

Food and temperature change photoperiodic responses in two vole species

Laura van Rosmalen^{1,2*}, Roelof A. Hut¹

¹Chronobiology Unit, Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands.

²Present address: Salk Institute for Biological Studies, La Jolla, CA 92037, USA

*Corresponding author: Laura van Rosmalen, Lauravanrosmalen@hotmail.com

Keywords: Seasonal reproduction, photoperiodism, ambient temperature, food scarcity, hypothalamic gene expression, voles

Summary statement

This study provides a better understanding of the molecular mechanism through which metabolic cues can modify photoperiodic responses, to adaptively adjust timing of reproductive organ development.

Abstract

Seasonal timing of reproduction in voles is driven by photoperiod. Here we hypothesize 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 reveal that in common voles (*Microtus arvalis*) and tundra voles (*Microtus oeconomus*), photoperiodic induced pars tuberalis thyroid-stimulating hormone β -subunit (*Tsh β*) 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.

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 (for review, see Baker, 1938; Nakane and Yoshimura, 2019). In mammals, photoperiodic signals are perceived by retinal photoreception, and converted in the brain via the suprachiasmatic nucleus (SCN) and the pineal gland into melatonin signals regulating gonadal responses by the so called 'photoperiodic neuroendocrine system' (PNES) for review, see 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 (PT) produces thyroid-stimulating hormone β -subunit (TSH β) (Nakao et al., 2008), which binds to α -GSU to form an active dimer (TSH) (Magner, 1990) that regulates the balance between type 2/3 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 (T₃) in the mediobasal hypothalamus (Lechan and Fekete, 2005). T₃, which is required for creating seasonal rhythms (Hazlerigg and Loudon, 2008), possibly indirectly regulates 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 is largely unknown. Neurons expressing gonadotropin-releasing hormone (GnRH) are known to be the ultimate driver of the reproductive axis controlling the release of hormones (i.e. LH, FSH) from the pituitary gland (Guillemin, 1977; Schally et al., 1970). Prior studies in Siberian hamsters show that mediobasal hypothalamic (MBH) thyroid hormone triiodothyronine (T_3), which is increased under long photoperiods, does not regulate GnRH expression directly, yet facilitated 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 dorso-/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 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 regulates 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). Involvement of KISS1 and RFRP-3 expressing neurons in both 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*, Pallas 1778 and tundra vole, *Microtus oeconomus*, Pallas 1776). Voles may be the ideal species to study these questions since voles can have strong photoperiodic responses and a functional PNES system (Król et al., 2012; van Rosmalen et al., 2020; van Rosmalen et al., 2021). On the other hand, 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 (Dakette 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 proposed that the use of photoperiod, ambient temperature and food availability as cues for regulating reproduction depends on local climates, which varies with latitude (Bronson, 1988). 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 (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 (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 five years, our laboratory populations have been kept under long photoperiod (LP, 16h light: 8h dark) conditions and switched to a short photoperiod (SP, 8h light: 16h dark) for approximately two consecutive months at least twice a year.

All voles used in this study were gestated and born under a short photoperiod (SP, 8h light:16h dark) and transferred to a long photoperiod at the day of weaning at either 21±1°C or 10±1°C.

A photoperiodic transition from SP to LP simulates spring, during which voles become reproductive 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 both species at the start of the experiments (common voles: 16h light: 8h dark; tundra voles: 14h light:10h dark). Animals were transferred to cages (15 x 32 x 13 cm³) 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.

Work-for-food protocol

In the work-for-food 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 (630J 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), which is similar to *ad libitum* food conditions, since 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. Detailed description of the work-for-food protocol for this experiment was 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 - 0.5 gram/day) and the amount of earned pellets per day (>44 kJ/day). 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 collections

Animals were sacrificed by decapitation, with short prior CO₂ sedation, 17 ±1 hours after lights OFF (common voles: 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 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 pars tuberalis, DMH, VMH and Arcuate nucleus) and anterior hypothalamus (containing POA, PVN, PVz and SCN) were separated by a coronal cut at 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 dura mater. The sampled tissues were flash frozen within 2-4 minutes after death in liquid N₂ and stored at -80°C until RNA extraction. Reproductive organs were dissected and cleaned of fat, and wet masses of testes, paired ovary and uterus were measured (±0.0001 g).

RNA extractions/ 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 (~20 mg) were homogenized in 0.5 ml TRIzol in a TissueLyser II (Qiagen, Hilden, Germany) (2 x 2 min at 30 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% ETOH. RNA was diluted in RNase-free H₂O (range 20-100 µl) and quantified on a Nanodrop

2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, DNA was removed by a DNase I treatment (Invitrogen, Carlsbad, CA, USA), and equal RNA quantities were used for cDNA synthesis by using RevertAid H minus first strand cDNA synthesis reagents (Thermoscientific, Waltham, MA, USA). Reverse transcription (RT; 20 μ l) reactions were prepared using 1 μ g RNA, 100 μ mol l^{-1} Oligo(dT)₁₈, 5x Reaction buffer, 20 U μ l⁻¹ RiboLock RNase Inhibitor, 10 mmol l^{-1} dNTP Mix, RevertAid H Minus M-MuLV Reverse Transcriptase (200 U μ l⁻¹) (Table S1). RNA was reversed transcribed by using a thermal cycler (S1000; Bio-Rad, Hercules, CA, USA). Incubation conditions used for RT were: 45°C for 60 minutes followed by 70°C for 5 minutes. Transcript levels were quantified by real-time qPCR using SYBR Green (KAPA SYBR FAST qPCR Master Mix, Kapa Biosystems). 20 μ l reactions were carried out in duplo for each sample by 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 $\Delta\Delta$ CT method using *Gapdh* as reference gene (Pfaffl 2001).

Statistical analysis

Sample size (N=6-8) was determined by a power calculation (α =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 weight, body mass and gene expression levels were determined using type I two-way ANOVAs. Tukey HSD post-hoc pairwise comparisons were used to compare groups. Statistical significance was determined at α = 0.05. Statistical results can be found in the supplementary information (Table S4). Analyses were performed using RStudio (version 1.2.1335) (R Core Team, 2013), 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 ‘work-for-food’ 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 a 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, GSI) was not reduced by high workloads (Fig. 1K-P).

*Food scarcity under long photoperiod suppresses *Tshβ* 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, *Tshβ* expression can be exclusively attributed to the pars tuberalis. *Tshβ* expression was significantly reduced (50% reduction) in male voles at high workloads (Fig. 2A,C; Table S4). In males, this effect is 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°C (Fig. 2B,D; Table S4). *Tshβ* expression was for the most part unaffected by temperature, only a significant reduction was found in female common voles at 10°C (Fig. 2A-D; Table S4). Overall, *Tshβ* and gonadal weight show a positive relationship (Fig. 3A-D), suggesting that *Tshβ* may be involved in suppressing gonadal development when food is scarce.

After translation, TSH β and α 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 show slightly elevated *Tshr* expression at 10°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 *Tshβ* are not reflected in *Dio2* 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. *Npvf*) (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 (Hrvatin et al., 2020; Takahashi et al., 2020). *Kiss1* expression in the anterior hypothalamus is decreased at high workload in tundra vole males (Fig. 2G, Table S4), whereas *Kiss1* is close to zero in all groups of 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* shows a positive relationship with gonadal weight 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 nor temperature (Fig. 2E-H, Table S4).

Fitted linear models revealed that reproductive organ mass can be best predicted by: *Tshβ* and *Tshr* in common vole males ($F_{2,23} = 7.44, p < 0.01$); *Tshβ* in common vole females ($F_{1,23} = 4.89, p < 0.05$); *Tshβ*, *Rfrp*, anterior hypothalamic *Kiss1* in tundra vole males ($F_{3,28} = 8.47, p < 0.001$); *Tshβ* 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 *Gnrh* expression, but changes in gonadotropin release would be expected as KISS1 is the main drivers 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, since voles can reach sexual maturity within 40 days depending on environmental conditions. A negative energy balance under long photoperiod exerts similar effects on testis size of common (Fig. 1A) and tundra voles (Fig. 1D) as 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* 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 long photoperiods 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 weight may drop in summer while spermatogenic activity remains high (Adams et al., 1980). Therefore, testicular weight is a reliable indicator for 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 weight under long

photoperiods when food is 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 demands 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 bad indicator for hormonal secretory activity. Here, we did not perform histological analysis on ovaries, therefore, this data should be interpreted with caution. On the other hand, small uteri at low temperature are related to reduced height of secretory epithelium and the number of endometrial glands (Reiter, 1968). This confirms that the decline in uterine weight at high workload and low temperature, is the result of incomplete development of uterine glands. This may lead to infertility, since 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 to modify photoperiodic responses under energetically challenging conditions to reduce gonadal activation. The short duration of pineal melatonin release under long photoperiods lead to increased pars tuberalis *Tsh β* , which serves a pivotal role in the PNES (Hanon et al., 2008; Ono et al., 2008). The present study reveals that the photoperiodic induced *Tsh β* 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,B,C). Thus, reduced food availability decreases *Tsh β* 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 PT-*Tsh β* , remains to be investigated.

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

Pars tuberalis derived TSH binds locally to TSH receptors (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 *Tsh β* suppression caused by a negative energy balance is not reflected in tanycyte *Dio2* expression (Fig. 2A-D). On one hand, this suggests that TSH modulates central T_3 levels and ultimately gonadal development, via pathways parallel to the *DIO2/DIO3* system.

However, *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 functioning, hence T_3 metabolism, which impacts seasonal breeding in quails (Ikegami et al., 2015). On the other hand, 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, 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 two-fold higher *Dio2* levels in this study compared to our previous experiments might be explained by the fact that here animals were born at short photoperiod and transferred to long photoperiod at weaning, whereas our previous study used constant long photoperiod 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, two to three-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 β* and *Dio2* levels at different temperatures at low workload under long photoperiods in spring programmed voles are 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 Siberian hamsters (Paul et al., 2009), no effect of food scarcity was detected (Fig. 2A-D). In seasonal rodents, RFRP-3 synthesis appeared to be primarily regulated by photoperiod, but effects of RFRP3 on reproduction is highly variable between species, sex and photoperiodic condition (for review, see 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 *KissI* 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). *KissI* expression in the posterior hypothalamus, where the ARC is located, was not affected by either food or temperature (Fig. 2A-D). ARC *kissI* 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 *KissI* 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). Since 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 over 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; when ovulation is triggered by copulation, during which tactile stimulation of the cervix causes an ovulatory LH surge (Breed, 1967; Chitty and Austin, 1957; Hoyte, 1955). Since animals were not exposed to copulation/mating in our experiments, the lack of an ovulatory LH surge, and therefore the lack of positive sex-steroid feedback on KISS1 neurons in females may explain that preoptic *Kiss1* neurons are 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 weight, 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, in tundra voles *Kiss1* expression levels were considerably higher 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 (*Tshβ*, *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 is an important candidate for this. Transcriptomic analysis of the Siberian hamster hypothalamus confirmed that *Pomc* is a primary target for long term T₃-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 covers 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 photoperiodic regulated *Tsh β* 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.

List of abbreviations

ARC	arcuate nucleus
<i>Dio2</i>	iodothyronine deiodines 2
<i>Dio3</i>	iodothyronine deiodines 3
DMH/VMH	dorso-/ventromedial hypothalamus
Gnrh	gonadotropin-releasing hormone
<i>Kiss1</i>	kisspeptin
PNES	photoperiodic neuroendocrine system
POA	preoptic area

<i>Rfrp</i> (<i>Npvf</i>)	RF-amide related peptide
<i>Tshβ</i>	thyroid-stimulating hormone β -subunit
<i>Tshr</i>	thyroid-stimulating hormone receptor

Acknowledgements

We thank L. de Wit for help during tissue collections, G.J.F. Overkamp for technical assistance, Prof.dr. M.P. Gerkema for establishing the common vole colony, and Dr. C. Dijkstra and Dr. L. van de Zande for establishing the tundra vole colony at the University of Groningen. We thank Dr. H. Dardente for his critical and valuable comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

LvR and RAH designed the research, LvR performed the research and analyzed the data, LvR and RAH wrote the manuscript.

Funding

This research was supported by an Adaptive Life Program grant awarded to Prof.dr. Roelof A. Hut made possible by the Board of the University of Groningen, the Faculty of Science and Engineering and the Groningen Institute for Evolutionary Life Sciences (GELIFES).

Data availability

The data that support the findings of this study are openly available in Figshare at <https://doi.org/10.6084/m9.figshare.13522700.v1>.

References

- Adams, M. R., Tamarin, R. H. and Callard, I. P.** (1980). Seasonal changes in plasma androgen levels and the gonads of the beach vole, *Microtus breweri*. *Gen. Comp. Endocrinol.* **41**, 31–40.
- Ancel, C., Bentsen, A. H., Sébert, M. E., Tena-Sempere, M., Mikkelsen, J. D. and Simonneaux, V.** (2012). Stimulatory effect of RFRP-3 on the gonadotrophic axis in the male Syrian hamster: The exception proves the rule. *Endocrinology* **153**, 1352–1363.

- Angelopoulou, E., Quignon, C., Kriegsfeld, L. J. and Simonneaux, V.** (2019). Functional Implications of RFRP-3 in the Central control of daily and seasonal rhythms in reproduction. *Front. Endocrinol. (Lausanne)*. **10**, 1–15.
- Ansel, L., Bolborea, M., Bentsen, A. H., Klosen, P., Mikkelsen, J. D. and Simonneaux, V.** (2010). Differential regulation of kiss1 expression by melatonin and gonadal hormones in male and female syrian hamsters. *J. Biol. Rhythms* **25**, 81–91.
- Baker, J.** (1938). The evolution of breeding seasons. *Evol. Essays Asp. Evol. Biol.* 161–177.
- Banks, R., Delibegovic, M. and Stevenson, T. J.** (2016). Photoperiod- and triiodothyronine-dependent regulation of reproductive neuropeptides, proinflammatory cytokines, and peripheral physiology in Siberian hamsters (*Phodopus sungorus*). *J. Biol. Rhythms* **31**, 299–307.
- Bao, R., Onishi, K. G., Tolla, E., Ebling, F. J. P., Lewis, J. E., Anderson, R. L., Barrett, P., Prendergast, B. J. and Stevenson, T. J.** (2019). Genome sequencing and transcriptome analyses of the Siberian hamster hypothalamus identify mechanisms for seasonal energy balance. *Proc. Natl. Acad. Sci.* **116**, 13116–13121.
- Breed, W. G.** (1967). Ovulation in the genus *Microtus*. *Nature* **214**, 826–826.
- Bronson, F. H.** (1988). Mammalian reproductive strategies: genes, photoperiod and latitude. *Reprod. Nutr. Dev.* **28**, 335–347.
- Caro, S. P., Schaper, S. V., Hut, R. A., Ball, G. F. and Visser, M. E.** (2013). The case of the missing mechanism: How does temperature influence seasonal timing in endotherms? *PLoS Biol.* **11**, e1001517.
- Cázares-Márquez, F., Milesi, S., Laran-Chich, M.-P., Klosen, P., Kalsbeek, A. and Simonneaux, V.** (2019). Kisspeptin and RFRP3 modulate body mass in *Phodopus sungorus* via two different neuroendocrine pathways. *J. Neuroendocrinol.* **31**, e12710.
- Cázares-Márquez, F., Laran-Chich, M. P., Klosen, P., Kalsbeek, A. and Simonneaux, V.** (2020). RFRP3 increases food intake in a sex-dependent manner in the seasonal hamster *Phodopus sungorus*. *J. Neuroendocrinol.* **32**, 1–9.
- Cázares-Márquez, F., Eliveld, J., Ritsema, W. I. G. R., Foppen, E., Bossenbroek, Y., Pelizzari, S., Simonneaux, V. and Kalsbeek, A.** (2021). Role of central kisspeptin and RFRP-3 in energy metabolism in the male Wistar rat. *J. Neuroendocrinol.* **33**, 1–15.
- Chitty, H. and Austin, C. R.** (1957). Environmental modification of oestrus in the vole. *Nature* **179**, 592–593.

- Cooke, P. S., Spencer, T. E., Bartol, F. F. and Hayashi, K.** (2013). Uterine glands: Development, function and experimental model systems. *Mol. Hum. Reprod.* **19**, 547–558.
- Coppola, A., Meli, R. and Diano, S.** (2005). Inverse shift in circulating corticosterone and leptin levels elevates hypothalamic deiodinase type 2 in fasted rats. *Endocrinology* **146**, 2827–2833.
- Daksetse, M.-J. and Martinet, L.** (1977). Effect of temperature on the growth and fertility of the field-vole, *Microtus arvalis*, raised in different daylength and feeding conditions. *Ann Biol Anim Biochim Biophys* **17**, 713–721.
- Dardente, H., Wood, S., Ebling, F. and Sáenz de Miera, C.** (2018). An integrative view of mammalian seasonal neuroendocrinology. *J. Neuroendocrinol.* **31**, e12729.
- Dardente, H., Lomet, D., Chesneau, D., Pellicer-Rubio, M. T. and Hazlerigg, D.** (2019). Discontinuity in the molecular neuroendocrine response to increasing daylengths in Ile-de-France ewes: Is transient Dio2 induction a key feature of circannual timing? *J. Neuroendocrinol.* **31**, 1–10.
- De Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L. and Milgrom, E.** (2003). Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 10972–10976.
- Diano, S., Naftolin, F., Goglia, F. and Horvath, T. L.** (1998). Fasting-induced increase in type II iodothyronine deiodinase activity and messenger ribonucleic acid levels is not reversed by thyroxine in the rat hypothalamus. *Endocrinology* **139**, 2879–2884.
- Ergon, T., Lambin, X. and Stenseth, N. C.** (2001). Life-history traits of voles in a fluctuating population respond to the immediate environment. *Nature* **411**, 1043–1045.
- Gerkema, M. P., Daan, S., Wilbrink, M., Hop, M. W., van der Leest, F. and Gerkema, M. P.** (1993). Phase control of ultradian feeding rhythms in the common vole (*Microtus arvalis*): The roles of light and the circadian system. *J. Biol. Rhythms* **8**, 151–171.
- Greives, T. J., Humber, S. A., Goldstein, A. N., Scotti, M. A. L., Demas, G. E. and Kriegsfeld, L. J.** (2008). Photoperiod and testosterone interact to drive seasonal changes in kisspeptin expression in siberian hamsters (*Phodopus sungorus*). *J. Neuroendocrinol.* **20**, 1339–1347.
- Guerra, M., Blázquez, J. L., Peruzzo, B., Peláez, B., Rodríguez, S., Toranzo, D., Pastor, F. and Rodríguez, E. M.** (2010). Cell organization of the rat pars tuberalis. Evidence for open communication between pars tuberalis cells, cerebrospinal fluid and tanycytes. *Cell Tissue Res.* **339**, 359–381.

- Guillemin, R.** (1977). Purification, isolation, and primary structure of the hypothalamic luteinizing hormone-releasing factor of ovine origin. A historical account. *Am. J. Obstet. Gynecol.* **129**, 214–218.
- Han, S. K., Gottsch, M. L., Lee, K. J., Popa, S. M., Smith, J. T., Jakawich, S. K., Clifton, D. K., Steiner, R. A. and Herbison, A. E.** (2005). Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J. Neurosci.* **25**, 11349–11356.
- Han, S. Y., McLennan, T., Czielesky, K. and Herbison, A. E.** (2015). Selective optogenetic activation of arcuate kisspeptin neurons generates pulsatile luteinizing hormone secretion. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 13109–13114.
- Hanon, E. A., Lincoln, G. A., Fustin, J.-M., Dardente, H., Masson-Pévet, M., Morgan, P. J. and Hazlerigg, D. G.** (2008). Ancestral TSH mechanism signals summer in a photoperiodic mammal. *Curr. Biol.* **18**, 1147–1152.
- Hazlerigg, D. and Loudon, A.** (2008). New insights into ancient seasonal life timers. *Curr. Biol.* **18**, 795–804.
- Hazlerigg, D., Lomet, D., Lincoln, G. and Dardente, H.** (2018). Neuroendocrine correlates of the critical day length response in the Soay sheep. *J. Neuroendocrinol.* e12631.
- Henningsen, J. B., Gauer, F. and Simonneaux, V.** (2016). RFRP neurons – the doorway to understanding seasonal reproduction in mammals. *Front. Endocrinol. (Lausanne)*. **7**, 1–10.
- Henningsen, J. B., Ancel, C., Mikkelsen, J. D., Gauer, F. and Simonneaux, V.** (2017). Roles of RFRP-3 in the daily and seasonal regulation of reproductive activity in female Syrian hamsters. *Endocrinology* **158**, 652–663.
- Herwig, A., Wilson, D., Logie, T. J., Boelen, A., Morgan, P. J., Mercer, J. G. and Barrett, P.** (2009). Photoperiod and acute energy deficits interact on components of the thyroid hormone system in hypothalamic tanycytes of the Siberian hamster. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **296**, 1307–1315.
- Hileman, S. M., McManus, C. J., Goodman, R. L. and Jansen, H. T.** (2011). Neurons of the lateral preoptic area/rostral anterior hypothalamic area are required for photoperiodic inhibition of estrous cyclicity in sheep. *Biol. Reprod.* **85**, 1057–1065.
- Hoyte, H. M. D.** (1955). Observations on reproduction in some small mammals of arctic Norway. *J. Anim. Ecol.* **24**, 412–425.

- Hrvatin, S., Sun, S., Wilcox, O. F., Yao, H., Lavin-Peter, A. J., Cicconet, M., Assad, E. G., Palmer, M. E., Aronson, S., Banks, A. S., et al.** (2020). Neurons that regulate mouse torpor. *Nature* **583**, 115–121.
- Huang, X. F., Koutcherov, I., Lin, S., Wang, H. Q. and Storlien, L.** (1996). Localization of leptin receptor mRNA expression in mouse brain. *Neuroreport* **7**, 2635–2638.
- Hut, R. A.** (2011). Photoperiodism: Shall EYA compare thee to a summers day? *Curr. Biol.* **21**, R22–R25.
- Hut, R. A., Pilorz, V., Boerema, A. S., Strijkstra, A. M. and Daan, S.** (2011). Working for food shifts nocturnal mouse activity into the day. *PLoS One* **6**, 1–6.
- Hut, R. A., Dardente, H. and Riede, S. J.** (2014). Seasonal timing: How does a hibernator know when to stop hibernating? *Curr. Biol.* **24**, 602–605.
- Ikegami, K., Atsumi, Y., Yorinaga, E., Ono, H., Murayama, I., Nakane, Y., Ota, W., Arai, N., Tega, A., Iigo, M., et al.** (2015). Low temperature-induced circulating triiodothyronine accelerates seasonal testicular regression. *Endocrinology* **156**, 647–659.
- Janati, A., Talbi, R., Klosen, P., Mikkelsen, J. D., Magoul, R., Simonneaux, V. and El Ouezzani, S.** (2013). Distribution and seasonal variation in hypothalamic RF-amide peptides in a semi-desert rodent, the jerboa. *J. Neuroendocrinol.* **25**, 402–411.
- Jaroslawska, J., Chabowska-Kita, A., Kaczmarek, M. M. and Kozak, L. P.** (2015). Npvf: Hypothalamic biomarker of ambient temperature independent of nutritional status. *PLoS Genet.* **11**, 1–23.
- Klosen, P., Sébert, M. E., Rasri, K., Laran-Chich, M. P. and Simonneaux, V.** (2013). TSH restores a summer phenotype in photoinhibited mammals via the RF-amides RFRP3 and kisspeptin. *FASEB J.* **27**, 2677–2686.
- Król, E., Douglas, A., Dardente, H., Birnie, M. J., van der Vinne, V., Eijer, W. G., Gerkema, M. P., Hazlerigg, D. G. and Hut, R. A.** (2012). Strong pituitary and hypothalamic responses to photoperiod but not to 6-methoxy-2-benzoxazolinone in female common voles (*Microtus arvalis*). *Gen. Comp. Endocrinol.* **179**, 289–295.
- Lechan, R. M. and Fekete, C.** (2005). Role of thyroid hormone deiodination in the hypothalamus. *Thyroid* **15**, 883–897.
- Lincoln, G. A. and Fraser, H. M.** (1979). Blockade of episodic secretion of luteinizing hormone in the ram by the administration of antibodies to luteinizing hormone releasing hormone. *Biol. Reprod.* **21**, 1239–1245.

- Linzey, A. V., Shar, S., Lkhagvasuren, D., Juskaitis, D., Sheftel, B., Meinig, H., Amori, G. and Henttonen, H.** (2016). *Microtus oeconomus*. *IUCN Red List Threat. Species*.
- Lomet, D., Druart, X., Hazlerigg, D., Beltramo, M. and Dardente, H.** (2020). Circuit-level analysis identifies target genes of sex steroids in Ewe seasonal breeding. *Mol. Cell. Endocrinol.* 110825.
- Magner, J. A.** (1990). Thyroid-stimulating hormone: biosynthesis, cell biology, and bioactivity. *Endocr. Rev.* **11**, 354–385.
- Nakane, Y. and Yoshimura, T.** (2019). Photoperiodic regulation of reproduction in vertebrates. *Annu. Rev. Anim. Biosci.* **7**, 173–94.
- Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., Yasuo, S., Katou, Y., Kageyama, S., Uno, Y., et al.** (2008). Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature* **452**, 317–322.
- Negus, N. C. and Berger, P. J.** (1977). Experimental triggering of reproduction in a natural population of *Microtus montanus*. *Science* **196**, 1230–1231.
- Nelson, R. J., Dark, J. and Zucker, I.** (1983). Influence of photoperiod, nutrition and water availability on reproduction of male California voles (*Microtus californicus*). *J. Reprod. Fertil.* **69**, 473–477.
- Nelson, R. J., Frank, D., Smale, L. and Willoughby, S. B.** (1989). Photoperiod and temperature affect reproductive and nonreproductive functions in male prairie voles (*Microtus ochrogaster*). *Biol Reprod* **40**, 481–485.
- Nelson, R. J., Marinovic, A. C., Moffatt, C. A., Kriegsfeld, L. J. and Kim, S.** (1997). The effects of photoperiod and food intake on reproductive development in male deer mice (*Peromyscus maniculatus*). *Physiol. Behav.* **62**, 945–950.
- Nieminen, P., Hohtola, E. and Mustonen, A.-M.** (2013). Body temperature rhythms in *Microtus* voles during feeding, food deprivation, and winter acclimatization. *J. Mammal.* **94**, 591–600.
- Ono, H., Hoshino, Y., Yasuo, S., Watanabe, M., Nakane, Y., Murai, A., Ebihara, S., Korf, H.-W. and Yoshimura, T.** (2008). Involvement of thyrotropin in photoperiodic signal transduction in mice. *Proc. Natl. Acad. Sci.* **105**, 18238–18242.
- Paul, M. J., Pyter, L. M., Freeman, D. A., Galang, J. and Prendergast, B. J.** (2009). Photic and nonphotic seasonal cues differentially engage hypothalamic kisspeptin and RFamide-related peptide mRNA expression in Siberian hamsters. *J. Neuroendocrinol.* **21**, 1007–1014.

- Pfaffl, M. W.** (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 16–21.
- Rasri-Klosen, K., Simonneaux, V. and Klosen, P.** (2017). Differential response patterns of kisspeptin and RFamide-related peptide to photoperiod and sex steroid feedback in the Djungarian hamster (*Phodopus sungorus*). *J. Neuroendocrinol.* **29**, 1–14.
- Reiter, R. J.** (1968). Changes in the reproductive organs of cold-exposed and light-deprived female hamsters (*Mesocricetus auratus*). *J. Reprod. Fertil.* **16**, 217–222.
- Revel, F. G., Saboureau, M., Masson-Pévet, M., Pévet, P., Mikkelsen, J. D. D. and Simonneaux, V.** (2006). Kisspeptin mediates the photoperiodic control of reproduction in hamsters. *Curr. Biol.* **16**, 1730–1735.
- Revel, F. G., Ansel, L., Klosen, P., Saboureau, M., Pévet, P., Mikkelsen, J. D. and Simonneaux, V.** (2007). Kisspeptin: A key link to seasonal breeding. *Rev. Endocr. Metab. Disord.* **8**, 57–65.
- Revel, F. G., Saboureau, M., Pévet, P., Simonneaux, V. and Mikkelsen, J. D.** (2008). RFamide-related peptide gene is a melatonin-driven photoperiodic gene. *Endocrinology* **149**, 902–912.
- Ruffino, L., Salo, P., Koivisto, E., Banks, P. B. and Korpimäki, E.** (2014). Reproductive responses of birds to experimental food supplementation: A meta-analysis. *Front. Zool.* **11**, 1–13.
- Sáenz de Miera, C., Bothorel, B., Jaeger, C., Simonneaux, V. and Hazlerigg, D.** (2017). Maternal photoperiod programs hypothalamic thyroid status via the fetal pituitary gland. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 8408–8413.
- Sáenz De Miera, C., Monecke, S., Bartzen-Sprauer, J., Laran-Chich, M. P., Pévet, P., Hazlerigg, D. G. and Simonneaux, V.** (2014). A circannual clock drives expression of genes central for seasonal reproduction. *Curr. Biol.* **24**, 1500–1506.
- Sanders, E. H., Gardner, P. D., Berger, P. J. and Negus, N. C.** (1981). 6-methoxybenzoxazolinone: A plant derivative that stimulates reproduction in *Microtus montanus*. *Science* **214**, 67–69.
- Schally, A. V., Parlow, A. F., Carter, W. H., Saito, M., Bowers, C. Y. and Arimura, A.** (1970). Studies on the site of action of oral contraceptive steroids. II. Plasma LH and FSH levels after administration of antifertility steroids and LH-releasing hormone (LH-RH). *Obstet. Gynecol. Surv.* **25**, 953–954.

- Schneider, J. E.** (2004). Energy balance and reproduction. *Physiol. Behav.* **81**, 289–317.
- Seminara, S. B., Messenger, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S., Shagoury, J. K., Bo-Abbas, Y., Kuohung, W., Schwinof, K. M., Hendrick, A. G., et al.** (2004). The GPR54 gene as a regulator of puberty. *Obstet. Gynecol. Surv.* **59**, 351–353.
- Simonneaux, V.** (2020). A Kiss to drive rhythms in reproduction. *Eur. J. Neurosci.* **51**, 509–530.
- Smith, J. T., Dungan, H. M., Stoll, E. A., Gottsch, M. L., Braun, R. E., Eacker, S. M., Clifton, D. K. and Steiner, R. A.** (2005a). Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* **146**, 2976–2984.
- Smith, J. T., Cunningham, M. J., Rissman, E. F., Clifton, D. K. and Steiner, R. A.** (2005b). Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology* **146**, 3686–3692.
- Steinlechner, S., Stieglitz, A., Ruf, T., Heldmaier, G. and Reiter, R. J.** (1991). Integration of environmental signals by the pineal gland and its significance for seasonality in small mammals. In *role of melatonin and pineal peptides in neuroimmunomodulation*, pp. 159–163.
- Takahashi, T. M., Sunagawa, G. A., Soya, S., Abe, M., Sakurai, K., Ishikawa, K., Yanagisawa, M., Hama, H., Hasegawa, E., Miyawaki, A., et al.** (2020). A discrete neuronal circuit induces a hibernation-like state in rodents. *Nature* **583**, 109–114.
- Talbi, R., Laran-Chich, M. P., Magoul, R., El Ouezzani, S. and Simonneaux, V.** (2016). Kisspeptin and RFRP-3 differentially regulate food intake and metabolic neuropeptides in the female desert jerboa. *Sci. Rep.* **6**, 1–10.
- Tamarkin, L., Baird, C. J. and Almeida, O. F. X.** (1985). Melatonin: A coordinating signal for mammalian reproduction? *Science* **227**, 714–720.
- Team, R. C.** (2013). R: A language and environment for statistical computing. *R Found. Stat. Comput. Vienna, Austria*.
- Ubuka, T., Inoue, K., Fukuda, Y., Mizuno, T., Ukena, K., Kriegsfeld, L. J. and Tsutsui, K.** (2012). Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. *Endocrinology* **153**, 373–385.

- van de Zande, L., van Apeldoorn, R. C., Blijdenstein, A. F., de Jong, D., van Delden, W. and Bijlsma, R.** (2000). Microsatellite analysis of population structure and genetic differentiation within and between populations of the root vole, *Microtus oeconomus* in the Netherlands. *Mol. Ecol.* **9**, 1651–1656.
- van der Vinne, V., Riede, S. J., Gorter, J. A., Eijer, W. G., Sellix, M. T., Menaker, M., Daan, S., Pilorz, V. and Hut, R. A.** (2014). Cold and hunger induce diurnality in a nocturnal mammal. *Proc. Natl. Acad. Sci.* **111**, 15256–15260.
- van der Vinne, V., Gorter, J. A., Riede, S. J. and Hut, R. A.** (2015). Diurnality as an energy-saving strategy: energetic consequences of temporal niche switching in small mammals. *J. Exp. Biol.* **218**, 2585–2593.
- van der Vinne, V., Tachinardi, P., Riede, S. J., Akkerman, J., Scheepe, J. and Hut, R. A.** (2019). Maximising survival by shifting the daily timing of activity. *Ecol. Lett.* **22**, 2097–2102.
- van Rosmalen, L. and Hut, R. A.** (2021). Negative energy balance enhances ultradian rhythmicity in spring-programmed voles. *J. Biol. Rhythms* **36**, 359–368.
- van Rosmalen, L., van Dalum, J., Hazlerigg, D. G. and Hut, R. A.** (2020). Gonads or body? Differences in gonadal and somatic photoperiodic growth response in two vole species. *J. Exp. Biol.* **223**, jeb.230987.
- van Rosmalen, L., van Dalum, J., Appenroth, D., Roodenrijs, R., de Witt, L., Hazlerigg, D. and Hut, R.** (2021). Mechanisms of temperature modulation in mammalian seasonal timing. *FASEB J.* **35**, e21605.
- Wang, D., Li, N., Tian, L., Ren, F., Li, Z., Chen, Y., Liu, L., Hu, X., Zhang, X., Song, Y., et al.** (2019). Dynamic expressions of hypothalamic genes regulate seasonal breeding in a natural rodent population. *Mol. Ecol.* **28**, 3508–3522.
- Wickham, H.** (2016). Elegant graphics for data analysis. *Elegant Graph. Data Anal.* 3–10.
- Yeo, S. and Colledge, W. H.** (2018). The role of Kiss1 neurons as integrators of endocrine, metabolic, and environmental factors in the hypothalamic–pituitary–gonadal axis. *Front. Endocrinol. (Lausanne)*. **9**,.
- Yigit, N., Hutterer, R., Krystufek, B. and Amori, G.** (2016). *Microtus arvalis*. *The IUCN red list of threatened species*.
- Young, K. A., Zirkin, B. R. and Nelson, R. J.** (2000). Testicular regression in response to food restriction and short photoperiod in white-footed mice (*Peromyscus leucopus*) is mediated by apoptosis. *Biol. Reprod.* **62**, 347–354.

Figures

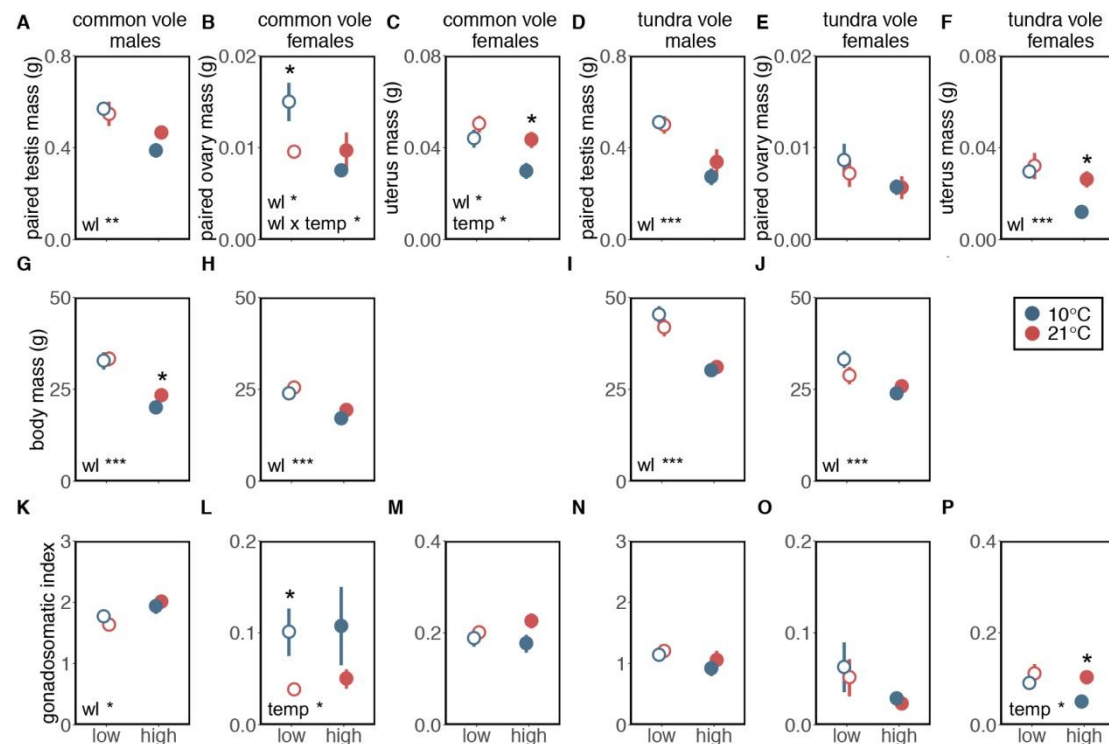


Figure 1. Food scarcity and ambient temperature effects on gonadal weight and body mass in male and female voles. (A, D) paired testis mass, (B, E) paired ovary mass, (C, F) uterus mass, (G-J) body mass and (K-P) gonadosomatic index for common and tundra voles respectively at low (open symbols) or high 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 (temp) and interactions (wl x temp) are shown: *p < 0.05, **p < 0.01, ***p < 0.001. Statistic results for ANOVAs can be found in Table S4.

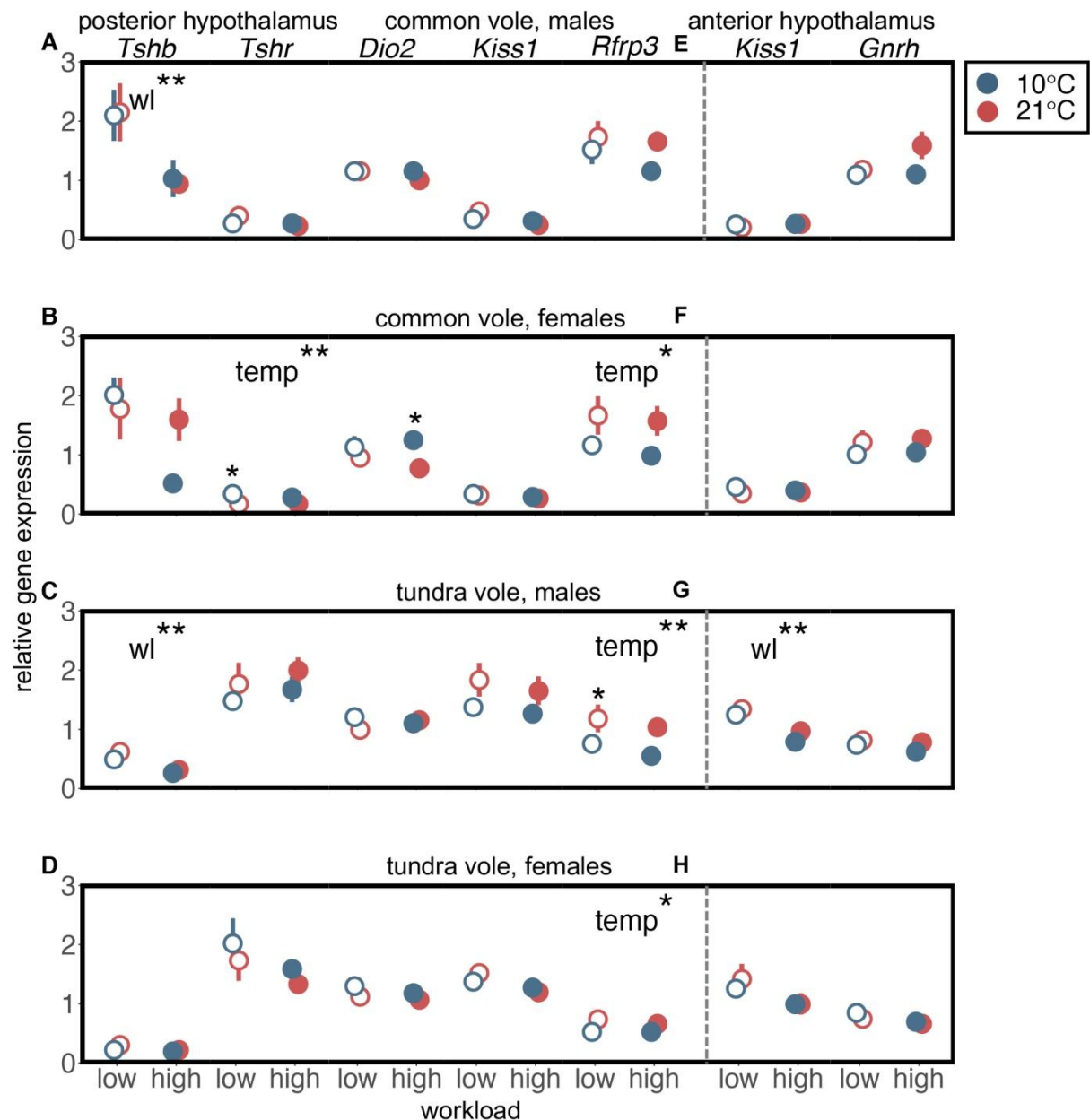


Figure 2. Food scarcity and ambient temperature affect gene expression in the posterior and anterior hypothalamus. Relative gene expression levels of *Tshb*, *Tshr*, *Dio2*, *Kiss1* and *Rfrp* in the posterior hypothalamus and *Kiss1* and *Gnrh* in the anterior hypothalamus for (A, E) common vole males, (B, F) common vole females, (C, G) tundra vole males and (D, H) tundra vole females respectively, 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) and temperature (temp) are shown: *p < 0.05, **p < 0.01, ***p < 0.001. Significant differences between groups (one-way ANOVA) are indicated by asterisks. Statistic results for ANOVAs can be found in Table S4.

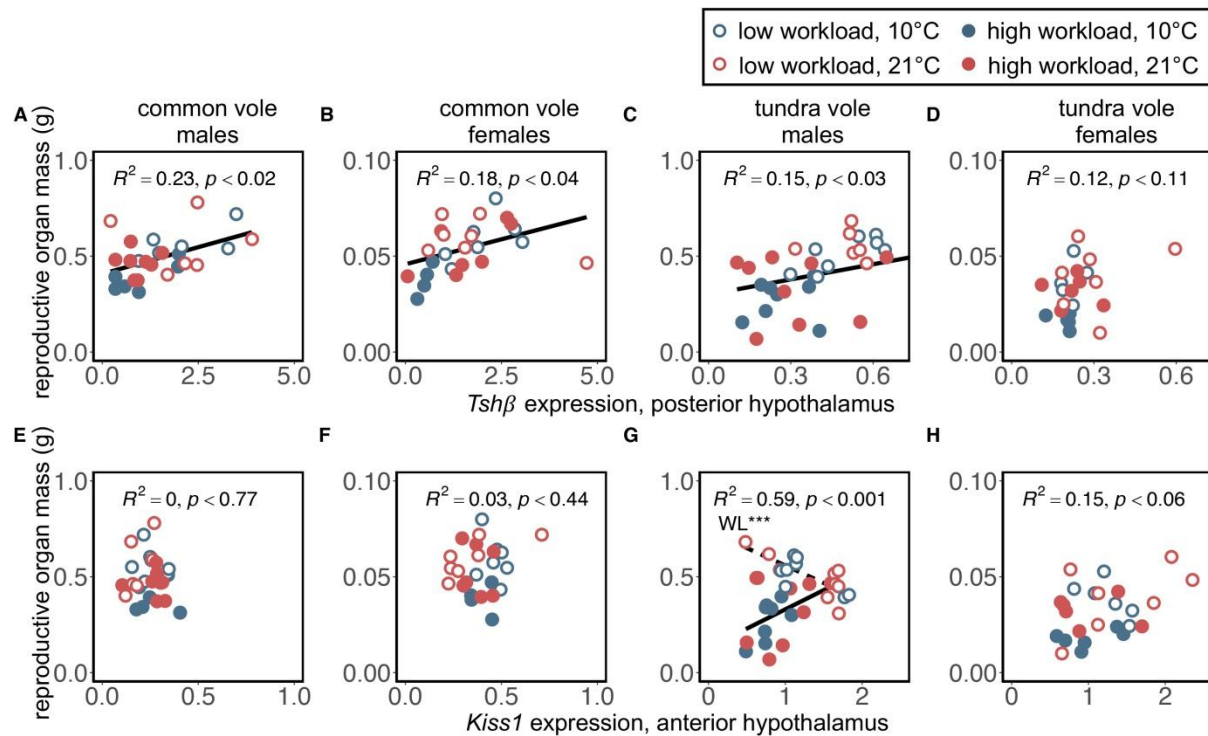


Figure 3. Relationship between reproductive organ mass, *Tshβ* and *Kiss1* expression.

Correlations between (A-D) *Tshβ* expression in the posterior hypothalamus and reproductive organ mass (male: paired testis weight, females: paired ovary + uterus weight), and between (E-H) *Kiss1* expression in the anterior hypothalamus and reproductive organ mass for common vole males, common vole females, tundra vole males and tundra vole females respectively 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.

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

Component	Stock concentration	Final concentration
Oligo(dT) ₁₈	100 μ M	5 μ M
5X REACTION buffer	5X	1X
RiboLock RNase Inhibitor	20 U/ μ l	1 U/ μ l
dNTP Mix	10 mM	1 mM
RevertAid H Minus Reverse Transcriptase	200 U/ μ l	10 U/ μ l
Template RNA	0.1 μ g/ μ l	1 μ g/ μ 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>Tshβ</i>	GCTTATGGCAACAGGGTAGGA	AATACGCGCTCTCCCAGGAT
<i>Tshr</i>	ATCCCCAGTCTCGCGTTTTTC	GCTTCTGGTGTTCGCGATTT

Table S4. Statistics for two-way ANOVAs

	Common vole (<i>Microtus arvalis</i>)				Tundra vole (<i>Microtus oeconomus</i>)			
	paired testis mass (m)				paired testis mass (m)			
	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
	paired ovary mass (f)				paired ovary mass (f)			
	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
	uterus mass (f)				uterus mass (f)			
	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
	body mass (m)				body mass (m)			
	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
	body mass (f)				body mass (f)			
	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
	Tshb, posterior hypothalamus (m)				Tshb, posterior hypothalamus (m)			
	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
	Tshb, posterior hypothalamus (f)				Tshb, posterior hypothalamus (f)			
	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
	Tshr, posterior hypothalamus (m)				Tshr, posterior hypothalamus (m)			
	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
	Tshr, posterior hypothalamus (f)				Tshr, posterior hypothalamus (f)			
	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
	Dio2, posterior hypothalamus (m)				Dio2, posterior hypothalamus (m)			
	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
	Dio2, posterior hypothalamus (f)				Dio2, posterior hypothalamus (f)			
	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
	<i>Kiss1</i>, posterior hypothalamus (m)				<i>Kiss1</i>, posterior hypothalamus (m)			
	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
	<i>Kiss1</i>, posterior hypothalamus (f)				<i>Kiss1</i>, posterior hypothalamus (f)			
	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
	<i>Rfrp3</i>, posterior hypothalamus (m)				<i>Rfrp3</i>, posterior hypothalamus (m)			
	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
	<i>Rfrp3</i>, posterior hypothalamus (f)				<i>Rfrp3</i>, posterior hypothalamus (f)			
	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
	<i>Kiss1</i>, anterior hypothalamus (m)				<i>Kiss1</i>, anterior hypothalamus (m)			
	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
	<i>Kiss1</i>, anterior hypothalamus (f)				<i>Kiss1</i>, anterior hypothalamus (f)			
	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
	<i>Gnrh</i>, anterior hypothalamus (m)				<i>Gnrh</i>, anterior hypothalamus (m)			
	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
	<i>Gnrh</i>, anterior hypothalamus (f)				<i>Gnrh</i>, anterior hypothalamus (f)			
	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