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Conservation of the photoperiodic neuroendocrine axis among vertebrates: Evidence from the teleost fish, *Gasterosteus aculeatus*

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ABSTRACT

Photoperiod, or length of day, has a predictable annual cycle, making it an important cue for the timing of seasonal behavior and development in many organisms. Photoperiod is widely used among temperate and polar animals to regulate the timing of sexual maturation. The proper sensing and interpretation of photoperiod can be tightly tied to an organism's overall fitness. In photoperiodic mammals and birds the thyroid hormone pathway initiates sexual maturation, but the degree to which this pathway is conserved across other vertebrates is not well known. We use the threespine stickleback *Gasterosteus aculeatus*, as a representative teleost to quantify the photoperiodic response of key genes in the thyroid hormone pathway under controlled laboratory conditions. We find that the photoperiodic responses of the hormones are largely consistent amongst multiple populations, although differences suggest physiological adaptation to various climates. We conclude that the thyroid hormone pathway initiates sexual maturation in response to photoperiod in *G. aculeatus*, and our results show that more components of this pathway are conserved among mammals, birds, and teleost fish than was previously known. However, additional endocrinology, cell biology and molecular research will be required to define precisely which aspects of the pathway are conserved across vertebrates.

1. Introduction

Proper timing of life-history events is critical to fitness [12]. Photoperiod, or length of day, has a highly reliable annual cycle that makes it an ideal environmental signal that organisms can use to anticipate and prepare for seasonal changes. The use of photoperiod for the timing of sexual maturation and reproduction is widespread among polar and temperate animals [10,26]. The extensive use of photoperiod across diverse organisms in order to time critical life-history events underscores the importance of this environmental signal for fitness, and leads to the hypothesis that organismal systems that sense and respond to photoperiod have been molded by the action of natural selection for millennia.

Photoperiodic induction of sexual maturation in vertebrate animals begins with reception of a stimulatory photoperiod regime that leads to induction of gonadotropin release, which in turn stimulates production of gonadal sex hormones [11]. In photoperiodic mammals and birds, the thyroid hormone (TH) pathway initiates the release of gonadotropins [5,46,62,65]. Although the mechanisms of photoperiod signal reception and transduction differ between mammals and birds, the initial hormonal cascade and its location within the brain are conserved (Fig. 1). These findings lead to the hypothesis that involvement of TH in reproduction is conserved among vertebrates and therefore may have originated in chordates prior to the diversification of vertebrates [32,54]. Support for this hypothesis is limited, however, because most studies have occurred on organisms from just the tetrapod clade of vertebrates. A further test of this hypothesis requires comparable studies in other vertebrates, particularly teleost fishes, which is the most speciose vertebrate clade but for which we have precious little data regarding this hypothesis.

Photoperiodic control of sexual maturation is widespread among teleost fishes [8,10,13] and the function of the TH pathway is conserved in fish [52,57]. However, photoperiodic control of the TH pathway remains unclear (Fig. 1C). Our current understanding is limited by an inability to compare studies directly due to differences in measurement techniques, the variety of species examined, and the ability to relate hormonal changes solely to photoperiodic response.

In seasonally reproducing fishes, the bioactive form of thyroid hormone, triiodothyronine (T3), tends to increase during early sexual maturation [7,21,49] and, in at least rainbow trout, photoperiod is the specific trigger for this increase in T3 [20]. T3 can stimulate GnRH secretion from GnRH neurons in the Nile tilapia [53], but this stimulation of GnRH has not been tested in a photoperiodic fish.

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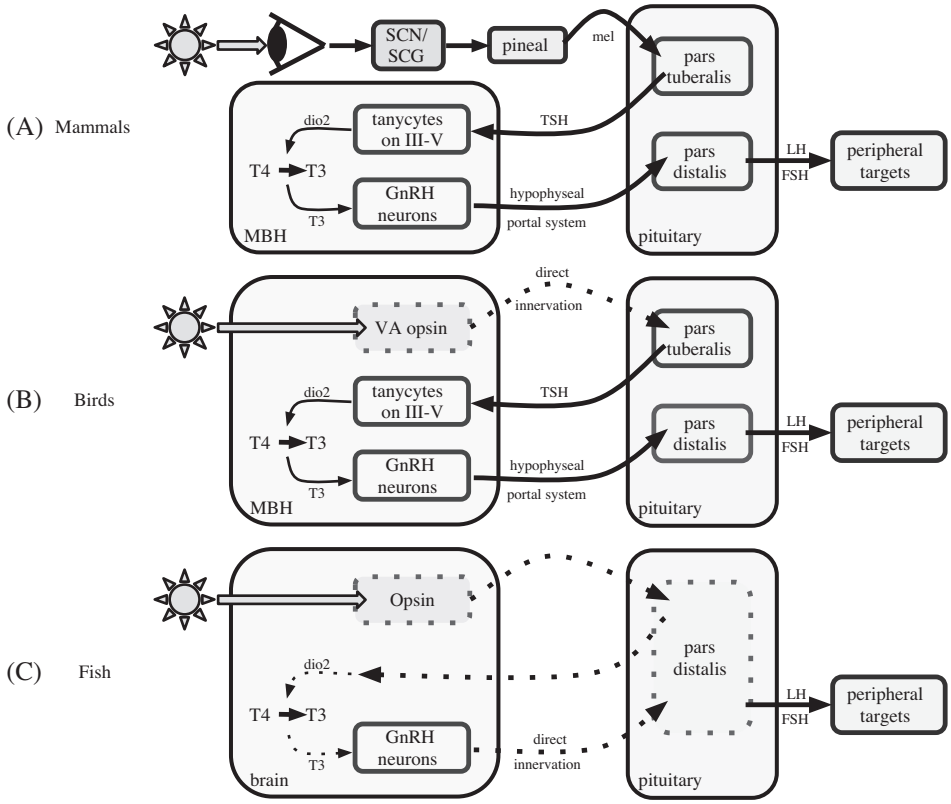

Fig. 1. The photoperiodic TH pathway in fish as inferred from mammals and birds. Solid lines and borders indicate established steps and known neuroanatomical locations, respectively. Dashed lines and borders indicate suggested or inferred steps and neuroanatomical locations. *Signal reception in mammals and birds*: (A) In mammals, the photoperiod signal is received by the retina and neuronally communicated via the suprachiasmatic nuclei (SCN) and superior cervical ganglion (SCG) to the pineal gland [44]. Melatonin (mel) produced by the pineal encodes the signal [14]. (B) In birds, the signal is received by extraretinal photoreceptors, most likely hypothalamic opsins [28,45]. Communication of the signal is neuronal and does not involve melatonin [58]. *The neuroendocrine response in mammals and birds*: In both (A) mammals and (B) birds, the earliest hormonal response to a stimulatory photoperiod occurs in the pars tuberalis, where production of thyroid stimulating hormone beta (TSHβ) and chorionic gonadotropin alpha (CGα) increase [29,46,63]. TSHβ and CGα heterodimerize to form thyroid-stimulating hormone (TSH), which stimulates deiodinase 2 (dio2) production in tanycytes lining the third ventricle of the hypothalamus (III-V). Dio2 catalyzes the conversion of the thyroid hormone thyroxin (T4) to the bioactive triiodothyronine (T3) [29,66]. T3 stimulates production of gonadotropin releasing hormone (GnRH) from neurons in the mediobasal hypothalamus (MBH). GnRH is transported via the pituitary portal system to the pars distalis, where it stimulates production of the gonadotropins follicle stimulating hormone (FSHβ) and luteinizing hormone (LHβ) [61]. These heterodimerize with CGα and are released into the bloodstream where they act upon the gonads and other peripheral targets. *Signal reception and neuroendocrine response in fish*: (C) In fish, photoperiodic signal reception is extraretinal [8,39] and may be an hypothalamic opsin [55]. Melatonin does not appear to encode the signal [8], as it can affect sexual maturation in some species, but its targets may be downstream of reception and initial response to photoperiod [41,42]. The early responses of TSHβ and dio2 to a stimulatory photoperiod have not been studied in fish. In general, plasma T3 increases during sexual maturation in photoperiodic fishes [7,21,49], but its effects on GnRH in photoperiodic fishes are unknown. Photoperiodic manipulation stimulates GnRH, LH and FSH production [3,19,31,43]. LH and FSH stimulate the gonads to produce sex hormones [8].

GnRH orthologs are often referred to by the species in which they were first discovered, but can also be referenced by their paralog name to facilitate comparison of their roles among species. We adopted the latter convention for our work. GnRH1 is expressed in neurons located in the preoptic area and GnRH2 neurons are found in the midbrain tegmentum. GnRH3 is unique to teleosts and is expressed in the ventral telencephalon [17,18]. GnRH1 is considered the hypophysiotropic form, (acting on the pituitary) as it is capable of stimulating gonadotropin production and gonadal development and is expressed in neurons that innervate the pituitary [17]. In fishes where GnRH1 is not present, GnRH3 is the hypophysiotropic form [17,18]. In masu salmon exposed to a stimulatory photoperiod regime, GnRH3 neurons increase in number [2], but the response of GnRH3 in other photoperiodic fishes is unknown.

Extending the role of the TH pathway in photoperiodic induction of sexual maturation to teleosts requires a species with a strong photoperiodic response that can be manipulated in controlled conditions and that can be measured using techniques that make the results comparable to those in mammals, birds and other fishes. These criteria are met in the threespine stickleback, *Gasterosteus aculeatus*, in which we are able to isolate hormonal responses to photoperiod from other environmental variables using controlled laboratory experiments.

The threespine stickleback is a small teleost fish with a wide latitudinal and environmental range that uses photoperiod to initiate sexual maturation [6,9,31,64]. Like birds [23,45] and other fishes [8], recent research indicates that reception of light related to photoperiodism is most likely extraretinal and extrapineal [9]. A stimulatory photoperiod increases gonadotropin production [30] and wild-caught sticklebacks have an annual cycle of gonadotropin production that peaks early in the reproductive season [31]. As in mammals and birds, androgens exert a feedback effect on gonadotropin production in both stimulatory and non-stimulatory photoperiod regimes [9]. Photoperiodically-induced modifications in morphology can also be measured in controlled-photoperiod conditions in the laboratory [50].

The goals of this study were to determine if the TH pathway is involved in the photoperiodic initiation of teleost sexual maturation and, if so, whether the dynamics of the response of the pathway are conserved among mammals, birds and teleosts. To accomplish these goals, we quantified gene expression levels of

key TH pathway genes in the brains and pituitaries of threespine stickleback during exposure to a stimulatory photoperiod regime. In the mammal and bird models, an increase in thyroid stimulating hormone (TSH) is the first known response of the photoperiodic neuroendocrine cascade (Fig. 1A and B). An increase in hypophysiotropic gonadotropin releasing hormone (GnRH) is the first indicator of the initiation of sexual maturation. Luteinizing hormone (LH) is one of the two gonadotropins that are secreted by the pituitary into circulation to stimulate sex hormone production (Fig. 1). By measuring these hormones in controlled conditions we determined the effects of photoperiod on expression of these genes independently of other environmental factors. We were then able to make direct comparisons between *G. aculeatus* and mammals and birds. In addition, evaluating multiple populations allowed us to determine the robustness of the results within a single species.

2. Materials and methods

2.1. Gasterosteus aculeatus stocks

Two northern populations were established from Alaska, Rabbit Slough (AK1: 61°34′N, 149°15′W) and Boot Lake (AK2: 61°43'N, 149°7'W). One southern population was established from Oregon, Eel Creek (OR: 43°35'N, 124°11'W). We will refer to these populations as AK1, AK2, and OR, respectively, throughout the rest of the text.

Crosses were made via *in vitro* fertilization using established laboratory procedures, and then the fish were reared under standard laboratory conditions ([64], stickleback.uoregon.edu/index.php/Crossing_and_Rearing_Protocols). Experimental fish were reared to adulthood at 20 °C on a non-stimulatory 10L:14D photoperiod cycle for 11–12 months (L:D = Light:Dark). They were at least 50 mm standard length (SL), as measured from the dorsum of the pre-maxilla to the caudal peduncle before they were subjected to any experimental treatment. All fish care and experimental procedures complied with University of Oregon IACUC-approved animal care protocols.

2.2. Experimental design

Conditions were identical to those previously used in measure the phenotypic effects of photoperiod [63]. One adult male and one adult female were paired in a single aquarium that was visually separated from other aquaria to avoid confounding visual cues. Aquaria were cleaned separately to avoid the possibility of transferring hormonal cues. The fish were fed twice a day *ad libitum*. All experiments were run in light-tight, air-cooled cabinets at 20 °C. Photoperiods were programmed with Chrontrol XT electronic timers (www.chrontrol.com).

Upon being placed in the experimental aquaria, male–female pairs were given two short-day cycles of light:dark = 10:14 (hereafter: 10L:14D) before being exposed to 17L:7D stimulatory long days. This long-day regimen was chosen because it is the shortest photoperiod at which phenotypic indicators of sexual maturation in threespine stickleback plateau [64]. We used the males exclusively for all of the following experiments.

For *in situ* mRNA hybridization of TSHβ, male stickleback from the AK2 line were sampled six hours after dawn during a short-day regimen or six hours after dawn after exposure to a single 17L:7D long day regimen. Fish were anesthetized in MS-222 (Sigma) and the entire brain, including the pituitary, was dissected out and stored in 4% paraformaldehyde solution (Sigma Aldrich) at 4 °C. The brains with pituitaries were then cryostat sectioned along the coronal plane, and placed on slides that were stored at −80 °C until use.

For quantitative real-time PCR measurement of target genes, males from the three populations were sampled six hours after dawn following exposure to 0, 1, 2, 5 or 10 long days. Fish were anesthetized in MS-222 (Sigma) and the entire brain including the pituitary was dissected out and stored in Trizol (Invitrogen) at −80 °C. Total RNA was extracted following a standard phenol chloroform protocol. Synthesis of cDNA was performed using random hexamers (Invitrogen) and SuperScript III (Invitrogen). Sample sizes per treatment ranged from 8 to 13 adult males (Supplementary Table 1).

2.3. Target gene identification

Thyroid stimulating hormone (TSH) and luteinizing hormone (LH) are both heterodimers consisting of a protein-specific β subunit and an α subunit common to TSH and LH. Therefore, we targeted the β subunits to ensure hormone specificity. First, *Homo sapiens and* zebrafish *Danio rerio* TSHβ, GnRH and LHβ orthologs were compared to the stickleback genome using BLAST to produce a set of candidate genes for further analysis.

Second, we performed phylogenetic reconstructions of the gene families to confirm the identity of the candidate genes, and in the cases of TSHβ and GnRH3, we annotated all paralogs found in the stickleback genome. Complete amino acid sequences of orthologs from the three gene families were downloaded from the NCBI protein database. If a species contained multiple paralogs, all were included. Alignments of the three gene families were made using Muscle [24]. PhyML 3.0 [27] was used to estimate phylogenies and compute their likelihood scores. The parameters of the phylogenetic model were searched and optimized using M3L (code.google.com/m3l/), which implements the Broyden–Fletcher–Goldfarb–Shanno algorithm [47]. The best-fitting model for each gene family was selected using the Akaike information criterion test [1]. Approximate likelihood ratio tests for each node were scaled using Shimodaira and Hasegawa (SH-like) support [5]. For the phylogenetic reconstruction of all three gene families, the best model was JTT [34] with a gamma-distributed set of evolutionary rates [61] (Supplementary Fig. 1).

Third, we used syntenic analysis of the target orthologs to confirm the results of the phylogenetic reconstructions. The Synteny Database uses Reciprocal Best Hit Analysis (RBH) to detect synteny between two genomes [15,16]. Here, genes from a target genome and an outgroup genome are compared to one another using BLAST. Genes in the two genomes are considered orthologous if they are each other's best BLAST matches. If regions of the two genomes have a high number of orthologs, syntenic conservation due to common descent is inferred [15,16]. We compared the regions around our target orthologs in the stickleback genome to the spotted green puffer fish *Tetraodon nigroviridis* and *Homo sapiens* genomes using the Synteny Database [15].

Table 1
A two-way population * photoperiod ANOVA for TSHβ1, GnRH3, and LHβ expression in the brain and pituitary.

Gene	Effect	df	*F*-ratio	*P*
TSHβ1	Photoperiod	4, 131	25.52	<<.0001
	Population	2, 131	1.37	0.26
	Photoperiod * Population	8, 131	2.14	0.037
GnRH3	Photoperiod	4, 131	21.32	<<.0001
	Population	2, 131	7.09	.0012
	Photoperiod * Population	8, 131	1.62	0.124
LHβ	Photoperiod	4, 131	38.89	<<.0001
	Population	2, 131	4.35	0.015
	Photoperiod * Population	8, 131	1.74	0.096

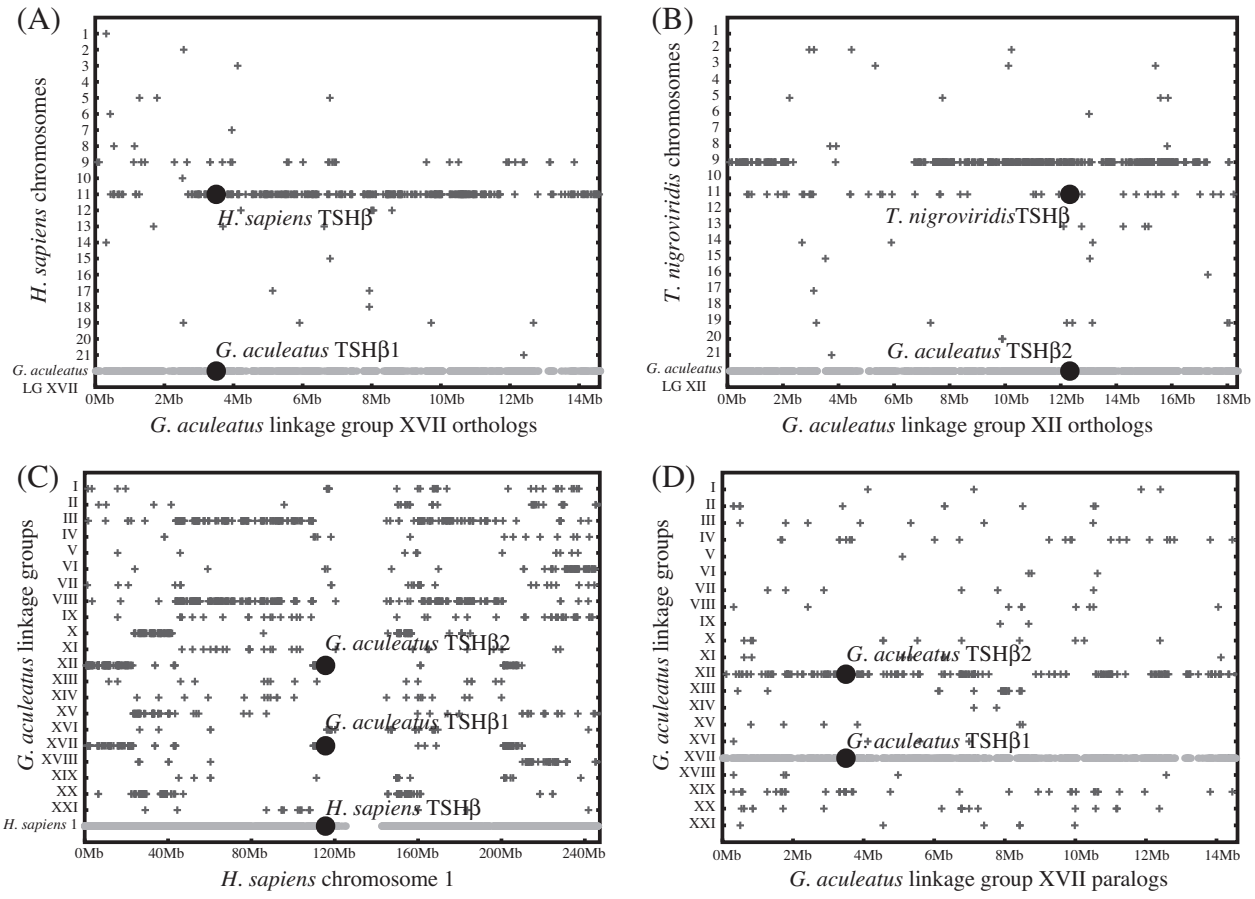

Fig. 2. The threespine stickleback genome contains two thyroid stimulating hormone beta subunit (TSHβ) paralogs. Genes along the x-axis and their orthologs are labeled with dots and crosses respectively. Black circles indicate TSHβ orthologs. (A) Threespine stickleback TSHβ1 (ENSGACG00000005276), orthologous to the single TSHβ in the green spotted puffer fish *Tetraodon nigrovirdis* (ENSTNIG00000018284). (B) Threespine stickleback TSHβ2 (ENSGACG00000009897), orthologous to the single *T. nigrovirdis* TSHβ. (C) *H. sapiens* TSHβ (ENSG00000134200), orthologous to the two threespine stickleback TSHβ paralogs. (D) Syntenic relationships within the threespine stickleback genome show that linkage groups XVII and XII have a high number of paralogs. TSHβ1 (ENSGACG00000005276) and TSHβ2 (ENSGACG00000009897) are labeled.

2.4. mRNA in situ hybridization

A riboprobe complementary to stickleback TSHβ1 mRNA was synthesized using digoxigenin-labeled UTP (Roche Applied Science). It was hybridized to coronal sections of brains and pituitaries removed from males from the AK2 population to visualize the location of the TSHβ expression. The hybridization protocol was adopted from Thisse et al. [60] with the following modifications: sections were not dehydrated prior to hybridization, and incubation with the anti-digoxigenin antiserum solution was performed at room temperature.

2.5. Quantitative real-time PCR (qPCR)

First, target and housekeeping gene primer sets were tested using serial dilutions of cDNA to ensure specificity and consistent amplification across a wide range of concentrations. CDNA concentrations of biological samples were quantified using a Qubit fluorometer (Invitrogen). Two hundred nanograms of cDNA were added to individual qPCR reactions. Reactions were performed in 10 μl volumes using a Kapa SYBR® Fast kit (Kapa Biosystems). Three technical replicates were performed per gene per biological sample.

Two normalization steps created ΔΔCt values. First, Ct values for technical replicates were averaged and normalized to expression of the housekeeping gene β-actin (Ensembl ID# ENSGACG00000007836) [33]. The resulting value was then normalized to the Day 0 population means for each individual gene.

Data were analyzed in R using a two-way population * photoperiod treatment with a Tukey HSD correction for multiple comparisons [59] and Dunnett's test for comparison of treatment means with a control [67]. Both photoperiod and population were treated as fixed effects.

3. Results

3.1. The genomic location and annotation of hypothesized targets of photoperiodism

We identified two TSHβ paralogs in the stickleback genome, confirming previous results [37]. TSHβ1 (ENSGACG00000005276) is on linkage group XVII and TSHβ2 (ENSGACG00000009897) is on linkage group XII (Fig. 2). Phylogenetic reconstruction places them within their expected clades with high support (Supplementary Fig. 1A). TSHβ2 is nested within the teleost TSHβ1 clade with the Siberian sturgeon *Acipenser baerii*, the as an immediate outgroup to the clade containing both TSHβ paralogs (Supplementary Fig. 1A). This topology indicates that the TSHβ duplication resulted from the teleost-specific genome duplication, as the sturgeon lineage is known to have diverged prior to the teleost-specific genome duplication [56]. Furthermore, there is a high number of paralogs between the genomic regions where TSHβ1 and TSHβ2 are found,

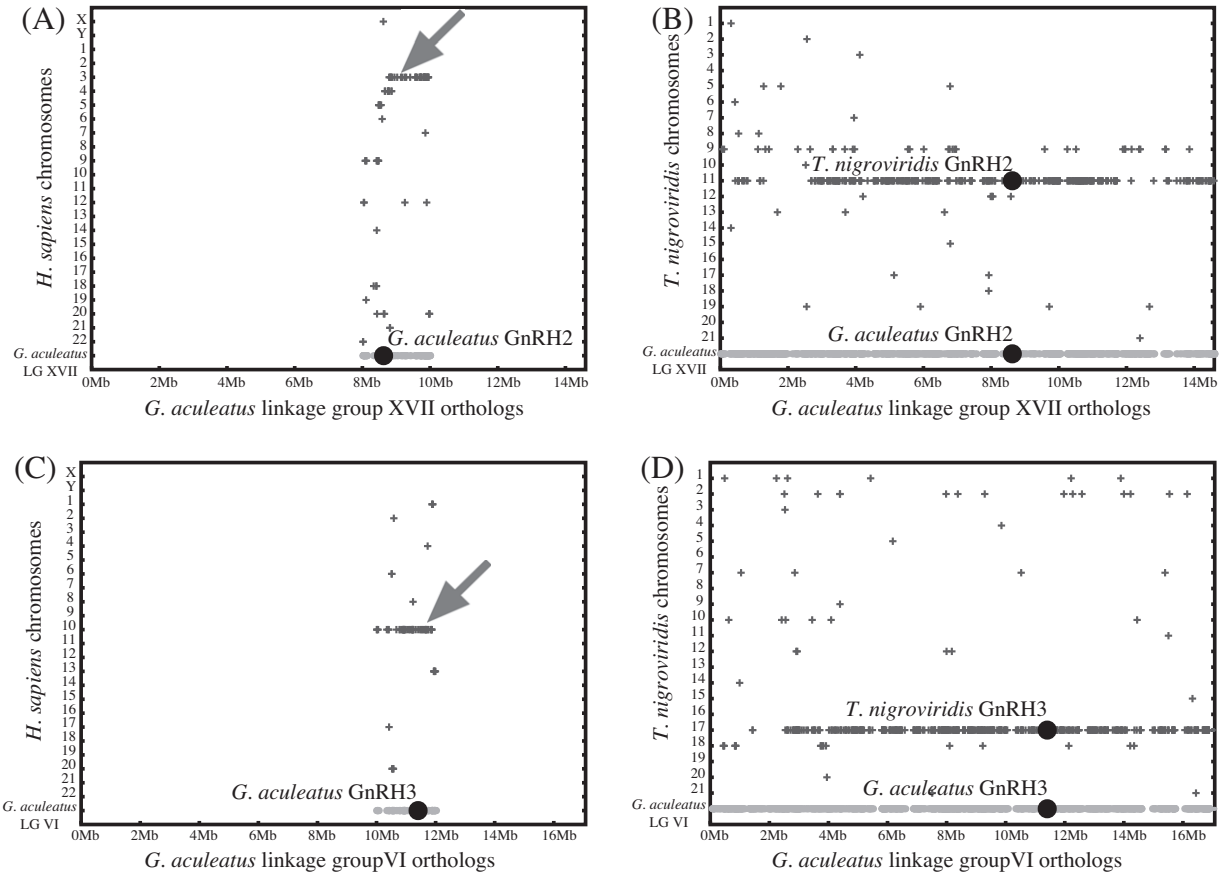

Fig. 3. The threespine stickleback genome contains two of the three vertebrate gonadotropin releasing hormone (GnRH) orthologs. Genes along the x-axis and their orthologs are labeled with dots and crosses respectively. Black circles indicate GnRH orthologs. Grey arrows indicate the expected positions of missing orthologs. (A) Threespine stickleback GnRH2 (ENSGACG00000009021), which has no ortholog in the *H. sapiens* genome. The two megabase region surrounding threespine stickleback GnRH2 is isolated to show syntenic conservation, but the absence of an ortholog. (B) Threespine stickleback GnRH2 has a single ortholog in the *T. nigrovirdis* genome (ENSTNIG00000002767). (C) Threespine stickleback GnRH3 (ENSGACG00000009582), which has no ortholog in the *H. sapiens* genome. The two megabase region surrounding Threespine stickleback GnRH3 is isolated to show syntenic conservation, but the absence of an ortholog. (D) Threespine stickleback GnRH3 has a single ortholog in the T. *nigrovirdis* genome (ENSTNIG00000013337).

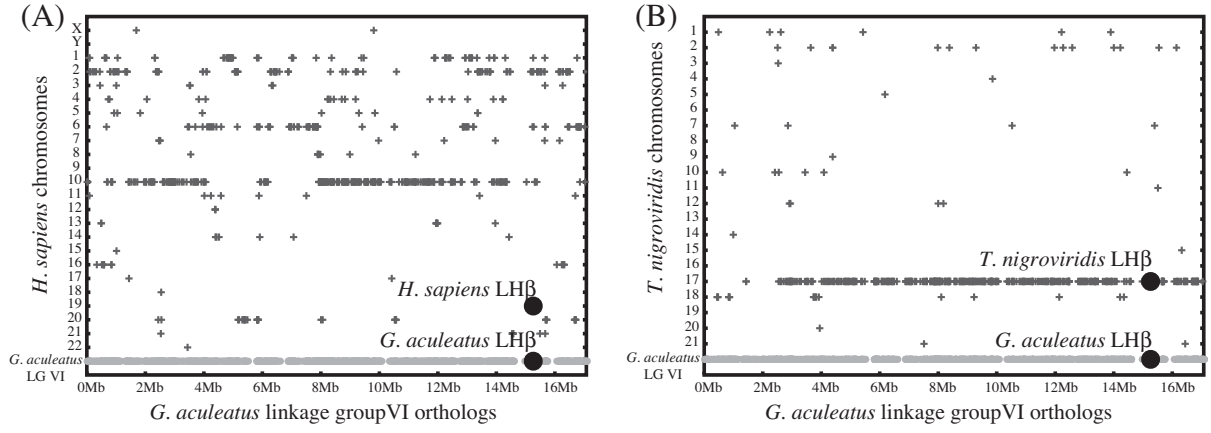

Fig. 4. The threespine stickleback genome contains a single luteinizing hormone beta subunit (LHβ). Genes along the x-axis and their orthologs are labeled with dots and crosses respectively. Black circles indicate LHβ orthologs. Synteny dot plots for threespine stickleback LHβ (ENSGACG00000011475), which has a single ortholog in (A) *H. sapiens* (ENSG00000104826) and (B) *T. nigrovirdis* (ENSTNIG00000009862).

indicating that they originated from a single chromosomal region (Fig. 2D). As TSHβ1 is the most conserved paralog among teleosts (Fig. 2 and Supplementary Fig. 1A), and we could not detect TSHβ2 expression in our biological samples, only the photoperiodic response of TSHβ1 was measured.

Two GnRH paralogs (GnRH2 and GnRH3) were found in the stickleback genome, but GnRH1 is absent. GnRH2 (ENSGACG0000 0009021) is located on linkage group XVII and GnRH3 (ENSGACG00000009582) is on linkage group VI (Fig. 3). The phylogenetic reconstruction shows strong support for separation between

the three GnRH paralog clades, with the stickleback GnRH paralogs placed in their expected clades (Supplementary Fig. 1B). GnRH3 is unique to teleosts and nested within the GnRH1 clade, confirming previous results [18]. As GnRH3 is the hypophysiotropic form in fish when GnRH1 is absent [17,18], the photoperiodic response of GnRH3 was measured.

A single LHβ ortholog (ENSGACG00000011475) was identified in the stickleback genome, on linkage group VI (Fig. 4 and Supplementary Fig. 1C). The phylogenetic reconstruction shows strong support for separation between the teleost and tetrapod clades with the LHβ clade, with stickleback LHβ placed in the expected clades. Interestingly, synteny of the surrounding genomic region is conserved between stickleback and the spotted green pufferfish, *T. nigroviridis* (Fig. 4B), but not between stickleback and *H. sapiens* (Fig. 4A), that the genomic location of LHβ changed after the divergence of teleosts and tetrapods, but prior to the divergence of stickleback and *Tetraodon nigrovirdis* from their most recent common ancestor.

3.2. TSHβ1. expression is localized to expected regions of the brain

TSHβ1 mRNA is expressed in the pars distalis of the pituitary, as measured by visual inspection of brain section slides after *in situ* hybridization (Fig. 5). TSHβ1 may also be expressed around the third ventricle (III–V in Fig. 5), although the latter is too faint to distinguish from background staining with certainty. We expected expression to be localized to the pars distalis, as it is the region of the teleost pituitary that produces the gonadotropins [35]. TSHβ1 mRNA expression appeared to increase after exposure to a single long day (Fig. 5).

3.3. Quantitative real-time PCR (qPCR) shows rapid response of the target genes to stimulatory photoperiods

ΔΔCt treatment means for TSHβ1, GnRH3 and LHβ are illustrated in Fig. 6. Results of the two-way ANOVA are reported in Table 1. Photoperiod has a significant effect on the expression of all three genes (for all three, $P < 0.0001$). There is a significant difference among the populations for GnRH3 ($P = 0.001$) and LHβ expression ($P = 0.015$). There is only a photoperiod * population interaction term for TSHβ1 expression ($P = 0.037$). The significant interaction term for TSHβ1 requires a closer examination of the main effect of photoperiod. As can be seen in Fig. 6, the general trend of the effect of photoperiod is still clear across populations, and the significant interaction term is due to a lower level of expression on day 1.

There is a significant difference among the three populations in the response of TSHβ1 to photoperiod. A single long day causes a pulse in TSHβ1 expression in AK1 and AK2, but the response of OR is not significantly different from baseline values (Fig. 6 and Table 1; photoperiod * population effect: $P = 0.037$). This pulse demonstrates a significant effect of photoperiod on TSHβ1 expression (Fig. 6 and Table 1; photoperiod effect: $P << 0.0001$), although subsequent long days produce no response that is significantly different from baseline values in any of the populations (Fig. 6).

There is no difference among the three populations in response of GnRH3 to photoperiod (Fig. 6 and Table 1; photoperiod *population effect: $P = 0.124$). Long days cause a gradual decrease in GnRH3 expression in the brain and pituitary of all populations, with an eventual return to baseline values after six to ten long days (Fig. 6 and Table 1; photoperiod effect: $P << 0.0001$). This decrease is first significant after two to five long days, and the return to baseline levels occurs after five to ten long days (Fig. 6). There are significant differences among the populations in overall GnRH3 expression (Fig. 6 and Table 1; population effect: $P = 0.0012$).

Long days cause a gradual increase in LHβ expression in the brain and pituitary of all populations (Fig. 6 and Table 1; photoperiod effect: $P << 0.0001$). Differences in the timing of this increase among the populations are not significant (Table 1; photoperiod * population: $P = 0.096$). There are significant differences among the populations in overall LHβ expression (Fig. 6; population effect: $P = 0.015$).

4. Discussion

4.1. Answers to primary questions

The primary questions addressed in this study were to ask (1) whether photoperiodic control of sexual maturation occurred via the thyroid hormone (TH) pathway in a teleost fish and, hence, whether the use of this inductive pathway was conserved from fishes to birds and mammals, (2) whether the order of hormonal events in this pathway coincided with birds and mammals, (3) whether this order of events was robust among different populations. In the threespine stickleback, *G. aculeatus*, the answer to all three questions is affirmative, but with variations.

4.2. Thyroid stimulating hormone

The cascade of response to gonad-stimulating long days begins with up-regulation of thyroid stimulating hormone (TSH). One day of exposure to gonad-stimulating long days elicits an increase in TSHβ1 in the pars distalis of the pituitary (Table 1, Figs. 5, and 6). In mammals and birds, the first short night elicits a similar response of TSHβ [22,40,46] but in the pars tuberalis of the pituitary [46,63]. As the pars tuberalis as a distinct region of the pituitary is found only in tetrapods [35], the pulse of TSHβ1 in the stickleblack pars distalis indicates that function of the tetrapod pars tuberalis is likely contained within the pars distalis of teleosts.

A previous study on the stickleback TSHβ paralogs found no difference in TSHβ1 pituitary expression between immature and sexually mature adults [37]. Our findings contrast with those results (Fig. 6), but the studies are not directly comparable. Whereas our study compared lab-raised adult males, Kitano et al. [37] compared TSHβ1 in lab-raised 8-month-old fish on a short day regimen to 12-month-old fish on a long-day regimen, potentially confounding age and time of exposure to long days. The important early pulse of TSHβ1 that we document (Fig. 6) would therefore not have been observed by Kitano et al. [37].

The early pulse TSHβ1 was higher in the two more northern populations where the growing season is shorter and the winters are longer and more severe (Table 1 and Fig. 6). Stickleback in populations that are ecologically similar and geographically proximal to the northern sites in this study breed strictly from mid-May through July [36], whereas in the southern population, where winters are mild [50], some sexually mature individuals are found nearly year round (Q. Yeates-Burghart and C. O'Brien, unpublished

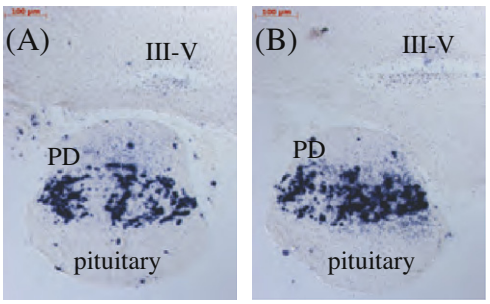

Fig. 5. TSHβ1 expression in the pars distalis of the pituitary labeled via mRNA *in situ* hybridization. Exemplar coronal sections of the ventral hypothalamus and pituitary from adult male threespine stickleback exposed to (A) short days and (b) one long day. Scale bar is 100 μm. III–V: Third ventricle; PD: pars distalis.

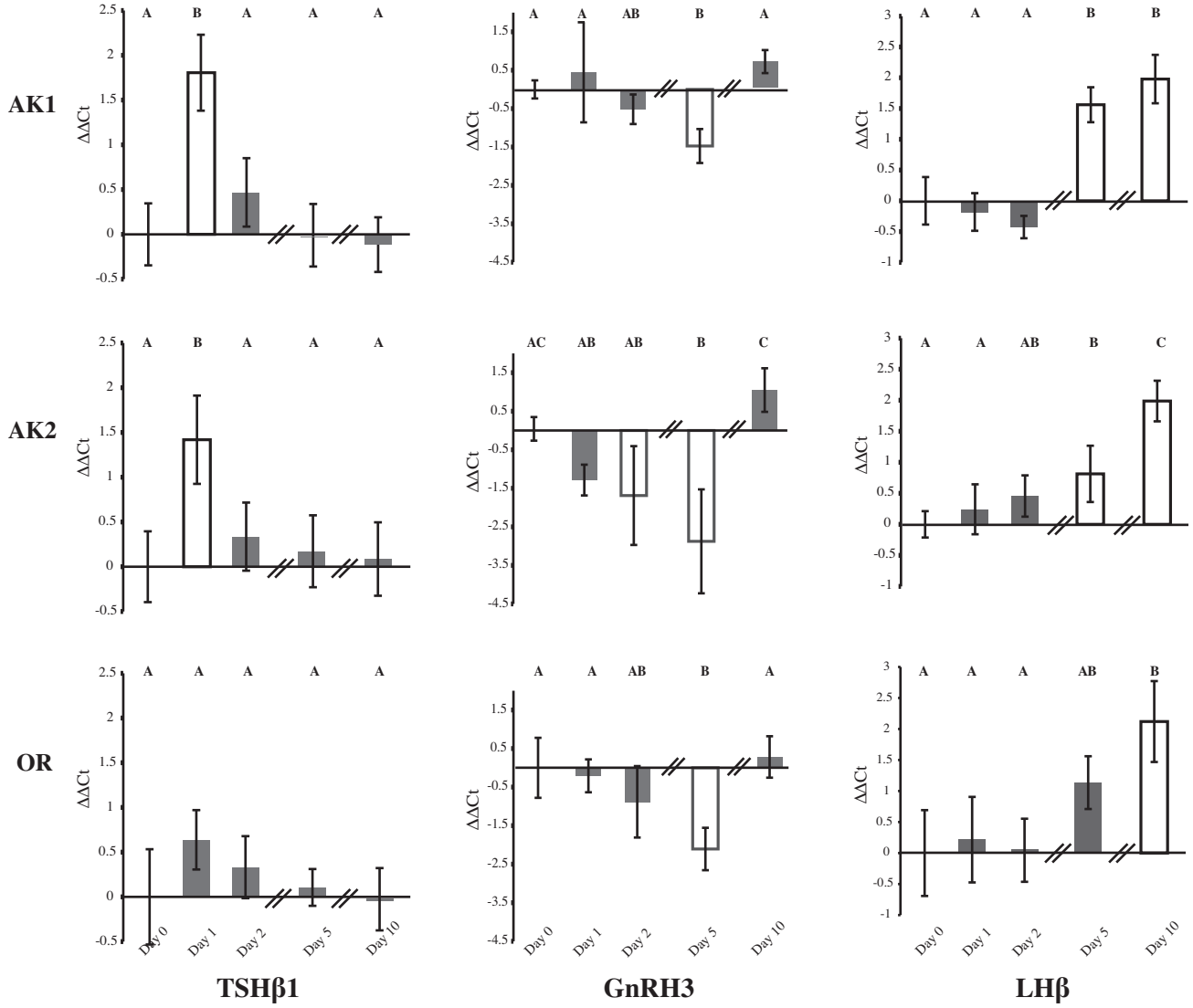

Fig. 6. The effect of photoperiod on the thyroid stimulating hormone pathway in the brain of adult male threespine stickleback, *Gasterosteous aculeatus*. Male sticklebacks from two populations in Alaska (AK1, AK2) and one population in Oregon (OR) were reared from hatch to adulthood on short days and then exposed to another short day (control, Day 0) or 1, 2, 5, or 10 long days, all at 20 °C. Expression of TSHβ1, GnRH3 and LHβ were quantified with qPCR and long-day treatments normalized to the short-day control. Error bars are ± 2S.E. Sample sizes are given in Supplementary Table 1.Time points that share a letter are not significantly different at $P < 0.05$ according to one-way ANOVA with a Tukey HSD correction for multiple a posteriori comparisons; open bars indicate hormone expression levels that differ significantly from the control at $P < 0.05$ according to Dunnett's test.

results). Future studies might consider whether a lower threshold expression of TSHβ1 is required to initiate the cascade of events leading sexual maturation in *G. aculeatus*.

4.3. Gonadotropin releasing hormone

The cascade of response to gonad-stimulating long days in *G. aculeatus* continues with a change in the level of gonadotropin releasing hormone (GnRH), but with a decrease (Fig. 6) rather than an increase in expression, as is seen in photoperiodic tetrapods [62]. We propose three potential explanations.

First, regulation of LH may be independent of GnRH in stickleback. However, GnRH stimulates LH secretion throughout vertebrates, including photoperiodic fishes [8]. In stickleback, the pars distalis has extensive innervation from GnRH neurons [4]. As the pars distalis is the site of gonadotropin production and secretion in the vertebrate pituitary [35], this innervation supports the concept of a direct control of gonadotropins by GnRH.

Second, GnRH3 may not be the actual hypophysiotropic paralog of GnRH. Although the distribution of GnRH2 and GnRH3 expression is similar to that in other teleost fishes that have lost GnRH1 [4,51], GnRH3 and not GnRH2 is the hypophysiotropic form in other species of fish that lack GnRH1 [17,18]. Future research should consider the possibility that GnRH2 may be the hypophysiotropic form in *G. aculeatus* as well as other teleosts.

Third, the hypophysiotropic function of GnRH1 and, in the fish species where it is absent, GnRH3, is well documented, but additional functions of either paralog are much less understood [18]. In species where GnRH3 has assumed the hypophysiotropic function, we would expect it to retain its other functions as well. If GnRH3 expression is inhibited by long days in areas of the brain related to these other functions, but simultaneously stimulated in areas related to its hypophysiotropic function, the net expression of GnRH3 in the brain and pituitary combined could still decrease during long days. Sexual maturation in the grey mullet is regulated by photoperiod [38] and it has retained GnRH1 as the hypophysiotropic GnRH [48]. GnRH3 expression decreases in the brain of

the grey mullet during sexual maturation [48], presumably in the context of its other, non-hypophysiotropic functions. Future research should probe the other functions of GnRH paralogs, unrelated to gonadal maturation, especially in photoperiodic fish.

4.4. Lutenizing hormone

LHβ is the third hormone to be expressed in the sequence of events leading downstream from gonadal stimulating long days (Table 1 and Fig. 6). LHβ is a gonadotropin that stimulates the sex hormones required to initiate sexual maturation in vertebrates. In the Japanese quail, a single long day stimulates LHβ release [46]. A single long day also affects phenotypic indicators of sexual maturation in at the photoperiodic Siberian hamster [25]. In the threespine stickleback, LHβ expression rises above baseline after 5–10 long days but, given the effects of a single long day on quail and hamsters, the later expression in LHβ does not necessarily mean that 5–10 long days are necessary for LHβ expression or to commit stickleback to sexual maturation. Future research should determine the number of long days required to activate the entire TSHβ to LHβ cascade in stickleback and whether, once increased above baseline, expression of LHβ is sufficient to commit stickleback to seasonal reproductive maturation.

5. Conclusions

Our results strongly support a direct role for the TH pathway in the photoperiodic initiation of sexual maturation in teleosts, therefore also supporting the conservation of the TH pathway in the photoperiodic response among vertebrates. Our use of lab-raised populations and controlled experimental conditions allowed us to eliminate the potential influence of other environmental or historical factors that have limited inference in previous studies of the physiological basis of photoperiodic response in teleost fish. We can conclude definitively that the transcriptional changes that we observe are initiated by a change in photoperiod.

Although our data support a conserved role of the entire pathway in the photoperiodic response across vertebrates, the precise degree to which each component of the pathway is conserved remains to be determined. As one would expect given the hundreds of millions of years of combined evolutionary divergence across the mammal, bird and fish lineages, some differences in aspects of the pathway are evident. For example, as pointed out previously, in birds and mammals TSH regulation happens in a subset of cells in the pars tuberalis region of the pituitary rather than the main PD, whereas this region is absent in teleosts. Our data themselves show some divergence in component function in that GnRH2, and not GnRH1 or GnRH3, may be the hypophysiotropic form in stickleback.

Despite these differences, our results indicate an overall conservation of the pathway, and point to the need for additional research to define precisely what aspects of the endocrinology, cell biology, and molecular networks are conserved, and which aspects are divergent. For example, it is known that the TSH mediated photoperiodic response in birds and mammals occurs through local actions of TSH-receptor expression, leading to localized hypothalamic regulation of DIO2 and DIO3 levels [11,65]. Whether these actions are also true for teleosts is not known, and similar well controlled experiments to the ones we present here could be used to determine the patterns of TSH-R and deiodinase gene expression. These data would help determine if the photoperiodic response of TSHb occurs evenly throughout the PD, or whether a subset of cells carries a function analogous to that of pars tuberalis cells in birds and mammals.

Although the photoperiodic responses of the populations in this study are phenotypically indistinguishable [50], their physiological responses demonstrate the benefits of replicating studies across multiple populations within a single species. First, the differences in early TSHβ1 response between the southern and northern populations may reflect differences in seasonal reproductive patterns. Second, the initial decrease in GnRH3 was unexpected, but is robust because this decrease was consistent among all three populations. Future work motivated by these GnRH3 results will illuminate functional variation in a highly conserved hormone family [18]. Finally, the gradual LHβ increases in all populations suggests differences between fish and birds in the timing of sexual maturation in response to photoperiod. To our knowledge, the ecological significance of the early LH release in birds has not been addressed. The results herein provide a motivation and a basis for such studies.

Taken together, our results further establish the threespine stickleback as a vertebrate model of photoperiodic response [9] and form a foundation for future investigations into the degree of conservation and amount of variation of the hormonal basis of vertebrate photoperiodic response in varied seasonal environments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2012.03.010.

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