Two transcripts of hypoxia inducible factor-1 (HIF-1) from *Scylla paramamosain* Estampador, 1950 (Brachyura: Portunidae) and their expression profiles under different hypoxic conditions

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ABSTRACT

In a hypoxic environment, hypoxia inducible factor 1 (HIF-1) plays a major role as a transcription factor that regulates the expression of many related genes, including those involved in angiogenesis, cell proliferation, apoptosis, and energy metabolism, so that organisms can adapt to an anoxic environment. Two full-length HIF-1 cDNAs (*SpHIF-1α* and *SpHIF-1β*) from *Scylla paramamosain* Estampador, 1950 are reported. The cDNA of *SpHIF-1α* is 4,059 bp, including a 5' untranslated region of 267 bp, an open reading frame (ORF) of 3,180 bp coding for a protein consisting of 1,059 amino acids, and a 3' untranslated region of 612 bp. *SpHIF-1β* is 2,244 bp in length encoding a protein of 656 amino acids, with a 5' untranslated region of 150 bp and a 3' untranslated region of 123 bp. SpHIF-1α consists of a signal peptide with 16 amino acids. Both SpHIF-1α and SpHIF-1β have two conservative structure domains, which are basic helix–loop–helix (bHLH) and Per-Arnt-Sim (PAS) domains, whereas HIF-1α exclusively has an oxygen-dependent degradation (ODDD) domain and a C-terminal transactivation domain (C-TAD), both playing crucial roles in hypoxia. *SpHIF-1α* and *SpHIF-1β* were constitutively expressed in all of the experimental tissues in a normoxic environment, as detected by using qRT-PCR. The results indicated that 1) there was a synergistic effect between various tissues in the oxygen balance mechanism, such as the gills, the hepatopancreas and the hemocytes; 2) *S. paramamosain* could only tolerate 2 mg/l hypoxia; 3) hemocytes could be the last line of defense to maintain the oxygen balance and determine the minimum concentration of dissolved oxygen that an individual can bear; and 4) within nine hours in hypoxic conditions, individuals were not in a life-threatening situation. In summary, the HIF-1 gene plays an essential role in regulating the hypoxic response.

Key Words: gene expression, HIF-1, *SpHIF-1α*, *SpHIF-1β*

INTRODUCTION

Scylla paramamosain Estampador, 1950 is a portunid crab that is one of the most important aquaculture species in the southern Pacific Ocean and the North Indian Ocean. In Asia, especially in China, the productivity of *S. paramamosain* has been consistently high (Wang *et al.*, 2005; Ma *et al.*, 2006). Aquaculture operations along coasts have nevertheless resulted in seawater eutrophication as a result of the discharge of nutrients and organic matter into the sea (Diaz & Rosenberg, 1995; Diaz, 2001; Wu, 2002). Eutrophication has resulted in hypoxic events of increasing magnitude, frequency, and duration (Diaz & Rosenberg, 2008). We hypothesize that hypoxia will become increasingly serious and will continue to threaten the survival of coastal species as a result of global climate

change. A hypoxic situation can also occur during the farming process (Rosas *et al.*, 1999). The impacts of an increasingly hypoxic environment in this species is therefore needed.

Hypoxia, even for brief periods, can have severe and persistent threats to the health of humans and most mammals, and might lead to death, as mammals possess little tolerance to hypoxia and their tissues are normally debilitated by any prolonged lack of oxygen (Semenza *et al.*, 2000). Sublethal hypoxia can trigger a series of behavioral, physiological, biochemical, and molecular responses and might severely reduce the overall health of marine organisms (Burnett & Stickle, 2001). Crustaceans and fishes are known to be affected by reduced food intake (Bell *et al.*, 2003), weak immune function (de la Vega *et al.*, 2007; Holman *et al.*, 2004; Paschke *et al.*, 2010; Tanner *et al.*, 2006), slow growth (Coiro *et al.*, 2000; Stierhoff *et al.*, 2006) and impaired reproduction (Brown-Peterson *et al.*, 2008; Thomas & Rahman, 2009) under hypoxic conditions.

Hypoxia inducible factor (HIF-1) is a transcription factor that acts as a global regulator of oxygen homeostasis and is part of the adaptive response to hypoxia (Semenza, 1999). It regulates many genes involved in the response to hypoxia, including erythropoietin, vascular endothelial growth factor, glycolytic enzymes, vasodilation, angiogenesis, and glucose transport proteins (Harris, 2002; Semenza, 2001a, b; Trenin *et al.*, 2003). HIF-1 is a heterodimeric DNA-binding complex, consisting of one hypoxia-regulated α-subunit and the oxygen-insensitive HIF-1β subunit (aryl hydrocarbon receptor nuclear translocator, or ARNT), and both of these are members of the basic helix-loop/Per-Arnt-Sim (bHLH/PAS) family of proteins (Wang *et al.*, 1995). HIF-1α and HIF-1β are constitutively expressed. Under normoxia, HIF-1α is selectively degraded and only HIF-1β persists. Under hypoxia, however, HIF-1α accumulates and is translocated to the nucleus to form a functional heterodimer with HIF-1β and then starts the hypoxic responses by regulating the expression of hypoxia-related genes (Maxwell *et al.*, 1999).

The function and response mechanism of HIFs in the regulation of hypoxia has been studied in mammals, birds, amphibians, fishes, and invertebrates (Catron *et al.*, 2001; De Beaucourt & Coumailleau, 2007; Soitamo *et al.*, 2001; Thomas & Rahman, 2009; Soñanez-Organis *et al.*, 2009). Among marine invertebrates, the HIF-1 hypoxia response has been investigated in mollusks and crustaceans (Li & Brouwer, 2007; Kodama *et al.*, 2011; Hardy *et al.*, 2012), such as the mollusks *Crossostrea virginica* (Piontkivska *et al.*, 2011) and *Crossostrea gigas* (Kawabe & Yokoyama, 2011), and the decapod crustaceans *Litopenaeus vannamei* Boone, 1931 and *Callinectes sapidus* (Rathbun, 1896). In these studies, the physiological and behavioral changes and the gene expression and protein synthesis were generally suppressed or upregulated to enhance survival under hypoxic conditions. Little is nevertheless known about the HIF signaling pathway and the transcriptional dynamics of marine organisms when they suffer fluctuating oxygen conditions on a daily, seasonal and spatial basis. It is therefore important to conduct more in-depth HIF gene research, especially in marine animals such as the farmed *S. paramamosain*.

MATERIALS AND METHODS

Materials and experimental gas exposure

Seventy-five healthy intermolt stage mud crabs, *S. paramamosain* (170 ± 10 g), were collected from Hainan Island, China. The crabs were farmed in seawater that was aerated, circulated, and UV-sterilized, with 30 ppt salinity and 25 °C. These individuals stayed at least one week or more in this environment and were fed with frozen clam on alternate days. We transferred the acclimated individuals into 30 l exposure tanks of seawater with a salinity of 30 ppt, under temperature control (25 °C), and UV-sterilized. After acclimating for 24 h, these individuals were exposed to different dissolved gas treatments as follows: a) normoxia 7 ± 0.2 mg/l DO for 3 h, b) 4 ± 0.1 mg/L DO for 3 h, c) 3 ± 0.3 mg/l DO for 3 h, d) 2 ± 0.1 mg/l DO for 3 h, and e) 1 ± 0.1 mg/l DO

for 3 h. In additional experiments, individuals were maintained at 2 ± 0.1 mg/l DO for 72 h to make comparisons with the normoxia control group. The water was bubbled with nitrogen gas and air to balance the specific oxygen contents. The dissolved oxygen, temperature, and salinity were continuously monitored by an oxygen meter during all of the experiments.

Different tissues (gills, heart, hepatopancreas, brain, muscle, and hemocytes) were collected from each individual and were preserved at –80 °C for RNA extraction. The hemolymph was obtained from the arthrodial membrane with a disposable syringe containing an anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA; pH 4.6), and the hemocytes were separated from the hemolymph by centrifugation (8000 rpm) at 4°C for 10 m.

RNA isolation and reverse transcription

Total RNA was extracted from *S. paramamosain* tissues using TRIZOL reagents (Takara, Shiga, Japan) with trichloromethane as the phase separation reagent. The genomic DNA was removed by DNase I. Spectrophotometry and 1% agarose gel electrophoresis were used to detect the quality and integrity of the RNA.

First-strand cDNA was synthesized in a 10-μl reverse transcription reaction by incubating 2.5 μg of total RNA (1.5 μl) with 1.0 μl of random primers, 1.0 μl of a dNTP mixture, and 6.5 μl of RNase-free water at 65 °C for 5 min. Subsequently, by using a Prime Script One Step RT-PCR Kit (Takara, Shiga, Japan), the above reaction liquid was mixed with 4 μl of buffer, 0.5 μl of RNase inhibitor, 4.5 μl of RNase-free water, and 1.0 μl of RTase, and the mixture was incubated at 30 °C for 10 m followed by 42 °C for 30 m and 95 °C for 5 m.

Cloning of the full-length cDNAs of SpHIF-1α *and* SpHIF-1β

The *SpHIF-1α* and *SpHIF-1β* fragments were identified from a cDNA library that was constructed from the thoracic ganglion tissue of *S. paramamosain* (Zhang *et al.*, 2011). The full-length *SpHIF-1α* and *SpHIF-1β* cDNAs were obtained by reverse-transcription polymerase chain reaction (RT-PCR), and the missing 5' and 3' ends of these two cDNAs were obtained by the rapid amplification of cDNA ends (RACE) method using RNA to construct the cDNA library by the SMARTTM RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The products were checked by agarose gel electrophoresis and purified by the agarose gel DNA purification kit version 2.0 (Takara) and were then cloned into the pMD19-T vector (Takara) and sequenced. The 5'- and 3'-RACE primers are provided in Table 1.

Table 1. Oligonucleotide primers used in the research.

Name	Sequence (5'-3')
SP-HIF-1α-3GSP1	GAACCAGTAGAGCCGCCAC
SP-HIF-1α-3GSP2	CAAAATGAGCAGCACGGAGG
SP-HIF-1α-5GSP1	AGCGAGTGTCTGCTCACGAAG
SP-HIF-1α-5GSP2	GCCAAGGAAACTGACGATGTTC
SP-HIF-1β-3GSP1	CGTCGGCGTCGGAACAAGATG
SP-HIF-1β-3GSP2	TGACTGGTTTGGGTCTAGTGTGTATG
SP-HIF-1β-5GSP1	GCCACCAAACAGCAATGAGAAGC
SP-HIF-1β-5GSP2	CATCTTGTTCCGACGCCGACGCTC
SP-HIF-1α-RTF	AGAACATCGTCAGTTTCCTTGG
SP-HIF-1α-RTR	GCTCCACTTCGCTGTGATCTACC
SP-HIF-1β-RTF	ACTCCAGTCCTTAATCAGCCACAG
SP-HIF-1β-RTR	CCGAACCTTGTCCACATCCTC
SP-18S-RT-F	GGGGTTTGCAATTGTCTCCC
SP-18S-RT-R	GGTGTGTACAAAGGGCAGGG

Sequence analysis

Identity searches for the nucleotide and protein sequences were performed using the BLASTN and BLASTP algorithm at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Protein prediction was performed by the open reading frame (ORF) finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequences of various species from the NCBI GenBank were retrieved and analyzed using the Vector NTI Suite 11.0, Clustal W Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). Neighbor-joining phylogenetic trees were constructed using MEGA software version 5.1 with the Kimura-2 parameters, and the confidence levels in the trees generated were obtained via 1000 bootstraps.

Real-time quantitative PCR analysis of SpHIF-1α *and* SpHIF-1β

Total RNA was reverse transcribed for the first-strand cDNA with the Rever Tra Ace qPCR RT Kit (Toyobo, Shiga, Japan), and the product was diluted 10 times and stored at –20 °C. The *SpHIF-1α* and *SpHIF-1β* transcripts were analyzed using the StepOne Plus real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) and were calculated by the standard curve method. *18S* rRNA was chosen as an internal control due to its relatively stable expression level, and a pair of primers was designed according to the *18S* rRNA sequence downloaded from the NCBI GenBank (GenBank accession no. FJ646616.1). A relative standard curve was prepared using 5-fold serial dilutions of the cDNA. The reactions were conducted in 10 μl as follows: 5 μl of SYBR Premix Taq TM, 0.4 μl of PCR Forward Primer (10 mM), 0.4 μl of PCR Reverse Primer (10 mM), 0.2 μl of ROX Reference DyeI, 1.0 μl of cDNA temple, and 3.0 μl of diethylpyrocarbonate water (DEPC-water). The 10:1 dilutions of the cDNA temple were subjected to reactions. The reaction mixture was predenatured for 10 min at 95 °C and was then amplified over 40 cycles of denaturation at 95 °C for 15 s and an annealing and extension at 60 °C for 1 min, followed by an extension at 72 °C for 10 m. The primers for qRT-PCR are shown in Table 1. The significance of the results of qRT-PCR were calculated using ANOVA followed by Tukey's multiple comparison test.

RESULTS

Characterization and homology analysis of SpHIF-1α *and* SpHIF-1β

The *SpHIF-1α* cDNA sequence (GenBank accession no. KU644140) was 4059 bp with the 5' and 3' untranslated regions (UTR), which were 261 bp and 612 bp, respectively, and the 3'-UTR with a poly-A tail (Fig. 1). The open reading frame (ORF) of 3180 bp coded for a protein consisting of 1,059 amino acids and a predicted molecular weight of 115.78 kDa with an isoelectric point of 6.04. The overall deduced amino acid sequence had high identity to *HIF-1α* of *Callinectes sapidus* Rathbun, 1896 (94%), *Metacarcinus magister* (Dana, 1852) (86%), *Eriocheir sinensis* H. Milne Edwards, 1853 (78%), *Litopenaeus vannamei* (Boone, 1931) (50%), *Tribolium castaneum* (Herbst, 1797) (47%), and humans (32%) (Table 2). The conserved bHLH domain in *SpHIF-1α* was from 33–80, and the regions from 117–167 and 242–339 formed the two PAS domains (Fig. 1). A comparison of the *SpHIF-1α* bHLH and PASA/B domain with the HIF-1α of other animals in these regions revealed similar identity (55%-95% and 49%-99%) (Table 2). In *SpHIF-1α*, there were two conserved prolines (Pro) that were located in the N-terminal oxygen-dependent degradation domain (N-ODDD, 481 aa) and the C-terminal oxygen-dependent degradation domain (C-ODDD, 671 aa). The C-terminal transactivation domain (C-TAD) had a conserved
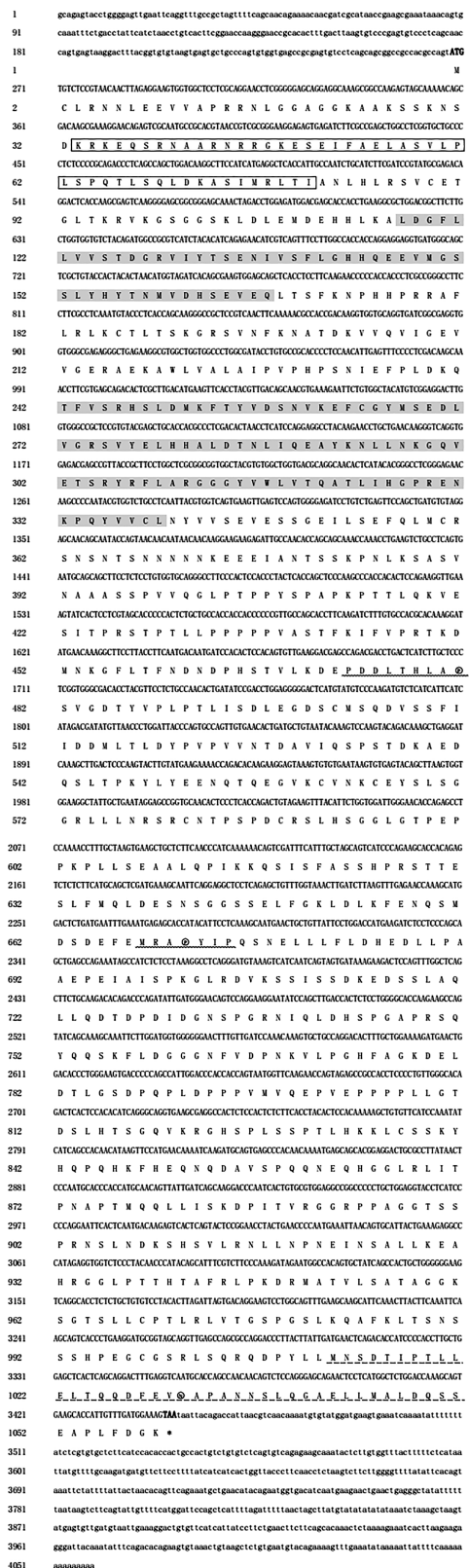

Figure 1. Nucleotide and predicted amino acid sequences of *SpHIF-1α*. The start (ATG) and stop (TAA) codons are in bold. The bHLH domain is boxed (33–80 aa), the PAS domains (117–167 aa and 242–339 aa) are labeled with a gray background, the curve is at the bottom of N-ODDD and C-ODDD, and the dotted line indicates the C-TAD domain. Two conserved proline residues are indicated by the circles (located in N-ODDD, 481 aa and C-ODDD, 671 aa) and a conserved aspartic residue (located in C-TAD, 1032 aa).

Table 2. Comparison of the predicted amino acid sequences of *HIF-1α* from *Scylla paramamosain* and other species.

Species	GenBank numbers	Identity (overall)	Identity (bHLH)	Identity (PAS-A/B)	Identity (C-TAD)
Callinectes sapidus	AEZ04012.1	94%	95%	99%/97%	99%
Metacarcinus magister	ABF83561.1	86%	92%	93%/92%	92%
Eriocheir sinensis	AHH85804.1	78%	69%	92%/88%	89%
Litopenaeus vannamei	ACU30154.1	50%	56%	70%/79%	54%
Apis mellifera	XP_392382.4	48%	60%	52%/55%	-
Tribolium castaneum	EFA04586.1	47%	61%	49%/60%	54%
Homo sapiens	NP_001521.1	26%	59%	36%/48%	48%

asparagine (Asp) that was located at position 1032 (Fig. 1). A comparison of the *SpHIF-1α* C-TAD domain (1012–1051 aa) with the HIF-1α of other animals in this region revealed 54%-99% identity.

SpHIF-1β (GenBank accession no. KU644141) was 2244 bp with a 5' untranslated region of 150 bp and a 3' untranslated region of 123 bp, excluding the poly-A tail (Fig. 2). The predicted protein of *SpHIF-1β* contains 656 amino acid residues and had a molecular weight of 71.95 kDa with an isoelectric point of 6.52. The *SpHIF-1β* deduced amino acid sequence had high overall identity to HIF-1β of *Litopenaeus vannamei* (87%), *Callosobruchus maculatus* (Fabricius, 1775) (63%), *Cimex lectularius* (Linnaeus, 1758) (64%), *Limulus polyphemus* (Linnaeus, 1758) (63%), and humans (44%) (Table 3). *SpHIF-1β* also had one bHLH (53–105) and two PAS domains (134–227 and 320–417) (Fig. 2) with 92%-100% and 60%-99% identity to other animals' bHLH and PAS regions of HIF-1β (Table 3). Both SpHIF-1α/β proteins had conserved residues in the bHLH and PAS domains, and the main differences in HIF-1α from the other animals were in their C-terminals (Tables 2 and 3).

Phylogenetic analysis of SpHIF-1α and SpHIF-1β

The deduced amino acid sequences from HIF-1α and HIF-1β were used in a neighbor-joining analysis with the Kimura-2 parameters. HIF-1 (α and β) was grouped into mammals, birds, fishes, crustaceans, and insects, which were all divided into invertebrate and vertebrate (Figs. 3, 4). In the invertebrate clade, the crustaceans (*Callinectes sapidus, Metacarcinus magister, Eriocheir sinensis, Litopenaeus vannamei, Oratosquilla oratoria* (De Haan, 1844), and *Portunus trituberculatus* (Miers, 1876)) and insects (*Tribolium castaneum, Solenopsis invicta* (Buren, 1972), and *Harpegnathos saltator* (Jerdon, 1851)) were divided from the vertebrates with 100% and 99% bootstrap support, respectively. The crustacean HIF-1α formed a group that was different from that of the insect HIF-1α. In the vertebrate clade, the mammals and birds clustered into a group, whereas the fishes clustered into another group (Fig. 3A). For HIF-1β, the invertebrate clade had 100% bootstrap support and was divided into two subclades as follows: 1) *S. paramamosain* HIF-1β was grouped with the crustaceans (*Portunus trituberculatus* and *Litopenaeus vannamei*) with 100% bootstrap support, and 2) the insects (*Apis florae* Fabricius, 1787, *Megachile rotundata* Fabricius, 1787, *Callosobruchus maculates* (Fabricius, 1775), *and Tribolium castaneum*) had 98% bootstrap support (Fig. 3B). Similar to the relationships of HIF-1α, the crustacean HIF-1β formed a cluster that was different from that of insect HIF-1β.

Gene expression of SpHIF-1α and SpHIF-1β

The expression of *SpHIF-1α* and *SpHIF-1β* was quantified in different tissues from healthy *S. paramamosain*. The mRNA transcripts of *SpHIF-1α* and *SpHIF-1β* from all of the examined tissues
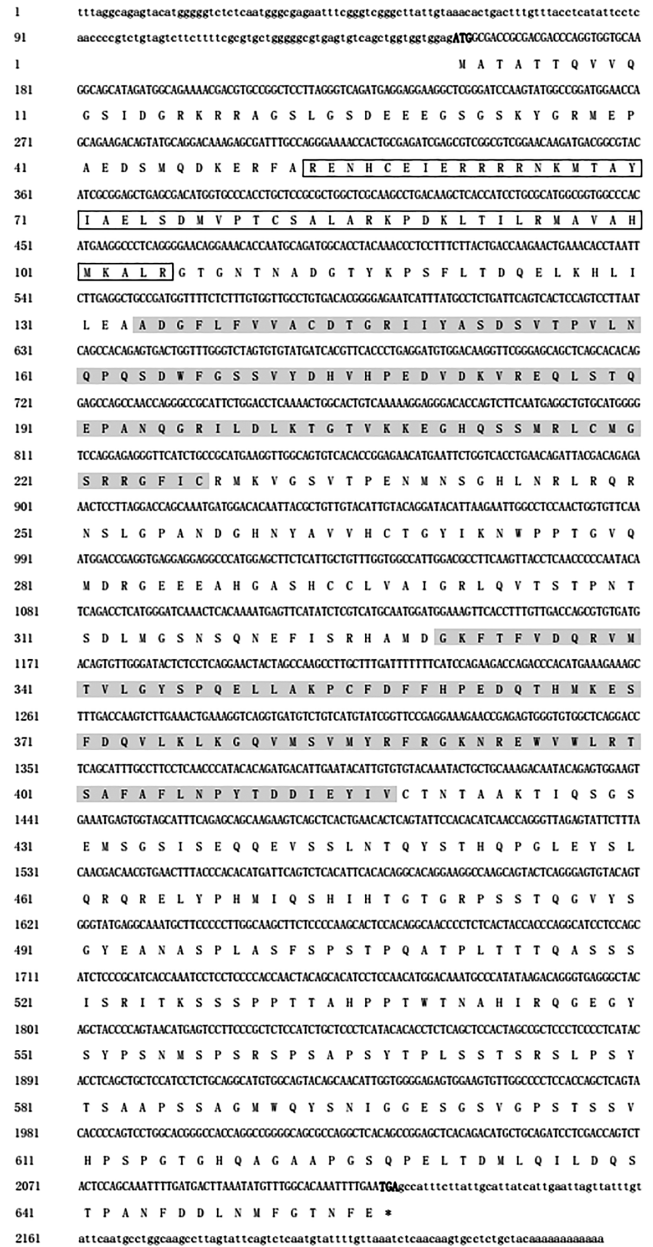

Figure 2. Nucleotide and predicted amino acid sequences of *SpHIF-1β*. The start (ATG) and stop (TGA) codons are in bold. The bHLH domain is boxed (53–105 aa), and the PAS domains (134–227 aa and 320–417 aa) are labeled with a gray background.

Table 3. Comparison of the predicted amino acid sequences of *HIF-1β* from *Scylla paramamosain* and other species.

Species	GenBank numbers	Identity (overall)	Identity (bHLH)	Identity (PAS-A/B)
Litopenaeus vannamei	ACU30155.1	87%	100%	94%99/%
Callosobruchus maculatus	AFL70632.1	63%	98%	86%/82%
Cimex lectularius	XP_014239879.1	64%	98%	87%/85%
Limulus polyphemus	XP_013776124.1	63%	98%	80%/89%
Homo sapiens	AAQ96598.1	44%	92%	70%/60%

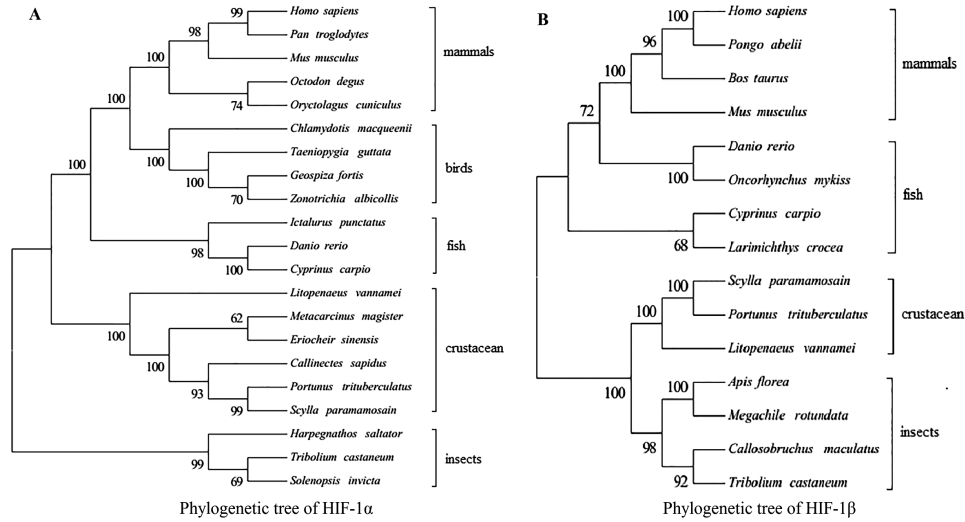Phylogenetic tree of HIF-1α

Phylogenetic tree of HIF-1β

Figure 3. Phylogenetic tree of HIF-1α (**A**) and HIF-1β (**B**). Deduced amino acid sequence constructed by the Neighbor-Joining method. The GenBank accession numbers of the protein sequences used for HIF-1α are as follows: *Oryctolagus cuniculus* (NP_001076251.1), *Pan troglodytes* (XP_001168948.1), *Octodon degus* (XP_004624861.1), *Mus musculus* (NP_034561.2), *Homo sapiens* (NP_001521.1), *Taeniopygia guttata* (XP_002200394.2), *Chlamydotis macqueenii* (XP_010114554.1), *Zonotrichia albicollis* (XP_014125772.1), *Geospiza fortis* (XP_005418082.1), *Ictalurus punctatus* (NP_001187230.1), *Danio rerio* (AAQ91619.1), *Cyprinus carpio* (ABV59209.1), *Callinectes sapidus* (AEZ04012.1), *Metacarcinus magister* (ABF83561.1), *Eriocheir sinensis* (AHH85804.1), *Litopenaeus vannamei* (ACU30154.1), *Tribolium castaneum* (XP_967427.2), *Solenopsis invicta* (XP_011171237.1), and *Harpegnathos saltator* (EFN75729.1). The GenBank accession numbers of the protein sequences used for HIF-1β are as follows: *Homo sapiens* (AAQ96598.1), *Pongo abelii* (NP_001125275.1), *Mus musculus* (AAH12870.1), *Pongo abelii* (NP_001125275.1), *Cyprinus carpio* (AGU12797.1), *Danio rerio* (XP_009290298.1), *Larimichthys crocea* (XP_010737471.1), *Oncorhynchus mykiss* (NP_001118182.1), *Litopenaeus vannamei* (ACU30155.1), *Tribolium castaneum* (XP_008190739.1), *Callosobruchus maculates* (AFL70632.1), *Apis florea* (XP_003698987.1), and *Megachile rotundata* (XP_012141424.1).
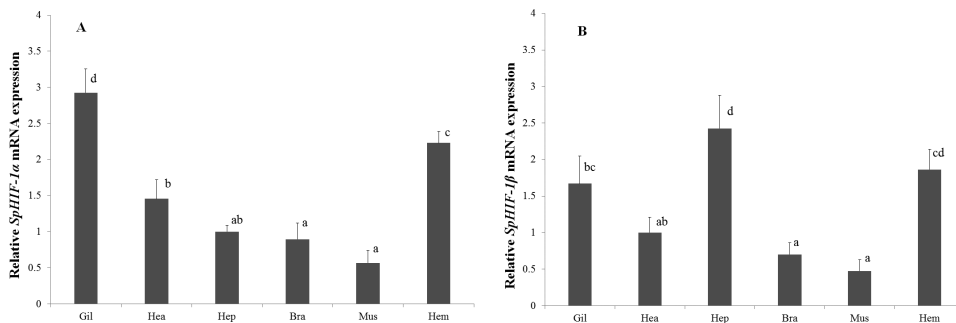

Figure 4. Relative expression profiles of *SpHIF-1α* (**A**) and *SpHIF-1β* (**B**) in the different tissues by quantitative real-time PCR. Gil, gills; Hea, heart; Hep, hepatopancreas; Bra, brain; Mus, muscle; Hem, hemocytes. A. The ratio of expression levels of *SpHIF-1α/18S* rRNA in the Hep was initiated as "1.0," and therefore the relative ratios of expression levels of *SpHIF-1α/18S* rRNA in the other tissues were determined by comparing with those in the Hep. B. The ratio of expression levels of *SpHIF-1β/18S* rRNA in the heart was initiated as "1.0," and therefore the relative ratios of expression levels of *SpHIF-1β/18S* rRNA in the other tissues were determined by comparing with those in the heart. Bars show mean (±) SD (*N* = 4), the significances (*P* < 0.05) are marked above columns with letters.

including the gills, heart, hepatopancreas, brain, muscle, and hemocytes were detected. Different expression levels were found in the examined tissues in normoxia. The qRT-PCR results revealed that the *SpHIF-1α* mRNA transcript levels were significantly higher in the gills than in the hemocytes, hepatopancreas, and muscle (Fig. 4A). For *SpHIF-1β*, the transcript levels were significantly higher in the hepatopancreas than in the hemocytes and gills (Fig. 4B).

The expression of *SpHIF-1α* in almost every tissue had an obvious increasing trend with a decreased oxygen concentration (7 mg/l, 4 mg/l, 3 mg/l, 2 mg/l and 1 mg/l) except in the brain and reached the highest in the 2 mg/l oxygen concentration, while at 1 mg/l, the expression decreased (Fig. 5A). In the hypoxic condition (2 mg/l), the expression of *SpHIF-1α* mRNA in the gills, hepatopancreas, and hemocytes was significantly increased at 3 h and 9 h (Fig. 6A). For *SpHIF-1β*, the expression level increased slightly but not significantly at the different oxygen concentrations (Fig. 5B) and remained relatively constant in the 2 mg/l hypoxic condition (Fig. 6B).

DISCUSSION

In vertebrates such as mammals and fishes, HIF-1α is considered to be the main regulator of cellular oxygen homeostasis (Wenger, 2002; Liu *et al.*, 2016), playing a positive role in inducing the target genes at the transcriptional level and during post-transcriptional modification (Pisani & Dechesne, 2005). Vertebrates have evolved three different homologous genes, including HIF-1α, HIF-2α, and HIF-3α, which have been identified in mammals (Heidbreder *et al.*, 2003), while HIF-4α, an individual ortholog to mammalian HIF-3α, was isolated from fishes (Law *et al.*, 2006; Rytkönen *et al.*, 2011). Invertebrates (including crustaceans) nevertheless seem to have evolved only one HIF-α gene (Soñanez-Organis *et al.*, 2009; Piontkivska *et al.*, 2011). Molecular phylogeny analysis indicated that the invertebrates (crustaceans and insects) clustered firmly with the HIFs from other vertebrates (Fig. 3). Limited research has been carried out on the HIF regulation mechanism of cellular oxygen homeostasis during low oxygen conditions in crustaceans (Li & Brouwer, 2007; Soñanez-Organis *et al.*, 2009). We hoped to elucidate *S. paramamosain* HIF-1α's role in hypoxia regulation by detecting changes in mRNA levels as well as to contribute to the study of crustacean HIF.

This was the first report of the cloning and characterization of *SpHIF-1α* and *SpHIF-1β* from *S. paramamosain*, and of the analysis of their transcript expressions. The ORF of *SpHIF-1α* was 3180 bp and coded for 1059 amino acid residues, which was relatively conserved in invertebrates and vertebrates (Fig. 1 and Table 2). Three typical functional domains were detected (bHLH and PASA/B) both in *SpHIF-1α* and *SpHIF-1β*. Furthermore, there was a conserved functional domain (C-TAD) and two oxygen-dependent degradation domains (ODDD) in *SpHIF-1α*. There was a conserved Asn residue in the C-TAD (EVNAP) and conserved Pro residues in the N-ODDD (LTHLAP) and C-ODDD (MRAPYIP), respectively. There were nevertheless some differences in the conserved Pro between *SpHIF-1α* and other HIF-1α. For human and grass shrimp (*Palaemonetes pugio* Holthuis, 1949), proline residues are located in the LXXLAP region in the ODDD and N-TAD (Li & Brouwer, 2007), while *SpHIF-1α* lacked an N-TAD. *SpHIF-1β* is a structural protein that combines with *SpHIF-1α* and then forms a functional heterodimeric complex. *SpHIF-1β* also has the bHLH (53–105 aa) and PASA/B (PASA, 134–227 aa and PASB, 320–417 aa) domains with high identity to other vertebrates and invertebrates.

In the qRT-PCR test, *SpHIF-1α* and *SpHIF-1β* transcripts were detected in all of the experimental tissues, and there were significant differences in the abundance of different tissues. Under normoxia, *SpHIF-1α* transcript levels were higher in the gills and hemocytes than in other tissues; *SpHIF-1β* was significantly higher in the hepatopancreas than in hemocytes and gills. This phenomenon might reveal the existence of a synergistic effect between various tissues in the oxygen balance mechanism, such as the gills, hepatopancreas, and hemocytes.

Under different oxygen concentrations, the transcripts increased in all of the tissues and were particularly significant in the gills, and the transcripts reached the highest levels in the 2 mg/l oxygen concentration; simultaneously, the motility and
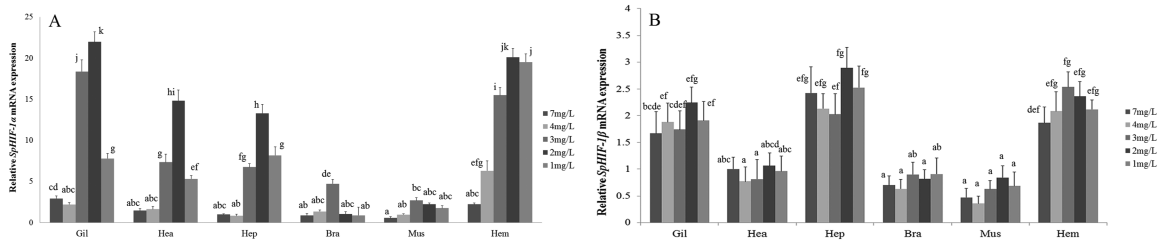
Figure. 5 The influence of the different hypoxic conditions on *SpHIF-1α* (**A**) and *SpHIF-1β* (**B**) expressions in the gills (Gil), heart (Hea), hepatopancreas (Hep), brain (Bra), muscle (Mus), and hemocytes (Hem). The ratio of expression levels of *SpHIF-1α/18S* rRNA in the hepatopancreas at an oxygen concentration of 7 mg/l was initiated as "1.0," and therefore the relative ratios of expression levels of *SpHIF-1α/18S* rRNA in the other tissues and under other hypoxic conditions were determined by comparing with that. B. The ratio of expression levels of *SpHIF-1β/18S* rRNA in the heart at an oxygen concentration of 7 mg/l was initiated as "1.0," and therefore the relative ratios of expression levels of *SpHIF-1β/18S* rRNA in the other tissues and under other hypoxic conditions were determined by comparing with that. Bars show mean (±) SD (*N* = 4), the significances (*P* < 0.05) are marked above columns with letters.
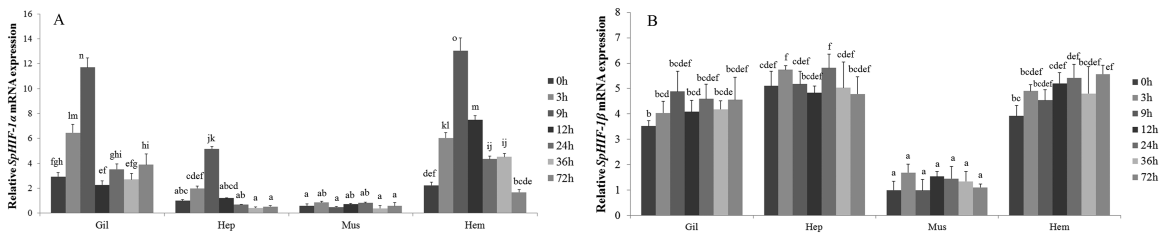

Figure. 6 *SpHIF-1α* (**A**) and *SpHIF-1β* (**B**) expressions in the gills (Gil), hepatopancreas (Hep), muscle (Mus), and hemocytes (Hem) under the hypoxic condition of 2 mg/l. A. The ratio of expression levels of *SpHIF-1α/18S* rRNA in the hepatopancreas at 0 h was initiated as "1.0," and therefore the relative ratios of expression levels of *SpHIF-1α/18S* rRNA at the other periods and in the other tissues were determined by comparing with that. B. The ratio of expression levels of *SpHIF-1β/18S* rRNA in the muscles at 0 h was initiated as "1.0," and therefore the relative ratios of expression levels of *SpHIF-1β/18S* rRNA at the other periods and in the other tissues were determined by comparing with that. Bars show mean (±) SD (*N* = 4), the significances (*p*<0.05) are marked above columns with letters.

appetite of individuals diminished under this hypoxic environment. This result indicated that *S. paramamosain* could only tolerate 2 mg/l hypoxia conditions. Furthermore, the *SpHIF-1α* expression decreased in the 1 mg/l condition, and this finding revealed that a long-term oxygen concentration of less than 2 mg/l could be life-threatening. We nevertheless found that the expression of the *SpHIF-1α* gene in the hemocytes still maintained a high level in 1 mg/l. This suggested that the hemocytes could be the last line of defense to maintain the oxygen balance and determine the minimum concentration of dissolved oxygen an individual can tolerate.

When in the 2 mg/l hypoxia condition, the expression of *SpHIF-1α* mRNA in the gills, hepatopancreas, and hemocytes was significantly increased at 3 h and 9 h (Fig. 6A). The expression level nevertheless gradually decreased after 9 h and was accompanied by an increased mortality of individuals. This meant that when *S. paramamosain* was in the 2 mg/l condition or lower oxygen environment, the living processes of individuals gradually weakened, and if these conditions were sustained more than 9 h, it could have resulted in death. In aquaculture, when faced with hypoxic conditions, it is therefore preferable to increase the amount of dissolved oxygen within nine hours; otherwise, it will affect the survival rate. For the expression of *SpHIF-1β,* there were no obvious changes in the different oxygen concentrations (Figs. 5B, 6B).

Our results revealed that the gills could play a major role in the hypoxic HIF response in crustaceans and other invertebrates because the tissue often had the highest levels of *SpHIF-1α* under both normoxic and hypoxic conditions (Piontkivska *et al.,* 2011). The gills are involved in respiration, osmoregulation, acid–base balance, detoxification, and immune function, whereas the hepatopancreas is a multifunctional organ that participates in carbohydrate metabolism, excretion, molting, lipid synthesis, secretion of digestive enzymes, absorption of nutrients, synthesis of hemocyanin and lipoproteins, and storage of energy reserves (Gibson & Barker, 1979; Yepiz-Plascencia *et al.,* 2000). Because of these functions of metabolic regulation, the transcript levels of HIF fluctuated in normoxia and hypoxia conditions. The experimental results are consistent with other studies in invertebrates showing that HIF-α transcripts levels are increased in hypoxia (Morin *et al.,* 2005; Gorr *et al.,* 2006). There were nevertheless different results in *Litopenaeus vannamei,* which showed that HIF had a significant decrease in HIF-α transcripts in response to low oxygen in the gills (Soñanez-Organis *et al.,* 2009). This finding suggested that HIF hypoxia regulation does exist, but that there were different mechanisms regulating it in different species.

As a first step to understand the role of *SpHIF-1α* and *SpHIF-1β* in mediating the hypoxia response in *S. paramamosain*, we isolated and characterized the full-length cDNAs. The predicted proteins of *SpHIF-1α and SpHIF-1β* had the bHLH and PASA/B domains. Furthermore, *SpHIF-1α* also had two prolines in the ODDD and one asparagine hydroxylation motif in the C-TAD domain. The special amino acids of these *SpHIF-1α* molecules were important for regulation in response to hypoxia. It was demonstrated that *SpHIF-1α* is expressed in multiple tissues and that its expression is tissue-temporal-specific in normoxia and hypoxia conditions. Meanwhile, the expression of *SpHIF-1β* did not obviously change in the different oxygen concentrations. These results suggest the HIF functions and its molecular mechanism in hypoxia stress. Our study has nevertheless not provided enough details of these mechanisms, as HIF-1α transactivational activity is exerted at the translational and post-translational level and not at the transcriptional level so that to determine the regulatory roles of HIF-1α and HIF-1β would require functional studies. Besides, given that coastal ecosystems are become increasingly hypoxic, further research of the functional characterization of the *SpHIF-1α* and *SpHIF-1β* genes and proteins is required.

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