

## RESEARCH ARTICLE

# Food and temperature change photoperiodic responses in two vole species

Laura van Rosmalen<sup>\*,†</sup> and Roelof A. Hut

## ABSTRACT

Seasonal timing of reproduction in voles is driven by photoperiod. We hypothesized that a negative energy balance can modify spring-programmed photoperiodic responses in the hypothalamus, controlling reproductive organ development. We manipulated energy balance by the 'work-for-food' protocol, in which voles were exposed to increasing levels of food scarcity at different ambient temperatures under long photoperiod. We found that in common voles (*Microtus arvalis*) and tundra voles (*Microtus oeconomus*), photoperiod-induced pars tuberalis thyroid-stimulating hormone  $\beta$ -subunit (*Tsh $\beta$* ) expression is reduced to potentially inhibit gonadal development when food is scarce. Reduction in gonadal size is more pronounced in tundra voles, in which anterior hypothalamic *Kiss1* is additionally downregulated, especially in males. Low temperature additionally leads to decreased hypothalamic *Rfrp* expression, which potentially may facilitate further suppression of gonadal growth. Shutting off the photoperiodic axis when food is scarce in spring may be an adaptive response to save energy, leading to delayed reproductive organ development until food resources are sufficient for reproduction, lactation and offspring survival. Defining the mechanisms through which metabolic cues modify photoperiodic responses will be important for a better understanding of how environmental cues impact reproduction.

**KEY WORDS:** Seasonal reproduction, Photoperiodism, Ambient temperature, Food scarcity, Hypothalamic gene expression, Voles

## INTRODUCTION

Seasonal mammals time their reproduction such that offspring will be born during the most optimal time of year, when temperatures are rising and food is abundant. Because of the absence of inter-annual variation in photoperiodic cycles, many vertebrates use photoperiod as a reliable cue to synchronize intrinsic annual timing mechanisms controlling seasonal adaptation of physiology and behavior (reviewed in Baker, 1938; Nakane and Yoshimura, 2019). In mammals, photoperiodic signals are perceived by retinal photoreceptors, and converted in the brain via the suprachiasmatic nucleus and the pineal gland into melatonin signals regulating gonadal responses by the so called 'photoperiodic neuroendocrine system' (PNES) (reviewed in Dardente et al., 2018; Hut, 2011;

Nakane and Yoshimura, 2019). Under long days, when there is a short period of melatonin release (Tamarkin et al., 1985), the pars tuberalis produces thyroid-stimulating hormone  $\beta$ -subunit (TSH $\beta$ ) (Nakao et al., 2008), which binds to glycoprotein hormone  $\alpha$ -subunit ( $\alpha$ -GSU) to form an active dimer (TSH) (Magner, 1990) that regulates the balance between type 2/3 iodothyronine deiodinase (DIO2/DIO3) in the tanycytes (Guerra et al., 2010; Hanon et al., 2008; Nakao et al., 2008), which subsequently controls the amount of available active thyroid hormone (triiodothyronine,  $T_3$ ) in the mediobasal hypothalamus (Lechan and Fekete, 2005).  $T_3$ , which is required for creating seasonal rhythms (Hazlerigg and Loudon, 2008), possibly indirectly regulates gonadotropin-releasing hormone (GnRH) pulsatility via other hypothalamic areas, leading to gonadotropin release by the anterior pituitary and ultimately reproductive activation.


Animals that experience food scarcity reduce their overall food consumption, while foraging activity is increased (van der Vinne et al., 2019). This induces a negative energy balance, in which there is less energy available for reproductive investment, because most energy ingested is needed for body tissue maintenance and thermoregulation. Energy balance and reproduction are closely related (Ruffino et al., 2014; Schneider, 2004), as food restriction in different species of seasonal breeders leads to sexual arrest (Nelson et al., 1997; Young et al., 2000), but its regulatory mechanisms remain to be disclosed. Seasonally breeding animals, such as voles, may use a combination of photic and non-photoc seasonal cues to control reproduction. Environmental factors such as ambient temperature, food availability and its behavioral foraging activity response can all affect energy balance and are expected to be involved in adaptive modification of the photoperiodic response to inhibit or accelerate reproductive development (Caro et al., 2013; Hut et al., 2014).

The neuroanatomical networks that underly integration of energy balance information into the photoperiodic response system are largely unknown. Neurons expressing GnRH are known to be the ultimate driver of the reproductive axis controlling the release of hormones (i.e. luteinizing hormone and follicle-stimulating hormone) from the pituitary gland (Guillemin, 1977; Schally et al., 1970). Prior studies in Siberian hamsters show that mediobasal hypothalamic thyroid hormone ( $T_3$ ), which is increased under long photoperiods, does not regulate GnRH expression directly, but facilitates gonadal growth, indicating an indirect effect on GnRH release (Banks et al., 2016). Perhaps  $T_3$  signals rather via other hypothalamic areas, such as the preoptic area (POA), the dorsomedial/ventromedial hypothalamus (DMH/VMH) and the arcuate nucleus (ARC), which are involved in regulating energy homeostasis (for review, see Hut et al., 2014). Neurons located in those hypothalamic regions communicate directly with GnRH neurons (Hileman et al., 2011), and express RF-amides: Kisspeptin (KISS1) (Smith et al., 2005a; Smith et al., 2005b) and RF-amide related peptide (RFRP-3). KISS1 functions as a strong activator of

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**List of abbreviations**

ARC	arcuate nucleus
<i>Dio2</i>	iodothyronine deionidase 2
<i>Dio3</i>	iodothyronine deionidase 3
DMH	dorsomedial hypothalamus
Gnrh	gonadotropin-releasing hormone
<i>Kiss1</i>	kisspeptin
LP	long photoperiod
PNES	photoperiodic neuroendocrine system
POA	preoptic area
<i>Rfrp</i> ( <i>Npvf</i> )	RF-amide related peptide
SP	short photoperiod
T <sub>3</sub>	triiodothyronine
<i>Tshβ</i>	thyroid-stimulating hormone β-subunit
<i>Tshr</i>	thyroid-stimulating hormone receptor
VMH	ventromedial hypothalamus
WFF	work-for-food

GnRH neurons, and therefore is an important regulator of puberty onset and reproduction (De Roux et al., 2003; Seminara et al., 2004). In Siberian hamsters and Wistar rats, RFRP-3 is involved in regulating food intake and modulating somatic growth (Cázares-Márquez et al., 2019; Cázares-Márquez et al., 2020; Cázares-Márquez et al., 2021). Furthermore, hypothalamic RFRP-3 and KISS1 are regulated by photoperiod and regulate activation of the reproductive axis (Ancel et al., 2012; Henningsen et al., 2016; Henningsen et al., 2017; Hut et al., 2014; Revel et al., 2006; Revel et al., 2007; Ubuka et al., 2012). The involvement of KISS1- and RFRP-3-expressing neurons in photoperiodic, metabolic and reproductive regulation suggests that these may be potential sites for integration of metabolic cues to modulate photoperiodic signals mediating seasonal reproductive responses (Janati et al., 2013; Klosen et al., 2013; Revel et al., 2006; Revel et al., 2008).

To investigate mechanisms of adaptive modification of the photoperiodic response, we decided to use two vole species as study organisms (common vole, *Microtus arvalis*; and tundra vole, *Microtus oeconomus*). Voles may be the ideal species to study these questions as voles can have strong photoperiodic responses and a functional canonical PNES (Król et al., 2012; van Rosmalen et al., 2020; van Rosmalen et al., 2021). However, small short-lived mammals such as voles are expected to have an opportunistic dimension to their breeding strategy. Moreover, voles are known to be able to respond to food availability, which is a characteristic of an opportunistic breeder (Daksete and Martinet, 1977; Ergon et al., 2001; Negus and Berger, 1977; Nelson et al., 1983; Sanders et al., 1981). Common voles are distributed in central Europe (38–62°N) (Yigit et al., 2016), whereas tundra voles are distributed at more northern latitudes (48–72°N) (Linzey et al., 2016). Voles from our two lab populations originate from the same latitude in The Netherlands (53°N), which is for the common vole at the center of its latitudinal range, and for the tundra vole at the southern boundary of its latitudinal range. For this reason, it is expected that our common vole lab population is better adapted to the local environment at 53°N than our tundra vole lab population, which may be better adapted to more northern latitudes. Bronson (1988) proposed that the use of photoperiod, ambient temperature and food availability as cues for regulating reproduction depends on the local climate, which varies with latitude. At temperate latitudes where seasonal climates are highly predictable, mammals may use photoperiod as a proxy to time reproduction when the environment is favorable. At more northern latitudes, weather conditions such as

snow fall and snow melt are less predictable, and therefore also the timing of food availability is less predictable. Perhaps, at northern latitudes, voles use food as a more reliable cue to become fertile. Presumably, differences in hypothalamic neurobiological mechanisms may underly the different breeding strategies of the common and the tundra vole.

In this study, voles were exposed to photoperiodic transitions mimicking spring, under which both ambient temperature and food availability were manipulated. By implementing the work-for-food (WFF) paradigm, we can induce different levels of natural food scarcity in the laboratory, leading to a negative energy balance in small rodents on a high workload (Hut et al., 2011; van der Vinne et al., 2014). We assessed how (i.e. which genes) and where in the brain photoperiodic and metabolic cues are integrated to mediate reproductive responses, and how this neurobiological system is differently shaped in two closely related vole species.

**MATERIALS AND METHODS****Animals**

All experimental procedures were carried out according to the guidelines of the animal welfare body (IvD) of the University of Groningen, and all experiments were approved by the Centrale Commissie Dierproeven of the Netherlands (CCD, license number: AVD1050020186147). Common voles, *Microtus arvalis* (Pallas 1778) were obtained from the Lauwersmeer area (The Netherlands, 53°24'N, 6°16'E) (Gerkema et al., 1993). Tundra or root voles, *Microtus oeconomus* (Pallas 1776) were obtained from four different regions in The Netherlands (described in van de Zande et al., 2000). All voles in this study were indoor bred as an outbred colony at the University of Groningen. Over the last 5 years, our laboratory populations have been kept under long photoperiod (LP, 16 h light:8 h dark) conditions and switched to a short photoperiod (SP, 8 h light:16 h dark) for approximately two consecutive months at least twice a year.

All voles used in this study were gestated and born under a SP and transferred to a LP on the day of weaning at either 21±1 or 10±1°C. A photoperiodic transition from SP to LP simulates spring, during which voles become reproductively active in nature. Based on photoperiodic dose–response curves for gonadal mass from our prior research (van Rosmalen et al., 2021), we selected the photoperiod where maximum gonadal responses were reached, to obtain identical physiological status for the two species at the start of the experiments (common voles: 16 h light:8 h dark; tundra voles: 14 h light:10 h dark). Animals were transferred to cages (15×32×13 cm<sup>3</sup>) provided with running wheels (14 cm diameter) when they were 35 days old. *Ad libitum* food was available for all animals until they were 40 days old. Animals were provided with water *ad libitum* throughout the course of the experiments.

**WFF protocol**

In the WFF protocol (starting when animals were 40 days old), animals had to make a set number of wheel revolutions in order to receive a 45 mg grain-based food pellet (630 J per pellet; F0165, Bio-Serv, Flemington, NJ, USA), using a computer-controlled food dispenser (Med Associates Inc., St Albans, VT, USA). All animals started on a low workload protocol (100 revolutions/pellet=0.07 m J<sup>-1</sup>), which is similar to *ad libitum* food conditions, as there were always pellets present in the cages. Half of the animals were subsequently exposed to an increasing workload paradigm in which workload was increased daily by an additional 10–30 revolutions per pellet. A detailed description of the WFF protocol for this experiment has been published elsewhere (van Rosmalen and

Hut, 2021). In short, the increase in workload per day was titrated to obtain moderate individual body mass loss ( $0\text{--}0.5\text{ g day}^{-1}$ ) and the amount of pellets earned per day ( $>44\text{ kJ day}^{-1}$ ). All voles were weighed every other day throughout the course of the experiments, in order to carefully monitor growth and to keep animals above 75% of their initial body mass (35 days old).

### Tissue collection

Animals were killed by decapitation, with short prior  $\text{CO}_2$  sedation,  $17\pm 1\text{ h}$  after lights off (common voles: zeitgeber time, ZT9; tundra voles: ZT7) at 75 days old. During brain dissection, special care was taken to include the intact pituitary stalk containing the pars tuberalis by cutting the stalk half-way between the hypothalamus and pituitary gland residing in the hypophysial fossa. To further dissect the hypothalamus, the cerebellum, bulbus olfactorius and frontal cortex were removed by coronal cuts. A sagittal hypothalamic tissue block was obtained by lateral sagittal cuts at the hypothalamic sulci and a horizontal cut at 4 mm distance from the ventral border of the hypothalamus. The posterior hypothalamus (containing the pars tuberalis, DMH, VMH and arcuate nucleus) and anterior hypothalamus (containing the POA, paraventricular nucleus, periventricular zone of hypothalamus and suprachiasmatic nucleus) were separated by a coronal cut at the posterior border of the optic chiasm and the mammillary bodies. After dissection, it was checked that the remainder of the pituitary gland, containing the pars nervosa, pars intermedia and pars distalis, was still intact in the sella turcica covered by the dura mater. The sampled tissues were flash frozen within 2–4 min of death in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until RNA extraction. Reproductive organs were dissected and cleaned of fat, and wet mass of testes, paired ovary and uterus were measured ( $\pm 0.0001\text{ g}$ ).

### RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA was extracted from the anterior and posterior hypothalamic area using TRIzol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Frozen pieces of tissue ( $\sim 20\text{ mg}$ ) were homogenized in 0.5 ml TRIzol in a TissueLyser II (Qiagen, Hilden, Germany) ( $2\times 2\text{ min}$  at  $30\text{ Hz}$ ) using tubes containing a 5 mm RNase free stainless-steel bead; 0.1 ml chloroform was added for phase separation. Following RNA precipitation by 0.25 ml of 100% isopropanol, the obtained RNA pellets were washed with 0.5 ml of 75% ethanol. RNA was diluted in RNase-free  $\text{H}_2\text{O}$  (range  $20\text{--}100\text{ }\mu\text{l}$ ) and quantified on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, DNA was removed by DNase I treatment (Invitrogen), and equal quantities of RNA were used for cDNA synthesis using RevertAid H Minus first strand cDNA synthesis reagents (Thermo Scientific). Reverse transcription (RT;  $20\text{ }\mu\text{l}$ ) reactions were prepared using  $1\text{ }\mu\text{g}$  RNA,  $100\text{ }\mu\text{mol l}^{-1}$  oligo(dT) $_{18}$ ,  $5\times$  reaction buffer,  $20\text{ U }\mu\text{l}^{-1}$  RiboLock RNase Inhibitor,  $10\text{ mmol l}^{-1}$  dNTP Mix and RevertAid H Minus M-MuLV Reverse Transcriptase ( $200\text{ U }\mu\text{l}^{-1}$ ) (Table S1). RNA was reversed transcribed using a thermal cycler (S1000; Bio-Rad, Hercules, CA, USA). Incubation conditions used for RT were  $45^\circ\text{C}$  for 60 min followed by  $70^\circ\text{C}$  for 5 min. Transcript levels were quantified by real-time quantitative PCR (qPCR) using SYBR Green (KAPA SYBR FAST qPCR Master Mix, Kapa Biosystems). Reactions ( $20\text{ }\mu\text{l}$  volume) were carried out in duplicate for each sample using 96-well plates in a Fast Real-Time PCR System (StepOnePlus, Applied Biosystems, Waltham, MA, USA) (Table S2). Primers for genes of interest were designed using Primer-BLAST (NCBI). All primers were designed based on the annotated *Microtus ochrogaster* genome (NCBI:

txid79684, GCA\_000317375.1), and subsequently corrected for gene specificity in the genomes of the common vole, *Microtus arvalis* (NCBI:txid47230, GCA\_007455615.1) and the tundra vole, *Microtus oeconomus* (NCBI:txid64717, GCA\_007455595.1), which both have been sequenced as a collaborative effort between the Hut lab (Groningen), the Hazlerigg lab (Tromsø) and the Sandve lab (Oslo) (Table S3). Relative mRNA expression levels were calculated based on the  $\Delta\Delta\text{CT}$  method using *Gapdh* as the reference gene (Pfaffl, 2001).

### Statistical analysis

Sample size ( $N=6\text{--}8$ ) was determined by a power calculation ( $\alpha=0.05$ , power=0.95) based on the effect size ( $d=2.26$ ) of our previous study, in which gonadal mass was assessed in female and male voles under two different photoperiods (van Rosmalen et al., 2020). Effects of workload, temperature and interactions on gonadal mass, body mass and gene expression levels were determined using type I two-way ANOVA. Tukey HSD *post hoc* pairwise comparisons were used to compare groups. Statistical significance was determined at  $\alpha=0.05$ . Statistical results can be found in Table S4. Analyses were performed using RStudio (version 1.2.1335; <http://www.R-project.org/>), and all figures were generated using the ggplot2 package (Wickham, 2016).

## RESULTS

### Food scarcity reduces reproductive organ mass even under long photoperiods

The reduced body mass in high workload voles (Fig. 1G–J; Table S4) confirms that a negative energy balance was induced by the WFF protocol. This negative energy balance also caused a 15–47% reduction in testes mass (Fig. 1A,D; Table S4), a 0–50% reduction in ovary mass (Fig. 1B,E; Table S4) and an 18–60% reduction in uterus mass (Fig. 1C,F; Table S4). This effect appeared to be stronger in tundra voles, and in female voles at low temperature. In contrast, reproductive organ mass corrected for body mass (gonadosomatic index) was not reduced by high workloads (Fig. 1K–P).

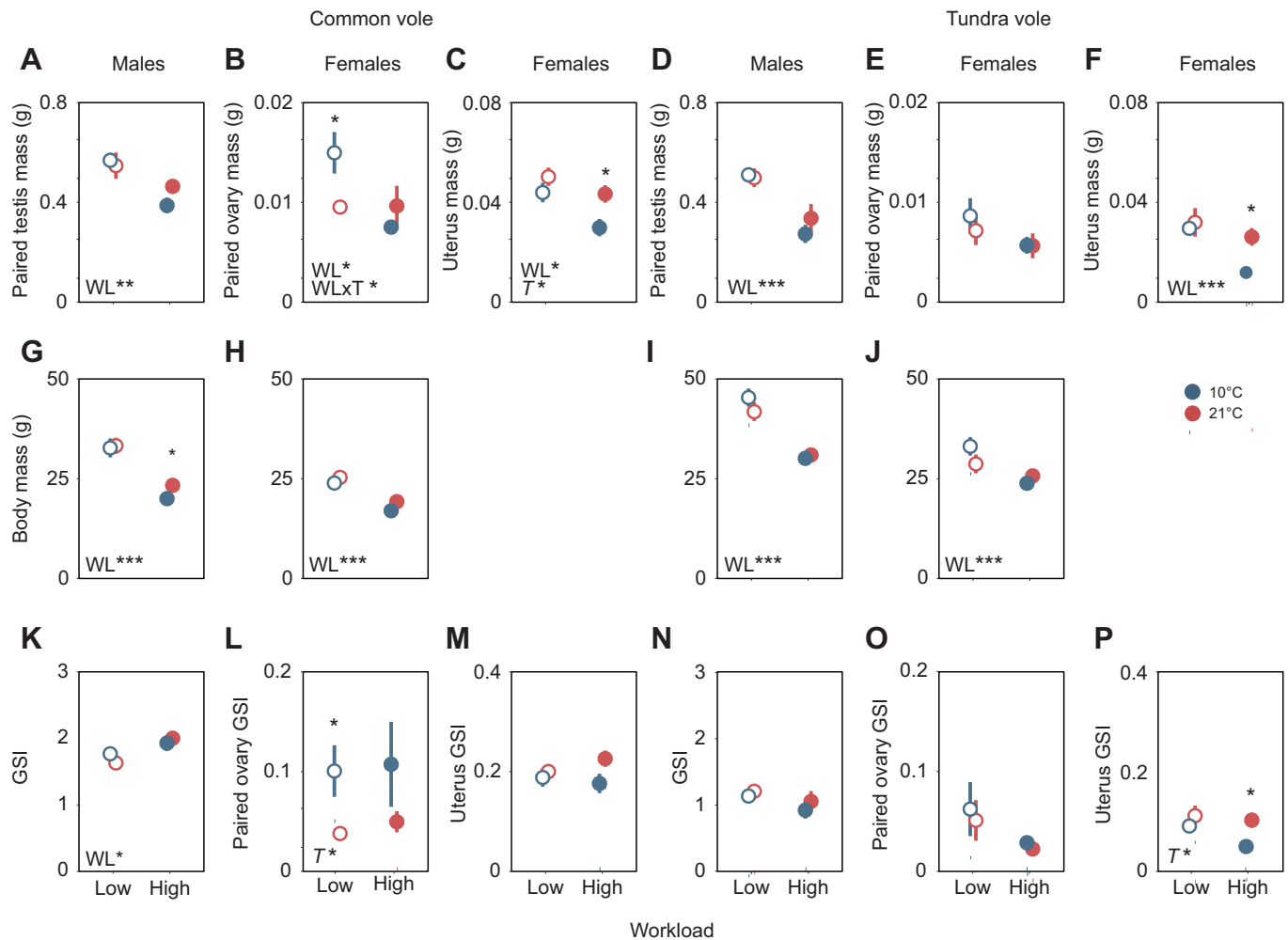
### Food scarcity under LP suppresses *Tshb* expression in the pars tuberalis

To test at what level of the signaling cascade metabolic cues may act to modify PNES output signals, we measured gene expression levels in the posterior and anterior hypothalamus. Because the posterior hypothalamic block did not contain the pars distalis, *Tshb* expression can be exclusively attributed to the pars tuberalis. *Tshb* expression was significantly reduced (50% reduction) in male voles at high workloads (Fig. 2A,C; Table S4). In males, this effect was stronger in common voles than in tundra voles (Fig. 2A,C; Table S4). In females, this effect was only observed in common voles at  $10^\circ\text{C}$  (Fig. 2B,D; Table S4). *Tshb* expression was for the most part unaffected by temperature; only a significant reduction was found in female common voles at  $10^\circ\text{C}$  (Fig. 2A–D; Table S4). Overall, *Tshb* and gonadal mass show a positive relationship (Fig. 3A–D), suggesting that *Tshb* may be involved in suppressing gonadal development when food is scarce.

After translation, TSH $\beta$  and  $\alpha$ -GSU locally dimerize to form active TSH, which can bind to its receptor (TSHr) located in the tanycytes around the third ventricle of the brain. Workload did not affect *Tshr* expression in both sexes of both species (Fig. 2A–D; Table S4). Although common vole females showed slightly elevated *Tshr* expression at  $10^\circ\text{C}$ , general *Tshr* levels were lower in common voles than in tundra voles (Fig. 2A–D; Table S4).

Although TSH generally leads to increased *Dio2* levels, workload-induced changes in *Tshb* were not reflected in *Dio2*





**Fig. 1. Food scarcity and ambient temperature effects on gonadal mass and body mass in male and female voles.** (A,D) Paired testis mass, (B,E) paired ovary mass, (C,F) uterus mass, (G–J) body mass and (K–P) gonadosomatic index (GSI) for common and tundra voles at low (open symbols) or high workload (filled symbols), at 10°C (blue) or 21°C (red). Data are presented as means  $\pm$  s.e.m. ( $n=6-8$ ). Significant effects (two-way ANOVA) of workload (WL), temperature (T) and their interactions (WL  $\times$  T) are shown: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Significant differences between groups (one-way ANOVA) are indicated by asterisks next to data points. Statistical results for ANOVA can be found in Table S4.

expression (Fig. 2A–D), suggesting that modifying factors other than TSH can affect posterior hypothalamic *Dio2* expression.

*Kiss1*-expressing neurons are located in the ARC nucleus in the posterior hypothalamus and are potentially involved in metabolic regulation and food intake depending on various conditions such as metabolic status and sex (reviewed in Hut et al., 2014; Simonneaux, 2020; Yeo and Colledge, 2018). Workload and temperature did not affect *Kiss1* expression in the posterior hypothalamus (Fig. 2A–D; Table S4).

The DMH and VMH of the posterior hypothalamus are nuclei that are involved in the regulation of feeding behavior and both these areas are capable of expressing *Rfrp* (i.e. *Npyf*) (Angelopoulou et al., 2019; Talbi et al., 2016). Although overall *Rfrp* expression was higher at 21°C, no effects of workload on *Rfrp* expression were observed (Fig. 2A–D; Table S4).

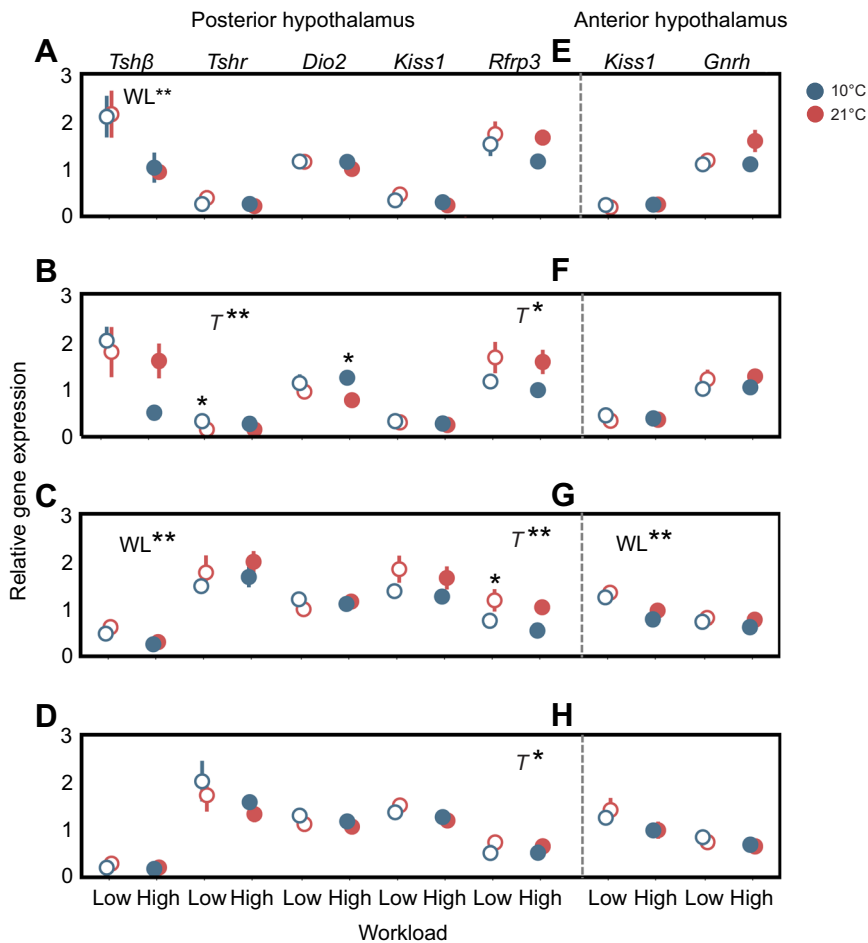
#### Food scarcity suppresses *Kiss1* expression in the anterior hypothalamus of tundra vole males

*Kiss1*-expressing neurons in the anterior hypothalamus are located in the POA, which is involved in temperature regulation (Hrvatín et al., 2020; Takahashi et al., 2020). *Kiss1* expression in the anterior

hypothalamus was decreased at high workload in tundra vole males (Fig. 2G; Table S4), whereas *Kiss1* expression was close to zero in both male and female common voles (Fig. 2E,F; Table S4). *Kiss1* expression in the anterior hypothalamus was not affected by temperature (Fig. 2E–H; Table S4). Anterior hypothalamic *Kiss1* showed a positive relationship with gonadal mass only in tundra vole males at high workload (Fig. 3E–H). This finding may indicate that the *Kiss1* system is potentially involved in modifying photoperiodic responses when food is scarce in tundra voles, but not in common voles.

Perikarya of GnRH neurons are also located in the POA and increased frequency of pulsatile release of GnRH through axonal projections into the median eminence is described to regulate gonadotropin release in the pars distalis of the pituitary gland (Lincoln and Fraser, 1979). *Gnrh* expression in the anterior hypothalamus was not affected by workload or temperature (Fig. 2E–H; Table S4).

Fitted linear models revealed that reproductive organ mass can be best predicted by: *Tshb* and *Tshr* in common vole males ( $F_{2,23}=7.44$ ,  $P < 0.01$ ); *Tshb* in common vole females ( $F_{1,23}=4.89$ ,  $P < 0.05$ ); *Tshb*, *Rfrp* and anterior hypothalamic *Kiss1* in tundra vole



**Fig. 2. Food scarcity and ambient temperature affect gene expression in the posterior and anterior hypothalamus.** Relative gene expression levels of *Tshb*, *Tshr*, *Dio2*, *Kiss1* and *Rfrp3* in the posterior hypothalamus (A–D) and *Kiss1* and *Gnhr* in the anterior hypothalamus (E–H) for (A,E) common vole males, (B,F) common vole females, (C,G) tundra vole males and (D,H) tundra vole females, at low (open symbols) or high workload (filled symbols), at 10°C (blue) or 21°C (red). Data are presented as means±s.e.m. ( $n=6-8$ ). Significant effects (two-way ANOVA) of workload (WL) and temperature (T) are shown: \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . Significant differences between groups (one-way ANOVA) are indicated by asterisks next to data points. Statistical results for ANOVA can be found in Table S4.

males ( $F_{3,28}=8.47$ ,  $P<0.001$ ); and *Tshb* and anterior hypothalamic *Kiss1* in tundra vole females ( $F_{2,21}=3.84$ ,  $P<0.05$ ).

## DISCUSSION

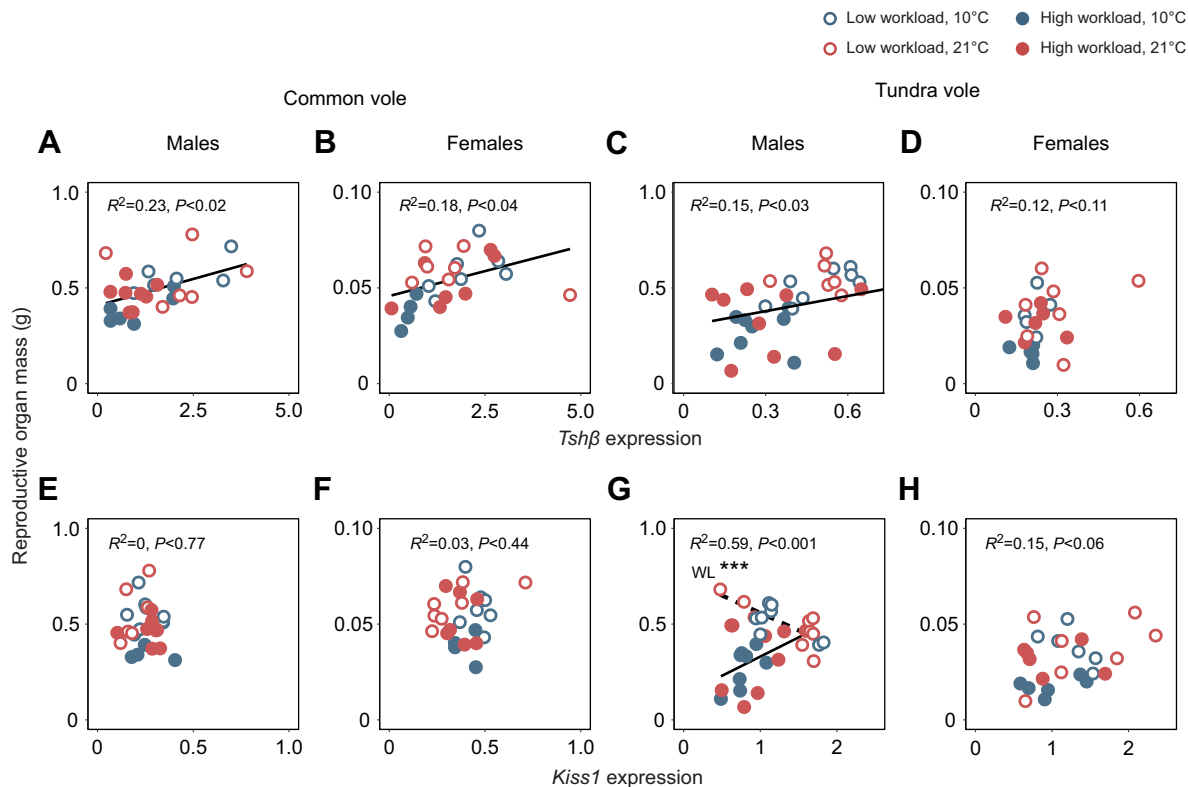
Our data demonstrate that photoperiodic responses driving gonadal activation can be modified by negative energy balance. Food scarcity seems to act in part via the pars tuberalis to downregulate local levels of TSH, which potentially may lead in turn to suppression of gonadal growth, especially in common voles. Tundra vole males additionally may use the hypothalamic *Kiss1* system to control reproduction when food is scarce at long photoperiods. Observed patterns in *Kiss1* expression were not reflected in *Gnhr* expression, but changes in gonadotropin release would be expected as KISS1 is the main driver for GnRH release (De Roux et al., 2003; Han et al., 2005; Han et al., 2015; Seminara et al., 2004). However, our data have to be interpreted with caution as the current study only considered gene expression levels and did not investigate protein levels. Furthermore, relaxation of natural selection in our laboratory colonies cannot be excluded.

Although, reproductive organ mass in tundra vole females is reduced at high workloads, no effects at the level of candidate genes have been observed. In general, low temperature enhances the inhibitory effects of reduced energy intake on gonadal size. Within the hypothalamus, we show that reduced *Rfrp* levels may correlate with the decreased reproductive organ mass observed at low temperature under high workloads.

Here, we chose to investigate the reproductive effects of a negative energy balance in young animals, as voles can reach sexual maturity

within 40 days depending on environmental conditions. A negative energy balance under LP exerts similar effects on testis size of common (Fig. 1A) and tundra voles (Fig. 1D) to those in deer mice, *Peromyscus maniculatus* (Nelson et al., 1997). The lack of this effect in Siberian hamsters, *Phodopus sungorus*, may be explained by the fact that food was minimally reduced to 80–90% of *ad libitum* levels in this study (Paul et al., 2009). Photoperiod seems to be the driving factor for gonadal development in animals under positive or neutral energy balance, or even with a moderate negative energy balance. A further reduction in food intake counteracts the stimulating effects of LP on gonadal development, leading to small testes, ovaries and uterus (Fig. 1). Although large testes are generally associated with high spermatogenic activity and high androgen levels, vole testicular mass may drop in summer while spermatogenic activity remains high (Adams et al., 1980). Therefore, testicular mass is a reliable indicator of fertility in spring, but less so in summer. Here, voles were exposed to spring photoperiod transitions; therefore, we assume that in our study, testes mass is a reliable predictor for fertility. Temperature did not affect testicular mass under LP when food was available *ad libitum* (Fig. 1A,D). This finding is consistent with prior reports in Siberian hamsters (Steinlechner et al., 1991) and Prairie voles, *Microtus ochrogaster* (Nelson et al., 1989).

Lowering ambient temperature under high feeding-related workloads further increases metabolic demand in females, as confirmed by reduced uterine size (Fig. 1C,F). Ovaries of Syrian hamsters (*Mesocricetus auratus*) at low temperature or in the absence of light did not change in size, but had fewer follicles and corpora lutea (Reiter, 1968). This indicates that ovary mass is a poor indicator



**Fig. 3. Relationship between reproductive organ mass, *Tshb* and *Kiss1* expression.** Correlation between reproductive organ mass (males: paired testis mass, females: paired ovary+uterus mass) and (A–D) *Tshb* expression in the posterior hypothalamus and (E–H) *Kiss1* expression in the anterior hypothalamus for common voles and tundra voles at low (open symbols) or high workload (filled symbols), at 10°C (blue) or 21°C (red). Linear models are fitted and  $R^2$  and  $P$ -values are shown. Significant effect of workload (WL) was found in G: \*\*\* $P$ <0.001.

of hormonal secretory activity. Here, we did not perform histological analysis on ovaries, so these data should be interpreted with caution. However, small uteri at low temperature are related to reduced thickness of secretory epithelium and the number of endometrial glands (Reiter, 1968). This confirms that the decline in uterine mass at high workload and low temperature is the result of incomplete development of uterine glands. This may lead to infertility, as uterine glands are essential for pregnancy (Cooke et al., 2013).

Energetically challenged voles do not enter torpor, as observed in house mice (Hut et al., 2011), but average body temperature is decreased by  $\sim 0.5^\circ\text{C}$ , yielding limited energy savings (Nieminen et al., 2013; van der Vinne et al., 2015; van Rosmalen and Hut, 2021). This results in reduced reproductive investment, because all ingested energy is needed for maintaining organ function crucial to survive. Reproductive organ development may persist as ambient temperature and food resources are sufficient for lactation and pup growth.

Our data show that photoperiodic genes expressed in the anterior and posterior hypothalamus are potential regulators modifying photoperiodic responses under energetically challenging conditions to reduce gonadal activation. The short duration of pineal melatonin release under LP leads to increased pars tuberalis *Tshb*, which serves a pivotal role in the PNES (Hanon et al., 2008; Ono et al., 2008). The present study reveals that the photoperiod-induced *Tshb* signal can be downregulated by a negative energy balance in common vole males at both temperatures, common vole females only at 10°C and in tundra vole males at both temperatures (Fig. 2A–C). Thus, reduced food availability decreases *Tshb* mRNA at the level of the pars tuberalis, either by decreasing transcription or by increasing post-transcriptional processes. This indicates that a negative energy balance can modify photoperiodic responses at the level of the pars

tuberalis or even more upstream in the photoperiodic axis, primarily in common voles. Potentially, the pars tuberalis receives information about an animal's fat content via leptin receptors which are highly expressed in the median eminence (Huang et al., 1996). However, whether indeed low leptin levels in voles under a negative energy balance cause reduced pars tuberalis *Tshb*, remains to be investigated.

The lower *Tshb* levels and therefore higher *Tshr* levels in tundra voles might be attributed to the different photoperiod regimens (16 h light:8 h dark for common voles; 14 h light:10 h dark for tundra voles). For this reason, it cannot be excluded that in tundra voles under 16 h light:8 h dark, a negative energy balance can decrease pars tuberalis *Tshb* levels to a similar extent to that observed in common voles.

Pars tuberalis-derived TSH binds locally to TSHr in the tanycytes, where it systematically leads to increased *Dio2* (Guerra et al., 2010; Hanon et al., 2008; Nakao et al., 2008). The observed *Tshb* suppression caused by a negative energy balance is not reflected in tanycyte *Dio2* expression (Fig. 2A–D). This suggests that TSH modulates central  $T_3$  levels and ultimately gonadal development, via pathways parallel to the *Dio2*/*Dio3* system.

*Dio3* and thyroid hormone levels were not measured in the current study, and therefore we cannot exclude that the environmental manipulations may have affected downstream thyroid functioning. Indeed, it has been shown that temperature affects thyroid function, and hence  $T_3$  metabolism, which impacts seasonal breeding in quails (Ikegami et al., 2015). However, sex steroid feedback on gene expression in the tanycytes, but not in the pars tuberalis, as observed in ewes, could provide an explanation for unaltered *Dio2* levels (Lomet et al., 2020). Furthermore, a

disconnection in the seasonal neuroendocrine response has also been shown in ewes, illustrating a multistep signaling cascade in the mammalian photoperiodic neuroendocrine system (Dardente et al., 2019; Hazlerigg et al., 2018). Interestingly, food restriction in LP-housed Siberian hamsters led to reduced *Dio2* and reduced serum  $T_3$  levels (Herwig et al., 2009), which suggests that integrating of metabolic cues in the PNES takes place at different levels of the pathway in hamsters and voles.

The 2-fold higher *Dio2* levels in this study compared with our previous experiments might be explained by the fact that here animals were born at SP and transferred to LP at weaning, whereas our previous study used constant LP conditions (van Rosmalen et al., 2020). This effect of maternal photoperiodic programming on tanycyte gene expression has previously been confirmed (Sáenz de Miera et al., 2017; van Rosmalen et al., 2021). In addition, 2–3 day fasted rats show elevated *Dio2* mRNA levels in tanycytes (Coppola et al., 2005; Diano et al., 1998). This might be an acute effect, which disappears when food is restricted for longer periods as in our study (i.e. 35 days). Stable *Tsh $\beta$*  and *Dio2* levels at different temperatures at low workload under LP in spring-programmed voles were confirmed by *in situ* hybridization in our prior experiments (van Rosmalen et al., 2021). This indicates that our brain dissections in combination with RT-qPCR are a reliable method to assess gene expression at the level of the pars tuberalis and the tanycytes.

At the level of the posterior hypothalamus, where the DMH/VMH are located, low temperature induced a small reduction in *Rfrp* expression, but consistent with findings in Siberian hamsters (Paul et al., 2009), no effect of food scarcity was detected (Fig. 2A–D). In seasonal rodents, RFRP-3 synthesis appears to be primarily regulated by photoperiod, but effects of RFRP-3 on reproduction are highly variable between species, sex and photoperiodic condition (reviewed in Angelopoulou et al., 2019; Henningsen et al., 2016). Here, we show that *Rfrp* expression may also be an important regulator of the vole PNES to adaptively respond to ambient temperature changes. This finding is consistent with previous reports, showing that the *Rfrp* gene is a hypothalamic biomarker of ambient temperature in mice (Jaroslawska et al., 2015). Moreover, our findings are consistent with a field study in wild Brandt's voles, *Lasiopodomys brandtii*, in which elevated *Rfrp* expression levels were observed during the warmest part of the year (June–August) (Wang et al., 2019). As RFRP-3 is expected to mediate reproductive axis function, our findings suggest that downregulation of *Rfrp* expression by low temperature may be responsible for decreased reproductive organ mass observed at low temperature under high feeding-related workloads (Fig. 1A–F).

We next focused on hypothalamic *Kiss1* expression, because of its potential role in the integration of photoperiodic and metabolic cues controlling reproductive activity (Caro et al., 2013; Hut et al., 2014; Simonneaux, 2020). Hypothalamic Kisspeptin neurons act on GnRH neurons driving gonadotropin release, which promotes gonadal development (for review, see Simonneaux, 2020). *Kiss1* expression in the posterior hypothalamus, where the ARC is located, was not affected by either food or temperature (Fig. 2A–D). ARC *Kiss1* expression can be reversed by strong negative sex steroid feedback (Greives et al., 2008; Rasri-Klosen et al., 2017; Sáenz De Miera et al., 2014), which may explain similar *Kiss1* and *Gnrh* levels in different experimental groups (Fig. 2). In Siberian hamsters, food restriction causes a decrease in ARC *Kiss1* expression (Paul et al., 2009). As whole coronal sections were used in the study of Paul et al. (2009), thalamic and cortical areas contribute to the detected *Kiss1* expression levels, whereas we exclusively used hypothalamic tissue.

It seems important to note that the involvement of *KISS1* in PNES regulation by various environmental factors may be less generalizable across different species. For instance, reversed photoperiodic effects on ARC *Kiss1* expression have been shown in Syrian versus Siberian hamsters (Klosen et al., 2013). Furthermore, hypothalamic *Kiss1* expression in common voles is extremely low (Fig. 2A,B,E,F). These findings may suggest that *KISS1* systems have species-specific functions in regulating reproduction. Although *Kiss1* expression in the posterior hypothalamus was not affected, food scarcity caused downregulation of *Kiss1* expression in the anterior hypothalamus of tundra vole males (Fig. 2G), but not females (Fig. 2H). Voles can be induced ovulators, i.e. ovulation can be triggered by copulation, during which tactile stimulation of the cervix causes an ovulatory luteinizing hormone surge (Breed, 1967; Chitty and Austin, 1957; Hoyte, 1955). As animals were not exposed to copulation/mating in our experiments, the lack of an ovulatory luteinizing hormone surge, and therefore the lack of positive sex-steroid feedback on *KISS1* neurons in females may explain why preoptic *Kiss1* neurons were unaffected by food in females. POA *Kiss1* expression is known to be stimulated by positive sex steroid feedback (Ansel et al., 2010). Although the decrease in anterior hypothalamic *Kiss1* expression may therefore be the result of lower circulating testosterone levels due to lower testis mass, direct effects of metabolic cues cannot be excluded. Contrary to our expectations, anterior hypothalamic *Kiss1* was not affected by temperature. Common voles exhibit extremely low *Kiss1* expression levels under all conditions. Interestingly, *Kiss1* expression levels were considerably higher in tundra voles than in common voles. This species difference may explain the greater reduction in reproductive organ mass at a negative energy balance observed in tundra voles. It would be important to investigate how gene silencing and overexpression (*Tsh $\beta$* , *Kiss1* and *Rfrp*) in specific hypothalamic regions may affect reproductive development, to disentangle causal relationships between hypothalamic gene expression and reproductive responses related to energy balance. Of course, there are more hypothalamic neurotransmitters that can play a role: *Npy*, *Pomc* and other genes involved in energy metabolism are important candidates for this. Transcriptomic analysis of the Siberian hamster hypothalamus confirmed that *Pomc* is a primary target for long-term  $T_3$ -dependent regulation of somatic growth (Bao et al., 2019).

Differences in responses to food scarcity between common and tundra voles suggest that tundra voles use food as a more important external cue to time reproduction. This seems to be in line with the hypothesis that tundra voles may use an opportunistic breeding strategy, while common voles use a breeding strategy that is more driven by photoperiod (van Rosmalen et al., 2020). At northern latitudes, where tundra voles are abundant, voles live for a large part of the year under snow cover where light input is attenuated. Reproducing whenever food is available, either as leaves in summer and autumn or as roots during winter and spring, may be a favorable breeding strategy in such environments.

Our findings show that a negative energy balance induced by food scarcity and ambient temperature modifies long day responses in the PNES. In general, food scarcity regulates the photoperiodically regulated *Tsh $\beta$*  response in the pars tuberalis (primarily in common voles), and the *Kiss1* response in the anterior hypothalamus (exclusively in tundra vole males), whereas temperature regulates *Rfrp* in the posterior hypothalamus. Shutting off the photoperiodic axis when food is scarce or temperatures are low is an adaptive response that favors individual somatic maintenance and survival at the expense of reproductive organ development and current



reproductive output. In addition, delaying reproductive onset will yield energy savings, which results in less required foraging time and reduced exposure to predation, which will further increase individual survival and the probability of successful future reproductive attempts (van der Vinne et al., 2019). Defining the mechanisms through which metabolic cues modify photoperiodic responses will be important for a better understanding of how annual cycling environmental cues shape reproductive function and plasticity in life history strategies.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: L.v.R., R.A.H.; Methodology: L.v.R., R.A.H.; Validation: L.v.R., R.A.H.; Formal analysis: L.v.R.; Investigation: L.v.R., R.A.H.; Resources: R.A.H.; Data curation: L.v.R.; Writing - original draft: L.v.R., R.A.H.; Writing - review & editing: L.v.R., R.A.H.; Visualization: L.v.R.; Supervision: R.A.H.; Project administration: L.v.R.; Funding acquisition: R.A.H.

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#### Data availability

Data supporting the findings of this study are openly available from figshare: <https://doi.org/10.6084/m9.figshare.13522700.v1>

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**Table S1.** Concentrations of components used for Reversed-Transcription reactions

Component	Stock concentration	Final concentration
Oligo(dT) <sub>18</sub>	100 $\mu$ M	5 $\mu$ M
5X REACTION buffer	5X	1X
RiboLock RNase Inhibitor	20 U/ $\mu$ l	1 U/ $\mu$ l
dNTP Mix	10 mM	1 mM
RevertAid H Minus Reverse Transcriptase	200 U/ $\mu$ l	10 U/ $\mu$ l
Template RNA	0.1 $\mu$ g/ $\mu$ l	1 $\mu$ g/ $\mu$ l

**Table S2.** Thermal cycling conditions for qPCR.

qPCR step	T ( $^{\circ}$ C)	Duration (seconds)	Cycles
Enzyme activation	95	180	Hold
Denaturation	95	3	40
Annealing/ extension/ data acquisition	60	20	40
Dissociation	95	3	
	65	5	
	95	15	

**Table S3.** Primers used for qPCR.

Gene	Forward primer sequence (‘5-‘3)	Reverse primer sequence (‘5-‘3)
<i>Dio2</i>	CAGCCAACTCCGGACTTCTT	GCCGACTTCCTGTTGGTGTA
<i>Gapdh</i>	GCTGCCCAGAACATCATCCCTG	GACGACGGACACATTGGGGGTA
<i>Gnrh</i>	AGCACTTCGAATGCACTGTC	GGGTGTTGTGGATCCTTCGG
<i>Kiss1</i>	AACCAGGGACCAGTGAGAGTA	AGTGCAGTCATTCTGGCAGG
<i>Rfrp3</i>	AGGCAGGGATCTTGAACCAC	TCTCTGTAGCCAGCGACTCA
<i>Tsh<math>\beta</math></i>	GCTTATGGCAACAGGGTAGGA	AATACGCGCTCTCCCAGGAT
<i>Tshr</i>	ATCCCCAGTCTCGCGTTTTC	GCTTCTGGTGTTCGCGATTT



**Table S4.** Statistics for two-way ANOVAs

	<b>Common vole (<i>Microtus arvalis</i>)</b>				<b>Tundra vole (<i>Microtus oeconomus</i>)</b>			
	<b>paired testis mass (m)</b>				<b>paired testis mass (m)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,24	0.11227	12.488	< 0.002**	1,30	0.3313	21.425	< 0.001***
temperature (temp)	1,24	0.00505	0.562	< 0.47	1,30	0.0055	0.357	< 0.56
wl x temp	1,24	0.01662	1.848	< 0.19	1,30	0.0118	0.765	< 0.39
	<b>paired ovary mass (f)</b>				<b>paired ovary mass (f)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,22	0.0000788	4.751	< 0.05*	1,21	2.962e-05	2.487	< 0.14
temperature (temp)	1,22	0.0000258	1.554	< 0.23	1,21	3.900e-06	0.327	< 0.58
wl x temp	1,22	0.0000911	5.492	< 0.03*	1,21	3.170e-06	0.266	< 0.62
	<b>uterus mass (f)</b>				<b>uterus mass (f)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,22	0.0005666	6.310	< 0.02*	1,21	0.0008776	8.885	< 0.008**
temperature (temp)	1,22	0.0005904	6.575	< 0.02*	1,21	0.0004102	4.153	< 0.06
wl x temp	1,22	0.0000813	0.905	< 0.36	1,21	0.0002190	2.218	< 0.16
	<b>body mass (m)</b>				<b>body mass (m)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,24	860.9	43.865	< 0.001***	1,30	1415.3	44.805	< 0.001***
temperature (temp)	1,24	25.6	1.303	< 0.27	1,30	15.6	0.493	< 0.49
wl x temp	1,24	13.3	0.676	< 0.41	1,30	41.0	1.297	< 0.27
	<b>body mass (f)</b>				<b>body mass (f)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,22	256.81	28.267	< 0.001***	1,21	221.7	9.907	< 0.005**
temperature (temp)	1,22	20.83	2.292	< 0.15	1,21	12.2	0.547	< 0.47
wl x temp	1,22	1.22	0.135	< 0.72	1,21	61.0	2.725	< 0.12
	<b>Tshb, posterior hypothalamus (m)</b>				<b>Tshb, posterior hypothalamus (m)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,22	8.467	11.089	< 0.004**	1,28	0.5397	13.213	< 0.002**
temperature (temp)	1,22	0.004	0.005	< 0.95	1,28	0.0603	1.477	< 0.24
wl x temp	1,22	0.032	0.042	< 0.84	1,28	0.0146	0.358	< 0.56
	<b>Tshb, posterior hypothalamus (f)</b>				<b>Tshb, posterior hypothalamus (f)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,21	2.981	3.04	< 0.10	1,20	0.02197	2.801	< 0.12
temperature (temp)	1,21	0.608	0.62	< 0.44	1,20	0.01920	2.448	< 0.14
wl x temp	1,21	2.549	2.60	< 0.13	1,20	0.00479	0.611	< 0.45
	<b>Tshr, posterior hypothalamus (m)</b>				<b>Tshr, posterior hypothalamus (m)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,22	0.0495	1.135	< 0.30	1,28	0.413	0.897	< 0.36
temperature (temp)	1,22	0.0079	0.181	< 0.68	1,28	0.739	1.604	< 0.22
wl x temp	1,22	0.0472	1.082	< 0.32	1,28	0.002	0.005	< 0.95
	<b>Tshr, posterior hypothalamus (f)</b>				<b>Tshr, posterior hypothalamus (f)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,21	0.0119	0.772	< 0.39	1,21	1.025	1.878	< 0.19
temperature (temp)	1,21	0.1319	8.547	< 0.009**	1,21	0.463	0.848	< 0.37
wl x temp	1,21	0.0045	0.289	< 0.60	1,21	0.003	0.005	< 0.95
	<b>Dio2, posterior hypothalamus (m)</b>				<b>Dio2, posterior hypothalamus (m)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,22	0.0518	0.414	< 0.53	1,28	0.005	0.048	< 0.83
temperature (temp)	1,22	0.0436	0.349	< 0.57	1,28	0.040	0.354	< 0.56
wl x temp	1,22	0.0326	0.261	< 0.62	1,28	0.132	1.164	< 0.30
	<b>Dio2, posterior hypothalamus (f)</b>				<b>Dio2, posterior hypothalamus (f)</b>			
	Df	SS	F	p	Df	SS	F	p
workload (wl)	1,21	0.0584	0.406	< 0.54	1,21	0.0414	0.291	< 0.60
temperature (temp)	1,21	0.5508	3.835	< 0.07	1,21	0.1329	0.933	< 0.35

wl x temp	1,21	0.1248	0.869	< 0.37	1,21	0.0070	0.049	< 0.83
<b><i>Kiss1</i>, posterior hypothalamus (m)</b>					<b><i>Kiss1</i>, posterior hypothalamus (m)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,21	0.1163	3.801	< 0.07	1,28	0.119	0.337	< 0.57
temperature (temp)	1,21	0.0043	0.141	< 0.72	1,28	1.428	4.050	< 0.06
wl x temp	1,21	0.0563	1.841	< 0.19	1,28	0.010	0.028	< 0.87
<b><i>Kiss1</i>, posterior hypothalamus (f)</b>					<b><i>Kiss1</i>, posterior hypothalamus (f)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,21	0.01875	2.393	< 0.14	1,21	0.2870	1.988	< 0.18
temperature (temp)	1,21	0.00434	0.554	< 0.47	1,21	0.0101	0.070	< 0.80
wl x temp	1,21	0.00002	0.003	< 0.96	1,21	0.0737	0.511	< 0.49
<b><i>Rfrp3</i>, posterior hypothalamus (m)</b>					<b><i>Rfrp3</i>, posterior hypothalamus (m)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,22	0.217	0.750	< 0.40	1,28	0.170	1.039	< 0.32
temperature (temp)	1,22	0.860	2.978	< 0.10	1,28	1.662	10.140	< 0.004**
wl x temp	1,22	0.133	0.459	< 0.51	1,28	0.007	0.042	< 0.84
<b><i>Rfrp3</i>, posterior hypothalamus (f)</b>					<b><i>Rfrp3</i>, posterior hypothalamus (f)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,21	0.020	0.051	< 0.83	1,21	0.0136	0.329	< 0.58
temperature (temp)	1,21	1.734	4.494	< 0.05*	1,21	0.1953	4.713	< 0.05*
wl x temp	1,21	0.012	0.030	< 0.87	1,21	0.0119	0.286	< 0.60
<b><i>Kiss1</i>, anterior hypothalamus (m)</b>					<b><i>Kiss1</i>, anterior hypothalamus (m)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,24	0.00776	1.387	< 0.26	1,30	1.463	11.090	< 0.003**
temperature (temp)	1,24	0.00360	0.643	< 0.44	1,30	0.167	1.266	< 0.27
wl x temp	1,24	0.00448	0.801	< 0.39	1,30	0.014	0.107	< 0.75
<b><i>Kiss1</i>, anterior hypothalamus (f)</b>					<b><i>Kiss1</i>, anterior hypothalamus (f)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,21	0.00383	0.334	< 0.57	1,21	0.769	3.446	< 0.08
temperature (temp)	1,21	0.03504	3.053	< 0.10	1,21	0.047	0.209	< 0.66
wl x temp	1,21	0.01071	0.933	< 0.35	1,21	0.039	0.177	< 0.68
<b><i>Gnrh</i>, anterior hypothalamus (m)</b>					<b><i>Gnrh</i>, anterior hypothalamus (m)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,24	0.405	1.807	< 0.20	1,30	0.0443	1.518	< 0.23
temperature (temp)	1,24	0.553	2.467	< 0.13	1,30	0.1153	3.953	< 0.06
wl x temp	1,24	0.289	1.292	< 0.27	1,30	0.0118	0.403	< 0.54
<b><i>Gnrh</i>, anterior hypothalamus (f)</b>					<b><i>Gnrh</i>, anterior hypothalamus (f)</b>			
	Df	SS	<i>F</i>	<i>p</i>	Df	SS	<i>F</i>	<i>p</i>
workload (wl)	1,21	0.036	0.225	< 0.65	1,21	0.0859	1.855	< 0.19
temperature (temp)	1,21	0.278	1.741	< 0.21	1,21	0.0294	0.634	< 0.44
wl x temp	1,21	0.001	0.006	< 0.95	1,21	0.0068	0.148	< 0.71