which temperature could be integrated with photoperiod to regulate seasonal rhythms. We used immunohistochemistry to assess the effects of low-temperature winter dormancy at 4 °C or 12 °C on thyroid-stimulating hormone (TSH) within the infundibulum of the pituitary as well as deiodinase 2 (Dio2) and 3 (Dio3) within the hypothalamus of red-sided garter snakes (*Thamnophis sirtalis*). Both the duration and, in males, magnitude of low-temperature dormancy altered deiodinase immunoreactivity within the hypothalamus, increasing the area of Dio2-immunoreactivity in males and females and decreasing the number of Dio3-immunoreactive cells in males after 8–16 weeks. Reciprocal changes in Dio2/3 favor the accumulation of triiodothyronine within the hypothalamus. Whether TSH mediates these effects requires further study, as significant changes in TSH-immunoreactive cell number were not observed. Temporal changes in deiodinase immunoreactivity coincided with an increase in the proportion of males exhibiting courtship behavior as well as changes in the temporal pattern of courtship behavior after emergence. Our findings mirror those of previous studies, in which males require low-temperature exposure for at least 8 weeks before significant changes in gonadotropin-releasing hormone immunoreactivity and sex steroid hormones are observed. Collectively, these data provide evidence that the neuroendocrine pathway regulating the reproductive axis via thyroid hormone metabolism is capable of transducing temperature information. Because all vertebrates can potentially use temperature as a supplementary cue, these results are broadly applicable to understanding how environment–organism interactions mediate seasonally adaptive responses.

1. Introduction

In many vertebrates, the predominant environmental cue used to synchronize extrinsic and intrinsic processes is photoperiod. Extensive research effort has been devoted to understanding how changes in daylength modulate physiology and behavior, especially within the context of seasonal reproductive cycles. In addition to the hypothalamus-pituitary-gonad axis, it has been well established that the thyroid hormone axis plays an important regulatory role in reproductive processes in all vertebrates, including humans [e.g., see reviews in (Habibi et al., 2012; Karsch et al., 1995; Krassas et al., 2010; Leatherland, 1987) (Norris, 2023)]. For example, treatment with exogenous thyroxine mimics the effects of long days on the reproductive axis in birds [reviewed in (Dawson et al., 2001)]. In addition, thyroidectomy can prevent the development of photorefractoriness in some species and maintain elevated levels of gonadotropin-releasing hormone (GnRH) within the hypothalamus (Dawson et al., 2001; Nakao et al., 2008b). This and similar observations suggested thyroid hormones were exerting their effects directly within the brain, but the specific target(s) and mechanisms of thyroid hormone modulation were elusive.

Although many questions remain, a solid foundation now exists for understanding the molecular mechanisms underlying photoperiodism as well as the role of thyroid hormones in seasonal reproduction [e.g., see reviews in (Brzezinski et al., 2021; Dardente and Simonneaux, 2022; Helfer et al., 2019; Liddle et al., 2022; Nakane and Yoshimura, 2019; Pérez, 2022; Reiter and Sharma, 2021; Stevenson, 2017)]. In spring breeding mammals, increasing daylength decreases the duration of elevated melatonin concentrations (Fig. 1), and this in turn dis-inhibits the production of thyrotropin (or thyroid stimulating hormone, TSH) within the pitutary pars tuberalis that surrounds the hypophyseal stalk

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(Hanon et al., 2008; Nakao et al., 2008a; Unfried et al., 2009). TSH modifies the mediobasal hypothalamus via retrograde signaling by binding to its receptor expressed on tanycytes lining the ventral walls of the third ventricle (Ono et al., 2008; Unfried et al., 2009). TSH receptor binding induces localized changes in thyroid hormone metabolism within the tanycytes by modifying the expression of deiodinases (Duarte-Guterman et al., 2014; Lewis and Ebling, 2017; Nakao et al., 2008a; Nakao et al., 2008b; Watanabe et al., 2004; Yasuo et al., 2009). The type 2 deiodinase (Dio2) enzyme converts thyroxine (T_4) to the more bioactive triiodothyronine (T_3) hormone, whereas type 3 deiodinase (Dio3) inactivates T_3 via deiodination (Yasuo et al., 2005). Reciprocal changes in Dio2 and Dio3 enhance the local accumulation of T_3 within tanycytes, which may induce remodeling of the tanycyte endfeet that surround GnRH neurons to facilitate GnRH release and the activation of the reproductive axis (Yamamura et al., 2006; Yasuo et al., 2006). Additional evidence suggests that changes in thyroid hormone metabolism modulate both the expression of GnRH mRNA and the synthesis of GnRH peptide [e.g., reviewed in (Nakane and Yoshimura, 2019)], which facilitate the seasonal brain changes required for the expression of reproductive behavior (Sáenz de Miera et al., 2014).

Thus far, the sensitivity of this pathway to photoperiod cues has been studied in mammals, birds, and fish [e.g., (Nakane and Yoshimura, 2014)], and virtually identical changes in hypothalamic thyroid hormone metabolism, including its regulation by TSH, have been described in these vertebrates. Although many seasonal breeders use photoperiod as their primary environmental cue, it is likely that most animals use additional environmental cues, such as temperature, social signals, food availability, and precipitation, to supplement photoperiod and fine-tune their responses to environmental conditions [e.g., (Ball and Ketterson, 2008; Stevenson et al., 2022)]. Relatively little is known, however, about how supplementary cues are integrated with photoperiodic responses. One challenge to understanding the role of supplementary environmental cues in seasonal biology is identifying an animal system in which the impacts of the supplementary cue can be isolated and interrogated. It is notable that the majority of the animal models used thus far to examine the role of altered thyroid hormone metabolism in seasonal reproduction have been endotherms. While advantageous for understanding photoperiodism, most endotherms present challenges to investigating the effects of prolonged, seasonally-relevant exposure to environmental temperatures outside the thermal neutral zone without also evoking significant modifications to energy metabolism and energy balance. In this context, ectotherms provide an excellent opportunity for isolating the relationship between temperature, neural thyroid hormone metabolism, and seasonal reproduction.

In this study, we asked if environmental temperature alters thyroid hormone metabolism within the hypothalamus, thereby providing a neuroendocrine mechanism by which temperature information could be integrated with photoperiod to regulate seasonal rhythms. We used immunohistochemistry to assess the effects of prolonged low-temperature winter dormancy on TSH within the infundibulum of the

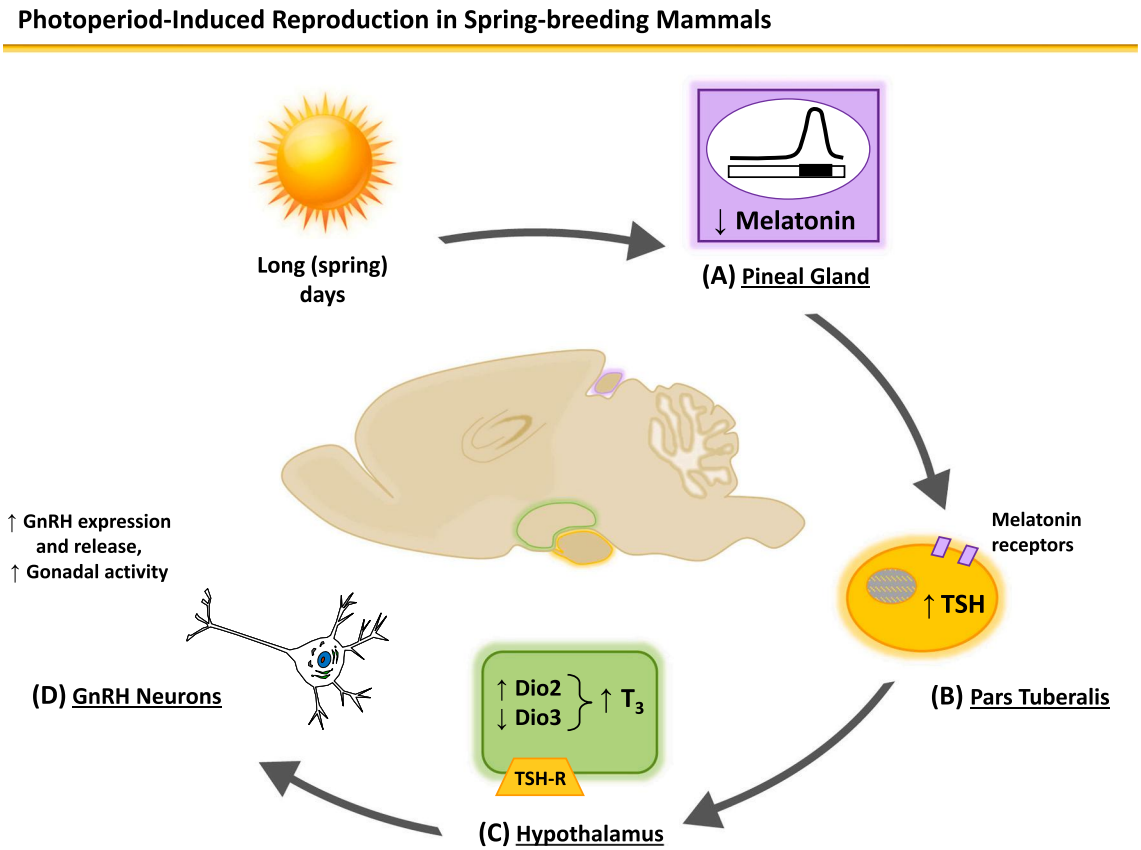

Fig. 1. Schematic showing the effects of seasonal changes in photoperiod on neural thyroid hormone metabolism and gonadotropin-releasing hormone (GnRH) in spring breeding mammals. During the spring, increasing daylength (A) decreases the duration of elevated melatonin synthesis by the pineal gland, (B) increases thyrotropin or thyroid stimulating hormone (TSH) within the pituitary pars tuberalis, (C) and alters local thyroid hormone metabolism within the mediobasal hypothalamus by modifying the expression of deiodinase enzymes (Dio2 and Dio3). The accumulation of triiodothyronine (T_3) within the hypothalamus is in turn linked to (D) increased expression and release of GnRH and activation of the reproductive axis. In red-sided garter snakes (*Thamnophis sirtalis parietalis*), low temperature winter dormancy similarly decreases melatonin concentrations and increases both GnRH immunoreactivity and courtship behavior (Lutterschmidt et al., 2022; Lutterschmidt and Mason, 2009). We therefore tested the hypothesis that low-temperature winter dormancy also alters thyroid hormone metabolism within the hypothalamus.
Figure modified from Winters et al. (2022).

pituitary gland as well as Dio2 and Dio3 within the median eminence and ventral hypothalamus of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Like many ectothermic vertebrates in temperate regions, red-sided garter snakes become refractory to warm temperatures during the spring and summer, and the seasonal re-activation of reproduction in subsequent years requires a period of low temperature exposure prior to the spring mating season (Duvall et al., 1982; Licht, 1972, 1984; Whittier et al., 1987b). Red-sided garter snakes are an exceptional comparative system for these studies because their seasonal sex behavior is regulated exclusively by temperature, with little photoperiod involvement during the transition from winter dormancy to spring emergence [reviewed in (Lutterschmidt, 2012)]. Here, we first highlight the results of previous studies demonstrating that temperature is a potent modulator of the endocrine and neuroendocrine pathways regulating seasonal reproduction.

Northern populations of red-sided garter snakes overwinter in underground dens for 8 months each year in complete darkness. Following emergence in late April through mid-May, males remain near the den searching for mates during the attenuated mating season, as females can emerge up to 3 weeks later than males (Crews and Garstka, 1982). Red-sided garter snakes exhibit a temporally dissociated reproductive pattern, in which peak mating behavior does not occur at the same time as maximal gonadal steroidogenesis or gametogenesis (e.g., Crews, 1984). As such, gonads are regressed and plasma concentrations of sex steroid hormones tend to be low during the mating season (Crews, 1984; Krohmer et al., 1987; Lutterschmidt and Mason, 2009; Moore et al., 2000; Moore and Mason, 2001; Whittier et al., 1987a). While these are hallmarks of a temporally dissociated reproductive pattern, current evidence indicates that both GnRH and sex steroid hormones are central to reproductive regulation in this species, but the timing of their action occurs during winter dormancy (Crews, 1991; Lutterschmidt, 2012). For example, in males, low temperature exposure both maintains elevated androgens throughout winter dormancy (Lutterschmidt and Mason, 2009) and increases the number of GnRH-immunoreactive cells in the preoptic area (Lutterschmidt et al., 2022). Further, aromatization of androgens during dormancy plays a critical role in activating male sex behavior (Krohmer, 2020; Krohmer et al., 2010). Thus, as in other vertebrates, GnRH and/or sex steroids play a role in priming the brain to respond to reproductive signals prior to mating activity.

Notably, previous studies examining the time-course of winter dormancy have established that a critical period of low-temperature exposure is necessary to elicit changes in hormones and behavior. For all parameters measured thus far, this critical period is between 4 and 8 weeks of low-temperature exposure. The effects of temperature on the neuroendocrine reproductive axis appear to be mediated, at least in part, by the pineal hormone melatonin, as low temperature exposure (4 °C versus 10 °C) during winter dormancy induced persistent changes in plasma melatonin concentrations of male red-sided garter snakes (Lutterschmidt and Mason, 2009). Most recently, Winters et al. (2022) manipulated melatonin signaling during winter dormancy using the synthesis precursor 5-hydroxytryptophan and the melatonin receptor antagonist luzindole. While their results suggest that both TSH in the infundibulum and GnRH in the hypothalamus are sensitive to melatonin, the observed changes depended on dormancy temperature (4 °C versus 12 °C), only one time point was examined (i.e., after 6 weeks of temperature ± melatonin manipulation), and Dio2 was not assessed. Thus, the timecourse of changes in TSH and/or Dio2 and Dio3 in response to prolonged low-temperature winter dormancy is unknown in any ectotherm.

We predicted that exposure to low-temperature winter dormancy for at least 8 weeks would increase TSH in the infundibulum of the pituitary gland and increase Dio2 and decrease Dio3 within the hypothalamus. We also hypothesized that temporal changes in TSH and/or deiodinase immunoreactivity would be associated with an increase in male courtship behavior. Given the sexually dimorphic responses of GnRH neurons observed previously (Lutterschmidt et al., 2022), we predicted that the temperature-induced responses of TSH and deiodinase would also vary between sexes.

2. Materials and methods

These experiments were conducted in the laboratory at Portland State University with red-sided garter snakes (*Thamnophis sirtalis parietalis*) collected from a den site in Inwood, Manitoba, Canada. All protocols were approved by the Institutional Animal Care and Use Committee and were in compliance with 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 Natural Resources and Northern Development.

2.1. Experimental design

A total of 212 male and 65 female snakes were collected from the den during the fall (16–18 Sept 2016), when snakes migrate back to the den site from summer feeding grounds in preparation for winter dormancy. Snakes arrived in the lab on 21 Sept 2016, where they were housed in groups of 4–6 females or 6–8 males in 40-liter aquaria within environmental chambers. Snakes were acclimatized to the photoperiod and temperature conditions shown in Table 1 during pre-hibernation (4.5 weeks), hibernation (16 weeks), and spring emergence (4 weeks). Water was provided ad libitum throughout the experiment. All snakes were clipped on the ventral scales with a unique number code to identify individuals throughout the experiments.

Animals were randomly assigned to one of two winter dormancy temperatures (4 °C or 12 °C) and hibernated in complete darkness (0:24 h L:D photoperiod) for up to 16 weeks. These temperature regimes were chosen based upon previous laboratory studies in this species [e.g., (Bona-Gallo and Licht, 1983; Krohmer and Crews, 1987; Lutterschmidt and Mason, 2008, 2009; Whittier et al., 1987b)] as well as recorded body temperatures of red-sided garter snakes during winter dormancy under natural field conditions, when body temperatures decrease to approximately 1 °C towards the end of hibernation (Lutterschmidt et al., 2006). The elevated temperature treatment, consisting of a constant temperature of 12 °C during winter 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). Snakes maintained at an elevated dormancy temperature of 10–12 °C exhibit disruptions in some, but not all, reproductive axis parameters, including plasma melatonin concentrations, sex steroid hormone metabolism, and patterns of male courtship behavior.

Table 1
Acclimatization regimes for investigating the influence of prolonged low-temperature winter dormancy on neural thyroid hormone metabolism in red-sided garter snakes *(Thamnophis sirtalis parietalis)*.

"Season"	Weeks in experiment	Dates	Acclimatization conditions (Photoperiod; Thermoperiod)
Fall pre-hibernation	−4.5 through −1.5	20 Sept – 10 Oct	11:13 h L:D; 18:12 °C
Fall pre-hibernation	−1.5 through 0	11 Oct – 20 Oct	11:13 h L:D; 15:12 °C
Hibernation	0 through 16	21 Oct – 11 Feb	0:24 h L:D; either 4 °C or 12 °C
Spring emergence	multiple	multiple	16:8 h L:D; 25:20 °C

2.1.1. Neural thyroid hormone metabolism

To establish a fall, pre-hibernation baseline for TSH and deiodinases within the hypothalamus, we euthanized a subset of 12 male and 8 female snakes at week 0 (i.e., on 20 Oct 2016), immediately prior to the induction of hibernation on 21 Oct 2016 and any temperature manipulation (Table 1). To determine the effect of dormancy temperature and duration on neural thyroid hormone metabolism, we euthanized a subset of 12 males within each temperature treatment after 4, 8, and 16 weeks in hibernation (n = total of 84 males for all sampling times). To reduce the number of female snakes euthanized for these studies, and because of a priori knowledge of sex differences in response to low temperature dormancy (Lutterschmidt et al., 2022), we decreased both the sample size and the number of sampling times for females. A subset of 8 females within each temperature treatment was euthanized after 8 and 16 weeks in hibernation (n = total of 40 females for all sampling times). Brains were collected and processed for immunohistochemistry as described below. We collected additional tissues from each snake for analyses not presented here.

2.1.2. Reproductive behavior

To determine if temperature-induced changes in thyroid hormone metabolism coincide with changes in male courtship behavior, we transferred a subset of 16 males in each temperature group to spring-like environmental conditions (Table 1) following low-temperature dormancy for 0, 4, 8, or 16 weeks ($n = 16 \times 2$ temperature treatments $\times$ 4 sampling times = total of 128 males for all behavior assays). Within each timepoint, 8 males were randomly assigned to each of 4 aquaria for courtship behavior assays. 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). Because male red-sided garter snakes exhibit facilitated courtship behavior, where the presence of a mating ball increases the probability of exhibiting courtship behavior, we ensured that males from each temperature group were equally distributed across all behavior arenas (i.e., each behavior arena contained 4 males from each of the 2 temperature treatments). Males in each cage received a unique colour marking on the rostral dorsal stripe using permanent markers; this allowed us to score courtship behavior without disturbing the males or having to read the ventral scale clips during the behavior assays. The observer was blind to the treatment group of each male.

Using an ethogram of male courtship behavior (Table 2), we measured the courtship behavior of each male 1, 3, 7, 10, 14, and 21 days after simulated spring 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. We covered each female's cloaca with medical adhesive tape to prevent mating during the courtship trial [e.g., (Lutterschmidt et al., 2004)], as female red-sided garter snakes become unattractive and unreceptive after mating. Thus, the highest courtship score each male could achieve was 4.0. We used the highest courtship score of each male during the 60 min courtship trial to categorize snakes as exhibiting courtship behavior or not on each day post-emergence. Because a courtship score of 2.0 (i.e., Male chin-rubs female with rapid tongue-flicks; Table 2) is exhibited only in the context of reproductive behavior, a male receiving a courtship score of 2.0 or higher during the 60 min behavior trial was characterized as exhibiting courtship behavior on that day post-emergence. We then calculated the proportion of males exhibiting courtship behavior on each day post-emergence within each temperature group for statistical analyses.

An additional 25 female snakes were collected from the field and served as stimulus females to assess male courtship behavior during the experiment. All stimulus females were hibernated at 4 °C in complete darkness for a minimum of 4 weeks prior to male courtship trials, as 4 weeks of low-temperature exposure facilitates estradiol-induced changes in the female sexual attractiveness pheromone (Lutterschmidt and LeMaster, unpublished data). At each sampling time of 0, 4, 8, and 16 weeks in hibernation, we transferred a randomly selected subset of 6 females to spring-like environmental conditions for use in behavior assays. To ensure all stimulus females were sexually attractive across all sampling time points, we treated stimulus females with 17β-estradiol (40 μg per 75 g body mass in 50 μl peanut oil) via daily intraperitoneal injection for 6 days prior to beginning male courtship trials (Crews, 1976, 1985). Distribution of 6 stimulus females among the 4 behavior arenas ensured that all males were exposed to novel, sexually attractive females throughout the spring emergence period (i.e., on each of day 1, 3, 7, 10, 14 and 21 post-emergence). Across all sampling times and behavioral assays, every stimulus female elicited courtship behavior from at least one male and every arena of male snakes exhibited courtship behavior. At the conclusion of the behavior experiments, males and stimulus females were maintained in the lab until they were returned to their site of capture and released.

Table 2
Ethogram of courtship behavior for male red-sided garter snakes *(Thamnophis sirtalis parietalis)*.

Courtship score	Description of behavior
0.0	No reproductive behavior
1.0	Male investigates female, increased tongue-flick rate
2.0	Male chin-rubs female with rapid tongue-flicks
3.0	Male aligns body with female
4.0	Male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves
5.0	Male copulates with female

Behaviors ≥2.0 are exhibited only in a reproductive context (ethogram modified from Crews et al. 1984).

2.2. Tissue collection and processing

Snakes were euthanized via injection with 300–500 μl of 1 % 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 16–18 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 intact and then decalcified by incubating tissues in 10 % ethylenediaminetetraacetic acid (EDTA) diluted in 0.1 M phosphate-buffer (pH 7.2) at 4 °C for three days. Tissues were transferred to fresh EDTA solution each day. The brain and decalcified ventral skull were then cryoprotected in 30 % sucrose in 0.1 M phosphate buffer and 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.

2.3. Immunohistochemistry

As in Winters et al. (2022), TSH immunoreactivity was examined using a polyclonal anti-garter snake TSH antiserum generated in rabbit (PAC 12291–92). Dio3 immunoreactivity was examined using a rabbit polyclonal, anti-garter snake Dio3 antiserum (PAC 59160). These antibodies were custom generated by Pacific Immunology, Inc. (Ramona, CA, USA) against the amino acid sequences for TSHβ 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 can be found in Winters et al. (2022). Dio2 immunoreactivity was examined using a commercially available, polyclonal goat anti-Dio2 antiserum (item ab-77481; Abcam, Cambridge, MA, USA).

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 preadsorption tests. Preadsorption tests for the rabbit anti-TSH and rabbit anti-Dio3 antisera are described

in Winters et al. (2022). To confirm the specificity of Dio2 staining, we incubated Dio2 antisera (diluted 1:2000) with 50 μg/ml synthetic Dio2 peptide (EVKKHQNQEDRC; item 131396, Abcam) overnight at 4 °C prior to immunohistochemistry. All tissues used in antibody omission and preadsorption tests were independent from the animals used in the hibernation experiments.

For each peptide, one series of tissue was processed in a single assay using the methods described in detail in Lutterschmidt and Maine (2014) and Winters et al. (2022). Briefly, slides containing the medial hypothalamus and pituitary were defrosted and dried on a slide warmer at 50 °C for a minimum of 30 min. Slides were incubated in 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) and then washed 3 × 5 min each with 0.1 M PBS. This washing procedure was completed after every step of the assay. Paraformaldehyde was neutralized with 0.1 % sodium borohydride (pH 8.5) and endogenous peroxidase activity quenched with 3 % hydrogen peroxide. Slides were washed in 0.3 % Triton X solution in 0.1 M PBS (PBS-T) and then incubated 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. Tissues were then incubated with primary antibody at a dilution of 1:1000 (TSH, Dio3) or 1:2000 (Dio2) 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 TSH and Dio3 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; the Dio2 antibody signal was amplified with biotinylated horse anti-goat secondary antibody (item BA-9500; Vector Labs). Tissues were then incubated with avidin conjugated to horseradish peroxidase (Elite ABC peroxidase kit, item PK-6100, Vector Labs). For Dio2 immunohistochemistry, the primary antibody signal was further amplified using 100 μl biotin-labeled tyramide (item SAT700, Perkin Elmer, Piscataway, NJ, USA) followed by a second incubation with avidin-conjugated horseradish peroxidase. Primary antibody binding was visualized with 0.25 mg/ml diaminobenzidine (item 0430-5G; BioExpress, Kaysville, Utah, USA) in 0.2 % hydrogen peroxide in 0.05 M Tris-HCL buffer (pH 7.2). The reaction was terminated by immersion in nanopure H_2O (3 × 5 min). Tissues were dehydrated in a series of graded ethanol washes, cleared with Citrasolv (Fisher Scientific), and sealed with permount and glass coverslips.

2.4. Quantification of immunoreactive staining

Analysis of immunoreactive staining was performed using an Olympus BX40 microscope with a QImaging QIClick digital camera and Qcapture software (QImaging; Surrey, B.C., Canada). 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. 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 cell counts inflated.

As in Winters et al. (2022), TSH-immunoreactive cells were counted manually in all sections containing the infundibulum and median eminence of the hypothalamus under x200 magnification and again at x400 magnification. If the counts were not identical the section was recounted under x400 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 analyses.

Due to the pattern of dense but diffuse Dio2 immunoreactive staining within the anterior and mediobasal hypothalamus, it was difficult to consistently distinguish individually labeled cells via manual cell counting methods. We therefore utilized FIJI Software [ImageJ; (Schindelin et al., 2012)] to quantify Dio2 immunoreactive staining. For each individual snake, we first identified the sagittal midline, marked by the simultaneous presence of the third ventricle, optic chiasm, pineal gland and intact infundibulum. Within the hypothalamus, Dio2-labeling is concentrated around the lateral margins of the third ventricle. We collected three images side by side at 400× magnification to capture Dio2 staining around the third ventricle at the midline (Fig. 2). Each image was captured with QCapture's Image Averaging function using three z-positions to reduce random noise and increase dynamic range. There was no tissue overlap between or among the captured images, and the size of each imaged area was fixed and held constant for all animals. For all images, the microscope manual brightness setting was held constant and the settings on QCapture were: White Balance $R = 1.0$, B = 1.5; Gain = 1; Gamma = 1.350; Offset = 0. Image analysis of Dio2-immunoreactive staining was then performed using FIJI software to create a mask using image thresholding (lower threshold level = 220, upper threshold level = 237) and particle analysis (size = 92–740 pixels, circularity = 0.76–1.00) to determine the total area of Dio2 immunoreactive staining (in pixels) within the imaged area for each individual. Cells cut off by the border of the image frame were not counted to avoid double-counting of cells.

Similar to Winters et al. (2022), the number of Dio3-labeled cells was quantified manually in the median eminence, rostral infundibulum, and ventral hypothalamus, from the rostral aspect of the median eminence through the caudal mediobasal hypothalamus. Studies in photoperiodic mammals and birds demonstrate that changes in Dio2 and Dio3 mRNA expression occur within the same regions (and in some studies, the same cells) lining the third ventricle of the hypothalamus [e.g., (Yasuo et al., 2005)]. To mirror our analysis of Dio2-immunoreactive staining and increase the likelihood of detecting reciprocal changes in Dio2 and Dio3 within the same brain regions, we quantified the total number of Dio3-immunoreative cells in the same sagittal midline section used to quantify Dio2-immunoreactive staining. For each snake, the sagittal midline was identified by the simultaneous presence of the third ventricle, optic chiasm, pineal gland and intact infundibulum.

2.5. Statistics

We used a two-way analysis of variance (ANOVA) within each sex to confirm that there were no differences in the initial body mass or snout-vent-length of animals between temperature groups or among sampling times. We also investigated if body mass loss during winter dormancy varied significantly with temperature or sampling time using a two-way ANOVA within each sex.

For each peptide of interest, we used a two-way ANOVA within each sex to determine if immunoreactive staining changes during winter dormancy; temperature and weeks in hibernation (i.e., sampling time) were included in the analyses as between-subjects factors. To assess the effects of hibernation duration and temperature on patterns of reproductive behavior, we used a three-way ANOVA on the proportion of males courting with hibernation duration, temperature, and days post-emergence as between-subjects factors. Because the main effect of hibernation temperature was not statistically significant in this analysis, we collapsed the temperature groups and reanalyzed the data using a two-way ANOVA with weeks in hibernation and days post-emergence as factors.

We used SigmaPlot 14.5 (Systat Software 2022, Inpixon, Palo Alto, CA, USA) to perform all statistical analyses. Data were transformed where necessary using square root to meet the assumptions of parametric analysis. Statistical comparisons were considered significant at $p \leq 0.05$ and effect sizes are reported as η^2 (SS_{factor}/SS_{total}). Unless otherwise noted, significant main effects identified by multifactor

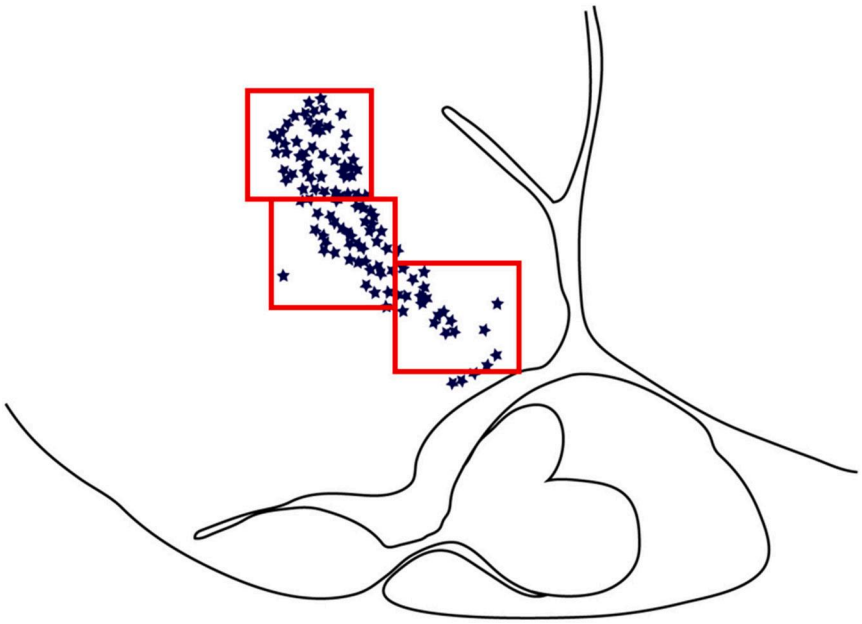

Fig. 2. Schematic showing the placement of captured images around the third ventricle to quantify the area of deiodinase 2 (Dio2)-immunoreactive staining within the sagittal midline of the hypothalamus in red-sided garter snakes (*Thamnophis sirtalis parietalis*). There was no tissue overlap between or among the three captured images, and the size of each imaged area was fixed and held constant for all animals. Image analysis of Dio2-immunoreactive staining was performed using FIJI software to determine the total area of Dio2 immunoreactive staining (in pixels) within the imaged area for each individual. Cells cut off by the border of the image frame were not counted to avoid double-counting of cells.

ANOVAs were further investigated using an all-pairwise Student-Newman-Keuls multiple comparisons test, which uses step-wise comparisons to detect differences between groups.

3. Results

Initial morphometric data of male and female snakes and changes during hibernation are listed in Table 3. There were no differences in the initial body mass or snout-vent length of animals among sampling times or between temperature treatments for either males (all p values $\geq$0.203 and $\eta^2 \leq 0.057$ for body mass; all p values $\geq$0.173 and $\eta^2 \leq 0.021$ for snout-vent length, from two-way ANOVAs) or females (all p values $\geq$0.297 and $\eta^2 \leq 0.067$ for body mass; all p values $\geq$0.162 and $\eta^2 \leq 0.101$ for snout-vent length, from two-way ANOVAs). As expected, body mass decreased after 16 weeks in winter dormancy in both males ($F_{(3,74)} = 14.160$, $p < 0.001$, $\eta^2 = 0.353$) and females ($F_{(2,34)} = 2.584$, $p = 0.090$, $\eta^2 = 0.107$). However, body mass loss did not differ significantly between the two temperature treatments at any sampling time in males ($F_{(1,74)} = 0.100$, $p = 0.753$, $\eta^2 = 0.001$). In females, snakes maintained at 4 °C for 16 weeks showed a significantly greater loss of body mass compared to those maintained at 12 °C for 16 weeks ($F_{(1,34)} = 6.176$, $p = 0.018$, $\eta^2 = 0.128$). The percent body mass loss after 16 weeks in hibernation for females was 14.2 % ± 1.30 SEM in the 4 °C group versus 10.4 % ± 1.68 SEM in the 12 °C group. For males, the percent body mass loss after 16 weeks in hibernation was 16.1 % ± 0.91 SEM in the 4 °C group versus 15.2 % ± 1.44 SEM in the 12 °C group. The main effect of the interaction between duration of winter dormancy and temperature on percent body mass loss was not statistically significant for either males ($F_{(3,74)} = 1.388$, $p = 0.253$, $\eta^2 = 0.035$) or females ($F_{2,34)} = 0.775$, $p = 0.469$, $\eta^2 = 0.032$).

3.1. TSH-immunoreactivity

As in Winters et al. (2022), we observed a distinct population of TSH-immunoreactive cells located within the median eminence and infundibulum of the pituitary gland (Fig. 3); we refer to this cell population collectively as the infundibulum from this point forward. Positive TSHβ staining in the anterior pituitary gland was also observed as expected. Omission of the primary antibody and control tests with preadsorbed TSH antiserum eliminated all immunoreactive staining in both the

Table 3
Initial morphometric data and changes in body mass during hibernation in red-sided garter snakes *(Thamnophis sirtalis parietalis)*.

Sex	Week	Hibernation temperature	Sample size	Snout-vent length (cm)	Initial mass (g)	Final mass (g)	Body mass loss (%)
	0	–	8	56.3 ± 1.37	83.3 ± 7.21	77.2 ± 6.68	−7.3 ± 0.63
	8	4 °C	8	55.2 ± 1.06	71.0 ± 3.50	63.0 ± 3.42	−11.4 ± 0.92
Female	8	12 °C	8	58.0 ± 1.54	82.6 ± 4.40	75.7 ± 3.98	−8.3 ± 0.42
	16	4 °C	8	59.1 ± 1.42	78.4 ± 4.83	67.1 ± 3.74	−14.2 ± 1.30
	16	12 °C	8	55.1 ± 1.59	73.4 ± 7.17	65.3 ± 5.79	−10.4 ± 1.68
	0	–	12	44.4 ± 1.15	34.2 ± 2.10	31.7 ± 2.02	−7.4 ± 1.41
	4	4 °C	12	43.4 ± 1.48	31.6 ± 2.66	28.9 ± 2.47	−8.8 ± 0.75
	4	12 °C	12	43.5 ± 1.32	32.2 ± 2.62	29.2 ± 2.40	−9.3 ± 0.47
Male	8	4 °C	12	42.6 ± 1.49	30.9 ± 3.35	28.0 ± 3.10	−9.8 ± 0.56
	8	12 °C	12	44.9 ± 0.99	34.8 ± 2.59	31.4 ± 2.31	−9.3 ± 1.58
	16	4 °C	12	45.1 ± 0.81	37.5 ± 2.17	31.4 ± 1.81	−16.1 ± 0.91
	16	12 °C	12	45.0 ± 1.66	36.3 ± 2.87	30.5 ± 2.20	−15.2 ± 1.44

Snout-vent length and initial body mass were measured after collection in the field (16–18 Sept 2016) and prior to transport of snakes to the lab. Final body mass was recorded on the day animals were euthanized and tissues collected. All data are reported as the group mean ± 1 SEM.

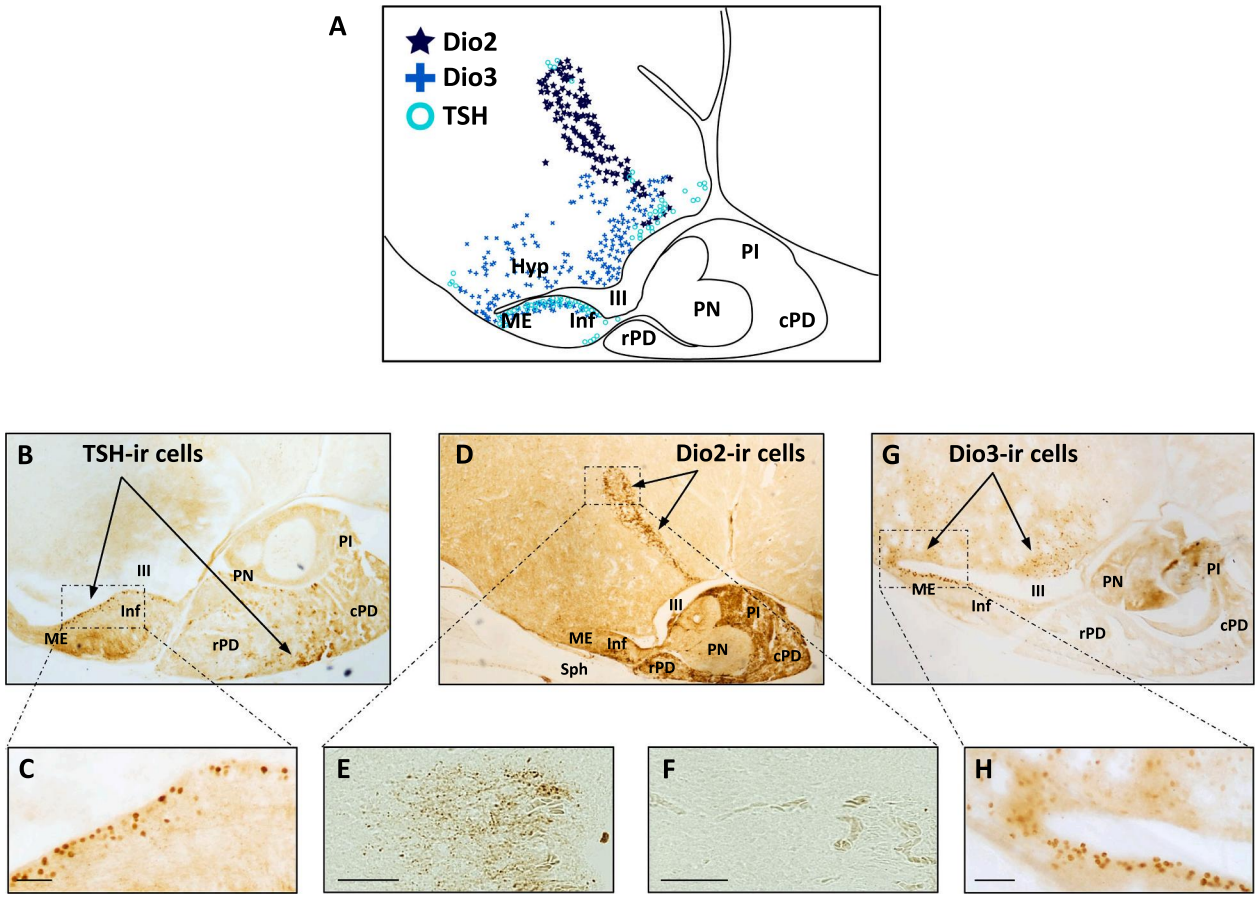

Fig. 3. Localization of thyroid stimulating hormone (TSH), deiodinase 2 (Dio2), and deiodinase 3 (Dio3) immunoreactivity in the brain of red-sided garter snakes (*Thamnophis sirtalis parietalis*). (A) Representative schematic showing the anatomical relationship between TSH, Dio2, and Dio3 labeling within the hypothalamus and infundibulum of the pituitary gland in garter snakes in the sagittal plane [schematic adapted from (Schreibman, 1986)]. Both zones of the pars distalis are shown. (B) Example photomicrograph showing the distribution of TSH-labeled cells in the median eminence, infundibulum, and anterior pituitary gland (rostral pars distalis). The box delineates the magnified space in (C) the infundibulum. (D) Example photomicrograph of Dio2-immunoreactive staining around the third ventricle. The box delineates the magnified space showing Dio2 labeling (E) prior to preadsorption of the Dio2 antiserum and (F) following control tests with preadsorbed antiserum. (G) Example photomicrograph showing the distribution of Dio3-labeled cells in the median eminence, infundibulum, and ventral hypothalamus; the box delineates the magnified space in (H). Results from preadsorption tests for TSH and Dio3 are reported in Winters et al. (2022). The rostral brain is oriented to the left in each sagittal section; all scale bars are 50 μm. Abbreviations: Caudal pars distalis (cPD), Hypothalamus (Hyp), Infundibulum (Inf), Median eminence (ME), Pars intermedia (PI), Pars nervosa (PN), Rostral pars distalis (rPD), Sphenoid bone (Sph), Third ventricle (III).

infundibulum and the anterior pituitary gland [see (Winters et al., 2022)].

In male red-sided garter snakes, the number of TSH-immunoreactive cells did not vary significantly with duration of low-temperature dormancy (Fig. 4A; $F_{(3,65)} = 0.755$, $p = 0.523$, $\eta^2 = 0.034$) or hibernation temperature ($F_{(1,65)} = 0.179$, $p = 0.674$, $\eta^2 = 0.003$). The interaction between duration of winter dormancy and temperature was not statistically significant ($F_{(3,65)} = 0.0476$, $p = 0.986$, $\eta^2 = 0.002$).

In females, neither the duration of low-temperature dormancy ($F_{(2,28)} = 0.388$, $p = 0.682$, $\eta^2 = 0.025$) nor dormancy temperature ($F_{(1,28)} = 0.532$, $p = 0.472$, $\eta^2 = 0.017$) significantly influenced TSH-immunoreactive cell number (Fig. 4B). The interaction between factors was statistically nonsignificant ($F_{(2,28)} = 0.864$, $p = 0.432$, $\eta^2 = 0.056$).

3.2. Dio2-immunoreactivity

We observed Dio2-immunoreactive staining in the anterior and mediobasal hypothalamus, concentrated around the lateral margins of the third ventricle. As expected, omission of the primary antibody from the immunohistochemistry assay eliminated immunoreactive staining. Control tests with the antiserum preabsorbed with Dio2 peptide prior to immunohistochemistry also eliminated immunoreactive staining (Fig. 3F).

The area of Dio2-immunoreactive staining within the imaged region of the hypothalamus of male snakes varied significantly with the duration of low-temperature dormancy (Fig. 4C; $F_{(3,75)} = 4.065$, $p = 0.010$, $\eta^2 = 0.131$). Although Dio2-immunoreactive area did not vary significantly with the main effects of hibernation temperature ($F_{(1,75)} = 0.009$, $p = 0.924$, $\eta^2 \leq 0.001$), results from an all-pairwise multiple comparisons test indicate that significant changes in Dio-2 immunoreactivity during hibernation occurred only within the 4 °C temperature group (Fig. 4C). Further, males hibernated at 4 °C for 16 weeks tended to have a larger area of Dio2-positive labeling than males hibernated at 12 °C ($p = 0.079$). The interaction between factors was not statistically significant ($F_{(3,75)} = 1.774$, $p = 0.159$, $\eta^2 = 0.057$).

In female red-sided garter snakes, the area of Dio2-immunoreactive staining within the hypothalamus also increased significantly with the duration of low-temperature dormancy (Fig. 4D; $F_{(2,34)} = 3.450$, $p = 0.043$, $\eta^2 = 0.160$). Hibernation temperature did not significantly influence the area of Dio2 immunoreactivity ($F_{(1,34)} = 0.704$, $p = 0.407$, $\eta^2 = 0.016$). The interaction between factors was also statistically nonsignificant ($F_{(2,34)} = 0.542$, $p = 0.586$, $\eta^2 = 0.025$).

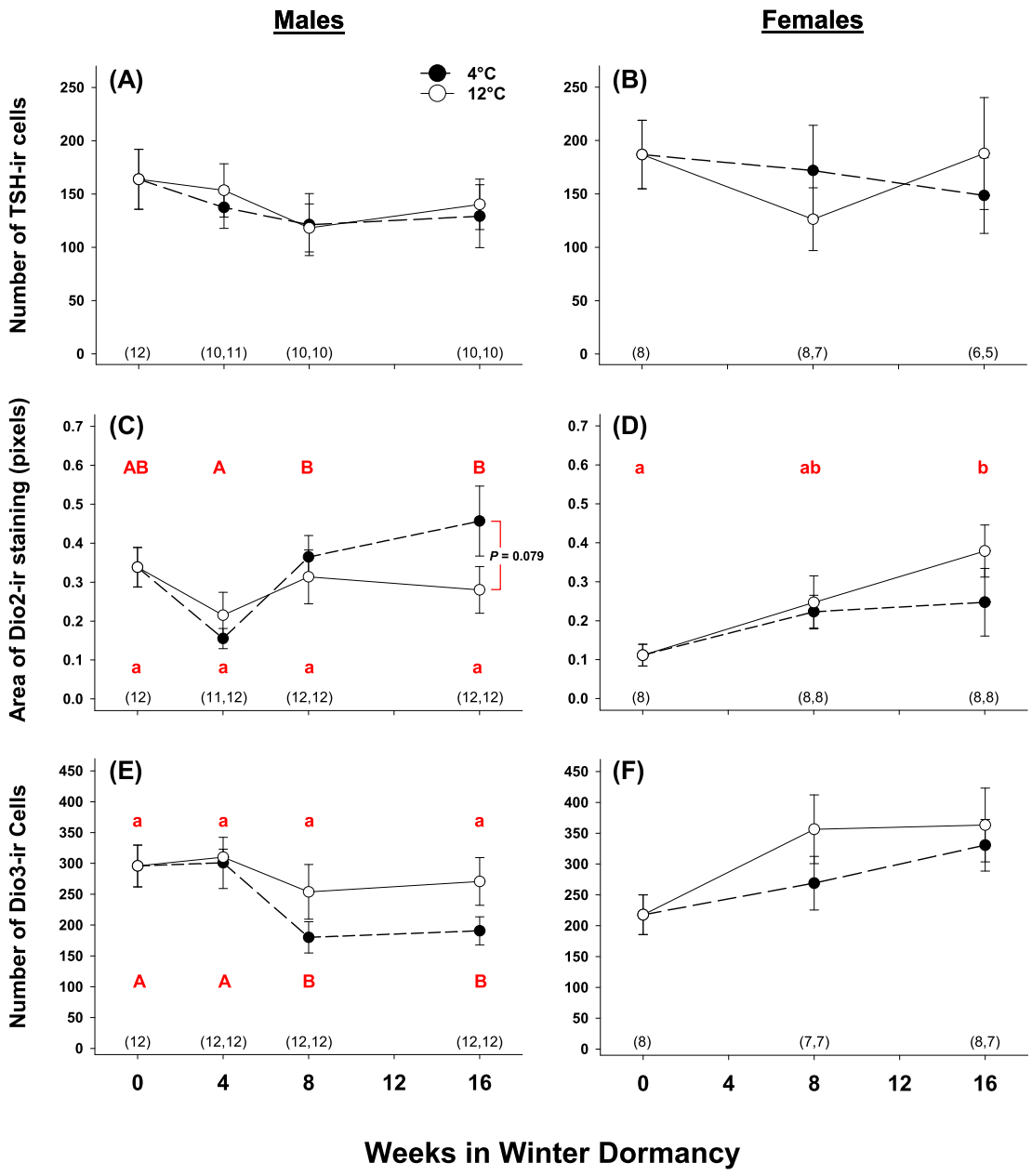

Fig. 4. Influence of low temperature dormancy on thyroid hormone metabolism within the hypothalamus of male and female red-sided garter snakes (*Thamnophis sirtalis parietalis*). Snakes were collected from the field during the fall and hibernated in the lab in complete darkness at 4° or 12 °C. We assessed possible changes in thyroid hormone metabolism by quantifying (A, B) the total number of thyroid stimulating hormone (TSH)-immunoreactive (ir) cells in the infundibulum of the pituitary gland and median eminence, (C, D) the area of deiodinase 2 (Dio2)-ir staining around the third ventricle in the sagittal midline of the hypothalamus, and (E, F) the number of deiodinase 3 (Dio3)-ir cells in the median eminence, rostral infundibulum, and ventral hypothalamus in the sagittal midline of male and female snakes, respectively. Dio2 enzymatically converts thyroxine (T_4) into triiodothyronine (T_3), while Dio3 enzymatically inactivates T_3 by converting it to reverse T_3 and/or diiodothyronine (T_2). Similar to the effects of long photoperiods in spring breeders, we observed increased Dio2 and decreased Dio3 immunoreactivity with increasing duration of low-temperature dormancy in males, changes that would favor the accumulation of T_3 within the hypothalamus. Each data point is the mean ± 1 SEM; sample sizes are listed along the x-axes as (4 °C, 12 °C). Samples collected at time 0 occurred prior to decreasing ambient temperature for winter dormancy and were therefore used to establish a fall prehibernation baseline for each peptide. Letters indicate statistical differences among sampling times. When temporal changes differ between the two temperature treatments, capital letters denote differences within the 4 °C group while lowercase letters denote differences within the 12 °C group.

3.3. *Dio3-immunoreactivity*

Similar to Winters et al. (2022), we observed Dio3-labeled cells in the median eminence, rostral infundibulum of the pituitary gland, and ventral hypothalamus, from the rostral aspect of the median eminence through the caudal mediobasal hypothalamus (Fig. 3). Omission of the primary antibody and control tests with preadsorbed Dio3 antiserum eliminated all immunoreactive staining [see (Winters et al., 2022)].

In male red-sided garter snakes, the number of Dio3-immunoreactive cells within the midline significantly decreased with increasing duration of low-temperature dormancy (Fig. 4E; $F_{(3,76)} = 3.976$, $p = 0.011$, $\eta^2 = 0.125$). Dio3-immunoreactive cell number also varied significantly with hibernation temperature ($F_{(1,76)} = 6.975$, $p = 0.010$, $\eta^2 = 0.073$). The interaction between duration of winter dormancy and temperature was not statistically significant ($F_{(3,76)} = 0.446$, $p = 0.721$, $\eta^2 = 0.014$).

In females, neither the duration of low-temperature dormancy

($F_{(2,31)} = 2.797$, $p = 0.076$, $\eta^2 = 0.139$) nor dormancy temperature ($F_{(1,31)} < 0.001$, $p = 0.993$, $\eta^2 \leq 0.001$) significantly influenced Dio3-immunoreactive cell number (Fig. 4F). The interaction between factors was statistically nonsignificant ($F_{(2,31)} = 1.803$, $p = 0.182$, $\eta^2 = 0.089$).

3.4. Male courtship behavior

The proportion of males courting increased significantly with increasing duration of winter dormancy (Fig. 5; $F_{(3,38)} = 8.268$, $p < 0.001$, $\eta^2 = 0.340$), but neither hibernation temperature ($F_{(1,38)} = 1.650$, $p = 0.207$, $\eta^2 = 0.023$) nor days post-emergence ($F_{(5,38)} = 1.714$, $p = 0.155$, $\eta^2 = 0.118$) significantly affected the proportion of males exhibiting courtship behavior in this analysis (statistics from a three-way ANOVA). After collapsing the temperature groups, a two-way ANOVA revealed that the main effects of hibernation duration ($F_{(3,24)} = 20.262$, $p < 0.001$, $\eta^2 = 0.299$) and days post-emergence ($F_{(5,24)} = 6.555$, $p < 0.001$, $\eta^2 = 0.161$) on the proportion of males courting were statistically significant. Moreover, a significant interaction occurred between the duration of winter dormancy and days post-emergence ($F_{(15,24)} = 5.730$, $p < 0.001$, $\eta^2 = 0.422$), indicating that the pattern of male courtship behavior after emergence changed significantly with and depended upon the duration of low temperature dormancy. Indeed, results from a Holm-Sidak multiple comparisons test with day 1 post-emergence as the control group indicate that males exhibit courtship behavior significantly earlier after emergence as the duration of winter dormancy increases (Fig. 5).

4. Discussion

Photoperiod-induced modulation of hypothalamic thyroid hormone metabolism is well documented, with the resulting local accumulation of triiodothyronine (T3) mediating seasonal changes in the hypothalamus-pituitary-gonad axis [e.g., (Nakane and Yoshimura, 2019)]. Nearly identical neuroendocrine pathways have been described in several bird and mammal species, and some aspects of this pathway have been identified in fishes and a reptile [reviewed in (Chmura and Williams, 2022)], further supporting the hypothesis that neural thyroid hormone metabolism is a common pathway for the environmental control of reproduction in all vertebrates.

Given taxonomic variation in reproductive biology, particularly in regard to its environmental regulation within varied ecological niches, it is likely that any pathway conserved across vertebrate evolution would be capable of integrating multiple environmental cues into a fine-tuned response. We tested this hypothesis explicitly by examining whether environmental temperature modulates thyroid hormone metabolism within the brain of red-sided garter snakes (*Thamnophis sirtalis parietalis*). Our results demonstrate that both the duration and, in males, the magnitude (i.e., 4 °C but not 12 °C) of low-temperature winter dormancy alter deiodinase immunoreactivity within the hypothalamus. We observed an increase in the area of Dio2-immunoreactivity in male and female snakes with increasing duration of low-temperature winter dormancy. In males, the number of Dio3-immunoreactive cells decreased significantly after 8 weeks in winter dormancy at 4 °C. Together, the reciprocal changes in Dio2 and Dio3 immunoreactivity would favor the accumulation of triiodothyronine (T_3) within the hypothalamus in response to at least 8 weeks of low-temperature exposure. Prior studies in this population of garter snakes demonstrated that low-temperature winter dormancy significantly decreased plasma melatonin concentrations (Lutterschmidt and Mason, 2009), altered TSH-immunoreactive cell number within the infundibulum (Winters et al., 2022), significantly increased both the number and size of GnRH-immunoreactive cells in male snakes (Lutterschmidt et al., 2022), significantly increased and/or maintained elevated plasma sex steroid hormone concentrations in both males and females (Lutterschmidt et al., 2022; Lutterschmidt and Mason, 2009), and significantly increased the proportion of males expressing courtship behavior upon spring emergence (Garstka et al., 1982; Krohmer and Crews, 1987, 1989; Lutterschmidt and Mason, 2009); this study). Taken together, these data provide evidence that the neuroendocrine pathway regulating the reproductive axis via thyroid hormone metabolism (Fig. 1) is capable of transducing temperature information.

4.1. Influence of temperature on deiodinase immunoreactivity and courtship behavior in males

Similar to the effect of low-temperature dormancy on the number and size of GnRH-immunoreactive neurons in males (Lutterschmidt et al., 2022), we observed a significant increase in the area of Dio2-immunoreactive staining from week 4 to weeks 8 and 16 in winter dormancy (Fig. 4C). Although the main effects of temperature were not statistically significant, an all-pairwise multiple comparisons test revealed that the significant increase in Dio2 immunoreactivity with increasing duration of low-temperature dormancy ($p = 0.010$, main effect from a two-way ANOVA) was driven by snakes hibernated at 4 °C. A similar pattern was observed in Dio3 immunoreactivity: a significant decrease in Dio3-immunoreactive cell number was observed following 8 weeks in hibernation only when males were maintained at 4 °C (Fig. 4E). When snakes were maintained at an elevated dormancy temperature of 12 °C, there were no significant changes in Dio2 or Dio3 immunoreactivity over time.

While plasma levels of thyroid hormones have not been measured in red-sided garter snakes, data from other reptiles suggest that seasonal and/or temperature-induced changes in circulating thyroid hormone concentrations could contribute to the activation of GnRH and the reproductive axis. For example, in aspic vipers (*Vipera aspis*), plasma thyroxine levels increased towards the end of hibernation (Naulleau et al., 1987) and were elevated at spring emergence (Fleury and Naulleau, 1987). Not only does the hypothalamus-pituitary-thyroid axis respond directly to cold temperature exposure at multiple levels [see review in (Zhang et al., 2018)], but Little and Seebacher (Little and Seebacher, 2014) argue that thyroid hormones were a central mechanism in the evolution of endothermy, because they mediate metabolic and thermogenic responses to cold temperature exposure in both endotherms and ectotherms. Thyroid hormone responses to season and temperature, however, are not consistent even among reptiles. Thyroid gland activity, assessed histologically as epithelial cell height, was lowest during hibernation in both adder snakes (*Vipera berus*) and western fence lizards (*Sceloporus occidentalis*) (Nilson, 1982; Wilhoft, 1958). Further, plasma thyroxine levels were low during hibernation and peaked during summer in male Chinese cobras (*Naja naja*) and during spring in male painted turtles (*Chrysemys picta*) (Bona-Gallo et al., 1980; Licht et al., 1985). Thyroid hormones likely play a more permissive role in regulating the reproductive axis (Dawson et al., 2001), because changes in plasma thyroxine levels are not necessary for deiodinases to alter local thyroid hormone concentrations within specific cells or brain regions. Nevertheless, it would be very informative to know whether low temperature dormancy alters plasma thyroid hormone concentrations in garter snakes.

Our current findings mirror those of previous studies, in which males require exposure to low temperature for at least 8 weeks before significant changes in GnRH immunoreactivity, plasma sex steroid hormones, and courtship behavior are observed (Garstka et al., 1982; Lutterschmidt et al., 2022). The current data further support the hypothesis that there is a threshold effect for the duration of low temperature dormancy on reproductive physiology and behavior. In this study, increasing duration of low-temperature dormancy not only increased the proportion of males exhibiting courtship behavior, but also impacted the pattern of courtship behavior after spring emergence (Fig. 5). Specifically, males exhibited courtship behavior significantly earlier after emergence as the duration of winter dormancy increased. In both lab and field studies, male red-sided garter snakes in northern populations typically display

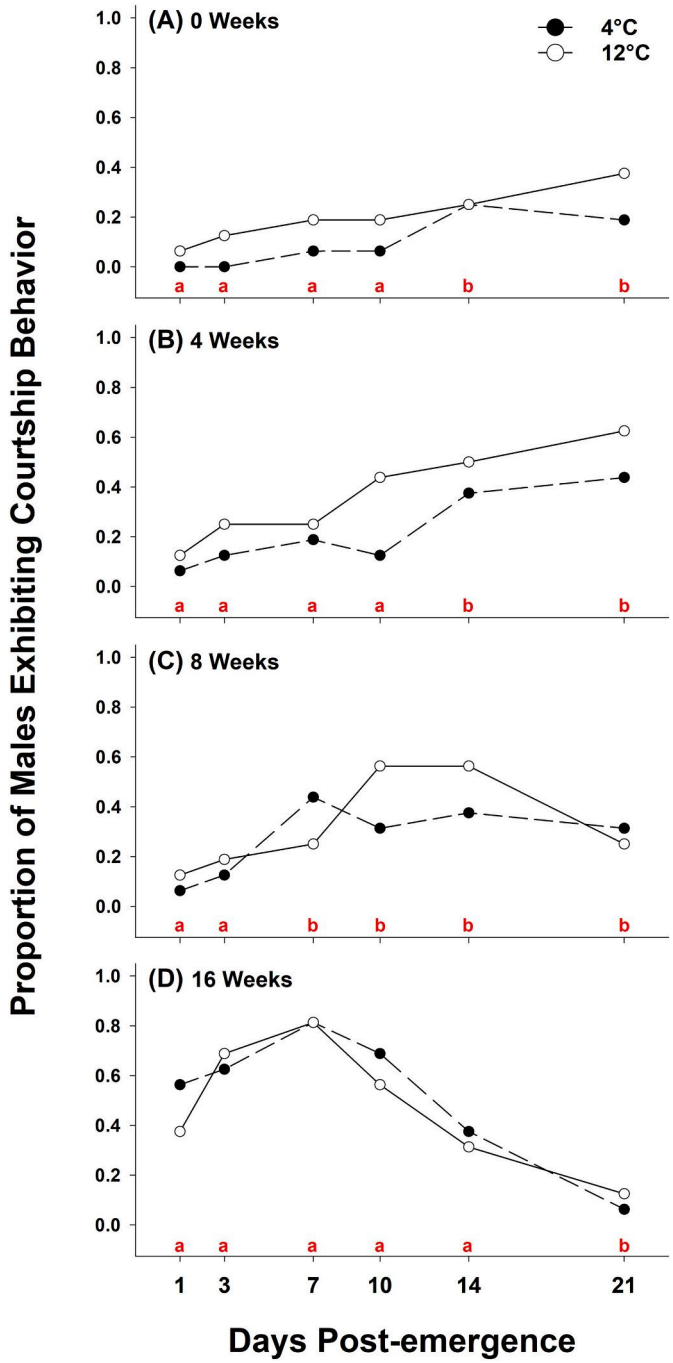

Fig. 5. Influence of low temperature dormancy on the proportion of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) exhibiting courtship behavior. Snakes were collected from the field during the fall and hibernated in the lab in complete darkness at 4° or 12 °C. Following low-temperature dormancy for (A) 0, (B) 4, (C) 8, or (D) 16 weeks, males were subjected to simulated spring emergence and courtship behavior was measured on days 1, 3, 7, 10, 14, and 21 post-emergence. Males receiving a courtship score $\geq$ 2.0 were categorized as courting; these data were then used to calculate the proportion of males exhibiting courtship behavior on each day post-emergence within each temperature group. Each data point represents a sample size of 16 male snakes. After collapsing the temperature groups (the main effect of temperature from a three-way ANOVA: $p = 0.207$), a two-way ANOVA revealed a statistically significant interaction ($p < 0.001$) between increasing duration of winter dormancy (i.e., the factor represented across panels A through D) and temporal changes in courtship behavior post-emergence (i.e., the factor represented by the x-axis). This significant interaction indicates that the pattern of male courtship behavior after emergence changed significantly with and depended upon the duration of low temperature dormancy. Letters along the x-axes indicate statistically significant differences in the proportion of males courting within each hibernation duration relative to day 1 post-emergence (statistics from a Holm-Sidak multiple comparisons test with day 1 post-emergence as the control group).

intense courtship behavior for approximately 14 days after emergence from winter dormancy, with peak intensity occurring between 3 and 7 days post-emergence (Lutterschmidt and Mason, 2008, 2009; Winters et al., 2022). Our results demonstrate that this intra-seasonal pattern of reproductive behavior also depends on the duration of low-temperature dormancy. Because red-sided garter snakes exhibit an extremely abbreviated mating season, any perturbation that increases hibernation temperatures (Lutterschmidt and Mason, 2009) or shortens the duration of winter dormancy (this study) could disrupt natural patterns of seasonal reproductive behavior and have a disproportionately large influence on reproductive fitness. While the temporal increases in male reproductive behavior coincided with increased Dio2 and decreased

Dio3 immunoreactivity, further experimental manipulations of deiodinase activity and/or expression are needed to determine if this relationship is causal.

Interestingly, we observed an initial decrease in the area of Dio2-immunoreactive staining after 4 weeks in winter dormancy at 4 °C, followed by a significant increase at both 8 and 16 weeks (Fig. 4C). An identical pattern was observed in the effect of low-temperature dormancy on the soma size (a proxy for relative cell activity) of GnRH neurons in male snakes (Lutterschmidt et al., 2022). Although the initial decrease in neither Dio2 nor GnRH was statistically significant compared to week 0, the pattern is worth highlighting because it is similar to that reported in photorefractory birds, in which GnRH synthesis and release decreases prior to the reproductive axis regaining sensitivity to photoperiod stimulation [reviewed in (MacDougall-Shackleton et al., 2009)]. It is possible that the initial decrease in Dio2-immunoreactive area and/or GnRH cell area is related to the release of snakes from temperature refractoriness, in which the sensitivity of the hypothalamus to temperature cues is re-established. Additional studies are needed to test this hypothesis.

4.2. Sexually dimorphic responses to low-temperature dormancy

Population-level differences in reproductive timing are well documented in many vertebrates, but the mechanisms underlying such differences are poorly understood (Ball and Ketterson, 2008). Similarly, males of many species (including red-sided garter snakes) typically emerge from winter dormancy or arrive at breeding grounds prior to females, yet sex differences in the sensitivity to environmental cues have not been studied extensively (Tolla and Stevenson, 2020). We previously observed sex differences in the response of GnRH neurons to low-temperature winter dormancy (Lutterschmidt et al., 2022). In contrast to males, neither hibernation temperature nor the duration of winter dormancy significantly altered GnRH cell number or soma size in female red-sided garter snakes. A similar sexual dimorphism was observed in Dio3-immunoreactive cell number in this study (Fig. 4E, F).

In contrast to the sexually dimorphic responses of Dio3 and GnRH, we found that the area of Dio2-immunoreactive staining increased significantly in response to low-temperature winter dormancy in both male and female snakes (Fig. 4C, D). These results suggest that the effects of low-temperature dormancy on the neuroendocrine reproductive axis are similar between male and female red-sided garter snakes, and instead point to sexually dimorphic responses being a result of more subtle differences in the sensitivity to temperature, the duration of low-temperature exposure, and/or the magnitude of changes in each target peptide. For example, although significant changes in GnRH-positive cell number or cell area were not observed in female snakes, plasma estradiol concentrations did increase significantly during low-temperature dormancy (Lutterschmidt et al., 2022). A transient but significant increase in estradiol concentrations immediately prior to spring mating is hypothesized to regulate female receptivity (Mendonca and Crews, 1996; Whittier et al., 1987a). Thus, it is possible that the synthesis and/or release of GnRH is indeed altered by the effects of low-temperature dormancy on thyroid hormone metabolism within the hypothalamus of female snakes, but more sensitive methods are required to detect such changes. Future studies focused on temperature-induced changes in mRNA expression and/or peptide concentrations are needed to understand the nature of the observed sex differences in Dio3 in this study as well as GnRH.

4.3. TSH and regulation of deiodinases

In spring breeding birds and mammals, photoperiod-induced changes in deiodinase expression within tanycytes are mediated by TSH produced by the pituitary pars tuberalis. Despite the fact that snakes lack a histologically distinct pars tuberalis (Chester-Jones et al., 1987; Schreibman, 1986), as discussed in Winters et al. (2022), our results indicate that a population of TSH-positive cells resides within the infundibulum. Our findings are similar to those in fish, which also do not possess an anatomically distinct pars tuberalis. Rather, TSHβ is localized to the coronet cells of the saccus vasculosus, and these cells both express deiodinases and respond to changes in photoperiod [(Nakane et al., 2013), also see (Irachi et al., 2021)]. Thus, the presence of an evolutionarily conserved pathway mediating the effects of environmental cues on hypothalamic thyroid hormone metabolism is supported, regardless of whether a distinct pars tuberalis is present.

In contrast to our predictions, which stem from the well-established effects of dormancy temperature and especially duration on hormones and behavior in this species, neither hibernation temperature nor the duration of low-temperature dormancy significantly altered TSH-immunoreactive cell number in the infundibulum of male or female snakes (Fig. 3A, B). It is possible that immunohistochemistry could not detect small changes in TSHβ synthesis and/or release, particularly when combined with a multifactor experimental design. The latter hypothesis is supported by Winters et al. (2022), who reported that TSH-immunoreactive cell number in the infundibulum decreased significantly in vehicle-treated males following 6 weeks of winter dormancy at 4 °C. Moreover, treatment of snakes with a melatonin synthesis precursor to elevate endogenous melatonin concentrations blocked the effects of low-temperature dormancy on TSH-immunoreactive cell number. Thus, it appears that the TSH-positive cells within the infundibulum are sensitive to both environmental temperature and melatonin, similar to the case in mammals. Further studies directed at measuring changes in mRNA expression are needed to determine if TSHβ is indeed altered during low-temperature dormancy. It would also be valuable to compare the responses of TSH-positive cells among other reptiles that do develop a pituitary pars tuberalis (e.g., turtles and crocodiles).

An alternative explanation is that the TSH-positive cells within the infundibulum are not temperature sensitive, and either the deiodinase-positive cells respond directly to environmental temperature or another, as-yet unidentified factor relays temperature cues to the hypothalamus. This hypothesis is supported by studies in mammals and birds. For example, in common voles (*Microtus arvalis*), warm temperatures (21 °C versus 10 °C) enhanced Dio2 mRNA expression and testosterone synthesis during long days but did not significantly alter TSHβ mRNA expression (van Rosmalen et al., 2021). In tundra voles (*Microtus oeconomus*), van Dalum et al. (2023) showed that Dio2 mRNA expression exhibited the highest sensitivity to ambient temperature, while Dio3 and TSHβ mRNA expression were relatively insensitive to changes in temperature. Similarly, arctic ground squirrels (*Urocitellus parryii*) warmed to 30 °C relative to control animals maintained at 2 °C during midwinter showed no change in TSHβ mRNA expression within the pars tuberalis, while Dio2 mRNA expression within the hypothalamus increased (Chmura et al., 2022b). However, a subsequent study in the same species found that increasing ambient temperature from −6 °C to −1 °C late in hibernation tended to increase pars tuberalis TSHβ mRNA expression in males ($p = 0.068$) but did not significantly alter Dio2 mRNA expression (Chmura et al., 2022a). Current data from the few studies examining the pars tuberalis TSHβ-temperature relationship in birds are similarly inconclusive [reviewed in (Chmura and Williams, 2022)], with photoperiod + temperature manipulations altering TSHβ mRNA expression in Eurasian tree sparrows [*Passer montanus*; (Renthlei et al., 2021)] and redheaded buntings [*Emberiza bruniceps*; (Trivedi et al., 2019)] but not Japanese quail [*Coturnix japonica*; (Ikegami et al., 2015)]. It is very likely that the effects of photoperiod-temperature interactions (as well as other supplementary cues) on TSHβ, Dio2, and/or

Dio3 are specific to the ecological niche of the animal and the species-specific pattern of reproductive regulation. Indeed, exciting new evidence shows that genetic differences (i.e., single nucleotide polymorphisms) in the TSH receptor gene among populations of common voles (*M. arvalis*) in Western Europe are more related to local photoperiod-temperature patterns than latitude alone (van Rosmalen et al., 2023).

4.4. Conclusions

Relative to photoperiod, few studies have investigated if and how supplementary environmental cues modulate hypothalamic thyroid hormone metabolism and subsequent reproductive activity. In this study, we found that both the duration and, in males, the magnitude (i.e., 4 °C but not 12 °C) of low-temperature winter dormancy altered deiodinase immunoreactivity within the hypothalamus, increasing the area of Dio2-immunoreactivity in males and females and decreasing the number of Dio3-immunoreactive cells in males after 8–16 weeks in winter dormancy. The temporal changes in deiodinase immunoreactivity were coincident with an increase in male reproductive behavior (this study) as well as a significant increase in the number and size of GnRH-immunoreactive neurons (Lutterschmidt et al., 2022). Whether TSH-positive cells within the infundibulum mediate the effects of temperature on deiodinase 2/3 requires further study, as we did not observe significant changes in TSH-immunoreactive cell number in this study. While the relative importance of temperature to reproductive regulation varies among species, the data presented here suggest that animals could rely on a single neuroendocrine pathway to integrate environmental temperature with photoperiod to fine-tune their reproductive responses to environmental conditions. Because all vertebrates could potentially supplement photoperiod information with temperature cues, these results are broadly applicable to understanding how environment–organism interactions mediate seasonally adaptive responses to a changing environment.

CRediT authorship contribution statement

Deborah I. Lutterschmidt: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Kalera Stratton:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Treven J. Winters:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Stephanie Martin:** Investigation, Methodology. **Lauren J. Merlino:** Data curation, Investigation, Methodology, Software, Validation.

Data availability

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

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