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Period gene expression in relation to seasonality and circadian rhythms in the linden bug, *Pyrrhocoris apterus* (Heteroptera)

MAGDALENA HODKOVÁ, ZDEŇKA SYROVÁ, DAVID DOLEŽEL and IVO ŠAUMAN

Institute of Entomology, Academy of Sciences, České Budějovice, CZ-370 05 Czech Republic; e-mail: magda@entu.cas.cz

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Abstract. Wild females of *Pyrrhocoris apterus* exhibit seasonal changes in neuroendocrine activity and, consequently, reproduction. Long days (18 h light/6 h dark) (LD) stimulate reproduction, whereas short days (12 h light/12 h dark) (SD) induce reproductive arrest (diapause). This study reveals how photoperiod influences the expression of the circadian clock gene, *period* (*per*) in the insect's head. There is only a weak diurnal rhythm in *per* mRNA expression under LD and SD. However, levels of *per* mRNA are consistently higher (up to 10-fold) under SD than under LD. The influence of photoperiod on *per* gene expression is linked to a developmental output (diapause vs. reproduction); mutant females, reproducing under both LD and SD, show low *per* mRNA levels under both photoperiodic conditions. Thus, the magnitude of *per* gene expression may be important to the translation of photoperiodic signals into a hormonal message. Levels of *per* mRNA are related to properties of locomotor activity rhythms. Low *per* mRNA levels (displayed by wild females in LD and mutant females in both LD and SD) are associated with long free-running periods (τ~26–27 h) and late peaks of activity ($\psi_{R,L}$~10–12 h), whereas high *per* mRNA levels coincide with short free-running periods (τ~24 h) and early peaks of activity ($\psi_{R,L}$~4-6 h). Overall, the data provide a background for a molecular approach to the long-standing question about the role of the circadian system in insect photoperiodism.

INTRODUCTION

It is generally accepted that biological timing depends not only on the generation of circadian rhythms, but also on the measurement of time intervals (day length/length of night). While the expression of circadian output determines the circadian activities of the organism, the interval timing results in seasonal changes in the developmental pathways. The relationship between these two phenomena is still largely unresolved. Formal properties of photoperiodic responses indicate that photoperiodic time measurement is a function of the circadian system (Saunders, 1998; Vaz Nunes & Saunders, 1999). Recently, Veerman (2001) suggested that downstream events, such as synthesis of a clock substrate, are under circadian control in insects and mites. While our understanding of molecular mechanisms of circadian rhythms has greatly advanced, particularly due to the enormous power of *Drosophila melanogaster* genetics (Schotland & Sehgal, 2001), molecular events, from photoreception through time measurement and accumulation of photoperiodic "information" to neuroendocrine activity, controlling the onset of diapause or continuation of development/reproduction are still unknown.

Although the *period* gene (*per*) has been shown to form a central part of the circadian clock of insects as well as vertebrates (Dunlap, 1999; Reppert & Weaver, 2000; Schotland & Seghal, 2001), its role in the photoperiodic regulation of development is uncertain. Saunders (1990) concluded that the *per* gene in not causally involved in the photoperiodic induction of ovarian diapause in *D. melanogaster*; flies in which the *per* locus was missing (per^0) were still able to discriminate between diapause-inducing short days and diapause-averting long days, although the critical daylength was altered. Studies of latitudinal clines in photoperiodic responses show only weak correlation between critical daylength and period (τ) of eclosion rhythm (e.g. Pittendrigh et al., 1984 - *D. auraria*; Lankinen, 1986 - *D. litoralis*) or τ of the Nanda-Hamner rhythm (Vaz Nunes et al., 1990 - *Tetranychus urticae*). Furthermore, Nanda-Hamner experiments indicate that τ for the photoperiodic oscillator differs from that for locomotor rhythm in *Calliphora vicina* (Saunders, 1987a; Hong & Saunders, 1998). The above examples indicate that overt behavioural rhythmicity and photoperiodic timing involve separate circadian pacemakers with different characteristics (Saunders, 2001). On the other hand, day length affects the expression of *per* mRNA (Majercak et al., 1999), as well as the τ of locomotor rhythm in *D. melanogaster* (Tomioka et al., 1997). The effect of day length is mediated through the photosensitive timeless protein (Majercak et al., 1999), and the *timeless* locus is thought to be causally involved in the photoperiodic induction of larval diapause in another drosophilid, *Chymomyza costata* (Kostal & Shimada, 2001; Pavelka et al., 2003). These results indicate that the circadian clock governing overt rhythms and mechanisms decoding photoperiodic time may share common molecular components.

A heteropteran insect, *Pyrrhocoris apterus*, exhibits an adult diapause controlled by photoperiod; long days stimulate the activity of the corpus allatum (CA) and reproduction, whereas short days are inhibitory. Effect of daylength on the CA is mediated through the pars intercerebralis (PI) of the brain (Hodková, 1976) and *per* mRNA is expressed in neurosecretory cells of the PI (Syrová et al., 2001). Although it is unlikely that a difference in any single clock component, such as *per* mRNA

TABLE 1. Characteristics of locomotor activity rhythms in females of *P. apterus* under different photoperiods.

Insects	Photoperiod	Food conditions	Replicate	Free-running period (τ)(h)[1)]	Activity peak ($\psi_{R,L}$)(h)[2)]	Activity level (mesor)
Photoperiodically sensitive	long	feeding	1	26.4**	10.6*	5.5
		feeding	2	27.2*	12.6**	6.2
		starving	3	26.4**	10.2*	6.4
	short	feeding	1	?	8.1*	0.6
		feeding	2	?	5.6*	2.7
		starving	3	24.2*	3.8*	1.9
Photoperiodically insensitive	long	feeding	1	27.6**	12.8**	6.9
		starving	2	26.0**	10.8*	11.3
	short	feeding	1	26.0**	10.0*	9.8
		feeding	2	27.0**	12.6*	15.9
		starving	3	26.2**	11.2*	8.8

τ and $\psi_{R,L}$ were calculated for 5–8 cycles, mesor was calculated for 10–13 cycles.
1)**p < 0.005–0.01, *p < 0.05
2)**excellent, *good

level, is responsible for decoding photoperiodic time, it may reflect an underlying difference in the circadian structure. We have investigated, using an RNase protection assay, how day length affects the expression of *per* mRNA in the insect's head. A potential relationship of *per* mRNA levels to developmental mode (diapause vs. reproduction) and properties of circadian rhythms in locomotor activity have been estimated using two laboratory strains of *P. apterus* differing in their diapause photoresponsiveness: wild-type insects, reproducing under long days and entering diapause under short days, and mutant insects, reproducing under both long day and short day conditions.

MATERIAL AND METHODS

Insects

Wild-type and mutant strains of *P. apterus* (L.) (Heteroptera) used in this study originated from adults collected from the field near České Budějovice, Czech Republic. Insects were reared at 26 ± 1°C on linden seed and water ad libitum. The wild-type strain (referred to as photoperiodically sensitive insects) was maintained under diapause-preventing long days (LD) (18 h light / 6 h dark). A selected non-diapause strain (referred to as photoperiodically insensitive) was maintained under short days (SD) (12 h light / 12 h dark). Experimental insects were reared from the egg under either LD or SD. In addition to feeding females, locomotor activity rhythms were monitored in starving females, provided with only water, to discriminate a potential masking effect of feeding and oviposition behaviour. In *P. apterus*, feeding is essential for ovarian maturation. Only feeding females were used for RNase protection assay analysis.

Locomotor activity rhythms

Locomotor activity was monitored by placing individual females in Petri dishes and using a recording device comprised of an infrared beam passing horizontally through the dish to a phototransistor. Activity was monitored in 12 min bins as the number of interruptions of the infrared light beam by moving insects and the data were recorded by computer. Monitoring was performed at 26 ± 1°C. Light was provided by a fluorescent lamp (9 watts) controlled by a 24-h timer. Females aged 1–2 days were exposed to 5–8 light cycles before being transferred to constant darkness (DD) for 5–8 days. The average rhythm characteristics were calculated by pooling data for 5–8 females. Three independent measurements (two with feeding females, one with starving females) were analysed. The phase difference between the time of peak activity and light on (ψ_{RL}) was calculated using an acrophase program, fitting a cosine wave to the data (Refinetti, 2000). The free-running period (τ) under DD was determined by chi-square periodogram analysis (Refinetti, 2000). The activity level was calculated as the arithmetic mean of all values (mesor) in the light cycles and DD. In the figures, the mean of 5 values (5 × 12 min = 1 h) was calculated for every hour.

RNase protection assay

Heads (without antennae and rostrum) were cut every 4 h around the clock and immediately placed on dry ice, and kept at –85°C until analysis. For each time point 25 heads were used for total RNA isolation. Total RNA was extracted using the RNA Blue isolation system (Top-Bio). [^{32}P]UTP-labelled *per* cRNA antisense and sense probes were generated by subcloning PCR-amplified cDNA fragments into pBluescript, followed by *in vitro* transcription driven from T3 and T7 RNA polymerase promoters, respectively. An antisense *RP49* reference probe cloned from *P. apterus* (unpublished data) was included in each RNase protection reaction as an internal control for the amount of RNA loaded in each lane. To equalize the signal intensity of the *per* and *RP49* bands on the gel, the specific activity of the *RP49* probe was reduced by diluting the [^{32}P]UTP 1:750 with nonradioactive UTP in the *in vitro* transcription reaction. RNase protection assays were performed using a RPA III kit (Ambion) according to the supplied protocol. Quantification was performed on a Storm PhosphorImager (Molecular Dynamics) and ImageQuant software (Molecular Dynamics). Each protection assay was performed at least three times with similar results. The results were replicated with at least two sets of animals for each experiment.

RESULTS

Effect of photoperiod on locomotor rhythms

Photoperiodically sensitive insects

Females showed significant diurnal locomotor activity rhythms under both LD and SD conditions with periods close to 24 h, similar to that of the light cycle. The peak of activity in females held in LD was in late afternoon in all experimental groups ($\psi_{R,L}$ = 10.2–12.6 h) (Table 1, Fig. 1A, 2A). Under DD, the rhythm free ran with a period longer than 26 h (τ = 26.4–27.2 h) (Table 1, Fig. 1B).

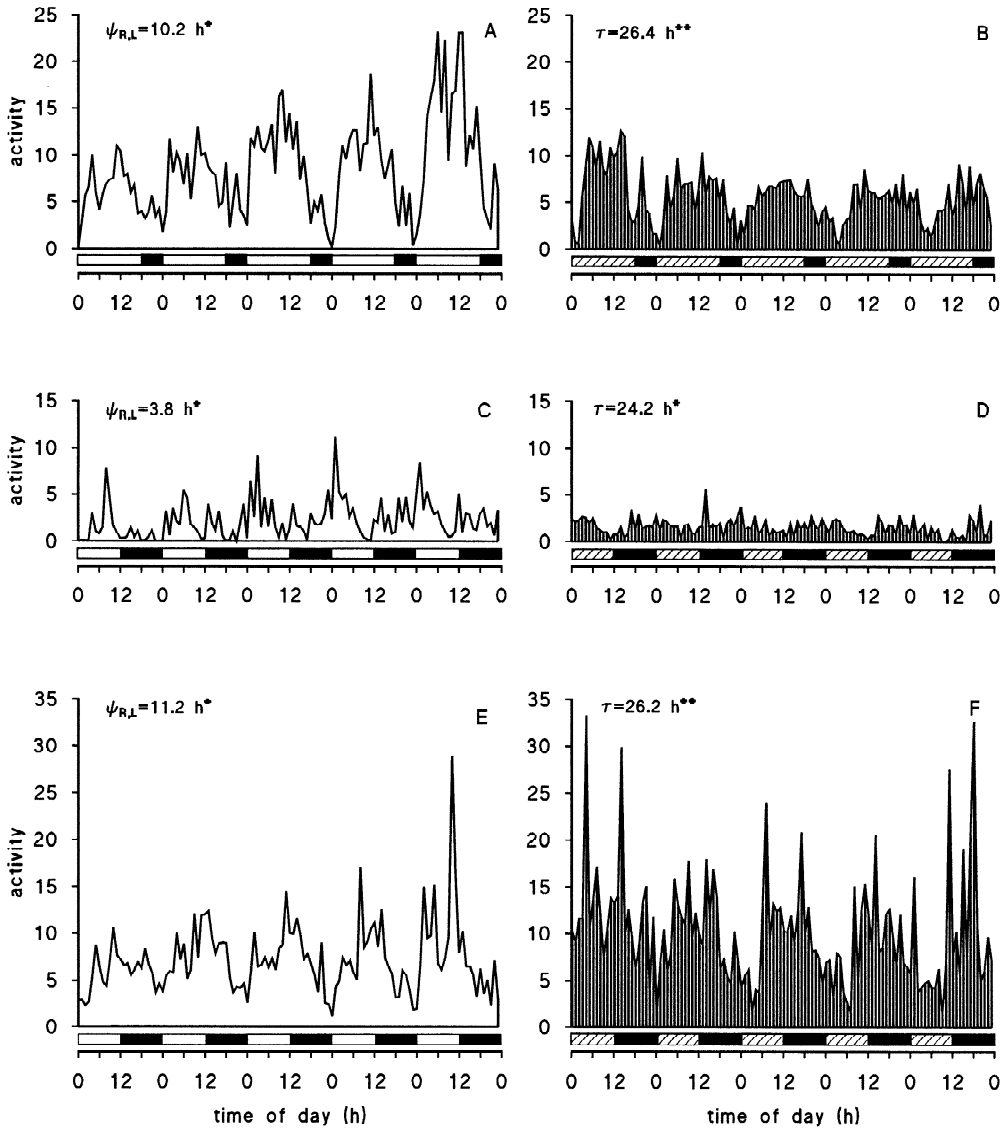

Fig. 1. Diurnal and circadian patterns of locomotor activity in starving females of *P. apterus* under different photoperiods. Photoperiodically sensitive females were held in long day (A), constant darkness after long day entrainment (B), short day (C), constant darkness after short day (D). Photoperiodically insensitive females were held in short day (E), constant darkness after short day (F). White and black horizontal bars represent times in daily cycles when the lights were either on or off, respectively. Hatched bars represent subjective day. For other explanations see Table 1.

Females held at SD showed a considerable difference in rhythm properties relative to SD females. The activity level under SD was about one-third of that under LD (Table 1, Fig. 1A-D, 2A,B). Furthermore, the peak of activity under SD was several hours earlier relative to LD ($\psi_{R,L}$ = 3.8–8.1 h) (Table 1, Fig. 1C, 2B). Feeding SD females appeared arrhythmic under DD (Table 1), probably due to a low locomotor activity associated with a masking effect of feeding activity. In starving SD females, however, the free-running period was shorter by about two hours compared to starving LD females (τ = 24.2 h) (Table 1, Fig. 1D).

Photoperiodically insensitive insects

Rhythm properties in photoperiodically insensitive females showed fewer differences between LD and SD compared to photoperiodically sensitive females. In both LD and SD females, the peak of activity occurred in the late afternoon ($\psi_{R,L}$ = 10.8–12.8 h for LD, 10.0–12.6 h for SD) (Table 1, Fig. 1E). The free-running period was 26 h or longer under DD in both LD females (τ = 26.0–27.6) (Table 1) and SD females (τ = 26.0–27.0 h) (Table 1, Fig. 1F). Under both LD and SD, the rhythm assumed a period close to the 24-h period of the light cycle. The activity level was high under both photoperiods; it tended to be higher under SD than under LD in feeding females, while in starving females an opposite trend was observed (Table 1). It is noteworthy that the rhythm characteristics (τ , $\psi_{R,L}$) in photoperiodically insensitive females held in SD were similar to those for photoperiodically sensitive females under LD. The overall activity tended to be

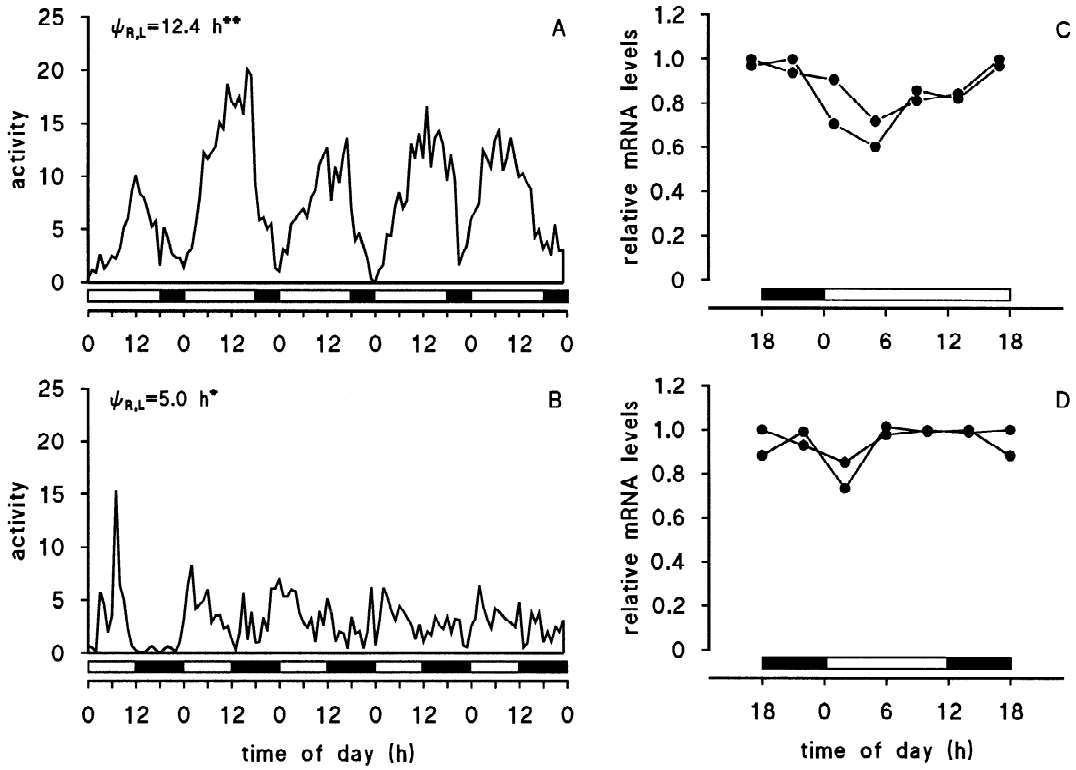

Fig. 2. Diurnal rhythms in locomotor activity and relative levels of *per* mRNA in feeding females of *P. apterus* under different photoperiods. Photoperiodically sensitive females were held in long day (A,C) or short day (C,D). Relative *per* mRNA levels refer to *per*/*RP49* ratio. For other explanations see Fig. 1 and Table 1.

higher in photoperiodically insensitive relative to sensitive females (Table 1).

Effect of photoperiod on *per* mRNA expression

Temporal changes in *per* mRNA levels in heads of photoperiodically sensitive females revealed a weak diurnal rhythm under LD, with a peak of expression at Zeitgeber time (ZT) 17–21 and minimum expression at ZT 5 (Fig. 2C). Under SD, there was no distinct oscillation in *per* mRNA expression. Levels of *per* mRNA were consistently high, with the exception of a slight decrease at ZT 2 (Fig. 2D). A delayed increase in the *per* mRNA level under LD relative to SD (Fig. 2 C,D) corresponds to a delayed peak in the locomotor rhythm under LD (Table 1, Fig. 2 A,B). The most striking difference between LD and SD females was a considerably higher expression of *per* mRNA under SD. Levels of *per* mRNA were consistently higher under SD (up to 10-fold) than under LD (Fig. 3 A, B).

To see whether photoperiod influences the magnitude of *per* gene expression in photoperiodically insensitive females, *per* mRNA levels were compared between LD and SD at two time points (Table 2). These preliminary data show that *per* mRNA levels detected in photoperiodically insensitive females were low under both SD and LD and similar to those found in photoperiodically sensitive females held in LD. Low *per* mRNA levels in the three groups of females coincided with a higher activity level, later activity peak, and longer free-running period of their locomotor rhythms compared to photoperiodically sensitive females held in SD, which showed high *per* mRNA levels (see above).

TABLE 2. Relative *per* mRNA levels in feeding females of *P. apterus* under different photoperiods.

Insects	Photoperiod	Zeitgeber time (h)	*per*/RP49
Photoperiodically sensitive	long	5	0.08
		17	0.20
	short	6	1.00
		18	0.87
Photoperiodically insensitive	long	5	0.13
		17	0.22
	short	6	0.21
		18	0.18

DISCUSSION

The conventional view that the circadian system is involved in the transduction of photoperiodic signals into a developmental output (diapause vs. continued development/reproduction) (see Introduction) implies that molecular components of the circadian system somehow respond to alterations in photoperiod. Here we show how photoperiod regulates the expression of the essential circadian clock gene, *period*, in heads of *P. apterus* females. Photoperiod is translated into the magnitude of *per* gene expression, with a considerably higher expression under SD than under LD. However, the strong up-regulation of *per* gene expression under SD occurs only in wild-type (photoperiodically sensitive) females that enter diapause

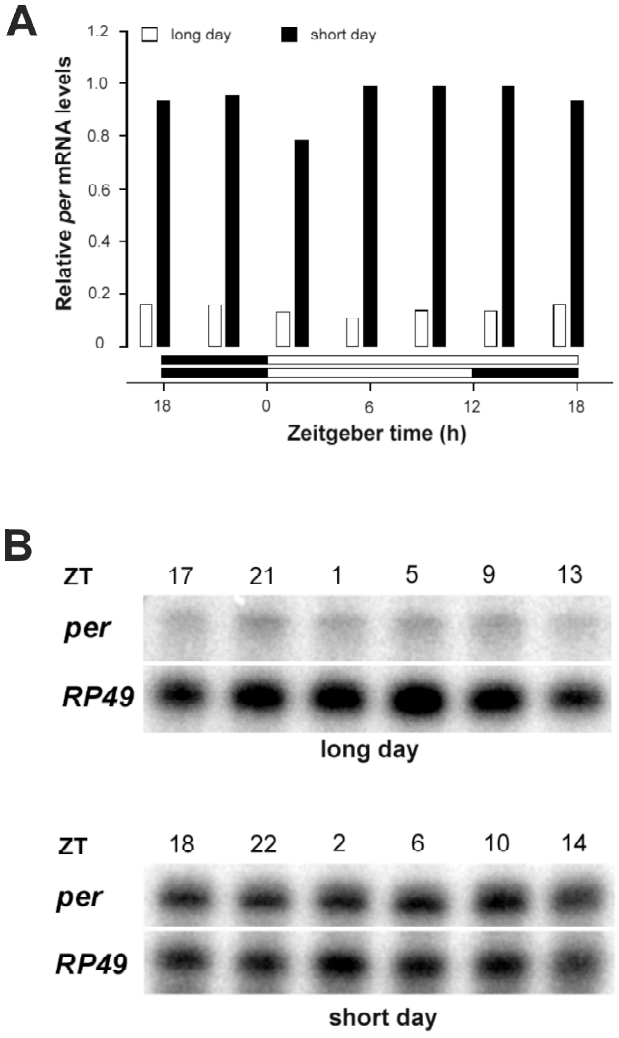

Fig. 3. Effect of photoperiod on *per* mRNA levels in feeding females of *P. apterus*. A – relative *per* mRNA levels were compared between long day and short day females sensitive to photoperiod. Values represent means of two determinations; B – RNAs were visualised by autoradiography. For other explanations see Fig. 1 and 2.

under SD. Mutant (photoperiodically insensitive) females, reproducing under both LD and SD, fail to increase *per* mRNA levels under SD (Table 3). Because *per* mRNA levels are linked to both photoperiod and developmental output, the magnitude of *per* gene expression may be important to the transduction of photoperiodic signals into a hormonal message. This view is supported by findings that *per* mRNA is expressed in the neurosecretory cells of the PI of the brain (Syrová et al., 2001) that are implicated in the translation of photoperiod into neurohormonal signals (Hodková, 1976). It is not clear, however, whether the *per* gene is a component of the photoperiodic clock or its target. The photoperiodic response of *P. apterus* presents a few unusual features. This species appears to "measure" day length rather than length of night and Nanda-Hamner results indicate an extremely short free-running period (16 h) of the constituent oscillators (Saunders, 1987b). On the other hand, free-running periods of locomotor rhythms are longer than 24 h (Table 1, Figs 1, 2). However, there is only a weak diurnal rhythm of *per* mRNA under SD and LD (Fig. 2). The difference in the level of *per* mRNA between SD and LD suggests, perhaps, that the *per* gene plays no central role as a clock component, but might be on the output pathway to diapause/reproduction. Although there may be no causal relationship between the *per* gene itself and photoperiodic regulation of diapause, as is suggested for *D. melanogaster* (Saunders, 1990), it is likely that the photoperiodic regulation of *per* gene expression, as revealed in *P. apterus*, reflects responses of other molecular components of the circadian system to photoperiod. It will be important to study how photoperiod affects other circadian clock-related genes, particularly in the view of a recent finding that the photoperiodic insensitivity in a drosophilid fly, *C. costata*, may be caused by an inability to transcribe the *timeless* (*tim*) gene (Pavelka et al., 2003). A higher (about twice) peak level of *tim* mRNA under short daylength relative to long daylength was demonstrated in adult heads of the flesh fly, *Sarcophaga crassipalpis* (Goto & Denlinger, 2002). It is not clear, however, whether the level of *tim* gene expression in adults has any relation to pupal diapause in this species. The molecular basis for the absence of diapause photoresponsiveness in the photoperiodically insensitive strain of *P. apterus* is not known. If the *per* gene is part of a photoperiodic transduction chain, the block to diapause photoresponsiveness may be expected upstream from the *per* mRNA expression. Diapause photoresponsiveness also disappears during diapause development in wild-type females of *P. apterus* (Hodek, 1971). It will be interesting to study how these ontogenetic changes in photoperiodic response affect *per* mRNA expression.

The *period* and *timeless* genes seem to be essential components of the circadian clock underlying overt rhyth-

TABLE 3. *Per* mRNA levels and activity rhythm properties in relation to photoperiod and developmental program (summary).

	Photoperiodically sensitive insects		Photoperiodically insensitive insects	
Photoperiod	long	short	long	short
Developmental program	reproduction	diapause	reproduction	reproduction
per mRNA level	low	high	low	low
Free-running period	long	short	long	long
Activity peak	late	early	late	late
Activity level	high	low	high	high

For details see Table 1,2, Fig. 1-3, and text.

micity in *D. melanogaster* (Dunlap, 1999; Sauman & Hashimi, 1999; Schotland & Sehgal, 2001). In *P. apterus*, diurnal rhythms in the relative abundance of *per* mRNA show no robust peaks (particularly under SD) that would indicate a clear relation to locomotor activity rhythms. On the other hand, the magnitude of *per* gene expression seems to be related to the free-running period of the activity rhythm. It was shown in *D. melanogaster* that the free-running period for circadian rhythms is inversely related to the log of *per* mRNA titre (Baylies et al., 1987). Similarly, in *P. apterus*, high levels of *per* mRNA were associated with a short free-running period (τ~24 h), while low levels of *per* mRNA under LD coincided with a long free-running period (τ~26–27 h) of the activity rhythm (Table 3). The mechanism by which *per* mRNA levels are regulated by photoperiod is not known. The rhythm assumed a 24- hour period (i.e. the period of light cycle) with a phase angle difference ($\psi_{R,L}$) depending on τ (the longer the τ, the later the peak - Table 1), in accordance with general rules of the entrainment of circadian rhythms (Pittendrigh, 1981), in both wild and mutant females of *P. apterus*. Therefore, the failure of mutant females to increase *per* mRNA levels under SD cannot be explained by decoupling of circadian system from the entraining effect of light. Conversely, it may be assumed that the influence of photoperiod on *per* mRNA levels in wild females is not executed through an entrainment pathway. The downstream pathway from the magnitude of *per* gene expression to the free-running period of activity rhythm is not known either. Properties of locomotor activity rhythms (τ, ψ_{RL}, activity level) were related to a developmental program determined by photoperiod (Table 3). It is unlikely, however, that final outputs, such as vitellogenesis and oviposition, are responsible for the difference in rhythm properties between LD and SD; the difference was also found in starving females, although vitellogenesis was prevented (Fig. 1A-D). The CA is inhibited from the PI in both SD females and starving LD females of *P. apterus*, but the inhibition caused by SD is qualitatively different from that caused by starvation (Hodková et al., 2001). Findings that removing the neurosecretory cells of the PI influences properties of locomotor rhythms (unpublished data) and mating rhythms (Hodková, 1994) indicate a neurohormonal regulation of the circadian rhythms in *P. apterus*. A juvenile hormone analogue (methoprene) affected the τ of the eclosion rhythm in the southwestern corn borer, *Diatraea grandiosella* (Yin et al., 1987). There is evidence that light cycles perceived during development affect properties of circadian rhythms, particularly their free-running period (e.g. Barrett & Page, 1989; Tomioka et al., 1997; Wattari, 2002), but the physiological and molecular mechanisms of these alterations are still largely unknown.

In addition to the PI, *per* expressing cells were found in the compound eyes (Syrová et al., 2001), where the circadian pacemaker for locomotor rhythmicity was identified in *P. apterus* (Hodková, 1999). It is possible that similar molecular components are used by different tissues for different functions: Translation of day length into a neuroendocrine message by the PI and entrainment of the circadian rhythms by the compound eyes. In a mammal, the Syrian hamster, photoperiod affects the amplitude of an early-response gene, *Per1*, expression in the pars tuberalis that is implicated in decoding day length. In contrast, photoperiod has no effect on the gene expression in the suprachiasmatic nucleus that is the site of the circadian pacemaker (Messager et al., 1999). Studies are still needed to determine how photoperiod affects clock gene expression in individual *per*-expressing tissues in *P. apterus*.

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