

Differential temperature effects on photoperiodism in female voles: An explanation for species-specific declines in vole populations

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Abstract

The photoperiodic neuroendocrine system drives seasonal rhythms in reproduction. To get a better understanding of how annual cycling environmental cues impact reproductive function and plasticity in small, short-lived herbivores with different geographic origins, we investigated the mechanisms underlying integration of temperature in the photoperiodic-axis regulating female reproduction in tundra voles and in common voles. We show that photoperiod and temperature interact to determine appropriate physiological responses; there is species-dependent annual variation in the sensitivity to temperature for reproductive organ development. These findings are in line with our census data, showing an earlier onset of spring reproduction in cold springs, while reproductive offset in autumn is synchronized to photoperiod. The reproductive organs of tundra voles were relatively insensitive to temperature, whereas hypothalamic gene expression was generally upregulated at 10°C. Due to global warming, spring reproduction in common voles will be delayed, perhaps resulting in shorter breeding seasons and thus declining populations.

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[†]Dedicated to Dr. Cor Dijkstra (1950-2017)

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Abstract

The photoperiodic neuroendocrine system drives seasonal rhythms in reproduction. To get a better understanding of how annual cycling environmental cues impact reproductive function and plasticity in small, short-lived herbivores with different geographic origins, we investigated the mechanisms underlying integration of temperature in the photoperiodic-axis regulating female reproduction in tundra voles and in common voles. We show that photoperiod and temperature interact to determine appropriate physiological responses; there is species-dependent annual variation in the sensitivity to temperature for reproductive organ development. These findings are in line with our census data, showing an earlier onset of spring reproduction in cold springs, while reproductive offset in autumn is synchronized to photoperiod. The reproductive organs of tundra voles were relatively insensitive to temperature, whereas hypothalamic gene expression was generally upregulated at 10°C. Due to global warming, spring reproduction in common voles will be delayed, perhaps resulting in shorter breeding seasons and thus declining populations.

Introduction

In most terrestrial temperate zone regions, winter represents an annual period with short photoperiod, decreased ambient temperature and reduced food availability, which induces increased energetic challenges for non-hibernating mammals. Reproduction under these circumstances is not beneficial for survival of small, short-lived mammals, because pregnancy and lactation are energy-consuming processes (Speakman 2008), and newly born offspring is vulnerable to harsh environmental conditions. Hence, many temperate species have evolved intrinsic timing mechanisms to predict major seasonal changes and accurately time morphology, physiology and reproductive behavior. Because of its absence in inter-annual variation, many organisms use the purely proximate predictor photoperiod, as reliable signal to prepare metabolically for upcoming seasons. In several species it has been demonstrated that the rate of postnatal maturation is set *in utero* through transmission of maternal melatonin (van Dalum *et al.* 2020). In long-day-breeders, prenatal exposure to short photoperiods and postnatal exposure to intermediate photoperiods (i.e., spring programmed) facilitates accelerated postnatal reproductive development in juveniles (Hoffmann 1973; Horton 1984; Yellon & Goldman 1984; Horton 1985; Stetson *et al.* 1986; Horton & Stetson 1992; Prendergast *et al.* 2000; Sáenz de Miera *et al.* 2017). This phenomenon is named ‘maternal photoperiodic programming’, reviewed in (Horton 2005; Sáenz De Miera 2019; van Dalum *et al.* 2020), and operates through the hypothalamic photoperiodic neuroendocrine system (PNES) for seasonal synchronization (Sáenz de Miera *et al.* 2017; van Rosmalen *et al.* 2021).

The PNES measures photoperiod and subsequently drives annual rhythms in reproduction, and has been described in detail in several mammal and bird species (Baker 1938; Dardente *et al.* 2003, 2010, 2018; Hanon *et al.* 2008; Nakao *et al.* 2008; Ono *et al.* 2008; Masumoto *et al.* 2010;

Hut 2011; Sáenz De Miera *et al.* 2014; Wood *et al.* 2015; Nakane & Yoshimura 2019), including the common (*Microtus arvalis*) and tundra vole (*Microtus oeconomus*) (Król *et al.* 2012; van Rosmalen *et al.* 2020, 2021).

Energetic demands, such as costs of cellular maintenance, thermoregulation and foraging all compete with reproduction (Bronson 1989; Schneider 2004; Speakman 2008; Ruffino *et al.* 2014). Ambient temperature largely affects thermoregulatory costs and energy balance in non-hibernating small mammals, due to the large surface-to-volume ratio. It has been demonstrated that ambient temperature influences timing of breeding in birds (Crick *et al.* 1997; Verhagen *et al.* 2020), hibernation ending in mammals (Inouye *et al.* 2000), and gonadal activation in small rodents (Dakotse & Martinet 1977; Nelson *et al.* 1989; Steinlechner *et al.* 1991; Kriegsfeld *et al.* 2000; Larkin *et al.* 2001). Thermal cues are expected to be involved in modulation of photoperiodic responses to inhibit or accelerate reproductive development (Caro *et al.* 2013; Hut *et al.* 2014), but underlying regulatory mechanisms need to be clarified.

This led us to ask how photoperiod and temperature interact to regulate reproductive activation in voles, herbivorous species in which plasticity in onset of spring reproduction has been observed in nature (Negus *et al.* 1986; Ergon *et al.* 2001), in which 3-year population cycles have been widely documented (Huntington 1931; Krebs *et al.* 1973; Krebs 2013; Myers 2018), and in which food and ambient temperature are significant modifiers of female (Baker *et al.* 1932; Dakotse & Martinet 1977; Simons *et al.* 2011), and male reproductive activation (Baker *et al.* 1932; Negus & Berger 1977; Sanders *et al.* 1981; Nelson *et al.* 1983, 1989; Kriegsfeld *et al.* 2000; Larkin *et al.* 2001; Steinlechner & Puchalski 2003). For this reason, an opportunistic dimension (i.e., sensitive to both photic and non-photoc annual cues) to reproductive strategies of voles might be expected.

The neurobiological basis that underlies (thermo) energetic modification of the photoperiodic axis is largely unknown. Gonadotropin-releasing hormone (GnRH) neurons are important drivers of reproduction regulating hormonal release (i.e., LH, FSH) from the pituitary gland (Schally *et al.* 1970; Guillemin 1977). Long day induced T₃ in the mediobasal hypothalamus may control GnRH neurons via hypothalamic areas that are involved in thermo- and metabolic regulation, reviewed in (Hut *et al.* 2014). The preoptic area (POA) is the primary site for thermoregulation, reviewed in (Morrison & Nakamura 2019), where both internal and external thermal cues are integrated. Furthermore, the arcuate nucleus (ARC) and the dorso/ventromedial hypothalamus (DMH/VMH) are involved in sensing energy balance. Neurons expressing RF-amides: Kisspeptin (KISS1) and RF-amide related peptide (RFRP-3), are located in the POA, ARC, DMH and VMH (Smith *et al.* 2005a, b; Oakley *et al.* 2009; Parhar *et al.* 2012; Henningsen *et al.* 2016), and are neuropeptides known to function as strong activators of GnRH neurons controlling puberty onset and reproduction (De Roux *et al.* 2003; Seminara *et al.* 2004; Hileman *et al.* 2011). KISS1 and RFRP-3 are therefore possibly involved in integrating thermal cues, regulating reproduction in mammals (Revel *et al.* 2008; Klosen *et al.* 2013; Simonneaux *et al.* 2013; Hut *et al.* 2014; Sáenz De Miera *et al.* 2014), albeit that evidence for this concept remains limited.

Local variation in annual food patterns depend on annual photoperiod-temperature patterns, and might be a driving force in the evolution of breeding seasons in small mammals. The sensitivity to photoperiod and ambient temperature as a breeding cue can differ depending on the predictability of the environment, which varies with latitude (Bronson 1989). On one hand, temperate latitudes are highly predictable environments that allow photoperiod to be the main driver for short windows of permitting reproductive success. On the other hand, latitudes further

away from the equator may display unpredictable snowfall which leads to unpredictable changes in food availability, predation risk and thermoregulatory costs. Therefore, opportunistic reproductive strategies may have evolved in short-lived mammals living at extreme latitudes.

To understand how thermal cues integrate in the PNES, and subsequently modify female gonadal responses in Northern and Southern species, we investigated photoperiodic responses in both physiology and hypothalamic gene expression levels by exposing Northern voles (tundra or root vole, *Microtus oeconomus*) and Southern voles (common vole, *Microtus arvalis*) to different photoperiod and ambient temperature regimes. To compare the balance of integration of photoperiodic-temperature interactions affecting PNES function in Northern and Southern vole species, we present time series of vole population dynamics in relation to ambient temperature, and subsequently investigated the mechanisms underlying integration of thermal cues in the photoperiodic-axis that subsequently regulates female reproduction.

Material and methods

Study area and trapping

Two-month interval census data were collected from March 1980 to November 1986 at the Lauwersmeer area, the Netherlands (53° 24' N, 6° 16' E). Common voles were snap trapped using fixed locations consisting of ten traps each. In total 500 traps with carrots were set up for three consecutive nights every other month, and checked at daytime for the three consecutive days, amounting to a constant trapping effort during each census session. Common voles have no protected status according to IUCN red list, and are considered locally as an agricultural pest (Yigit *et al.* 2016). Reproductive status of trapped voles was determined by several reproductive traits,

and animals were classified as sexually active if there was evidence of enlarged testes, pigmented scrotal area, open vulva, pregnancy or enlarged nipples indicating lactation.

Animals and experimental procedures

All experimental procedures were carried out according to the guidelines of the animal welfare body (IvD) of the University of Groningen conform to Directive 2010/63/EU, and approved by the CCD (Centrale Commissie Dierproeven) of the Netherlands (CCD license number: AVD1050020171566). 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 (van de Zande *et al.* 2000). Weaned voles were individually housed in transparent plastic cages (15x40x24 cm) provided with sawdust, dried hay, an opaque pvc tube and *ad libitum* water and food (Standard rodent chow; Altromin #141005).

The female voles used in the experiment (63 common voles; 52 tundra voles) were kept in temperature-controlled chambers. Voles were gestated and raised to weaning under either a short photoperiod (SP, 8h light:16h dark: early breeding season, winter/spring programmed) or a long photoperiod (LP, 16h light:8h dark: late breeding season, summer/autumn programmed) at $21 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ relative humidity. At weaning (21 days old), voles were transferred to either 10°C or 21°C under a range of different photoperiods. Photoperiods applied after weaning at 21°C were (hours light: hours dark): 6:18, 10:14, 12:12, 14:10, 16:8, 18:6. Photoperiods applied after weaning at 10°C were: 10:14, 12:12, 14:10, 16:8 (Fig. 1).

Tissue collections

Voles were sacrificed by decapitation, 17 ± 1 -h after lights OFF when 50 days old. Reproductive organs were dissected and cleaned of fat, and wet masses of paired ovary and uterus were measured (± 0.0001 g). Whole brains were removed with special care to visually include the intact proximate pituitary stalk containing the pars tuberalis. Within 5 minutes after decapitation, brains were slowly frozen on a brass block surrounded by liquid N₂, and stored at -80°C until further dissection. Posterior and anterior hypothalamic areas were dissected on ice as described in (van Rosmalen & Hut 2021a), and tissues were transferred to tubes containing Trizol immediately after dissection. Subsequently, RNA extractions, reverse transcription and real-time quantitative PCR was performed.

An extensive description of methodological details is available in the Supporting Information.

Results

Timing of spring reproduction in wild common voles is associated with ambient temperature

Population dynamics of common voles in the Lauwersmeer area, the Netherlands reveal annual cycling patterns with growing populations in spring and summer, and declining populations in autumn and winter (Fig. 2A). In each year, population density peaks in September (shortly after summer solstice), while large inter-annual variation in vole density levels in September were observed, generating so called population cycles with an enigmatic period of three years (Fig. 2A,E,H,K). Furthermore, annual timing of female and male reproductive status largely fluctuates between years, which resulted in different onsets for spring reproduction (Fig. 2C,F,I,L). The proportion of sexually active voles' peaks in May or in July depending on year (Fig. 2C,F,I,L).

This indicates that vole reproductive status can be modified by non-phototic, unpredictable environmental signals.

Higher proportions of sexually active female voles (i.e., pregnant or lactating females) in May were detected when average March temperature in that same year was low ($F_{1,4}=11.52$, $P<0.03$, $R^2=0.74$; Fig. 2G). A similar negative relationship was found for the proportion of total sexually active voles (females and males) in May ($F_{1,4}=10.06$, $P<0.04$, $R^2=0.72$; Fig. 2M). In males, a trend in the same direction was found, however, this effect was non-significant ($F_{1,4}=0.27$, $P = 0.64$, $R^2=0.06$). Higher proportions of sexually active voles in May led to higher population densities in subsequent months, except for 1984, in which vole density was remarkably low while average March temperature was also low (Fig. 2D,J)

Temperature overrides photoperiodic spring response in common voles, but not in tundra voles

Spring-programmed common voles at 10°C had a 1.5 to 4-fold higher uterus mass (Fig. 3A), 2 to 2.5-fold higher ovary mass (Fig. 3E), 1 to 1.3-fold higher body mass (Fig. 3I) and 1.2 to 2.3-fold higher gonadosomatic index, GSI (Fig. 3M) than common voles at 21°C (Table S4). Contrast analysis revealed that temperature effects on body mass corrected organ mass (GSI) were strongest at short photoperiods (i.e., 10L:14D and 12L:12D) (Fig. 3M). Interestingly, temperature effects were stronger in spring-programmed voles ($F_{1,44}=37.74$, $P<0.0001$) than in autumn-programmed voles ($F_{1,30}=5.77$, $P<0.04$).

Common voles at 21°C under long photoperiods showed significantly higher uterus mass ($F_{4,19}=4.97$, $P=0.007$; Fig. 3A), ovary mass ($F_{4,19}=3.29$, $P=0.04$; Fig. 3E), body mass ($F_{4,19}=4.99$, $P=0.007$; Fig. 3I) and GSI ($F_{4,19}=4.17$, $P=0.02$; Fig. 3M) than common voles at 21°C under shorter photoperiods. In common voles at 10°C, photoperiodic effects on physiological outputs were small

or absent (uterus: $F_{3,25}=0.76$, $P=0.53$; ovary: $F_{3,25}=1.78$, $P=0.18$; body mass: $F_{3,25}=2.55$, $P=0.08$; GSI: $F_{3,25}=0.55$, $P=0.66$), indicating that temperature can overrule photoperiodic signals in this species.

To assess whether female voles' program offspring photoperiodic sensitivity through transfer of maternal photoperiod *in utero*, voles in this study were either photoperiodical spring-programmed (gestated and raised to weaning under SP) or photoperiodical autumn-programmed (gestated and raised to weaning under LP) (Fig. 1). Photoperiodic-history dependent effects were found in common voles, with uterus mass and GSI being slightly higher in spring-programmed voles (uterus: $F_{1,74}=5.26$, $P<0.03$; GSI: $F_{1,74}=4.81$, $P<0.04$; Fig. S1A,M,C,O). Although autumn-programmed common voles significantly increased ovary and body mass at 10°C (ovary: $F_{1,30}=10.08$, $P<0.004$; body mass: $F_{1,30}=10.28$, $P<0.004$; Fig. 3F,J), this temperature effect was not reflected in GSI ($F_{1,30}=1.09$, $P=0.31$; Fig. 3N).

In tundra voles, ovary mass and GSI were affected by photoperiod, with increased values at long photoperiods (ovary: $F_{4,72}=2.78$, $P<0.04$; GSI: $F_{4,72}=6.36$, $P<0.0002$; Fig. 3C,O). In spring-programmed tundra voles, temperature did not affect physiological outputs (Fig. 3C,G,K,O; Table S4), while in autumn-programmed tundra voles, low temperature enhances reproductive organ and body mass (uterus: $F_{1,32}=4.85$, $P<0.04$; ovary: $F_{1,32}=16.05$, $P<0.0004$; body mass: $F_{1,33}=11.17$, $P<0.003$; GSI: $F_{1,32}=4.27$, $P<0.05$; Fig. 3D,H,L,P). These data indicate that there is species dependent annual variation in the sensitivity to temperature for timing of reproductive onset.

Temperature affects hypothalamic gene expression in tundra voles, but not in common voles

To assess at what level of the reproductive axis thermal cues act to modify photoperiodic output signals, we measured gene expression levels in posterior and anterior hypothalamus in a subset of

experimental groups: spring-programmed 10L:14D and 16L:8D at 10°C and 21°C, here temperature and photoperiod largely affected reproductive organ mass.

Our results show that *Tshβ* expression is higher in voles at long photoperiod (16L:8D) than in voles at short photoperiod (10L:14D) ($F_{1,42}=21.52$, $P<0.0001$), and was not affected by temperature ($F_{1,42}=0.42$, $P<0.53$; Fig. 4A,H). Under 16L:8D, *Tshβ* levels were approximately 2-fold higher in common voles than in tundra voles ($F_{1,19}=5.23$, $P < 0.05$).

Although in common voles, *Tshr* levels were relatively low, a small reduction was observed at 16L:8D ($F_{1,17}=8.51$, $P<0.01$; Fig. 4B), but *Tshr* was not affected by temperature ($F_{1,42}=0.09$, $P=0.77$). In tundra voles, photoperiod did not affect *Tshr* ($F_{1,21}=0.11$, $P=0.75$; Fig. 4I), while *Tshr* expression was enhanced at 10°C ($F_{1,9}=6.01$, $P<0.05$; Fig. 4I). Furthermore, overall *Tshr* levels were approximately 3-fold higher in tundra voles than in common voles ($F_{1,44}=103.2$, $P<0.001$).

Indeed, *Dio2* follows similar responses to photoperiod as observed in *Tshβ* ($F_{1,42}=21.52$, $P<0.0001$; Fig. 4C,J). In tundra voles, *Dio2* was further enhanced at 10°C ($F_{1,20}=6.83$, $P<0.03$), while in common voles, temperature did not affect *Dio2* ($F_{1,18}=1.14$, $P=0.32$; Fig. 4C).

In both vole species, *Kiss1* expression in the posterior hypothalamus was affected by temperature (common vole: $F_{1,18}=8.79$, $P<0.009$; tundra vole: $F_{1,20}=9.59$, $P<0.006$; Fig. 4D,K). In common voles, posterior *Kiss1* was elevated at 10°C under 16L:8D ($F_{1,8}=22.32$, $P<0.002$), indicating that temperature affects *Kiss1* only at long photoperiods. In tundra voles, posterior *Kiss1* was elevated at 10°C under 10L:14D ($F_{1,11}=15.09$, $P<0.003$), indicating that temperature affects *Kiss1* only at short photoperiods. Furthermore, general posterior hypothalamic *Kiss1* levels were >2-fold higher in tundra voles than in common voles ($F_{1,38}=141.46$, $P<0.0001$).

In common voles, *Rfrp* levels were higher at long photoperiod independent of temperature ($F_{1,18}=6.56$, $P<0.02$; Fig. 4E). In tundra voles, a significant photoperiod-temperature interaction was found, with increased *Rfrp* levels at 10°C under 10L:14D, and decreased *Rfrp* levels at 21°C under 16L:8D ($F_{1,20}=7.27$, $P<0.02$; Fig. 4L).

Common voles under 10L:14D showed slightly increased *Kiss1* levels at 10°C ($F_{1,11}=5.82$, $P<0.05$; Fig. 4F). However, in tundra voles, temperature and photoperiod did not affect *Kiss1* in the anterior hypothalamus (PP: $F_{1,21}=0.62$, $P=0.44$; temp: $F_{1,21}=0.52$, $P=0.48$; Fig. 4M). As observed in the posterior hypothalamus, also general anterior hypothalamic *Kiss1* levels were higher in tundra voles than in common voles ($F_{1,44}=109.39$, $P<0.0001$).

GnRH expressing neurons are located in the POA and act on the pituitary gland where the release of gonadotropins is regulated to drive reproduction. For this reason, it was unexpected that in common voles, *Gnrh* was reduced under long photoperiods ($F_{1,16}=5.07$, $P<0.04$; Fig. 4G), and unaffected by temperature ($F_{1,16}=1.34$, $P=0.27$). In tundra voles, *Gnrh* was rather stable and not affected by photoperiod ($F_{1,16}=0.12$, $P=0.73$; Fig. 4N) or temperature ($F_{1,16}=0.34$, $P=0.57$).

Discussion

Our results confirm the importance of thermal cues for female reproduction in small mammals. Lowering ambient temperature caused accelerated reproductive organ maturation in spring-programmed common voles, while temperature effects were not reflected in hypothalamic gene expression (Fig. 5D). In contrast, temperature in tundra voles did not influence physiological spring responses, while hypothalamic gene expression was affected by temperature (Fig. 5E).

Annual population cycle dynamics in common vole populations revealed that cold springs are associated with advanced onset of spring reproduction (Fig. 2G,M). This eventually may lead

to higher vole densities in subsequent months (Fig. 2D,J). These data confirm that seasonal reproductive cycles in common voles are plastic, and are therefore not exclusively controlled by photoperiod, but also depend on ambient temperature. This is in agreement with previous studies reporting large inter-annual variation in the ratio of reproductive females in spring, which has been shown to be positively or negatively related to temperature depending on ecological context (Giraudoux *et al.* 2019; McLean & Guralnick 2020). Field transplant experiments revealed that the immediate environment drives the onset of spring reproduction in field voles, *Microtus agrestis* (Ergon *et al.* 2001). Thus, annual variation in cycle dynamics within vole populations may be attributed to local environmental breeding cues, such as food, temperature, rain, predators and parasites (Huntington 1931).

Our laboratory experiments confirmed our observation that cold springs advance reproductive onset, since lowering ambient temperature caused accelerated reproductive organ development in spring-programmed female common voles (Fig. 3). In Syrian hamsters (*Mesocricetus auratus*) at low temperatures, ovary mass was not affected, while fewer follicles and corpora lutea were observed (Reiter 1968). For this reason, ovary mass may be an unreliable indicator for hormonal secretion. Histological analyses were not performed in our study; therefore, this data should be interpreted with caution. In contrast, uterine size is positively related to thickness of secretory epithelium and the number of endometrial glands (Reiter 1968). Therefore, low uterus mass observed at high temperature is presumably related to incomplete maturation of uterine glands, indicating infertility, because uterine glands are crucial for pregnancy (Cooke *et al.* 2013).

In addition, common vole males also accelerated gonadal growth at 10°C (van Rosmalen *et al.* 2021), confirming that in nature, female and male voles may be synchronized in their

reproductive status. Similar findings have been reported in an earlier study, showing that the largest and most fertile female and male common voles were those raised at 5°C under 15L:9D, while the smallest and least fertile animals were raised at 33°C under 10L:14D (Dakette & Martinet 1977). In contrast, hamsters and other vole species show decreased gonadal size, and decreased reproductive output at low temperatures (Nelson *et al.* 1989; Steinlechner *et al.* 1991). Opposite temperature effects may be explained by species differences in optimal ambient temperature for reproduction and different photoperiodic histories of the animals. Here we show that maternal photoperiod affects postnatal growth of reproductive organs in female voles, as was previously demonstrated in male long-day breeders (Hoffmann 1973; Horton 1984, 1985; Yellon & Goldman 1984; Stetson *et al.* 1986; Horton & Stetson 1992; Prendergast *et al.* 2000; Sáenz de Miera *et al.* 2017), including common voles (van Rosmalen *et al.* 2021).

Although large variation in the onset of the breeding season could be observed between years, the offset of the breeding season in autumn was rather synchronized with photoperiod (Fig. 2C,F,I,L). It is therefore possible, that temperature sensitivity changes throughout the season. Although, in autumn-programmed common voles, ovary and body mass were also elevated at 10°C (Fig. 3F,J), GSI was unaffected by temperature (Fig. 3N). These results demonstrate that in common vole females, photoperiodical spring-programmed responses can be modulated by temperature, whereas photoperiodical autumn-programmed responses are relatively insensitive to modulation by temperature.

Because grass growth is initiated when temperatures are rising, it is counterintuitive that voles, an herbivorous species, accelerate reproductive development when spring temperatures are low. However, grass growth is initiated at 5-10°C (Cooper 1964; Peacock 1975, 1976), and at 53°N latitude (from where our common voles originate) an average ambient temperature of 10°C

occurs in spring (Hut *et al.* 2013). It is therefore likely that when food is abundant, common voles perceive 10°C as an additional environmental cue indicating spring, which therefore further facilitates reproductive activation. Given a specific photoperiod, autumn, is generally warmer than spring (Hut *et al.* 2013), and 21°C may therefore be perceived as an additional environmental cue indicative of autumn, causing a reduced reproductive sensitivity to photoperiod. It has previously been shown that there is an optimal ambient temperature for breeding performance in deer mice, *Peromyscus maniculatus borealis* (Bronson & Pryor 1983). Our dataset did not contain extremely cold springs (Fig. 2B); therefore, we may only have data for the right side of the parabolic relationship between ambient temperature and reproductive status. Extremely cold and extremely warm springs are both expected to delay reproductive onset, since under these circumstances all energy is needed for thermoregulatory functions and tissue maintenance. This hypothesis is line with the heat dissipation limit theory, which suggests that heat generated during metabolism limits energy intake, and therefore decreases reproductive output when temperatures are high (Speakman & Król 2010; Simons *et al.* 2011; Zhao *et al.* 2020). This effect has been confirmed in common voles (Simons *et al.* 2011). Endotherms can maintain their body temperature under a large range of ambient temperatures, and many mammalian species can reduce their energy expenditure by entering daily torpor when food is scarce or temperatures are low (Heldmaier *et al.* 2004; Hut *et al.* 2011; van der Vinne *et al.* 2015). In contrast, voles do not enter torpor when energetically challenged (Nieminen *et al.* 2013; van Rosmalen & Hut 2021b), yielding limited energy savings. For this reason, the vole reproductive strategies and the underlying PNES may be more sensitive to temperature.

Ambient temperatures that belong to a certain photoperiod can be deduced from the ellipse-like relationships between photoperiod and monthly average temperatures at specific locations

(Fig. 5C). Groningen (53°N) is the center of the latitudinal distribution range for the common vole (Fig. 5A), and Oslo (60°N) for the tundra vole (Fig. 5B). Assuming that latitudinal adaptation is optimal in the center of its distributional range, it is expected that common voles are better adapted to the local seasonal environment of Groningen, the Netherlands (53°N, current latitudinal center of distribution) while tundra voles are better adapted to the local seasonal environment of Oslo, Norway (60°N, current latitudinal center of distribution). Groningen and Oslo differ in the ellipse-like relationship between photoperiod and ambient temperature, leading to different ambient temperatures that belong to specific photoperiods in spring (Fig. 5C). Our data demonstrated that spring-programmed common voles are sensitive to temperature, whereas tundra voles are insensitive to temperature (Fig. 3). However, broader temperature ranges under different photoperiodic conditions need to be applied in order to confirm whether tundra voles are also insensitive to more extreme temperatures as was shown for deer mice living at different latitudes (Bronson & Pryor 1983). In that study, reproductive output in house mice was insensitive to temperature, whereas deer mice showed narrow temperature ranges at which breeding took place, with high temperatures leading to reproductive success at 56°N and low temperatures leading to reproductive success at 31°N. In 1988, Bronson proposed that the use of photoperiod and ambient temperature as a cue to time breeding might depend on local habitats which change with latitude (Bronson 1988). Natural selection might either inhibit or promote the use of photoperiod, nutritional and thermal cues to control seasonal reproduction, which will result in species-specific reproductive strategies (Hut *et al.* 2014). Although we did not find major species differences in photoperiodic responsiveness, only common voles are sensitive to temperature in spring (Fig. 3).

The latitudinal distribution range of tundra voles is far up North, where they live under isolating snow covers for a large part of the year. The rather stable ambient temperatures under

snow covers in winter and early spring, may make temperature an unreliable seasonal cue for tundra voles. Furthermore, timing of spring reproduction in wild tundra vole populations may still correlate with temperature, but this does not necessarily mean that temperature has a direct effect on reproductive responses. However, there can be an indirect effect of temperature on vegetation growth, and tundra voles may use an opportunistic reproductive strategy in which the use of food availability as a cue is driving reproductive onset (van Rosmalen & Hut 2021a). Because common and tundra voles are both sensitive to photoperiod, but only common voles are sensitive to spring temperature (Fig. 3), comparisons between these two vole species may be used as models to investigate temperature modification of neurobiological mechanisms underlying photoperiodic responses.

In the posterior hypothalamus, where the pars tuberalis is localized, PNES genes (i.e., *Tsh β* , *Tshr* and *Dio2*) all respond to photoperiod (Fig. 4), which has previously been confirmed in other rodent species (Dardente *et al.* 2010; Masumoto *et al.* 2010; Sáenz de Miera *et al.* 2017; Wang *et al.* 2019), and in our captive vole populations (Król *et al.* 2012; van Rosmalen *et al.* 2020). Increased *Rfrp* under long photoperiods as observed in common voles and in tundra voles at 21°C (Fig. 4E,L), has previously been observed in both short and long-day breeders, and is believed to be important in controlling seasonal reproduction (Henningsson *et al.* 2016).

Although common voles strongly respond to temperature in physiological outputs (Fig. 3A,E,I,M), hypothalamic gene expression was mostly insensitive to temperature (Fig. 4A-G). The posterior hypothalamus contains kisspeptin neurons localized in the ARC controlling daily timing of food intake (Padilla *et al.* 2019), and is involved in sensing fat reserves and may subsequently be involved in decreasing fertility when food is scarce (Fu & van den Pol 2010; Harter *et al.* 2018). The anterior hypothalamus contains kisspeptin neurons localized in the POA, which receives

projections from thermoreceptors in the skin and also contains thermosensitive neurons (Morrison & Nakamura 2019). Therefore, it was rather unexpected that *Kiss1* in common voles was only slightly upregulated by low temperature. On one hand, this suggests that other factors more downstream or outside the PNES are responsible for temperature modulations of photoperiodic responses. In mammals, cold exposure leads to upregulation of *DIO2* in brown adipose tissue (BAT), leading to elevated peripheral T_3 levels (Silva & Larsen 1985; Lowell & Spiegelman 2000; De Jesus *et al.* 2001). Whether, circulating T_3 can act on the hypothalamus to activate GnRH neurons and subsequently control uterine growth remains unclear. In addition, uterine nuclei contain receptors for T_3 , and may therefore be a target organ for low temperature-induced circulating T_3 affecting seasonal uterine growth (Evans *et al.* 1983).

Surprisingly, downstream *GnRH* expression in the anterior hypothalamus does not reflect gonadal weight (Fig. 4G,N). Steroid feedback mechanisms on hypothalamic areas and the phase of the estrous cycle are highly involved in initiating the GnRH surge and may play an important role in our observations. Furthermore, negative sex steroid feedback on ARC kisspeptin neurons (Greives *et al.* 2008; Sáenz De Miera *et al.* 2014; Rasri-Klosen *et al.* 2017), may explain similar *Kiss1* and *GnRH* levels observed in different experimental groups. This is an important issue for future studies, and may be solved by shortening the interval between changing environmental conditions and tissue collection.

Interestingly, tundra voles have increased hypothalamic *Tshr*, *Dio2* and *Kiss1* levels at low temperature (Fig. 4I-K), however this is not reflected in reproductive organs. Therefore, it is conceivable that spring-programmed tundra voles are also sensitive to temperature, but later in development than common voles. Because of the substantial delay between gene expression and physiological responses, cold exposure experiments for extended periods with time-series

sampling are necessary to reveal whether indeed upregulated *Tshr*, *Dio2* and *Kiss1* are responsible for the accelerated reproductive organ maturation at 10°C. In agreement with our previous study, *Rfrp* was not affected by temperature under LP when food was available *ad libitum*, while *Rfrp* was downregulated at low temperature when food was scarce (van Rosmalen & Hut 2021a). In the experiments described here, food was available *ad libitum*. Thus, animals could increase their food intake to compensate for increased thermoregulatory costs at low temperatures. Our previous experiments revealed that temperature has different effects on the photoperiodic axis when food is scarce (van Rosmalen & Hut 2021a).

Our findings show that reproductive responses of both vole species are sensitive to photoperiod, whereas particularly the spring response of the common vole is determined by temperature. This is in line with our common vole census data, which reveals that warm springs are associated with later reproductive onset. Accelerating reproductive organ maturation when born in a relatively cold spring, but with abundant food available, is an adaptive response that facilitates reproduction and increases fitness. Due to a cold temperature in spring, reproductive onset is advanced, and pups will be born early in spring under increasing photoperiod, resulting in juveniles being programmed to accelerate reproductive organ development. This will lead to exponential growth of the population, resulting in more winter survivors. Due to increased vole density, more animals will advance their spring reproduction, and therefore these animals can produce an additional generation of pups, leading to a peak in vole density in the subsequent summer. This cycle will continue until vole density is extremely high, and therefore food resources are getting scarce and mortality increases. Food scarcity in winter/spring results in slower reproductive organ development (van Rosmalen & Hut 2021a). This might result in a population collapse and might be an explanation for the three-year population cycles documented in voles.

Furthermore, warmer springs due to global warming (Fig. 2B) may cause delayed onset of spring reproduction, while the offset of the breeding season appears to be relatively unaffected by temperature (Fig. 2,3), leading to a dramatic shortening of the breeding season. This observation provides an explanation for recent decline in vole populations and population cycles observed in Europe (Ims *et al.* 2008; Cornulier *et al.* 2013). Defining the molecular mechanisms through which thermal cues modify maternally photoperiodical-programmed responses to adaptively adjust timing of reproductive organ development will be important for a better understanding of how seasonal cycling environmental breeding cues shape female reproductive function and plasticity.

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Figures

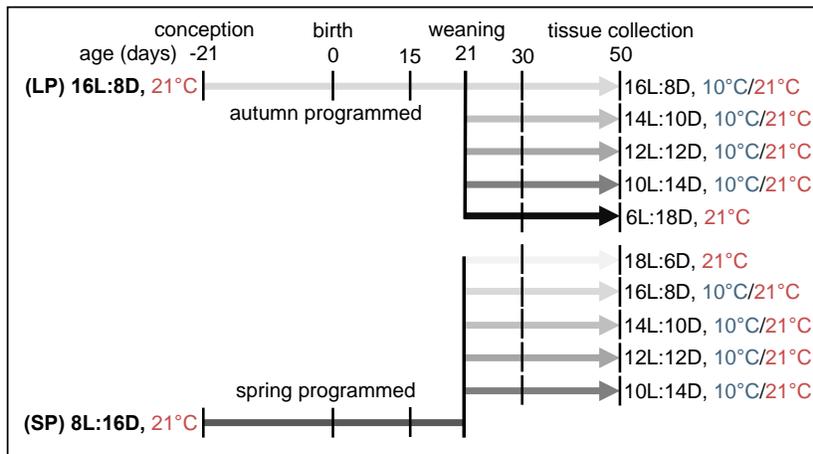


Figure 1. Experimental design. Conception, gestation, birth and lactation took place under either LP (i.e., autumn programmed) or SP (i.e., spring programmed) at 21°C. At weaning (21 days old) animals were transferred to either 10°C or 21°C at a range of different photoperiods. 6L:18D was only applied in autumn programmed voles, 18L:6D was only applied in spring-programmed voles. Tissues were collected at an age of 50 days.

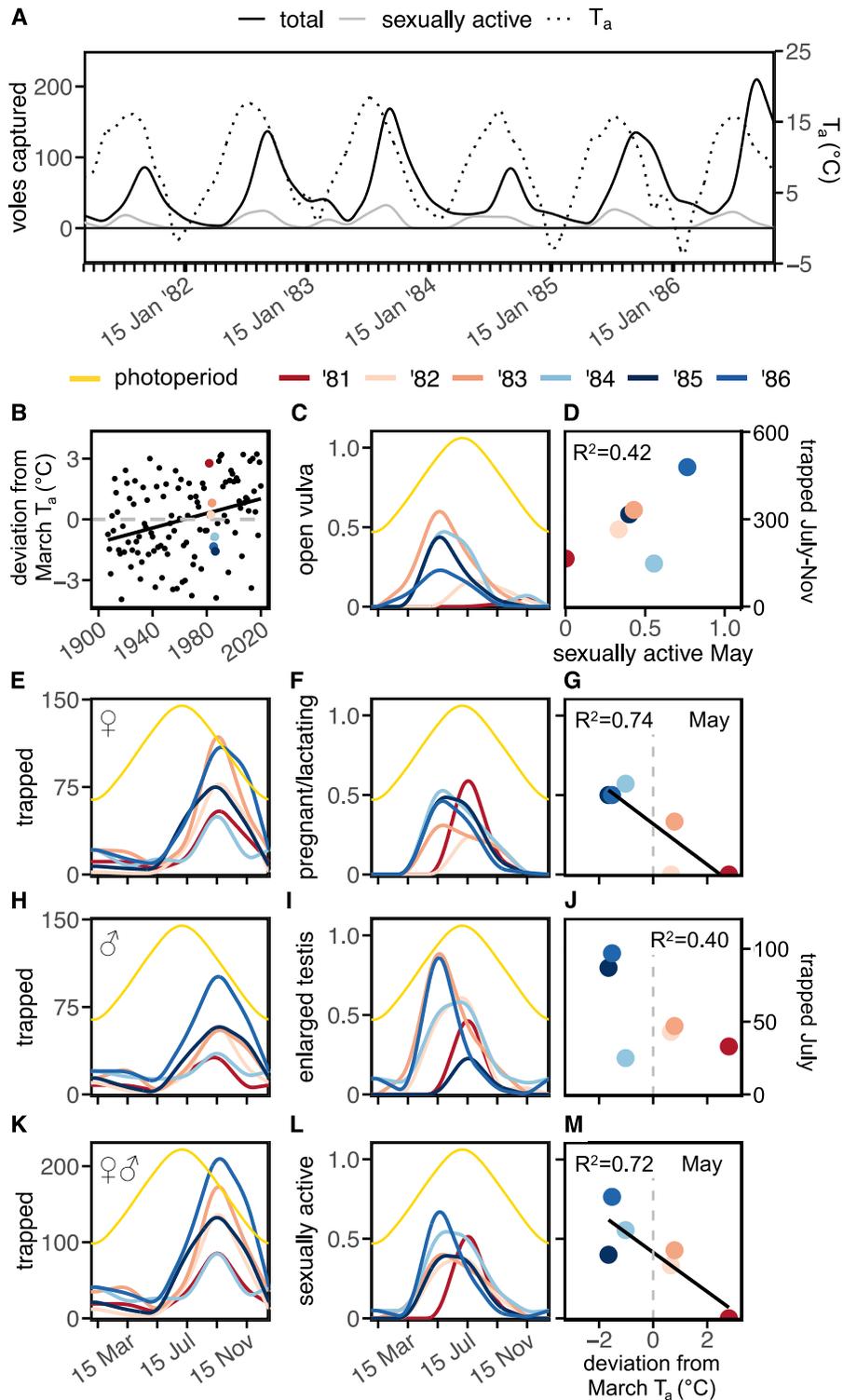


Figure 2. Onset of spring reproduction in common voles is associated with ambient temperature. Time series of common vole population dynamics in the Lauwersmeer area, the Netherlands (53.38°N, 6.22°E) between 1981-1986. Reanalysis of a previously published dataset (Dijkstra *et al.* 1988) (A) Two-month interval census data for the total

number of snap-trapped voles /1500 trap nights (solid, black line), number of sexually active voles (solid, grey line), and monthly average ambient temperature (T_a) (dashed, black line). (B) Deviation from average March T_a is increasing over the years (1907-2020). T_a is retrieved from the Eelde airport weather station, the Netherlands (53.13°N, 6.59°E) (<https://weerstatistieken.nl/eelde>). Annual changes in the total number of trapped (E) female (H) male and (K) total voles/ 1500 trap nights related to civil twilight-based photoperiod (yellow), which varies annually between 8.92h and 18.77h. Annual changes in the proportion of (C) females with open vulva (F) pregnant or lactating females, (I) males with enlarged testes and (L) sexually active males (enlarged testis) and females (pregnant/lactating). (D) Proportion of sexually active voles in May in relation to the total number of trapped voles in July, September and November ($F_{1,4} = 2.89$, $P = 0.17$). Deviation from average March T_a in relation to (G) the proportion of pregnant or lactating females ($F_{1,4} = 11.52$, $P < 0.03$), (J) the total number of trapped voles in July ($F_{1,4} = 2.66$, $P = 0.18$), and (M) the proportion of sexually active voles ($F_{1,4} = 10.06$, $P < 0.04$). Significant linear regression models for average T_a of previous months are indicated with a black line and details can be found in Table S5.

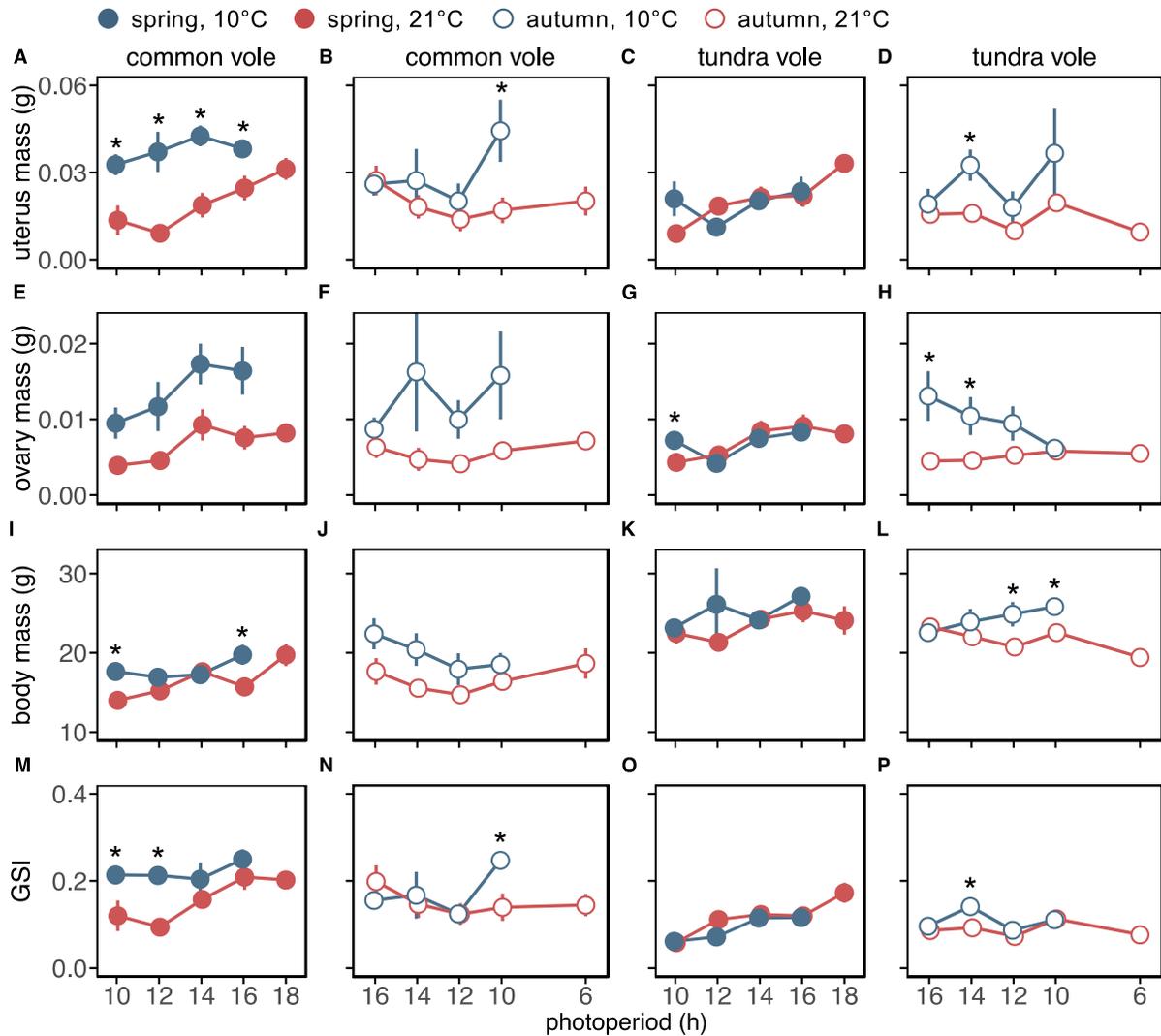


Figure 3. Temperature-dependent modulation of photoperiodic responses in physiological outputs of female voles. Responses to photoperiod for (A-D) uterus mass, (E-H) paired ovary mass, (I-L) body mass and (M-P) total reproductive organ mass corrected for body mass (gonadosomatic index, GSI) in 50-day old common and tundra voles respectively, photoperiodical spring programmed (closed symbols; gestated and raised to weaning under SP) or photoperiodical autumn programmed (open symbols; gestated and raised to weaning under LP) at 10°C (blue) or 21°C (red). X-axis of autumn-programmed data is plotted in the reversed direction to illustrate annual photoperiodic changes. Data are presented as means \pm SEM (n=4-8). Significant effects of contrast analysis are indicated: $*P < 0.05$. In short, significant photoperiodic effects were found in: B, E, G, I, O and P, significant temperature effects were found in A, B, D, E, F, H, I, J, L, M and P (Table S4).

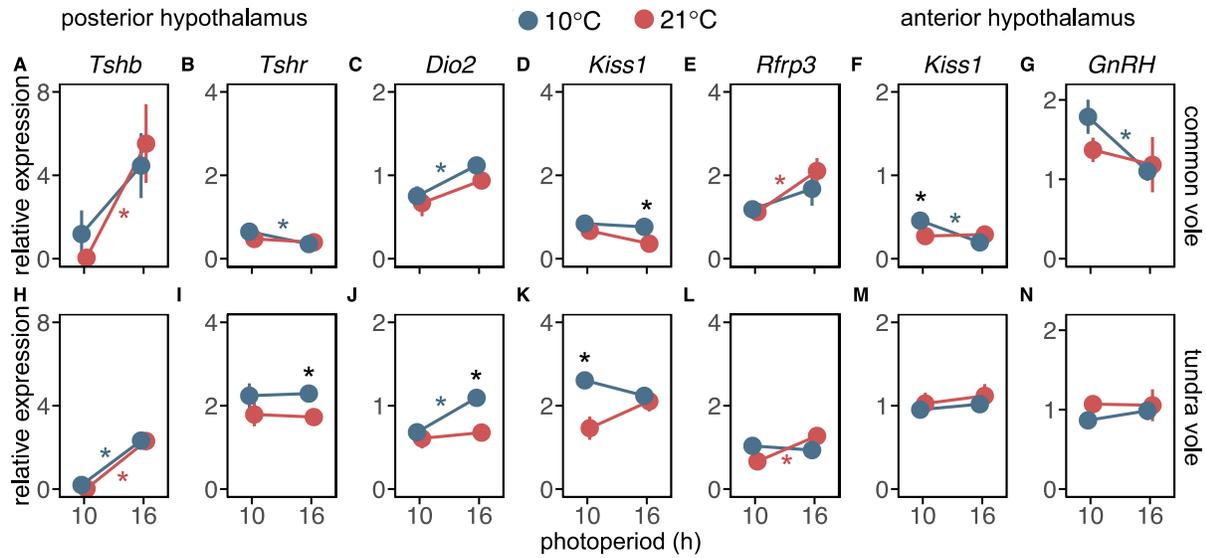


Figure 4. Temperature-dependent modulation of photoperiodic spring responses in hypothalamic gene expression. Spring-programmed responses to photoperiod for relative gene expression levels in the posterior hypothalamus: (A, H) *Tshb*, (B, I) *Tshr*, (C, J) *Dio2*, (D, K) *Kiss1*, (E, L) *Rfrp3*, and anterior hypothalamus: (F, M) *Kiss1*, (G, N) *GnRH* in 50-day old common and tundra voles respectively, at 10°C (blue) or 21°C (red). Data are presented as means \pm SEM (n=4-8). Significant effects of contrast analysis are indicated: $*P < 0.05$. In short, significant photoperiodic effects were found in: A, B, C, E, F, G, H, J, L, significant temperature effects were found in: D, J, K, and significant interactions between photoperiod and temperature were found in: F, K, L (Table S4).

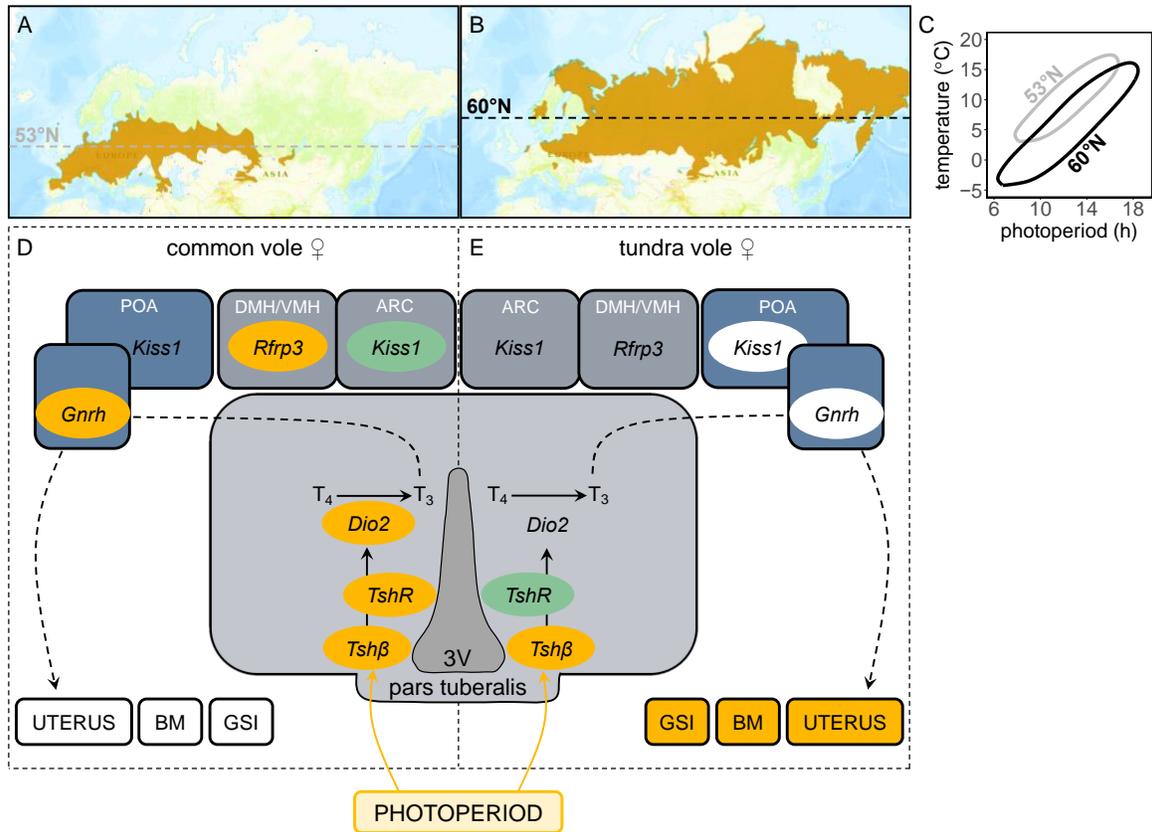


Figure 5. Graphical summary showing the effects of photoperiod and ambient temperature on the photoperiodical spring-programmed PNES in 50-day old female common and tundra voles. Geographic range in orange for (A) common voles and (B) tundra voles (obtained from: <https://IUCNredlist.org>). (C) Ellipse-like annual relationship between photoperiod and ambient temperature for Eelde (53°N; grey) and Oslo (60°N; black). Ambient temperature is retrieved from the Eelde airport weather station, the Netherlands (53.13°N, 6.59°E) and Oslo airport weather station, Norway (60.19°N, 11.10°E) (obtained from: <https://weerstatistieken.nl/eelde> and <https://wunderground.com>). Effects of photoperiod (yellow) and ambient temperature (green) on the PNES in photoperiodical spring-programmed (D) common and (E) tundra voles.