Evolutionary Divergence of Circadian and Photoperiodic Phenotypes in the Pitcher-Plant Mosquito, *Wyeomyia smithii*

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Abstract For decades, chronobiologists have investigated the relationship between the circadian clock that mediates daily activities and the photoperiodic timer that mediates seasonal activities. The main experiment used to infer a circadian basis for photoperiodic time measurement is the Nanda-Hamner protocol (NH). Herein, the authors compare additive and nonadditive (dominance and epistasis) genetic effects that lead to the divergence of populations of the pitcher-plant mosquito, *Wyeomyia smithii*, for critical photoperiod (CPP) and amplitude of the rhythmic response to NH for 3 temporal-geographic scales: 1) Over geological time between populations in northern and southern clades, 2) over millennial time between populations within the northern clade, and 3) over generational time between lines selected for long and short CPP from within a single population. The authors show that the pattern of additive, dominance, and epistatic effects depends on the time scale over which populations or lines have diverged. Patterns for genetic differences between populations for CPP and response to NH reveal similarities over geological and millennial time scales but differences over shorter periods of evolution. These results, and the observation that neither the period nor amplitude of the NH rhythm are significantly correlated with CPP among populations, lead the authors to conclude that the rhythmic response to NH has evolved independently of photoperiodic response in populations of *W. smithii*. The implication is that in this species, genetic modification of the circadian clock has not been the basis for the adaptive modification of photoperiodic time measurement over the climatic gradient of North America.

Key words circadian rhythm, critical photoperiod, evolutionary divergence, genetic architecture, Nanda-Hamner, photoperiodic time measurement

A wide variety of plants and animals use the length of day or photoperiod to anticipate and prepare for future seasonal events in their life histories. Among temperate arthropods, the day length (critical photoperiod [CPP]) used to switch from active development to dormancy (diapause) increases with latitude and altitude (Bradshaw, 1976; Taylor and Spalding, 1986; Danks, 1987, Table 24). The CPPs from different localities "breed true" and hence represent evolutionary (genetically determined) adaptations to the local changing seasons.

Two basic mechanisms have been proposed to account for the evolution of critical photoperiod over climatic gradients. First, photoperiodic response could

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 21 No. 2, April 2006 132-139
DOI: 10.1177/0748730406286320

be the result of an interval or hourglass timer that assesses the length of day or night independently of the circadian clock. Such a timer needs to be reset by a dawn or dusk transition each 24-h cycle, and sensitivity to light does not repeat or cycle during a long dark period. Second, photoperiodic response could be the result of the underlying circadian clock that free runs under constant conditions and does not require resetting by a dawn or dusk transition each 24-h cycle. In this scenario, sensitivity to light would repeat or cycle endogenously during a long dark period. A circadian basis for photoperiodic time measurement has had an enduring appeal because, if true, it would mean that the circadian clock that controls daily activities would also form the basis of the photoperiodic timer that regulates seasonal activities. Understanding circadian rhythmicity would then provide insight into the mechanism by which organisms adapt to new seasonal environments during periods of range expansion or rapid climate change. The role of circadian rhythmicity in photoperiodic time measurement has been investigated by chronobiologists for more than 65 years, but the issue remains unresolved (Bradshaw and Holzapfel, 2001a; Veerman, 2001; Saunders, 2002, pp 479-481; Bradshaw et al., 2003; Danks, 2003, 2005).

The principal experiment used to implicate involvement of the daily, circadian clock in the seasonal, photoperiodic timer is the Nanda-Hamner protocol (NH). In NH experiments, organisms are exposed to a short day and a long night of varying duration to create a total cycle length (T) of 1 to several days, typically T = L + D = 24-72 h (Fig. 1). The proposition is that, if photoperiodic response were determined by an hourglass, each of these cycles would be a short day and a long night and should sustain only a short-day response since a timer reset by dawn or dusk would always run out in the dark. By contrast, if photoperiodic response were determined by the circadian clock, there should be an underlying rhythmic sensitivity to light and, consequently, a rhythmic long-day response as T is increased from 24 to 72 h (Fig. 1). Such a rhythmic long-day response has been observed among plants and animals and has been used to infer a circadian basis for photoperiodic time measurement (Pittendrigh, 1981; Takeda and Skopik, 1997; Vaz Nunes and Saunders, 1999; Tauber and Kyriacou, 2001). There is general consensus that a rhythmic response to NH represents an overt expression of the circadian clock; there is less agreement as to whether this expression of the circadian clock is actually a causal and necessary component of photoperiodic time measurement (Bradshaw and Holzapfel, 2001a; Veerman, 2001; Saunders, 2002, pp 479-481; Bradshaw et al., 2003; Danks, 2003, 2005). In this study, we address the question of genetic causality.

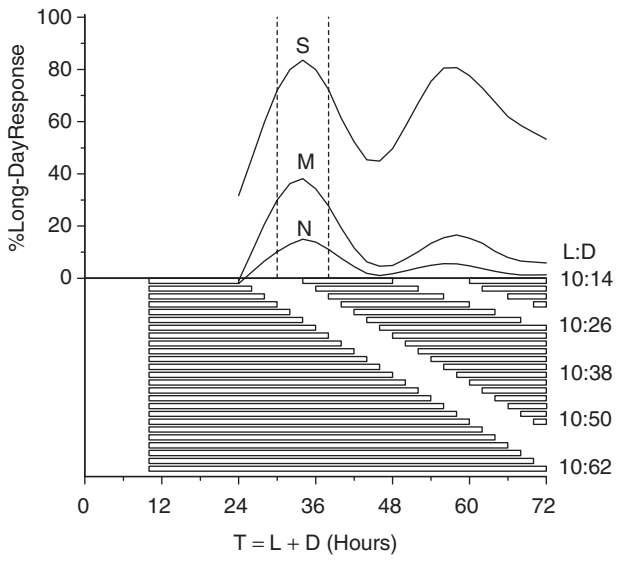

Figure 1. Examples of the rhythmic response to the Nanda-Hamner protocol in *Wyeomyia smithii*. Cohorts of diapausing larvae were exposed for 8 weeks to L:D = 10:D, where D = 14-62 h in 2-h increments in 25 different experiments. The declining amplitudes of the rhythmic responses represent, from top to bottom, either 1) southern (S), midlatitude (M), and northern (N) populations; or 2) lines from a midlatitude population selected for short critical photoperiod, unselected control, and selected for long critical photoperiod (Bradshaw et al., 2003). The dashed vertical lines in the top portion show the range of total cycle lengths (T-cycles) from 30 to 38 h used in the line crosses.

The perception of any rhythm depends upon 2 properties, period and amplitude. The period of a rhythm is the interval over which it repeats itself. The period of the rhythmic, long-day response to NH does not change with geography and is not significantly correlated with CPP among widespread populations of either mites in Europe (Vaz Nunes et al., 1990) or mosquitoes in North America (Bradshaw et al., 2003). The period component of circadian rhythmicity does not appear to be involved in the adaptive evolution of CPP at temperate latitudes. Consequently, we now focus on amplitude.

The amplitude of a rhythm is the magnitude of the peaks. A number of studies have shown an apparent decline in the amplitude of the rhythmic response to NH in northern as compared to southern arthropods (Bradshaw and Holzapfel, 2001a). However, CPP increases with altitude as well as latitude because winter arrives earlier at higher elevations. Multiple

regression of amplitude of the rhythmic response to NH on both altitude and latitude of population origin in the mosquito, *Wyeomyia smithii*, reveals that amplitude decreases with altitude but not latitude; furthermore, when altitude as well as latitude of population origin is included, CPP is no longer significantly correlated with amplitude (Bradshaw et al., in press). Hence, in a single species in which CPP is tightly correlated with both latitude and altitude, with R^2 regularly >90% (Bradshaw and Holzapfel, 2001b), CPP is not correlated with period or amplitude of the rhythmic response to NH. The implication is that CPP can and has evolved independently of circadian rhythmicity.

For independent evolution to occur, there must be genetic differences between the 2 processes. This point is essential because both CPP and NH experiments quantify the percentage of individuals that resume active development following a larval diapause, only under different photic conditions. Assuming that the hormonal and developmental steps required to terminate diapause are the same in both experiments, genetic differences between responses are most likely in the interpretation of the photic environment, that is, genetic differences between the circadian clock and the photoperiodic timer. No study has previously examined the genetic basis of geographic variation in the rhythmic response to NH or compared the genetic architecture of CPP with the genetic architecture of the rhythmic response to NH among populations from over a wide geographic range.

Herein we shall 1) determine the genetic effects (i.e., additive, dominance, or epistatic[a]) underlying differences in the amplitude of the rhythmic response to NH between populations and selected lines and 2) compare the patterns of additive, dominance, and epistatic effects among populations and selected lines between CPP and amplitude of the rhythmic response to NH.

EXPERIMENTAL APPROACH

First, our approach was to perform a series of crosses between natural populations and between selected lines of *W. smithii* that vary in both CPP (reflecting the seasonal timer) and amplitude of the rhythmic response to NH (reflecting the circadian clock) and then to use the joint scaling test[b] to examine the genetic effects underlying evolutionary divergence over 3 time scales: 1) geological, between populations in northern and southern clades; 2) millennial, between populations within the more recently derived northern clade; and 3) generational, between lines of a single population divergently selected over 13 generations for long and short CPP. If, as expected, the genetic architectures for both traits are different at any time scale, such a result would indicate that the 2 processes are genetically distinguishable and would underscore the independent evolution of photoperiodic time measurement and circadian rhythmicity in *W. smithii*. If the genetic architectures for both processes are similar at all time scales, such a result would indicate that the 2 processes are genetically interdependent despite their lack of correlation among populations.

WYEOMYIA SMITHII

The pitcher-plant mosquito, *W. smithii*, ranges in North America from the Gulf of Mexico (30°N) to eastern and central Canada (57°N). Throughout its range, *W. smithii* oviposit into and complete their preadult development within the water-filled leaves of the carnivorous purple pitcher plant, *Sarracenia purpurea*. *Wyeomyia smithii* overwinter in the evergreen leaves of their host in a larval diapause that is initiated, maintained, and terminated by photoperiod (Bradshaw and Lounibos, 1977).

Populations of *W. smithii* comprise a southern clade found at low elevations from 30-36°N and a northern clade found at higher elevations at 35-36°N, at low elevations from 38-40°N, and at higher latitudes (41-57°N) formerly covered by the Laurentide Ice Sheet until 8000 to 20,000 years ago (Armbruster et al., 1998). Populations from throughout this range are fully interfertile when crossed.

Hybridization experiments (line crosses) between more distantly related populations in the southern and northern clades consistently show genetic differences in CPP due to additive, dominance, and epistatic effects. Line crosses between populations within the more recently-derived northern clade show genetic differences in CPP due to additive effects alone, additive and dominance effects alone, or additive, dominance, and epistatic effects (Hard et al., 1993; Lair et al., 1997). Crosses between lines divergently selected for long and short critical photoperiod within each of 3 demes from a single midlatitude population all differ in additive, dominance, and epistatic effects in critical photoperiod (Bradshaw et al., 2005). Hence, all of the additive and nonadditive genetic effects by which CPP has evolved

over millennial (within the northern clade) and geological (between the northern and southern clade) time scales are already represented within a single midlatitude population.

Selection on CPP within the midlatitude population also elicited a correlated response to NH that mimics the geographic pattern (Fig. 1): Lines selected for long critical photoperiod show a reduced amplitude (northern), and lines selected for short critical photoperiod show an increased amplitude (southern) rhythmic response to NH (Bradshaw et al., 2003). We now use line crosses between populations and between selected lines of *W. smithii* to ask whether the genetic divergence of the amplitude of the rhythmic response to NH over the same geological, millennial, and generational time scales involves the same sorts of additive and nonadditive (dominance and epistasis) genetic effects as critical photoperiod.

Table 1. Crosses between Natural Populations of *Wyeomyia smithii* and between Lines Selected for Short and Long Critical Photoperiods

Cross (Ref [a]*)*	*Latitudinal Difference*	*Area Difference*[b]	*CPP Difference*[c]
Southern × Northern			
FL × NJ (WI, PB)	10°	0.2061	1.4
NC × NJ (GS, PB)	6°	0.3301	0.8
NC × MD (GS, NP)	4°	0.3652	0.7
Northern × Northern			
MD × NJ (NP, PB)	2°	–0.0351	0.1
NJ × ME (PB, KC)	6°	0.0976	1.9
Short-selected lines × Long-selected lines from 3 demes within a 300-m radius in NJ (PB)			
Streamside	0°	0.2204	1.5
Backwater	0°	0.2467	1.3
Sandy Bog	0°	0.1767	1.3

NOTE: Populations are designated by state of origin using 2-letter abbreviations; selected lines are designated by subpopulation of origin.
a. Reference to populations from specific localities used in previous studies from this laboratory.
b. Area under the rhythmic-response curve for Nanda-Hamner experiments (T = 30-38 h) from experiments in this study.
c. CPP = critical photoperiod. SOURCE: Bradshaw et al. (2003).

MATERIALS AND METHODS

Collection and Maintenance of *Wyeomyia smithii*

Two thousand larvae were collected from each locality (Table 1) and, prior to experiments, reared through at least 2 generations under long-day conditions (18L:6D) with 80% humidity and a sine-wave thermoperiod ranging from 13 to 29 °C each day.

Selected Lines

Lines selected for divergent CPPs were derived from 3 demes sampled from within 300 m of one another at a midlatitude locality (Table 1). Divergent selection for long and short CPP was applied within each of the 3 demes for 13 generations, with minimum effective population sizes of $N_e > 100$ each generation and cumulative inbreeding less than 4% (Bradshaw et al., 2003). Following selection, populations were reared through 11 additional generations with $N > 1000$ per generation without selection to reduce linkage disequilibrium before performing the line crosses described below.

Crosses between Populations and Selected Lines

The 2 parent populations or selected lines were hybridized to produce 14 "generations": the 2 parents, F_1, F_2, both backcrosses, and all but 2 of their possible reciprocals, as in Lair et al. (1997). The experimental generations were reared on short days to induce dormancy and to synchronize development.

Nanda-Hamner Experiments

Previous experiments showed that the greatest variation in amplitude among populations or lines occurs at the 1st peak in long-day response, between L + D = T from 30 to 38 h (Fig. 1; Bradshaw et al., 2003). For all 14 generations from the crosses between populations or selected lines in Table 1, larvae were exposed to 10L + 20-28D in 5 separate treatments, each treatment increasing in night length by 2 h. At least 100 larvae from each line, in each generation and in each T-cycle (i.e., ≥7000 larvae per cross) were exposed to the experimental regimen for 8 weeks at 23 °C and then transferred to short days (8L:16D) at 21 °C for 2 additional weeks to allow and record development of larvae that had been stimulated by the experimental conditions but had not pupated by day 56. At the end of 2 weeks on short days, the remaining larvae were censused and scored as remaining in diapause. Long-day response to NH was measured as fraction of larvae developing (pupating) and was calculated at the end of the experiment for each replicate as [total number of

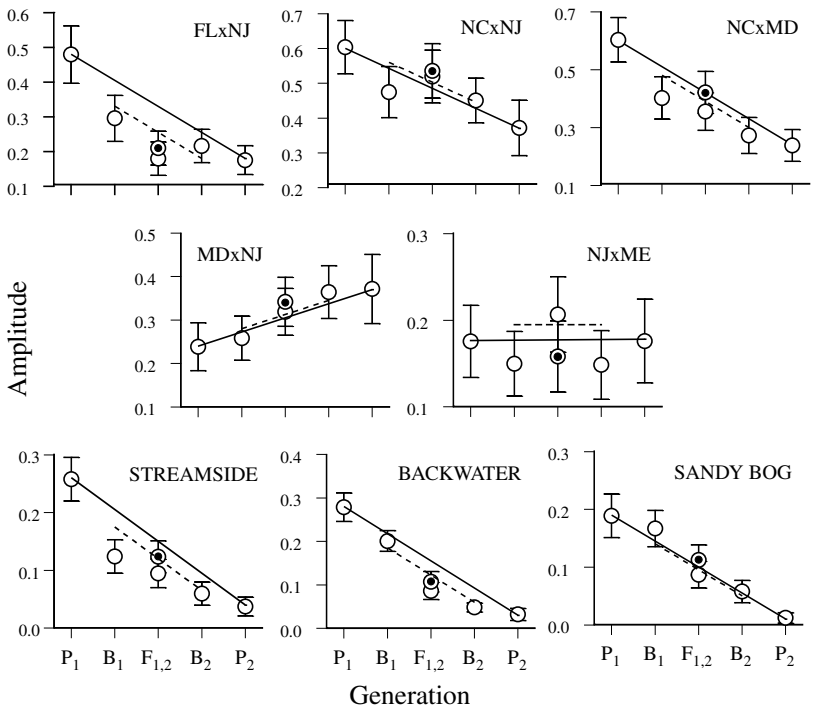

Figure 2. Amplitude of the rhythmic response to the Nanda-Hamner protocol measured as frequency of development (long-day response) from total cycle length (T) = 30-38 h in 2-h increments in parental and hybrid lines from crosses: Top row, between populations in the southern and northern clades; middle row, between populations within the northern clade; bottom row, between lines selected for long and short critical photoperiod in 3 demes within a single midlatitude population (New Jersey [NJ]). The P_1 generation represents either the more southern parent (top, middle) or the parent selected for short critical photoperiod (bottom); conversely, the P_2 generation represents either the more northern parent (top, middle) or the parent selected for long critical photoperiod (bottom). The F_1 generation is simply a hybrid between P_1 and P_2 generations, while the F_2 generation represents offspring of the F_1. The B_1 and B_2 generations represent backcrosses between the F_1 and either the P_1 or P_2 generations, respectively. Error bars show ± 2 SE. The solid line shows the additive expectation based on the 2 parents; the dashed line shows the additive-dominance expectation based on the 2 parents and their F_1s. The symbol with the central solid circle represents the F_2. Reciprocal crosses are pooled for clarity.

larvae having pupated] ÷ [total number of larvae having pupated + number of larvae remaining alive on day 70] across all 5 T-cycles within each generation of each cross. Variances were calculated using the binomial approximation (Zar, 1996). Response to NH was not determined for all crosses concurrently, but within each cross, all 14 generations and 5 T-cycles were run concurrently as a single block.

Line Cross Analysis and the Joint Scaling Test

By crossing populations or lines with divergent phenotypes, the means and variances of recombinant (F_2 and backcross generations) and nonrecombinant (F_1 generation) hybrid phenotypes can be used via the joint scaling test to assess the contribution of additive, dominance, and epistatic effects to the phenotypic differences between populations or selected lines. The joint scaling test uses the method of least squares to test hierarchically for goodness-of-fit of generation means and variances to increasingly inclusive models (Lair et al., 1997).

RESULTS

The genetic architecture of CPP and the amplitude of the rhythmic response to NH differed among time scales.

The crosses between southern and northern clades revealed that additive, dominance, and epistatic effects all contribute to the geographic differentiation in the amplitude of the rhythmic response to NH over the range of *W. smithii* (Fig. 2, top; Table 2). For the 2 crosses between populations within the northern clade, additive effects alone were sufficient to explain divergence in amplitude of the rhythmic response to NH between the Maryland (MD) and New Jersey (NJ)

Table 2. Results of the Joint Scaling Test for Genetic Differences in Amplitude of the Rhythmic Response to the Nanda-Hamner Protocol

Cross	*A* (4 df)	*AD* (3 df)	*ADM* (4 df)	*ADME* (1 df)
Southern × Northern				
FL × NJ				
χ^2	32.06*	16.42*	14.45*	1.30
p	<0.001	<0.001	0.006	0.254
NC × NJ				
χ^2	13.82*	13.15*	15.77*	0.21
p	0.008	0.004	0.003	0.647
NC × MD				
χ^2	16.53*	13.32*	24.93*	4.87*
p	0.002	0.004	<0.001	0.027
Northern × Northern				
MD × NJ				
χ^2	3.69	—	—	—
p	0.450			
NJ × ME				
χ^2	12.80*	10.60*	6.36	—
p	0.012	0.014	0.174	
Short-selected lines × Long-selected lines				
Streamside				
χ^2	30.90*	21.76*	22.59*	0.87
p	<0.001	<0.001	<0.001	0.351
Backwater				
χ^2	55.32*	6.52	—	—
p	<0.001	0.089		
Sandy Bog				
χ^2	9.31	—	—	—
p	0.054			

NOTE: Values are based on generation means and variances from crosses between natural populations and lines of *W. smithii* selected for short and long critical photoperiods. Goodness-of-fit tests (χ^2) were run for A, an additive model; AD, an additive-dominance model; ADM, an additive-dominance-maternal model; and ADME, an additive–dominance–maternal–digenic epistasis model. *df* = degrees of freedom.
*Statistically significant at $p < 0.05$. Significance is not changed by applying a sequential Bonferroni correction.

Table 3. Summary of Genetic Differences (A, additive; D, dominance; E, epistatic) between Populations and Selected Lines in Critical Photoperiod and Amplitude of the Rhythmic Response to the Nanda-Hamner Protocol

Cross	*Critical Photoperiod*	*Amplitude of Rhythm*[a]
Southern × Northern	A, D, & E (8/8)[b,c]	A, D, & E (3/3)
Northern × Northern	A (1/3); A & D (1/3); A, D, & E (1/3)[c]	A (1/2); A & D (1/2)
Short × Long within NJ	A, D, & E (3/3)[d]	A (1/3); A & D (1/3); A, D, & E (1/3)

NOTE: Numbers in parentheses = (number of crosses showing the genetic effect/total number of crosses).
a. See Table 2.
b. SOURCE: Hard et al., 1993.
c. SOURCE: Lair et al., 1997.
d. SOURCE: Bradshaw et al., 2005.

populations, while additive, dominance, and maternal effects alone accounted for differences in amplitude of the rhythmic response to NH between NJ and Maine (ME) (Fig. 2, middle; Table 2).

For the crosses between lines selected for long and short critical photoperiods, the joint scaling test (Fig. 2, bottom; Table 2) demonstrated that divergence of the amplitude in the rhythmic response to NH may involve additive effects alone (Sandy Bog), additive and dominance effects alone (Backwater), or a more complex genetic architecture involving additive, dominance, and epistatic effects (Streamside). Mean fractions developing in the P_2 generations ranged from 0.01 to 0.05, and the significant effects of epistasis came from all 3 kinds of digenic epistasis: additive × additive, additive × dominance, and dominance × dominance (data not shown).

DISCUSSION

Among populations of *W. smithii*, the pattern of genetic effects for the amplitude of rhythmic response to NH parallels that of CPP (Hard et al., 1993; Lair et al., 1997) over both geological and millennial time scales (Table 3). Additive, dominance, and epistatic effects all contribute to the genetic divergence of northern from southern populations; and genetic divergence among the more closely related northern populations involves only additive or additive and dominance effects without epistasis. In contrast, over generational time scales the pattern of genetic effects for the amplitude of rhythmic response to NH differs distinctly from that of CPP, demonstrated by data from crosses between selected lines derived from 3 demes within a single population (Table 3). Genetic differences in CPP among selected lines uniformly involve additive, dominance, and epistatic effects (Bradshaw et al., 2005), while genetic differences in the rhythmic response to NH involve additive effects alone, additive and dominance effects alone, or a combination of additive, dominance, and epistatic effects (Table 3).

These findings show that the correlated response of NH to direct selection on CPP involved different genetic pathways in demes undergoing uniform selection, resulting in very similar phenotypes in the derived

lines. There are at least 2 nonexclusive explanations for these results. First, genetic subdivision of the population is occurring over a very fine spatial scale. Second, unique genetic trajectories (i.e., unique allelic combinations) underlie similar phenotypic trajectories in response to a uniform selection gradient. Regardless of the relative roles of these 2 alternatives, our results show that diverse and distinct pathways of additive and nonadditive genetic variation contribute to response to selection over short generational time scales for both CPP and the amplitude of the rhythmic response to NH (Table 3). These data, and the fact that neither the period nor amplitude of the rhythmic response to NH is significantly correlated with CPP among populations (Bradshaw et al., in press), lead us to conclude that the rhythmic response to NH has evolved independently from CPP over the climatic gradient of North America.

The implication of our conclusion is that genetic changes in the circadian clock have not been responsible for the adaptive, genetic modification of photoperiodic time measurement. Originally, Bünning (1936) proposed that the endogenous daily rhythm forms the basis (*Grundlage*) of the photoperiodic reaction—in other words, that circadian rhythmicity constitutes a causal, necessary element of photoperiodic time measurement. We find it difficult to support this proposition when the adaptive, geographic variation in photoperiodic response in *W. smithii* is not correlated with the period, amplitude, or genetic architecture of the rhythmic response to NH, the main experiment historically used to infer a circadian basis of photoperiodic time measurement. Our results do not, however, permit us to reject the more equivocal stance that "a positive [rhythmic] Nanda-Hamner result indicates that the circadian system is *somehow* involved in the photoperiodic phenomenon" (Vaz Nunes and Saunders, 1999, p 91, emphasis theirs). To do so, one would have to show that the circadian system was in no way involved in the photoperiodic phenomenon, a position we consider unlikely since circadian rhythmicity has such a pervasive influence over cellular biochemistry in general. We view it as more likely that some of the genes comprising the 2 negative feedback loops of the circadian clock may be involved in photoperiodism, independently of their role in circadian rhythmicity. Indeed, it has been suggested that the gene *timeless* may play such a role (Tauber and Kyriacou, 2001; Mathias et al., 2005).

A major impediment to progress in determining the relationship between circadian rhythmicity and photoperiodic time measurement is the fact that there is currently no laboratory model system that can be used to investigate both photoperiodism and circadian rhythms comprehensively at the molecular level. *Drosophila melanogaster* is excellent for molecular studies of the circadian clock, but while "*D. melanogaster* with its unrivalled genetic background has provided a foundation for uncovering the molecular basis of the circadian mechanism in both locomotor and eclosion rhythmicity, . . . it is probably less useful as a model for photoperiodism. Further studies should examine species with a much more robust [photoperiodic] response" (Saunders, 2002, p 481). What is lacking are molecular data from multiple photoperiodic arthropods, preferably from forward genetic approaches that do not make a priori assumptions about candidate loci. Studies such as this one, as well as others we have undertaken with *W. smithii* (Bradshaw et al., 2003, 2005, in press; Mathias et al., 2005), provide the impetus to develop molecular tools in photoperiodic, non–model organisms. Such tools are neither quick nor inexpensive to develop, but resolution of the relationship between the circadian clock that mediates daily activities and the photoperiodic timer that mediates seasonal activities will undoubtedly depend on their development.

ACKNOWLEDGMENTS

The authors thank Lara Andrijasevich and Tanya McKitrick for helping to run experiments. They also gratefully acknowledge NIH Training Grant 2T32GM07413-26 and NSF IGERT Training Grant DGE-9972830 for supporting D.M. as well as NSF research grants DEB-9806278, DEB-0412573, IBN 9814438, and IBN-0415653 to W.E.B.

NOTES

a. The *additive* effect of an allele is its independent effect on the phenotype regardless of which other alleles are present at the same locus or at different loci. In contrast, the *dominance* effect depends on the expression of other alleles at the same locus, while the *epistatic* effect depends on the expression of other alleles at different loci.

b. The *joint scaling test* is a goodness-of-fit test of observed generation means (parents, F_1, F_2, backcross, etc.) to the generation means expected if the parents differ in additive, dominance, maternal, epistatic, or other genetic effects.

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