EXPRESSION OF CORE CIRCADIAN CLOCK GENES UNABLE TO EXPLAIN CHANGES IN THE PHOTOPERIODIC TIMER ACROSS LATITUDINAL AND ALTITUDINAL GRADIENTS IN *WYEOMYIA SMITHII*

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THESIS ABSTRACT

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Photoperiodism is the ability of plants and animals to utilize day length or night length to mitigate seasonal exigencies. The circadian clock allows organisms to organize daily demands. Both process are set by light, and for more than 80 years a functional relationship has been pursued. Previous experiments have revealed, through phenotypic expression, that the daily circadian clock and seasonal photoperiodic timer have evolved independently, yet molecular evidence is lacking. Herein, we use the mosquito, *Wyeomyia smithii*, to understand the relationship between the photoperiodic response, diapause, and the daily circadian clock. We measured variation in the formal properties of the core circadian clock over a latitudinal and altitudinal gradient which we compare to the critical photoperiod, a measure of diapause, over the same geographic gradient. We found that there is no correlation with any of the formal properties of the core circadian clock and critical photoperiod, indicating independent evolution.

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I dedicate this work to my mother and father, Jill and Wayne, who I would not be here without. I also dedicate this work to Mary Wood, who helped in so many ways I stopped counting and whose patience cannot be overstated. Without you this would not have been possible.

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CHAPTER I

INTRODUCTION

There are two principal environmental rhythms in nature: the daily cycle due to the Earth's rotation about its axis, bringing us night and day, and the annual revolution of the Earth around the Sun, bringing us seasons. As a result of the rotation, orbit, and 23.5° axial tilt of the Earth, there exist daily and seasonal fluctuations of light, temperature, and precipitation that are highly correlated to latitude (Hut et al 2013). Length of favorable growing season decreases with increasing latitude and altitude; the photoperiod amplitude increases with increasing latitude but not with altitude (Bradshaw and Holzapfel 2007). The robust natures of these rhythms serve as reliable cues allowing animals to track changes to their environment, such as avoiding dangerous parts of the day or unfavorable growing seasons. We see this in many organisms, such as mice who are primarily active at night, thus avoiding predation, or plants that go dormant during the winter, resulting in survival of the harsh growing conditions. The two major adaptations that allow coordination of organisms with their environment are the daily circadian clock and the seasonal photoperiodic timer; the circadian clock allows animals to track daily changes in their environment, and the photoperiodic timer allows animals to prepare for seasonal changes (Bradshaw and Holzapfel 2010; Hut et al. 2013). Although the clock and the timer have different temporal regulations, both are set by light, which is processed by sensory organs to produce a physiological response. Despite this similarity, research is ongoing to determine the degree to which these processes are related. More specifically, have these processes co-evolved concurrently or evolved independently? By measuring the expression of the core circadian clock genes over a geographical gradient among different populations of the pitcher-plant mosquito *Wyeomyia smithii*, this paper will attempt to determine the association of the circadian clock with the photoperiodic response.

Circadian Clock

The daily circadian clock, derived from the Latin *circa* meaning "about" and *dies* meaning "day", displays an endogenous daily rhythm of approximately 24 hours and organizes a multitude of behavioral and physiological daily functions, including locomotor behavior, eclosion rhythms, mating patterns, and a host of metabolic functions (Allada and Chung 2011; Claridge-Chang et al. 2001). The circadian clock is entrained by light, an incredibly consistent signal, on a continuous basis and is reset daily, persists or free-runs under constant conditions, and is temperature compensated (Pittendrigh 1960; Aschoff et al. 1965; Saunders 2002; Allada and Chung 2010). An important distinction is that the circadian clock does not count light:dark cycles, but rather interprets light and transmits information to appropriate systems continuously (Saunders 2002; Emerson et al. 2008b). Circadian timing systems have been found in most species, ranging from bacteria to mammals (Dunlap et al. 1999; Bradshaw and Holzapfel 2007; Hut and Beersma 2011) and have shown to be paramount for the survival and fitness of both prokaryotes and eukaryotes (Ouyang et al. 1998; Woelfle 2004; Emerson et al. 2008a).
Since the discovery of the first circadian gene in *Drosophila*, called *period*, a host of other genes have been identified (Konopka and Benzer 1971). The insect circadian clock is composed of a core set of five regulatory genes - *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), and *cryptochrome2* (*cry2*) - that act as a transcription-translation feedback loop to cycle throughout the day (Allada and Chung 2011; Yuan et al 2007). The regulatory cycle begins with a CLOCK (CLK) and CYCLE (CYC) heterodimer binding to an E-box promoter to upregulate the transcription of *per* and *tim* genes (**Figure 1**). In the cytosol, PER, TIM, and CRY2 form a complex that translocates back to the nucleus where they inhibit CLK and CYC activity, thereby reducing their own abundance. The input of light is mediated by CRYPTOCHROME1 (CRY1) which, when exposed to light, binds to TIM, leading to the degradation of both proteins and advancing or delaying the cycling nature of the core clock genes. There are numerous other post-translational genes involved in the core circadian clock but are not considered core clock genes.

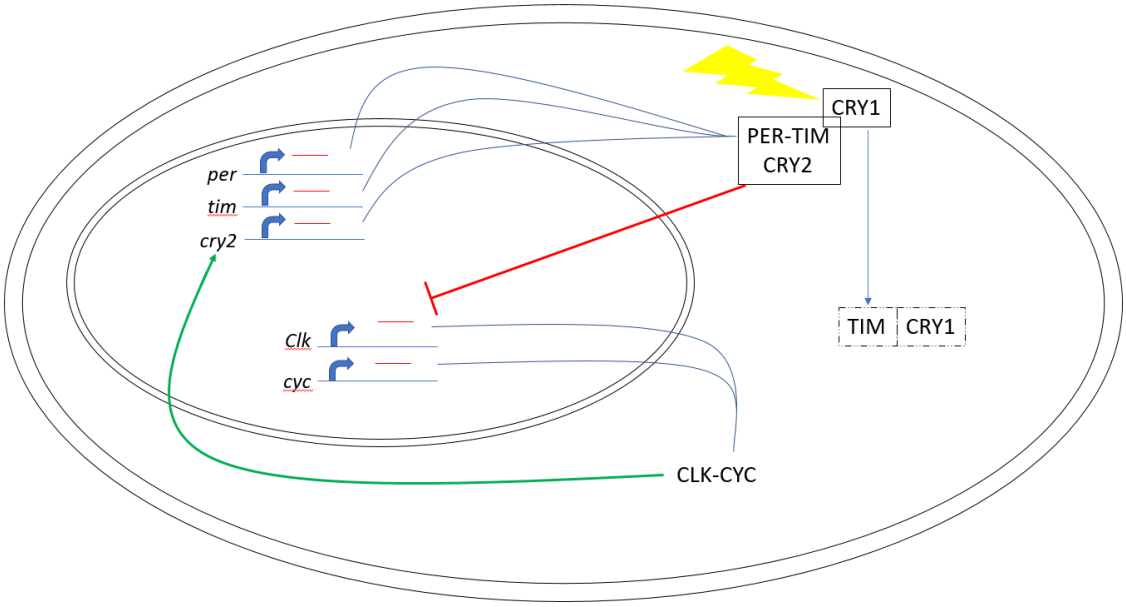

Figure 1. Simplified diagram of the core circadian clock. The clock represented here consists of two positive regulators (CLOCK and CYCLE) and three negative regulators (PERIOD, TIMELESS, and CRYPTOCHROME 2). The translated proteins of CLK and CYC form a heterodimer in the cytoplasm and translocate back to the nucleus. CLK-CYC bind to the E-box promoters of *per*, *tim*, and *cry2* to promote transcription. PER, TIM, and CRY2 form a complex in the cytoplasm and translocate back to the nucleus where PER actively represses CLK activity, thereby reducing expression of *per*, *tim*, and *cry2* (Sandrelli et al 2008).

Photoperiodism

Photoperiodism is a physiological response to day or night length that initiates major seasonal events of an organism including reproduction, development, and diapause (Tauber et al. 1986; Meuti and Denlinger 2013) and consists of two distinct components. The photoperiodic timer evaluates the length of day or night, and the photoperiodic counter, as suggested, counts the number of long or short day cycles (Saunders 2002; Emerson et al. 2008b; Emerson et al 2009). In contrast to the daily circadian clock, the seasonal photoperiodic response is irreversible once begun, runs to completion, and does not display rhythmicity (Nijhout 1994; Saunders 2002; Bradshaw and Holzapfel 2007; Bradshaw and Holzapfel 2010). As with the daily synchrony of the circadian clock, photoperiodism is crucial for temporal coordination with seasonality and preparing for the long-term exigencies experienced throughout the year, leading to increased fitness (Tauber et al. 1986; Bradshaw et al. 2004; Bradshaw and Holzapfel 2007).

Photoperiodism is utilized by a diverse range of organisms from plants, fish, birds, mammals, and arthropods, among others (Bradshaw and Holzapfel 2007), and one of most common photoperiodic responses is the onset of diapause in insects. Diapause is the cessation of development, which is hormonally regulated, and typically occurs during unfavorable conditions, allowing the insect to survive though dormancy or migration, contributing to the evolutionary success of insects (Denlinger 2008; Saunders 2011; Meuti and Denlinger 2013). Typically, short days and long nights lead to a high frequency of diapause, while long days and short nights lead to a low frequency of diapause. One metric of the photoperiodic response, known as the critical photoperiod (CPP), is the point, in hours of daylight, at which 50% of the population will remain in diapause, mainly used for temperate species. This measure is useful because it allows the comparison of the photoperiodic response among different populations of the same species. A consistent trend among populations of the same species is that CPP increases with latitude and altitude (Bradshaw and Lounibos 1977; Bradshaw and Holzapfel 1975; Bradshaw and Holzapfel 2001) allowing populations residing at higher latitudes to avoid the earlier onset of unfavorable winter conditions. For example, a population of *Wyeomyia smithii* mosquitoes in northern Maine has a CPP of approximately 15 hours. When length of day reaches 15 hours or below, the mosquitoes will enter diapause until day length increases past 15 hours, typically sometime in the spring. Compare this response to a population *W. smithii* in Florida that has a CPP of 12.14 hours. These mosquitoes will enter diapause much later in the year because the onset of winter arrives later in the year, and the day length resulting in the entry into diapause is much shorter. Hence, the mosquitoes will remain active until day length reaches approximately 12 hours (Bradshaw et al. 2003).

The hormonal cascade that is initiated through diapause is well annotated (Denlinger et al. 2012), yet the molecular mechanisms used to convey information from the input of light though sensory organs to the hormonal output are little understood. This lack of information is

one reason why there has been difficulty understanding the relationship between the circadian clock and the photoperiodic timer.

Relationship of the Circadian Clock and Photoperiodic Timer

Although the circadian clock and photoperiodic timer differ in their temporal control, both are regulated by light, therefore a causal connection was initially pursued. More than 80 years ago, Erwin Bünning proposed a theory that the circadian clock forms the functional basis of the photoperiodic response (Reviewed in Saunders 2011). The original hypothesis posits that light entrains the circadian clock, which determines a light sensitive phase; if light is experienced during this phase a photoperiodic response is triggered (Pittendrigh and Minis 1964). This is an appealing concept because it would mean that both the seasonal and daily timer are regulated by the same system. This hypothesis has been tested expansively (reviewed by Nunes and Saunders 1999; Tauber and Kyriacou 2001; Saunders and Bertossa 2011), primarily using two distinct protocols. The Bünsow protocol, involving night interruption experiments, exposes the organisms to short photoperiods in addition to brief pulses of light throughout the extended night. If light is exposed during a light sensitive phase, a long day response is initiated, suggesting circadian involvement (reviewed in Saunders 2011). The more extensively used Nanda-Hamner protocol (NH) exposes the organisms to a short period of light (10-12 hours) followed by increasing periods of darkness that cycle with a period of 24 to 72 hours (T = light + dark). For example, one treatment will be exposed to a L : D cycle of 10 : 14 (T = 10 + 14, over several cycles), another treatment will be exposed to a L : D of 10 : 24, another 10 : 30, and so on (Nanda and Hamner 1958). If the photoperiodic response (typically termination of diapause) is related to the circadian system, then the response will exhibit peaks approximately every 24 hours, displaying a daily rhythmic sensitivity to light. The results of these experiments have shown a rhythmic response and offer the main support for involvement of the circadian clock in the photoperiodic response (Nunes and Saunders 1999; Tauber and Kyriacou 2001; Bradshaw et al. 2006). However, these

experiments don't discern between a causal and correlative relationship or account for an arrhythmic or non-response to their protocols.

It has been pointed out that a rhythmic response to the Nanda-Hamner experiments do not alone support Bünning's hypothesis (Veerman 2001; Bradshaw et al. 2006). Emerson et al. 2009 writes:

> "When all experiments are run under the same environmental conditions… persistent phenotypic variation among populations represents evolved (genetic) differences among them. If the evolutionary modification of two physiological processes is causally (genetically) connected through common genes (pleiotropy), then there should be a strong correlation between the two processes among evolutionary lineages: If the evolution of the photoperiodic response is due to evolution of the circadian clock, then the formal properties of both processes should be correlated among populations within a single species."

To investigate this relationship further, Bradshaw et al. (2003, 2006) used the pitcher-plant mosquito, *Wyeomyia smithii*, with interfertile populations ranging from the Gulf Coast of the United States, along the East Coast into Newfoundland and up through the Midwest into Canada. In *Wyeomyia smithii*, CPP has a strong correlation with latitude and altitude of geographic origin ($R^2 \geq 0.92$), increasing towards higher latitudes and altitudes (Bradshaw and Holzapfel 2001). In a series of experiments, it was shown that the period and amplitude of the response to NH and CPP are not correlated among populations (Bradshaw et al. 2003; Bradshaw et al. 2006); within a population, the amplitude but not the period of the rhythmic response to NH is negatively genetically correlated with CPP. To determine if this negative correlation was the result of an underlying pleiotropic connection between the photoperiodic response and the circadian clock, a series of antagonistic selections were performed (Bradshaw et al. 2012) on amplitude and CPP. In only five rounds of selection, they were able to reverse the negative correlation, indicating that neither the period nor the amplitude of the rhythmic response to NH are causally correlated to CPP. This is an important distinction because period and amplitude are the two essential properties of any rhythm; Bradshaw et al. (2006) suggest that the rhythmic response to the Nanda-Hamner experiments may be an expression of basic circadian rhythmicity

and not a causal link to the photoperiodic response. If the photoperiodic response is being informed by the circadian clock then there would be some correlation between CPP and the rhythmic response to the NH experiments, which has not been supported by the data. Most experiments thus far have focused on the output of the circadian clock and photoperiodic response, but being able to compare the rhythmicity of the core genes and CPP would give more insight into this debate.

Other approaches have been taken to determine the relationship of the circadian clock and photoperiodic response. One approach, with interesting results, has been to disrupt certain circadian genes, thereby affecting the molecular machinery of the clock, and determine the effect on the photoperiodic response. In a series of experiments, it was shown that genes *Clock*, *cycle*, *period*, and *cryptochrome2* are all needed for the circadian controlled cuticle deposition of endocuticle layers in the bug *Riptortus pedestris*, yet when the clock genes were disrupted diapause was also disrupted (Ikeno et al. 2013). Another experiment used arrhythmic mutant strains, per^{01}, tim^{01}, and Clk^{Jrk} to determine the effect on the photoperiodically regulated chill comma recovery times in *Drosophila* (Pegoraro et al. 2014). They found that clock mutations do affect recovery times, suggesting an involvement of the circadian clock in the photoperiodic timer. However, it is important to note that this does not mean there is a causal relationship; there may be pleiotropic effects of the individual circadian genes that incidentally inform the photoperiodic timer either directly or through downstream effects on photoperiodic genes yet discovered. Also, these experiments were done on a single population of a single species; considering the variation in circadian clock models and photoperiodic phenotypes it is hard to make any definitive conclusions. The only conclusions one can make is that in this population of this species, the circadian clock seems to be intimately involved in the photoperiodic response. In addition to these molecular studies, there have been several studies that have found a correlation with circadian output (oviposition rhythms) and latitude (reviewed in Hut et al. 2013). These

experiments are some of the first to describe this trend, yet it is important to note that they are only looking at the phenotypic output of the clock and not the core clock itself.

Herein, we determine whether the formal properties of the core circadian clock genes vary over latitudinal and altitudinal gradients, giving insight into the evolutionary relationship with the photoperiodic timer in *Wyeomyia smithii.* This experiment provides an opportunity to understand the molecular mechanisms involved in the circadian clock over a geographic gradient as opposed to the phenotypic output experiments such as the Nanda-Hamner protocol. To our knowledge, no previous studies have investigated the variation of the core circadian clock genes over both latitude and altitude. One unique feature of this experiment is that we can utilize the mountain populations and the coastal plain populations which share a similar latitude (and thus similar photoperiod) but different altitudes (and therefore different seasonal environments). The mountain populations in this experiment show a longer, northern CPP compared to the coastal plain populations at the same latitude, which show a more southern, shorter CPP (Bradshaw et al. 2003). Thus, if the daily circadian clock and seasonal photoperiodic timer were causally involved then one would expect to see differences in the formal properties of the circadian clock between the two populations. By comparing the LD cycle, driven by light, to the DD cycle, the free-running rhythm, we can determine differences among populations of the period, amplitude, phase, and damping of the five core circadian clock genes (*period, timeless, Clock, cycle,* and *cryptochrome2*) over latitudinal and altitudinal gradients. If the circadian clock is in fact informing the photoperiodic timer we should see some correlation with some formal property of at least one core circadian clock gene with latitude and altitude, giving support to the theory originally proposed by Bünning. A lack of correlation in any of the core clock genes with latitude or altitude would indicate that, at the level of the gene, these two systems can and have evolved independently.

CHAPTER II

METHODS

Experimental organism:

Wyeomyia smithii completes its pre-adult development within the water-filled leaves of the pitcher-plant, *Sarracenia purpurea*, tightly following the range of its host plant throughout North America, ranging from the gulf coast of the United States, through the east coast and up into northern and central Canada. The ancestral southern populations enter diapause in the fourth instar and the derived northern and mountain populations enter diapause in the third instar (Bradshaw and Lounibos 1977; Armbruster et al. 1998). There is a linear increase in critical photoperiod with latitude and altitude; northern populations have a higher CPP than southern populations (Bradshaw and Lounibos 1977).

Collection and maintenance of *Wyeomyia smithii*:

Wyeomyia smithii mosquitos were collected as developing larvae in the fall of 2016 from 10 distinct geographic locations, ranging from 30°N to 46°N latitude and from 0 m to 900 m elevation, with 2 populations from each of five geographic zones giving geographic replication (**Figure 2, Table 1**). Throughout the text, the populations will be referred to by locality acronyms (**Table 1**). The larvae were immediately cleaned and randomized into dishes (n=35) and put on a long-day photoperiod (L : D = 18 : 6) and a sine wave thermoperiod of 18°C to 33°C to initiate development. Larval mosquitoes were fed a liquid diet of a 4:1 mixture of dry gerbil food and brine shrimp dissolved in water once a week. Mosquitoes were raised through one generation and hatch was placed into short-day conditions (L : D = 8 : 16) at a constant 21°C to initiate diapause (3^{rd} instar for northern populations and 4^{th} instar for southern populations). The F_1 generation was used for the purposes of sampling a population that is as close to the natural population as possible. Diapausing larvae were placed into separate sampling dishes (n=70) and transferred to light cabinets for photic entrainment.

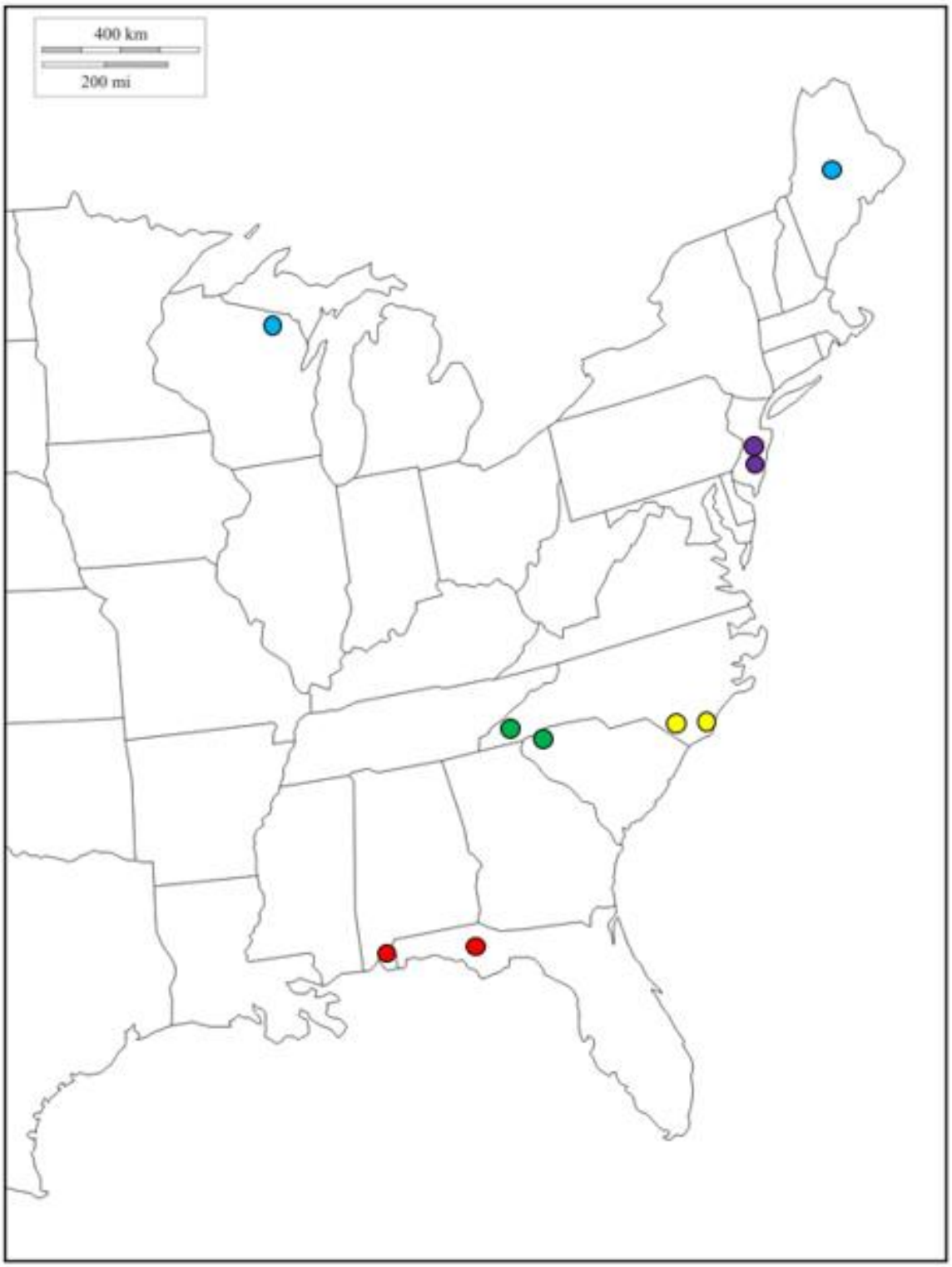

Figure 2. Geographic range of populations of *Wyeomyia smithii*. All 10 populations are color coded by geographic/phylogenetic zone; Northern (blue), Mid-Atlantic (purple), Appalachian (green), Coastal Plain (yellow), and Southern (red).

Table 1. Origin of experimental populations, * reference to names of populations by locality.

Population Origin	Reference*	Region	°N Lat	°W Long	m Alt
N Maine	KC	North	46.2	68.3	365
N Wisconsin	ML	North	46	89.6	500
Central New Jersey	PB	Mid-Atlantic	39.9	74.4	10
South New Jersey	HV	Mid-Atlantic	39.7	74.5	10
Appalachian Mts. NC	DB	Appalachia	35	83	900
Appalachian Mts. NC	SF	Appalachia	34.9	82.7	640
Coastal North Carolina	SH	Coastal Plain	35.3	77.5	107
Coastal North Carolina	GS	Coastal Plain	34.1	78.3	20
S Alabama	LI	South	30.5	87.5	15
N Central Florida	WI	South	30.1	85	10

Photic entrainment and tissue sampling

Animals were allowed to entrain for one full week in short-day conditions (L : D = 10 : 14) at a constant 21°C. Samples we collected in 3 hour increments, using first light exposure as ZT=0, then proceeding to ZT=3, 6, 9, etc. ending to ZT=60, a total of 21 time points for each population.

Sample preparation involved several distinct steps. Larvae were first transferred from the larval dish to cold 190 proof ethanol. They were then transferred to a sterile dish lid that was cooled on a bed of dry ice, removed of excess liquid, and allowed to freeze. Larval heads (n=70 per population) were then decapitated and put into 500 µl TRI-Reagent®, homogenized, and immediately placed in dry ice. At the end of the sampling day they were stored at -80°C. This process was replicated three times, giving three biological replicates per population. One complete replicate was sampled before the next to eliminate autocorrelation.

RNA extraction and quantitative PCR

RNA extraction, purification, and qPCR were performed at the Center for Genome Research and Biocomputing at Oregon State University. The circadian clock models vary by species and are reviewed in Tomioka and Matsumoto 2010; for the purposes of this paper we will

use the *A. gambiae* circadian model rather than the *Drosophila* model, which does not possess the *cry2* gene (Rund et al. 2011). All core clock genes were verified in *Wyeomyia smithii* through the assembly of a transcriptome and reciprocal BLAST with *Aedes aegypti* (Tormey et al. 2015).

RNA extraction:

Samples were removed from -80°C and thawed on ice, and RNA was extracted using the Direct-zol™ RNA Mini prep kit following protocol from Zymo Research. RNA was precipitated by adding 1ul glycogen, 5ul 3M NaOAc, and 150ul 95% ethanol to each tube and allowed to freeze approximately 30 mins at -80°C. Tubes were then centrifuged at 4°C for 30 mins to concentrate the RNA pellet, washed with 500ul of ice-cold 70% ethanol, centrifuged for 5 mins, and the supernatant was removed. Once dry, the pellet was resuspended in 30ul DEPC treated water.

First strand cDNA synthesis/purification:

RNA was quantified using Qubit® RNA BR Assay Kit and normalized to 1ug total RNA in 8ul water (125ng/ul). Reverse transcription was performed using the Invitrogen Superscript II Reverse Transcription kit. The Oligo$(dT)_{20}$ primer was used for the cycle of 50°C for 50 minutes, 85°C for 5 minutes, and 37°C for 20 minutes resulting in a cDNA volume of 20ul. The cDNA was purified using the Zymo DNA Clean and Concentrator 5 kit per the manufacturer's protocol. To increase sample volume, 18ul of reverse transcription reaction was added to 162ul nuclease free water for a total volume of 180ul.

Plate design:

qPCR was performed using the 96-well plate format. Due to plate size (96-well) each biological replicate, consisting of 21 time points (ZT 0, 3, 6…) was split into two plates (Sample plate 1 ZT 0 – 33 and Sample plate 2 ZT 33 – 60). Each biological replicate sample plates were divided into two technical replicates, using the same genetic material for technical replicates 1 and 2. This resulted in 12 plates per population (3 biological replicates x 2 Sample plates x 2 technical replicates). A normalizing control was included for each gene assayed (*per, cyc, tim,*

Clk, *cry2*, and *rp49*) which consisted of pooled RNA from that gene from all time points (ZT 0 – 60).

qPCR:

qPCR was performed on the Applied Biosystems 7500 Fast Real Time PCR Systems using the 96-well format. Each PCR reaction used 5ul of cDNA, 10ul of 2X SYBR Fast Master Mix, 4.2 nuclease free water, and 0.4ul of each forward and reverse primers (**Table 2).** The reaction conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 3 seconds and 60°C for 20 seconds. *rp49* was included as a housekeeping gene to normalize expression of core clock genes (*per*, *cyc*, *tim*, *Clk*, and *cry2*).

Table 2. Primer sequences used for qPCR for all clock genes and housekeeping gene.

Clock gene		Sequence
per	F	CCAGACACACGACGTGCGGA
per	R	TCCATTACCGAGCGTCCCAGG
cyc	F	CCAAAACGATGCTTCCAGTT
cyc	R	TTTTGCATTTCATCCGACAA
tim	F	GTGCATCATGGTGAAAATGC
tim	R	AAGTTCGCCACAATGGAAAT
Clk	F	CAACGGACTATCGGTTCGAG
Clk	R	CCTAGAGGCGGTCATCAGAG
cry2	F	CCATTTCCGATCGTATCCAC
cry2	R	CCGAGCCCCTCTTACCTATC
rp49	F	ATCGGTTACGGATCGAACAA
rp49	R	TTCTGCATCAGCAGCACTTC

Statistical methods

Data analysis was performed using Excel 2016 and JMP 4.0.

Expression data for all genes was represented as cycle number (CT = cycle threshold), the number of cycles needed to reach a quantitative threshold established by the Applied Biosystems qPCR system.

Raw data for the core clock genes was processed sequentially:

1. For each core clock gene and time point, the clock gene CT level was standardized by subtracting the *rp49* CT level
2. The CT level of the normalizing control (NC) for each clock gene was standardized by subtracting the *rp49* NC CT level
3. Core clock gene CT levels were further standardized by subtracting the CT level of their standardized NC for each gene and time point
4. To determine fold expression change for each clock gene and time point, the twice standardized CT level was raised to the base 2 (ex. $2^{\text{Processed gene expression}}$)

Fold expression change for technical replicates 1 and 2 was averaged for each biological replicate, resulting in 3 biological replicates for each population. Data were then divided into two distinct average tech (AVT) groups, the light cycle (ZT 0 – 24) called AVT-LD and the free running cycle (ZT 12 – 60) called AVT-DD. Expression levels for all time points in all the biological replicates were averaged for each AVT and subtracted from each time point. This procedure generated a mean fold-expression value of zero and allowed a comparison of deviation from the mean for each time point. Outliers were identified using the 99% CI of AVT-wide deviations from the mean across all time points and removed from subsequent steps of analysis. This method was derived a-priori using a sample population, equivalent to an experimental population (3 biological replicates and 2 technical replicates). The data were from a previously-collected sample and are not included in experimental data.

The next step in the data analysis process was performed in JMP 4.0 with the goal of generating a four parameter (slope of linear function, amplitude, phase, and period) or five parameter (slope of linear function, amplitude, damping, phase, and period) cosine model for each gene in the LD and DD data set respectively. The model output gives gene expression (GE) for each gene at each timepoint (ZT) $GE = b*ZT + c*\text{Cos}\left(\frac{ZT-e}{f}\right)*6.283$: *b* slope of linear function, *c* amplitude, *d* damping coefficient, *e* phase angle, and *f* period of rhythm (reviewed in

Emerson et al. 2009). To determine if the model fit the observed data, a correlation was performed comparing the observed data and the model data for each gene per population. Several genes did not converge in the model, reported in results, and were removed from further analysis.

Latitude and Altitude Variation:

To evaluate differences in latitude and altitude, a multiple regression was performed in Excel, allowing the comparison of the parameters, generated in JMP 4.0, of the core clock genes for each population. A coefficient of correlation was generated for each gene in each population through a comparison of each of the parameters for each gene and both latitude and altitude, as these factors are not separable. To avoid type-1 error due to the multiple comparisons, a Holm-Bonferroni sequential correction (Armstrong 2014) was performed for each of the LD and DD data sets.

Phylogenetic Variation:

To evaluate differences in phylogeny, a single factor ANOVA was performed in Excel, comparing the northern clade (KC, ML, PB, HV, DB, and SF) and the southern clade (WI, LI, GS, and SH) for differences in the formal properties for each gene for both LD and DD data sets. To qualify the amount of total variation, accounted for by differences between northern and southern populations, for each parameter, % reduction in total sum of squares was calculated as $\frac{Between\ Group\ SS}{Total\ SS}$. To avoid type-1 error due to the multiple comparisons, a Holm-Bonferroni sequential correction was performed for each of the LD and DD data sets.

CHAPTER III

RESULTS

Core clock genes respond to a driven light:dark cycle over 24 hours, and show decreased rhythmicity as follows *per, cyc,* and *tim* (**See Appendix, Figures 3.1, 3.2. and 3.4**), whereas *Clk* and *cry2* are arrhythmic (**Appendix, Figures 3.3 and 3.5**). The expression of the core clock genes to the dark:dark cycle show a free-running rhythm *per*, *cyc,* and *tim* (**Appendix, Figures 4.1, 4.2, and 4.4**), and as in the L : D cycle, *Clk* and *cry2* are arrhythmic (**Appendix, Figures 4.3 and 4.5**).

Between the regression analysis and ANOVA tests, we made 70 distinct comparison which has a high potential for type-1 error. Regressing the formal properties for each gene on Altitude-corrected Latitude for the LD data set resulted in no significant p-values before Bonferroni correction (**Figure 5.1**). Regressing the formal properties for each gene on altitude-corrected latitude for the DD data set resulted in one significant p-value (p=0.041), period for *cry2* (**Figure 5.2**). To correct for any false positives, we used the Holm-Bonferroni method on each of the LD and DD data sets. After corrections, there were no p-values that rise to the level of significance in the LD or DD data sets (**Appendix, Table 3**).

Analysis of variance comparing the formal properties of all clock genes of the Southern ancestral clade with the Northern derived clade revealed 3 significant differences: period of *cycle* for the LD data sets (**Appendix Table 4a**); for the DD data sets, period of *cry2,* and phase of *cycle* (**Appendix Table 4b**). Percent reduction in total sum of squares was also calculated to determine the total amount of variation due to differences in between the groups, mainly phylogenetic, for both LD (**Figure 6.1**) and DD (**Figure 6.2**) data sets, also shown in table form, **Appendix, Table 5a and 5b**. To correct for any false positives, we used the Holm-Bonferroni method on each of the LD and DD data sets. After corrections, there were no p-values that rise to the level of significance in any of the data sets (**Appendix, Table 6**).

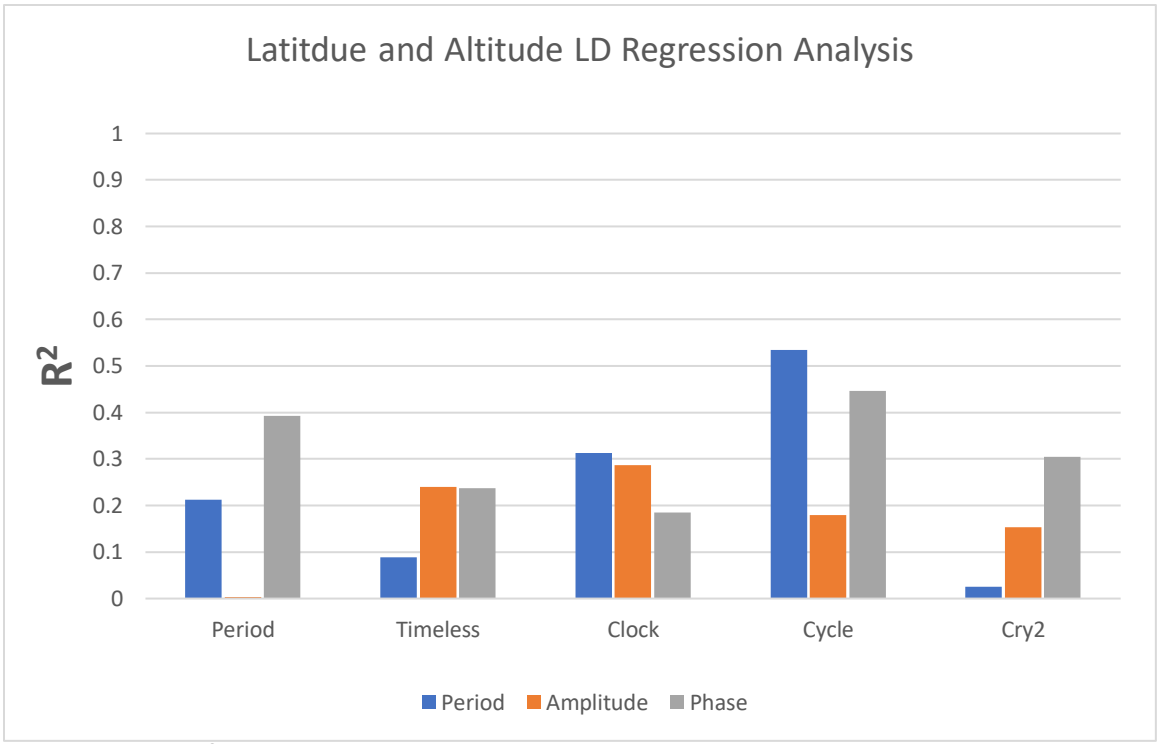

Figure 5.1. R^2 values for the regression analysis comparing the 3 parameters (period, amplitude, and phase) to latitude and altitude for the LD data set. There were no significant correlations before Bonferroni correction.

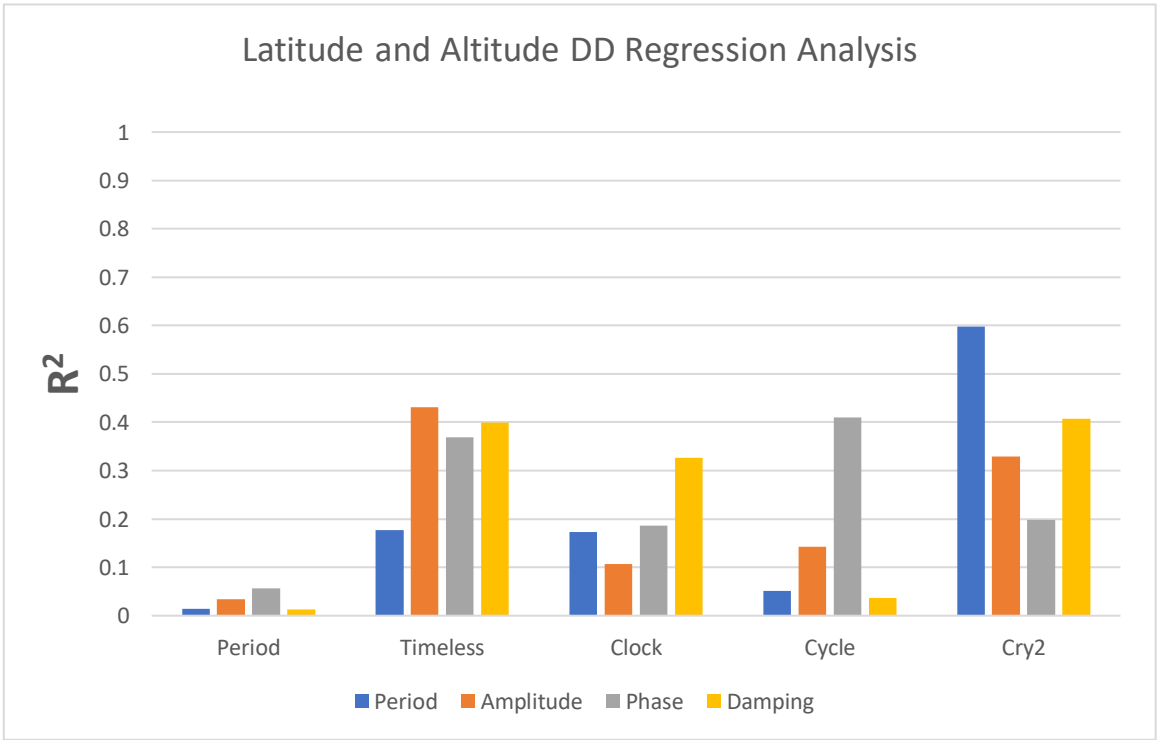

Figure 5.2. R^2 values for the regression analysis comparing the 4 parameters (period, amplitude, phase, and damping) to latitude and altitude for the DD data set. After Bonferroni correction, there were no significant correlations.

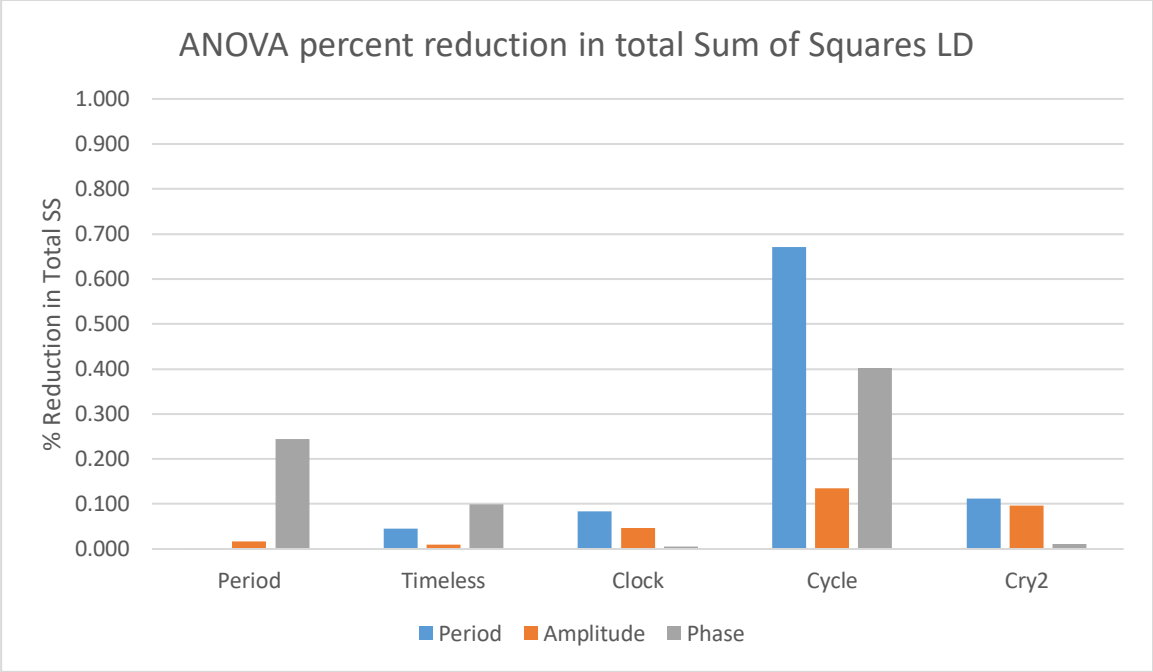

Figure 6.1. ANOVA % reduction in total sum of squares for LD data set comparing the 3 parameters of the northern (KC, ML, PB, HV, DB, and SF) and southern (WI, LI, GS, and SH) populations. A larger value indicates that there was a greater amount of variation between the Northern and Southern populations for that formal property.

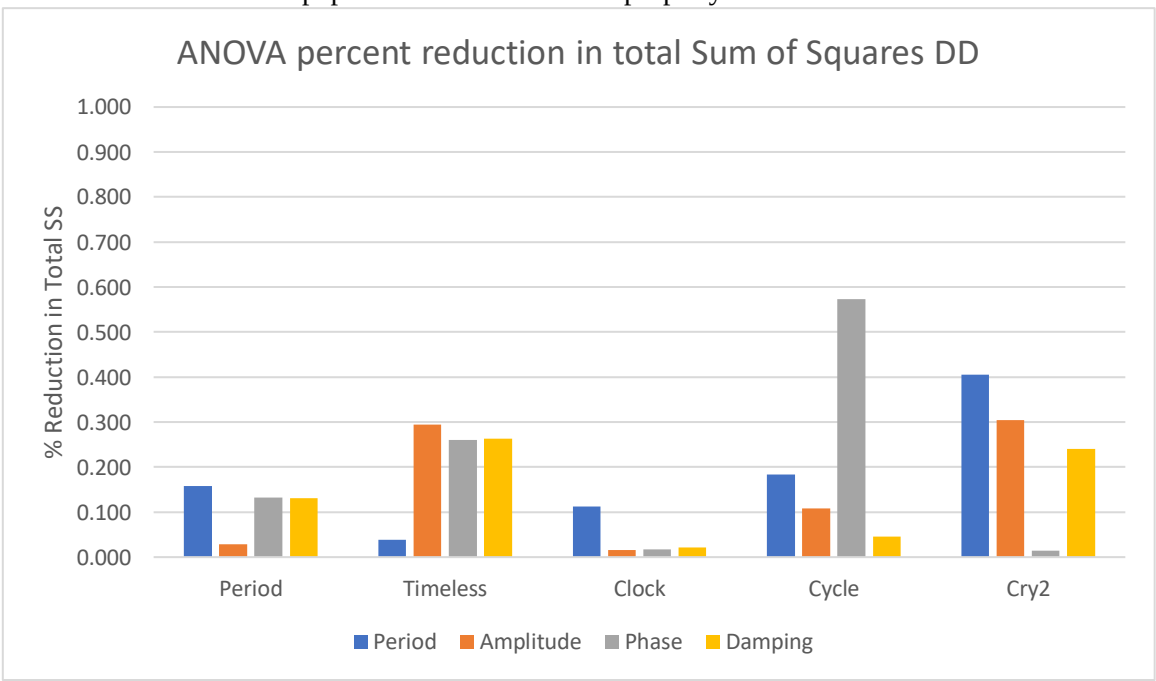

Figure 6.2. ANOVA % reduction in total sum of squares for DD data set comparing the 4 parameters of the northern (KC, ML, PB, HV, DB, and SF) and southern (WI, LI, GS, and SH) populations. A larger value indicates that there was a greater amount of variation between the Northern and Southern populations for that formal property.

CHAPTER IV

DISCUSSION

Photoperiodic time measurement allows organisms to accurately predict unfavorable seasonal conditions through a physiological response that manifests through dormancy or migration (Denlinger 2008; Saunders 2011). The circadian clock regulates a host of metabolic, behavioral, and reproductive processes on a daily basis (Allada and Chung 2011; Claridge-Chang et al. 2001). While the importance of a functioning circadian clock has been recognized for more than a century, the mechanistic relationship connecting it to photoperiodic time measurement is still unresolved. For more than 80 years, scientists have debated the validity of Bünning's hypothesis. There are several issues that have arisen in discerning the circadian clock from the photoperiodic timer. First, the circadian clock is well annotated, and much is known about the molecular mechanisms that make up the clock; alternatively, there is less known about the molecular mechanisms that comprise the photoperiodic timer (Meuti and Denlinger 2013). Second, much of our circadian clock knowledge derives from *Drosophila*, which is only weakly photoperiodic, making inferences drawn from this species likewise weakly compelling. This weakness has made it more difficult to draw any connections between the photoperiodic timer and circadian clock (Rivas et al 2016). These facts have given scientists the impetus to use other, non-model organisms to study these two systems.

One such organism, *Wyeomyia smithii,* is a North American temperate mosquito that originates from the tropics, invading through the Gulf of Mexico and extending northward along the eastern coast into Newfoundland, Canada and up through the Great Lakes into central Canada (Bradshaw and Lounibos 1977; Armbruster et al. 1998). *Wyeomyia smithii* shows a robust association between CPP and evolutionary history, with a positive correlation of both latitude and altitude and CPP (Bradshaw and Holzapfel 2001). This correlation is even more pronounced in the next generation (F_2) of the same experimental populations used in this experiment (0.992 R^2) (Travis Neuman, unpublished Honor's Thesis). The core circadian clock genes, along with the

post-translational modifiers, have also been identified (Tormey et al. 2015). The fact that *W. smithii* shows a strong photoperiodic response, has a fully functional circadian clock, and has a large geographic range makes it an ideal organism to study the connection between the circadian clock and photoperiodic timer.

To investigate this issue, we identified the formal properties (period, amplitude, phase, slope of the linear function, and damping) of the five core clock genes in *Wyeomyia smithii* (*per*, *tim*, *Clk*, *cyc*, and *cry2*) in all 10 populations over a geographic gradient (Supplemental Materials)**.** Our results show that the clock genes in *W. smithii* respond to a driving light cycle (**Appendix, Figures 3.1-3.5)** and free-run under constant conditions (**Appendix, Figures 4.1-4.5)**. These results are important because they not only establish a fully rhythmic core clock, but also affirm the validity of our method of gene expression analysis. When comparing the formal properties of all five core clock genes over a latitudinal and altitudinal gradient, we found that there were no significant differences in both the driven cycle (**Figures 5.1 and Appendix Table 3)** and free-running cycle (**Figures 5.2, and Appendix Table 3)** after correction for multiple comparisons**.** If the circadian clock was informing the photoperiodic timer, then we would expect to see differences in one or more of the formal properties over a geographic gradient, contrary to our results. Hence, none of the formal properties of any of the clock genes can account for any co-evolution of the circadian clock and photoperiodic timer. These results refute the damping hypothesis advocated by Lewis and Saunders, two major proponents of Bünning's theory. According to Saunders et al. (2004), experiments that have shown the presence of a strong photoperiodic response and no response to Nanda-Hamner protocol are due to immediate damping of the circadian clock during long dark periods. They claim that because there are built-in time-delay mechanisms in the form of post-translational modifiers in the circadian clock, these core clock oscillations tend to dampen out when exposed to long periods of dark. This rapid damping in turn will cause a lack of rhythmicity to the Nanda-Hamner protocol, seen in the mountain populations of *W. smithii* (Bradshaw et al. 2003). Our results show that there are no

differences in damping of core clock genes over the geographic gradient of *W. smithii* (**Figure 5.2 and Appendix Table 3)**. Hence, the hypothesized variation in damping of the core circadian clock cannot account for variations in response to NH or Bünsow protocols, as well as for the variation of the photoperiodic timer itself. Especially informative is the comparison between the mountain populations (DB and SF) and coastal plain populations (SH and GS) at similar latitudes but different altitudes, where we see no difference in damping. This is an important point because these populations experience similar photoperiods, due to similar latitudes, but different seasonality, due to different altitudes. If the daily circadian clock and seasonal photoperiodic timer were causally linked then one would expect to see differences in the formal properties of the core clock genes between these two population groups due to the different seasonal experiences; this is contrary to our results. There are no differences in the formal properties over altitude-corrected latitude (**Figure 5.1 and 5.2**). Therefore, damping is not responsible for the lack of short day Nanda-Hamner response seen in the mountain populations.

We also found no differences in the formal properties of the core clock genes when comparing populations of distinct evolutionary history of *W. smithii* (**Figures 6.1 and 6.2, and Appendix, Tables3, 4, and 5)**. Although there are no significant differences, when examining the ANOVA % reduction in total sum of squares, there are a few interesting observations. There is a large amount of variation between the Northern and Southern populations for period of the *cycle* gene in the LD data set (**Figure 6.1**). There is also a large amount of variation in the DD data sets when looking at the phase of *cycle* and the period of *cry2* (**Figure 6.2**). These may be interesting targets for future study, especially when considering the theory of a pleiotropically related circadian clock and photoperiodic timer (Reviewed in Emerson et al. 2009). Taken together, we can confidently claim that the differences in the formal properties of the circadian clock suggest independent evolution of the daily circadian clock and seasonal photoperiodic timer.

Advocates of Bünning's theory cite the rhythmic response to the Nanda-Hamner protocol as major support for the functional involvement of the circadian clock with the photoperiodic

timer (reviewed in Nunes and Saunders 1999; Tauber and Kyriacou 2001; Bradshaw et al. 2006). Two essential components of a rhythm are the period and amplitude of the oscillations. Both the amplitude and period of the rhythmic response to the Nanda-Hamner protocol are not correlated with CPP (Bradshaw et al. 2003, 2006, and 2012) leading to the conclusion that the circadian clock and photoperiodic timer have evolved independently. Our results not only support that position, based on physiological phenotypes, but also offers a deeper level of support because we are able to answer questions at the molecular level of the circadian clock. The Nanda-Hamner experiments are designed to test what Bünning described as the 'hands of the clock,' which is the phenotypic output of the circadian clock and photoperiodic timer. The described experiment herein was able to look at the direct, genetic mechanisms underlying the circadian clock to determine if any of their formal properties were potentially responsible for the co-evolution of the circadian clock and photoperiodic timer. We found no evidence to support the hypothesis of a direct mechanistic relationship.

Altogether, the data indicate that the circadian clock and the photoperiodic timer are two distinct process that have adapted to separate environmental pressures (Danks 2005). The rotation, orbit, and axial tilt of the Earth creates both a daily and seasonal cycle of light, temperature, and precipitation. Higher temperatures are experienced at the equator due to more perpendicular exposure to solar radiation, resulting in higher fluctuations of both photoperiod and temperature toward the poles (Hut et al. 2013). Because there is considerable variation in temperatures between years yet photoperiod remains stable, both daily and seasonally, organisms use photoperiod as a signal to predict unfavorable conditions rather than temperature (Hut et al. 2013). This variability in photoperiod over geographic gradients creates a different set of regional pressures, especially for organisms in temperate regions. Thus, the duration of winter and length of reproductive season can be entirely different for higher latitude or altitude populations compared to lower latitude populations. If there were a causal connection between the circadian clock and photoperiodic timer, then the rapid evolution of the photoperiodic timer seen in

invading species (Urbanski et al 2012) would involve significant tradeoffs between the circadian homeostasis and the evolution of photoperiodic time measurement, which has yet to be supported. Consequently, independent evolution should be presumed between the circadian clock and photoperiodic time measurement. Our results do not exclude the possibility of circadian involvement in the photoperiodic response within individual populations, but show that the circadian clock and photoperiodic timer have evolved independently at the molecular as well as the physiological level. Therefore, we support the "commensal model of biological timekeeping' proposed by Bradshaw et al. (2017, Unpublished). In this model, the photoperiodic timer can co-opt one or many downstream output proteins of the circadian clock to act as a time reference point, without disrupting the core circadian clock. These output proteins may serve as a point of evolution, with the photoperiodic timer using these proteins in response to selection imposed by changing seasonal environments. With this 'commensal model' in place, several questions remain. Does this model hold true for multiple species considering the variation in circadian clock models and photoperiodic phenotypes? What downstream proteins act as a reference point for the photoperiodic timer? What gene(s) underlie the photoperiodic response, and are these ubiquitous throughout the organisms that utilize photoperiodism? The answers to many of these questions will require a multifaceted approach, including not only molecular techniques and population biology, but the use of various organisms in both natural and laboratory settings.

CHAPTER V

APPENDIX: FIGURES

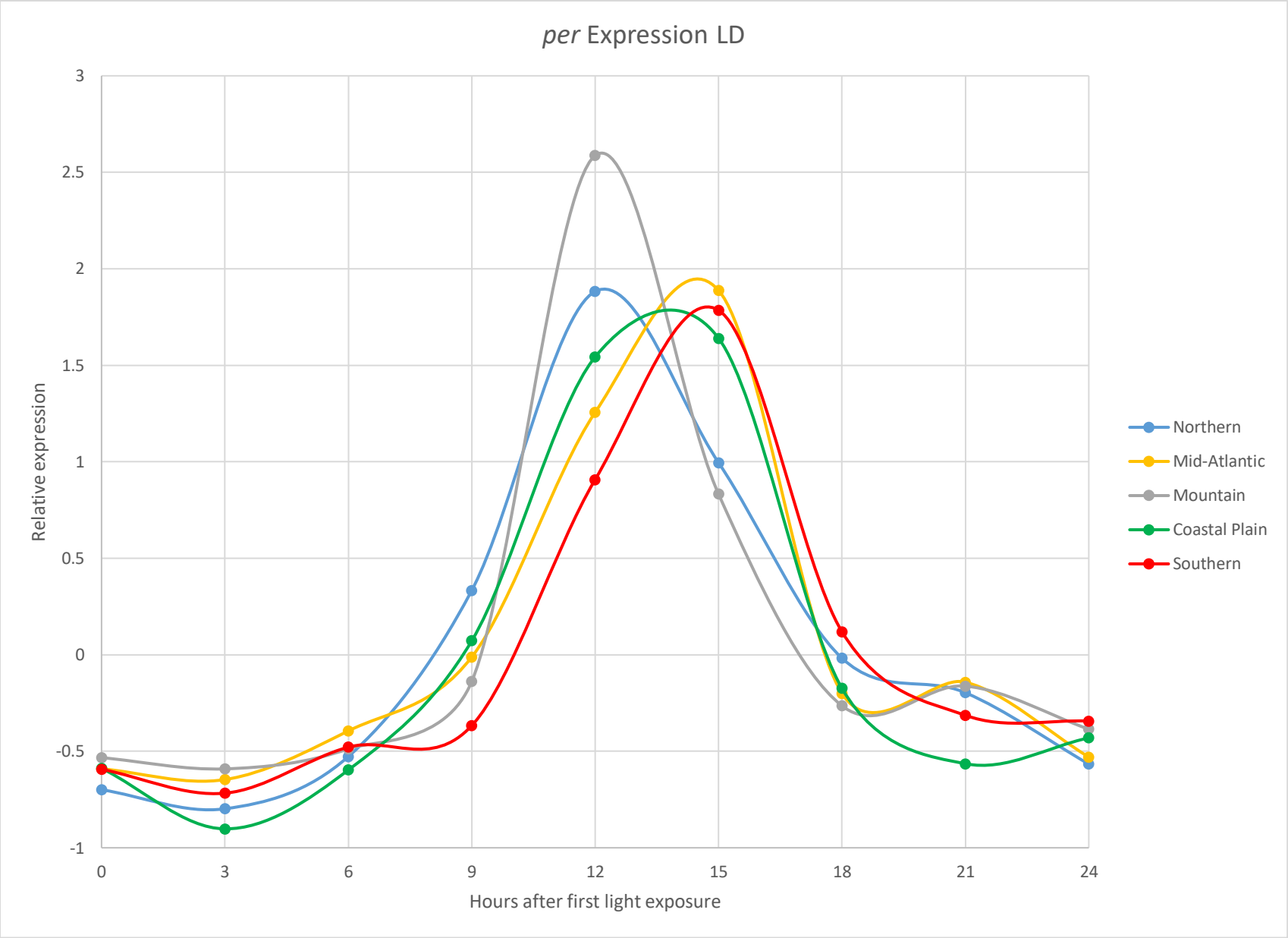

Figure 3.1. Relative expression of *period* over a 24 hour L : D cycle (10 : 12). Populations of similar region were combined making trends more discernable.

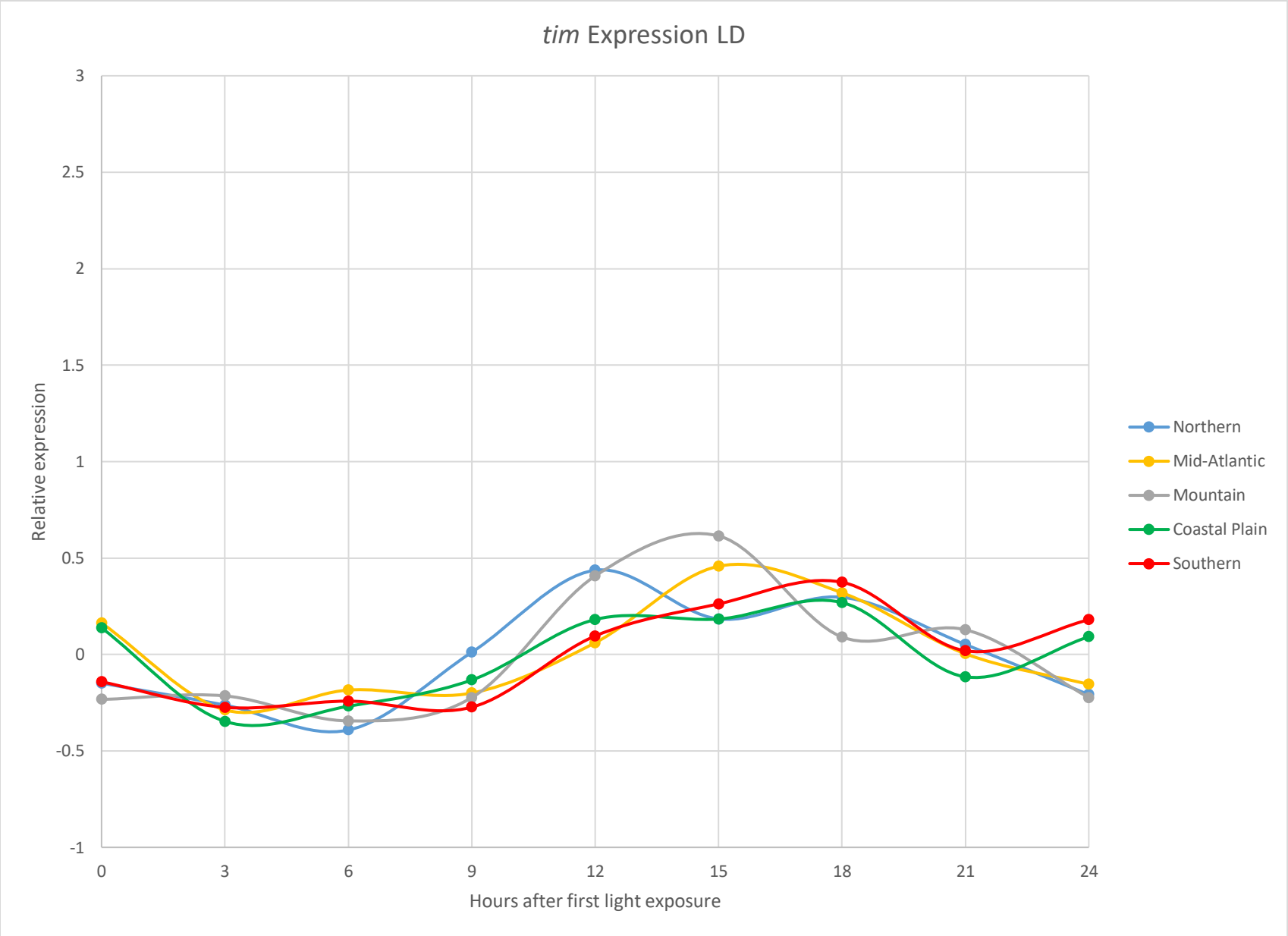

Figure 3.2. Relative expression of *timless* over a 24 hour L : D cycle (10 : 12). Populations of similar region were combined making trends more discernable.

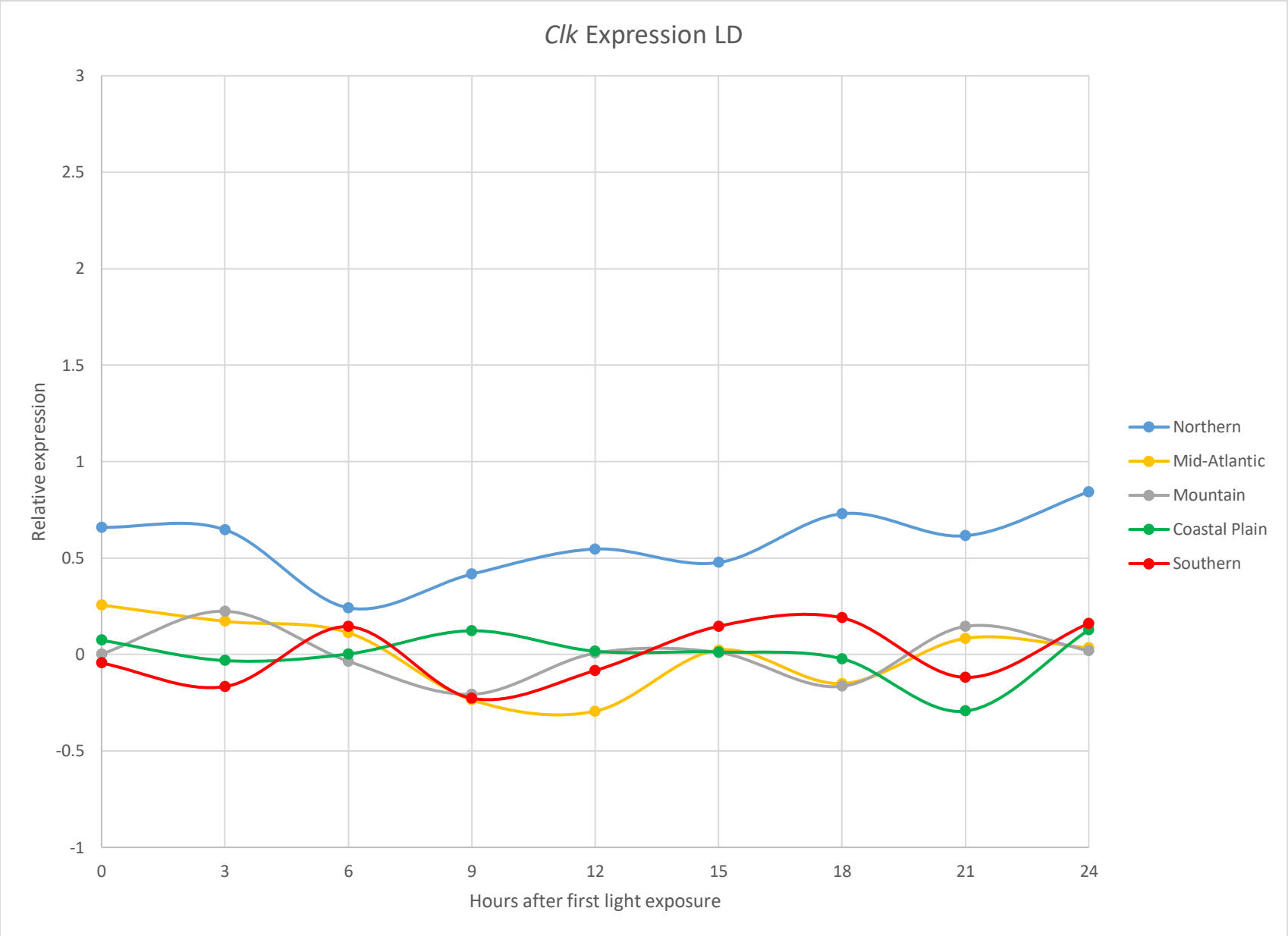

Figure 3.3. Relative expression of *Clock* over a 24 hour L : D cycle (10 : 12). Populations of similar region were combined making trends more discernable.

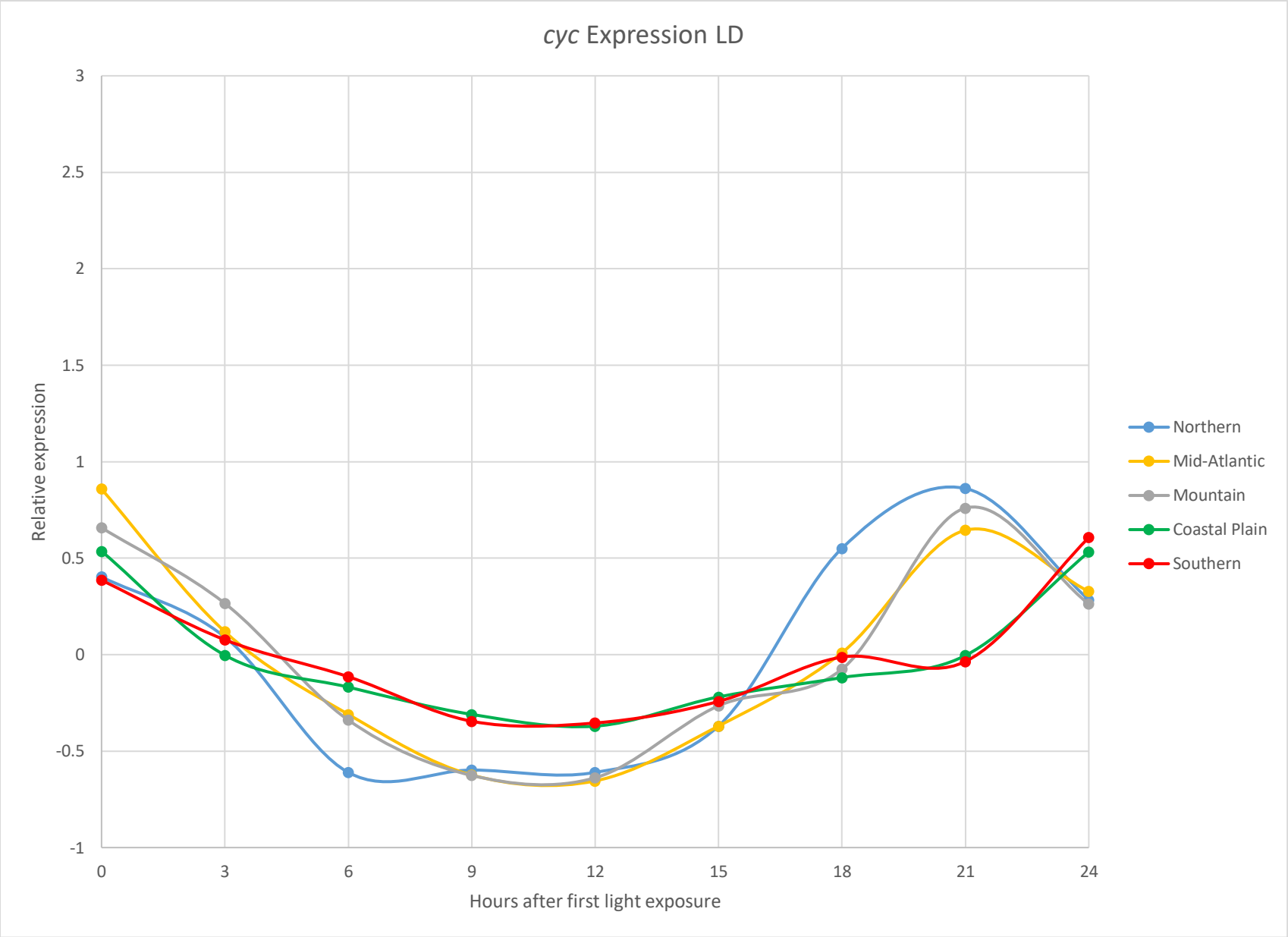

Figure 3.4 Relative expression of *cycle* over a 24 hour L : D cycle (10 : 12). Populations of similar region were combined making trends more discernable.

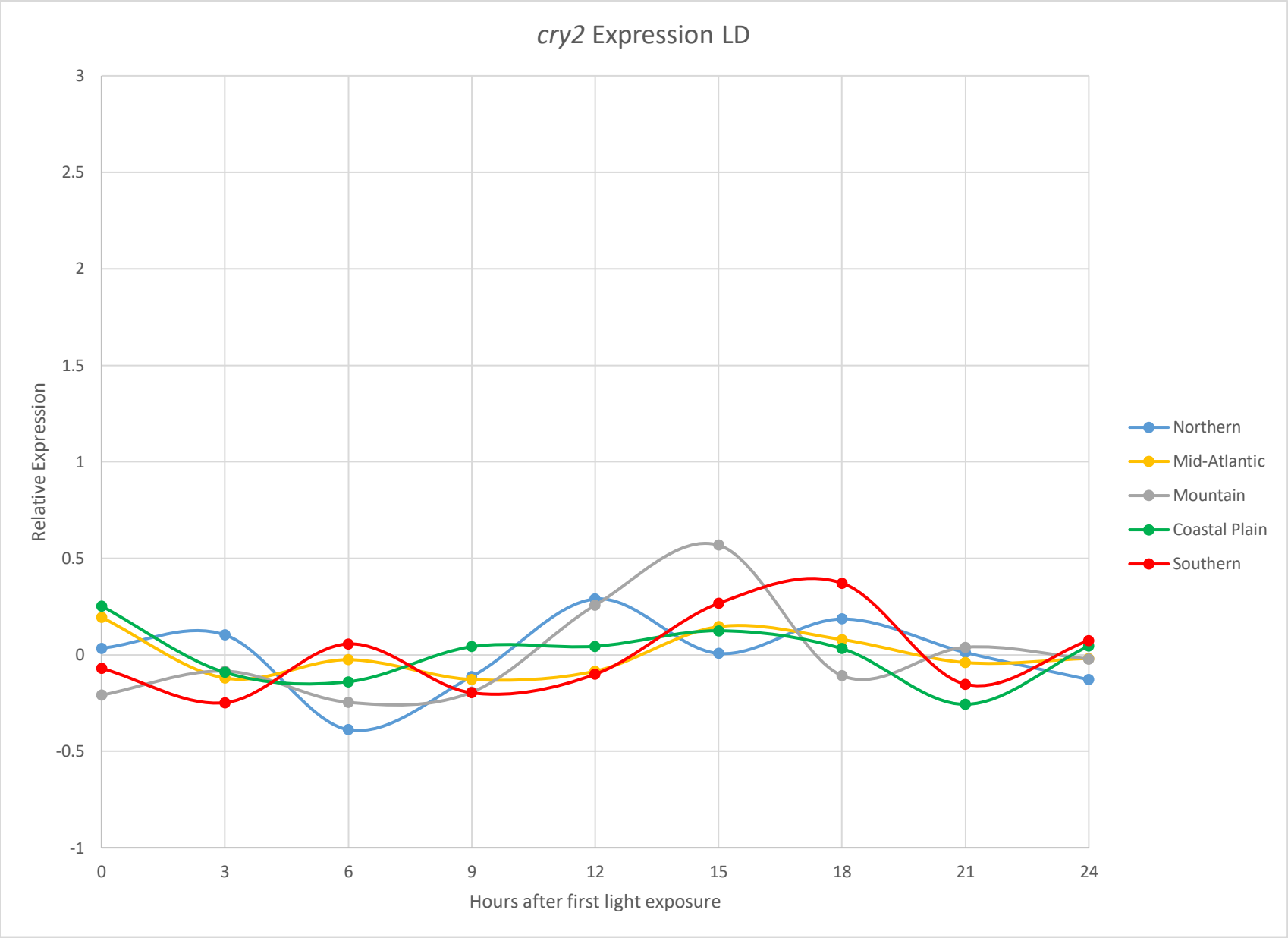

Figure 3.5. Relative expression of *cry2* over a 24 hour L : D cycle (10 : 12). Populations of similar region were combined making trends more discernable.

Figure 4.1. Relative expression of *period* over a 48 hour D : D cycle, exposed to 10 hours of light and 50 hours dark (10 : 50). Data starts from time point 12, the first time point after lights turn off. Populations of similar region were combined making trends more discernable.

Figure 4.2 Relative expression of *timeless* over a 48 hour D : D cycle, exposed to 10 hours of light and 50 hours dark (10 : 50). Data starts from time point 12, the first time point after lights turn off. Populations of similar region were combined making trends more discernable.

Figure 4.3. Relative expression of *Clock* over a 48 hour D : D cycle exposed, to 10 hours of light and 50 hours dark (10 : 50). Data starts from time point 12, the first time point after lights turn off. Populations of similar region were combined making trends more discernable.

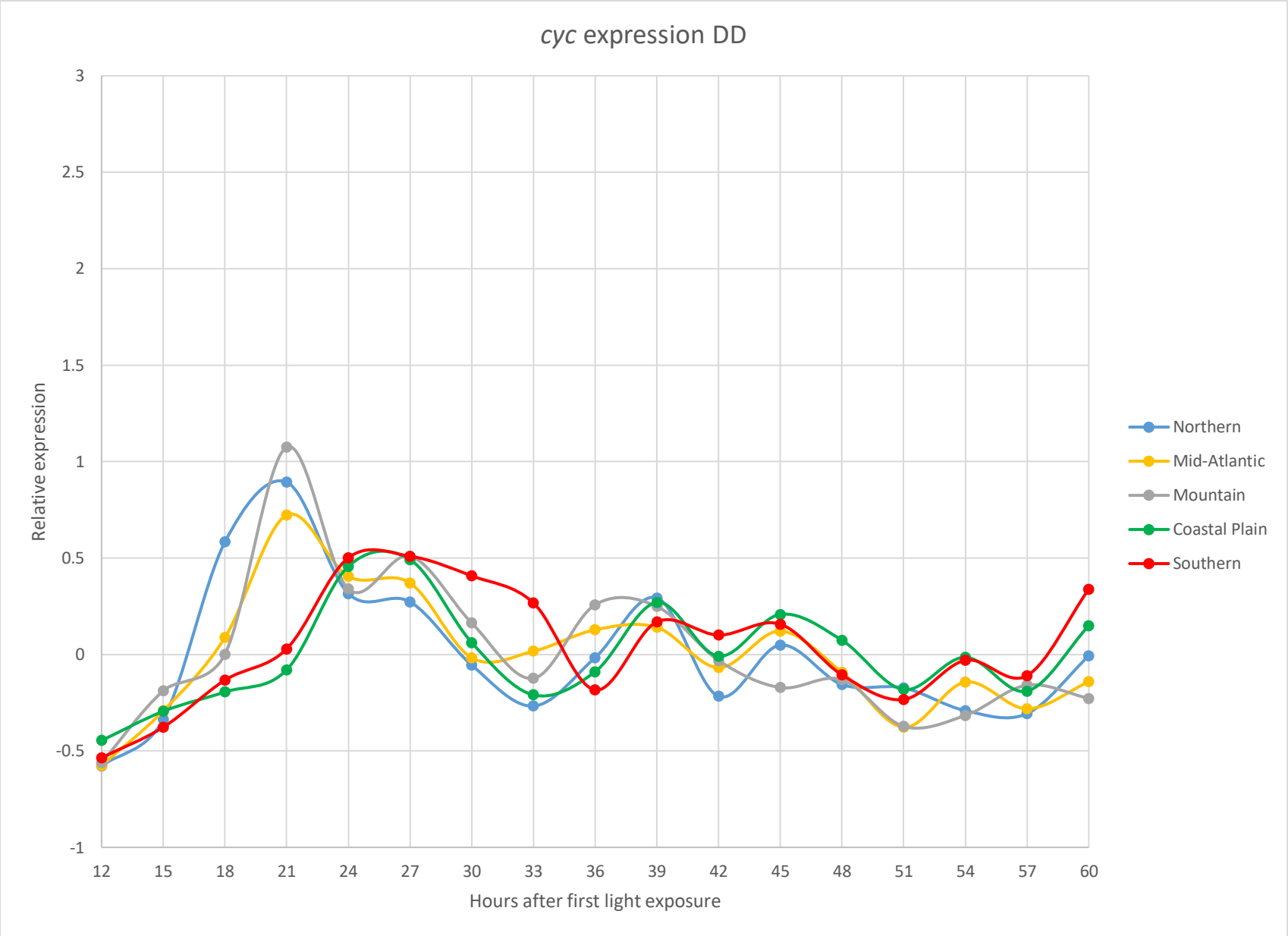

Figure 4.4. Relative expression of *cycle* over a 48 hour D : D cycle exposed, to 10 hours of light and 50 hours dark (10 : 50). Data starts from time point 12, the first time point after lights turn off. Populations of similar region were combined making trends more discernable.

Figure 4.5. Relative expression of *cycle* over a 48 hour D : D cycle exposed, to 10 hours of light and 50 hours dark (10 : 50). Data starts from time point 12, the first time point after lights turn off. Populations of similar region were combined making trends more discernable.

Table 3. Holm-Bonferroni Sequential correction for the regression analysis, calculated using p-values (SOM). A p-value is only considered significant if it is less than the adjusted p-value.

Holm-Bonferroni Sequential Correction - Regression					
Regression LD			Regression DD		
p-value	Ranked Comparisons	Adjusted p-value	p-value	Ranked Comparisons	Adjusted p-value
0.1475	15	0.0033	0.0413	20	0.0025
0.1749	14	0.0036	0.1581	19	0.0026
0.2277	13	0.0038	0.1605	18	0.0028
0.2687	12	0.0042	0.1843	17	0.0029
0.2799	11	0.0045	0.2167	16	0.0031
0.3056	10	0.0050	0.2472	15	0.0033
0.3826	9	0.0056	0.2511	14	0.0036
0.3872	8	0.0063	0.2521	13	0.0038
0.4330	7	0.0071	0.4614	12	0.0042
0.4895	6	0.0083	0.4852	11	0.0045
0.5568	5	0.0100	0.5151	10	0.0050
0.6100	4	0.0125	0.5578	9	0.0056
0.7231	3	0.0167	0.5834	8	0.0063
0.9124	2	0.0250	0.6734	7	0.0071
0.9891	1	0.0500	0.8175	6	0.0083
			0.8317	5	0.0100
			0.8779	4	0.0125
			0.8862	3	0.0167
			0.9525	2	0.0250
			0.9550	1	0.0500

Table 4. ANOVA p-values for LD (a) and DD (b) data sets comparing the 3 or 4 parameters of the northern (KC, ML, PB, HV, DB, and SF) and southern (WI, LI, GS, and SH) populations. Significant p-values, before Bonferroni correction, are in red. After Bonferroni correction, no p-values rise to the level of significance

ANOVA LD p-values (a)			
Gene	**Period**	**Amplitude**	**Phase**
Period	0.916	0.716	0.146
Timeless	0.554	0.788	0.375
Clock	0.418	0.549	0.846
Cycle	**0.013**	0.370	0.091
Cry2	0.345	0.383	0.778

ANOVA DD p-values (b)				
Gene	**Period**	**Amplitude**	**Phase**	**Damping**
Period	0.256	0.640	0.301	0.305
Timeless	0.611	0.131	0.161	0.158
Clock	0.343	0.728	0.718	0.689
Cycle	0.216	0.352	**0.011**	0.554
Cry2	**0.048**	0.098	0.740	0.150

Table 5. ANOVA % reduction in total sum of squares for LD (**a**) and DD (**b**) data sets comparing the 3 or 4 parameters of the Northern (KC, ML, PB, HV, DB, and SF) and Southern (WI, LI, GS, and SH) populations. Significant p-values, before Bonferroni correction, are in red. After Bonferroni correction, no p-values rise to the level of significance. A larger value indicates that there was a greater amount of variation between the Northern and Southern populations for that formal property.

ANOVA LD % reduction in Total SS (a)

Gene	**Period**	**Amplitude**	**Phase**
Period	0.001	0.017	0.245
Timeless	0.046	0.010	0.099
Clock	0.083	0.047	0.005
Cycle	**0.671**	0.135	0.402
Cry2	0.112	0.096	0.010

ANOVA DD % reduction in Total SS (b)

Gene	**Period**	**Amplitude**	**Phase**	**Damping**
Period	0.157	0.029	0.133	0.130
Timeless	0.039	0.295	0.260	0.263
Clock	0.113	0.016	0.017	0.021
Cycle	0.184	0.109	**0.574**	0.045
Cry2	**0.405**	0.305	0.015	0.241

Table 6. Holm-Bonferroni Sequential correction for the ANOVA, calculated using p-values (SOM). A p-value is only considered significant if it is less than the adjusted p-value.

Holm-Bonferroni Sequential Correction - ANOVA					
	ANOVA LD			ANOVA DD	
p-value	Ranked Comparisons	Adjusted p-value	p-value	Ranked Comparisons	Adjusted p-value
0.0128	15	0.0033	0.0111	20	0.0025
0.0913	14	0.0036	0.0479	19	0.0026
0.1460	13	0.0038	0.0979	18	0.0028
0.3450	12	0.0042	0.1309	17	0.0029
0.3700	11	0.0045	0.1500	16	0.0031
0.3750	10	0.0050	0.1582	15	0.0033
0.3829	9	0.0056	0.1609	14	0.0036
0.4184	8	0.0063	0.2161	13	0.0038
0.5493	7	0.0071	0.2564	12	0.0042
0.5537	6	0.0083	0.3007	11	0.0045
0.7161	5	0.0100	0.3051	10	0.0050
0.7784	4	0.0125	0.3427	9	0.0056
0.7879	3	0.0167	0.3523	8	0.0063
0.8460	2	0.0250	0.5543	7	0.0071
0.9160	1	0.0500	0.6115	6	0.0083
			0.6403	5	0.0100
			0.6887	4	0.0125
			0.7183	3	0.0167
			0.7278	2	0.0250
			0.7403	1	0.0500

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