Clock Gene *Timeless* in the Chagas Disease Vector *Triatoma infestans* (Hemiptera: Reduviidae)

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Abstract. To contribute to a better understanding of the molecular basis of the circadian biological rhythms in Chagas disease vectors, in this work we identified functional domains in the sequences of the clock protein TIMELESS (TIM) in *Rhodnius prolixus* and analyzed the expression of the *timeless* (*tim*) gene at the mRNA level in *Triatoma infestans*. The *tim* gene expression in nervous tissue of adult *T. infestans* revealed clear oscillations in the abundance of the transcript in both sexes in the group maintained under photoperiod with a daily canonical rhythm, showing a significant increase in expression at sunset. As expected, in the group maintained in constant light, no daily increase was detected in the *tim* transcript level.

INTRODUCTION

Triatoma infestans (Hemiptera, Reduviidae) is the main vector of Chagas disease in the Southern Cone of Latin America. Vector control has proven to be difficult, and, recently, insecticide resistance has emerged as one of the main explanations of unsatisfactory control observed.[1] Chronobiological studies could be a novel and relevant aspect that might contribute to the development of more effective control programs. Endogenous or circadian clocks make the temporary coordination between biological processes and environmental cycles. Among the most important external signals or synchronizers (*Zeitgebers*) are light/dark alternation, the variation in environmental temperature, the availability of food, and social interaction.[2–4] In *Drosophila melanogaster*, the clock is composed of two "loops" interrelated by feedback, period/timeless loop (per/tim loop), and clock loop, and encoded by the *period* (*per*), *timeless* (*tim*), *clock* (*Clk*), and *cycle* (*cyc*) genes.[5] Homologous circadian genes are found in all insect clocks, but their contribution to species-specific circadian timing systems differs.

Circadian rhythms in biological processes such as reproduction, foraging, breeding, oviposition, dispersion, and host seeking have been extensively studied in adults of *Rhodnius prolixus* and *T. infestans.*[6–11] In a previous work, the *per* gene expression showed a daily canonical rhythm in nervous tissue of groups of adult individuals of *T. infestans* maintained under photoperiod or light/dark cycle (LD) and constant dark.[12] With the propose to analyze the circadian expression profile of the *tim* gene in *T. infestans*, in this work the *tim* mRNA level in nervous tissue of adults specimens was determined under different dark/light regimes.

MATERIALS AND METHODS

Triatoma infestans were reared at 28 ± 1°C at a relative humidity of 60–70% and fed once every 2 weeks on restrained chickens. Since the fifth-instar nymph stage, *T. infestans* individuals were maintained under different dark/light regimes.

Two experimental groups were subjected either to 1) light/dark cycle (LD) or 2) constant light (LL). The LD cycle group consists of 12 hours of light and 12 hours of darkness. Time of day was reported in 24-hour *Zeitgeber time* (ZT), with ZT12 (20:00 hours) defined as time of lights off and ZT0 (08:00 hours) defined as end of the dawn transition under the LD cycle. For the LL group, subjective day was reported between ZT0 (8:00 hours) and ZT12 (20:00 hours). For the circadian study of the *tim* gene expression, the heads (brain nervous tissue) were excised between 40 and 45 days after molt from adult female and male specimens of the two experimental groups every 4 hours over 24 hours. In all cases, the tissues were dissected under aseptic conditions and stored in liquid nitrogen until used for RNA extraction.

Total RNA was isolated from pools of insect tissues using MasterPure RNA Purification Kit (Epicentre, Madison, WI). Samples from five adult females or five adult males were pooled. Synthesis of cDNA was performed from the total RNA using Oligo-dT$_{20}$ and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The amplification of a fragment of the *tim* gene (200 base pairs) was carried out by the polymerase chain reaction (PCR) using the primers forward ATTTGGTTGGAACAAAGCTGAT and reverse TGAAGGTGT TGCTTTATGTGAAC. After the PCR products were purified, they were sent for direct sequencing to the Genomics Unit/ Node CATG National Genomics Platform, Institute of Biotechnology (CICVyA-INTA's, Buenos Aires, Argentina). The transcript levels of the *tim* gen were measured by semi-quantitative PCR and expressed as described in a previous work carried out in our laboratory.[12] The experimental values represent mean ± SD of two independent experiments for each sample composed by pooled tissues from five specimens. The significance of differences in *tim* transcript gene levels was determined using analysis of variance. All statistical calculations were performed using Prism 5 software (Graph-Pad, San Diego, CA). The rhythms in *tim* mRNA expression were analyzed using the JTK_Cycle nonparametric algorithm.[13] In this statistical analysis, the *P*-value, period, phase (LAG), and amplitude (AMP) were evaluated.

To identify conserved functional domains, an alignment was carried out with the deduced TIMELESS (TIM) protein sequences from *R. prolixus* Contig 3.0.3-177.8 (GeneBank: ACPB03012942.1) and those from *Papilio xuthus* (GenBank: KPI93841.1), *Ephestia kuehniella* (GenBank: AJG06482.1), *Antheraea pernyi* (GenBank: AAF66996.1), *Tribolium castaneum*

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(GenBank: AOR83338.1), *D. melanogaster* (GenBank: NP_ 001334730.1), *Aedes aegypti* (GenBank: AAY40757.1), *Belgica antarctica* (GenBank: AGZ88039.1), *Clunio marinus* (GenBank: AFS34623.1), *Halyomorpha halys* (GenBank: XP_014278196.1), and *Cimex lectularius* (GenBank: XP_ 014260029.1). Multiple alignments of sequences were performed using the multiple alignment program Clustal W in MEGA version 6.06.[14] Phylogenetic analysis was performed by the neighbor-joining method using MEGA version 6.06 software.[14] The reliability of the trees was tested by the bootstrap procedure with 1,000 replications.

RESULTS AND DISCUSSION

A number of behaviors 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.[6–11] In *R. prolixus*, four groups of photosensitive clock cells were found expressing the clock proteins PERIOD (PER) and TIMLESS (TIM) rhythmically.[15] Their clock neurons are probably homologous to the side and dorsal neurons of *D. melanogaster* and are possibly responsible for controlling the rhythms in locomotor activity.[16] Lateral neurons of *R. prolixus* exhibit robust rhythms in the abundance of the PER and TIM proteins with a peak in the dark phase.[17] This clock receives outside information and generates rhythmicity in the release of neurohormones as prothoracicotropic hormone, providing temporal information to the peripheral oscillators.[18]

The comparative analysis among the deduced amino acid sequences from *R. prolixus* and other insect species revealed conserved sites and domains typical of TIM protein (Timeless pfam04821 domain, PER interaction domains 1 and 2, and the amino acids corresponding to the nuclear and the cytoplasmic localizations). Phylogenetic analysis was performed based on the complete amino acid sequences of TIM protein of the hemipteran *R. prolixus*, *C. lectularius*, and *H. halys*, and of eight representative species of the orders Diptera, Lepidoptera, and Coleoptera. As expected, insect TIM sequences from the same insect order were grouped together with significant bootstrap support (Figure 1). Within Hemiptera, *R. prolixus* clustered with *C. lectularius* with high bootstrap support (100%), as well as these two species with *H. halys*.

Similar to that observed for the clock gene *per*, the expression of the *tim* gene showed a daily canonical rhythm in the *T. infestans* group maintained under photoperiod (LD). In this group, the *tim* transcript level showed a significant increase at ZT12 and ZT16 in both sexes (Figure 2A and B).[12] The JTK Cycle analyses indicated a significant rhythm in the expression of *tim* transcript in both sexes (adjusted *P*-value for females: 0.00050 and males: 0.0026), with the same period of 28 hours. However, differences in *tim* transcript rhythmic expression were detected in the amplitude (AMP) and phase (LAG). Females showed a lower amplitude and a higher phase (AMP: 14, LAG: 0.13103) than males (AMP: 18, LAG: 0.06632). In the constant light group, no changes were detected in *tim* transcript levels at the ZTs analyzed (Figure 2C and D). The corresponding mRNA levels remain flat in constant light with relatively similar levels of expression in both sexes at all
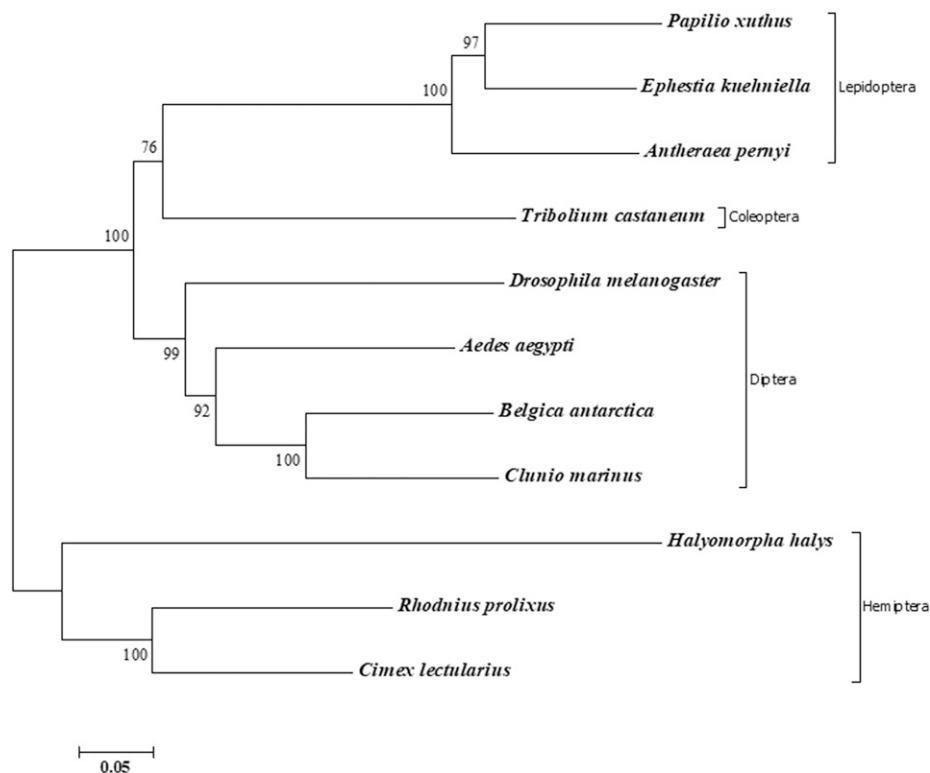

FIGURE 1. Neighbor-joining phylogeny of *Rhodnius prolixus* TIM deduced amino acid sequences, selected TIMs from other hemipteran species and representative species of the orders Lepidoptera, Coleoptera, and Diptera. Bootstrap values next to nodes represent the percentage of 1,000 replicate trees that preserved the corresponding clade.
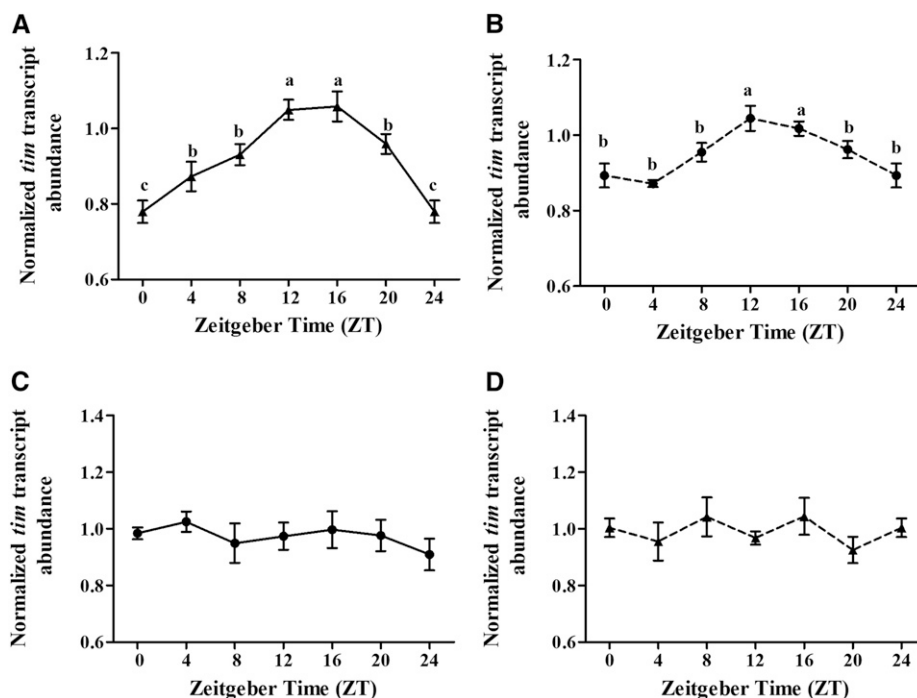
FIGURE 2.   Circadian expression of *tim* gene in the nervous tissue of *Triatoma infestans* adults under photoperiod (**A** and **B**) and constant light (**C** and **D**) conditions. Relative abundance of *tim* mRNA expression was referred to the ratio of *tim* mRNA vs. β-*actin* mRNA expression at each time point. Continuous and dash lines correspond to male and female groups, respectively. The error bars represent the SD of the mean. Different letters above the error bars indicate statistical differences determined by one-way analysis of variance followed by Bonferroni test ($P < 0.05$).

examined time points. The pattern of *per* and *tim* genes expression under LD, observed in adult females and males of *T. infestans*, agrees with the expression profile of this canonical clock gene in *D. melanogaster*. In this species, the mechanism of the circadian clock has been extensively studied and it has been observed that the clock genes are expressed in cycles close to 24 hours.[5] Moreover, in *R. prolixus*, an increase in levels of PER and TIM proteins in the central clock neurons during the dark phase of the photoperiod was detected.[15–17] Because *R. prolixus* and *T. infestans* are close species, it could be inferred that the increased level of *per* and *tim* mRNAs detected in *T. infestans* at sunset would promote a peak of PER and TIM protein levels at night, necessary for the function of the period/timeless loop in the clock cells. On the other hand, as expected, in the nervous tissue of the group maintained in constant light (LL), no daily increase was detected in *per* and *tim* transcript levels. The rhythms of expression of the *tim* and *per* genes showed visible damping in the LL group, confirming that prolonged exposure to light results in the loss of rhythmicity in the expression of these clock genes. The same effect of the light was demonstrated in studies about the levels of the PER and TIM proteins in clock neurons of *R. prolixus*.[17]

This kind of study would provide potentially useful information to analyze the temporal regulation of important biological processes, such as reproduction, dispersal, and insecticide resistance. For example, in adult mosquito *Ae. aegypti*, the major vector of dengue viruses in Taiwan, daily fluctuation of insecticide resistance was found. Existence of a clock control over sensitivity to insecticide was further indicated by reduced expression of a *cytochrome P450* (CYP9M9), involved in detoxification metabolism, and reduced mosquito resistance to insecticide after temporal

silencing of the *per* gene. These data provide the evidence on the circadian control of insect resistance to insecticides.[19] Because it was also detected that daily variations in the expression of genes related to insecticide resistance in *T. infestans*, the clock genes could also be involved in the circadian control of the metabolic pathways of insecticide detoxification and resistance.[20] Therefore, studies of circadian rhythms in Chagas disease vectors and their molecular basis, as well as the analysis of the central clock relationship with insecticide resistance, would promote the development of new control strategies.

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