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The biological clock of an hematophagous insect: Locomotor activity rhythms, circadian expression and downregulation after a blood meal

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Abstract Despite the importance of circadian rhythms in vector-borne disease transmission, very little is known about its molecular control in hematophagous insect vectors. In *Drosophila melanogaster*, a negative feedback loop of gene expression has been shown to contribute to the clock mechanism. Here, we describe some features of the circadian clock of the sandfly *Lutzomyia longipalpis*, a vector of visceral leishmaniasis. Compared to *D. melanogaster*, sandfly *period* and *timeless*, two negative elements of the feedback loop, show similar peaks of mRNA abundance. On the other hand, the expression of *Clock* (a positive transcription factor) differs between the two species, raising the possibility that the different phases of *Clock* expression could be associated with the observed differences in circadian activity rhythms. In addition, we show a reduction in locomotor activity after a blood meal, which is correlated with downregulation of *period* and *timeless* expression levels. Our results suggest that the circadian pacemaker and its control over the activity rhythms in this hematophagous insect are modulated by blood intake.

Keywords: *Period*; *Timeless*; *Clock*; Circadian rhythms; Blood-feeding; Insect vector; *Luzomyia longipalpis*

1. Introduction

A diversity of organisms has circadian variations of behavior and physiology that are indeed controlled by an internal biological clock. In *Drosophila melanogaster* a number of genes controlling this mechanism are known [1]. The core clock mechanism is based on two interacting molecular feedback loops in which the transcription factors encoded by the genes *Clock* (*Clk*) and *cycle* (*cyc*) form a heterodimer that binds to upstream E-boxes sequences (CACGTG) and activate transcription of *period* (*per*), *timeless* (*tim*), *vrille* (*vri*) and *PAR domain protein 1* (*Pdp1*) [1–4]. Whereas the heterodimer PER/TIM interacts with CLK/CYC, inhibiting its function, VRI and PDP1ε compete for the same site in the *Clk* promoter, regulating its transcription.

These two interlocked feedback loops create circadian rhythms of gene expression of its components, except for *cyc*, which is constitutively expressed [1,5]. The repressor genes *per* and *tim*, for example, have their peaks of mRNA expression around Zeitgeber Time (ZT) 13 [6,7], while the transcriptional activator *Clk* reaches its maximum abundance in the late night/early morning, around ZT22-2 [8]. These cycles in the expression of negative and positive elements of the clock are important for the generation of circadian rhythms. Besides its central role in the link between the two feedback loops, the CLK/CYC heterodimer is believed to bind to regulatory sequences of downstream genes, controlling different aspects of physiology and behavior, as suggested by microarray experiments with the loss-of-function Clk^{Jrk} strain (e.g. [9]).

In contrast to the abundance of information about the molecular control and circadian expression of clock genes in *D. melanogaster*, very little is known about it in hematophagous insect disease vectors. Nevertheless, for some mosquito species there is a large amount of data on circadian activity rhythms (reviewed in [10]).

Blood-feeding, which is critical to disease transmission, is tightly adjusted to a specific time of day and certainly controlled by the circadian pacemaker [10]. In addition, there is circumstantial evidence that it might have an effect on the clock. In the malaria vector *Anopheles stephensi*, for example, blood-feeding is followed by a marked reduction of circadian flight activity for 2–3 days [11]. Based on these findings and on molecular data from *D. melanogaster*, we hypothesized that the inhibitory effect of blood on circadian locomotor activity could involve regulation of clock gene expression levels.

For the sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae), despite its medical importance as the main vector of American visceral leishmaniasis [12–14], neither molecular nor behavioral data on circadian locomotor activity rhythms in controlled laboratory conditions are available.

To initiate the molecular study of circadian rhythms in insect vectors, we analyzed the circadian expression of *per*, *tim* and *Clk* in *L. longipalpis*. We also examine the effect of blood-feeding on the expression of these three genes and recorded the circadian locomotor activity of this species in controlled laboratory conditions.

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2. Materials and methods

2.1. Insects

Lutzomyia longipalpis sandflies from a Lapinha (Minas Gerais State, Brazil) laboratory colony were reared as previously described [15]. *D. melanogaster* from the Canton S strain was raised on oatmeal medium at room temperature.

2.2. Analysis of locomotor activity rhythms

Newly emerged males and females of *L. longipalpis* were kept together in an incubator at 25 °C and LD 12:12 (cycles of 12 h of light and 12 h of darkness). Two days before recording, 3–5 days old sandflies were transferred to the Drosophila Activity Monitoring System (Trikinetics Inc., Waltham, MA, USA). *Drosophila* males used in the activity experiments for comparison were treated in a similar way.

2.3. Cloning and sequence analysis of the gene fragments used in the expression experiments

Fragments homologous to the *Drosophila per*, *tim*, *Clk* and *rp49* genes used in the circadian gene expression experiments were initially amplified from *L. longipalpis* genomic DNA using the degenerate primer PCR technique. The amplified fragments were subcloned and sequenced at Instituto Oswaldo Cruz on an ABI 377XL using BigDye Terminator v3.0 (Applied Biosystems). Sequence analysis was conducted using the GCG software and the NCBI website (www.ncbi.nlm.nih.gov/BLAST). The sequences of the fragments used in the expression experiments were submitted to the GenBank (Accession No. DQ207732; DQ207733; DQ207734; DQ207735; DQ207736; DQ207737; DQ207738). The full sequences of the three *L. longipalpis* clock genes will be published elsewhere.

2.4. Sample preparation for quantitative RT-PCR analysis of circadian gene expression

Sandflies were entrained for 3 days in LD12:12. On the fourth day, we collected 3 independent replicates samples with circa 40 sandflies for each time point at ZTs 1, 5, 9, 13, 17 and 21. Because *per* mRNA levels are differentially expressed between head and body in females of *D. melanogaster* (but not in males) [16], female sandfly heads were manually dissected from bodies on a frozen dish in dry ice. Heads and bodies of each ZT were stored at −80 °C until mRNA extraction. Males were not dissected. mRNA was extracted from whole males, female heads and bodies with the QuickPreptrade Micro mRNA Purification kit (Amersham Biosciences) according to manufacturer instructions. Approximately 10 ng of each mRNA sample were reverse-transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems) using the oligo-dT primer.

2.5. Quantitative RT-PCR

Levels of *per*, *tim* and *Clk* RNA relative to non-cycling levels of *rp49* were assayed by quantitative real time PCR using an ABI PRISM® 7000 (Applied Biosystems). For all genes analyzed two different sets of primers were used, except for the *rp49* constitutive control (primer sequences are available upon request). At least one of each primer in the pair spanned an exon/intron boundary to prevent amplification from any genomic DNA contamination. Indeed, all PCR reactions generated only the expected amplicons as shown by the melting-temperature profiles of the final products and by gel electrophoresis (data not shown). Standard curves were used to confirm that primers pairs had similar reaction efficiencies. Reactions were carried out in quadruplicates in a final reaction volume of 30 μl using 2X SYBR® Green PCR Master Mix (Applied Biosystems) and primers at a final concentration of 500 nM. Amplifications were carried out for 50 cycles as follows: (i) 95 °C, 10 s; (ii) 60 °C, 60 s; (iii) 78 °C, 25 s (florescence recorded); (iv) repeat. Raw data were exported to EXCEL (Microsoft) for analysis.

2.6. Experiments testing the effect of blood-feeding in locomotor activity and gene expression

Two- to three-day-old females were blood-fed on an anaesthetized hamster during 10 min at the light–dark transition (the hamster was placed in the cage at ZT11:55 and removed at ZT12:05). This procedure allowed about half of the females in the cage to blood-feed. Afterwards blood-fed and unfed controls (from the same cage) were separated and kept in different cages in an incubator at 25 °C and LD12:12 until frozen (for the gene expression analysis) or directly placed in the activity monitor for behavioral analysis (with recording starting one day later). Since blood-fed and unfed controls had to be visually separated after the feeding period, they were subjected to a phase-delay of 2 h, that is, placed in a different incubator with lights turning on and off 2 h later than the previous one where they were entrained. In fact, this procedure did not alter sandfly circadian locomotor behavior after one-day entrainment (see Section 3). The sandflies used in the expression experiment were collected and frozen at ZT13 in the following day (27 h after the blood meal – the same 2 h needed to separate blood-fed and unfed controls plus 25 h to reach the ZT13 in the next day).

3. Results

3.1. Locomotor activity rhythms

Fig. 1 shows the locomotor activity of the sandfly *L. longipalpis* compared to that of *D. melanogaster*. The graphs show the mean normalized activity of males of each species during two consecutive days, one in LD12:12 followed by one day at constant darkness (DD). We can see that *L. longipalpis* has a nearly unimodal pattern with a small startle response to lights-on. The activity peak is centered around the light-to-dark transition, showing clearly the characteristic anticipation to lights-off and higher level of activity during the dark phase than during the day (Fig. 1). Sandfly females show a very similar pattern (see below). The first day of DD shows a peak about 23 h later than that observed in LD and the activity is nearly unimodal. In the same conditions *D. melanogaster* shows a bimodal activity pattern in LD, which dampens in the first day in DD (Fig. 1). This is in agreement with several reports showing that after some days in DD the morning activity peak tends to disappear while the evening peak is sustained (e.g. [17]). *D. melanogaster* also shows far more activity in the subjective day than *L. longipalpis*. That is consistent with the fact that the latter is a crepuscular/nocturnal insect in the wild [13,14]. Indeed the difference in the proportion of diurnal over total activity between *D. melanogaster* and *L. longipalpis* is highly significant in both LD ($t = -8.713$; d.f. 108; $P < 0.001$) and DD ($t = -4.034$; d.f. 99; $P < 0.001$).

3.2. Temporal clock gene expression patterns in males and female heads

Quantitative real time RT-PCR analysis of males and female heads have not revealed significant differences, therefore, the results were combined. Analysis of mRNA abundance indicates low amplitude circadian oscillation of *per*, *tim* and *Clk* in *L. longipalpis* relative to the *rp49* constitutive control. *per* and *tim* mRNAs oscillate with a peak at ZT13 (Fig. 2A and B) presenting a pattern somewhat similar to that observed in *D. melanogaster* [6,7,18]. A posteriori (LSD – least significant difference) comparisons indicate significant differences in *per* mRNA levels between ZT13 and ZTs 1 and 9 ($P < 0.05$). Although the difference in *tim* mRNA levels between peak (ZT13) and through (ZT1) fail to reach significance in the LSD analysis ($0.05 < P < 0.10$), *per* and *tim* show similar patterns with higher mRNA levels during the dark phase. Note that the amplitude of *per* and *tim* mRNA cycling in sandflies (1.5–2-fold difference between peak and through) is not as high as reported for *Drosophila*. However, similar results were found in other insects (e.g. [19]). Analysis of *Clk* mRNA revealed an expression pattern (Fig. 2C) that is very different