

Food and temperature change photoperiodic responses in two vole species: different roles for Tsh β , Kisspeptin and Rfrp3

Laura van Rosmalen¹ and Roelof Hut¹

¹University of Groningen

March 30, 2022

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 reveal that Common (*Microtus arvalis*) and Tundra voles (*Microtus oeconomus*), reduce photoperiodic induced pars tuberalis thyroid-stimulating hormone β -subunit (Tsh β) expression to inhibit gonadal development when food is scarce. Reduction in gonadal size is more pronounced in tundra voles, in which the hypothalamic Kisspeptin (Kiss1) system seems involved in downregulating gonadal development, especially in males. Low temperature additionally leads to decreased hypothalamic RF-amide related peptide (Rfrp3) levels, which 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.

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 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).

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. Energy balance and reproduction are closely related (Schneider 2004; Ruffino *et al.* 2014), but its regulatory mechanisms remain to be disclosed. Seasonally breeding animals, such as voles, may use a combination of photic and non-photic 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 main drivers of the reproductive axis controlling the release of hormones (i.e. LH, FSH) from the pituitary gland (Schally *et al.* 1970; Guillemin 1977). Prior studies suggest that mediobasal hypothalamic (MBH) thyroid hormone triiodothyronine (T₃), which is increased under long photoperiods, may not act

on GnRH neurons directly, but 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 metabolic regulation (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. 2005b, a) and RF-amide related peptide (*Rfrp3*), which are candidates for integrating environmental cues, mediating seasonal reproductive responses (Revelet et al. 2008; Krebs et al. 2009; Klosen et al. 2013; Simonneaux et al. 2013). *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).

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 since voles can have strong photoperiodic responses and a functional canonical PNEs system (Król et al. 2012; van Rosmalen et al. 2020). On the other hand, voles are also known to be able to respond to the environment in a more opportunistic way (Dakette & Martinet 1977; Negus & Berger 1977; Sanders et al. 1981; Nelson et al. 1983; Ergon et al. 2001). Common voles are distributed in central Europe (38-62°N), whereas Tundra voles are distributed at more northern latitudes (48-72°N). 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. At northern latitudes, Tundra voles live under isolating snow covers for a substantial part of the year, which may make photoperiod an unreliable cue for seasonal adaptation in this species. 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 was 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.

Methods

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*) were obtained from the Lauwersmeer area (Netherlands, 53° 24' N, 6° 16' E, (Gerkema et al. 1993). Tundra voles (*Microtus oeconomus*) 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.

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. Based on photoperiodic dose-response-curves for gonadal mass from our prior research (van Rosmalen et al., in preparation), 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 (12.5 x 12.5 x 20 cm) 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. 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 (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 = 0.03 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, submitted). 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 prior CO₂ sedation, 17 ± 1 hours after lights OFF 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 intermedialis and pars distalis, was still intact in the sella turcica covered by dura mater. The extracted 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 isolated from the anterior and posterior hypothalamic area using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). In short, frozen pieces of tissue (~0.02 g) were homogenized in 0.5 ml TRIzol reagent 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. Subsequently, 0.1 ml chloroform was added for phase separation. Following RNA precipitation by 0.25 ml of 100% isopropanol, the obtained RNA pellet was washed with 0.5 ml of 75% ethanol. Depending on size, RNA pellets were diluted in adequate volumes of RNase-free H₂O (range 20-100 µL) and quantified on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). After DNA removal by DNase I treatment (Invitrogen, Carlsbad, CA, USA), equal quantity of RNA from each sample was used for cDNA synthesis by using RevertAid H minus first strand cDNA synthesis reagents (Thermoscientific, Waltham, MA, USA). Reverse transcription (RT; 20 µL) reactions were prepared using 1 µg RNA, 100 µM Oligo(dT)₁₈, 5x Reaction buffer, 20 U/µL RiboLock RNase Inhibitor, 10 mM dNTP Mix, RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µL). Concentrations used for RT reactions can be found in supplementary information (Table S1). RNA was reversed transcribed by using a thermal cycler (S1000; Bio-Rad, Hercules, CA, USA). Incubation conditions use 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 µL (2 µL cDNA + 18 µL Mastermix) 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) and optimized annealing temperature (T_m) and primer concentration. All primers were designed based on the annotated *Microtus ochrogaster* genome (NCBI:txid79684, GCA_000317375.1), and subsequently checked 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 (housekeeping) gene (Pfaffl 2001).

Statistical analysis

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 $\alpha = 0.05$. 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.

Φοοδ σαρσιτιψ υνδερ λογγ πηοτοπεριοδ συππρεσσες Τσηβ εξπρεσσιον ιν τηε παρς τυβεραλις

To test at what level of the signaling cascade metabolic cues 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). Temperature did not affect *Tshβ* expression, but a significant reduction was found only in female common voles at 10°C (Fig. 2A-D; Table S4). Overall, *Tshβ* and gonadal weight show a positive relationship (Fig. 3A-D), indicating that *Tshβ* is involved in suppressing gonadal development when food is scarce.

After translation, TSHβ and αGSU 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 involved in metabolic regulation and food intake. For this reason, it was unexpected that 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 *Rfrp3*. Although overall *Rfrp3* expression was higher at 21°C, no effects of workload on *Rfrp3* expression were observed (Fig. 2A-D, Table S4).

Food scarcity suppresses Kiss1 signaling 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 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). Surprisingly, *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 indicates that the *Kiss1* system is 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 & Fraser 1979). Surprisingly, *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: $T\sigma\eta\beta$ and $Tshr$ in Common vole males ($F_{2,23} = 7.44, p < 0.01$); $T\sigma\eta\beta$ in Common vole females ($F_{1,23} = 4.89, p < 0.05$); $T\sigma\eta\beta$, $P\phi\phi\pi$, anterior hypothalamic *Kiss1* in Tundra vole males ($F_{3,28} = 8.47, p < 0.001$); $T\sigma\eta\beta$ 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 leads in turn to suppression of gonadal growth, especially in Common voles. Tundra vole males additionally seem to use the hypothalamic *Kiss1* system to control reproduction when food is scarce at long photoperiods. *Kiss1* is one of the main drivers for GnRH neuron activation (De Roux *et al.* 2003; Seminara *et al.* 2004; Han *et al.* 2005, 2015). It is therefore surprising that observed patterns in *Kiss1* expression are not reflected in GnRH expression. 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 *Rfrp3* levels may cause decreased reproductive organ mass observed at low temperature.

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; Wang *et al.* 2019). 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, submitted). 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 hypothalamic gene expression is related to modify the PNES axis under energetically challenging conditions to reduce gonadal activation. The short duration of pineal melatonin release under long photoperiods lead to increased pars tuberalis $T\sigma\eta\beta$, which serves a pivotal role in the PNES (Hanon *et al.* 2008; Ono *et al.* 2008). The present study reveals that the photoperiodic induced $T\sigma\eta\beta$ 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 $T\sigma\eta\beta$ 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.

Pars tuberalis derived TSH binds locally to TSH receptors (TSHr) in the tanycytes, where it systematically leads to increased DIO2 (Hanon *et al.* 2008; Nakao *et al.* 2008; Guerra *et al.* 2010). The observed $T\sigma\eta\beta$ 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. 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). 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 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.*, in preparation). In addition, two to three-day fasted rats show elevated *Dio2* mRNA levels in tanycytes (Diano *et al.* 1998; Coppola *et al.* 2005). This might be an acute effect, which disappears when food is restricted for longer periods as in our study (i.e. 35 days). Stable $T\sigma\eta\beta$ 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.*, in preparation). 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 *Rfrp3* (i.e. *Npvf*) expression, but consistent with Siberian hamsters (Paulet *et al.* 2009), no effect of food scarcity was detected (Fig. 2A-D). In seasonal rodents, *Rfrp3* synthesis appeared to be primarily regulated by photoperiod (for review, see Angelopoulou *et al.*, 2019), but here we show that *Rfrp3* may also be an important regulator of the PNES to adaptively respond to ambient temperature changes. This finding is consistent with previous reports, showing that *Rfrp3* is a hypothalamic biomarker of ambient temperature, independent of nutritional status 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 *Rfrp3* levels were observed during the warmest part of the year (June-August) (Wang *et al.* 2019). Since RFRP3 is expected to mediate reproductive-axis function, our findings suggest that downregulation of *Rfrp3* 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 the hypothalamic Kiss1-system, 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; Sáenz De Miera *et al.* 2014; Rasri-Klosen *et al.* 2017), 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 (Paulet *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* signal, whereas we exclusively used hypothalamic tissue.

It seems important to note that the role of Kisspeptin in PNES regulation 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 suggest that Kisspeptin systems have species-specific functions in regulating reproduction. Although *Kiss1* in the posterior hypothalamus was not affected, food scarcity caused downregulation of *Kiss1* expression in the anterior hypothalamus of Tundra vole males (Fig. 2G). Although, this effect may be caused by positive sex steroid feedback on POA *Kiss1* expression (Ansel *et al.* 2010), 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* levels under all conditions. This indicates that the Kiss1-system is not involved in metabolic neuroendocrine control of reproduction in Common voles and it is conceivable that *Rfrp3* in this species has taken over the role of *Kiss1*. Interestingly, Tundra voles have a functional Kiss-system, which may explain the greater reduction in reproductive organ mass at a negative energy balance. It would be important to investigate how gene-silencing and overexpression (*Tshb*, *Kiss1* and *Rfrp3*) in specific hypothalamic regions may affect reproductive development, to disentangle causal relationships between hypothalamic gene expression and reproductive responses related to energy balance.

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 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 blocked. 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 *Tshb* 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 *Rfrp3* 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.

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. This work was funded by the Adaptive Life program of the University of Groningen (B050216)

References

- Adams, M.R., Tamarin, R.H. & 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.
- Angelopoulou, E., Quignon, C., Kriegsfeld, L.J., Simonneaux, V. & Anderson, G. (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. & 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.
- Caro, S.P., Schaper, S. V., Hut, R.A., Ball, G.F. & Visser, M.E. (2013). The Case of the Missing Mechanism: How Does Temperature Influence Seasonal Timing in Endotherms? *PLoS Biol.* , 11.
- Cooke, P.S., Spencer, T.E., Bartol, F.F. & Hayashi, K. (2013). Uterine glands: Development, function and experimental model systems. *Mol. Hum. Reprod.* , 19, 547–558.
- Coppola, A., Meli, R. & Diano, S. (2005). Inverse shift in circulating corticosterone and leptin levels elevates hypothalamic deiodinase type 2 in fasted rats. *Endocrinology* , 146, 2827–2833.
- Dakotse, M.-J. & 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. & Sáenz de Miera, C. (2018). An integrative view of mammalian seasonal neuroendocrinology. *J. Neuroendocrinol.* , 31.
- Diano, S., Naftolin, F., Goglia, F. & 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. & 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. & 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. & 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., *et al.* (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., *et al.* (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. & 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., *et al.* (2008). Ancestral TSH mechanism signals summer in a photoperiodic mammal. *Curr. Biol.* , 18, 1147–1152.
- Hileman, S.M., McManus, C.J., Goodman, R.L. & 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.

- Hrvatin, S., Sun, S., Wilcox, O.F., Yao, H., Lavin-Peter, A.J., Cicconet, M., *et al.* (2020). Neurons that regulate mouse torpor. *Nature* , 583, 115–121.
- Hut, R.A. (2011). Photoperiodism: Shall EYA compare thee to a summers day? *Curr. Biol.* , 21, 22–25.
- Hut, R.A., Dardente, H. & Riede, S.J. (2014). Seasonal timing: How does a hibernator know when to stop hibernating? *Curr. Biol.* , 24, 602–605.
- Hut, R.A., Pilorz, V., Boerema, A.S., Strijkstra, A.M. & Daan, S. (2011). Working for food shifts nocturnal mouse activity into the day. *PLoS One* , 6, 1–6.
- Jaroslawska, J., Chabowska-Kita, A., Kaczmarek, M.M. & 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. & Simonneaux, V. (2013). TSH restores a summer phenotype in photoinhibited mammals via the RF-amides RFRP3 and kisspeptin. *FASEB J.* , 27, 2677–2686.
- Krebs, S., Fischaleck, M. & Blum, H. (2009). A simple and loss-free method to remove TRIzol contaminations from minute RNA samples. *Anal. Biochem.* , 387, 136–138.
- Król, E., Douglas, A., Dardente, H., Birnie, M.J., Vinne, V. van der, Eijer, W.G., *et al.* (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.
- Lincoln, G.A. & 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.
- Lomet, D., Druart, X., Hazlerigg, D., Beltramo, M. & Dardente, H. (2020). Circuit-level analysis identifies target genes of sex steroids in Ewe seasonal breeding. *Mol. Cell. Endocrinol.* , 110825.
- Nakane, Y. & 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., *et al.* (2008). Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature* , 452, 317–322.
- Negus, N.C. & Berger, P.J. (1977). Experimental Triggering of Reproduction in a Natural Population of *Microtus montanus*. *Science* , 196, 1230–1231.
- Nelson, R.J., Dark, J. & 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. & 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. & 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. & 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., *et al.* (2008). Involvement of thyrotrophin in photoperiodic signal transduction in mice. *Proc. Natl. Acad. Sci.* , 105, 18238–18242.
- Paul, M.J., Pyter, L.M., Freeman, D.A., Galang, J. & 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. & 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., Saboureaux, M., Pévet, P., Simonneaux, V. & Mikkelsen, J.D. (2008). RFamide-related peptide gene is a melatonin-driven photoperiodic gene. *Endocrinology* , 149, 902–912.
- van Rosmalen, L., van Dalum, J., Hazlerigg, D.G. & 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.
- De Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L. & 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.
- Ruffino, L., Salo, P., Koivisto, E., Banks, P.B. & 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. & Hazlerigg, D. (2017). Maternal photoperiod programs hypothalamic thyroid status via the fetal pituitary gland. *Proc. Natl. Acad. Sci.* , 114, 8408–8413.
- Sáenz De Miera, C., Monecke, S., Bartzen-Sprauer, J., Laran-Chich, M.P., Pévet, P., Hazlerigg, D.G., *et al.* (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. & 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. & 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., *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.
- Simonneaux, V., Ancel, C., Poirel, V.J. & Gauer, F. (2013). Kisspeptins and RFRP-3 act in concert to synchronize rodent reproduction with seasons. *Front. Neurosci.* , 7, 1–11.
- Smith, J.T., Cunningham, M.J., Rissman, E.F., Clifton, D.K. & Steiner, R.A. (2005a). Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology* , 146, 3686–3692.
- Smith, J.T., Dungan, H.M., Stoll, E.A., Gottsch, M.L., Braun, R.E., Eacker, S.M., *et al.* (2005b). Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* , 146, 2976–2984.
- Steinlechner, S., Stieglitz, A., Ruf, T., Heldmaier, G. & 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., *et al.* (2020). A discrete neuronal circuit induces a hibernation-like state in rodents. *Nature* , 583, 109–114.
- Team, R.C. (2013). R: A language and environment for statistical computing. *R Found. Stat. Comput. Vienna, Austria*.

van der Vinne, V., Gorter, J.A., Riede, S.J. & 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., Riede, S.J., Gorter, J.A., Eijer, W.G., Sellix, M.T., Menaker, M., *et al.* (2014). Cold and hunger induce diurnality in a nocturnal mammal. *Proc. Natl. Acad. Sci.* , 111, 15256–15260.

van der Vinne, V., Tachinardi, P., Riede, S.J., Akkerman, J., Scheepe, J. & Hut, R.A. (2019). Maximising survival by shifting the daily timing of activity. *Ecol. Lett.* , 22, 2097–2102.

Wang, D., Li, N., Tian, L., Ren, F., Li, Z., Chen, 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). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

van de Zande, L., van Apeldoorn, R.C., Blijdenstein, A.F., de Jong, D., van Delden, W. & 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.

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 and (G -J) body mass 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 SEM (n = 6-8). Significant effects (two-way ANOVA) of workload (wl), temperature (temp) and interactions (wlxtemp) 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 2. Food scarcity and ambient temperature affect gene expression in the posterior and anterior hypothalamus. Relative gene expression levels of $T\sigma\eta\beta$, $Tshr$, $Dio2$, $Kiss1$ and $Rfrp3$ 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 SEM (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, $Tsh \beta$ and $Kiss1$ expression. Correlations between (A -D) $T\sigma\eta\beta$ 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.



