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Transcriptomic analysis of juvenile cobia in response to hypoxic stress

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Abstract

Cobia is an important cultured marine fish species in southern China. It is characterised by fast growth. Due to the effects of climate change on water oxygen levels and seawater temperatures, understanding the influence of environmental challenges on cobia culture has become very important. In this study, to explore the stress and adaptability of cobia (*Rachycentron canadum*) during hypoxia-reoxygenation conditions, the fish were exposed to an oxygen-deficient environment with dissolved oxygen (DO) level of 2.64 ± 0.25 mg/L. Liver tissue transcriptome sequencing was detected in the fish at acute hypoxia stress, after which fish were returned to normal DO levels (6.34 mg/L) for 8, 24, and 48 h. Comparative analysis of liver transcriptomes revealed that there were 1689, 651, 236, and 1150 differential genes in the hypoxia stress group (SC), reoxygenation-8h group (R8), reoxygenation-24h group (R24), and reoxygenation-48h group (R48), respectively. The differentially expressed genes were compared with the GO database. The main aggregated genes were related to gene ontology functional elements such as ribosome structural components, matrix-dependent cell migration, hormone activity, and oxidoreductase activity. The differentially expressed genes were compared with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and a total of 43,054 differentially expressed genes were found to be enriched in 212 cases. For the first time, gene expression patterns in the liver of a juvenile's cobia were examined in response to hypoxia. The results of this study contribute to further clarifying hypoxia's effects on the liver of marine fish.

Keywords *Rachycentron canadum* · Hypoxia · Reoxygenation · Liver · Transcriptome analysis

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Introduction

The life activities of fish are affected by various environmental factors, and water's dissolved oxygen (DO) content is one of the critical factors. Decreased dissolved oxygen levels in water can have multiple effects on fish. Different fish have different adaptation strategies to environmental hypoxia, associated with different physiological and biochemical changes (Sun et al. 2015a, b; Zhang et al. 2017a, b). Studies have shown that hypoxia can significantly affect the interaction of some genes and signalling pathways (such as the HIF-1 signal pathway) in fish (Zhang et al. 2017a, b), which will affect the physiological, biochemical, and behavioural activities of fish and ultimately affect the ecological characteristics of the entire population. Fish are subjected to both chronic and acute stress responses due to hypoxia, which leads to changes in molecular, physiological, morphological, and behavioural markers (Mu et al. 2020; Abdel-Tawwab et al. 2019). In the fish culture, hypoxia has caused substantial economic losses because it impairs organ function, reduces fertility, and causes changes in skin colour, cell death, and even death. However, these effects can vary between individuals and depend on the amount of time that it occurs (Gong et al. 2020). It is a complex process that involves O_2 transport, metabolism, and cellular processes for fish to adapt to hypoxia (Gong et al. 2020). The liver is the central place for the metabolism of all fish energy substances, and it is also the primary organ for fish to excrete their waste (Heath 1988). When the environment is hypoxic, the liver plays a critical role in reducing or protecting the body from hypoxic stress and damage (Huang et al. 2015). In addition to storing energy and responding to immune stimuli, the liver also contributes to hepatocyte function under normal and disease conditions through hepatic tissue oxygenation (Gong et al. 2020; Sun et al. 2015a, b; Rankin et al. 2009).

It is possible to understand how climate change impacts fish by considering the correlation between fish's environment and its physiology (Crear et al. 2020). Hypoxia and hypoxia-reoxygenation involve complex gene expression regulatory networks (Mandic et al. 2009). Therefore, starting from the whole transcriptional level seems more applicable than studying the hypoxia adaptation mechanism through a gene. The transcriptome is the collection of all mRNAs transcribed from a specific tissue or cell in a particular developmental stage or functional state. Transcriptome research can study gene function and structure at the overall level, revealing specific biological processes and molecular mechanisms in the occurrence of diseases (Zhang et al. 2017a, b). A technique known as RNA sequencing (RNA-seq) can be used to determine quantitative expression levels in transcriptomes with high accuracy (Xu et al. 2015). Animal studies have widely used RNA-seq (Li et al. 2019; Zhang et al. 2017a, b). In recent years, researchers have used transcriptome sequencing technology to study the hypoxia adaptation mechanism of fish. Hypoxia tolerance has been studied using liver tissue transcriptome profiling in fish (Gong et al. 2020; Wang et al. 2015a, b, c; Li et al., 2015a; Olsvik et al. 2013; Liao et al. 2013b). Even though fish transcriptome profiling has often been used for hypoxia tolerance studies, little was known about the regulation of genes related to hypoxia in cobia.

Cobia (*Rachycentron canadum*) is a migratory carnivorous fish in tropical and subtropical waters. It belongs to the genus Rachycentron (Amenyogbe et al. 2021, 2022; Huang et al. 2021; Wang et al., 2021) and is widely cultivated in southern China (Zhou et al. 2006; Chi et al. 2011; Luo 2013; Chi et al. 2014). Hypoxia is an emerging problem in southern Chinese waters, mainly occurring from July to September. In the regions with poor water exchange capability, it lasted for a few days, weeks, or months, reaching maximum records in August (Li et al. 2014a, b). A few studies have examined the effects of hypoxia on cobia

(Cnaani and McLean 2009; Huang et al. 2021; Wang et al., 2021). When hypoxia occurs in the body, the most vulnerable organs will be the brain, liver, and heart (MedlinePlus. Respiratory failure). It may be possible to uncover new mechanisms of hypoxia acclimation and shed light on the evolution of these adaptive mechanisms in vertebrates by examining changes in gene expression in fish exposed to hypoxia. Therefore, this study's main purpose is to analyse gene expression profiles of the juveniles' cobia (*Rachycentron canadum*) livers under hypoxic conditions by means of transcriptomic sequencing analysis based on the transcriptomic data. These results contribute to further our understanding of the mechanisms used by the cobia fish to adapt to hypoxia.

Materials and methods

Experimental materials

Animals

The experimental juvenile fish were bred by the fish seed engineering and breeding team of the Fisheries College of Guangdong Ocean University in the biological research base of Donghai Island, China. Two-hundred healthy individuals were randomly selected and transported to the laboratory with a special fry transport vehicle. The mean body weight of the juveniles was 220.67 ± 20.73 g, and the mean total length was 29.37 ± 3.76 cm. In a self-made indoor circulating aquaculture system, the water exchange capacity was 90 L/h. The water tank size was 70 cm × 50 cm × 60 cm, and 20 juvenile fish were placed in each water tank. During the holding period (1 week), the DO level in the water (>6 mg/L), water temperature (26.3 ± 2.5 °C), the salinity (27.8 ± 0.47), and the total ammonia nitrogen (0.17 ± 0.03 mg/L) were maintained. At the end of the holding period, the fish were fasted for 24 h and randomly divided into two treatment groups, hypoxic stress and normoxic control groups, with 3 replicates per group and 20 fish per replicate. The tanks of the control group were continuously aerated and circulated with water. A dissolved oxygen meter (Hengxin, Taiwan, AZ8403) was used to monitor the changes in dissolved oxygen every 10 min. In the experimental group, dissolved oxygen levels were adjusted by controlling the flow of circulating water, closing the inflation, and covering the membrane above the water tank; a dissolved oxygen meter was used for real-time monitoring. Compound feed (Guangdong Yuequn Marine Biology Research and Development Co., Ltd., Jieyang, China) was provided twice a day, and then faeces were cleaned regularly by siphoning off from the water. The feed was calculated on 10% of their body weight. Feeding was stopped 1 day before experiment completion.

Experimental design

The concentration of DO was maintained via flowing the nitrogen gas into the circulating aquaculture system. The experiments were performed in 6 tanks of the circulating aquaculture system, divided into a test group and a control group, with 3 replicates each. The concentration of DO was set based on the previously published literature (Chen 2016; Wang et al. 2015a, b, c). The control group was continuously aerated and circulated with water. A dissolved oxygen meter (Hengxin, Taiwan, AZ8403) was used to monitor the DO change every 10 min. The DO level in the control group was 6.64 ± 0.35 mg/L. The DO

level was adjusted in the test group by controlling the flow of circulating water, closing the inflation, and covering the membrane above the water tank, and running water were turned off. The aeration was stopped so that DO in the water was naturally reduced to 3 mg/L. The DO was stabilised by adjusting the size of the flowing water and aeration for 3 h, following which the hypoxia stress experiment commenced. Other conditions were consistent with the temporary support period. The dissolved oxygen meter was used for real-time monitoring. The samples were taken when DO was maintained for 3 h with an average of 2.64 ± 0.25-mg/L hypoxia stress. After the sampling, the DO was restored to an average of 6.34 ± 0.15 mg/L within 0.5 h; samples were taken at 8, 24, and 48 h, respectively. The sampling times of the test and the control groups were the same.

Sample collection

All experimental processes were performed per the regulations for administering laboratory animals in Guangdong Province, China. This study was conducted in compliance with the Guangdong Ocean University Research Council's guide for the care and use of laboratory animals. For tissue collection, fish were euthanised using ethyl-3-aminobenzoate methanesulphonate (MS-222; Sigma, USA). Five cobia juveniles were randomly sampled from the control and hypoxic groups. The fish's liver was collected and frozen in liquid nitrogen and quickly kept at −80 °C for further studies. The experimental and control groups' sampling time was the same.

RNA extraction, cDNA library construction, and sequencing

The liver samples were quickly ground into powder in a pre-cooled mortar, and then 1 mL of TRIzol reagent was added to extract total RNA by the conventional TRIzol method. NanoDrop detected the purity and integrity of total RNA, Agilent 2100, and agarose gel electrophoresis apparatus, respectively, and the concentration of total RNA was accurately quantified by Qubit 2.0.

For transcriptome sequencing, the qualified RNA samples were sent to Frasergen Co., Ltd. (Wuhan, China). The approximate steps were took 2 μg of detection-qualified RNA from each sample, enriched mRNA with magnetic beads with Oligo (dT), and then added fragmentation buffer to break mRNA into short fragments. The first strand of cDNA was synthesised using random hexamers using the short fragments of mRNA as a template. Buffer, dNTPs, DNA polymerase I, and RNase H were added to synthesise the second strand of cDNA. Double-stranded cDNA was purified, end-repaired, A-tailed, ligated with sequencing adapters, and size-selected with AMPure XP beads (Beckman Coulter, Brea, CA). Finally, the cDNA library was obtained by PCR enrichment. The constructed library was initially quantified with Qubit 2.0 and then diluted to 1.5 ng/μL; then, the Agilent 2100 was used to check whether the insert size of the library was as expected. Qualified libraries were sequenced by paired-end (PE) on the IlluminaHiSeq™ 2500 sequencing platform, and the sequencing mode was 2 × 150 bp.

Gene function annotation

The Trinity program was used to assemble the cobia larva liver transcriptome data (clean reads), and each parameter was set as the default parameter. The obtained assembled transcripts were compared with the non-redundant protein (NR), Kyoto Encyclopedia of Genes

and Genomes Orthology (KEGG), and Swiss-Prot databases using the Diamond program, and the obtained assembled transcripts were compared with the Clusters of orthologous groups for complete eukaryotic genomes (KOG) database by NCBI blast 2.2.29+, and the transcripts were annotated. Finally, bowtie2 and RSEM programs were used to align the cobia reference transcriptome sequences and compare the alignment results.

Identification and enrichment analysis of differentially expressed genes

In this transcriptome sequencing, FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) was used as an indicator to measure the level of transcripts and gene expression. The formula for calculating the FPKM value was as follows:

$$FPKM = \frac{cDNA\ fragments}{Mapped\ fragments\ (millions) \times Transcript\ length\ (kb)}$$

In the formula, cDNA fragments represented the number of fragments aligned to a transcript and the number of paired-end reads. Mapped fragments (millions) represented the total number of fragments aligned to the transcript in units of 10^6. Transcript length (kb) indicated the transcript length in units of 10^3 bases. For samples with replicates between groups, DEseq was suitable for differential expression analysis between sample groups to obtain a differentially expressed gene set between two biological conditions. During the detection of differentially expressed genes, |log2 fold change| $\geq$ 2 and (FDR) <0.05 were used as screening criteria. Fold change represented the ratio of expression levels between the two groups. The False Discovery Rate (FDR) was obtained by correcting the *p*-value for the significance of the difference. Since differential expression analysis by transcriptome sequencing was an independent statistical hypothesis test on a large number of gene expression values, there was a problem of false positives. Therefore, in the process of differential expression analysis, the recognised Benjamini-Hochberg correction method was used to correct the significant *p*-value obtained by the original hypothesis test. Finally, FDR was used as the critical indicator for differentially expressed gene screening. GOseq method was used for the GO enrichment analysis (Young et al. 2010), based on Wallenius non-central hypergeometric distribution. Compared with ordinary hypergeometric distribution, the characteristic of this distribution is that the probability of extracting individuals from a particular category is different from that of extracting individuals from outside a particular category. This difference in probability is obtained by estimating the preference for gene length. Thus, the probability of the GO term being enriched by different genes can be calculated more accurately.

Validation of differentially expressed genes by real-time PCR

In order to verify the reliability of the results of differentially expressed genes by transcriptome sequencing, 9 differentially expressed genes were randomly selected to design primers for real-time quantitative PCR to detect their expression levels. Differentially expressed genes and β-actin primers are shown in Table 1. RNA was extracted from liver tissue of juvenile cobia and reverse transcribed into cDNA for RT-qPCR verification. Real-time PCR experiments were performed on a StepOnePlus™ instrument using the SYBR SELECT MASTER MIX kit. Using β-actin as the internal reference gene, the total amplification system was 20 μL, including 10 μL RT-qPCR master mix, 0.5 μL forward and reverse primers, 8 μL ddH2O, and 1 μL cDNA template. The

Table 1 Primer sequences of genes used for RT-qPCR

GenBank accession numbers	Genes	Forward primers	Reverse primers
OP627107	*AMPK*	CACATCCGTAGGAAGCACAACAG	GCCACGCAGTCACCACAATC
OP627108	*PDHA*	ACTTCGTCGTTACAGCAACATGG	CGGCAGAGGACAGAGTGAAGAT
OP627109	*RPL23*	GGTAATGTGTTGAACAGGCTT CCA	ACCGGACCAGTAATACCAGAACC
OP627110	*EGLN*	ATGTGCGTTCCTGCTCTATTGTG	ACCGTGGCGTTCCAGTTCTT
OP627111	*pckA*	TTGGCAGCGGTTATGGAGGAA	TCACCCACACACTCGACCTTC
OP627112	*FABP1*	GAGCAAGCCTAAACTTCACCA TCA	GACAGACCCTTCAGTCGGAATTTC
OP627113	*RPLP0*	TCCCGCTTCTGGTACTTTGTGT	TCTCGGTAGCACTGTGTGACTG
OP627114	*CDC20*	AGAACCGAGTCCGACTTCATCC	AGGCGACAGACCGTTCAAGG
OP627115	*ERG24*	CCAGAAGGAGCGGATGTAGAGG	TGCCAGTTGGAAAGGTGTCAGA
EU266539	*β-actin*	AGGGAAATTGTGCGTGAC	AGGCAGCTCGTAGCTCTT

reaction procedure was performed in three steps: pre-denaturation at 94 °C for 30 s and 40 cycles, including denaturation at 94 °C for 5 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. Each sample was tested 3 times, and the results were calculated by the $2^{-\Delta\Delta Ct}$ method. Finally, the results were drawn according to the data of the relative expression levels between the samples and the control group.

Results

Sequencing assembly results

In this experiment, the liver tissues of the control group (SC), hypoxia group (S), reoxygenation-8h group (R8), reoxygenation-24h group (R24), and reoxygenation-48h group (R48) were subjected to transcriptome sequencing analysis. The relevant statistical results are shown in Table 2. Transcriptome sequencing quality analysis showed that the GC content range of each group was 48.5–49.3%, the Q20 ratio of the base quality value of each sample was not less than 97.3%, and the Q30 ratio of the base quality value was not less than 93.4%, indicating that the sequencing quality was good, which can be used for assembly analysis for the next step.

Of the unigenes, 118176 were assembled and spliced by the Trinity program, of which the longest was 26,559 bp, the shortest was 180 bp, the average length was 1921 bp, and N50 was 3803 bp (Table 3). The length distribution of unigenes is shown in Fig. 1. Most unigenes were more than 2000 bp (about 33.9%), followed by less than 500 bp (about 33.7%), 500–1000 bp (about 16.3%), and 1000–2000 bp (about 16.0%). The results of the unigenes assembled in this experiment show that the sequencing quality and other splicing results were more accurate and reliable.

Table 2 Statistic summary of RNA-seq data

Sample	Clean base	Clean reads	GC	Q20%	Q30%
SC-1	6,715,767,600	22,385,892	49.2	97.6	93.4
SC-2	7,224,458,400	24,081,528	48.7	97.5	93.2
SC-3	6,161,010,300	20,536,701	49.2	97.7	93.6
S-1	5,903,160,900	19,677,203	48.9	97.8	93.9
S-2	6,199,123,800	20,663,746	49.0	97.5	93.3
S-3	6,608,339,400	22,027,798	48.8	97.3	92.9
R8-1	6,620,352,300	22,067,841	48.7	97.4	93.1
R8-2	6,315,776,400	21,052,588	48.8	97.5	93.2
R8-3	5,849,431,200	19,498,104	49.3	97.4	93.1
R24-1	6,610,329,000	22,034,430	48.8	97.6	93.5
R24-2	7,379,797,200	24,599,324	48.9	97.5	93.2
R24-3	6,705,639,000	22,352,130	49.3	97.5	93.3
R48-1	8,666,423,700	28,888,079	48.5	97.7	93.8
R48-2	7,016,580,000	23,388,600	48.5	97.4	93.2
R48-3	6,767,087,400	22,556,958	48.7	97.3	93.9
Total	100,743,276,600	335,810,922			
Average			48.9	97.5	93.3

GC: GC content; Q20%, Q30%: sequencing quality control value

Table 3 Length distribution of assembled transcripts and unigenes

Item	Unigene
Total sequence number	118,176
Total assembled bases	227,033,103
Average length	1921 bp
Max length	26,559 bp
Min length	180 bp
N50	3803 bp

Functional annotation of unigenes

In order to obtain complete gene function information, the gene function annotation of NR, KOG, KEGG, GO, and Swiss-Prot databases were performed on the assembled 118,176 unigenes. The results showed that 62,142 unigenes (about 52.58%) were the most annotated in the NR database, and 23,108 (19.55%) unigenes were the least annotated in the GO database (Table 4).

All unigenes in the cobia transcriptome were compared with the GO database, and a total of 23,108 unigenes were annotated into 57 functional items, including three subclasses of cellular component (CC), molecular function (MF), and biological function (biological process (BP)). The functions of the majority of unigenes were categorised into cells, metabolism, single-tissue processes, cells, cellular components, cell membranes, membrane components, binding, and catalytic activity (Fig. 2).

According to the annotation results of KOG, a total of 32,745 unigenes were successfully annotated to 26 categories. Signal transduction mechanisms were the most

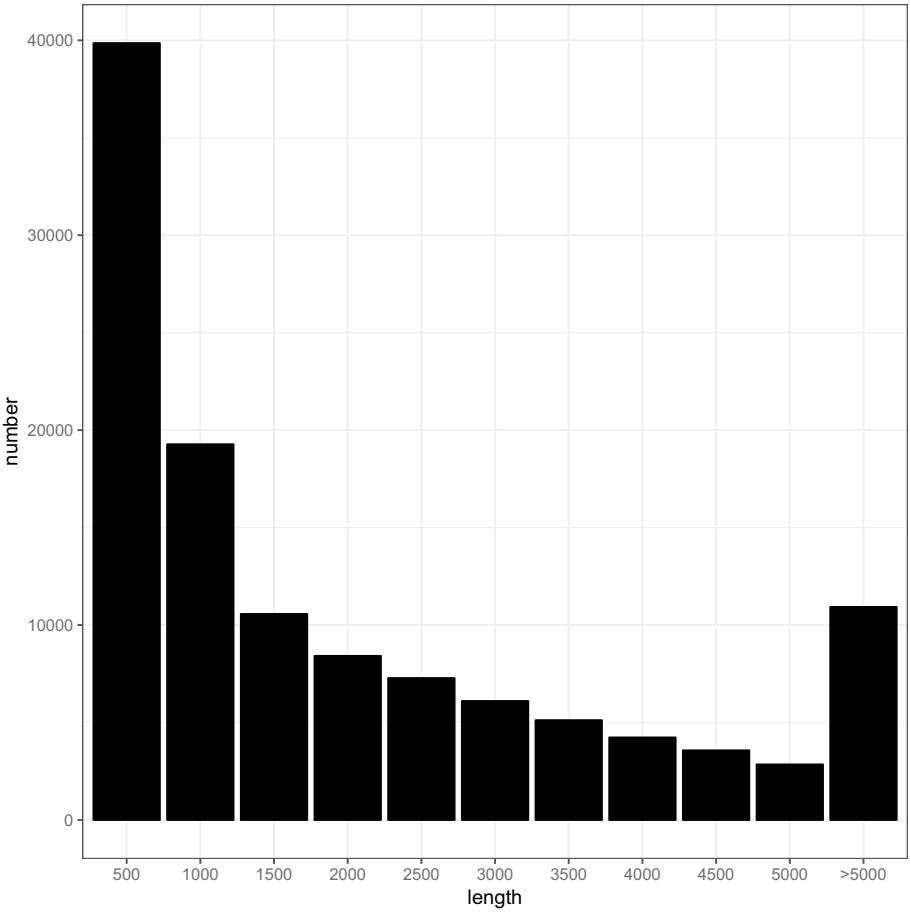

Fig. 1 Length frequency distribution of transcriptome unigenes

Table 4 Successful rates of gene annotation in cobia

Database	Annotated unigenes	Percentage (%)
KOG	32,745	27.71
KEGG	43,054	36.43
NR	62,142	52.58
GO	23,108	19.55
Swiss-Prot	54,670	46.26
Unknown	55,823	47.24
Total	118,176	100

annotated in the 26 categories of KOG, followed by general function prediction only and protein post-translational modification, protein turnover, and protein chaperones (post-translational modification, protein turnover, chaperones). The least unigenes were associated with cell motility and nuclear structure. In addition, a few unigenes were classified as of unknown function (Fig. 3).

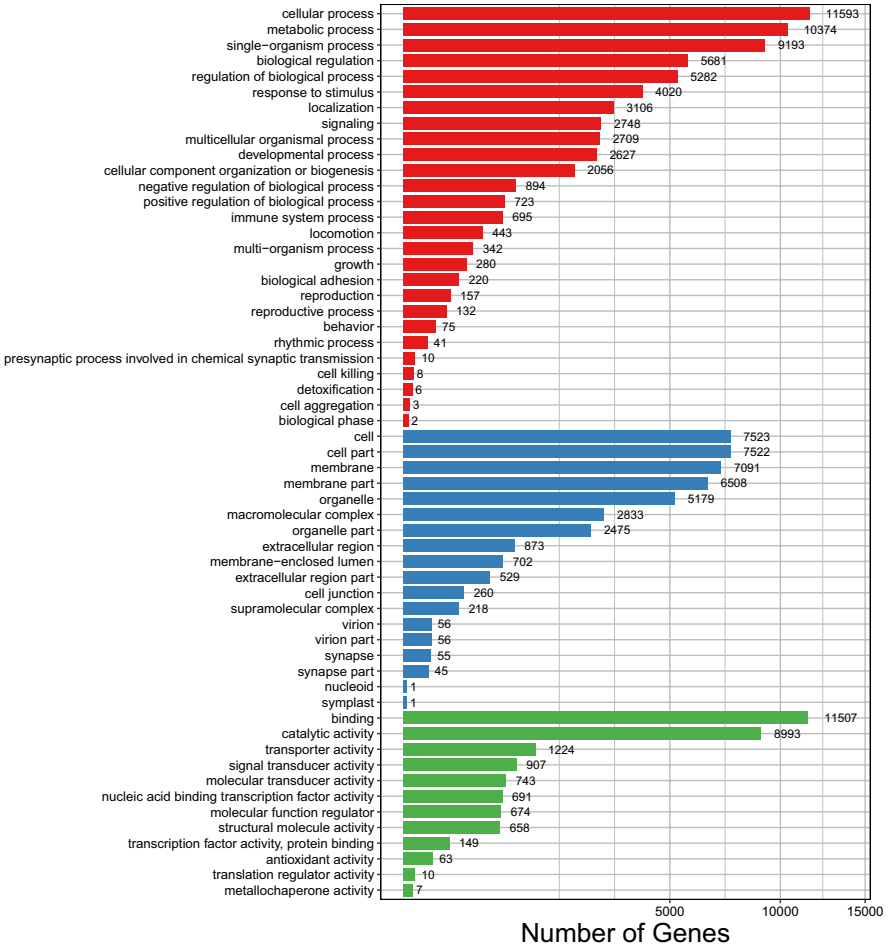

Fig. 2 GO classification

The results of the KEGG database showed that a total of 43,054 unigenes were significantly matched to the KEGG pathway of 5 categories and 34 subcategories. The five major categories were tissue systems (15,619 unigenes), metabolism (10,074 unigenes), cellular processes (9590 unigenes), environmental information processing (9167 unigenes), and genetic information processing (5203 unigenes) (Fig. 4).

Identification and enrichment analysis of differentially expressed genes

Differential gene expression analysis and screening

Using bowtie2 and RSEM programs, the second-generation sequences of each sample after quality control were aligned to the assembled reference transcripts, and the alignment rate of high-quality sequencing data was 85.32 to 88.46% (Table 5).

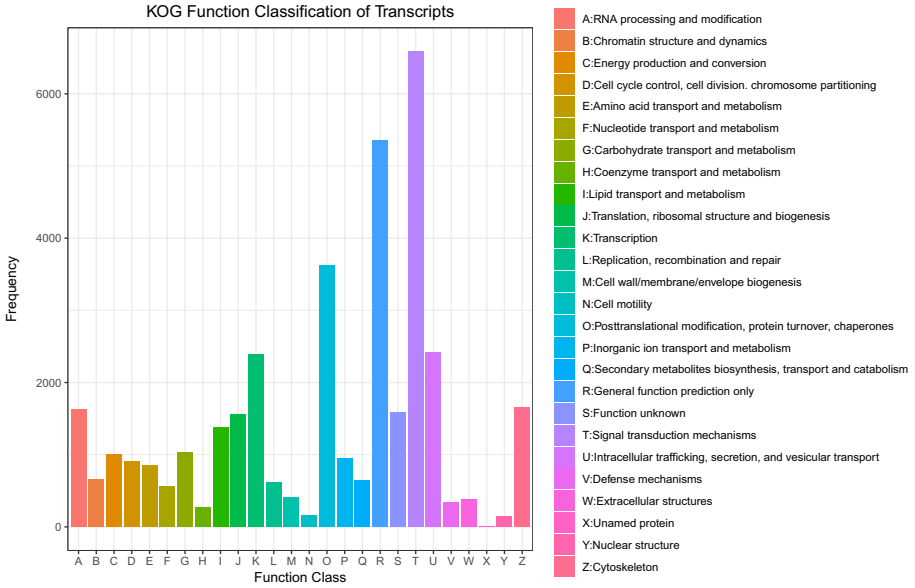

Fig. 3 KOG classification

According to the gene significance results analysed by the R language package edgeR program, the expression of differential genes in the liver tissue of cobia juveniles during hypoxia-reoxygenation was obtained (Fig. 5; Table 6). The results showed that 905 genes were up-regulated in 1689 unigenes, and 784 were down-regulated in the control and hypoxic stress groups (SC-vs-S). Amongst the 651 unigenes, 199 were up-regulated, and 452 were down-regulated in the hypoxic stress group and reoxygenation-8h group (S-vs-R8). Amongst the 236 unigenes, 71 were up-regulated, and 165 were down-regulated in the hypoxic stress group and reoxygenation-24h group (S-vs-R24). Amongst the 1150 unigenes, 429 were up-regulated, and 721 were down-regulated in the hypoxia stress group and reoxygenation-48h group (S-vs-R48). By drawing a Venn diagram, the data of the four databases were compared in pairs, and the common differential genes were counted. It was found that there were 34 common differential genes under different time treatments of hypoxia (H8, H24, and H48) and reoxygenation, accounting for 1.2% of the total (Fig. 6). In order to visualise the overall distribution of all genes in the liver of cobia juveniles during hypoxia-reoxygenation, the MA plot and volcano plot of sample differential genes were drawn according to the set threshold (Fig. 7).

Differential gene GO enrichment analysis

Through the enrichment analysis of the GO function of the differential genes, it was more intuitive and convenient to observe the annotation status and function description information of the differential genes of cobia hypoxia-reoxygenation adaptation. The number of GO enriched genes in the control, hypoxia groups, and reoxygenation-8h, 24h, and 48h groups were statistically significant. It was found that 34, 193, 50, and 51 GO categories were significantly enriched, respectively (p-value < 0.05).

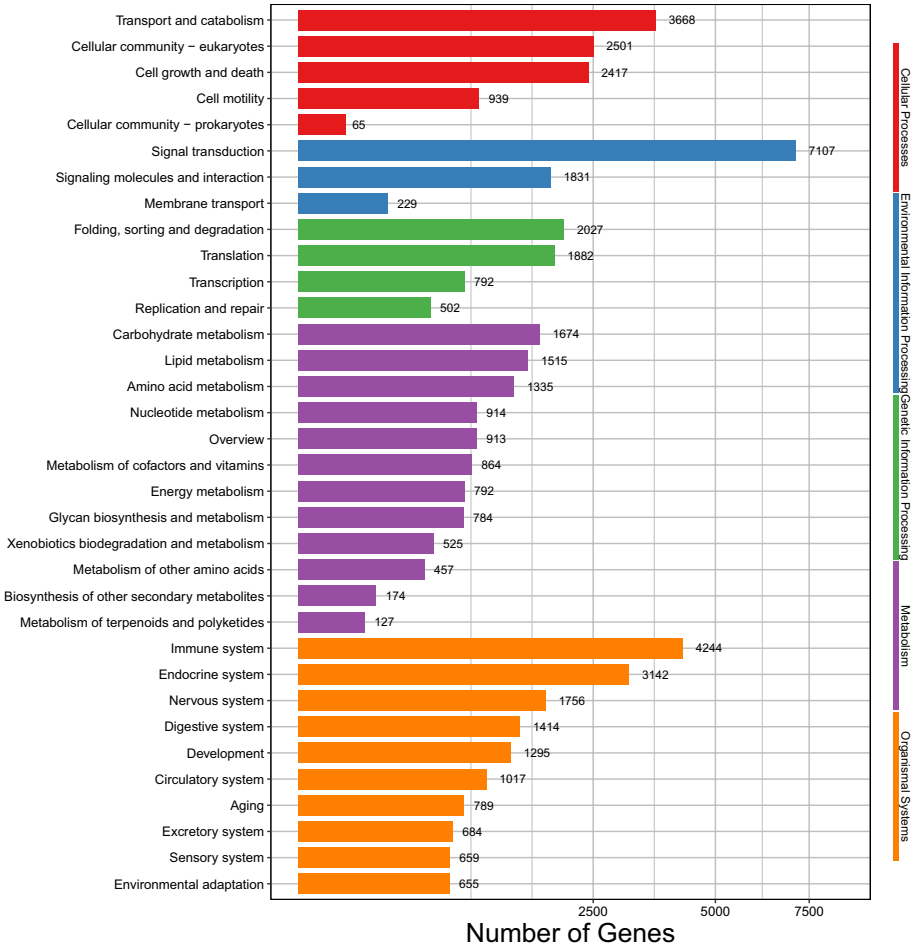

Fig. 4 KEGG classification

In the hypoxia group (SC-vs-S), the differential genes in the liver tissue of cobia were significantly enriched under different GO entries. The top 10 GO entries were closely related to ribosomes, structural components of ribosomes, and biological processes such as translation (Table 7), and all appeared in the GO function classification of molecular functions, cell components, and biological processes (Fig. 8a). In the reoxygenation-8h group (S-vs-R8), the GO entries in the top 10, which were significantly enriched in the differential genes of the cobia liver, were closely related to biological processes such as matrix-dependent cell migration, substrate-dependent cell migration, cell adhesion to the substrate, and calcium-dependent cell-matrix adhesion (Table 7). However, all GO entries only appeared in the classification of biological processes (Fig. 8b). In the reoxygenation-24h group (S-vs-R24), the GO entries in the top 10, which were significantly enriched in the differential genes of the cobia liver, were closely related to the biological processes such as matrix-dependent cell migration, substrate-dependent cell migration, cell adhesion to the substrate, and calcium-dependent cell-matrix adhesion

Table 5 Reads and reference sequence comparison statistics

Samples	Total reads	Total mapped
SC-1	22,385,892	19,678,218 (87.90%)
SC-2	24,081,528	21,082,767 (87.55%)
SC-3	20,536,701	18,153,410 (88.39%)
S-1	19,677,203	17,371,979 (88.28%)
S-2	20,663,746	17,871,780 (86.49%)
S-3	22,027,798	18,824,737 (85.46%)
R8-1	22,067,841	19,095,514 (86.53%)
R8-2	21,052,588	18,434,898 (87.57%)
R8-3	19,498,104	17,247,953 (88.46%)
R24-1	19,198,104	3,718,134 (86.01%)
R24-2	4,322,754	5,275,789 (86.26%)
R24-3	6,116,431	4,113,718 (87.49%)
R48-1	15,188,017	12,988,625 (85.52%)
R48-2	5,420,447	4,624,800 (85.32%)
R48-3	2,255,958	19,449,882 (86.23%)

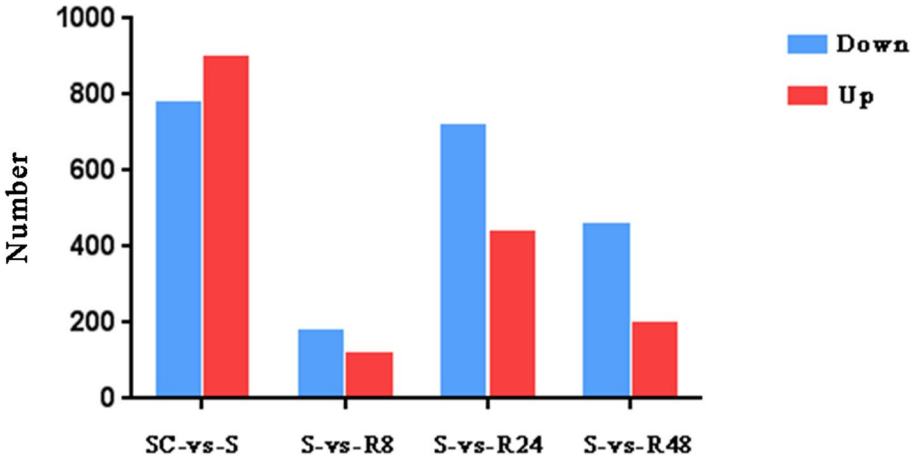

Fig. 5 Number of differentially expressed genes amongst groups

(Table 7), and all appeared in the molecular function, cell components, and biological processes (Fig. 8c). In the reoxygenation-48h group (S-vs-R48), the GO entries in the top 10, which were significantly enriched in the differential genes of the cobia liver, were closely related to biological processes such as redox activity, redox process, and monomer metabolism process (Table 7), and all appeared in the classification of molecular function, cell components, and biological processes (Fig. 8d).

Validation of gene expression levels by real-time quantitative PCR

In order to verify the accuracy of RNA-Seq results, five differentially expressed up-regulated genes (AMPK, FABP, PDHA, EGLN, PCKA) and four differentially expressed

Table 6 Statistical analysis of edge R gene differential expression

Group	Total	Up	Down
SC-vs-S	1689	905	784
SC-vs-R8	615	156	459
SC-vs-R24	2073	687	1386
SC-vs-R48	5719	2605	3114
S-vs-R8	651	199	452
S-vs-R24	236	71	165
S-vs-R48	1150	429	721
R8-vs-R24	528	254	274
R8-vs-R48	3516	1602	1914
R24-vs-R48	697	485	212

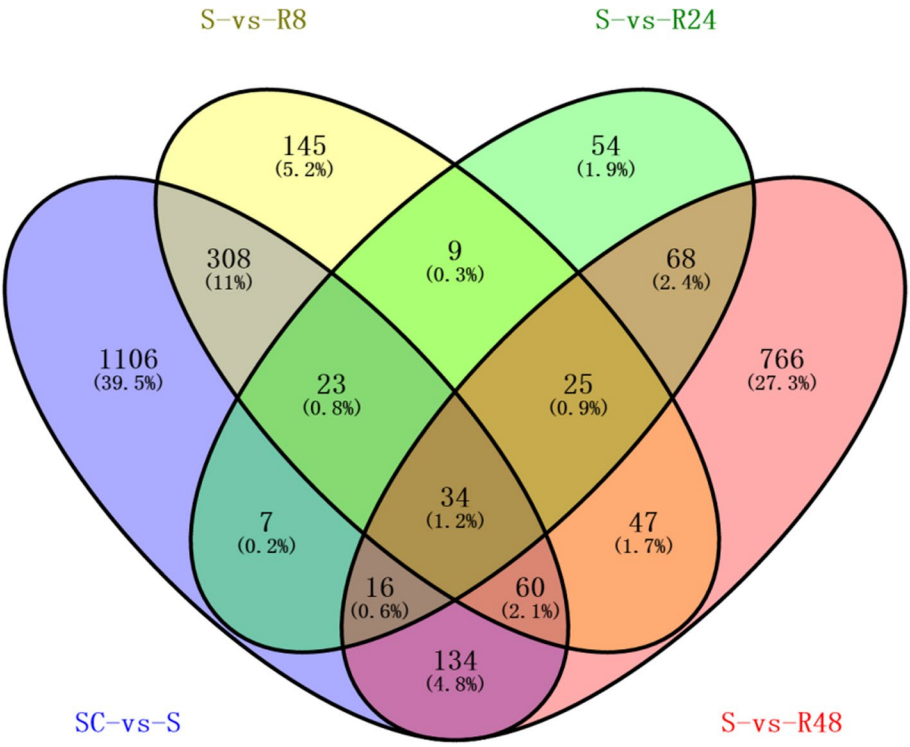

Fig. 6 Venn diagram analysis of differentially expressed genes amongst groups

down-regulated genes (RPL23, RPL0, CDC20, ERG24) were randomly selected for qRT-PCR. The results of real-time quantitative PCR verification and RNA-Seq results are shown in Fig. 9. Although there was a deviation in the expression level, the qRT-PCR expression levels of the nine differentially expressed genes in liver tissue were very similar

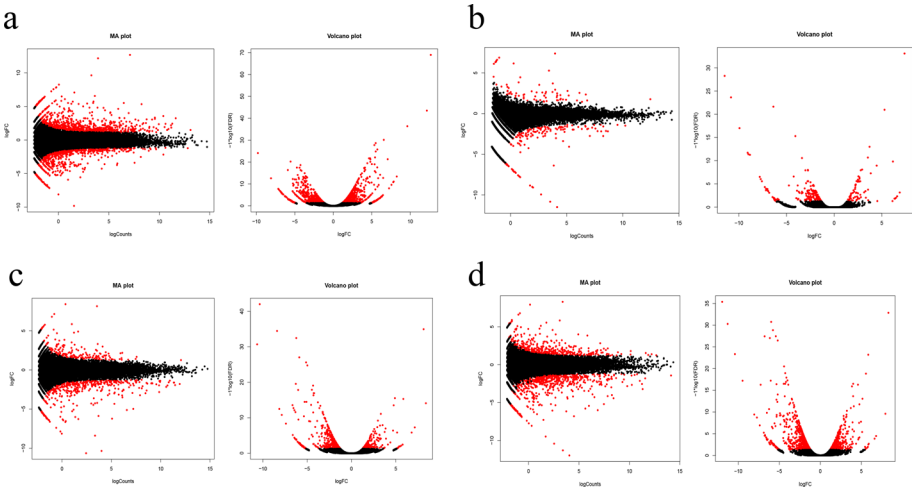

Fig. 7 Differences in expression of the genetics of MA plot and volcanic maps. **a** SC-vs-S, **b** S-vs-R8, **c** S-vs-R24, and **d** S-vs-R48. Note that the scattered dots in the figure represent each gene. Black dots represent genes with no differential expression, and red dots represent genes with differential expression

to the transcriptome results, indicating that the transcriptome sequencing results had certain reliability and repeatability.

Discussion

The transcriptome sequencing data quality analysis

Limitations in oxygen availability (hypoxia) have profound effects on aquatic life; their growth, reproduction, and development; and the entire aquatic ecosystem (Storz et al. 2010; Shang and Wu 2004). It has been reported that many fish species can respond to or cope with hypoxia by deploying a complex suite of molecular mechanisms that affect the transcription of genes (Qi et al. 2018; Liu et al. 2018; Kang et al. 2017; Zhong et al. 2009).

This study obtained 100,743,276,600 and 335,810,922 clean reads from the transcriptome high-throughput sequencing analysis of cobia liver in response to hypoxia-reoxygenation. The 118,176 unigenes were obtained by assembly and splicing, with an average length of 1921 bp and N50 of 3803 bp. Although the transcriptome sequencing sample was a single tissue, the results of the length of assembled unigenes showed that the sequencing quality and assembly results were relatively accurate and reliable. The comparison of transcripts successfully annotated in five databases, KOG, KEGG, NR, GO, and Swiss-Prot, found that the number of transcripts annotated in the NR database was the largest, accounting for about 52.58% of all transcripts. Whilst 47.24% of the transcripts were not annotated, this phenomenon of low alignment with known databases also appeared in the transcriptomes of other fish, such as *Gymnocypris eckloni* (Qi et al. 2018). This analysis may be due to the lack of alignment information of similar species genes in existing NCBI data databases. Since the size of the cobia genome is currently unknown, the molecular basis of hypoxic stress in cobia is still in the preliminary exploratory stage. Therefore, whether the gene sequences with low similarity in this transcriptome sequencing after comparison with the existing data

Table 7 The GO terms of significantly differential genes in the liver (TOP10)

GO term		GO ID	Gene number	Up	Down	*p*-value	*Q*-value
Biological processes (SC-vs-S)	Structural constituent of ribosome	GO:0003735	52	0	52	1.47E−12	4.67E−09
	Ribosome	GO:0005840	53	0	53	6.32E−12	1.01E−08
	Translation	GO:0006412	66	8	58	5.97E−11	4.75E−08
	Amide biosynthetic process	GO:0043604	68	10	58	5.39E−11	4.75E−08
	Peptide biosynthetic process	GO:0043043	66	8	58	9.94E−11	5.68E−08
	Structural molecule activity	GO:0005198	62	1	61	1.07E−10	5.68E−08
	Cellular amide metabolic process	GO:0043603	71	11	60	1.36E−10	6.18E−08
	Peptide metabolic process	GO:0006518	66	8	58	4.75E−10	1.89E−07
	Ribosomal subunit	GO:0044391	35	0	35	5.78E−09	2.05E−06
	Cytosolic part	GO:0044445	37	0	37	1.46E−08	4.65E−06
Biological processes (S-vs-R8)	Substrate-dependent cell migration	GO:0006929	3	3	0	7.44E−05	0.006891214
	Substrate-dependent cell migration, cell attachment to the substrate	GO:0006931	3	3	0	7.44E−05	0.006891214
	Calcium-independent cell-matrix adhesion	GO:0007161	3	3	0	7.44E−05	0.006891214
	Peptide cross-linking	GO:0018149	4	4	0	4.42E−05	0.006891214
	Extracellular matrix disassembly	GO:0022617	3	3	0	7.44E−05	0.006891214
	Integrin activation	GO:0033622	3	3	0	7.44E−05	0.006891214
	Transforming growth factor-beta secretion	GO:0038044	3	3	0	7.44E−05	0.006891214
	Negative regulation of cytokine secretion	GO:0006518	3	3	0	7.44E−05	0.006891214
	Regulation of substrate-dependent cell migration, cell attachment to the substrate	GO:1904235	3	3	0	7.44E−05	0.006891214

Table 7 (continued)

GO term		GO ID	Gene number	Up	Down	*p*-value	*Q*-value
Biological processes (S-vs-R24)	Hormone activity	GO:0005179	5	2	3	8.02E−07	0.000508179
	Steroid biosynthetic process	GO:0006694	2	1	1	3.85E−03	0.166762738
	Steroid metabolic process	GO:0008202	3	1	2	1.20E−03	0.166762738
	Aerobic respiration	GO:0043604	3	0	3	3.26E−03	0.166762738
	Lactate transport	GO:0015727	1	1	0	7.89E−03	0.166762738
	Sterol metabolic process	GO:0016125	2	0	2	4.52E−03	0.166762738
	Peptide cross-linking	GO:0018149	2	2	0	3.22E−03	0.166762738
	Lactate transmembrane transport	GO:0006518	1	1	0	7.89E−03	0.166762738
	Plasma membrane lactate transport	GO:0035879	1	0	1	7.89E−03	0.166762738
Biological processes (S-vs-R48)	Oxidoreductase activity	GO:0016491	61	12	49	6.75E−16	1.16E−12
	Oxidation-reduction process	GO:0055114	55	9	46	1.09E−13	9.38E−11
	Single-organism metabolic process	GO:0044710	88	19	69	2.98E−09	1.71E−06
	Heme binding	GO:0020037	15	4	11	6.06E−08	2.60E−05
	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	GO:0016705	16	9	7	9.89E−08	3.09E−05
	Tetrapyrrole binding	GO:0046906	15	4	11	1.08E−07	3.09E−05
	Monooxygenase activity	GO:0020037	12	4	8	3.99E−07	9.81E−05
	Extracellular region	GO:0005576	41	22	19	2.27E−06	4.87E−04
	Iron ion binding	GO:0005506	14	3	11	9.97E−06	1.91E−03
	Hormone activity	GO:0005179	7	3	4	1.57E−05	2.71E−03

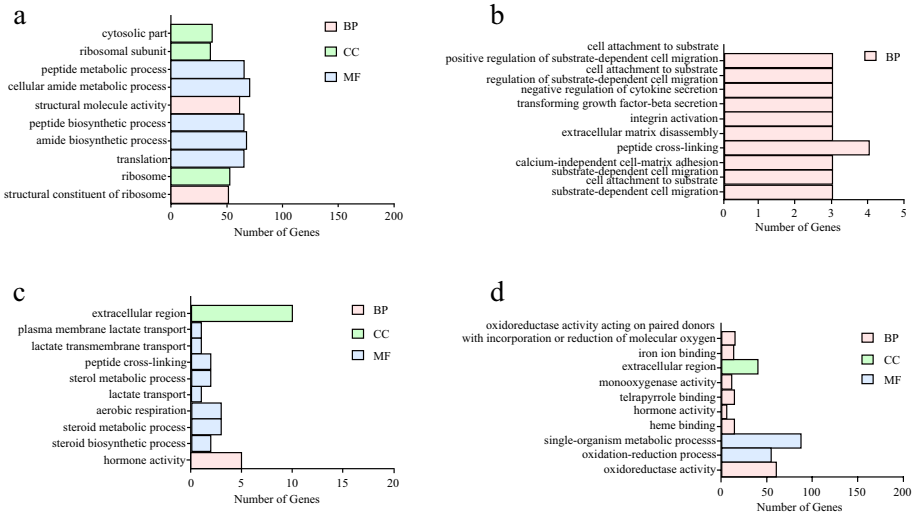

Fig. 8 The most enriched top 10 GO terms and corresponding differentially expressed gene numbers of each

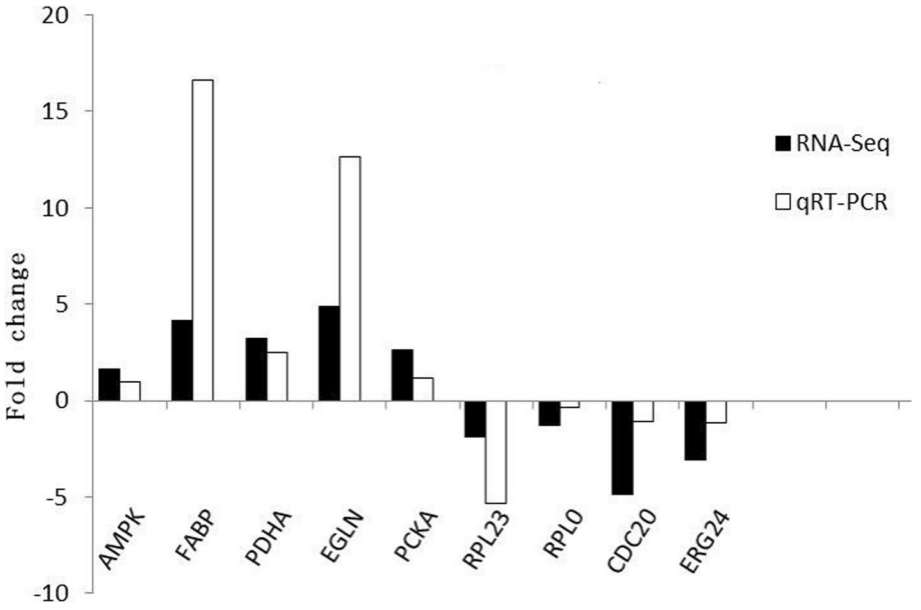

Fig. 9 qRT-PCR validation of the expression of selected genes in the liver of cobia

database are related to new genes unique to cobia hypoxia still need further research. Pairwise comparisons of hypoxia and reoxygenation were performed amongst five groups of juvenile cobia samples, and the DEGs of hypoxia and reoxygenation at 8 h, 24 h, and 48 h were screened. The number of more up-regulated genes than down-regulated ones were found in the hypoxia period, indicating that the gene expression levels were relatively active in the hypoxic period so that the juvenile fish could respond to the hypoxic environment.

Analysis of hypoxia-reoxygenation regulation of gene expression and metabolic response in cobia liver

The differentially expressed genes were mainly annotated into biological processes in the juvenile fish liver tissue in this study, and the cellular amide metabolism pathway closely related to the maintenance of intracellular redox homeostasis was shown to be activated. Therefore, the results suggest that cobia might initiate the metabolism of the body's cellular amide level in the hypoxia-reoxygenation stage. Previous studies have found that this response mode has effects on the regulation of key antioxidant enzyme activities, the regulation of related gene expression (Liu et al. 2016), and the improvement of non-specific immunity (Zhang et al. 2016) of juvenile fish under hypoxic stress. However, other studies have also found that this level of response will also cause hypoxia/reoxygenation damage to tissues and cells to a certain extent, increase pathogenic infection, the occurrence of hypoxia syndrome, and the risk of new mutations in oncogenes (Sun et al. 2013; Zhang and Qing 2013).

Compared with the control group, there were 1689 differentially expressed genes in the hypoxia treatment group, including 905 up-regulated genes and 784 down-regulated genes. This result is consistent with the study of hepatopancreas transcriptome in *Gymnocypris eckloni* (Qi et al. 2018) under hypoxia stress, which showed that the up-regulated genes of differentially expressed genes are significantly more than the down-regulated genes. It was speculated that there might be differences in how liver tissue and other tissues respond to hypoxia stress. Liver tissue may respond to hypoxia stress more by enhancing gene expression rather than inhibiting gene expression.

In order to respond to the hypoxic environment, fish mainly adopt strategies to reduce their own oxygen consumption (such as decreased metabolism) and increase oxygen supply (such as angiogenesis) (Yu 2017). However, little was known about the regulation of genes related to hypoxia in cobia, especially the molecular mechanism of adaptation to hypoxia in cobia. Some potentially related differentially expressed genes were found in this study by transcriptome analysis of cobia juveniles under hypoxia-reoxygenation experimental conditions. These include genes on signalling pathways such as PPAR, HIF-1, and Ribosome. These differential genes involve many key factors and jointly participate in the hypoxia regulatory network. Through the interaction of metabolic pathways, the transcriptional level of juvenile fish changed, and the molecular adaptation strategy of cobia to hypoxia was also determined in response to hypoxia stress (Detmar et al. 1997).

In this experiment, 16, 11, 3, and 9 differentially expressed genes were found to be significantly enriched in the PPAR signal pathway in the liver transcriptome of the juvenile cobia under hypoxia stress group, reoxygenation-8h group, reoxygenation-24h group, and reoxygenation-48h group, respectively ($p < 0.001$). PPAR signalling pathway was involved in regulating lipid metabolism, energy metabolism, and inflammatory response, and the activation level of the PPAR pathway is related to lipid metabolism (Zhang 2000). The PPAR family's physiological functions are mainly as intracellular lipid sensors in mediating metabolic regulation, which can bind to specific response elements in the promoter region of target genes and initiate transcriptional processes (Xu et al. 2011). Researchers have also demonstrated that PPARs prevent hypoxia-induced pulmonary hypertension, suggesting vasoprotective effects of PPARs under chronic hypoxia. These properties sustain vital physiological functions such as smooth muscle proliferation, anticoagulation, vasodilation, antioxidative capacity, and leucocyte adhesion (Xu et al. 2011; Zhang 2000).

In this experiment, through KEGG pathway analysis, it was found that the two genes CD36 (CD36 antigen) and hepatic fatty acid-binding protein (FABP1) are both distributed upstream of the PPAR signalling pathway; stearoyl-CoA dehydrogenase (SCD), phospholipid transporter protein (PLTP), and phosphoenolpyruvate carboxykinase (pckA) are distributed downstream of PPAR-α, PPAR-β, and PPAR-γ genes, respectively. During hypoxia, chylomicron VLDL/chylomicron activates the receptor CD36 on the membrane and transmits the signal to the second messenger, FABP1. The activated second messenger FABP1 enters the nucleus. It binds to the transcription factors PPAR-α, PPAR-β, and PPAR-γ, activating transcriptional activity that regulates the expression of target genes, resulting in a significant down-regulation of PLTP gene expression and a significant up-regulation of SCD and pckA gene expression. The opposite is true for gene expression during reoxygenation. A study has shown that CD36, PPAR-β, and PPAR-γ can regulate lipid metabolism in rat liver under hypoxic training induction (Zhu et al. 2019). Therefore, it was assumed that CD36, PPAR-β, and PPAR-γ mediated up- or down-regulation of PLTP and SCD, and pckA gene expression further confirmed that cobia was more prone to attenuate lipid transport and lipid oxidation enhance lipid synthesis. In general, under this mechanism, juvenile fish reduced the oxygen demand of the body cells during hypoxia and improved the oxygen utilisation rate (Cameron et al. 2013).

After annotating the KEGG pathway of differentially expressed genes between the hypoxia treatment group and the normoxia control group, it was found that the number of genes involved in the ribosome signalling pathway (Ribosome) was the largest. Ribosomal pathway up-regulated genes were mainly RPS27, and down-regulated genes mainly included RPLP0, RPLP1, RPL34, and RPS5. The RPLP0 gene encodes P0, the largest subunit of the acidic ribosomal phosphoprotein, and is part of the composition of the ribosomal 60S subunit (Xiao et al. 2007). RPLP1 and RPLP0 can interact and form the main part of the active centre of ribosomal GTPase. RPLP1 protein can also covalently combine with ubiquitin to form a fusion protein, which is involved in regulating biological processes such as apoptosis and transcription. Studies have found that loss of function or mutation of RPS27 can cause symptoms of anaemia (Wang et al. 2015a, b, c). The significant up-regulation of ribosomal protein RPS27 and the significant down-regulation of RPL34 and RPS5 indicated the expression strategy of ribosomal proteins in response to hypoxia stress. On the other hand, hypoxia inhibits protein translation and mRNA synthesis in hepatocytes, reduces the level of protein metabolism in the body, reduces oxygen consumption, and reduces hypoxic injury (Chee et al. 2019; Casey 2002).

In fact, in addition to the differentially expressed genes involved in the PPAR as mentioned above and Ribosome signalling pathways, more genes jointly respond to hypoxia stress in fish by participating in more signalling pathways (Di et al. 2018). HIF-1, as a signal transduction pathway of cells under hypoxic stress, was a classic hypoxic pathway related to adaptive strategies when fish respond to hypoxic stress. The expression of related genes in the HIF-1 signalling pathway not only mediates the regulation of basic metabolism but also inhibits erythrocyte proliferation and apoptosis by increasing blood turnover and respiratory rate (Sun 2006), stimulates compensatory angiogenesis (Qu and Zhao 2009), and increases blood oxygen affinity (Guo 2005) and energy demand (Cai et al. 2014), so as to reduce oxygen consumption (Cameron et al. 2013; Smith et al. 1996). It was generally believed that HIF-1α and HIF-2α were the main transcriptional initiators in the HIF-1 signalling pathway. Under the influence of proline hydroxylase (PHD), expressions under normoxia, HIF-1α and HIF-2α, are degraded, whilst under hypoxia, HIF-1a and HIF-2α were accumulated due to PHD inactivation (Lando 2002; Nicholas and Sumbayev 2010; Yasuda et al. 2014). However, in this experiment, the regulation of HIF-1α

and HIF-2α was not detected in the liver transcriptome during hypoxia or reoxygenation, which is consistent with the research results of *Gymnocypris eckloni* (Qi et al. 2018) and *Megalobrama amblycephala* (Chen 2016). According to the results of this experiment, proline hydroxylase (PHD) is still highly expressed under hypoxic stress, so this may be the most fundamental reason why HIF-1α and HIF-2α are not regulated. Therefore, the result suggests that different tissues of different fish were destined to have different expression patterns of HIF-1α and HIF-2α under hypoxia-reoxygenation conditions.

In addition, 13 differentially expressed genes were significantly enriched in the signalling pathway HIF-1 in this study ($p < 0.001$). Hypoxic stress caused high expression of HIF-1 signalling pathway-related genes PDHA, PHD, and PAI1 in the liver of cobia juveniles, whilst genes such as INS and SLC2A1 were inhibited, and their expression levels decreased. In the reoxygenation stage of juvenile fish, the SLC2A1 gene down-regulated by hypoxia was significantly up-regulated, the PhD gene up-regulated by hypoxia was down-regulated, and the expression of transferrin (TF) and transferrin receptor (TFRC) genes were significantly down-regulated at reoxygenation-48h, suggesting that the way of promoting anaerobic metabolism in the liver is significantly affected by hypoxia stress. However, in the face of transporting and supplying oxygen, the expression of related genes is up-regulated, which induces the activation of PAI1 to promote the occurrence of new blood vessels to obtain more oxygen.

The result also shows that young fish reduce oxygen consumption, store oxygen by weakening basic metabolism under a hypoxic environment, and meet their energy needs by enhancing lipid synthesis and liver glycogen conversion to glucose. Therefore, membrane receptor SLC2A1 related to anaerobic glycolysis is not activated, and PDHA phosphorylation inhibits the expression of SLC2A1 (Rojas et al. 2007). Finally, in the reoxygenation stage, in order to replenish the energy lost during the hypoxia period, the juveniles reactivated SLC2A1 to store new energy (Fig. 10).

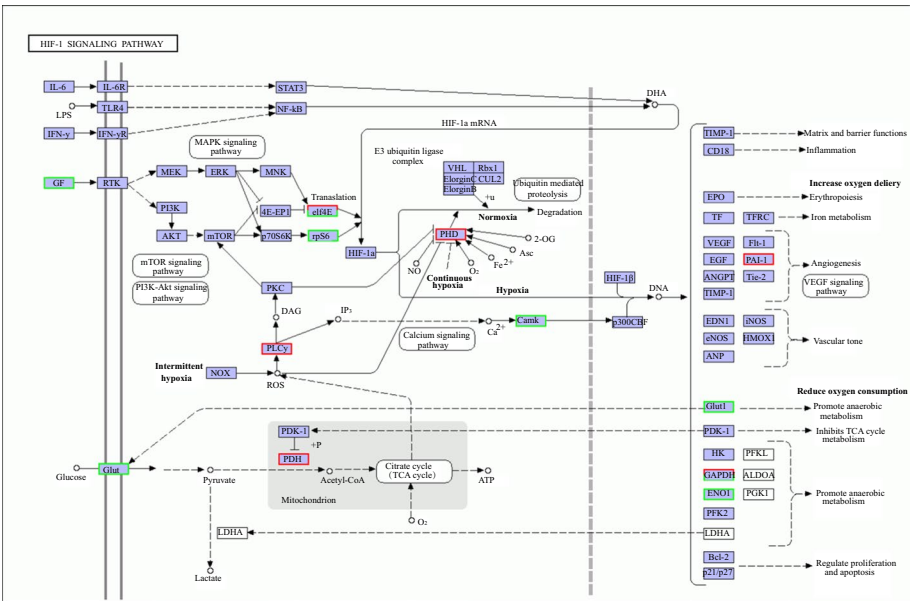

Fig. 10 KEGG pathway enrichment analysis of differentially expressed genes (HIF-1)

Conclusion

DO is one of the critical environmental factors in aquaculture, where hypoxia poses a significant threat to the survival of aquatic organisms. The fish liver is crucial for its existence since it plays an imperative role in the uncountable characteristics of fish's biological functions, such as synthesising proteins, detoxification of the organism, and producing biochemicals needed for digestion and growth. At present, there is scarce literature on the fluctuations in the cobia liver during hypoxic exposure. This study assessed the effects of hypoxia stress on the cobia juveniles' fish *Rachycentron canadum* liver. Hypoxia affected liver tissue. Under hypoxia, *Rachycentron canadum* underwent changes in gene expression, suggesting several key adaptive strategies. For the first time, gene expression patterns in the liver of a larval cobia were examined in response to hypoxia. The results of this study may contribute to further clarifying hypoxia's effects on the liver of ocean fish. However, additional physiological and molecular studies are necessary to verify the transcriptomic response of cobia fish to hypoxia.

Author contribution Jian-Sheng Huang participated in the data curation, project administration, data analysis, and original article writing. Zhi-Xiong Guo participated in the data collection, Jian-Dong Zhang participated in the data collection, Wei-Zheng Wang participated in the data collection, Zhong-Liang Wang supervised the project, and Rui-Tao Xie supervised the project. Eric Amenyogbe did the writing, review, and edited the original article. Gang Chen planned and designed the experiments and acquired funding.

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Declarations

Competing interests The authors declare no competing interests.

Ethics approval This study was conducted in accordance with the guidelines of Guangdong Ocean University Research Council for the care and use of laboratory animals (approval number: GDOU-LAE-2020-013).

Consent to participate All authors voluntarily consent to participate.

Consent for publication All authors voluntarily consent for publication.

Conflict of interest The authors declare no competing interests.

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