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Insect Clocks: What are They Telling Us Besides Time?

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Abstract. Circadian biological clocks are fascinating time-keeping devices that are found in all studied eukaryotic organisms and some prokaryotes as well. Our understanding of molecular mechanisms underlying the function of circadian clocks has developed tremendously within the past few years. Several different insect species, from both endopterygota and exopterygota subdivisions, served as important model organisms in physiological and molecular studies of circadian biological oscillators. The aim of this review is to summarize the current understanding of the fundamental molecular mechanisms of insect circadian clocks and stress the similarities as well as the differences found among the studied insect model species.

Key words: Circadian clock, insect, *Drosophila,* silkmoth, *period, timeless.*

Introduction

Most, if not all, living organisms show biological cycles at a molecular, physiological and behavioral level. These cycles are controlled by endogenous biological clocks, and in general can be divided into three major categories: circadian rhythms with a period close to 24 h, ultradian rhythms with a period considerably shorter than 24 h and infradian rhythms with period much longer than 24 h. There is no doubt that due to astonishing advancement in molecular genetics, the best studied biological oscillators are the circadian clocks. Circadian biological clocks among all organisms share three common characteristics. First, as mentioned before the biological clock is endogenous, genetically determined, with a period about 24 h. Unlike oscillations, which are driven by periodical changes in the environment, the endogenous biological clock can "free-run"in constant conditions without any surrounding environmental oscillations. Second, the clock can be entrained, or reset by environmental stimuli, the most prominent being light and temperature. Entrainment occurs on daily basis and ensures synchronization of the internal biological clock with external environmental oscillations. Third, the circadian clock is temperature insensitive within a certain range of physiologically plausible temperatures. This means, that for instance in the giant silkmoth, the clock is "ticking" with exactly the same speed in diapausing pupa at sub-zero temperatures and in the adult moth at temperatures over 25 or 30℃. This phenomenon, which seemingly escapes the laws of thermodynamics, is referred to as temperature compensation.

Each circadian timing system consist of three parts: the input pathway, the core oscillator, and the output pathway. The clock input pathway provides the connection of the clock with the surrounding environment and is necessary for entrainment. The important part of the input system are photoreceptors, which in many cases are distinct from the visual system, the so called extraocular photoreceptors. The core oscillator represents the time-keeping device, the endogenous clock, which can free-run without any external stimuli. The output pathway ensures the transfer of the timing information from the core oscillator to the rest of the organism.

Within the last few years, the analysis of circadian clocks at the biochemical, molecular, and genetic level has accelerated at a phenomenal rate. A central issue to the field of circadian biology is to elucidate the molecular "gears" underlying circadian oscillators. Several gene candidates have been cloned in a variety of species and many are still to be identified. Due to the tremendous advancement in the field, many recent reviews have been written about the subject (Dunlap, 1998a, b; Hall, 1998; Reppert, 1998; Sassone-Corsi, 1998; Schibler, 1998; Wilsbacher & Takahashi, 1998; Young, 1998). This fact makes the task of writing a new review very challenging. The danger is even higher considering the speed of the development of the circadian field and the considerable "phase shift" between writing the review article and its actual appear-

ance in press. Our role is even more complicated by the recent appearance of an excellent and extensive review by Jay Dunlap (1999) summarizing very coherently the entire field of "molecular circadian biology". For this reason and also considering the main field of our research, we will focus on circadian clocks in insects, stressing the main similarities and also surprising differences between the *Drosophila* timing system and some other insect model organisms.

The research of circadian clocks has a long tradition and insects very often served as important model systems. There is no doubt that the fruitfly, *Drosophila melanogaster,* due to the enormous power of its genetics, represents the premier insect species for the molecular analysis of the circadian rhythms. The first paper providing direct evidence of the genetic basis of the biological clock came from the study by Konopka & Benzer (1971). They used chemical mutagenesis in *Drosophila* and subsequently screened for genetic variants with unusual circadian rhythms. As a result, they have isolated three mutant lines in circadian rhythmicity. All three mutations mapped to the same locus on the X-chromosome, which was named the *period* (*per*) gene (Konopka & Benzer, 1971). The first of the three mutations, called per^0, exhibited completely arrhythmic behavior. The second mutation had behavioral rhythms much shorter than wild-type flies, the period (τ) being about 19 h, and was thus named per^S. The last mutant line, per^L, exhibited long behavioral rhythms of about 28 h. Strikingly, this last mutation not only affected the period length, but the per^L flies also lost temperature compensation, a fundamental feature of all circadian timing systems. This finding strongly suggested that even this crucial clock property is genetically determined.

The Drosophila Clock

The classic paper by Konopka and Benzer initiated the intensive molecular study of the *Drosophila melanogaster per* gene in the 1980's. An early observation revealed that the PER protein oscillates in its abundance with a clear circadian rhythm in the fruitfly heads. The most pronounced PER cycling was detected in the photoreceptors of the visual system and also in specific lateral neurons in the central brain (Siwicki *et al.,* 1988). PER protein staining reached peak intensity late at night and disappeared during the day phase of the light–dark (LD) cycle. Soon afterwards, it was demonstrated that the *per.* mRNA also oscillates in the fruitfly heads (Hardin *et al.,* 1990). Interestingly, the peak level of the *per* mRNA was reached early at night, about six hours earlier than the peak of PER protein. This striking temporal delay between *per* mRNA and protein levels indicated the presence of a mechanism, which uncouples *per* transcription from immediate translation. This also suggested some form of negative feedback, in which the PER protein either directly or indirectly inhibits its own transcription. The key parameters for this negative feedback oscillator model is the temporal delay between the accumulation of *per* mRNA and protein and the stability of PER product. A variety of mathematical models of *per* oscillation taking into account the tem*per*ature compensation have confirmed that these are indeed the two critical variables leading to a *per*petual molecular cycle (Goldbeter, 1995; Ruoff, 1998).

If it is assumed that PER protein is directly involved in the negative regulation of its own transcription, then it should fulfill the following two criteria: first, it should move to the nuclei of the *per* expressing cells to turn off its own gene; second, the PER protein should contain a DNA binding domain, which would allowed PER to bind to *per* gene promoter. The former prediction was confirmed by immunocytochemical observation that PER protein indeed moves to the nucleus of *Drosophila* eye photoreceptors, as well as lateral neurons in the central brain (Curtin *et al.,* 1995). In both cases, the nuclear translocation occurs late at night when the PER protein level reaches its maximum. However, amino acid sequence analysis of PER protein failed to confirm the latter prediction. The PER protein does not contain any known DNA binding motif and therefore is highly unlikely to bind to its own promoter and act directly on *per* transcription. Database comparison revealed, however, that PER contains so called PAS domain (according to its presence in PER, in the human aryl hydrocarbon receptor nuclear translocater protein, ARNT, Hoffman *et al.,* 1991, and in the *Drosophila single-minded* protein, SIM, Crews *et al.,* 1988). The PAS domain is a protein-protein dimerization motif found in a large family of proteins (Pellequer *et al.,* 1998). This finding initiated a search for a potential PER partner, which, if also equipped by a proper DNA binding domain, could mediate PER's transcriptional function. The hunt for PER's dimerizing partner was successful and its corresponding cDNA was independently cloned by two different molecular approaches reported in the same issue of Science (Myers *et al.,* 1995; Gekakis *et al.,* 1995). Myers and his colleagues (1995) reported positional cloning of a second *Drosophila* clock gene, *timeless* (*tim*). Using a yeast two-hybrid system as a screen for potential

PER-interacting partners, Gekakis and colleagues (1995) reported cloning of a putative PER dimerization partner. Remarkably, the protein turned out to be identical to TIM. Sequence analysis revealed that *tim* encodes a novel protein and shows no homology to PER. Surprisingly, TIM does not contain a PAS domain thus suggesting a heterotypic interaction between TIM and PER (Reppert & Sauman, 1995). Mutations of the *tim* locus produce phenotypes similar to those of *per* mutants (Sehgal *et al.*, 1994). Temporal analysis of *tim* expression showed that both the *tim* mRNA and the protein undergo striking circadian oscillations in their abundance, with approximately identical phases as the corresponding products of the *per* gene. Both genes are also co-expressed in the same population of cells in *Drosophila* central brain and visual system (Sehgal *et al.*, 1994; Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996). Furthermore, the arrhythmic *tim* mutation also abolishes the molecular oscillations of the *per* gene products (Sehgal *et al.*, 1995) indicating that *tim* and *per* form interdependent molecular feedback loops. Further spatial analysis revealed that PER and TIM proteins indeed dimerize in the cytoplasm of *per* and *tim* co-expressing cells and both undergo translocation to the nucleus late at night (Zeng *et al.*, 1996; Lee *et al.*, 1996). PER product is progressively phosphorylated during the night phase (Edery *et al.*, 1994) suggesting this phosphorylation is largely responsible for the delay between the appearance of PER and TIM monomers in the cytoplasm early at night and the formation of PER-TIM dimers late at night shortly before their nuclear translocation. Recently, a new gene involved in the *Drosophila* circadian timing system, *double-time* (*dbt*), was cloned and characterized (Kloss *et al.*, 1998; Price *et al.*, 1998). The DBT protein is closely related to the human casein kinase and mutations in the *dbt* gene alter period length of *Drosophila* circadian rhythms. Remarkably, it was found that DBT is involved in phosphorylation of PER monomers as soon as they are translated in the cytoplasm. The phosphorylation destabilizes PER protein preventing its premature accumulation in the cytoplasm (Kloss *et al.*, 1998; Price *et al.*, 1998). As TIM levels start to rise in the cytoplasm during the night, the effect of DBT is suppressed via an unknown mechanism and as a result enough PER monomers become available to dimerize with TIM. After dimerization, the PER-TIM complex is translocated to the nucleus. As mentioned above, the tim^{01} mutation eliminates circadian oscillation of both *per* transcript and protein (Vosshall *et al.*, 1994; Price *et al.*, 1995) and similarly per^{01} mutation has the same effect on the products of the *tim* gene (Sehgal *et al.*, 1995). Furthermore, both mutations also block nuclear localization of their respective dimerization partner (Vosshall *et al.*, 1994; Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996). Although both proteins contain a nuclear localization signal, they are restricted to the cytoplasm when expressed individually (Saez *et al.*, 1996). Deletion mutagenesis of PER and TIM proteins revealed a functional domain on both proteins, called the cytoplasmic localization domain (CLD), which anchors the proteins in the cytoplasm and promotes cytoplasmic accumulation (Vosshall *et al.*, 1994; Saez *et al.*, 1996). The heterodimerization of PER and TIM proteins overcomes the cytoplasmic blockade and the dimer translocates to the nucleus. This regulation of PER/TIM nuclear entry can thus serve as one of the critical checkpoints in the circadian molecular oscillation and can also contribute to the delay between *per* and *tim* transcription and translation that is essential for a stable 24 h molecular oscillation (Saez *et al.*, 1996; Kay & Millar, 1995; Reppert & Sauman, 1995). After entering the nucleus, PER/TIM complex is presumably involved in negative regulation of *per* and *tim* transcription. This transcriptional inhibition is lifted after ultimate degradation of the complex.

One of the fundamental features of all circadian clocks is their ability to be entrained by environmental stimuli, particularly by light. It has been demonstrated that even very short pulses of light can reset the circadian clock. In many species including *Drosophila,* light pulses administered under free-running conditions (constant darkness and temperature) cause phase delays early evening and phase advances late at night, generating a so called phase response curve (PRC; Saunders, 1982; Saunders *et al.*, 1994). If *per* and *tim* are *bona fide* clock genes, parts of the core clock oscillator, one would predict that the molecular oscillations of their respective products (mRNA and protein) will be reset by light pulses. This prediction was confirmed by finding that TIM protein is sensitive to light and is rapidly degraded by acute exposure to illumination (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). In turn, the levels of PER protein start declining shortly after TIM because its stability greatly depends on TIM (Lee *et al.*, 1996; Zeng *et al.*, 1996). Coming back to the PRC, the mechanism of the phase delay as well as the phase advance can be explained by the acute sensitivity of TIM protein to light. Light pulse administered early at night will cause degradation of TIM in the cytoplasm. Because of the high level of

tim mRNA at this time of the day, the cytoplasmic TIM is recovered by translation of its available transcript. The time necessary to reach the pre-pulse TIM level generates the phase delay of the clock. Similarly, when a light pulse is given late at night, nuclear TIM is degraded, but no *tim* mRNA is available in the cytoplasm at this time of day. Therefore TIM protein drops prematurely to the level that is characteristic for subjective morning. Thus the circadian clock as well as the locomotor activity rhythms appear to advance to a phase that corresponds with a low level of TIM protein.

Since neither PER nor TIM possess any known DNA binding domain, the remaining question is how the PER/TIM dimer represses transcription of *per* and *tim* genes. A logical prediction would be the existence of an additional, unknown partner(s) of PER and TIM, which would possessed DNA binding ability. Recently, two additional mutations disrupting *Drosophila* circadian rhythms have been isolated (Allada *et al.*, 1998; Rutila *et al.*, 1998). The first is *cycle* (*cyc*), also called *dBmal1*, and the second was initially named *Jerk* (*Jrk*), but now is referred to as *dClock*. The cDNAs of both *dBmal1* (*cyc*) and *dClock* (*Jrk*) genes have been cloned and the following sequence analysis revealed that both proteins not only carry a PAS domain, but also contain a basic-helix-loop-helix (bHLH) DNA binding motif. In addition, biochemical analysis showed that both dBMAL1 and dCLOCK proteins can dimerize with PER via its PAS domain (Darlington *et al.*, 1998; Gekakis *et al.*, 1998). The promoters of both *per* and *tim* genes contain a short sequence called an E-box that serves as a binding site for bHLH transcription factors. It has been demonstrated that deletion of the E-box from the *per* promoter considerably reduces *per* transcription and abolishes PER cycling (Hao *et al.*, 1996). A significant progress in our understanding of *Drosophila* clock came from experiments showing that dBMAL1/dCLOCK heterodimer physically binds to the *per* and *tim* E-boxes and constitutively stimulates their expression (Darlington *et al.*, 1998; Gekakis *et al.*, 1998). Furthermore, it has been shown that PER protein directly represses the dBMAL1/dCLOCK-

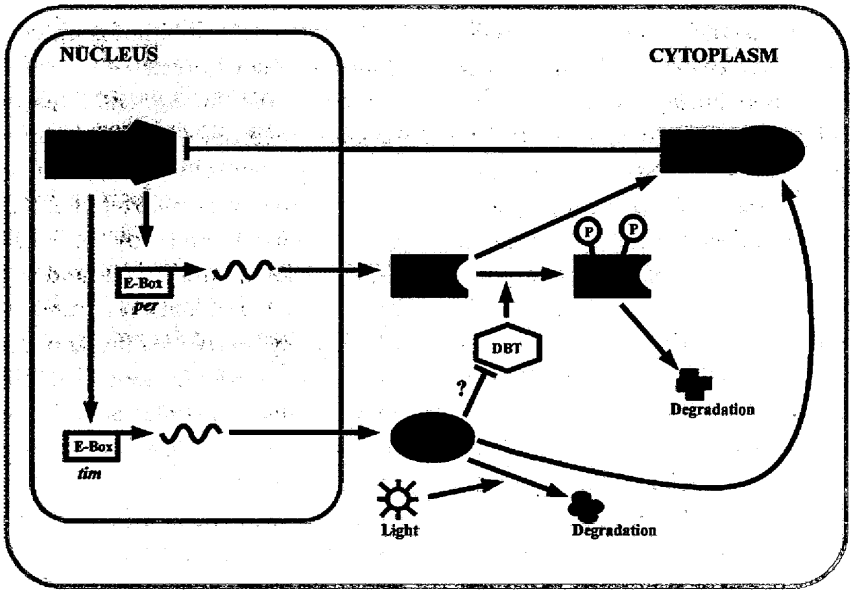

Fig. 1. A cartoon depicting the major biochemical gears in the *Drosophila* clock. Heterodimers of dBMAL1 (CYC) and dCLK (JRK) bind to the E-boxes located in the promoters of the *per* and *tim* genes, constitutively driving their expression. The mRNA levels of both peak early at night. Both are translated into their respective proteins, PER and TIM, in the cytoplasm. PER monomers are immediately phosphorylated by the kinase DBT, signaling the protein for its eventual degradation. The action of DBT fine tunes the clock by preventing premature accumulation of PER in the cytoplasm. DBT is inhibited as TIM levels rise, although it remains unknown whether TIM acts directly or indirectly, or if these two events are coincidental. TIM is also susceptible to degradation by exposure to light, thus enabling the clock to be entrained. PER and TIM form a stable complex late at night, about six hours after the peak in mRNA levels, just as the accumulation of these proteins reaches their maximum. This step serves as a vital checkpoint for maintaining the periodicity of the clock. The PER/TIM heterodimer is then traslocated into the nucleus where it inhibits the action of the dBMAL1/dCLK complex, thus closing the loop of the negative feedback mechanism in the *Drosophila* clock. Arrows indicate positive regulation or direct modulation. Lines terminating in bars exclusively denote negative regulation.

stimulated *per* transcription (Darlington *et al.*, 1998). These last experiments thus closed the molecular loop of the Drosophila core circadian oscillator (Fig. 1).

The Silkmoth Clock

The giant silkmoth, *Antheraea pernyi,* served as another insect model system in classical physiological studies of circadian rhythms initiated more than three decades ago (Williams & Adkisson, 1964; Truman & Riddiford, 1970; Truman, 1971a, b, 1972, 1974). The silkmoth manifests robust circadian behavior in egg hatching, photoperiodic termination of pupal diapause, adult eclosion, and the adult flight rhythm (Truman and Riddiford, 1970; Truman, 1972, 1974; Sauman *et al.*, 1996). The size and the neurohormonal properties of the silkmoth brain greatly facilitated elegant brain lesion and transplantation studies determining the location of the silkmoth circadian pacemaker in the dorsal lateral protocerebrum (Truman, 1972, 1974). The molecular dissection of the silkmoth circadian clock was initiated by cloning the homolog of the *Drosophila* clock gene *period* (Reppert *et al.*, 1994). Besides *A. pernyi, per* homologs were also cloned from other insect species: another silkmoth, *Hyalophora cecropia*; the hawkmoth, *Manduca sexta*; and a hemimetabolous insect, the American cockroach, *Periplaneta americana.* The cloning of *per* from these insect species was the first evidence that the molecular elements forming the basis of the circadian clock may be evolutionarily conserved, at least in the class Insecta. The initial study in *A. pernyi* revealed a prominent circadian oscillation of *per* transcript in the silkmoth heads. Similar to *Drosophila,* PER protein was found to oscillate in the silkmoth eyes and also undergoes nuclear translocation in the photoreceptor cells (Reppert *et al.*, 1994). Subsequent transgenic studies demonstrated that silkmoth PER, when expressed under a control of *Drosophila per* promoter in per^0 flies, exhibits the same nuclear-cytoplasmic dynamic in *Drosophila* lateral neurons, the presumed pacemaker cells of the fruitfly (Levine *et al.*, 1995). The silkmoth *per* transgene was also able to rescue the arrhythmic locomotor behavior of the per^0 flies, clearly demonstrating that silkmoth *per* can function as a molecular component of the *Drosophila* circadian timing system (Levine *et al.*, 1995). The surprising results came from the silkmoth central brain. The expression of PER protein in *A. per*nyi brain is restricted to eight large neurosecretory cells in the dorsal lateral protocerebrum (Sauman & Reppert, 1996), a strikingly different pattern than *Drosophila,* where dozens of brain neurons and hundreds of glial cells express PER protein (Ever *et al.*, 1992; Frisch *et al.*, 1994). The location of the PER expressing cells corresponds to the brain region which was identified in brain lesion and transplantation studies as the location of the circadian clock controlling the silkmoth flight behavior and adult eclosion rhythm (Truman, 1972, 1974). Even more surprising is the fact that the PER protein is confined to the cytoplasm of the neurosecretory cells and is also found in their axonal projections terminating in the ipsilateral corpora allata of the moth retrocerebral complex (Sauman & Reppert, 1996). The axonal transport of PER to these neurohemal organs suggests a possibility that PER may be released into the hemolymph or play some regulatory role in the corpora allata. Interestingly, the *per* expressing cells and their axonal trajectories are strategically positioned in close vicinity to several groups of neurosecretory cells, e.g. prothoracicotropic hormone and eclosion hormone producing cells, whose secretion is known to be under a circadian control (Williams, 1969; Truman, 1992; Sauman & Reppert, 1996). Examination of PER in the eight neurons in the moth central brain revealed a pronounced circadian oscillation of PER protein level, however, there was no detectable PER product in the nuclei of these cells at any time. TIM, the partner of PER in the *Drosophila* timing system, is co-expressed in the same eight neurosecretory cells in the silkmoth central brain. TIM protein also oscillates in these cells, in phase with PER cycling, and like PER, does not undergo the nuclear translocation (Sauman & Reppert, 1996). The eight neurons also co-express *per* mRNA with pronounced circadian oscillation. Unlike *Drosophila,* however, there is no time delay between *per* transcription and translation and both mRNA and protein rhythms are virtually superimposable. Completely unprecedented was the finding that the eight PER positive cells also express antisense *per* RNA (Sauman & Reppert, 1996). Moreover, this antisense *per* transcript undergoes striking circadian oscillations with an opposite phase compared to the *per* sense mRNA and PER (and TIM) protein. As expected for molecules forming the circadian clock, the exposure to constant light that abolishes rhythmic behavior in *A. pernyi* (Truman, 1971; Sauman & Reppert, 1996; Sauman *et al.*, 1996), also disrupts the molecular oscillations of *per* mRNA as well as its corresponding protein. The rhythm of the *per* antisense RNA is also abolished under constant light conditions (Sauman & Reppert, 1996).

As mentioned before, the egg hatching behavior in

A. pernyi is under the control of the circadian clock (Sauman *et al.*, 1996; Sauman & Reppert, 1998). The functional circadian clock controlling this behavior is formed by mid-embryogenesis. This coincides with the appearance of PER and TIM proteins in aforementioned eight cells in the embryonic brain. Similar to the adult brain, PER and TIM are restricted to the cytoplasm of these cells and is never detected in the nuclei. However, PER protein is also expressed in the midgut epithelial cells with striking oscillation and nuclear translocation (Sauman *et al.*, 1996), identical to its behavior in the adult moth ocular photoreceptors. Brain transplantation studies of pharate larvae have determined that the location of the circadian clock gating egg hatching behavior is indeed in the brain (Sauman & Reppert, 1998). In addition, it was shown that PER oscillation in the midgut epithelial cells depends on an intact embryonic brain. The transplantation studies strongly suggest existence of an unknown, humoral factor from the brain that initiates the egg hatching behavior (Sauman & Reppert, 1998).

The results from the silkmoth, *Antheraea pernyi,* are in clear contradiction with the molecular mechanism of the circadian clock in *Drosophila.* If neither silkmoth PER nor TIM enter the nuclei of the putative pacemaker cells, it is difficult to explain their circadian clock function by the negative feedback oscillator model. It has been suggested that perhaps the *per* antisense RNA, which cycles in antiphase with the *per* sense mRNA, can generate the PER oscillation by negatively regulating its translation (Sauman & Reppert, 1996; Hall, 1996; Sassone-Corsi, 1996). If this is true, the function of PER protein in the cytoplasm of clock cells remains a mystery. One possibility mentioned earlier is that PER can play some neurohumoral role by being secreted from the corpora allata into the hemolymph. However, there is no evidence for such a function and this suggestion is purely speculative. It is also feasible that neither silkmoth *per* nor *tim* are central components of the moth circadian clock and are only part of the clock output pathway (Hall, 1996). This conjecture seems less likely, however, considering all the evidence about moth *per* function that has accumulated over the last five years.

Conclusion and Future Prospectives

It is obvious that molecular regulation of circadian clock gene *period* in the silkmoth, *Antheraea pernyi* is strikingly different from *Drosophila* (see Table 1). Perhaps *A. pernyi* represents just some exceptional example in the evolution of circadian clock mechanism in insects. However, the same results were obtained with seven other giant silkmoth species (Sauman & Reppert, 1996; Sauman, unpublished data), perhaps extending this "exception" to all saturniid moths. In addition, we have used several different available antibodies against PER protein to screen all major insect orders for PER-like expression in the adult central nervous system (CNS). In most cases we have obtained clear staining in a restricted population of brain cells, but the PER-like immunoreactivity was always confined to the perikaryal cytoplasm at all times and was never detected in the nuclei (unpublished data). Surprisingly, even the housefly, *Musca domestica,* a species phylogenetically closely related to *Drosophila* (both belong to higher dipterans, the Cyclorrhapha group) exhibits a strikingly different pattern of PER staining. Unlike in *Drosophila, per*-like expression is restricted to a small population of neurons in housefly brain with no indication of nuclear translocation, a critical feature of fruitfly PER function (unpublished data; Kyriacou, personal communication). These preliminary results indicate that the molecular mechanisms underlying the circadian clock in these two dipteran species may be fundamentally different, and that *Musca* exhibits PER staining more similar to the silkmoth. So, the question could be is *Drosophila* the exception in insects after all? Could time really be running out on the *Drosophila* clock?

Table 1. Expression patterns of circadian clock genes in adult central brain.

	Fruitfly	Silkmoth
PER		
Cell No.	>100	8
Cycling	Yes	Yes
Nuclear	Yes	No
Axons	No	Yes
per mRNA cycling	Yes	Yes
per/PER phase	Phase delay	Synchronous
per antisense RNA	No	Yes
TIM		
Co-localization	Yes	Yes
Cycling	Yes	Yes
Nuclear	Yes	No
Axons	No	Yes

The table contrasts the major differences in the circadian timing system between the fruitfly, *Drosophila melanogaster,* and the giant silkmoth, *Antheraea pernyi.* The data included in this table can be found in the following references: Siwicki *et al.*, 1988; Hardin *et al.*, 1990; Reppert *et al.*, 1994; Reppert and Sauman, 1995; Hall, 1996; Sauman & Reppert, 1996; Dunlap, 1998a, 1990.

Apparently, more detailed comparative studies are needed to understand the general concept of the molecular regulation of circadian clocks in insects. So, it is clear that the game is still far from being over . . .

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