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Geographic and developmental variation in expression of the circadian rhythm gene, *timeless*, in the pitcher-plant mosquito, *Wyeomyia smithii*

D. Mathias*, L. Jacky, W.E. Bradshaw, C.M. Holzapfel

Center for Ecology and Evolutionary Biology, University of Oregon, Eugene, OR 97403-5289, USA

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Abstract

Expression of the circadian rhythm gene *timeless* was investigated in the pitcher-plant mosquito, *Wyeomyia smithii* (Coq.), and was found to vary with time of day, instar of diapause, and latitude of origin. The temporal pattern of *timeless* expression differed between the two diapausing instars and was significantly higher in southern (38–40 °N) than in northern (50 °N) populations, when diapausing instar was held constant. Expression of *timeless* is therefore both developmentally and evolutionarily variable. This result provides the first example of a latitudinal difference in the expression of *timeless*, suggesting that, along with evidence from other insects, *timeless* has the potential to affect photoperiodic response and its adaptive evolution in temperate seasonal environments.

Keywords: Photoperiodism; *timeless*; Circadian rhythm; Stage of diapause; Latitude

1. Introduction

At temperate latitudes, organisms are confronted with daily and seasonal variations in light, temperature, and resources. Circadian rhythms and photoperiodic time measurement, respectively, enable insects to anticipate and prepare for these daily and seasonal variations in their environment (Danilevskii, 1965; Saunders, 2002). In 1936, Bünning proposed that circadian rhythms play a causal, necessary role in photoperiodic response. This concept has had enduring appeal because, if true, it would mean that a single physiological mechanism orchestrates both daily and seasonal activities. Physiological experiments over the last 60 years have revealed parallel patterns in the formal properties of circadian rhythms and photoperiodic response (Withrow, 1959; Menaker, 1971; Beck, 1981; Takeda and Skopik, 1997; Vaz Nunes and Saunders, 1999; Saunders, 2002) but also inconsistencies that have prompted some to question the circadian basis of photoperiodism (Bradshaw and Holzapfel, 2001a; Veerman, 2001; Bradshaw et al., 2003a, b; Danks, 2003).

A great deal is now known about the molecular basis of circadian rhythmicity in Dipterans (Dunlap, 1999; Young, 2000; Panda et al., 2002) and how the circadian clock adjusts to different temperatures and to long and short day lengths (Majercak et al., 1999; Goto and Denlinger, 2002; Collins et al., 2004). In *Drosophila* the central, autoregulatory feedback loop of the circadian clock involves interaction between proteins encoded by the genes *timeless* and *period*. *period* null mutants are behaviorally arrhythmic under constant darkness but still photoperiodic (Saunders et al., 1989; Saunders, 1990), i.e., a normally functioning circadian clock is unnecessary for photoperiodic response (Saunders et al., 1989; Saunders, 1990; Kostál and Shimada, 2001). These results do not mean, however, that none of the circadian rhythm genes is involved in photoperiodism

*Corresponding author. Tel.: +1 541 346 4542; fax: +1 541 346 2364.
E-mail address: dmathias@darkwing.uoregon.edu (D. Mathias).

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independently of its role in circadian rhythmicity. *timeless* may be just such a gene. Levels of the TIMELESS protein are negatively regulated by light through an interaction with the photoreceptor CRYPTOCHROME (Ceriani et al., 1999). In the case of *period* null mutants, *timeless* is expressed constitutively and at elevated levels (Claridge-Chang et al., 2001). Although the levels of *timeless* mRNA do not oscillate in these *period* null mutants, the TIMELESS protein should continue to interact with CRYPTOCHROME and to be degraded by light as in wild-type flies, resulting in an increase in TIMELESS protein following lights-off. Thus, an hourglass-type signal involving *timeless* could persist in the absence of a normally functioning circadian clock. Indeed, there is evidence from another drosophilid fly, *Chymomyza costata,* that a mutation interrupting a normal photoperiodic response in this species may be associated with the *timeless* locus (Pavelka et al., 2003).

Some labs are now looking at the potential role of *timeless* in photoperiodism in laboratory strains of insects (Goto and Denlinger, 2002; Pavelka et al., 2003), but to our knowledge only one other lab is focusing on variation in photoperiodic response among natural populations (C. Kyriacou, personal communication, March, 2005). Herein, we present results from this novel approach, using natural populations recently collected from the field that have evolved divergent photoperiodic responses and testing for associated differences in the expression of *timeless* among these populations. If two populations differ in photoperiodic response but not in the expression of a given gene (*timeless*, in this case), then the expression of that gene cannot be responsible for variation in photoperiodic response.

Photoperiodic response varies according to seasonality. The relative length of the summer and winter seasons change with latitude and altitude, and the day length at which insects switch from continuous development to diapause (the critical photoperiod) is closely correlated with latitude and altitude (Danilevskii, 1965; Bradshaw, 1976; Taylor and Spalding, 1986; Danks, 1987; Saunders, 2002). Relative to the south, northern winters arrive earlier when days are longer so that, in order to enter diapause in a timely manner, northern insects use a longer critical photoperiod than southern insects. The critical photoperiod is a highly heritable trait (Dingle et al., 1977; Hoy, 1978; Hard et al., 1993; Bradshaw and Holzapfel, 2001a) that responds rapidly to climate change (Bradshaw and Holzapfel, 2001b). Hence, stabilizing selection results in a high degree of genetically determined local adaptation and correlations between critical photoperiod and geography of origin can exceed 90% (Bradshaw and Holzapfel, 2001a, b). By using naturally evolved populations, we now ask (1) whether the temporal pattern of *timeless* expression varies between developmental stages that are sensitive to photoperiod and (2) whether the expression of *timeless* varies among populations from different latitudes with different critical photoperiods in the mosquito, *Wyeomyia smithii*.

Wyeomyia smithii ranges from along the Gulf of Mexico (30 °N) to eastern and central Canada (57 °N). Throughout its range *W. smithii* oviposit into and complete their pre-adult 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 (Smith and Brust, 1971; Bradshaw and Lounibos, 1972; Evans and Brust, 1972). Larvae enter primarily a fourth instar diapause in the south (30–36 °N) and primarily a third instar diapause further north (Bradshaw and Lounibos, 1977). Critical photoperiod tracks closely both latitude and altitude with R^2 repeatedly greater than 90% (Bradshaw and Holzapfel, 2001b).

Herein, we examine developmental and latitudinal variation in the expression of *timeless* in naturally evolved populations of *W. smithii*. We show that the expression of *timeless* varies with diapausing instar and then, by holding diapausing instar constant, that the expression of *timeless* varies with latitude of origin.

2. Experimental approach

Over its geographic range, *W. smithii* diapauses in two separate instars. Our first question was straightforward: Is the expression of *timeless* the same in both diapausing instars? To answer this question, we measured *timeless* expression in a Maryland (38 °N) population that is polymorphic for stage of diapause with 30–40% of the population diapausing as fourth instars and the remainder as third instars. After finding that the pattern of *timeless* expression does indeed vary between diapausing instars (see Section 4.1), we restricted our geographical study to populations where we could use *W. smithii* that diapause in the third instar.

Our second question was likewise straightforward: Does the expression of *timeless* in diapausing third instars vary with latitude of origin in naturally occurring populations of *W. smithii*? We chose four representative localities (Table 1), two relatively northern populations in Newfoundland and Ontario and two relatively southern populations in Maryland and New Jersey. Northern and southern populations differ by about five standard deviations in mean critical photoperiod (Lair et al., 1997).

In insects in which *timeless* expression has been measured, the peak occurs at lights-off or during the dark portion of the light:dark regimen (Goto and Denlinger, 2002; Majercak et al., 1999; Pavelka et al., 2003); however, the position of the peak can vary within

Table 1
Origin and critical photoperiods of populations used in this study

State/Province	°N Lat	°W Lon	m Alt	Reference[a]	Critical photoperiod
Maryland	38	75	20	NP	13.4[b]
New Jersey	40	74	10	PB	13.5[b]
Newfoundland	50	58	55	GM	15.1[c]
Ontario	50	94	405	DL	15.1[d]

[a]Specific locality designation from prior studies from this lab.
[b]Bradshaw et al. (2003a).
[c]Estimated from regression of critical photoperiod on latitude and altitude (Bradshaw et al., 2003a).
[d]Bradshaw et al. (1998).

species according to factors such as day length and temperature. Therefore, when designing the latitude experiment we could not a priori assume that the peak of *timeless* expression would be at the same time for each population at both latitudes. Consequently, we sampled at several time points throughout the dark period in order to detect a peak whether it came early or late in the subjective night. We then compared expression of *timeless* relative to a reference "housekeeping gene" between early and late during the dark period of an L : D = 10 : 14 light:dark cycle.

3. Materials and methods

3.1. Mosquito collection and maintenance

Mosquitoes were collected from four localities (Table 1). Collection, transportation, and basic husbandry in the lab were as described previously (Lair et al., 1997). To minimize field effects, populations were maintained for at least three generations on long days with a simulated natural thermoperiod (Bradshaw et al., 2003a).

3.2. Stage of diapause

Eggs for the experimental generation of the Maryland population were collected and placed on short day (L : D = 8 : 16) at a constant temperature of 21 °C and allowed to hatch. Larvae were maintained under these conditions for 35–50 days to ensure entry into diapause and then segregated according to instar (3rd or 4th) into dishes of 30. For each instar, 18 of these dishes were transferred to a photoperiod cabinet with an L : D = 10 : 14 photoperiod at a constant temperature of 23 °C. The larvae were then allowed to entrain to the new conditions for 5 days. Immediately following the entrainment period, samples were collected for RNA extraction every 4 h over a 24 h period. With time 0 equal to lights-on, sampling occurred at time points 1, 5, 9, 13, 17, and 21. At each time point, three replicates of 24 larvae per instar were transferred to 1.5 mL microcentrifuge tubes. The water was removed, 250 μl of RNAwiz RNA isolation reagent (Ambion) were added, and the larvae were ground with a motorized pestel. Once ground, each replicate was flash frozen in liquid nitrogen to minimize the chance for RNA degradation and then stored at −80 °C.

3.3. Latitude

Eggs of the experimental generation from all four populations used in this experiment were collected, allowed to hatch, and reared on short days as above (Section 3.2). After 35–50 short days, diapausing third instars were sorted from each population and transferred to the L : D = 10 : 14 photoperiod at 23 °C. After five days of entrainment, six replicates of 24 larvae were sampled as above either early (hours 11, 13, and 15 after lights-on) or late (hours 19, 21 and 23 after lights-on) in the subjective night.

3.4. RNA extraction and quantitative real-time PCR

Prior to extracting RNA, samples were removed from −80 °C and thawed on ice. While thawing, an additional 250 μl of RNAwiz RNA isolation reagent (Ambion) was added to each sample to bring the final volume up to 500 μl. Total RNA was then extracted according to the RNAwiz protocol. Following extraction, each sample was immediately treated with DNase I (DNA-*Free* DNase treatment and removal reagents; Ambion) to remove any genomic DNA carried over from the extraction process. For each sample, 2 μg of total RNA was then used as template for cDNA synthesis using oligo $d(T)_{16}$ as the primer (TaqMan Gold RT-PCR kit; Applied Biosystems). Reverse transcription was carried out at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. Following cDNA synthesis, quantitative real-time PCR was performed on an Applied Biosystems 7900 sequence detection system using the 96-well format. Each PCR reaction had a total volume of 40 μl with 4 μl of cDNA as the template. Other components of the reaction included 20 μl of 2X SYBR Green master mix (Applied Biosystems), 2.4 μl of

Table 2
Primers used for timeless and the reference genes, *RpL8* and *Rp49*

Gene	Direction	Sequence	GenBank Accession no.
timeless	Forward	5′ GTGCATCATGGTGAAAATGC 3′	AY943312
	Reverse	5′ AAGTTCGCCACAATGGAAAT 3′	
RpL8[a]	Forward	5′ GGCGTTCCTCGCTTAACA 3′	AY943310
	Reverse	5′ CGAAAGTGCGTGGTGT TG 3′	
Rp49[b]	Forward	5′ ATCGGTTACGGATCGAACAA 3′	AY943311
	Reverse	5′ TTCTGCATCAGCA GCACTTC 3′	

[a]Used for stage of diapause experiments.
[b]Used for latitude experiments.

both forward and reverse primers (300 nM final concentration), and 11.2 μl of water. Reaction conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Prior to these experiments all conditions and concentrations of PCR components were rigorously optimized as recommended by the SYBR Green master mix protocol. The primer sets in Table 2 produced amplicons of 101 (*timeless*), 99 (*RpL8*), and 103 (*Rp49*) bp. The reference gene was changed from *RpL8* to *Rp49* to make the results of the latitude study more comparable with studies in other insects that used *Rp49* as a reference gene.

3.5. Statistical methods

Expression of *timeless* between stages of development was subjected to two-way ANOVA with stage of diapause and time of day as treatments using replicates within treatments for the error term. Expression of *timeless* between latitudes was subjected to two-way ANOVA with time of night (early vs. late) and latitude as treatments, populations within latitude as the error term for the latitude effect, and time points within populations and time of night as the error term for the interaction effect (time of night by latitude) and for populations within latitudes.

4. Results

4.1. Stage of diapause

Mean expression of *timeless* relative to *RpL8* (Table 3A; Fig. 1) over all 24 h did not vary between instars. Expression of *timeless* did vary according to time of the L : D = 10 : 14 cycle, and there was a significant time of the cycle by instar interaction. The latter result means that the temporal pattern of *timeless* expression in diapausing larvae depends on diapausing instar.

Table 3
ANOVA of *timeless* expression

Source of variation	*df*[a]	*SS*	*MS*	*F*	*df*	*P*
(A) *Stage of diapause*						
Instar	1	0.0283	0.0283	0.91	1,5	0.351
Time	5	0.4175	0.0835	2.68	5,23	0.047
Instar × time	5	0.4179	0.0836	2.68	5,23	0.047
Error	23	0.7165	0.0312			
(B) *Latitude*						
Latitude	1	0.3271	0.3271	23.67	1,4	0.008
Early vs. late	1	0.0612	0.0612	9.65	1,16	0.007
Lat × E vs. L	1	<0.000	<0.000	0.00	4,16	0.997
Populations (Lat)	4	0.0553	0.0138	2.18	4,16	0.118
Time points (Pop)	16	0.1015	0.0063			

[a]*df*, degrees of freedom; *SS*, sum of squares; *MS*, mean square.

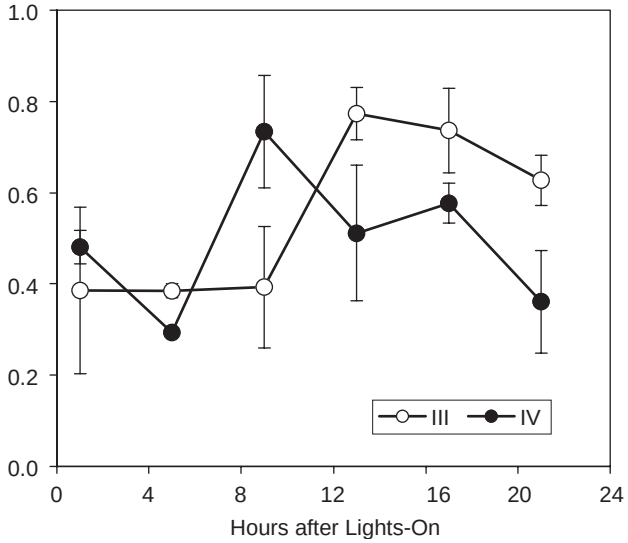

Fig. 1. Expression of *timeless* relative to *RpL8* in diapausing III and IV instar *W. smithii* from Maryland exposed to a L : D = 10 : 14 cycle at 23 °C. The *Y*-axis is scaled to values of 1.0 and 0.0 for the maximum and minimum relative concentrations, respectively. Error bars show ± SE.

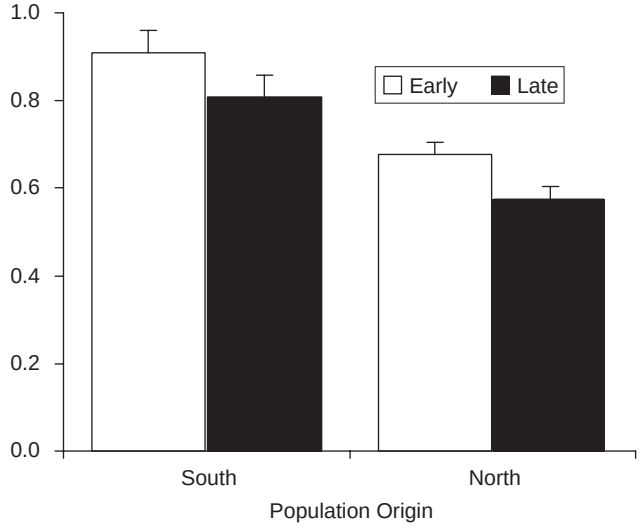

Fig. 2. Expression of *timeless* relative to *Rp49* in southern (38–40 °N) and northern (50°N) populations early (11,13, and 15 h after lights-on) and late (19, 21, and 23 h after lights-on) in the subjective night of a L : D = 10 : 14 cycle at 23 °C. The *Y*-axis is scaled to values of 1.0 and 0.0 for the maximum and minimum relative concentrations, respectively. Error bars show ± SE.

4.2. Latitude

Expression of *timeless* relative to *Rp49* (Table 3B; Fig. 2) showed no interaction between time of night and latitude of origin, meaning that time of night and latitude of origin had independent effects on the expression of *timeless*. Expression of *timeless* was higher in the early than late subjective night and higher in southern populations with short critical photoperiods (Table 1) than in northern populations with long critical photoperiods. These results show that the expression of *timeless* varies consistently according to latitude of origin and, hence evolved differences in critical photoperiod.

5. Discussion

5.1. Stage of diapause

Even within a single population, the temporal pattern in the expression of *timeless* differs between adjacent stages of larval diapause (Fig. 1). *Wyeomyia smithii* are photoperiodic while in diapause (Bradshaw and Lounibos, 1977) so that the diapausing instar is also the stage at which daylength is perceived and integrated into a go, no-go response for development. These results mean that stage of the life cycle must be considered when comparing gene expression between species, between populations within a species, or between photoperiod treatments within populations, especially when looking for associations between transcript levels and phenotypes.

The stage of the life cycle at which diapause is programmed is quite variable among insect taxa (Lees, 1955; Tauber et al., 1986; Saunders, 2002). However, most data on circadian gene expression in insects where photoperiodism is of primary interest come from studies of adults that are non-photoperiodic (Goto and Denlinger, 2002; Pavelka et al., 2003). Our point here is that developmental stage is clearly an important factor in the expression of circadian clock genes (Fig. 1; Kaneko et al., 1997; Kaneko and Hall, 2000) and in order to relate expression of those genes to photoperiodism, their expression needs to be assessed in the stages in which photoperiodic response is actually taking place.

5.2. Latitude

In laboratory strains of *Drosophila* and *Sarcophaga*, studies of *timeless* expression have demonstrated that both long and short days, as well as temperature, affect levels of mRNA transcript (Majercak et al., 1999; Goto and Denlinger, 2002). In addition, the expression of *period* varies among species of Diptera (Rosato and Kyriacou, 2001) or between worker castes of honey bees (Toma et al., 2000). However, to our knowledge, no one has compared the expression of circadian rhythm genes between natural populations over geographic ranges that show different photoperiodic responses, i.e., critical photoperiods. Since correlations between latitude and critical photoperiod have been observed in numerous species (see Section 1), molecular genetic changes affecting photoperiodic response should also correlate with latitude. Finding identical levels of *timeless* expression in northern and southern populations would exclude expression of *timeless* as a factor affecting the evolution of critical photoperiod; the converse result does not necessarily demonstrate a causal role for *timeless* in the evolution of critical photoperiod, only that it could play such a role. Although *W. smithii* provides no functional data linking the expression of *timeless* to the evolution of photoperiodic time measurement, it does show a consistent association between latitude and level of expression (Fig. 2). This result provides the first example of a latitudinal difference in the expression of *timeless*, suggesting that, along with evidence from within laboratory strains of fruit and flesh flies (Majercak et al., 1999; Goto and Denlinger, 2002; Pavelka et al., 2003) *timeless* has the potential to affect photoperiodic response and its adaptive evolution in temperate seasonal environments. This effect of *timeless* on photoperiodism could be totally independent of and incidental to its functional role in circadian rhythmicity.

We are now determining whether this latitudinal pattern of expression remains consistent in other photoperiods and whether polymorphisms in *timeless* itself are associated with variation in critical photoperiod.

We believe that experiments designed from an evolutionary perspective focusing on naturally occurring variation in photoperiodic phenotypes (e.g. critical photoperiod) will be key to uncovering the genetics of photoperiodic time measurement. Combining this approach with functional genomic techniques (e.g. RNAi, microarrays) that can be applied to non-model organisms will prove especially powerful.

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