REVIEW ARTICLE

Comparative analysis of circadian clock genes in insects

F. Sandrelli*, R. Costa*, C. P. Kyriacou† and E. Rosato†

**Department of Biology, University of Padova, Padova 35131, Italy; †Department of Genetics, University of Leicester, Leicester, LE1 7RH UK*

Summary

After a slow start, the comparative analysis of clock genes in insects has developed into a mature area of study in recent years. Brain transplant or surgical interventions in larger insects defined much of the early work in this area, before the cloning of clock genes became possible. We discuss the evolution of clock genes, their key sequence differences, and their likely modes of regulation in several different insect orders. We also present their expression patterns in the brain, focusing particularly on Diptera, Lepidoptera, and Orthoptera, the most common non-genetic model insects studied. We also highlight the adaptive involvement of clock molecules in other complex phenotypes which require biological timing, such as social behaviour, diapause and migration.

Keywords: circadian, genes, insects, expression, comparative.

Introduction

Circadian rhythms are important regulatory processes, which, almost universally, are used to harmonise physiology and behaviour with the 24 h geophysical cycles of light and temperature of our planet. The clock therefore is at the interface between the organism and the external environment and, as for other signalling pathways, it probes the outside world, transducing external changes to downstream effectors. In the last 25 years or so, the phenomenology which contributed to most of the circadian literature before about 1984, was replaced by the more pragmatic molecular genetic analysis of the clock, initially in *Drosophila* (Hall, 2003). At the cellular level, molecules which exemplify the

progression of time are cyclically transformed. Phosphorylation, degradation and altered subcellular localisation of clock proteins, result in modified expression of clock and clock controlled genes, ultimately coordinating the activities of the organism with its environment (aka 'entrainment') (Hall, 2003; Zheng & Sehgal, 2008). The same chain of events persists, with minor differences, also under constant 'free-running' conditions, suggesting that the internal milieu functions as a self-sustaining oscillation that continues in the absence of any periodic input. Such a design is a common feature of all circadian clocks (Dunlap, 1999) and it underlines the mechanistic constraints encountered (more than once) in the evolution of circadian systems.

The identification of the mammalian genes *Clock* (Antoch *et al.*, 1997; King *et al.*, 1997b) and *Period* (Sun *et al.*, 1997; Tei *et al.*, 1997) revealed that vertebrate clocks have a common evolutionary origin with that of insects, although some interesting differences have also emerged (Reppert & Weaver, 2000; Yu & Hardin, 2006). This evolutionary plasticity is observed between insect species and even within-species (Costa & Kyriacou, 1998; Helfrich-Forster, 2005c; Yuan *et al.*, 2007; Kyriacou *et al.*, 2008). Our review will focus on recent developments in the identity of functional molecular and neuronal components of the clock in insects.

A molecular model for the clock

The cloning of the *Drosophila melanogaster period* (*per*) gene paved the way for the modern molecular dissection of the circadian system (Hall, 2003). However, it also biased the way we think about the functioning and the evolution of the circadian clock. For many years *Drosophila* was the only tractable system among animals (in terms of genetics and molecular biology of the clock) until the identification of the mammalian clock genes in 1997 (Antoch *et al.*, 1997; King *et al.*, 1997a,b; Sun *et al.*, 1997; Tei *et al.*, 1997). At first glance the clocks of flies and mice are well conserved, the main molecular components are the same and there is only some variation on the general theme. However, these differences are intriguing, in particular the apparent swapping of the starring roles of key components (Clayton *et al.*, 2001). At first this seemed to correlate with the phylogenetic distance between flies and mice, but in fact, some of the
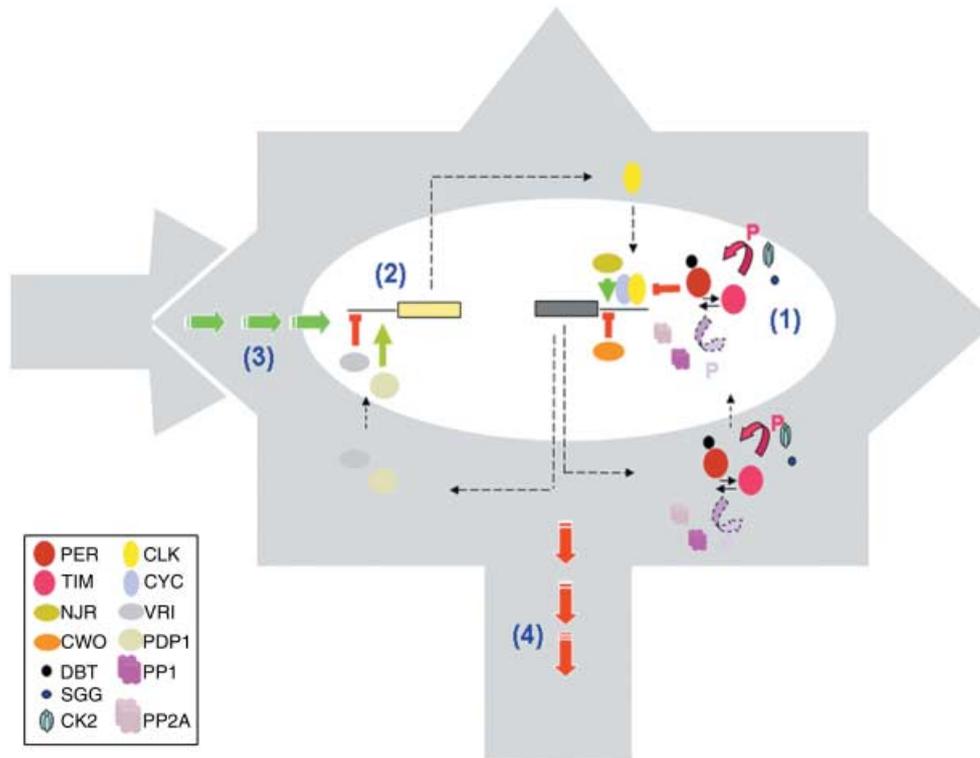
Figure 1. The *Drosophila* molecular clock within a clock neuron. (1) The first negative feedback loop revolves around the cytoplasmic regulation of PER and TIM. Formation of a dynamic complex between the two molecules regulates phosphorylation/dephosphorylation by kinases [DBT, SGG, CK2] and phosphatases [PP1 and PP2A] and nuclear translocation and accumulation. In the nucleus PER inhibit CLK perhaps by facilitating its phosphorylation by DBT. CLK together with CYC binds E-box sequences on the promoter of clock and clock controlled genes. NJR helps transcriptional activation whereas CWO likely contributes to CLK/CYC repression. (2) The second loop is less well understood. VRI and PDP1ε are expressed under control of CLK. They accumulate with different kinetics and then feed back by repressing (VRI) and activating (PDP1ε) *Clk* transcription. Other factors likely contribute to this loop. (3) Post-synaptic pathways also mediate the regulation of the clock with modalities that are still unknown. (4) The clock generates rhythmic signals that are passed on by the neuron to downstream effectors. *In this diagram some factors have not been included for the sake of simplicity.*

mouse-like features of these genes are already evident within the insects (Zhu *et al.*, 2005; Yuan *et al.*, 2007).

The *Drosophila* model

Traditionally the fly clock is described as an ensemble of interlocked negative transcription/translation feedback loops (Hall, 2003). In each loop positive elements drive the transcription of negative elements that rhythmically feed back to inhibit the action of the former. However, recent work suggests that post-translational modifications of clock proteins are more important than originally thought and they might provide the fulcrum for the whole machinery (Zheng & Sehgal, 2008). This new wave of thinking has not produced, as yet, a better 'model', thus we will describe the clock in the canonical fashion.

At the core of the clock are the transcriptional activators CLOCK (CLK) and CYCLE (CYC), that, as heterodimers, bind to E-box sequences on the promoters of *period* and *timeless* (*tim*) initiating their transcription (Hall, 2003). CLK, CYC and PER have a sequence similarity in that they all contain a

PAS domain, a dimerizing region that is found in an enormous family of proteins, many of which are involved in environmental sensing (Gu *et al.*, 2000). Seemingly, CLK/CYC are facilitated in their function by NEJIRE (NEJ), the *Drosophila* orthologue of the CBP/p300 family of transcription co-activators, although an alternative scenario has also been suggested (Hung *et al.*, 2007; Lim *et al.*, 2007a) (see Fig. 1).

After translation PER and TIM interact with each other (via the PER-PAS domain, among others) and with a number of kinases and phosphatases (see below) that regulate stability, timing of nuclear entry and accumulation, and their ability to interact and repress the CLK/CYC dimer (Zheng & Sehgal, 2008). As a consequence, abundance, phosphorylation levels, nuclear/cytoplasmic ratio and the repressor function of these two proteins cycle in synchrony, reaching a peak at the end of the night (Zheng & Sehgal, 2008). Although the details are still unclear, the formation of a complex between PER and TIM is a dynamic process, as exemplified by the fact that the two proteins can accumulate in the nucleus with different kinetics, but still require each other in order to do so (Shafer *et al.*, 2002; Meyer *et al.*, 2006).

As mentioned above the complex also involves kinases, such as DOUBLETIME (DBT), SHAGGY (SGG) and CASEIN KINASE II (CKII), and phosphatases, such as protein phosphatases 1 (PP1) and 2A (PP2A), that phosphorylate/dephosphorylate PER and/or TIM and, once transported inside the nucleus, also CLK (Kloss *et al.*, 1998; Price *et al.*, 1998; Martinek *et al.*, 2001; Lin *et al.*, 2002a; 2005; Akten *et al.*, 2003; Sathyanarayanan *et al.*, 2004; Kim & Edery, 2006; Yu *et al.*, 2006; Fang *et al.*, 2007). Unlike PER and TIM, CLK levels are constant throughout the 24 h but there are PER-dependent rhythmic changes in its phosphorylation status that have implications for its transcriptional activity (Houl *et al.*, 2006; Kim & Edery, 2006; Yu *et al.*, 2006). PER likely facilitates CLK phosphorylation by DBT, resulting in diminished CLK/CYC affinity for DNA (Yu *et al.*, 2006). This observation might explain why TIM is not an efficient repressor of CLK/CYC whereas PER, once in the nucleus, is a more active repressor when alone (Rothenfluh *et al.*, 2000).

CYC is also a constitutively expressed protein (Rutila *et al.*, 1998) hence it is particularly important that several mechanisms intervene to impart rhythmicity to CLK/CYC activity. Another transcriptional regulator CLOCKWORK ORANGE (CWO) contributes to the inhibition of CLK/CYC perhaps by facilitating changes in chromatin structure (Kadener *et al.*, 2007; Lim *et al.*, 2007b; Matsumoto *et al.*, 2007). However a more complicated mode of action has also been suggested, where CWO functions as a transcriptional activator at the beginning of the night, when PER is low and cytoplasmic, and as a repressor in the late night-early morning, when PER levels are high and nuclear (Richier *et al.*, 2008).

Another feedback loop is centered around the rhythmic expression of *Clk*, although its practical contribution to the functioning of the whole system is at present unclear. Initially CLK/CYC dimers bind to E-boxes on the promoters of *PAR domain protein 1ε* (*Pdp1ε*) and *vrille* (*vri*), driving their robust rhythmic transcription. PDP1ε and VRI are transcriptional regulators that cycle also at the protein level, but with different phases. VRI peaks before PDP1ε, outcompeting the latter for the same binding sites on the *Clk* promoter and causing repression of *Clk* transcription. A few hours later increased PDP1ε displaces VRI and promotes *Clk* mRNA production (Blau & Young, 1999; Cyran *et al.*, 2003; Glossop *et al.*, 2003). However, as mis-expression of *Pdp1ε* in clock cells does not halt the cycling of *Clk* mRNA (Benito *et al.*, 2007), this loop is probably more complex than originally thought, perhaps requiring additional, but as yet unknown elements.

Finally, light-resetting of the clock is mediated via the canonical visual transduction system, as well as a blue-light sensitive protein, CRYPTOCHROME (CRY) (Helfrich-Forster *et al.*, 2001). CRY is particularly important in that it also acts as a light gateway into the clock because, unlike

wild-type, flies carrying a nearly null mutation, *cry^b*, are rhythmic in constant light (LL), even though their visual system is intact (Emery *et al.*, 2000).

Various *cry* mutant genotypes also show reduced circadian responses to brief light pulses (Emery *et al.*, 1998; Stanewsky *et al.*, 1998; Rosato *et al.*, 2001; Dissel *et al.*, 2004). One of the modes of action of CRY is to mediate the light-degradation of TIM through the E3 ligase protein, JETLAG (JET) (Koh *et al.*, 2006; Peschel *et al.*, 2006). The degradation of TIM exposes PER to the kinase DBT, with repercussions for PER stability and the feedback loop (Young, 1998). However, not all TIM containing neurons express CRY at quantifiable levels, yet TIM is nevertheless rapidly degraded by light also in those cells (Picot *et al.*, 2007; Yoshii *et al.*, 2008). This suggests that communication among neurons might be the most important factor for the light-degradation of TIM and, in general, for entrainment (Nitabach *et al.*, 2002; Peng *et al.*, 2003), so that one of the functions of CRY may be to mediate cross-talk among clock cells. In this regard, CRY can be found in the axonal projections of the neurons in which it is expressed (Klarsfeld *et al.*, 2004; Yoshii *et al.*, 2008). While this hypothesis has yet to be verified, a recent knock-out of the gene suggests a dark function for CRY, highlighting its multiple roles in the *Drosophila* clock (Dolezelova *et al.*, 2007).

Differences between *Drosophila* and mammalian clocks

Although in this review we focus on the clock of insects, it is convenient to refer briefly to the distinctive features of the mammalian clock. This is because mammalian-like clock characteristics are found in insects other than *Drosophila*, suggesting that both flies and mammals specialised by diverging from a common design. There are several differences in the architecture of the mammalian and fly clocks, the most obvious being that in mammals, most clock components are present in multiple copies, increasing the complexity and the redundancy of the system (Clayton *et al.*, 2001). The mammalian CRY proteins (CRY1 and CRY2) substitute for *Drosophila* TIM as the partners of PER proteins. CRY1 and CRY2 do not show circadian light responsiveness, but instead, they act as the main transcriptional repressors. *Bmal1* (the homologue of *Drosophila cyc*) substitutes for *Clk* as the rhythmic component of the second loop and contains the main transactivation domain of the CLK/BMAL1 complex (Reppert & Weaver, 2000; Yu & Hardin, 2006), which in *Drosophila* is carried by CLK (Allada *et al.*, 1998; Rutila *et al.*, 1998). Moreover, mammalian *Tim* (*mTim*) actually corresponds to the fly paralogue *timeout/tim2* (Benna *et al.*, 2000; Gotter *et al.*, 2000). Although both the fly (F. Sandrelli and R. Costa unpublished observations) and the mammalian (Barnes *et al.*, 2003; Unsal-Kacmaz *et al.*, 2005) *timeout/tim2* genes seem to be

	CRY-m	CRY-d	TIM-m	TIM-d	BTR	CPQ
Mus musculus	✓	✗	✓	✗	✓	✓
Apis mellifera	✓	✗	✓	✗	✓	✓
Tribolium castaneum	✓	✗	✓	✓	?	?
Antheraea pernyi	✓	✓	?	✓	✓	✗
Bombyx mori	✓	✓	✓	✓	✓	✗
Danaus plexippus	✓	✓	?	✓	?	?
Anopheles gambiae	✓	✓	✓	✓	✓	✓
Drosophila melanogaster	✗	✓	✓	✓	✗	☑

Figure 2. *Drosophila*-like (-d) and mammalian-like (-m) clock components in representative insects. Insect orders: Hymenoptera, Coleoptera, Lepidoptera, Diptera. *Mus musculus* (mouse) is the outgroup. BTR = BMAL1 transactivation domain. CPQ = CLOCK poly-Q repeats. ? = unknown. ☑ = extensive poly-Q repeats from (Chang & Reppert, 2003; Zhu *et al.*, 2005, 2008b; Rubin *et al.*, 2006; Yuan *et al.*, 2007).

involved with the circadian clock, at present their role is not completely clear. For the interested reader, several recent reviews offer a more detailed description of the mammalian clock (Ko & Takahashi, 2006; Levi & Schibler, 2007; Maywood *et al.*, 2007).

The ancestral clock of insects

Clock genes sequences are available for several non-Drosophilid insect species. Figure 2 shows that in all Lepidoptera analysed there are two *cry* genes, one sensitive to light, hence *Drosophila*-like (*cry*-d), the other able to repress CLK/CYC mediated transcription *in vitro* as in mammals (*cry*-m) (Yuan *et al.*, 2007). However, mammalian-like *cry* genes are also found in the honeybee *Apis mellifera* and in the flour beetle *Tribolium castaneum*, both curiously lacking a copy of *cry*-d, whereas both types of *cry's* are found instead in the mosquitos *Anopheles gambiae* and *Aedes aegypti* (Zhu *et al.*, 2005; Rubin *et al.*, 2006; Yuan *et al.*, 2007).

The mammalian-like TIM is an essential gene both in mouse (Gotter *et al.*, 2000) and in *Drosophila* (F. Sandrelli and R. Costa, unpublished observations), and unlike its paralogue, fly *tim*, it has been identified in every animal that has been studied. Orthologues to fly *tim* are almost ubiquitous among insects, with the exception of the honeybee (Rubin *et al.*, 2006) and so *tim* would appear to be the result of a more recent duplication of the ancestral essential gene *timeout/tim2*, even though its presence in sea urchins

dates the duplication event to pre-Cambrian times (Rubin *et al.*, 2006).

Finally the main transactivation domain of the CLK/CYC(BMAL1) complex is generally found at the C-terminus of BMAL1 type proteins, whereas the poly-Q repeats found in CLK only serve an ancillary function in transcriptional activation. However, *Drosophila* CYC is shorter and lacks the transactivation domain and conversely, the poly-Q repeats on CLK are expanded and provide the activation domain necessary for the functioning of the complex (Chang *et al.*, 2003; Rubin *et al.*, 2006). In conclusion, the ancestral clock of insects seemingly consisted of two CRYs (-m and -d), two TIMs (-m and -d) and has CYC as the main transcriptional activator. In *Drosophila*, loss of *cry*-m (although a transcriptional repressor function might persist in CRY-d, – Collins *et al.*, 2006), perhaps favoured by the loss of the CYC C-terminus and the rise of CLK as the dominant element of the complex, has brought TIM-d to acquire an important role in the main feedback loop and boosted the relevance of PER as a transcriptional repressor.

Anatomy of the clock in *Drosophila*

The anatomy of the clock is based predominantly on staining with anti-PER and anti-TIM antibodies. There are six main clusters of brain neurons, that are divided into three groups of lateral and three groups of dorsal neurons (Hall, 2003) (see Fig. 3). The lateral neurons (LNs) are further subdivided into dorsal (1 cluster of ~6 cells, LNd),
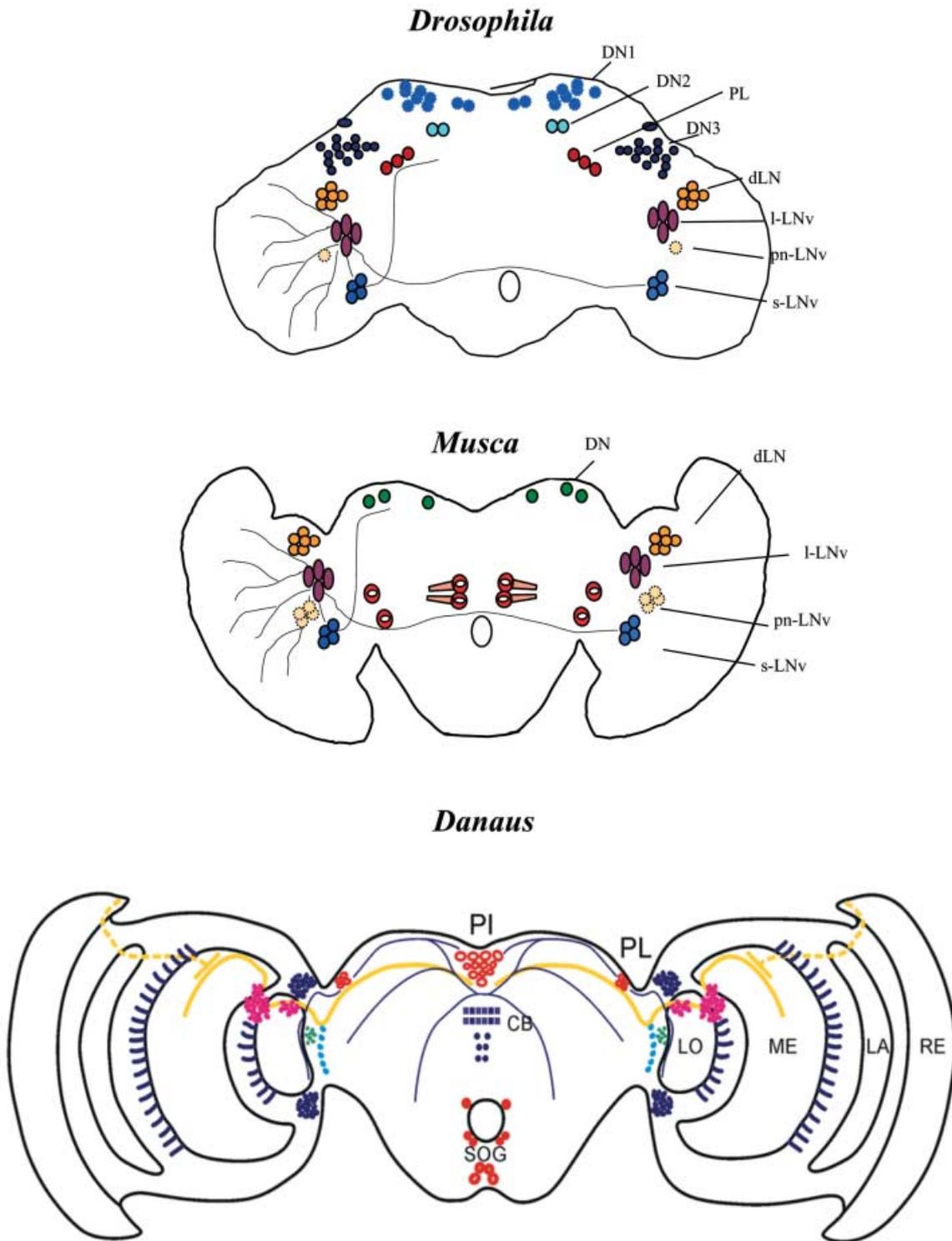
Figure 3. Schematic representation of clock molecule expression in brains of Diptera and Lepidoptera. Top panel. *Drosophila melanogaster*; middle panel, *Musca domestica*. Neurons expressing PER in *Musca* and *Drosophila* brains. Key: DN1, DN2 and DN3, dorsal neurons; PLNs, posterior lateral neurons; LNds, lateral neurons dorsal; s-LNvs, small lateral neurons ventral; l-LNvs, large lateral neurons ventral; Pn-LNv, PDF-null lateral neuron ventral; MNs, medial neurons; MLNs, medio-lateral neurons (Codd *et al.*, 2007). Lower panel. *Danaus plexippus*. Regions expressing TIM, PER, CRY1 and/or CRY2 are highlighted in red. In these areas the four clock proteins are partially colocalized. Areas expressing TIM or CRY1 are indicated in magenta. In these regions the two clock proteins do not colocalise. CRY1 positive fibers are represented by continuous yellow lines. Projections of the dorsal rim area photoreceptors are indicated by dotted yellow lines. Neurons and fibres expressing CRY2 exclusively are represented in blue. Areas staining exclusively for TIM and PER are indicated in green and light blue, respectively. PL, Pars lateralis; PI, Pars intercerebralis; SOG, Subesophageal ganglion; CB, central body; LO, lobula; ME, medulla; LA, lamina; RE, retina (Modified from Sauman *et al.*, 2005; Reppert, 2006; Zhu *et al.*, 2008b).

and ventral (LNv) with the latter also further differentiated into four large (l-LNv) and four small (s-LNv) cells. The s-LNv and l-LNv neurons express the neuropeptide, pigment dispersing factor (PDF) (Helfrich-Forster & Homberg, 1993). The more posterior and dorsal brain neurons (DNs), are subdivided into DN1 (~16 cells), DN2 (2 cells) and DN3 (~40 cells in a very dorso-lateral position, Kaneko & Hall, 2000; Helfrich-Forster, 2005a; Helfrich-Forster *et al.*, 2007) (see Fig. 3).

This general clock neuron classification, which is based on anatomical criteria, although useful, hides some further complexity. For example, among the LNv group there is an additional single small neuron, which is usually found very close to the l-LNvs but does not express PDF (Kaneko & Hall, 2000). This pdf-null LNv (pn-LNv), unlike the l-LNvs, but as in the s-LNvs, is already present in the larva, and possibly represents a remnant of larger cluster of clock cells, that is observed in other diptera, such as *Musca domestica* (Codd *et al.*, 2007). Furthermore only two out of the ~16 neurons clustered in the DN1 group are found in the larva, and only those two do not express the transcription factor GLASS (Shafer *et al.*, 2006) which generally directs the differentiation of opsin-based photoreceptors (Moses *et al.*, 1989). Finally, in the adult there is a further cluster consisting of three lateral posterior neurons (LPNs) which express high levels of TIM (Kaneko & Hall, 2000) and only low levels of PER (Helfrich-Forster, 2005a,b; Shafer *et al.*, 2006; Helfrich-Forster *et al.*, 2007), and seem to be involved in temperature entrainment (Miyasako *et al.*, 2007). More information on the physiological differences among those neurons and their role in the circadian network of the fly, can be found in a recent review (Nitabach & Taghert, 2008).

Functional analysis of clock genes in other fruit flies

The basic circadian plan for *Drosophila* has also been examined in other species of fruitflies. Probably the most comprehensive of these studies has been carried out in the sibling species of the Queensland fruitfly, *Bactrocera tryoni* and *Bactrocera neohumeralis.* These species show a temporal separation by time of mating, in that the former mate at dusk whereas the latter prefer to mate during the brighter parts of the day (Tychsen & Fletcher, 1971; Smith, 1979). Whereas the *per* orthologues of both species show a similar transcript cycle to that of their *Drosophila* cousins any *Cry* mRNA cycle appears to be severely dampened in *Bactrocera* heads (An *et al.*, 2002, 2004). However dissected out brains or antennae reveal significantly high levels of mid-daytime *Cry* mRNA compared to middle of the night, suggesting a cycle. In both tissues, *B. tryoni* have significantly less *Cry* mRNA than *B. neohumeralis*, and this is also reflected in corresponding hybrids which have been selected for dusk (*tryoni*-like) vs. day (*neohumeralis*-like)

mating, revealing a putative association between *Cry* levels and temporal sexual isolation (An *et al.*, 2004). *In situ* hybridisation revealed *Cry* transcripts in lateral and some dorsal cells. In addition, using a *Drosophila* reagent, anti-PER staining was detected in lateral cells, plus weaker staining in dorsal areas (An *et al.*, 2004).

The relationship of *Cry* levels to possible day vs. dusk mating is particularly interesting given CRY's circadian light function and is relevant to another tephritid, the melon fly *Bactrocera curcurbitae*. Populations of *B. curcurbitae* that were initially selected for rapid development time during the successful Okinawa Prefectural Fruit Fly Eradication Project, in which millions of sterile males were produced and released, showed a highly significant change in their free-running circadian periods (Shimizu *et al.*, 1997; Miyatake, 2002; Koyama *et al.*, 2004). The fast-developing S-Line had a 22 h free-running period, whereas a slower developing L-line, showed a 29 h behavioural cycle (Shimizu *et al.*, 1997). These remarkable observations parallel an old and forgotten finding in the *Drosophila* literature, namely that the *per*-mutants also significantly affect development time (Kyriacou *et al.*, 1990). The S- and L-lines also showed a ~5 h difference in the phase of their *per* mRNA cycles in LD cycles, and also revealed a change in mating time, with S-lines mating at dusk, and L-lines during the night phase, so they were in effect temporally sexually isolated (Miyatake *et al.*, 2002). By cleverly manipulating the photoperiods so that the two sets of mating times were in synchrony, this assortative mating between the two lines was *lost,* clearly revealing that *only* the mating time was contributing to the isolation, and not some other associated ethological factor (Miyatake *et al.*, 2002).

Similar results have also been generated in *Drosophila*, where *Drosophila pseudoobscura* shows a later time of mating than *D. melanogaster* (Tauber *et al.*, 2003). When transgenic *per*-null *D. melanogaster* hosts carrying *D. pseudoobscura per* were compared to control flies carrying the conspecific *melanogaster per* transgene, they expressed the later mating times of the donor species. When mixed together, a significant assortative mating between the two types of transformants was observed which according to the genotypes involved, reflected their temporal isolation (Tauber *et al.*, 2003). Mating rhythms in the sibling species *D. melanogaster* and *Drosophila simulans* have also been reported in LD and DD to be slightly out of phase with each other (Sakai & Ishida, 2001). Not surprisingly, in *D. melanogaster*, this mating rhythm disappears in *per*-null mutants in DD (Sakai & Ishida, 2001). These mating rhythms are dictated by the female, but mating tests of *per*-null transformants carrying *D. melanogaster* or *D. simulans per* transgenes have failed to observe any systematic differences in *D. melanogaster* female choice patterns (Ritchie & Kyriacou, 1994). Having said this, these latter experiments were done for another

purpose, and the time of the mating tests was not controlled, so no firm conclusions can be drawn.

Clock genes and photoperiodism

Another fruitfly, *Chymomyza costata*, has also received some attention due mainly to its photoperiodic phenotypes, in which a mature larval diapause is programmed by short days (Kostal & Shimada, 2001). A naturally occurring non-diapausing strain (*npd*, non-photoperiodic diapause) has been mapped to the *timeless* locus (Pavelka *et al.*, 2003). Subsequent molecular analysis reveals a large number of amino acid differences between the wild-type and the mutant strain, as well as a large deletion (~2 Kb) in the 5′ UTR which removes the transcription start signal plus other regulatory sequences (Stehlik *et al.*, 2008).

The mature *npd* larva shows very low levels of *Cctim* mRNA, and no evidence for immunoreactivity to a conspecific *Cc*TIM antiserum. Circadian cycles of *Cctim* are found in the wild-type larval brain that are more robust in short than long days, and two lateral dorsal neurons in each hemisphere appear to express *Cc*TIM, with more intense staining at ZT2 and weakest at ZT20 in long days, but with more intense (apparently cytoplasmic) staining during the night in short days (Stehlik *et al.*, 2008). Thus there seems to be a correlation between the presence of photoperiodic diapause and *Cctim* expression in this species, as well as changes in *Cc*TIM expression with different photoperiods. This is also seen in the diapausing pitcher plant mosquito, *Wyomia smithii*, in which *Wstim* expression changes consistently with latitude in North America (Mathias *et al.*, 2005). While linkage analysis may suggest that the *Wstim* locus is associated with photoperiodism, it has been argued that this is unlikely to involve the circadian clock directly (Bradshaw *et al.*, 2006; Mathias *et al.*, 2007).

The long standing debate on whether clock genes can contribute to photoperiodic diapause in insects (Nunes & Saunders, 1999; Bradshaw *et al.*, 2006) has been further stoked by the findings that in *D. melanogaster*, a new *tim* natural variant, *ls-tim* appears to be under natural selection because it enhances the level of diapause in the European seasonal environment (Tauber *et al.*, 2007). The molecular basis for this appears to be that the binding of the new TIM variant protein to CRY is attenuated, leading to a more stable, light insensitive TIM, that leads to a reduced light responsiveness for both circadian and photoperiodic phenotypes (Sandrelli *et al.*, 2007a). Such changes would be favourable under the colder habitats, and seasonally varying exotic photoperiods of Europe, to which *D. melanogaster* migrated, out of eastern Africa ~15 000 years ago (Kyriacou *et al.*, 2008). The new *ls-tim* variant, which originated in southern Italy about 8000 years ago, thus came under selection and spread throughout Europe (Tauber *et al.*, 2007). However it is not yet completely clear whether circadian behaviour and diapause induction are simply sharing the same TIM-based light input mechanism, or whether the new *tim* variant affects diapause through the circadian system (Bradshaw & Holzapfel, 2007; Kyriacou *et al.*, 2008).

Clocks in other Diptera

The clock genes of a number of other flies have been compared, the most detailed study so far is probably that in *Musca domestica*, the housefly (Codd *et al.*, 2007). In this species, the RNAs for *Musca per*, *tim*, and *vri*, are cycling and peak early in the subjective night, whereas the *Clk* transcript cycles with an opposite phase. In LL, these cycles were lost, and so these data are completely consistent with those from *Drosophila*, including the observation that *cyc* does not cycle in DD in the housefly. However, as in *Bactrocera* (An *et al.*, 2004), and in contrast to *Drosophila*, *Mdcry* does not cycle in DD. It is not known whether *Mdcry* would cycle in brains if, like *Bactrocera*, these were dissected out from the heads.

While *Mdper* transcript cycles in the head, the *Md*PER protein is, surprisingly, produced constitutively as assessed by Western blots in both heads and thoraces, and analysed separately in males and in females (Codd *et al.*, 2007). A similar situation exists in the medfly, *Ceratitis capitata* (Mazzotta *et al.*, 2005), whereas in the sheep blowfly, *Lucilia cuprina*, both gene products appear to cycle (Warman *et al.*, 2000). *Md*TIM on the other hand shows the expected cycles in LD and DD. In LL however, *Md*PER remains highly stable, whereas *Md*TIM degrades immediately, so unlike *Drosophila*, *Md*PER does not apparently require *Md*TIM for stability.

Initial immunohistochemistry (IHC) using head sections revealed various *Md*PER staining neurons, particularly in medial and lateral areas in which the signal was always cytoplasmic at every time point (Codd *et al.*, 2007). A more detailed confocal analysis was then carried out using whole mounts with fluorescent secondary antibodies. Initially, groups of medial and medial lateral neurons were again detected that appeared to be expressing high levels of constitutive cytoplasmic *Md*PER, but not *Md*TIM. An anti-PDH reagent also detected a group of neurons that seemed homologous to the s-LNvs and l-LNvs of *Drosophila* (Pyza *et al.*, 2003; Codd *et al.*, 2007), and, when re-examined carefully, these cells were also observed to co-express *Md*TIM and *Md*PER (see Fig. 3). Furthermore the *Musca* s-LNvs showed nuclear *Md*PER/TIM staining at ZT24, whereas the l-LNvs showed a generally lower and more diffuse staining for both reagents in both cytoplasmic and nuclear compartments, rather different to the situation in *Drosophila*. A single sLNv neuron which in *Drosophila* does not express PDF (Pn-lNv) and is found closer to the l-LNv cluster, appears to have expanded to four neurons in

Musca and shows nuclear localisation of *Md*PER/TIM at ZT 24. *Musca* neurons equivalent to the *Drosophila* LNds were also detected and these showed nuclear PER/TIM staining at the end of the night. A small number of dorsal neurons were also observed to colocalise *Md*PER and *Md*TIM. It thus appears that the constitutive *Md*PER expression observed in Western blots comes from those very highly, constitutively cytoplasmically expressing medial/medial-lateral *Md*PER neurons that do not co-express *Md*TIM (see Fig. 3).

This study reveals that the housefly has considerable similarities in clock gene expression to *Drosophila*, but also some notable differences. What, for example is the function of the *Md*PER-reacting material in the medial-lateral cells which do not co-express *Md*TIM and therefore cannot be required for *Md*TIM stability? Indeed, is it *Md*PER at all, or just non-specific cross-reacting material? In the absence of a negative genetic control, this is always a difficult question to answer. The *Md*PER band in a *Musca* head Western blot, which is constitutively expressed, likely reflects the dynamics of constitutive '*Md*PER' in these medial neurons. This band is the same size as the band seen in *Drosophila per*-null transformant carrying the *Mdper* transgene, so it seems very likely that this antigenic material in both blots and IHC is indeed *Md*PER (Piccin *et al.*, 2000; Codd *et al.*, 2007).

In addition, transformation of the *Mdper* gene into *D. melanogaster per*[01] mutants, rescues rhythmicity very robustly, indeed far more strongly than the more closely phylogenetically related *D. pseudoobscura per* transgene (Piccin *et al.*, 2000). This intriguing observation correlates with a higher amino acid identity in the PAS domain between *Musca* and *D. melanogaster* PER than between the two *Drosophila* species (Piccin *et al.*, 2000). As this region interacts physically with TIM (Gekakis *et al.*, 1995) this might represent a case of intergenic coevolution, something that can be tested when the full *Md*TIM sequences become available. In fact one might expect under such circumstances that the PER interacting region of TIM to also be more similar between *Musca* and *D. melanogaster*, than between *D. melanogaster* and *D. pseudoobscura*.

The *Musca* study also provides something of a cautionary tale, in that, based on enzymatic-reactions, initial IHC revealed only cytoplasmic, constitutive PER expression. When fluorescence based methods were used with confocal microscopy, the *Md*PER and *Md*TIM colocalising neurons with nuclear staining at appropriate phases of the circadian cycle, were revealed. Thus reports based on enzymatic IHC on a number of insect orders which generally report non-cycling 'cytoplasmic PER' (e.g. Zavodska *et al.*, 2003b) may have to be revised when more sensitive methods are utilised.

Other Diptera that have been studied include the fleshfly, *Sarcophaga crassipalpis*, in which *Scper* and *Sctim* mRNA cycles have been detected in fly heads, but again, neither *Sccry* nor *Sccyc* transcripts show any significant temporal

pattern (Goto & Denlinger, 2002). In the yellow fever and dengue vector, *Aedes aegypti*, *Aatim* shows a weak cycling with a peak in mRNA abundance around the light-dark transition (Gentile *et al.*, 2006). The hematophagous sandfly *Lutzomya longipalpis* is unusual compared to *Drosophila*, in that both the *Llclk* and *Llcyc* transcripts cycle with higher levels during the light phase (Meireles-Filho *et al.*, 2006a,b). *Llper* and *Lltim* are also down regulated by blood-feeding, which also causes a reduction in locomotor activity (Meireles-Filho *et al.*, 2006b). *Lutzomya* appears to have a C-terminal transactivation domain that is characteristic of CYC in mosquitos (Gentile *et al.*, 2006), bees (Rubin *et al.*, 2006), and moths (Chang *et al.*, 2003), but is absent in *Drosophila* (Rutila *et al.*, 1998). Thus, and as mentioned earlier, the evolution of an extensive polyQ transactivation domain in CLK might relieve similar pressure on the CYC partner molecule to maintain its own.

Circadian clocks in Lepidoptera

In Lepidoptera, rhythmic behaviour in egg-hatching and adult eclosion (as well as a number of other rhythms such as the behavioural activities in preparation for pupation observed in *Manduca sexta*), appear to be driven by a photosensitive circadian clock. In fact, circadian behavioural rhythmicity in LD as well as in constant conditions has been observed in a number of species (Sauman & Hashimi, 1999; Froy *et al.*, 2003). Most of the comparative circadian studies conducted on Lepidoptera involve the giant silk-moth, *Antheraea pernyi*, the domestic silk moth *Bombyx mori* and the monarch butterfly, *Danaus plexippus*, (Sauman & Hashimi, 1999; Chang *et al.*, 2003; Sehadova *et al.*, 2004; Takeda *et al.*, 2004; Zhu *et al.*, 2005, 2008a,b; Iwai *et al.*, 2006; Reppert, 2006; Trang le *et al.*, 2006; Iwai *et al.*, 2008). The latter species is particularly important given the possible link between the circadian clock and annual long distance migrations characteristic of this organism.

Molecular cloning and bioinformatic studies have identified Lepidopteran orthologues and paralogues for most of the known *Drosophila* and/or mammalian clock genes (see Fig. 2). The *A. pernyi* genome harbors a family of three sex-linked *per* genes, of which only one (*ApperZ*) is essential for circadian clock functions (Gotter *et al.*, 1999). Conversely, *B. mori* has only a single copy of the *per* gene in both males and females (Takeda *et al.*, 2004; Iwai *et al.*, 2006; Sandrelli *et al.*, 2007b), that produces two *Bm*PER isoforms, differing by 5 aa in length (Takeda *et al.*, 2004) whereas in the monarch butterfly only a single *Dpper* cDNA has been isolated (Froy *et al.*, 2003). The PER proteins of these Lepidoptera show the canonical *Drosophila* PER domains, including the nuclear localization signal (NLS), PAS A and B regions, cytoplasmic localization domain (CLD) and nuclear export signal (NES) (Reppert *et al.*, 1994; Takeda *et al.*, 2004).

All three species appear to encode orthologues for *Drosophila tim* (Chang *et al.*, 2003; Iwai *et al.*, 2006) *Dptim* AY36059 (http://rana.lbl.gov/*Drosophila*/), and *timeout/tim2* has been reported in *B. mori* (Rubin *et al.*, 2006). Bioinformatic comparison among *Drosophila*, *Antheraea* and *Bombyx* TIM reveal the presence of highly conserved functional domains, such as the NLS and PER interaction sites, and a lower degree of conservation in for the CLD domain (Chang *et al.*, 2003; Iwai *et al.*, 2006). These species encode a single orthologue of *Drosophila Clock* and *cyc/Bmal1.* Lepidopteran CLK/BMAL1 heterodimers are potent transcription factors, with a C-terminal BMAL1 transactivation domain (Chang *et al.*, 2003; Markova *et al.*, 2003; Zhu *et al.*, 2005; Rubin *et al.*, 2006). As stated above, both *cry-d* and *cry-m* like genes have been identified in the three species (Zhu *et al.*, 2005; Yuan *et al.*, 2007).

Clock gene expression

In the three species mentioned above, when *per* and *tim* mRNA cycling has been studied, cycles have been detected in moth/butterfly heads in LD and DD, with higher mRNA levels during the subjective dark phase (Reppert *et al.*, 1994; Froy *et al.*, 2003; Iwai *et al.*, 2006; Zhu *et al.*, 2008b). *Bma1l/cyc* mRNA expression in *B. mori* does not show any significant daily oscillation (Markova *et al.*, 2003). In *D. plexippus* heads, *cry1* and *cry2* mRNA expression profiles in DD are similar to those described for *per* and *tim* mRNAs, but the rhythms are not as statistically robust (Zhu *et al.*, 2008b).

Brain lesion and transplantation experiments performed in the 1970's in *A. pernyi* mapped the Lepidopteran circadian clock to the dorsolateral protocerebrum (Truman, 1972, 1974). To localize the core of the circadian clock at the cellular level, *in situ* hybridization and IHC experiments were carried out on brains for all three model species. Although species-specific differences in brain expression profiles are observed, one cluster of four large neurons in each dorsolateral hemisphere (Pars Lateralis, PL) expresses the different clock factors, in all three species. These neurons (type Ia$_1$ neurosecretory cells), co-express corazonin, a neuropeptide implicated in the activation of ecdysis, which is currently considered a phenotypic marker for the presence of circadian clock cells in the Lepidopteran dorsal protocerebrum (Wise *et al.*, 2002; Sehadova *et al.*, 2004; Sauman *et al.*, 2005; Zhu *et al.*, 2008b).

In *A. pernyi*, type Ia$_1$ neurons express *Ap*PER throughout pupal and adult development. In adults, these neurons show cycling expression of *Ap*PER and *Ap*TIM in LD, with lower levels during the day than the night (Sauman & Reppert, 1996). Intriguingly, and in contrast to *Drosophila*, there is no lag between the cycles of *Ap*per mRNA and proteins. All these molecular oscillations were obliterated in LL conditions, when *Antheraea* also shows an arrhythmic adult eclosion. Subcellular localization studies revealed that both *Ap*PER and *Ap*TIM signals were restricted to the cytoplasm and the axonal projections (Sauman & Reppert, 1996). In *B. mori* the four PL neuronal cells coexpress PER-, CYC-, DBT-, and CRY-like proteins (Sehadova *et al.*, 2004). For all these proteins, signals were restricted to the cytoplasm and only the *Bm*PER signal oscillated, with higher levels at dusk and lower levels around dawn. *Bm*PER was also detected in the axonal projections of these neurons, with an oscillating profile similar to that observed in the cytoplasm, and, occasionally, in 2–4 small neurons, immediately ventral to the large type-Ia$_1$ PL neurons.

A systematic effort to map the brain expression of many clock elements in the monarch butterfly has revealed that the PL neuronal cells co-express *Dp*TIM and *Dp*CRY2 (see Fig. 3). In each cluster, two out of the four PL neurons also co-express *Dp*PER and *Dp*CRY1 (Sauman *et al.*, 2005; Zhu *et al.*, 2008b) (see Fig. 3). *Dp*PER and *Dp*TIM show a circadian oscillation in LD and DD, with a peak of expression during the middle of the night. In LL, *Dp*PER levels were reduced or absent and *Dp*TIM decreased significantly after a light pulse (Sauman *et al.*, 2005; Zhu *et al.*, 2008b). No such light-dependent expression was shown for *Dp*CRY1 and, as in *A. pernyi* and *B. mori*, *Dp*PER, *Dp*TIM and *Dp*CRY1 signals were restricted to the cytoplasm. However, *Dp*CRY2 cycled in DD, peaking in the early to middle night, and nuclear staining was detected only in the early part of the day which corresponds to the timing of the repression of *Dpper* mRNA (Sauman *et al.*, 2005; Zhu *et al.*, 2008b).

Functional characterization of Lepidopteran clock components

Function analysis of Lepidopteran clock elements was studied *ex in vivo* in *Dp*N1 cells, a monarch butterfly embryonic line (Zhu *et al.*, 2008b). In these cells, *Dp*CRY2 was able to inhibit *Dp*CLK/*Dp*BMAL-mediated transcription, while *Dp*PER and *Dp*TIM did not, a result consistent with *Dp*CRY2's nuclear localization in neurosecretory cells. In addition, both in monarch brains and *Dp*N1 cells, *Dp*CRY2, TIM, and PER appear to form a complex, as indicated by immunoprecipitation experiments. However, in other cellular environments, such as *Drosophila* embryonic S2 cells, *Dp*PER, as well as *Ap*PER and *Dm*PER, are capable of acting within the nucleus to inhibit the CLK:BMAL1-mediated transcription (Chang *et al.*, 2003; Zhu *et al.*, 2008b). Furthermore, when *Ap*PER is expressed in the clock neurons of *per*[01] *Drosophila* transformants, it shows the nuclear/cytoplasmic oscillation typical of *Dm*PER and is able to modestly rescue *per*[01] arrhythmic behaviour (Levine *et al.*, 1995). These results indicate that even though the Lepidopteran PER homologues may have retained their negative regulatory roles in heterospecific milieus, it is

*Dp*CRY2 that acts as the major clock transcriptional repressor within the monarch. The *Dp*CRY2 interaction with *Dp*PER and *Dp*TIM is probably cytoplasmic and may have a stabilizing function before nuclear translocation (Zhu *et al.*, 2008b), as occurs in mammals (Lee *et al.*, 2001).

In *Dp*N1 cells, *Dp*CRY1 is able to induce light-dependent *Dp*TIM degradation (Zhu *et al.*, 2008b). These results are consistent with the ability of a *Dpcry1* transgene (but not *Dpcry2*) to partially rescue the circadian light responses of the *cry*^b mutant in transgenic *Drosophila* (Sauman *et al.*, 2005, Zhu *et al.*, 2008b).

A feature that seems to be common at least to *A. pernyi* and *B. mori* is the expression of different clock elements in the axonal projections of the PL neurons. This is particularly evident for PER, which shows a daily oscillation in LD in both species (Sauman & Reppert, 1996; Sehadova *et al.*, 2004). In *A. pernyi* and *B. mori*, the dorsolateral PER-expressing neurons are very close to two neurons expressing the prothoracicotropic hormone (PTTH), a critical regulator of Lepidoptera postembryonic development (Kawakami *et al.*, 1990; Sauman & Reppert, 1996). In addition, *Bm*PER staining has been identified in the *B. mori* nerve fibre arborizations in the *corpora cardiaca* and *corpora allata*, organs involved in moulting and metamorphosis (Sehadova *et al.*, 2004). These patterns suggest a regulatory activity for PER at the level of the neurohormones involved in postembryonic development. A role for *Bm*PER in *B. mori* development has been suggested also by the observed reduction in egg-to-adult developmental time induced by *Bmper* dsRNAi (Sandrelli *et al.*, 2007b) and might be due to a pleiotropic effect on development, as already demonstrated for the *Drosophila per* clock gene (Kyriacou *et al.*, 1990). This post-transcriptional manipulation of *Bmper* also generated a mild disruption of the egg hatching rhythm (Sandrelli *et al.*, 2007b), something which was also initially seen in *A. pernyi* with *Apper* antisense injections (Sauman *et al.*, 1996), but this finding was subsequently withdrawn (Sauman *et al.*, 2000). Ironically, the corresponding results with *Bombyx* thus provide some additional support for the initial finding in *Antheraea*.

In *Bombyx*, PER-, CYC-, DBT-, CRY-like signals have been observed also in some neurons of the pars intercerebralis (PI), where these proteins apparently co-localize, and in two large neurons of the frontal ganglion, considered a 'peripheral' component of the circadian oscillator that may be implicated in some rhythmic activities controlled by the stomatogastric nervous system (Sehadova *et al.*, 2004). In the monarch, *Dp*PER, *Dp*TIM, *Dp*CRY1 and *Dp*CRY2 staining has also been detected in the suboesophageal ganglion and PI, where a cluster of neurons co-express *Dp*TIM, *Dp*CRY1 and *Dp*PER (Sauman *et al.*, 2005; Zhu *et al.*, 2008b). *Dp*PER expression was also identified in the *corpora cardiaca* and for *Dp*CRY2, in both *corporae cardiaca* and *allata*. Moreover, clock proteins were also detected

in different regions of the optic lobe (Sauman *et al.*, 2005; Reppert, 2007; Zhu *et al.*, 2008b). Finally, a complex series of both *Dp*CRY1- or CRY2-positive nerve fibers have been revealed in the brain (Sauman *et al.*, 2005; Reppert, 2007; Zhu *et al.*, 2008b) (see Fig. 3). These neuronal pathways seem to link the four PL clock neurons with different brain and eye structures implicated in the monarch's annual 3500−4000 km migration from North America to Mexico. The migratory state is characterized by reproductive diapause and increased longevity, induced by a reduction in juvenile hormone (JH) levels, and circumstantial evidence implicates a role for the circadian clock (Reppert, 2006). *Dp*CRY1 positive pathways can be traced from the four PL neurons to PI, a neuronal region expressing clock proteins and able to regulate the production of JH in the corpora allata (Reppert, 2006). It has been proposed that seasonal day-length variations could be registered by the four circadian PL neurons, and communicated via these *Dp*CRY1 positive fibers to the PI, which in turn activates the migratory state (Reppert, 2006).

Additional *Dp*CRY1 positive pathways connect the PL clock neurons with the dorsal rim area of the eye, a structure able to perceive UV polarized light, one of the input signals for orientation during the long flight (Sauman *et al.*, 2005; Reppert, 2006). Finally, *Dp*CRY2 positive fibres can be traced from the four PL neurons to the central brain complex (Zhu *et al.*, 2008b), a region that has been recently identified as the site of the sun compass in locusts (Heinze & Homberg, 2007). Zhu *et al.* (2008b) thus propose a dual role for *Dp*CRY2: that of a key factor in the feedback loop which generate the circadian oscillation in the four dorsolateral clock neurons, and that of an output element that, with unknown mechanisms, regulates the circadian activity in the central complex.

Cockroaches, crickets and other insects

It has been 40 years since the first surgical lesion studies were published in which localisation of the circadian oscillator to the optic lobes was demonstrated in the cockroach *Leucophaea maderae* (Nishiitsutsuji-Uwo & Pittendrigh, 1968). Subsequent and very elegant transplantation experiments revealed that optic lobes from a donor brain that had been entrained to a different circadian period than the host, could, after several weeks, impart these altered periods to the recipient (Page, 1982). A more recent modification of this theme has been the transplantation of the accessory medulla (AMe), a small region of the optic lobe that shows immunoreactivity to anti-PDH. When placed in hosts whose own AMe had been removed, and who had been entrained to a different LD cycle than the donor, behavioural rhythmicity could be restored, and this appeared to correlate with the regeneration of PDH-ir fibres to the central brain (Reischig & Stengl, 2003). Similar correlations were obtained

when optic stalks were cut bilaterally, leading to arrhythmic behaviour, which was restored in those animals that also showed regenerated PDH-ir fibres to the central brain (Stengl & Homberg, 1994). In addition, GABA and Mas-allatotropin innervations in the accessory medulla have been strongly implicated in the photic entrainment pathway (Petri *et al.*, 2002).

However, in the cricket *Gryllus bimaculatus*, it does not appear to be the AMe that is so important, but a more distal region of the optic lobe, the lamina (Tomioka & Abdelsalam, 2004). In other cricket species, *Acheta domesticus*, lesion and transplantation studies have implicated the neurose-cretory *pars intercerebralis* (Cymborowski, 1981). In the cricket *Teleogryllus* some neurons in the AMe are immuno-reactive for both anti-PER and anti-PDH, suggesting a *Drosophila*-centric view of clock anatomy (Lupien *et al.*, 2003). In the ground cricket, *Dianemobius nigrofasciatus* immuno-reactivity to PER, DBT, CRY and CLK was observed pre-dominantly in the optic lobes, but intriguingly CYC was not observed in the CLK expressing neurons in this region. CLK and CYC were also expressed in the *pars intercere-bralis* and suboesophageal ganglion (Shao *et al.*, 2006, 2008a). In contrast, in another ground cricket, *Allenomo-bius allardi*, these clock proteins appear to be particularly enriched in the suboesophageal ganglion (Shao *et al.*, 2006, 2008b). What does appear in all insect orders studied with antibodies to clock proteins, mostly from *Drosophila*, or with anti-PDH, is that optic lobe staining is a common feature (Helfrich-Forster, 2004, 2005c). However, so far, it is only in *Drosophila* and other diptera such as *Musca*, *Teleogryllus*, and the blood feeding hemipteran, *Rhodnius prolixus*, that PDF/PER co-localisation has been observed. In the latter species, a concerted effort by Steel, Vafopoulou and co-workers have documented the circadian basis for the rhythm in PTTH, which controls steroidogenesis from the prothoracic glands (PG) and insect moulting (Steel & Vafopoulou, 2006). Eight lateral neurons in the optic lobes of *Rhodnius* adults co-express rhythmic PER/TIM and PDF, and the axons of these 'clock' neurons associate with PTTH cells, providing a link between the clock and the neurohormonal system (Vafopoulou *et al.*, 2007).

Finally, Hymenoptera reveal yet another dimension to the clock mechanism. In nurse bees which provide round the clock brood care, indirect measurements of their activity in the hive suggest that circadian behaviour may be attenu-ated (Shemesh *et al.*, 2007). However when placed in isolation, nurses revert back to a behavioural locomotor cycle within a couple of days. Temporal expression analysis of the 'usual suspects' putative clock molecules, reveals that even under such optimal circadian conditions, molecular oscillations in nurse heads are severely compromised com-pared to foragers (Shemesh *et al.*, 2007). An analysis of clock protein cycling in the brain of such nurse bees would be welcomed to investigate this apparent uncoupling of molecular and behavioural rhythms. Nevertheless, the take home message of these studies is that there is a certain developmental plasticity in the circadian system of bees. This is related to the social structuring demands of the col-ony, so that older bees that take on foraging roles have robust behavioural and molecular cycles whereas younger nurses have their circadian cycles largely suppressed within the hive (Toma *et al.*, 2000; Bloch *et al.*, 2001).

Conclusions and future prospects

Hemi-metabolous insects such as cockroaches and crick-ets, have thus provided the best material for surgical manip-ulations, and together with expression studies of clock molecules, they have significantly extended the 'evo-devo' type of analysis of clock neuroanatomy. However, one com-mon 'fly in the ointment', so-to-speak, is that when clock protein staining has been 'demonstrated' in insects, and this usually means PER and typically with heterospecific anti-fly PER antibodies, the localisation is commonly exclu-sively cytoplasmic (Zavodska *et al.*, 2003a,b). One notable exception is the hawkmoth, *Manduca sexta*, where PER nuclear antigenicity is observed in putative circadian neurons (Wise *et al.*, 2002; Schuckel *et al.*, 2007).

However, in general, one can surmise that either a novel form of clock gene regulation is present (as discussed ear-lier with some *Musca* and *Antheraea* neurons), or that the anti-PER reagents are identifying cross-reacting material. The absence of a negative control (ie a *per-null* mutant) in non-model insects provides a caveat that has already been discussed earlier in relation to the *Musca* PER story (Codd *et al.*, 2007). This cautionary element can most practically be alleviated by colocalising both the mRNA and protein to the neuronal regions in question (Zhu *et al.*, 2008b). One further important consideration that comes from these var-ious studies that we have discussed, will probably turn out to be a general rule of thumb. In insects that have both fly-like (CRY1) and vertebrate-like (CRY2) CRYs, they are likely to use CRY2 rather than PER or TIM as the main negative regulator, as elegantly demonstrated recently in Lepidoptera (Zhu *et al.*, 2008b).

One of the most exciting parts of this comparative work however, apart from examining how evolution has tweaked the roles of the various clock genes, is the way the clock may impact on other complex phenotypes such as devel-opment (Kyriacou *et al.*, 1990; Shimizu *et al.*, 1997; Miyat-ake, 2002; Koyama *et al.*, 2004; Sandrelli *et al.*, 2007b), mating (Tychsen & Fletcher, 1971; Smith, 1979; Sakai & Ishida, 2001; An *et al.*, 2002, 2004; Miyatake *et al.*, 2002; Tauber *et al.*, 2003), navigation (Sauman *et al.*, 2005; Zhu *et al.*, 2008b) and social (Toma *et al.*, 2000; Bloch *et al.*, 2001) behaviour in different species, revealing that clock proteins will have extensive pleiotropic effects. This has

Table 1. Summary of species and references

SPECIES	ORDER	REFERENCES
Acheta domesticus	Orthoptera	Cymborowski, 1981
Aedes aegypti	Diptera (Nematocera)	Zhu *et al.*, 2005; Gentile *et al.*, 2006; Rubin *et al.*, 2006; Yuan *et al.*, 2007
Allenomobius allardi	Orthoptera	Shao *et al.*, 2006, 2008b
Anopheles gambiae	Diptera (Nematocera)	Zhu *et al.*, 2005; Rubin *et al.*, 2006; Yuan *et al.*, 2007
Antheraea pernyi	Lepidoptera	Truman, 1972, 1974; Gotter *et al.*, 1999; Levine *et al.*, 1995; Reppert *et al.*, 1994; Sauman *et al.*, 1996, 2000; Sauman & Hashimi, 1999; Chang *et al.*, 2003
Apis mellifera	Hymenoptera	Toma *et al.*, 2000; Bloch *et al.*, 2001; Shemesh *et al.*, 2007
Bactrocera curcurbitae	Diptera (Brachycera)	Shimizu *et al.*, 1997; Miyatake, 2002; Miyatake *et al.*, 2002; Koyama *et al.*, 2004
Bactrocera neohumeralis	Diptera (Brachycera)	Tychsen & Fletcher, 1971; Smith, 1979; An *et al.*, 2004
Bactrocera tryoni	Diptera (Brachycera)	Tychsen & Fletcher, 1971; Smith, 1979; An *et al.*, 2004
Bombyx mori	Lepidoptera	Kawakami *et al.*, 1990; Markova *et al.*, 2003; Sehadova *et al.*, 2004; Takeda *et al.*, 2004; Iwai *et al.*, 2006; Trang le *et al.*, 2006; Sandrelli *et al.*, 2007b; Iwai *et al.*, 2008
Ceratitis capitata	Diptera (Brachycera)	Mazzotta *et al.*, 2005
Chymomyza costata	Diptera (Brachycera)	Kostal & Shimada, 2001; Pavelka *et al.*, 2003; Stehlik *et al.*, 2008
Danaus plexippus	Lepidoptera	Froy *et al.*, 2003; Sauman *et al.*, 2005; Reppert, 2006, 2007; Zhu *et al.*, 2008a,b
Dianemobius nigrofasciatus	Orthoptera	Shao *et al.*, 2006, 2008a
Drosophila pseudoobscura	Diptera (Brachycera)	Tauber *et al.*, 2003; Piccin *et al.*, 2000
Drosophila simulans	Diptera (Brachycera)	Sakai & Ishida, 2001
Gryllus bimaculatus	Orthoptera	Tomioka & Abdelsalam, 2004
Leucophaea maderae	Blattaria	Nishiitsutsuji-Uwo & Pittendrigh, 1968; Page, 1982; Stengl & Homberg, 1994; Petri *et al.*, 2002; Reischig & Stengl, 2003
Lucilia cuprina	Diptera (Brachycera)	Warman *et al.*, 2000
Lutzomya longipalpis	Diptera (Brachycera)	Meireles-Filho *et al.*, 2006a,b
Manduca sexta	Lepidoptera	Wise *et al.*, 2002; Schuckel *et al.*, 2007
Musca domestica	Diptera (Brachycera)	Piccin *et al.*, 2000; Codd *et al.*, 2007
Rhodnius prolixus	Hemyptera	Steel & Vafopoulou, 2006; Vafopoulou *et al.*, 2007
Sarcophaga crassipalpis	Diptera (Brachycera)	Goto & Denlinger, 2002
Teleogryllus commodus	Orthoptera	Lupien *et al.*, 2003
Teleogryllus oceanicus	Orthoptera	Lupien *et al.*, 2003
Wyomia smithii	Diptera (Nematocera)	Mathias *et al.*, 2005, 2007; Bradshaw *et al.*, 2006

been brought to focus most dramatically by microarray studies in *Drosophila* when a clock mutant background is used. The vast majority of genes that normally show circadian mRNA cycles in wild type, become non-rhythmic, as expected. However many non-rhythmic genes, that likely have non-circadian functions, also have their basic transcript levels altered in clock mutant backgrounds (Claridge-Chang *et al.*, 2001; McDonald & Rosbash, 2001; Ceriani *et al.*, 2002; Lin *et al.*, 2002b; Ueda *et al.*, 2002). Conseqeuntly we should perhaps not be surprised if more than just timing phenotypes are eventually linked to clock genes.

In our review we have focused on behavioural rhythms and 'brain' clocks which, likely, are unique in their position of being entrained directly by retinal input. However peripheral clocks are common in insects and, at least in many cases, can be entrained by light. It would be interesting for future studies to address how light entrainment of peripheral clocks is achieved in species that do not have a light sensitive CRY and to compare the molecular organisation of peripheral and central clocks. Conversely, in insects such as cockroaches and crickets where available data suggest that retinal photoreceptors are both necessary and sufficient for entrainment and that there are no extra-retinal photoreceptors involved, it would be interesting to analyse

the implications on the molecular organization of the clock, particularly in relation to CRY's photoreceptive function.

In conclusion, after a slow start, the comparative analysis of clock molecules in insects is rapidly gaining pace. Unfortunately due to space limitations we could not cover all of this work. However we hope to have given a flavour of what is out there, and of the evolutionary plasticity of the circadian system that exists within the insects. Table 1 provides a summary of the different species we have discussed.

Note

An interesting analysis of PER and PDF colocalisation in three neurons within the optic lobes of two sibling species of cockroaches (*Blattella germanica* and *B. bisignata*) has just appeared (Wen & Lee, 2008).

References

Akten, B., Jauch, E., Genova, G.K., Kim, E.Y., Edery, I., Raabe, T. *et al.* (2003) A role for CK2 in the *Drosophila* circadian oscillator. *Nat Neurosci* **6**: 251–257.

Allada, R., White, N.E., So, W.V., Hall, J.C. and Rosbash, M. (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell* **93**: 791–804.

An, X., Wilkes, K., Bastian, Y., Morrow, J.L., Frommer, M. and Raphael, K.A. (2002) The *period* gene in two species of tephritid fruit fly differentiated by mating behaviour. *Insect Mol Biol* **11**: 419–430.

An, X., Tebo, M., Song, S., Frommer, M. and Raphael, K.A. (2004) The *cryptochrome* (*cry*) gene and a mating isolation mechanism in tephritid fruit flies. *Genetics* **168**: 2025–2036.

Antoch, M.P., Song, E.J., Chang, A.M., Vitaterna, M.H., Zhao, Y., Wilsbacher, L.D. *et al.* (1997) Functional identification of the mouse circadian *Clock* gene by transgenic BAC rescue. *Cell* **89**: 655–667.

Barnes, J.W., Tischkau, S.A., Barnes, J.A., Mitchell, J.W., Burgoon, P.W., Hickok, J.R. *et al.* (2003) Requirement of mammalian *timeless* for circadian rhythmicity. *Science* **302**: 439–442.

Benito, J., Zheng, H. and Hardin, P.E. (2007) PDP1epsilon functions downstream of the circadian oscillator to mediate behavioral rhythms. *J Neurosci* **27**: 2539–2547.

Benna, C., Scannapieco, P., Piccin, A., Sandrelli, F., Zordan, M., Rosato, E. *et al.* (2000) A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim. Curr Biol* **10**: R512–R513.

Blau, J. and Young, M.W. (1999) Cycling *vrille* expression is required for a functional *Drosophila* clock. *Cell* **99**: 661–671.

Bloch, G., Toma, D.P. and Robinson, G.E. (2001) Behavioral rhythmicity, age, division of labor and period expression in the honey bee brain. *J Biol Rhythms* **16**: 444–456.

Bradshaw, W.E. and Holzapfel, C.M. (2007) Tantalizing *timeless. Science* **316**: 1851–1852.

Bradshaw, W.E., Holzapfel, C.M. and Mathias, D. (2006) Circadian rhythmicity and photoperiodism in the pitcher-plant mosquito: can the seasonal timer evolve independently of the circadian clock? *Am Nat* **167**: 601–605.

Ceriani, M.F., Hogenesch, J.B., Yanovsky, M., Panda, S., Straume, M. and Kay, S.A. (2002) Genome-wide expression analysis in *Drosophila* reveals genes controlling circadian behavior. *J Neurosci* **22**: 9305–9319.

Chang, D.C. and Reppert, S.M. (2003) A novel C-terminal domain of *Drosophila* PERIOD inhibits dCLOCK:CYCLE-mediated transcription. *Curr Biol* **13**: 758–762.

Chang, D.C., McWatters, H.G., Williams, J.A., Gotter, A.L., Levine, J.D. and Reppert, S.M. (2003) Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi. J Biol Chem* **278**: 38149–38158.

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N. and Young, M.W. (2001) Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron* **32**: 657–671.

Clayton, J.D., Kyriacou, C.P. and Reppert, S.M. (2001) Keeping time with the human genome. *Nature* **409**: 829–831.

Codd, V., Dolezel, D., Stehlik, J., Piccin, A., Garner, K.J., Racey, S.N. *et al.* (2007) Circadian rhythm gene regulation in the housefly *Musca domestica. Genetics* **177**: 1539–1551.

Collins, B., Mazzoni, E.O., Stanewsky, R. and Blau, J. (2006) *Drosophila* CRYPTOCHROME is a circadian transcriptional repressor.*Curr Biol.* **16**: 441–449.

Costa, R. and Kyriacou, C.P. (1998) Functional and evolutionary implications of natural variation in clock genes. *Curr Opin Neurobiol* **8**: 659–664.

Cymborowski, B. (1981) Transplantation of circadian pacemaker in the house cricket, *Acheta domesticus* L. *J Interdiscipl Cycle Res* **12**: 133–140.

Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R., Hardin, P.E. *et al.* (2003) *Vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. *Cell* **112**: 329–341.

Dissel, S., Codd, V., Fedic, R., Garner, K.J., Costa, R., Kyriacou, C.P. *et al.* (2004) A constitutively active cryptochrome in *Drosophila melanogaster. Nat Neurosci* **7**: 834–840.

Dolezelova, E., Dolezel, D. and Hall, J.C. (2007) Rhythm defects caused by newly engineered null mutations in *Drosophila's cryptochrome* gene. *Genetics* **177**: 329–345.

Dunlap, J.C. (1999) Molecular bases for circadian clocks. *Cell* **96**: 271–290.

Emery, P., So, W.V., Kaneko, M., Hall, J.C. and Rosbash, M. (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95**: 669–679.

Emery, P., Stanewsky, R., Hall, J.C. and Rosbash, M. (2000) A unique circadian-rhythm photoreceptor. *Nature* **404**: 456–457.

Fang, Y., Sathyanarayanan, S. and Sehgal, A. (2007) Post-translational regulation of the *Drosophila* circadian clock requires protein phosphatase 1 (PP1). *Genes Dev* **21**: 1506–1518.

Froy, O., Gotter, A.L., Casselman, A.L. and Reppert, S.M. (2003) Illuminating the circadian clock in monarch butterfly migration. *Science* **300**: 1303–1305.

Gekakis, N., Saez, L., Delahaye-Brown, A.M., Myers, M.P., Sehgal, A., Young, M.W. *et al.* (1995) Isolation of *timeless* by PER protein interaction: defective interaction between timeless protein and long-period mutant PERL. *Science* **270**: 811–815.

Gentile, C., Meireles-Filho, A.C., Britto, C., Lima, J.B., Valle, D. and Peixoto, A.A. (2006) Cloning and daily expression of the *timeless* gene in *Aedes aegypti* (diptera:Culicidae). *Insect Biochem Mol Biol* **36**: 878–884.

Glossop, N.R., Houl, J.H., Zheng, H., Ng, F.S., Dudek, S.M. and Hardin, P.E. (2003) VRILLE feeds back to control circadian transcription of clock in the *Drosophila* circadian oscillator. *Neuron* **37**: 249–261.

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.

Gotter, A.L., Levine, J.D. and Reppert, S.M. (1999) Sex-linked *period* genes in the silkmoth, *Antheraea pernyi:* implications for circadian clock regulation and the evolution of sex chromosomes. *Neuron* **24**: 953–965.

Gotter, A.L., Manganaro, T., Weaver, D.R., Kolakowski, L.F., Jr, Possidente, B., Sriram, S. *et al.* (2000) A time-less function for mouse *timeless. Nat Neurosci* **3**: 755–756.

Gu, Y.Z., Hogenesch, J.B. and Bradfield, C.A. (2000) The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* **40**: 519–561.

Hall, J.C. (2003) Genetics and molecular biology of rhythms in *Drosophila* and other insects. *Adv Genet* **48**: 1–280.

Heinze, S. and Homberg, U. (2007) Maplike representation of celestial E-vector orientations in the brain of an insect. *Science (New York, NY)* **315**: 995–997.

Helfrich-Forster, C. (2004) The circadian clock in the brain: a structural and functional comparison between mammals and insects. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **190**: 601–613.

Helfrich-Forster, C. (2005a) Techniques that revealed the network of the circadian clock of *Drosophila. Meth Enzymol* **393**: 439–451.

Helfrich-Forster, C. (2005b) Neurobiology of the fruit fly's circadian clock. *Genes Brain Behav* **4**: 65–76.

Helfrich-Forster, C. (2005c) Organization of endogenous clocks in insects. *Biochem Soc Trans* **33**: 957–961.

Helfrich-Forster, C. and Homberg, U. (1993) Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and of several mutants with altered circadian rhythmicity. *J Comp Neurol* **337**: 177–190.

Helfrich-Forster, C., Winter, C., Hofbauer, A., Hall, J.C. and Stanewsky, R. (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron* **30**: 249–261.

Helfrich-Forster, C., Yoshii, T., Wulbeck, C., Grieshaber, E., Rieger, D., Bachleitner, W. *et al.* (2007) The lateral and dorsal neurons of *Drosophila melanogaster*: new insights about their morphology and function. *Cold Spring Harbor Symp Quant Biol* **72**: 517–525.

Houl, J.H., Yu, W., Dudek, S.M. and Hardin, P.E. (2006) *Drosophila* CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells. *J Biol Rhythms* **21**: 93–103.

Hung, H.C., Maurer, C., Kay, S.A. and Weber, F. (2007) Circadian transcription depends on limiting amounts of the transcription co-activator nejire/CBP. *J Biol Chem* **282**: 31349–31357.

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.

Iwai, S., Thi Dieu Trang, L., Sehadova, H. and Takeda, M. (2008) Expression analyses of casein kinase 2alpha and casein kinase 2beta in the silkmoth, *Bombyx mori. Comp Biochem Physiol B Biochem Mol Biol* **149**: 38–46.

Kadener, S., Stoleru, D., McDonald, M., Nawathean, P. and Rosbash, M. (2007) Clockwork orange is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. *Genes Dev* **21**: 1675–1686.

Kaneko, M. and Hall, J.C. (2000) Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J Comp Neurol* **422**: 66–94.

Kawakami, A., Kataoka, H., Oka, T., Mizoguchi, A., Kimura-Kawakami, M., Adachi, T. *et al.* (1990) Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science (New York, NY)* **247**: 1333–1335.

Kim, E.Y. and Edery, I. (2006) Balance between DBT/CKIepsilon kinase and protein phosphatase activities regulate phosphorylation and stability of *Drosophila* CLOCK protein. *Proc Natl Acad Sci USA* **103**: 6178–6183.

King, D.P., Vitaterna, M.H., Chang, A.M., Dove, W.F., Pinto, L.H., Turek, F.W. *et al.* (1997a) The mouse *Clock* mutation behaves as an antimorph and maps within the W19H deletion, distal of *kit. Genetics* **146**: 1049–1060.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P. *et al.* (1997b) Positional cloning of the mouse circadian *Clock* gene. *Cell* **89**: 641–653.

Klarsfeld, A., Malpel, S., Michard-Vanhee, C., Picot, M., Chelot, E. and Rouyer, F. (2004) Novel features of cryptochrome-mediated photoreception in the brain circadian clock of *Drosophila. J Neurosci* **24**: 1468–1477.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S. *et al.* (1998) The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase iepsilon. *Cell* **94**: 97–107.

Ko, C.H. and Takahashi, J.S. (2006) Molecular components of the mammalian circadian clock. *Hum Mol Genet* **15 Spec No 2**: R271–7.

Koh, K., Zheng, X. and Sehgal, A. (2006) JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. *Science* **312**: 1809–1812.

Kostal, V. and Shimada, K. (2001) Malfunction of circadian clock in the non-photoperiodic-diapause mutants of the drosophilid fly, *Chymomyza costata. J Insect Physiol* **47**: 1269–1274.

Koyama, J., Kakinohana, H. and Miyatake, T. (2004) Eradication of the melon fly, *Bactrocera cucurbitae*, in Japan: importance of behavior, ecology, genetics, and evolution. *Annu Rev Entomol* **49**: 331–349.

Kyriacou, C.P., Oldroyd, M., Wood, J., Sharp, M. and Hill, M. (1990) Clock mutations alter developmental timing in *Drosophila. Heredity* **64** (Pt 3): 395–401.

Kyriacou, C.P., Peixoto, A.A., Sandrelli, F., Costa, R. and Tauber, E. (2008) Clines in clock genes: fine-tuning circadian rhythms to the environment. *Trends Genet* **24**: 124–132.

Lee, C., Etchegaray, J.P., Cagampang, F.R., Loudon, A.S. and Reppert, S.M. (2001) Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**: 855–867.

Levi, F. and Schibler, U. (2007) Circadian rhythms: mechanisms and therapeutic implications. *Annu Rev Pharmacol Toxicol* **47**: 593–628.

Levine, J.D., Sauman, I., Imbalzano, M., Reppert, S.M. and Jackson, F.R. (1995) Period protein from the giant silkmoth *Antheraea pernyi* functions as a circadian clock element in *Drosophila melanogaster. Neuron* **15**: 147–157.

Lim, C., Lee, J., Choi, C., Kim, J., Doh, E. and Choe, J. (2007a) Functional role of CREB-binding protein in the circadian clock system of *Drosophila melanogaster. Mol Cell Biol* **27**: 4876–4890.

Lim, C., Chung, B.Y., Pitman, J.L., McGill, J.J., Pradhan, S., Lee, J. *et al.* (2007b) *Clockwork orange* encodes a transcriptional repressor important for circadian-clock amplitude in *Drosophila. Curr Biol* **17**: 1082–1089.

Lin, J.M., Kilman, V.L., Keegan, K., Paddock, B., Emery-Le, M., Rosbash, M. *et al.* (2002a) A role for casein kinase 2alpha in the *Drosophila* circadian clock. *Nature* **420**: 816–820.

Lin, J.M., Schroeder, A. and Allada, R. (2005) *In vivo* circadian function of casein kinase 2 phosphorylation sites in *Drosophila* PERIOD. *J Neurosci* **25**: 11175–11183.

Lin, Y., Han, M., Shimada, B., Wang, L., Gibler, T.M., Amarakone, A. *et al.* (2002b) Influence of the *period*-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **99**: 9562–9567.

Lupien, M., Marshall, S., Leser, W., Pollack, G.S. and Honegger, H.W. (2003) Antibodies against the PER protein of *Drosophila* label neurons in the optic lobe, central brain, and thoracic ganglia of the crickets *Teleogryllus commodus* and *Teleogryllus oceanicus*. *Cell Tissue Res* **312**: 377–391.

Markova, E.P., Ueda, H., Sakamoto, K., Oishi, K., Shimada, T. and Takeda, M. (2003) Cloning of *cyc* (*Bmal1*) homolog in *Bombyx mori*: structural analysis and tissue specific distributions. *Comp Biochem Physiol B Biochem Mol Biol* **134**: 535–542.

Martinek, S., Inonog, S., Manoukian, A.S. and Young, M.W. (2001) A role for the segment polarity gene *shaggy/GSK-3* in the Drosophila circadian clock. *Cell* **105**: 769–779.

Mathias, D., Jacky, L., Bradshaw, W.E. and Holzapfel, C.M. (2005) Geographic and developmental variation in expression of the circadian rhythm gene, *timeless*, in the pitcher-plant mosquito, *Wyeomyia smithii*. *J Insect Physiol* **51**: 661–667.

Mathias, D., Jacky, L., Bradshaw, W.E. and Holzapfel, C.M. (2007) Quantitative trait loci associated with photoperiodic response and stage of diapause in the pitcher-plant mosquito, *Wyeomyia smithii*. *Genetics* **176**: 391–402.

Matsumoto, A., Ukai-Tadenuma, M., Yamada, R.G., Houl, J., Uno, K.D., Kasukawa, T. *et al.* (2007) A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the *Drosophila* circadian clock. *Genes Dev* **21**: 1687–1700.

Maywood, E.S., O'Neill, J.S., Reddy, A.B., Chesham, J.E., Prosser, H.M., Kyriacou, C.P. *et al.* (2007) Genetic and molecular analysis of the central and peripheral circadian clockwork of mice. *Cold Spring Harbor Symp Quant Biol* **72**: 85–94.

Mazzotta, G.M., Sandrelli, F., Zordan, M.A., Mason, M., Benna, C., Cisotto, P. *et al.* (2005) The clock gene *period* in the medfly *Ceratitis capitata*. *Genet Res* **86**: 13–30.

McDonald, M.J. and Rosbash, M. (2001) Microarray analysis and organization of circadian gene expression in *Drosophila*. *Cell* **107**: 567–578.

Meireles-Filho, A.C., Amoretty, P.R., Souza, N.A., Kyriacou, C.P. and Peixoto, A.A. (2006a) Rhythmic expression of the cycle gene in a hematophagous insect vector. *BMC Mol Biol* **7**: 38.

Meireles-Filho, A.C., da S Rivas, G.B., Gesto, J.S., Machado, R.C., Britto, C., de Souza, N.A. *et al.* (2006b) The biological clock of an hematophagous insect: locomotor activity rhythms, circadian expression and downregulation after a blood meal. *FEBS Lett* **580**: 2–8.

Meyer, P., Saez, L. and Young, M.W. (2006) PER-TIM interactions in living *Drosophila* cells: an interval timer for the circadian clock. *Science (New York NY)* **311**: 226–229.

Miyasako, Y., Umezaki, Y. and Tomioka, K. (2007) Separate sets of cerebral clock neurons are responsible for light and temperature entrainment of *Drosophila* circadian locomotor rhythms. *J Biol Rhythms* **22**: 115–126.

Miyatake, T. (2002) Pleiotropic effect, clock genes and reproductive isolation. *Popul Ecol* **44**: 201–207.

Miyatake, T., Matsumoto, A., Matsuyama, T., Ueda, H.R., Toyosato, T. and Tanimura, T. (2002) The *period* gene and allochronic reproductive isolation in *Bactrocera cucurbitae*. *Proc R Soc Lond B Biol Sci* **269**: 2467–2472.

Moses, K., Ellis, M.C. and Rubin, G.M. (1989) The *glass* gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. *Nature* **340**: 531–536.

Nishiitsutsuji-Uwo, J. and Pittendrigh, C.S. (1968) Central nervous system control of circadian rhythmicity in the cockroach. III. the optic lobes, locus of driving oscillation? *Zeitschrift for Vergleichende Physiologie* **58**: 14–46.

Nitabach, M.N. and Taghert, P.H. (2008) Organization of the *Drosophila* circadian control circuit. *Curr Biol* **18**: R84–R93.

Nitabach, M.N., Blau, J. and Holmes, T.C. (2002) Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. *Cell* **109**: 485–495.

Nunes, M.V. and Saunders, D. (1999) Photoperiodic time measurement in insects: a review of clock models. *J Biol Rhythm J Biol Rhythm* **14**: 84–104.

Page, T.L. (1982) Transplantation of the cockroach circadian pacemaker. *Science (New York, NY)* **216**: 73–75.

Pavelka, J., Shimada, K. and Kostal, V. (2003) TIMELESS: a link between fly's circadian and photoperiodic clocks? *Eur J Entomol* **100**: 255–265.

Peng, Y., Stoleru, D., Levine, J.D., Hall, J.C. and Rosbash, M. (2003) *Drosophila* free-running rhythms require intercellular communication. *PLoS Biol* **1**: E13.

Peschel, N., Veleri, S. and Stanewsky, R. (2006) *Veela* defines a molecular link between cryptochrome and timeless in the light-input pathway to *Drosophila's* circadian clock. *Proc Natl Acad Sci USA* **103**: 17313–17318.

Petri, B., Homberg, U., Loesel, R. and Stengl, M. (2002) Evidence for a role of GABA and mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. *J Exp Biol* **205**: 1459–1469.

Piccin, A., Couchman, M., Clayton, J.D., Chalmers, D., Costa, R. and Kyriacou, C.P. (2000) The clock gene *period* of the housefly, *Musca domestica*, rescues behavioral rhythmicity in *Drosophila melanogaster*: evidence for intermolecular coevolution? *Genetics* **154**: 747–758.

Picot, M., Cusumano, P., Klarsfeld, A., Ueda, R. and Rouyer, F. (2007) Light activates output from evening neurons and inhibits output from morning neurons in the *Drosophila* circadian clock. *PLoS Biol* **5**: e315.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B. and Young, M.W. (1998) Double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* **94**: 83–95.

Pyza, E., Siuta, T. and Tanimura, T. (2003) Development of PDF-immunoreactive cells, possible clock neurons, in the housefly *Musca domestica*. *Microsc Res Tech* **62**: 103–113.

Reischig, T. and Stengl, M. (2003) Ectopic transplantation of the accessory medulla restores circadian locomotor rhythms in arrhythmic cockroaches (*Leucophaea maderae*). *J Exp Biol* **206**: 1877–1886.

Reppert, S.M. (2006) A colorful model of the circadian clock. *Cell* **124**: 233–236.

Reppert, S.M. (2007) The ancestral circadian clock of monarch butterflies: role in time-compensated sun compass orientation. *Cold Spring Harbor Symp Quant Biol* **72**: 113–118.

Reppert, S.M. and Weaver, D.R. (2000) Comparing clockworks: mouse vs. fly. *J Biol Rhythms* **15**: 357–364.

Reppert, S.M., Tsai, T., Roca, A.L. and Sauman, I. (1994) Cloning of a structural and functional homolog of the circadian clock gene *period* from the giant silkmoth *Antheraea pernyi*. *Neuron* **13**: 1167–1176.

Richier, B., Michard-Vanhee, C., Lamouroux, A., Papin, C. and Rouyer, F. (2008) The clockwork orange *Drosophila* protein functions as both an activator and a repressor of clock gene expression. *J Biol Rhythms* **23**: 103–116.

Ritchie, M.G. and Kyriacou, C.P. (1994) Reproductive isolation and the *period* gene of *Drosophila*. *Mol Ecol* **3**: 595–599.

Rosato, E., Codd, V., Mazzotta, G., Piccin, A., Zordan, M., Costa, R. *et al.* (2001) Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY. *Curr Biol* **11**: 909–917.

Rothenfluh, A., Young, M.W. and Saez, L. (2000) A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron* **26**: 505–514.

Rubin, R., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H.M. and Bloch, G. (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. *Genome Res* **16**: 1352–1365.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M. and Hall, J.C. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell* **93**: 805–814.

Sakai, T. and Ishida, N. (2001) Circadian rhythms of female mating activity governed by clock genes in *Drosophila*. *Proc Natl Acad Sci USA* **98**: 9221–9225.

Sandrelli, F., Tauber, E., Pegoraro, M., Mazzotta, G., Cisotto, P., Landskron, J. *et al.* (2007a) A molecular basis for natural selection at the *timeless* locus in *Drosophila melanogaster. Science (New York, NY)* **316**: 1898–1900.

Sandrelli, F., Cappellozza, S., Benna, C., Saviane, A., Mastella, A., Mazzotta, G.M. *et al.* (2007b) Phenotypic effects induced by knock-down of the *period* clock gene in *Bombyx mori. Genet Res* **89**: 73–84.

Sathyanarayanan, S., Zheng, X., Xiao, R. and Sehgal, A. (2004) Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell* **116**: 603–615.

Sauman, I. and Hashimi, H. (1999) Insect clocks: what are they telling us besides time? *Entomol Sci* **2**: 589–596.

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.

Sauman, I., Tsai, T., Roca, A.L. and Reppert, S.M. (1996) Period protein is necessary for circadian control of egg hatching behavior in the silkmoth *Antheraea pernyi. Neuron* **17**: 901–909.

Sauman, I., Tsai, T., Roca, A.L. and Reppert, S.M. (2000) Erratum of period protein is necessary for circadian control of egg hatching behaviour in the silkmoth *Antheraea pernyi. Neuron* **27**: 189.

Sauman, I., Briscoe, A.D., Zhu, H., Shi, D., Froy, O., Stalleicken, J. *et al.* (2005) Connecting the navigational clock to sun compass input in monarch butterfly brain. *Neuron* **46**: 457–467.

Schuckel, J., Siwicki, K.K. and Stengl, M. (2007) Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta. Cell Tissue Res* **330**: 271–278.

Sehadova, H., Markova, E.P., Sehnal, F. and Takeda, M. (2004) Distribution of circadian clock-related proteins in the cephalic nervous system of the silkworm, *Bombyx mori. J Biol Rhythms* **19**: 466–482.

Shafer, O.T., Rosbash, M. and Truman, J.W. (2002) Sequential nuclear accumulation of the clock proteins period and timeless in the pacemaker neurons of *Drosophila melanogaster. J Neurosci* **22**: 5946–5954.

Shafer, O.T., Helfrich-Forster, C., Renn, S.C. and Taghert, P.H. (2006) Reevaluation of *Drosophila melanogaster's* neuronal circadian pacemakers reveals new neuronal classes. *J Comp Neurol* **498**: 180–193.

Shao, Q.M., Sehadova, H., Ichihara, N., Sehnal, F. and Takeda, M. (2006) Immunoreactivities to three circadian clock proteins in two ground crickets suggest interspecific diversity of the circadian clock structure. *J Biol Rhythms* **21**: 118–131.

Shao, Q.M., Bembenek, J., Trang le, T.D., Hiragaki, S. and Takeda, M. (2008a) Molecular structure, expression patterns, and localization of the circadian transcription modulator CYCLE in the cricket, *Dianemobius nigrofasciatus. J Insect Physiol* **54**: 403–413.

Shao, Q.M., Hiragaki, S. and Takeda, M. (2008b) Co-localization and unique distributions of two clock proteins CYCLE and CLOCK in the cephalic ganglia of the ground cricket, *Allonemobius allardi. Cell Tissue Res* **331**: 435–446.

Shemesh, Y., Cohen, M. and Bloch, G. (2007) Natural plasticity in circadian rhythms is mediated by reorganization in the molecular clockwork in honeybees. *FASEB J* **21**: 2304–2311.

Shimizu, T., Miyatake, T., Watari, Y. and Arai, T. (1997) A gene pleiotropically controlling developmental and circadian periods in the melon fly *Bactrocera cucurbitae* (diptera: Tephritidae). *Heredity* **79**: 600–605.

Smith, P.H. (1979) Genetic manipulation of the circadian clock's timing of sexual behavior in the queensland fruit flies, *Dacus tryoni* and *Dacus neohumeralis. Physiol Entomol* **4**: 71–78.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A. *et al.* (1998) The *cryb* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila. Cell* **95**: 681–692.

Steel, C.G. and Vafopoulou, X. (2006) Circadian orchestration of developmental hormones in the insect, *Rhodnius prolixus. Comp Biochem Physiol A Mol Integr Physiol* **144**: 351–364.

Stehlik, J., Zavodska, R., Shimada, K., Sauman, I. and Kostal, V. (2008) Photoperiodic induction of diapause requires regulated transcription of timeless in the larval brain of *Chymomyza costata. J Biol Rhythms* **23**: 129–139.

Stengl, M. and Homberg, U. (1994) Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. *J Comp Physiol [A]* **175**: 203–213.

Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. and Lee, C.C. (1997) *RIGUI*, a putative mammalian ortholog of the *Drosophila period* gene. *Cell* **90**: 1003–1011.

Takeda, Y., Chuman, Y., Shirasu, N., Sato, S., Matsushima, A., Kaneki, A. *et al.* (2004) Structural analysis and identification of novel isoforms of the circadian clock gene period in the silk moth *Bombyx mori. Zoolog Sci* **21**: 903–915.

Tauber, E., Roe, H., Costa, R., Hennessy, J.M. and Kyriacou, C.P. (2003) Temporal mating isolation driven by a behavioral gene in *Drosophila. Curr Biol* **13**: 140–145.

Tauber, E., Zordan, M., Sandrelli, F., Pegoraro, M., Osterwalder, N., Breda, C. *et al.* (2007) Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster. Science (New York, NY)* **316**: 1895–1898.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. *et al.* (1997) Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. *Nature* **389**: 512–516.

Toma, D.P., Bloch, G., Moore, D. and Robinson, G.E. (2000) Changes in *period* mRNA levels in the brain and division of labor in honey bee colonies. *Proc Natl Acad Sci USA* **97**: 6914–6919.

Tomioka, K. and Abdelsalam, S. (2004) Circadian organization in hemimetabolous insects. *Zoolog Sci* **21**: 1153–1162.

Trang le, T.D., Sehadova, H., Ichihara, N., Iwai, S., Mita, K. and Takeda, M. (2006) Casein kinases I of the silkworm, *Bombyx mori*: their possible roles in circadian timing and developmental determination. *J Biol Rhythms* **21**: 335–349.

Truman, J.W. (1972) Physiology of insect rhythms 2 Silkmoth brain as location of biological clock controlling eclosion. *J Comp Physiol* **81**: 99–114.

Truman, J.W. (1974) Physiology of insect rhythms, IV: role of the brain in regulation of the flight rhythm of the giant silkmoths. *J Comp Physiol* **95**: 281–296.

Tychsen, P.H. and Fletcher B.S., (1971) Studies on the rhythm of mating in the queensland fruit fly, *Dacus tryoni. J Insect Physiol* **17**: 2139–2156.

Ueda, H.R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T. and Hashimoto, S. (2002) Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila. J Biol Chem* **277**: 14048–14052.

Unsal-Kacmaz, K., Mullen, T.E., Kaufmann, W.K. and Sancar, A. (2005) Coupling of human circadian and cell cycles by the timeless protein. *Mol Cell Biol* **25**: 3109–3116.

Vafopoulou, X., Steel, C.G. and Terry, K.L. (2007) Neuroanatomical relations of prothoracicotropic hormone neurons with the circadian timekeeping system in the brain of larval and adult *Rhodnius prolixus* (hemiptera). *J Comp Neurol* **503**: 511–524.

Warman, G.R., Newcomb, R.D., Lewis, R.D. and Evans, C.W. (2000) Analysis of the circadian clock gene *period* in the sheep blow fly *Lucilia cuprina. Genet Res* **75**: 257–267.

Wen, C.-H. and Lee, H.-J. (2008) Mapping the cellular network of the circadian clock in two cockroach species. *Arch Insect Biochem & Physiol* **68**: 215–231.

Wise, S., Davis, N.T., Tyndale, E., Noveral, J., Folwell, M.G., Bedian, V. *et al.* (2002) Neuroanatomical studies of *period* gene expression in the hawkmoth, *Manduca sexta. J Comp Neurol* **447**: 366–380.

Yoshii, T., Todo, T., Wulbeck, C., Stanewsky, R. and Helfrich-Forster, C. (2008) Cryptochrome is present in the compound eyes and a subset of *Drosophila's* clock neurons. *J Comp Neurol* **508**: 952–966.

Young, M.W. (1998) The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila. Annu Rev Biochem* **67**: 135–152.

Yu, W. and Hardin, P.E. (2006) Circadian oscillators of *Drosophila* and mammals. *J Cell Sci* **119**: 4793–4795.

Yu, W., Zheng, H., Houl, J.H., Dauwalder, B. and Hardin, P.E. (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev* **20**: 723–733.

Yuan, Q., Metterville, D., Briscoe, A.D. and Reppert, S.M. (2007) Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol Biol Evolution* **24**: 948–955.

Zavodska, R., Sauman, I. and Sehnal, F. (2003a) The cycling and distribution of PER-like antigen in relation to neurons recognized by the antisera to PTTH and EH in *Thermobia domestica. Insect Biochem Mol Biol* **33**: 1227–1238.

Zavodska, R., Sauman, I. and Sehnal, F. (2003b) Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. *J Biol Rhythms* **18**: 106–122.

Zheng, X. and Sehgal, A. (2008) Probing the relative importance of molecular oscillations in the circadian clock. *Genetics* **178**: 1147–1155.

Zhu, H., Yuan, Q., Briscoe, A.D., Froy, O., Casselman, A. and Reppert, S.M. (2005) The two CRYs of the butterfly. *Curr Biol* **15**: R953–R954.

Zhu, H., Casselman, A. and Reppert, S.M. (2008a) Chasing migration genes: a brain expressed sequence tag resource for summer and migratory monarch butterflies (*Danaus plexippus*). *PLoS ONE* **3**: e1345.

Zhu, H., Sauman, I., Yuan, Q., Casselman, A., Emery-Le, M., Emery, P. *et al.* (2008b) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. *PLoS Biol* **6**: e4.