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Geography of the circadian gene *clock* and photoperiodic response in western North American populations of the threespine stickleback *Gasterosteus aculeatus*

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Abstract

The gene *clock* is a core component of the daily circadian oscillator in flies and mammals. This gene gained renewed interest over a decade ago when the C-terminus of the Clock protein was found to include polyglutamine repeat domains (PolyQ). Since that time, several studies have used variation in PolyQ as a proxy for variation in circadian function. Furthermore, conjectures were made about the possible role of this variation in photoperiodic control of seasonal timing in birds and fishes, generally with questionable results. Herein, we use controlled laboratory experiments to show that Oregon and Alaskan threespine stickleback, collected from populations that differ by 18° of latitude, show no significant variation in length of the polyglutamine domain of *clock*, or in photoperiodic response within or between latitudes despite the fact that male and female sticklebacks are photoperiodic at both latitudes. Hence, we urge caution when interpreting variation in the PolyQ domain of the *clock* gene in the context of seasonal activities or in relationship to photoperiodism along geographical gradients.

Keywords

geographic variation; polyglutamine domain; PolyQ; seasonality; circadian clock; photoperiodism

INTRODUCTION

Proper timing of seasonal events in the life histories of organisms is a key component of fitness at temperate and polar latitudes. A wide variety of animals use the length of day (photoperiodism) to anticipate and prepare in advance for future seasonal changes (Bradshaw & Holzapfel, 2007a). Over 70 years ago, Erwin Bünning (1936) proposed that the circadian clock that organizes the daily activities of organisms also formed the basis of the seasonal photoperiodic timer. Evidence for this proposition is strongest in plants (Kobayashi & Weigel, 2007; Wilczek et *al.*, 2009) and highly inbred strains of the golden hamster (Shimomura *et al.*, 1997; Lowrey *et al.*, 2000). Otherwise, the connection between the two physiological processes remains highly contentious (Hazlerigg & Loudon, 2008; Goto et al., 2010; Bradshaw & Holzapfel, 2010a,b; Saunders, 2010; Koštál, 2011; Schiesari et al., 2011). Historically, causal connections between the daily circadian clock and the

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seasonal photoperiodic timer were inferred from parallel peculiarities of their physiological behavior to exotic light:dark cycles (vaz Nunes & Saunders, 1999; Tauber & Kyriacou, 2001; Goldman, 2001; Saunders, 2002, 2010, 2011).

With the advent of tractable molecular techniques, a common approach to examine the relationship of circadian and photoperiodic timers has been to use circadian clock genes as candidate loci and then to seek a correlation between mutations or knockdowns of those genes and variation in diapause response in photoperiodic insects (Saunders, 1990; Goto et al., 2006; Stehlík et al., 2008; Han & Denlinger, 2009; Ikeno et al., 2010). The expression of diapause involves a neuroendocrine pathway and it is not clear whether variation in diapause response is due to the effect of the circadian clock on photoperiodism, which is the desired result by the authors, or to an individual clock gene somewhere in the neuroendocrine pathway leading to diapause independently of photoperiodism (Bradshaw & Holzapfel, 2007b; Emerson *et al.*, 2009; Bradshaw & Holapfel, 2010a, 2010b; Schiesari et al., 2011).

Three logical associations have led investigators to ask whether evolution of the photoperiodic timer, especially over latitudinal gradients, is connected with allelic variation in candidate circadian clock genes segregating in natural populations (Tauber *et al.*, 2007; Mathias *et al.*, 2007; Liedvogel *et al.*, 2009; O'Malley *et al.*, 2010). First, photoperiodism is a physiological mechanism for anticipating seasonal change and preparing in advance for that seasonal change. Second, seasonal environments change with latitude. Third, the timing of seasonal activities (phenology) changes with latitude. The speculative leap in logic is then to assume that any correlation between a circadian clock gene and latitude or phenology implies a causal connection between the circadian clock and photoperiodism. While this conjecture may be true, it is none-the-less a hypothesis that needs to be tested and not assumed.

The canonical circadian gene *clock* has been the focus of several studies seeking to relate variation in C-terminal polyglutamine domain length within this gene to photoperiodism in order to infer a role of the circadian clock. In *Drosophila melanogaster* deletion of two of the three PolyQ domains of clock resulted in altered circadian behavior (Darlington et al., 1998). In mice, excision of a glutamine-rich exon also resulted in altered circadian behavior (King et al., 1997). These findings provided the point of departure for studies aimed at correlating variation in PolyQ with latitude (Johnsen et al., 2007) or with seasonal events acting as a presumptive proxy for photoperiodism in nature (Liedvogel et al., 2009; O'Malley & Banks, 2008a; O'Malley et al., 2010). However, correlation does not demonstrate causation (Kingsolver & Schemske, 1991; Petraitis et al., 1996; O'Brien et al., 2011). In fact, none of the aforementioned studies actually determined photoperiodic response directly or sought to determine the relationship between PolyQ and photoperiodism under controlled conditions free from maternal or field effects. The hypothesis of a causative relationship between *clock* gene PolyQ variation and differences in the photoperiodic response across latitudes therefore remains to be tested.

Herein, we determine variation in PolyQ and in photoperiodic response as measured by sexual maturation of the threespine stickleback *Gasterosteus aculeatus* L. in northwestern North American populations from Oregon and Alaska (18° difference in latitude).

Gasterosteus aculeatus is found from marine to freshwater habitats (Bell & Foster 1994), shows extensive population-level variation in phenology in natural populations (Borg, 1982; Crivelli & Britton, 1987), and has been shown to be photoperiodic in both wild-caught (Baggerman, 1985; Bornestaf & Borg, 2000) and laboratory-reared populations (Yeates-Burghart et al., 2009). Among wild-caught fishes from the Baltic Sea (c. 56–59°N), long days promote reproduction in the late spring and early summer (Borg, 1982; Borg & Van Veen, 1982; Borg et al., 2004). In males, sexual maturation is manifest through increased bright body coloration, territoriality, nest building, courtship, and hypertrophy of the kidney to produce spiggin, the glue used for nest construction (Borg, 1982; Borg et al., 2004; Mayer et al., 2004). Kidney hypertrophy is therefore a reliable indicator of sexual maturity in males. In females, sexual maturation is manifest through increased ovarian mass as a consequence of oocyte maturation (Baggerman, 1972, 1985; Bornestaf et al., 2001; Mayer et al., 2004).

MATERIALS AND METHODS

PHOTOPERIODIC RESPONSE

Northern (Alaskan) stocks were established from Bear Paw Lake (61°37′N, 149°45′W) and Rabbit Slough (61°34′ N, 149°15′W). Southern stocks (Oregon) were established from Cushman Slough (43°36′N, 124°2′W) and Eel Creek (43°35′N, 124°11′W). The animals used for these experiments were G_7 (AK), G_1 (Eel Creek, OR), and G_2 (Cushman Slough, OR) outbred descendants of wild-caught individuals. All collection and care of fish conformed to approved animal care protocols.

The experimental fish were produced, hatched and reared using standard protocols (Cresko et al., 2004; Yeates-Burghart et al., 2009). Briefly, experimental fish were reared on a 10L: 14D cycle for 11 – 12 months (Alaska fish) or 11 months (Oregon fish). All fish used in the experiment were at least 50 mm standard length (SL), measured from the dorsum of the pre-maxilla to the end of the caudal peduncle. Within each stock, fish from several parental lines were pooled and split into male-female pairs for the experiments. Experiments were run in light-tight air-cooled cabinets in climate-controlled rooms at 20°C. Aquaria were visually separated and cleaned separately to avoid the possibility of transferring visual or hormonal cues between aquaria. Fish from each population were exposed to six different photoperiod regimes, ranging from 8L:16D to 23L:1D. Fish that died were not replaced. At the end of six weeks, all surviving fish were included in the data set.

To quantify sexual maturation, the ovary-somatic index (I_O) and the kidney-somatic index (I_K) were determined. Kidneys or ovaries were dissected out and transferred to 37°C with the respective soma in a desiccator containing Drierite (www.drierite.com) until there was no decrease in mass between two successive weighings. Ovaries, kidneys and soma were weighed using a Mettler AT261 DeltaRange electronic balance (mt.com). I_O and I_K were calculated as the ratio of ovary and kidney to total body mass, respectively. I_O and I_K values were raised by 10^3 before log transformation to ensure positive values on a log scale.

clock POLYGLUTAMINE DOMAIN

Northern (Alaskan) collections were made from Bear Paw Lake, Rabbit Slough, Hidden Lake (60°29′N, 150°16′W), and Anchor River (59°45′N, 151°30′W). Rabbit Slough and Anchor River are populations in oceanic environments, whereas Bear Paw Lake and Hidden Lake are isolated freshwater populations. Southern (Oregon) collections were made from Eel Creek, Winchester Marsh (43°16′N, 124°19′W), Miner Creek (43°20′N, 124°22′W), and the junction of the Smith and Umpqua Rivers (43°43′N, 124°05′W). All fish were collected using unbaited minnow traps, anesthetized in MS-222 (Aquatic Eco-systems) and preserved in 200 proof ethanol. DNA was extracted from caudal fin clips using a MasterPure DNA Purification Kit (Epicentre).

The human *clock* ortholog (Ensembl ID ENSG00000134852) was compared via BLAST against the threespine stickleback reference genome to find the gene *clock*. Reciprocal Best Hit (RBH) analysis was then conducted to ensure that the resulting gene was the only *clock* paralog in the stickleback genome. To do so the putative stickleback ortholog was compared via BLAST against the human genome. The best match that it returned was reciprocally compared via BLAST against the stickleback genome to ensure that its best match was stickleback *clock*. As an additional check, syntenic analysis of the genomic regions surrounding the *clock* orthologs was performed. The synteny database detects synteny between a specified genomic region (in this case, the genomic region surrounding stickleback *clock*) and regions from an outgroup genome (the human genome) using automated RBH analysis (Catchen *et al.*, 2009).

All further sequence annotation and analysis used Geneious Pro 4.7.6 software. The stickleback clock gene was annotated by identifying exons using Ensembl's automatic gene annotation (Curwen *et al.*, 2004), and then confirmed by comparing the translated protein against the amino acid sequence of other, annotated paralogs. The PolyQ domain was apparent in the reference sequence as a region containing only glutamines and a single arginine.

To sequence the PolyQ domain, we used flanking primers: a forward primer (CAGGGAGGTCAAACCCAGAC) located on exon 19 of clock and a reverse primer (TACTGTGGTTGGCTGCTGAC) located in the 3′ UTR. These primers were designed using NCBI Primer Design (NCBI). PCR products were amplified in an MJ Research PTC-200 (Applied Biosystems) using the following protocol: 95 C three minutes, 32 cycles of 95 C 30s, 60 C 30s, 72°C 60s, single cycle of 72°C 7 minutes. Because of a high degree of heterozygosity, PCR products were not sequenced directly, but instead were cloned into a pCR® 4-TOPO® vector (Invitrogen) and sequenced using a 3130x Genetic Analyzer (Applied Biosystems). In order to capture variation in PolyQ length among alleles within individuals, multiple TOPO clones were sequenced from each individual.

Resulting sequences were translated and the polyQ domain was manually annotated in ten fish from each population. Sequences with low quality scores in the domain were discarded and re-sequenced. The number of glutamines within the polyQ domain was counted and the positions of the arginine within the polyQ domain were recorded.

ANALYSES

Linear and quadratic regressions were performed to relate indices of sexual maturation to input variables. For regressions of I_O or I_K on day length, linear regression was always significant ($P < 0.003$); in no case did the addition of a quadratic term significantly increase the reduction in total sum of squares. We therefore used linear regression for all analyses. We used JMP IN 4 (Sall et al., 2005) for ANOVAs. In the latter case, we modeled latitude (AK = north vs. OR = south) and day lengths as fixed effects. Variation between populations within latitudes was incorporated into the error term.

RESULTS

PHOTOPERIODIC RESPONSE IS VERY SIMILAR ACROSS POPULATIONS AT NORTHERN AND SOUTHERN LATITUDES

Sexual maturation in both males and females from both northern and southern latitudes increased with day length (Fig. 1). The kidney:somatic index (I_K) depended on day length (Two-way ANOVA: $F_{5,219} = 19.4$; $P < 0.001$), did not differ between northern and southern males ($F_{1,219} = 2.31$; $P = 0.130$) and there was no latitude by photoperiod interaction ($F_{5,219} = 0.43$; $P = 0.829$). The ovary:somatic index (I_O) depended upon day length ($F_{5,230} = 20.26$; $P < 0.001$) and was higher in southern than northern females ($F_{1,230} = 9.28$; $P = 0.023$) but there was no significant latitude by photoperiod interaction ($F_{5,230} = 0.91$; $P = 0.477$). These results show that while sexual maturation increased with day length at both latitudes (Fig. 1) photoperiodic response did not differ between northern and southern latitudes (no significant photoperiod by latitude interaction).

THE POLYGLUTAMINE DOMAIN OF *CLOCK* VARIES ACROSS INDIVIDUAL STICKLEBACK BUT SHOWS NO POPULATION STRUCTURING

The BLAST search and syntenic analysis found one *H. sapiens clock* ortholog in the stickleback genome (Ensembl ID ENSGACG00000015939) (Supplementary Fig. 1). *Gasterosteous aculeatus clock* contains 20 exons from bp 489,361 – 499,374 on linkage group IX (Ensembl). Examination of the sequence shows that the PolyQ domain is located in exon 20.

The PolyQ domains (Fig. 2a) contained between 22 and 38 glutamine repeats and did not differ between latitudes (Nested ANOVA: $F_{1,6} = 0.74$; $P = 0.422$) or among populations within latitudes ($F_{6,72} = 1.162$; $P = 0.336$). An arginine residue (Fig. 2b) occurred within each of the PolyQ domains between positions 2 and 26. Mean position of the arginine residue did not differ between latitudes ($F_{1,6} = 0.533$; $P = 0.493$) or among populations within latitudes ($F_{6,72} = 0.907$; $P = 0.495$). These results show that there is no significant difference in either length of the PolyQ domain or position of the arginine residue within the PolyQ domain between latitudes or among populations within the northern (AK) and southern (OR) latitudes.

DISCUSSION

STICKLEBACK HAVE SIMILAR PHOTOPERIODIC RESPONSES AT NORTHERN AND SOUTHERN LATITUDES

Previously (Yeates-Burghart et al., 2009), we found that photoperiodic response of a single southern (Oregon) population exhibited no significant variation with photoperiod in either ovarian development or male kidney enlargement whereas a single northern (Alaska) population exhibited a strong photoperiodic response. After using replicate populations within Oregon and Alaska (Fig. 1), it is now clear that threespine stickleback are photoperiodic at both latitudes and do not differ in photoperiodic response between latitudes. This pattern is inconsistent with other vertebrates where photoperiodic response tends to increase with latitude and northern populations typically exhibit a stronger photoperiodic response than southern populations (Bradshaw & Holzapfel, 2007a). In both Yeates-Burghart et al. (2009) and the present study all experiments were run at 20°C using laboratory-reared fishes where field and maternal effects were minimized. Experimental fishes consisted of a single male paired with a single female that were visually and chemically isolated from other experimental fish and, hence, represented independent replicates. Consequently, the similarity in their photoperiodic responses cannot be ascribed to phenotypically plastic responses to a variable environment or to visual or water-borne cues. We therefore conclude that genetically determined photoperiodic responses do not differ between Oregon and Alaskan populations separated by ~18° of latitude.

Constancy of photoperiodic response in a common laboratory environment does not necessarily translate into a constant physiological response to natural environments over a latitudinal gradient. In threespine stickleback from the field, gonadal maturation is accelerated both by increasing day lengths and warmer temperatures, and phenological differences have be noted across stickleback populations in the wild (Borg, 1982; Borg *et al.*, 1987; Andersson *et al*., 1992; Hellqvist *et al*., 2004). In addition, cold-acclimated fishes have greater facility in adjusting to warm temperatures with increasing day lengths (Guderley *et al.*, 2001). These physiological responses to day length and temperature need to be considered in the context of the photic and thermal environments of Alaska and Oregon. We only manipulated one of these variables, photoperiod, while keeping the others constant. Although climates are colder in coastal Alaska than Oregon (U. S. Department of Commerce, 1968), spring and summer day lengths are longer and spring temperatures rise faster in Alaska than Oregon (Fig. 3). We therefore propose that the accelerating effects of longer day lengths and increasing temperatures in the more northern environment may compensate for the lower average temperature in Alaska than Oregon. Hence, northern fishes would be reproductively prepared to exploit the shorter northern growing season during the brief period when summer waters are warmest. Finally, we encourage rearing animals from different localities in a common environment before using them to infer an underlying genetic basis for differences in functional phenotypes.

ABSENCE OF CLOCK POLYGLUTAMINE DOMAIN LENGTH (POLYQ)

In *Drosophila melanogaster*, the Clock protein heterodimerizes with the Cycle protein to promote the transcription of the genes *period* and *timeless*. Heterodimerization of Period and

Timeless and their migration into the nucleus lead to the inhibition of their own transcription by Clock and Cycle (Darlington et al., 1998). The interest in PolyQ comes from the observation that "a truncated dCLOCK protein lacking two of the three polyglutamine repeats [dCLOCK (ΔQ)] only weakly activates *per* and *tim*" (Darlington *et al.*, 1998, p. 1602). In the mouse, the clock$^{\Delta 19}$ mutant results in a long circadian period (Gekakis et al., 1998; Jin et al., 1999; Lowrey & Takahashi, 2004). King et al. (1997), found that "an AàT transversion at the third base position of the 5′ splice donor site of intron 19" results in skipping the exon immediately upstream, i.e., exon 19. Exon 19 is in the "glutamine-rich region of the C-terminus of the predicted Clock protein (amino acids 514-564)," but not in the downstream PolyQ region (amino acids 739-837) (King *et al.*, 1997). These studies provided new and interesting insights into *clock* in the context of daily circadian timing, but they revealed nothing about any relationship between circadian rhythmicity and photoperiodism. The tractability of measuring PolyQ provided a convenient proxy for variation in the circadian clock that potentially could create functional differences in circadian rhythmicity. Unfortunately, various investigators made a logical error by seeking a causative relationship between the circadian clock and photoperiodic timer by demonstrating correlation between variation in PolyQ and latitude or phenology as assumed proxies for the photoperiodic timer.

Our findings of a lack of correlation between polyQ domain and aspects of photoperiodic response are not unique. We found no association between PolyQ and latitude in western North American populations of stickleback (Fig. 2). Similarly in the European blue throat *Luscinia svecica* there is no correlation between PolyQ and latitude from Armenia to Norway (40°30′ – 70°30′N) (Johnsen et al., 2007). Hence, in both species, there is no evidence of a connection between PolyQ and local or regional variation in phenology or photoperiodic response.

Photoperiodism, more than any other proximal factor, is responsible for the onset of first clutches among populations of the blue tit *Cyanistes caeruleus*, and photoperiodic response can vary between island and mainland populations at the same latitude (Lambrechts et al., 1997). In a transect from Italy to Finland (36°44′ – 62°37′N), Johnsen et al. (2007) found a significant correlation between latitude and PolyQ but only when an atypical, monomorphic, southernmost population was entered into the correlation. Johnsen *et al*. (2007) did not provide any correlation between PolyQ variation and phenological events and, in fact, made the appropriate warning (p. 4878): "Determination of the phenotypic effects of different ClkpolyQcds alleles described here would require detailed studies of both circadian and photoperiod-related behaviours of birds of differing ClkpolyQ genotypes."

Within a single site (Wytham Woods, UK; 51°47′N), Liedvogel et al. (2009) sought to correlate PolyQ with laying date, hatch date, and incubation duration of 950 blue tits over a two-years period. No "significant overall year*genotype interaction was found for any of the timing traits in focus (all results with $P > 0.213$)." However, when the authors continued their search for significance within the observed "non-significant" data, they found that by considering the second year in isolation, they could find a significant correlation between PolyQ and laying date and hatch date ($P = 0.047$ and $P = 0.033$, respectively, but without any table-wide adjustment for a-posteriori multiple comparisons). A follow up study on a

great tit *Parus major* population at the same site found no association between PolyQ and the same measures of reproductive timing (Liedvogel & Sheldon, 2010). Hence, studies among birds over a large latitudinal range or within a single locality with a large sample size provide at best equivocal evidence for an association between clock polyglutamine repeat length and the timing of phenological events, much less photoperiodism.

Among teleost fishes, the molecular basis of daily circadian rhythmicity has been studied in the zebrafish *Danio rerio*. In zebrafish, the core loop of the circadian clock involves three paralogs of *clock* whose proteins form heterodimers with three paralogs of *bmal* that drive rhythmic expression of three paralogs of period and *cryptochrome* (Vatine et al., 2011). No connection has been made between any core circadian rhythm genes and photoperiodically controlled seasonal life histories in zebrafish.

Salmonids as a family are photoperiodic for many seasonal life-cycle transitions, such as smolting, precocious sexual maturation, migration to sea, and the initiation of migration back to freshwater (Bromage et al., 2001). Two paralogs of *clock* have been identified in Chinook salmon *Oncorhyncus tshawytscha*, *OtsClock1a* and *OtsClock1b*, which arose from a tetraploidation event during divergence of salmonids from other teleost fishes (O'Malley & Banks, 2008b). No functional connection has yet been made between either of these paralogs and circadian rhythmicity in salmonids. Likewise, their functional role in photoperiodism, if any, has not been established. There is no evidence for polyglutamine length polymorphism in the OtsClock1a paralog among four species in the genus Oncorhyncus. In the OtsClock1b paralog, polyglutamine length is polymorphic within and among populations of Chinook, chum *O. kita*, 2010). Mean length of the glutamine domain (PolyQ) is not significantly correlated with latitude among 19 populations of coho or 16 populations of pink salmon, but is correlated with latitude in Chinook and chum salmon (O'Malley & Banks, 2008a; O'Malley et al., 2010). O'Malley et al. (2010) used univariate regression trees to identify correlations between the frequency of the most common polyglutamine domain length allele of *OtsClock1b* and day length on the date of peak spawn and a freshwater migration index over a wide latitudinal range of Chinook, coho, chum and pink salmon. They found that the ability of the univariate regression tree "to assign populations to groups correctly on the basis of these factors" (day lengh and migration index) was not significant (O'Malley *et al.*, 2010, p. 3711) and significant ($P < 0.05$) only in pink salmon where length of the most common allele varied with day length on the date of peak spawn but not the freshwater migration index. They did not test for a persistent correlation between the frequency of most common *OtsClock1b* allele and latitude after their common covariation with latitude was factored out (O'Brien et al., 2011).

Hence, in fishes as in birds, there is little evidence for a correlation between polyglutamine domain length and latitude or the timing of phenological events. Even if there had been a general pattern of correlation, correlation is not causation (Kingsolver & Schemske, 1991; Petraitis et al., 1996; O'Brien et al., 2011). In neither the birds nor the fishes was there any determination of the actual effect of PolyQ on circadian function or any actual direct measurement of photoperiodic response. Our results in threespine stickleback, in context with these previous findings from other fishes and birds, should give pause to those assuming a functional molecular relationship between the circadian clock and photoperiodic

timer. Instead, this hypothesis should be directly tested by identifying the genes and physiological pathways that contribute to each of these core mechanisms that are used to utilize daily and annual cycles across a wide range of organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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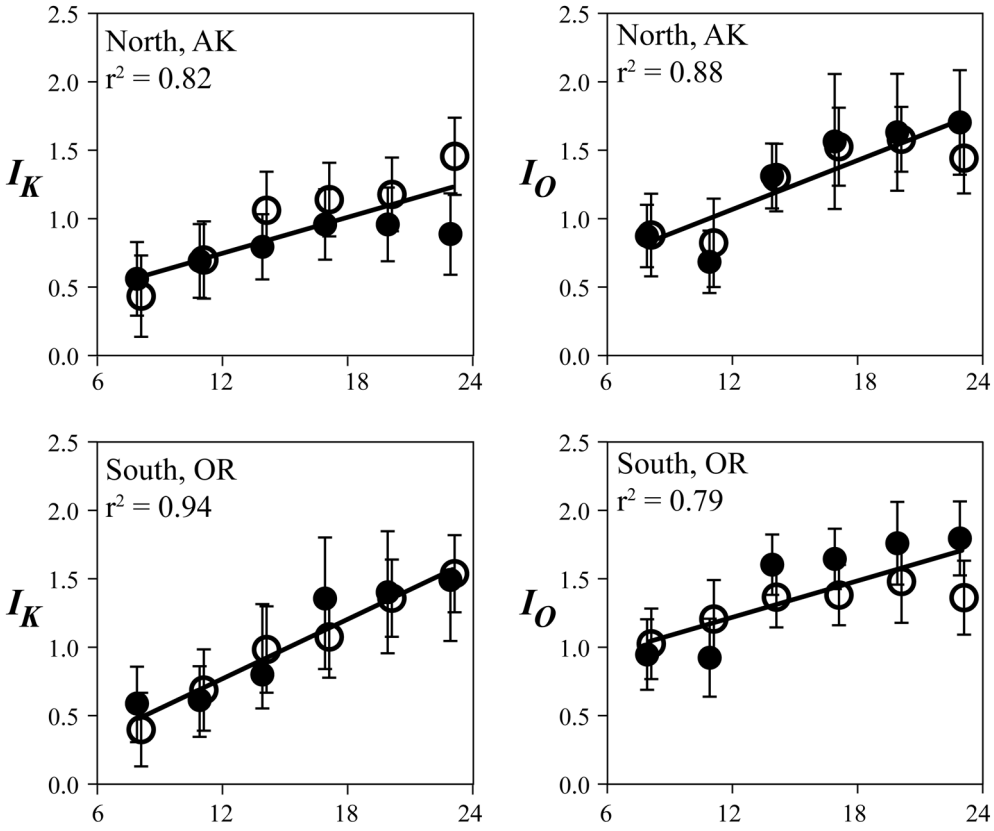

Fig. 1.

Photoperiodic response of male and female threespine stickleback (*Gasterosteus aculeatus*) in Oregon (43.5°N) and Alaska (61.5°N) in western North America. Male response is represented by kidney:body mass ratio (I_K); female response is represented by the ovary:body mass ratio (I_O). Open circles show results from Yeates-Burghart et al. (2009); closed circles show results from the present study. Error bars are ± 2SE.

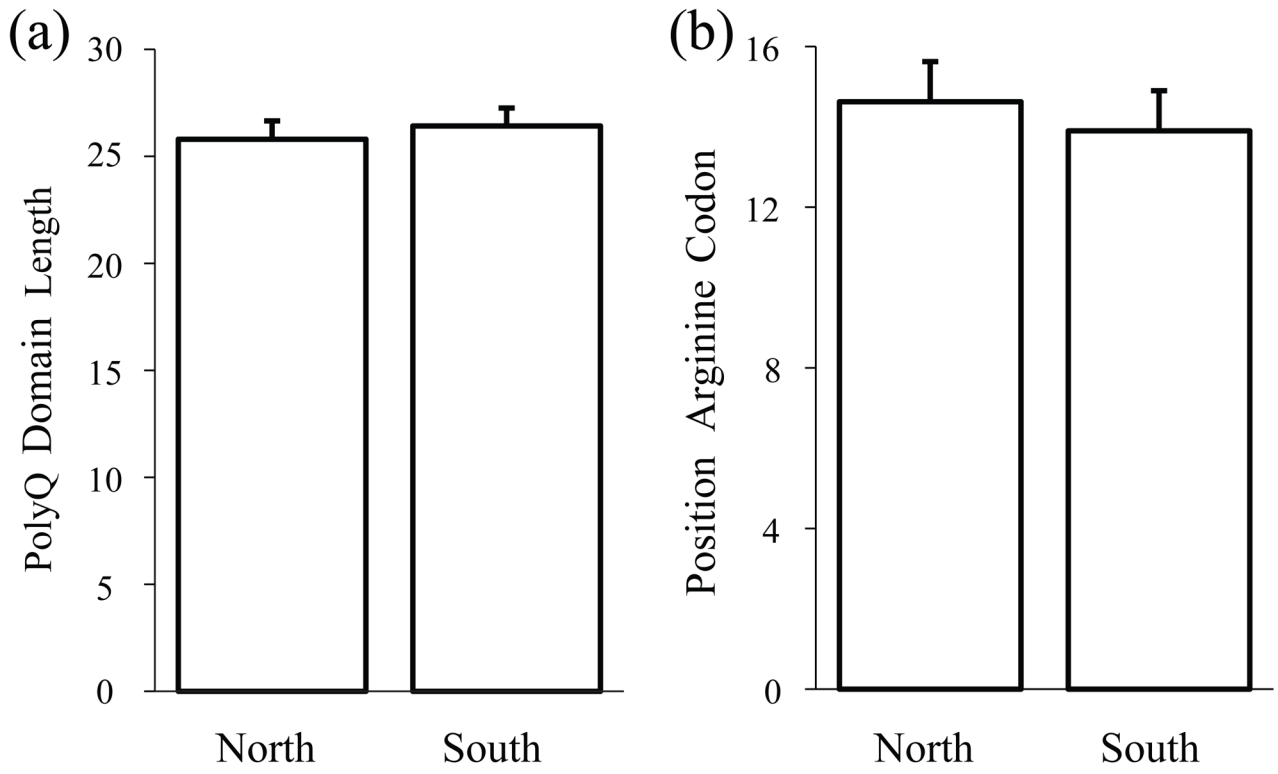

Fig. 2.

Polyglutamine domain (PolyQ) in the clock gene in southern (Oregon) and northern (Alaska) populations of *Gasterosteus aculeatus*. (a) Domain length in number of glutamine repeats; (b) position of the arginine codon within the polyglutamine domain. Error bars are ± 2SE.

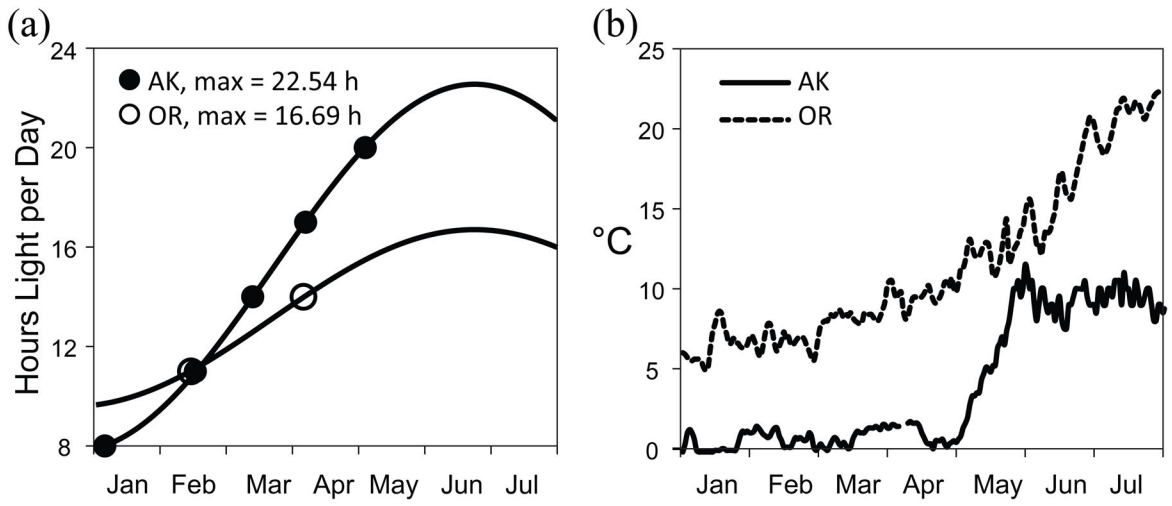

Fig. 3.

Day length and temperature profiles during the winter and spring in Oregon (OR) and Alaska (AK). (a) Circles show the day lengths at which photoperiodic responses were determined. Note that the Oregon populations do not experience day lengths as short as eight hours or as long as 17 hours light per day. Day lengths are calculated as the time from the onset of civil twilight in the dawn until the end of civil twilight in the dusk for Florence, OR, and Seward, AK (http://www.sunrisesunset.com). (b) Water temperatures in the Rogue River near Agness, OR (42° 34.7′ N, USGS 14372300), and Wasilla Creek, near Palmer, AK (61°38.5′ N, USGS 15285000), based on data from 2010 and 2011 (http://waterdata.usgs.gov/usa/nwis/).