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Research Article

Endocrine-dependent expression of circadian clock genes in insects

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Abstract. Current models state that insect peripheral oscillators are directly responsive to light, while mammalian peripheral clock genes are coordinated by a master clock in the brain via intermediate factors, possibly hormonal. We show that the expression levels of two circadian clock genes, *period* (*per*) and *Par Domain Protein 1* (*Pdp1*) in the peripheral tissue of an insect model species, the linden bug *Pyrrhocoris apterus*, are inversely affected by contrasting photoperiods. The effect of photoperiod on *per* and Pdp1 mRNA levels was found to be mediated by the corpus allatum, an endocrine gland producing juvenile hormone. Our results provide the first experimental evidence for the effect of an endocrine gland on circadian clock gene expression in insects.

Keywords. *Period* gene, *Pdp1* gene, photoperiodism, corpus allatum, endocrine gland.

Introduction

In multicellular organisms, circadian clock genes are expressed not only in the central nervous system, but also in peripheral organs and tissues. In mammals, peripheral oscillators are not directly responsive to light, but are (in part) entrained by the central oscillator in the suprachiasmatic nucleus by still unidentified neuronal and hormonal mechanisms [1–3]. Several observations indicate that glucocorticoid signaling is one of these pathways [4, 5]. In contrast, the peripheral oscillators of insects can be directly entrained by light, independently of the brain clock and hormonal milieu [6–9]. In addition to entrainment of circadian rhythms, photoperiod also acts as an important seasonal cue, inducing adaptive changes in tissue physiology (*e.g.*, with respect to the diapause or reproduction) *via* the neuroendocrine pathway [10], but the question of whether seasonal changes in the hormonal output alter the expression of circadian clock genes in insect peripheral tissues has not yet been addressed. The reason for this lacuna may be the weakness and fragility of the reproductive diapause in *Drosophila melanogaster*, which makes this powerful genetic model species unsuitable for the study of photoperiodism [11–13].

The linden bug *Pyrrhocoris apterus* exhibits robust diapause response to photoperiod, whereby the corpus allatum (CA), an endocrine gland producing juvenile hormone (JH), plays a key role [14, 15]. The effect of JH on the fat body metabolism in adult females of *P. apterus* is well documented. Under diapause-preventing long days (LD), synthesis of vitellogenins by the fat body and their uptake by the developing oocytes are stimulated by JH [16]. Under

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diapause-inducing short days (SD), the CA is inhibited by a signal from the pars intercerebralis in the brain [14, 15]. In SD-females or LD-females deprived of their CA, JH is absent, the fat body synthesizes hexameric storage proteins and other metabolic reserves (instead of vitellogenins) and the ovaries remain undeveloped [16, 17]. During quantitative assessment of circadian-clock gene expressions, we found a remarkable effect of the photoperiod on *per* [18, 19] and *Pdp1* mRNA (unpublished data) levels in the head of *P. apterus*. Yet no differences were observed in the levels of additional circadian products encoded by *shaggy*, *double-time*, *casein kinase 2alpha* and *casein kinase 2beta* genes (data not shown). Therefore, we concentrated on the two transcripts, *period* (*per*) and *Par Domain Protein 1* (*Pdp1*) [20], and examined a potential effect of the CA on their expression in the fat bodies of LD- and SD-females of *P. apterus*.

Material and methods

Animals. Colonies of *P. apterus* (L.) (Heteroptera) were reared at 25±2°C and a diapause-preventing LD photoperiod of 18 h light/6 h darkness or diapause-promoting SD photoperiod of 12 h light/12 h darkness and supplied *ad libitum* with linden seeds and water. Adult females were used for all analyses. Females destined for surgical manipulations were deprived of food for 12 h after adult ecdysis and operated 2 days later. CA was removed through a neck membrane incision under Ringer insect saline. The neck membrane was cut in the sham-operated females. Females were given food immediately after operation. Abdominal fat bodies were dissected out under Ringer insect saline and immediately placed on dry ice, and stored at –85°C until analysis.

Molecular techniques. RNA from individual fat bodies was isolated with TRIzol (Sigma). Total RNA (0.1–1 μg) was used for cDNA synthesis (Superscript II, Invitrogen) with oligo dT_{24} primer. *per* and *Pdp1* PCR primers were designed to anneal to exon sequences separated by large (~1 kb) intron and conditions were optimized so that only the cDNA product (~200 bp for *per*; ~360 bp for *Pdp1*) was amplified. Primers: *per* forward: 5′-ACAGCTAGTGGTGGTGAAGAGG, *per* reverse: 5′-AAAAGTTGTTCAGTAAGAGCAGTAG, *Pdp1* forward: 5′-CTTAAGTTAGGGGCAGTAG, and *Pdp1* reverse: 5′-TCTTCATCAGAAAAGGCTCTTG. As a reference transcript we amplified ribosomal protein 49 (RP49) with forward primer designed to anneal specifically only to cDNA (intron position is marked with ^): 5′-CCGATATGTAAAACTGAGG^AGAAAC and reverse primer: 5′-GGAGCATGTGCCTGGTCTTTT. To reduce pipetting errors, 5 μl diluted cDNA was added to tube containing 15 μl PCR mastermix [final reaction concentrations: Ex Taq HS polymerase (Takara) 1.6 U/100 μl, Ex Taq buffer 1×, dNTPs 200 μM each, Syber green 1:25 000, primers 400 nM each]. PCRs for *per* and RP49 were done in separate tubes (20 μl/tube) in triplicates for each primer combination and each cDNA sample. Real-time PCR (Rotor-Gene 3000, Corbett research) started with an initial 5-min cDNA denaturation and enzyme activation (95°C), followed by 40–50 cycles each consisting of denaturation (94°C, 15 s), primer annealing (59°C, 30 s), extension and acquiring on Syber green channel (74°C, 40 s), followed by acquiring on Syber green channel at 80°C (15 s). Melting analysis was performed when all cycles were completed; PCR product sizes were verified by 2 % agarose gel electrophoresis. We always ran three to four reactions without cDNA (substituted with 5 μl H_2O) for each primer combination as a negative control. Data were analyzed and quantified with the Rotor-Gene 6 analysis software. Relative values were standardized to *RP49* and normalized to the sample with highest expression. GraphPad PRISM (Version 4) software was used for statistical analysis.

Results

Daily rhythms in *per* and *Pdp1* mRNA levels were not apparent, or exhibited only a very low amplitude, and ANOVA analysis did not reveal significant daily changes in gene expression, irrespective of photoperiod (Fig. 1). On the other hand, photoperiod had a remarkable effect ($p<0.0001$) on both *per* mRNA and *Pdp1* mRNA levels. Even more interesting was the fact that these two transcripts were inversely regulated by photoperiod. While *per* mRNA levels were higher in SD than in LD (Fig. 1A), *Pdp1* mRNA levels were lower in SD than in LD (Fig. 1B). Thus, day length seems to affect basal levels and/or amplitude of circadian clock gene transcripts rather than their cycling.

To determine whether the effect of photoperiod on *per* and *Pdp1* expression levels is mediated by the CA, we compared the levels of clock gene transcripts in fat bodies of LD- and SD-females deprived of this gland (Fig. 2). Extirpation of the CA from LD-females resulted in a clear change in the abundance of both transcripts: An increase ($p<0.05$) of *per* mRNA (Fig. 2A) and a decrease ($p<0.01$) of *Pdp1* mRNA (Fig. 2B). On the other hand, there was no significant difference in the level of either transcript between

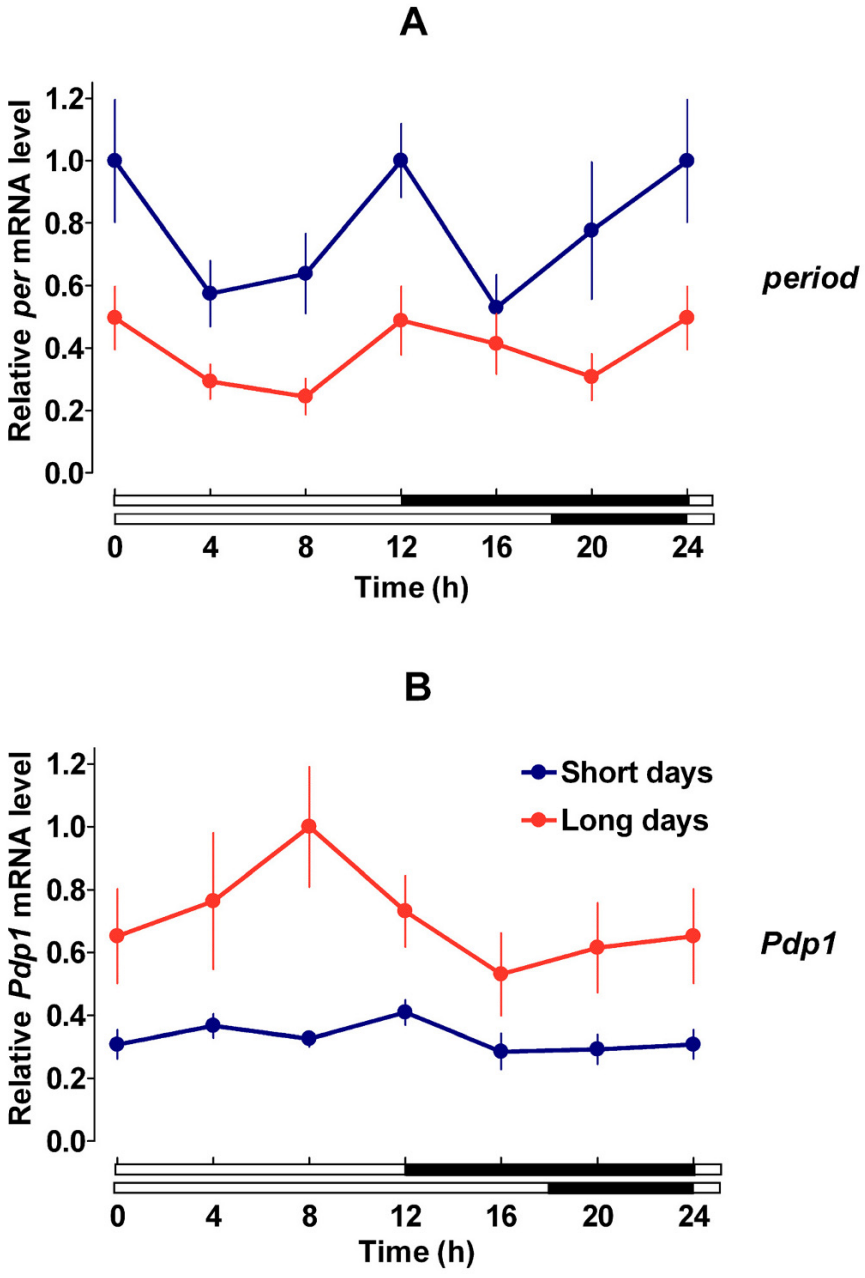

Figure 1. Effect of photoperiod on *per* (*A*) and *Pdp1* (*B*) expression in the fat body of *Pyrrhocoris apterus*. Gene expression was analyzed by quantitative real-time PCR and was plotted relative to *RP49*. Mean ± SEM of five females per data point are plotted for long day (LD) (red) and short day (SD) (blue). Fat bodies were dissected every 4 h (around the clock) out from females aged 1 week. The white and black indicate the light and dark phases, respectively. Two-way ANOVA analysis revealed a significant effect of time on *per* expression ($p<0.05$), but a non-significant effect of time on *Pdp1* expression. There was an extremely significant effect of photoperiod on the expression of both genes ($p<0.0001$). Photoperiod-time interactions were not significant for either gene. Subsequent one-way ANOVA analysis demonstrated that there are no significant time-dependent changes in *per* and *Pdp1* expression in either LD or SD.

allatectomized LD-females and any group of SD-females (Fig. 2). In contrast to LD-females, extirpation of the CA from SD-females had no significant effect on either *per* (Fig. 2B) or *Pdp1* mRNA (Fig. 2D) levels in the fat bodies. Given that the CA is active at LD but not at SD [14, 15], the data strongly suggest that the reciprocal responses of *per* and *Pdp1* to photoperiod are mediated by hormonal signals from the CA.

Discussion

We tested the effect of ablating the CA (the source of JH) on the expression levels of two circadian clock genes in the fat body of *P. apterus* held under two contrasting photoperiods. The most important finding is that the absence of an active CA in LD-females has strong reciprocal effects on the expression levels of *per* and *Pdp1* (Fig. 2A, C). This effect is not attributable to a non-specific response of circadian clock genes to surgical intervention, because the absence of an inactive CA in SD-females has no significant effect on transcript levels (Fig. 2B, D). Prothoracicotropic

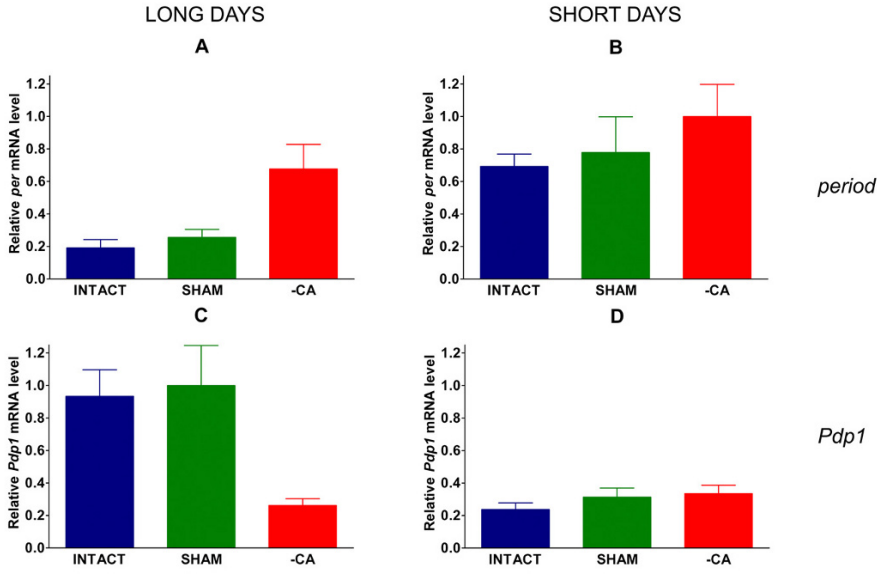

Figure 2. Effects of allatectomy on *per* (*A, B*) and *Pdp1* (*C, D*) expression in the fat body of *Pyrrhocoris apterus* held at long days (*A, C*) or short days (*B, D*). Gene expression was analyzed by quantitative real-time PCR and was plotted relative to *RP49*. Mean ± SEM of seven to nine animals per column are plotted for intact, sham-operated (SHAM) and allatectomized (-CA) females. Fat bodies were dissected out 1 week after operation at Zeitgeber time 7–10. One-way ANOVA and Tukey's post test demonstrated a significant effect of allatectomy on both *per* ($p<0.05$) and *Pdp1* ($p<0.01$) expression in LD-females. There was no significant difference in the expression of either gene between allatectomized LD-females and any group of SD-females or among groups of SD-females.

hormone, ecdysteroids, ecdysteroid receptors and JHs exhibit circadian rhythms, and are expected to drive rhythms in tissue-specific activities [21, 22], but until now these hormones were not shown to affect the expression of circadian clock genes.

Although the results reported here suggest that the expression levels of two circadian clock genes are strongly affected by the CA, the challenge of identifying the involved molecular pathway remains. A definitive demonstration of this pathway will require, for example, identification of JH receptors [23] and chemical structure of the JH in Heteroptera [24]. The functional significance of the observed effect of the CA on the magnitude of clock gene expression is not clear either. Setting the phase of peripheral oscillators, a role suggested for glucocorticoid signaling in mammals [4, 5], is probably not the function of JH, given the absence of significant daily changes in clock gene transcripts and differences between their basal levels in LD and SD (Fig. 1). Although it is possible that the resulting clock proteins cycle despite the lack of their respective transcripts oscillations, quantitative Western blots with available antibodies did not generate any conclusive results (unpublished data). The observed reciprocal regulation of *per* and *Pdp1* points to a different activity of transcription factors responsible for the expression of these genes and may reflect changes in the expression of other genes related to diapause or reproduction physiology. A recent study of mouse shows that, in response to physical stress, glucocorticoid signaling triggers transient induction of *Per1* transcription *via* a glucocorticoid-responsive element in its promoter without resetting of the circadian time of the molecular rhythm, thus indicating a non-clock function of *Per1* transcription in mammalian peripheral tissues [25]. In response to photoperiod, CA hormones may have an analogous role in a non-clock function of *per* and *Pdp1* transcripts in *P. apterus* fat body. This view is supported by "abnormal behavior" of clock molecules observed in other species, including non-rhythmic levels of *per* mRNA [26] and proteins [27] in the follicle cells of ovaries of adult *D. melanogaster*. It is noteworthy that follicle cells, similar to the fat body, are important targets of JH [28]. A possibility of the functional versatility of at least a fraction of clock molecules has been discussed previously [29].

Distinct daily patterns of *per* and / or *timeless* under different photoperiodic conditions have been recorded in two dipteran species, *D. melanogaster* [30–32] and the flesh fly, *Sarcophaga crassipalpis* [33], and in the silk moth, *Bombyx mori* [34]. However, it is not known whether these patterns depend on endocrine glands. Interestingly, photoperiod modulates the expression of *Per1* in peripheral tissues of a seasonal mammal, the Syrian hamster, with a short-duration, low-amplitude rhythm in SD and a prolonged, high-amplitude rhythm in LD [35]. Furthermore, this *Per1*

rhythm reverts to an LD-like, high-amplitude rhythm, in SD-refractory hamsters, thus reflecting LD-like physiology irrespective of SD-photoperiod and implicating unknown intermediate factor(s) not directly driven by the central oscillator in the suprachiasmatic nucleus [35]. In LD-allatectomized females of *P. apterus*, *per* and *Pdp1* mRNA levels assume SD-like characteristics, thus reflecting SD-like hormonal milieu (without CA hormones) rather than the ambient (LD) photoperiod (Fig. 2). Hypothetically, different amplitudes of clock gene rhythmicities in hamster and basal levels of clock molecules in *P. apterus* may have similar roles in endocrine regulation of tissue physiology. Whatever the explanation, if we are to gain a full understanding of the biological function of circadian clock genes in peripheral tissues, we must take into account not only their roles in daily changes of tissue metabolism, but also their roles in metabolic switches. Here the question is not when within the 24-h cycle an event is to occur, but instead which type of event (adaptation) is to occur.

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References

1 Yamazaki S., Numano R., Abe M., Hida A., Takahashi R., Ueda M., Block G. D., Sakaki Y., Menaker M. and Hajime, T. (2000) Resetting central and peripheral circadian oscillators in transgenic rats. Science 288, 682–685.
2 Reppert S. M. and Weaver D. R. (2002) Coordination of circadian timing in mammals. Nature 418, 935–941.
3 Schibler U. and Sassone-Corsi P. (2002) A web of circadian pacemakers. Cell 111, 919–922.
4 Balsalobre A., Brown S. A., Marcacci F. T., Tronche F., Kelledonk C., Reichardt H. M., Schütz G. and Schibler U. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289, 2344–2347.
5 Le Minh N., Damiola F., Tronche F., Schutz G. and Schibler U. (2001) Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. EMBO J. 20, 7128–7136.
6 Plautz J. D., Kaneko M., Hall, J. C. and Kay S. A. (1997) Independent photoreceptive circadian clocks throughout *Drosophila.* Science 278,1632–1635 (1997).
7 Giebultowicz J. M. (1999) Insect circadian clocks: Is it all in their heads? J. Insect Physiol. 45, 791–800.
8 Giebultowicz J. M., Ivanchenko M. and Vollintine T. (2001) Organization of the insect circadian system: Spatial and developmental expression of clock genes in peripheral tissues of *Drosophila melanogaster.* In: Insect Timing: Circadian Rhythmicity to Seasonality, pp. 31–42, Denlinger D. L., Giebultowicz, J. and Saunders D. S. (eds.), Elsevier Science B. V., Amsterdam.
9 Tanoue S., Krishnan P., Krishnan B., Dryer S. E. and Hardin P. E. (2004) Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila.* Curr. Biol. 14, 638–649.
10 Saunders, D. S. (2002) Insect Clocks, 3rd edn., Elsevier, Amsterdam.
11 Saunders D. S., Henrich V. C. and Gilbert L. I. (1989) Induction of diapause in *Drosophila melanogaster* – Photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. Proc. Natl. Acad. Sci. USA 86, 3748–3752.
12 Denlinger D. L. (2002) Regulation of diapause. Annu. Rev. Entomol. 47, 93–122.
13 Tauber E. and Kyriacou P. (2001) Insect photoperiodism and circadian clocks: Models and mechanisms. J. Biol. Rhythms 16, 381–390.
14 Hodkova M. (1976) Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus.* Nature 263, 521–523.
15 Hodkova M., Okuda T. and Wagner R. M. (2001) Regulation of corpora allata in females of *Pyrrhocoris apterus* (Heteroptera) (a mini-review). *In vitro* Cell. Dev. Biol. Anim. 37, 560–563.
16 Socha R., Sula J., Kodrik D. and Gelbic I. (1991) Hormonal control of vitellogenin synthesis in *Pyrrhocoris apterus* (L.) (Heteroptera). J. Insect Physiol. 37, 805–816.
17 Sula J., Kodrik D. and Socha R. (1995) Hexameric haemolymph protein related to adult diapause in the red firebug, *Pyrrhocoris apterus* (L.) (Heteroptera). J. Insect Physiol. 41, 793–800.
18 Syrova Z., Dolezel D., Sauman I. and Hodkova M. (2003) Photoperiodic regulation of diapause in linden bugs: Are period and Clock genes involved? Cell. Mol. Life Sci. 60, 2510–2515.
19 Dolezel D., Sauman I., Kostal V. and Hodkova M. (2007) Photoperiodic and food signals control expression pattern of the clock gene, period, in the linden bug, *Pyrrhocoris apterus.* J. Biol. Rhythms 22, 335–342.
20 Cyran S. A., Buchsbaum A. M., Reddy K. L., Lin M. C., Glossop N. R., Hardin P. E., Young M. W., Storti R. V. and Blau J. (2003) *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell 112, 329–341.
21 Steel C. G. H. and Vafopoulou X. (2002) Physiology of circadian systems. In: Insect Clocks, 3rd edn., pp. 115–188, Saunders D. S. (ed.), Elsevier, Amsterdam.
22 Vafoupoulou X. and Steel C. G. H. (2006) Ecdysteroid hormone nuclear receptor (EcR) exhibits circadian cycling in certain tissues, but not others, during development in *Rhodnius prolixus* (Hemiptera). Cell Tissue Res. 323, 443–455.
23 Flatt T., Moroz L. L., Tatar M. and Heyland A. (2006) Comparing thyroid and insect hormone signaling. Integr. Comp. Biol. 46, 777–794.
24 Davey K. G. (2000) The modes of action of juvenile hormones: Some questions we ought to ask. Insect Biochem. Mol. Biol. 30, 663–669.
25 Yamamoto T., Nakahata Y., Tanaka M., Yoshida M., Soma H., Shinohara K., Yasuda A., Mamine T. and Takumi T. (2005) Acute physical stress elevates mouse *Period1* mRNA expression in mouse peripheral tissues *via* a glucocorticoid-responsive element. J. Biol. Chem. 280, 42036–42043.
26 Hardin P. E. (1994) Analysis of *period* mRNA cycling in *Drosophila* head and body tissues indicates that body oscillators behave differently from head oscillators. Mol. Cell. Biol. 14, 7211–7218.
27 Beaver L. M., Rush B. L., Gvakharia B. O. and Giebultowicz J. M. (2003) Noncircadian regulation and function of clock genes *period* and *timeless* in oogenesis of *Drosophila melanogaster.* J. Biol. Rhythms 18, 463–472.
28 Wyatt G. R. and Davey K. G. (1996) Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. Adv. Insect Physiol. 26, 1–155.
29 Hall J. C. (1996) Are cycling gene products as internal zeitgebers no longer the zeitgeist of chronobiology? Neuron 17, 799–802.
30 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.

31 Collins B. H., Rosato E. and Kyriacou C. P. (2004) Seasonal behavior in *Drosophila melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C. Proc. Natl. Acad. Sci. USA 101, 93–122.
32 Shafer O. T., Levine R. D. and Warman G. R. (2004) Flies by night: Effects of changing day length in *Drosophila*'s circadian clock. Curr. Biol. 14, 424–432.
33 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.
34 Iwai S., Fukui Y., Fujiwara Y. and Takeda M. (2006) Structure and expressions of two circadian clock genes, *period* and *timeless* in the commercial silkmoth, *Bombyx mori.* J. Insect Physiol. 52, 625–637.
35 Carr A.-J. F., Johnston J. D., Semikhodski A. G., Nolan T., Cagampang F. R. A., Stirland J. A. and Loudon S. I. (2003) Photoperiod differentially regulates circadian oscillators in central and peripheral tissues of the Syrian hamster. Curr. Biol. 13, 1543–1548.