What Season Is It Anyway? Circadian Tracking vs. Photoperiodic Anticipation in Insects

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REVIEW

What Season Is It Anyway? Circadian Tracking vs. Photoperiodic Anticipation in Insects

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Abstract The daily rhythm of 24 h and the annual rhythm of 12 mo constitute the 2 major, highly predictable rhythms of the biosphere. The internal circadian clock enables organisms to track daily changes in their environment; the photoperiodic timer, alone or in concert with a circannual clock, enables organisms to anticipate and prepare in advance for seasonal changes in their environment. The circadian clock entrains to dawn and dusk and tracks light and temperature on a day-to-day basis, while the photoperiodic timer serves as a long-term, physiological go/no-go switch that commits an animal to development, reproduction, dormancy, or migration on a seasonal or even lifetime basis. In 1936, Erwin Bünning proposed that circadian rhythms formed the basis (*Grundlage*) for photoperiodic response to day length. Historical inertia generated by correlative evidence from early physiological studies and a proliferating number of descriptive models has resulted in the widespread assumption that the circadian clock constitutes the necessary, causal basis of photoperiodism in general. This historical inertia has also restricted the search for genes involved in insect photoperiodism to genes central to the circadian clock in *Drosophila* and has led investigators to conclude that any behavior, process, or gene expression that varies with day length represents photoperiodism or a gene involved in photoperiodism. The authors discuss how blinders imposed by the circadian imperative have retarded progress toward identifying the genetic basis of photoperiodism and how the insights gained from geographic variation in photoperiodic response have been used to show the independent evolution of the circadian clock and photoperiodism. When geographic variation is found in circadian genes, the most immediate and parsimonious search for adaptive significance should be in circadian function, not in extrapolation to photoperiodism. Finally, the authors propose that circadian-unbiased, forward genetic approaches should be used to identify genes involved in photoperiodism within extant populations and among populations over evolutionary time.

Key words circadian rhythms, photoperiodism, diapause, evolution, forward genetics

There are 2 major rhythms of the biosphere: a daily rhythm of 24 h and an annual rhythm of 12 mo. The internal circadian clock enables animals to track daily changes in their environment; the photoperiodic timer, either alone or in conjunction with an internal circannual clock, provides a physiological mechanism

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that enables animals to anticipate and prepare for seasonal changes in their environment that are often far into the future. The distinction between the circadian clock and the photoperiodic timer is important because mechanism matters. Daily temporal organization by the circadian clock is important for the integration of hundreds of metabolic events on a daily basis (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Matsumoto, 2006) and for the maintenance of fitness in both prokaryote and eukaryote systems (Sharma, 2003; Emerson et al., 2008a); seasonal temporal organization by the photoperiodic timer is important for maintaining synchrony of life-historical events with changing seasonal exigencies and opportunities (Danilevskii, 1965; Tauber et al., 1986; Danks, 1987; Leather et al., 1993; Bradshaw et al., 2004; Bradshaw and Holzapfel, 2007a). The close correlation between photoperiodism and geographically variable seasonal environments in arthropods (Danilevskii, 1965; Danks, 1987, table 24) attests to the importance of photoperiodism in the dispersal of animals in temperate and polar habitats. Circadian rhythm genes can play a role in plant photoperiodism (Shultz and Kay, 2003) and also in the *tau* hamster that, to date, is the only mammal in which the effects of a genetically disrupted circadian clock on photoperiodism have been tested (Hazlerigg and Loudon, 2008). Herein, we focus on insects, review functional and formal properties of both circadian rhythmicity and photoperiodism, describe experiments that have been used to probe possible genetic connections between and coevolution of the 2 processes, and propose future directions for research.

FUNCTIONAL AND FORMAL PROPERTIES

First and foremost, circadian rhythms perform the function of a daily clock; photoperiodism performs the function of a seasonal timer. 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 (Fig. 1). Entrainment of the circadian clock resets on a day-to-day basis (Pittendrigh, 1960, 1965, 1981b; Aschoff, 1965) while a photoperiodic response, once executed, is irreversible within a seasonal context or even within the lifetime of an individual (Nijhout, 1994; Bradshaw and Holzapfel, 2007a). Finally, the

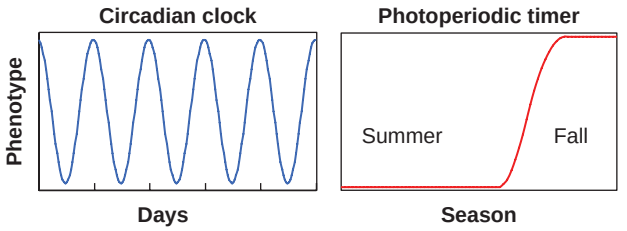

Figure 1. Properties of the daily circadian clock and the seasonal photoperiodic timer. The circadian clock cycles and can be reset on a day-to-day basis; the photoperiodic timer acts as a go/no-go physiological switch that, once flipped, initiates a cascade of events that runs to completion and is irreversible in a seasonal, annual, or lifetime context.

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 (Saunders, 1981, 2002; Vaz Nunes and Saunders, 1999; Emerson et al., 2008b).

The formal properties of circadian rhythms are well established and constitute rhythms that persist under constant conditions with a duration of about a day, whose phase can be reset by a brief interruption in the constant regimen, and whose period is relatively independent of temperature within the physiological range of normal growth (Pittendrigh, 1960; Aschoff et al., 1965; Dunlap et al., 2004, p 387). By contrast, photoperiodic response has no intrinsic rhythmicity, and once the go/no-go switch is flipped, the photoperiodically induced results in a cascade of physiological or developmental events that runs to completion (Tauber et al., 1986; Nijhout, 1994; Bradshaw and Holzapfel, 2007a; Emerson et al., 2009a).

THE BÜNNING HYPOTHESIS AND ITS AFTERMATH

The search for a connection between circadian rhythmicity and photoperiodism has its roots with the plant physiologist Erwin Bünning's (1936) hypothesis that the circadian clock constituted the basis (*Grundlage*) of photoperiodism, that is, it played a necessary, causal role in the photoperiodic switch.[1] A necessary, causal connection between circadian rhythmicity and photoperiodism has intrinsic appeal. Suggestive evidence for a circadian basis of photoperiodism has been drawn from many physiological

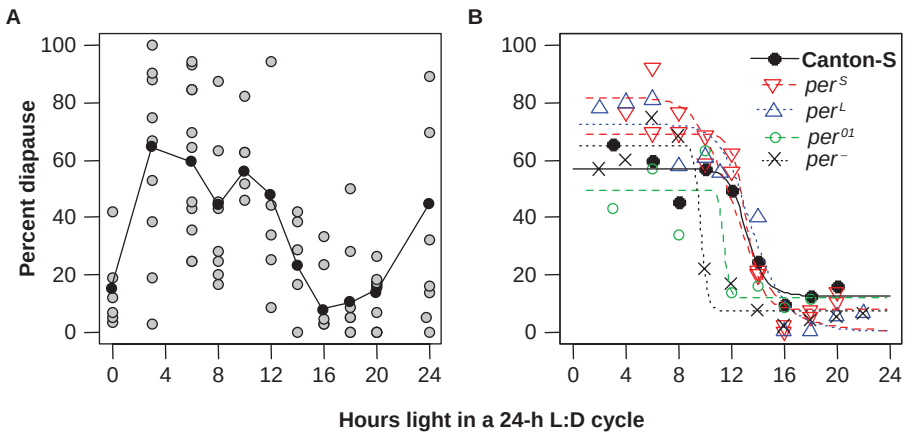

Figure 2. Photoperiodic response of Canton-S *Drosophila melanogaster* and various *period* mutants. (A) Diapause in Canton-S flies. Individual points represent the incidence of diapause in groups of flies; the line connects the mean of the groups at a given day length. (B) Diapause in Canton-S (black dots) and *period* mutants (colored symbols). Only means of groups were available. Curves were fit by logistic regression (R Development Core Team, 2007). The median responses (critical photoperiods) of the *period* mutants do not differ significantly from Canton-S (see SOM). Recall that the solid black line (Canton-S) in Figure 2B is based on the widely scattered points in Figure 2A. Data from Saunders (1990) as plotted in Emerson et al. (2009a) with permission from Elsevier.

studies showing what Minis (1965) called "parallel peculiarities in the entrainment of a circadian rhythm and photoperiodic induction" and from a proliferating number of descriptive models seeking to explain these parallelisms in different species (Vaz Nunes and Saunders, 1999; Saunders, 2002). Rumblings to the contrary started with Lees' studies on aphids and spider mites, leading him to conclude that "arthropod photoperiodic mechanisms do not appear to depend on an endogenous 24-h rhythm of light sensitivity. They are here regarded as one of the many kinds of [non-circadian] 'interval timers' which undoubtedly exist in living organisms" (Lees, 1960, p 267).[2] Pittendrigh (Pittendrigh and Minis, 1971, pp 238-239) found that in red light ≥600 nm, populations of the moth *Pectinophora*

> in which all known circadian rhythms (and thence their underlying circadian oscillations) are totally asynchronous nevertheless measure—with complete efficacy—the difference between 12 and 14 hr of light. . . . We were then confronted, in other words, with the most telling, unequivocally positive evidence that Bünning's proposition in any form is invalid at least in this one species: that the clock measuring photoperiod is neither a circadian oscillator nor any of its slave rhythms.[3]

These early disconnects between the circadian clock and the photoperiodic timer continued in more recent studies at the molecular level in *Drosophila melanogaster*.

In *D. melanogaster*, the molecular basis of the circadian clock consists of both positive and negative feedback loops (Hall, 2003; Price, 2004). Central to the circadian timing mechanism are the genes *clock*, *cycle*, *period*, and *timeless* (*clk*, *cyc*, *per*, & *tim*). Different mutants at the *per* locus result in locomotor and eclosion rhythms that run fast, run slow, or are arrhythmic (Hall, 2003, table 1). The discovery by Saunders and colleagues (Saunders et al., 1989; Saunders, 1990) that a Canton-S laboratory line of *D. melanogaster* was weakly photoperiodic for the induction of adult ovarian diapause at 12 °C provided the opportunity to test for a connection between the circadian clock and the photoperiodic timer at the molecular level. Wild-type Canton-S exhibit a sigmoid photoperiodic response curve in light:dark (L:D) cycles from L:D = 4:20 to L:D = 20:4 (Fig. 2A). However, the distribution of replicate groups of flies is widely scattered about the mean responses, and each point upon which the mean is based is itself the mean of a group of flies. This range in variation needs to be kept in mind when comparing the photoperiodic response of different *period* mutants (Fig. 2B). Figure 2B shows photoperiodic response curves of 4 *period* mutants that alter clock function (Saunders, 1990) fitted by logistic regression (R Development Core Team, 2007). per^s, per^l (later per^{L1}), per^{01}, and per^- result in short, long, or arrhythmic eclosion and activity rhythms with respect to wild-type Canton-S[4] (Saunders, 1990). First, a robust photoperiodic response curve persists in all mutants regardless of their altered clock function. Second, the critical photoperiods scored as the transition point or median of the photoperiodic response curves do not differ among the mutants and wild-type in all pairwise comparisons, in pairwise comparisons only between wild-type and each of the 5 *per* mutants, or in pairwise comparisons only between wild-type and just the 2 arrhythmic per^0 and per^- mutants (see SOM for statistics). These results

mean that a functional circadian clock is not necessary for photoperiodic response in Saunders' line of Canton-S *D. melanogaster* and that the critical photoperiods (left-right shift of the photoperiodic response curves) are not significantly different from each other, either collectively or in an a posteriori comparison between wild-type and the null mutants. Neither long, short, nor null *period* mutants have a significant effect on photoperiodic response of Canton-S *D. melanogaster*, despite their moderate to drastic effect on circadian rhythmicity. Even though a normally functioning circadian clock is not necessary for a robust photoperiodic response, individual circadian rhythm genes, independently of their role in circadian rhythmicity, may still be involved in photoperiodism (Tauber and Kyriacou, 2001; Mathias et al., 2005; Emerson et al., 2009a). The suspects would likely reside on the pathway between the input of light into the circadian system and *per* (Tauber and Kyriacou, 2001; Mathias et al., 2005), that is, the genes *cryptochrome* (*cry*), *shaggy* (*sgg*), or *tim*. The focus of other labs, as well as our own, has been on *tim* for several reasons. First, levels of TIMELESS protein (TIM) are negatively regulated by light through an interaction with the blue-light receptor CRYPTOCHROME (CRY; Ceriani et al., 1999; Price, 2004). Second, TIM continues to be expressed constitutively at high levels in *per* null mutants (Claridge-Chang et al., 2001) but still binds to and is degraded by CRY in the presence of light (Myers et al., 1996; Zeng et al., 1996). Consequently, even in flies with a dysfunctional circadian clock, TIM could be providing a noncircadian, interval-timing signal to the flies. Correlative evidence for just such a role for *tim* comes from its variation in expression in mosquitoes in our lab (Mathias et al., 2005), in flesh flies (Goto and Denlinger, 2002; Goto et al., 2006), and in another drosophilid *Chymomyza costata* (Stehlík et al., 2008), a correlation that has appealed to many researchers. It is important to recall, however, that the correlative evidence is equally strong that *tim* is involved elsewhere along the photoperiodism-diapause developmental axis (Emerson et al., 2009a).

In European *D. melanogaster*, there are 2 naturally segregating alleles of *tim*: an ancestral *s-tim* that transcribes only a short form of the mRNA and a derived *ls-tim* that transcribes both a long and a short form of the mRNA (Rosato et al., 1997). Short and long forms of the mRNA are translated into short (S-TIM) and long (L-TIM) TIMELESS proteins, respectively. L-TIM binds less tightly to CRY and renders phase shifting of the circadian clock less sensitive to light than S-TIM (Sandrelli et al., 2007). European *D. melanogaster* are photoperiodic for the initiation of adult ovarian dormancy. Short days and *ls-tim* promote diapause, while long days or *s-tim* promote continuous development, but there is no photoperiod by genotype interaction in either natural populations or in transformant lines (Tauber et al., 2007; Sandrelli et al., 2007). Hence, variation in *timeless* alleles segregating in natural populations of *D. melanogaster* affects expression of the circadian clock and the expression of diapause but does so without involving the photoperiodic timer (Tauber et al., 2007; Bradshaw and Holzapfel, 2007b).

From the above, we know that arrhythmic *period* mutants do not prevent the expression of a robust photoperiodic response (Fig. 2B) and that, at any locality, there is a greater effect between 2 *timeless* alleles on the expression of diapause than there is among 6 day lengths from 8 to 18 h within a single population (Kyriacou et al., 2007). If knockouts or null mutants of *clock*, *period*, *timeless*, and *cycle* each and all rendered otherwise photoperiodic *D. melanogaster* both arrythmmic and nonresponsive to day length and did not modify temperature-dependent diapause, then the circadian clock itself as a functional module, and not just the pleiotropic effects of one of its genes, would be positively implicated in photoperiodism. This distinction between pleiotropic effects of individual genes versus a pleiotropic effect of the principal integrated module to which those genes contribute is essential. For example, this distinction has been crucial in establishing the insulin-signaling pathway as an integral part of the brain-gonad axis in the determination of ovarian maturation and diapause in Diptera (Emerson et al., 2009a).

The drosophilid fly *Chymomyza costata* undergoes a photoperiodically mediated larval diapause. The *npd* mutant in *C. costata* was isolated from a natural population in northern Japan. The *npd* flies do not undergo diapause in response to short days, have a 10-fold decrease in *timeless* expression, have 37 nonsynonymous substitutions out of 1356 in their coding region, and have an 1855 base-pair deletion in the 5′ untranslated region, removing the start transcription and all regulatory motifs found in the wild-type strain from northern Japan (Stehlík et al., 2008). Stehlík et al. (2008) are aware of the results in *D. melanogaster* (Tauber et al., 2007; Sandrelli et al., 2007) and point out, correctly, that the effect of this massive *timeless* mutation in *C. costata* could be due to the effect of *timeless* on the photoperiodic timer or to the effect of *timeless* on diapause directly without involving the photoperiodic timer.

A similar conclusion holds for the effect of circadian rhythm gene variation on diapause in flesh flies (*Sarcophaga*). Han and Denlinger (2009) show exactly what their title says: "Length variation in a specific region of the *period* gene correlates with differences in pupal diapause incidence in the flesh fly, *Sarcophaga bullata*." However, they then make the leap that because short days induce diapause in *S. bullata*, the effect of length variation in *per* must be having its effect through the photoperiodic timer and not on diapause directly. Consequently, they describe this region of *per* as the "C-terminal photoperiod (CP) region." In fact, they have shown that length variation in the C-terminal region of *per* affects the expression of diapause itself; they do not show whether or not this length variation actually involves the photoperiodic timer. This region of *per* would be better named "C-terminal diapause region" until the downstream pathways from *per* have been determined. What we do know is that genetic variation in both *per* and *tim* can affect the expression of diapause; what we do not know is where on the complex neuroendocrine pathway between the input of light and the expression of diapause these genes are having their effect (Emerson et al., 2009a).

Despite the above observations, why does there remain a persistent desire to ascribe the effects of circadian clock mutants on diapause as synonymous with a causal effect of those mutants on the photoperiodic timer? We believe there are several reasons. First, it is undeniable that both daily entrainment of the circadian clock and seasonal switching of the photoperiodic timer are cued primarily by light. Second, if circadian rhythmicity were to form the basis of the photoperiodic timer, photoperiodism would become an appealing adaptive significance of the circadian clock. Third, the Bünning (1936) hypothesis is supported by numerous correlates between the 2 physiological processes, but correlation is not causation, and changes in the phase or amplitude of circadian gene expression at different day lengths do not causally link the circadian clock and the photoperiodic timer. Fourth, use of the term *photoperiodic clock* to describe the photoperiodic timer conceptually links the daily circadian clock with the seasonal photoperiodic timer.

Finally, and more subtly, there is a natural line of thought that follows from the imprecise use of the term *photoperiod* itself. As originally coined by Garner and Allard (1920), *photoperiod* meant day length; as defined by Aschoff et al. (1965, p xvi), "a light-dark cycle is composed of light time (L) and dark time (D): the term photoperiod is synonymous with light time." Colloquially and incorrectly, *photoperiod* has been used to denote the entire light-dark cycle so that an L:D = 14:10 cycle is often referred to as an "L:D = 14:10 photoperiod." The misleading conclusion then is that any phenomena that vary with day length constitute a photoperiodic response. Hence, by further false extension, any gene whose expression varies in phase or amplitude among different day lengths becomes photoperiodic or a "photoperiod gene." Since chronobiologists, including ourselves, who work with photoperiodism on a daily basis are sometimes careless in our terminology, biogeographers, climatologists, ecologists, and evolutionary biologists who use our conclusions as their "baselines" promulgate and extend our imprecise language into far reaches of the literature.

Does the lack of a convincing causal connection between the daily circadian clock and the seasonal photoperiodic timer in insects preclude an adaptive role for circadian rhythmicity in the tracking of daily variation in light and temperature? After all, as we have discussed above, the circadian clock is linked to hundreds of behavioral and metabolic events that are adjusted on a daily basis. Majercak et al. (1999) provide an elegant example of the circadian clock tracking light and temperature conditions that might be encountered during different times of year. In Figure 3, flies are active during early morning at high temperature and long days; flies are active throughout the day at low temperatures and short days. However, there is no evidence for a go/no-go switch characteristic of a photoperiodic response between long-day and short-day phenotypes; rather, there is a continuum of phenotypes that is exquisitely tuned to tracking day-to-day changes in the environment. Clearly, it is essential that a fly be able to fine-tune behavioral and metabolic processes to be in concert with immediate daily exigencies and opportunities afforded by variation in light and temperature. Importantly, a single fly, if it lived long enough, could exhibit the full range of all of the behaviors illustrated in Figure 3. These behaviors do not represent a seasonal photoperiodic timer; these behaviors do represent phenotypic plasticity due to variation in phase of the circadian clock relative to dawn and dusk at different temperatures (Dubruille and Emery, 2008). That these behaviors are not photoperiodic does not exclude their being adaptive (see Glossary in the SOM): "Indeed, with cold weather, it is probably beneficial for flies to be active during the warmth of the day. In contrast, in hot weather, flies

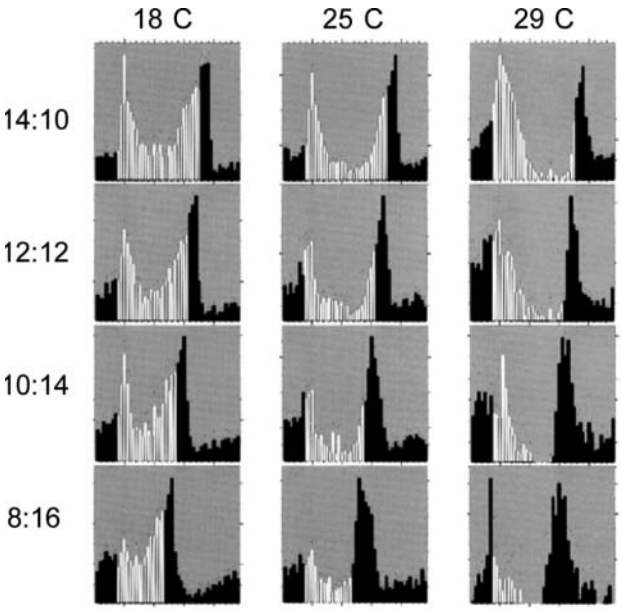

Figure 3. Activity rhythms of *Drosophila melanogaster* at different temperatures and day and night lengths (L:D). White bars, activity during the light; black bars, activity during the dark. These activity patterns represent phenotypic plasticity, the continuously adjustable behavior of individual flies. These patterns do not represent an example of seasonal genetic change (adaptation) or an example of a seasonal timer (photoperiodism). Adapted from Majercak et al. (1999) with permission from Elsevier.

should avoid midday heat and be mostly active around dawn and dusk, when temperatures are cooler" (Dubruille and Emery, 2008, p 135). The circadian clock can clearly contribute to fitness as a continuous tracker of the annual change in dawn, dusk, and temperature without acting as a go/no-go seasonal switch mechanism, that is, without providing the basis of or even contributing to or involving the photoperiodic timer.

"NOTHING MAKES SENSE IN BIOLOGY EXCEPT IN THE LIGHT OF EVOLUTION, *SUB SPECIE EVOLUTIONIS*" (DOBZHANSKY, 1964, P 449)

In this section, we rephrase Dobzhansky's quote to ask, "What can evolution tell us about the relationship between the circadian clock and photoperiodism?" Geographic gradients in climate provide geographic gradients in selection on photoperiodic response and on properties of circadian rhythmicity, leading to inherited (evolved) differences in both mechanisms. We now explore what geographic covariation, or the lack thereof, can tell us about evolution of circadian rhythmicity as a causal factor in the evolution of photoperiodism.

One of the most robust ecogeographic rules is that switching day length or critical photoperiod for the determination of hibernal diapause increases with latitude and altitude (Danileveskii, 1965; Danks, 1987, table 24). The question then remains: Do properties of the circadian clock covary with critical photoperiod over geographic (evolutionary) gradients? A positive correlation between the circadian clock and photoperiodism leaves open the question as to whether the correlation is due to an underlying causal connection or to independent (i.e., parallel) evolution. However, the lack of such a correlation precludes a causal connection between evolution of the circadian clock and the photoperiodic timer.

In *Drosophila littoralis*, Lankinen observed that both the phase of pupal eclosion and critical photoperiod were correlated with latitude in eastern Europe and, within populations, were genetically correlated with each other (Lankinen, 1986a, 1986b). Insightfully, he also calculated that if each trait were separately regressed on latitude, the residuals from the 2 regressions were not correlated (Lankinen, 1986b). This latter result suggested that the latitudinal covariation of the 2 traits was due to parallel evolution (independent selection on each trait) and not a causal relationship between the evolution of the 2 traits. Twenty years later, Lankinen and Forsman (2006) showed that the genetic correlation was due to linkage and not pleiotropy, that is, that there was no causal connection between the evolution of photoperiodic response and evolution of the circadian eclosion rhythm in *D. littoralis*.

In the pitcher-plant mosquito, *Wyeomyia smithii*, *timeless* was used as a codominant marker to construct a QTL map for the evolution of photoperiodic response. The QTL map shows 6 to 9 QTL, accounting for 62% of the difference in critical photoperiod between Florida and Alberta (Mathias et al., 2007). On the 3rd chromosome, *timeless* does not lie under a QTL, but *timeless*, or a gene closely linked to it, does exhibit epistatic interactions with 4 AFLP markers and a gene predicted to have isocitrate dehydrogenase activity (Flybase: l(1)G0156). Even if the epistatic interaction is actually due to *timeless*, there is no evidence as to whether or not such an epistatic effect on the evolution of critical photoperiod is due to the role of *timeless* in the circadian clock or to pleiotropic

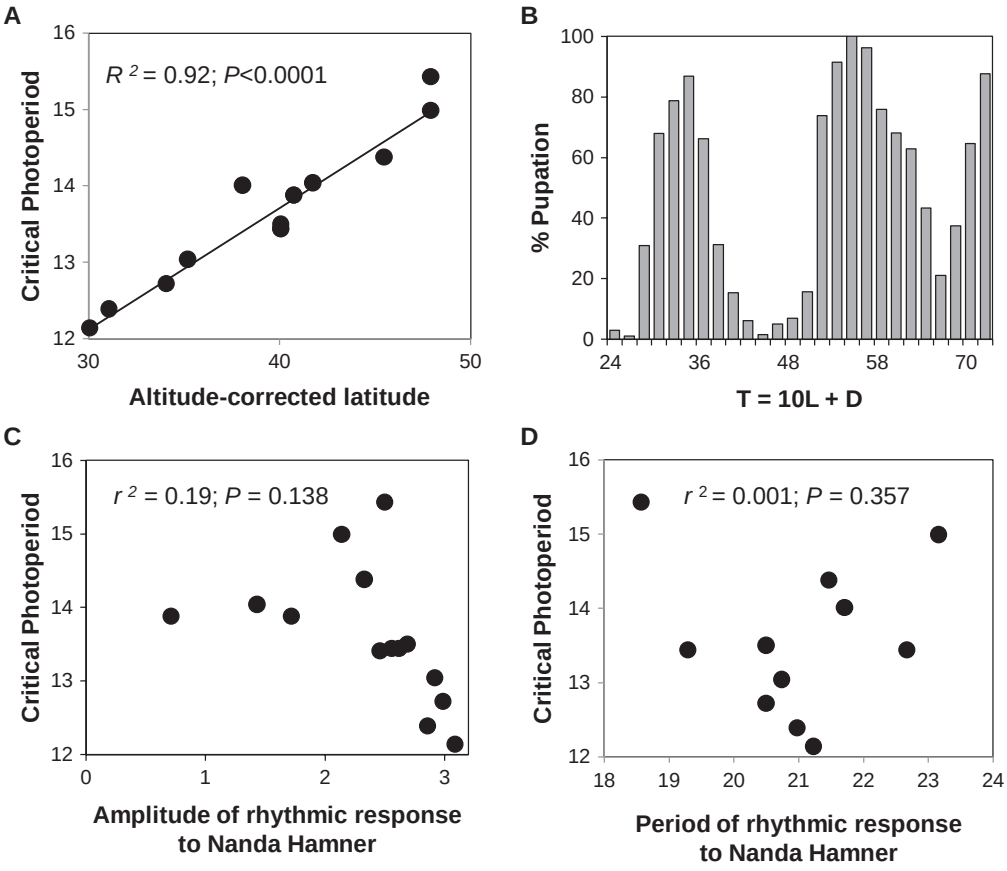

Figure 4. Photoperiodic response of *Wyeomyia smithii* and its relationship to the period and amplitude of the circadian clock. (A) Critical photoperiod is tightly correlated with latitude from the Gulf of Mexico to Canada (30-50°N) in North America. (B) Response to the Nanda-Hamner protocol of a population from the Gulf Coast (30°N). The amplitude of the rhythmic response is measured by log (area under the curve) and the period of the rhythm by the peak-to-peak interval. Evolution of critical photoperiod (A) is not significantly correlated with either the amplitude (C) or period (D) of the circadian rhythm as measured from response to the Nanda-Hamner protocol in the same respective populations. Data from Bradshaw et al. (2003, 2006).

effects of *timeless* acting as a single gene independently of its role in the circadian clock.

Our other work with *W. smithii* leads us to a conclusion similar to Lankinen and Forsman (2006). The most widely used experiment to infer a circadian basis of photoperiodism is the Nanda-Hamner protocol (Nanda and Hamner, 1958; Pittendrigh, 1981a; Saunders, 2002, pp 351-358). The basic premise is that there is a sensitivity to light that is rhythmic and free runs in the dark portion of a light:dark cycle, and if dawn occurs during the sensitive portion of the rhythm, a long-day response would occur. In the Nanda-Hamner protocol, animals are exposed to a short day and a long night, typically an L:D = 10:14 regimen. Then, in separate experiments, day length is held constant but night length is increased. The concept is that if night ends when the rhythm is in the light-sensitive phase, then the L:D cycle would be interpreted as a long day; if night ends when the rhythm is in the light-insensitive phase, then the L:D cycle would be interpreted as a short day. Hence, if the long-day response is plotted as a function of total cycle length, T = L + D, then a rhythmic long-day response has been used historically to imply a circadian basis of photoperiodic response (Saunders, 1968, 2009; Pittendrigh, 1981a). The time interval from one peak of long-day response to the next is interpreted as the period of the underlying circadian rhythm. But, to be a rhythm, there must be amplitude as well as a period, and the peak-to-valley difference in phenotype or area under the response curve is a measure of the amplitude of the rhythm (Pittendrigh et al., 1991; Bradshaw et al., 2003). In *W. smithii*, the critical photoperiod (an expression of the photoperiodic timer) mediating the onset and maintenance of larval diapause increases with latitude and altitude, with R^2 regularly ≥92% (Fig. 4A), but neither the period nor the amplitude of response to the Nanda-Hamner protocol (Fig. 4B) is correlated with critical photoperiod across the range of *W. smithii* (Fig. 4C, D).

One cannot simultaneously argue that the rhythmic response to the Nanda-Hamner protocol represents an expression of the circadian clock and that the circadian clock is responsible for the evolution of photoperiodic response when the critical photoperiod is not correlated with either the period or amplitude of the circadian clock. We agree that rhythmic response to Nanda-Hamner represents an expression of the circadian clock analogous to pupal

eclosion but conclude that rhythmic response to the Nanda-Hamner protocol does not represent a causal relationship between the circadian clock and photoperiodism (Emerson et al., 2009b). Consequently, in *W. smithii*, adaptive evolution of the photoperiodic response over the ecoclimatic gradient of North America has taken place independently of the circadian clock.

Mutational or allelic variation in clock genes should and can be interpreted in the context of circadian function, that is, daily not seasonal timing. Figure 3 shows how entrainment of the circadian clock may be adaptive in a varying photothermal environment without invoking photoperiodism or a photoperiodic timer. Another excellent example of this approach is the geographic cline in naturally occurring alleles of threonine-glycine repeats in the *period* gene in European *D. melanogaster*. Interestingly, the more prevalent northern allele results in a less accurate but more temperature-compensated clock; the prevalent southern allele results in a more accurate but less temperature-compensated clock. The former is postulated to be adaptive in the daily and seasonally more variable northern thermal environment, while the latter is postulated to be adaptive in the daily and seasonally more uniform southern thermal environment (Sawyer et al., 1997; Costa and Kyriacou, 1998; Kyriacou et al., 2007). When geographic variation is found in circadian genes, the most immediate and parsimonious search for adaptive significance should be in circadian function.

The daily circadian clock and the seasonal photoperiodic timer have distinct formal properties and serve 2 separate adaptive functions (Fig. 1). Independent evolution of these 2 physiological processes should be expected over ecoclimatic gradients that vary in the amplitude of seasonal day length, in the mean and amplitude of daily temperature, in the length of the growing season, in the duration and severity of winter cold and summer heat, and in ecological contexts that are not the same between times of day and between times of year (Danks, 2005; Emerson et al., 2009b).

FUTURE DIRECTIONS

The daily circadian clock can be highly adept at tracking annual changes in day length and temperature on a day-to-day basis, which are intimately tied to the clock's arbiter of cellular, metabolic, and behavioral functions (Fig. 3). The seasonal photoperiodic timer is highly precise in measuring day/night length and counting L:D cycles in the anticipation of the changing environments over ecoclimatic gradients (Fig. 4A). Most attempts at discovering a molecular basis of the photoperiodic switch have used circadian rhythm genes as candidate loci, including studies from our own lab (Mathias et al., 2005). There are several concerns. First, if one looks only for effects of circadian genes on photoperiodism, then only effects of circadian genes will ever be found. Second, circadian rhythmicity is pervasively involved in the behavior, physiology, and cellular biochemistry of organisms. Since the photoperiodic timer is undoubtedly a product of cellular physiology and biochemistry, it would be highly unusual if circadian rhythmicity had zero effect on the photoperiodic timer, but having an effect is not the same as being a causal, necessary component of the photoperiodic timer as envisioned by Bünning. Third, circadian genes may affect the expression of other genes, independently of their role in circadian rhythmicity (Emerson et al., 2009a).

Instead of being restricted by circadian clock genes as candidate loci, forward genetic approaches, unbiased by a circadian perspective, should provide a more fruitful avenue of endeavor to discover the molecular basis of photoperiodism (Bradshaw and Holzapfel, 2007b; Tauber and Kyriacou, 2008). QTL mapping of the critical photoperiod shows 6 to 9 regions of the *Wyeomyia smithii* genome involved in the evolution of photoperiodic response (Fig. 5A), and expression microarrays reveal a number of specific genes involved in differential response to day length (Fig. 5B). One of the latter, *ppdrg1* (*photoperiodic response gene 1*, formerly *Ws13043*), maintains strong linkage disequilibrium between northern and southern alleles and critical photoperiod after 25 generations of free recombination from a cross between extreme phenotypes (Emerson et al., 2010). Hence, 3 independent forward genetic approaches all point to a gene of previously unknown function, *ppdrg1*, or a gene very tightly linked to it, as a candidate locus for involvement in the photoperiodic switch mechanism or a tightly connected neuroendocrine process immediately downstream from the photoperiodic switch.

The forward genetic approaches that we have used with *W. smithii* have taken advantage of genetic variation among natural populations having evolved over seasonal climatic gradients in North

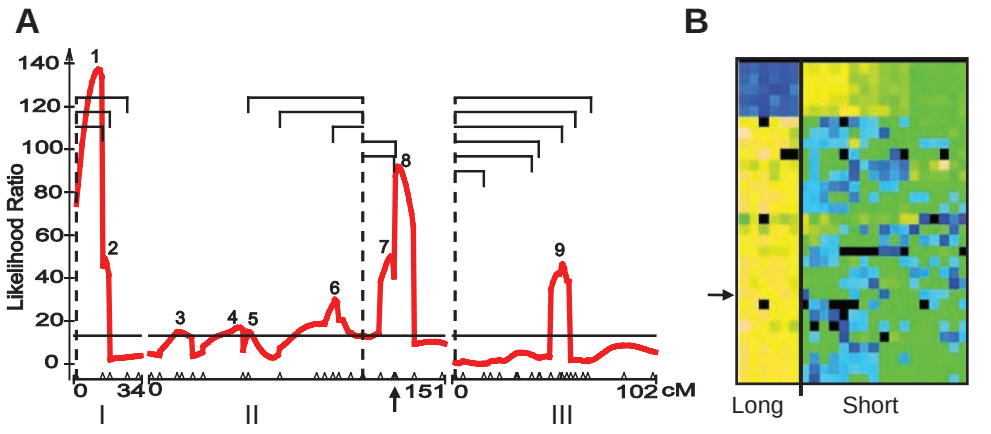

Figure 5. Circadian unbiased, forward genetic approaches to identifying photoperiodism genes in *Wyeomyia smithii*. (A) QTL map derived from the F2 hybrid between a single northern Alberta (58°N) male and a single Florida (31°N) female (Mathias et al., 2007). The map plots regions of the mosquito's 3 chromosomes contributing to the evolution of photoperiodic response. Peaks above the horizontal black line are considered significant, indicating that there are 6 to 9 QTL across all 3 chromosomes that contribute to the evolution of critical photoperiod. The dashed lines and brackets indicate epistatic interactions affecting differences in critical photoperiod between the 2 populations. (B) Gene expression that is regulated by differential physiological response to day length based on a cDNA microarray (Emerson et al., 2010). Blue squares represent down-regulated genes; yellow squares represent up-regulated genes. In both (A) and (B), the black arrow indicates the gene *ppdrg1*.

America. The increasing availability of molecular tools for nonmodel organisms is making available species and populations of animals whose biogeography, physiology, ecology, and evolutionary background make them more suitable for revealing the genetic basis of the photoperiodic switch. At the same time, the focus on photoperiodic control of seasonal development provides a window into evolutionary processes taking place over long-term dispersal of animals on a continental scale (Danilesvskii, 1965) to short-term responses to recent rapid climate change on a local scale (Bradshaw and Holzapfel, 2008).

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NOTES

Supplementary material (SOM) for this article is available on the *Journal of Biological Rhythms* Web site at http://jbr.sagepub.com/supplemental.

1. See SOM.
2. Lees (1973) provides a more comprehensive view of his studies on aphids.
3. It is interesting that after this time, Pittendrigh ceased working with *Pectinophora*.
4. *per*$^{-}$ is a double, overlapping deletion that lacks the *per* locus altogether and, unfortunately, occurs in a different genetic background [Df(y64j4)/Df(y^{2}TEM202/w^{+}Y)], confounding comparisons with Canton-S.

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