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Phenotypic Variation in Growth and Gene Expression Under Different Photoperiods in Allopatric Populations of the Copepod *Tigriopus californicus*

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Abstract. Daylength is a major environmental condition that varies seasonally and predictably along a latitudinal cline, where higher latitudes exhibit greater ranges in total daylengths. Generally, the circadian clock acts as a network of genes whose expression dynamics are known to control daily rhythms in response to daylength, and it enables the control of many physiological processes such as growth and development. While well studied in many model animals, the influence of daylength variation on phenotypic evolution is poorly examined in marine species. In this study we demonstrate that two allopatric populations of the intertidal crustacean *Tigriopus californicus* exhibit plastic and divergent phenotypic responses to changes in daylength. Using common-garden experiments, we discovered that shorter daylengths promoted decreased adult body size and faster growth rates in the two divergent populations, suggesting a plastic response to shortened days. In addition, the higher-latitude population exhibited a faster growth rate at any daylength condition, indicating a fixed response, possibly as a result of adaptation to respective natural light regimes. Gene expression profiles of several circadian clock genes, monitored throughout the day by quantitative polymerase chain reaction, revealed that the key core clock genes reach higher daily transcription maxima in the southern population compared to the northern population, pointing to divergent strategies used to respond to changes in daylength. Many modifier genes to the circadian clock showed similar plastic responses to the different daylengths, supporting the existence of at least some conserved gene expression across both populations. Ultimately, our results suggest that photoperiod and daylength exert a potent selective pressure underexplored in marine systems and warranting further future research.

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Abbreviations: ASW, artificial seawater; BR, Bird Rock, California; HSD, honestly significant difference; LD, long day (12h:12h light:dark cycle); qPCR, quantitative polymerase chain reaction; SD, short day (8h:16h light:dark cycle); SH, Strawberry Hill, Oregon; ZT, zeitgeber time.

Online enhancement: data file.

Introduction

Many aspects of organismal fitness rely on adapting to environmental conditions that vary daily. Factors that fluctuate daily, however, can widely differ, both spatially and temporally (such as the seasonal variation in temperature), and can impose significant effects on organismal fitness. Therefore, organisms must evolve coordinated cellular pathways to deal with multiple temporal scales of abiotic environmental changes (Emerson *et al.*, 2008; Goto, 2013). In natural populations, temporal and geographic variations in temperature have received the most attention, since these factors are thought to be strong determinants of species distributions.

Much of an organism's daily physiological processes, nevertheless, are governed by intrinsic rhythms that revolve around daylight. For example, the length and peak of daily activity (Roberts, 1956; Konopka and Benzer, 1971), developmental cues like pupal emergence (Pittendrigh, 1960; Allada and Chung, 2010), longevity (Hurd and Ralph, 1998), fecundity and gametic quality (Beaver *et al.*, 2002), and growth (Dodd *et al.*, 2005) have all been tied to organisms maintaining daily rhythms. Failing to acclimate or adapt to the patterns of daylength, or photoperiod, hence affects individual fitness at least in the short term.

The circadian clock comprises a core set of genes that are involved in keeping organisms in sync with their daily light

rhythms and that work as a network operated by a series of transcriptional-translational positive and negative feedback loops. Post-transcriptional and post-translational regulatory mechanisms help maintain rhythms by imposing temporal delays between transcription activation of core clock genes (Allada and Chung, 2010). Different model systems may use slightly different nomenclature, but the main components of the clock remain the same. Based on the work of Nesbit and Christie (2014), *Tigriopus* contains what is generalized as an ancestral arthropod clock. This type of clock contains a light-sensitive gene, such as *cryptochrome*, that acts to entrain the organism when light is present at dawn (Cashmore *et al.*, 1999). Another major component is a set of core genes that operate on a roughly 24-hour cycle, setting a pacemaker for the organism. Within *Drosophila*, arguably the most extensively studied arthropod clock, these genes are *clock*, *cycle*, *period*, and *timeless*. The protein products from these genes form the CLK-CYC and PER-TIM heterodimers that promote or inhibit transcription of each other to maintain the daily rhythm (Allada and Chung, 2010). The more ancestral arthropod clocks differ from this, as they contain a second copy of the *cryptochrome* gene referred to as *cryptochrome-2*, which is photo-insensitive. In these systems, the photo-sensitive *cryptochrome* is designated *cryptochrome-1* (Yuan *et al.*, 2007; Christie *et al.*, 2013). The *cryptochrome-2* gene has been lost in *Drosophila* clocks but has been found to be present in multiple marine organisms, including *Tigriopus* (Nesbit and Christie, 2014). *Timeless* has also evolved a paralogous copy, *timeout*, which bears higher sequence similarity with the canonical mammalian *timeless* gene and has been implicated to have numerous functions in other organisms, ranging from embryonic development and DNA replication to its role in the circadian clock (Benna *et al.*, 2000, 2010; Gotter *et al.*, 2007). The presence of *timeless* and/or *timeout* varies widely by species (Zhan *et al.*, 2011; Gu *et al.*, 2015).

The remainder of the clock includes numerous modifier genes that aid the core clock in maintaining its pace (Collins and Blau, 2007; Allada and Chung, 2010; Tataroglu and Emery, 2014). The clock must be able to accommodate for the seasonal changes in daylength that occur over the course of a year. A separate photoperiodic seasonal timer, independent of the circadian clock, is responsible for the seasonal switch in physiological cues (Bradshaw and Holzapfel, 2010). While there is presumably a link between these two timers, their complex interactions are not fully understood (Bradshaw and Holzapfel, 2010). Nevertheless, since daylength and the timing of sunrises vary in a predictable manner across latitudes (Hut *et al.*, 2013), the circadian clock must still entrain appropriately under the natural variations in daylength in order to maintain fitness of the organism. Mutations to genes within the clock may disrupt it, however, and may have a cascading effect downstream in the organism's biology. In some cases, mutations in circadian genes have been found to cause metabolic abnormalities (Tao *et al.*, 2017), irregularities to the phototaxic response (Mazzoni *et al.*, 2005), and general loss of rhythmic behaviors (Sehgal *et al.*, 1994).

Recent efforts have focused not only on light-driven rhythms but also on tidal-driven rhythms (referred to as circatidal rhythms). These rhythms persist at about 12-hour periods and are especially relevant when studying intertidal systems, where rhythmic inundation of tidal water brings changes in temperature, salinity, and other factors (Naylor, 2010). Many organisms that live within tidal zones will often exhibit both circadian and circatidal rhythms to varying degrees as a result (Bulla *et al.*, 2017). Studies aimed at researching transcriptional rhythms within circadian clock genes have shown that circadian and circatidal rhythms are largely regulated by distinct sets of genes but may share some regulators (Zhang *et al.*, 2013). This interaction between distinct rhythmically driven processes can become more complex when comparing locations with different tidal magnitudes or those that are also affected by lunar cycles.

The patterns of circadian clock gene expression in relation to light changes have been studied extensively in the field of chronobiology in many terrestrial systems (*Mus musculus*, *Drosophila melanogaster*, and *Arabidopsis*, among others), with increasing study in marine systems, including crustaceans (Strauss and Dircksen, 2010). The recent studies in marine species focus on the characterization, molecular basis, or identification of the genes associated with daily (Tilden *et al.*, 2011; Christie *et al.*, 2013; Nesbit and Christie, 2014; O'Grady *et al.*, 2016; Perrigault and Tran, 2017; Tarrant *et al.*, 2019), lunar (Zantke *et al.*, 2013; Schenk *et al.*, 2019), and tidal (Zhang *et al.*, 2013; Schnytzer *et al.*, 2018) rhythms. Notably, many of these studies examined rhythms and changes in phenotypes in one species without testing how organismal phenotypes of different populations may respond to changes in these rhythms.

Tigriopus californicus (Baker, 1912) provides an excellent system to study the evolutionary consequences of geographic isolation of populations along a wide latitudinal range. Isolated populations of this harpacticoid copepod live in supralittoral tidepools that experience little inundation of water to aid in dispersal. Despite the lack of dispersal for established populations, the species can be found throughout the west coast of North America, ranging from Baja, Mexico, to Alaska (Edmands, 2001; Willett, 2010). This isolation has allowed each population to respond to its own local environment independent of other populations, and multiple studies have documented strong levels of local adaptation to abiotic factors such as temperature and salinity (Willett, 2010; Kelly *et al.*, 2012; Leong *et al.*, 2017). As a consequence of isolation, a high degree of genetic and physiological differentiation has evolved among populations. *Tigriopus californicus* genes associated with the circadian clock have been identified from transcriptomic data, including the core genes, a potentially light-sensitive *cryptochrome-1*, as well as many modifier genes, with the exception of the gene *par domain protein 1* (Nesbit and Christie, 2014).

We aimed to quantify the levels of phenotypic plasticity and divergence associated with variation in daylength in two copepod populations isolated at different latitudes. We tested the hypothesis that, as documented in previous literature, growth rates would slow under shortened photoperiods (Pistole and Cranford, 1982; Kukita *et al.*, 2015) while body size would decrease. In this study, we show that developmental rate, body size, and core circadian gene expression respond to experimental changes in daylength. We also detected fixed differences between the populations in these phenotypes, thus providing a potential connection between differences in daylength and phenotypes as they evolve at different latitudes.

Materials and Methods

Copepod culturing

Populations of *Tigriopus californicus* (Baker, 1912) collected from supralittoral tidepools in Bird Rock, California (BR; 32.8143°, −117.2734°), and Strawberry Hill, Oregon (SH; 44.25425°, −124.11287°), were maintained en masse (~200–500 individuals) in 400-mL beakers with 35-ppt artificial seawater (ASW; Instant Ocean, Spectrum Brands, Blacksburg, VA) in incubators (20 °C, 12h:12h light:dark cycle). Populations were fed a mixture of dry fish food (ground Spirulina and TetraMin flakes) and live microalgae (*Isochrysis galbana* and *Nannochloropsis aculata*) for a minimum of two months (about two generations) before experiments were performed. Once a month, water and food were changed, and beakers were mixed to maintain outbreeding.

Photoperiod experimental setup

Gravid females from both populations were transferred to new 400-mL beakers containing fresh ASW and were put in an incubator set to 20 °C with an 8h:16h light:dark cycle (herein referred to as SD, for short day), with a light phase relative to the zeitgeber time (ZT, the environmental signal perceived when lights are on) of ZT 0 (lights first turn on) to ZT 8 (lights turn off) and a dark phase from ZT 8 to ZT 24. An equal number of gravid females were kept in the 12h:12h light:dark cycle (herein referred to as LD, for long day), with a light phase from ZT 0 to ZT 12 and a dark phase from ZT 12 to ZT 24. A whole generation was allowed to reproduce and turn over in these beakers before sampling, in order to reduce maternal effects from the stock culture conditions. After a full generation was born, raised, and mated in their respective conditions, 48 gravid females from each population and light condition had their egg sacs removed and individually placed in separate wells of 6-well plates containing 10 mL of ASW (one egg sac per well). All eggs hatched within a few hours of picking, and their hatch date was noted. To reduce the effect of diet differences, all samples were fed at the same schedule, starting with ~1 mg of the dry food being administered once a week and then transitioning to receiving an additional 3 drops of an equal mix of the live microalgae every 4 days from week 2 onward.

Growth rate and size

Plates were monitored daily, and growth rate for each clutch was scored as the number of days since hatched until the first nauplius larva metamorphosed into the copepodid stage. After confirmation of non-normally distributed data, a nonparametric Kruskal-Wallis test was performed in R version 3.5 (R Core Team, 2018) to test for variation between the four groups tested (two populations under two light regimes). Significant Kruskal-Wallis tests (at $\alpha = 0.05$) were followed by Dunn's *post hoc* test to examine for specific differences between the groups.

After all clutches reached age 25 days, body size was measured using adult male copepods. One individual from each clutch was chosen haphazardly to be measured. Individuals were placed on grid paper (1-mm^2 grids), and photos were taken using a Zeiss Axiocam ERc5s camera (Oberkochen, Germany). Individual pictures were inputted into ImageJ software (Schneider *et al.*, 2012) for body measurement, using the paper grids for scale. Body size was measured as the area of the cephalothorax, where the cephalothorax was treated as a half oval with a length (L) and width (W) as calculated below:

$$Area\ (\mathrm{mm}^2) = \frac{1}{2} * \left(\pi * L * \frac{1}{2} W\right).$$

Variation in body size was assessed with an ANOVA after normality was confirmed and then followed with a Tukey's honestly significant difference (HSD) *post hoc* test, to test for specific variation between groups ($\alpha = 0.05$).

Tissue sampling and RNA extraction

In order to examine levels of plasticity (between light regimes) and divergence (between populations) in the transcription of circadian clock genes, we sampled copepods at multiple ZT points. Before the experiments began, ~400 equal-age adult male copepods cultured from each light regime were transferred to a beaker and acclimated for 2 days. About 18 hours before the first collecting time point, 20–24 pools of 20 individuals were distributed into 2-mL screw-cap tubes containing 1.5 mL of ASW, with no food; and they were maintained at the respective experimental condition. Each pool was treated as a replicate. At each time point, 3–4 replicates were removed from the incubator and immediately processed, which involved removal of ASW with a pipette and then tissue homogenization in 250 μL of TRI Reagent (Molecular Research Center, Cincinnati, OH) with 1-mm zirconia silica beads. Samples were stored at −80 °C until RNA extraction. Tissues sampled in the light phase were collected under ambient lighting, while

dark phase samples were collected under a dim red-light headlamp. The LD and SD experiments were performed on the same day, using different incubator units of the same model. Samples from the LD treatment were sampled at five time points in total: three in the light phase (ZT 1, ZT 5, ZT 11) and two in the dark phase (ZT 13 and ZT 23). Samples in the SD treatment were sampled at six time points: three in the light phase (ZT 1, ZT 4, ZT 7) and three in the dark phase (ZT 9, ZT 13, ZT 23). Total RNA was isolated from each replicate, following the Direct-zol RNA MicroPrep (Zymo, Irvine, CA); and samples were then DNase treated with Turbo DNase-free kit (Life Technologies, Carlsbad, CA). RNA quality and quantity were assessed by nano-spectrophotometry, using 3 μL of RNA from each sample.

Gene sequence analysis

Eight genes associated with the circadian clock were identified from the *T. californicus* genome, based on their initial annotation (Barreto *et al.*, 2018). The genes *period* (National Center for Biotechnology Information [NCBI] accession no. TRY80873), *cryptochrome-2* (TRY67732), and *timeless* (TRY67893) were chosen to represent the canonical core clock, where these genes are rhythmically transcribed on daily cycles (Sehgal *et al.*, 1995; Allada and Chung, 2010). *Timeout* (TRY62970) was also selected in order to compare which gene within the *timeless* family functioned like the canonical circadian clock gene. The gene *cryptochrome-1* (TRY63317) was chosen because it acts as the canonical input to the circadian clock through blue-light entrainment (Cashmore *et al.*, 1999). Last, the three genes *shaggy* (TRY63873), *doubletime* (TRY64180), and *jetlag* (TRY81040) were chosen because they act as modifiers to the circadian clock. The genes *shaggy* and *doubletime* modify the clock through the phosphorylation of *timeless* and *period*, respectively (Kloss *et al.*, 1998; Martinek *et al.*, 2001; Allada and Chung, 2010), while *jetlag* promotes the light-induced degradation of the *timeless* protein TIM (Koh *et al.*, 2006). The gene identities were validated by additional BLASTX analyses against the NCBI "nr" database as well as a manually curated database compiled from Nesbit and Christie (2014) that first described the repertoire of circadian genes found in *T. californicus*.

While the sequences for the BR population were obtained from its genome assembly (Barreto *et al.*, 2018), the SH orthologous sequences were identified *via* BLASTN searches of the SH transcriptome assembly generated by Graham and Barreto (2019) (NCBI Transcriptome Shotgun Assembly accession no. GHUE00000000). Sequences for both BR and SH can be found in data supplement S1, available online. Their coding sequences were aligned in Geneious version 10 (Biomatters, Auckland, New Zealand), and MEGA7 (Kumar *et al.*, 2016) was used to calculate total amino acid divergence as well as the ratio of the rates of nonsynonymous and synonymous substitutions (d_N/d_S).

Primer design and quantitative polymerase chain reaction analyses

Alignments of SH and BR orthologs were also used to design quantitative polymerase chain reaction (qPCR) primers along fully conserved regions, so that the same primers could be used in both population templates. In addition to the eight target genes, we designed primers for two reference genes previously shown not to vary in expression across multiple conditions (Ribosomal Protein P1, *RPLP1*, and serine/threonine-protein phosphatase 2A, *Pp2A*). Primers were verified to have at minimum 85% efficiency by assessing the slope of standard curves from a series of dilutions from cDNA samples (Table A1). First-strand cDNA was synthesized from 2 μL (100 ng) of RNA, using the high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA); and these were then diluted 10-fold to 1 ng μL^{-1} (RNA equivalent). Quantitative PCR reactions were performed in 15 μL, using 1X iTaq SYBR Green Supermix (BioRad, Hercules, CA), 0.5 μmol L^{-1} of each primer, and 3 μL of cDNA. Reactions were carried out in a BioRad CFX96 Real-Time System, using a two-step amplification phase (95 °C/2 min, followed by 40 cycles of 95 °C/10 s and 58°C/30 s), followed by a melting curve analysis to confirm the presence of single amplicons.

For each sample, the geometric mean of the two reference genes was used for normalization. Then the normalized expression for each target gene was calculated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$. All genes were compared between populations at the same time points, while the response of each population was compared between light treatments as the combined expression over the 24-hour period. ANOVA tests were performed on all gene sets, and Tukey's HSD was used as a *post hoc* test of variance.

Results

Effect of daylength on size and development

Copepods raised in the SD condition grew to significantly smaller size than those in the LD condition, as measured by adult cephalothorax area, with an average decrease of 0.018 mm^2 in both populations (Fig. 1A; BR: $P = 6.8 \times 10^{-6}$, SH: $P = 1.00 \times 10^{-7}$). No significant differences were detected when comparing populations exposed to the same daylengths (SD, mean ± SD: SH = 0.096 ± 0.022 mm^2, BR = 0.093 ± 0.016 mm^2, $P = 0.721$; LD: SH = 0.116 ± 0.020 mm^2, BR = 0.113 ± 0.018 mm^2, $P = 0.798$; Fig. 1A).

Nauplii from the SH population developed faster into copepodids by an average of ~11 hours when raised in the SD condition (average, 6.13 days) compared to those raised in LD (average, 6.58 days) (Fig. 1B, $P = 0.0107$). In the SD treatment, SH showed little variation in developmental rate, with all but two clutches reaching a copepodid stage by day 6. In addition, BR saw a significant decrease in time to metamorphosis, with

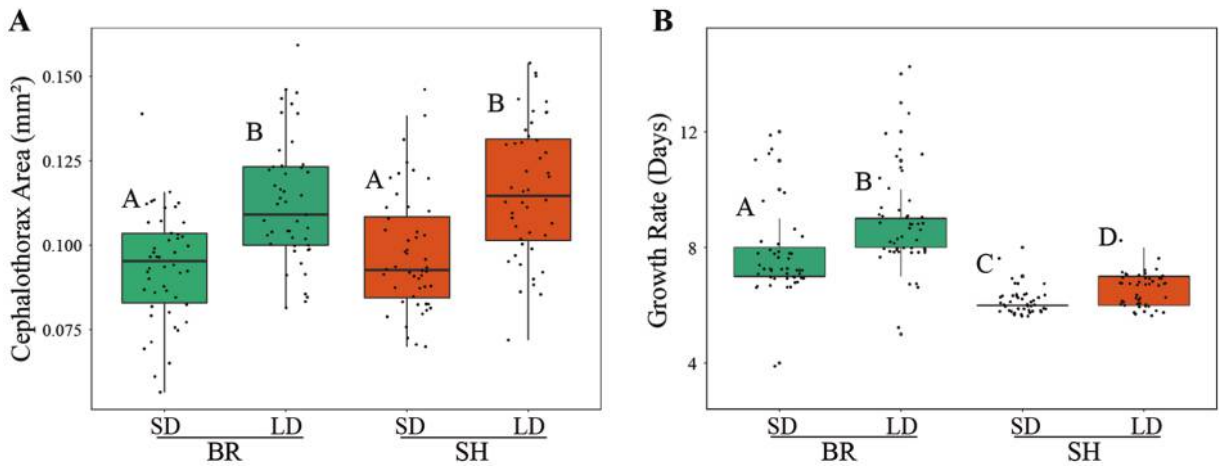

Figure 1. Growth traits of two populations of *Tigriopus californicus*—Bird Rock, California (BR), and Strawberry Hill, Oregon (SH)—raised under two photoperiods. SD (short day) treatment had the 8h:16h light:dark cycle, while LD (long day) had the 12h:12h light:dark cycle. Boxplots represent median value with 25th and 75th percentiles for each treatment. Individual jitter points represent measurements for individuals within each treatment, with *x*- and *y*-dimension noise to allow for easier visualization of data clusters. (A) Adult body size (cephalothorax area, mm^2) reached in each photoperiod. (B) Growth rate (days from hatch to copepodid metamorphosis) under each photoperiod. Uppercase letters above boxplots report the results of statistical tests comparing the groups, with different letters denoting significant difference ($\alpha = 0.05$). For body size, an ANOVA followed by a Tukey's honest significant difference (HSD) *post hoc* test was performed; for growth rate, a Kruskal-Wallis nonparametric test followed by Dunn's *post hoc* test was applied.

the SD population (average, 8.06 days) achieving metamorphosis ~20 hours sooner than LD counterparts (average, 8.92 days) (Fig. 1B, $P = 0.00135$). The two populations differed significantly in their development rate under the same daylength conditions, with SH reaching the copepodid stage first in both SD and LD treatments (Fig. 1B; LD: $P = 5.57 \times 10^{-4}$, SD: $P = 1.21 \times 10^{-11}$).

Effect of daylength on circadian gene expression

From our qPCR data, we examined the expression of 4 potential core clock genes, *cryptochrome-2*, *period*, and two genes of the *timeless* family (*timeless* and *timeout*), across 5–6 time points over the span of a 24-hour period under both light conditions. In *cryptochrome-2*, there were no significant differences in expression under the LD condition between each population, while the SD condition showed significant differences in three time points measured: ZT 4, ZT 7, and ZT 12 (Fig. 2; ZT 1: $P = 0.0784$, ZT 4: $P = 0.00133$, ZT 7: $P = 0.0151$, ZT 9: $P = 0.076$, ZT 12: $P = 0.044$, ZT 23: $P = 0.248$). For the gene *timeless*, the BR population expressed it to a higher level than SH at the maxima of ZT 13 in the LD regime (Fig. 2; $P = 0.012$), while there were no significant differences in the SD regime. The gene *timeout* showed no significant differences in expression under the LD regime, but the SH population showed consistently greater expression in the SD regime, with significant differences in five of the six time points (Fig. 2; ZT 1: $P = 0.0085$, ZT 4: $P = 7.11 \times 10^{-4}$, ZT 7: $P = 6.88 \times 10^{-5}$, ZT 9: $P = 0.025$, ZT 12: $P = 0.248$, ZT 23: $P = 5.63 \times 10^{-3}$). Last, *period* did not show any significantly different expression under the LD regime but showed greater expression in the BR population at time points ZT 9 ($P = 0.0087$) and ZT 12 ($P = 0.0207$) relative to SH in the SD regime (Fig. 2).

We also examined the relative expression of genes that act as the inputs or modifiers to the circadian clock. With the exception of *shaggy*, these genes showed no significant differences between populations within each light regime (Fig. 3). The gene *shaggy* was differently expressed in the SD regime at time points ZT 7 and ZT 23 (Fig. 3; $P = 0.0067$ and $P = 0.003$, respectively), with SH showing threefold higher expression than BR at those times.

We also compared overall expression (*i.e.*, averaged across the 24-hour period) between light regimes for each population. In three of the genes assayed, the SD light regime resulted in higher overall expression than LD in both populations (Fig. A1): *cryptochrome-1* (BR: $P = 4.18 \times 10^{-6}$, SH: $P = 4.06 \times 10^{-10}$), *doubletime* (BR: $P = 3.08 \times 10^{-10}$, SH: $P = 3.09 \times 10^{-10}$), *shaggy* (BR: $P = 7.15 \times 10^{-3}$, SH: $P = 3.08 \times 10^{-10}$). For *cryptochrome-2*, there were no significant differences between photoperiods for either population (SH: $P = 0.994$, BR: $P = 0.07$). In SH, the gene *jetlag* showed significantly lower expression in the LD treatment compared to the SD treatment ($P = 0.031$), while BR showed no differences across treatments ($P = 0.400$). In the gene *timeout*, the SH population showed lower expression

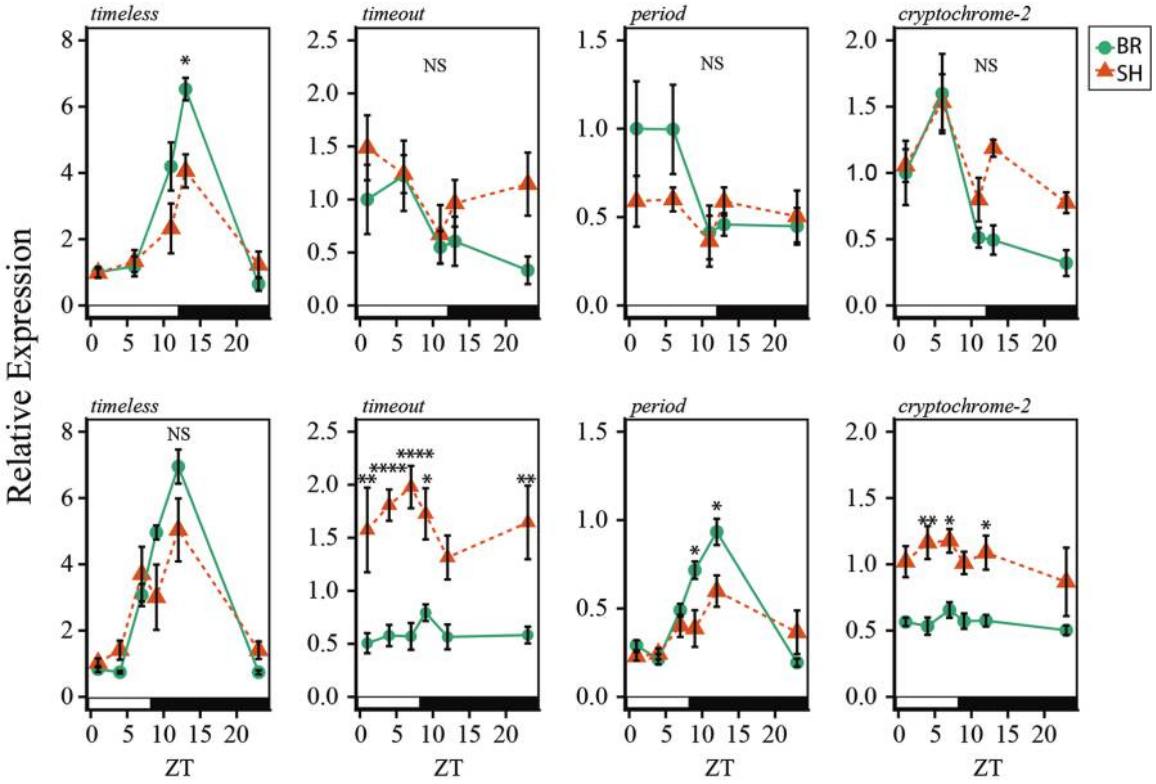

Figure 2. Relative gene expression for putatively canonical core clock genes in *Tigriopus californicus* quantified over a 24-hour period and 2 light regimes. Rectangle along the *x*-axis denotes the light phase (white) and the dark phase (black) within each treatment. Top panels show gene expression during the long day (LD) treatment (12h:12h light:dark cycle), and bottom panels show expression of the same genes during the short day (SD) treatment (8h:16h light:dark cycle). Points plotted as mean ± SEM ($n \geq 3$). Expression of each point is relative to the expression of the Bird Rock, California (BR), population at ZT 1 (zeitgeber time 1) in the LD treatment (*i.e.*, the relative expression of BR in the LD treatment at ZT 1 is set to 1). Results of Tukey's honest significant difference (HSD) test: NS = not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. SH, Strawberry Hill, Oregon.

in the LD treatment compared to SD ($P = 0.00132$), while BR did not ($P = 0.598$). *Period* also showed a significant difference in overall expression in the BR population when comparing the photoperiods, where the LD population had greater expression ($P = 0.0391$), while there was no significance within SH ($P = 0.281$). There were no differences noted in *timeless* when expression was compared between photoperiods within SH or BR when averaged over 24 hours (SH: $P = 0.684$, BR: $P = 0.999$).

Comparison of gene coding sequences

With the exception of *doubletime*, the predicted protein sequence of all genes showed multiple amino acid substitutions when comparing the genes of the 2 transcriptomes, with *timeout* having as many as 21 substitutions (Table 1). Proportionally, the gene *timeout* had the highest number of substitutions relative to length, with 0.65% of the total sites having a substitution. While d_N/d_S ratios are consistent with purifying selection (*i.e.*, $\ll 1$), the genes showed variation in relative functional evolution, with the highest being *cryptochrome-1* with $d_N/d_S = 0.141$.

Discussion

We tested the hypothesis that growth rate would slow as photoperiod was decreased. Our common-garden experiments revealed that populations of *Tigriopus californicus* showed a combination of fixed (between-population) and plastic (within-population) responses to perceived daylength associated with changes in growth rate. Our results showed that growth rate quickens under decreased perceived daylength (Fig. 1B), in contrast to various studies that have found shorter daylength corresponding to slower growth rates (Pistole and Cranford, 1982; Kukita *et al.*, 2015). Certain photoperiod-induced changes can increase metabolic rates and speed up developmental times in systems such as *Drosophila*, where short-day flies had higher metabolic rates than their longer-day counterparts (Lanciani *et al.*, 1990). Nylin and Gotthard (1998) proposed that potentially shortened reproductive

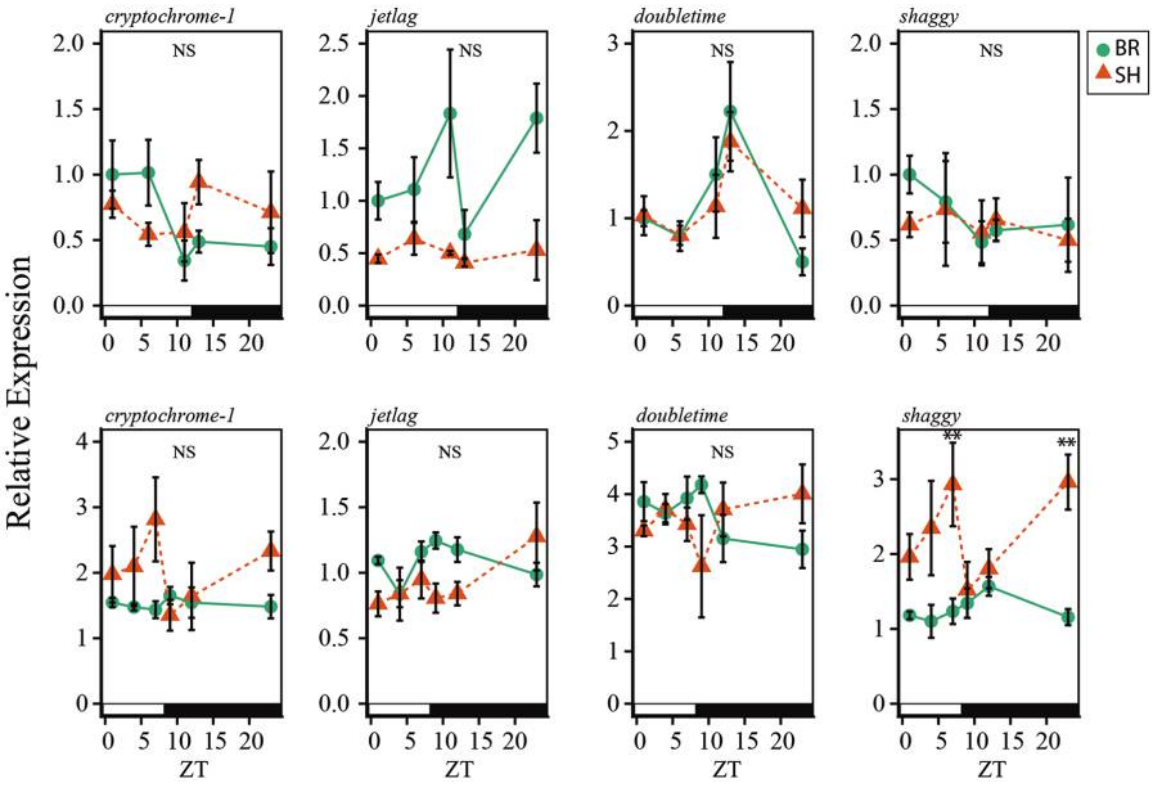

Figure 3. Relative gene expression of accessory circadian clock genes in *Tigriopus californicus* over a 24-hour period and 2 light regimes. Rectangle along the *x*-axis denotes the light phase (white) and the dark phase (black) within each treatment. Top panels show gene expression during the long day (LD) treatment (12h:12h light:dark cycle), and bottom panels show expression of the same genes during the short day (SD) treatment (8h:16h light:dark cycle). Points plotted as mean ± SEM ($n \geq 3$). Expression of each point is relative to the expression of the Bird Rock, California (BR), population at ZT 1 (zeitgeber time 1) in the LD treatment (*i.e.*, the relative expression of BR in the LD treatment at ZT 1 is set to 1). Results of Tukey's honest significant difference (HSD) test: NS = not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. SH, Strawberry Hill, Oregon.

periods are due to the onset of a shorter day or colder temperature, consistent with other studies (Lee *et al.*, 2010). This may explain why the northern *T. californicus* population showed an overall faster growth rate compared to the southern population.

While growth rate varied both between and within populations, we found evidence for body size variation only when comparing different daylengths within populations (Fig. 1A). Overall body size significantly decreased with

Table 1

Genetic divergence between Bird Rock and Strawberry Hill populations of Tigriopus californicus *across eight circadian clock genes*

Gene	No. of amino acid changes	Alignment length	d_N	d_S	d_N/d_S
cryptochrome-1	8	1686	0.008	0.0566	0.141
cryptochrome-2	7	2208	0.0041	0.0722	0.056
timeless	11	3516	0.0046	0.0503	0.091
timeout	21	3246	0.0092	0.0834	0.110
shaggy	2	1335	0.0019	0.0484	0.039
period	4	4380	0.0015	0.0549	0.027
jetlag	3	1002	0.0045	0.0535	0.084
doubletime	0	1101	0	0.0216	0

Data are based on the alignment of transcriptomes from both populations. Paralogous copies are denoted with 1 and 2 for their variant. d_N/d_S calculations (rates of nonsynonymous and synonymous substitutions, respectively) followed the model of Yang and Nielsen (2000).

a shorter daylength. Based on previous work, we expected that there would possibly be a trade-off of faster growth rates, leading to a smaller body size (Blanckenhorn, 2000), which was consistent with the comparison of populations at different daylengths; however, this does not explain how the two populations reach the same body size while varying in growth rates. The amount of food present in each experiment was not limiting, nor were there any significant differences in clutch size, consistent with previous literature (Vittor, 1971), thus eliminating competition as a source of growth variation within clutches. Thus, we expected that the faster-growing SH population would be smaller, on average, than the BR population because of its accelerated growth rate. We hypothesize that divergence in growth rates between populations from different latitudes has evolved trade-offs with other traits in order to accommodate the changes in energy expenditure used to grow faster.

We also demonstrated that the two populations regulate gene expression differently across their perceived daytime, with significant differences in the amplitude of gene expression in *timeless* and *period* (Fig. 2). The overexpression of *timeless* and *period* has been linked to extend lifespans through delayed aging in *Drosophila* (Katewa *et al.*, 2016; Hunt *et al.*, 2019). In particular, Katewa *et al.* (2016) found that overexpression in *timeless* and *period* altered fat metabolism and extended lifespan in *Drosophila* populations. This may aid in explaining growth rate differences observed in our findings, as overexpression was seen in the slower-developing BR population, where a trade-off between growth rate and lifespan may exist. The *timeout* variant showed a strikingly different pattern from *timeless*, suggesting that only *timeless* functions as the canonical core clock gene in this system, since it has consistent expression across each population and treatment. The expression of *timeout* was consistently and more highly expressed in SH than BR along the entire day. This may potentially still represent divergent strategies, as *timeout* has numerous functions ranging from DNA repair and development to, possibly, photoreception that may require different responses in these populations under different photoperiods (Benna *et al.*, 2000, 2010). A northern population may spend more energy in a faster growth rate to reach reproductive age sooner but may die sooner as a result. Currently, longevity differences among populations are unknown, and lifespan was examined in only one population (Foley *et al.*, 2019).

In the *cryptochrome* family, *cryptochrome-2* exhibited an increase in expression under the SD treatment for the SH population compared to that of the BR population, with the exception of ZT 23. Because *cryptochrome-2* is believed to be a part of the core cycling genes, an increased expression may lead to a stronger repression of *clock* and *cycle* activity. For *cryptochrome-1*, there was a pattern linked to the photoperiod, where the SD condition resulted in increased expression compared to the LD condition across populations. Higher expression of *cryptochrome-1* has been tied to increases in photosensitivity, suggesting that *T. californicus* may be more photosensitive under a shortened day. Several studies have shown *cryptochrome* to affect development and growth, specifically affecting timing of developmental events and size, such as height, most notably in plants such as *Arabidopsis thaliana* (Wang *et al.*, 2014). The higher expression observed here may then be causing downstream effects within the circadian clock or elsewhere, resuting in the smaller body size growth, as the expression is consistent with that pattern.

In the case of *doubletime* we observed a strong plastic response across photoperiods, with a near threefold change in both populations through their perceived day (Fig. A1). There was a clear signal of a photoperiod effect in both populations, suggesting that daylength plays a strong role in determining total expression of this gene. Studies have shown that *doubletime* exhibits a conserved circadian function in phosphorylating the PER protein for degradation (Yu *et al.*, 2006). An increased expression in *doubletime* observed here could increase degradation of PER in order to maintain an entrained rhythm to a shorter period of light availability.

For *shaggy* there was an observable pattern of higher expression in the SD regime compared to the LD regime across populations (Fig. A1). This supports a consistent plastic response to photoperiod in these populations. The exact effect of the higher expression is unknown; however, *shaggy* has been implicated in shortening the period of the locomotor activity cycle, when overexpressed through *timeless* phosphorylation (Martinek *et al.*, 2001). The higher expression observed here may correlate with a shortened activity period where less foraging could be occurring, possibly aiding in the smaller body size observed in the SD condition.

In *jetlag* we observed no differences in expression (Fig. 3). Because *jetlag* is believed to aid in resetting the clock through the light-induced degradation of the TIM protein (Peschel *et al.*, 2009), it is likely that this function is relatively conserved across the populations on the basis of their relative expression. The total amount of expressed *jetlag* was proportional to the relative amount of expressed *timeless* (the lower overall amount of *timeless* corresponded to the lower amounts of *jetlag*), possibly as a result of needing proportionally equal amounts of *jetlag* to aid in degrading TIM.

While changes in patterns of gene expression were seen only when differences in photoperiod were introduced, there is still a possibility of masking effects from other natural rhythms, such as tidal or lunar, that also may modulate the expression of circadian genes. *Tigriopus californicus* lives in tidepools that are at or above the high tide line, and their pools do not suffer daily inundation from high tides. This may result in a lesser role of tidal cycles in the evolution of genetic rhythms in this species. Nevertheless, recorded associations of this copepod with other organisms that do exhibit lunar and tidal behaviors may allow for at least some form of a tidal rhythm to be present in *Tigriopous* (Egloff, 1966; Flores *et al.*, 2007). Differences in local tidal amplitudes between sites could also further complicate this issue,

because high and low tides do not always occur within the same time of day at both locations. Regulations of these rhythms, however, have been shown to be largely through distinct gene networks (Zhang *et al.*, 2013). Interestingly, organismal tidal rhythms were shown to be sensitive to inhibition of the gene *casein kinase 1* (*doubletime*), suggesting that there may be at least some shared substrates between tidal and circadian cycles (Zhang *et al.*, 2013). The interactions of genes associated with the different rhythms warrant further study.

Many species display variation in life histories as well as morphologies through latitudinal clines (Endler, 1977; Vermeij, 1978), often as a result of local adaptation to cope with the varying degrees of change they experience (Blanckenhorn and Fairbairn, 1995; Stinchcombe *et al.*, 2004). Light has been demonstrated to act as a cue for changes in many seasonal responses (Heideman *et al.*, 1999; Bradshaw and Holzapfel, 2001; Uller and Olsson, 2003), but its more direct effects on various types of growth fitness are not well described in this system. In *Tigriopus*, there is prior evidence for local adaptation in heat-shock protein gene expression (Schoville *et al.*, 2012), thermal tolerance (Willett, 2010; Kelly *et al.*, 2012), and salinity tolerance (Leong *et al.*, 2017). The results presented in this paper also suggest similar evidence for growth rate and expression of clock genes in relation to differing light cues as another potential form of local adaptation *T. californicus*.

Ultimately, we observed two divergent marine populations responding to varying light regimes differently. While changes in body size were consistent across populations, the population that was native to both light regimes (SH) showed an overall faster growth rate compared to the BR population, which does not experience the shortened daylength naturally. Changes in the light regime itself did show a significant effect on the speed of growth, motivating further study into how light acts as a cue for these organisms. Both *doubletime* and *shaggy* showed consistent plastic responses when exposed to different light regimes, suggesting fixed responses to photoperiod across populations for certain circadian genes that may be associated with growth and development. The southern population (BR) showed upregulation of the genes *period* and *timeless*, which are associated with increased longevity, supporting the idea that this population may expend more energy into its total lifespan rather than reaching reproductivity sooner, compared to the northern population. Whether this response changes consistently across its broad latitudinal range is yet to be explored. There is also the potential for divergence due to differences in local light pollution. Bird Rock is located near San Diego, California, where levels of artificial light are relatively high, whereas Strawberry Hill is a remote waypoint on the Oregon coast with no major cities or buildings nearby. Ultimately, the patterns we observed warrant further study into the effects of different photoperiods and entrainment to daily rhythms. This system can provide ample potential avenues to understand broader impacts of light's role in driving evolutionary processes. Specifically, studies in this system of longevity, potential foraging habits, and fitness trade-offs will be useful for examining hybridization, its potential breakdown, and the process of speciation in the context of daily rhythms and photoperiod.

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Literature Cited

Allada, R., and B. Y. Chung. 2010. Circadian organization of behavior and physiology in *Drosophila*. *Annu. Rev. Physiol.* **72:** 605–624.

Barreto, F. S., E. T. Watson, T. G. Lima, C. S. Willett, S. Edmands, W. Li, and R. S. Burton. 2018. Genomic signatures of mitonuclear coevolution across populations of *Tigriopus californicus*. *Nat. Ecol. Evol.* **2:** 1250–1257.

Beaver, L. M., B. O. Gvakharia, T. S. Vollintine, D. M. Hege, R. Stanewsky, and J. M. Giebultowicz. 2002. Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* **99:** 2134–2139.

Benna, C., P. Scannapieco, A. Piccin, F. Sandrelli, M. Zordan, E. Rosato, C. P. Kyriacou, G. Valle, and R. Costa. 2000. A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim*. *Curr. Biol.* **10:** R512–R513.

Benna, C., S. Bonaccorsi, C. Wülbeck, C. Helfrich-Förster, M. Gatti, C. P. Kyriacou, R. Costa, and F. Sandrelli. 2010. *Drosophila timeless2* is required for chromosome stability and circadian photoreception. *Curr. Biol.* **20:** 346–352.

Blanckenhorn, W. U. 2000. The evolution of body size: What keeps organisms small? *Q. Rev. Biol.* **75:** 385–407.

Blanckenhorn, W. U., and D. J. Fairbairn. 1995. Life history adaptation along a latitudinal cline in the water strider *Aquarius remigis* (Heteroptera: Gerridae). *J. Evol. Biol.* **8:** 21–41.

Bradshaw, W. E., and C. M. Holzapfel. 2001. Genetic shift in photoperiodic response correlated with global warming. *Proc. Natl. Acad. Sci. U.S.A.* **98:** 14509–14511.

Bradshaw, W. E., and C. M. Holzapfel. 2010. What season is it anyway? Circadian tracking *vs*. photoperiodic anticipation in insects. *J. Biol. Rhythms* **25:** 155–165.

Bulla, M., T. Oudman, A. I. Bijleveld, T. Piersma, and C. P. Kyriacou. 2017. Marine biorhythms: bridging chronobiology and ecology. *Philos. Trans. R. Soc. B Biol. Sci.* **372:** 20160253.

Cashmore, A. R., J. A. Jarillo, Y. J. Wu, and D. Liu. 1999. Cryptochromes: blue light receptors for plants and animals. *Science* **284:** 760–765.

Christie, A. E., T. M. Fontanilla, K. T. Nesbit, and P. H. Lenz. 2013. Prediction of the protein components of a putative *Calanus finmarchicus* (Crustacea, Copepoda) circadian signaling system using a *de novo* assembled transcriptome. *Comp. Biochem. Physiol. D Genom. Proteom.* **8:** 165–193.

Collins, B., and J. Blau. 2007. Even a stopped clock tells the right time twice a day: circadian timekeeping in *Drosophila*. *Pflueg. Arch. Eur. J. Physiol.* **454:** 857–867.

Dodd, A. N., N. Salathia, A. Hall, E. Kévei, R. Tóth, F. Nagy, J. M. Hibberd, A. J. Millar, and A. A. R. Webb. 2005. Cell biology: Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309:** 630–633.

Edmands, S. 2001. Phylogeography of the intertidal copepod *Tigriopus californicus* reveals substantially reduced population differentiation at northern latitudes. *Mol. Ecol.* **10:** 1743–1750.

Egloff, D. A. 1966. Ecological aspects of sex ratio and reproduction in experimental and field populations of the marine copepod *Tigriopus californicus*. Ph.D. dissertation, Stanford University, Stanford, CA.

Emerson, K. J., W. E. Bradshaw, and C. M. Holzapfel. 2008. Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations. *Evolution* **62:** 979–983.

Endler, J. A. 1977. Geographic variation, speciation, and clines. *Monogr. Popul. Biol.* **10:** 1–246.

Flores, A. A. V., A. C. A. Mazzuco, and M. Bueno. 2007. A field study to describe diel, tidal and semilunar rhythms of larval release in an assemblage of tropical rocky shore crabs. *Mar. Biol.* **151:** 1989–2002.

Foley, H. B., P. Y. Sun, R. Ramirez, B. K. So, Y. R. Venkataraman, E. N. Nixon, K. J. A. Davies, and S. Edmands. 2019. Sex-specific stress tolerance, proteolysis, and lifespan in the invertebrate *Tigriopus californicus*. *Exp. Gerontol.* **119:** 146–156.

Goto, S. G. 2013. Roles of circadian clock genes in insect photoperiodism. *Entomol. Sci.* **16:** 1–16.

Gotter, A. L., C. Suppa, and B. S. Emanuel. 2007. Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. *J. Mol. Biol.* **366:** 36–52.

Graham, A. M., and F. S. Barreto. 2019. Loss of the HIF pathway in a widely distributed intertidal crustacean, the copepod *Tigriopus californicus*. *Proc. Natl. Acad. Sci. U.S.A.* **116:** 12913–12918.

Gu, H.-F., J.-H. Xiao, L.-M. Niu, B. Wang, G.-C. Ma, D. W. Dunn, and D.-W. Huang. 2015. Adaptive evolution of the circadian gene *timeout* in insects. *Sci. Rep.* **4:** 4212.

Heideman, P. D., T. A. Bruno, J. W. Singley, and J. V. Smedley. 1999. Genetic variation in photoperiodism in *Peromyscus leucopus*: geographic variation in an alternative life-history strategy. *J. Mammal.* **80:** 1232–1242.

Hunt, L. C., J. Jiao, Y.-D. Wang, D. Finkelstein, D. Rao, M. Curley, M. Robles-Murguia, A. Shirinifard, V. R. Pagala, J. Peng *et al.* 2019. Circadian gene variants and the skeletal muscle circadian clock contribute to the evolutionary divergence in longevity across *Drosophila* populations. *Genome Res.* **29:** 1262–1276.

Hurd, M. W., and M. R. Ralph. 1998. The significance of circadian organization for longevity in the golden hamster. *J. Biol. Rhythms* **13:** 430–436.

Hut, R. A., S. Paolucci, R. Dor, C. P. Kyriacou, and S. Daan. 2013. Latitudinal clines: an evolutionary view on biological rhythms. *Proc. R. Soc. B Biol. Sci.* **280:** 20130433.

Katewa, S. D., K. Akagi, N. Bose, K. Rakshit, T. Camarella, X. Zheng, D. Hall, S. Davis, C. S. Nelson, R. B. Brem *et al.* 2016. Peripheral circadian clocks mediate dietary restriction-dependent changes in lifespan and fat metabolism in *Drosophila*. *Cell Metab.* **23:** 143–154.

Kelly, M. W., E. Sanford, and R. K. Grosberg. 2012. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. B Biol. Sci.* **279:** 349–356.

Kloss, B., J. L. Price, L. Saez, J. Blau, A. Rothenfluh, C. S. Wesley, and M. W. Young. 1998. The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. *Cell* **94:** 97–107.

Koh, K., X. Zheng, and A. Sehgal. 2006. *Jetlag* resets the *Drosophila* circadian clock by promoting light-induced degradation of *timeless*. *Science* **312:** 1809–1812.

Konopka, R. J., and S. Benzer. 1971. Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* **68:** 2112–2116.

Kukita, S., M. Gouda, S. Ikeda, S. Ishibashi, T. Furuya, and K. Nakamura. 2015. Effects of photoperiod and temperature on growth and development in clouded salamander (*Hynobius nebulosus*) larvae. *Zool. Sci.* **32:** 266–271.

Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33:** 1870–1874.

Lanciani, C. A., J. T. Giesel, J. F. Anderson, and S. S. Emerson. 1990. Photoperiod-induced changes in metabolic response to temperature in *Drosophila*. *Funct. Ecol.* **4:** 41–45.

Lee, W. S., P. Monaghan, and N. B. Metcalfe. 2010. The trade-off between growth rate and locomotor performance varies with perceived time until breeding. *J. Exp. Biol.* **213:** 3289–3298.

Leong, W., P. Y. Sun, and S. Edmands. 2017. Latitudinal clines in temperature and salinity tolerance in tidepool copepods. *J. Hered.* **109:** 71–77.

Martinek, S., S. Inonog, A. S. Manoukian, and M. W. Young. 2001. A role for the segment polarity gene *shaggy*/GSK-3 in the *Drosophila* circadian clock. *Cell* **105:** 769–779.

Mazzoni, E. O., C. Desplan, and J. Blau. 2005. Circadian pacemaker neurons transmit and modulate visual information to control a rapid behavioral response. *Neuron* **45:** 293–300.

Naylor, E. 2010. *Chronobiology of Marine Organisms*. Cambridge University Press, Cambridge.

Nesbit, K. T., and A. E. Christie. 2014. Identification of the molecular components of a *Tigriopus californicus* (Crustacea, Copepoda) circadian clock. *Comp. Biochem. Physiol. D Genom. Proteom.* **12:** 16–44.

Nylin, S., and K. Gotthard. 1998. Plasticity in life-history traits. *Annu. Rev. Entomol.* **43:** 63–83.

O'Grady, J. F., L. S. Hoelters, M. T. Swain, and D. C. Wilcockson. 2016. Identification and temporal expression of putative circadian clock transcripts in the amphipod crustacean *Talitrus saltator*. *PeerJ* **4:** e2555.

Perrigault, M., and D. Tran. 2017. Identification of the molecular clockwork of the oyster *Crassostrea gigas*. *PLoS One* **12:** e0169790.

Peschel, N., K. F. Chen, G. Szabo, and R. Stanewsky. 2009. Light-dependent interactions between the *Drosophila* circadian clock factors *cryptochrome*, *jetlag*, and *timeless*. *Curr. Biol.* **19:** 241–247.

Pistole, D. H., and J. A. Cranford. 1982. Photoperiodic effects on growth in *Microtus pennsylvanicus*. *J. Mammal.* **63:** 547–553.

Pittendrigh, C. S. 1960. Circadian rhythms and the circadian organization of living systems. *Cold Spring Harbor Symp. Quant. Biol.* **25:** 159–184.

R Core Team. 2018. R: a language and environment for statistical computing. [Online]. R Foundation for Statistical Computing, Vienna. Available: http://www.R-project.org [2020, March 4].

Roberts, S. K. 1956. "Clock" controlled activity rhythms in the fruit fly. *Science* **124:** 172.

Schenk, S., S. C. Bannister, F. J. Sedlazeck, D. Anrather, B. Q. Minh, A. Bileck, M. Hartl, A. von Haeseler, C. Gerner, F. Raible *et al.* 2019. Combined transcriptome and proteome profiling reveals specific molecular brain signatures for sex, maturation and circalunar clock phase. *Elife* **8:** e41556.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9:** 671–675.

Schnytzer, Y., N. Simon-Blecher, J. Li, H. Waldman Ben-Asher, M. Salmon-Divon, Y. Achituv, M. E. Hughes, and O. Levy. 2018. Tidal and diel orchestration of behaviour and gene expression in an intertidal mollusc. *Sci. Rep.* **8:** 4917.

Schoville, S. D., F. S. Barreto, G. W. Moy, A. Wolff, and R. S. Burton. 2012. Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod *Tigriopus californicus*. *BMC Evol. Biol.* **12:** 170.

Sehgal, A., J. L. Price, B. Man, and M. W. Young. 1994. Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant *timeless*. *Science* **263:** 1603–1606.

Sehgal, A., A. Rothenfluh-Hilfiker, M. Hunter-Ensor, Y. Chen, M. P. Myers, and M. W. Young. 1995. Rhythmic expression of *timeless*: a basis for promoting circadian cycles in period gene autoregulation. *Science* **270:** 808–810.

Stinchcombe, J. R., C. Weinig, M. Ungerer, K. M. Olsen, C. Mays, S. S. Halldorsdottir, M. D. Purugganan, and J. Schmitt. 2004. A latitudinal cline in flowering time in *Arabidopsis thaliana* modulated

by the flowering time gene *FRIGIDA*. *Proc. Natl. Acad. Sci. U.S.A.* **101:** 4712.

Strauss, J., and H. Dircksen. 2010. Circadian clocks in crustaceans: identified neuronal and cellular systems. *Front. Biosci.* **15:** 1040.

Tao, H., X. Li, J.-F. Qiu, W.-Z. Cui, Y.-H. Sima, and S.-Q. Xu. 2017. Inhibition of expression of the circadian clock gene period causes metabolic abnormalities including repression of glycometabolism in *Bombyx mori* cells. *Sci. Rep.* **7:** 46258.

Tarrant, A. M., R. R. Helm, O. Levy, and H. E. Rivera. 2019. Environmental entrainment demonstrates natural circadian rhythmicity in the cnidarian *Nematostella vectensis*. *J. Exp. Biol.* **222:** jeb205393.

Tataroglu, O., and P. Emery. 2014. Studying circadian rhythms in *Drosophila melanogaster*. *Methods* **68:** 140–150.

Tilden, A. R., M. D. McCoole, S. M. Harmon, K. N. Baer, and A. E. Christie. 2011. Genomic identification of a putative circadian system in the cladoceran crustacean *Daphnia pulex*. *Comp. Biochem. Physiol. D Genom. Proteom.* **6:** 282–309.

Uller, T., and M. Olsson. 2003. Life in the land of the midnight sun: Are northern lizards adapted to longer days? *Oikos* **101:** 317–322.

Vermeij, G. J. 1978. *Biogeography and Adaptation: Patterns of Marine Life*. Harvard University Press, Cambridge, MA.

Vittor, B. 1971. Effects of the environment on fitness-related life history characters in *Tigriopus californicus*. Ph.D. dissertation, University of Oregon, Eugene.

Wang, X., Q. Wang, P. Nguyen, and C. Lin. 2014. Cryptochrome-mediated light responses in plants. *Enzymes* **35:** 167–189.

Willett, C. S. 2010. Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. *Evolution* **64:** 2521–2534.

Yang, Z., and R. Nielsen. 2000. Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. *Mol. Biol. Evol.* **17:** 32–43.

Yu, W., H. Zheng, J. H. Houl, B. Dauwalder, and P. E. Hardin. 2006. Per-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev.* **20:** 723–733.

Yuan, Q., D. Metterville, A. D. Briscoe, and S. M. Reppert. 2007. Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol. Biol. Evol.* **24:** 948–955.

Zantke, J., T. Ishikawa-Fujiwara, E. Arboleda, C. Lohs, K. Schipany, N. Hallay, A. D. Straw, T. Todo, and K. Tessmar-Raible. 2013. Circadian and circalunar clock interactions in a marine annelid. *Cell Rep.* **5:** 99–113.

Zhan, S., C. Merlin, J. L. Boore, and S. M. Reppert. 2011. The monarch butterfly genome yields insights into long-distance migration. *Cell* **147:** 1171–1185.

Zhang, L., M. H. Hastings, E. W. Green, E. Tauber, M. Sladek, S. G. Webster, C. P. Kyriacou, and D. C. Wilcockson. 2013. Dissociation of circadian and circatidal timekeeping in the marine crustacean *Eurydice pulchra*. *Curr. Biol.* **23:** 1863–1873.

Appendix

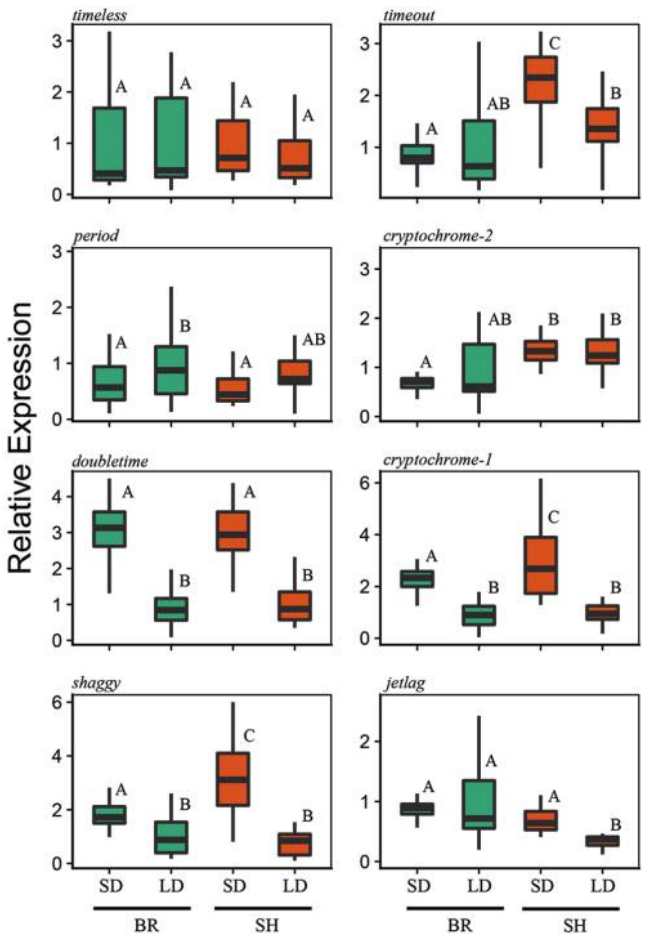

Figure A1. Average expression over a 24-hour period for genes associated with the circadian clock in 2 populations of *Tigriopus californicus*—Bird Rock, California (BR), and Strawberry Hill, Oregon (SH)—raised under two photoperiods. SD (short day) treatment had the 8h:16h light:dark cycle, while LD (long day) had the 12h:12h light:dark cycle. Boxplots represent median value with 25th and 75th percentiles for each treatment. Significantly different groups from an ANOVA followed by a Tukey's honest significant difference (HSD) *post hoc* test ($\alpha = 0.05$) shown in uppercase letters above boxplots.

Table A1

Primer sequences for eight genes associated with the circadian clock with their associated product size in base pairs and primer efficiency

Gene	Sequence (5′-3′)	Product size (bp)	Efficiency (%)
cryptochrome-1	F: GGGTAATGCCACTTCACCCA	162	100
	R: GCCTTGCGCTCTAAATGACG		
cryptochrome-2	F: GGTGAGCATCCTCTGGTTCC	141	91.6
	R: AATTTGGTGCCAGCCGTTTC		
doubletime	F: GACCACGGATTAATGAAGCAGC	142	94
	R: TGCCCTATCTGAAGGAGGTGTA		
jetlag	F: ACCATTTGACAGCCGGATGT	147	93.3
	R: TAAGGTCGATCATCCGCAGC		
period	F: GAGACCTTATGCAAAGGAACGC	101	95.7
	R: TCCCGCAGATAACTGTAATCCG		
shaggy	F: CTCTCCAGGCAAGGCTTCC	188	96.2
	R: AGAGGGAGCTCTTTGGCTTG		
timeless	F: AACTCCAGCACCCAAACCAA	100	92.1
	R: TCCAAGGAACCACGGGAATG		
timeout	F: GGAGCTCTTTGGCTTGGAGT	184	88
	R: CTCTCCAGGCAAGGCTTCC		
Pp2A	F: GTGGAGCAATTTGGAACCGAC	127	93.7
	R: AAGCCTCGGCCAATACATTGA		
RPLP1	F: ACTATTCTCAAGGCCGCCAAG	106	94.8
	R: AACCCACGTTCGTAATGAGCT		

The forward and reverse primer sequences are denoted with F and R, respectively. Efficiency was determined from the slope of standard curves by using a series of five fourfold dilutions from cDNA samples.