Comparative analysis of circadian clock genes in insects.

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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.
INTRODUCTION

Our fascination with the 24 h (circadian) clock is probably due to our own personal experiences when we disrupt it, unpleasantly vivid after intercontinental travel, shift work or simply a good night out with friends. 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, Song, Chang et al., 1997; King, Zhao, Sangoram et al., 1997) and Period (Sun, Albrecht, Zhuchenko et al., 1997; Tei, Okamura, Shigeyoshi 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, Metterville, Briscoe et al., 2007; Kyriacou, Peixoto, Sandrelli 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, Song, Chang et al., 1997; King, Vitaterna, Chang et al., 1997; King, Zhao, Sangoram et al., 1997; Sun, Albrecht, Zhuchenko et al., 1997; Tei, Okamura, Shigeyoshi 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, Kyriacou, Reppert, 2001). At first this seemed to correlate with the phylogenetic distance between flies and mice, but in fact, some of the mouse-like features of these genes are already evident within the insects (Zhu, Yuan, Briscoe et al., 2005; Yuan, Metterville, Briscoe 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, Hogenesch, Bradfield, 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, Maurer, Kay et al., 2007; Lim, Lee, Choi et al., 2007) (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, Rosbash, Truman, 2002; Meyer, Saez, Young, 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, Price, Saez et al., 1998; Price, Blau, Rothenfluh et al., 1998; Martinek, Inonog, Manoukian et al., 2001; Lin, Kilman, Keegan et al., 2002; Akten, Jauch, Genova et al., 2003; Sathyanarayanan, Zheng, Xiao et al., 2004; Lin, Schroeder, Allada, 2005; Kim, Edery, 2006; Yu, Zheng, Houl et al., 2006; Fang, Sathyanarayanan, Sehgal, 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, Yu, Dudek et al., 2006; Kim, Edery, 2006; Yu, Zheng, Houl et al., 2006). PER likely facilitates CLK phosphorylation by DBT, resulting in diminished CLK/CYC affinity for DNA (Yu, Zheng, Houl 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, Young, Saez, 2000).

CYC is also a constitutively expressed protein (Rutila, Suri, Le 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, Stoleru, McDonald et al., 2007; Lim, Chung, Pitman et al., 2007; Matsumoto, Ukai-Tadenuma, Yamada 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, Michard-Vanhee, Lamouroux 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, Buchsbaum, Reddy et al., 2003; Glossop, Houl, Zheng et al., 2003). However, as mis-expression of Pdp1ε in clock cells does not halt the cycling of Clk mRNA (Benito, Zheng, Hardin, 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, Winter, Hofbauer 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, cryb, are rhythmic in constant light (LL), even though their visual system is intact (Emery, Stanewsky, Hall et al., 2000).
Various cry mutant genotypes also show reduced circadian responses to brief light pulses (Emery, So, Kaneko et al., 1998; Stanewsky, Kaneko, Emery et al., 1998; Rosato, Codd, Mazzotta et al., 2001; Dissel, Codd, Fedic 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, Zheng, Sehgal, 2006; Peschel, Veleri, Stanewsky, 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, Cusumano, Klarsfeld et al., 2007; Yoshii, Todo, Wulbeck 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, Blau, Holmes, 2002; Peng, Stoleru, Levine 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, Malpel, Michard-Vanhee et al., 2004; Yoshii, Todo, Wulbeck 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, Dolezel, Hall, 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, Kyriacou, Reppert, 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, White, So et al., 1998; Rutila, Suri, Le et al., 1998). Moreover, mammalian *Tim (mTim)* actually corresponds to the fly paralogue *timeout/tim2* (Benna, Scannapieco, Piccin et al., 2000; Gotter, Manganaro, Weaver et al., 2000). Although both the fly (F. Sandrelli and R. Costa unpublished observations) and the mammalian (Barnes, Tischkau, Barnes et al., 2003; Unsal-Kacmaz, Mullen, Kaufmann et al., 2005) *timeout/tim2* genes seem to be 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, O'Neil, Reddy 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, Metterville, Briscoe 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 mosquitoes Anopheles gambiae and Aedes aegypti (Zhu, Yuan, Briscoe et al., 2005; Rubin, Shemesh, Cohen et al., 2006b; Yuan, Metterville, Briscoe et al., 2007).

The mammalian-like TIM is an essential gene both in mouse (Gotter, Manganaro, Weaver 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, Shemesh, Cohen et al., 2006b) 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, Shemesh, Cohen et al., 2006b).

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, McWatters, Williams et al., 2003; Rubin, Shemesh, Cohen et al., 2006b). 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, Mazzoni, Stanewsky 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), 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, Yoshii, Wulbeck 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, Dolezel, Stehlik 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, Helfrich-Forster, Renn et al., 2006) which generally directs the differentiation of opsin-based
photoreceptors (Moses, Ellis, Rubin, 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; Helfrich-Forster, 2005b; Shafer, Helfrich-Forster, Renn et al., 2006; Helfrich-Forster, Yoshii, Wulbeck et al., 2007), and seem to be involved in temperature entrainment (Miyasako, Umezaki, Tomioka, 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 Queensland fruitfly, *Bactrocera tryoni* and *B. 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, P. H. Fletcher, B. S., 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, Wilkes, Bastian et al., 2002; An, Tebo, Song et al., 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) versus day (*neohumeralis*-like) mating, revealing a putative association between *Cry* levels and temporal sexual isolation (An, Tebo, Song 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, Tebo, Song et al., 2004).

The relationship of Cry levels to possible day versus 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, Miyatake, Watari et al., 1997; Miyatake, 2002; Koyama, Kakinohana, Miyatake, 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, Miyatake, Watari 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, Oldroyd, Wood 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, Matsumoto, Matsuyama 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, Matsumoto, Matsuyama et al., 2002).

Similar results have also been generated in Drosophila, where D. pseudoobscura shows a later time of mating than D. melanogaster (Tauber, Roe,
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, Roe, Costa *et al.*, 2003). Mating rhythms in the sibling species *D. melanogaster* and *D. 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, Shimada, Kostal, 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, Zavodska, Shimada *et al.*, 2008).
The mature npd larva shows very low levels of \textit{Cctim} mRNA, and no evidence for immunoreactivity to a conspecific \textit{CcTIM} antiserum. Circadian cycles of \textit{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 \textit{CcTIM}, 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, Zavodska, Shimada \textit{et al.}, 2008). Thus there seems to be a correlation between the presence of photoperiodic diapause and \textit{Cctim} expression in this species, as well as changes in \textit{CcTIM} expression with different photoperiods. This is also seen in the diapaus ing pitcher plant mosquito, \textit{Wyomia smithii}, in which \textit{Wstim} expression changes consistently with latitude in North America (Mathias, Jacky, Bradshaw \textit{et al.}, 2005). While linkage analysis may suggest that the \textit{Wstim} locus is associated with photoperiodism, it has been argued that this is unlikely to involve the circadian clock directly (Bradshaw, Holzapfel, Mathias, 2006; Mathias, Jacky, Bradshaw \textit{et al.}, 2007).

The long standing debate on whether clock genes can contribute to photoperiodic diapause in insects (Nunes, Saunders, 1999; Bradshaw, Holzapfel, Mathias, 2006) has been further stoked by the findings that in \textit{D. melanogaster}, a new \textit{tim} natural variant, \textit{ls-tim} appears to be under natural selection because it enhances the level of diapause in the European seasonal environment (Tauber, Zordan, Sandrelli \textit{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, Tauber, Pegoraro \textit{et al.}, 2007). 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 ~15000 years ago (Kyriacou, Peixoto, Sandrelli *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, Zordan, Sandrelli *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, Peixoto, Sandrelli *et al.*, 2008).

**CLOCKS IN OTHER DIPTERA**

The clock genes of a number of other flies have been compared, the most comprehensive study so far is probably that in *Musca domestica*, the housefly (Codd, Dolezel, Stehlik *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, Tebo, Song *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 *MdPER* protein is, surprisingly, produced constitutively as assessed by Western blots in both heads and thoraces, and analysed separately in males and in females (Codd, Dolezel, Stehlik *et al.*, 2007). A similar situation exists in the medfly, *Ceratitis capitata* (Mazzotta, Sandrelli, Zordan *et al.*, 2005), whereas in the sheep blowfly, *Lucilia cuprina*, both gene products
appear to cycle (Warman, Newcomb, Lewis et al., 2000). MdTIM on the other hand shows the expected cycles in LD and DD. In LL however, MdPER remains highly stable, whereas MdTIM degrades immediately, so unlike Drosophila, MdPER does not apparently require MdTIM for stability.

Initial immunohistochemistry (IHC) using head sections revealed various MdPER staining neurons, particularly in medial and lateral areas in which the signal was always cytoplasmic at every time point (Codd, Dolezel, Stehlik 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 MdPER, but not MdTIM. An anti-PDH reagent also detected a group of neurons that seemed homologous to the s-LNvs and l-LNvs of Drosophila (Pyza, Siuta, Tanimura, 2003; Codd, Dolezel, Stehlik et al., 2007), and, when re-examined carefully, these cells were also observed to coexpress MdTIM and MdPER (see Fig 3). Furthermore the Musca s-LNvs showed nuclear MdPER/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 MdPER/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 MdPER and MdTIM. It thus appears that the constitutive MdPER expression observed in Western blots comes from those very
highly, constitutively cytoplasmically expressing medial/medial-lateral \textit{MdPER} neurons that do not co-express \textit{MdTIM} (see Fig 3).

This study reveals that the housefly has considerable similarities in clock gene expression to \textit{Drosophila}, but also some notable differences. What, for example is the function of the \textit{MdPER}-reacting material in the medial-lateral cells which does not co-express \textit{MdTIM} and therefore cannot require \textit{MdTIM} for stability? Indeed, is it \textit{MdPER} 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 \textit{MdPER} band in a \textit{Musca} head Western blot, which is constitutively expressed, likely reflects the dynamics of constitutive ‘\textit{MdPER}’ in these medial neurons. This band is the same size as the band seen in \textit{Drosophila per}-null transformant carrying the \textit{Mdper} transgene, so it seems very likely that this antigenic material in both blots and IHC is indeed \textit{MdPER} (Piccin, Couchman, Clayton et al., 2000).

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

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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 *MdPER* and *MdTIM* 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’ (eg Zavodska, Sauman, Sehnal, 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, Meireles-Filho, Britto *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, Amoretty, Souza *et al.*, 2006; Meireles-Filho, da S Rivas, Gesto *et al.*, 2006). *Llper* and *Lltim* are also down regulated by blood-feeding, which also causes a reduction in locomotor activity (Meireles-Filho, da S Rivas, Gesto *et al.*, 2006) *Lutzomya* appears to have a C-terminal transactivation domain that is characteristic of CYC in mosquitos (Gentile, Meireles-Filho, Britto *et al.*, 2006), bees (Rubin, Shemesh, Cohen *et al.*, 2006a), and moths (Chang, McWatters, Williams *et al.*, 2003), but is absent in *Drosophila* (Rutila, Suri, Le *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, Gotter, Casselman *et al.*., 2003). Most of the comparative circadian studies conducted on Lepidoptera involve the giant silkworm, *Antheraea pernyi*, the domestic silk moth *Bombyx mori* and the monarch butterfly, *Danaus plexippus*, (Sauman, Hashimi, 1999; Chang, McWatters, Williams *et al.*, 2003; Sehadova, Markova, Sehnal *et al.*, 2004; Takeda, Chuman, Shirasu *et al.*, 2004; Zhu, Yuan, Briscoe *et al.*, 2005; Iwai, Fukui, Fujiwara *et al.*, 2006; Reppert, 2006; Trangle, Sehadova, Ichihara *et al.*, 2006; Iwai, Thi Dieu Trang, Sehadova *et al.*, 2008; Zhu, Casselman, Reppert, 2008; Zhu, Sauman, Yuan *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, Levine, Reppert, 1999). Conversely, *B. mori* has only a single copy of the *per* gene in both males and females (Takeda, Chuman, Shirasu *et al.*, 2004; Iwai, Fukui, Fujiwara *et al.*, 2006; Sandrelli, Cappellozza, Benna *et al.*, 2007), that produces two BmPER isoforms, differing by 5 aa in length (Takeda, Chuman, Shirasu *et al.*, 2004) whereas in the monarch butterfly only a single *Dpper* cDNA has been isolated (Froy,
Gotter, Casselman 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, Tsai, Roca et al., 1994; Takeda, Chuman, Shirasu et al., 2004).

All three species appear to encode orthologues for Drosophila tim (Chang, McWatters, Williams et al., 2003; Iwai, Fukui, Fujiwara et al., 2006) Dptim AY36059 (http://rana.lbl.gov/Drosophila/), and timeout/tim2 has been reported in B. mori (Rubin, Shemesh, Cohen et al., 2006a). 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, McWatters, Williams et al., 2003; Iwai, Fukui, Fujiwara 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, McWatters, Williams et al., 2003; Markova, Ueda, Sakamoto et al., 2003; Zhu, Yuan, Briscoe et al., 2005; Rubin, Shemesh, Cohen et al., 2006a). As stated above, both cry-d and cry-m like genes have been identified in the three species (Zhu, Yuan, Briscoe et al., 2005; Yuan, Metterville, Briscoe 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, Tsai, Roca et al., 1994; Froy, Gotter, Casselman et al., 2003; Iwai, Fukui, Fujiwara et al., 2006; Zhu,
Sauman, Yuan et al., 2008). Bmal1/cyc mRNA expression in B. mori does not show any significant daily oscillation (Markova, Ueda, Sakamoto 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, Sauman, Yuan et al., 2008).

Brain lesion and transplantation experiments performed in the 1970’s in A. pernyi mapped the Lepidopteran circadian clock to the dorsolateral protocerebrum (Truman, 1972; Truman, 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 Ia₁ 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, Davis, Tyndale et al., 2002; Sehadova, Markova, Sehnal et al., 2004; Sehadova, Markova, Sehnal et al., 2004; Sauman, Briscoe, Zhu et al., 2005; Zhu, Sauman, Yuan et al., 2008).

In A. pernyi, type Ia₁ neurons express ApPER throughout pupal and adult development. In adults, these neurons show cycling expression of ApPER and ApTIM 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 Apper mRNA and proteins. All these molecular oscillations were obliterated in LL condition, when Antheraea also shows an arrhythmic adult eclosion. Subcellular localization studies revealed that both ApPER and ApTIM 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, Markova, Sehnal *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-Ia1 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, Briscoe, Zhu *et al.*, 2005; Zhu, Sauman, Yuan *et al.*, 2008) (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, Briscoe, Zhu *et al.*, 2005; Zhu, Sauman, Yuan *et al.*, 2008). 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, Briscoe, Zhu *et al.*, 2005; Zhu, Sauman, Yuan *et al.*, 2008).

**Functional characterization of clock components**

Function analysis of Lepidopteran clock elements was studied *ex in vivo* in *Dp*N1 cells, a monarch butterfly embryonic line (Zhu, Sauman, Yuan *et al.*, 2008). In these cells, *Dp*CRY2 was able to inhibit *Dp*CLK/*Dp*BMAL-mediated transcription,
while \textit{DpPER} and \textit{DpTIM} did not, a result consistent with \textit{DpCRY2}’s nuclear localization in neurosecretory cells. In addition, both in monarch brains and \textit{DpN1} cells, \textit{DpCRY2}, TIM, and PER appear to form a complex, as indicated by immunoprecipitation experiments. However, in other cellular environments, such as \textit{Drosophila} embryonic S2 cells, \textit{DpPER}, as well as \textit{ApPER} and \textit{DmPER}, are capable of acting within the nucleus to inhibit the CLK:BMAL1-mediated transcription (Chang, McWatters, Williams \textit{et al.}, 2003; Zhu, Sauman, Yuan \textit{et al.}, 2008). Furthermore, when \textit{ApPER} is expressed in the clock neurons of \textit{per}^{01} \textit{Drosophila} transformants, it shows the nuclear/cytoplasmic oscillation typical of \textit{DmPER} and is able to modestly rescue \textit{per}^{01} arrhythmic behaviour (Levine, Sauman, Imbalzano \textit{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 \textit{DpCRY2} that acts as the major clock transcriptional repressor within the monarch. The \textit{DpCRY2} interaction with \textit{DpPER} and \textit{DpTIM} is probably cytoplasmic and may have a stabilizing function before nuclear translocation (Zhu, Sauman, Yuan \textit{et al.}, 2008), as occurs in mammals (Lee, Etchegaray, Cagampang \textit{et al.}, 2001).

In \textit{DpN1} cells, \textit{DpCRY1} is able to induce light-dependent \textit{DpTIM} degradation (Zhu, Sauman, Yuan \textit{et al.}, 2008). These results are consistent with the ability of a \textit{Dpcry1} transgene (but not \textit{Dpcry2}) to partially rescue the circadian light responses of the \textit{cry}^{b} mutant in transgenic \textit{Drosophila} (Sauman, Briscoe, Zhu \textit{et al.}, 2005; Zhu, Sauman, Yuan \textit{et al.}, 2008).

A feature that seems to be common at least to \textit{A. pernyi} and \textit{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, Markova, Sehnal \textit{et al.}, 2004). In \textit{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, Kataoka, Oka et al., 1990; Sauman, Reppert, 1996). In addition, BmPER 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, Markova, Sehnal et al., 2004). These patterns suggest a regulatory activity for PER at the level of the neurohormones involved in postembryonic development. A role for BmPER in B. mori development has been suggested also by the observed reduction in egg-to-adult developmental time induced by Bmper dsRNAi (Sandrelli, Cappellozza, Benna et al., 2007) and might be due to a pleiotropic effect on development, as already demonstrated for the Drosophila per clock gene (Kyriacou, Oldroyd, Wood et al., 1990). This post-transcriptional manipulation of Bmper also generated a mild disruption of the egg hatching rhythm (Sandrelli, Cappellozza, Benna et al., 2007), something which was also initially seen in A. pernyi with Apper antisense injections (Sauman, Tsai, Roca et al., 1996), but this finding was subsequently withdrawn (Sauman, Tsai, Roca 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, Markova, Sehnal et al., 2004). In the monarch, DpPER, DpTIM, DpCRY1 and DpCRY2 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., 2008). $Dp$PER expression was also identified in the corpora cardiaca and for $Dp$CRY2, in both corpora cardiaca and allata. Moreover, clock proteins were also detected in different regions of the optic lobe (Sauman, Briscoe, Zhu et al., 2005; Reppert, 2007; Zhu, Sauman, Yuan et al., 2008). Finally, a complex series of both $Dp$CRY1- or CRY2-positive nerve fibers have been revealed in the brain (Sauman, Briscoe, Zhu et al., 2005; Reppert,2007; Zhu, Sauman, Yuan et al., 2008)(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, Briscoe, Zhu et al., 2005; Reppert, 2006). Finally, $Dp$CRY2 positive fibres can be traced from the four PL neurons to the central brain complex (Zhu, Sauman, Yuan et al., 2008), a region that has been recently identified as the site of the sun compass in locusts (Heinze, Homberg, 2007). Zhu et al (2008) thus propose a dual role for $Dp$CRY2: that of a key
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* (Nishiitsu-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, Homberg, Loesel *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 neurosecretory *pars intercerebralis*.
In the cricket *Teleogryllus* some neurons in the AMe are immunoreactive for both anti-PER and anti-PDH, suggesting a *Drosophila*-centric view of clock anatomy (Lupien, Marshall, Leser *et al.*, 2003). In the ground cricket, *Dianemobius nigrofasciatus* immunoreactivity to PER, DBT, CRY and CLK was observed predominantly 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 intercerebralis* and suboesophageal ganglion (Shao, Sehadova, Ichihara *et al.*, 2006; Shao, Bembenek, Trang le *et al.*, 2008). In contrast, in another ground cricket, *Allenomobius allardi*, these clock proteins appear to be particularly enriched in the suboesophageal ganglion (Shao, Sehadova, Ichihara *et al.*, 2006; Shao, Hiragaki, Takeda, 2008). 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; Helfrich-Forster, 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, Steel, Terry, 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 attenuated
(Shemesh, Cohen, Bloch, 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 compared to foragers (Shemesh, Cohen, Bloch, 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 colony, 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, Bloch, Moore et al., 2000; Bloch, Toma, Robinson, 2001).

CONCLUSIONS AND FUTURE PROSPECTS.

Hemi-metabolous insects such as cockroaches and crickets, have thus provided the best material for surgical manipulations, and together with expression studies of clock molecules, they have significantly extended the ‘evo-devo’ type of analysis of clock neuroanatomy. However, one common ‘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 exclusively cytoplasmic (Zavodska, Sauman, Sehnal, 2003a; Zavodska, Sauman, Sehnal, 2003b). One notable exception is the hawkmoth, Manduca sexta, where PER nuclear antigenicity is observed in putative circadian neurons (Wise, Davis, Tyndale et al., 2002; Schuckel, Siwicki, Stengl, 2007).
However, in general, one can surmise that either a novel form of clock gene regulation is present (as discussed earlier 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, Dolezel, Stehlik *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, Sauman, Yuan *et al.*, 2008). One further important consideration that comes from these various 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, Sauman, Yuan *et al.*, 2008).

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 development (Kyriacou, Oldroyd, Wood *et al.*, 1990; Shimizu, Miyatake, Watari *et al.*, 1997; Miyatake, 2002; Koyama, Kakinohana, Miyatake, 2004; Sandrelli, Cappellozza, Benna *et al.*, 2007), mating (Tychsen, P. H. Fletcher, B. S., 1971; Smith, 1979; Sakai, Ishida, 2001; An, Wilkes, Bastian *et al.*, 2002; Miyatake, Matsumoto, Matsuyama *et al.*, 2002; Tauber, Roe, Costa *et al.*, 2003; An, Tebo, Song *et al.*, 2004), navigation (Sauman, Briscoe, Zhu *et al.* 2005; Zhu, Sauman, Yuan *et al.* 2008) and social (Toma, Bloch, Moore *et al.*, 2000; Bloch, Toma, Robinson, 2001) behaviour in different species, revealing that clock proteins will have extensive pleiotropic effects. This has 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, Wijnen, Naef et al., 2001; McDonald, Rosbash, 2001; Ceriani, Hogenesch, Yanovsky et al., 2002; Lin, Han, Shimada et al., 2002; Ueda, Matsumoto, Kawamura et al., 2002). Consequently 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.
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REFERENCES


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<td><em>Rhodnius prolixus</em></td>
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<td>Steel, Vafopoulou, 2006; Vafopoulou, Steel, Terry, 2007</td>
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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 feedback 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.

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, Yuan, Briscoe et al., 2005; Rubin, Shemesh, Cohen et al., 2006a; Yuan, Metterville, Briscoe et al., 2007; Zhu, Sauman, Yuan et al., 2008)

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, Dolezel, Stehlik 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, Briscoe, Zhu et al., 2005; Reppert, 2006; Zhu, Casselman, Reppert, 2008)
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