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SYMPOSIUM

Effects of Spring Warming on Seasonal Neuroendocrinology and Activation of the Reproductive Axis in Hibernating Arctic Ground Squirrels

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Synopsis Many animals adjust the timing of seasonal events, such as reproduction, molt, migration, and hibernation, in response to interannual variation and directional climate-driven changes in temperature. However, the mechanisms by which temperature influences seasonal timing are relatively under-explored. Seasonal timing involves retrograde signaling in which thyrotropin (TSH) in the pars tuberalis (PT) alters expression of thyroid hormone (TH) deiodinases (*Dio2*/*Dio3*) in tanycyte cells lining the third ventricle of the hypothalamus. This, in turn, affects the availability of triiodothyronine (T3) within the mediobasal hypothalamus—increased hypothalamic T3 restores a summer phenotype and activates the reproductive axis in long-day breeders. Recently, we showed that retrograde TH signaling is activated during late hibernation in arctic ground squirrels (*Urocitellus parryii*) held in constant darkness and constant ambient temperature. Sensitivity of seasonal pathways to nonphotic cues, such as temperature, is likely particularly important to hibernating species that are sequestered in hibernacula during spring. To address this issue, we exposed captive arctic ground squirrels of both sexes to an ecologically relevant increase in ambient temperature (from −6 to −1°C) late in hibernation and examined the effects of warming on the seasonal retrograde TSH/Dio/T3 signaling pathway, as well as downstream elements of the reproductive axis. We found that warmed males tended to have higher PT *TSHβ* expression and significantly heavier testis mass whereas the TSH/Dio/T3 signaling pathway was unaffected by warming in females, although warmed females exhibited a slight decrease in ovarian mass. Our findings suggest that temperature could have different effects on gonadal growth in male and female arctic ground squirrels, which could lead to mismatched timing in response to rapid climate change.

Introduction

Animals have evolved central nervous system timing mechanisms that, through seasonal programs, allow them to synchronize critical life-history stages, such as reproduction, with periods of high resource availability (Perrins 1970; Drent 2006). These seasonal programs are regulated by changes in photoperiod (the daily duration of light and dark), which drive cyclic phenotypic changes and/or entrain circannual clock mechanisms (e.g., Gwinner 1996; Dawson et al. 2001). Seasonal change in photoperiod is consistent from year-to-year, making it a highly reliable cue that enables

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animals to anticipate predictable changes in their environment and initiate costly and time-consuming phenotypic changes well before they are required (Wingfield 2008).

While photoperiod is a critical cue for coarsely scheduling seasonal activities, animals must also adjust or fine-tune seasonal timing to account for interannual variations in weather and do so using a variety of supplemental cues including ambient temperature, snow cover, availability of food and water, and social cues (reviewed in: Boutin 1990; Helm et al. 2006; Ball and Ketterson 2008; Davies and Deviche 2014; Chmura et al. 2020). The ability of an organism to use nonphotic cues to time seasonal phenotypic transitions has long been appreciated in equatorial habitats (e.g., Hau et al. 2000; O'Brien and Hau 2005; Goymann and Helm 2014), where photoperiod is relatively invariant across the year and rainfall may be the most salient cue predicting favorable breeding conditions (Moore et al. 2005). Opportunistic breeders that take advantage of ephemeral pulses in resource availability are also well known for utilizing nonphotic cues to time reproduction (Hahn 1998; Perfito et al. 2008). More recently, responsiveness to nonphotic cues has been seen as an important factor determining whether an organism can respond to the rapid pace of anthropogenic climate change using phenotypic plasticity instead of, or in addition to, evolution (Merilä and Hendry 2014). Yet, there are relatively few studies investigating the integration of these cues in seasonal time-keeping pathways, especially at higher levels of regulation (Ball 1993; Chmura et al. 2020; Tolla and Stevenson 2020).

The neuroendocrine integration of temperature cues in vertebrates is poorly understood (Caro et al. 2013; Chmura and Williams 2022) and this is a vexing problem as environmental temperature may be a particularly informative cue that could afford adaptive phenotypic plasticity in a climate-altered world. To date, studies in diverse taxa report plasticity in seasonal timing in response to global climate change (reviewed in: Boutin and Lane 2014; Charmantier and Gienapp 2014; Crozier and Hutchings 2014; Urban et al. 2014), and at least one provides evidence of selection on phenotypic plasticity (Nussey et al. 2005).

A retrograde thyrotropin/deiodinase/triiodothyronine (TSH/Dio/T3) neuroendocrine pathway from the pars tuberalis (PT) to the hypothalamus is critical to seasonal timing of metabolism and reproduction (Fig. 1). In mammals, changing photoperiods alter the duration of nocturnal melatonin secretion from the pineal gland. Melatonin binds to receptors in the PT (Dardente et al. 2003) and alters circadian cycles of transcription and degradation of transcriptional coactivator *EYA3* (a member of the eyes absent family of proteins) (Dardente et al. 2010; Dupré et al. 2010; Wood et al. 2015). In addition, the PT is thought to be the site of (or closely controlled by) the endogenous circannual clock, although its precise neural substrates have yet to be fully described (Wood and Loudon 2018). High levels of *EYA3* in the second half of the circadian day elevate transcription of *TSHβ* in the PT (Dardente et al. 2010). TSH released from the PT alters the expression of deiodinase enzymes (increasing *DIO2* and decreasing *DIO3*) within ependymal β-tanycytes lining the third ventricle—these cells have long radial processes that terminate at the basal lamina separating the median eminence from the PT (Hanon et al. 2008; Rodríguez et al. 2019). These changes in deiodinase expression increase the local availability of T3, the most bioactive form of thyroid hormone (Watanabe et al. 2004), which, in turn, drives seasonal changes in reproduction and metabolism (Viguié et al. 1999; Watanabe et al. 2004; Barrett et al. 2007; Freeman et al. 2007). This phylogenetically ancient pathway is also

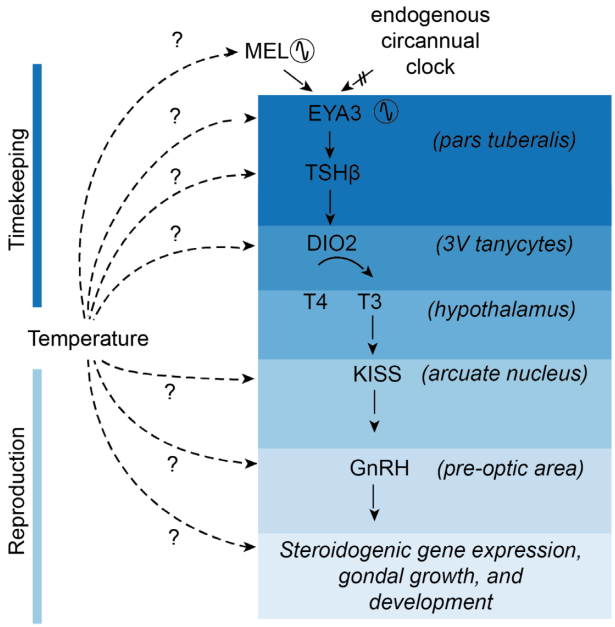

Fig. 1 Schematic illustrating potential pathways by which temperature cues (detected either peripherally or centrally and then relayed to the pre-optic area) could influence seasonal timing mechanisms in the hypothalamus. Dashed arrows in the top half of the diagram indicate temperature effects on melatonin and retrograde T3 signaling pathway mechanisms (EYA3, TSHβ, and DIO2). Dashed arrows in the bottom half of the diagram indicate temperature effects specifically on activation of seasonal reproduction via the HPG axis. Sinusoidal curves in circles are icons representing that a product is expressed in a circadian cycle and that temperature may affect the amplitude or duration of this signal. DIO3 (not depicted) inactivates thyroid hormone and is also expressed in third ventricle tanycytes but is downregulated as one of the earliest steps in the photorefractory response (Milesi et al. 2017).

present in birds (e.g., Yoshimura et al. 2003; Yasuo et al. 2005; Nakao et al. 2008) and fish (Nakane et al. 2013), although there are important taxonomic differences in modes of photoperiod perception and transduction upstream of TSH signaling and in the role of melatonin across these groups (Wilson 1991; Falcón et al. 2010).

To date, research on temperature perception and transduction is much more advanced than research on integration of temperature cues into seasonal pathways (Caro et al. 2013; Chmura and Williams 2022). Peripheral TRP (transient-receptor potential) channels (reviewed in Gracheva and Bagriantsev 2015) and the pre-optic area (POA) of the hypothalamus (reviewed in Morrison and Nakamura 2011) are thought to be important for temperature detection and transduction. Two basic and non-mutually exclusive hypotheses are that temperature signals could (1) influence canonical seasonal pathways (e.g., melatonin, EYA3, and retrograde TH signaling) or (2) act on pathways regulating specific seasonal phenotypes downstream (e.g., HPG axis) (reviewed in: Chmura and Williams 2022). This could happen either directly (i.e., temperature cues exert the effect) or indirectly via temperature effects on metabolism. Indeed, the importance of metabolically driven temperature effects on reproduction is suggested by work indicating that low temperatures suppress reproduction more in food limited environments (Manning and Bronson 1990).

In this study, we test the hypothesis that ecologically relevant temperature increases during hibernation affect seasonal neuroendocrine signaling and to identify what components of these pathways are modulated. We did so using the arctic ground squirrel *(Urocitellus parryii)*, a medium-sized, seasonally obligate hibernator native to boreal and arctic landscapes. Across the hibernation season, arctic ground squirrels experience minimum hibernacula temperatures as low as −23.4°C, and typically soils remain frozen until well after animals end hibernation (Buck and Barnes 1999). While hibernating in frozen hibernacula at subfreezing temperatures, arctic ground squirrels prevent tissues from freezing by generating heat in brown adipose tissue using non-shivering thermogenesis. Although, midwinter hibernacula temperatures fluctuate between ∼ −5 and −23°C, depending on the burrow (Buck and Barnes 1999), core body temperature during torpor remains stable at approximately −2°C (Williams et al. 2012). In the field, males terminate the heterothermic phase of hibernation prior to females (Sheriff et al. 2011) and seasonal warming of soils does not typically start until a few weeks later when females end hibernation (Williams et al. 2012). We predicted that ground squirrels would respond to higher ambient temperatures (−1 vs −6°C) by altering hypothalamic *DIO2*/*DIO3* signaling in tanycytes and initiating activation of the reproductive axis without changes to upstream EYA3/TSH signaling in PT thyrotrophs. This is because targets in the PT are thought to be tightly regulated by the endogenous circannual clock, whereas downstream DIO signaling may receive input from multiple hypothalamic pathways.

Material and methods

Husbandry and experimental design

The University of Alaska IACUC (protocol # 864,841) approved all procedures and the Alaska Department of Fish and Game permitted trapping activities (permit #18–188). In July 2018, male and female arctic ground squirrels (*U. parryii*) were captured along the Dalton Highway in northern Alaska south of the Atigun River (68°27′N, 149°21′W, elevation 812 m). On arrival at the University of Alaska Fairbanks Animal Facility, animals were maintained in individual hanging metal cages (48 × 32 × 32 cm) under 16L:8D photoperiod and 20°C. Cages contained cotton batting that squirrels could use for nesting material. Animals were fed 10 pellets of Mazuri Rodent Chow (Land O'Lakes, Inc; St. Louis, MO, USA) daily and provided water *ad libitum*. On August 1st, photoperiod was decreased 30 min/day to 4L:20D simulating a declining fall photoperiod. This does not perfectly match the photoperiod these squirrels would have experienced above ground in a natural setting, which would be 24 L for 2 months midsummer and a decline of about 15 min/day around the fall equinox. However, we note that arctic ground squirrels are semi-fossorial animals, so their photoperiodic exposure is inextricably linked to behavioral choices to enter and exit the burrow. At the end of August, most males are only aboveground for a maximum of 8 h/day under high temperature and low precipitation conditions (and much less or not at all during rainy or snowy weather), while most females are already hibernating and are not exposed to light (Williams et al. 2014; Chmura et al. 2020). Thus, we provide animals with 4 h of light per day by the end of August, which reflects an intermediate value between what would be typically experienced by each sex. Once animals exhibited behaviors characteristic of hibernation readiness, such as building large nests from cotton batting in combination with the absence of feeding behavior and activity, they were transferred to a single room where they were housed at 2°C and constant dark conditions. At the end of August, soil temperatures at a depth of 1 m hover just above freezing (Buck and Barnes 1999) as burrows remain cool throughout the year as they overlay continuous permafrost.

Once torpid, squirrels were maintained in plastic tubs (Nalgene, Rochester, NY, USA) and provided hydration gel packs (HydroGel, ClearH2O, Portland, ME, USA). Squirrels entered hibernation between September 8th and October 24th, which is somewhat later than females would be expected to enter hibernation in a field setting (Sheriff et al. 2011), but not unusual for the captive environment.

Beginning December 5th, ambient temperatures were gradually lowered from 2 to −2°C at 0.5°C/day over 8 days with a further drop from −2 to −6°C at 0.5°C/day over 8 days beginning January 10th. Soils in hibernacula typically freeze over a multi-week or multi-month period in the fall or winter, which may occur anywhere between mid-September and late January (Chmura *et al.* unpublished data). Within each sex, animals were assigned to experimentally warmed or control groups using a randomized design balanced by date of hibernation entry. Beginning February 11th, treatment animals were moved into a different room where they were warmed by 1°C/day from −6 to −1°C to simulate warming spring conditions. Control animals remained at −6°C. These temperatures were selected to minimize torpid body temperature differences between groups (Richter et al. 2015) and to be within the range of what squirrels encounter in their burrows during hibernation (Buck and Barnes 1999). Once frozen, burrow soil temperatures may fluctuate by several degrees over a few days in response to cold snaps, although the rate of the warming treatment likely exceeded that of typical spring warming.

Tissue sampling

Animals were sampled while euthermic because prior studies showing seasonal changes in neuroendocrine measures during hibernation sampled euthermic animals (Chmura et al. 2022). Sampling occurred either during a natural arousal from torpor or approximately 16 h after a manually stimulated arousal. Samples were collected from March 4th to 7th, except for one final sample on March 12th. Animals were anesthetized with isoflurane under dark conditions until they achieved a deep anesthetic plane, a blood sample was taken via cardiac puncture under dim light, and then animals were euthanized by decapitation. Brains were extracted, frozen on dry ice, and stored at −80°C. Gonads were removed, washed in sterile PBS, frozen in isopentane cooled with dry ice, and stored at −80°C. Plasma was separated from whole blood by centrifugation and stored at −80°C. Samples were collected from 13 males ($n = 5$ for −6°C, $n = 8$ for −1°C) and 14 females ($n = 7$ for −6°C, $n = 7$ for −1°C).

In situ hybridization

Frozen coronal brain sections were sliced at 12 µm on a cryostat (CM2050s, Leica Microsystems, Ltd., Milton Keynes, UK), thaw mounted on poly-l-lysine coated slides, and fixed in 4% paraformaldehyde. Expression of *EYA3, TSHβ, DIO2, DIO3, and KISS* (kisspeptin), which is involved in reproductive activation, was measured using in-situ hybridization with custom P33 α-UTP radio-labeled riboprobes as described in Chmura et al. (2022). Exposed film images of radio-labeled slides were scored in ImageJ for mean pixel grayness corrected for background staining. Regions examined were the PT (*EYA3, TSHβ*), ependymal lining of the third ventricle (*DIO2, DIO3*), and arcuate nucleus (*KISS*). Three sections per individual were included in analyses of targets in the PT, whereas two sections per individual were included in analyses of targets adjacent to the third ventricle or in the arcuate nucleus.

qPCR and gonad mass

Prior to dissecting tissues for qPCR, mass of frozen ovaries and testes was measured on an analytical balance (Sartorius, Göttingen, Germany). Length and width of testes were measured with calipers to calculate testicular volume using the formula: $4/3\ \pi\ L^2W$. Next, mRNA was extracted from up to 30 mg of gonadal tissue per animal using an RNAeasy mini kit (#74106, Qiagen Inc., Valencia, CA, USA). Tissues were homogenized in buffer RLT with a Bead Bug microtube tissue homogenizer (#D1030, Benchmark Scientific, Edison, NJ, USA) and treated with RNAse-free DNAse (#79254, Qiagen) to prevent genomic contamination. A Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) with a Qubit Broad Range Assay Kit (#Q10211, Invitrogen) was used to quantify mRNA concentration in each sample and an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to ensure that mRNA was of sufficient quality (RIN > 9.8 for all samples). A high-capacity cDNA reverse transcription kit (#436881, Applied Biosystems, Carlsbad, CA, USA) was used to synthesize cDNA from 1 µg of sample RNA in a 40 µL reaction. The reaction was conducted using the following profile: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and a 4°C terminal hold.

Primer design and selection is described in Chmura et al. (2022). Assays were run in triplicate using a 15 μL reaction volume (3 μL of cDNA in a 1:15 water dilution) and Power SYBR™ Green PCR Master Mix (Applied Biosystems) on an ABI-7900 HT system (Applied Biosystems). Reactions were run using the following cycle parameters: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplification of a single product was verified using a final dissociation curve (95°C

for 15 s, 60°C for 15 s, and 95°C for 15 s). Absence of contamination was checked with no-template and no-reverse transcription controls. cDNA pools from ovarian and testicular tissue representing both warmed and control animals were used to create a six-point standard curve (1:5 dilution) and calculate reaction efficiency. Reaction efficiencies are reported in Supplementary Table S1.

Genes of interest were selected for their known role in biosynthetic pathways for reproductive hormones [e.g., steroidogenic acute regulatory protein *(STAR)*, cholesterol side-chain cleavage enzyme (*CYP11A*)] or in gonadal development [estrogen receptor beta (*ERβ*), and nerve growth factor receptor (*NGFR*)]. Additional genes of interest were tested [follicle stimulating hormone receptor (*FSHR*) and nerve growth factor (*NGF*)], but are not reported here because they did not meet standards for reaction efficiency. Four potential reference genes were evaluated to ensure that expression did not differ between control and experimental animals and that Ct standard deviation was less than one (Pfaffl 2001). Three genes met these criteria for each sex: males (*RSP3*, *CYPA*, and *HPRT*) and females (*RSP3*, *HPRT*, and *B2M*). For each sex, the geometric mean of expression for the three reference genes and the Pfaffl method were used to normalize target gene expression, while accounting for differences in reaction efficiency (Pfaffl 2001). Expression levels are reported relative to the mean expression of the control group.

Hormone assays

Column solid phase extraction (BondElut #12,102,046; Agilent, Santa Clara, CA, USA) was used to extract hormones from 500 µL of plasma and then 90% methanol was used to elute samples, which were dried at 35°C in a ThermoSavant SpeedVac Concentrator (model SDP121P; Thermo Fisher Scientific, Waltham, MA, USA). Samples were stored at −80°C until the day before assays were conducted, when they were reconstituted in 1 mL assay buffer at a 1:2 dilution and shaken for 1 h on a multi-tube vortexer (Glas-Col Large Capacity Mixer, speed set on 50; Glas-Col, Terre Haute, IN, USA). Resuspended samples were stored at 4°C overnight and shaken an additional hour before steroid assays began. Testosterone (kit # K032, Arbor Assays, Ann Arbor, MI, USA) was quantified in males and estradiol (kit # KB30, Arbor Assays, Ann Arbor, MI, USA) and progesterone (kit # K025, Arbor Assays, Ann Arbor, MI, USA) were measured in females using Enzyme Immunoassays (EIAs). We validated the assays for use in arctic ground squirrels by demonstrating dilution linearity with spiked samples (i.e., standard addition) and by verifying parallelism between the standard curve and serially diluted plasma samples (i.e., tests of parallelism). Samples and standards were run in duplicate with a single plate per hormone and a standard curve. Intra-assay CV of duplicates was: 1.38 ± 0.86% (testosterone), 3.09 ± 1.10% (estradiol), and 3.27 ± 1.97% (progesterone).

Statistical analyses

Data from males and females were analyzed separately and all analyses were conducted in R (R Core Team 2021). The sexes were analyzed separately due to sex-specific patterns of hibernation timing in the field (Sheriff et al. 2011; Williams et al. 2017), and due to differences in targets measured such as gonadal mass and circulating hormones; additionally, power to detect interactive effects between sex and treatment would be low. mRNA expression quantified by in situ hybridization was analyzed with the lme4 package (Bates et al. 2015) using a linear mixed effects model with a random effect for individual id and a fixed effect for treatment group. *P*-values were estimated using the package lmerTest (Kuznetsova et al. 2017). Gonadal mass, testicular volume, plasma hormone concentrations, and gonadal tissue qPCR data were analyzed with a non-parametric Kruskal–Wallis test given that our sample size for each group ranged from 5 to 8.

Results

Experimentally warmed males at −1°C tended to have higher PT *TSHβ* expression than control males at −6°C ($\beta = 0.095$, df = 11.030, $P = 0.068$, Fig. 2). Third ventricle *DIO3* expression ($\beta = -0.003$, df = 11.000, $P = 0.28$, Supplementary Fig. S1) was undetectable in both groups, as expected for sampling points late in hibernation (Chmura et al. 2022). In contrast, *EYA3* expression in the PT ($\beta = 0.008$, df = 11.212, $P = 0.40$, Supplementary Fig. S1) and *DIO2* expression in the third ventricle ($\beta = -0.572$, df = 11.000, $P = 0.25$, Supplementary Fig. S1) were detectable in both warmed and control animals, but highly variable within groups and not significantly different between groups. The expression of *KISS* in the arcuate nucleus was strong, but similar between groups ($\beta = -0.007$, df = 11.000, $P = 0.73$, Supplementary Fig. S1). Despite, the overall similarity of hypothalamic expression for genes of interest between warmed and control animals, warmed males tended to have higher plasma testosterone (ChiSq = 2.045, df = 1, $P = 0.15$, Supplementary Fig. S2) and testis volume (ChiSq = 3.225, df = 1, $P = 0.07$, Supplementary Fig. S2) and had significantly higher gonad mass (ChiSq = 4.821, df = 1, $P = 0.03$, Fig. 2). There was a trend for higher expression of *STAR* in warmed animals (ChiSq = 2.593, df = 1, $P = 0.11$,

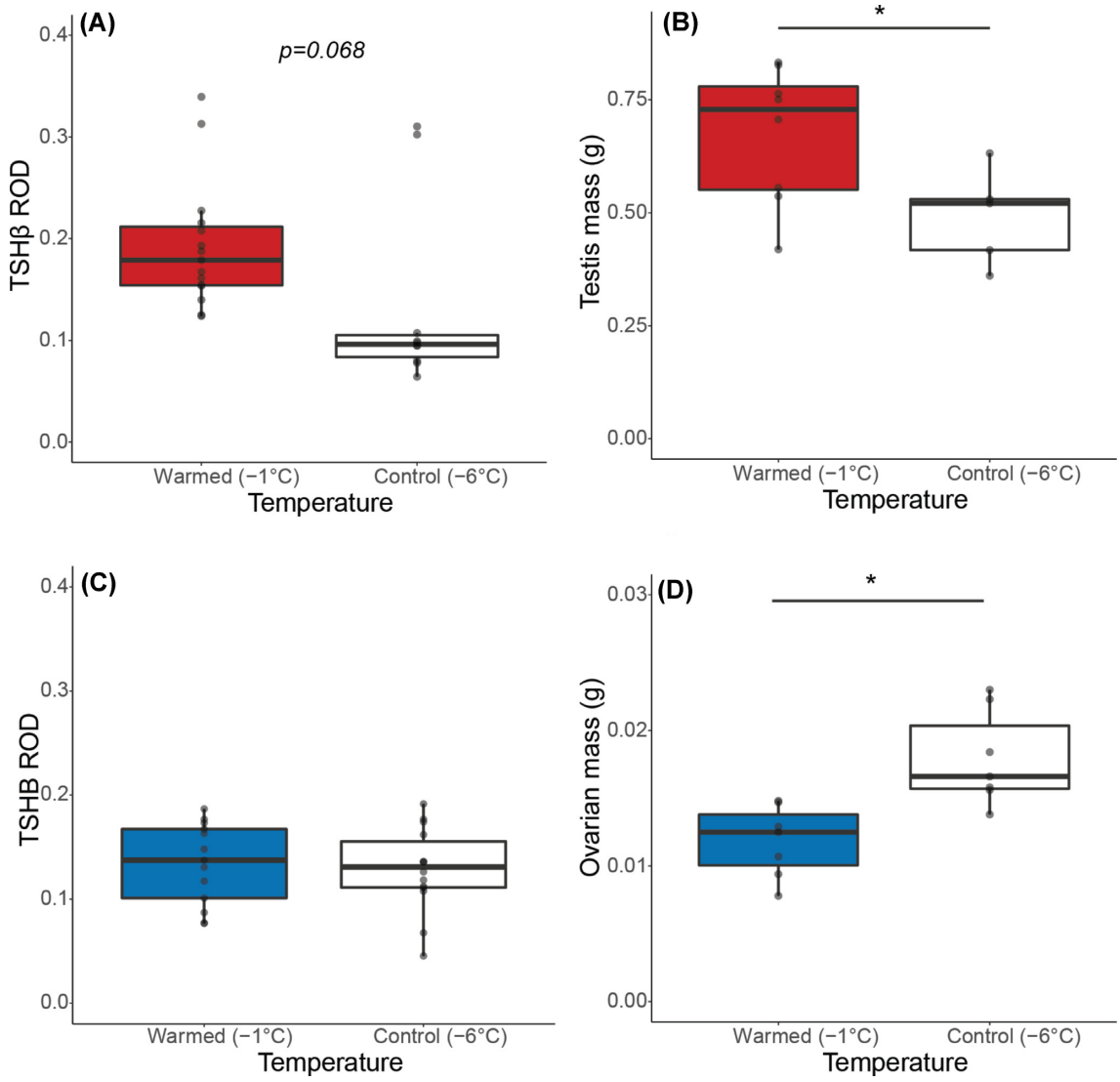

Fig. 2 *TSHß* expression in the pars tuberalis of hibernating male **(A)** and female **(C)** arctic ground squirrels and gonad mass (**B** and **D**). Data for in situ hybridization analyzed with a linear mixed effects model (multiple slices per individual). Data for mass analyzed with a Kruskal–Wallis test. Error bars represent the 25th and 75th percentiles ± 1.5 times the interquartile range. Significantly different comparisons are indicated with an asterisk (∗).

Supplementary Fig. S3), but this represented only a 2-fold difference relative to control animals. No additional trends or significant differences in gene expression were observed in testicular tissue for other genes of interest ($P > 0.5$, Supplementary Fig. S3).

Females exhibited no differences in PT *TSHß* expression ($\beta = 0.007$, df = 12.053, $P = 0.83$, Fig. 2). Like males they exhibited extremely low levels of *DIO3* ($\beta = 0.008$, df = 12.00, $P = 0.18$, Supplementary Fig. S4) and moderate to high levels of *EYA3* ($\beta = -0.009$, df = 12.293, $P = 0.48$, Supplementary Fig. S4), *DIO2* ($\beta = -0.003$, df = 12.000, $P = 0.96$, Supplementary Fig. S4), and *KISS* ($\beta = 0.016$, df = 12.000, $P = 0.44$, Supplementary Fig. S4), but showed no significant differences between groups. Warmed females had smaller ovaries than control females (ChiSq = 8.265, df = 1, $P = 0.004$, Fig. 2), but did not show significant differences in plasma progesterone (ChiSq = 0.494, df = 1, $P = 0.48$, Supplementary Fig. S5) or estradiol (ChiSq = 1.474, df = 1, $P = 0.22$, Supplementary Fig. S5). No significant differences were observed in gene expression in ovarian tissue between control and experimentally warmed females ($P > 0.25$, Supplementary Fig. S6).

Discussion

We found that, ecologically relevant warming late in the hibernation season resulted in a slight increase in gonadal size in male arctic ground squirrels. This

environmental warming may be mediated by changes in thyrotropin signaling between the PT and hypothalamus; however, while there was a trend for increased PT *TSHβ* expression in warmed males, corresponding changes in *DIO2* and *DIO3* in the third ventricle of the hypothalamus were not observed. Surprisingly, females showed no differences in TSH/Dio/T3 between temperature treatments and warmed females actually had smaller gonads.

Interestingly, prior work indicates that phenological responses to hibernacula warming can differ between the sexes, as males ended torpor earlier when exposed to warming conditions, whereas females did not (Barnes and Ritter 1993). The response of males in this earlier study may be due to the production of testosterone, which inhibits hibernation (Lee et al. 1990). We advocate for more studies examining physiological and phenological responses in both sexes. In particular, there is a need for larger studies with sufficient power to explicitly test for sex-differences in cue sensitivity.

Previously, we showed that retrograde TSH/Dio/T3 signaling, *KISS* expression, and the reproductive axis of arctic ground squirrels housed at 2°C are activated during constant darkness late in hibernation, consistent with preparing for spring reproduction (Chmura et al. 2022). We predicted arctic ground squirrels would be sensitive to warming late in hibernation, based on their need to adjust seasonal timing to local conditions and had expected that the PT would be unaffected by warming temperatures, whereas "downstream" hypothalamic targets (i.e., deiodinases and *KISS*) would exhibit thermal sensitivity. This is because seasonality in hibernating ground squirrels is regulated by a strong endogenous clock entrained by photoperiod during the active season (Pengelley and Fisher 1957, 1963; Pengelley et al. 1976; Lee and Zucker 1991), which is hypothesized to exhibit temperature compensation and to regulate PT TSHß expression directly (Wood and Loudon 2018). We also thought that temperature responses would be observed "downstream" of the PT because the neuron populations that sense and integrate thermal cues lie within the pre-optic area (POA) of the hypothalamus (reviewed in: Caro et al. 2013). Instead, we detected a trend for higher levels of TSHß in males from the warmed group, without changes in deiodinase expression or *KISS* expression, although warmed males had larger gonads. Whether this indicates that TSHß was having effects independent of *DIO2*/*DIO3*, whether *DIO2*/*DIO3* was affected but this was missed by our sampling regime, or whether temperature was having direct effects at the level of the gonads, remains unclear.

It is unclear how PT *TSHß* expression may be affected by perceived changes in temperature. To our knowledge, communication between temperature integration mechanisms in the POA and the PT has not been described. Given that there are small differences in metabolic rate between ground squirrels held at ambient temperatures of −1 and −6°C (Richter et al. 2015), metabolic signals to the PT cannot be excluded, although we would have expected to see metabolic signals converge on DIO signaling. One alternative possibility is that PT *TSHß* exhibits seasonal sensitivity to temperature cues. In a prior experiment with male arctic ground squirrels, we found that exposure to very warm temperatures (30 vs. 2°C control) in mid-hibernation had no effect on PT TSHß, but did cause an increase in *Dio2* expression along the third ventricle (Chmura et al. 2022). Given the methodological differences between these studies, it is not possible to determine whether differences between the prior study and present study are due to the scope of the change in temperature or seasonal changes in the temperature sensitivity of time keeping mechanisms, although these possibilities should be investigated further.

Females showed no changes in TSH/Dio/T3 signaling in response to warming and a slight decrease in ovarian mass. This is consistent with an earlier study, that found warming the hibernacula of animals housed outdoors in semi-natural conditions resulted in an earlier termination of hibernation in males, but not females (Barnes and Ritter 1993). In contrast, studies of free-living arctic ground squirrels indicate that females have greater phenological plasticity in when they end heterothermy than sexually maturing males (Sheriff et al. 2011; Williams et al. 2017). One potential explanation for differences observed in field studies vs. captive experiments is that thermal sensitivity, and any sex-differences therein, may vary across the hibernation season. In the present study, we manipulated the ambient temperature starting on the same calendar date for both sexes and similarly collected samples from both sexes at the same time. In the wild, females end heterothermy 3–5 weeks after males (Sheriff et al. 2011), which means we may have missed the window of thermal sensitivity for females by sampling too early. Future work to examine seasonal sex-differences in temperature sensitivity would be illuminating.

Relatively few studies have investigated the impacts of warming on seasonal retrograde TSH/Dio/T3 signaling, and across these studies, experimental paradigms differ slightly and address distinct hypotheses (Ikegami et al. 2015; Trivedi et al. 2019; Renthlei et al. 2021; van Rosmalen 2021; van Rosmalen et al. 2021; Chmura and Williams 2022). Important methodological differences, include selection of photoperiodic conditions, which ranged from constant darkness consistent with the hibernaculum environment to long-day conditions mimicking either early or late spring or summer.

Temperature selection also differed across these studies with one designed to investigate the impact of a noxiously high temperature treatment (Renthlei et al. 2021) and others focusing on presumably favorable mild warming treatments. Given the fundamentally different paradigms and taxonomic variation represented in these studies, it is perhaps unsurprising that a clear narrative about the impact of temperature on seasonal TH signaling is still emerging: some studies report effects on TSHß only (present study), others on one or multiple deiodinases (vole spp: van Rosmalen et al. 2021; arctic ground squirrels: Chmura et al. 2022) a few affect both TSHß and deiodinases (Trivedi et al. 2019; Renthlei et al. 2021) and one reports no PT TSHß or hypothalamic deiodinase response (Japanese quail: Ikegami et al. 2015). At present, it is premature to make conclusions regarding the effects of temperature and TSH/Dio/T3 signaling, although it seems clear that this seasonal signaling pathway is modulated by temperature cues.

Conclusion

Elucidating the mechanisms by which temperature influences seasonal timing remains an important component of predicting the adaptive responses of organisms to interannual differences in seasonality and to ongoing climate change. As has been argued previously (Caro et al. 2013; Chmura and Williams 2022), this mechanism has proven stubbornly elusive, which impedes our ability to understand the capacity of organisms to respond to climate change using existing phenotypic plasticity. Here, we show relationships between warming, TH signaling, and reproductive development in male, but not female, hibernating ground squirrels. This highlights the importance of not only continuing to investigate the mechanisms of temperature integration in seasonal timekeeping but also incorporating sex differences into these studies (Caro 2012; Williams et al. 2022)

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Supplementary data

Supplementary data available at *ICB* online.

Data availability statement

Raw data and images are available from the Dryad repository: https://doi.org/10.25338/B8QD1H.

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