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Hourglass and rhythmic components of photoperiodic time measurement in the pitcher plant mosquito, *Wyeomyia smithii*

Abstract The mosquito, *Wyeomyia smithii*, enters a larval dormancy or diapause that is initiated, maintained, and terminated by photoperiod. The median or critical photoperiod regulating diapause increases from 12 h of light per day along the Gulf of Mexico, USA (30° N), to over 15 h in southern Canada (49° N). Photoperiodic time measurement in *W. smithii* comprises both rhythmic and hourglass (interval timer) components. Using interrupted-night and resonance experiments, we show that both the rhythmic and hourglass components are prominent in the southern (ancestral) populations and that the influence of the rhythmic component declines with increasing latitude, while the hourglass component remains strong in northern (derived) populations. Previously, it has been shown that the genetic differences in critical photoperiod between northern populations and their southern ancestors involve not only the additive (independent) effects of genes, but also gene-gene interaction (epistasis). We therefore conclude that adaptive evolution of *W. smithii* has probably involved the progressive epistatic masking of the ancestral rhythmic component resulting in photoperiodic time measurement in northern populations accomplished principally through a day-interval timer. A comparison of *W. smithii* with previous studies indicates that the decline in critical photoperiod with increasing latitude represents an overall decrease in response to light rather than a shift in the timing of photosensitivity among arthropods in general. We propose that the underlying functional components of photoperiodic time measurement, as well as the overt photoperiodic response, are either homologous or are themselves responding directly to selection over latitudinal gradients in seasonality.

Abbreviations, symbols, and terms *ASPP* Asymmetric skeleton photoperiod with two light and two dark periods per day; herein, a longer main photophase with a shorter light break during the scotophase *CPP* Critical photoperiod, hours of light per day that stimulate 50% development and initiate or maintain 50% diapause in a sample population *Diapause* Arthropod dormancy, herein assumed to be hibernal $L:D=X:Y$ A cycle consisting of X hours of light and Y hours of darkness *Photophase* and *scotophase* The periods of light and dark, respectively, in an L:D cycle *PPRC* Photoperiodic response curve, a dose-response relationship between percent development and hours of light per day; this curve is usually sigmoid in shape at ecologically relevant photoperiods; extreme long or extreme short days may elicit intermediate responses *PTM* Photoperiodic time measurement, the ability of organisms to assess the duration or length of day or night *T* The period of the external driving cycle, the combined photophase and scotophase τ The period of an organism's free-running (unentrained) rhythm or oscillator

Introduction

A wide variety of plants and animals uses the length of day or photoperiod to synchronize development and dormancy with the changing seasons (Withrow 1959; Anonymous 1960; Bünning 1964; Aschoff 1965; Menaker 1971). One of the most robust ecogeographic generalizations is that the median or critical photoperiod (CPP) mediating the seasonal onset of diapause in temperate arthropods increases regularly with latitude or altitude (Andrewartha 1952; Danilevskii 1965; Bradshaw 1976; Taylor and Spalding 1986; Danks 1987, Table 24). The occurrence of this correlation in unrelated arthropods and the rapid evolution of latitude-specific photoperiod response by invading species (Hoy

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1978; Tauber et al. 1986, pp. 238–245; Fochs et al. 1994) argue that the geographic clines in photoperiodic response are adaptive. That is, these clines are the evolutionary product of natural selection acting on the timing of seasonal development locally for each population. Along with Taylor and Spalding (1986), we consider that the geographic cline in photoperiodic response has been established and needs no further testing to certify its generality. Still, over the range of a single temperate species, CPP may vary by as much as ten phenotypic standard deviations (Lair et al. 1997), representing an enormous evolutionary response to selection. The question then remains as to how populations accomplish this evolutionary diversification at the genetic and physiological levels.

Here, we consider two aspects of the physiological basis for the latitudinal adaptation of photoperiodic time measurement (PTM) in the pitcher plant mosquito, *Wyeomyia smithii*. First, we ask to what extent latitudinal adaptation of PTM reflects the variable expression of an underlying circadian pacemaker or the variable expression of an hourglass (interval) timer. Second, we ask to what extent latitudinal adaptation of PTM represents a measurement of time or a more generalized response to light.

Rhythmic and hourglass (interval) timers

Historically, it has been assumed that the photoperiodic response is effected through a day-interval timer, but, over 60 years ago, Bünning (1936) proposed that the measurement of photoperiod is executed not by an hourglass but by an endogenous circadian oscillation. An hourglass model is then posited as the null hypothesis, and the contribution of a rhythmic or circadian component has to be shown experimentally. Most insect photoperiodic responses have now been shown to possess a rhythmic component by one of two types of experiments (Saunders 1982; Takeda and Skopik 1997). First, and most directly, in night-interruption experiments (the Bünsow protocol), insects are exposed to a diapause-promoting short photophase and a long scotophase for a total period (*T*) of light plus dark of $T = L + D = 24, 48$, or 72 h. The long scotophase is then interrupted at progressively later times in a series of successive individual experiments by a short pulse of light, generally 1–2 h. A positive result is obtained when periodic peaks of long-day response appear, the interval between successive peaks being taken to represent the period (τ) of the underlying pacemaker. Second, in resonance or *T*-experiments (the Nanda-Hamner protocol), insects are exposed to a short-day photophase and scotophases of increasing duration to produce a total period ranging from $T = 20$–75 h in a series of individual experiments. When *T* is concordant or resonates with the period, τ, of the underlying pacemaker, the appropriate short-day response is observed; when *T* is discordant or out of phase with τ, a long-day response is observed. The result is a succession of response peaks and valleys as *T* is varied from 20 h to progressively longer cycles. Again, peak-to-peak intervals are taken to represent τ. A positive periodic response to either the Bünsow or Nanda-Hamner protocol is accepted as definitive evidence for a circadian component of PTM; failure of both protocols to evoke a rhythmic response is required for evidence of an hourglass mechanism. Below, we compare responses of the pitcher plant mosquito, *W. smithii*, originating from populations at 30–50 N in eastern North America to both interrupted-night and modified *T*-experiments. We show that they use both rhythmic and hourglass timers and then show how the interplay of these timers has contributed to the evolution of PTM in this species.

PTM as a response to light

Pittendrigh and co-workers (Pittendrigh and Takamura 1989, 1993; Pittendrigh et al. 1991) have proposed that PTM is not so much a measure of time as it is a response to light. Hence, the latitudinal increase in CPP should be due to a latitudinal decrease in long-day (light) response and should therefore result in a vertical, rather than a lateral shift in the entire PPRC from 0 to 24 h. Pittendrigh did not extend the predictions to Bünsow or Nanda-Hamner protocols. Below, we propose ways to use interrupted-night and *T*-experiments to test further whether the latitudinal increase in CPP represents a shift in the timing of response to daylength or a more general decrease in response to light. We shall then show how the evolution of rhythmic and hourglass components of PTM in *W. smithii* also involves the evolution of their response to light.

W. smithii

W. smithii is the sole temperate species of a large Neotropical genus (Lane 1953; Stone et al. 1959). Morphological, physiological, and behavioral characters all argue that *W. smithii* invaded North America from South America and since its establishment has diversified northwards from the Gulf of Mexico to Canada (Ross 1964; Bradshaw and Lounibos 1977; Istock and Weisburg 1987). Throughout its range, *W. smithii* is photoperiodic for the initiation, maintenance, and termination of larval diapause (Smith and Brust 1971; Evans and Brust 1972; Bradshaw and Lounibos 1972, 1977). The CPP for the initiation and maintenance of diapause is tightly correlated with latitude and altitude ($R^2 = 0.96$) of the locality of origin (Bradshaw 1976). CPP has evolved from 12 h of light per day along the Gulf Coast to over 15 h in the northern USA and southern Canada, representing 9–10 SDs of evolution from the ancestral (southern, short CPP) to the derived (northern, long CPP) state (Hard et al. 1993; Lair et al. 1997).
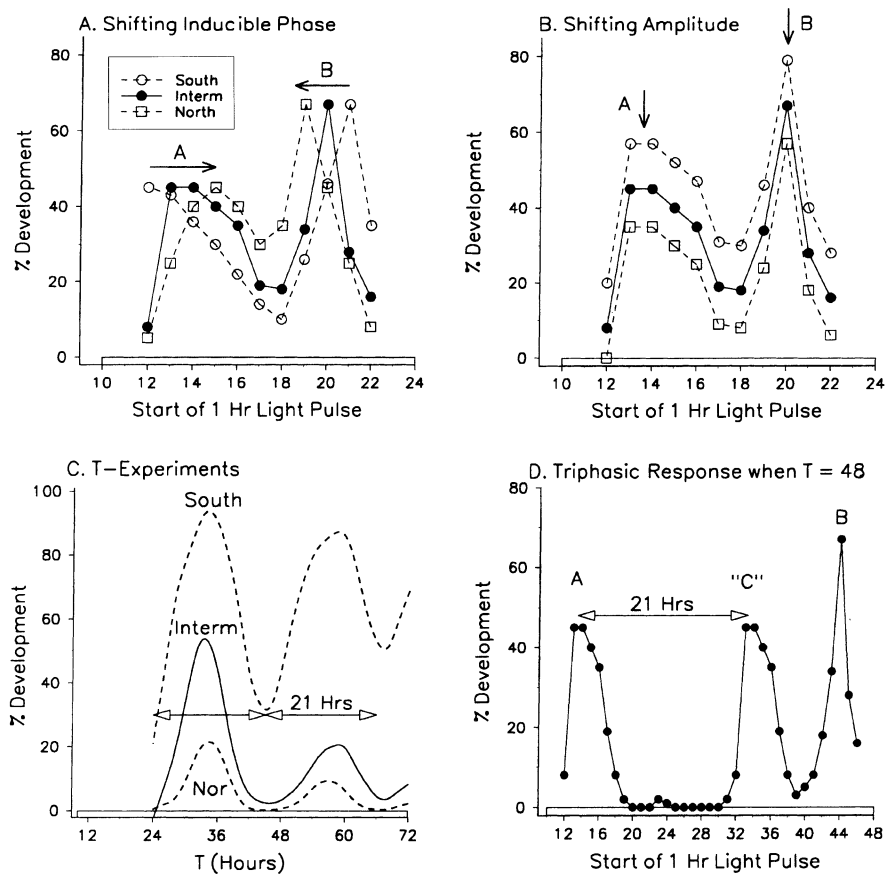
Fig. 1A–D Predicted patterns of developmental (long-day) response to asymmetric skeleton photoperiods with $T = 24$ and $T = 48$ h by *Wyeomyia smithii* from southern (*South*), intermediate (*Interm*), and northern (*North*) populations. **A, B** If the latitudinal increase in CPP is effected though shifting the position of the inducible phase, then the *A* peak should delay and the *B* peak advance with increasing latitude (*arrows*); if the latitudinal increase in CPP is effected though a decreasing response to light, then the height of both the *A* and *B* peaks should decline with increasing latitude (*arrows*). *Solid circles* plot the response of an intermediate population at 40 N (Bradshaw 1980). **C** Developmental (long-day) response of *W. smithii* to *T*-experiments with a 10-h photophase and *T* varied from 24 to 72 h (Wegis et al. 1997). Note that $\tau = 21$ h (valley-to-valley interval) for all latitudes and that the magnitude of the long-day response (peak height) declines with increasing latitude. **D** Predicted response to asymmetric skeleton photoperiods with a short main photophase (10 h) and $T = 48$ h, assuming a rhythmic sensitivity to light that creates a third *C* peak in long-day response at $\tau = 21$ h after the *A* peak

Diapause in *W. smithii* is always photoreversible. Laboratory stocks can be stored for 6 months as diapausing larvae on short days at 21°C and then stimulated to resume development by exposing them to long days. The CPP in *W. smithii* is the same for both the onset and maintenance of diapause (Bradshaw and Lounibos 1972). *W. smithii* is therefore an ideal organism for studying the effects of photoperiod with $T > 24$ h because diapausing larvae can accumulate photoperiodic information over an indefinitely long period, in contrast with the relatively brief sensitive period determining the onset of diapause in most insects (Saunders 1982).

Predictions

We use as our working hypothesis the external coincidence model (Pittendrigh and Minis 1964). In this model, light has two actions: (1) it sets or entrains the oscillation of photosensitivity and (2) it effects photoperiodic induction when it "coincides" with peak sensitivity or the "photoinducible phase" (ϕ_i). This model is best illustrated by considering the developmental (long-day) response of larvae experiencing an asymmetric skeleton photoperiod (ASPP). The solid lines in Fig. 1A, B show the developmental response of *W. smithii* from New Jersey (40° N) when receiving a 10-h (short-day) main photophase and a 1-h supplemental light pulse at different times (in separate experiments) during the 14-h scotophase (Bradshaw 1980). The developmental response is biphasic. There are two peaks of sensitivity, one in the early and one in the late subjective nights, the A and B peaks, respectively. The A peak occurs at hours 13–14 after dawn. Then, if dawn of the main photophase sets the photosensitivity rhythm, the pulse 13–14 h later effects photoperiodic induction. The B peak occurs at 20 h after dawn, which is 14 h before dusk of the main photophase. In this case, the 1-h pulse serves to set the photosensitivity rhythm and the end of the main pho-

tophase effects photoperiodic induction. We propose two ways by which this system might respond to selection for modification of the CPP.

First, modification of the CPP might be effected by shifting the position of the inducible phase. In Fig. 1A, the solid line represents the response of *W. smithii* from 40° N, an intermediate latitude. If the A peak were shifted earlier in the early subjective night or the B peak were shifted later in the subjective night, then the time interval between the initiation of the photosensitivity rhythm and the inducible phase would be shorter, i.e., resulting in a shorter CPP. Analogously, a later A peak or an earlier B peak would result in a longer CPP. Hence, as one proceeds from south to north, the positions of the A and B peaks should converge (Fig. 1A, arrows), with little or no change in the height of the response curves.

Second, modification of the CPP might be effected by raising or lowering responsiveness to light. In Fig. 1B, the solid line again represents the population from an intermediate latitude. An increase in response to light should raise the level of the whole response curve, and thereby result in a shorter critical photoperiod (Fig. 1B, open circles). Analogously, a decrease in responsiveness to light should lower the entire response curve, and result in a longer critical photoperiod (Fig. 1B, open squares). Hence, as one proceeds from south to north, the positions of the A and B peaks should decline in height (Fig. 1B, arrows), with little or no change in their positions.

Below, we shall test the following explicit hypotheses that when diapausing larvae experience a L:D = 10:14 (short-day) cycle with 1-h light breaks during the dark period, (1) H_0: the A and B peaks do not vary with latitude in either their position or their height; (2) H_{A1}: the A and B peaks converge closer to each other with increasing latitude; (3) H_{A2}: the area under the A and B peaks declines with increasing latitude.

The response curves in Fig. 1A,B could result from an interval (hourglass) timer as well as from the expression of an underlying rhythmic process. When $T = 24$, there is no opportunity to determine whether a rhythmic response to light persists though a long dark period unless that dark period is longer than the period of the underlying pacemaker. When the photophase is fixed at 10 h (short days) and the scotophase is varied systematically to produce $T = 24$–72, *W. smithii* shows a rhythmic response (Fig. 1C). In Fig. 1C, the "valleys" or short-day responses occur when $T = 24, 45$, and 66 h, indicating a 21-h period ($\tau = 21$ h) of the underlying pacemaker (Wegis et al. 1997). Note also that the height (long-day response) of the response curves declines among populations from progressively more northern latitudes. This declining height might result from an increase in the amplitude of the pacemaker or from a declining role of the rhythmic component of PTM and a concomitant increasing role of an interval timer. To investigate these alternatives, we make two tests.

First, in an extended ASPP with a 10-h photophase and $T = 48$, we expect the A and B peaks to appear as

before (Fig. 1D). In addition, if there is a rhythmic component of PTM as indicated by the previous T experiment (Fig. 1C), then there would be a tertiary "C" peak about 21 h after the A peak. Since the period of photosensitivity does not change with latitude of origin (Fig. 1C), the C peak may occur at varying times during the dark period but always at $\tau = 21$ h after the A peak. The absence of a C peak would indicate that the rhythmic component of PTM was not self-sustaining during the longer dark period and, consequently, that PTM was functionally an interval timer.

We test the explicit hypotheses that when diapausing larvae are exposed to a 10-h (short-day) photophase followed by a 38-h scotophase interspersed with 1-h light pulses at various times during the scotophase, (1) H_0: the developmental (long-day) response is represented solely by the A and B peaks whose position and height do not vary with latitude of origin; (2) H_{A1}: the developmental response includes a tertiary peak 21 h after the A peak; (3) H_{A2}: the positions of the A and B peaks converge at higher latitudes; (4) H_{A3}: the area under the A and B peaks, and under the C peak if it is present, declines with increasing latitude.

Second, if photoperiodic time measurement is functionally an interval timer, the timer could be measuring the length of the day or the length of the night. From the T experiment (Fig. 1C), it is clear that the effects of a short day are affected in a periodic manner by the length of the following night, at least in populations from southern and intermediate latitudes. In these cases, the rhythmic component of PTM can override or mask the effects of a night-interval timer. If the photophase in Fig. 1C were to be replaced by a long photophase (longer than the CPP), then the developmental (long-day) response should still vary with T if the rhythmic component predominates over an interval timing component. If, by contrast, a long photophase induces a long-day response, regardless of T, then the day-interval timing component can override or mask the rhythmic component.

We test the explicit hypotheses that when larvae are provided short to long photophases followed by a long night (>16 h) of variable duration to create $T = 34, 45$, or 72 h, (1) H_0: at all latitudes, the developmental response is independent of the duration of either the preceding photophase or the period (T) of the driving cycle; (2) H_{A1}: at some latitudes, photophases greater than the latitude-specific CPP stimulate development, regardless of night length >16 h; (3) H_{A2}: at some latitudes, driving cycles of 45, 72, and 34 h induce increasing long-day responses, respectively, regardless of photophase duration.

Materials and methods

We collected overwintering *W. smithii* from six localities in North America in spring 1993 (Fig. 2). Populations from Alabama (AL, 31° N), Florida (FL, 30° N), New Jersey (NJ, 40° N; two popu-

Fig. 2 Origin of *W. smithii*
populations in North America,
with the latitudes of origin (*Lat*)
and critical photoperiods (*CPP*)
for each location (*Loc*) from
Wegis et al. (1997)

Loc	Lat	CPP
AL	31	11.81
FL	30	11.87
NJ	40	13.61
NJ	40	13.76
ME	46	15.18
ON	49	15.06
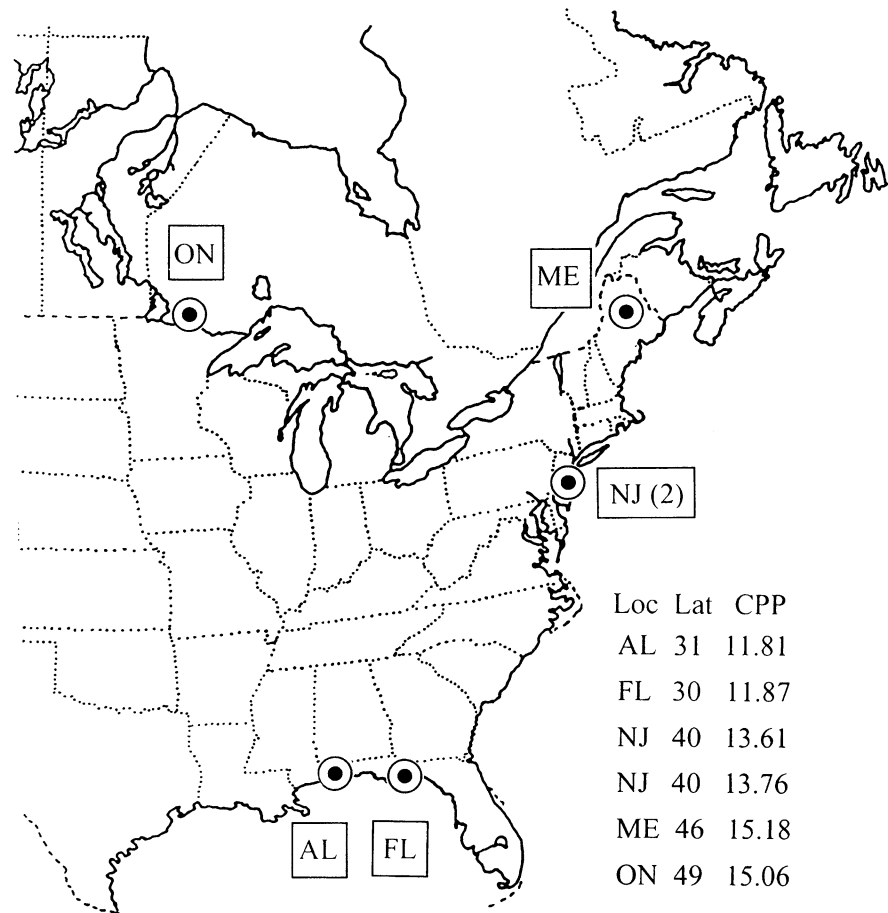
lations), Maine (ME, 46° N), and Ontario (ON, 49° N) correspond to LI, WI, MM, PB, KC, and DL, respectively, of earlier publications from this laboratory. All populations were reared for at least five but no more than eight generations in the laboratory prior to the start of experiments. Experiments used larvae reared on a short-day photoperiod (L:D = 8:16) at 21 ± 0.5 C and that had been on short days for 1 month prior to initiating the experiment. Within any one population, diapausing larvae were pooled from a number of petri dishes into a large pan, stirred, and then allocated to make up two replicate cohorts of 50 animals each for each experimental treatment. Experiments were run with 25 larvae per 150 × 25 mm petri dish. Prior to the start of experiments, two of the dishes (50 total larvae) were assigned to an A replicate and two additional dishes to a B replicate.

All experiments were run in a controlled-environment room at 23 ± 0.5° C inside light-tight photoperiod cabinets where diapausing larvae were exposed to a 4-W, cool-white fluorescent lamp at a distance of 10–20 cm. To avoid a parallel thermoperiod, the ballasts were located outside the cabinet and each lamp was housed in a clear polycarbonate tube though which air was forced at 100 ft³/min. L:D regimens were programmed on Chrontrol electronic timers. Within any experimental treatment, the four dishes of larvae from each of the six populations were placed within the same chamber of the photoperiod cabinet. The dishes were stacked in the chamber haphazardly without regard to latitude of origin at the start of the experiment and again twice per week when the dishes were cleaned, larvae were fed, and pupae were removed and scored.

Larvae were exposed to the experimental regimens for 30 days, then transferred to short days (L:D = 8:16) at 21 ± 0.5° C for an additional 2 weeks to permit development of stimulated larvae. At the end of 44 days, percentage development was calculated for each replicate as (cumulative number of pupae) ÷ (cumulative number of pupae + number of larvae alive on day 44).

All ASPPs used a main photophase of 10 h with either a 14 h ($T = 24$) or 38 h ($T = 48$) scotophase. The scotophase was scanned with 1-h light pulses starting 1 h after dusk in 1-h increments up to 2 h before dawn of the main photophase. ASPPs for $T = 24$ were run in a single block. ASPPs for $T = 48$ were run in two sequential blocks, the first scanning the early scotophase with 1-h pulses starting at hours 11–22, and the second scanning the middle and late scotophase with 1-h light pulses starting at hours 23–46.

We scored the position of the A or B peaks in a hierarchical manner. First, if in five sequential points, the first three (first, second, third) plotted as rising and the last three (third, fourth, fifth) as declining, we scored the middle point as a peak. Second, if no such peak was defined, we used a locally weighted linear regression ("lowess plots," TriMetrix 1992) of percent development with hours after dawn (in the early or late scotophase) as the predictor variable. In lowess plots, an iterative, non-parametric algorithm is used to obtain smoothed values for each value of y, given the values for x. The regression becomes more locally weighted as the number of iterations increases and the percentage of total points used in smoothing each value of y decreases. We obtained the best definition of peaks with ten iterations and using 20% of the points for each local regression. This procedure took into account variability in the vicinity of a local minimum as well as nearby rises to higher maxima. When $T = 24$, if the smoothed plot through the interval 11–18 h or through the interval 18–22 h displayed a distinct maximum, we scored the maximum as a peak in the early or late scotophase (A and B peaks), respectively. Similarly, when $T = 48$, if the smoothed plot through the interval 11–18 h or through the interval 36–46 h displayed a distinct maximum, we scored the maximum as a peak in the early or late scotophase (A and B peaks), respectively. Third, if neither of the above procedures defined a maximum, the replicate was scored as indeterminate and the replicate treated as a missing value in the subsequent analyses.

To quantify the area under a peak, we summed the percentages of development at the peak, 1 h earlier, and 1 h later.

Both the positions of and areas under the peaks frequently departed from the assumptions of parametric ANOVA. Consequently, we tested for differences in position or area using the Kruskal-Wallis test, adjusting for tied ranks where necessary (Zar 1996, section 10.4). To evaluate geographic variation in position or area, we treated each replicate as an independent observation, regardless of population within latitudinal zones, using geography (south, intermediate, north) as treatments.

To examine the effects of varying the length of both the photophase and T, we performed a full-factorial experiment. We used $T = 34$ h, enhancing a long-day response, $T = 72$ h, sustaining an intermediate response, and $T = 45$ h, reducing a long-day response (Fig. 1C). Within each of these Ts, we provided photophases of 10, 13, 14 3/4, or 18 h. Photophases of 10 and 18 h represent short ($<$CPP) and long ($>$CPP) photophases, respectively, for all populations. A photophase of 13 h represents a long photophase for southern populations and a short photophase for intermediate and northern populations. A photophase of 14 3/4 h represents a long photophase for southern and intermediate populations and a short photophase for northern populations (Wegis et al. 1997). The experiment was run in a single block in one 12-chamber photoperiod cabinet.

Percentages of development were first arcsine transformed and then subjected to a three-way ANOVA with T (34, 72, 45), photophase ($<$CPP, $>$CPP), and latitude (south, intermediate, north) as treatments. For the error estimate, we used the mean square between populations nested within T, photophase, and latitude with 18 df. ANOVAs were performed using SAS procedure GLM and type III sums of squares (SAS 1985). We used Ryan's Q multiple-comparison tests to determine significant differences between individual means (Day and Quinn 1989).

Results

Asymmetric skeleton photoperiods

When $T = 24$ h (Fig. 3), the peak developmental response ranged from 13 to 14 h in the early subjective night and from 20 to 21 h in the late subjective night. The position of these peaks did not differ among latitudes in either the early ($H_c = 0.50$, $P = 0.777$) or the late ($H_c = 1.53$, $P = 0.216$; omitting both northern populations in which the position of the B peak was not well defined) subjective night. The area under the peak declined with increasing latitude during both the early ($H_c = 9.88$, $P = 0.007$) and the late ($H_c = 10.21$, $P = 0.006$) subjective night. When $T = 48$ h (Fig. 4), peak development ranged from 13 to 14 h in the early subjective night and from 40 to 44 h in the late subjective night. As above, the position of these peaks did not differ among latitudes in either the early ($H_c = 0.49$, $P = 0.782$) or the late ($H_c = 3.28$, $P = 0.194$; omitting the ME populations in which the position of the B peak was not well defined) subjective night. In southern populations, there was a shoulder on the B peak at 34 h but no distinct peak at this time. There was a clear tertiary peak (C) at 24 h in both southern populations and this peak disappeared in the intermediate and northern

Fig. 3 Developmental (long-day) response of *W. smithii* to ASPPs with a 10-h main photophase and a 14-h scotophase ($T=24$). The two plots within each graph show two replicates from the same population. Times are given as hours from the start of the main photophase
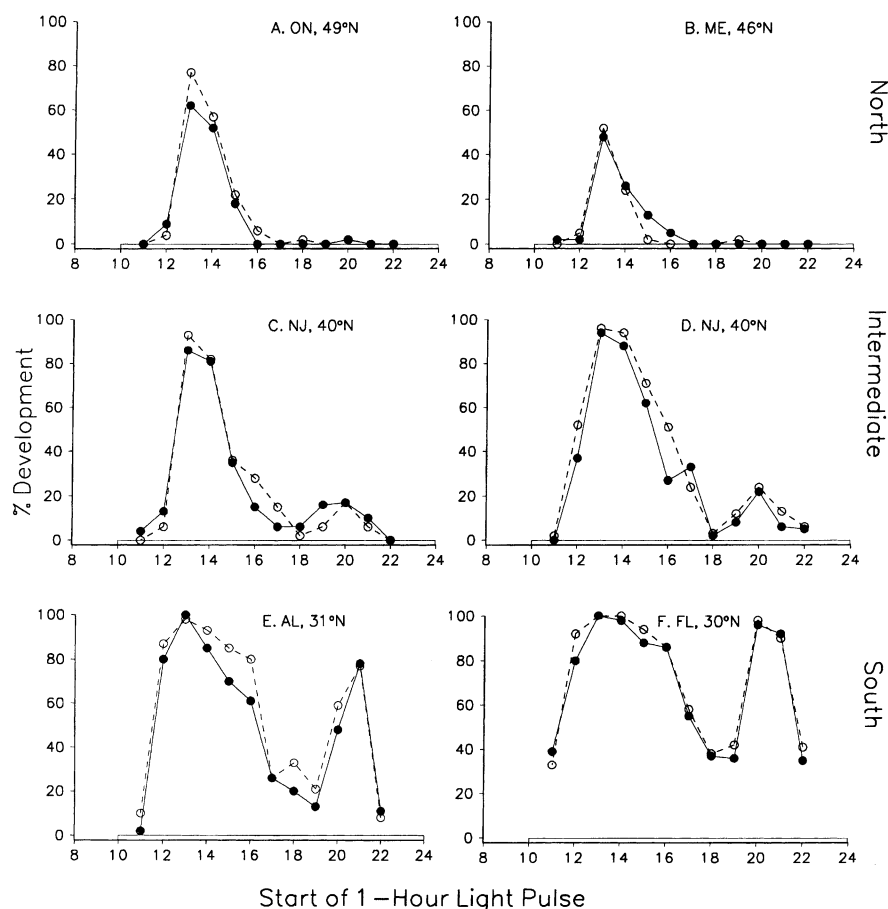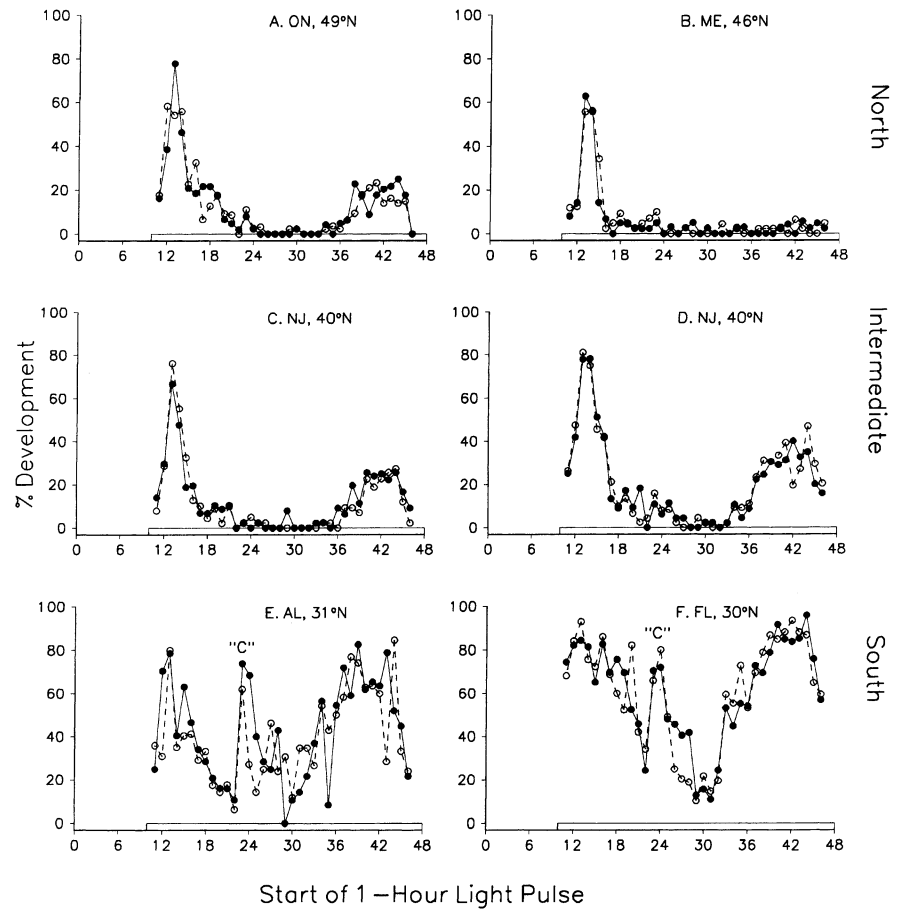
Start of 1−Hour Light Pulse

Fig. 4 Developmental (long-day) response of *W. smithii* to ASPPs with a 10-h main photophase and a 38-h scotophase (*T*=48). Plots as in Fig. 3

Start of 1–Hour Light Pulse

populations. The area under the curve early in the subjective night did not differ among latitudes ($H_c = 2.78$, $P = 0.249$) but declined with increasing latitude late in the subjective night ($H_c = 9.01$, $P = 0.011$).

These results show that the responses to light breaks did not vary among latitudes in their position but that their overall level of response declined with increasing latitude. A tertiary peak in response to light breaks did occur when $T = 48$, but only in the southern populations and at 24, not 34 h.

Variable day and night length

Within each latitude, development (long-day response) of *W. smithii* depended upon both the duration of the photophase and the period (*T*) of the driving cycle (Fig. 5A–C). Three-way ANOVA (Table 1) showed significant effects of all treatments and their two- and three-way interactions. Although all of the interaction terms were significant ($P < 0.05$), it is clear from Table 1 that the main effects of *T* and photophase had the greatest impact on development (long-day response). When latitudes are pooled within *T* and photophase (Fig. 5D), the differences between means show three patterns. First, within each *T*, photophases longer than

the CPP always promoted significantly greater development ($P < 0.01$) than photophases shorter than the CPP. Second, within photophases shorter than the CPP, the developmental response to variable *T* was always significantly greater in the direction expected: $T = 45 > 72 > 34$. Third, within photophases longer than the CPP, the trend in developmental response to variable *T* was in the direction expected but the only significant differences occurred between $T = 45$ and either $T = 72$ or $T = 34$. The difference in significance of developmental response to variable *T* between photophases is reflected by the relatively small ($\% \downarrow TSS = 4.7\%$) but highly significant ($P < 0.001$) effect of the *T* by photophase interaction (Table 1).

These results show that the effects of a photophase longer than the CPP persist through a long night, regardless of the period of the driving cycle (*T*). At photophases shorter than the CPP, a long-day response is enhanced when $T = 34$ and reduced when $T = 45$, as predicted by the original *T* experiments (Fig. 1C).

Discussion

Previous results with *W. smithii* (Wegis et al. 1997) have shown that in populations from more northern latitudes,

Fig. 5A–D Developmental (long-day) response of *W. smithii* to variable photophase (L = 10, 13, 14 3/4, 18 h) and a scotophase to create T = 45, 72, and 34 h. **A, B, C** Each *bar* shows the mean arcsine-transformed response across two replicates and two populations within each latitude. the *solid circle* on the photophase axis denotes the critical photoperiod at the given latitude. **D** Mean arcsine-transformed response after latitudes were pooled and after photophases were pooled according to whether they were shorter (< CPP) or longer (> CPP) than the latitude-specific CPP. *Error bars* show +2 SE. *Bars with the same letter* on their front faces do not differ ($P < 0.05$) in mean response after Ryan's Q multiple-comparisons test
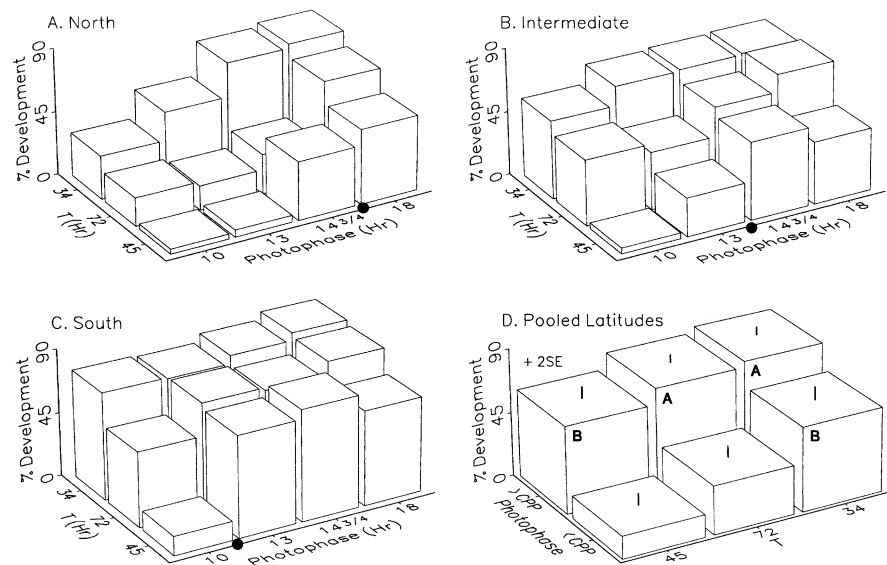

Table 1 Three-way ANOVA of percent development (long-day response) of *Wyeomyia smithii* with T (45, 72, 34), photophase (< CPP, > CPP), and latitude (south, intermediate, north) as treatments and using variation among localities nested within T, photophase, and latitude for the error mean square with 18 *df* (%↓TSS percent reduction in total sum of squares)

Source of variation	df	Sum of squares	%↓TSS	F	P
T	2	22,023	23.1	203.97	<0.001
Photophase	1	25,776	27.0	477.44	<0.001
Latitude	2	3,817	4.0	35.35	<0.001
T × photophase	2	4,489	4.7	41.57	<0.001
T × latitude	4	647	0.7	2.99	0.047
Photophase × latitude	2	1,076	1.1	9.97	0.001
T × photophase × latitude	4	2,015	2.1	9.33	<0.001
Localities (T, photophase, latitude)	18	972	1.0		

the whole PPRC shifts vertically downward, not laterally towards longer photophases. This same pattern is apparent in the responses to ASPPs (Figs. 3, 4), where the early and late peaks do not change position relative to dawn or dusk, but the area under them declines with increasing latitude. Hence, the increase in CPP at the more northern latitudes is associated with a decline in overall responsiveness to light breaks during an otherwise long night as predicted in Fig. 1B, and not with a shift in the photoinducible phase to a later time in the early subjective night or an earlier time in the late subjective night as predicted in Fig. 1A.

When T = 48, a tertiary peak appears in southern populations, but at 24 h (C in Fig. 4E,F), not at 34 h as predicted (Fig. 1D). In this experiment, the 1-h light pulse at 24 h is followed, sequentially, by a 24-h dark period and a 10-h light period; this regimen may then be mimicking a 10:24 = L:D cycle, which evokes a long-day response (Fig. 1C). In this case, we would also expect a peak in response to an ASPP with T = 48 at 34 h, which represents a 10-h light period followed by a 24-h dark period. We can discern a distinct shoulder in long-day response at 34 h in both southern populations (Fig. 4E, F) and the suggestion of a shoulder in long-day response at 34 h in one of the intermediate populations (Fig. 4D). The results shown in Fig. 4 are therefore still consistent with the previous conclusion (Wegis et al. 1997) that there is a rhythmic component to photoperiodic time measurement in at least the southern populations.

At all latitudes, a photophase longer than the latitude-specific CPP induces a strong developmental response, regardless of the following night length (Fig. 5). This observation indicates that, in addition to a rhythmic component, photoperiodic time measurement in *W. smithii* also includes a day-interval timer. The persistent peak of long-day response in the early subjective night (A peak) but disappearing responsiveness in the late subjective night (B peak) (Figs. 3, 4) indicates that the 1-h light pulses are sufficient to act as a terminator but not an initiator of the day interval. The rhythmic component still exists at a subliminal level in northern

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populations as shown (1) by the weak but still persistent response to the *T* experiments in Fig. 1C and (2) by the fact that *T* still has an influence on photoperiodic response at daylengths shorter than the CPP (Fig. 5A). Photophases longer than the CPP, however, largely or entirely override the effects of variation in *T* at all latitudes. Hence, while PTM in most populations contains both rhythmic and hourglass components, the responsiveness of the underlying pacemaker declines in influence in more northern populations, and its functional role is taken over by the day-interval timer.

This latitudinal shift in the underlying function from a rhythmic hourglass timer in the south to a day-interval timer in the north parallels the evolutionary trajectory of *W. smithii* in North America (Bradshaw and Lounibos 1977; Istock and Weisburg 1987; Bradshaw and Holzapfel 1990). At the same time, the genetic differences between northern, derived populations and their southern ancestors involve not only the independent (additive) effects of genes, but also the effects of gene-gene interactions (epistasis: Hard et al. 1992, 1993). We therefore conclude that the adaptive evolution of CPP has probably involved the progressive epistatic masking of the ancestral rhythmic component so that PTM in northern, derived populations is accomplished principally through the remaining day-interval timer.

Pittendrigh and co-workers (Pittendrigh and Takamura 1989, 1993; Pittendrigh et al. 1991) have proposed that PTM is not so much the measure of time as a response to light, and that the increasing CPP of more northern populations reflects a decreasing response to light. They predicted that the latitudinal increase in CPP should be accompanied by a downward (decreased long-day response), rather than a lateral shift in the entire PPRC from 0 to 24 h. They provided support for their prediction in *Drosophila auraria* and there is substantiating experimental support in *W. smithii* (Wegis et al. 1997) and in previously published studies with Lepidoptera (Danilevskii 1965; Kikukawa and Chippendale 1983). Pittendrigh and co-workers did not extend their predictions to *T* or interrupted-night experiments and we now do so. First, responses to short photophases and variable scotophase (*T* experiments) are expected to yield a short-day response when the period of the driving cycle (*T*) approximates (resonates with) that of the underlying pacemaker (τ). When the driving cycle is out of phase with the pacemaker, i.e., when $T = \tau + 0.5\tau$, the internal temporal organization of the individual is disrupted and a discordant, long-day response is expected (Pittendrigh 1966; Saunders 1982; Vaz Nunes and Veerman 1986; Takeda and Skopik 1997). We argue that the degree of long-day response represents the degree of perturbability of the system to light. A declining response to light should then be manifest as a declining long-day response to *T* experiments with increasing latitude and we observe this pattern in *W. smithii* (Figs. 1C, 5) and it can also be seen in Coleoptera (Thiele 1977), Lepidoptera (Takeda and Skopik 1985), and Acari (Vaz Nunes et al. 1990). Sec-

ond, we argue that the response to an ASPP represents the degree to which light can perturb an otherwise long scotophase. A declining response to light should then be manifest as a declining response to light breaks, and we observe this pattern in *W. smithii* (Figs. 3, 4).

Our results show that the latitudinal increase in critical photoperiod in *W. smithii* is accomplished by a declining response to light, not only in the entire PPRC as predicted earlier (Pittendrigh and Takamura 1989, 1993; Pittendrigh et al. 1991; Wegis et al. 1997) but also, as we now argue, in both *T* and interrupted-night experiments. Only consistent patterns of PPRCs and responses to T experiments are found in Coleoptera, Lepidoptera, and Acari. We therefore conclude that the latitudinal clines of declining CPP probably result from a declining response to light by arthropods in general. These same experiments also lead us to conclude that the functional components of PTM show a decline in the rhythmic component of PTM and a concomitant increase in reliance on an hourglass timer with an increase in latitude among arthropods in general. The pervasiveness of these geographic patterns in a wide variety of arthropods implies that the functional components of PTM are either homologous, i.e., derived from a similar mechanism in a shared common ancestor, or are the result of convergent evolution. If the mechanisms are convergent, the implication is that the photic environment or season length is imposing direct selection not only on the overt CPP, but also on its underlying functional components.

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