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## Transcriptome analysis reveals the molecular basis of the response to acute hypoxic stress in blood clam *Scapharca broughtonii*

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### Abstract

Hypoxia tolerance and adaptive regulation are important for aquatic animals, especially for species with poor mobility, such as most bivalves. Previous studies have confirmed that the blood clam *Scapharca broughtonii* has strong hypoxia resistance. However, the molecular mechanism supporting its hypoxic tolerance is still largely limited. To further screen the genes and their potential regulation of hypoxia tolerance, the transcriptome changes of *S. broughtonii* after acute hypoxic stress were explored by RNA sequencing. In this study, the average value of Q30 is 92.89%, indicating that the quality of sequencing is relatively high. The Unigenes obtained were annotated using four databases, namely Interpo, KEGG, Swisspro and TrEMBL. The annotation rates in these four databases were 71.82%, 75.95%, 92.98%, and 79.26%, respectively. And also, there were 649 DEGs in group B (dissolved oxygen (DO) of 2.5 mg/L) compared with group D (DO of 7.5 mg/L), among which 252 were up-regulated, and 397 were down-regulated. There were 965 DEGs in group A (DO of 0.5 mg/L), 2.5 mg/L, and 7.5 mg/L, compared with group B, among which 530 were up-regulated, and 435 were down-regulated. Meanwhile, there were 2,040 DEGs in group A compared with group D, among which 901 were up-regulated, and 1,139 were down-regulated. The main metabolic-related pathways of KEGG enriched in this study included Insulin secretion, Insulin signaling pathway, MAPK signal transduction pathway, and PPAR signaling pathway. These pathways may be critical metabolic pathways to solve energy demand and rebuild energy balance in *S. broughtonii* under hypoxic conditions. This study preliminarily clarified the response of *S. broughtonii* to hypoxia stress on the molecular levels, providing a reference for the following study on the response laws of related genes and pathways under environmental stress of *S. broughtonii*.

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## Introduction

Aquatic organisms will encounter various threats in the complex and changeable aquaculture water environment, and hypoxia is considered one of the main threats (Saha N. et al., 2022). In aquaculture, the hypoxia problem has caused more attention to the continuous deterioration of the water environment. It is worth noting that excessive nutrient enrichment and human activities may lead to the eutrophication of water bodies. Anoxic layers often appear near the seabed in the estuary and coastal areas in summer, resulting in the generation of anoxia or the formation of an anoxic bottom environment, and causing a large number of deaths of benthic animals (Zhang Y. et al., 2017; Nie H. et al., 2020; Sun X. et al., 2021). Different degrees of hypoxia have a significant impact on aquatic animals. It is reported that several marine mollusks show corresponding responses in behavior, physiology, and biochemistry when subjected to hypoxic stress (Nakano T. et al., 2017; Khan F. et al., 2020). Organisms in the ocean's intertidal zone often encounter hypoxia when exposed to low tide. Most benthos can adapt well to the low-oxygen aquatic environment, while some shellfish living in the intertidal zone must survive. Indeed, shellfish in those habitats are not passive to environmental changes. Their physiology and metabolism, including energy metabolism, respiratory metabolism, and immune response, will change dynamically to adapt to environmental changes. When shellfish are subjected to hypoxic stress, they need to undergo many physiological and molecular reactions to cope with hypoxia stress. One of the responses of bivalves to extremely low levels of hypoxia is to regulate their internal environment by closing their shells. Their respiratory activity usually slows down to regulate oxygen absorption, reducing oxygen consumption and energy consumption (Sun X. et al., 2021). Through the research on the half-lethal hypoxia tolerance time of fish (Rebeca T. et al., 2022), crustaceans (Zheng C. et al., 2021), echinoderms and other benthos (Lucey N. et al., 2020), it is found that mollusks can tolerate hypoxia stress for a long time than most marine animals, which may be related not only to the physiological protection of their shells but also to a set of genetic and physiological hypoxia adaptation mechanisms.

More and more studies have described the adaptive response of marine mollusks to hypoxia, indicating that aquatic animals can gradually change their hypoxia adaptive phenotype, showing in different aspects such as the expression level of RNA and protein, variation in enzyme activity, and regulation of cell volume and ion content (Yu B. et al., 2018; Chen L. et al., 2022). Living organisms have formed a series of regulatory mechanisms to cope with the anoxic environment. For example, the improvement of hypoxia-inducible factor level in organisms will lead to the increase of hypoxia performance of organisms and downstream influence on various physiological processes (Parker J., 2021). Mitogen-activated protein Kinase (MAPK) signaling pathway plays an important role in cell response to environmental stress (Hu Z. et al., 2022). During hypoxia and relative hyperoxia transfer (such as reoxygenation), MAPK expression will also mutate, which indicates that these kinases play an essential role in cell adaptation to hypoxia. PI3K-AKT signal pathway is reported to be an important intracellular signal pathway related to a cell stress response. The hypoxic response of aquatic organisms is mainly regulated by the PI3K-AKT-HIF-1 signal axis (Lai X. et al., 2022). In addition, it showed that hypoxia could induce the express change of many genes such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in *Litopenaeus vannamei* (Nuñez D. et al., 2020).

In the past decade, aquatic animal genome resources have increased dramatically with the development of high-throughput sequencing technology. RNA-seq is an effective technology to screen new genes, analyze differentially expressed genes (DEGs) and understand specific molecular mechanisms (Liu A. et al., 2019; Gong J. et al., 2020; Ren Z et al., 2020). In recent years, transcriptomics has been used in many marine mollusks to study hypoxia stress response. DEGs related to hypoxia stress, and hypoxia transcription has been found in *Ruditapes philippinarum* (Yan X. et al., 2019), *Mytilus edulis* (Sokolov E. et al., 2021),

*Haliotis discus hannai* (Kim C. et al., 2021) and *Patinopecten yessoensis* (Mao J. et al., 2022). Yan et al. (2019) analyzed the main adaptation of *R. philippinarum* to hypoxia, acidification, and parasite stress through transcriptome sequencing analysis of different tissues and development stages to study its adaptation to the environment. Some studies have shown that the mitochondria of mussels have an internal mechanism, including regulation through reversible protein phosphorylation to ensure high respiratory flux and reduce oxidative damage in hypoxia/reoxygenation(H/R) stress. Some studies found that the DEGs in scallops increased significantly with the temperature increase and the dissolved oxygen decrease, indicating that the gene regulation in scallops was enhanced in response to severe environmental changes. (Mao J. et al., 2022). It is proved that comparative transcriptomics analysis can better reveal the molecular mechanism of bivalve adaptation to the environment. Still, there are few reports on adapting blood clam *S. broughtonii* to hypoxia.

The *S. broughtonii* is a marine bivalve with important economic and scientific research value. In recent years, the increasing demand for the *S. broughtonii* has driven the rapid development of seedling production and aquaculture models such as pond and shallow sea cage culture. The aquaculture area and output are also expanding, making it one important specie cultured in northern China. Though the tolerance of bivalves to hypoxia or anoxic conditions has been studied before (Sun X. et al., 2021), the studies on the molecular mechanism of hypoxia tolerance and adaptation remain limited. This study aimed to screen the key candidate genes and related pathways in *S. broughtonii* related to hypoxic stress, explore the role of DEGs in hypoxia tolerance and analyze the possible molecular mechanism of acute hypoxic stress.

## Materials and methods

### *Experimental animals, hypoxia stress, and samples preparation*

The experimental blood clams collected from the Qingdao Sea area of Shandong province were acclimatized and cultured in aerated seawater with a temperature of  $17 \pm 1^\circ\text{C}$  and salinity of 30 for one week. During the acclimation stage, those calms were fed with unicellular algae twice, and the water was renewed by 50% daily. And then, the healthy adult clams (2 years old) with an average shell length of 42.87 mm were selected randomly for subsequent experiments.

The selected clam individuals were divided into groups A, B, and D, respectively, treated with different dissolved oxygen (DO) of 0.5 mg/L, 2.5 mg/L, and 7.5 mg/L. In contrast, group D was set as the control. The DO was regulated by aerating nitrogen into the water and monitored by a real-time DO analyzer. After treatment for 16 h, the hemolymph of three individuals from each experimental group was collected and rapidly centrifuged at 800 g at  $4^\circ\text{C}$  for 10 min. After removing the supernatant, the sediments were stored in liquid nitrogen for RNA extraction.

### *RNA sequencing*

A total of nine samples were collected from three groups for transcriptome sequencing. The total RNAs were extracted using Trizol reagent extraction method following the manufacturer's instructions, and the quantity, purity, and integrity were assessed by spectrophotometry ( $A_{260}/A_{280}$ ) and 1.5% agarose gel electrophoresis. The eligible RNAs were used to construct sequencing libraries. The magnetic beads with Oligo (dT) enriched the eukaryotic mRNA. Then those enriched RNAs were cut into short fragments using fragmentation buffer for reversing into the first cDNA strand using random hexamers, and then the second cDNA strand was synthesized. Afterward, the cDNA was successfully purified using the AMPure XP beads, followed by end-repaired, added poly (A), and ligated to sequencing adapters. Then AMPure XP beads were selected for size selection, and finally, the cDNA sequencing libraries were obtained by PCR amplification. After completing library

construction, Qubit 2.0 and Agilent 2100 were used to detect library concentration and insert size. Then quantitative real-time PCR (qRT-PCR) was used to measure library concentrations for library quantification accurately. After being qualified, sequencing was performed using the Illumina HiSeq 2000 platform following the manufacturer's recommendations.

#### *Sequencing data filtering and functional annotation*

Sequencing data contains some low-quality Reads with connectors, which will cause great interference to subsequent information analysis. Therefore, further filtering of sequencing data is required. There were mainly two criteria for data filtering. The first one was that the 3' - Cutadapt removed the end joint, and the removed part had at least 10 bp overlap (AGATCGGAAG) with the known joint, allowing a 20% base mismatch. And then, the reads with an average mass fraction lower than Q20 were removed.

The function of Unigenes was annotated by four databases, including NR (NCBI non-redundant protein sequences), GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genome), eggNOG (Evolutionary genealogy of genes: Non-supervised Orthologous Groups), and Swiss-Prot. Through comparison and annotation of the NR library, we can obtain the similarity between the gene sequence of this species and that of its relatives, as well as the functional information of the gene of this species. The GO annotation of Unigenes is completed by BLAST2GO software with the default parameters. Carry out eggNOG comparison annotation on Unigene, and assign the eggNOG number of the best comparison result to the corresponding Unigene. The Pathway annotations of Unigene is mainly completed by KEGG's KAAS online automatic annotation system <http://www.genome.jp/tools/kaas/>.

#### *Expression analysis*

The HTSEQ statistics were used to compare each gene's Read Count value as the gene's original expression amount. To make the gene expression levels of different genes and samples comparable, we used FPKM to normalize the expression level. The genes with FPKM > 0.5 were considered to express in the transcriptome with reference.

The DESeq (Version 1.18.0) was used to screen the DEGs and analyze their expression according to the fold change and P-value of the expression change. The selection standard is  $|\log_2(\text{FoldChange})| > 1$  and  $P\text{-value} < 0.05$ .

The GO functional significance enrichment analysis included mapping all DEGs to each term in the Gene Ontology database, calculating the number of genes in each term, and finding the terms significantly enriched. According to the DEGs obtained through analysis and screening, the DEGs included in different levels of KEGG pathways can be counted, which can intuitively determine the main metabolic pathways and signal transduction pathways involved.

#### *qRT-PCR analyses*

Seven genes, including insulin-like growth factors (*IGF*), heme oxygenase-1 (*HO-1*), Hemoglobin IIB (*HB IIB*), Transferrin (*Tf*), the transferrin receptor (*Tfr*), toll-like receptor 4 (*TLR4*) and *GAPDH* were selected to validate the transcriptome quality using the qRT-PCR. The cDNA obtained from the above experiment was diluted 15 times as the reaction template, specific primers were designed based on the sequences of selected genes, and  $\beta$ -*actin* gene was used as the internal reference gene. The reaction system included 10  $\mu$ l TB Green Premium Ex TaqII, 0.8  $\mu$ l forward primer, 0.8  $\mu$ l reverse primer, 0.4  $\mu$ l ROX ReferenceDye II (50x), 2  $\mu$ l template, and 6  $\mu$ l ddH<sub>2</sub>O. The reaction procedure was: 95 °C for 30s; 95 °C for 5s, 60 °C for 34s, 40 cycles; 95°C for 15s, 60°C for 1min, 95°C for 15s. The applied biosystems 7500 Fast Real-Time PCR system was used for amplification. The data results were calculated by the  $2^{-\Delta\Delta CT}$  method. SPSS 17.0 software was used for one-way ANOVA analyses. The difference was significant when  $p < 0.05$  and extremely significant when  $p < 0.01$ . GraphPad

Prism 6.0 software was used for mapping analysis. The primer information is shown in **Table 1**. And three replicates were made for each sample in this study.

**Table 1** The primers used in this experiment

<i>Genes</i>	<i>Primer name</i>	<i>Sequence (5'-3')</i>
<i>β-actin</i>	β-actin-F	GGTTACACTTTCACCACCACAG
	β-actin-R	ACCGGAAGTTTCCATACCTAAGA
<i>IGF</i>	IGF-F	TTAAGACAAGTCCATCAGCCAAG
	IGF-R	CATCCAGAGCATCAACGAAGT
<i>HO-1</i>	HO-1-F	AGGACAACCAAGCAATGACAA
	HO-1-R	GAATACCTGTTTCGCACACTGA
<i>HB IIb</i>	HB II B-F	AAGCAGACGAACAACAAAATC
	HB II B-R	TTTCCAATACCTTACACCCAA
<i>Tf</i>	Tf-1-F	TTCACAGTTCATCCGCAA
	Tf-1-R	CCACGCTTCTTAGTTGTTTCT
<i>Tfr</i>	Tfr-F	AGCTCTGAAGGAAGCCACC
	Tfr-R	AGCCTCAGGACATGGGATCT
<i>GAPDH</i>	GAPDH-F	ATTTCTCTGACCAGCTGGC
	GAPDH-R	GAGGAGGCAGTTGCAGGATT
<i>TLR4</i>	TLR4-R	TGAGCTGCTGGAGACACAAG
	TLR4-R	GGGTTGCAGGTATTGAGGCT

## Results

### Overview of sequencing data and comparative analysis

Nine cDNA libraries (A1, A2, A3, B1, B2, B3, D1, D2, D3) were constructed by using Illumina HiSeq. The average value of Q30 is 92.89% (**Table 2**). After removing the adapter sequence, fuzzy nucleotides, and low-quality sequences, sequencing obtained 373,296,520 reads, including 124,531,130 in the group D and 129,475,810 in group D group B, and 119,289,580 in group A (**Supplementary Table S1 – see Data Sets under Save article as...**). The transcriptome data has been uploaded to the NCBI database (NO. PRJNA910700) (<http://www.ncbi.nlm.nih.gov/bioproject/910700>).

**Table 2** Data filtering table

<i>Sample</i>	<i>Reads number</i>	<i>Bases(bp)</i>	<i>Q30(bp)</i>	<i>N(%)</i>	<i>Q20(%)</i>	<i>Q30(%)</i>	<i>GC(%)</i>
A1	40921430	6138214500	5659983082	0.0047222	96.25	92.2	38.26
A2	41480612	6222091800	5747371821	0.004682	96.34	92.37	38.52
A3	46103702	6915555300	6336614652	0.004678	95.97	91.62	39.04
B1	46379712	6956956800	6388305771	0.004248	96.06	91.82	39.5
B2	46673116	7000967400	6524288084	0.001605	97.08	93.19	40.55
B3	47404514	7110677100	6655932557	0.00159	97.23	93.6	40.14
D1	45285988	6792898200	6385612825	0.001248	97.39	94	38.73
D2	44972636	6745895400	6300072987	0.001629	97.12	93.39	39.32
D3	45194292	6779143800	6361781144	0.001462	97.35	93.84	39.34

After obtaining clean reads, *HISAT* was used to compare clean reads to the reference genome sequence. The uniform comparison rate indicated that the data among samples were comparable. The statistics of comparison results are shown in **Supplementary Table S1**.

### *Annotation of genes*

A total of 185,366 Unigenes were obtained by transcriptome sequencing of nine hemolymph RNA samples under hypoxic stress, of which 143,284 were annotated successfully, including 46,123 (24.88%) were annotated in the Nr database, 38,720 (20.89%) in the eggNOG database, 36,036 (19.44%) in the Swissprot database, and 4,151 (2.24%) were intersection in all databases (**Supplementary Table S2**). The results showed that the 46,123 genes annotated in Nr were compared with that of 935 species. Of these, 18,172 genes had the highest homology with *Crassostrea gigas*, accounting for 39.4%, which further proved the credibility of the database annotation.

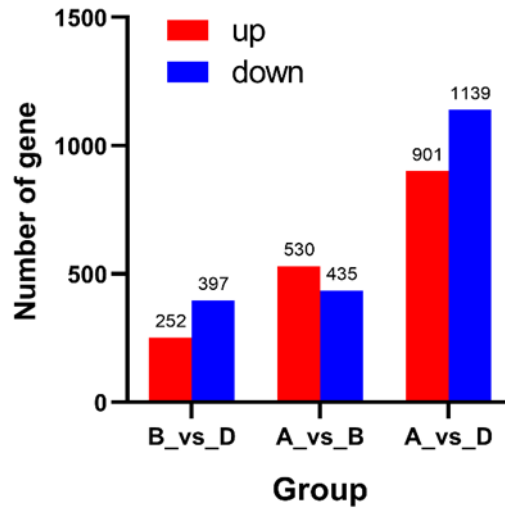
By mapping the GO annotation results to GO Term, the number of annotations on the second-level classification is shown in **Supplementary Figure S1**. It showed that in the top six biological categories, 10,312 genes were related to reproduction, 9,526 were related to cell killing, 8,544 were related to an immune system process, 8,487 were related to behavior, 8,469 related to metabolic process and 8,453 were related to the cellular process.

The statistical result of the KEGG pathway is shown in **Supplementary Figure S2**. It indicated that 6,488 genes were divided into five major biological and chemical pathways, including 1,880 genes related to metabolism, 1,514 genes related to tissue system, 115 genes related to genetic information processing, 1,031 genes related to cell process, and 888 genes related to environmental information processing. Many of these major categories of KEGG were carbohydrate metabolism, translation signal transduction, transport and catabolism, and the endocrine system. These results indicated that the above biological processes might play a certain role in regulating hypoxia adaptation.

As shown in **Supplementary Figure S3**, 38,720 genes are divided into 26 homologous groups in the eggNOG database. General function prediction is the main group, followed by Function unknown and Signal transduction mechanisms.

### *Identification of DEGs*

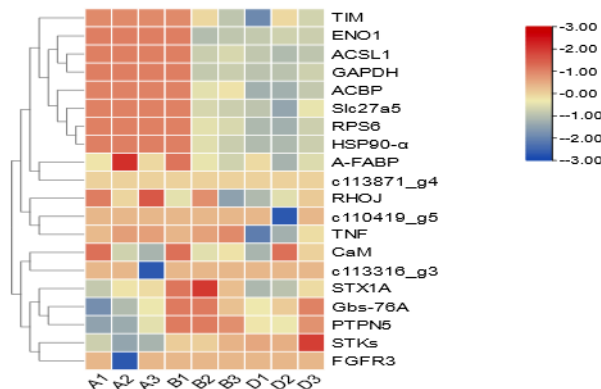
According to the gene expression level of each sample, the DEGs among the three groups were detected. As shown in **Figure 1**, there were 649 DEGs in group B compared with group D, among which 252 were up-regulated, and 397 were down-regulated. There were 965 DEGs in group A compared with group B, among which 530 were up-regulated, and 435 were down-regulated. Meanwhile, there were 2,040 DEGs in group A compared with group D, among which 901 were up-regulated, and 1,139 were down-regulated. These results fully indicated that hypoxia stress caused the expression changes of many genes.



**Figure 1** Statistic of DEGs.

According to annotation, those DEGs were mainly involved in maintaining energy metabolism, oxidative stress, and immune response. Under hypoxic stress, HSPs were mainly up-regulated to adapt to the environment. Among them, genes related to hypoxia emergency, especially *STKs* genes in MAPKs signal pathway, were significantly up-regulated. Genes in the energy metabolism pathway and immune pathway were induced. From the data of *TIM*, *ENO1*, *ACSL1*, *GAPDH*, and other genes in the figure, the gene expression increases after hypoxia, indicating that hypoxia induces gene expression in the energy metabolism pathway and immune pathway.

Hypoxic stress significantly induced a change in gene expression in *S. broughtonii*. We analyzed the DEGs involved in the anti-hypoxia defense system and found many genes related to immunity, metabolism, and hypoxia. As shown in **Figure 2**, we found that compared with the control group, some genes were increased with the increase of hypoxia concentration, such as *TIM*, *ENO1*, *ACSL1*, *GAPDH*, *ACBP*, *Slc27a5*, *RPS6*, and *HSP90-α*. Some genes decreased with the increase of hypoxic concentration: *STKs*, *PTPN5*, *GMS-76A*, and *STX1A*. It is worth noting that some genes did not change with low oxygen concentrations. For example, *FGFR3*, *c113871-g5*, *TNF*, *c113871-g4*, and *c113871-g3* were not significantly changed.



**Figure 2** Partial differential gene heat map under hypoxia stress.

### *GO enrichment analyses of DEGs*

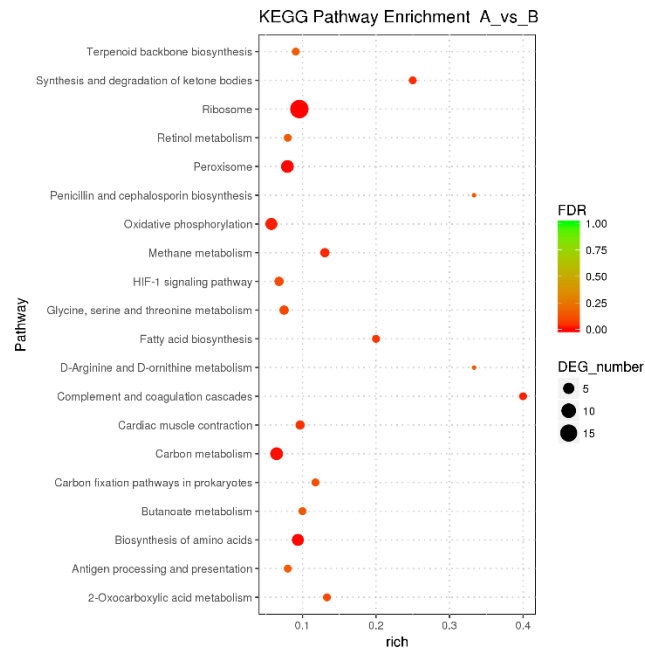
Time-course GO enrichment analyses were performed to evaluate patterns of variation in mRNA expression. As shown in **Supplementary Tables S3, S4, and S5**, three main GO terms were selected from the enriched gene list in the results of the hypoxia group compared with the other two groups. In addition, after hypoxia stress, immune response, regulation of response to stimulus, and oxidoreductase activity were screened in group A vs. B, and oxidoreductase activity, regulation of stress response, and metabolic process were screened in group A vs. D. These results suggest that the physiological process may play an important role in the regulation of hypoxia adaptation.

### *KEGG pathway enrichment analyses of DEGs*

The KEGG enrichment analyses were carried out to explore the signaling pathways associated with DEGs induced by hypoxic stress. 2,040 DEGs from group A vs. D were enriched to 153 pathways (**Supplementary Table S6**). These signal pathways included that in the other two groups, which indicated that gene expression changes increased with the intensity of hypoxia stress. KEGG pairwise comparison of the three groups of DEGs was shown in **Figure 3, Figure 4, and Figure 5**. It mainly included RNA transport, Carbon metabolism, Endocytosis, Complement, and coagulation cascades, HIF-1 signaling pathway, Butanoate metabolism, Fatty acid biosynthesis, Synthesis and degradation of ketone bodies, PI3K-Akt signaling pathway, Lysosome, RAS signaling pathway, Regulation of actin cytoskeleton and oocytes meiosis. The main metabolic pathways of KEGG include insulin secretion, insulin signaling pathway, and PPAR signaling pathway, which are closely related to carbohydrate catabolism and anabolism in the body. In addition, this study not only significantly enriched the energy-related pathways, such as carbon metabolism and fatty acid biosynthesis pathway but also enriched some pathways closely related to hypoxia metabolism, including the HIF-1, PI3K-Akt signaling pathway, and MAPK signaling pathway.

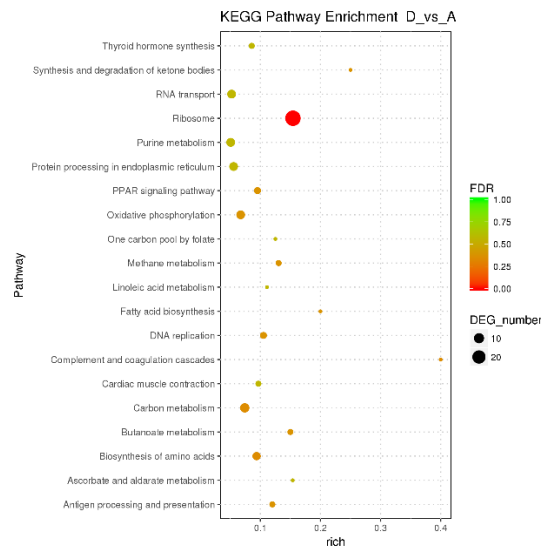
These results suggested that the above biological processes have been affected or destroyed under hypoxic stress. These signal transmission paths might play essential roles in the hypoxic adaptation regulation of *S. broughtonii*. And some of these signal transduction pathways might play a role in regulating hypoxia adaptation, such as the HIF signaling pathway and PI3K-Akt signaling pathway.





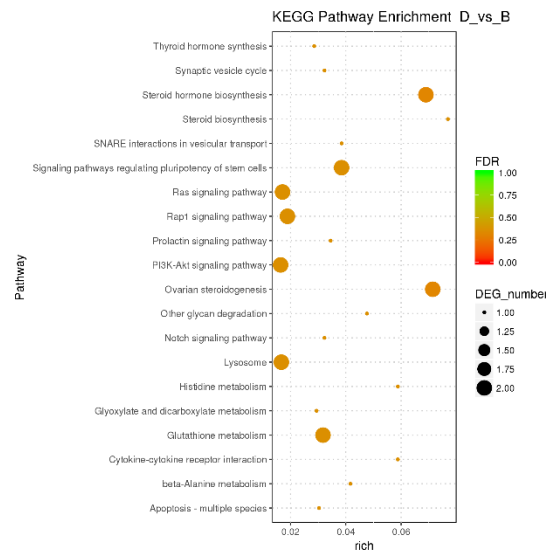
**Figure 3** KEGG enrichment analysis of A\_VS\_B.

Note: Red indicates a high correlation, and green indicates a low correlation. A large bubble indicates a high number of differential genes, and a small bubble indicates a low number of differential genes.



**Figure 4** KEGG enrichment analysis of D\_VS\_A.

Note: Red indicates a high correlation, and green indicates a low correlation. A large bubble indicates a high number of differential genes, and a small bubble indicates a low number of differential genes.

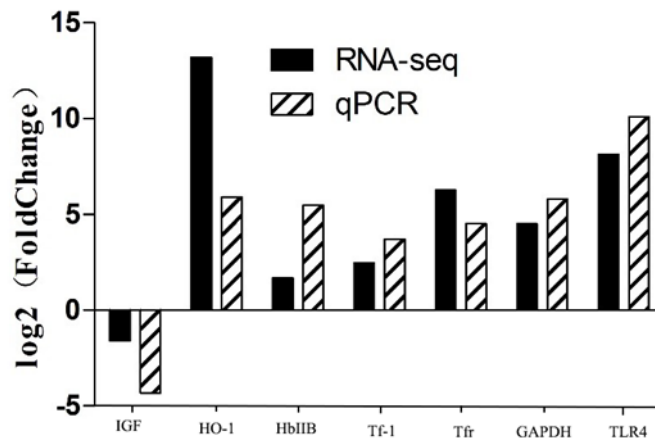


**Figure 5** KEGG enrichment analysis of D\_VS\_B.

Note: Red indicates a high correlation, and green indicates a low correlation. A large bubble indicates a high number of differential genes, and a small bubble indicates a low number of differential genes.

#### qRT-PCR verification

The results of qRT-PCR verification of seven DEGs are shown in **Figure 6**. These DEGs were involved in cell proliferation, hypoxia adaptation, glycolysis, and other biological processes, which could suggest that hypoxia stress might affect many physiological processes in the body. Although the gene expression obtained by qRT-PCR results and RNA sequencing showed a slight difference, the up-down trend was the same, which verified the credibility of transcriptome sequencing analysis.



**Figure 6** Results of fluorescent quantitative verification of DEGs.

Note: The horizontal coordinate indicates the gene, and the vertical coordinate indicates the relative expression of the gene. Those above the horizontal coordinate indicate up-regulation, and those below indicate down-regulation.

## Discussion

When Q30 is greater than 85%, the sequencing quality is qualified. This study's average value of Q30 was 92.89%, suggesting the high sequencing quality. In addition, the data obtained from nine transcripts were annotated. Among those, 46,123 annotated genes in the Nr database were matched to 935 species. And also, 18,172 genes have the highest homology with *C. gigas*, accounting for 39.4% of the total. Most of the top species in the comparison were marine mollusks, which further proves the credibility of the database annotation. After screening and analyzing the spliced genes, 2,040 DEGs from group A vs. D were enriched to 153 pathways, and major metabolic pathway and signal transduction pathway genes were obtained. These genes related to energy metabolism, hypoxia, and other immune pathways are screened by qRT-PCR, and the expression trend of genes was consistent with the transcriptome analysis results.

Organisms can usually cope with hypoxia by changing two metabolic modes: reducing the basal metabolic rate and changing from aerobic to anaerobic metabolism (Hardy K. et al., 2012). The main metabolic-related KEGG pathways enriched in this study included Insulin secretion, Insulin signaling pathway, and PPAR signaling pathway, which were closely related to carbohydrate catabolism and anabolism in the body. These pathways may be important metabolic pathways to solve energy demand and rebuild energy balance under hypoxic conditions. Some studies have shown that some fish can use glycolysis or gluconeogenesis to obtain more energy under hypoxic conditions (Li M. et al., 2018). These results are consistent with the results of the above-enriched pathways. In addition, the carbon metabolism pathway was significantly enriched in this study, which is also closely related to sugar metabolism. It has also been reported that under hypoxic stress, lipid metabolism in higher animals will increase lipid synthesis, thus leading to a massive accumulation of fatty acids (Long Y. et al., 2015). This study's fatty acid biosynthesis pathway was significantly enriched, indicating that hypoxia might reduce lipid metabolism and result in the synthesis of fatty acids. Therefore, it is speculated that the clam may also have a similar regulatory effect on hypoxia. In addition, the cell cycle signaling pathway significantly enriched by DEGs in this study can also inhibit cell apoptosis and promote angiogenesis by participating in cell proliferation to cope with the changes of a hypoxic environment (Li M. et al., 2017).

This study enriched the HIF-1 signaling pathway, which is very conservative through screening the DEGs caused by hypoxia treatment, which may activate the expression of homologous genes in the above-mentioned pathways, thus regulating the expression of homologous genes in the above-mentioned pathways oxygen transport and erythropoiesis (Kopp R. et al., 2014). Previous research shows that the HIF-1 can regulate more than 150 target genes (Alam H. et al., 2008). The discovery of the HIF-1 signaling pathway in this study also reflects that *S. broughtonii* might respond to environmental hypoxia by activating the HIF-1 signaling pathway to trigger a series of physiological and biochemical reactions, which can pay more attention in future research. It has been reported that the insulin signaling pathway can mediate the PI3K-AKT signaling pathway and insulin secretion signal pathway (Zhu C. et al., 2013). And the PI3K-AKT signal pathway is reported to increase the synthesis and transcription of the hypoxia-inducible factor gene (*HIF-1a*) to amplify the hypoxia signal through the HIF-1 signaling pathway (Yuan Z. et al., 2019). In this study, both the insulin secretion signal and PI3K-AKT signal pathway were enriched and might be activated by hypoxic stress. In addition, the MAPK family is an enzyme involved in oxygen sensing. It has been proved that it can regulate the HIF-1 signal pathway by changing the expression of MAPK under hypoxia stress in zebrafish (Zhou Y. et al., 2020). In this study, 37 DEGs were enriched in this pathway, indicating that the MAPK signal transduction pathway has been significantly activated in this experiment, which also preliminarily indicates that the MAPK signal transduction pathway is involved in the hypoxic adaptation regulation of *S. broughtonii*.

In this study, a total of nine samples of *S. broughtonii* under different concentrations of hypoxia stress were sequenced, and genes related to hypoxia adaptation were excavated to explore those related pathways involved in the regulation of hypoxia tolerance. Although this study cannot fully explain the molecular mechanism of shellfish hypoxia adaptation, it provides a reference for the following study on the response laws of related genes and pathways under the environmental stress of *S. broughtonii*.

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