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ORIGINAL PAPER

Extrinsic light:dark cycles, rather than endogenous circadian cycles, affect the photoperiodic counter in the pitcher-plant mosquito, *Wyeomyia smithii*

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Abstract A wide diversity of organisms use photoperiod (daylength) as an environmental cue to anticipate the changing seasons and to time various life-history events such as dormancy and migration. Photoperiodic time measurement consists of two main components, (1) the photoperiodic timer that discriminates between long and short days, and (2) the photoperiodic counter that accumulates and stores information from the timer and then induces the phenotypic output. Herein, we use extended night treatments to show that light is necessary to accumulate photoperiodic information across the geographic range of the mosquito, *Wyeomyia smithii* and that the photoperiodic counter counts extrinsic (external) light:dark cycles and not endogenous (internal) circadian cycles.

Keywords Evolution · Diapause · Circadian rhythm · Photoperiodism · Geographic variation

Abbreviations

LDC_{50}	Number of light:dark cycles necessary to elicit 50% development
L:D	Light:dark

Introduction

Photoperiod, or the length of the day, is a highly reliable predictor of seasonal change in the temperate regions and a wide diversity of organisms including plants, fish, birds, mammals, insects, and other arthropods use photoperiod to cue the appropriate timing of life-history events with respect to variation in the seasons (Withrow 1959; Anonymous 1960; Bünning 1964; Aschoff 1965; Menaker 1971; Bradshaw and Holzapfel 2007). Photoperiodic time measurement in arthropods is generally considered to be the result of two separate, though related, components: (1) a photoperiodic timer that distinguishes between long and short days (or nights), and (2) a photoperiodic counter that accumulates information from the timer and then triggers downstream processes when some threshold of information has been reached (vaz Nunes and Saunders 1999; Saunders 2002). Herein, we are concerned with the role of light in the accumulation of inductive ("long-day") cycles by the photoperiodic counter used to terminate diapause and how that role changes with latitude and altitude.

Models of the physiological mechanism of the photoperiodic counter fall into two categories: (1) models that require the repeated input of light for the counter to accumulate inductive cycles and, (2) models that require light only to set internal circadian oscillators that are then able to accumulate inductive cycles in the absence of light (vaz Nunes and Saunders 1999). The first category includes two classes of models that do or do not rely on circadian rhythmicity. First, in the "hourglass" model, the circadian clock plays no role and the photoperiodic counter measures an inductive cycle if there is a period of light followed by a period of darkness (Lees 1973; Veerman 2001; Bradshaw et al. 2003b; Veerman and Veenendaal 2003). Second, in the external coincidence model, light plays a dual role: light both entrains a circadian oscillation and triggers the photoperiodic counter if light occurs during the appropriate "inducible" phase of the oscillation (Bünning 1936; Pittendrigh and Minis 1964; Saunders 2002). In both of these

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models, light is required for the photoperiodic counter to accumulate inductive cycles.

The second category is represented by the internal coincidence model. In this model, dawn and dusk each entrain separate circadian oscillators that trigger the counter to accumulate inductive cycles if the appropriate phases of the two rhythms overlap in time (Danilevsky et al. 1970; Pittendrigh 1972; Saunders 1978). Light is required only to set the phases of the dawn and dusk oscillators and repeated inductive events are accumulated even during extended darkness when the appropriate phases of the dawn-set and dusk-set endogenous rhythms overlap.

The first category can be distinguished from the second by using a long-day followed by nights of varying duration. For example, a long day of 18 h followed by a dark period of 6, 30 or 54 h would count as a single cycle under either the hourglass or external coincidence model but would count as 1, 2 or 3 cycles, respectively, under the internal coincidence model. This comparison has been used to determine the role of light in single populations (Veerman and vaz Nunes 1987) but, to our knowledge, no study has considered how the role of light in the photoperiodic counter varies among populations representing climatic extremes due to latitude and altitude within the species' range. It is important to use geographic variation in the photoperiodic counter in these studies to show that the results are not specific to single populations, but rather generally applicable to the species as a whole.

Herein, we discriminate between single and repeated measurements by the photoperiodic counter in extended night environments using southern, northern, lowland and mountain populations of the pitcher-plant mosquito, *Wyeomyia smithii* (Coq). We use 24, 48 and 72 h cycles to distinguish between an hourglass/external coincidence and an internal coincidence mechanism for the photoperiodic counter among geographically disparate populations of *W. smithii*.

We test two predictions: first, if the mechanistic basis of the photoperiodic counter is due to internal coincidence, then multiple inductive events will be recorded by the photoperiodic counter in extended night environments. Thus the number of light:dark (L:D) cycles to induce 50% development (LDC_{50}) among diapausing larvae should be greater for an L:D = 18:06 than either an L:D = 18:30 or an L:D = 18:54 cycle; alternatively, if the mechanistic basis is due to an hourglass or to external coincidence then LDC_{50} should not differ among cycles of varying duration. Second, if the basis of the photoperiodic counter is due to an internal coincidence mechanism, then the ratio between LDC_{50} in response to an L:D = 18:06 cycle and LDC_{50} in response to an L:D = 18:30 or to an L:D = 18:54 cycle should equal 2.0 or 3.0, respectively, due to the counter measuring two inductive events in the 18:30 treatment and three events in the 18:54 treatment; alternatively, if the basis of the photoperiodic counter is due to an hourglass or to external coincidence, then these ratios should both equal 1.0.

Materials and methods

W. smithii lays its eggs and completes all of its pre-adult development within the water-filled leaves of the purple pitcher-plant, *Sarracenia purpurea*, and its range closely follows that of its host plant in North America. Throughout its range, short days induce and maintain diapause while long days avert or terminate diapause (Bradshaw and Lounibos 1977). The phenotypic output of the photoperiodic timer is represented by the critical photoperiod, the number of hours of light per day that initiates 50% diapause or 50% development. Critical photoperiod in *W. smithii* increases with latitude and altitude of population origin and has evolved independently of the circadian clock (Bradshaw et al. 2003a, b, 2006). The phenotypic output of the photoperiodic counter is represented by the depth of diapause, the number of long days necessary to terminate diapause in 50% of a cohort. Depth of diapause also increases with latitude and altitude but depth of diapause has heretofore been evaluated only under 24-h light:dark cycles (Bradshaw and Lounibos 1977).

Mosquitoes were collected as diapausing larvae from geographic extremes of latitude and altitude (Table 1) and passed through two to ten generations in the laboratory with effective population sizes $N_e = \frac{4N_M N_F}{N_M + N_F} > 200$ in order to reduce field and maternal effects and to maintain genetic variability (N_M and N_F equal the number of males and females, respectively). With $N_e > 200$ each generation, the cumulative inbreeding after ten generations would be $F_{10} < 1 - \left(1 - \frac{1}{2\times 200}\right)^{10} = 2.5\%$ (Hartl and Clark 1989), and thus the samples used represent naturally occurring levels of genetic variation. Each of the four geographic regions (southern, lowland, mountain, and northern) was represented by two independent populations.

At the start of the experiment, larvae were synchronized into diapause by being raised from eggs under short-day conditions for at least 30 days. Diapausing larvae were then placed into one of three experimental long-day cycles, L:D = 18:06, 18:30, or 18:54 for a pre-determined number of cycles in light-tight environmental chambers maintained in a controlled-environment room at 21 ± 0.5°C. After the pre-determined time under the experimental conditions larvae were transferred to short-day conditions (L:D = 10:14) for at least 14 days to allow for the expression of any development that might have been induced by the experimental treatments. Samples of at least

Table 1 Source of experimental populations, stage of diapause (III or IV larval instar), LDC_{50} ± SE (the number of long-day L:D cycles, followed by short day L:D cycles, that terminates diapause in 50% of a population) and observed ratios for LDC_{50} 18:06/18:30 and for LDC_{50} 18:06/18:54 with expected ratios of 2.0 and 3.0, respectively, for an internal coincidence model

Region	Loc[a]	Latitude (°N)	Altitude (m)	Stage of diapause	LDC_{50} 18:06	LDC_{50} 18:30	LDC_{50} 18:54	Expected ratio	
								2.0	3.0
Southern	WI	30.1	10	IV	4.38 ± 0.30	5.54 ± 0.12	2.34 ± 0.13	0.79	1.87
	CR	30.8	67	IV	3.56 ± 0.32	4.51 ± 1.22	2.92 ± 0.28	0.79	1.22
Lowland	GS	34.2	20	IV	5.37 ± 0.39	3.27 ± 0.27	3.15 ± 0.05	1.64	1.70
	SH	35.0	107	IV	7.25 ± 0.21	6.66 ± 0.52	6.94 ± 0.83	1.09	1.04
Mountain	DB	35.0	900	III	5.20 ± 0.88	7.33 ± 0.51	7.18 ± 1.42	0.71	0.72
	HS	35.1	1,190	III	9.89 ± 0.26	11.77 ± 2.31	10.81 ± 1.06	0.84	0.91
Northern	RY	45.8	295	III	6.72 ± 0.21	11.36 ± 0.63	11.23 ± 0.38	0.59	0.60
	KC	46.2	365	III	9.63 ± 0.33	13.92 ± 0.50	14.08 ± 0.84	0.69	0.68

[a] Locality code referred to in previous publications from this lab

105 larvae were used for each combination of population, experimental cycle and number of cycles, totaling over 43,000 larvae used in this study. This experiment was performed in two blocks over the course of one year. The first block was used to define the broader range of response and the second block was used to fill in or extend the results of the first block. To ensure consistency, there was at least one treatment in the second block that duplicated a treatment in the first block.

Logistic regression of long-day response (pupation) as a function of number of experimental cycles was performed using the DRC package of the statistical computing package R (Ritz and Streibig 2005; R Development Core Team 2007). A three-parameter logistic regression was fit to each set of data, assuming a lower bound of 0% long-day response and an upper bound of 100%. LDC_{50} was computed as the 50% intercept along with its standard error for each curve. To test the first prediction, LDC_{50} was calculated after pooling populations within regions; to test the second prediction, LDC_{50} was calculated for each population.

Results

All three L:D treatments used in this study were interpreted as long days by the mosquitoes. Long-day response was >96% in cohorts of larvae maintained for 15 (southern, lowland) or 25 (northern, mountain) cycles at L:D = 18:06, 18:30 or 18:54.

Within regions, long-day response increased as a sigmoid function of the number of long-day cycles experienced by an experimental cohort (Fig. 1). LDC_{50} was higher for northern and higher altitude populations, which diapause as third instars (9.93 ± 2.31, mean ± SE), than for southern and low altitude populations, which diapause as fourth instars (4.66 ± 0.82) ($F_{1,\ 23} = 30.73$, $P < 0.001$). These results confirm previous studies in *W. smithii* (Bradshaw and Lounibos 1972, 1977).

For each region, LDC_{50} under the L:D = 18:06 regimen was not greater than under the L:D = 18:30 regimen (Table 2) and LDC_{50} under the L:D = 18:06 regimen was not greater than under the L:D = 18:54 regimen, except in the southern region (Table 2). Hence, with the exception of one of the two comparisons (L:D = 18:06 vs. 18:54) in the southern region, our data support the predictions of an hourglass/external coincidence timer within each region.

To determine whether the L:D = 18:06 versus 18:54 comparison in the southern region reflected a consistent or inconsistent response of the two replicate populations, we made this comparison in each of the two populations individually (Table 1, lines 1–2). LDC_{50} for one population, CR, was not significantly greater for an L:D = 18:06 than an L:D = 18:54 cycle ($t = 1.51$, $df = 19$, $P_{\text{one-tail}} = 0.074$), supporting predictions for an hourglass/external coincidence timer. LDC_{50} for WI was indeed higher for an L:D = 18:06 than for an L:D = 18:54 cycle ($t = 6.24$, $df = 18$, $P_{\text{one-tail}} < 0.001$), supporting predictions for an internal coincidence model. Hence, the apparent exception of the southern region is due to the response of a single population to only one of four tests within that region. The results are therefore generally consistent with predictions for an hourglass/external coincidence counter mechanism within each region.

There was no evidence that, across all eight populations, the counter accumulated two inductive cycles in the L:D 18:30 treatment as the ratio, LDC_{50} (18:06) ÷ LDC_{50} (18:30), was significantly less than 2.0 ($t = 9.33$, $df = 15$, $P_{\text{one-tail}} < 0.001$) and was not significantly different from 1.0 ($t = 0.90$, $df = 15$, $P_{\text{two-tail}} = 0.397$). A similar result was found in the L:D 18:54 treatment where the ratio,

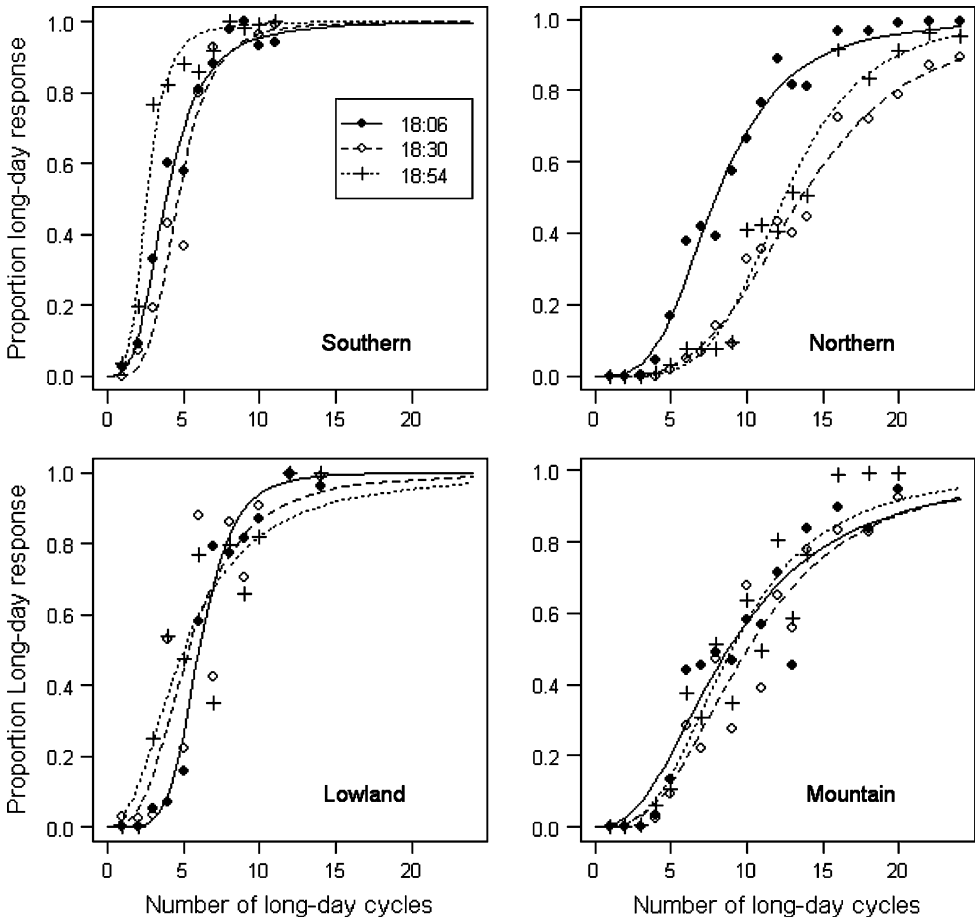

Fig. 1 Developmental response curves for the four geographic regions in three different long-day L:D regimens (18:06, 18:30 and 18:54). Animals were exposed to the specified number of long-day L:D cycles and then transferred to short days for at least 14 days before scoring pupation (long-day response)

Table 2 LDC_{50} by geographic region (Table 1) under different light:dark regimens (L:D) and tests for inequality of LDC_{50} for L:D = 18:06 > L:D = 18:30 and L:D = 18:06 > L:D = 18:54, which are the predictions based on an internal coincidence model

Region	L:D	LDC_{50} ± SE	18:06 > 18:30			18:06 > 18:54		
			t	*df*	$P_{\text{one-tail}}$	*t*	*df*	$P_{\text{one-tail}}$
Southern	18:06	3.83 ± 0.20	**−2.80**	27	0.995	5.18	27	<0.001
	18:30	4.63 ± 0.20						
	18:54	2.57 ± 0.14						
Lowland	18:06	6.03 ± 0.35	1.17	30	0.126	1.59	30	0.061
	18:30	5.34 ± 0.47						
	18:54	5.00 ± 0.54						
Mountain	18:06	8.82 ± 0.50	**−1.80**	45	0.958	**−0.28**	45	0.390
	18:30	10.07 ± 0.50						
	18:54	9.01 ± 0.45						
Northern	18:06	7.99 ± 0.22	**−15.0**	51	0.999	**−13.44**	51	0.999
	18:30	13.57 ± 0.31						
	18:54	12.41 ± 0.25						

Bold *t* values indicate that the relationship between the two LDC_{50} values is opposite of the direction expected under an internal coincidence model

LDC_{50} (18:06) ÷ LDC_{50} (18:54) was significantly less than 3.0 ($t = 9.32$, $df = 15$, $P_{\text{one-tail}} < 0.001$) and was not significantly different from 1 ($t = 0.57$, $df = 15$, $P_{\text{two-tail}} = 0.578$). These results uniformly support predictions for an hourglass/external coincidence counter across all populations as the basis for keeping track of the number of long days.

Discussion

Our results conform to the predictions of an hourglass/external coincidence model for the mechanistic basis of the photoperiodic counter in *W. smithii*. Both the observed inequalities and observed ratios of LDC_{50} between L:D 18:06 and either L:D 18:30 or L:D 18:54 across a broad

range of latitudes and altitudes support this category of models and rejects the internal coincidence model. These results do not distinguish between an hourglass model or an external coincidence model as the basis for the photoperiodic counter in *W. smithii*; but, these results do mean that light is necessary to accumulate photoperiodic information and that the photoperiodic counter of *W. smithii* counts external light:dark cycles and not internal circadian cycles. Despite variation among populations in the stage and depth of diapause (Table 1), the basic importance of light for the physiological mechanism underlying the photoperiodic counter prevails throughout the geographic range of *W. smithii*, including southern, northern, lowland and mountain populations.

In the northern hemisphere, as one proceeds northwards or increases in elevation, winter arrives progressively earlier in the year and becomes progressively harsher. Critical photoperiod, a measure of the photoperiodic timer, increases with latitude and altitude among a wide variety of arthropods (Taylor and Spalding 1986; Danks 1987, Table 24; Saunders 2002, Table 10.1), including *W. smithii* (Bradshaw and Lounibos 1977). Similarly in *W. smithii*, LDC_{50}, a measure of the photoperiodic counter, increases with latitude from southern to lowland to northern localities and increases with altitude from lowland to mountain localities under either 24-h (Danks 1987, Table 25). Hence, as populations encounter earlier, harsher winters, they become more prone to enter and remain in diapause. The positive correlation among populations between critical photoperiod and depth or intensity of diapause (LCD_{50}) is also reflected by their positive genetic correlation within populations and forms part of the evolutionarily flexible diapause syndrome in *W. smithii* (Campbell and Bradshaw 1992).

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