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Regulation of glyceraldehyde-3-phosphate dehydrogenase by hypoxia inducible factor 1 in the white shrimp *Litopenaeus vannamei* during hypoxia and reoxygenation

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ABSTRACT

Hypoxia is a frequent source of stress in the estuarine habitat of the white shrimp *Litopenaeus vannamei*. During hypoxia, *L. vannamei* gill cells rely more heavily on anaerobic glycolysis to obtain ATP. This is mediated by transcriptional up-regulation of glycolytic enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The hypoxia inducible factor 1 (HIF-1) is an important transcriptional activator of several glycolytic enzymes during hypoxia in diverse animals, including crustaceans. In this work, we cloned and sequenced a fragment corresponding to the 5′ flank of the GAPDH gene and identified a putative HIF-1 binding site, as well as sites for other transcription factors involved in the hypoxia signaling pathway. To investigate the role of HIF-1 in GAPDH regulation, we simultaneously injected double-stranded RNA (dsRNA) into shrimp to silence HIF-1α and HIF-1β under normoxia, hypoxia, and hypoxia followed by reoxygenation, and then measured gill HIF-1α, HIF-1β expression, GAPDH expression and activity, and glucose and lactate concentrations at 0, 3, 24 and 48 h. During normoxia, HIF-1 silencing induced up-regulation of GAPDH transcripts and activity, suggesting that expression is down-regulated *via* HIF-1 under these conditions. In contrast, HIF-1 silencing during hypoxia abolished the increases in GAPDH expression and activity, glucose and lactate concentrations. Finally, HIF-1 silencing during hypoxia-reoxygenation prevented the increase in GAPDH expression, however, those changes were not reflected in GAPDH activity and lactate accumulation. Altogether, these results indicate that GAPDH and glycolysis are transcriptionally regulated by HIF-1 in gills of white shrimp.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is an evolutionarily conserved enzyme that catalyzes the sixth step of glycolysis, the reversible conversion of glyceraldehyde-3-phosphate (G3P) into 1,3-biphosphoglycerate (1,3BPG) coupled to NAD^+ reduction and substrate phosphorylation using inorganic phosphate (Fothergill-Gilmore and Michels, 1993). GAPDH also participates in gluconeogenesis and is an intersection point for multiple pathways, including the pentose phosphate pathway, glycerol synthesis and fructose catabolism; therefore GAPDH influences the carbon flux through central metabolism (White and Garcin, 2017). GAPDH is ubiquitous in animal tissues, and thus it is routinely used as reference in quantitative analysis of the expression of other genes and proteins (Barber et al., 2019; Ferguson et al., 2005). However cumulative evidence obtained in vertebrates has demonstrated that GAPDH expression can be subject to significant regulation; its gene promoter has binding sites for many transcription factors, and its expression is responsive to diverse physiological and environmental stimuli (Seidler, 2013). Additionally, the enzyme can be post-translationally regulated resulting in changes in sub-cellular localization and function (Sirover, 2014). Furthermore, GAPDH is involved in several roles in addition to energy metabolism; for example, in the control of gene expression, in nucleic acid repair, endocytosis, autophagy, redox signaling, and apoptosis. This plethora of roles depends on the physiological status of the cell, and some functions are induced by stress (Sirover, 2011).

In mammalian cell lines GAPDH expression and protein abundance are up-regulated during hypoxic stress (Graven et al., 1998;

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Higashimura et al., 2011; Yamaji et al., 2003). Deficiencies in intracellular oxygen impair ATP production by mitochondrial oxidative phosphorylation and induce a compensatory anaerobic ATP production. A common characteristic among cells of hypoxia-tolerant animals is the enhancement of anaerobic glycolysis by inducing the expression of the pathway enzymes *via* the hypoxia-inducible factor 1 (HIF-1) (Hoogewijs et al., 2007). HIF-1 is a heterodimeric protein that belongs to the basic helix-loop-helix/Per-ARNT-Sim family of transcription factors, composed by α and β subunits. HIF-1α is primarily regulated by intracellular oxygen. Under normoxia, HIF-1α is degraded at the proteasome, but when oxygen decreases, it accumulates and translocates into the nucleus, where it dimerizes with HIF-1β and binds to hypoxia response elements (HREs) located at the promoter region of many genes involved in the response to hypoxia (Ke and Costa, 2006). The well-characterized human GAPDH gene promoter has six HREs, some already demonstrated to be functional (Higashimura et al., 2011; Lu et al., 2002).

Environmental hypoxia is a natural stressor in diverse marine habitats that is becoming even more extensive due to the impacts of global warming and pollution, representing a threat to animal populations (Breitburg et al., 2018). Aquatic hypoxia is defined as a dissolved oxygen (DO) concentration < 2 mg/L in the water body (Diaz, 2001). Aquatic animal species have large differences in hypoxia vulnerability that are determined by their ability to match energy supply and demand (Hochachka and Somero, 2002). For example, benthic crustaceans are in general more vulnerable to oxygen limitations than other benthic marine invertebrates and benthic fishes (Vaquer-Sunyer and Duarte, 2008). On the other hand, estuarine species are more tolerant to episodic hypoxia resulting from circadian, tidal and seasonal regimes. In addition, estuarine species usually experience a sudden increase in DO concentrations in the post-hypoxic period, which is known as reoxygenation (Freire et al., 2012). The white shrimp *Litopenaeus vannamei* is a commercially-important crustacean that spends a large part of its life cycle in estuaries of the Eastern Pacific coast (Holthuis, 1976) and is able to cope with DO concentrations as low as 0.2 mg/L for some time (Pérez-Rostro et al., 2004). Similar to hypoxia-tolerant mammalian cells, shrimp cells withstand fluctuations in oxygen by adjusting energy metabolism. During exposure to moderate hypoxia (~1.5 mg/L O_2), *L. vannamei* experiences tissue-specific changes in expression and activity of the glycolytic enzymes hexokinase (HK) (Soñanez-Organis et al., 2011), lactate dehydrogenase (LDH) (Soñanez-Organis et al., 2012; Ulaje et al., 2019) and phosphofructokinase (PFK) (Cota-Ruiz et al., 2015; Ulaje et al., 2019). In addition, moderate hypoxia resulted in lactate accumulation and changes in glucose content of hemolymph and tissues, indicating an increase in anaerobic metabolism rate (Pérez-Rostro et al., 2004; Racotta et al., 2002; Soñanez-Organis et al., 2010). Double-stranded RNA (dsRNA) mediated interference experiments in shrimp revealed that HK, PFK and LDH are regulated by HIF-1 in a tissue-specific manner (Cota-Ruiz et al., 2016; Soñanez-Organis et al., 2012, 2011). These studies highlight the importance of HIF-1 on modulating the metabolism of aquatic invertebrates during hypoxia, and establish *L. vannamei* as an interesting crustacean model for comparative studies on the molecular bases of physiological tolerance to changes in environmental oxygen.

Recently, the GAPDH gene from *L. vannamei* was cloned and sequenced, and its expression in gills was shown to be up-regulated during hypoxia (Camacho-Jiménez et al., 2018). Gills are involved in respiration, and energy-requiring processes like osmoregulation, detoxification and acid-base balance (Henry et al., 2012). Thus, gills must have an active metabolism and should be one of the first tissues to sense changes in DO. Interestingly, transcripts of the oxygen-sensitive HIF-1α are abundant in gills of *L. vannamei* (Soñanez-Organis et al., 2009) and other crustaceans like *Callinectes sapidus* (Hardy et al., 2012), *Macrobrachium nipponense* (Sun et al., 2016) and *Scylla paramamosain* (Wei et al., 2017), even in normoxic conditions, suggesting that pathways regulated by HIF-1 are key for the tissue functioning upon oxygen fluctuations. However, the putative role of HIF-1 on the transcriptional control of GAPDH expression has not been investigated yet in crustaceans. In this work, we report the presence of a potential HRE and other regulatory elements for transcription factors with roles in hypoxia signaling upstream of the translation start site in the GAPDH gene. Furthermore, we examined the specific role of HIF-1 in regulating GAPDH by dsRNA mediated silencing of HIF-1α and HIF-1β and analyzed the effects on gill GAPDH expression and enzyme activity during normoxia, hypoxia and reoxygenation. Finally, we also measured glucose and lactate concentrations in gills of shrimp from those conditions as indicators of changes in anaerobic metabolism.

2. Materials and methods

2.1. Cloning of partial 5′ flanking sequence of the GAPDH gene

A sequence upstream of the translation start codon in the GAPDH gene was obtained using the Universal Genome Walker kit (Clonetech, Palo Alto, CA, USA) according to the manufacturer's instructions with some modifications. Concisely, to produce genomic libraries, genomic DNA (gDNA) was extracted from shrimp muscle (Bradfield and Wyatt, 1983), digested with restriction enzymes (*Dra*I, *Stu*I and *Ssp*I), and ligated to the kit primers adapters. These libraries were used as template in separate PCR reactions using adapter primers (AP) from the Genome Walker kit and specific primers designed from the GAPDH gene sequence (GenBank accession no. MG878889). The first reaction was performed with AP1 and GAPDHRv6 primers (Table 1). Each reaction mixture (20 μL) contained 0.2 μM of each primer, 2 μL of 10× Advantage 2 PCR buffer (Clontech), 0.4 μL of Advantage 2 polymerase mix (Clontech), and 1 μL of gDNA library. The cycling conditions were: 94 °C, 25 s, 72 °C, 3 min (7 cycles); 94 °C, 5 min, 67 °C, 3 min (35 cycles); 67 °C, 7 min (1 cycle) using a DNA Dyad Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The primary PCR products were used as template in nested reactions using the AP2 and GAPDHRv5 primers (Table 1). Each reaction mixture (20 μL) contained 0.2 μM of each primer, 10 μL of Hot Start Taq 2× Master Mix (New England BioLabs, Ipswich, MA, USA) and 1 μL of PCR products (diluted 1:50). The cycling conditions were: 95 °C, 30 s, 68 °C, 3 min (5 cycles); 95 °C, 25 s, 68 °C, 3 min (20 cycles); 67 °C 7 min (1 cycle). The amplicons were cloned in the pGEM®-T Easy Vector (Promega, Madison, WI, USA) using TOP10 *Escherichia coli*. The cloned fragments were sequenced in both strands at the Laboratory of Genomic Analysis and Technology Core of the University of Arizona (Tucson, AZ, USA). Eukaryotic TATA-box and CCAAT-box motifs were predicted with HCtata (http://bioinfo.itb.cnr.it/~webgene/wwwHC_tata.html) and GPMiner (http://gpminer.mbc.nctu.edu.tw/) using default parameters. Potential HIF-1 binding sites were identified by searching the HRE core sequence (5′-NCGTG-3′) (Semenza et al., 1997). Additional eukaryotic regulatory motifs in the coding strand were predicted with Alibaba 2.1 (http://gene-regulation.com/pub/programs/alibaba2/index.html) (with 80% of matrix

Table 1
Primers used in PCR and qPCR.

Primer	Sequence (5′-3′)
AP1	GTAATACGACTCACTATAGGG
AP2	ACTATAGGGCACGCGTGGT
GAPDHFw2	GTGTTCACCACCATCGAGAAG
GAPDHRv1	GACACCACCTTCATGTCCTTG
GAPDHRv5	AGCCATTGATACCGATCTTCGA
GAPDHRv6	ACACTTACCTCGGCTCCCTTCTGGAG
HIFrtF	GGAG AGCGAGATCTTCACG
HIFrtR	GCCTCCTTCCGTGATCTTC
ARNTrtF	CAAGAGCCAGCCAACCAAG
ARNTrtR1	CTGCGTCAGAGAAATTCC
L8F2	TAGGCAATGTCATCCCCATT
L8R2	TCCTGAAGGGAGCTTTACACG

similarity cut-off), TFBIND (http://tfbind.hgc.jp/) (90% of cut-off), and Nsite 6.2014 (http://www.softberry.com/) (90% of cut-off). Only sites with a minimal length of four bases were considered as a match.

2.2. Synthesis of HIF-1 dsRNA

Two dsRNA were separately prepared to silence the expression of HIF-1α and HIF-1β in shrimp by RNA interference according to the procedures fully described by Cota-Ruiz et al. (2016). Briefly, to produce each interfering RNA, two complementary single stranded RNA (ssRNA) were synthetized by *in vitro* transcription with the T7 RNA polymerase using the T7 RiboMAX Large Scale RNA Production System (Promega, Madison, WI, USA) and as templates specific PCR products previously amplified from gills cDNA and containing at the 5′ end of the sequence of the T7 promoter (5′-GGTGGTTAATACGACTCACTATA GGG-3′). The region selected as template for silencing HIF-1α correspond to positions 243–822 of the coding sequence (CDS) in the cDNA (GenBank accession no. FJ807918), whereas in HIF-1β cDNA spans the 152–776 portions of the CDS (GenBank accession no. FJ807919). The concentration of each ssRNA was measured at 260 nm in a NanoDrop Lite spectrophotometer (Thermo Scientific, Wilmington, DE, USA). To produce both dsRNA corresponding to each HIF-1 subunit, equal amounts of the specific sense and antisense ssRNA were hybridized by heating at 80 °C for 10 min and slow cooling down to room temperature. The formation and integrity of the dsRNAs were confirmed by 1% agarose gel electrophoresis. Both dsRNAs were stored at −20 °C until injection in the shrimp.

2.3. Assay for HIF-1 silencing in normoxia, hypoxia and reoxygenation

A biological assay was performed with 160 juvenile healthy shrimp (14.27 ± 1.10 g) in intermolt maintained in seawater (28 °C, 35‰, 5.0 ± 0.50 mg/L DO) with constant aeration through air-diffusing stones for at least two weeks in the laboratory. Before the experiment, the shrimp were distributed in sixteen 100 L closed aquaria (ten shrimp per aquarium) filled with seawater in normoxic conditions (4.77 ± 0.27 mg/L DO) and the DO was monitored with a portable oximeter (YSI model 55, Yellow Spring, OH, USA) before feeding the shrimp. The animals were fed with commercial shrimp diet equivalent to 3% of their wet weight (distributed in three rations per day), and the residual food and feces were removed from aquaria before each feeding. After two days of acclimation and before the assay, the shrimp were fed only once, and the water of the tanks was fully exchanged. The shrimp were injected in the abdominal muscle either with 100 µL of sterile saline solution (SS) (20 mM Tris, 400 mM NaCl, pH 7.5) used as a control for the handling of the animals or 14 µg of HIF-1α and HIF-1β dsRNA dissolved in 100 µL of SS (~1 µg of dsRNA/g of shrimp wet weight). Twenty-four hours after injection and prior to the stress exposure, four shrimp injected with SS kept in normoxia were sampled as control group and the rest of the shrimp were assigned to the following treatments: 1) normoxia injected with dsRNA; 2) hypoxia injected with SS; 3) hypoxia injected with dsRNA; 4) hypoxia followed by reoxygenation injected with SS; 5) hypoxia followed by reoxygenation injected with dsRNA. The hypoxic conditions were established by bubbling nitrogen gas into the aquaria to reach a DO concentration of 1.53 ± 0.05 mg/L. DO conditions were monitored each hour with the portable oximeter and maintained by regulating the air or nitrogen supply when necessary. Four animals in normoxia (injected with dsRNA) and hypoxia (injected with SS and dsRNA) were sampled at 3, 24 and 48 h of exposure, while the shrimp in the treatments including hypoxia-reoxygenation (SS and dsRNA injected) were sampled after 3, 24 and 48 h of hypoxia plus 1 h of reoxygenation in normoxia (4.60 ± 0.47 mg/L DO), settled by fully opening the air supply. Gills of each shrimp were dissected. Approximately 50 mg, were submerged in ten volumes of TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), and a second aliquot was placed in a microcentrifuge tube; all the samples were immediately frozen in a dry ice/ethanol (96%) bath. The samples were stored at −80 °C until they were further processed.

2.4. mRNA quantification by RT-qPCR

Total RNA was isolated from each gill sample according to the TRI Reagent manufacturer's protocol. The concentration and purity of RNA was assessed by measuring the absorbance at 260 nm and the 260/280 nm ratio respectively, in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The integrity of total RNA was validated by 1% agarose gel electrophoresis. Contaminating gDNA was eliminated from total RNA by digestion with RNAse-free DNAse I, (Roche, Mannheim, Germany) and was verified by qPCR using the specific primers for the GAPDH gene GAPDHFw2/GAPDHRv1 (Table 1) that generate a product of 143 bp (Camacho-Jiménez et al., 2018). Each qPCR reaction (15 µL) contained: 7.5 µL of 2× SyBr green qPCR Master Mix (Biotool, Houston, TX, USA), 25 ng total RNA, and 0.5 µM each primer. As positive controls, reactions containing 25 ng muscle gDNA or gill cDNA were included per run. Non-template control (NTC) reactions were also included in duplicates to monitor reagent contamination. The qPCR analyses were performed in a CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA) under the following cycling conditions: 95 °C, 5 min (1 cycle); 95 °C, 30 s, 60 °C, 35 s, 72 °C, 55 s (40 cycles); 68 °C 5 min (1 cycle). To confirm the specificity of amplification and no primer dimer formation, at the end of each run, a melting curve analysis was done by measuring the fluorescence from 65 to 95 °C with an increase of 0.3 °C each 5 s.

Two complementary DNA (cDNA) were synthesized from each DNAse-treated RNA sample in separate reactions (0.5 µg of total RNA in each) by reverse transcription (RT) using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). Expression of HIF-1α, HIF-1β, and GAPDH was measured by quantitative PCR (qPCR) in the cDNA samples from each experimental group. Each gene was analyzed in two separate qPCR reactions in the two cDNA samples of each shrimp (four technical replicates per animal). The specific primers used for qPCR were (Table 1): HIFrtF/HIFrtR for HIF-1α (217 bp) (Cota-Ruiz et al., 2016), ARNTrtF/ARNTrtR1 for HIF-1β (189 bp) (Hernández-Palomares et al., 2018), and GAPDHFw2/GAPDHRv1. Additionally, a fragment (166 bp) of L8 ribosomal protein gene (Table 1) was amplified to normalize gene expression data, since its expression has been validated as stable in gills of *L. vannamei* under the same environmental conditions used in this study (Cota-Ruiz et al., 2015; García-Triana et al., 2010). The qPCR reactions were prepared as mentioned before but containing 1 µL of cDNA (equivalent to 25 ng of total RNA) as template, and 0.5 µM of each primer for HIF-1α, HIF-1β, and GAPDH or 1.2 µM of each primer for L8. Duplicated NTC were also included and a melting curve analysis at the end of each run. The amplification efficiency for each primer set was calculated from standard curves ($R^2 \geq 0.99$) ranging from 2.5×10^{1}–2.5×10^{-3} ng of cDNA for L8 and GAPDH, 5.0×10^{-4}-5×10^{-8} ng of specific PCR product for HIF-1α, and 5.0×10^{-4}-5×10^{-9} ng of specific PCR product for HIF-1β. The amplification efficiency of the primers was ~101% for HIF-1α and HIF-1β, ~93% for GAPDH and ~98% for L8. The relative expression of each gene was calculated by the Pfaffl method (Pfaffl, 2001).

2.5. GAPDH activity

GAPDH enzyme activity was analyzed in gill samples (30–50 mg) from each control and treatment. Each tissue sample was homogenized 1:4 (*w/v*) in cold homogenization buffer (100 mM sodium phosphate, 1 mM EDTA, 1 mM PMSF, pH 7.6), using a Branson sonifier 250 (Branson Ultrasonic Corporation, Danbury, CT, USA), and then centrifuged at 9000 xg, 4 °C for 15 min. The supernatants were collected (crude extracts) and protein concentration was quantified by the Bradford (1976) method in a Model 680 microplate reader (Bio-Rad) using bovine serum albumin as standard. Crude extracts were diluted

(1:20) in sodium phosphate buffer (200 mM, pH 7.6) before measuring activity.

Enzyme activity was assayed in the forward direction according to the method modified from Bisswanger (2011). The assay uses arsenate as a substitute for inorganic phosphate during the oxidation of G3P in the presence of NAD^+, rendering 1-arseno-3-phosphoglycerate and NADH. The reaction mixture (200 µL) contained 94 mM triethanolamine (pH 7.6), 0.9 mM DL-G3P, 3 mM potassium dihydrogen arsenate, 1 mM β-NAD^+, and 4 µL of crude extract. Each crude extract was analyzed in duplicate to monitor the NADH formation at 340 nm and 25 °C for 18.7 min using a FLUOstar Omega microplate reader (BMG Labtech, Durham, NC, USA). Specificity of the assay was validated in crude extract by monitoring the absorbance in two separate reactions without DL-G3P and NAD^+. As blank, a reaction without tissue sample was included in each run. As positive control, a reaction with 4 µg of GAPDH from rabbit muscle (Sigma-Aldrich) dissolved in phosphate buffer was included in each run. Enzyme activity was estimated from the ratio of absorbance change in a linear range over time and the extinction coefficient of NADH (6.22×10^{-3} L·mol^{-1}). One unit of enzyme activity is equivalent to the amount of enzyme necessary to produce 1 µmol of NADH per minute. The specific GAPDH activity is reported as units (U) per mg of protein.

2.6. Glucose and lactate in tissues

Glucose and lactate concentrations in gills were measured in crude extracts of tissue (30–50 mg) (for preparation see Section 2.5.) from shrimp of each experimental condition. Glucose was quantified using the Glucose GOD/PAP reagent (Randox, United Kingdom) and lactate with the Lactate Assay kit (Randox). The methods were adapted to microplate volume (200 µL) and each sample was analyzed in duplicate with the Model 680 microplate reader by measuring the absorbance at 490 nm and 550 nm for glucose and lactate, respectively. Metabolite concentrations were normalized with respect to the protein concentration in each sample.

2.7. Statistical analyses

Data from all technical replicates of all samples were tested for normality and homoscedasticity with the Shapiro-Wilk and Levene's tests, respectively. Glucose data were analyzed by One-Way ANOVA with Fisher's LSD test to assess significant differences between control and treatments, and between treatments at each sampling point. Because the data sets of expression, activity and lactate were not normally distributed or failed for equality of variances, non-parametric analyses were performed. Significant differences between experimental groups were assessed by Kruskal-Wallis test followed by Mann-Whitney *U* tests. The data were plotted as means ± standard error (SE). Analyses were done using SigmaPlot 12.0 (Systat, San Jose, CA, USA) at a significance level of $p \leq .05$.

3. Results

3.1. The 5′ flanking sequence of the GAPDH gene contains transcription regulatory elements

A sequence of 548 bp of the GAPDH gene including 522 bp upstream of the translation start site (ATG) (GenBank accession no. MK129177) was obtained from genomic DNA libraries (Fig. 1) and analyzed using bioinformatics tools to identify potential regulatory elements. The analyses predicted two TATA-box like motifs at −312 to −307 and − 287 to −278 relative to the translation start site and a putative CCAAT-box motif at −144 and − 139. Several *cis*-regulatory elements for transcription factors binding were identified; including a binding motif for HIF-1 with the core sequence 5′-CCGTG-3′ located at −76 to −72 upstream the start codon. Other sites for binding of transcription factors involved also in the response to hypoxia were identified: five motifs for Sp1, four sites for CCAAT-enhancer-binding proteins (C/EBP), three sites for heat shock factors (HSF), one motif for metal transcription factor 1 (MTF-1), two sites for activating protein 2 (AP-2), two sites for E26 transformation-specific (Ets) family, one element for nuclear factor kappa-B (NF-κB), and a site for cyclic AMP response element-binding protein 1 (CRE-BP1, also known as ATF-2), among others.

3.2. HIF-1 expression and silencing during normoxia, hypoxia and reoxygenation

Relative expression of HIF-1α and HIF-1β from shrimp injected with saline solution (SS, injection control) or with HIF-1 dsRNA (silencing treatment) is shown in Fig. 2. The efficiency of HIF-1 silencing was high, especially during conditions that induced HIF-1α and HIF-1β expression (*i.e.*, 3 h and 24 h of hypoxia) (Table 2). During normoxia, in response to dsRNA injection, there was a gradual decrease in HIF-1α expression (Fig. 2A) by 4.9-fold below initial control after 48 h ($p = .005$), equivalent to an 86.6% of silencing. Under the same conditions, HIF-1β expression (Fig. 2B) was significantly increased in dsRNA-injected shrimp by 4.3-fold at 3 h ($p < .001$), followed by a progressive decrease to minimal values after 48 h, being lower than those in initial control by 8.2-fold and equivalent to 87.6% of silencing ($p < .001$).

In shrimp exposed to hypoxia for 3 h, there was a significant ~10-fold increase in both HIF-1α and HIF-1β transcripts compared to initial normoxic control by ($p = .001$, in both cases). After the 3 h peak, HIF-1α and HIF-1β expression decreased, reaching values below normoxic control after 48 h of hypoxia. The increase in HIF-1α and HIF-1β mRNA abundance during hypoxia was abolished by dsRNA injection. After 3 h of hypoxia, HIF-1α expression in dsRNA-injected shrimp was significantly lower than SS-treated animals by ~16-fold (96.3% of silencing) ($p = .001$). At the same time, HIF-1β transcripts in dsRNA treated shrimp decreased by ~12-fold compared to SS-injected group (91.3% of silencing) ($p = .001$). The suppressive effect of dsRNA injection on HIF-1α expression was still present after 48 h of hypoxia ($p = .026$), whereas HIF-1β knockdown lasted only during 24 h ($p < .001$).

Reoxygenation for 1 h following 3 h of hypoxia stimulated a significant increase in HIF-1α and HIF-1β transcripts above the normoxic control value ($p = .029$ for HIF-1α and $p = .003$ for HIF-1β). However, the expression of both subunits was significantly lower than the respective hypoxic treatment by ~5-fold for HIF-1α ($p < .001$) and by ~6-fold for HIF-1β ($p = .003$). The expression of both subunits decreased after 24–48 h to reach values lower than those in initial control. In addition, by 48 h, there were no longer differences between hypoxic and reoxygenated shrimp. Thus, under these conditions, HIF-1α silencing was effective only at 3 h, reducing expression by ~63% with respect to SS-injected shrimp ($p = .003$). Conversely, no significant silencing was detected for HIF-1β during hypoxia plus reoxygenation at any sampling point.

3.3. HIF-1 silencing affects GAPDH expression

The effects of HIF-1 knockdown on GAPDH expression in gills from shrimp exposed to normoxia, hypoxia, and hypoxia plus reoxygenation are shown in Fig. 3. Under normoxia, GAPDH expression in dsRNA-injected animals increased by ~45-fold (3 h) compared to the initial control ($p = .002$). GAPDH expression in normoxic dsRNA-injected shrimp progressively decreased, reaching control values by 48 h ($p = .162$). Hypoxic stress also promoted a similar transient induction of GAPDH expression by ~40-fold compared to the initial normoxic control ($p = .002$). This hypoxia induced increase in GAPDH expression was considerably attenuated by the dsRNA injection, which was ~8-fold lower than in non-dsRNA-injected shrimp under the same conditions ($p = .002$).

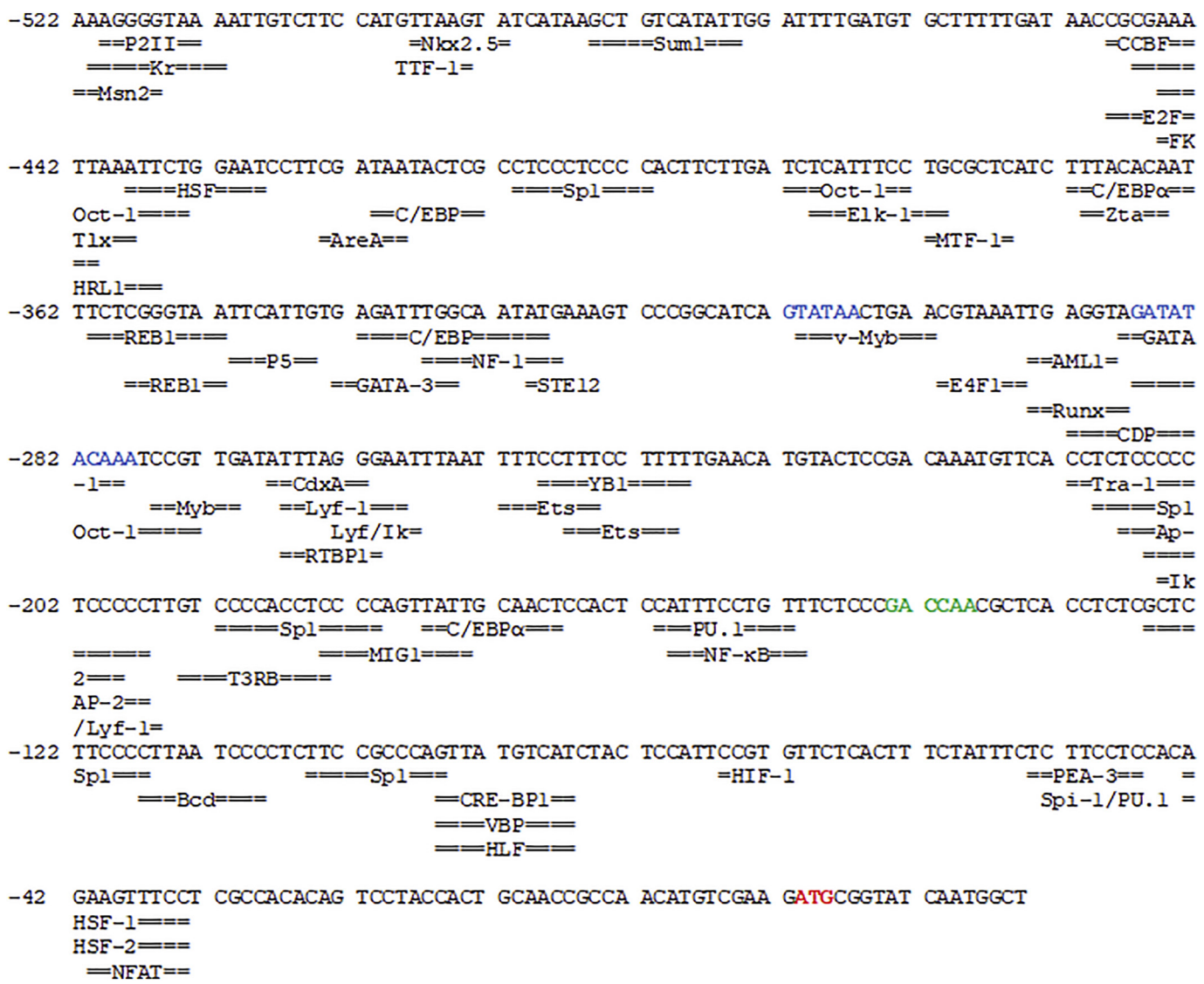

Fig. 1. Partial 5′ flanking sequence of the GAPDH gene. The numbers indicate nucleotide bases positions relative to translation the start codon (+1, bold red letters). Predicted TATA-box motives are indicated in bold blue letters. Potential CCAAT-box sequence is indicated in bold green. Putative transcription factor biding sites are indicated.

Reoxygenation following 3 h or 24 h of hypoxia induced a significant up-regulation of GAPDH expression, with a highest increase of ~15-fold after 3 h compared to initial control values (p = .002), went down to ~4-fold by 24 h (p = .005), and reached initial values by 48 h (p = .162). The increases in GAPDH expression were abolished by dsRNA treatment at both 3 h and 24 h, since the increase was ~4-fold lower than in SS-injected animals (p = .002 for 3 h and p = .004 for 24 h).

3.4. Effect of HIF-1 silencing on GAPDH activity

The time-course effects of HIF-1 silencing on GAPDH specific activity in gills from shrimp under the different DO treatments are shown in Fig. 4. GAPDH activity in normoxic shrimp injected with SS was $3.6 \pm 0.14 \times 10^{-2}$ U/mg of protein, and it did not change in any experimental group after 3 h of exposure (p = .211). However, dsRNA injection under normoxia induced a 38% increase at 24 h compared to initial control (p = .002). The stimulatory effect on GAPDH activity was still present after 48 h, however it was only 18% higher than initial values (p = .043). Exposure to hypoxia for 24 h induced a 31% increase in GAPDH activity compared to initial normoxic control (p = .004), which was abolished by dsRNA injection. Finally, reoxygenation only induced changes in GAPDH activity after 48 h of hypoxia, that decreased 33% compared to the initial control (p = .026). This reduction did not occur in HIF-1 silenced animals under the same conditions that presented values similar to normoxic shrimp injected with SS (initial control) (p = .491).

3.5. Effect of HIF-1 knockdown on glucose and lactate concentrations

Glucose concentrations in gills tissue throughout the experiment are shown in Fig. 5A. Values of glucose in shrimp from initial control in normoxia were $1.60 \pm 0.59 \times 10^{-2}$ mg/mg of protein. Glucose concentration in normoxic shrimp injected with dsRNA increased ~41% ($p < .001$) compared to the initial control and ~51% after 24 h ($p < .001$). By 48 h glucose concentration returned to initial control values (p = .361). Hypoxia induced an increase that was most evident after 3 h equivalent to ~62% greater than initial control concentration ($p < .001$) and was still evident after 24 h of hypoxia ($p < .006$). Glucose concentration returned to initial control values after 48 h of hypoxia (p = .523). HIF-1 dsRNA injection slightly (although not significantly) reduced the rise in gill glucose concentration seen after 3 h

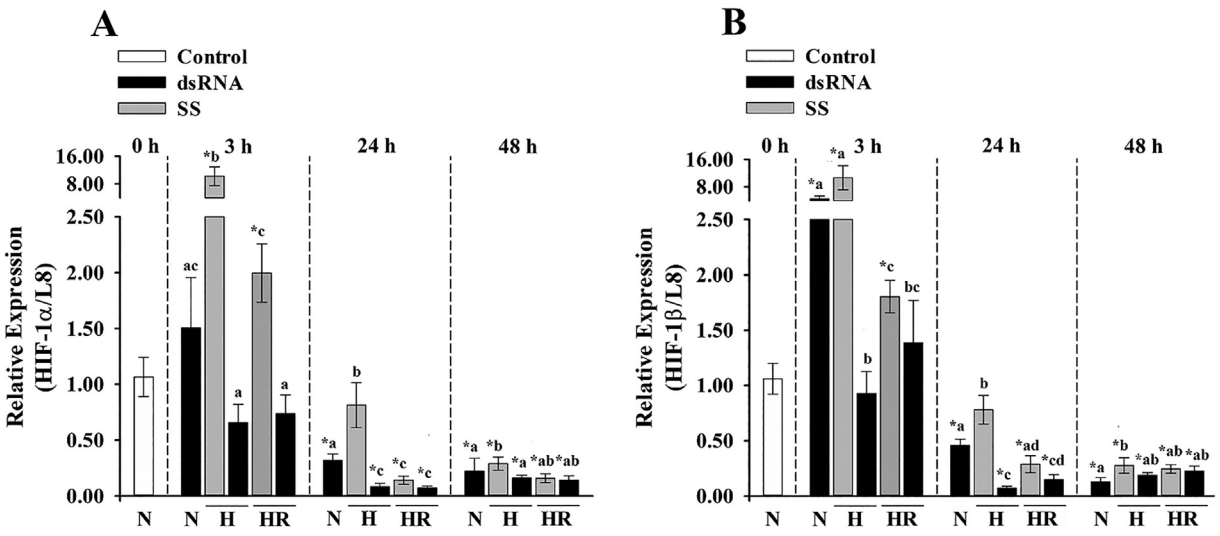

Fig. 2. Expression of HIF-1α and HIF-1β in gills during normoxia (N), hypoxia (H), and hypoxia followed by reoxygenation (HR). Panel A: HIF-1α at 3 h, 24 h, and 48 h of exposure. Panel B: HIF-1β at 0 h, 3 h, 24 h, and 48 h of exposure. In all: Control, shrimp injected with saline solution and sampled after 24 h, just before the exposure to hypoxia; dsRNA, shrimp injected with dsRNA; SS, shrimp injected with saline solution. Data are shown as mean ± SE (n = 3–4). Significant differences to initial control are indicated by an asterisk ($p < .05$) and differences between treatments at each sampling time are indicated by letters ($p < .05$).

Table 2
Silencing percentage in gills of shrimp injected with dsRNA.

	HIF-1α			HIF-1β		
	3 h	24 h	48 h	3 h	24 h	48 h
N	–	70.0%	86.6%	–	56.7%	87.4%
H	93.6%	89.5%	43.4%	91.3%	90.8%	–
HR	63.1%	–	–	–	–	–

N, normoxia; H, hypoxia, HR, hypoxia followed by reoxygenation (1 h).
(−) no significant decrease in expression was found (n = 3–4, $p < .05$).

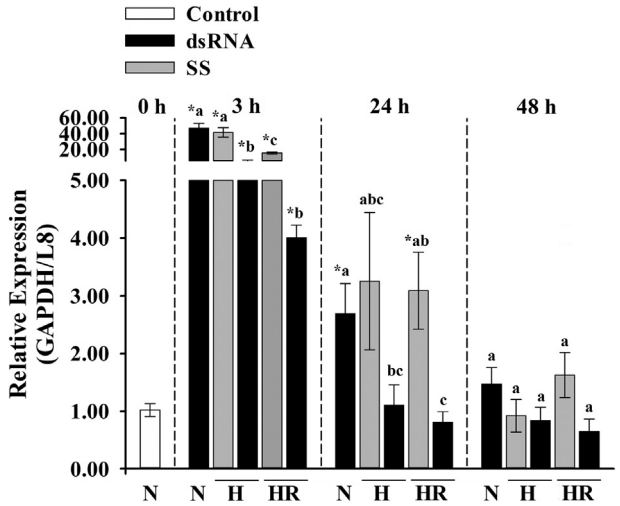

Fig. 3. Effect of HIF-1 silencing on GAPDH expression in gills of shrimp exposed to normoxia (N), hypoxia (H), and hypoxia followed by reoxygenation (HR) during 3 h, 24 h, and 48 h of exposure. In all: Control, shrimp injected with saline solution and sampled after 24 h, just before the exposure to hypoxia; dsRNA, shrimp injected with dsRNA; SS, shrimp injected with saline solution. Data are shown as mean ± SE (n = 3–4). Significant differences to initial control are indicated by an asterisk ($p < .05$) and differences between treatments at each sampling time are indicated by letters ($p < .05$).

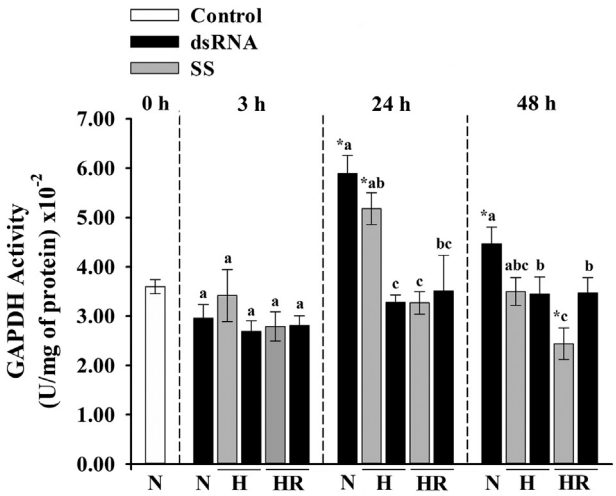

Fig. 4. GAPDH activity in gills after dsRNA injection during normoxia (N), hypoxia (H), and hypoxia followed by reoxygenation (HR) after 3 h, 24 h, and 48 h of hypoxia. In all: Control, shrimp injected with saline solution and sampled after 24 h, just before the exposure to hypoxia; dsRNA, shrimp injected with dsRNA; SS, shrimp injected with saline solution. Data are shown as mean ± SE (n = 3–4). Significant differences to initial control are indicated by an asterisk ($p < .05$) and differences between treatments at each sampling time are indicated by letters ($p < .05$).

of hypoxia (p = .146), and completely prevented the rise at 24 h (p = .0159), while it had no effect at 48 h (p = .746). Reoxygenation following 3 h of hypoxia also increased glucose concentration compared to the initial normoxic controls (~39%, $p < .001$), but to levels that were significantly lower (~24%) than in shrimps exposed to hypoxia for 3 h (p = .002). There were no differences in glucose concentration after reoxygenation following 24 h of hypoxia compared to initial normoxic controls (p = .432). However, there was a ~38% glucose increase from shrimp after 1 h of reoxygenation following 48 h of hypoxia ($p < .001$). This latter increase did not occur in HIF-1 dsRNA-injected shrimp, as glucose concentration was similar to normoxic controls (p = .879).

Lactate concentrations in gills from shrimp from the different treatments and sampling times are shown in Fig. 5B. In shrimp from

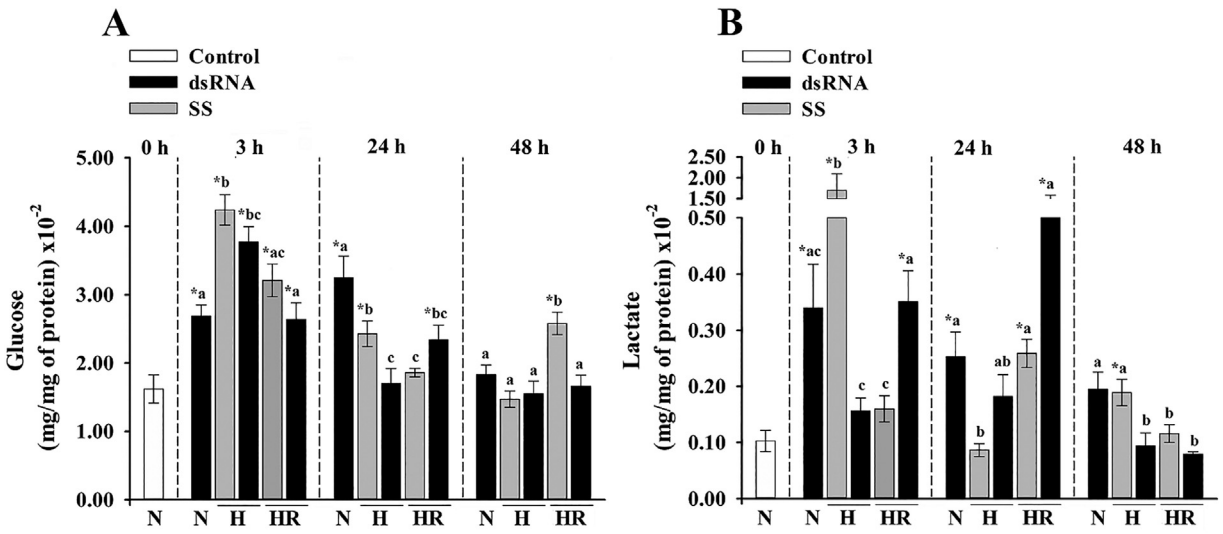

Fig. 5. Glucose and lactate concentration in gills of shrimp in normoxia (N), hypoxia (H), and hypoxia followed by reoxygenation (HR). Panel A: Glucose at 3 h, 24 h, and 48 h of exposure. Panel B: Lactate at 3 h, 24 h, and 48 h of exposure. In all: Control, shrimp injected with saline solution and sampled after 24 h, just before the exposure to hypoxia; dsRNA, shrimp injected with dsRNA; SS, shrimp injected with saline solution. Data are shown as mean ± SE (n = 3–4). Significant differences to initial control are indicated by an asterisk ($p < .05$) and differences between treatments at each sampling time are indicated by letters ($p < .05$).

initial normoxic control, lactate concentration was $0.10 \pm 0.05 \times 10^{-2}$ mg/mg of protein. The dsRNA injection in normoxic animals significantly increased lactate in normoxic animals sampled at 3 and 24 h, reaching a maximum of ~3-fold higher than initial controls after 3 h (p = .043). There were no significant differences at the 48 h normoxia sampling point (p = .142). Hypoxia for 3 h triggered a ~17-fold lactate accumulation (p = .002), that was completely abolished by dsRNA injection (p = .228). While no significant differences in lactate concentration were observed after 24 h of hypoxia compared to initial normoxic controls (p = .491), there was a significant ~1.9-fold increase in SS-injected animals after 48 h of hypoxia (p = .029) that was not detected in shrimp injected with dsRNA (p = .589). Reoxygenation only caused an increase in lactate after 24 h of hypoxia, which was ~2.6-fold higher than initial normoxic control ($p < .001$). HIF-1 dsRNA injection induced a ~3-fold increase during reoxygenation after 3 h of hypoxia (p = .002). In samples from the reoxygenation treatment after 24 h of hypoxia, the increase in dsRNA-injected shrimp was ~12-fold higher than initial control ($p < .001$). However, lactate concentration was not significantly different in the injected group SS under the same conditions due to sample variability (p = .279). Gill lactate concentrations in SS and dsRNA-injected shrimps that were exposed to reoxygenation after 48 h of hypoxia were similar to each other (p = .083), and both were also similar to initial normoxic control (p = .755 for SS-injected and p = .345 for dsRNA-injected).

4. Discussion

Even though GAPDH has been classically considered as a housekeeping protein, it is now known that its expression depends on the physiological status of the cells and it may change in response to stress (White and Garcin, 2017). Furthermore, although GAPDH is a key glycolytic enzyme, studies from prokaryotes to vertebrates have shown that it has multiple roles unrelated to its enzyme activity in glycolysis in the cells, and thus, it is considered a "moonlighting protein" (White and Garcin, 2017). In crustaceans, GAPDH gene expression and protein activity are sensitive to several stressors such as diseases (Liu et al., 2011; Wang et al., 2007), high hydrostatic pressure (Morris et al., 2015), hydrocarbons (Pasquevich et al., 2013), water acidification (Chang et al., 2016; Hauton et al., 2009), cold stress (Fan et al., 2013), hypercapnic hypoxia (Johnson et al., 2015) and hypoxia (Camacho-Jiménez et al., 2018). Although most of the knowledge on GAPDH molecular biology in animal models can be translated from the findings in vertebrates, the specific mechanisms underlying the transcriptional control of GAPDH are poorly understood in crustaceans as well as in most aquatic invertebrates.

In the present work, we report a partial 5′ flanking sequence of the GAPDH gene containing several putative regulatory elements. *In silico* analysis predicted two TATA-box like motifs and a CCAAT-box like element upstream of the translation start site, which are characteristic of the core promoter of many eukaryotic genes transcribed by RNA polymerase II (Burley, 1996; Dolfini et al., 2009). Thus, although the transcription initiation site in the GAPDH gene has not been determined yet, the sequence obtained contains at least part of the promoter region. In agreement with this, many putative sites for binding of diverse transcription factors were also found. Because hypoxia is one of the major challenges that shrimp face in estuarine environments (Freire et al., 2012), and since hypoxia induces GAPDH expression (Camacho-Jiménez et al., 2018), we focused on binding sites for factors with known roles in hypoxia. HIF-1 is a master regulator of oxygen homeostasis in animal cells that controls the expression of genes necessary to respond to hypoxia (Ke and Costa, 2006). A potential HRE is located in the 5′ flank of the GAPDH gene with the sequence 5′-CCGTG-3′. Functional HREs with the same sequence are present in mammalian genes including toll-like receptors (2 and 6) (Kuhlicke et al., 2007), lipin 1 (Mylonis et al., 2012) and choline kinase α (Bansal et al., 2012). Moreover, the 5′ flanking sequence contains other predicted binding elements for factors that in non-crustacean animals participate in HIF signaling, including motifs for Sp1 (Higashimura et al., 2011), NF-κB (Taylor, 2008), HSF-1 (Baird et al., 2006), HSF-2 (Chen et al., 2011), MTF-1 (Murphy, 2004), AP-2 (Niebler et al., 2015), CRE-BP1 (ATF-2) (Choi et al., 2009), Ets (Oikawa et al., 2001), and C/EBPα (Yang et al., 2008). These factors may act together or independently from HIF-1; for example, NF-κB controls HIF-1α expression and additionally regulates inflammatory and apoptotic pathways during hypoxia (Taylor, 2008). Further studies could investigate the functionality of these putative binding sites, and their role in GAPDH regulation may be validated, for example, by analyzing protein-DNA and protein-protein interactions or by promoter activity assays with reporter genes.

Previous studies in other crustaceans such as *C. sapidus* (Hardy et al., 2012), *M. nipponense* (Sun et al., 2016) and *S. paramamosain* (Wei et al., 2017) reported that HIF-1α expression is sensitive to DO changes,

whereas HIF-1β remained fairly constant. In *L. vannamei* both HIF-1α and HIF-1β expression are regulated by oxygen concentration in a tissue-specific manner (Soñanez-Organis et al., 2009; Wei et al., 2016). Those findings are in agreement with studies in other hypoxia-tolerant invertebrates such as *Crassostrea gigas* (Kawabe and Yokoyama, 2012), *Crassostrea virginica* (Piontkivska et al., 2011) and *Mytilus galloprovicialis* (Giannetto et al., 2015), which suggest that increases in gene expression are key for hypoxic HIF-1α stabilization in this species. In the current study, we found significant up-regulation of both HIF-1α and HIF-1β expression after 3 h of hypoxia, which was completely abolished by the simultaneous injection of dsRNA for both subunits. The highest (93.6%) and lowest (43.4%) silencing efficiencies obtained were comparable to those detected in hepatopancreas by Cota-Ruiz et al. (2016) using the same RNA interference strategy (82–34%). Additionally, the highest silencing efficiencies were observed in the hypoxic treatment after 3 and 24 h exposure, which correspond to the period with maximum HIF-1α and HIF-1β up-regulation, and also, when the regulatory effects of HIF-1 are expected to be more pronounced. Altogether, our results clearly indicate that HIF-1α and HIF-1β participate in the response to hypoxia in gills from *L. vannamei*.

Comparable to a previous study (Camacho-Jiménez et al., 2018), hypoxia strongly induced GAPDH expression in gills at 3 h, a response that was completely blunted in HIF-1 silenced animals. This suggests that, similar to mammalian cells (Higashimura et al., 2011; Lu et al., 2002), GAPDH expression is induced *via* HIF-1 by binding to HREs upon oxygen deficiency. Alternatively, HIF-1 could regulate other transcription factors in the hypoxia pathway that control GAPDH transcription. Exposure to hypoxia also induced an increase in gill GAPDH specific activity in a HIF-1 dependent manner, which was evident after 24 h. Enzyme activity assays are a proxy for the amount of functional protein, so the different timing compared to peak mRNA levels reflect a lag between transcription and translation, and further confirm physiologically relevant HIF-1 dependent transcriptional control of GAPDH in gills from *L. vannamei* in response to hypoxia.

Unexpectedly, HIF-1 silencing also resulted in noticeable up-regulation in GAPDH expression and specific activity after 24–48 h of normoxia. The mechanistic reasons for those results are unclear, however, we can think of at least two potentials, not mutually exclusive, explanations. The first one is that HIF-1 has hundreds of target genes (Hoogewijs et al., 2007), so HIF-1 silencing could have resulted in the release of down-regulation of some pathway that down-regulates GAPDH expression in normoxia. The second possible explanation is self-regulation of HIF-1 expression. Indeed, we observed a significant increase in HIF-1β mRNA expression from shrimp injected with HIF-1 dsRNA and maintained in normoxia for 3 h (as well as a minor increase in HIF-1α mRNA). If those mRNA increases were reflected in protein levels, it could have resulted in HIF-1α stabilization even in normoxic conditions, leading to HIF-1α and HIF-1β co-recruitment and up-regulation of GAPDH transcription. In fact, the human HIF-1α promoter has a functional HRE suggesting its self-regulation (Minet et al., 1999), and non-hypoxic HIF-1α stabilization in vertebrates occurs in response to diverse physiological stimuli (growth factors, cytokines, nitric oxide and hormones), independently of its degradation rate, but related to the enhancement of gene transcription (Kuschel and Simon, 2011). Clearly, the HIF-1 pathway is complex and the effects of silencing are not always predictable.

When shrimp were exposed to normoxia following 3 h and 24 h hypoxia, there was marked down-regulation of HIF-1α and HIF-1β expression compared to samples taken from hypoxic shrimp at the same point. Reoxygenated shrimp also exhibited lower GAPDH expression levels at the 3 h time point, and lower GAPDH specific activity at the 24 h time point, always compared to samples taken at the respective hypoxic time. Thus, restoring of normoxic conditions rapidly reverses the effects of hypoxia on HIF-1 signaling. A similar response was reported for the mussel *M. galloprovincialis*, in which up-regulation of prolyl hydroxylase, the major regulator of HIF-1α protein in normoxia, was proposed to down-regulate HIF-1α expression during reoxygenation (Giannetto et al., 2015).

We measured glucose and lactate concentrations in gill homogenates as proxies for respiratory fuel utilization. In particular, lactate is perhaps the most reliable indicator of anaerobic utilization of glucose and has been widely used in crustaceans (Bonvillain et al., 2012; da Silva-Castiglioni et al., 2010; Geihs et al., 2013). During normoxia, the increased abundance of both metabolites in shrimp injected with HIF-1 dsRNA suggests down-regulation of aerobic respiration and up-regulation of glycolytic rate related to the increase in HIF-1α and HIF-1β described above. Interestingly, HIF-1 silencing did not significantly affect mRNA expression or activity of HK (Soñanez-Organis et al., 2011) or LDH (Soñanez-Organis et al., 2012) in gills of *L. vannamei* during normoxia, suggesting that glycolysis is not accelerated under these conditions and the effects on metabolism are mainly due to the multifunctionality of GAPDH (Sirover, 2011). However, we cannot venture whether those effects are physiologically relevant for shrimp in their environment or an artifact of HIF-1 silencing.

In the early stages of hypoxia, glucose and lactate concentrations in gills of shrimp were significantly increased, as reported in a previous study (Soñanez-Organis et al., 2010). Lactate accumulation is a clear sign of increased anaerobic metabolism rate while the increase in glucose may be caused by local glycogen breakdown or its uptake from hemolymph as stress triggers the fast release of glucose to hemolymph from glycogen rich tissues of crustaceans (*i.e.* hepatopancreas and muscle) (Lorenzon, 2005). After 24–48 h in hypoxia, glucose and lactate concentrations decreased and reached initial control levels, probably due to a combination of glucose utilization by anaerobic metabolim and lactate clearance through excretion (Henry et al., 2012) or gluconeogenesis. However, despite the gluconeogenic capacity of shrimp gills, it has been demonstrated that expression or activity of enzymes in this pathway like fructose-1,6-biphosphatase (Cota-Ruiz et al., 2015) and phosphoenolpyruvate carboxykinase (Reyes-Ramos et al., 2018) do not increase in this tissue under short-term moderated hypoxia. On the other hand, the main effect of HIF-1 silencing was a complete blunting of lactate accumulation after 3 h of hypoxia. Although this time point coincides with a decrease in GAPDH mRNA abundance and activity in HIF-1 silenced shrimp, the effects can be also explained by the lack of HIF-1-dependent up-regulation of LDH abundance during 1 h of hypoxia reported by Soñanez-Organis et al. (2012) (in fact, LDH expression and activity were lower compared to normoxic controls).

During reoxygenation, the most interesting observation was the lactate build up that took place from HIF-1 silenced shrimps when normoxic conditions were restored after 24 h of hypoxia. As explained above, HIF-1 silencing decreases LDH activity during 1 h of hypoxia (Soñanez-Organis et al., 2012). Therefore, the increase in lactate concentration must be due to impaired lactate clereance, or to HIF-1 independent up-regulation of LDH abundance within the first 24 h, since lactate accumulation was also detected in non-silenced shrimp. Thus, alternatively this results suggest a “Warburg effect”, in which anaerobic metabolism is still active because mitochondrial respiration is not fully restored yet. This is supported by a recent report in which PFK and LDH mRNAs increased in gills of white shrimp during the first 12 h of normoxic reoxygenation after a similar hypoxic exposure (2 mg/L, 3 h) (Ulaje et al., 2019). However, our data show a disparity between GAPDH expression and activity in the hypoxia-reoxygenation treatment, making improbable an important contribution to glycolytic rate under these conditions. Nonetheless, up-regulation of GAPDH mRNA may reflect a higher protein need for other cellular functions. For example, in mammalian cells during ischemia-reperfusion (which causes cell damage by hypoxia-reoxygenation) GAPDH participates in apoptosis (Li et al., 2012), autophagy and antioxidant response (Liang et al., 2015). Potential roles of GAPDH outside energy metabolism will be examined in future studies in shrimp. In summary, this study provides valuable experimental evidence about transcriptional regulation of

GAPDH by HIF-1 especially during hypoxia, but also during normoxia and reoxygenation, in the white shrimp *L. vannamei*. We discussed the implications for regulation of energy metabolism, which is the canonical role of GAPDH. In addition, the results provided evidence for GAPDH "moonlighting" functions during normoxia, hypoxia and reoxygenation, which will form the basis of future research.

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Author contributions

LCJ and GYP conceived and designed the experiments. LCJ, LLC, ABPU and JLDG performed the experiments. LCJ analyzed the data. LCJ, MT and GYP drafted the MS and all the authors critically reviewed and approved the published MS.

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