



Effects of severe hypoxia and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) knock-down on its gene expression, activity, subcellular localization, and apoptosis in gills of the shrimp *Penaeus vannamei*

Laura Camacho-Jiménez, Alma B. Peregrino-Uriarte, Lilia Leyva-Carrillo, Silvia Gómez-Jiménez, Angus B. Thies, Martin Tresguerres & Gloria Yepiz-Plascencia

To cite this article: Laura Camacho-Jiménez, Alma B. Peregrino-Uriarte, Lilia Leyva-Carrillo, Silvia Gómez-Jiménez, Angus B. Thies, Martin Tresguerres & Gloria Yepiz-Plascencia (2023): Effects of severe hypoxia and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) knock-down on its gene expression, activity, subcellular localization, and apoptosis in gills of the shrimp *Penaeus vannamei*, Marine and Freshwater Behaviour and Physiology, DOI: [10.1080/10236244.2023.2216346](https://doi.org/10.1080/10236244.2023.2216346)

To link to this article: <https://doi.org/10.1080/10236244.2023.2216346>



View supplementary material [↗](#)



Published online: 26 May 2023.



Submit your article to this journal [↗](#)



View related articles [↗](#)



View Crossmark data [↗](#)



Effects of severe hypoxia and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) knock-down on its gene expression, activity, subcellular localization, and apoptosis in gills of the shrimp *Penaeus vannamei*

Laura Camacho-Jiménez^a, Alma B. Peregrino-Uriarte^a, Lilia Leyva-Carrillo^a, Silvia Gómez-Jiménez^a, Angus B. Thies^b, Martin Tresguerres^b and Gloria Yepiz-Plascencia^a

^aLaboratorio de Biología Molecular y Bioquímica, Centro de Investigación en Alimentación y Desarrollo (CIAD), Hermosillo, Sonora, Mexico; ^bScripps Institution of Oceanography, University of California San Diego, La Jolla, CA, USA

ABSTRACT

Penaeus vannamei, experiences hypoxia in its natural habitat and in aquaculture. Under hypoxia, cells enhance anaerobic energy production through glycolysis dependent on the up-regulation of glycolytic enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in gills. In vertebrates, GAPDH translocates into the nucleus and displays “moonlighting” functions including apoptosis. These alternative localizations and functions have not been described in crustaceans. We examined the effect of severe hypoxia and GAPDH silencing by RNA interference (RNAi) on its mRNA expression localization and glycolytic activity in *P. vannamei* gills. Expression and cytosolic activity were up-regulated only in hypoxia-exposed shrimp, but not in hypoxia-silenced specimens. GAPDH was immunodetected in cytosol and nucleus regardless of oxygen conditions. Hypoxia and RNAi decreased activity in cytosol and nucleus without affecting protein abundance, which suggests that nuclear GAPDH may have non-glycolytic functions. Moreover, Caspase-3 (Casp-3) expression increased with GAPDH silencing, suggesting alternative roles for GAPDH in apoptosis evasion.

ARTICLE HISTORY

Received 3 February 2023
Accepted 17 May 2023

KEYWORDS

Penaeus vannamei; hypoxia; GAPDH; Caspase-3; RNAi; cell fractionation

1. Introduction

The white shrimp (*Penaeus vannamei*) is a commercially important penaeid species for fisheries and aquaculture worldwide, and it is naturally distributed along the Eastern Pacific coast from Sonora, Mexico, to Tumbes, Peru (Holthuis 1980). This crustacean spends a large part of its life cycle in estuaries and coastal lagoons, where it can experience diel and seasonal decreases in dissolved oxygen (DO) concentrations below 2 mg O₂/L, a condition known as environmental hypoxia (Holthuis 1980; Diaz and

CONTACT Gloria Yepiz-Plascencia ✉ gyepiz@ciad.mx Laboratorio de Biología Molecular y Bioquímica, Centro de Investigación en Alimentación y Desarrollo (CIAD), Carretera Gustavo Enrique Astiazarán Rosas, no. 46, col La Victoria, Hermosillo, Sonora C.P. 83304, Mexico

Supplemental data for this article can be accessed online at <https://doi.org/10.1080/10236244.2023.2216346>

© 2023 Informa UK Limited, trading as Taylor & Francis Group

Rosenberg 2008). Hypoxia in coastal ecosystems has many natural causes, including changes in photosynthesis/respiration rates, stratification of water column, rises in water temperature, eutrophication, and organic matter mineralization (Rabalais et al. 2010). In addition, oxygen depletion has been increasing in marine environments since at least the middle of the 20th century due to the effects of global warming that has been rising ocean surface temperatures and anthropogenic contamination that exacerbates nutrient enrichment of coastal areas (Breitburg et al. 2018). In shrimp farms, hypoxia is also commonly caused by poor aeration, insufficient water exchange, deficient water mixing, high stocking density, overfertilization, algal blooms, increased microbial activity, among other factors (Boyd 2017). Even though the white shrimp can survive episodic drops in DO to as low as 0.2 mg O₂/L (Pérez-Rostro et al. 2004), prolonged exposure to sub-lethal hypoxia can affect immune and antioxidant responses (Zhou et al. 2014; Li et al. 2016), and decrease growth and survival (Han et al. 2017), overall having a negative impact on the yield of shrimp cultures.

The ability of *P. vannamei* to withstand hypoxia depends on changes at molecular and biochemical level that allow cells to adjust its metabolism to the shortage in oxygen supply. One of the major changes triggered by hypoxia is the transition from oxidative phosphorylation to anaerobic glycolysis as the dominant metabolic pathway to produce ATP (Hoogewijs et al. 2007). This switch is reflected in the accumulation of lactate in tissues and hemolymph as a consequence of higher expression and activity of lactate dehydrogenase (LDH) that catalyzes the last step of anaerobic glycolysis, namely the conversion of pyruvate to lactate (Soñanez-Organis et al. 2010, 2012; Ulaje et al. 2019). In addition to LDH, during hypoxia other genes encoding glycolytic enzymes of *P. vannamei* are up-regulated in a tissue-specific manner, such as hexokinase (HK) in gills and muscle (Soñanez-Organis et al. 2011), phosphofructokinase (PFK) in hepatopancreas (Cota-Ruiz et al. 2015, 2016) and gills (Ulaje et al. 2019), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in gills (Camacho-Jiménez et al. 2018, 2019). Experiments using RNA interference (RNAi) for genes knock-down have shown that the hypoxic induction of these enzymes is regulated by the hypoxia inducible factor 1 (HIF-1) (Soñanez-Organis et al. 2011, 2012; Cota-Ruiz et al. 2016; Camacho-Jiménez et al. 2019), an oxygen-sensitive transcription factor that coordinates diverse cellular processes as part of the responses of the white shrimp to hypoxia (Han et al. 2018; Duarte-Gutiérrez et al. 2021).

GAPDH (EC 1.2.1.12) catalyzes the sixth reaction of glycolysis, which consists in the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate (G3P) into 1,3-biphosphoglycerate (1,3-BPG) with the simultaneous reduction of NAD⁺. GAPDH typically exists in the cytosol of somatic cells as a homotetramer that accounts for 10–20% of total protein content (Kosova et al. 2017). In vertebrate cells, GAPDH has been found in other sub-cellular localizations, such as the endoplasmic reticulum, Golgi, and nucleus, where it has ‘moonlighting’ action alternative to its glycolytic activity (Sirover 2012; Kosova et al. 2017). Multiple roles have been found for nuclear GAPDH, including DNA repair, regulation of gene transcription, tRNA export, telomere maintenance, histone biosynthesis and induction of apoptosis (Kosova et al. 2015). For these reasons, GAPDH is now considered a crucial effector protein in the cellular responses to diverse endogenous and external stimuli (Sirover 2012).

Experiments in mammalian cell lines have revealed that knock-down of GAPDH expression prevents apoptosis during hypoxia/reoxygenation injury (Liang et al. 2015). Hypoxia induces the translocation of GAPDH into the nucleus, where it promotes apoptosis through signaling pathways mediated by the tumor suppressor protein p53, a stress-responsive transcription factor (Gong et al. 2019). The mechanisms through which GAPDH participates in p53-dependent apoptotic pathways are not clear, but p53 can up-regulate GAPDH expression, while acetylation of nuclear GAPDH can enhance the pro-apoptotic functions of p53 (Colell et al. 2009). Moreover, GAPDH forms a nuclear complex with p53 that seems to be essential for apoptosis (Zhai et al. 2014).

Even though it has been shown that hypoxia may promote apoptosis in crustaceans like *Scylla paramamosain* (Xu et al. 2021), *Macrobrachium nipponense* (Sun et al. 2017, 2020), *Marsupenaeus japonicus* (Wang et al. 2022), and *P. vannamei* (Felix-Portillo et al. 2016; Han et al. 2018; Nuñez-Hernandez et al. 2018; Duarte-Gutiérrez et al. 2021), the molecular mechanisms of this process have been poorly studied in these species. Programmed cell death occurred in hemocytes, hepatopancreas, gills, and midgut of *P. vannamei* exposed to low DO conditions, indicated by increases in DNA fragmentation, apoptotic cell count and up-regulation of caspase-3 (Casp-3) expression or activity (Felix-Portillo et al. 2016; Han et al. 2018; Nuñez-Hernandez et al. 2018; Duarte-Gutiérrez et al. 2021). In shrimp cells, hypoxia-driven apoptosis is triggered through a mechanism that is partially dependent on p53 (Felix-Portillo et al. 2016; Nuñez-Hernandez et al. 2018) and seems to involve changes in GAPDH expression (Nuñez-Hernandez et al. 2021). This suggests that in addition to its glycolytic role, GAPDH could participate in the regulation of apoptosis in shrimp cells exposed to hypoxia. However, there is still no evidence that GAPDH can translocate into the nucleus of cells from crustaceans and exert a pro-apoptotic role in response to hypoxia, as occurs in vertebrates (Gong et al. 2019).

To find out if GAPDH can be localized at the nucleus of *P. vannamei* cells in response to changes in DO conditions, we examined the effects of severe hypoxia (1 mg O₂/L) on GAPDH expression, protein abundance, and glycolytic activity in cytosolic and nuclear fractions of gills. In addition, to determine a possible participation of GAPDH in apoptosis, we evaluated the effects of GAPDH silencing by RNAi on its own sub-cellular distribution and Casp-3 expression as apoptotic marker.

2. Materials and methods

2.1 Synthesis of GAPDH dsRNA

A double-stranded RNA (dsRNA) was prepared to silence the expression of GAPDH in *P. vannamei* by RNAi. The target region (499 bp) corresponds to the positions 499–997 of the GAPDH mRNA (GenBank accession no. MG787341) encoding 166 residues of the catalytic domain (Camacho-Jiménez et al. 2018). First, the DNA fragments were obtained by PCR using the GAPDHFw6 and GAPDHRv5 primers (Table 1) and complementary DNA (cDNA) from gills as template. The PCR reaction (25 µL) contained 12.5 µL of Hot Start Taq 2× Master Mix (New England BioLabs, Ipswich, MA, USA), 0.4 µM of each primer and 1 µL of cDNA (corresponding to 25 ng of total RNA). The amplification conditions were 95°C, 3 min (1 cycle); 95°C, 30 s,

Table 1. Primers used in PCR and qPCR.

Primer	Sequence (5'-3')
GAPDHFw2	GTGTTCAACCACCATCGAGAAG
GAPDHFw6	CTGGAGGGTCTCATGACCA
GAPDHRv1	GACACCACCTTCATGTCCTTG
GAPDHRv5	AGGCATCGACGGTCTGCATG
T7GAPDHF2*	GTAATACGACTCACTATAGGG CTGGAGGGTCTCATG
T7GAPDHR2*	GTAATACGACTCACTATAGGG AGGCATCGACGGTCT
Casp3-F	AGTTAGTACAAACAGATTGGAGCG
Casp3-R	TTGTGGACAGACAGTATGAGGC
L8F2	TAGGCAATGTCATCCCCATT
L8R2	TCCTGAAGGGAGCTTTACACG

*The T7 promoter sequence is bold.

60°C, 30 s, 68°C 1 min (35 cycles); 68°C, 10 min (1 cycle). Using the amplicon as template, two separated PCR reactions were prepared to add a T7 promoter sequence at the 5' end of the fragments in both sense and antisense orientation. The sense fragment was produced with the T7GAPDHF2 and GAPDHRv5 primers (Table 1), while the antisense fragment was amplified with the GAPDHFw6 and T7GAPDHR2 primers (Table 1). Both reactions were done as the previous one but containing 1 µL of the first PCR product (1:100 dilution) instead of cDNA. The amplification conditions were 95°C, 3 min (1 cycle); 95°C, 30 s, 60°C, 30 s, 68°C 1 min (5 cycles); 95°C, 30 s, 64°C, 30 s, 68°C 1 min (30 cycles); 68°C, 10 min (1 cycle). The two PCR products (sense and antisense) were used as templates to synthesize two complementary single-stranded RNA (ssRNA) by *in vitro* transcription using the RiboMAX Large-Scale RNA Production System (Promega, Madison, WI, USA). The concentration of each ssRNA was measured at 260 nm in a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To form dsRNA, equal amounts of the sense and antisense ssRNA were heated at 90°C for 3 min and 80°C for 20 min, followed by a slow cooling down step to room temperature for hybridization. The dsRNA hybrid was confirmed by 1.2% agarose gel electrophoresis and stored at -20°C until the silencing bioassay.

2.2 Shrimp

The *P. vannamei* shrimp used in these experiments were obtained as post-larvae (PL) from a commercial farm in Sonora, Mexico. The PL were grown in tanks (100 L) with a continuous flow of seawater (30.00 ± 1.00°C, 35.00 ± 1.00 ‰) and constant aeration (5.00 ± 1.00 mg O₂/L). The tanks were connected to a recirculation system to purify the seawater through sand filters, bag filters (5 and 10 µm) and ozone treatment. Shrimp were fed with commercial pellets (3% of their wet weight) three times a day, and the bottom of the tanks was siphoned to remove feces and residual feed once a day. The quality of seawater in the recirculation system was monitored daily by measuring total ammonia levels (Hunter and Uglow 1993) and water was exchanged when necessary to keep ammonia concentration <3 mg/L, which is below the 'safety level' for *P. vannamei* at 35 ‰ (3.95 mg/L) (Lin and Chen 2001). The PL were maintained under these conditions until they reached the juvenile stage.

2.3 Biological assay for GAPDH silencing in normoxia and hypoxia

A biological assay was performed with 240 juvenile shrimp (8.69 ± 0.80 g) in intermolt stage, that were placed in 16 aquaria (15 shrimp/aquarium) filled with 50 L of seawater ($28.24 \pm 0.48^\circ\text{C}$, 35.00 ± 1.00 ‰) in normoxia (5.04 ± 0.17 mg O_2/L). Specimens were acclimated for 3 days and fed with a commercial diet corresponding to 3% of their wet weight divided in two rations. After the acclimation period, the shrimp were fed for the last time, and the seawater in the aquaria was fully exchanged. The shrimp were injected through the abdominal muscle with a sterile syringe (30 G) either with 100 μL of sterile saline solution (SS) (20 mM Tris, 400 mM NaCl, pH 7.5) or 16 μg of GAPDH dsRNA dissolved in 100 μL of SS (~ 2 μg of dsRNA/g of wet weight). The injected shrimp were assigned to the following treatments: 1) normoxia injected with SS; 2) normoxia injected with GAPDH dsRNA; 3) hypoxia injected with SS; 4) hypoxia injected with GAPDH dsRNA. Twenty-four hours after injections, the DO was lowered in the hypoxic treatments by bubbling nitrogen gas into the seawater to reach 1.05 ± 0.09 mg O_2/L . The DO conditions were monitored each hour using a portable oximeter ProODO (YSI, Yellow Spring, OH, USA) and adjusted, when necessary, by regulating the air or nitrogen supply. Six shrimp in each experimental condition were sampled at 1, 6, 24, and 48 h after DO change and the gills were dissected. One part of the tissue was submerged in 10 volumes of TRIsure reagent (Bioline, London, UK), and another part was stored without solutions. The samples were immediately frozen in a dry ice/ethanol (96%) bath and stored at -80°C until further analysis. An additional section of gills from each shrimp was submerged in 10 mL of fixative solution A containing 3% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.8) and 10% glucose.

2.4 mRNA quantification by RT-qPCR

Total RNA was isolated from individual gill samples (~ 50 mg) of each experimental condition ($n = 4$) following the TRIsure reagent (Bioline) manufacturer's protocol. The concentration and purity of total RNA was evaluated by measuring the absorbance at 260 nm, as well as the 260/280 and 260/230 ratios in a NanoDrop One spectrophotometer (Thermo Fisher Scientific). The integrity of the RNA was analyzed by 1% agarose gel electrophoresis. Isolated RNA was decontaminated of genomic DNA with RNase-free DNase I (Roche, Mannheim, Germany). Two cDNA were synthesized by reverse transcription (RT) from each DNA-free RNA sample (0.5 μg of total RNA/reaction) with the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). Quantitative PCR (qPCR) was used to measure the mRNA expression of GAPDH and Casp-3. The analysis was performed in each cDNA in duplicated reactions (four technical replicates per shrimp) using the GAPDHFw2 and GAPDHRv1 primers (Table 1) to amplify a 143 bp fragment of the GAPDH gene (Camacho-Jiménez et al. 2018), whereas the Casp3-F and Casp3-R primers (Table 1) were used to amplify a Casp-3 gene fragment (156 bp) (Wang et al. 2013). Also, a L8 ribosomal protein gene fragment (166 bp) was amplified with the L8F2 and L8R2 primers (Table 1) to normalize expression data, since its expression is stable in shrimp under hypoxia (Cota-Ruiz et al. 2015). The qPCR reactions (15 μL) contained 7.5 μL of $2\times$ SyBr green qPCR Master Mix (Biotool, Houston, TX, USA) for

GAPDH or 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) for Casp-3, 1 µL of cDNA (equivalent to 25 ng of total RNA) as template and 0.5 µM of each GAPDH primer or 0.2 µM of each Casp-3 primer. The reactions were run in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following amplification conditions: 95°C, 5 min (1 cycle); 95°C, 30 s, 55.5°C for Casp-3, 60°C for GAPDH and L8, 35 s, 72°C, 55 s (40 cycles). A melting curve analysis (65 to 95°C with increases of 0.3°C each 5 s) was performed at the end of each run to confirm the specificity of the reaction and no primer-dimer formation. Non-template controls were included in each run to monitor reagent contamination. The amplification efficiency was estimated from standard curves ($R^2 \geq 0.99$) ranging from 2.5×10^{-1} – 2.5×10^{-3} ng of total RNA derived cDNA. The efficiency of the reactions was $92.36 \pm 1.73\%$ for GAPDH, $97.40 \pm 2.96\%$ for Casp-3 and $98 \pm 1.33\%$ for L8. The relative expressions of GAPDH and Casp-3 mRNA were calculated by the $2^{-\Delta C_q}$ method (Schmittgen and Livak 2008).

2.5 Anti-GAPDH antibody production and Western blot analysis

Full-length recombinant *P. vannamei* GAPDH protein (rGAPDH) was overexpressed in *Escherichia coli* and used to produce polyclonal antibodies in rabbits (GenScript, Piscataway, NJ, USA). The antibodies were used for immunodetecting GAPDH by Western blotting and fluorescence microscopy. Nuclear and cytosolic protein extracts were prepared from frozen gill samples (~100 mg) of each treatment ($n = 3$) using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). Total protein was measured in duplicates of each fraction by the Bradford method (Bradford 1976) using a Model 680 microplate reader (Bio-Rad) and bovine serum albumin as standard. The protein histone H3 (nuclear marker) was immunodetected by Western blot in cytosolic and nuclear extract to validate cell fractionation of the gills. For Western blot analysis, 8 µg of protein for each extract were separated by SDS-PAGE (15%) and transferred (22 V for 30 min) to a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Immobilon-P) (Millipore, Billerica, MA, USA) in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membrane was incubated (overnight at 4°C) in blocking solution containing 5% skim milk and 1% gelatin in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20). The membrane was washed four times (5 min each) in TBST and incubated (4 h, room temperature) with a primary rabbit polyclonal anti-Histone H3 antibody (GeneTex, Irvine, CA, USA, Catalog no. GTX122148) diluted (1:2000) in incubating solution (1% skim milk in TBST). The membrane was washed three times with TBST and incubated (2 h, room temperature) with a secondary antibody goat anti-rabbit IgG-AP conjugate (Bio-Rad, Catalog no. 170–6518) diluted (1:3000) in incubating solution. Finally, the membrane was washed three times and the signal was developed with the alkaline phosphatase colorimetric substrate from AP Conjugate Substrate Kit (Bio-Rad).

GAPDH protein content in nuclear and cytosolic fractions of each treatment from the silencing experiment was analyzed by Western blot as described for histone H3, with some modifications. Briefly, cytosolic, and nuclear fractions of each treatment were pooled to have 9 µg of total protein (3 µg per shrimp). The proteins were separated by SDS-PAGE (12%) and transferred to a PVDF membrane. After blocking, the membrane

was incubated (1 h, room temperature) with anti-*P. vannamei* GAPDH antibodies diluted (1:5000) in incubation solution, followed by incubation with secondary antibody Goat anti-Rabbit IgG-AP Conjugate (1:3000). The bands were developed with the AP substrate and analyzed in the ChemiDoc MP Imaging System with Image Lab Software version 5.2.1 (Bio-Rad). The absolute amount of GAPDH protein in each pooled fraction was quantified based on a standard curve constructed of 2-fold dilutions of *P. vannamei* rGAPDH ranging from 300 to 18.75 ng of protein (Figure S1). The abundance of GAPDH (ng of protein) is presented as the average of two duplicated Western blot analyses.

2.6 GAPDH activity

GAPDH enzymatic activity was assayed in cytoplasmic and nuclear fractions of individual gills according to previously reported methods with minor modifications (Camacho-Jiménez et al. 2019). The cytoplasmic fractions were diluted (1:20) in 0.2 M sodium phosphate buffer (pH 7.6) while the nuclear fractions were analyzed without dilution. NADH formation was followed during 20 min at 340 nm and 25°C in a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific). All the samples were analyzed in duplicates and the enzyme activity was calculated from the absorbance increase in a linear range over time and using the extinction coefficient of NADH ($6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$). One unit (U) of enzyme activity is equivalent to the amount of enzyme necessary to convert 1 μmol of NAD^+ in NADH per minute. The GAPDH specific activity in the extracts is represented as U/mg of protein.

2.7 Immunolocalization

Shrimp gills were fixed in solution A (Section 2.3) for 17–22 h at 4°C. Fixative was removed and replaced with 50% ethanol (overnight at 4°C). Subsequently, 50% ethanol was discarded and replaced with 70% ethanol. Gill samples were stored at 4°C until further processing as previously described in detail (Barott and Tresguerres 2015). Briefly, samples were rehydrated in serial baths of 95% ethanol for 20 min, 100% ethanol three times for 20 min, SafeClear three times for 20 min before embedding in liquid paraffin wax (55°C; three times for 30 min incubations). Paraffin blocks solidified for 48 h at room temperature before microtome sectioning onto glass slides. Tissue sections were deparaffinized and rehydrated in serial baths (SafeClear three times for 10 min, 100% ethanol for 10 min, 95% ethanol for 10 min, 70% ethanol for 10 min, and PBS with 0.2% (v/v) Triton-X-100 (PBS-TX) for 10 min) prior to blocking for 1 h at room temperature in blocking buffer (4 mL PBS-TX, 80 μL normal goat serum, 0.8 μL keyhole limpet hemocyanin solution). Sections were incubated overnight at 4°C with either anti-GAPDH antibodies (0.654 $\mu\text{g/mL}$) or blocking buffer as a no-primary negative control. Slides were washed three times for 5 min in PBS-TX to remove unbound anti-GAPDH antibodies. Secondary antibodies (goat anti-rabbit-Alexa Fluor 555, 4 $\mu\text{g/mL}$ in blocking buffer; Invitrogen) were then added for 1 h at room temperature followed by DAPI DNA Stain (1 $\mu\text{g/mL}$, Invitrogen) for 5 min at room

temperature. Slides were again washed in PBS-TX to remove unbound secondary antibody (three times for 5 min).

Samples were imaged using a confocal microscope with Airyscan capabilities connected to a laser scanning module with 405-, 488-, 561-, and 640-nm laser lines (Zeiss AxioObserver Z1 with LSM 800 with Airyscan, Carl Zeiss AG, Oberkochen, Germany). Anti-GAPDH antibodies and nuclear signals were visualized with goat anti-rabbit-Alexa Fluor 555 secondary antibodies and DAPI stain, respectively (Alexa Fluor 555—ex: 561 nm, em: 540 to 700 nm; DAPI – ex: 465 nm, em: 400 to 600 nm).

2.8 Statistical analyses

Gene expression, protein abundance, and enzyme activity data were tested for normality and variance equality by the Shapiro-Wilk and Brown-Forsythe test, respectively. As data sets failed at least one of those assumptions, non-parametric analyses were performed. Kruskal-Wallis followed by Mann-Whitney U-tests were used to assess significant differences among experimental groups at each sampling time with a significance level at $p \leq 0.05$. The data were plotted as mean $\pm$ standard error of mean (SEM). The statistical analyses and plots were done with SigmaPlot 12 (Systat, San Jose, CA, USA).

3. Results and discussion

3.1 Mortality during hypoxia and RNAi

The mortality of the organisms was recorded during the experiment and deaths only occurred in the hypoxic treatments. As expected, the maximal mortality (~67%) was found in silenced shrimp exposed to hypoxia for 48 h (Table 2). These results indicate that in silenced shrimp, prolonged exposure to a DO concentration ≤ 1 mg O₂/L is lethal and confirms the important role of GAPDH to survive the stress due to its key enzymatic role in carbohydrate metabolism and ATP production during anaerobic conditions (Kosova et al. 2017).

3.2 GAPDH mRNA expression

The effects of dsRNA-mediated silencing and DO conditions on GAPDH mRNA expression were analyzed in gills of *P. vannamei* by RT-qPCR (Figure 1). The dsRNA injection significantly reduced GAPDH transcripts in all experimental conditions, except in

Table 2. Mortality during GAPDH silencing experiment.

	1 h	6 h	24 h	48 h
N (SS)	0.00%	0.00%	0.00%	0.00%
N (dsRNA)	0.00%	0.00%	0.00%	0.00%
H (SS)	0.00%	0.00%	0.00%	13.33%
H (dsRNA)	0.00%	0.00%	13.33%	66.67%

N, normoxia; H, hypoxia; SS, shrimp injected with saline solution; dsRNA, shrimp injected with dsRNA. Each data point has an $n=15$.

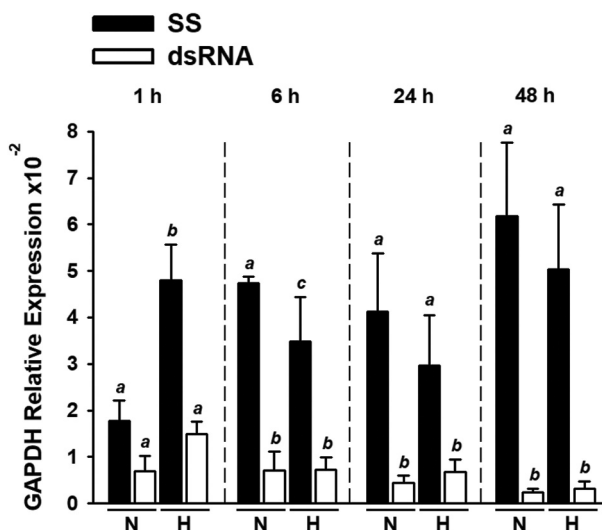


Figure 1. Expression and silencing of GAPDH mRNA in gills during normoxia (N) and hypoxia (H). SS, shrimp injected with saline solution; dsRNA, shrimp injected with dsRNA. Data are shown as mean $\pm$ SEM ($n = 4$). Significant differences between treatments at each sampling time are indicated by letters ($p \leq 0.05$).

normoxia for 1 h ($p = 0.065$). The efficiency of GAPDH silencing was $>68\%$ and increased with time reaching $>93\%$ after 48 h.

As previously reported, (Camacho-Jiménez et al. 2018, 2019; Nuñez-Hernandez et al. 2021), GAPDH mRNA (Figure 1) was significantly up-regulated after 1 h exposure to severe hypoxia (1 mg O₂/L) by 2.7-fold ($p = 0.005$). This change did not occur in the silenced specimens ($p < 0.001$). The GAPDH gene of *P. vannamei* is transcriptionally regulated via HIF-1 (Camacho-Jiménez et al. 2019) and p53 (Nuñez-Hernandez et al. 2021), which controls a myriad of genes that mediate the response to hypoxia, including those of the glycolytic pathway (Sermeus and Michiels 2011). Hypoxia has been shown to boost glycolysis in gills through HIF-1 dependent induction of HK and LDH-1 expression, leading to a local lactate accumulation in just a few hours (Soñanez-Organis et al. 2011, 2012; Camacho-Jiménez et al. 2019). This fast activation of anaerobic glycolysis should aid in sustaining ATP at a compensatory level, enough to maintain the vital functions performed by gills including ion transport, excretion and acid-base regulation (Henry et al. 2012).

However, GAPDH mRNA expression (Figure 1) in hypoxic treatment decreased by 0.74-fold with respect to normoxia ($p = 0.015$), and there were not significant differences in GAPDH mRNA expression between normoxic and hypoxic shrimp at 24–48 h of exposure ($p \geq 0.05$). Moreover, the highest mRNA levels (Figure 1) in non-silenced shrimp were detected at 48 h of exposure regardless of DO conditions, which were strongly reduced ($>90\%$) by dsRNA injection ($p \geq 0.05$). A potential explanation is that shrimp were last fed 24 h before the hypoxia exposure and starved shrimp tap into carbohydrate reserves to support energy demands, and hence, the glycolytic pathway can become accelerated (Sánchez-Paz et al. 2008). In addition, GAPDH expression in vertebrates is highly dynamic and responds to

a variety of non-stressful factors, for example, cell cycle progression, iron concentration, influx of calcium and growth (White and Garcin 2017). Thus, the up-regulation of the GAPDH gene could be part of the normal physiology of shrimp cells and could indicate a potential functional pleiotropy, as occurs in vertebrates (White and Garcin 2017; Sirover 2020). However, aside from its well-known role in carbohydrate metabolism, no other functions of GAPDH have been assessed yet in crustaceans.

3.3 Immunodetection of GAPDH in gills

The anti-GAPDH antibodies immunodetected a specific ~35.5 kDa band in gill extracts (Figure 2A) that matched the predicted molecular mass of *P. vannamei* GAPDH (Camacho-Jiménez et al. 2018). Immunohistochemistry and confocal microscopy revealed that the GAPDH protein was ubiquitously expressed throughout gill tissue of both control and hypoxic *P. vannamei* (Figure 2B-C). Higher magnification images demonstrated GAPDH was in the cytosol of all cells as well as in some nuclei (Figure 2D-E).

After cell fractionation of gills, the successful enrichment of nuclei proteins in nuclear extract was confirmed by immunodetecting a ~16k Da band that agrees with the molecular mass predicted for *P. vannamei* histone H3 (Patat et al. 2004), which was not present in the cytosolic fraction (Figure S2). In agreement with immunohistochemical analyses, cytosolic and nuclear extracts from all normoxic and hypoxic shrimps were positive to anti-GAPDH antibodies (Figure S3). Silencing induced a decrease in GAPDH protein abundance (Figure 3) under normoxia in both the cytosolic (6–24 h) and nuclear (1 h) fractions. However, there were no significant differences in GAPDH cytosolic or nuclear protein abundance between control and hypoxic shrimp at any of the sampling points ($p \geq 0.05$). These results evidence that gill cells have a pool of nuclear GAPDH even during normal oxygen conditions, suggesting that constitutive translocation of the enzyme can happen in shrimp, as has been proposed in vertebrates, in which basal levels of nuclear GAPDH appear to be necessary for ‘normal’ nuclear functions (nuclear tRNA export, maintenance of genomic integrity) (Sirover 2012, 2014). However, more studies are needed to elucidate the possible multifunctional role of GAPDH in the nucleus of gill cells, both under hypoxic stress as well under normal oxygen conditions.

The mechanisms governing the shuttling of GAPDH to the nucleus are not totally clear yet, but it is known that in mammalian models is prompted by diverse posttranslational modifications (PTM) like acetylation, glycosylation, oxidation, phosphorylation, and S-nitrosylation of specific residues. Moreover, shrimp GAPDH seems to lack a nuclear localization signal (NLS), as occurs in other animal species (Camacho-Jiménez et al. 2018). Therefore, it cannot translocate by itself into the nucleus meaning that the association with proteins harboring a NLS is necessary (Sirover 2012). PTMs allow GAPDH to form complexes with other proteins with an own NLS (Sirover 2020). Interestingly, residues susceptible of PTM in mammalian GAPDH are totally conserved in the *P. vannamei* enzyme, suggesting that the mechanisms for nuclear translocation could be similar to those found in other species (Camacho-Jiménez et al. 2018).

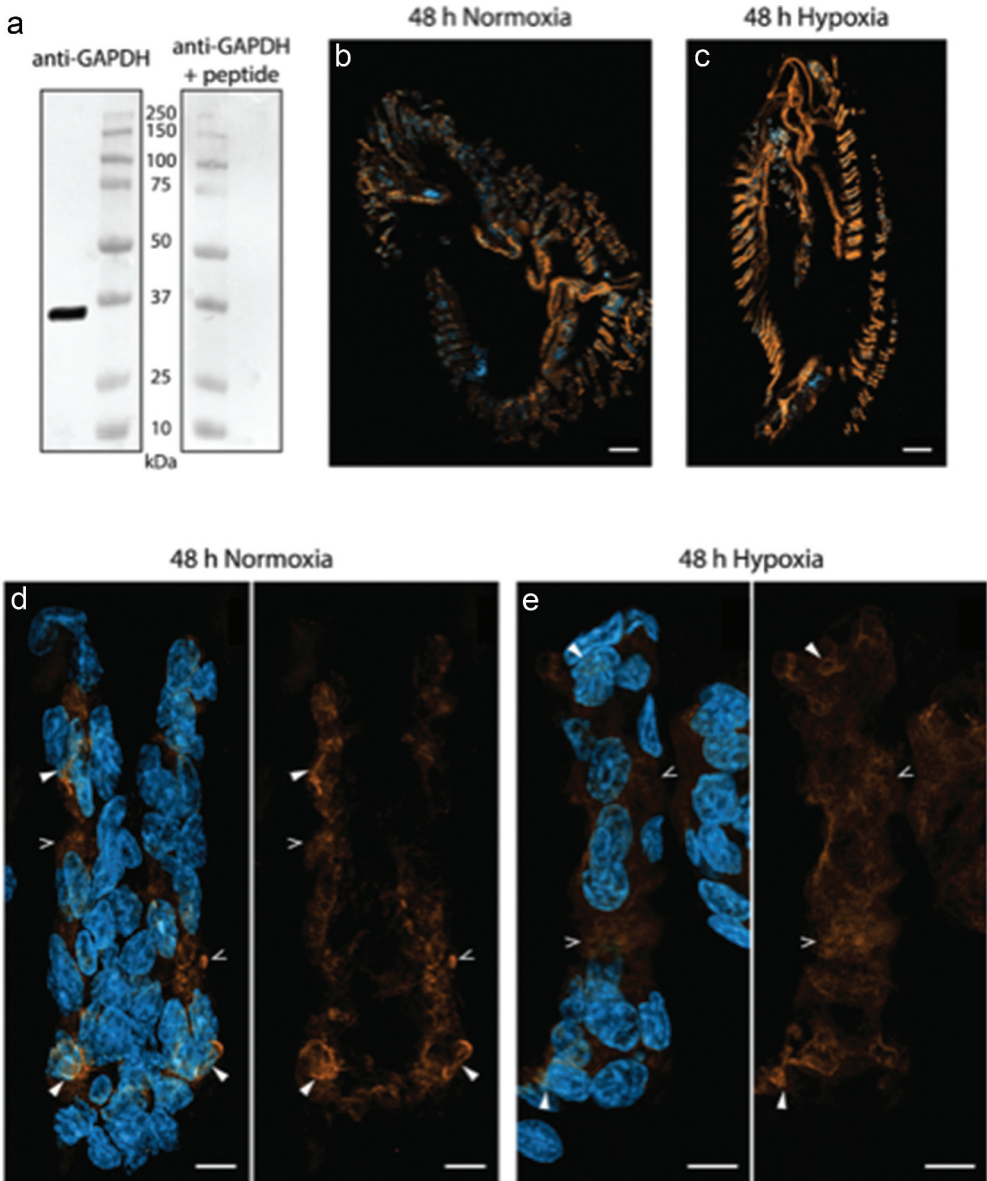


Figure 2. Immunolocalization of GAPDH protein in *P. vannamei* gills. A) Western blot showing a specific ~35.5 kDa band that is absent in the peptide preabsorption control. B, C) Ubiquitous GAPDH immunolocalization in gills from shrimps exposed to normoxia or hypoxia for 48 h. Scale bar= 100 μ m. D, E) GAPDH presence in the cytosol (open arrowheads) and nucleus (filled arrowheads). Scale bar= 5 μ m. GAPDH immunolocalization is shown in orange, nuclear staining is shown in blue.

3.4 GAPDH activity in gill cytosolic and nuclear fractions

To further explore the significance of GAPDH in the nucleus of gill cells during hypoxia, we measured GAPDH NADH-forming activity. Interestingly, GAPDH activity was 3–8 times lower in the nuclear fraction than in the cytosolic fraction (Figure 4) despite

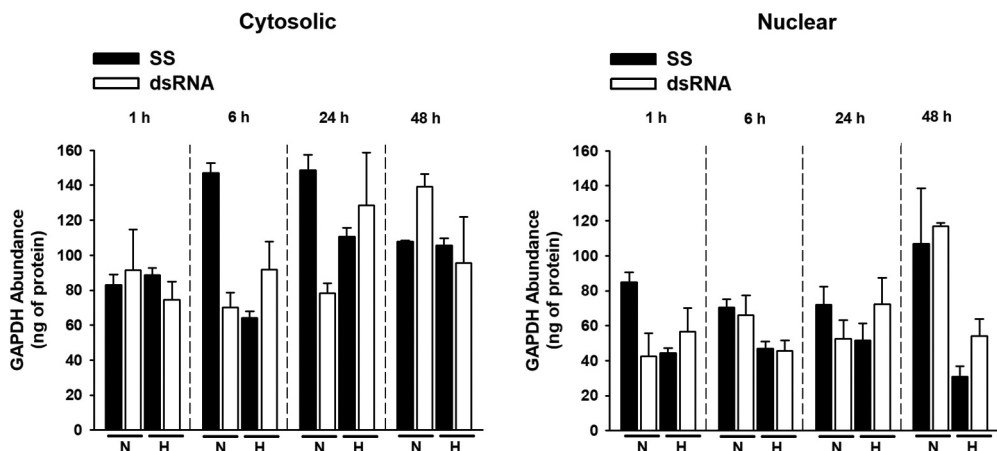


Figure 3. Quantification of GAPDH protein abundance in cytosolic and nuclear fractions from gills tissue during normoxia (N) and hypoxia (H). Data are shown as mean $\pm$ SEM ($n = 3$). No significant differences were found between treatments at any sampling time ($p \geq 0.05$).

comparable protein levels (Figure 3), which would mean a loss of glycolytic capacity when GAPDH is localized at the nucleus.

Cytosolic GAPDH activity (Figure 4) was 1.76-fold lower in non-silenced shrimp after 6 h of hypoxia than in normoxia ($p = 0.041$), while it was higher after 48 h (by 0.64-fold), even though protein abundance (Figure 3) did not have any significant disparity. In mammalian skeletal muscles, GAPDH activity is modulated through reversible phosphorylation by protein kinases and phosphatases (V et al. 2014). Thus, the differences in activity detected at 6 h and 48 h (Figure 4) could have resulted from PTM of preexistent enzyme instead of the synthesis of new protein, since the early induction in mRNA

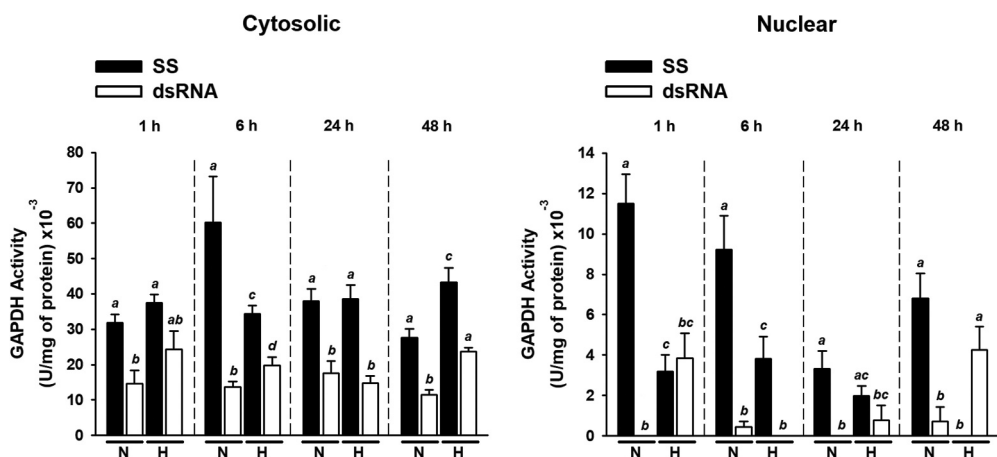


Figure 4. GAPDH enzyme activity in cytosolic and nuclear fractions from gills tissue during normoxia (N) and hypoxia (H). SS, shrimp injected with saline solution; dsRNA, shrimp injected with dsRNA. Data are shown as mean $\pm$ SEM ($n = 3$). Significant differences between treatments at each sampling time are indicated by letters ($p \leq 0.05$).

(Figure 1) under hypoxia (1 h) did not result in a further increase in protein levels compared to normoxia. On the other hand, the nuclear activity (Figure 4) in all SS-injected specimens was lower in hypoxia than in normoxia ($p \leq 0.05$), except at 24 h ($p = 0.310$). The highest difference was found after 48 h, when the nuclear activity was equal to zero in hypoxic treatment ($p = 0.002$). During oxidative stress, GAPDH is target of different oxidative PTMs (i.e. sulfenylation, S-thiolation, nitrosylation, and sulphydration) that may alter its conformation and attenuate glycolytic activity at cytosol, but modify its subcellular localization and promote other non-glycolytic functions (Tossounian et al. 2020). Therefore, hypoxia is an stressor that causes oxidative stress in shrimp tissues (Li et al. 2016; González-Ruiz et al. 2021), and might have exacerbated the impairment in nuclear enzyme activity, which in turn, may have allowed GAPDH to perform other nuclear functions.

Also, significant decreases in GAPDH activity (Figure 4) occurred in normoxia by the effect of dsRNA ($p \leq 0.05$). The decline in cytosolic activity was significant after 1 h ($p = 0.002$) and the maximal decrease of 77.21% occurred at 6 h ($p = 0.002$), while nuclear activity was depressed in all sampling times ($p \leq 0.05$), reaching 100% at 1 h ($p = 0.002$) and 24 h ($p = 0.002$). Therefore, somehow, RNAi may have affected the abundance of GAPDH (and activity) in cells, considering that the mRNA was effectively down-regulated. Under hypoxia, cytosolic activity (Figure 4) in silenced shrimp was also reduced to a maximal after 24 h (61.69%) ($p = 0.002$), while in the nuclear fraction it was totally abolished after 6 h ($p = 0.015$). Because dsRNA did not cause any perceptible reduction in hypoxic protein expression (Figure 3), enzyme regulation possibly occurred at posttranslational level, considering that hypoxia has numerous effects on the physiology of cells (Semenza 2013). However, the strongest gene knock-down (Figure 1) was detected after 48 h of hypoxia. Thus, it is possible that the effects in protein abundance need more time to be measurable, considering the existence of multiple (and complex) posttranscriptional and translational mechanisms regulating gene expression.

3.5 Effect of GAPDH silencing on Casp-3 expression

Casp-3 expression in gills (Figure 5) of SS-injected shrimp was lower in hypoxia than in normoxia by 6.49-fold at 6 h ($p < 0.001$) and 9.71-fold after 24 h ($p < 0.001$). These results indicate that severe hypoxia did not trigger apoptosis in gills of shrimp, as the cell death program relies on the sequential activation of caspases, being Casp-3 considered as a 'no return point' (Yadav et al. 2021). These findings are in agreement with a previous study in *P. vannamei* that reported the down-regulation of Casp-3 activity in the same tissue in response to a DO concentration of 1.5 mg O₂/L (González-Ruiz et al. 2021). By contrast, Duarte-Gutiérrez et al. (2021) found that Casp-3 expression is increased in hemocytes and gills during moderate hypoxia. Moreover, in hepatopancreas and hemocytes of white shrimp, hypoxia induces apoptosis associated with an increase in expression and activity of Casp-3, which is positively regulated by p53 (Felix-Portillo et al. 2016; Nuñez-Hernandez et al. 2018). Considering these precedents, we expected that GAPDH might be involved in p53-dependent apoptosis in gills, however, it seems that under severe hypoxia the enzyme could have in fact, a pro-survival role, also supported by the Casp-3 levels found in shrimp injected with RNAi (Figure 5).

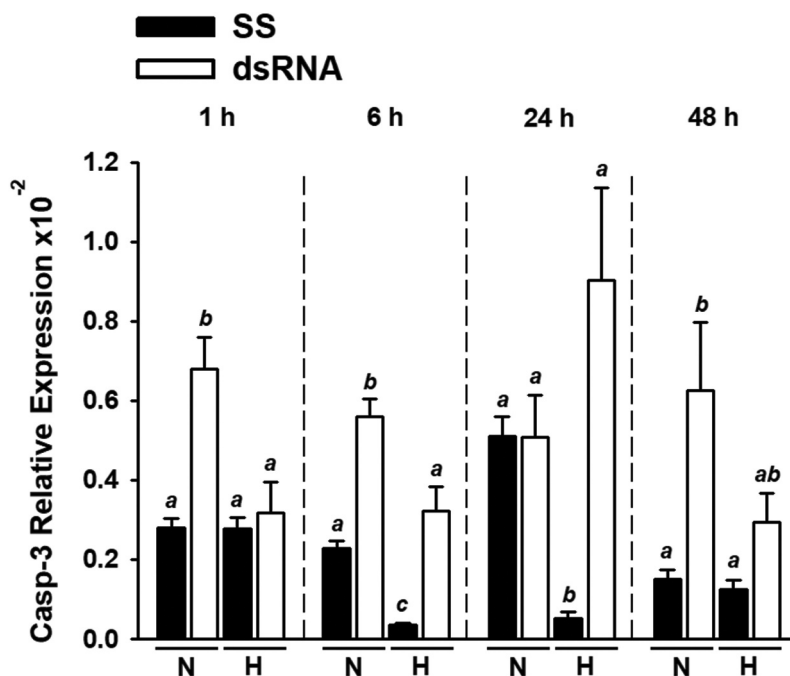


Figure 5. Effect of GAPDH silencing on Casp-3 mRNA expression in gills during normoxia (N) and hypoxia (H). SS, shrimp injected with saline solution; dsRNA, shrimp injected with dsRNA. Data are shown as mean $\pm$ SEM ($n = 4$). Significant differences between treatments at each sampling time are indicated by letters ($p \leq 0.05$).

Under hypoxia, GAPDH silencing increased Casp-3 expression (Figure 5) by 9.15-fold at 6 h ($p = 0.001$) and 15.10-fold at 24 h ($p < 0.001$) with respect to non-silenced specimens. Likewise, Casp-3 was up-regulated in dsRNA-injected shrimp kept in normal DO ($p \geq 0.05$), except at 24 h ($p = 0.613$). The maximal difference between silenced and non-silenced groups in normoxia was 7.38-fold after 48 h ($p = 0.005$). The positive effect of GAPDH knock-down on Casp-3 expression, indicated that GAPDH is somehow participating in the inhibition of apoptosis in gill cells of shrimp exposed to 1 mg O₂/L. Apoptotic pathways in shrimp are activated or suppressed in a tissue-specific manner, and some tissues appear to be more resilient to hypoxia than others. In shrimp muscle, hypoxia induces the expression of cyclin-dependent kinase 2 (Cdk-2) (Nuñez-Hernandez et al. 2019), a gene under the control of p53 (Nuñez-Hernandez et al. 2018). Because Cdk-2 regulates DNA replication and the transition through the G1/S phases, an increase in expression indicates cell cycle progression and cell survival (Nuñez-Hernandez et al. 2019).

The low apoptotic rates in gills of stressed shrimp indicated by low Casp-3 expression could be in part explained by the increase in cytosolic GAPDH activity in non-silenced shrimp after 48 h of hypoxia (Figure 4) and indicates an augmentation in glycolytic flux, as has been reported in organisms exposed to similar conditions (Camacho-Jiménez et al. 2018, 2019). Simultaneously, GAPDH was detected in nucleus without enzymatic activity (Figures 2, 3, and 4), suggesting its participation in other processes that might promote the resistance of gill cells to hypoxia, for example, the repair of DNA lesions, delay in

telomere shortening, as well as the acceleration of cell cycle progression (Icard et al. 2019). Interestingly, in mammalian cells, GAPDH can exert a protective role by rescuing cells from mitochondrial outer-membrane permeabilization (MOMP) in caspase-independent apoptosis. The mechanism involve the enhancement of the glycolytic rate and the up-regulation of the autophagy-related genes (ATGs) by nuclear GAPDH to remove the terminally damaged mitochondria (Colell et al. 2007). Also, GAPDH can also be associated with damaged mitochondria for mitophagy in a process dependent on reversible enzyme phosphorylation (Yogalingam et al. 2013). In the brain of the crayfish *M. nipponense*, the exposure to hypoxia increases anaerobic glycolysis and the expression of ATGs. The knock-down of ATGs by RNAi decreased cell viability, suggesting that autophagy is an adaptive response protecting against hypoxia in crustaceans (Sun et al. 2019). Thus, it is possible that similar protective mechanisms are activated in some *P. vannamei* cells upon the stress caused by low DO, and GAPDH may be involved.

On the other hand, apoptosis stimulated in gills by GAPDH knock-down, could be related to the slow-down of glycolytic activity (in both sub-cellular fractions) (Figure 4), as has been found in mammalian cardiomyocytes (Kimata et al. 2010). Nonetheless, other alternative functions of GAPDH may be promoting caspases activation, considering that protein abundance was not significantly affected by hypoxia or silencing (Figure 3). The role of GAPDH in apoptosis is not completely clear, but in vertebrate cells, it appears to participate in multiple effector pathways. For example, the S-nitrosylation of GAPDH promotes its binding to Siah1 (an E3 ubiquitin ligase with a NLS), leading to nuclear translocation of the complex and the subsequent degradation of nuclear proteins. Nuclear GAPDH can be acetylated by p300/CREB-binding protein (CBP), resulting in the activation of downstream targets in apoptotic cascade as p53 (Sen et al. 2008). Also, nuclear GAPDH also can form a complex with p53 that leads to apoptosis (Zhai et al. 2014). However, we do not know if those events occur in apoptotic cells of crustaceans.

4. Conclusions

In summary, we show that the GAPDH protein is present both, in the cytosol and nucleus of *P. vannamei* gill cells both during normoxia and severe hypoxia (1 mg O₂/L). Hypoxia induced an up-regulation in GAPDH mRNA expression (1 h) and cytosolic activity (48 h), which is related to an enhancement of anaerobic glycolysis for ATP production. These increases did not happen in shrimp in which GAPDH was silenced with dsRNA. However, the glycolytic activity of GAPDH in nuclear fractions was lower than in the cytosolic ones. Since GAPDH protein abundance was not significantly affected during the experiment, the presence in the nucleus could be related to non-glycolytic functions, especially during low DO conditions, in which NADH-dependent activity was progressively abolished.

Hypoxia down-regulated the expression of Casp-3, whereas in silenced shrimp, it increased. These outcomes revealed that apoptosis is probably suppressed in gills in response to hypoxia. Survival of gills cells in hypoxia could be favored by a higher glycolytic flux and potentially by alternative functions of GAPDH (i.e., genetic material integrity and autophagy). This work adds knowledge on the basic biology of GAPDH and the molecular mechanisms of crustacea to survive hypoxia. More studies are needed to understand how GAPDH is translocated into the nucleus, as well as the interactions with

other effectors involved in inhibition or stimulation of apoptotic pathways in multiple tissues of shrimp exposed to hypoxia and other stressors.

Acknowledgments

We thank Biol. Adrian Gamez-Alejo, Guadalupe Cota and Elizabeth Cota from the Laboratory of Marine Invertebrates Physiology at CIAD for their technical support in shrimp experiments. We also thank Dr. José Angel Huerta for his help in Western blot densitometric analysis. LCJ had a postdoctoral fellowship from CONACyT. ABT is a Ph.D. student at Scripps Institution of Oceanography-UCSD.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by UC-MEXUS CONACyT grant CN-18-108 to MT and GYP) and CONACyT Ciencia Básica grant A1-S-24557 to GYP.

Author's contributions

LCJ, MT, and GYP conceived and designed the experiments. LCJ, LLC, and ABPU performed the experiments and analyzed the data. ABT and MT performed and analyzed the immunostaining assays. SGJ coordinated the shrimp maintenance and biological assay. LCJ drafted the manuscript. GYP and MT edited the manuscript. All the authors reviewed and approved the final version of the manuscript.

References

- Barott K, Tresguerres M. 2015. Immunolocalization of proteins in corals: the V-type H⁺ATPase proton pump. *Bio-Protocol*. 5(17). doi:[10.21769/bioprotoc.1573](https://doi.org/10.21769/bioprotoc.1573).
- Boyd CE. 2017. General relationship between water quality and aquaculture performance in ponds. In: Jeney G, editor. *Fish Dis Prev Control Strateg* [Internet]. London, UK: Elsevier Academic Press; pp. 147–166. doi:[10.1016/B978-0-12-804564-0.00006-5](https://doi.org/10.1016/B978-0-12-804564-0.00006-5).
- Bradford MM. 1976. A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72(1–2):248–254. doi:[10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- Breitburg D, Levin LA, Oschlies A, Grégoire M, Chavez FP, Conley DJ, Garçon V, Gilbert D, Gutiérrez D, Isensee K, et al. 2018. Declining oxygen in the global ocean and coastal waters. *Science*. 359(6371):eaam7240. doi:[10.1126/science.aam7240](https://doi.org/10.1126/science.aam7240).
- Camacho-Jiménez L, Leyva-Carrillo L, Peregrino-Uriarte AB, Duarte-Gutiérrez JL, Tresguerres M, Yepiz-Plascencia G. 2019. Regulation of glyceraldehyde-3-phosphate dehydrogenase by hypoxia inducible factor 1 in the white shrimp *Litopenaeus vannamei* during hypoxia and reoxygenation. *Comp Biochem Physiol Part A*. 235:56–65. Internet. doi:[10.1016/j.cbpa.2019.05.006](https://doi.org/10.1016/j.cbpa.2019.05.006).
- Camacho-Jiménez L, Peregrino-Uriarte AB, Martínez-Quintana JA, Yepiz-Plascencia G. 2018. The glyceraldehyde-3-phosphate dehydrogenase of the shrimp *Litopenaeus vannamei*: molecular cloning, characterization and expression during hypoxia. *Mar Environ Res*. 138(April):65–75. doi:[10.1016/j.marenvres.2018.04.003](https://doi.org/10.1016/j.marenvres.2018.04.003). Internet.

- Colell A, Green DR, Ricci J-E. 2009. Novel roles for GAPDH in cell death and carcinogenesis. *Cell Death Differ.* 16(12):1573–1581. doi:[10.1038/cdd.2009.137](https://doi.org/10.1038/cdd.2009.137). Internet.
- Colell A, Ricci JE, Tait S, Milasta S, Maurer U, Bouchier-Hayes L, Fitzgerald P, Guio-Carrion A, Waterhouse NJ, Li CW, et al. 2007. GAPDH and Autophagy Preserve Survival after Apoptotic Cytochrome c Release in the Absence of Caspase Activation. *Cell.* 129(5):983–997. doi:[10.1016/j.cell.2007.03.045](https://doi.org/10.1016/j.cell.2007.03.045).
- Cota-Ruiz K, Leyva-Carrillo L, Peregrino-Uriarte AB, Valenzuela-Soto EM, Gollas-Galván T, Gómez-Jiménez S, Hernández J, Yepiz-Plascencia G. 2016. Role of HIF-1 on phosphofructokinase and fructose 1,6-bisphosphatase expression during hypoxia in the white shrimp *Litopenaeus vannamei*. *Comp Biochem Physiol Part A.* 198:1–7. doi:[10.1016/j.cbpa.2016.03.015](https://doi.org/10.1016/j.cbpa.2016.03.015).
- Cota-Ruiz K, Peregrino-Uriarte AB, Felix-Portillo M, Martínez-Quintana JA, Yepiz-Plascencia G. 2015. Expression of fructose 1,6-bisphosphatase and phosphofructokinase is induced in hepatopancreas of the white shrimp *Litopenaeus vannamei* by hypoxia. *Mar Environ Res.* 106(1):1–9. doi:[10.1016/j.marenvres.2015.02.003](https://doi.org/10.1016/j.marenvres.2015.02.003).
- Diaz RJ, Rosenberg R. 2008. Spreading dead zones and consequences for marine ecosystems. *Science.* 321(5891):926–929. doi:[10.1126/science.1156401](https://doi.org/10.1126/science.1156401).
- Duarte-Gutiérrez J, Peregrino-Uriarte AB, Gómez-Jiménez S, Mata-Haro V, Yepiz-Plascencia G. 2021. HIF-1 is involved in the regulation of expression of metallothionein and apoptosis incidence in different oxygen conditions in the white shrimp *Litopenaeus vannamei*. *Comp Biochem Physiol A Mol Integr Physiol.* 262(May):111072. doi:[10.1016/j.cbpa.2021.111072](https://doi.org/10.1016/j.cbpa.2021.111072).
- Felix-Portillo M, Martínez-Quintana JA, Arenas-Padilla M, Mata-Haro V, Gómez-Jiménez S, Yepiz-Plascencia G. 2016. Hypoxia drives apoptosis independently of p53 and metallothionein transcript levels in hemocytes of the whiteleg shrimp *Litopenaeus vannamei*. *Chemosphere.* 161:454–462. doi:[10.1016/j.chemosphere.2016.07.041](https://doi.org/10.1016/j.chemosphere.2016.07.041).
- Gong Y, Zou B, Peng S, Li P, Zhu G, Chen J, Chen J, Liu X, Zhou W, Ding L, et al. 2019. Nuclear GAPDH is vital for hypoxia-induced hepatic stellate cell apoptosis and is indicative of aggressive hepatocellular carcinoma behavior. *Cancer Manag Res.* 11:4947–4956. doi:[10.2147/cmar.s202268](https://doi.org/10.2147/cmar.s202268).
- González-Ruiz R, Peregrino-Uriarte AB, Valenzuela-Soto EM, Cinco-Moroyoqui FJ, Martínez-Téllez MA, Yepiz-Plascencia G. 2021. Mitochondrial manganese superoxide dismutase knock-down increases oxidative stress and caspase-3 activity in the white shrimp *Litopenaeus vannamei* exposed to high temperature, hypoxia, and reoxygenation. *Comp Biochem Physiol - Part A Mol Integr Physiol.* 252:110826. Internet. doi:[10.1016/j.cbpa.2020.110826](https://doi.org/10.1016/j.cbpa.2020.110826).
- Han S, Wang B, Liu M, Wang M, Jiang K, Qi C, Wang L. 2017. Effect of cyclic serious/medium hypoxia stress on the survival, growth performance and resistance against *Vibrio parahaemolyticus* of white shrimp *Litopenaeus vannamei*. *Invertebr Surviv J.* 14:259–270.
- Han S, Wang M, Liu M, Wang B, Jiang K, Wang L. 2018. Comparative sensitivity of the hepatopancreas and midgut in the white shrimp *Litopenaeus vannamei* to oxidative stress under cyclic serious/medium hypoxia. *Aquaculture.* 490(November 2017):44–52. Internet. doi:[10.1016/j.aquaculture.2018.02.021](https://doi.org/10.1016/j.aquaculture.2018.02.021).
- Henry RP, Lucu C, Onken H, Weihrauch D. 2012. Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. *Front Physiol.* 3(November):1–33. doi:[10.3389/fphys.2012.00431](https://doi.org/10.3389/fphys.2012.00431).
- Holthuis LB. FAO species catalogue. Vol.1-shrimps and prawns of the world. FAO Fish Synopsis. 1980;1(125):284.
- Hoogewijs D, Terwilliger NB, Webster KA, Powell-Coffman JA, Tokishita S, Yamagata H, Hankeln T, Burmester T, Rytknen KT, Nikinmaa M, et al. 2007. From critters to cancers: bridging comparative and clinical research on oxygen sensing, HIF signaling, and adaptations towards hypoxia. *Integr Comp Biol.* 47(4):552–577. doi:[10.1093/icb/icm072](https://doi.org/10.1093/icb/icm072).
- Hunter DA, Uglow RF. 1993. A technique for the measurement of total ammonia in small volumes of seawater and haemolymph. *Ophelia.* 37(1):31–40. doi:[10.1080/00785326.1993.10430375](https://doi.org/10.1080/00785326.1993.10430375).
- Icard P, Fournel L, Wu Z, Alifano M, Lincet H. 2019. Interconnection between metabolism and cell cycle in cancer. *Trends Biochem Sci.* 44(6):490–501. doi:[10.1016/j.tibs.2018.12.007](https://doi.org/10.1016/j.tibs.2018.12.007).

- Kimata M, Matoba S, Iwai-Kanai E, Nakamura H, Hoshino A, Nakaoka M, Katamura M, Okawa Y, Mita Y, Okigaki M, et al. **2010**. and TIGAR regulate cardiac myocyte energy homeostasis under hypoxic stress. *Am J Physiol - Hear Circ Physiol*. 299(6):53. doi:[10.1152/ajpheart.00250.2010](https://doi.org/10.1152/ajpheart.00250.2010).
- Kosova AA, Khodyreva SN, Lavrik OI. **2015**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) interacts with apurinic/aprimidinic sites in DNA. *Mutat Res - Fundam Mol Mech Mutagen* [Internet]. 779:46–57. doi:[10.1016/j.mrfmmm.2015.06.005](https://doi.org/10.1016/j.mrfmmm.2015.06.005).
- Kosova AA, Khodyreva SN, Lavrik OI. **2017**. Role of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in DNA repair. *Biochemistry*. 82(6):859–872. doi:[10.1134/S0006297917060013](https://doi.org/10.1134/S0006297917060013).
- Li Y, Wei L, Cao J, Qiu L, Jiang X, Li P, Song Q, Zhou H, Han Q, Diao X. **2016**. Oxidative stress, DNA damage and antioxidant enzyme activities in the pacific white shrimp (*Litopenaeus vannamei*) when exposed to hypoxia and reoxygenation. *Chemosphere*. 144:234–240. Internet. doi:[10.1016/j.chemosphere.2015.08.051](https://doi.org/10.1016/j.chemosphere.2015.08.051).
- Liang S, Aiqun M, Figtree G, Ping Z. **2015**. GAPDH-silence preserves H9C2 cells from acute hypoxia and reoxygenation injury. *Int J Biol Macromol*. 81:375–386. Internet. doi:[10.1016/j.ijbiomac.2015.08.028](https://doi.org/10.1016/j.ijbiomac.2015.08.028).
- Lin YC, Chen JC. **2001**. Acute toxicity of ammonia on *Litopenaeus vannamei* boone juveniles at different salinity levels. *J Exp Mar Bio Ecol*. 259(1):109–119. doi:[10.1016/S0022-0981\(01\)00227-1](https://doi.org/10.1016/S0022-0981(01)00227-1).
- Nuñez-Hernandez D, Camacho-Jiménez L, Leyva-Carrillo L, Peregrino-Uriarte AB, Valenzuela-Soto EM, Gómez-Jiménez S, González-Ríos H, Yepiz-Plascencia G. **2021**. Expression of p53 isoforms in response to two levels of hypoxia and effects of p53 knock-down in glyceraldehyde-3-phosphate dehydrogenase in the white shrimp *Litopenaeus vannamei*. *Aquac Res*. 52(5):1–11. doi:[10.1111/are.15083](https://doi.org/10.1111/are.15083).
- Nuñez-Hernandez DM, Camacho-Jiménez L, González-Ruiz R, Mata-Haro V, Ezquerro-Brauer JM, Yepiz-Plascencia G. **2019**. Cyclin-dependent kinase 2 (Cdk-2) from the White shrimp *Litopenaeus vannamei*: molecular characterization and tissue-specific expression during hypoxia and reoxygenation. *Comp Biochem Physiol -Part A Mol Integr Physiol*. 230 (December 2018):56–63. doi:[10.1016/j.cbpa.2018.12.013](https://doi.org/10.1016/j.cbpa.2018.12.013). Internet.
- Nuñez-Hernandez DM, Felix-Portillo M, Peregrino-Uriarte AB, Yepiz-Plascencia G. **2018**. Cell cycle regulation and apoptosis mediated by p53 in response to hypoxia in hepatopancreas of the white shrimp *Litopenaeus vannamei*. *Chemosphere*. 190:253–259. doi:[10.1016/j.chemosphere.2017.09.131](https://doi.org/10.1016/j.chemosphere.2017.09.131).
- Patat SA, Carnegie RB, Kingsbury C, Gross PS, Chapman R, Schey KL. **2004**. Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. *Eur J Biochem*. 271(23–24):4825–4833. doi:[10.1111/j.1432-1033.2004.04448.x](https://doi.org/10.1111/j.1432-1033.2004.04448.x).
- Pérez-Rostro CI, Racotta IS, Ibarra AM. **2004**. Decreased genetic variation in metabolic variables of *Litopenaeus vannamei* shrimp after exposure to acute hypoxia. *J Exp Mar Bio Ecol*. 302 (2):189–200. doi:[10.1016/j.jembe.2003.10.010](https://doi.org/10.1016/j.jembe.2003.10.010).
- Rabalais NN, Díaz RJ, Levin LA, Turner RE, Gilbert D, Zhang J. **2010**. Dynamics and distribution of natural and human-caused hypoxia. *Biogeosciences*. 7(2):585–619. doi:[10.5194/bg-7-585-2010](https://doi.org/10.5194/bg-7-585-2010).
- Sánchez-Paz A, Soñanez-Organis JG, Peregrino-Uriarte AB, Muhlia-Almazán A, Yepiz-Plascencia G. **2008**. Response of the phosphofructokinase and pyruvate kinase genes expressed in the midgut gland of the Pacific white shrimp *Litopenaeus vannamei* during short-term starvation. *J Exp Mar Bio Ecol*. 362(2):79–89. doi:[10.1016/j.jembe.2008.06.002](https://doi.org/10.1016/j.jembe.2008.06.002).
- Schmittgen TD, Livak KJ. **2008**. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*. 3(6):1101–1108. doi:[10.1038/nprot.2008.73](https://doi.org/10.1038/nprot.2008.73).
- Semenza GL. **2013**. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest*. 123(9):3664–3671. doi:[10.1172/JCI67230](https://doi.org/10.1172/JCI67230).
- Sen N, Hara MR, Kornberg MD, Cascio MB, Bae B, Shahani N, Thomas B, Dawson TM, Dawson VL, Snyder SH, et al. **2008**. Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. *Nat Cell Biol*. 10(7):866–873.
- Sermeus A, Michiels C. **2011**. Reciprocal influence of the p53 and the hypoxic pathways. *Cell Death Disease*. 2(5):164. Internet. doi:<https://doi.org/10.1038/cddis.2011.48>.

- Sirover MA. 2012. Subcellular dynamics of multifunctional protein regulation: mechanisms of GAPDH intracellular translocation. *J Cell Biochem.* 113(7):2193–2200.
- Sirover MA. 2014. Structural analysis of glyceraldehyde-3-phosphate dehydrogenase functional diversity. *Int J Biochem Cell Biol.* 0:1–7. Internet. doi:[10.1016/j.biocel.2014.09.026](https://doi.org/10.1016/j.biocel.2014.09.026).
- Sirover MA. 2020. Moonlighting glyceraldehyde-3-phosphate dehydrogenase: posttranslational modification, protein and nucleic acid interactions in normal cells and in human pathology. *Crit Rev Biochem Mol Biol.* 0(0):1–18. doi:[10.1080/10409238.2020.1787325](https://doi.org/10.1080/10409238.2020.1787325). Internet.
- Soñanez-Organis JG, Peregrino-Uriarte AB, Sotelo-Mundo RR, Forman HJ, Yepiz-Plascencia G. 2011. Hexokinase from the white shrimp *Litopenaeus vannamei*: cDNA sequence, structural protein model and regulation via HIF-1 in response to hypoxia. *Comp Biochem Physiol Part B: Biochem Mol Biol.* 158(3):242–249. Internet.
- Soñanez-Organis JG, Racotta IS, Yepiz-Plascencia G. 2010. Silencing of the hypoxia inducible factor 1 -HIF-1- obliterates the effects of hypoxia on glucose and lactate concentrations in a tissue-specific manner in the shrimp *Litopenaeus vannamei*. *J Exp Mar Bio Ecol.* 393(1–2):51–58. doi:[10.1016/j.jembe.2010.06.031](https://doi.org/10.1016/j.jembe.2010.06.031).
- Soñanez-Organis JG, Rodriguez-Armenta M, Leal-Rubio B, Peregrino-Uriarte AB, Gómez-Jiménez S, Yepiz-Plascencia G. 2012. Alternative splicing generates two lactate dehydrogenase subunits differentially expressed during hypoxia via HIF-1 in the shrimp *Litopenaeus vannamei*. *Biochimie.* 94(5):1250–1260. doi:[10.1016/j.biochi.2012.02.015](https://doi.org/10.1016/j.biochi.2012.02.015).
- Sun S, Chen Y, Hu R. 2020. Aquatic hypoxia disturbs oriental river prawn (*Macrobrachium nipponense*) testicular development: a cross-generational study. *Environ Pollut.* 266:115093. Internet. doi:[10.1016/j.envpol.2020.115093](https://doi.org/10.1016/j.envpol.2020.115093).
- Sun S, Wu Y, Fu H, Ge X, You H, Wu X. 2019. Identification and characterization of four autophagy-related genes that are expressed in response to hypoxia in the brain of the oriental river prawn (*macrobrachium nipponense*). *Int J Mol Sci.* 20(8):1–20. doi:[10.3390/ijms20081856](https://doi.org/10.3390/ijms20081856).
- Sun S, Xuan F, Fu H, Zhu J, Ge X, Wu X. 2017. Molecular cloning, characterization and expression analysis of caspase-3 from the oriental river prawn, *Macrobrachium nipponense* when exposed to acute hypoxia and reoxygenation. *Fish Shellfish Immunol.* 62:291–302. Internet. doi:[10.1016/j.fsi.2017.01.045](https://doi.org/10.1016/j.fsi.2017.01.045).
- Tossounian MA, Zhang B, Gout I. 2020. The writers, readers, and erasers in redox regulation of GAPDH. *Antioxidants.* 9(12):1–21. doi:[10.3390/antiox9121288](https://doi.org/10.3390/antiox9121288).
- Ulaje SA, Rojo-Arreola L, Lluch-Cota SE, Ascencio F, Cruz-Hernández P, Sicard MT. 2019. Gene expression and energetic metabolism changes in the whiteleg shrimp (*Litopenaeus vannamei*) in response to short-term hypoxia. *Aquac Res.* 50(4):994–1004. doi:[10.1111/are.13960](https://doi.org/10.1111/are.13960).
- V BR, Smith C, Storey KB. 2014. Purification and properties of glyceraldehyde-3-phosphate dehydrogenase from the skeletal muscle of the hibernating ground squirrel, *Ictidomys tridecemlineatus*. *PeerJ.* 2:e634. doi:[10.7717/peerj.634](https://doi.org/10.7717/peerj.634).
- Wang P, Liu H, Zhao S, Yu S, Xie S, Hua S, Yan B, Xing C, Gao H. 2022. Hypoxia stress affects the physiological responses, apoptosis and innate immunity of Kuruma shrimp, *Marsupenaeus japonicus*. *Fish Shellfish Immunol.* 122(January):206–214. doi:[10.1016/j.fsi.2022.02.016](https://doi.org/10.1016/j.fsi.2022.02.016). Internet.
- Wang PH, Wan DH, Chen YG, Weng SP, Yu XQ, He JG. 2013. Characterization of four novel caspases from *Litopenaeus vannamei* (Lvcaspase2-5) and their role in WSSV infection through dsRNA-mediated gene silencing. *PLoS One.* 8(12):1–14. doi:[10.1371/journal.pone.0080418](https://doi.org/10.1371/journal.pone.0080418).
- White MR, Garcin ED. 2017. D-glyceraldehyde-3-phosphate dehydrogenase structure and function. *Subcell Biochem.* 83:413–453. Internet. doi:[10.1007/978-3-319-46503-6_15](https://doi.org/10.1007/978-3-319-46503-6_15).
- Xu Z, He J, Wang J. 2021. Hypoxia affects the resistance of *Scylla paramamosain* to *Vibrio alginolyticus* via changes of energy metabolism. *Aquac Reports.* 19:100565. Internet. doi:[10.1016/j.aqrep.2020.100565](https://doi.org/10.1016/j.aqrep.2020.100565).
- Yadav P, Yadav R, Jain S, Vaidya A. 2021. Caspase-3: a primary target for natural and synthetic compounds for cancer therapy. *Chem Biol Drug Des.* 98(1):144–165. doi:[10.1111/cbdd.13860](https://doi.org/10.1111/cbdd.13860).
- Yogalingam G, Hwang S, Ferreira JCB, Mochly-Rosen D. 2013. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) phosphorylation by protein kinase Cδ (PKCδ) inhibits mitochondria

- elimination by lysosomal-like structures following ischemia and reoxygenation-induced injury. *J Biol Chem.* 288(26):18947–18960. doi:[10.1074/jbc.M113.466870](https://doi.org/10.1074/jbc.M113.466870). Internet.
- Zhai D, Chin K, Wang M, Liu F. 2014. Disruption of the nuclear p53-GAPDH complex protects against ischemia-induced neuronal damage. *Mol Brain.* 7(1):1–12. doi:[10.1186/1756-6606-7-20](https://doi.org/10.1186/1756-6606-7-20). Internet.
- Zhou H, Li Y, Wei L, Zhang Z, Huang H, Diao X, Xiang J. 2014. Sensitivity of larvae and adult and the immunologic characteristics of *Litopenaeus vannamei* under the acute hypoxia. null. 2014:574534. doi:[10.1155/2014/574534](https://doi.org/10.1155/2014/574534).