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Natural Variation and Genetics of Photoperiodism in *Wyeomyia smithii*

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Contents

Abstract

Seasonal change in the temperate and polar regions of Earth determines how the world looks around us and, in fact, how we live our day to day lives. For biological organisms, seasonal change typically involves complex physiological and metabolic reorganization, the majority of which is regulated by photoperiodism. Photoperiodism is the ability of animals and plants to use day length or night length, resulting in life-historical transformations, including seasonal development, migration, reproduction, and dormancy. Seasonal timing determines not only survival and reproductive success, but also the structure and organization of complex communities and, ultimately, the biomes of Earth. Herein, a small mosquito, *Wyeomyia smithii* that lives only in the water-filled leaves of a carnivorous plant over a wide geographic range, is used to explore the genetic and evolutionary basis of photoperiodism. Photoperiodism in *W. smithii* is considered in the context of its historical biogeography in nature to examine the startling finding that recent rapid climate change can drive genetic change in plants and animals at break-neck speed, and to challenge the ponderous 80+ year search for connections between daily and seasonal time-keeping mechanisms. Finally, a model is proposed that reconciles the seemingly disparate 24-h daily clock driven by the invariant rotation of Earth about its axis with the evolutionarily flexible seasonal timer orchestrated by variable seasonality and driven by the rotation of Earth about the Sun.

1. INTRODUCTION

1.1 Raison d'être of photoperiodism

Photoperiodism is essential for the maintenance of plant and animal fitness in temperate and arctic climates. Photoperiodism is the ability of plants and animals to use the length of day or night resulting in modification of their activities (Bradshaw & Holzapfel 2007; Kubota et al. 2014; Lucas-Reina et al. 2015). Photoperiodism orchestrates such seasonal activities as growth, development, reproduction, migration and dormancy that make a direct contribution to survivorship and reproductive success. Hence, being at the right place at the right time of year is essential for optimizing fitness at temperate latitudes. The trick is being able to anticipate and prepare in advance for seasonal change. "Food, temperature, moisture, and air pressure are all very much less regular in their seasonal procession and are therefore likely to be less effective as "clocks" than photoperiod" (Andrewartha & Birch 1954, p. 294). Indeed, in comparison with temperature (**Fig. 1**) the length of day, or photoperiod, fluctuates regularly with the changing seasons and at any given spot on Earth, is the same today as it was 10,000 years ago and will be 10,000 years into the future. Hence, photoperiod constitutes the most reliable environmental cue for predicting future seasonal change through evolutionary time. It should be no surprise that plants from algae to angiosperms and animals from rotifers to rodents use photoperiodism to regulate their seasonal activities (Nelson, Denlinger, & Somers 2010).

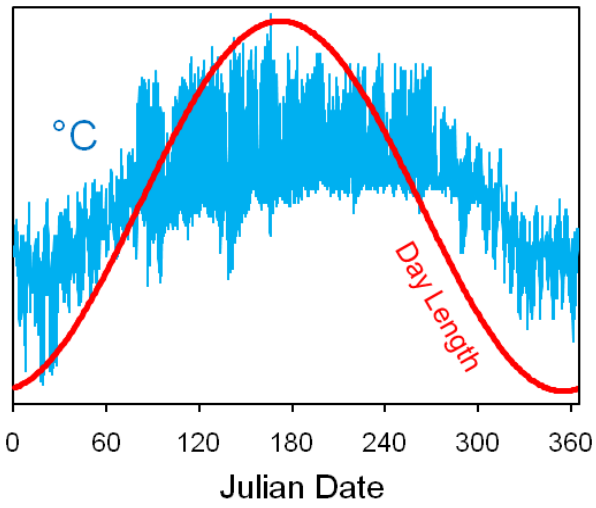

Figure 1*. Annual variation of day length and temperature.* Temperature in a pitcher-plant leaf measured every two hours for one year. For comparison, day length is shown for the equivalent date.

1.2 Geographic clines

Geographically variable climates impose concomitant selective pressure on the timing of seasonal activities and, hence, on photoperiodic response. Length of the growing season favorable for growth, development and reproduction decreases with increasing latitude and altitude; at the same time, the annual fluctuation in day length increases in amplitude with increasing latitude, but not altitude (Bradshaw & Holzapfel 2007a). Hence, the timing of seasonal activities is dependent upon length of the local growing season, the local flux in day length, and the locality-appropriate response to day length. It is important to emphasize that the target of local selection is not day length itself. Rather, selection acts on the genetically determined response of individual organisms to length of day. Genetic variation underlying response to day length is tested in the crucible of the local seasonal environment, forming a feedback loop from the genome through photoperiodic response to concordance of resulting phenotypes with season-specific opportunities and exigencies. Seasonal selection then winnows out early or late phenotypes and alters allelic frequencies in genes programming photoperiodic response, thereby altering the timing of seasonal events. In sum, photoperiodic response represents the physiological connection between the genome and the seasonal phenotype and, as seasonal climate changes in space and time, so also do the observed photoperiodic responses of organisms that live in that climate.

1.3 Photoperiodism in *Wyeomyia smithii*

1.3.1 Photoperiodism & phylogeography

Wyeomyia smithii overwinters as diapausing larvae in the evergreen leaves of the carnivorous purple pitcher plant. The robustness of its photoperiodic response and the consistency of its habitat over a broad geographic range results in a clear signal of seasonal and photoperiodic adaptation. In order to understand the evolution and genetics of photoperiodism, it is important to use as an experimental organism one that is robustly photoperiodic in the first place. The

pitcher-plant mosquito enters an hibernal diapause as larvae: Short days initiate and maintain diapause; long days promote continuous development or the resumption of development in diapausing larvae. The day length promoting 50% development and 50% diapause (the critical photoperiod) is the same for the initiation and termination of diapause in unchilled larvae (Bradshaw & Lounibos 1972). For the initiation of diapause, insects pass through a "sensitive period" during which day length is physiologically interpreted as long or short resulting in a diapause/no-diapause response (Beach 1978; Saunders 1981; Taylor 1985). *Wyeomyia smithii* and some other insects are robustly photoperiodic while in diapause. For these insects the sensitive period is indefinitely long and the effect of manipulating different light:dark cycles can be assessed over weeks or months, instead of a few days, greatly enhancing their value in exploring photoperiodism.

Wyeomyia smithii oviposit into and complete their entire pre-adult development only within the water-filled leaves of the carnivorous pitcher plant *Sarracenia purpurea* (Smith 1902), which is common in swamps and bogs from the Gulf of Mexico to northern Canada and from sea level to high mountain seeps in the Appalachian Mountains. Throughout this range, *W. smithii* occupies a uniform microhabitat whose community composition remains highly consistent (Bradshaw 1983; Bradshaw & Creelman 1984; Buckley et al. 2003, 2010),

Dispersal of *W. smithii* in North America has been coincident with the historical biogeography of peatlands during and following retreat of the Laurentide Ice Sheet (Istock & Weisburg 1987; Halsey, Vitt, & Gignac 2000; Gorham, et al. 2007; Merz et al. 2013). Consistent with this distinction between the descendents of more ancient populations in the south and more recent populations in the north, isolation-by-distance is about six times greater among southern than northern populations (Armbruster et al. 1998). Hence, the phylogeography of *W. smithii* presents diverse end points of evolutionary dispersal in space and time. Concomitantly, photoperiodic response has been exposed to diverse seasonal changes, both *in situ* and during post-glacial dispersal. This history of populations over climatic gradients in space and time provides the necessary background for interpreting the evolutionary genetics of photoperiodism in *W. smithii*.

1.3.2 Geography of photoperiodic response

Geographically variable seasonal climates select for geographically variable photoperiodic responses. Critical photoperiod in W. smithii *tracks the climatic gradient of North America more closely than any other known eco-geographic trait. Comparing the action spectra of light regulating diapause with light availability in pitcher plant leaves during twilight defines a photoperiodic day and enables accurate prediction of the timing of diapause in nature from photoperiodic response determined under appropriately controlled conditions in the laboratory.* As one proceeds northwards in latitude or upwards in altitude, winters arrive earlier and springs arrive later in the year, shortening the length of the growing season. The optimal time to enter diapause is just over one full generation in advance of the onset of winter. The reason is that, at any given point in developmental time, an insect has either to continue developing or to enter

diapause. If the insect continues to develop beyond its sensitive period, it must complete an additional full generation before its next sensitive period and be able to replace itself with at least one diapausing offspring in the overwintering population (Taylor 1980). There are two important consequences of this reality. First, the diapause/no-diapause switch must be made when temperatures are declining. The optimal time to enter diapause then occurs when there remains more than the minimal number of day-degrees above threshold required to complete an entire generation. This go/no-go point can occur at a time of year when current environmental conditions are otherwise optimal for growth, development, and reproduction. This scenario underscores the importance of photoperiodism: Photoperiodically controlled regulation of diapause can override favorable temperature, humidity, and resources, ensuring that the individual will be in diapause when winter arrives.

Second, the earlier winter arrives, the earlier is the optimal time to enter diapause. Winter arrives earlier at higher than lower latitudes or altitudes. Hence, the optimal time to enter diapause should be earlier and at longer day lengths at higher than lower latitudes or altitudes. Consequently, the critical photoperiod mediating diapause should increase with increasing latitude and altitude. The correlation between critical photoperiod and latitude has long been recognized (Danilevskii 1965), but the relationship between altitude and latitude was first established in *W. smithii* (Bradshaw 1976). Photoperiodic response curves mediating diapause in *W. smithii* show remarkably parallel sigmoid patterns across populations from the Gulf of Mexico to Canada and from sea level to over 1,000m in the Appalachians (Bradshaw, Holzapfel & Mathias 2006). The photoperiodic response curves shift consistently from left to right with increasing latitude, with the exception of populations from the southern Appalachian Mountains at 35°N whose curves approximate those from low elevations at 40-41°N. These earlier studies, run at a constant temperature, were extended by using a sine-wave thermoperiod that varied from 13-29°C each day and lagged the photoperiod by 3h as in nature (Lair, Bradshaw & Holzapfel 1997). Again, critical photoperiod among eight independently collected populations increased with latitude and altitude of population origin (R^2 = 0.0.98, P<0.001). In total, the regression of critical photoperiod on latitude and altitude has been repeated on four separate occasions using two different approaches and consistently yields $R^2 \geq 0.92$ (Bradshaw & Holzapfel 2001). Critical photoperiod evolved 10 standard deviations across the range of populations sampled, indicating directional selection on a continental scale. By contrast, standard deviations of critical photoperiod did not vary with either latitude or altitude (R^2 = 0.14, P = 0.682), indicating stabilizing selection on a local scale (Hard, Bradshaw, & Holzapfel 1993; Lair, Bradshaw, & Holzapfel 1997).

The question remains as to whether critical photoperiods determined in the laboratory even under carefully controlled conditions are relevant to the timing of development in nature. To answer this question, Bradshaw & Phillips (1980) determined the astronomic units defining a photoperiodic "day" in nature by comparing the action spectra for photoperiodic response with the light-availability spectra within pitcher-plant leaves during twilight in nature. They determined that a day began at the onset of civil twilight in the dawn and extended through the

end of civil twilight in the dusk. These results enabled using the critical photoperiods from Bradshaw, Quebodeaux, & Holzapfel (2003a), adjusted for twilight duration, to plot subjective day lengths as perceived by *W. smithii* on latitude and time of year, generating lines of iso-day lengths (**Fig. 2**). Actual time of entry into diapause in the field at four localities separated by more than 15° of latitude was accurately predicted by critical photoperiods determined in the laboratory.

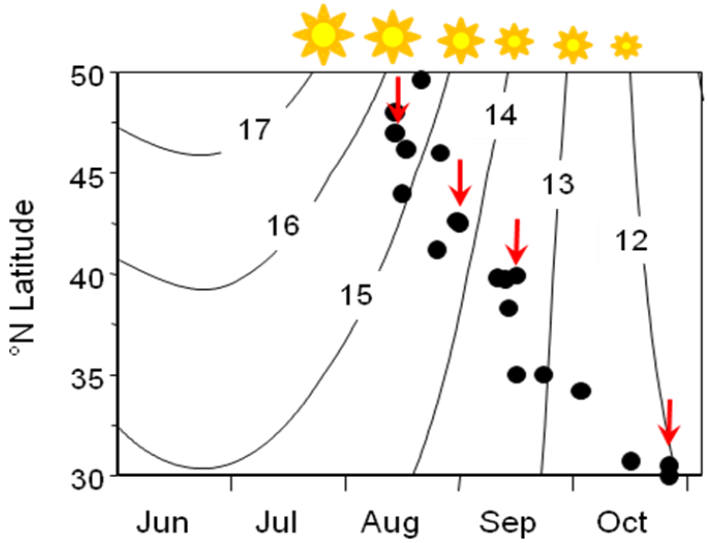

Figure 2. ***Day length, latitude and initiation of diapause.*** "Iso-day lengths" (sunrise-sunset with flanking civil twilights) vary with latitude and time of year. Critical photoperiods determined in computer-controlled environment rooms (black circles) are plotted according to latitude. The actual date of entry into diapause in the field is known for four localities. The red arrows indicate the date predicted from lab critical photoperiods for the initiation of diapause at the four localities to which they point.

2. GENETICS OF PHOTOPERIODIC RESPONSE

Photoperiodic response among populations "breeds true" under constant conditions in the laboratory and is highly heritable. Extant populations in nature harbor sufficient genetic variation in photoperiodic response to respond to rapidly changing seasonal environments. Photoperiodism is a polymorphic trait, determined by many genes of varying effect. The additive effect of genes is immediately available to respond to selection; other genetic effects appear only in changing environments (genotype by environment interaction) or are hidden by interaction between alleles at the same locus (dominance relationships) or at different loci (gene-gene interaction or epistasis). The genes themselves remain elusive, but their combined effects and regions of the chromosome where they reside can be determined statistically using quantitative genetics. Evolution of photoperiodic response within and between populations of W. smithii has involved a complex web of both additive and non-additive effects, resulting in unique genetic architectures underlying individual populations. There is no "wild-type" photoperiodic response in W. smithii *and any evaluation of genetic effects must take the population-specific genetic background into account. Unraveling the genetics of the seasonal photoperiodic timing mechanism is going to be far more complex than that of the daily circadian clock.*

2.1 Genetic potential to respond to selection

Heritable genetic variation for photoperiodic response in W. smithii *exists within and among populations. Heritabilities within populations are all non-zero, but increase dramatically in northern, post-glacial populations.*

Genetic variation in photoperiodic response among populations is readily evidenced by the fact that differences among populations persist in "common garden" experiments. In common garden experiments, populations are reared for two or more generations under the same conditions in the lab and then are exposed to identical experimental conditions. Since the environment is being held constant, differences that persist among populations are genetic in origin. In addition, like Danilevskii's (1965) pioneering experiments, hybrids between populations of *W. smithii* differing in photoperiodic response exhibit intermediate phenotypes (Bradshaw & Lounibos 1977; Hard, Bradshaw, & Holzapfel 1992, 1993; Lair, Bradshaw & Holzapfel, 1997).

The precise geographic cline in photoperiodic response and phenotypic variation within populations predicts that there should be substantial genetic variation in populations that is available to respond to selection in climates that change in space and time. Genetic potential to respond to selection depends on the heritability of a trait. The problem in estimating heritability for photoperiodic response is that this trait is typically assessed by the incidence of diapause in a cohort of individuals exposed to a given experimental regimen. Hence, critical photoperiod is described as a property of a group of organisms, not individual organisms. Because *W. smithii* responds to day length while in diapause, diapausing larvae can be exposed to short days that increment by 2-3min per day as at mid-latitudes in nature. At some point, each individual interprets the day length as long and develops. The day an individual pupates is then scored as its individual critical photoperiod. Critical photoperiod is then directly expressed as a continuously distributed trait with mean and variance. Mean and standard errors of heritabilities are then estimated from the slope (*b*) from regression of offspring mean critical photoperiod on parent mean critical photoperiod across multiple parent-offspring cohorts (Bradshaw & Holzapfel 1990). Critical photoperiods estimated from this approach include recognition of long day lengths and accumulation of photoperiodic information required to initiate development. Heritabilities of critical photoperiod ranged from 0.15-0.27 in three southern populations and 0.35-0.79 in three northern populations. Hence, extant populations harbor the genetic potential to respond to selection on photoperiodic response and this potential is higher in northern than southern populations.

2.2 Hidden genetic variation

Additive genetic variance is the primary, major source of phenotypic response to selection in large, outbreeding populations. When environmental conditions change, so also can the additive effects of genes (genotype by environment interaction or GxE). GxE can itself be altered by selection and can be a source of genetic variation contributing to adaptation. During migration

and the founding of new isolated populations, population size is initially small and genetic drift becomes an important component of the genetic composition of populations. Isolation and drift can unmask recessive and epistatic alleles formerly hidden within ancestral populations. During population expansion following a founder event, selection assumes an ever-increasing role not only on phenotypes under selection, but also on novel combinations of genes contribution to evolving phenotypes. (Mayr 1954; Fisher 1958; Wright 1968, 1977; Wallace 1991; Gibson & Dworkin 2004; Phillips 2008; Paaby & Rockman 2014; Paaby & Gibson 2016; Yadav, Dhole & Sinha, 2016).

2.2.1 Genotype by environment interaction

Differential response to day length represents a form of developmental phenotypic plasticity (phenotypic variation of an individual in different environments) and the heritability of photoperiodic response constitutes genetic variation in phenotypic plasticity, i.e., genotype by environment interaction (GxE). Selection on critical photoperiod exposes responses to exotic day lengths not ordinarily seen by populations in nature, thereby revealing GxE as a potential source of genetic variation in changing seasonal environments.

The first "hiding place" of genetic variation lies in the interaction between genotype and environment (GxE). Diapause in *W. smithii* represents a form of phenotypic plasticity: depending on the photic environment (day length), individuals may or may not enter diapause and critical photoperiod is a heritable trait, i.e., diapause is determined by interaction between the genotype and the environment (Chevin & Lande 2015). One of the hallmarks of GxE's contribution to hidden genetic variation is a phenotype that is differentially expressed under conditions to which the population is never exposed. After imposition of truncation selection for long and short critical photoperiods in three sub-populations from New Jersey (Bradshaw, Quebodeaux & Holzapfel 2003b), developmental response to exotic short days never experienced in nature is enhanced in the short-selected lines and reduced in the long-selected lines. Even more unexpected is marked reduction in diapause at exotic long day lengths in both the short- and long-selected lines. Selection has clearly revealed hidden genetic variation for diapause-response to exotic day lengths. Hence, GxE can increase the ability of populations to respond to changing seasonal climates, including the advance and regression of glaciers or recent rapid climate change.

2.2.2 Dominance and epistasis

Interaction between alleles both within and among populations provides novel genetic variation to explain complex genetic architectures underlying photoperiodism and its evolutionary flexibility. The second hiding place of genetic variation lies in allelic and genic interactions, i.e., dominance and epistasis that are revealed by independent assortment and recombination in hybrid phenotypes. Additive and non-additive genetic effects on differences in critical photoperiod among latitudes, longitudes, and altitudes are shown in **Table 1** based on 14-generation line crosses (Lair, Bradshaw & Holzapfel 1997). Expected generation means were

estimated from F-∞ parameterization as in Hayman (1958, 1960ab) and Mather & Jinks (1982, Chs. 5, 10). Models of genetic effects were tested, sequentially, for adequacy of additive, additive-dominance, additive-dominance-maternal-and additive-dominance-maternal-digenic epistasis models. Rejection of the most inclusive model indicated significant higher order epistasis and/or linkage effects.

Table 1. ***Differences in genetic effects among populations***

Cross	Add	Dom	Amat	Dmat	AxA	AxD	DxD	ADME
FLxAL	***	***	*	***	***	°	**	°
FLxMt	***	°	°	**	°	***	*	°
NCxMt	***	***	°	°	°	°	***	°
MtxME	***	°	***	*	***	°	°	***
NJxON	***	°	°	**	–	–	–	–
WIxME	***	***	°	°	–	–	–	–
MExON	***	**	**	**	°	°	***	°

Significance of genetic effects given by: ° P>0.05; * P<0.05; **P<0.01; ***P<0.001 after row-wide sequential Bonferoni correction. Add, additive; Dom, dominance; Amat, additive maternal; Dmat, dominance maternal; AxA, additive by additive digenic epistasis; AxD, additive by dominance digenic epistasis; DxD, dominance by dominance digenic epistasis. ADME, test for adequacy of the combined additive-dominance-maternal model. A dash (**–**) indicates no tests for digenic epistasis were made because the additive-dominance-maternal model was adequate to explain genetic differences in critical photoperiod between parent populations. Mt indicates a population from the southern Appalachian Mountains. Summarized from Lair, Bradshaw & Holzapfel (1997).

Several patterns emerge. First, there are always strong additive effects contributing to the genetic differences in critical photoperiod among populations. Second, either dominance or maternal dominance effects contributed to genetic differences in critical photoperiod among populations. Third, digenic epistasis made significant contribution to genetic differences in critical photoperiod in five of the seven crosses; the two exceptions both occurred in crosses between two populations within the northern clade (WIxME and NJxON) and not between the cross within the southern clade (FLxAL) that was separated by less geographic and phenotypic distance. Third, there was no consistent pattern of digenic epistasis among crosses, supporting Hard, Bradshaw, & Holzapfel's (1992, 1993) conclusion that different genetic architectures contributed to the evolutionary divergence of critical photoperiod among populations. What Table 1 shows is that rampant and diverse hidden genetic variation for photoperiodic response exists without any apparent pattern throughout the range of *W. smithii*. Evolution of photoperiodic response in *W. smithii* is founded on a rugged genetic landscape of allelic and genic interactions.

During dispersal of *W. smithii* in North America, the additive genetic variance for critical photoperiod has increased exponentially; over the same range, average heterozygosity of individual protein-coding loci has declined, as would be expected from sequential founder events

following recession of the Laurentide Ice Sheet 20,000 years ago (Armbruster et al. 1998). Based on a consistent involvement of epistasis in south to north differentiation of post-glacial populations, Hard, Bradshaw, & Holzapfel (1993) attributed this increase in additive genetic variance to a release of additive from non-additive genetic variance during isolation and drift following successive founder events.

For there to be a release of additive from non-additive genetic variance during the differentiation of populations, there has to be non-additive genetic variation within populations in the first place. To test this proposition, Bradshaw, Haggerty, & Holzapfel (2005) tested for non-additive genetic variance in a single New Jersey meta-population (40°N). They collected from three interconnected sub-populations within a 200m radius in this large population and within each sub-population, imposed selection for divergent critical photoperiod for 13 generations. After selection, they performed a 14-generation line cross within each. In each case, the joint scaling test rejected an additive-dominance-maternal model and the signature of digenic epistasis was unique to each sub-population. These results showed first that non-additive genetic variance for photoperiodic response existed in all three of these sites, consistent with the concept of there being non-additive genetic variance available within populations to be converted to additive genetic variance. These results also showed either that there was spatial genetic structure within the New Jersey meta-population over a range of just 200m, that early events in their responses to selection sent them down different genetic trajectories, or a combination of both.

Short-term selection within a single meta-population of *W. smithii* revealed sufficient non-additive genetic variance to explain the release of additive from non-additive variance during their post-glacial dispersal. More importantly, there exists within a single meta-population of *W. smithii* the full range of potential allelic and genic interactions observed across the entire species from Florida to Canada shown in **Table 1.**

2.3 Mapping genotype to phenotype

The number of genes and position of genes within the genome that contribute to the evolution of photoperiodic time measurement can be estimated from line crosses and quantitative trait loci (QTL), respectively. The latter approach reveals polymorphism in QTL within and between populations and confirms that the perception of genes contributing to photoperiodic response are crucially dependent on genetic background.

While a great deal is known about the genetics of the daily circadian clock in insects, including at the molecular level within species and between orders of insects (Tormey et al. 2015), the actual genes involved in the seasonal photoperiodic timer, apart from being *somehow* connected to the circadian clock (Sec. 4) remain elusive. The number of genes can be estimated from line cross means and variances via the Castle-Wright-Lande model that estimates the minimum number of "effective factors" contributing to genetic differences among populations or lines (Roff, 1997, p. 11; Lynch & Walsh 1998, p. 233). For photoperiodic response, these

estimates range from 0-19, and increase with phenotypic distance between the parent populations (Hard, Bradshaw & Holzapfel 1992; Lair, Bradshaw & Holzapfel 1997).

An alternate approach is to use quantitative trait locus mapping (QTL) to estimate the number and effect of regions in the genome containing the genes or groups of genes contributing to genetic differences in critical photoperiod. In the first QTL for photoperiodic response in any animal, Mathias et al. (2007) made an F_2 hybrid cross between geographic extremes of *W. smithii*, a Florida ♀ (31°N) × an Alberta ♂ (57°N). The critical photoperiods of both the parents and F_2's were determined with incremental day lengths and genotyped using AFLP and gene-based markers. The QTL map was based on 45 marker loci spanning 287cM from 264 F_2 individuals. *Wyeomyia smithii*, like other culicine mosquitoes, has three chromosomes (Moeur & Istock 1982). Composite interval mapping (CIM) revealed nine QTL: two on the first chromosome bearing the sex locus, six on the second chromosome, and one on the third, accounting for an estimated 62% of the phenotypic variance. The latter portion of the second chromosome includes an overlapping region of QTL for photoperiodic response as well as stage of diapause. Also included in this region are epistatic interactions between one of the markers and five other markers within this region, including two ecdysteroid activated genes, *Pep* and *Impl3*. This region of the second chromosome is then a candidate "hot spot" for genes integrating continuous development, photoperiodic response, diapause and metabolism in *W. smithii* (Mathias et al. 2007)

Individual QTL represent a region of the genome, and may include multiple genes. In addition, resolution of QTL is limited by the number of markers, the number of individuals phenotyped and genotyped, and the number of recombination events preceding the genotyping generation (Beavis 1999; Xu 2003; Phillips 2005; Mackay, Stone, & Ayroles 2009). In a separate series of crosses involving entirely different localities, Bradshaw et al. (2012) examined QTL for photoperiodic response among three populations based on single-pair matings of *W. smithii*: New Jersey♂ × Maine ♀ (NJ×ME) separated by ~13,000 y of evolutionary time, and reciprocal single-pair matings of Alabama × Maine (AL×ME, ME×AL) separated by ~200,000 years of evolutionary time (Merz et al. 2013). RAD markers greatly increased the resolution of QTL with an average of 220 markers and 660cM per cross.

Composite interval mapping (CIM) of photoperiodic response between the New Jersey and Maine populations revealed two QTL, one on the second and one on the third chromosome. CIM of the AL♂×ME♀ revealed three QTL, all on the first chromosome. Finally, CIM of the reciprocal ME×AL revealed two QTL, one on the first and one on the second chromosome. There was no overlap in QTL among the three maps. Both of the QTL in NJ×ME showed dominance deviation towards the southern parent; all of the QTL in AL×ME showed dominance deviation towards the northern parent; the one QTL in the reciprocal ME×AL showing significant dominance deviation was towards the northern parent. Hence, there was no consistent position of QTL or sign of additive or dominance effects among the three crosses.

Reciprocal crosses between different parents within the same population indicated as much variation in regions of the genome contributing to differences in photoperiodic response as

among all crosses separated over wide geographic space and evolutionary time. Perception of regions of the genome, much less individual genes contributing to the evolution of photoperiodic response depends crucially on the genetic background within individual populations.

2.4 Lessons from quantitative genetic approaches in *Wyeomyia smithii*

Line crosses and QTL mapping of genomic regions contributing to divergence of photoperiodic response reveal complex underlying genetic architectures within populations, between nearby populations, and among populations that are more remote in space and time. In a sense, there is no standard genetic background or unique "wild type." Any evaluation of standing genetic variation within a population, genetic structure among nearby sub-populations, or potential genetic processes underlying current patterns of phenotypic variation or even phenotypic similarities has to take genetic background into account.

Both line-cross analyses and QTL mapping have revealed a complex genetic architecture underlying genetic variation within local populations as well among populations having dispersed over the climatic gradient of North America. Genotype by environment interaction, maternal effects, dominance, and epistasis provide hidden sources of genetic variation that potentially can be recruited during dispersal across climatic zones or within local populations during periods of climate change. Most importantly, convergent phenotypes can have divergent underlying genetic architectures and even local populations contain additive and non-additive genetic variation sufficient to generate novel genetic architectures in descendent populations of a dispersing species.

3. PHOTOPERIODISM & CLIMATE CHANGE

Recent rapid climate change has been imposing primarily seasonal, not thermal selection on the biotic world. Response to this selection has resulted in the expansion of animal activities during progressively longer growing seasons and in a genetic (evolutionary) shift in photoperiodic response towards more southern-like critical photoperiods.

The last five decades have brought a challenge to research scientists: Earth has been warming at an accelerated rate, unprecedented in the last millennium (Stott 2000). Polar and temperate winters have become milder and shorter, and growing seasons have become longer. The abiotic effects of global warming have resulted in melting glaciers and rising sea levels (Karl & Trenberth 2003; IPCC 2007); but, animals are not glaciers and the main effects on temperate and polar biota have been new opportunities resulting from the opening of new climatic zones poleward and from longer growing seasons without imposing appreciable heat stress (Bradshaw & Holzapfel 2010b, 2010c). The result is that animals have been expanding their ranges poleward, and exploiting longer growing seasons by increasing time spent growing, developing, and reproducing, and by migrating earlier in the spring and later in the fall. In short, animals have been entering new photic zones and experiencing selection on the timing of seasonal activities in their life histories (Hughes 2000; Peñuelas & Filella 2001; Root et al. 2003; Parmesan & Yohe 2006). Initially, these patterns could be ascribed to phenotypic plasticity, but

genetic shifts in photoperiodic response in *Wyeomyia smithii* provided the first evidence that recent rapid climate change has actually been driving genetic change (evolution) in natural populations (Bradshaw & Holzapfel 2001). Over a 24-yr period, there has been a genetic shift towards shorter, more southern-like critical photoperiods. This shift is detectable after only five years, representing evolution at break-neck speed in nature. The shift is also more pronounced in the north than in the south, reflecting both the higher additive genetic variance for photoperiodic response in northern populations and the trend of climate change to be faster in the north than in the south. Evolutionary responses were subsequently confirmed in plants, other insects, birds, and mammals (Bradshaw & Holzapfel 2006, 2008, 2010b; Franks, Sim & Weis 2007; Gomi et al. 2007; Urbansky et al. 2012), all reflecting seasonal, not thermal adaptation.

Definitive evidence of the relative importance of photic vs. thermal adaptation imposed by rapid climate change was determined experimentally by Bradshaw, Zani and Holzapfel (2004). They programmed year-long climates in computer-controlled environmental rooms capable of reproducing daily and annual changes in light, temperature and humidity from the tropics to polar regions of Earth. As an index of fitness, they used the year-long cohort replacement rate, i.e. net performance integrated through all four seasons of *W. smithii* reared in the leaves of intact pitcher plants and fed freeze-dried Drosophila to mimic the prey-capture behavior of plants in nature. As a baseline they determined fitness of four northern populations in a natural 50°N thermal and photic year throughout all four seasons (**Fig. 3**). They then

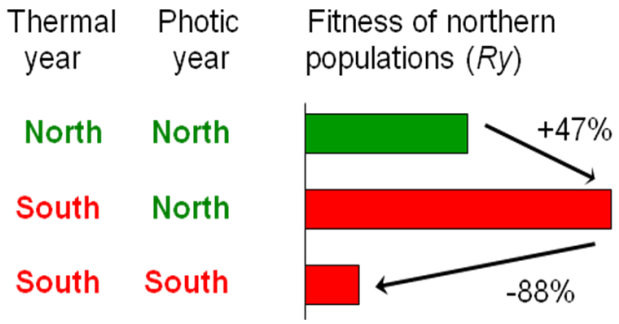

Figure 3. ***Photoperiodism, temperature and fitness*****.** Year-long climates were programmed in computer-controlled environment rooms, replicating daily and annual changes in light, temperature and humidity in nature. Fitness (*Ry*) was determined for four northern populations under three different conditions: (1) northern thermal and photic years (Green), (2) southern thermal and northern photic years (Red), and (3) southern thermal and photic years (Red). *Ry* represents the year-long cohort replacement rate integrated over all four seasons of *Wyeomyia smithii* living in the leaves of intact pitcher plants.

programmed the same northern photic year but a mid-latitude thermal year equivalent to 180 years of global warming at its present rate through all four seasons. Fitness actually increased by 47% -- warmer was better. When they then programmed the same benign mid-latitude thermal year, but also programmed the mid-latitude photic year, fitness declined by 88%. Hence, the immediate response to climatically incorrect photic cues resulted in a path to rapid extinction. Thermal adaptation does occur across latitudinal and altitudinal gradients over long time scales, but photic responses track geography more closely than thermal tolerance or thermal optima

(Zani et al. 2005; Bradshaw & Holzapfel 2008). The important conclusion is that the immediate evolutionary response to rapid climate change in the biological world involves seasonal (photoperiodic) adaptation, with thermal adaptation occurring over a longer time span in already established populations.

4. PHOTOPERIODISM AND CIRCADIAN RHYTHMICITY

The evolutionary response of photoperiodism in W. smithii *during both millennial and recent climate change offers decades if insight into the evolutionary and functional connection between daily and seasonal timekeeping. Both the daily circadian clock and the seasonal photoperiodic timer use the input of light, but serve different functional roles in biological timekeeping. Experimentation and intense discourse surrounding their relationship has persisted for over 80 years.* Wyeomyia smithii *addresses this discourse from an evolutionary perspective and this perspective is used to formulate a model reconciling more recent molecular experiments and the independent evolution of the clock and timer in* W. smithii.

4.1 Functionality, definitions, & the evolutionary perspective

There are two great rhythms of the biosphere: a daily, 24-hour rhythm created by the rotation of the earth about its axis and a seasonal rhythm created by the rotation of the earth about the sun. Temporal organization of daily biochemistry, physiology, and activities is orchestrated by an internal, self-sustaining circadian clock. Temporal organization of seasonal biochemistry, physiology, development, and activities is orchestrated by day length. Concordance with both the daily cycle of dawn and dusk and the seasonal cycle of day length are crucial for the maintenance of fitness in *Wyeomyia smithii* (Emerson, Bradshaw & Holzapfel 2007; Bradshaw, Zani & Holzapfel 2004). The circadian clock and the photoperiodic timer are functionally distinct (Danks 2005; Bradshaw & Holzapfel 2010a; Hut et al. 2013):

> Circadian rhythms entrain to dawn and dusk on a continuous basis while photoperiodism acts as a go/no-go seasonal switch that commits an animal to migration, dormancy, development or reproduction that may be separated from the present environment in time or space by months or thousands of kilometers... Entrainment of the circadian clock resets on a day-to-day basis …while a photoperiodic response, once executed, is irreversible within a seasonal context or even within the lifetime of an individual … Finally, the circadian clock does not "count" light:dark cycles. The photoperiodic counter both counts and accumulates light:dark cycles that the photoperiodic timer has interpreted as long or short and then triggers the corresponding physiological response when some threshold number of inductive cycles has been exceeded. (Bradshaw & Holzapfel 2010a, p. 156)

Precise definitions are important in this discourse. Confucius called it the rectification of names: "That is, things in actual fact should be made to accord with the implication attached to them by

names" (Fung 1960, p. 41). For example, the often used term "photoperiodic clock" implies *a priori* unity of photoperiodism and circadian rhythmicity. Hence, the discussion below will make the consistent distinction, unless in a direct quote from the literature, between the daily circadian clock (the clock) and the seasonal photoperiodic timer (the timer) and between "photoperiod" (day length or light portion of the daily cycle) and "photoperiodism" (ability to use day or night length to regulate some biological, generally seasonal process).

Biological timekeeping in *W. smithii* has been interpreted in the light of geographic variation that represents diverse endpoints of evolution in space and time. The major perspective to keep in mind is that regardless of variable seasonal selection on photoperiodic response, organisms on Earth live in a constant 24-hour world through evolutionary time. A major theme of this discourse revolves around the question: "If the circadian clock forms the basis of the photoperiodic timer, how can the clock exposed to an invariant 24-hour world maintain precise integration of daily events while the timer maintains genetic and evolutionary flexibility through highly variable space and time?"

4.2 Daily vs. seasonal timing

Classical physiological experiments show that *Wyeomyia smithii* assesses the length of day, not night, and that tests for circadian rhythmicity as an integral component of photoperiodism yield negative or contrary results within and among populations. Unique comparisons between lowland and mountain populations of *W. smithii* at the same latitude (hence, same photic environment, but different seasonal environments) show independent evolution of the photoperiodic timer and the circadian clock, the latter measured by overt circadian behavior and by the rhythmic expression of the core circadian clock gene *period*. However, accumulating evidence from other insects supports the concept that the clock and timer are "somehow related." Herein, we propose a model based on gene pleiotropy that reconciles these seemingly disparate results and viewpoints.

4.2.1 Bünning's proposition then and subsequently

Because both circadian rhythmicity and photoperiodism are involved in biological timing and both rely on the input of light, there has been avid conjecture, discussion, and experimentation revolving around the mechanistic connection between them ever since Bünning (1936, p. 590) proposed that the circadian clock formed the *Grundlage* of photoperiodic time measurement: A great deal hangs on the meaning of *Grundlage*, which can be translated as groundwork, foundation, base, rudiment, matrix or basement (De Vries 1959). In an English edition, Bünning (1973) is not clear what he meant by *Grundlage*, but, based on the observation that critical photoperiod is often independent of temperature, "This [independence] suggests the assumption that here, too, [photoperiodic] time is measured by means of the circadian clock" (p. 203) and, in retrospect (p. 223),"it is no longer surprising to us that certain developmental processes may or may not be coupled to the clock." Bünning's proposition has historically elicited voluminous and creative physiological and genetic experiments with an increasing appreciation of an

evolutionary context. Investigations with *Wyeomyia smithii* emphasize evolutionary and physiological genetics, raise fundamental questions about the interpretation of data and the formulation of hypotheses, and conclude that Bünning's "coupling," if carefully defined, may achieve reality by invoking gene pleiotropy.

4.2.2 How W. smithii assesses day length

Photoperiodic timing in Wyeomyia smithii relies on the day length and not night length, and requires the repeated input of external light rather than an internal circadian clock to count photoperiodic inductive cycles. However, comparisons among populations of this single species reveal a more complex evolution of photoperiodism than would be perceived from consideration of a single population.

There are two fundamental questions relating to photoperiodism in any organism. First, does the photoperiodic timer assess the length of day or the length of night (Saunders 2013) and, second, does the accumulation of photoperiodic information (the photoperiodic counter) continue under constant conditions (darkness) or require the repeated input of light? In diapausing populations of *W. smithii* from the Gulf of Mexico to Canada, day lengths longer than the critical photoperiod invariably induce development, regardless of night length (Bradshaw, Holzapfel & Davison 1998; Emerson et al. 2009). Photoperiodic response in *W. smithii* relies on day length, not night length.

In an experiment with eight populations from the Gulf of Mexico to Canada, diapausing larvae were exposed to a diapause-terminating long day every 24, 48, or 72 hours (Emerson et al. 2008). If the counter were continuing to run in the longer nights of the 46- and 72-h cycles, each of the longer cycles should count twice or three times as many long days as a 24-h cycle, respectively. In fact, all three cycle durations counted an equivalent number of long days. In addition, when Bradshaw, Quebodeaux, & Holzapfel (2003b) exposed 14 populations from the Gulf Coast to Canada to day lengths from 10-18 hours with at total cycle of 24 or 72h, the critical photoperiods were the same for both treatments. These results show that the photoperiodic counter in *W. smithii* relies on the repeated input of light to accumulate photoperiodic information, but the photoperiodic timer does not lose track of day length during long nights.

Pittendrigh and Minis (1964) proposed that light played two roles: setting the phase of the sensitivity rhythm and triggering a long-day response if light occurred during the sensitive phase of the rhythm (the external coincidence model). The model predicts that in an otherwise diapause-maintaining short day regimen, there should be two peaks of diapause-terminating sensitivity to light pulses during the long night in *W. smithii*: an early night "A" peak in effect simulating a delayed dusk after the main short day, and a late night "B" peak simulating an advanced dawn before the main short day. With increasing critical photoperiod, the A peak should occur progressively later in the night and the B peak progressively earlier (Saunders & Bertossa 2011).

This effect of light pulses was tested by exposing six populations of diapausing *W. smithii* from the Gulf of Mexico to Canada to a short day and a long night with both T = 24 (L:D = 10:14) and T = 48 (L:D = 10:38) interrupted by 1-hr light pulses every hour (Bradshaw, Holzapfel & Davison 1998). As expected, southern populations showed both A and B peaks, but the peaks did not change position with either T=24 or T = 48. Furthermore, the A peak persisted at all latitudes; the B peak declined with increasing latitude. When T = 48, there appears an unpredicted third peak between the A and B peaks in the southern populations only.

While these results are consistent with Pittendrigh and Minis' (1964) concept of the dual action of light (setting the clock and triggering the long-day response), it is also clear that photoperiodic response in *W. smithii* is more complex than envisioned by this model. These results also demonstrate the importance of the evolutionary perspective: had only a mid-latitude population been investigated, the complexities of the disappearing B peak in the north and the appearance of the C peak in the south would not have been revealed.

4.2.3 Resonance

As evidenced by resonance (tracking external Light:Dark cycles) experiments, variation in expression of the daily circadian clock is not a driving force in the evolution of the seasonal photoperiodic timer in Wyeomyia smithii.

Short days and long nights should, at first glance, maintain diapause regardless of night length. However, this condition exists only if the circadian clock can track, or resonate with the total light:dark (L:D) cycle. Conceptually, a circadian driven light-sensitivity rhythm should resonate with cycles of ~24, 48 or 72h, i.e., short days and long nights should maintain diapause. With cycles of ~36 or 60h that depart radically from the normal 24-h period of the environment, the circadian clock should fail to synchronize with the L:D cycle and the sensitive phase of the cycle should drift into the short day, resulting in a discordant long-day response. When exposed to short days and increasing night lengths (in different experiments) to create cycles of T = 10:14, 10:16, 10:18… 10:62, a circadian driven photoperiodic timer should encounter resonant cycles that maintain diapause and discordant cycles that stimulate development. Hence, a plot of percent development as a function of T = 10+D should oscillate between diapause and development as the underlying circadian sensitivity rhythm resonates with or drifts through the external L:D cycle. Such experiments are known as resonance, T, or Nanda-Hamner experiments (Pittendrigh 1981; Saunders 2002, pp. 351-358).

Response by *Wyeomyia smithii* to resonance experiments shows that there is the expected rhythmic response in Gulf and Carolina lowland populations. The period of this rhythm remains constant at ~21hr throughout the latitudinal and altitudinal range of *W. smithii*, but the amplitude of this rhythm varies from robust to non-existent (Wegis et al. 1987; Bradshaw, Quebodeaux & Holzapfel 2003). Amplitude of the rhythmic response to resonance experiments is correlated with altitude, but not with latitude and not with critical photoperiod, showing that the photoperiodic timing of seasonal events in *Wyeomyia smithii* can evolve independently of the daily circadian clock (Bradshaw, Holzapfel & Mathias 2006).

The declining amplitude of resonance rhythmicity might be explained by a rapidly damping circadian oscillator (Saunders 2009, 2016). If, instead of exposing diapausing larvae to a resonance experiment with short days, they are exposed to a resonance experiment with diapause-terminating long days as in Sec. 4.4.2, >92% of all larvae develop and eventually pupate (Emerson et al. 2009a). Development time measured as days to pupation increases with T = L+D but, in some populations, there is rhythmic variation about the regression of development time on T. Notably, low elevation populations at 35°N have a robust short critical photoperiod, a robust resonance when L = 10h, and a robust rhythmic number of days to pupation when L = 18h. By contrast high elevation populations at the same latitude have a long critical photoperiod, a totally arrhythmic, zero resonance response, but a robust rhythmic number of days to pupation when L = 18. All experiments were run at the same temperature. The rhythmic days to pupation do not differ in period or amplitude between the low and high elevation populations. As evidenced by resonance of development time with varying external Light:Dark cycles, a robust circadian oscillator persists in populations at both elevations; consequently, a damped circadian oscillator cannot be invoked to explain the absence of a rhythmic response to resonance experiments in *W. smithii*.

Pittendrigh and Minis recognized the power of an indefinitely long sensitive period when photoperiod controls the maintenance and termination of diapause as they observed in the pink bollworm: "In general, resonance experiments are (1) always powerful when a positive resonance effect is found; but (2) powerful when one is missing only if the system remains for a sufficiently long time in the inducible state for the resonance effect to occur" (Pittendrigh & Minis, 1971, p. 243). The sensitive period in *W. smithii* persists throughout diapause for as long as year and the resonance experiments were of eight weeks duration. The eight weeks provided ample time for >95% development to long-day control 18:54 = L:D cycles, and therefore any positive resonance effect to occur. The absence of a positive resonance effect in independent mountain populations is not an anomaly, but instead constitutes the powerful experiment called for by Pittendrigh and Minis and demonstrates that something other than a simple concept of the daily circadian clock being an integral component of photoperiodism is driving evolution of the seasonal photoperiodic timer in *Wyeomyia smithii*.

4.3 Molecular connections & pleiotropy

Increasing evidence from mutations and RNA interference (RNAi) of circadian clock genes support Pittendrigh's (1972, p. 2734) concluding assertion that "circadian rhythmicity is often somehow involved in the physiology of photoperiodic induction" (emphasis ours). Over four subsequent decades of physiological, genetic and molecular research have not substantially improved on the "somehow involved." Below, we present a model that reconciles the diverse evolutionary, physiological, functional and molecular viewpoints of how the circadian clock is "somehow involved" in photoperiodism. This reconciliation depends on the recognition that evolution, physiology, function and mechanism are, in fact, different entities and, as such may be subject to different selective pressures, regardless of their inter-relationships.

The circadian clock and the photoperiodic timer, while both cued by light, regulate different functions, daily and seasonal timing, respectively (Danks 2005; Bradshaw & Holzapfel 2010a). As discussed in **Sec 4.1**, whereas the earth's rotation about its axis imposes a 24-h world regardless of geography or climate, the rotation of the earth about the sun generates seasonal climates that vary directly with geography (MacArthur 1972) and that have profound effect on photoperiodism (Danilevskii 1965; Danks 2005; Bradshaw & Holzapfel 2006, 2007, 2010b; Hut et al. 2013). There is now accumulating evidence from molecular data supporting the proposition that the circadian clock is "somehow involved" in the mechanism of photoperiodic time measurement (Koŝtál 2011; Hut et al. 2013; Meuti & Denlinger 2013; Goto 2013; Numata, Miyazaki & Ikeno 2015; Doležel 2015; Omura, Numata & Goto 2016). What remains to be resolved 80 years after Bünning's (1936) proposal and Pittendrigh's refinements of the concept (Pittendrigh & Minis 1964; Pittendrigh 1966, 1972) assertion is still what "somehow involved" means in terms of mechanism at the genetic and genomic levels.

Although many experiments spanning decades of research with *Wyeomyia smithii* show the signature of independent evolution of the circadian clock and the photoperiodic timer, the most direct evidence for this conclusion comes from comparing photoperiodic and circadian properties over an elevation gradient at the same latitude. In this comparison, both high and low elevation populations experience the exact same annual change in day length, each day of the year. However, lower elevations have earlier springs, later onsets of winter, and longer growing seasons than higher elevations. Hence, there is selection for more northern-like photoperiodic responses at high mountain altitudes as compared with low elevation. *Wyeomyia smithii* from 35°N in North Carolina illustrates just this expected result (Bradshaw, 1976; Bradshaw, Quebodeaux, & Holzapfel 2003). The question then remains as to what are the properties of the circadian clock over this same elevation gradient?

At the physiological level, diapausing *W. smithii* from high and low elevations exposed to a diapause-terminating long day followed by night lengths varying from 6-54h (T = L + D = 24-72h) in 26 different experiments exhibited rhythmic development time with an average period of 23h (**Sec. 4.2.3**). However, neither period nor amplitude differed between the mountain and lowland populations, indicating that the overt properties of the circadian clock were the same regardless of differences in seasonality and photoperiodic response.

At the molecular level, (**Fig. 4**) rhythmic expression of the core circadian clock gene *period* in constant dark did not differ between the mountain and lowland populations in period, amplitude, or degree of damping.

These experiments show that among populations, when the photic environment is held constant while the seasonal environment is varied, physiological and molecular properties of the circadian clock remain invariant and constant while photoperiodic response follows the seasonal environment. Within a single population, the sign of a genetic correlation between critical photoperiod and rhythmic response to resonance experiments can be reversed by antagonistic selection even more readily in *W. smithii* (Bradshaw, Emerson & Holzapfel 2012) than in *Drosophila littoralis* (Lankinen & Forsman 2006). Therefore, these two great biological timing

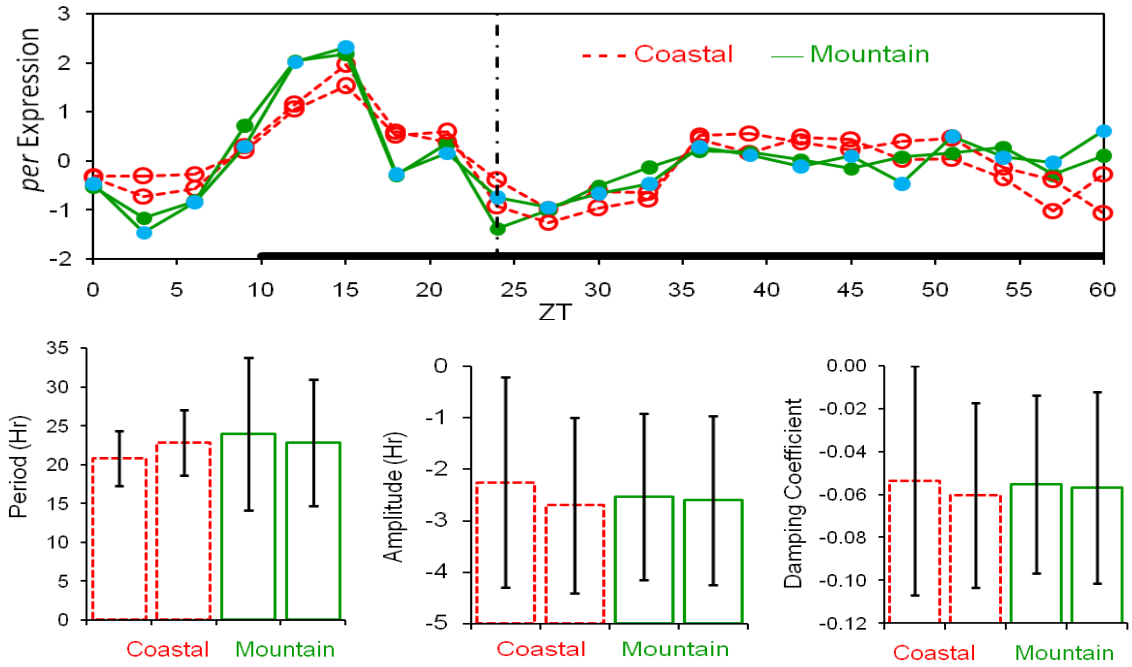

Figure 4. *Rhythmic properties of* period. The top plot shows *per* expression in diapausing *Wyeomyia smithii* larvae as $\log_2$ fold variation about mean expression for each population. Two populations each were from either the coastal region or from the Appalachian Mountains in North Carolina at the same latitude, 35°N. In nature, these mosquitoes and localities encounter the same year-round day lengths but very different seasonal environments. When the photic environment is held constant, but the seasonal environment is varied, molecular properties of the circadian clock remain invariant while the photoperiodic response follows the seasonal environment. Error bars ± 2SE.

mechanisms are clearly capable of independent response to selection and, hence, independent evolution. This conclusion returns us to the conundrum: how does the circadian clock maintain close control of daily functions in a 24-h world if it is tightly coupled to a genetically flexible photoperiodic timer finely attuned to geographically local seasonal environments? The answer depends on how one asks the question. If one asks, "How does the circadian clock control photoperiodism" one is already assuming *a priori* that the circadian clock controls photoperiodism. Koštál (2011) and Doležel (2015) pose the more neutral question: "What is the relationship between the circadian clock and the photoperiodic timer – is it one of unity, cooperation, or independence?" Given the evolutionary independence of the clock and timer in *W. smithii*, the question is more appropriately asked: "How can the photoperiodic timer connect with the circadian clock?" In other words, how can a variable photoperiodic timer co-opt the time cues provided by the circadian clock without disrupting daily temporal organization? Emerson, Bradshaw & Holzapfel (2009b) and Bradshaw & Holzapfel (2010a) contrasted the circadian clock acting as a unit or module (modular pleiotropy) with individual circadian clock genes acting individually, and incidentally of their circadian function on photoperiodic timing (gene pleiotropy). Tormey et al. (2015) and Denlinger et al. (2017) proposed a variant of the gene pleiotropy model (**Fig. 5**) that falls between Koštál's (2011) and Doležel's (2015) concepts of independence and cooperation. Basically, it posits that the circadian clock must communicate

with the rest of the organism and it is most likely to do so through the transcription factors and transcription regulators involved in the core transcription-translation feedback loop. All it takes is for the photoperiodic timer to cue in on, or co-opt one or a few of the output proteins of these genes to provide a time-reference point. Herein, we further refine this proposition with the "commensal model of biological timekeeping." In this model, the relationship is one of commensalism (one entity, the photoperiodic timer, benefits without cost or benefit to the other entity, the circadian clock), not parasitism or mutualism. Evolution of photoperiodic response can then be the result of the photoperiodic timer co-opting different titers of the same protein, different proteins, or different combinations of proteins in response to selection imposed by seasonal contexts that vary in space and time.

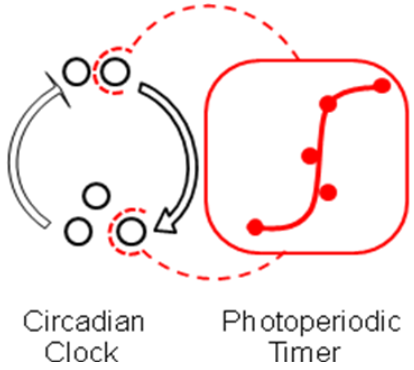

Figure 5. ***The Commensal Model of Biological Timekeeping*****.** (commensal: one entity of the relationship benefits without cost or benefit to the other). In this reconciliation model, the photoperiodic Timer co-opts one or a few of the output proteins of one or a few circadian Clock genes, thus providing a time reference point to the Timer. Communication with downstream photoperiodic time measurement would then occur without altering basic circadian clockworks. Downstream communication may be through the co-option of different titers of the same Clock protein, or even different combinations of Clock proteins in response to variable seasonal contexts. This model accommodates both stability of the circadian Clock and independent evolution of Timer and Clock mechanisms.

In sum, the commensal model provides a genetic mechanism for the independent evolution of the daily circadian clock and the seasonal photoperiodic timer that incorporates evolution of photoperiodism in *Wyeomyia smithii* with more general conclusions and models across diverse insect taxa (Koŝtál 2011; Goto 2013; Doležel 2015). Some gene or combination of genes in the circadian clock would then communicate with downstream processes, including photoperiodic time measurement, and those downstream processes can respond to selection without altering the basic circadian clockworks. The important remaining questions relate to (1) how alleles segregating in natural populations provide the genetic variation in and covariation between the circadian clock and the photoperiodic timer and (2) how this genetic architecture provides the opportunities for or impediments to their independent or joint evolution. The answer to these questions will continue to require not only molecular approaches, but also approaches involving physiology, population genetics, and quantitative genetics, both within and among populations having evolved in the crucible of nature.

5. CONCLUSIONS

The evolutionary perspective provided by Wyeomyia smithii *demonstrates that the genetic basis of photoperiodism and its relation to circadian rhythmicity is a population, not species level consideration. The experiments and results discussed herein generate a model of the commensal relationship between the clock and the timer that permits canalization or homeostatic stability of the circadian clock and, at the same time, evolutionary flexibility of the photoperiodic timer.*

The pioneering work of Danilevskii (1965) and colleagues treated photoperiodic response as a population-level character that, importantly, varied over geographic clines and with the degree of hybridity between populations. In spite of Danilevskii's broad vision, subsequent research into the mechanistic basis of photoperiodic response has generally emphasized detailed physiological and molecular studies of an investigator-specific species and population within that species. This research focus has generally led to the tacit assumptions that stage and formal properties of circadian-photoperiod relationships are a species-level character and that the specific population under consideration is representative of that entire species using but a single population (Saunders 2010, Table 13.1). Although Pittendrigh did not challenge this narrow mind set, in his heart, he remained a true evolutionary biologist, interpreting variation in circadian rhythmicity, photoperiodism, and their connection "in the light of evolution" (Dobzhansky 1964):

> The last 25 years have yielded abundant proof that there is indeed a circadian component in the photoperiodic responses of a great many very different organisms; but we still lack any sure understanding of what that component is. We are not even sure it is always (indeed ever!) the clock that effects the photoperiodic time-measurement; in some species it may be the clock, and elsewhere not; and even when it does serve to measure photoperiod we cannot be sure it always does so in the same way. Moreover there is growing empirical support for our own intuitive preference that extensive convergent evolution probably underlies the phenomenological similarities between different taxa. (Pittendrigh, Elliott, & Takamura 1984, p. 37)

The major contributions of research on photoperiodism in *Wyeomyia smithii* originates from this evolutionary perspective with the added dimension of focusing on multiple populations in their natural habitat: (1) to explore the genetic basis of photoperiodism in the context of historical biogeography through space and time by using multiple populations from over 100 localities in North America, (2) to intensify and clarify research on the circadian-photoperiodism connection by invoking the power of gene pleiotropy, and (3) to accelerate research on the genetics of photoperiodism by demonstrating the complexities of variation and covariation within and among populations of a single species. Among populations of *W. smithii* alone, these complexities encompass the range of variation associated with the entire pantheon of insect species. Even within a single population of *W. smithii*, there is in fact no "wild type" photoperiodic response and every attribute of photoperiodic response or covariation with any

putative underlying mechanism is critically dependent upon the genetic background in which it is assessed. A complex genetic architecture underlies photoperiodism within and among populations, underlies classic experiments to determine Pittendrigh's "circadian component," and therefore underlies a genetically and evolutionarily flexible interaction between photoperiodism and the circadian clock.

> Evolutionary theory, which is our clearest guide to and explanation of unities in the living world also has another voice: It warns that functional similarity (functional unity) may often be the outcome of convergent evolution, of the fact that natural selection – Darwin's demon – is indifferent to precisely how the functional prerequisite is met. (Pittendrigh & Minis 1971, p. 245).

Darwin's demon haunts those who advocate a genetic unity of photoperiodism or an invariant circadian-photoperiodic interaction, but delights those of us who pursue the diversity of natural systems and the intriguing variety of ways their underlying genetic architectures enable continuing adaption to the ever-changing natural world around them.

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