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Research Article

Photoperiodic regulation of diapause in linden bugs: are *period* and *Clock* genes involved?

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Abstract. Although photoperiodism is undoubtedly one of the most important functions of the circadian system, the role of circadian clock genes remains unclear. We compared the expression of *period* and *Clock* genes in the head of the linden bug, *Pyrrhocoris apterus*, kept under diapause promoting short days (SD) and diapause-preventing long days (LD), using an RNase protection assay. There was only a weak diurnal rhythm in both *period* and *Clock* mRNA under LD and no rhythm under SD. Under SD, however, the level of *period* mRNA was about tenfold and that of *clock* mRNA about twofold higher than under LD. In a mutant that does not undergo diapause, even under SD, levels of both transcripts were low in both photoperiods. The differential regulation of the levels of two clock gene transcripts in a photoperiodic mutant, demonstrated for the first time in an animal species, strongly indicates a link between photoperiod, the magnitude of clock gene expression, and developmental outputs.

Key words. *Period* gene; *Clock* gene; photoperiodism; circadian clock; photoperiodic mutant.

Many organisms use photoperiodic signals to anticipate seasonal changes in the external environment. Photoperiodic regulation of development (diapause vs continuous development/reproduction) is thought to be a function of the circadian system. Formal models of photoperiodic induction propose that circadian oscillators have either a 'clock role' and are directly involved in the measurement of night (or day) length (photoperiodic clock) or a 'non-clock' role operating at a downstream level, e.g. accumulation of successive nights (long or short) by a photoperiodic counter [1]. While the role of clock genes is well understood in relation to overt circadian rhythms, due to the power of *Drosophila melanogaster* genetics [2], their role, if any, in relation to photoperiodism remains unclear. However, *D. melanogaster* is not very suitable for the study of photoperiodism, because this species has a weak reproductive dormancy induced only at a low temperature (~12 °C) [3, 4].

In contrast to *D. melanogaster*, our model species, the linden bug *Pyrrhocoris apterus*, shows a robust diapause response to photoperiod at a high temperature of 25 °C, i.e. diapause is not caused by unfavourable environmental conditions [5, 6]. Recently, we found that *per* gene expression is strongly up-regulated in heads of diapausing adults of *P. apterus* [7]. In *D. melanogaster*, *Clk* positively regulates *per* and *tim* and, directly or indirectly, other genes that are expressed in a circadian way [2, 8]. Therefore, we extended this study by performing a simultaneous analysis of the levels of *per* and *Clk* mRNA in the heads of *P. apterus* kept in either diapause-promoting short days or diapause-preventing long days. In addition to wild-type insects, the analysis was performed in mutant insects that do not undergo diapause, irrespective of photoperiod.

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Material and methods

Animals

Adult females of *P. apterus* (L.) (Heteroptera) were used in all experiments. Insects were reared at 26 ± 2 °C, and supplied ad libitum with linden seed and water. The wild-type strain was maintained under a diapause-preventing long-day photoperiod of 18 h light/6 h darkness (LD). A mutant strain, lacking the diapause response at 26 °C, was selected under a diapause-promoting short-day photoperiod of 12 h light/12 h darkness (SD) from a few reproducing individuals. The diapause response to SD became apparent at lower temperatures (70% and 41% diapause at 17 °C and 20 °C, respectively) [9]. Thus, selection has apparently been for gene(s) controlling the temperature dependence of the diapause response to photoperiod. Genetic analysis revealed that, under SD and 26 °C, the non-diapause trait behaves as a single autosomal recessive [10]. Experimental insects were reared from eggs under either LD or SD. The mean duration of larval development was similar under the two photoperiods (34–35 days). Adults aged 1 week were used for analysis.

Period and *Clock* genes from *P. apterus*

Molecular cloning and detailed characterization of *P. apterus per* and *Clk* genes, will be reported elsewhere. Using the MegAlign program, the *Pyrrhocoris per* cDNA fragment (~1 kb) showed 35% and 45% overall identity at the amino acid level with the corresponding regions of *Drosophila* and giant silkmoth (*Antheraea pernyi*) *per*, respectively. The overall identity between the linden bug *Clk* cDNA fragment (~0.9 kb) and the corresponding fruitfly region at the amino acid level was 55%. Sequences of both cDNAs were deposited in GenBank under accession numbers AY247968 and AY256442, respectively, and will be released upon publication of the article.

RNase protection assay

Every 4 h around the clock, heads of insects (without antennae and rostrum) were cut off 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. [^{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. The *per* probe (224 bp) was derived from the region between the PER nuclear localization signal (NLS) and the PAS domain. The *Clk* probe (197 bp) corresponded to the CLK region between the basic helix-loop-helix (bHLH) domain and the PAS domain. 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 non-radioactive UTP in the in vitro transcription reaction. RNase protection assays were performed using an 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 two to three times, with similar results. The results were replicated with at least two sets of animals for each experiment.

In situ hybridization

Dioxenin-labelled antisense and sense probes were generated by subcloning a PCR-amplified fragment of the bug *per* and *Clk* cDNA into pCR II TOPO TA vector (*per*) and pGEM-T Easy vector (*Clk*), respectively, followed by in vitro transcription in the presence of dioxigenin-labelled UTP driven from Sp6 and T7 RNA polymerase promoters, respectively. The efficiency of dioxigenin incorporation and sensitivity of the labelled probes was assessed by detection on nylon membranes. Similar to the silkmoth, *A. pernyi* [11], the cells in the linden bug brain which express *per* and *Clk* also express corresponding antisense RNAs. In control experiments, we used an irrelevant control labelled probe from the DIG RNA labelling kit (Boehringer Mannheim). As an additional control, we used sense and antisense probes of the same length against *per* and *Clk* from a different insect species, the housefly *Musca domestica*. In all cases, no specific staining above background was observed. Since we obtained clear signals with both antisense and sense probes, we performed the following control experiment to eliminate the possibility that both RNA strands were synthesized in the labelling in vitro transcription reaction due to 'leaky' promoters (even though each plasmid was verified for its complete linearization by agarose gel electrophoresis prior to the labelling reaction). Each individual RNA strand was heat-denaturated for 10 min at 85 °C, annealled for 1 h at 37 °C and subjected to RNase A treatment for 1 h at 37 °C. Subsequent agarose gel analysis revealed no detectable contamination of the single-stranded RNAs with their respective complementary strands.

Results and discussion

An RNase protection assay revealed consistently higher levels of both *per* and *Clk* mRNA in SD relative to LD insects (fig. 1), suggesting that the expression of both genes is controlled by photoperiod. However, the response of the two transcripts to photoperiod showed important quantitative differences. While levels of *per* mRNA were about tenfold higher (fig. 1 A), those of *Clk* mRNA were

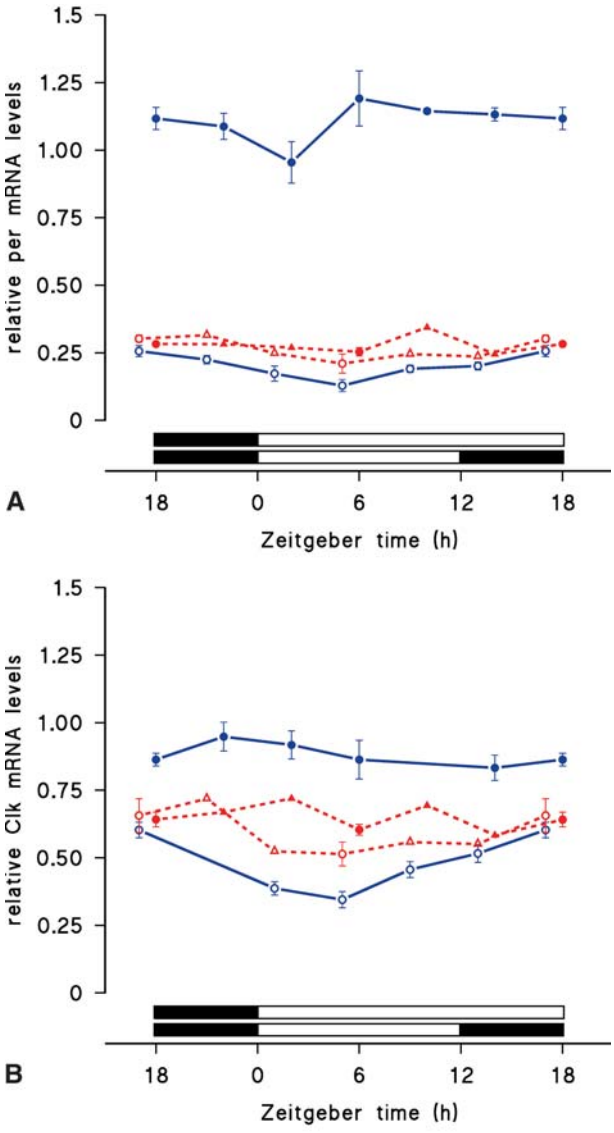

Figure 1. Relative levels of *per* and *Clk* mRNA in the heads of *P. apterus* kept under LD or SD photoperiod. Relative mRNA levels refer to the *per*/RP49 (*A*) and *Clk*/RP49 (*B*) ratio. Blue, mRNA levels in wild-type insects; red, mRNA levels in photoperiodic mutant; open symbols, LD; solid symbols, SD. Each circle represents a mean of two to three analyses ± SD (1 analysis = 25 heads). In wild-type insects, the difference between LD and SD is significant (Student's t test) at all time points for both *per* mRNA ($p \ll 0.0001 - p < 0.001$) and *Clk* mRNA ($p < 0.02 - p < 0.05$). In the photoperiodic mutant, there was no significant difference between LD and SD for either *per* or *Clk* mRNA ($p = 0.2 - 0.9$) at time points indicated by circles. Triangles represent 1 analysis (25 heads) and were not subjected to statistical evaluation. White and black horizontal bars represent times in daily cycles when the lights were either on or off, respectively.

only about twofold higher (fig. 1B) under SD relative to LD. The difference between LD and SD did not result from a direct effect of light. The levels of *per* and *Clk* mRNAs at the first day in constant darkness (DD) or constant light (LL) after LD (at time points corresponding to ZT 5 and 17) were similar to those during the light cycle (*per*/RP49 was 0.205 at ZT 5 vs 0.208 at DD or 0.229 at LL, and 0.201 at ZT 17 vs 0.208 at DD or 0.229 at LL; *Clk*/RP49 was 0.464 at ZT 5 vs 0.418 at DD or 0.558 at LL, and 0.542 at ZT 17 vs 0.433 at DD or 0.513 at LL).

The diapause response of *P. apterus* to photoperiod disappears during diapause development in the field [12]. We found undetectable levels of *per* and *Clk* mRNAs in heads of *P. apterus* females collected in the field in February, still prior to the disappearance of the diapause syndrome and the expression of reproductive activity. Females started to oviposit within 1 week after the transfer to the laboratory at 26 °C under both LD and SD (the photoperiodic response was lost) and levels of both transcripts remained undetectable under both photoperiods. Thus the absence of the photoperiodic response after the termination of diapause seems to be associated with the down-regulation of *per* and *Clk* mRNAs.

To determine whether the up-regulation of *per* and *Clk* gene expression under SD was related to the induction of diapause, mutant insects, which reproduce under both LD and SD, were also examined. The results showed that not only was the photoperiodic response absent, but *per* and *Clk* mRNA levels in both SD and LD insects were similar to those found under LD in wild-type insects (fig. 1). This suggests that the influence of photoperiod on the expression of both genes is linked to a developmental output (diapause vs reproduction). Downstream consequences of either *per* or *Clk* mRNA expression are not yet understood, but the Clock protein in known to act as a transcription factor regulating the expression of several genes, in addition to the clock genes, *per* and *tim* [2].

Upstream events influencing the levels of *per* and *Clk* mRNAs are also not clear. Reproducing females, i. e. wild females under LD and mutant females under both photoperiods, showed clear circadian rhythms in locomotor activity with similar free-running periods under constant darkness (n = at least 15 individuals per group, χ^2 periodogram analysis [13], $\tau = 26.4 - 26.8$, $p < 0.01$) and peaks of activity (11.3 – 11.8 h after light on, an acrophase program [13]). Rhythms assumed the period of the light cycle (24 h), under both SD and LD [7]. These results suggest that the mutation does not prevent the entrainment of circadian rhythms and, therefore, the failure of mutant females to increase *per* and *Clk* mRNA levels under SD cannot be explained by decoupling of the circadian system from the entraining effect of light. Conversely, one may assume that the influence of photoperiod on the levels of *per* and *Clk* mRNAs in wild females is executed through a pathway independent of the entrainment of circadian rhythms in locomotor activity. Consistent with this view, Nanda-Hamner experiments indicate an extremely short free-running period (16 h) of a photoperiodic oscillator in *P. apterus* [14], while the free-running period of locomotor rhythms is longer than 24 h (see above). Different characteristics of photoperi-

odic oscillators and circadian oscillators governing behavioural rhythms have also been reported for other insect species [1]. Furthermore, the photoreceptor for photoperiodism in insects and mites is probably an opsin and not a cryptochrome, which is the most likely candidate photoreceptor for circadian entrainment in insects [15].

Wild diapausing females held in SD showed a lower (about threefold) level of locomotor activity, shorter free-running period (τ = 24.2 h, $p < 0.05$) and earlier peak of activity (4.4 h after light on) compared to reproducing females [7]. This effect of SD seems to be a component of the diapause response; no influence of photoperiod on properties of locomotor activity was observed in a non-diapause mutant [7]. In *D. melanogaster,* the free-running period of circadian rhythms is inversely related to the log of *per* mRNA titre [16], and the level of *per* mRNA was higher in diapausing than in reproducing females of *P. apterus* (fig. 1 A). On the other hand, properties of circadian rhythms may be influenced by endocrine activities [17] that are different in diapausing and reproducing *P. apterus* [18].

In contrast to *Drosophila*, diurnal rhythms in the abundance of *per* and *Clk* mRNA in the head of *P. apterus* show no robust peaks (particularly under SD) that would indicate a clear relation to locomotor rhythms (fig. 1). A circadian pacemaker for locomotor rhythms has been localised in the compound eyes [19] showing relatively weak hybridisation signals with both *per* and *Clk* mRNAs probes (fig. 2). Therefore, a clear circadian rhythm in *per* and *Clk* mRNAs in the compound eyes might be lost in large amounts of 'total brain' mRNAs. However, the semi-quantitative in situ hybridisation analysis revealed no significant fluctuations in the levels of *per* and *Clk* transcripts in the compound eyes. The role of clock genes in circadian timing remains unclear, because rhythmic mutants are not yet available in *P. apterus*.

Few studies have addressed the question about the role of clock genes in relation to photoperiodism, and no consensus is yet available. In the head of the adult flesh fly, *Sarcophaga crassipalpis*, the peak level of *timeless* (*tim*) mRNA, but not that of *per* mRNA, was about two times higher under SD than under LD, but whether the level of *tim* gene expression is related to pupal diapause in this species is not clear [20]. In a mammal, the Syrian harmster, the peak level of *Per1* mRNA in the pars tuberalis of the pituitary (a site of the action of melatonin that conveys the photoperiodic signal) was about three times higher under LD than under SD [21]. In *P. apterus*, the most convincing evidence for involvement of the *per* and *Clk* genes in photoperiodism is their differential response to photoperiod in a mutant lacking diapause photoresponsiveness and a wild-type insect (fig. 1). Noteworthy is that most of the *per* and *Clk* mRNAs in the central nervous system of *P. apterus* are expressed in the pars intercerebralis of the brain (fig. 2) that is responsible for the translation of photoperiod into hormonal output and the storage of photoperiodic information [6, 18, 22, 23].

The effect of clock gene mutations on the photoperiodic response has been studied in two drosophilid species, *D. melanogaster* and *Chymomyza costata*. Saunders [3] concluded that the *per* gene is not causally involved in the photoperiodic induction of ovarian diapause in *D. melanogaster*: flies in which the *per* locus is missing (per^0) were still able to discriminate between diapause-inducing SD and diapause-averting LD, although the critical

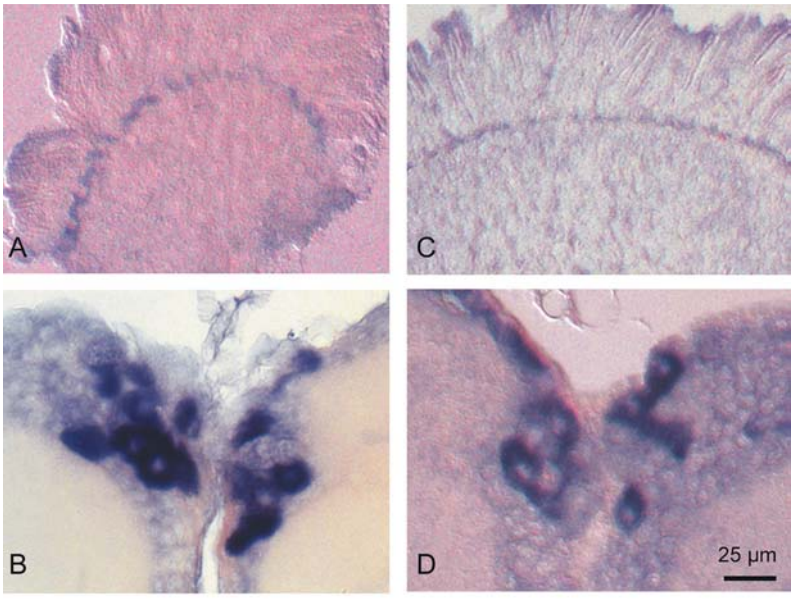

Figure 2. Identification of *period* (*A*, *B*) and *Clock* (*C*, *D*) mRNA by in situ hybridization in compound eyes (*A*, *C*) and pars intercerebralis (*B*, *D*) of the brain of *P. apterus*. A representative adult LD female aged 1 week.

daylength was altered. A shift in the peak of *per* and *tim* mRNA at different photoperiods has been reported in heads of non-diapausing adults of *D. melanogaster*, but no effect of photoperiod on the peak levels of the two transcripts has been mentioned [24]. In a mutant of *C. costata* that does not undergo diapause, the transcription of *tim* as well as the circadian rhythmicity in adult eclosion was suppressed, suggesting a role for the *tim* locus in both the circadian and photoperiodic time measurement [25]. The block to diapause photoresponsiveness in a mutant of *P. apterus* is not associated with defects in the circadian timing, but effects of photoperiod on the levels of *per* and *Clk* mRNAs, developmental mode and circadian properties appear to be connected (see above).

In contrast to the above organisms, in which photoperiod affects the amplitude and/or timing of the clock gene cycle, wild females of *P. apterus* show constantly enhanced levels of *per* and *Clk* mRNAs under SD (fig. 1). Daylength measurement itself is unlikely to result in a constantly high (or low) level of clock components. The difference in the levels of *per* and *Clk* gene expression between SD and LD suggests that these genes play no central role as photoperiodic clock components, but may be important in the output pathway (e.g. in a photoperiodic counter) to diapause/reproduction. This interpretation is in concert with the conclusion of a recent review that a 'photoperiodic clock role for the circadian system in insects and mites is highly unlikely' [15].

P. apterus is, to our knowledge, the first animal species in which a photoperiodic mutant has been used to demonstrate a link between photoperiod, the levels of clock gene transcripts and developmental outputs. Recently, flowering of *Arabidopsis* was shown to require a coincidence of a threshold level of *CONSTANS* (*CO*) gene, transcribed in a circadian way, with light. Mutants with reduced or elevated *CO* expression show early or late flowering, respectively, independent of daylength [26]. Saunders [27] stressed difficulties in providing a completely satisfactory explanation for the insect circadian clock and pointed out how much more difficult is unravelling the complexities of seasonality. Photoperiodic induction includes photoreception, measurements of night length, accumulation of successive long or short night signals by a 'counter', storage of such information, its transfer from a sensitive to a responsive stage and, finally, regulation of neuroendocrine activities controlling the onset of diapause or continuing development [27]. Furthermore, the process leading to the termination of diapause is not just a reversal of the induction of diapause. The results reported here demonstrate the potential for the level of *per* and *Clk* gene expression to participate in the translation of photoperiod into a developmental mode. The next major challenge is to discover the function of these and other clock-related genes and their products in the transmission cascade.

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1 Saunders D. S. (2002) Insect Clocks, 3rd edn, Elsevier, Amsterdam
2 Stanewski R. (2002) Clock mechanisms in *Drosophila*. Cell Tissue Res. **309:** 11–26
3 Saunders D. S. (1990) The circadian basis of ovarian diapause regulation in *Drosophila melanogaster*: is the period gene causally involved in photoperiodic time measurement ? J. Biol. Rhythms **5:** 315–331
4 Denlinger D. L. (2002) Regulation of diapause. Annu. Rev. Entomol. **47:** 93–122
5 Hodek I. (1968) Diapause in females of *Pyrrhocoris apterus* L. (Heteroptera). Acta Entomol. Bohemoslov. **65:** 422–435
6 Hodková M. (1976) Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*. Nature **263:** 521–523
7 Hodková M., Syrová Z., Doležel D. and Šauman I. (2003) *Period* gene expression in relation to seasonality and circadian rhythms in a heteropteran insect, *Pyrrhocoris apterus*. Eur. J. Entomol. **100:** 267–273
8 McDonald M. J. and Robash M. (2001) Microarray analysis and organization of circadian gene expression in *Drosophila.* Cell **107:** 567–578
9 Hodková M. and Socha R. (1995) Effect of temperature on photoperiodic response in a selected 'non-diapause' strain of *Pyrrhocoris apterus* (Heteroptera). Physiol. Entomol. **20:** 303–308
10 Socha R. and Hodková M. (1994) Selection for non-diapause in the heteropteran *Pyrrhocoris apterus*. Hereditas **120:** 81–85
11 Sauman I. and Reppert S. M. (1996) Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. Neuron **17:** 889–900
12 Hodek I. (1971) Termination of adult diapause in *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) in the field. Entomol. Exp. Appl. **14:** 212–222
13 Refinetti R. (2000) Circadian Physiology, CRC Press, Boca Raton
14 Saunders D. S. (1987) Insect photoperiodism: the linden bug, *Pyrrhocoris apterus*, a species that measures daylength rather than nightlength. Experientia **43:** 935–937
15 Veerman A. (2001) Photoperiodic time measurement in insects and mites: a critical evaluation of the oscillator-clock hypothesis. J. Insect Physiol. **47:** 1097–1109
16 Baylies M. K., Bargiello T. A., Jackson F. R. and Young M. W. (1987) Changes in abundance or structure of the *per* gene product can alter periodicity of the *Drosophila* clock. Nature **326:** 390–392
17 Steel C. G. H. and Vafoupoulou X. (2002) Physiology of circadian system. In: Insect Clocks, 3rd edn., pp. 115–188, Saunders D. S. (ed), Elsevier, Amsterdam
18 Hodková M., Okuda T. and Wagner R. M. (2001) Regulation of corpora allata in females of *Pyrrhocoris apterus* (Heteroptera). In Vitro Cell. Dev. Biol. Anim. **37:** 560–563
19 Hodková M. (1999) Regulation of diapause and reproduction in *Pyrrhocoris apterus* (L.) (Heteroptera) – neuroendocrine outputs. Entomol. Sci. **2:** 563–566
20 Goto S. G. and Denlinger D. L. (2002) Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*. J. Insect Physiol. **48:** 803–816
21 Messager S., Ross A.W., Barett P. and Morgan P. J. (1999) Decoding photoperiodic time through *Per1* and ICER gene amplitude. Proc. Natl. Acad. Sci. USA **96:** 9938–9943
22 Hodková M. (1979) Hormonal and nervous inhibition of reproduction by brain in diapausing females of *Pyrrhocoris apterus* L. (Hemiptera). Zool. Jahrb. Physiol. **83:** 126–136

23 Hodková M. (1992) Storage of the photoperiodic 'information' within the implanted neuroendocrine complex of the linden bug *Pyrrhocoris apterus* (L.) (Heteroptera). J. Insect Physiol. **38:** 357–363
24 Majercak J., Sidote D., Hardin P. E. and Edery I. (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron **24:** 219–230
25 Pavelka J., Shimada K. and Koštál V. (2003) Timeless: a link between fly's circadian and photoperiodic clocks ? Eur. J. Entomol. **100**: 255-265
26 Davis S. J. (2002) Photoperiodism: the coincidental perception of season. Curr. Biol. **12:** R841–R843
27 Saunders D. S. (2001) The blow fly *Calliphora vicina*: a 'clockwork' insect. In: Insect Timing: Circadian Rhythmicity to Seasonality, pp. 1–14, Denlinger D. L., Giebultowicz J. M. and Saunders D. S. (eds), Elsevier, Amsterdam