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

C. O'Brien, L. Unruh, C. Zimmerman, W. E. Bradshaw, C. M. Holzapfel and W. A. Cresko*

Institute of Ecology and Evolution, 5289 University of Oregon, Eugene, OR, 97403-5289, U.S.A.

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Controlled laboratory experiments were used to show that Oregon and Alaskan three-spined stickleback *Gasterosteus aculeatus*, collected from locations differing by 18° of latitude, exhibited no significant variation in length of the polyglutamine domain of the clock protein or in photoperiodic response within or between latitudes despite the fact that male and female *G. aculeatus* are photoperiodic at both latitudes. Hence, caution is urged when interpreting variation in the polyglutamine repeat (PolyQ) domain of the gene *clock* in the context of seasonal activities or in relationship to photoperiodism along geographical gradients.

Key words: circadian clock; geographic variation; photoperiodism; polyglutamine domain; PolyQ; seasonality.

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, 2007*a*). 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 *Mesocricetus auratus* (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, 2010*a*,*b*; Saunders, 2010; Koštál, 2011; Schiesari *et al*., 2011). Historically, causal connections between the daily circadian clock and the seasonal photoperiodic timer were inferred from parallel peculiarities of their physiological behaviour to exotic light:dark cycles (vaz Nunes & Saunders, 1999; Tauber & Kyriacou, 2001; Goldman, 2001; Saunders, 2002, 2010; Saunders & Bertossa, 2011).

*Author to whom correspondence should be addressed. Tel.: +1 541 346 4779; email: wcresko@uoregon.edu

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 preceding authors, or to an individual clock gene somewhere in the neuroendocrine pathway leading to diapause independently of photoperiodism (Bradshaw & Holzapfel, 2007*b*; Emerson *et al*., 2009; Bradshaw & Holzapfel, 2010*a*, *b*; Schiesari *et al*., 2011).

Three logical associations have led investigators to ask whether evolution of the photoperiodic timer, especially over latitudinal gradients, is associated 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.

The canonical circadian gene *clock* has been the focus of several studies seeking to relate variation in C-terminal polyglutamine (PolyQ) domain length of this gene with variation in photoperiodic response to infer a role of the circadian clock in photoperiodism. In *Drosophila melanogaster*, deletion of two of the three PolyQ domains of *clock* resulted in altered circadian behaviour (Darlington *et al*., 1998). In mice *Mus musculus*, excision of a glutamine-rich exon also resulted in altered circadian behaviour (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, 2008*a*; O'Malley *et al*., 2010). Correlation, however, 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.

Variation in the length of the *clock* PolyQ and in photoperiodic response as measured by sexual maturation of the three-spined stickleback *Gasterosteus aculeatus* L. 1758 in north-western North American populations from Oregon and Alaska (18° difference in latitude) was determined. *Gasterosteus aculeatus* is found from marine to freshwater habitats (Bell & Foster, 1994), showing 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 fish 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 colouration, 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 (AK) stocks were established from Bear Paw Lake (61° 37′ N; 149° 45′ W) and Rabbit Slough (61° 34′ N; 149° 15′ W). Southern stocks (OR) 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 University of Oregon animal care and use 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 (AK fish) or 11 months (OR fish). All fish used in the experiment were at least 50 mm standard length (L_S), 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 6 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 (www.mt.com/balance). 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 $\log_{10}$ transformation to ensure positive values on a log scale.

CLOCK POLYGLUTAMINE DOMAIN

Northern (AK) 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, anaesthetized in MS-222 and preserved in 100% ethanol. DNA was extracted from caudal fin clips using a MasterPure DNA Purification Kit (Epicentre; www.epibio.com).

The human *clock* orthologue (Ensembl ID ENSG00000134852) was compared *via* basic local alignment search tool (BLAST) to the *G. aculeatus* genome (Ensembl) to identify its gene *clock*. Reciprocal best hit (RBH) analysis was then conducted to ensure that the resulting gene was the only *clock* paralogue in the *G. aculeatus* genome. To do so the putative s *G. aculeatus* orthologue was again compared *via* BLAST to the human genome. The best match that it returned was reciprocally compared *via* BLAST to the *G. aculeatus*

genome to ensure that its best match was the *G. aculeatus clock*. As an additional check, syntenic analysis of the genomic regions surrounding the *clock* orthologues was performed. The synteny database detects synteny between a specified genomic region (in this case, the genomic region surrounding the *G. aculeatus 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 (Invitrogen; www.invitrogen.com). The *G. aculeatus 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 paralogues. The polyQ domain was apparent in the reference sequence as a region containing only glutamines and a single arginine.

To sequence the PolyQ domain, flanking primers were used: a forward primer (CAGGGAGGTCAAACCCAGAC) located on exon 19 of *clock* and a reverse primer (TACTGTGGTTGGCTGCTGAC) located in the 3′ untranslated region. These primers were designed using NCBI Primer Design (NCBI). Polymerase chain reaction (PCR) products were amplified in an MJ Research PTC-200 (Applied Biosystems; www.appliedbiosystems.com): 95° C for 3 min, 32 cycles of 95° C for 30 s, 60° C for 30 s, 72° C for 60 s, single cycle of 72° C for 7 min. 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).

Resultant sequences were translated and the Polyq domain was manually annotated in 10 fish from each population. Sequences with low quality scores in the domain were discarded and resequenced. The number of glutamines within the Polyq domain was counted and the positions of the arginine within the Polyq domain were recorded.

ANALYSES

Microsoft Excel (Microsoft; www.microsoft.com) was used for linear and quadratic regressions. For regressions of I_O or I_K on day length, linear regression was always significant ($P < 0{\cdot}01$); in no case did the addition of a quadratic term significantly reduce the total sum of squares. Linear regression was therefore used for all analyses. JMP 4 (Sall *et al*., 2005) was used for ANOVAs. In the latter case, latitude (AK = north *v*. OR = south) and day lengths were modelled 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 I_K depended on day length (two-way ANOVA, $F_{5,219} = 19{\cdot}4$, $P < 0{\cdot}001$) and did not differ between northern and southern males ($F_{1,219} = 2{\cdot}31$; $P > 0{\cdot}05$). There was no latitude by photoperiod interaction ($F_{5,219} = 0{\cdot}43$; $P > 0{\cdot}05$). The I_O depended upon day length ($F_{5,230} = 20{\cdot}26$, $P < 0{\cdot}001$) and was higher in southern than northern females ($F_{1,230} = 9{\cdot}28$, $P < 0{\cdot}05$) but there was no significant latitude by photoperiod interaction ($F_{5,230} = 0{\cdot}91$; $P > 0{\cdot}05$). 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). Sample sizes ranged from eight to 14 individuals per treatment (Table I).

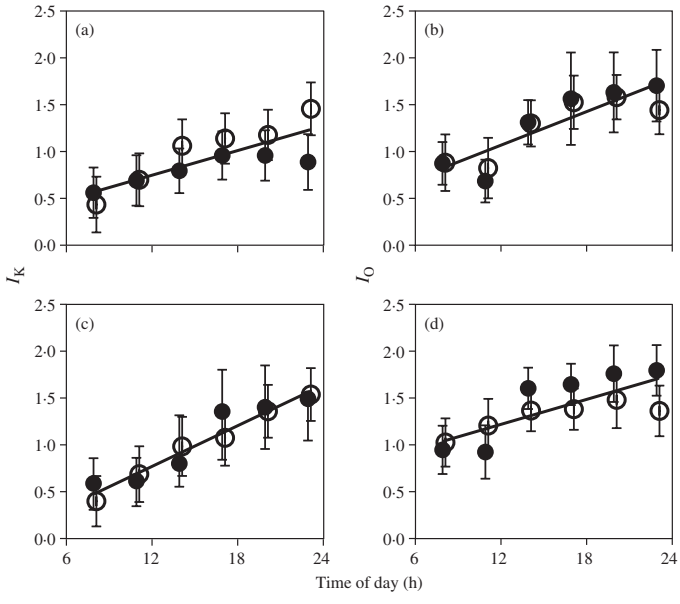

FIG. 1. Photoperiodic response of male and female *Gasterosteus aculeatus* in (a, b) Alaska (61·5° N) and (c, d) Oregon (43·5° N) in north-western North America. Male response is represented by (a, c) kidney:body mass ratio (I_K) and female response is represented by (b, d) the ovary:body mass ratio (I_O) [○, results from Yeates-Burghart *et al*. (2009); ●, results from the present study]. Values are mean ± 2 S.E. The curves were fitted by: (a) $y = 0{\cdot}0439x + 0{\cdot}2204$ ($r^2 = 0{\cdot}82$), (b) $y = 0{\cdot}0597x + 0{\cdot}3509$ ($r^2 = 0{\cdot}88$), (c) $y = 0{\cdot}0725x - 0{\cdot}0994$ ($r^2 = 0{\cdot}94$) and (d) $y = 0{\cdot}0444x + 0{\cdot}6875$ ($r^2 = 0{\cdot}79$).

THE POLYGLUTAMINE DOMAIN OF *CLOCK* VARIES ACROSS INDIVIDUAL *G. ACULEATUS* BUT SHOWS NO POPULATION STRUCTURING

The BLAST search and syntenic analysis found one *H. sapiens clock* orthologue in the *G. aculeatus* genome (Ensembl ID ENSGACG00000015939) (Fig. 2). *Gasterosteus aculeatus clock* gene contains 20 exons from 489 361 to 499 374 bp on

TABLE I. Photoperiodic response sample sizes of *Gasterosteus aculeatus*. Fish that died during the course of the experiment were not replaced. Only fish that survived until the end of the experiment were analysed. Photoperiod treatments are listed by the hours of light per day

			Photoperiod treatment					
Latitude	Population	Sex	8	11	14	17	20	23
North (Alaska)	Rabbit Slough	Male	12	11	13	9	10	7
		Female	11	14	11	7	9	8
	Boot Lake	Male	9	8	10	13	7	9
		Female	8	7	12	9	12	11
South (Oregon)	Cushman Slough	Male	9	10	8	9	11	10
		Female	11	9	13	12	8	10
	Eel Creek	Male	10	10	9	8	8	7
		Female	11	12	11	11	8	7

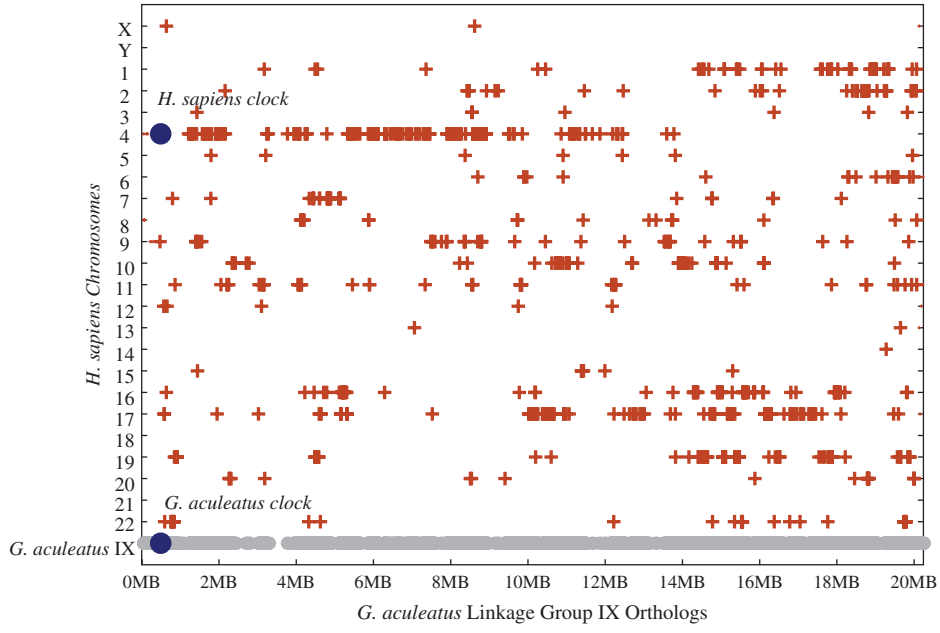

FIG. 2. Syntenic analysis of the *Gasterosteus aculeatus clock* gene: ○, genes found on *G. aculeatus* linkage group IX; ●, orthologues in the *Homo sapiens* genome; ●, *H. sapiens* (Ensembl ID: ENSG00000134852) and *G. aculeatus* (Ensembl ID: ENSGACG00000015939) *clock* homologues.

linkage group IX (Ensembl). Examination of the sequence shows that the *clock* gene contains a single Polyq domain located in exon 20.

Ten individuals each from eight total populations were analysed, for a total of 80 individuals. The Polyq domains [Fig. 3(a)] contained between 22 and 38 glutamine repeats and did not differ between latitudes (nested ANOVA, $F_{1,6} = 0{\cdot}74$; $P > 0{\cdot}05$) or among populations within latitudes ($F_{6,72} = 1{\cdot}162$, $P > 0{\cdot}05$). An arginine residue [Fig. 3(b)] occurred within each of the PolyQ domains between positions 2 and 26. Mean position of the arginine residue did not differ between latitudes ($F_{1,6} = 0{\cdot}533$, $P > 0{\cdot}05$) or among populations within latitudes ($F_{6,72} = 0{\cdot}907$; $P > 0{\cdot}05$). 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

GASTEROSTEUS ACULEATUS HAVE SIMILAR PHOTOPERIODIC RESPONSES AT NORTHERN AND SOUTHERN LATITUDES

Previously (Yeates-Burghart *et al.*, 2009), it was 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.

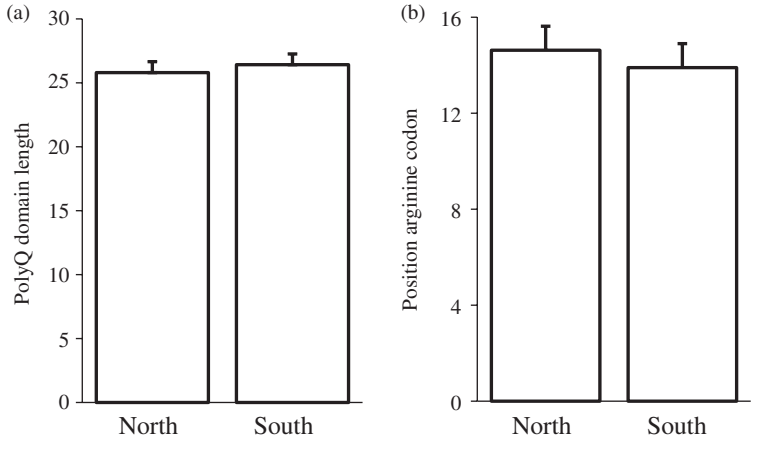

FIG. 3. Polyglutamine domain (PolyQ) in the *clock* gene in southern (Oregon) and northern (Alaska) populations of *Gastersteus aculeatus*. (a) Domain length in number of glutamine repeats and (b) position of the arginine codon within the polyglutamine domain. Values are means ± 2 S.E.

After using replicate populations within Oregon and Alaska (Fig. 1), it is now clear that *G. aculeatus* are photoperiodic at both latitudes and do not differ significantly 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, 2007*a*). 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 fish 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. It is therefore concluded that genetically determined photoperiodic responses do not differ between Oregon and Alaskan populations separated by *c*. 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 *G. aculeatus* from the field, gonadal maturation is accelerated by both increasing day length and warmer temperatures (Borg, 1982, 1987; Andersson *et al*., 1992; Hellqvist *et al*., 2004) and cold-acclimated fishes have greater facility in adjusting to warm temperatures with increasing day length (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. Only one of these variables, photoperiod, was manipulated while keeping the others constant. Although climate is 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. 4). It is therefore proposed that the accelerating effects of longer day length and increasing temperatures in the more northern environment may compensate for the lower average temperature in Alaska than Oregon. Hence, northern fish would be reproductively

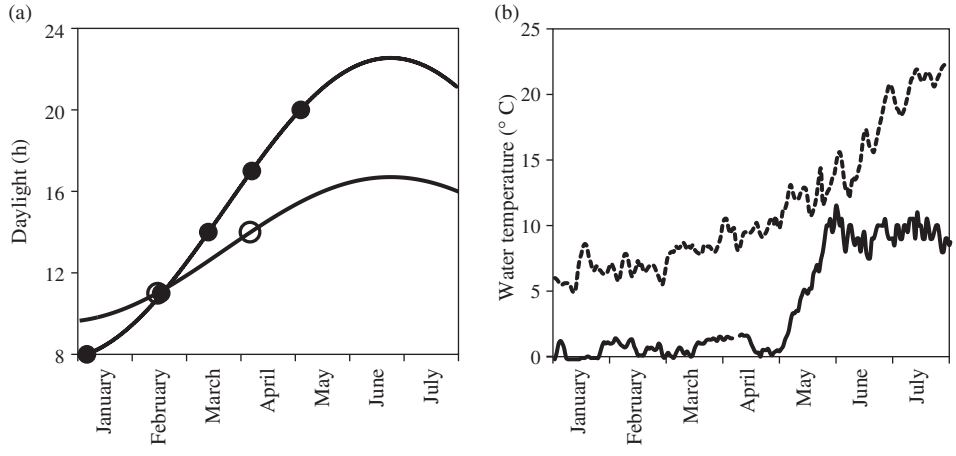

FIG. 4. (a) Day length at which photoperiodic responses were determined during the winter and spring in Oregon (○) and Alaska (●). Note that the Oregon populations do not experience day lengths as short as 8 h or as long as 17 h light per day (Oregon maximum 16·69 h; Alaska maximum 22·54 h). Day lengths 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 (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/).

prepared to exploit the shorter northern growing season during the brief period when summer waters are warmest. Finally, rearing animals from different localities in a common environment before using them to infer an underlying genetic basis for differences in functional phenotypes is encouraged.

ABSENCE OF *CLOCK* POLYGLUTAMINE DOMAIN LENGTH (PolyQ)

In *D. melanogaster*, the Clock protein heterodimerizes with the Cycle protein to promote the transcription of the genes *period* and *timeless*. Heterodimerization of Period and Timeless proteins, and their migration into the nucleus, leads to the inhibition of their own transcription by Clock and Cycle (Darlington *et al*., 1998). The interest in the *clock* gene 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 *M. musculus*, 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 A to T 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.

The present findings of a lack of correlation between PolyQ domain and aspects of photoperiodic response are not unique. No association was found between PolyQ and latitude in western North American populations of *G. aculeatus* (Fig. 3). 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. In fact, a brief re-examination of several other studies relating PolyQ length to photoperiodic response exhibit a general lack of correlation, and puts the present results into context.

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: '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 Wood, U.K.; 51° 47′ N), Liedvogel *et al*. (2009) sought to correlate PolyQ with egg-laying date, hatch date and incubation duration of 950 blue tits *C. caeruleus* over a 2 year period. No 'significant overall year × genotype interaction was found for any of the timing traits in focus (all results with $P > 0{\cdot}213$).' When the authors continued their search for significance within the observed non-significant data, however, they found that by considering the second year in isolation, they could find a significant correlation between PolyQ and laying date and hatch date (both $P < 0.05$ 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 teleosts, the molecular basis of daily circadian rhythmicity has been studied in the zebrafish *Danio rerio* (Hamilton 1822). In *D. rerio*, the core loop of the circadian clock involves three paralogues of *clock* whose proteins form heterodimers with three paralogues of *bmal* that drive rhythmic expression of three paralogues 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 *D. rerio*.

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 fresh water (Bromage *et al*., 2001). Two paralogues of *clock*

have been identified in Chinook salmon *Oncorhyncus tshawytscha* (Walbaum 1792), *otsclock1a* and *otsclock1b* that arose from a tetraploidation event during divergence of salmonids from other teleosts (O'Malley & Banks, 2008*b*). No functional connection has yet been made between either of these paralogues 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* paralogue among four species in the genus *Oncorhyncus*. In the *otsclock1b* paralogue, polyglutamine length is polymorphic within and among populations of *O. tshawytscha*, chum salmon *Oncorhynchus keta* (Walbaum 1792), coho salmon *Oncorhynchus kisutch* (Walbaum 1792) and pink salmon *Oncorhynchus gorbuscha* (Walbaum 1792) (O'Malley & Banks, 2008*a*; O'Malley *et al*., 2010). Mean length of the glutamine domain (PolyQ) is not significantly correlated with latitude among 19 populations of *O. kisutch* or 16 populations of *O. gorbuscha*, but is correlated with latitude in *O. tshawytscha* and *O. keta* (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 *O. tshawytscha*, *O. kisutch*, *O. keta* and *O. gorbuscha*. They found that the ability of the univariate regression tree 'to assign populations to groups correctly on the basis of these factors' (day length and migration index) was not significant (O'Malley *et al*., 2010) and significant ($P < 0{\cdot}05$) only in *O. gorbuscha* where length of the most common allele varied with photoperiod on the date of peak spawning 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.

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