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

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Abstract. To contribute to a better understanding of the molecular bases of the circadian biological rhythms in Chagas disease vectors, in this work we identified functional domains in the sequences of the clock protein PERIOD (PER) in *Rhodnius prolixus* and *Triatoma infestans* and analyzed the expression of the PER gene at mRNA level in *T. infestans*. The PER protein sequences comparison among these species and those from other insects revealed that the most similar regions are the PAS domains and the most variable is the COOH-terminal. On the other hand, the *per* gene expression in nervous tissue of adult *T. infestans* varies with a daily canonical rhythm in groups of individuals maintained under photoperiod (light/dark, LD) and constant dark (DD), showing a significant peak of expression at sunset. The pattern of expression detected in LD persists under the DD condition. As expected, in the group maintained in constant light (LL), no daily increase was detected in *per* transcript level. Besides, the presence of *per* transcript in different tissues of adult individuals and in nervous tissue of nymphs evidenced activity of peripheral clocks in adults and activity of the central clock in nymphs of *T. infestans*.

INTRODUCTION

The Chagas disease or American trypanosomiasis, considered among the most important neglected tropical diseases, is a serious parasitic endemic illness in terms of its social and economic impact and affects 6–7 million people in Latin America and the Caribbean.[1] The disease is caused by infection with the protozoan *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae), transmitted by the blood-sucking insects of the subfamily Triatominae (Hemiptera, Reduviidae). In South America, among the main species vectors of Chagas are *Triatoma infestans* and *Rhodnius prolixus*.[2] Because there are no effective vaccines against *T. cruzi*, most efforts to control the disease is focused on eliminating vector population using pyrethroid insecticides. However, the goals of current vector control policies are compromised by several factors, including the abundance of other vector species and the extension of endemic areas, which hampers regularity in entomological surveillance.[3] In addition, resistance to pyrethroid insecticides has been reported as a fact that renders difficult vector control strategies.[4] Chronobiological studies could be a novel and relevant aspect that might contribute to the development of more effective control programs. The circadian regulation of insect activity is an area of behavioral science particularly relevant for the understanding of epidemiology related questions as it affects the time and the degree of contact between either the vector and the host or the vector and insecticide-sprayed surfaces.

Endogenous or circadian clocks make the temporary coordination between biological processes and environmental cycles. These clocks keep time even in the absence of external signals, although the environmental cycles are needed to synchronize the clocks and activate certain rhythmic processes at appropriate times.[5] In animals, circadian clocks reside in a variety of tissues (brain, sensory structures, internal organs, etc.) and are organized forming a highly interdependent hierarchical network in which a central oscillator sends temporal information to the peripheral oscillators.[5] Among the most important external signals or synchronizers (*Zeitgebers*) are light/dark (LD) alternation, the variation in environmental temperature, the availability of food, and social interaction.[6–8] In *Drosophila melanogaster* the clock is composed of two "loops" interrelated by feedback, period/timeless loop (per/tim loop) and clock loop, encoded by the *period* (PER), *timeless* (TIM), *clock*, and *cycle* genes.[9]

Circadian rhythms of triatomines, with a periodicity of approximately 24 hours, have been extensively studied in the adults of *R. prolixus* and *T. infestans*. In both species, these rhythms seem to control basic biological processes such as reproduction, foraging, breeding, oviposition, dispersion, and host-seeking.[10–14] The LD and temperature cycles, and the presence of a host seem to be important *Zeitgebers*.[13] The nocturnal activity patterns displayed by these bugs are generally bimodal. The first peak occurs just after dusk and corresponds to host-seeking activities, whereas the second is at dawn, interpreted as an effort by the insect to search for an appropriate daytime shelter.[13,15–17]

In *R. prolixus* were found four groups of photosensitive clock cells expressing the clock proteins PER and TIM rhythmically.[18] 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.[19] Lateral neurons of *R. prolixus* exhibit robust rhythms in the abundance of the PER and TIM proteins, with a peak in the dark phase.[20,21] This clock receives outside information and generates rhythmicity in the release of neurohormones as prothoracicotropic hormone, providing temporal information to the peripheral oscillators.[18]

In our laboratory was detected daily variation in *T. infestans* expression of glycerol-3-phosphate dehydrogenase (GPDH), the enzyme that plays a central role in the metabolism of flight. It was demonstrated that GPDH isoforms present a pattern of temporal expression, tissue-specific, differentiated by sex, and show changes in relation to temperature and the amount of intake.[22,23] In flight muscles of adult *T. infestans* under different conditions of photoperiod, the expression of GPDH isoforms presented rhythmic variations differentiated by sex.[24] GPDH-1, the isoform involved in the energetic metabolism

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of the flight, showed a rhythmic expression profile and synchronized in constant darkness, suggesting an endogenous clock regulation. The knowledge of the temporal structure and the underlying molecular mechanisms of the biological clock in triatomine insects could contribute to a better understand of their metabolic rhythms, nocturnal activity, and dispersion. To investigate the molecular bases of the circadian rhythms, we explored the biological central clock at the molecular level in triatomine insects. With this purpose, we identified functional domains in the sequences of the clock protein PER in *R. prolixus* and *T. infestans* and analyzed the PER gene expression at mRNA level in *T. infestan*s. The PER mRNA level was determined in the nervous tissue of adult specimens under different dark/light regimes. Besides, it was investigated at sunset the presence of PER transcript in different tissues of adult individuals and in nervous tissue of fifth instars' nymphs restrained under photoperiod.

MATERIALS AND METHODS

Laboratory colony. The laboratory colonies were initiated with individuals collected from Chuña, Ischilin Department, Córdoba Province, Argentina (30°28′ S, 64°40′ W), during May 2010. The specimens were reared at 28°C ± 1°C at a relative humidity of 60–70% and fed 60 minutes on chickens, one time every 2 weeks after molt. Because fifth instar nymph stage, *T. infestans* individuals were maintained under the different dark/light regimes. The heads (nervous tissue), thoracic muscles, gonads, and fat bodies were extracted between 40 and 45 days after molt from female and male specimens pooled separately. The tissues were dissected under aseptic conditions and stored in liquid air (–194°C). For the circadian studies, the adult heads were excised every 4 hours over 24 hours. Three experimental groups were subjected either to 1) LD cycle, 2) constant light (LL), and 3) constant dark (DD). The LD cycle group consists of 12 hour light and 12 hour darkness. Time of day was reported in 24-hour *zeitgeber time* (ZT) with ZT12 (20:00 hour) defined as time of lights off and ZT0 (08:00 hour) defined as end of the dawn transition under the LD cycle. For LL and DD groups subjective day was reported between ZT0 (08:00 hour) and ZT12 (20:00 hour).

Analysis of the clock protein PER. The deduced PER protein sequences were obtained from the genoma of *R. prolixus* (GenBank, SuperContig KQ034059: 5,280,851–5,304,066) and from a transcriptoma of *T. infestans* (GenBank accession number: JAC18341.1), using the program BLAST x.v.2.1.14.[25]

To identify conserved functional domains, an alignment was carried out with the deduced PER protein sequences from *R. prolixus*, *T. infestans*, and those from *D. melanogaster* (GeneBank accession number: NP_525056.2), *Cimex lecticularis* (GeneBank accession number: BAG07407.1), *Riptortus pedestris* (GeneBank accession number: BAG07407.1), *Blatella germanica* (GeneBank accession number: AAN02439.2), *Gryllus Bimaculatus* (GeneBank accession number: BAG48878.1), and *Laupala paranigra* (GeneBank accession number: ADO24377.1) using Clustal W Omega program (Analysis Tool Web Services from the EMBL-EBI, 2013). The sequences were analyzed with databases of GeneBank accessible on the website of NCBI SWISSPROT.

Amplification and sequencing of *per* gene fragments. Total DNA was isolated from 1 to 5 mg of *R. prolixus* and *T. infestans* thoracic muscles. The extraction was performed using the purification kit MasterPure™ DNA following the manufacturer's instructions (Complete DNA Purification Kit, Epicentre Biotechnologies, Madison, WI). Amplification of *per* gene fragments of *R. prolixus* and *T. infestans* was carried out by polymerase chain reaction (PCR), using primers designed from the *per* gene coding regions characterized in *R. prolixus* (Fper1: 5′-AACGGTTACACGCACATCGG-3′, Rper1: 5′-CCGTGGTTTTCGGTTTACTGT-3′, Fper2: 5′-CGTCTCCAGTACTTGAACCAGAA-3′, Rper2: 5′-AGGTTTGG TAATCTCGTCCATTAGA-3′). As a positive control of reaction, it was amplified a fragment of β-actin gene with primers AF2: 5′-ATTGCCCCACGCCATCCTT-3′ and AR2: 5′-AGCGGTAGC CATTTCCTCTTCA-3′. PCR amplifications were carried out in a Thermocycler (MyCycler; Bio-Rad, Hercules, CA) in 25 µL of a solution containing 1 µL of DNA, 0.2 µM of each specific primer, 0.5 U of Taq Platinum DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2.5 µL of 10× PCR Buffer minus M (Invitrogen). The PCR was performed with initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 45 seconds of annealing gradient, 72°C for 1 minute, and a final incubation at 72°C for 7 minutes. The PCR products (10 µL) were separated by electrophoresis on a 10-cm 1.5% agarose gel (Tris-acetate EDTA buffer, pH 8) containing 0.5 µg/mL of ethidium bromide. Digital images were obtained under ultraviolet illumination with a Chemi Doc System (Bio-Rad). After the PCR products were purified, they were sent for direct sequencing to the Genomics Unit/Node CATG National Genomics Platform, Institute of Biotechnology (ClCVyA-INTA's, Buenos Aires, Argentina). Sequences were reviewed with the software Chromas (version 1.45) and analyzed using the resources mentioned in the section "Identification and analysis of the sequence of PER gene in *R. prolixus*."

Phylogenetic relationship. Phylogenetic analysis was conducted to investigate evolutionary relationships among the deduced PER protein from *R. prolixus*, the putative PER protein recently identified in *T. infestans* (GeneBank accession number: JAC18341.1), and other selected sequences from *R. pedestris* (GeneBank accession number: BAG07407.1), *C. lectularius* (GeneBank accession number: XP_014250733.1), *B. germanica* (GeneBank accession number: AAN02439.2), *Pediculus humanus corporis* (GeneBank accession number: XP_002426301.1), *G. bimaculatus* (GeneBank accession number: BAG48878.1), *L. paranigra* (GeneBank accession number: ADO24377.1), *Rhyparobia maderae* (GeneBank accession number: AGA01525.1), *Apis mellifera* (GeneBank accession number: NP_001011596.1), *D. melanogaster* (GeneBank accession number: NP_525056.2), *Belgica antárctica* (GeneBank accession number: AGZ88038.1), *Tribolium castaneum* (GeneBank accession number: XP_008194040.1), *Bombyx mori* (GeneBank accession number: XP_012548895.1), *Halyomorpha halys* (GeneBank accession number: XP_014285208.1), and *Apteronemobius asahinai* (GeneBank accession number: BAL72155.1). Multiple alignments of sequences was performed using the multiple alignment program Clustal W in MEGA version 6.06.[26] Tree construction was performed by the neighbor-joining method, using MEGA version 6.06 software.[26] The reliability of the trees was tested by the bootstrap procedure with 1,000 replications.

Semiquantitative reverse transcription-PCR (RT-PCR) analysis in *T. infestans*. Total RNA was isolated from pools of insect tissues using MasterPure RNA Purification Kit (Epicentre Biotechnologies) according to the manufacturer's

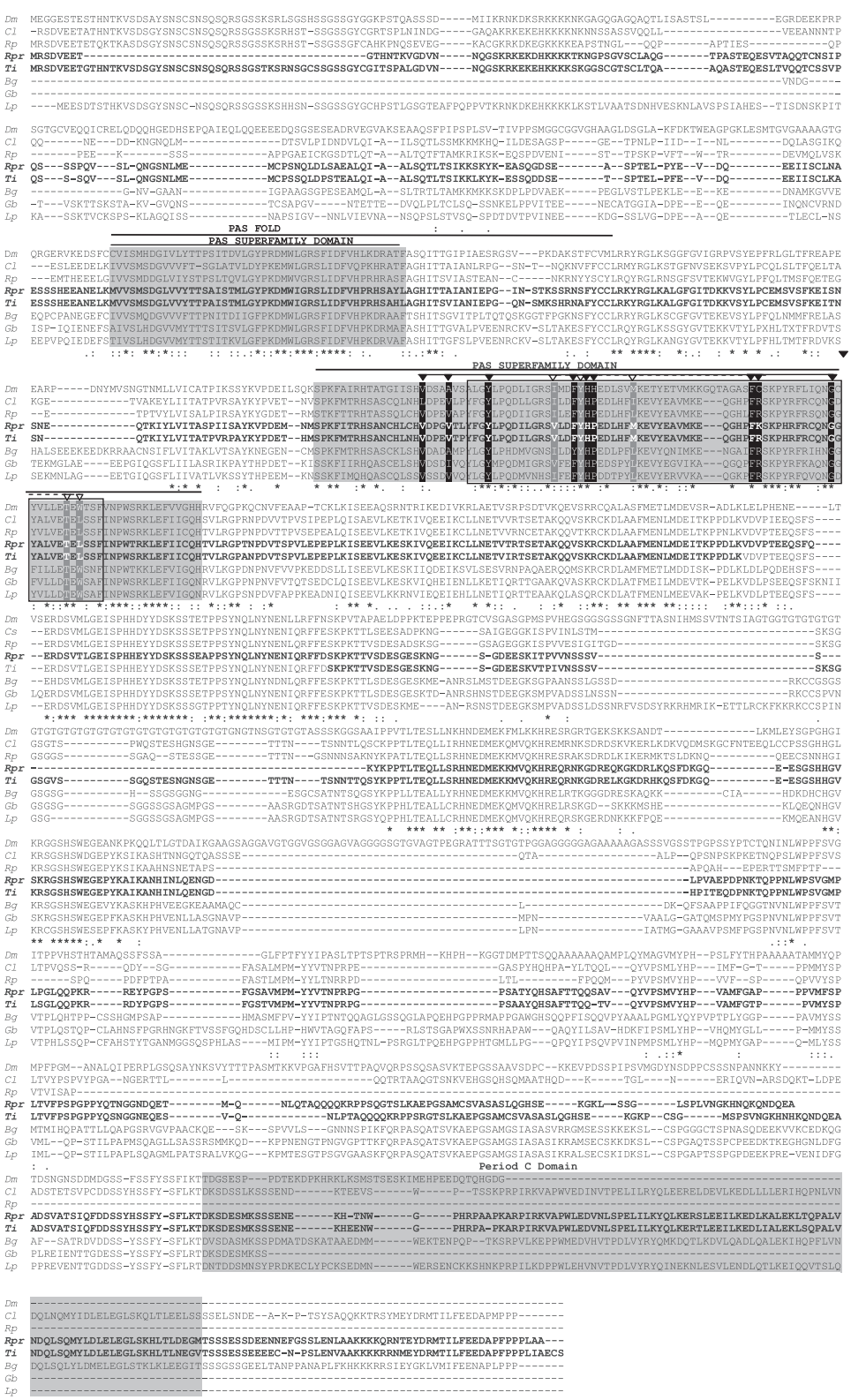

FIGURE 1. Period amino acid sequence alignment and conserved domain structure assignment. The alignment was performed using Clustal W program. *Bg = Blatella germanica*; *Cl = Cimex lecticularis*; *Dm = Drosophila melanogaster*; *Gb = Gryllus bimaculatus*; *Lp = Laupala paranigra*; *Rp = Riptortus pedestris*; *Rpr = Rhodnius prolixus*; *Ti = Triatoma infestans*. The conserved regions predicted by the National Center for Biotechnology Information Conserved Domain Search are indicated 1) the PAS FOLD is shown below a black bar, 2) the PAS superfamily domains are shown as gray-shaded regions below a black line, 3) the Period C domain is shown as gray-shaded region at the carboxy terminus, 4) the sensory box is shown with a frame, inside the putative active site residues are shaded in black and the residues of the heme pocket (binding site) are shaded in dark gray.

protocol. Samples from five adult females or five adult males were pooled. Extracts were diluted 1:100 with nuclease-free water containing 0.1% diethylpyrocarbonate.

Reverse transcription-PCR (RT-PCR) was performed from the total RNA isolated. First-strand cDNA synthesis was performed with 1 µL of Oligo-dT20 (50 µM) (Invitrogen), 3 µg of total RNA, and 300 U of SuperScript III-RT (Invitrogen) in a 20 µL reaction volume that was incubated at 55°C for 1 hour. The PCR mix contained 1 µL of first-strand cDNA as template, 0.2 µM of each specific primer, 0.5 U of Taq Platinum DNA polymerase (Invitrogen), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2.5 µL of 10× PCR buffer minus M (Invitrogen) in a 25 µL reaction. The PCR was performed in a Thermocycler (MyCycler) with initial denaturation at 94°C for 5 minutes; followed by 35 cycles at 94°C for 30 seconds, 60°C for 40 seconds, 72°C for 1 minute, and a final incubation at 72°C for 7 minutes. The primers Fper2 and Rper2 designed from PER gene coding region of the *R. prolixus* genome resulted also specific for *T. infestans* and were used for PER gene expression determination in *T. infestans*. The RT-PCR products (10 µL) were separated by electrophoresis on a 10-cm 1.5% agarose gel (Tris-acetate EDTA buffer, pH 8) containing 0.5 µg/mL of ethidium bromide. Digital images were obtained under ultraviolet illumination with a Chemi Doc System (Bio-Rad). Semiquantification of PCR bands was performed with the ImageJ Launcher (http//imagej.1557.n6.nabble.com/The-new-ImageJ-launcher-td370302.html) through a graphical method that measures peak areas. The results were expressed as a ratio calculated from the integrated signal bands over β-actin gene amplicon bands obtained with the specific primers AF2 and AR2.

Statistical analysis. The experimental values represent mean ± standard deviation of two independent experiments for each sample composed by pooled tissues from five specimens. The significance of differences in PER transcript gene levels was determined using analysis of variance. All statistical calculations were performed using Prism 5 software (GraphPad, San Diego, CA).

RESULTS

Analysis of the clock protein PER. The alignment of the deduced PER amino acid sequences from *R. prolixus*, *T. infestans*, *R. pedestris*, *Cimex lectularis*, *D. melanogaster*, *G. bimaculatus*, *B. germanica*, and *L. paranigra* is shown in the Figure 1. The comparative analysis of the amino acid sequences from these species revealed 91%, 61%, 61%, 37%, 50%, 50%, and 45% of identity, respectively. Conserved sites and domains typical of PER protein (PAS superfamily domains, the sensory box, and Period C domain) were localized in the predicted PER protein primary structure. Interspecies comparison revealed that the major region of sequence similarity in the PER protein are the PAS domains; otherwise, the -COOH end is very variable with low sequence homology.

Phylogenetic analysis was performed based on the complete amino acid sequences of PER protein of the hemipteran *T. infestans*, *R. prolixus*, *C. lectularius*, *R. pedestris*, and *H. halys*, and of 11 representative species of the orders Diptera, Lepidoptera, Coleoptera, Orthoptera, Blatodae, Phthiraptera, and Hymenoptera. As expected, insect PER sequences from the same insect order were grouped together with significant bootstrap support (Figure 2). Within Hemiptera, *T. infestans* clustered with *R. prolixus* with high bootstrap support (100%), as well as these two species of the subfamily Triatominae with *C. lecticularus* (97% bootstrap value). These three members clustered with strong support with *R. pedestris* and *H. halys* (100% bootstrap value), and

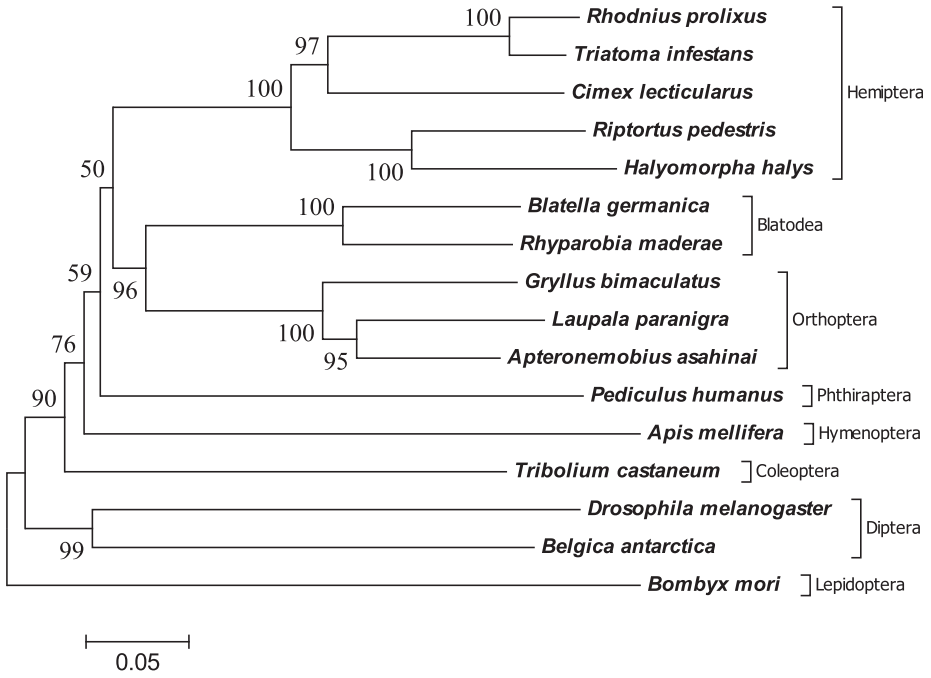

FIGURE 2. Neighbor-joining phylogeny of *Triatoma infestans* and *Rhodnius prolixus* PER deduced amino acid sequences, selected PERs from other hemipteran species, and representative species of the orders Diptera, Lepidoptera, Coleoptera, Orthoptera, Blatodae, Phthiraptera, and Hymenoptera. Bootstrap values next to nodes represent the percentage of 1,000 replicate trees that preserved the corresponding clade.

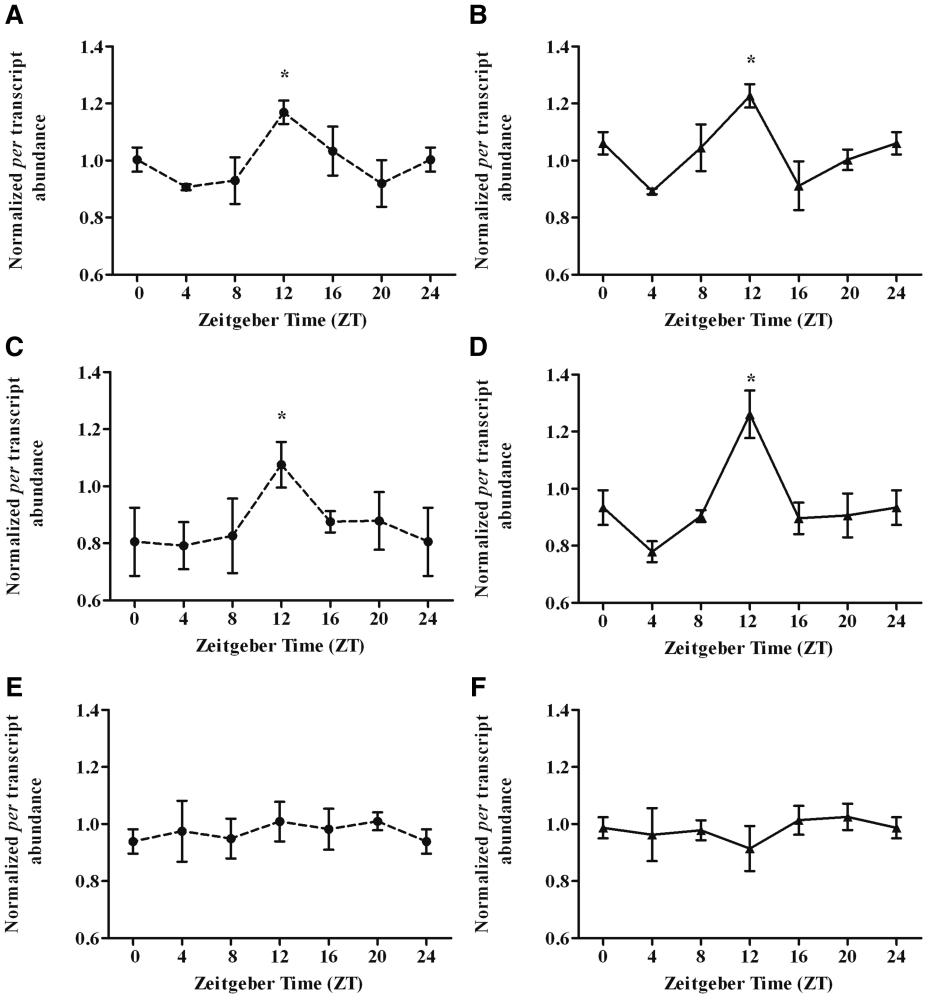

FIGURE 3. Expression profile of PER transcript in nervous tissue from male and female adults of *Triatoma infestans*. Abundance of PER transcript in a panel of cDNA derived from LD (**A** and **B**), DD (**C** and **D**), and LL (**E** and **F**) groups were measured using semiquantitative reverse transcription-polymerase chain reaction using the specific primers for PER and β-actin. Dash and continuous lines correspond to male and female groups respectively. The error bars represent the standard deviation of mean. Statistical significance: $^*P < 0.01$.

both species were also closely related (100% bootstrap value).

PER gene expression in *T. infestans*. The determination of PER gene expression at the nervous tissue was performed in groups of adult females and males of *T. infestans* maintained under strict photoperiod (LD), LL, and DD. The semiquantitative analysis of samples collected at 4-hour intervals for a period of 24 hours (ZT0 corresponds to lights-on) revealed clear oscillations in the abundance of PER transcript in both sexes of the LD and DD groups (Figure 3A–D). In these groups, the transcript level showed a significant increase at ZT12 ($P < 0.01$), and no differences were found in the remaining ZTs. Besides, no differences in PER oscillation were detected between the two sexes. By contrast, in the LL group, no changes were detected in *per* transcript levels at the ZTs analyzed (Figure 3E and F). The corresponding mRNA levels remain flat in constant light with relatively similar levels of expression in both sexes at all examined time points, comparable with the average values in LD and DD.

On the other hand, in adult individuals of both sexes restrained under photoperiod 12-hour light and 12-hour dark, it was detected at dusk (ZT12), the presence of PER transcript in gonads, thoracic muscles, and fat bodies. In addition, in male and female, fifth instar nymphs maintained in the same condition was detected PER transcript expression in nervous tissue (Figure 4).

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.[10,12–14,18] During the day, the Chagas disease vectors *R. prolixus* and *T. infestans* remains aggregate in dark shelters, such as holes or cracks. During the night, they show two peaks of locomotor activity, one at dusk to develop their activities such as finding food, feeding, dispersal, reproduction, etc., and the second at dawn to take shelter.[17] Minoli et al.,[16] demonstrated that the photoperiod contributes to the synchronization of daily activity and aggregation rhythm in *T. infestans*.

To further characterization of the circadian clock components of triatomine insects, a comparative analysis of the PER amino acid sequences from *R. prolixus* and *T. infestans* with those from other insects was carried out. The PER deduced amino acid sequences from *R. proluxis* and *T. infestans* possess all the important functional domains known in

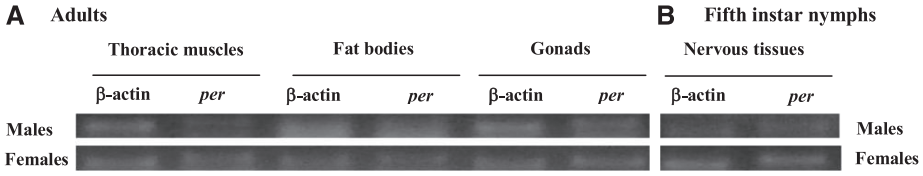

FIGURE 4. Polymerase chain reaction products of PER and β-actin transcripts from *Triatoma infestans* under light/dark regimen: (**A**) female and male adult thoracic muscles, fat bodies and gonads, and (**B**) female and male fifth instar nymph nervous tissue.

D. melanogaster and in other insects, including hemipteran species. Because of the interspecific variability detected in *Drosophila*, PER gene has been considered a "speciation gene."[27,28] Comparative analysis of the amino acid sequences of the protein PER from *Drosophila* species revealed the existence of variable regions in the -COOH terminal. PER sequence comparison between *R. prolixus* and *T. infestans* also showed differences in the -COOH end. On the other hand, in both species were detected conserved domains typical of PER protein as members of the PAS superfamily, which were previously identified in *D. melanogaster* and the hemipteran *R. pedestris*.[29,30] PAS domains were also found in the PER protein homologues in mammals (PER 1 and PER 2), suggesting the existence of conserved functions through large phylogenetic distances.[31] Among the functions of this domains are highlighted the dimerization with other similar binding domains in transcription factors, the function as sensors and transducers of signals of light and oxygen, and ligands binding.[32] Particularly, in *D. melanogaster* has been described with the function of the TIM protein dimerization.[29]

The PER gene expression at transcriptional level varies in nervous tissue with a daily rhythm in the groups maintained under photoperiod (LD) and DD, showing a significant peak of expression at sunset (Figure 3A–D). The pattern of PER gene expression under LD, observed in adult females and males of *T. infestans*, agree with the expression profile of this canonical clock gene in *D. melanogaster*. In this species has been extensively studied the mechanism of the circadian clock and has been observed that the clock genes are expressed in cycles close to 24 hours.[9] Moreover, in *R. prolixus* was detected an increase of levels of PER protein in the central clock neurons during the dark phase of the photoperiod.[20,21] Because *R. prolixus* and *T. infestans* are close species, it could be inferred that the increased level of PER mRNA detected in *T. infestans* at sunset would promote a peak of PER protein levels at night, necessary for the function of the period/timeless loop in the clock cells. On the other hand, also in agreement with the observed in *D. melanogaster*, the pattern of PER gene expression detected in LD persists under the DD condition in the nervous tissue of *T. infestans*.[9] Besides, as expected, in the nervous tissue of the group maintained in LL, no daily increase was detected in *per* transcript level. The same effect of the light was demonstrated in studies about the levels of the PER protein in clock neurons of *R. prolixus*.[21]

Insect clocks are classified into central and peripheral clocks based on their anatomical locations. Peripheral circadian clocks have also been shown to regulate the circadian rhythm in the function of the tissue where they reside.[33] The PER transcript is present at sunset in flight muscles, gonads, and fat bodies of adult individuals of *T. infestans* under 12:12 dark/light (LD) hours regimen. These results of the expression at transcriptional level of this clock gene are an evidence of activity of peripheral clocks in those tissues. The role of peripheral circadian clocks in various rhythmic phenomena should be examined in the future. In addition, the presence of PER transcript at dusk in the nervous tissue of fifth instar nymphs demonstrated the activity of the biological central clock during this development stage of *T. infestans*.

As the clock is fundamentally important to every aspect of behavior 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. For example, in adult mosquito *Aedes aegypti*, the major vector of dengue viruses in Taiwan, was found daily fluctuation of insecticide resistance. 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.[34] Because it was also detected that overexpression of a *cytochrome P450 (CYP4EM7*) gene in a highly insecticide–resistant strain of *T. infestans*, the clock gene PER could be also involved in the circadian control of the metabolic pathways of insecticide detoxification and resistance.[35]

This is the first work related to the circadian clock in *T. infestans*. This kind of study would provide potentially useful information to analyze the temporal regulation of important biological processes, such as reproduction, dispersal, insecticide resistance, etc. The studies of circadian rhythms in Chagas disease vectors and their molecular bases, 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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