Differential Expression of Glycerol-3-Phosphate Dehydrogenase Isoforms in Flight Muscles of the Chagas Disease Vector *Triatoma infestans* (Hemiptera, Reduviidae)

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Abstract. Flight muscles of *Triatoma infestans* have two glycerol-3-phosphate dehydrogenase (GPDH) isoforms: GPDH-1 is involved in flight metabolism and GPDH-2 provides lipid precursors. Total GPDH activity was greater in the natural population and almost only due to GPDH-1. Different expression and activity observed between GPDH isoforms in the natural population and the first laboratory generation was not detected in the second laboratory generation. This pattern may be caused by gradual adaptation to laboratory nutritional conditions. During development, the expression of GPDH-2 increased with a longer time of intake, which would imply an increment in lipid biosynthesis. The GPDH-1 transcript predominated with respect to that of GPDH-2 in the lower nutritional condition, suggesting the necessity of insects to fly during this nutritional status. The transcriptional pattern showed a delay at 22°C. The isoforms activities and transcript patterns in flight muscles suggest transcriptional adaptation to metabolic requirements originated by alternative splicing.

INTRODUCTION

Chagas' disease or American trypanosomiasis is well recognized as the most serious human parasitic disease of Latin America in terms of its social and economic impact; 10–18 million persons are infected and approximately 90 million are living in disease-endemic areas.^{1–3} The disease is produced by infection with *Trypanosoma cruzi*, a protozoan transmitted by hematophagous insects of the subfamily Triatominae (Hemiptera, Reduviidae). Among them, *Triatoma infestans* is the main vector in the Southern Cone of Latin America between latitudes 10°S and 46°S.⁴

Members of the subfamily Triatominae are hemimetabolous insects with incomplete metamorphosis. Their nymphal period has five stages, each ending in a molt or ecdysis. The wings appear after the last molt from the fifth instar nymph to an adult. The ability to fly is important for dispersion of adults.⁵ In this respect, the temperature and nutritional conditions are the most important adaptive determinants of flight activity. An empirical model predicted that flight initiation of laboratory-reared *T. infestans* is associated with a low nutritional status and high temperatures.^{6,7} Active dispersal of *T. infestans* has seasonal variation with peaks in summer and males outnumber females as potential fliers.^{8,9} Moreover, flight dispersal is the most important mechanism for reinfestation of houses at a village scale after insecticide spraying.^{10–12}

In the flight metabolism of insects, glycerol-3-phosphate dehydrogenase (GPDH: NAD⁺ 2 oxidoreductase, EC 1.1.1.8) plays a central role in α -glycerophosphate cycle.¹³ This enzyme is soluble cytosolic NAD⁺-dependent and is a dimer of two identical subunits present in all eukaryotic organisms.^{10,14} It is usually present in several isozymic forms that show different properties and specific tissue and developmental distribution.¹⁵ In *Drosophila melanogaster*, three GPDH isozymes (GPDH-1, GPDH-2 and GPDH-3) were charac-

terized, and these isozymes originated by alternative splicing from the primary transcript of a single gene.¹⁶ The two major isozymes in *D. melanogaster*, GPDH-1 and GPDH-3, exhibit a differential tissue distribution in adults and a different temporal expression throughout development.^{17–19} Each isozyme performs a distinct metabolic function: GPDH-1 is involved in the flight muscle metabolism. GPDH-2 and GPDH-3 provide precursor for lipid biosynthesis in the gonads, fat bodies, and abdomen in larvae and nymphs.^{18,20}

In *T. infestans*, it was demonstrated that GPDH involved in the glycerophosphate shuttle increases its activity 30-fold in adult thoracic muscles.²¹ *Triatoma infestans* adult muscles should have higher glycolytic and respiratory capacity to support fly activity. Electrophoretic studies showed two isoforms of GPDH in *T. infestans*. The predominant isoform in thoracic muscles and gonads of nymphs has less mobility than the isoform in thoracic muscles of adults.

We have characterized two transcripts corresponding to GPDH isozymes from *T. infestans* and investigated the transcript patterns in adult tissues and during flight muscles development.²² We have demonstrated that GPDH-1 and GPDH-2 exhibit a unique temporal and tissue-specific transcription pattern. The GPDH-1 transcript is predominant in thoracic muscles of pools of both sexes of 1–5-day-old adults, and the GPDH-2 transcript is present in thoracic muscles, adult gonads, and fat bodies of pools of both sexes of fifth instar nymphs. Both transcripts are present in thoracic muscles of 30-day-old adults. The transcript patterns during flight muscles development show differences between sexes. Semiquantification shows that the GPDH-1 transcript is later in males and GPDH-2 is higher expressed in female after the last molt to adults. These results were consistent with transcripts requirement for GPDH isozymes expression according to the metabolic role described for GPDH isozymes in flying insects.^{17,18,20}

We explored expression at the mRNA level and enzyme activity of the two GPDH isoforms in natural populations and laboratory generations of *T. infestans*. Moreover, we studied the effect of nutritional and temperature variation on the transcriptional pattern, during the muscle development in laboratory colony of this species. We also

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infer the possible process involved in the origin of the GPDH isoforms.

MATERIALS AND METHODS

Laboratory colony. The *T. infestans* were reared at $28 \pm 1^\circ\text{C}$ at a relative humidity of 60–70% with a 6-hour light:18-hour dark cycle and fed once every two weeks after molt on restrained chickens. Six experimental groups of insects were analyzed: 1) individuals from first and second generations from laboratory (LG1 and LG2, respectively), which were fed for 60 minutes; 2) females fed for 30 minutes (In1) and for 120 minutes (In2); and 3) females insects reared at $22 \pm 1^\circ\text{C}$ (T1) and $28 \pm 1^\circ\text{C}$ (T2), which were fed for 60 minutes.

Individuals included in the experimental groups were those that gained the same average weight in the time of intake established (Table 1). Flight muscles were extracted from 30-day-old fifth instar nymphs and 1–5- and 30-day-old adult specimens. Tissues were dissected under aseptic conditions and stored in liquid air. Fifth instar nymphs were sexed according to the differences reported by Espinola.²³

Natural population. Individual *T. infestans* were collected from peridomestic and domestic environments of the department of Capayán, Catamarca Province, Argentina, during May 2010. Flight muscles of adults from this population were dissected under aseptic conditions and stored in liquid air.

Isolation of total RNA. Total RNA was isolated from pools of insect tissues by using TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Each sample was a pool of thoracic muscles from 10 adults or fifth instar nymph specimens. 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 pools and first-strand cDNA synthesis was performed and subjected to reverse transcription–polymerase chain reaction (RT-PCR) analysis. First-strand cDNA synthesis was performed with 1 μL of Oligo-dT20 (50 μM) (Invitrogen), 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_2 , and 2.5 μL of $10\times$ PCR Buffer minus M (Invitrogen) in a 25- μL reaction volume. The PCR was performed using a thermocycler Mycycler; Bio-Rad, Hercules, CA) with an 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 1.0 minutes; and a final incubation at 72°C for 7 minutes. Specific primers for GPDH were F: 5'-CTGG TTTCATTGATGGCTTAGG-3', and different reverse primer corresponding to GPDH-1 Rm: 5'-TTCTTCACT GGGTGTTC-3' and GPDH-2 Rg: 5'-GTCCAGCAGTT AACCCCCGTAGA-3'. The β -actin-specific primers AF2: 5'-ATTGCCCCACGCCATCCTT-3' and AR2: 5'-AGCG GTAGCCATTTCCTCTTCA-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 $\mu\text{g}/\text{mL}$ 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-td3703902.html>) through a graphical method that measures peak areas. Results were expressed as a ratio calculated from integrated signal bands over β -actin gene amplicon bands.

Non-denaturing polyacrylamide gel electrophoresis. Muscle pools of 100 mg were homogenized in 0.1 M phosphate buffer, pH 7.4 (1:2 v/v) with an Ultra Turrax T25 disruptor (Thistle Scientific, Glasgow, Scotland) at 4°C . Non-denaturing polyacrylamide gel 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^+ , disodium alpha-glycerolphosphate, 0.1 M phosphate 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.²⁴ 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 absorbance at 340 nm in a 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 over 2 minutes. One unit of enzyme is the amount that uses 1 μmol of substrate per minute in the assay conditions. The molar extinction coefficient of 6.22 $\text{cm}^2/\mu\text{mol}$ was used to calculate NADH oxidation. Activity is expressed as specific activity units per gram of wet tissue. Controls were included in all determinations by omitting the substrate in the assay mixture.

Statistical analysis. Experimental values are the mean $\pm$ SD of 3 independent experiments for each sample. Significance of differences between the bands intensity/area of the samples was determined by using Student's *t*-test. Analysis of variance

TABLE 1
Triatoma infestans included in experimental groups according to weight gained over time of intake*

Characteristic	Females					
	Fifth instar nymphs			Adults		
Stage						
Time of intake (minutes)	30	60	120	30	60	120
Mean $\pm$ SD weight (mg) gained at time of intake	120 $\pm$ 4.8	205 $\pm$ 8.6	240 $\pm$ 9.4	220 $\pm$ 10.5	310 $\pm$ 14.3	430 $\pm$ 24.1

FIGURE 1. Total glycerol-3-phosphate dehydrogenase (GPDH) activity in a natural population (NP) and the first and second laboratory generations (LG1 and LG2) of *Triatoma infestans* flight muscles. Underlined * point indicates a statistically significant difference ($P < 0.05$). Error bars indicate SD.

was used. All statistical calculations were made using Prism 5 software (GraphPad, San Diego, CA).

RESULTS

Expression of GPDH isoforms in a laboratory colony and natural population of *T. infestans*. The GPDH-1 and GPDH-2 activities and transcription patterns were determined from adult flight muscles of *T. infestans*. In the natural population, total GPDH activity was significantly higher ($P < 0.05$) than in the laboratory colony (Figure 1), in which no significant difference was observed between the two generations analyzed.

Levels of GPDH transcripts showed relative differences in two experimental groups (Figure 2A). In the natural population, the GPDH-1 transcript level was approximately 50% higher than that of GPDH-2. The first laboratory generation also showed a higher prevalence of the GPDH-1 transcript, but the difference compared with that of GPDH-2 decreased

to a value close of approximately 25%. No significant difference was found between the GPDH isoform transcript levels in the second laboratory generation group and the other two groups. Comparative analysis among the three groups showed similar GPDH-1 transcript levels in the natural population and the first laboratory generation, but both were different ($P < 0.05$) from that of the GPDH-1 transcript level detected in the second laboratory generation. No significant variation in the GPDH-2 level was observed.

The natural population had only GPDH-1 activity, and the first and second laboratory generations had GPDH-1 and GPDH-2 activities (Figure 2B). The GPDH-1 activity detected in the first laboratory generation was approximately 50% higher than that of GPDH-2, and there was no difference between both isoforms in the second laboratory generation. Comparative analysis among the three groups showed a significant GPDH-1 activity difference only between the natural population and the second laboratory generation group. The two groups that showed GPDH-2 activity were different (40% more activity in the second laboratory generation than in the first laboratory generation).

Some concordance was observed among GPDH expression at the mRNA level (GPDH-1 and GPDH-2 transcripts) and specific activity (Figure 2). The GPDH-1 isoform transcript and GPDH-1 isozyme activity were predominant with respect to GPDH-2 in the natural population and the first laboratory generation, but similar in the second laboratory generation. Conversely, concordance was observed between GPDH-1 transcript patterns and GPDH-1 specific activities of the natural population and the laboratory generations (Figure 2). In contrast, the GPDH-2 transcript level was similar in the three groups (Figure 2A), and although GPDH-2 activity showed variations, it was not detected in the natural population and showed an increase from the first laboratory generation to the second laboratory generation (Figure 2B).

Transcription patterns of GPDH in different intake conditions during development. The GPDH isoform transcript patterns from *T. infestans* flight muscles showed changes with different times of intake during development (Figure 3).

FIGURE 2. Glycerol-3-phosphate dehydrogenase (GPDH) transcript patterns and isozymes activities in a natural population (NP) and first and second laboratory generations (LG1 and LG2) of *Triatoma infestans* flight muscles. **A**, reverse transcription–polymerase chain reaction (RT-PCR) semiquantification and agarose gel electrophoresis (lane = β -actin control; lane 2 = RT-PCR GPDH-1 product, lane 3 = RT-PCR GPDH-2 product of GPDH-1 and GPDH-2 transcripts). **B**, GPDH-1 and GPDH-2 semiquantification activities and non-denaturing polyacrylamide gel electrophoresis (arrows indicate electrophoretic migration of GPDH isoforms). Underlined * point indicates a statistically significant difference ($P < 0.05$) between GPDH-1 and GPDH-2 transcript levels. Error bars indicate SD.

FIGURE 3. Glycerol-3-phosphate dehydrogenase (GPDH) transcripts in *Triatoma infestans* flight muscles with different time of intake during development. Reverse transcription-polymerase chain reaction (RT-PCR) semiquantification and agarose gel electrophoresis (lane 1 = β -actin control; lane 2 = RT-PCR GPDH-1 product; lane 3 = RT-PCR GPDH-2 product of GPDH-1 and GPDH-2 transcripts). **A**, 30 minutes of intake (In1); **B**, 120 minutes of intake (In2). Underlined * point indicates a statistically significant difference ($P < 0.05$) between GPDH-1 and GPDH-2 transcript levels. Error bars indicate SD.

Comparative analysis between transcript levels of the fifth instar nymphs showed that GPDH-1 predominated with respect to GPDH-2 in the 30- and 120 minute intake groups (In1 and In2). However, the difference was greater in individuals fed for 30 minutes (Figure 3A). The GPDH-1 transcript level was approximately 30% higher than that of GPDH-2 in young adults (1–5 days post-molt) and 30-day-old adults in the In1 group (Figure 3A). In contrast, in young adults in the In2 group, the GPDH-2 transcript predominated, and there were no differences between the GPDH-1 and GPDH-2 transcript levels in 30-day-old adults in the same group (Figure 3B). When intake time was increased from 30 to 120 minutes (Figure 3), the level of GPDH-1 transcript was nearly identical and the level of GPDH-2 transcript was more than 25 times higher in fifth instar nymphs.

In young adults, the GPDH-1 transcript decreased approximately 20% and the GPDH-2 transcript increased approximately 40%. No significant changes were observed in the GPDH-1 transcript levels in 30-day-old adults in the two intake groups, and the GPDH-2 transcript increased approximately 18%.

Transcription patterns of GPDH at different temperatures during development. The GPDH isoform transcript patterns in *T. infestans* flight muscles showed changes in response to temperature conditions during development (Figure 4). The GPDH-2 transcript level was five times higher than that of GPDH-1 in the fifth instar nymphs group at 22°C (T1) (Figure 4A). Conversely, GPDH-1 predominated in the fifth instar nymphs group at 28°C (T2) (Figure 4B). Young adults in both groups (T1 and T2) showed higher levels of GPDH-1

FIGURE 4. Glycerol-3-phosphate dehydrogenase (GPDH) transcripts in *Triatoma infestans* flight muscles under different temperature (T1 and T2) during development. Reverse transcription-polymerase chain reaction (RT-PCR) semiquantification and agarose gel electrophoresis (lane 1 = β -actin control; lane 2 = RT-PCR GPDH-1 product; lane 3 = RT-PCR GPDH-2 product of GPDH-1 and GPDH-2 transcripts). **A**, 22°C (T1); **B**, 28°C (T2). Underlined * point indicates a statistically significant difference ($P < 0.05$) significant difference between GPDH-1 and GPDH-2 transcript levels. Error bars indicate SD.

transcript than GPDH-2 transcript, and a higher difference in T2 group (Figure 4). Thirty-day-old adults showed a GPDH-1 transcript level three times higher than that for GPDH-2 in the T1 group (Figure 4A). However, in the T2 group, GPDH-2 predominated (Figure 4B).

When the temperature was increased from 22°C to 28°C (Figure 4), the GPDH-1 transcript increased four times and the GPDH-2 transcript decreased 50% in fifth instar nymphs. In young adults, the GPDH-1 transcript was similar in both groups, and the GPDH-2 transcript decreased approximately 50%. The GPDH-1 transcript decreased approximately 60% and the GPDH-2 transcript did not change in the 30-day-old adults. Moreover, results showed that young adults and 30-day-old adults at 22°C had similar GPDH-1 and GPDH-2 transcript patterns to those of 30-day-old fifth instar nymphs and young adults at 28°C.

DISCUSSION

Total GPDH activity was greater in the natural population than in the first and second generations of the laboratory colony (Figure 1). Although this difference was approximately 10%, it is important to point out that the GPDH activity is predominately that of the GPDH-1 isoform (Figure 2B), which is involved in flight energetic metabolism. In the laboratory generations, an increase of GPDH-2 activity was observed, which is involved in provision of precursors for lipids biosynthesis. Conversely, the differences in transcript levels observed between GPDH-1 and GPDH-2 in the natural population and the first laboratory generation was not detected in the second laboratory generation (Figure 2A). These results were consistent with activities of GPDH isoforms (Figure 2B). However, GPDH-2 transcript levels were not significantly different in the three groups and showed activity only in the laboratory colony, suggesting a post-transcriptional regulation process.

The patterns observed may be originated by gradual adaptation to nutritional conditions in the laboratory, in which regular blood feeding would favor lipid accumulation and the need of flight dispersal should be reduced. It is known that the nutritional status of insect populations affects flight dispersal. López and others reported that *T. infestans* from chicken coops had higher feeding rates and nutritional status during the spring and summer, and were predicted to have a low potential to flight dispersal.²⁵ Moreover, the probability of flight initiation of *T. infestans* was significantly associated with low nutritional status and high temperatures.⁶

The increase of intake time from 30 to 120 minutes during development promoted changes in the expression of GPDH isoforms at the mRNA level (Figure 3). The variation in the transcription patterns could be caused by a transcriptional adaptation in response to intake. In all instances, expression of the GPDH-2 isoform increases with a longer time of intake, which would imply an increment in lipid biosynthesis. In adults, the GPDH-1 transcript predominated with respect to that of GPDH-2 in the lower nutritional condition, suggesting the necessity of insects to fly during this nutritional status (Figure 3). With regard to the different temperature analyzed (Figure 4), a delay in GPDH-1 and GPDH-2 expression patterns at 22°C was observed. Expression of both isoforms in 30-day-old fifth instar nymphs at 22°C corresponds to expression observed in a previous study

in 1–5-day-old old fifth instar nymphs at 28°C.²² This result is consistent with those obtained for triatomine insects in which temperature affected development.²⁶

Enzyme activity can be modified by several mechanisms. These mechanisms include changes in concentration of mRNA for a specific enzyme, varying rates of transcription or mRNA processing, or mRNA degradation. In this regard, insect GPDH isoforms expression occurs by alternative splicing of a single gene.¹⁶ Bioinformatics analysis of *T. infestans* GPDH isoform complete cDNA sequences and sequence from the genome of another triatomine species (*Rhodnius prolixus*) suggests that expression of GPDH isoforms in these insects is generated by alternative splicing from a single gene. Comparative analysis of full-length GPDH-1 (1,253 basepairs) and GPDH-2 (1,262 basepairs) of *T. infestans* cDNA showed 84% homology with GPDH gene of *R. prolixus* and 97% homology with the deduced amino acid sequence.²² This analysis enabled detection of eight exons in the *R. prolixus* GPDH gene, stop codons in exons seven and eight, and polyadenylation signals, which suggest alternative splicing (Stroppa MM and others, unpublished data).

Alternative splicing is well-known mechanism for providing tissue-specific function and performing key roles in development, but there has been little effort to examine how quantitative intra-specific variation in alternative splicing within a tissue and developmental stage affects phenotype and fitness.²⁷ In this regard, in previous studies in *T. infestans*, we demonstrated that the GPDH isoforms are not distributed equally in space or time. It was determined that expression is related to insect tissue or development stage and the pattern of expression differs between sexes.²²

Although approximately 50–75% of animal genes undergo alternative splicing at the pre-mRNA level, there is fairly rudimentary knowledge regarding how alternative splicing varies among individuals or responds to body condition, environmental variation, or extracellular signals in general.^{28–30} We detected changes in expression of GPDH transcripts under different nutritional status and temperature during development that could be induced by adaptative splicing modification. In this regard, Marden and others showed that evolutionarily conserved alternative splicing of troponin-t in flight muscle of adult moths responds in a quantitative fashion to experimental manipulation of larval nutrition and adult body weight.³¹

Overall, these results showed that alternative splicing of a gene expressed in muscle responds in a quantitative fashion to whole-organism variables, which apparently serves to coordinate muscle strength and energy expenditure with body condition and life history. Consistent with these findings, our results support the hypothesis that isoform expression responds to organism conditions to produce different isoform profiles that are associated with energetic and life-history traits. Thus, expression of isozyme control depends on energy required for flight muscle or lipid accumulation.

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