Seasonal adaptation: Geographic photoperiod-temperature patterns explain genetic variation in the common vole Tsh receptor

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Abstract

The vertebrate photoperiodic neuroendocrine system uses photoperiod as a proxy to time annual rhythms in reproduction. To investigate seasonal adaptation in mammals, the hinge region and the first part of the transmembrane domain of the *Tshr* gene were sequenced for 278 common vole (*Microtus arvalis*) specimens from 15 localities in Western Europe, and 28 localities in Eastern Europe. Forty-nine single nucleotide polymorphisms (SNPs; 22 intronic, 27 exonic) were found, with weak or zero correlation with pairwise geographical distance, latitude, longitude and altitude. By applying a temperature threshold to the local photoperiod-temperature ellipsoid, we obtained a predicted critical photoperiod (pCPP) as a proxy for spring onset of local primary food production (grass). The pCPP obtained explains the distribution of genetic variation in *Tshr* in Western Europe through highly significant correlations. Thus, *Tshr*, which plays a pivotal role in the sensitivity of the mammalian photoperiodic neuroendocrine system, was targeted by natural selection, resulting in optimized timing of seasonal reproduction.

Introduction

Herbivores in mid to high latitudes, are dependent on seasonal food availability and use photoperiod (i.e. day length) to synchronize their reproduction with primary food production. Primary production in the food web is temperature dependent(Malyshev, Henry, & Kreyling, 2014; Peacock, 1976; Robson, 1967). Since temperature is a notoriously noisy environmental signal, herbivores use photoperiod as a proxy to adjust seasonal timing of reproduction(Baker, 1938). The annual relationship between photoperiod and ambient temperature is primarily dependent on latitude(Hut, Paolucci, Dor, Kyriacou, & Daan, 2013), but will also change with altitude and longitude, depending on local climatic conditions. As a consequence, for the Northern hemisphere, primary food production and biological spring will generally start later in the year at higher latitudes, coinciding with longer photoperiods than at low latitudes. Therefore, selection pressure on timing of reproduction has caused adaptive evolution of seasonal timing mechanisms in herbivores, such that reproduction in spring starts at longer photoperiods in more northern populations in the Northern hemisphere.

Latitudinal clines in annual timing have been described in many insect species and in some bird and plant species, but rarely in mammals(Hut et al., 2013). For photoperiodic induction of diapause in insects, the critical photoperiod (CPP) increases with latitude(W. E. Bradshaw, Holzapfel, & Mathias, 2006; William E. Bradshaw & Holzapfel, 2010; William E. Bradshaw & Lounibos, 1977; Hut et al., 2013; S. Paolucci, van de Zande, & Beukeboom, 2013; Saunders, 1973). Furthermore, bird species change their annual breeding frequency patterns at different latitudes, with the peak shifting to a later time point in the year at higher latitudes(Baker, 1938). These findings suggest that there is latitudinal adaptation of photoperiodic timing mechanisms driving reproduction. A genetic basis for variation in photoperiodic responsiveness has been demonstrated in the parasitoid *Nasonia vitripennis* where it is associated with clinal allelic variation of the*period* gene(Benetta, Beukeboom, & van de Zande, 2019; Silvia Paolucci, Salis, Vermeulen, Beukeboom, & van de Zande, 2016), in pitcher-plant mosquitoes *Wyeomyia smithii* (William E. Bradshaw & Holzapfel, 2001b; D. Mathias, Jacky, Bradshaw, & Holzapfel, 2005; Derrick Mathias, Jacky, Bradshaw, & Holzapfel, 2007), in the deer mice*Peromyscus leucopus* (Heideman & Bronson, 1991), and*Peromyscus maniculatus* (Desjardines, Bronson, & Blank, 1986), and in Siberian hamsters *Phodopus sungorus* (Kliman & Lynch, 1992; Lynch, Lynch, & Kliman, 1989). Although, latitude of origin influences photoperiodic responses in deer mice(Dark, Johnston, Healy, & Zucker, 1983), the underlying genetics for adaptation of such photoperiodic mechanisms in mammals is not clear.

Laboratory experiments have revealed that annual rhythms in physiology and reproduction are driven by the photoperiodic neuroendocrine system(Nakane & Yoshimura, 2019). This mechanism is well conserved among vertebrates, including the common vole, *Microtus arvalis* (Król et al., 2012; van Rosmalen et al., 2021; van Rosmalen, van Dalum, Hazlerigg, & Hut, 2020), and comprises a seasonal timing mechanism which synchronizes to changes in photoperiod using the central circadian clock (i.e. the suprachiasmatic nucleus, SCN). Photoperiod is inversely related to the duration of the nocturnal melatonin release by the pineal gland(Bittman, Dempsey, & Karsch, 1983; Carter & Goldman, 1983; Hoffman & Reiter, 1965). Under short photoperiod, pineal melatonin is present in the morning hours (12h after lights OFF) and binds to melatonin receptors in the pars tuberalis of the pituitary, causing suppression of thyroid-stimulating hormone beta-subunit (TSHβ)(Dardente et al., 2010; Masumoto et al., 2010). Under long photoperiod, melatonin is absent in the morning (12h after lights OFF) which allows the transcriptional coactivator EYA3 and subsequently TSHβ to increase. TSHβ forms an active heterodimer with glycoprotein hormone alpha-subunit (αGSU)(Magner, 1990), which then binds as TSH to its receptor, TSHR, in the tanycytes around the third ventricle of the brain where it increases the production of iodothyronine deiodinase 2 (DIO2)(Guerra et al., 2010; Hanon et al., 2008; Nakao et al., 2008; Ono et al., 2008; Yoshimura et al., 2003). This leads to increased active thyroid hormone levels (T_3)(Lechan & Fekete, 2005) acting on GnRH neurons regulating gonadotropin release to control reproductive behavior(Hanon et al., 2008; Nakao et al., 2008; Yoshimura et al., 2003).

TSH dependent sensitivity to photoperiod, which is defined as a shift of photoperiodic response curves, can be modulated by TSHR abundance and function. TSHR is therefore an essential protein in the mammalian seasonal reproduction pathway. In addition, Ho and colleagues showed that *Tshr* mutations can change the signaling efficiency of the receptor(Ho, Sande, Lefort, Vassart, & Costagliola, 2001). Selection on the *Tshr* gene has been shown in domestic chicken and suggests that the transmembrane domain is especially important in modulating photoperiodic responses(Karlsson et al., 2016; Rubin et al., 2010). Therefore, to assess seasonal adaptation of photoperiodic mechanisms, we focus on the TSH receptor (*Tshr*).

The protein encoded by the *Tshr* gene belongs to the glycoprotein hormone receptor family(Smits et al., 2003). TSHR is a transmembrane domain (TMD) G protein-coupled receptor (GPCRs) with a large extracellular N-terminal part, containing leucine-rich repeats (LRRs). LRRs form a baseball glove-like structure, which is responsible for hormone (TSH) recognition and binding(Kleinau, Neumann, Grüters, Krude, & Biebermann, 2013). TSH binding causes a conformational change in the TSHR, which activates G-protein dependent signaling transduction. The extracellular LRR domain is connected to a large transmembrane helix, the hinge region.

The *Tshr* gene is very large owing to its exceptionally large introns. This offers many possibilities for cis-regulatory elements, that may modulate transcription(Wittkopp & Kalay, 2012). Mutations in intronic regions may disrupt transcription factor binding, which may lead to altered *TSHR* expression. Human *TSHR* mRNA splice variants encoding a TSHR without TMD have been reported(Graves, Tomer, & Davies, 1992), which may hint at alternative splice sites within intronic regions prior to the exon encoding for the TMD. Since the hinge region and the first TMD are important for ligand binding and for signaling transduction(Mizutori, Chen, McLachlan, & Rapoport, 2008) and mutations in the hinge region of the *TSHR* are known to change the signaling efficiency of the receptor(Ho et al., 2001), we consider this region as a potential target for natural selection and functional adaptive variation.

In this study we evaluate genetic adaptation to local climate conditions in the common vole (*Microtus arvalis*) *Tshr* gene by comparing genetic variance over the large European geographical range of this herbivorous rodent. For this purpose, we focus on the end of intron 8 and the beginning of exon 9, encoding for the hinge region and the first part of the transmembrane domain of TSHR. Because the reproductive response at high latitudes in the Northern hemisphere requires longer photoperiods, and therefore higher TSH levels, allelic variation of the*Tshr* gene may be associated with reduced TSHR signaling at high latitudes. Tissue samples were collected from 43 different localities over a large European geographical distribution (Fig. 1A, Table S1). The large variation in latitude (42° 21' 36" N - 59° 17' 60" N), longitude (5° 31' 48" W - 38° 23' 24" E) and altitude (4 – 2146 m above sea level), allowed us to assess whether location specific annual photoperiod-temperature ellipsoid patterns can explain the distribution of genetic variation in coding and non-coding parts of *Tshr* .

Materials and Methods

Tissue samples

We obtained tissue samples from 278 previously-collected specimens of female and male common vole, *Microtus arvalis* (Pallas 1778) from both Western Europe (France, Great Britain and Spain) and Eastern Europe (Czech Republic, Hungary, Poland, Russia, Serbia and Ukraine) (Fig. 1A, Table S1). Specimens were collected between 1995 – 2015 (voles from 1 site were caught simultaneously) and consisted of appendages (legs, toes, ears, tail tips and muscle tissues) preserved in 96-99% ethanol at 4-15 °C. Sex was not documented for all specimens, therefore, we could not account for sex in the model.

DNA isolation, PCR and Sanger sequencing

Total genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) for Western European samples, and using the Syngen Tissue DNA Mini Kit (Syngen Biotech, Wrocław, Poland) for Eastern European samples, according to the manufacturer's protocols. Primers (Table S2) were designed based on the *Microtus arvalis*genome (NCBI:txid47230, GCA_007455615.1) using Primer-BLAST (NCBI), in two overlapping fragments, each 1100-1200 bp in length. Using those primers, the last ˜829 bp of intron 8 and the first ˜849 bp of exon 9 of the *Tshr* gene was amplified by PCR using DreamTaq (Thermoscientific^{TM}, Waltham, Massachusetts, United States). A mastermix containing: 15.7 µL ultrapure H_2O, 2 µL Dreamtaq Buffer (10x), 0.4 µL dNTP mix (10 mM), 0.4 µL Forward primer (10 µM), 0.4 µL Reverse primer (10 µM), 0.1 µL DreamTaq DNA polymerase (5U/ µL) was prepared for each reaction. Twenty µL-reactions (1 µL DNA + 19 µL mastermix) were carried out for each sample by using a thermalcycler (S1000^{TM}, Bio-Rad, Hercules, California, United States) (Table S3). Following PCR, an enzymatic clean-up with ExoSAP-IT reagent (Applied Biosystems^{TM}, Foster City, California, United Sates) was performed in order to remove excess primers and nucleotides. Five µL of cleaned PCR product and 5 µL of the forward or reverse primer (5 µM) were transferred to a new 1.5 ml tube and sent out for Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). The intronic fragment was sequenced in two directions, while the exonic fragment was sequenced in the forward direction only.

Data analysis and statistical analysis

The distribution map of common vole samples used in this study (Fig. 1A) was generated using the fol-

lowing R packages: 'rworldmap'(South, 2011), 'rworldxtra'(South, 2012), 'RcolorBrewer'(Neuwirth, 2014), 'maptools'(Bivand & Lewin-Koh, 2019), and 'classInt'(Bivand, 2019). Ellipse-like annual relationships between temperature and photoperiod (Fig. 1B, C) were built using ˜10-year (between 2000-2019) average monthly ambient temperatures obtained from local weather stations (within 110km of sample location) at http://www.wunderground.com. Photoperiod, based on civil twilight times at dawn and dusk at different locations were retrieved from https://www.timeanddate.com/. Grass growth in spring is used as a*proxy* for the onset of the favorable reproductive season. Grass growth is initiated at 5-10°C air temperature(Cooper, 1964; Peacock, 1975, 1976). To include all locations in our analysis, a temperature threshold at 6.6°C was used to deduce for further analysis the corresponding predicted critical photoperiod (pCPP) that would initiate optimal timing of reproduction.

The 'Phyre2' web portal for protein modeling was used to predict the TSHR protein 3D structure (Fig. 2D)(Kelley, Mezulis, Yates, Wass, & Sternberg, 2015). SNPs were detected by sequence alignments using 'CLC Sequence Viewer' (version 8.0) (QIAGEN, Aarhus, Denmark). Chromatograms were checked for sequencing quality and heterozygosity of SNPs in the Mac OS software '4-peaks' (Nucleobytes, Aalsmeer, the Netherlands). Variation in DNA sequences were classified as SNPs if >3 of the specimens contained the mutation. Putative transcription factor bindings sites were predicted using AliBaba2(Grabe, 2002). To statistically test gene-environment associations, we used a population-based approach, in which an environmental variable was modelled as a linear function of population allele frequency(Rellstab, Gugerli, Eckert, Hancock, & Holderegger, 2015). Pearson's correlation tests were carried out: pairwise distances of allele frequencies were correlated to pairwise geographical distance, pairwise latitudinal difference, pairwise longitudinal difference, pairwise altitudinal difference, and pairwise critical photoperiod difference.P -values were adjusted according to the Benjamini-Hochberg procedure(Benjamini & Hochberg, 1995; Yekutieli & Benjamini, 1999), which is one of the strongly recommended method to use in environmental association analysis(Rellstab et al., 2015). Pairwise linkage disequilibrium heatmaps (Fig. S3) were generated using the R-package 'LDheatmap'(Shin, Blay, McNeney, & Jinko Graham, 2006). The constructed phylogenetic tree (Fig. S2) from SNP frequency data by using the neighbor-joining method(Saitou & Nei, 1987) was generated using 'POPTREEW'(Takezaki, Nei, & Tamura, 2014). All other analyses were performed using 'RStudio' (version 1.2.1335) and figures were generated using the R-package 'ggplot2'(Wickham, 2016).

Results

The *Tshr* gene of the common vole is 113,629 bp long and consists of 8 introns and 9 exons (Fig. 2A). In this study we sequenced a ˜1700 bp region around the beginning of exon 9, comprising 829 bp of intronic and 849 bp of exonic sequences (Fig. 2B). The predicted TSHR protein structure, based on the *Microtus arvalis* genome (NCBI: txid47230, GCA_007455615.1), comprises seven leucine-rich repeats, a hinge region and seven transmembrane domains (Fig. 2C, D).

Forty-nine single nucleotide polymorphisms (SNPs) were detected (Table S4, S5). Twenty-two intronic SNPs and 27 exonic SNPs were found, from which 23 were synonymous. These SNPs were used to calculate the genetic differentiation of the sampled populations. Pairwise multilocus F_{ST} estimates ranged from F_{ST} = 0.000 to 0.978 (mean F_{ST} = 0.366, with 0 denoting no difference and 1 referring to completely different populations), and reveal high genetic differentiation between the populations sampled (Fig. 3). The high F_{ST} values in this species are in agreement with previous studies(Heckel, Burri, Fink, Desmet, & Excoffier, 2005; Martínková et al., 2013). The structure of the observed population differentiation suggests that not only population subdivision but also natural selection may be an explanatory factor. Therefore, it was tested whether the observed SNPs are associated with geographical location. Genetic differentiation between Eastern and Western European populations was larger (F_{ST} = 0.247 to 0.978, mean: 0.640) than differentiation among Western European populations (F_{ST}= 0.016 to 0.467, mean: 0.234) and among Eastern European populations (F_{ST} = 0.000 to 0.648, mean: 0.132), and strongly depends on geographical distance and longitude (Figs. 3, S2, Table S5). The constructed distance tree based on *Tshr* haplotypes (Fig. S2) together with pairwise multilocus F_{ST} analysis (Fig. 3), confirmed that the Western and Eastern European populations belong to different genetic lineages. Given the genetic separation of these lineages, it is appropriate to analyze Western

and Eastern European samples separately. To reveal possible patterns of association between SNPs, heatmaps of pairwise linkage disequilibrium (LD) measurements were generated (Fig. S3).

Western European lineage

Forty-three SNPs (22 intronic, 21 exonic) were found in Western European populations, of which 5 are non-synonymous and 16 synonymous (Fig. 4 and Table S6). To assess whether variation in the *Tshr* gene can be explained by local seasons, SNP frequencies were correlated to different environmental proxies. The non-synonymous SNPs were not associated with the tested environmental proxies (Table S6). In the Western European lineage, 3/43 SNPs (exonic, synonymous) significantly correlated with pairwise geographical distance (Fig. 4A, C, Table S6), indicating that there is a lack of regional equilibrium(Hutchison & Templeton, 1999), and that an alternative approach may be used to detect selection, classifying SNPs that show clinal variation(Endler, 1977). SNP frequency weakly correlated to pairwise latitudinal, longitudinal and altitudinal difference for the majority of the observed SNPs (latitude: 2 exonic SNPs, longitude: 11 intronic and 5 exonic SNPs, altitude: 3 intronic and 3 exonic SNP) (Fig. 4D-L, Table S6). These findings show that geographical distance, latitude and altitude by themselves are bad predictors for genetic variation in the *TSHR* gene, despite the fact that annual photoperiod-food abundance patterns depend on all these parameters. Therefore, for each sample location, the pCPP at which grass growth is initiated in spring (at 5-10°C ambient temperature; Cooper, 1964; Peacock, 1975; Peacock, 1976) was deduced from local annual photoperiod-ambient temperature ellipsoids. pCPP at 6.6°C achieved highest number of significant SNPs. Therefore, the temperature threshold for grass growth initiation in spring was set at 6.6°C, and was used to deduce corresponding pCPP, which were calculated to vary between 10.19 and 15.40 hours of light /24 hours (Fig. 1 and Table S1). In Western European samples, 5 intronic and 7 exonic SNPs strongly correlated to pairwise difference in pCPP (Fig. 4M, Table S6). F_{ST}values for these specific SNPs were high (ranging from F_{ST} = 0.032 to 0.310, mean: 0.166). All these significant mutations were, however, synonymous SNPs. Strongest associations with pCPP were found for intronic SNP-158 (G>C), -128 (T>C), and exonic SNP126 (A>G) (Fig. 4M-O, Table S6). It is expected that between Orkney Island and between mainland, some of the variation reflects isolation and genetic drift. Therefore, the same analysis was performed excluding the Orkney Island populations and revealed similar results. Pairwise multilocus F_{ST}-values were high for populations that differ highly in pCPP, while F_{ST}-values were low for populations with similar pCPP (Fig. 3).

Eastern European lineage

Thirty-four SNPs (14 intronic, 20 exonic) were found in Eastern European populations, from which 3 were non-synonymous and 17 synonymous (Fig. 5 and Table S7). Although highly significant p -values for some correlations between *Tshr* SNP frequencies and environmental proxies were found in the Eastern European lineage, R^2-values are extremely low (0.1±0.02, mean±SD). This indicates that in Eastern European voles, *Tshr* SNP frequencies weakly correlate with geographical distance, latitude, longitude, altitude and pCPP (Fig. 5, Table S7). This observation indicates that genetic *Tshr* variation in Eastern Europe is unlikely to be explained by natural selection due to seasonal variance.

Discussion

The *M. arvalis* populations are characterized by large-scale genetic differentiation of *Tshr* , reflecting local adaptation to annual temperature-photoperiod patterns, rather than latitude *per*se. Variation in *Tshr* sequence indicates that the *M. arvalis* population can be subdivided into Eastern and Western European clusters, indicating that they may belong to distinct genetic lineages (Figs. 3, S3). This phylogeographical structure is consistent with that found for mitochondrial cytochrome *b* gene sequences, and microsatellite loci (representing nuclear DNA)(Haynes, Jaarola, & Searle, 2003; Martínková et al., 2013; Stojak et al., 2016; Stojak, Mcdevitt, Herman, Searle, & Wójcik, 2015), and justifies the analysis of Western and Eastern European populations separately. The Western versus Eastern divide could well be due to re-invasion of Northern Europe from separate glacial refugia, and therefore separate evolutionary events(Hewitt, 1999).

For insights into the geographical variation in *Tshr* , the association of SNP frequencies with local climatic

conditions was examined. Here we showed that genetic variation in the vole *Tshr*is better explained by local photoperiod-temperature patterns than by latitude only. This may be caused by the temperature dependence of vegetation growth. In house mice, genes (but not the *Tshr*) that show signals of selection are also associated with local average annual ambient temperature, and are linked with clinal variation in phenotypic aspects, such as body mass and metabolism(Ferris et al., 2021; Phifer-Rixey et al., 2018). Interestingly, SNPs found in thyroid hormone receptors, which are involved in regulation of seasonal reproduction in the hypothalamus(Yoshimura et al., 2003), significantly correlated with variation in average annual temperature(Ferris et al., 2021). This suggests that genomic evolution of seasonal adaptation in house mice and voles involves unique responses to genetic selection. Annual temperature patterns not only depend on latitude, but also on longitude, altitude, and other regional climatic variables like the Gulf Stream warming European Atlantic coastal regions. Critical photoperiods in pitcher-plant mosquitoes strongly correlated with altitude-corrected latitude (r = 0.96), however, this measure does not integrate local temperature patterns(W. E. Bradshaw et al., 2006; William E. Bradshaw, 1976; William E. Bradshaw & Lounibos, 1977). Deriving the regional photoperiod-temperature ellipsoids may be better to account for such regional climatic differences than latitude or altitude-corrected latitude only. We post-hoc tested photoperiods at other temperature thresholds, however this did not improve the results. Moreover, 6.6°C is not an unreasonable temperature since grass growth is initiated at 5-10°C air temperature(Cooper, 1964; Peacock, 1975, 1976).

In addition, several SNPs correlated well with longitude and altitude (Fig. 4G,J). Altitudinal gradients in seasonal timing of breeding have been observed in deer mice (*Peromyscus maniculatus borealis*), with shorter breeding seasons at high elevations(Millar & Innes, 1985). The pCPP at which a temperature threshold for grass growth initiation is reached can be deduced from local photoperiod-temperature patterns, and is here confirmed to be a strong determinant for distributional variation in *Tshr* SNP frequency in Western European common vole populations (Fig. 4M). Pairwise multilocus F_{ST} analysis revealed that populations which differ in pCPP, also show greater genetic distance in *Tshr* haplotypes (Fig. 3). These findings indicate that seasonality is likely to be a selective force for*Tshr* evolution in common voles, and imply that *Tshr* is an important gene for genetic adaptation of the photoperiodic response systems.

The observed genetic *Tshr* variation is unlikely to be caused by isolation only, with the possible exception of the Orkney island populations, which are geographically isolated from each other and from mainland populations by the sea. Therefore isolation and genetic drift may be a more important evolutionary force than natural selection in the Orkney populations. Interestingly, the same SNPs appear to be related to pCPP when the Orkney Island populations are excluded from the analysis. This indicates that the results in Western Europe are not dominated by the Orkney population's data, and that the observed distribution of*Tshr* variation may be a sign of adaptive evolution likely operating in response to photoperiod.

In Eastern European populations, none of the tested environmental proxies are good predictors for *Tshr* SNP frequencies (Fig. 5). These results indicate that the *Tshr* in the Eastern European lineage is not linked to seasonal adaptation as observed in the Western European lineage. Oceanic climates (Western Europe) are known for their small annual temperature amplitudes, while continental climates (Eastern Europe) are known for their large annual temperature amplitudes. These climatic differences may have led to divergent evolutionary adaptation of TSHR function, which may provide an explanation for the observed longitudinal separation in genetic *Tshr* differentiation. Another hypothesis is that photoperiodic genes other than the *Tshr* are under selection for seasonal adaptation in Eastern European vole populations.

SNPs associated with local pCPP were all synonymous or intronic mutations. This suggests that these sites may be involved in regulatory rather than structural variation. Five intronic SNPs were strongly associated with pCPP in Western Europe (Fig. 4), of which two (i.e. SNP-144 and -158) were strongly associated with altitude in Eastern Europe (Fig. 5). Putative regulatory protein binding sites were predicted for the intronic region, and revealed that intronic SNP-128, which strongly correlates to pCPP (Fig. 4O), is located in a potential SP1 (specificity protein 1) binding site(Höller, Westin, Jiricny, & Schaffner, 1988; Ji, Casinghino, McCarthy, & Centrella, 1997). Interestingly, SNPs closely located to this enhancer region, such as SNP-158, are related to different environmental proxies in Eastern and Western Europe (Fig. 4, 5, S2).

It is tempting to speculate that variation in and around this SP1 binding site sequence may influence *Tshr* transcription. Furthermore, there is strong evidence that synonymous SNPs are not necessarily neutral as they can alter mRNA expression, splicing, and structure, therefore having downstream effects on protein expression(Chamary, Parmley, & Hurst, 2006; Hunt, Sauna, Ambudkar, Gottesman, & Kimchi-Sarfaty, 2009). Synonymous polymorphisms require different transfer RNAs (tRNA) to recruit the same amino acids and may cause codon-bias. Synonymous tRNA vary strongly in frequency between species and tissues (i.e. codon bias)(Dittmar, Goodenbour, & Pan, 2006; Goodenbour & Pan, 2006). It is therefore possible that the observed synonymous mutations in the *TSHR* may alter translation efficiency within a species and tissue by changing the elongation rate(Quax, Claassens, Soll, & Oost, 2015). Reduced elongation rate may therefore result in lower protein abundance. Hence synonymous SNPs in the *Tshr* gene could result in altered receptor abundance, changed sensitivity to TSH and modified photoperiodic response. It is therefore conceivable that synonymous SNPs in the *Tshr* gene are subject to natural selection, and reflect local geographical adaptation. TSHR plays a pivotal role in photoperiodic response in the pars tuberalis, but also in thyroid hormone metabolism in the thyroid gland. Tissue-specific functions of TSHR may benefit from genetic adaptation in photoperiodism through synonymous SNPs, since tissue-specific tRNA expression, which has been demonstrated in human and mouse tissues(Dittmar et al., 2006; Pinkard, McFarland, Sweet, & Coller, 2020), may perhaps lead to altered TSHR function in the pars tuberalis, but not in the thyroid gland.

Photoperiodic regulation of the reproductive system in deer mice has been shown to vary with latitude, with weaker photoperiodic responses in animals originating from lower latitudes(Dark et al., 1983). Moreover, photoperiodic sensitivity in pitcher-plant mosquitoes correlated with global warming, indicating the importance of season-length driving evolution (genetic change) of photoperiodism during recent rapid climate change(W. E. Bradshaw & Holzapfel, 2008; William E. Bradshaw & Holzapfel, 2001a, 2006; William E. Bradshaw, Zani, & Holzapfel, 2004). Our findings confirm that the *Tshr* gene is under selection, which has previously been reported in chicken domestication in relation to photoperiodic responsiveness(Karlsson et al., 2016; Rubin et al., 2010). Future studies should determine whether the SNPs identified as seasonal timing dependent genetic variation in the vole *Tshr* can indeed alter, genetically based, photoperiodic responses. Such an approach will confirm whether habitat-specific photoperiodic responses are indeed regulated by means of functional TSHR adaptation. In vole populations with later onsets of reproduction and shorter breeding seasons(Tkadlec, 2000), our results predict lower concentrations in the tanycytes of *Tshr* or lower TSH-binding affinities of *Tshr* haplotypes.

Optimal timing of reproduction, enhancing energetically demanding pregnancy, and parental care, is necessary to maximize fitness in temperate and northern seasonal environments. *Tshr* is an essential gene in the pathway programming seasonal reproduction in mammals. Herein, we show how onset of the favorable season over a wide geographical range of the common vole, *Microtus arvalis* , explains much of the genetic variation in the TSH binding site, hinge region, and transmembrane domain of TSHR in Western but not Eastern Europe. Yet, vole populations thrive in both regions. We therefore conclude that different genetic mechanisms have been important in enabling vole populations to exploit geographically distinct regions. Such distinctions of how the genetic underpinnings of seasonal timing have evolved over climatic gradients in nature will be important in predicting how animals will adapt to new seasonal environments during ongoing rapid climate change.

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Data accessibility : Raw sequence reads and metadata will be deposited in a public repository (FigShare, https://doi.org/10.6084/m9.figshare.14695356.v1) after acceptance.

Benefits Generated: A research collaboration was developed with scientists from countries providing genetic samples, all collaborators are included as co-authors, the results of research have been shared with the providers of the samples. Benefits from this research accrue from the sharing of our data and results on public databases as described above.

Author Contributions: L.v.R., L.v.d.Z. and R.A.H. conceived and designed the experiments. J.S. and J.M.W. provided the Eastern European samples. J.S.H. and J.B.S. provided the Western European samples. L.v.R., R.S., W.H. and A.S.P. conducted the experiments. L.v.R. analyzed the data. L.v.R. wrote the manuscript. L.v.d.Z., J.B.S and R.A.H revised it; all authors commented on the paper and approved it.

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Figure 1. Sample locations and local annual photoperiod-temperature ellipsoids. (A) Distribution map of localities of common vole samples used in this study. Location numbers with corresponding environmental proxies are listed in Table S1. (B) Annual patterns (counter-clockwise) of photoperiod and 10-year average monthly ambient temperature for each sample location in Western Europe and (C) in Eastern Europe. Temperature data were obtained from the closest weather station (always within 110 km of sample location) obtained from Wunderground (https://www.wunderground.com/). Photoperiod was obtained from https://www.timeanddate.com, and is based on civil twilight times at dawn and dusk, which is the timing at which log light intensities change most rapidly (Daan & Aschoff, 1975; Hut et al., 2013). Civil twilight incorporates geographical and seasonal variation in the duration of twilight (Nielsen, 1961), and is therefore considered as the moment of 'lights on' and 'lights off' for biological systems (Hut et al., 2013). Dotted lines indicate a temperature threshold at 6.6°C from which the corresponding predicted critical photoperiod (pCPP) in spring (ellipse crossing the 6.6 °C line for the second time) is used as a *proxy* for onset of grass growth and consequently as a proxy for onset of the favorable season. Regional pCPP's are listed in Table S1. Colors indicate latitude, ranging from 42°N (yellow) to 59°N (purple).

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Figure 2. The *Tshr* gene and predicted protein for the common vole. (A) The *Tshr* gene for the common vole consisting of 8 introns and 9 exons. (B) The magnified region, including the last part of intron 8 and the first part of exon 9, have been sequenced in this study. (C) The predicted TSHR protein and (D) its 3D-structure. All mutations found in the current study are labeled and listed in Table S4-S7. SP = signal peptide, LRR = Leucine-rich repeats, TMD = transmembrane domain, ICD = intra cellular domain, ECD = extra cellular domain, CDS = coding sequence.

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Figure 3. Pairwise multilocus F_{ST} heatmap for *Tshr* haplotypes. F_{ST} values were calculated using the original method for estimation of genetic distance with correction for sample size bias (Nei, 1978). Colors indicate pairwise multilocus F_{ST} values ranging from 0 (yellow) to 1 (dark blue). Western and Eastern European populations are ordered from long to short predicted critical photoperiod (pCPP).

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Figure 4. *Tshr* mutations in Western European common vole populations. Manhattan-type plots ($-\log_{10}(p)$) for the sequenced *Tshr* region for (A) geographical distance, (D) latitude, (G) longitude, (J) altitude and (M) predicted critical photoperiod (pCPP). Grey bars indicate Benjamini-Hochberg adjusted p-values, black bars indicate R^2-values. SNPs that meet the threshold for significant correlations ($p < 0.05$) cross the red dashed line. Pairwise difference in SNP frequency for two representative mutations (SNP-158 and SNP126) related to (B,C) pairwise geographical distance, (E,F) pairwise latitudinal difference, (H,I) pairwise longitudinal difference, (K,L) pairwise altitudinal difference and (N,O) pairwise difference in pCPP. Significant correlations are indicated by linear regression lines. All statistic results of linear models for SNP frequency related to environmental proxies can be found in Table S6.

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Figure 5. *Tshr* mutations in Eastern European common vole populations. Manhattan-type plots ($-\log_{10}(p)$) for the sequenced *Tshr* region for (A) geographical distance, (D) latitude, (G) longitude, (J) altitude and (M) predicted critical photoperiod (pCPP). Grey bars indicate Benjamini-Hochberg adjusted p-values, black bars indicate R^2-values. SNPs that meet the threshold for significant correlations ($p < 0.05$) cross the red dashed line. Pairwise difference in SNP frequency for two representative mutations (SNP-158 and SNP729) related to (B,C) pairwise geographical distance, (E,F) pairwise latitudinal difference, (H,I) pairwise longitudinal difference, (K,L) pairwise altitudinal difference and (N,O) pairwise difference in pCPP. Significant correlations are indicated by linear regression lines. All statistic results of linear models for SNP frequency related to environmental proxies can be found in Table S7.