



The gluconeogenic glucose-6-phosphatase gene is expressed during oxygen-limited conditions in the white shrimp *Penaeus (Litopenaeus) vannamei*: Molecular cloning, membrane protein modeling and transcript modulation in gills and hepatopancreas

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Abstract

The white shrimp *Penaeus (Litopenaeus) vannamei* is the most economically important crustacean species cultivated in the Western Hemisphere. This crustacean shifts its metabolism to survive under extreme environmental conditions such as hypoxia, although for a limited time. Glucose-6-phosphatase (G6Pase) is a key enzyme contributing to maintain blood glucose homeostasis through gluconeogenesis and glycogenolysis. To our knowledge, there are no current detailed studies about cDNA or gene sequences of G6Pase from any crustacean reported. Herein we report the shrimp *P. (L.) vannamei* cDNA and gene sequences. The gene contains seven exons interrupted by six introns. The deduced amino acid sequence has 35% identity to other homolog proteins, with the catalytic amino acids conserved and phylogenetically close to the corresponding invertebrate homologs. Protein molecular modeling predicted eight transmembrane helices with the catalytic site oriented towards the lumen of the endoplasmic reticulum. G6Pase expression under normoxic conditions was evaluated in hepatopancreas, gills, and muscle and the highest transcript abundance was detected in hepatopancreas. In response to different times of hypoxia, G6Pase mRNA expression did not change in hepatopancreas and became undetectable in muscle; however, in gills, its expression increased after 3 h and 24 h of oxygen limitation, indicating its essential role to maintain glycemic control in these conditions.

Keywords G6Pase · Gluconeogenesis · Glycogenolysis · Hepatopancreas · Gills · Dissolved oxygen

Introduction

Glucose-6-phosphatase (G6Pase) (EC 3.1.3.9) is a gluconeogenic and glycogenolytic enzyme that removes phosphate from glucose-6-phosphate (G6P) to release glucose into the bloodstream in the final step of each pathway (van Schaftingen and Gerin 2002). G6Pase is found mainly in liver and kidney of vertebrates and plays the important

role of providing glucose during starvation. Unlike most phosphatases acting on water-soluble compounds, it is a membrane-bound enzyme associated with the endoplasmic reticulum. In 1975, W. Arion and co-workers proposed a model for the G6Pase as an unspecific phosphatase with its catalytic site oriented towards the lumen of the endoplasmic reticulum (ER) (Arion et al. 1975), and that the substrate was provided by a translocase specific for G6P, thereby accounting for the specificity of the phosphatase for G6P in intact microsomes. Distinct transporters would allow inorganic phosphate and glucose to leave the vesicles. At variance with this substrate-transport model, other models propose that conformational changes play an important role in the properties of G6Pase. The last 10 years have witnessed important progress in the knowledge of the glucose-6-phosphate hydrolysis system. The genes encoding G6Pase and the glucose-6-phosphate translocase have been cloned

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and shown to be mutated in glycogen storage disease type Ia and type Ib, respectively. A gene encoding a G6Pase-related protein is specifically expressed in pancreatic islets. Specific potent inhibitors of G6Pase and of the glucose-6-phosphate translocase have been synthesized or isolated from microorganisms. This information as well as other findings support the model initially proposed by Arion. Much progress has also been made about the regulation of the expression of G6Pase by insulin, glucocorticoids, cAMP and glucose (van Schaftingen and Gerin 2002).

G6Pase is necessary to maintain glucose homeostasis and regulates G6P reserves in response to low blood glucose levels or during high energy demands in mammals (Trandaburu 1977; Surholt and Newsholme 1981; Hah et al. 2002; Varga et al. 2019). There is limited information about G6Pase in invertebrates. In the crab *Carcinus maenas*, the G6Pase enzyme from the digestive gland (hepatopancreas) has high metabolic plasticity and low specificity and plays an important role in systemic glucose homeostasis (Loret and Devos 1992). Changes in G6Pase have been reported in invertebrates. For example, G6Pase activity increased during fasting in hepatopancreas in the crab *Menippe mercenaria*, without changes in muscle (Scholnick et al. 2006). In contrast, G6Pase decreased in hepatopancreas of WSSV infected shrimp *Penaeus monodon* (Mathew et al. 2007), while in the chordate amphioxus *Branchiostoma japonicum*, its expression and activity increased in the digestive diverticulum during fasting (Wang et al. 2015); thus, it is important to understand the specific regulation and G6Pase contribution to maintain cellular processes (Hah et al. 2002).

As mentioned before, the integral membrane G6Pase activity is in the lumen of the ER formed by a complex of the catalytic subunit and a transporter of G6P, producing glucose and inorganic phosphate (Pi) (Marcolongo et al. 2013). Most G6Pases genes have five or six exons with sizes from 800 to 6,000 bp; in humans, the genes are located at 17q21 in the genome (Wang et al. 2015). There are three isoforms of G6Pase in vertebrates and they share structural features, but generally differ in organ expression in specific conditions (Marcolongo et al. 2013).

Low dissolved oxygen (DO) or hypoxia is one of the most essential variables that affect aquatic environments. The DO at which the animals begin to suffer hypoxia varies between species, but generally, adverse effects such as reduced feeding and growth rate, decrease culture yield, and mass mortality of crustaceans may appear when the DO concentration falls below 2 mg/L (Diaz 2001; Wu 2002); however, most organisms have biochemical and physiological mechanisms to respond to oxygen limitations for survival.

The shrimp *P.(L.) vannamei* is one of the most important crustacean species for global aquaculture, generating high economic resources for shrimp farming countries (Bostock et al. 2010). This shrimp species is an oxyconformer organism

that can adjust its metabolism depending on the oxygen available to survive (Wannamaker and Rice 2000) and can withstand DO concentrations as low as 0.2 mg/L for a short time (Pérez-Rostro et al. 2004).

Several studies have reported that shrimp exposure to hypoxia changes the expression of genes encoding enzymes of glycolysis and the Krebs cycle pathways (Ulaje et al. 2019). In addition, the activity of enzymes of anaerobic metabolism is increased as a strategy to maintain the energy balance to survive hypoxic stress (Soñanez-Organis et al. 2010, 2011; Cota-Ruiz et al. 2015; Camacho-Jiménez et al. 2018). Furthermore, higher expression of the genes for the gluconeogenic enzymes fructose-1,6-bisphosphatase (FBP), (Cota-Ruiz et al. 2015, 2016) and phosphoenolpyruvate carboxykinase (PEPCK) (Reyes-Ramos et al. 2018) occurs in hepatopancreas in response to prolonged periods of hypoxia in an organ-specific manner. This is an indication that the gluconeogenic pathway is stimulated. Likewise, the consumption of glycogen reserves in crustaceans due to hypoxia, and its subsequent restoration in post-hypoxic recovery through the recycling of lactate generated in anaerobic metabolism, indicates that these animals are capable of performing both, synthesis and catabolism of glycogen (Ellington 1983; Hervant et al. 1999). However, the metabolic adaptation mechanisms have just started to be unraveled.

Current knowledge about the expression and regulation of G6Pase gene in invertebrates is minimal and, to our knowledge, there are no profound studies about G6Pases genes sequences in crustaceans. In this work, we report the gene structure, coding sequence (CDS), phylogenetic analysis, topology prediction and gene expression in three organs under normoxic conditions and during different periods of hypoxia in hepatopancreas and gills of G6Pase from the white shrimp *P. (L.) vannamei* to contribute to the knowledge of crustacean shrimp energy metabolism and their responses to hypoxia.

Materials and methods

G6Pase cDNA sequence

Total RNA was extracted from gills of healthy juvenile shrimp using TRI® (Sigma-Aldrich, San Luis, Missouri, USA) according to the manufacturer instructions and to remove genomic DNA (gDNA) traces, it was treated with DNase I (Roche, Mannheim, Germany). RNA integrity was confirmed by 1% gel agarose. The first-strand cDNA synthesis from RNA was done using the QuantiTect Reverse transcription system (Qiagen, Hilden, Germany). For this, 1000 ng of total RNA were reverse transcribed with RT Primer Mix. Specific primers (Table 1) were designed for our study for the G6Pase sequence from the partially

Table 1 Specific primers used

Primers	Sequence (5'3')
qG6PF1	GCTGCAAGGGGCATTACTAC
qG6PR1	GCGATGACCCCCATAATACA
qG6PF2	GAAACTAGACCACTTCTGCGT
qG6PR2	TCCAAGCGAAATGCCCAACA
G6PF2	ATGGCAGAGCCTGATCTTCA
G6PR2	ACACATGGTCAAAAAGAAAGTGC
L8F2	TAGGCAATGTCATCCCCATT
L8R2	TCCTGAAGGGAGCTTTACACG

annotated *P. (L.) vannamei* genome (Zhang et al. 2019) and transcriptomic data (Ghaffari et al. 2014) using Primer3 (<https://primer3.ut.ee/>) and Oligo Calc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>).

The complete coding sequence (CDS) was obtained by PCR using the G6PF2/G6PR2 primers. For a 20 µL reaction, the mix contained 10 µL Hot Start Taq 2× Master Mix (New England BioLabs, Ipswich, Massachusetts, USA), 500 nM of Fw and Rv primers, 6 µL milliQ water, and 2 µL of cDNA (equivalent to 50 ng of total RNA) and was amplified under the following conditions: 94 °C for 3 min, followed by 94 °C for 30 s, 55 °C for 1 min and 68 °C for 1 min and 40 s per cycle for 42 cycles and finally, 72 °C for 10 min in a PTC 200 Thermal Cycler (Bio-Rad, Hercules, California, USA). Both strands of the PCR products were sequenced in the Laboratory of Genomic Analysis and Technology Core of the University of Arizona (Tucson, Arizona, USA) by the dideoxy chain-termination method. cDNA sequence and deduced protein were compared to nucleotide and protein GenBank databases using the BLAST algorithm (Altschul et al. 1990) at the National Center for Biotechnology Information, Bethesda, MD. CDS analyses were done using ExPASy (<http://web.expasy.org/translate/>), Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) bioinformatics tools.

G6Pase gene cloning and structure

Genomic DNA was isolated from the muscle of a healthy juvenile shrimp using proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation (Bradfield and Wyatt 1983). The complete *G6Pase* gene sequence was obtained by overlapping two PCR fragments, the 5' fragment using G6PF2/qG6PR1 primers and the 3' fragment with qG6PF1/G6PR2 primers (Table 1). The reaction was done using 12.5 µL of Hot Start Taq 2× Master Mix, 500 nM of each Fw and Rv primer, 25 ng of gDNA, and 9.5 µL of milliQ water to a final volume of 25 µL under the following conditions: a first denaturing step of 75 °C for 15 min

and 94 °C for 3 min, followed by 94 °C for 30 s, 59 °C for 1 min and 68 °C for 2 min per cycle for 36 cycles and finally, 72 °C for 10 min in a PTC-200 Thermal Cycler. PCR products were cloned in the pGEM-T Easy Vector System I (Promega, Madison, WI, USA) and used to transform TOP10 *Escherichia coli* competent cells. Recombinant plasmids were sequenced thoroughly in both DNA strands. The exon–intron identification was done with Splign (<https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi>) and SnapGene (GSL Biotech).

Structural and phylogenetic analysis of protein sequence

Molecular weight and isoelectric point were calculated with ExPASy (https://web.expasy.org/compute_pi/). Search for domains and conserved motifs was done with Pfam 32.0 (<https://pfam.xfam.org/>). The topological model of the protein was predicted with the HMMTOP transmembrane topology prediction server (Tusnady and Simon 2001) and the representation in two dimensions was constructed with TMRPrest2D (Spyropoulos et al. 2004). The amino acid sequences alignment included 25 G6Pase amino acid sequences obtained from the NCBI database (Table 2) and was performed in CLUSTAL W (Larkin et al. 2007). The phylogenetic analyses were conducted using MEGA version X (Kumar et al. 2018) and the Maximum Likelihood method, based on the Le Gascuel model (Le and Gascuel 2008) with 1000 bootstrap replicates. All positions containing gaps and missing data were eliminated and the tree was rooted with two G6Pase halobacterial sequences from *Haloferax gibbonsii* and *Haloarcula hispanica*.

Hypoxia bioassay

Juvenile shrimp obtained from a local farm were acclimated in 100 L tanks connected to a closed circulation filtration system with seawater under normoxic conditions [5 mg DO/L], constant salinity and temperature (35 ppt and 28 °C, respectively), in the Laboratory of Marine Invertebrate Physiology. The shrimp were transported to the laboratory as postlarvae and maintained in quarantine for approximately two months until they reached the appropriate size. In this period, the animals were analyzed by molecular and microbiological tests (PCR and bacteriological count) to discard White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), Infectious Hypodermal Hematopoietic Necrosis Virus (IHHNV), *Vibrio parahaemolyticus* and *Hepatobacter penaei* infected animals. Shrimp were fed with commercial feed equivalent to 3% of their dry weight in three portions per day with daily removal of residual food and feces and constant monitoring of temperature, salinity, ammonia, and dissolved

Table 2 G6Pase amino acid sequences used for phylogenetic analysis

Scientific name	Isoform	Gen Bank accession No
<i>Macrobrachium nipponense</i>	G6Pase*	ALK82315.1
<i>Varroa jacobsoni</i>	G6Pase 2	XP_022687819.1
<i>Centruroides sculpturatus</i>	G6Pase 2	XP_023224655.1
<i>Rhipicephalus microplus</i>	G6Pase*	AIW65716.1
<i>Tetranychus urticae</i>	G6Pase*	XP_015793132.1
<i>Rattus norvegicus</i>	G6Pase 1	NP_037230.2
<i>Danio rerio</i>	G6Pase*	NP_001157278.1
<i>Bos taurus</i>	G6Pase*	NP_001069592.1
<i>Astyanax mexicanus</i>	G6Pase*	XP_007234056.2
<i>Mus musculus</i>	G6Pase 1	NP_032087.2
<i>Mus musculus</i>	G6Pase 2	NP_067306.1
<i>Mus musculus</i>	G6Pase 3	NP_787949.2
<i>Cricetulus griseus</i>	G6Pase 2	RLQ65030.1
<i>Cricetulus griseus</i>	G6Pase 3	RLQ62406.1
<i>Homo sapiens</i>	G6Pase 1	NP_000142.2
<i>Homo sapiens</i>	G6Pase 2	Q9NQR9.1
<i>Homo sapiens</i>	G6Pase 3	Q9BUM1.2
<i>Globicephala melas</i>	G6Pase 2	XP_030705606.1
<i>Chanos chanos</i>	G6Pase 2	XP_030642465.1
<i>Cervus elaphus hippelaphus</i>	G6Pase 3	OWK14193.1
<i>Castor canadensis</i>	G6Pase 3	JAV36574.1
<i>Haloferax gibbonsii</i> ATCC 33,959	G6Pase*	ELZ83903.1
<i>Haloarcula hispanica</i> ATCC 33,960	G6Pase*	AEM55699.1
<i>Galendromus occidentalis</i>	G6Pase*	XP_003747144.1
<i>Penaeus (Litopenaeus) vannamei</i>	G6Pase	MW246958▲

*Not defined isoform

oxygen. Then, the shrimp (13.8 ± 1.4 g) were randomly distributed in 4 aquaria (11–12 shrimp/tank) and kept for three days in these tanks. Before the test, the shrimp were fed only once, and afterward, a total exchange of seawater was done in each aquarium. The molting stage of the shrimp was not identified but clearly recently molted specimens were discarded. The following treatments were performed: 0 h normoxia (control at the beginning of the test) [4.99 ± 0.16 mg DO/L, 28 ± 0.1 °C], 3 h hypoxia [1.5 ± 0.2 mg DO/L, 27.4 ± 0.2 °C], 24 h hypoxia [1.5 ± 0.2 mg DO/L, 27.2 ± 0.2 °C], and 48 h hypoxia [1.5 ± 0.3 mg DO/L, 27.1 ± 0.3 °C] in separate tanks. Normoxia was maintained by air bubbling with air diffuser stones, while hypoxia was produced by bubbling nitrogen decreasing the oxygen concentration progressively for 1 h. Dissolved oxygen and temperature were monitored every hour with a portable oximeter (model YSI proODO, Yellow Spring, OH, USA) and adjusted when necessary. Ammonia was monitored daily, and if necessary, water exchanges were done to keep levels below 3.0 mg/L. After time elapsed, 4–5 shrimp were sampled per treatment starting with the control group. The organs of each shrimp were dissected and placed in a cold microtube containing

10 volumes of TRI reagent (w/v), immediately frozen in a dry ice/96% ethanol mixture, and then stored at -80 °C until the analysis.

G6Pase mRNA quantification

G6Pase expression in normoxia was evaluated in hepatopancreas, gills, and muscle, while hepatopancreas and gills were used to analyze the effect of hypoxia by RT-qPCR (see below). Total RNA was isolated from 50 mg of each organ and treated with DNase I to remove gDNA remnants. The qG6PF2/qG6PR2 primers were used to amplify the G6Pase transcript to generate a 307 bp amplicon. One qPCR product per organ was sequenced in both directions to confirm the specificity of the quantification. The ribosomal protein L8 (GenBank accession no. DQ316258.1), that has been previously used as reference gene to normalize expression data for hypoxia studies in shrimp (Cota-Ruiz et al. 2015), was used with the primers L8F2/L8R2 previously designed in our laboratory to amplify a 166 bp fragment (Trasviña-Arenas et al. 2013) (Table 1). The G6Pase standard curve was performed using as a template a 765 bp purified PCR product previously obtained with the qG6PF2/G6PR2 primers with

1:10 serial dilutions (from 8×10^{-5} to 8×10^{-9} ng/ μ L) while the L8 standard curve was performed with serial dilutions 1:5 (from 25 to 4×10^{-2} ng/ μ L derived from total RNA) of gills cDNA to calculate amplification efficiency. As mentioned before, a 307 bp fragment was used to quantitate expression for G6Pase and a 166 bp fragment for ribosomal protein L8. cDNAs were synthesized for each organ using 500–750 ng of total RNA as a template with the QuantiTect Reverse Transcription Kit. Four technical replicates (two cDNA reactions for each RNA and two PCR reactions per cDNA) were analyzed in 4–5 shrimp per treatment. Due to the low abundance of G6Pase mRNA in hepatopancreas in hypoxia, the expression experiments were done in two stages. For normoxia expression in hepatopancreas, gills and muscle, and hypoxia in gills in the first stage, each qPCR reaction included 3 μ L of 5 \times HOT FIREPol[®] EvaGreen[®] (Solis BioDyne, Tartu, Estonia), 500 or 700 nM of each primer for G6Pase or L8, respectively, 1 μ L of cDNA (equivalent to 25 ng of total RNA) and nuclease-free water for a final volume of 15 μ L. For hypoxia expression in hepatopancreas in the second stage, each qPCR reaction included 7.5 μ L of 2 \times Sso Advanced universal SYBR[®] Green supermix (Bio-Rad, Hercules, CA, USA), 500 or 600 nM of each primer for G6Pase or L8, respectively, 2 μ L of cDNA (equivalent to 100 ng of total RNA) and nuclease-free water for a final volume of 15 μ L; a non-template control reaction was included in each run to check PCR contamination. The reactions were performed in a CFX96 Touch Real-Time PCR Detection System thermal cycler (Bio-Rad) with an initial cycle of 95 °C for 12 min, followed by 43 cycles of 95 °C for 15 s, 60.2 °C for 20 s and 72 °C for 20 s in the first stage, and the protocol for the second stage was an initial cycle of 94 °C for 3 min, followed by 45 cycles of 95 °C for 15 s, 60.2 °C for 35 s and 72 °C for 55 s. Finally, a melting curve analysis was done with a gradual increase of temperature (0.5 °C/3 s) from 65 °C to 95 °C to examine the presence of primers dimers or non-specific amplifications. The expression of G6Pase relative to L8 was calculated with the $2^{-\Delta C_t}$ method (Schmittgen and Livak 2008).

Statistical analysis

Data are presented as means \pm SD or medians and ranges as indicated. The distribution of data was tested with the Shapiro–Wilk normality test. Homogeneity of variance was verified with Levene or Brown–Forsythe test. Data of G6Pase mRNA expression in different shrimp organs in normoxia were analyzed using one-way ANOVA and differences between means were detected with Tukey test. Because data of hypoxia effect in G6Pase mRNA expression in hepatopancreas and gills fail in normality assumption, Kruskal Wallis and Dunn tests were performed to assess differences between medians. All the analyses were done

in the NCSS 2020 software (NCSS LLC, Kaysville, Utah, USA) with a significance level of $p < 0.05$. Graphics were made in GraphPad Prism version 9.

Results

G6Pase gene, CDS, and deduced protein

The gene and cDNA sequences of G6Pase obtained from the white shrimp were deposited in GenBank with the accession number MW246958 (Fig. 1). The gene nucleotide sequence we obtained is 99.07% identical to the genome sequence deposited in GenBank by Zhang et al. (2019), and 99.8% identical to the transcript included in the transcriptomic data of Ghaffari et al. (2014). The gene sequence starting from the initial codon for MET, ATG, is 2823 bp long. The genomic sequence was obtained with two overlapping fragments from genomic DNA. It comprises seven exons of 86, 165, 207, 131, 252, 119, and 117 bp each, interrupted by six introns with five split codons. All the introns contain the consensus 5'-GT-AG-3' splice junctions necessary for correct RNA splicing of most eukaryotic genes (Breathnach et al. 1978) and the number and size of exons and introns correspond with the ones reported in other species (Wang et al. 2015). The average base composition A/T and G/C of the exons are 56.43% and 43.57%, respectively, whereas for the introns are 73% and 27%, respectively. The CDS was initially identified from gills cDNA and is 1,077 bp long encoding 358 amino acids residues from the initial methionine (ATG) to the stop codon (TAA) at position 1075, resulting in calculated values for the isoelectric point and molecular weight of 9.25 and 40.74 kDa, respectively. The catalytic amino acids were identified by sequence alignments. Analysis with the Pfam 32.0 tool revealed that shrimp G6Pase has the PAP2-glucose-6-phosphatase domain characteristic of all G6Pase isoforms and no signal peptide presence was predicted with signalP, which is typical of known G6Pases.

Homology and phylogenetic analysis of P. (L.) vannamei G6Pase protein sequence

BLAST analysis of the deduced protein indicated an identity of 23.7 to 35.9% with the G6Pase sequences from the fruit fly (*Drosophila melanogaster*, GenBank accession no. NP_001097063), human (*Homo sapiens*, GenBank accession no. P35575), zebrafish (*Danio rerio*, GenBank accession no. NP_001157278.1) and eastern river shrimp (*Macrobrachium nipponense*, GenBank accession no. ALK82315.1). The highest homology of the white shrimp G6Pase was found with *M. nipponense* and *D. rerio* with the catalytic amino acids contained in highly conserved regions (Fig. 2). The phylogenetic analysis of 25 G6Pase

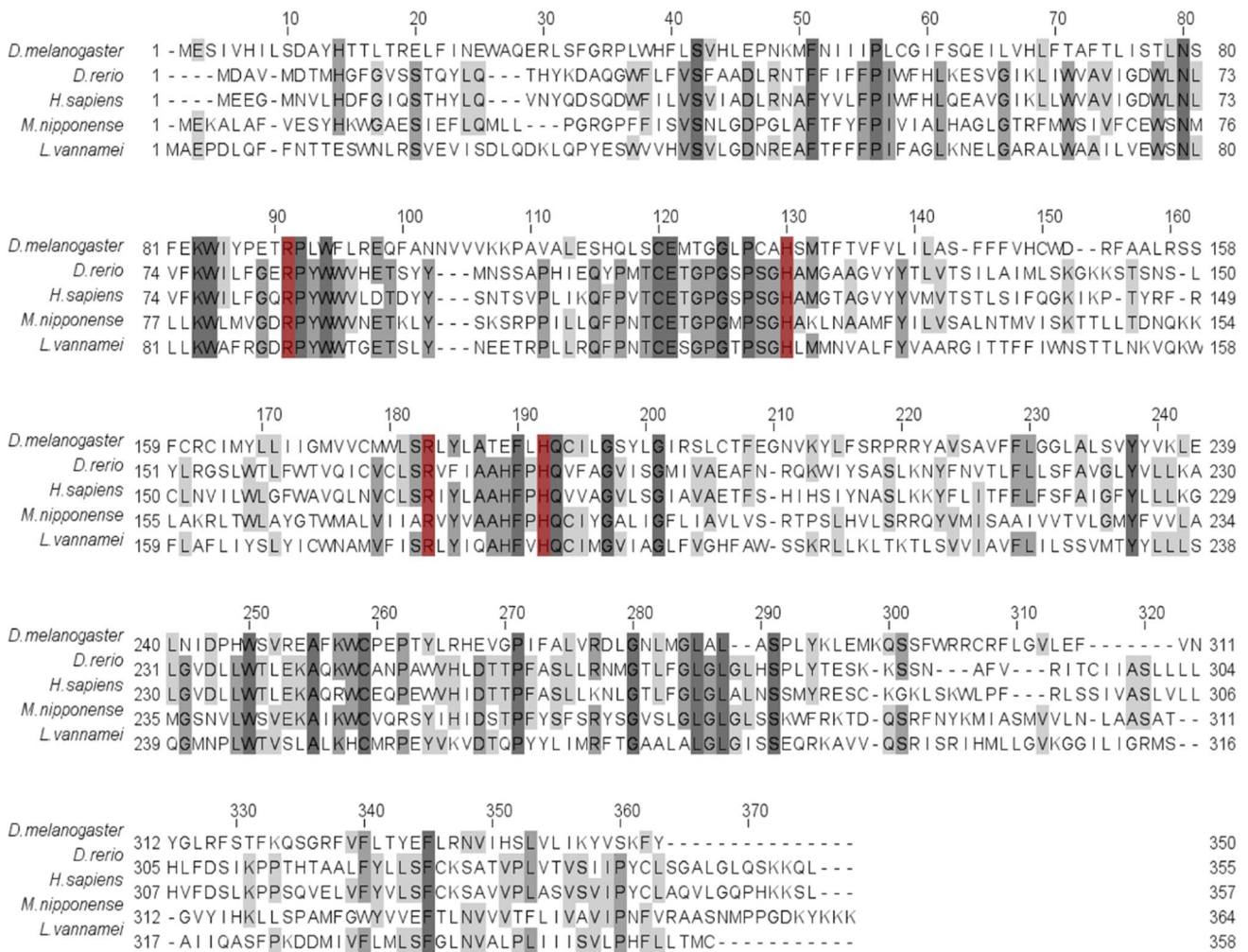


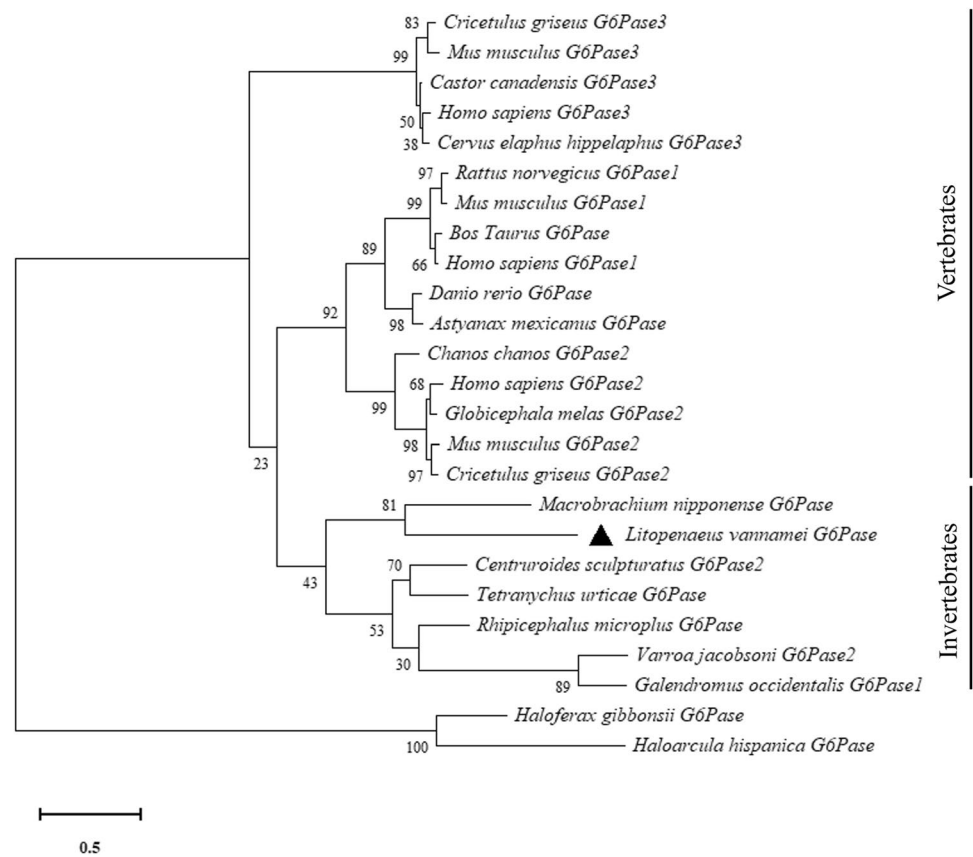
Fig. 2 Multiple alignment of the deduced amino acid sequences from *L. vannamei* G6Pase and selected species. The intensity of the gray color indicates higher conservation between sequences aligned, catalytic amino acids are highlighted in red

the shrimp G6Pase gene was detected in the three evaluated organs with the highest expression in hepatopancreas ($p < 0.05$), with 6.1 and 3.5-fold than in gills and muscle, respectively. Similar results were reported for the amphioxus *B. japonicum* that had a higher G6Pase expression in digestive diverticulum than in muscle and gills under developmental conditions (Wang et al. 2015), *B. japonicum* occupies a nodal evolutionary position from vertebrates and invertebrates. Likewise, G6Pase activity was 20 times higher in hepatopancreas than in claw muscle under feeding conditions in the stone crab *M. mercenaria* (Scholnick et al. 2006).

The effect of hypoxia on G6Pase relative gene expression was evaluated in hepatopancreas and gills by RT-qPCR (Fig. 6) and initially also evaluated in muscle, but expression in hypoxic conditions was undetectable. The PCR of amplification efficiencies and correlation coefficient in real

time were 91.1%, 0.99 and 90.5%, 0.99 for G6Pase and L8, respectively. No significant differences were detected within the treatments in hepatopancreas due to the high variability among the shrimp, although the means for the hypoxia groups are lower by approximately 0.5-fold compared to the normoxia control. In gills, there was a significant increase ($p < 0.05$) of 11.3 and 5.2-fold at 3 h and 24 h respectively compared to normoxia and no significant changes were detected at 48 h, but with a tendency to increase (2.1-fold) with respect to normoxia. The effect of hypoxia on the expression of G6Pase has not been studied in crustaceans or other invertebrates, but it is known that in vertebrates species such as the fish *Sparus aurata* (Metón et al. 2004; Wang et al. 2015; Zhang et al. 2020) and in the stone crab *M. mercenaria* (Scholnick et al. 2006), starvation stress results in increased activity of the enzyme in the liver and hepatopancreas, respectively.

Fig. 3 Phylogenetic tree based in a multiple alignment of 25 G6Pase amino acid sequences from different species. Numbers show the percentages of support based on 1000 bootstraps steps. The scale bar equals 0.5 substitutions per site



Discussion

The analysis of enzymes involved in carbohydrate metabolism is essential to understand their regulation and physiological significance in different species. The enzymes that catalyze irreversible reactions in central metabolism are particularly interesting to comprehend the pathways and their regulators that provide vital physiological responses for the cells. In the white shrimp, the genes of four enzymes that catalyze irreversible reactions of glycolysis and the corresponding for gluconeogenesis have been identified: pyruvate carboxylase (PC) (our unpublished data), PEPCK (Reyes-Ramos et al. 2018), FBP (Cota-Ruiz et al. 2015) and herein we report the G6Pase sequence, a key enzyme involved in glucose homeostasis. To our best knowledge, this is the first detailed report of this gene in crustaceans.

There are three isozymes for *G6Pase* in vertebrates: *G6Pase-I*, *G6Pase-II*, and *G6Pase-III* (Hutton and O'Brien 2009); however, information about characterization, expression, and regulation of *G6Pase* genes is rather limited in invertebrates. The cDNA coding sequence was initially obtained from gills in our laboratory, and the complete gene sequence was experimentally determined from genomic DNA. When the genome sequence of *P. (L.) vannamei* was uploaded to the NCBI database (but not fully annotated),

the cDNA nucleotide sequence that we had determined was carefully compared to the genomic data and found the interrupted *G6Pase* gene sequence. Analysis of *G6Pases* genes from different species included in GenBank (not fully complete or annotated) showed that the exon–intron structure of the shrimp *G6Pase* is more similar to arthropods such as in the moth *Ostrinia furnacalis* (Gene ID: 114,364,740) and the silk moth *Bombyx mori* (Gene ID: 101,742,080) which contain seven exons interrupted by six introns, while most of the genes in mammals are composed of five or six coding exons. The shrimp gene shares the same size and position of the second and fourth coding exons of the above mentioned arthropods, likely due to their phylogenetic proximity, although the length of the introns and complete genes are different.

Even though the overall identity between the *G6Pase* proteins is not very high, the regions containing the catalytic amino acids are conserved (Ghosh et al. 2002; Metón et al. 2004). The *G6Pase* protein from *B. japonicum* has 20–40% identity with other *G6Pases* of different species, and this enzyme plays a key role in glucose homeostasis (Wang et al. 2015). The 358 amino acid shrimp *G6Pase* has the conserved phosphatases sequence motif PAP2 (KXXXXXXXXRP-(X12-54)-PSGH-(X31-54)-SRXXXXX HXXXX) characteristic of all *G6Pases* (Stukey and Carman 1997). Additionally,

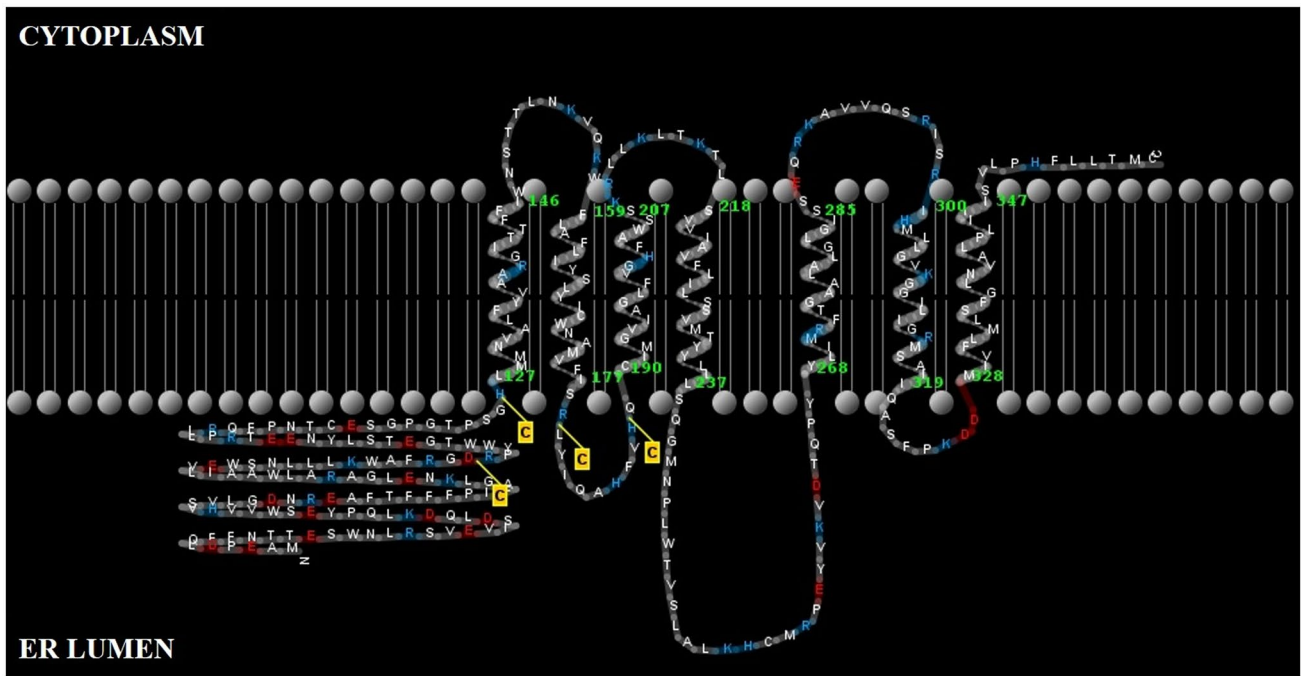


Fig. 4 Transmembrane topology prediction of *L. vannamei* G6Pase by HMMTOP server. The amino acids are coloring by electrostatic potential and the catalytic residues are indicated with C in yellow squares

the prediction of secondary structure based on hydropathy analysis using HMMTOP indicates the presence of transmembrane helices in G6Pase and coincides with homologs from vertebrates (Marcolongo et al. 2013), but the number of helices differs. The presence of seven TM helices separated by six hydrophilic loops in white shrimp G6Pase is presented in the model (Fig. 4).

In the phylogenetic analysis, the shrimp G6Pase is placed in the invertebrate group, with proximity only to the other shrimp species including the *M. nipponense* G6Pase and separated from the vertebrate sequences. It is worth noting that while isoforms in vertebrates can be grouped into different clades, in invertebrates there is no notable difference, indicating the higher proximity of G6Pases in this group. However, although the overall percentage of identity is low, there are highly conserved regions and the shrimp sequence is more similar to isoforms 1 and 2 of the fishes *Astyanax mexicanus* (30.72%), and *Chanos chanos* (30.52%) respectively.

In vertebrates, the liver is the central organ responsible for glucose homeostasis by *de novo* synthesis from non-carbohydrate precursors such as lactate or by hydrolysis of glycogen (Ghosh et al. 2002). In both metabolic pathways, the final reaction is catalyzed by G6Pase; therefore, this enzyme has been widely studied as an indicator of energy flow regulation, especially under stressful conditions such as starvation or under periods of high energy demand (Hah et al. 2002; Melkonian and Schury 2019).

Under optimal development conditions, little has been studied about G6Pase at a transcriptional level in invertebrates. However, in vertebrates as spotted gar, zebrafish, medaka, and rainbow trout, the liver is the main organ with the highest G6Pase expression compared to gills, muscle, kidney, demonstrating that it has a similar organ-specific expression pattern (Marandel et al. 2017). The same behavior was found in shrimp, indicating that hepatopancreas could be responsible for glycemic control in normoxia, coinciding with previous reports (Cervellione et al. 2017). Hence, it is possible that G6Pase functions as a good indicator of shrimp metabolic and physiological state.

In contrast, the expression of the gluconeogenic enzymes FBP and PEPCK is lower in the hepatopancreas compared to the muscle and the gills (Cota-Ruiz et al. 2015; Reyes-Ramos et al. 2018), which could indicate the activation of glycogenolysis instead of gluconeogenesis in this organ as a result of the fasting period to which the organisms were subjected (24 h at the sampling of this experiment). Consequently, the lack of energy substrates could induce the breakdown of the stored glycogen aiding to distribute glucose through the hemolymph to the target tissues for its use in the ATP rapid generation pathways as previously described, linking oxidative metabolism with the use and mobilization of carbohydrates between organs in shrimp (Sánchez-Paz et al. 2007; Ulaje et al. 2019). On the other hand, muscle and gills could play a secondary role in this metabolic process during normoxia, but it has been reported that G6Pase

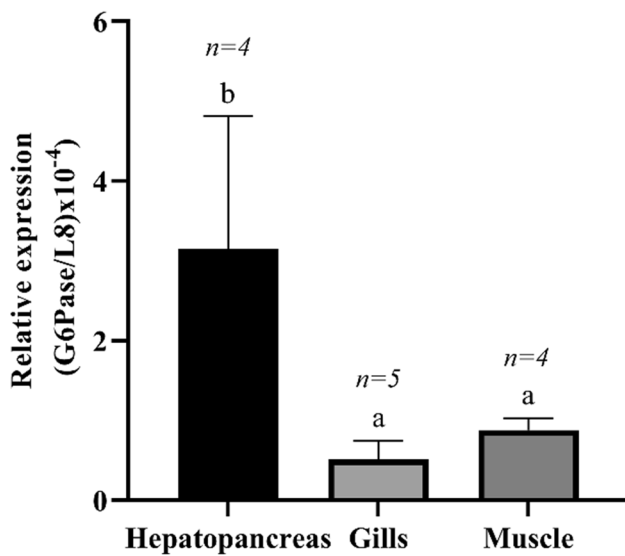


Fig. 5 Relative gene expression of G6Pase in white shrimp organs under normoxia. Data were normalized with the mRNA expression of the ribosomal protein L8 per the $2^{-\Delta\Delta Ct}$ method. Bars represent means \pm SD. The differences between the treatments are represented by literals ($p < 0.05$). The n values are included for each group

activity in starvation increases in both organs (Thabrew et al. 1971; Mathew et al. 2007), which suggests a condition and organ-specific regulation of the glycemic control to compensate the stress suffered by the animals.

The studies about shrimp have shown a complex metabolic regulation system to resist different stressors. Until now, hypoxia has received ample interest since climate change and anthropogenic activities are affecting marine and shrimp farm waters. Carbohydrate metabolism enzymes and their genes appear to respond in a specific organ-condition manner as mentioned before. Hypoxia does not seem to affect G6Pase expression in the hepatopancreas in this study conditions. This could indicate that glucose synthesis in this organ is independent of oxygen deficiency-induced stress. Nevertheless, gluconeogenesis appears to be activated in crustaceans by prolonged hypoxia (Cota-Ruiz et al. 2015; Reyes-Ramos et al. 2018) or post-hypoxic recovery to synthesize glucose using the end products of anaerobic metabolism such as lactate as precursors (Hervant et al. 1999). Thus, we cannot discard the participation of other G6Pase isoforms involved in glucose homeostasis, as is known in mammals (Lord-Dufour et al. 2009). The expression of G6Pase in gills increased after 3 h and 24 h of hypoxia, suggesting that glucose is generated *de novo*; in contrast, the expression of FBP and PEPCK decreased (Cota-Ruiz et al. 2015; Reyes-Ramos et al. 2018), while the concentration of glucose and relative expression of its transporter increased (Martínez-Quintana et al. 2014, 2016) under similar stressful conditions, which could again indicate catabolism of

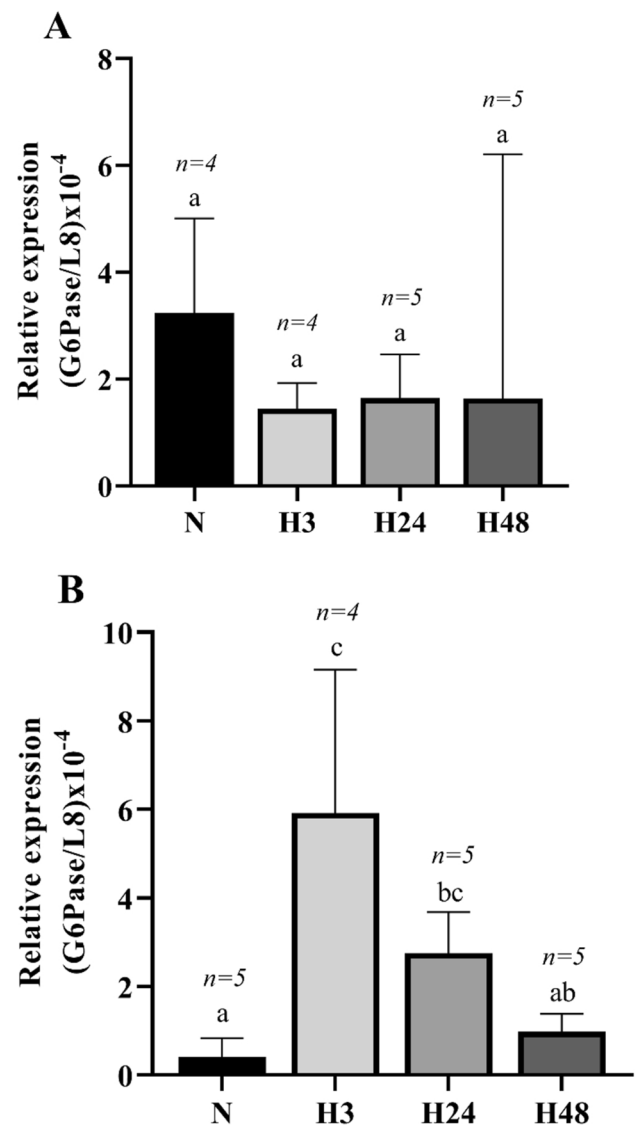


Fig. 6 Effect of hypoxia on G6Pase gene expression in hepatopancreas (A) and gills (B) of white shrimp. Data were normalized with the mRNA expression of the ribosomal protein L8 per the $2^{-\Delta\Delta Ct}$ method. N: control normoxia, H3: 3 h in hypoxia, H24: 24 h in hypoxia, H48: 48 h in hypoxia. Bars denote medians and ranges. The differences between the treatments are represented by literals ($p < 0.05$). The n values are included for each group

glycogen. Since it is known that crustaceans with different tolerance to hypoxia increase their hemolymph glucose and decrease their glycogen stores in the organs (Taylor and Spicer 1987), there is likely glycogenolysis activation in the white shrimp. It might be possible that gills exposed to low oxygen levels at the first exposed hours require additional energy *vs* other organs to perform essential processes such as respiration, detoxification, and osmotic regulation (Henry et al. 2012; Ulaje et al. 2019). Hence, G6Pase through glycogenolysis is generating enough glucose for the processes mentioned above.

Additionally, at 48 h of hypoxia in gills, the expression of G6Pase decreases, and it is reset at the level found in normoxia. Likewise, in this organ, the expression of FBP and PEPCK also decreases, which likely indicates that gluconeogenesis is not occurring, in contrast to the activation of gluconeogenesis in hepatopancreas that might occur at 48 h of hypoxia (Cota-Ruiz et al. 2015; Reyes-Ramos et al. 2018). This is probably the result of depression of metabolism (or hypometabolism) (Gorr et al. 2006) as a physiological strategy for the survival of shrimp to hypoxia to conserve energy through a coordinated decrease of the functions that mainly use ATP in the cell (for example, the synthesis and degradation of proteins) and prioritize essential and survival processes as mentioned above, to prevent lethal drops in cellular ATP levels. This could also be an indication of the synergistic regulation between gluconeogenesis and glycogenolysis not only as a glucose generation pathway to obtain fuel, but also as a strategy to take advantage of the carbon skeletons generated as lactate during anaerobic glycolysis.

Conclusions

This work reinforces the important role of the use and mobilization of carbohydrates in shrimp to survive and resist the low concentration of dissolved oxygen in different periods. The white shrimp G6Pase appears to contain all the necessary components of an active enzyme and the expression patterns in the three studied organs in normoxic and hypoxic conditions demonstrate its important role in glucose regulation probably mainly in gills by a glycogenolytic pathway in oxygen limited condition. Further research considering analysis of the promoter sequence, enzyme activity, and the implication of other G6Pase isoforms in response to different stressors in aquaculture will be helpful to the understanding of the physiological implications of this gene in these marine organisms.

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Authors contributions LEHA performed experiments, data analysis and writing of original draft. KCR performed initial experiments and data analysis. SGJ, provided shrimp and bioassay facilities. ABPU supervised experiments and methodology, gave helpful suggestions, critically revised and approved the final manuscript. GYP conceived, designed and supervised the experiments, critically revised, edited and approved the final manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing or financial interest.

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