

## Daily Variations in the Glycerol-3-Phosphate Dehydrogenase Isoforms Expression in *Triatoma infestans* Flight Muscles

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**Abstract.** *Triatoma infestans*, the main vector of Chagas disease, is a blood-sucking insect. Flight dispersal of adults is the most important mechanism for reinfestation of houses after insecticide spraying. Flight muscles have two glycerol-3-phosphate dehydrogenase (GPDH) isoforms: GPDH-1 is involved in flight metabolism and GPDH-2 provides lipid precursors. In this study, we explored the profile of GPDH expression in females and males adult flight muscles under light/dark cycle, constant light, and constant dark conditions. Under constant dark conditions, GPDH-1 flight muscles of *T. infestans* showed a rhythmic pattern of transcription synchronous with a rhythmic profile of activity suggesting regulation by the endogenous circadian clock. Otherwise, the GPDH-2 expression analysis showed no regulation by the endogenous clock, but showed that an external factor, such as the dark/light period, was necessary for synchronization of GPDH-2 transcription and activity.

### INTRODUCTION

Chagas' disease (American trypanosomiasis) is produced by infection with *Trypanosoma cruzi*, a protozoan transmitted by hematophagous insects of the subfamily Triatominae (Hemiptera: Reduviidae). The disease is a public health problem and has high socioeconomic effects in Latin America, where approximately 7–8 million persons are affected by this disease.<sup>1,2</sup> Transmission requires contact of host skin or mucosa with feces of the vector containing metacyclic forms of *T. cruzi*. The triatomines are potential vectors of *T. cruzi* from their earliest stages because they may acquire the infection during their first meal.

From an epidemiologic standpoint, the most important species of triatomines involved in the transmission of Chagas' disease are those that combine a high degree of adaptation to the domestic environments, have a wide geographic distribution, possess a high vectorial capacity, and are anthrophilic.<sup>3,4</sup> Considering their different habitats, only a few species, those with a high degree of adaptation to the domestic environment, have been recognized as effective vectors of trypanosomiasis in humans. Among them, *Triatoma infestans* (Klug) is the main vector species of Chagas' disease in the Southern Cone of Latin America between latitudes 10°S and 46°S. Across its distribution, *T. infestans* is primarily restricted to domestic and peridomestic environments, such as human dwellings, chicken coops, and pig or goat corrals.<sup>5</sup> This vector is described as being predominantly nocturnal, and flight dispersal is believed to take place at night.<sup>6–15</sup>

The ability to fly is important for adult dispersal.<sup>16,17</sup> Flight dispersal is the most important mechanism for reinfestation of houses at a village scale after insecticide spraying.<sup>18,19</sup> In the flight metabolism of insects, the glycerol-3-phosphate dehydrogenase (GPDH: NAD<sup>+</sup> 2 oxidoreductase, EC 1.1.1.8) plays a central role in  $\alpha$ -glycerophosphate cycle.<sup>20</sup> This enzyme is soluble, cytosolic, NAD<sup>+</sup>-dependent and is a dimer of two identical subunits present in all eukaryotic organ-

isms.<sup>21,22</sup> It is usually present in several isozymic forms that show different properties and specific tissue and developmental distribution.<sup>23</sup> Each isozyme performs a distinct metabolic function: GPDH-1 is involved in the flight muscle metabolism. GPDH-2 and GPDH-3 provide precursors for lipid biosynthesis in the gonads, fat bodies, and abdomen in larvae and nymphs.<sup>23,24</sup>

In *T. infestans*, it was demonstrated that GPDH involved in the glycerolphosphate shuttle increases its activity 30-fold in adult thoracic muscles. *Triatoma infestans* adult muscles should have higher glycolytic and respiratory capacity to support flight activity. Electrophoretic studies showed two isoforms of GPDH in *T. infestans*.<sup>25</sup> In a previous study, we characterized two transcripts corresponding to GPDH isoforms from *T. infestans* and investigated the transcript patterns in adult tissues and during flight muscle development.<sup>26</sup> In another study, we showed that GPDH-1 and GPDH-2 exhibited a tissue-specific pattern of expression and differentiated by sex and that the expression pattern of GPDH changes with different temperatures and amount of intake.<sup>27</sup> These results were consistent with transcripts requirement for GPDH isoforms expression according to the metabolic role described for GPDH isoforms in flying insects.<sup>23,24</sup>

Daily rhythms have been observed in a wide variety of behavioral, physiological, and metabolic process in many insects.<sup>28</sup> *Triatoma infestans* exhibits 24-hour time-of-day specific rhythms in flight activity, feeding, and reproductive behaviors.<sup>29</sup> However, biological timing at the molecular level has not yet been studied. Improved understanding of the molecular basis for light/dark regulated rhythms that underlie key physiological aspects of insect vectors may prove to be important to successful implementation of established and novel vector control methods.

The purpose of this study was to evaluate the molecular basis of flight habits of *T. infestans* by analyzing GPDH mRNA expression and enzyme activity under different dark/light regimens.

### MATERIALS AND METHODS

**Laboratory colony.** *Triatoma infestans* were reared at 28 ± 1°C at a relative humidity of 60–70% and fed 60 minutes on chickens, one time every two weeks after molt.

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TABLE 1  
Time of day in 24-hr Zeitgeber Time (ZT)\*

Regimen	ZT0	ZT4	ZT8	ZT12	ZT16	ZT20	ZT24
LD	End of dawn transition	Morning	Afternoon	Dusk (time of lights off)	Midnight	Early morning	End of dawn transition
DD	Subjective day			Subjective night			
LL							

\*LD = light/dark; DD = constant dark; LL = constant light.

Three experimental groups were subjected to a light/dark (LD) cycle, a constant light (LL) cycle, and a constant dark (DD) cycle. The LD cycle group consisted of 12 hours of light and 12 hours of darkness. Time of day is reported in 24-hour Zeitgeber Time (ZT); ZT12 is defined as time of lights off and ZT0 is defined as end of the dawn transition under the LD cycle. For LL and DD groups, subjective day was reported between ZT0 and ZT12 (Table 1).

Since the fifth instar nymph stage, insects were reared under different dark/light regimens. The flight muscles (thoracic muscles) were extracted 40–45 days after molt from female and male adult specimens and pooled separately every 4 hours over a 24-hour period under LD, LL, and DD conditions. Tissues were dissected under aseptic conditions and stored in liquid air (−194°C).

**Isolation of total RNA.** Total RNA was isolated from pools of insect tissues by using the Master Pure™ RNA Purification Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocol. Thoracic samples from 10 adult females or 10 adult males were pooled. For each experimental group, RNA isolation was performed in triplicate. Extracts were diluted 1:100 with nuclease-free water containing 0.1% diethylpyrocarbonate, and RNA concentration was determined by absorbance at 260 nm.

**Expression of glycerol-3-phosphate dehydrogenase isozymes at the mRNA level.** Isolation of total RNA from tissue pooled and first-strand cDNA synthesis was performed and subjected to reverse transcription-polymerase chain reaction (RT-PCR). First-strand cDNA synthesis was performed with 1 µL of Oligo-dT20 (50 µM) (Invitrogen, Carlsbad, CA), 3 µg of total RNA, and 300 units of SuperScript III-RT (Invitrogen) in a 20-µL reaction volume that was incubated at 55°C for 1 hour. Subsequent PCRs used 1 µL of first-strand cDNA as template, 0.2 µM of each specific primer and β-actin primers as positive controls, 0.5 units of Taq Platinum DNA polymerase (Invitrogen), 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, and 2.5 µL of 10× PCR Buffer minus M (Invitrogen) in a 25-µL reaction volume. The PCR was performed using a thermocycler (Mycycler; Bio-Rad, Hercules, CA) with a initial denaturation at 94°C for 5 minutes; followed by 35 cycles at 94°C for 30 seconds, 60°C for 40 seconds, and 72°C for 1 minute; and a final incubation at 72°C for 7 minutes. Specific primer for GPDH was F: 5'-CTGTTTCATTGATGGCTTAGG-3'. Different reverse primers corresponding to GPDH-1 Rm: 5'-TTCTTCACTGGGTGTTCTC-3' and GPDH-2 Rg: 5'-GTCCAGCAGTTAACCCCGTAGA-3' were used. The β-actin-specific primers AF2: 5'-ATTGCCCCACGCCATCC TT-3' and AR2: 5'-AGCGGTAGCCATTTCTCTTCA-3' were designed based on a sequenced 300-basepair PCR product amplified with the β-actin universal primer pair. 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). Semi-quantification of PCR bands was performed with the ImageJ Launcher (<http://imagej.1557.n6.nabble.com/The-new-ImageJ-launcher-t370302.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.

**Non-denaturing polyacrylamide gel electrophoresis.** Muscles pools of 100 mg were homogenized in 0.1 M phosphate buffer, pH 7.4 (1:5 w:v) by using Ultra Turrax T25 disrupter (Thistle Scientific, Glasgow, Scotland) at 4°C. Non-denaturing electrophoresis was conducted in a 7% polyacrylamide continuous gel system, pH 6.8. The running buffer was Tris-glycine, pH 8.3. Activity was determined by using a specific enzyme activity reaction at 37°C with 10 mg/mL of NAD<sup>+</sup> (disodium salt), 0.1 M alpha-glycerolphosphate, 0.1 M Tris-HCl buffer, pH 7.4, 1 mg/mL of phenazine methosulfate, and 1.6 mg/mL of nitroblue tetrazolium.

**Total enzyme activity.** Total GPDH activity was determined by using the protocol of Fink and Brosemer.<sup>30</sup> The assay medium was composed of 50 mM MOPS buffer, pH 6.6, 0.17 mM NADH (disodium salt) and 0.20 mM dihydroxyacetone phosphate (lithium salt). The final volume was 1 mL. Enzyme activity was determined in the supernatant by following the change in the absorbance at 340 nm in spectrophotometer with the cuvette compartment thermostabilized at 30°C. The reaction was started by addition of the enzyme preparation, diluted to give an absorbance change between 0.050 and 0.250 per minute, and absorbance was read every 10 seconds for 3 minutes. One unit of enzyme is the amount that utilizes 1 µmol of substrate per minute in the assay conditions. The molar extinction coefficient of 6.22 cm<sup>2</sup> µmol was used to calculate NADH oxidation. Activity is expressed as specific activity units per milligram of total protein. Controls were included in all determinations by omitting the substrate in the assay mixture.

**Total proteins.** Total protein quantifications were performed by using a fluorescence-based method with the Quant-it™ protein reagent kit (Invitrogen) in the Qubit® 2.0 Fluorometer (Invitrogen).

**Statistical analysis.** Experimental values represent the mean ± SD of three independent experiments for each ZT sample. The significance of differences between GPDH-1 and GPDH-2 transcript levels, between females and males transcript levels, and between total GPDH activities were determined by using Student's *t* test and analysis of variance. All statistical calculations were made by using Prism 5 software (GraphPad, San Diego, CA).

## RESULTS

Over 24 hours, biological material was collected every 4 hours from adult males and females maintained under strict LD cycle, LL cycle, and DD cycle conditions.

**Transcription profiles of GPDH.** The GPDH isoforms transcript patterns in *T. infestans* flight muscles showed different daily variations in both sexes. In females, GPDH-1 and GPDH-2 transcripts were present, but in males, only GPDH-1 transcript was detected (Figure 1).

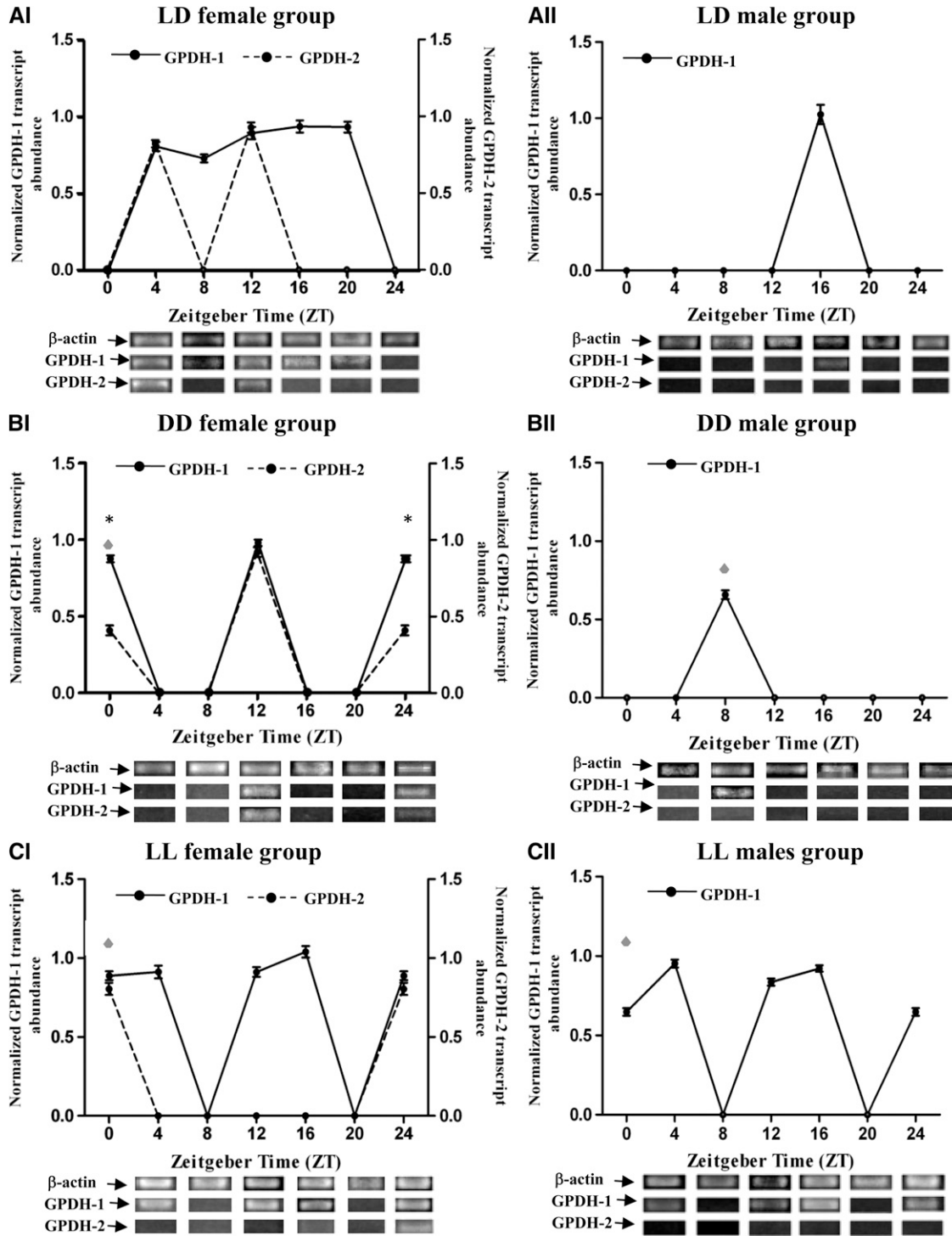


FIGURE 1. Reverse transcription–polymerase chain reaction (RT-PCR) semiquantification and agarose gel electrophoresis of glycerol-3-phosphate dehydrogenase (GPDH) transcripts in *Triatoma infestans* flight muscles from light/dark (LD) female and male groups (AI and AII), constant dark (DD) female and male groups (BI and BII), and constant light (LL) female and male groups (CI and CII) groups. \* indicates a statistically significant difference ( $P < 0.001$ ) between GPDH-1 and GPDH-2 transcript levels. indicates a statistically significant difference ( $P < 0.001$ ) between females and males transcript levels. Error bars indicate SD. Arrows indicate RT-PCR products:  $\beta$ -actin, GPDH-1, and GPDH-2.

In the LD group (Figure 1, AI), females had similar GPDH-1 transcript levels at Zeitgeber time (ZT) between ZT4 and ZT20, but the transcript was not detected in the dawn transition (ZT0/ZT24). In males, GPDH-1 transcript was expressed only at midnight (ZT16) at the same level as females (Figure 1, AI and AII). Females had two similar GPDH-2 transcripts peaks corresponding to the morning (ZT4) and the dusk (ZT12) (Figure 1, AI).

In the DD group, females had the same profile for both transcripts, which were expressed during the subjective dusk (ZT12) and during the subjective dawn transition (ZT0/ZT24). However, the level of expression of GPDH-2 was half that of GPDH-1 during the subjective dawn transition ( $P < 0.001$ ) (Figure 1, BI). In males, GPDH-1 was detected only during the subjective afternoon (ZT8) and was significantly less expressed ( $P < 0.001$ ) than in females (Figure 1, BI and BII).

In the LL group, females and males showed the same GPDH-1 transcript profile, but expression was not detected at ZT8 and ZT20. The only difference was that males had a lower transcript level than females during the subjective dawn transition ( $P < 0.001$ ). Conversely, GPDH-2 transcription was observed only in females during the subjective dawn transition (ZT0/ZT24) at levels similar to that of GPDH-1 (Figure 1, CI and CII).

#### Total glycerol-3-phosphate dehydrogenase activity profiles.

Total GPDH activity was determined from female and male flight muscles of *T. infestans* and showed different daily variations in both sex. In males, the DD and LL conditions generated similar total GPDH activity profiles during day and the dusk (between ZT0 and ZT12). The total GPDH activity was lower ( $P < 0.001$ ) between the subjective morning (ZT4) and the subjective afternoon (ZT8) in the female LL group than in the female LD and DD groups (Figure 2, AI, AII, BI, BII, CI, and CII).

In the LD group, females had the highest total GPDH activity in the morning (ZT4) ( $P < 0.001$ ) and similar activities during the rest of the ZT analyzed. In contrast, males (Figure 2, AI and AII) showed a lower total activity than females in the morning (ZT4) and had a low value at midnight (ZT16) ( $P < 0.001$ ).

In the DD group, females showed two peaks; the highest total GPDH activity was at ZT8 and ZT20 ( $P < 0.001$ ). The lowest value of total females GPDH activity was detected at the subjective midnight (ZT16). However, males had a peak of GPDH activity at ZT16 (Figure 2, BI and BII) ( $P < 0.001$ ).

In the LL group (Figure 2, CI), the highest total activity ( $P < 0.001$ ) was observed in females between subjective midnight (ZT16) and the end of the subjective dawn transition (ZT0/ZT24). During this period, males had two peaks ( $P < 0.001$ ), one during the subjective afternoon (ZT8) and the other during the subjective early morning (ZT20) (Figure 2, CII).

**GPDH-1 and GPDH-2 activities.** GPDH-1 activity was present in all ZT analyzed in both sexes, but GPDH-2 activity was observed only in some of the ZT of females (Figure 2A–C). Therefore, the total GPDH activity generally reflects only the activity of the GPDH-1 isoform.

The LD group showed GPDH-2 activity during midnight and early morning (ZT16 and ZT20) and a lower activity than GPDH-1 (Figure 2, AI). GPDH-2 activity in the DD group was observed during the subjective day between ZT4 and ZT8 when total activity is increasing. Also, GPDH-2 activity was

lower than GPDH-1 (Figure 2, BI). In the LL group, GPDH-2 activity was present during the subjective morning (ZT4), when females showed the lowest total GPDH activity, and during the subjective midnight (ZT16). GPDH-2 activity was lower than GPDH-1 activity at ZT4 and similar to GPDH-1 activity at ZT16 (Figure 2, CI).

## DISCUSSION

GPDH-1 is constantly transcribed between ZT4 and ZT20 in females in the LD group and is sufficient to maintain GPDH-1 activity over 0.4 IU/mg of protein during dark and light periods (Figure 1, AI and Figure 2, AI). LD males, which showed one peak of transcription at midnight (ZT16), had GPDH-1 activity two values below 0.4 IU/mg of protein (Figure 1, AII and Figure 2, AII). These GPDH-1 profiles of transcription and activities showed differences in DD and LL groups. In DD group females, a synchronous fluctuation was observed between transcription and activity peaks (Figure 1, BI and Figure 2, BI). Similar to the LD group, males also showed one peak of transcription, which was during the subjective afternoon (ZT8), and fluctuation of GPDH-1 activity (Figure 1, BII and Figure 2, BII). It is important to highlight that there is a peak of GPDH activity in female and male DD groups eight hours after transcription peaks (Figure 1, BI and BII, and Figure 2, BI and BII). In female and male LL groups, there was an oscillation in levels of the GPDH-1 transcript. However, these fluctuations were not reflected in the activity profiles (Figure 1, CI and CII, and Figure 2, CI, and CII).

In the mosquito *Anopheles gambiae*, many genes are rhythmic only during an environmental LD cycle, suggesting that light directly regulates gene expression. However, other genes are rhythmic under DD conditions, indicating regulation by the endogenous circadian clock.<sup>31</sup> Several circadian rhythms in behavior and physiological processes are under endogenous control in *T. infestans*, such as locomotor activity, negative phototaxis, egg hatching, ecdysis, and thermopreference. However, the oscillators controlling these rhythms has not yet been studied.<sup>32</sup> Under DD conditions, GPDH-1 flight muscles of *T. infestans* showed a rhythmic pattern of transcription synchronous with a rhythmic profile of activity, which also suggests regulation by the endogenous circadian clock. We hypothesize that light would have a direct action in suppressing rhythmic gene expression.

In females in the LD group, GPDH-2 was transcribed in the morning (ZT4) and at dusk (ZT12) (Figure 1, AI), and activity was detected at midnight (ZT16) and in the early morning (ZT20) (Figure 2, AI). This pattern of GPDH-2 transcription and activity changed in DD and LL conditions. The DD group also showed two GPDH-2 peaks of transcription, one at the subjective dawn transition (ZT0/ZT24) and the other at subjective dusk (ZT12) (Figure 1, BI). In this instance, GPDH-2 activity was observed during the subjective day (ZT4 and ZT8) (Figure 2, BI). GPDH-2 is transcribed only during the subjective dawn transition (ZT0/ZT24) in the LL group (Figure 1, CI), but its activity is present during the subjective morning (ZT4) and at subjective midnight (ZT16) (Figure 2, CI). These findings of different GPDH-2 patterns of activity detected between the LD group and the DD and LL groups are consistent with the hypothesis that the dark-light period is necessary to synchronize GPDH-2 transcription and activity.



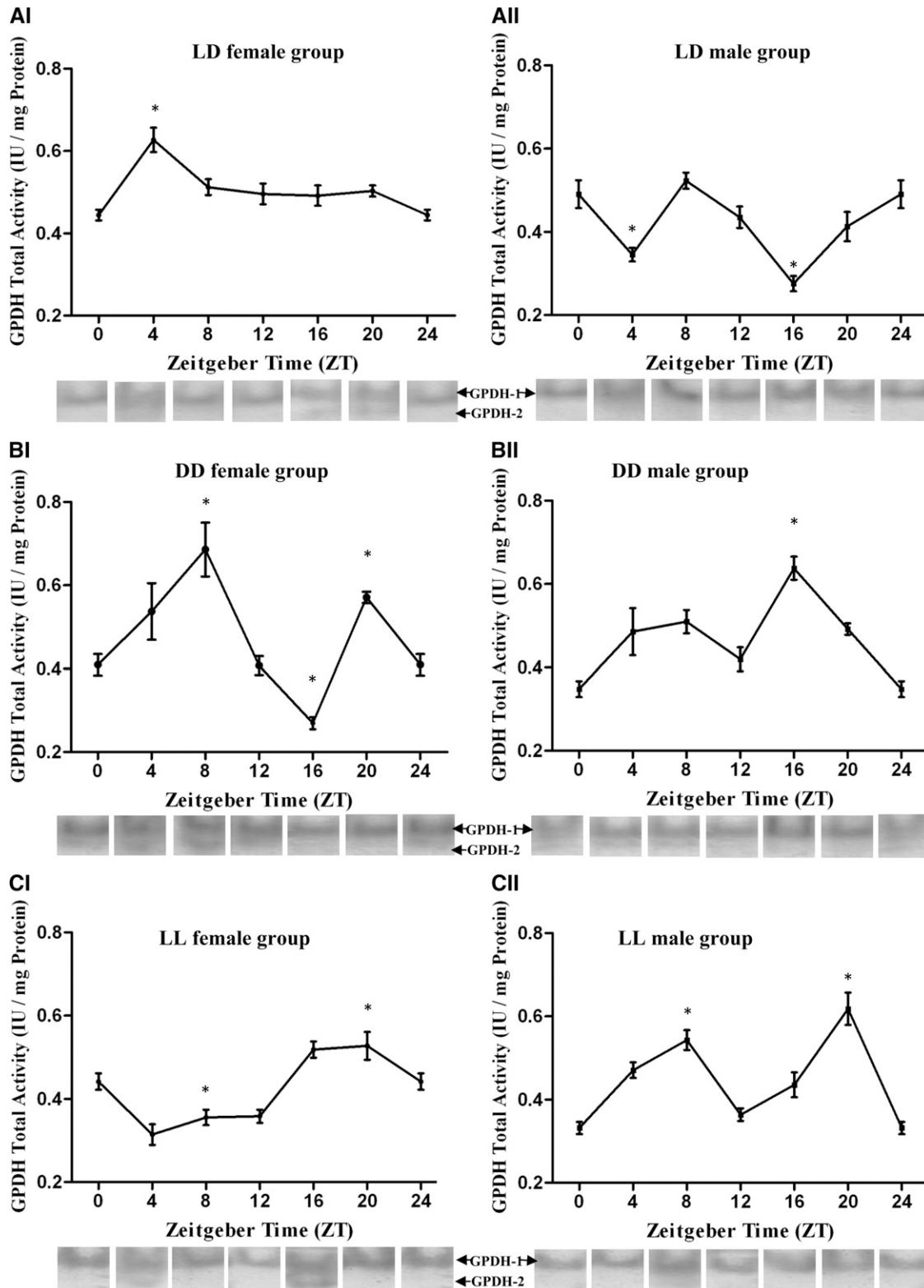


FIGURE 2. Total glycerol-3-phosphate dehydrogenase (GPDH) activity and non-denaturing polyacrylamide gel electrophoresis in *Triatoma infestans* flight muscles from light/dark (LD) female and male groups (AI and AII), constant dark (DD) female and male groups (BI and BII), and constant light (LL) female and male groups (CI and CII). Arrows indicate electrophoretic migration of GPDH isoforms. \* indicates a statistically significant difference ( $P < 0.001$ ) between activity levels. Error bars indicate SD.

*Triatoma infestans* spends a great deal of time during its life aggregated inside dark refuges, such as crevices, hollows, cracks, or fissures. Individuals of this species leave their refuges at the beginning of the night and display the major part of their activities, such as host search, feeding, and dispersion, in discrete temporal windows during this dark period.<sup>32</sup> Thus, this insect stays naturally in the dark during the day. The GPDH-1 isoform, which is involved in flight energetic metabolism, showed a synchronous expression in the DD group that would reflect the living condition of *T. infestans*. In the dark, the endogenous clock would regulate the GPDH-1 synchronous expression that could be involved in nocturnal flight habits of the vector. However, analysis of GPDH-2 expression showed no regulation by the endogenous circadian clock. Rather, an external factor such as the photoperiod was relevant in modulation of the rhythmic GPDH-2 expression (Figure 1, AI and Figure 2, AI). The finding of GPDH-2 activity during the subjective day in the DD condition would favor lipid accumulation during this period. This finding is expected in insects such as *T. infestans* that usually remain in an akinetic state inside their shelters during daylight hours.

Our study constitutes the first study related to biological timing of flight muscle metabolism at the molecular level in the Chagas disease vector *T. infestans*. GPDH-1 is associated with flight metabolism and GPDH-2 provides precursors for lipid biosynthesis. The activity of GPDH-1 suggests that expression of this enzyme is associated with an endogenous circadian clock, whereas GPDH-2 expression seems to be associated more with the dark/light period. Understanding the patterns of GPDH isoforms expression rhythms, which are important in triatomine flight muscle metabolism, has implications for development of novel vector control strategies, particularly in the context of infestation/re-infestations of houses by vector flight dispersal.

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