

Circadian rhythms in insect disease vectors

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Organisms from bacteria to humans have evolved under predictable daily environmental cycles owing to the Earth's rotation. This strong selection pressure has generated endogenous circadian clocks that regulate many aspects of behaviour, physiology and metabolism, anticipating and synchronising internal time-keeping to changes in the cyclical environment. In haematophagous insect vectors the circadian clock coordinates feeding activity, which is important for the dynamics of pathogen transmission. We have recently witnessed a substantial advance in molecular studies of circadian clocks in insect vector species that has consolidated behavioural data collected over many years, which provided insights into the regulation of the clock in the wild. Next generation sequencing technologies will facilitate the study of vector genomes/transcriptomes both among and within species and illuminate some of the species-specific patterns of adaptive circadian phenotypes that are observed in the field and in the laboratory. In this review we will explore these recent findings and attempt to identify potential areas for further investigation.

Key words: circadian clock - disease vectors - gene expression - genomics

The Earth's rotation around its own axis as well as its angular tilt creates predictable cyclical changes in light and temperature with periods of 24 h and one year. These constant and predictable cycles have imposed a strong selective pressure on organisms to tune their internal physiology, metabolism and behaviour to these rhythmically fluctuating external conditions. Organisms have therefore evolved internal timekeepers to predict these daily and seasonal changes. The first report indicating that these rhythms were endogenously generated was published in 1729 by the French Astronomer Jean Jacques d'Ortous de Mairan. He observed that a sensitive heliotrope plant was able to show daily leaf movements even in the absence of environmental clues, i.e., in controlled laboratory conditions of constant darkness (de Mairan 1729). Because these rhythms are close to, but not exactly 24 h, they are called circadian (Latin: *circa* = about, *dies* = day) and have been observed in organisms ranging from archaea to humans (Edgar et al. 2012), synchronising their physiology, metabolism and behaviour to the Earth's geophysical cycles.

Mosquitoes have contributed to chronobiology with many studies that characterise circadian phenotypes such as oviposition, sugar and blood feeding and locomotor activity (Clements 1999). As early as 1918, self-sustainable circadian flight activity rhythms in the mosquito *Anopheles maculipennis* were reported in controlled laboratory conditions (Roubaud 1918). In a study of spatial and temporal differences in the distribution of two Anopheline mosquitoes in Trinidad, West

Indies, Pittendrigh (1950) observed that although highly correlated with moisture conditions, the blood feeding activity persisted day after day in spite of overall humidity variation. The observation of this endogenous component in the wild, associated with evidence that circadian rhythms were genetically encoded (Bünning 1936), led Pittendrigh to study this phenomenon in detail using *Drosophila* as a model. After describing the circadian pattern of pupal-adult eclosion (Pittendrigh 1954), he artificially selected flies that eclosed either very early or very late in the daily distribution of emergence activity (Pittendrigh 1967). In the following years, numerous studies conducted by Pittendrigh and others helped to build up the conceptual model of the clock that gives rise to circadian rhythms [reviewed in Moore-Ede et al. (1982)]. Its main components are: (i) the "input or zeitgeber", which is any external stimulus that is transmitted to the internal pacemaker to adjust it with the environment, (ii) the pacemaker or biological clock, which is the endogenous oscillator that is self-sustainable, synchronisable and temperature compensated, and (iii) the "output", which is the behavioural and physiological periodic outcome from the interaction between the pacemaker and the input.

Main properties of the biological clocks - (i) Self-sustainability or free-running: even in the absence of periodical input from external factors, the endogenous rhythms persist with a period close to 24 h; (ii) synchronisation: although independent from external factors, light, temperature and chemicals can "entrain" or adjust the clock period to match altered environmental conditions; (iii) temperature compensation: the free-running period does not change over a wide range of temperatures, in contrast to most biochemical processes. Although this feature is conserved in all animals, it is especially relevant for poikilothermic animals such as insects, which change their body temperature over a day or among seasons.

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Over the last 30 years, many advances have been made towards understanding the molecular mechanisms of the circadian clock in the different kingdoms, including cyanobacteria (*Synechococcus elongatus*), filamentous fungi (*Neurospora crassa*), fruit flies (*Drosophila melanogaster*), plants (*Arabidopsis thaliana*), rodents and humans [reviewed in Dong et al. (2010), Baker et al. (2012), Nagel & Kay (2012), Ozkaya & Rosato (2012), Eckel-Mahan & Sassone-Corsi (2013)]. On the other hand, although insect vectors were widely studied at the behavioural level in the last century, only recently have their clock molecules been elucidated. In the present review we will give a brief description of the *Drosophila* circadian clock as a comparative model to study the molecular clock of insect vector species and update several of the recent advances obtained in three insect vectors groups: triatomine bugs, phlebotomine sandflies and mosquitoes, highlighting their similarities and differences. We also discuss how the rich field of natural variation studies conducted in the past could be combined with recent next generation sequencing technologies to better understand these vector's clocks, which have an impact in the dynamics of diseases they transmit and therefore should be considered in future prevention and control programs (Klowden & Zwiebel 2004).

The insect model: D. melanogaster - The isolation of the first *period* (*per*) clock mutants in *D. melanogaster*, heralded the first "behavioural" gene ever to be identified in any species by mutagenesis (Konopka & Benzer 1971). As *D. melanogaster* is by far the best-understood "higher" organism at the genetic and molecular levels, with a bag of molecular/genetic tricks that cannot be equalled, much of what we know about the clock mechanism has understandably focused on this model species. The presence of *Drosophila* clock gene homologues in mammals indicates that the basic features of the circadian pacemaker are well conserved, although interactions among specific clock components vary (Gallego & Virshup 2007). Insects thus share most of the fundamental clock features and the phylogenetic distance among them allows the use of *D. melanogaster* as a good starting point for dissecting the molecular clock in these species. In the following sections, we give a brief description of the *D. melanogaster* molecular clock, but as this is not our main focus we refer readers to a recent review that covers this topic in depth (Ozkaya & Rosato 2012).

Clock anatomy - The circadian clock operates in a cell autonomous manner in almost all tissues in *Drosophila* (Plautz et al. 1997). While the central clock neurons located in the fly lateral brain regulate locomotor activity and mating rhythms (Helfrich-Förster 2004, 2005, Sakai et al. 2004, Helfrich-Förster et al. 2007), peripheral clocks found in the antennae, prothoracic glands (PGs), gut, Malpighian tubules etc. independently control their own circadian programs (Tomioka et al. 2012). Therefore, rather differently from mammals (and some insects) where the central clock in the brain synchronises the peripheral clocks, in *Drosophila* this control seems to be more relaxed. For example, electrophysiological responses to olfactory stimuli display a rhythm driven by

clock genes expressed locally in the antenna, even when the fly's central clock is genetically ablated (Tanoue et al. 2004). Similarly, transplanted Malpighian tubules are able to keep self-sustained out-of-phase cycling for days within the host animal (Giebultowicz et al. 2000). These results suggest that multiple oscillators throughout the fly operate independently from a hierarchical system controlled by the brain via neural or humoral signals as observed in mammals. Nevertheless, internal temporal order is assured by direct light synchronisation (Plautz et al. 1997) assuring that their defined outputs act in unison to maintain physiological homeostasis.

The intracellular circadian pacemaker - Endogenous circadian rhythmicity is generated at the molecular level by interlocked transcriptional and translational feedback loops, which are autonomously operational in many cell types (Fig. 1). In the two main loops, the transcription factors coded by *Clock* (*Clk*) and *cycle* (*cyc*) heterodimerise and bind to E-box (CACGTG) upstream regulatory regions of the genes *per*, *timeless* (*tim*), *vri* (*vri*) and *PAR-domain protein 1* (*Pdp1*), activating their expression (Fig. 2) (Hao et al. 1997, Allada et al. 1998, Darlington et al. 1998, Rutilla et al. 1998, Cyran et al. 2003, Glossop et al. 2003). In the first loop, PER and TIM proteins start to accumulate in the cytoplasm at ZT12 [zeitgeber time where ZT0 is lights on and ZT12 is lights off in a light/dark (LD)12:12 cycle], about 6-8 h after their respective mRNAs. As PER alone is unstable, its accumulation is dependent of its heterodimerisation with TIM (Curtin et al. 1995, Gekakis et al. 1995, Zeng et al. 1996). Upon PER stabilisation by TIM, the PER/TIM heterodimer shuttles into the nucleus and indirectly shuts down *tim* and *per* expression by sequestering CLK/CYC from about ZT18-ZT4 (Lee et al. 1998, 1999, Bae et al. 2000, Yu et al. 2006). Without CLK/CYC mediated activation, *per* and *tim* levels start to decrease concomitantly with PER and TIM degradation, which consequently releases CLK/CYC inhibition, allowing a new round of transcription to occur. In a second loop, *Clk* transcription is regulated by VRI and PDP1, which compete for the same VRI/PDP1-boxes in the *Clk* promoter. While VRI represses *Clk* transcription at around ZT14, PDP1 activates it around ZT18, which creates cyclical production of *Clk* (Cyran et al. 2003, Glossop et al. 2003) (Fig. 1). The pacemaker is also regulated at the posttranslational level by the kinases *doubletime*, *shaggy*, *casein kinase 2* and the phosphatase *PP2A*, which have important roles in controlling the stability and nuclear entry of clock proteins (Price et al. 1998, Martinek et al. 2001, Lin et al. 2002, Sathyanarayanan et al. 2004). These transcriptional/translational regulatory loops generate circadian gene expression of its component molecules in different phases, except for *cyc*, which is constitutively expressed (Fig. 2). The periodic production of TFs is of vital importance for the clock output, as we discuss below.

Input - Light and temperature - Since endogenous rhythms are close to, but not equal to 24 h, the pacemaker has to be adjusted on a daily basis by external factors such as light, temperature, chemicals and social cues to synchronise it to the environmental cycles (Ozkaya &

Rosato 2012). Among them, light was considered until recently to be the most important for entrainment and is the one best characterised at the molecular level.

The mechanism that light uses to reset the clock is through the blue light sensor Cryptochrome (CRY) (Emery et al. 1998, Stanewsky et al. 1998). Upon light activation, CRY binds to TIM and irreversibly commits it to degradation by the proteasome through the E3 ligase protein Jetlag (Ceriani et al. 1999, Naidoo et al. 1999, Busza et al. 2004, Koh et al. 2006, Peschel et al. 2006). In addition BRWD3, a substrate receptor for CRL4 (cullin 4 ring finger E3 ligase), has also been implicated in light induced CRY ubiquitinyation and degradation (Ozturk et al. 2013). As PER is unstable without TIM, PER levels decrease and CLK/CYC inhibition is released, allowing a new cycle of transcription to take place (Hunter-Ensor et al. 1996, Myers et al. 1996, Zeng et al. 1996). Besides resetting the clock every morning, cells that autonomously express CRY can use this mechanism to phase-shift the clock to adapt to new environmental schedules (Ivanchenko et al. 2001, Rush et al. 2006) (Fig. 1).

Although light input was always recognised as the most important aspect for the clock entrainment, recent data suggests that in the wild temperature might be the most prominent zeitgeber (Vanin et al. 2012). Indeed, given its small size and poikilothermic nature, even small differences of 2-3°C are able to phase-shift the *Drosophila* rhythmic locomotor behaviour in constant darkness (Wheeler et al. 1993). So far, mutations in four genes have been isolated that impair temperature synchronisation: *no-receptor-potential-A* (*norpA*), *nocte*, *transient receptor potential (TRP) cation channel A1* and *pyrexia* (*Pyx*). Although their precise molecular

mechanisms are not entirely clear, it is known that *norpA* acts on the thermal-dependent regulation of *per* splicing, which increases PER levels that consequently advances the locomotor activity phase (Collins et al. 2004, Majercak et al. 2004). On the other hand, *nocte* appears to be important for proper structure and function of chordotonal organs (cho), which send temperature information to the brain, highlighting the importance of this sensory structure for temperature synchronisation (Sehadova et al. 2009). *Pyx* and *TRP1* are TRP channels that function as thermo-receptors and, while *Pyx* is expressed in the cho, *TRP1* is expressed in the brain (Lee & Montell 2013, Wolfgang et al. 2013). Importantly, temperature reception in the brain is dependent on cho input (Sehadova et al. 2009), which highlights an interesting difference between light and temperature entrainment: while light can activate CRY in a cell autonomous manner, temperature acts on different receptors in different tissues and their coordination is important for the clock's thermal entrainment.

Output - Expression patterns - One of the ways by which the circadian pacemaker drives rhythmic output rhythms in behaviour, physiology and metabolism is by the circadian expression of downstream target genes driven by the pacemaker transcription factors. Studies using DNA microarrays have conservatively estimated 10% of the *Drosophila* genome to be rhythmically expressed, covering a broad spectrum of cellular pathways (Claridge-Chang et al. 2001, McDonald & Rosbash 2001, Ceriani et al. 2002, Lin et al. 2002, Ueda et al. 2002, Keegan et al. 2007). Among them, one study went further and compared the rhythmic expression in different

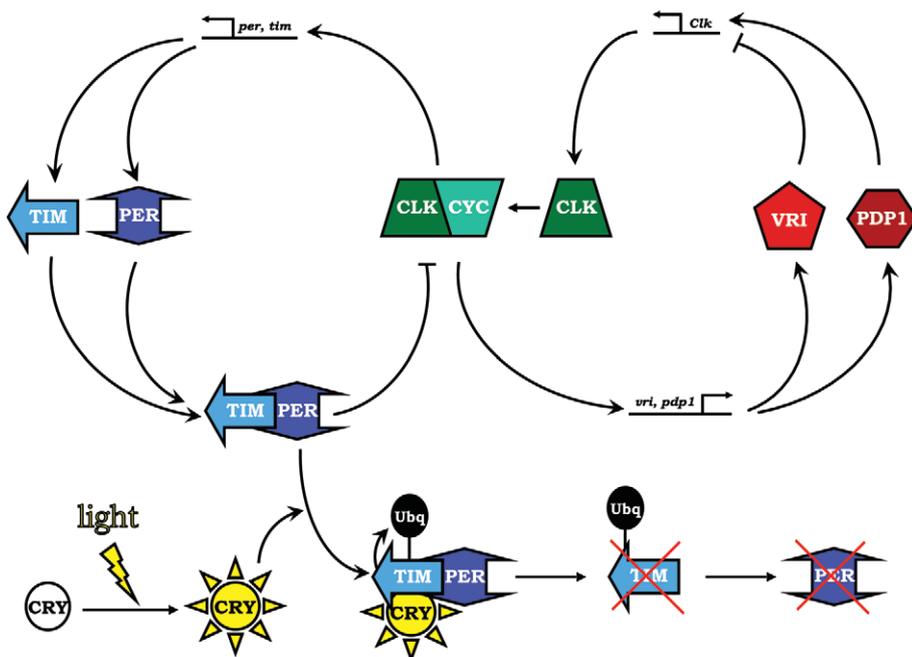


Fig. 1: model of the *Drosophila melanogaster* molecular clock comprising the two transcriptional/translational feedback loops mechanism and the light input pathway. CLK: Clock; CYC: Cycle; PDP1: PAR-domain protein 1; PER: Period; TIM: Timeless; Ubq: ubiquitin; VRI: Vrille.

tissues, i.e. heads and bodies and identified that less than 10% of the cycling genes were shared between these datasets (Ceriani et al. 2002). This suggests that the clock control over output rhythms is highly tissue-specific and that genes that do not cycle in one tissue might cycle in another to drive tissue-specific physiological outputs. In agreement with that, more recent datasets on circadian expression in specific tissues have identified cycling genes correlated with tissues' specific functions. For example, most of the genes controlled by the circadian clock in the fly fat body are involved in physiological processes such as metabolism, steroid hormone regulation, detoxification and the immune response (Xu et al. 2011). In addition, the extent of the cycling transcriptome in clock cell neurons has revealed that the amplitude of cycling mRNAs in pigment dispersing factor expressing key pacemaker neurons is much higher than in other regions of the head (Kula-Eversole et al. 2010). Furthermore, in fly heads, more mRNAs cycle than nascent RNAs, suggesting a strong post-transcriptional component contributing to the cycling transcriptome (Rodriguez et al. 2013). These data highlight the importance of using more defined cell populations for a comprehensive “omics” understanding of the circadian clock.

Insect vector clocks - Insect vectors have revealed that a diverse number of events in their life cycle are controlled by an endogenous pacemaker. They have evolved particular circadian outputs that reflect adaptations for exploring specific environmental conditions as well as for coping with the resting periods of hosts and predators. Thus, the behavioural activity generally referred as “locomotor” in *Drosophila* comprises in insect vectors of a plethora of actions including sugar feeding, blood feeding, mating swarms and oviposition behaviours (Clements 1999, Saunders 2002, Barrozo et al. 2004b, Lazzari et al. 2004), all of which are important for de-

termining the amount and quality of time spent with their hosts and thus critical for the dynamics of pathogen transmission. Recent years have witnessed a progression in moving from behavioural data (in the wild and controlled laboratory conditions) into physiology, genetics and molecular biology in these species. In the following sections, we will cover the advances in these fields for three groups of insect vectors: triatomine bugs, phlebotomine sandflies and mosquitoes.

Triatomine bugs - Triatomine bugs (Hemiptera: Reduviidae: Triatominae) are large bloodsucking insects that occur mainly in Latin America and the southern United States of America, where they are vectors of Chagas disease. Also known as American trypanosomiasis, this life-threatening zoonosis affects about seven-eight million people worldwide, most of them in Latin America where the disease is endemic. It is caused by the flagellated protozoan parasite *Trypanosoma cruzi*, which is transmitted by the faeces of infected triatomines through the insect sting.

Triatomine bugs are normally found in the burrows and nests of wild animals, on which they feed during the night by sucking blood. A number of species, among them the two major Chagas disease vectors *Triatoma infestans* and *Rhodnius prolixus*, have adapted to living in and near houses, where they feed on humans and domestic animals. They spend most of the daytime in an inactive state, or akinesis, hidden in their refuges. During the night they show two peaks of locomotor activity: one at dusk to search for the host and the second at dawn to take shelter (Lorenzo & Lazzari 1998, 1999). Both peaks of activity are driven by the circadian clock and can be synchronised by light and temperature, but light is the predominant zeitgeber (Lazzari 1992). Interestingly, triatomines show an increased host's odour (carbon dioxide) perception around dusk, which persists in constant

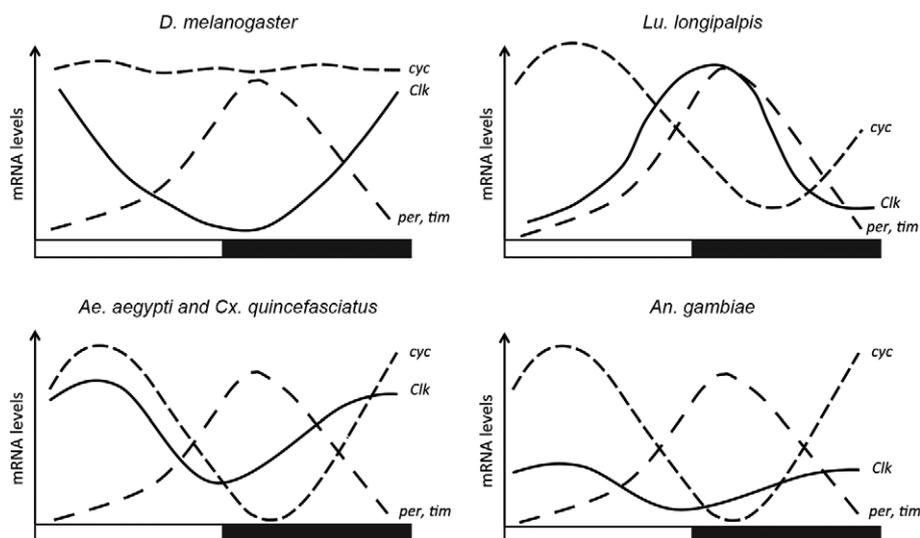


Fig. 2: circadian transcriptional profiles of the canonical clock genes in the main feedback loop from *Drosophila melanogaster*, *Lutzomyia longipalpis*, *Aedes aegypti* and *Culex quinquefasciatus* and *Anopheles gambiae*. Clk: clock; cyc: cycle; per: period; tim: timeless.

conditions and is therefore also endogenously generated (Barrozo et al. 2004a, Bodin et al. 2008).

Besides locomotor activity, a number of other behaviours within the triatomines' life cycle are controlled by the circadian clock such as egg hatching, oviposition, ecdysis, sensitivity to light, thermopreference and light/dark adaptation of compound eyes (Ampleford & Steel 1982, Ampleford & Davey 1989, Lazzari 1992, Reisenman et al. 1998, 2002, Minoli & Lazzari 2003). One of the most dramatic changes in both morphological features and physiological regulatory mechanisms that are controlled by the clock in these species are moulting and metamorphosis (Steel & Vafopoulou 2006), where in addition to their endocrine functions, prothoracicotropic hormone (PTTH) and ecdysteroids hormones are also key circadian outputs.

At the anatomical level, the circadian pacemaker in *R. prolixus* is composed of dorsal and lateral photosensitive clock neurons that rhythmically express PER and TIM and are probably analogous to *D. melanogaster* clock neurons that control rhythms in locomotor activity (Helfrich-Förster 2004, 2005, Helfrich-Förster et al. 2007). These neurons project close to axons of a group of neurosecretory cells that express the PTTH, providing timing information to the PTTH cells and other neurosecretory cells in the medial region of the protocerebrum (Vafopoulou & Steel 1996, 2001, Vafopoulou et al. 2010). PTTH is a neuropeptide whose function is to stimulate the release of ecdysteroids by PGs (Warren et al. 1988). PG produces ecdysones, which are converted in peripheral tissues into 20-hydroxyecdysone (the major moulting hormone) whose titre leads to the initiation of the moulting process. The rhythmic release of PTTH not only stimulates, but also entrains the release of ecdysteroids by PG, which also possess light-entrainable autonomous oscillators (Steel & Vafopoulou 2006). The resulting rhythm in haemolymph ecdysteroid titre is read by the target tissue's ecdysteroid nuclear receptors, which leads to internal tissue synchronisation and also triggers the rhythmic expression of downstream genes on different tissues such as fat body, rectal epithelium, oenocytes, neurosecretory cells and even the lateral clock neurons (Vafopoulou & Steel 2006).

Besides controlling important aspects of triatomines development, this system also constitutes an interesting example of tissue temporal synchronisation that has striking differences compared to *D. melanogaster* and most insects analysed so far, where most peripheral oscillators are entrained directly by light (Giebultowicz 2000). In this sense, the *R. prolixus* circadian system resembles the mammalian one, where tissue-specific peripheral oscillators require input from the central clock in the brain to generate coherent systemic rhythms (Dibner et al. 2010), highlighting the potential importance of hormones as time signals in insects.

Phlebotomine sandflies - The sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) is the main vector of visceral leishmaniasis in the Americas (Deane & Deane 1962). Leishmaniasis is caused by protozoan parasites from the genus *Leishmania*, which

are believed to infect around 12 million people in 88 different countries. The transmission occurs when the infected females lacerate capillaries, inoculating pharmacologically active compounds to preserve the haemorrhagic pool upon which they feed, thereby releasing the *Leishmania* parasites into the bloodstream.

In the wild sandflies are more active at dusk, which corresponds to the period when females seek a blood-meal (Felicciangeli 2004). In fact, this pattern is also observed in controlled laboratory conditions and persists in constant darkness (Meireles-Filho et al. 2006b, Rivas et al. 2008). Blood-feeding, which is a known modulator of several mosquito species' behaviour (Clements 1999) also affects *Lu. longipalpis* locomotor activity, reducing it at dusk in controlled laboratory conditions. This apparently involves the central pacemaker, since *per* and *tim* expression levels were not only reduced in the female body, but also in the head (Meireles-Filho et al. 2006b).

Lu. longipalpis was the first insect vector to have a clock gene identified (*per*) (Peixoto et al. 2001) and the subsequent cloning of other clock genes and expression analyses of molecular members from the first feedback loop showed considerable similarities, but also interesting differences compared to *D. melanogaster*. While *per* and *tim* mRNAs cycle, peaking around ZT13 in sandflies, as in flies and other insects (So & Rosbash 1997, Goto & Denlinger 2002, Meireles-Filho et al. 2006b, Sandrelli et al. 2008), *Clk* expression peaks around ZT 9-13, about half a day later than in *D. melanogaster* (Bae et al. 1998, Lee et al. 1998, Meireles-Filho et al. 2006b). In addition, *cyc* expression cycled robustly, beginning to rise at the end of the night and peaking in the middle of the day ZT 5-9 (Meireles-Filho et al. 2006a), in sharp contrast with *Drosophila* where *cyc* is constitutively expressed (Bae et al. 2000) (Fig. 2). Another notable difference observed in the sandfly clock is the C-terminal transactivation domain found encoded in the *cyc* gene that is absent in *Drosophila* (Rutila et al. 1998, Meireles-Filho et al. 2006a). It is believed that during the course of evolution *Drosophila* has compensated for the lack of this domain by acquiring an extensive poly-Q transactivation domain in the *Clk* gene, thereby keeping CLK/CYC heterodimer functionality (Allada et al. 1998).

But although several transcriptome studies have identified many new sandfly molecules (Anderson et al. 2006, Dillon et al. 2006, Jochim et al. 2008, Oliveira et al. 2009, Pitaluga et al. 2009, Azevedo et al. 2012), the lack of the full sequence genome of this vector has hindered further advances in the molecular details of its circadian clock. As the current *Lu. longipalpis* genome project has already sequenced about 22.6 million reads (representing a coverage of 38.9x) (vectorbase.org), we expect that these efforts will help to boost our knowledge in the sandfly circadian clock in the future.

Mosquitoes - Mosquitoes (Diptera: Culicidae) are remarkably adaptive species and some species are important vectors of harmful human diseases such as malaria, yellow fever and filariasis. Their circadian rhythms have been extensively studied, both in the wild and in laboratory conditions. They show a remarkable variation of

locomotor activity behaviours that can be affected by several factors such as nutritional and mating status, temperature and light intensity (Clements 1999). In addition, there is increasing evidence that mosquito behaviour can also be modulated by parasites to increase transmission rates (Hurd 2003, Schaub 2006). This makes the study of these insects' circadian rhythms of epidemiological relevance and important for disease control. In addition, the availability of fully sequenced genomes from phylogenetically closely related species with different circadian rhythms makes mosquitoes valuable for the molecular analysis of these processes. Given the large amount of data that could be potentially cited, we have decided to focus the following section on the species that have their genomes fully sequenced and whose circadian clocks are best characterised at the molecular level: *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles gambiae*.

C. quinquefasciatus - Mosquitoes from the *Culex* genus are the most diverse and geographically widespread of these three mosquito genera. They are the main vectors of West Nile encephalitis, St. Louis encephalitis and most importantly nematodes that cause lymphatic filariasis (LF). LF is currently endemic in 72 countries and is caused by three species of nematode: *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, which are transmitted to the hosts through the bite of infected female mosquitoes. Interestingly, the interaction between *Culex* and the filarial worm is one of the best examples of coevolution of circadian rhythms of parasitism and its adaptive value: the worms reach their highest density in the human peripheral blood at the time when the vector shows its biting activity peak, increasing their chances of transmission into the insect (Hawking 1975). *Culex* presents a nocturnal pattern of activity in the wild that can be reproduced under controlled laboratory conditions (Jones 1982, Gentile et al. 2009) and can be synchronised by temperature cycles (Chiba et al. 1993). In addition it is and modulated by insemination status (Chiba et al. 1990), which is driven by a pheromone produced by the male accessory gland (Jones & Gubbins 1979).

The molecular analysis of the biological clock in this species assessed the structure and circadian expression of genes of the two main loops. Besides the presence of *per*, *tim*, *Clk*, *cyc*, *vri* and *Pdpl*, two *cry* genes were cloned in *Culex*. This was also observed in other species and this is apparently the most ancestral organisation of circadian systems in insects where CRY1 serves as a photic synchroniser as in *Drosophila* and CRY2 participates in the core clock as a repressor of CLK/CYC-mediated gene transcription as in mammals (Zhu et al. 2005, 2008, Yuan et al. 2007). In general, the circadian expression patterns were similar with those observed in other insects, with some exceptions. For example in the first loop, while *Clk* expression peaked around ZT22-ZT2, similar to *Clk* expression in *Drosophila* and opposite to *Lu. longipalpis*, *cyc* expression was rhythmic as in sandflies and peaked slightly earlier at ZT1-5 (Fig. 2). Interestingly, the expression of the *cry* genes differed: while the photoreceptor *cry1* was constitutive (in contrast to *Drosophila cry* that peaks at ZT5) (Zheng et al. 2008), *cry2* cycled with a peak at ZT15-19 (Gentile

et al. 2009). The molecular structure of the *Culex* clock with its two *cry* genes suggests that *cry2* will represent a negative regulator, but whether the two *cry*'s could be involved in the differences observed in the locomotor activity rhythms among species remains open (Gentile et al. 2009) (see below).

Ae. aegypti is the best-characterised species within the Culicinae and is the primary vector for yellow fever and dengue. Dengue fever is caused by the dengue virus, which is transmitted to humans through the bites of infected female mosquitoes. The disease occurs in tropical and sub-tropical regions and has recently advanced into urban and semi-urban areas. It is estimated that 50-100 million dengue infections occur worldwide every year, which makes this disease a major international public health problem.

Ae. aegypti - *Ae. aegypti* is considered an urban mosquito and shows endophilic and anthropophilic behaviour, being frequently caught inside houses and feeding on human blood. It has diurnal rhythms of blood-feeding activity that are modulated by reproductive state (i.e., insemination) and feeding status. Virgin females show a bimodal pattern of activity, which decreases after they mate. When mated and blood-fed, they become inactive for the first two days after the blood-meal, but recover activity on subsequent days, probably reflecting the search for a place to oviposit (Taylor & Jones 1969, Jones 1981).

As with *Culex*, orthologues of *Drosophila* circadian genes were also cloned in *Ae. aegypti* and had their daily expression analysed (Gentile et al. 2006, 2009). Analysis of circadian abundance of the genes from the two loops revealed striking similarities with that observed for *Culex*, despite the fact that they have different locomotor activity patterns (*Aedes* diurnal vs. *Culex* nocturnal) (Fig. 2). *Aedes* also has two *cry* genes and the expression of *cry2* was the main difference between these two species: while *Culex* had a peak of expression at ZT15-19, *Aedes cry2* had a bimodal peak at ZT1-5 and ZT15-19, raising the interesting possibility that *cry2*, which is homologous to the mammalian-like repressor *cry*, might be responsible for the differences observed in locomotor activity behaviour between these two species and among other insect species in general (Gentile et al. 2009).

The publication of the *Ae. aegypti* genome provoked a number of studies that have addressed the transcriptional profile of this species in development, insecticide challenge and after blood-feeding [reviewed in Severson & Behura (2012)]. Ptitsyn et al. (2011) analysed genome-wide rhythmic expression in female heads using Agilent microarrays under LD conditions and have found that molecules in a number of important pathways can cycle. For example, genes related to detoxification pathways such as xenobiotic metabolism and cytochrome P450 family were expressed with a period of 24 h (Ptitsyn et al. 2011), which might explain the daily fluctuation of permethrin-resistance (Yang et al. 2010) and therefore be considered in insecticide control programs. As this study did not assess cyclic expression in constant conditions [dark/dark (DD)], it is likely that many of the observed rhythmically expressed gene are a direct response to light instead of being controlled by the circa-

dian clock. Indeed, 59% of rhythmic genes in a similar study with *An. gambiae* were rhythmic only under LD conditions (Rund et al. 2011) (see below).

An. gambiae - The mosquito *An. gambiae* is the primary African malaria vector, which is caused by parasites from the *Plasmodium* genus. As for LF and dengue, the parasites are transmitted through the bites of infected *Anopheles* female mosquitoes and about 219 million cases of malaria were estimated in 2010 with approximately 660,000 deaths.

The mosquito daily behavioural rhythms include dusk mating swarms, nocturnal flight activity, sugar feeding, blood feeding and oviposition. It is a nocturnal species and, as for *Culex* and *Aedes*, its rhythms are also modulated by insemination and blood-feeding (Jones et al. 1972, Jones & Gubbins 1978, Gary & Foster 2006, Das & Dimopoulos 2008).

Analysis of clock gene circadian expression in *An. gambiae* revealed a pattern indistinguishable from that observed with *Culex* (Fig. 2), including constitutive *cry1* expression and unimodal *cry2* rhythmic expression (Rund et al. 2011). Since *An. gambiae* is a nocturnal species as is *Culex*, it is tempting to speculate that *cry2* expression indeed might be responsible for the differences observed in locomotor patterns among insect species. However, this can only be definitively answered with the type of interspecific transformation experiments that have been carried out in *Drosophila* to dissect the molecular basis of species-specific locomotor profiles (Tauber et al. 2003).

To better understand the molecular interactions among the circadian clock, light sensing system and host seeking, Das and Dimopoulos (2008) measured genome wide expression changes after the application of light pulses at the beginning of night, which is known to inhibit blood-feeding behaviour. They observed that while short light pulses inhibit blood-feeding in a clock independent manner, long light stimulation induced phase advances in blood-feeding propensity in a clock dependent manner. Interestingly, both light regimes altered the expression of clock related genes such as *tim* and the *takeout* gene family (which links circadian locomotor rhythms and feeding behaviour in *Drosophila*) (Sarov-Blat et al. 2000, So et al. 2000). Likewise, silencing of *tim*, *cry* (which like *tim* is also involved in the clock's synchronisation by light) and *takeout* genes promoted an increase propensity for the mosquito to feed on blood, suggesting that they are important components of the circadian clock that drives blood-feeding activity (Das & Dimopoulos 2008). Besides these genes, the light treatment induced transcriptome changes in a variety of physiological systems similar to the ones caused by blood-feeding, highlighting the possible role of the circadian clock in linking these two processes.

Several other studies have addressed the global circadian regulation of gene expression in this species also in DD, where rhythmic gene expression in diverse biological functions such as metabolism, detoxification, olfaction, vision, cuticle/peritrophic membrane regulation and immunity have been uncovered, all of them tissue-specific (Rund et al. 2011, 2013). The comparison between the

An. gambiae and *Ae. aegypti* circadian transcriptomes revealed that genes related to visual signalling pathways cycled with somewhat similar phases, suggesting that sensitivity tuning to photic activation does not depend on the temporal niche (i.e. nocturnal vs. diurnal). In contrast, odorant binding protein (OBP) genes, important components of the olfactory system, differed in that *An. gambiae* OBPs expression showed a very tight regulation with all 17 genes clustered around ZT9-13, while in *Ae. aegypti* the 15 mRNAs identified peaked in expression at various times of the day (Rund et al. 2013).

Are species-specific differences in behaviour and physiology directly controlled by the core pacemaker or reflect differences in how the information of the central clock is translated into output signals? In light of these datasets, it is tempting to speculate that changes in the transcriptional downstream programs are more likely due to the differences in the regulatory sequences of upstream clock target genes than changes in the main clock components as seen in other systems (Meireles-Filho & Stark 2009). On the other hand, *cry2* differential expression might be an important factor in this equation and was also found to affect output rhythms in diverse species. For example, in two strains of *Bactrocera cucurbitae* that differed in mating time, the allele frequency at two polymorphic sites in *cry*, as well as *cry* gene expression itself, correlated with differences in circadian locomotor period, suggesting that variation in the *cry* gene is related to differences in the circadian behaviour in the two strains (Fuchikawa et al. 2010). Even in plants a single amino-acid substitution from methionine to valine in the *cry2* gene was associated with an early flowering quantitative trait loci (El-Din El-Assal et al. 2001). Future studies on the biochemical and molecular interactions between clock genes as well as interspecific transformation experiments in loss-of-function *D. melanogaster* mutants [e.g. Tauber et al. (2003)], might further illuminate the role of clock genes on activity and their modulation by mating and feeding status.

Future directions - Although the circadian clock of insect vectors control a number of aspects of their behaviour and physiology important for their survival, including sensory perception and susceptibilities to insecticide or immune challenge, the temporal organisation of the vectors' life has not yet been exploited by vector-control programs. This is in part hindered by the fact that it is still unclear how the circadian clock connects to output rhythms, and therefore any strategy considering manipulation of physiology and behaviour through the clock would probably not succeed.

The advancement in next generation sequencing technologies provides means by which association of a phenotype to its causal polymorphism is now relatively straightforward [e.g. Massouras et al. (2012)]. Allied to the extensive natural circadian activity variation of insect vectors found in the wild that can be reproducibly observed in the laboratory [e.g. Rivas et al. (2008), Rund et al. (2012)], this scenario configures an exciting opportunity to link genetic and phenotypic variation, which might lead us to better understand the molecular

underpinnings of the circadian clock, its variation and how plasticity is achieved. As the clock is fundamentally important to every aspect of behaviour and physiology of almost all higher organisms, the circadian analysis of insect vectors, given their epidemiological importance, must not be undervalued by the vector community.

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To the memory of Alexandre Afranio Peixoto (1963-2013), student of CPK and mentor of ACAMF. Alex lit up the population genetic and molecular evolution study of insect clock genes in the 1990's with a series of outstanding papers, mostly on the *period* gene of *Drosophila*. He used the newly developed neutrality tests of that time to study the evolutionary signatures present in the patterns of molecular variation in and around *clock* genes and was able to determine whether natural selection was shaping that variation. However unlike almost all others, he allied this statistical approach to DNA sequences with phenotypic analyses and generated a number of classic case studies involving natural selection and clines, intramolecular coevolution and repeated sequence evolution. His star burned particularly brightly during the 1990's, but he consolidated this success with evolutionary and functional studies of ion channel genes and later applied what he had learned as a graduate student and then as a postdoc, to vector biology at the Oswaldo Cruz Institute (IOC). He initiated the molecular analysis of vector clocks and used *clock* gene sequences that evolve rapidly, to determine the evolutionary relationships among closely related vector populations, particularly in sandflies. Apart from his outstanding intellect (I never had a conversation about science with him when I didn't learn something - CPK/I have never met anyone brighter, more open-minded and willing to discuss when it comes to science - ACAMF), his enthusiasm for science and his easy-going outlook on life, provided his group at IOC with a mentor who was always approachable, helpful and fun to be around. His kindness, humour and generosity will always be remembered by his family, friends and colleagues and he is sadly missed by us all.

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