



# RNA-seq analysis reveals divergent adaptive response to hyper- and hypo-salinity in cobia, *Rachycentron canadum*

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**Abstract** Salinity is an important abiotic stress that affects metabolic and physiological activities, breed, development, and growth of marine fish. Studies have shown that cobia (*Rachycentron canadum*), a euryhaline marine teleost fish, possesses the ability of rapid and effective hyper/hypo ionic- and osmoregulation. However, genomic studies on this species are lacking and it has not been studied at the transcriptome level to identify the genes responsible for salinity regulation, which affects the understanding of the fundamental mechanism underlying adaptation to fluctuations in salinity. To describe the molecular response of cobia to different salinity levels, we used RNA-seq analysis to identify

genes and biological processes involved in response to salinity changes. In the present study, 395,080,114 clean reads were generated and then assembled into 65,318 unigenes with an N50 size of 2758 bp. There were 20,671 significantly differentially expressed genes (DEGs) including 8805 genes adapted to hypo-salinity and 11,866 genes adapted to hyper-salinity. These DEGs were highly represented in steroid biosynthesis, unsaturated fatty acid metabolism, glutathione metabolism, energy metabolism, osmoregulation, and immune response. The candidate genes identified in cobia provide valuable information for studying the molecular mechanism of salinity adaptation in marine fish.

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Furthermore, the transcriptomic sequencing data acts not only as an important resource for the identification of novel genes but also for further investigations regarding cobia biology.

**Keywords** Transcriptome · Salinity adaptation · *Rachycentron canadum* · RNA-seq · Differential gene expression

## Background

Salinity is one of the most important environmental factors affecting the survival, breed, development, growth, and physiological functions of teleost fish (Zhang et al. 2017). Owing to variation in the salinity of the aquatic environment, teleost fish have evolved various physiological strategies for salinity adaptation (Chong-Robles et al. 2014; Bayly n.d.). Euryhaline fish species can adapt to a wide range of salinities and cope with both chronic and rapid osmotic stress; hence, it acts as an excellent model for studying the mechanisms of salinity adaptation during acclimation to various aquatic environments (Si et al. 2018). *Rachycentron canadum* is a euryhaline teleost fish that can survive at a wide range of salinities (Denson et al. 2003), and cobia is currently considered a promising candidate for aquaculture because of its rapid growth, effective feed utilization, and high market value (Arnold et al. 2010; Chou et al. 2001). Therefore, together with its economic importance, cobia is a valuable template for investigating the mechanisms of salinity adaptation. Moreover, understanding the potential mechanisms of salinity adaptation can also prevent germplasm degeneration and further expansion of cobia culture.

With the rapid development of molecular technologies, it is possible to study the ecological and physiological mechanisms of teleost fish using gene expression analysis (Xu et al. 2015). As enzymes and transporters maintain internal osmotic and ionic homeostasis in an environment of fluctuating salinity by participating in salinity adaptation and osmoregulation (Hwang and Lee 2007; Tseng and Hwang 2008), identification of candidate genes involved in salinity change is critical for understanding the molecular basis of this core physiological process. The transcriptome is a group of dynamically expressed genes, the expression of which change depending on the physiological state of the organisms and is sensitive

to the external environment (Wang et al. n.d.). As a result, transcriptome analysis is a powerful tool for interpreting the functional genomic elements and for unraveling the molecular mechanisms in cells and tissues (Nguyen et al. 2016). Next-generation high-throughput RNA sequencing technology (RNA-seq) now provides a cost- and time-effective method of generating transcriptomic data, which has many distinct advantages over other traditional methods for generating these types of data (Wang et al. n.d.; Yi et al. 2014; Alina et al. 2012). Over the past few years, significant advancements have been made in understanding the transcript expression profiles after exposure to salinity changes of various euryhaline teleost fish, such as half-smooth tongue sole (*Cynoglossus semilaevis*) (Si et al. 2018), Mozambique tilapia (*Oreochromis mossambicus*) (Ronkin et al. 2015), medaka (*Oryzias melastigma*) (Lai et al. 2015), striped catfish (*Pangasianodon hypophthalmus*) (Nguyen et al. 2016; Thanh et al. 2015), and Siberian sturgeon (*Acipenser baerii*) (Guo et al. n.d.). These studies have revealed some of the differentially expressed genes and pathways related to salinity changes (Zhang et al. 2017). However, the existing investigations in cobia have been limited to studies on the influence of salinity on survival, larval and juvenile development, oxygen consumption, ammonia excretion, growth, and energy budget (Denson et al. 2003; Faulk and Holt 2006; Doi and Barbieri 2012; Wu et al. 2009), and little is known regarding the molecular pathways related to the salinity change-associated adaptive mechanisms. Therefore, it is necessary to study the salinity adaptability of cobia at the transcriptional level to understand the more fundamental mechanisms of salinity adaptation.

As salinity adaptation in cobia is still largely unexplored, and as the genomic and transcriptomic data for this species is still limited, we aimed to identify and assess the genes that play latent roles in salinity adaptation using RNA-seq and to compare their expression patterns in three chief osmoregulatory organs, namely, the gill, intestine, and kidney, under three experimental conditions (10 ppt, 30 ppt (control), and 35 ppt). Our comparative approach revealed the mechanism via which cobia regulates its genes under salinity adaptation, and identified important genes involved in salinity adaptation. This study will be a valuable resource for understanding the mechanisms of salinity adaptation and for promoting genetic studies in cobia.

## Methods

### Salinity adaptation and sampling

*Rachycentron canadum* (initial weight  $29.65 \pm 1.18$  g) were obtained from the marine biological research base of Guangdong Ocean University located in Donghai Island, Zhanjiang, China. After acclimation in normal seawater (28–30 ppt) for 1 week, the fish were stocked in nine normal seawater tanks (500 L) with 20 fish each. One set of three tanks with normal seawater was used as the control, and the other two sets of three tanks were adapted to the salinities of 10 and 35 ppt, respectively, using crystal sea salt and dechlorinated tap water to increase or decrease the salinity by 4 ppt per day. Fish were kept at three final salinities 10, 30, and 35 ppt in triplicate for 1 month. Half the amount of water was replaced with water of the same salinity every day. Fish were fed with a commercial diet (46% crude protein, 8% crude lipid) twice a day at 08:00 a.m. and 16:00 p.m. At the end of the experiment, fish were euthanized with clove oil (200 mg/L) and samples were taken from the gill, kidney, and intestine. These tissues were selected as they are considered to be the major osmoregulatory organs in most teleost fish (Higashimoto et al. 2001). The tissue samples were placed in RNA-Later stabilization reagent (Qiagen) and stored at  $-80$  °C for later use. The samples were then transported to the GENE DENOVO Biotechnology Limited Company for transcriptome analysis.

### RNA extraction

Total RNA was extracted from each sample using TRIzol reagent (Ambion) according to the manufacturer's instructions. RNA quality (degradation and contamination) was analyzed using 1% agarose gel electrophoresis. In order to make sure the veracities and universalities, equal amounts of RNA from nine individual fish in each treatment group were mixed as one sample. Total RNA was further purified using an ISOLATE II RNA mini kit (Bioline). The concentration and quality of total RNA were checked using Agilent 2100 and NanoDrop 2000 prior to subsequent experiments. Only high-quality RNA samples with optical density (OD) 260/280 values  $> 1.8$  and  $< 2.2$ , RIN  $\geq 7$ , and RNA yield  $> 5$   $\mu$ g were used for subsequent transcriptome analysis.

### cDNA library construction and sequencing

A qualified RNA sample was used for subsequent processing. First, the mRNA was enriched using magnetic beads containing oligo (dT), and then the enriched mRNA was fragmented by adding a fragmentation buffer. The first-strand cDNA was synthesized using mRNA templates and random primers. The second-strand cDNA was synthesized by adding buffer, dNTPs, RNase H, and DNA polymerase I. The cDNA was purified, repaired at its ends, followed by the addition of poly(A) and ligation of the fragment. The ligation products were selected using agarose gel electrophoresis, PCR amplified, and sequenced using Illumina Hiseq™ 2000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

### De novo assembly of sequencing reads

Following the sequencing step, raw data of FASTQ format were first processed using in-house Perl scripts. In this step, clean data were obtained by removing reads containing adapter, reads containing more than 10% of unknown nucleotides (N), and low-quality reads containing more than 50% low-quality ( $Q$  value  $\leq 10$ ) bases. All the downstream analyses were based on high-quality clean data.

The de novo assembly was performed on all the clean reads using the Trinity package (v2012-10-05) (Grabherr et al. 2011). The transcripts were assembled and clustered using the chrysalis clusters software. Finally, the longest transcripts of each gene were selected as unigenes. This study also proposed N50 to initially estimate assembly quality.

### Functional annotation of transcripts

To annotate the unigenes, we used the BLASTx program (<http://www.ncbi.nlm.nih.gov/BLAST/>) with an  $E$  value threshold of  $1.0E^{-5}$  with the NCBI non-redundant protein (Nr) database (<http://www.ncbi.nlm.nih.gov>), the Swiss-Prot protein database (<http://www.expasy.ch/sprot>), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg>), and the COG/KOG database (<http://www.ncbi.nlm.nih>).

gov/COG). Protein functional annotations were obtained based on the optimal alignment results.

#### Gene differential expression and enrichment analysis

The rRNA-removed high-quality clean reads were mapped to the reference transcriptome using the default parameters of the short reads alignment tool Bowtie2 (Li et al. 2009). The mapped reads from the alignments were counted and then normalized to determine RPKM (reads per kb per million reads) (Mortazavi et al. n.d.). Differentially expressed genes were identified across samples or groups using the edgeR package (<http://www.r-project.org/>). Fold change  $\geq 2$  and false discovery rate (FDR)  $< 0.05$  were set as the threshold for significant DEGs. The DEGs were then subjected to enrichment analysis of GO functions and KEGG pathways. GO functional annotations of unigenes could be obtained from Nr annotation results. GO annotation of unigenes were analyzed by the Blast2GO software (Ana et al. 2005). The WEGO software was used to perform functional classification of unigenes (Jia et al. 2006).

#### Real-time PCR validation

To validate the Illumina sequencing results, ten genes with high level of significance were selected randomly for real-time PCR analysis with  $\beta$ -actin as the reference gene. The primers were designed based on the assembled transcriptome sequence using the Primer 5 software (Premier Biosoft International) and listed in the S1 Appendix. Real-time PCR experiments were performed using the TransStart® Tip Green qPCR SuperMix kit, which was performed in a three-step method on a LightCycler 96 real-time PCR instrument, and each test sample was tested thrice. The amplification system consisted of 0.4  $\mu$ L cDNA, 0.4  $\mu$ L forward primer, 0.4  $\mu$ L reverse primer, 10  $\mu$ L 2  $\times$  TransStart® Tip Green qPCR Super Mix, and 8.8  $\mu$ L ddH<sub>2</sub>O in a total volume of 20  $\mu$ L. The reaction procedure was as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 60 °C for 15 s, and 72 °C for 10 s. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction. The relative expression of these genes was calculated using the  $2^{-\Delta\Delta CT}$  method. Statistical analysis was performed using SPSS 18.0 statistical software (IBM SPSS, USA).

## Results

#### Transcriptome sequencing, de novo assembly, and alignment

To obtain a comprehensive understanding of the differences in the expression profile of cobia under 10 ppt, 30 ppt, and 35 ppt, we performed deep sequencing of RNA samples from fish gills, intestines, and kidneys. In total, 40,623,5674 raw reads (150 bp) were obtained from the nine libraries on the Illumina HiSeq™ 2000 platform. After preprocessing and removal of low-quality sequences, 395,080,114 clean reads were generated (Table 1).

After de novo assembly based on all Illumina clean reads, 65,318 unigenes (ranging from 201 to 27,143 bp) were generated for cobia with an N50 size of 2758 bp (Table 2). For all nine sequencing libraries, the percentages of reads that could be mapped to assembled reference sequences were higher than 90.10% (Table 1).

#### Annotation and function analysis of all unigenes

The unigenes were subjected to annotation analysis by comparing with Nr, KOG, Swiss-Prot, KEGG, and GO databases. Results showed that 28,058 annotated unigenes (42.96%) had significant BLAST hit against the Nr database. The detailed results of the annotation are listed in Table 2.

For top-hit species matched against the Nr database, the species distribution revealed that 51.30% of the hits matched sequences from *Lates calcarifer*, followed by *Larimichthys crocea* (9.59%), *Stegastes partitus* (7.05%), *Paralichthys olivaceus* (6.86%), *O. niloticus* (2.52%), *Notothenia coriiceps* (1.63%), *Neolamprologus brichardi* (1.12%), and other species (19.93%) (Fig. 1).

Based on the results of Nr annotation, the GO annotations of unigenes were generated using the BLAST2GO program, and 9349 genes were annotated and assigned into three categories: molecular function (MF), cellular component (CC), and biological process (BP). Among those assigned to the category of BP, cellular process (20.5%), single-organism process (18.61%), metabolic process (15.67%), and biological regulation (11.95%) were the most highly represented. The other unigenes were assigned into 19 other biological processes, including response to stimulus (6.5%), localization (6.2%), signaling (4.81%), developmental

**Table 1** Summary statistics of Illumina RNA-seq reads

Sample	Raw reads	Clean reads	After alignment rRNA reads <sup>a</sup>	Q20 (%) <sup>b</sup>	Q30 (%) <sup>c</sup>	Mapping ratio (%) <sup>d</sup>
G-10 ppt	47,594,944	46,346,820	45,046,828	98.30	94.68	90.12
I-10 ppt	38,816,938	37,573,278	36,745,190	98.11	94.22	91.18
K-10 ppt	41,626,462	40,408,218	39,894,314	98.20	94.47	90.71
G-30 ppt	40,717,652	39,620,758	38,701,464	98.25	94.58	90.74
I-30 ppt	44,153,326	42,898,846	42,038,318	98.23	94.49	91.09
K-30 ppt	42,860,956	41,579,256	40,537,074	98.20	94.50	90.44
G-35 ppt	52,401,580	51,096,472	50,095,228	98.38	95.08	90.73
I-35 ppt	53,287,852	51,857,976	51,019,144	98.36	95.02	91.36
K-35 ppt	44,775,964	43,698,490	42,830,764	98.41	95.17	90.81
Total	406,235,674	395,080,114				

<sup>a</sup> The number of clean reads that mapped to ribosome RNA (rRNA)

<sup>b</sup> Q20: the percentage of bases with a Phred value > 20

<sup>c</sup> Q30: the percentage of bases with a Phred value > 30

<sup>d</sup> The number of after alignment rRNA reads that mapped onto the assembled reference transcriptome

process (4.36%), multicellular organismal process (4.34%), and cellular component organization or biogenesis (3.45%). In addition, 19 GO functional groups were assigned to the category of CC, with cell (19.29%), cell part (19.29%), membrane (16.22%), membrane part (14.47%), and organelle (14.18%) being represented

**Table 2** Summary of assembly and annotation statistics of the transcriptome analysis

Category	Number of unigenes
Total number of genes	65,318
Average length of all transcripts (bp)	1255
N50 length of all transcripts (bp)	2758
Max length (bp)	27,143
Min length (bp)	201
Total number of annotation genes	28,220 (43.20%)
Total number of without annotation genes	37,098 (56.80%)
Total number of annotated unigenes in Nr database	28,058 (42.96%)
Total number of annotated unigenes in KEGG database	17,164 (26.28%)
Total number of annotated unigenes in KOG database	18,950 (29.01%)
Total number of annotated unigenes in the GO database	9349 (14.31%)
Total number of annotated unigenes in Swiss-Prot database	23,600 (36.13%)

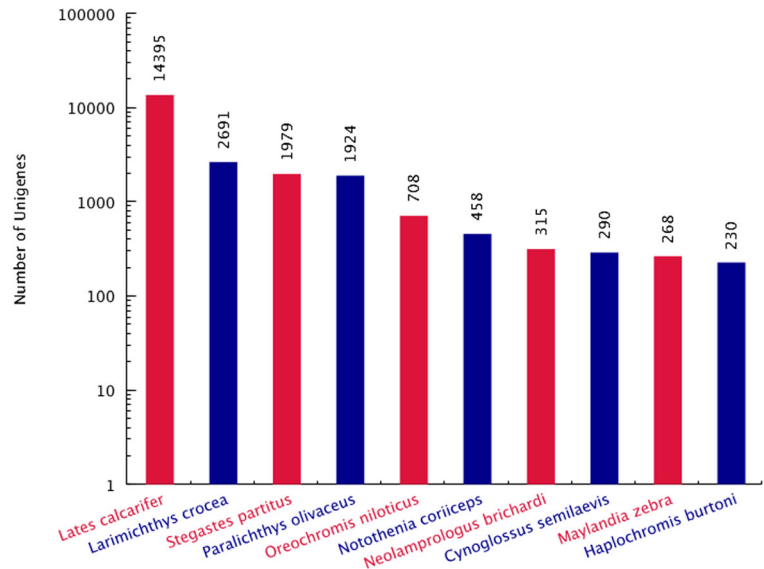
most highly. Similarly, 12 GO functional groups were assigned to the category of MF, with binding (44.67%) and catalytic activity (34.32%) being represented most highly (Fig. 2).

To assess and classify the possible functions, the assembled transcripts were aligned to the KOG database in which orthologous gene products were classified. In total, 18,950 (29.01%) unigenes were assigned to 25 KOG categories. Among the matched sequences, 9411 unigenes were assigned to the KOG category of signal transduction mechanisms, which represented the largest functional group, followed by general function prediction (6868), post-translational modification, protein turnover, chaperones (3657), transcription (2627), intracellular trafficking, secretion, and vesicular transport (2275), and other classifications (Fig. 3).

KEGG pathway analysis was performed to elucidate the probable functional status of the assembled transcripts. In total, 17,164 (26.28%) annotated unigenes of cobia with significant matches were assigned to five main categories that included 227 KEGG pathways. Among the five main categories that identified, metabolism accounted for the highest number of unigenes (7404), followed by environmental information processing (3683), cellular processes (3184), organismal systems (2415), and genetic information processing (2113). As shown in the S2 Appendix, the five main categories contained 33 sub-categories, unigenes in which were significantly engaged in global and overview (3423), signal transduction (2486), signaling molecules and



**Fig. 1** Top 10 species distribution matched against the Nr database



interaction (1115), cellular community (1066), and endocrine system (1037).

#### Identification of DEGs

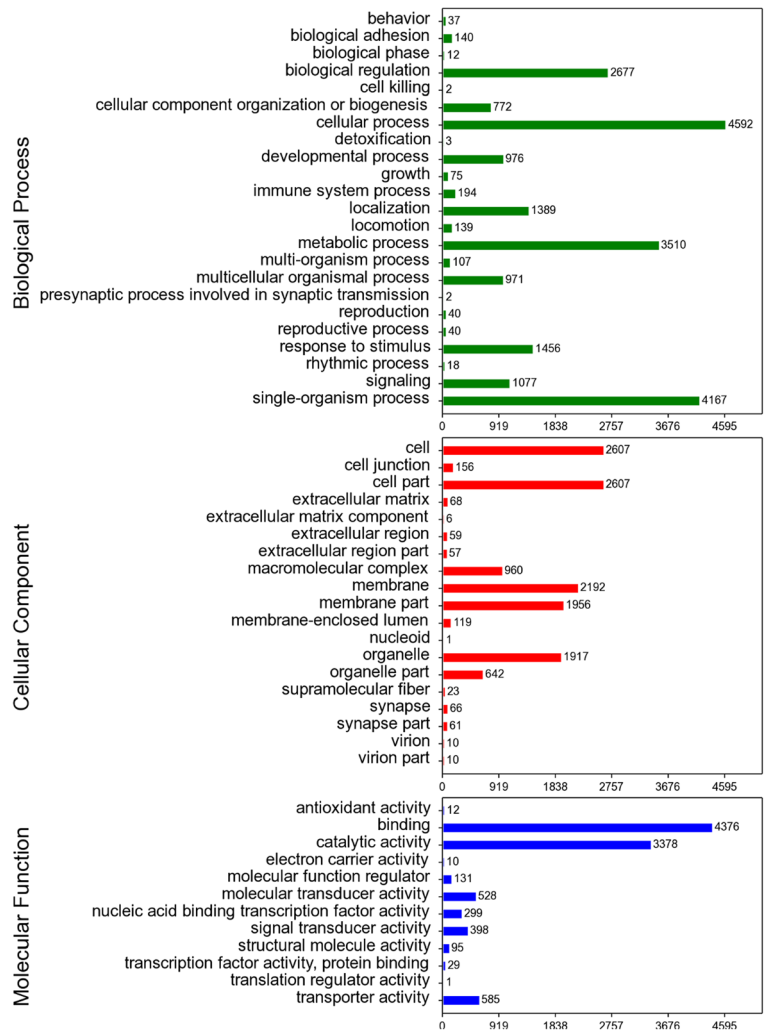
To identify the DEGs, the transcriptome data of cobia from the 30 ppt vs. 10 ppt group and 30 ppt vs. 35 ppt group were analyzed. Between the 30 ppt vs. 10 ppt and 30 ppt vs. 35 ppt groups, we recorded 8805 and 11,866 unigenes, respectively, which were differentially expressed in three experimental osmoregulatory organ libraries. Under high-salt conditions, the three osmoregulatory organs showed similar differential gene expression (Fig. 4a). However, in a low-salt condition, the gill showed the largest effect on gene expression, more than the intestine and kidney (Fig. 4b). Figure 4a and b consisted of unigenes sorted based on FDR-adjusted  $P$  value  $< 0.05$  and  $|\text{Log}_2(\text{fold change})| > 1$ . A Venn diagram describes the shared up- and downregulated genes among tissues (Fig. 5).

GO enrichment analysis was performed for all DEGs belonging to CC, MF, and BP. According to biological process, isoprenoid metabolic process (GO:0006720), actin filament-based movement (GO:0030048), and actin-mediated cell contraction (GO:0070252) were highly ranked in the 30 ppt vs. 10 ppt group GO enrichment, whereas gas transport (GO:0015669), glutamine family amino acid metabolic process (GO:0009064), and glutamine metabolic process (GO:0006541) were highly ranked in the 30 ppt vs. 35 ppt group GO

enrichment. According to molecular function, tetrapyrrole binding (GO:0046906) was markedly enriched in both 30 ppt vs. 10 ppt and 30 ppt vs. 35 ppt groups. As for cellular component, membrane (GO:0016020) and intrinsic component of membrane (GO:0031224) was markedly enriched in the 30 ppt vs. 10 ppt group, non-membrane-bounded organelle (GO:0043228), and intracellular non-membrane-bounded organelle (GO:0043232) were markedly enriched in the 30 ppt vs. 35 ppt group. The results of the pathway enrichment test were included in S3 Appendix.

KEGG pathway analysis identifies molecular interaction networks within cells and assists in elucidating the potential biological functions of the analyzed genes (Du et al. 2014). The top 15 KEGG pathways in response to hypo- and hyper-salinity are listed in Tables 3 and 4, respectively. Hypo-salinity regulated many metabolic pathways or processes, which include steroid biosynthesis, nitrogen metabolism, glutathione metabolism, and biosynthesis of amino acids. Concomitantly, several immune pathways were also influenced by hypo-salinity, including cytokine-cytokine receptor interaction, cell adhesion molecules (CAMs), insulin secretion, and antigen processing and presentation (Table 3). However, more metabolic pathways or processes were detected in hyper-salinity cobia, and there were 13 pathways related to metabolic processes. In addition, ribosome biogenesis in eukaryotes and neuroactive ligand-receptor interaction were enriched significantly (Table 4).

**Fig. 2** Histogram of GO classifications of cobia consensus sequences. Results are summarized for the three main GO categories: biological process, cellular component, and molecular function. The left axis indicates the number of genes in each category



Validation of differentially expressed genes

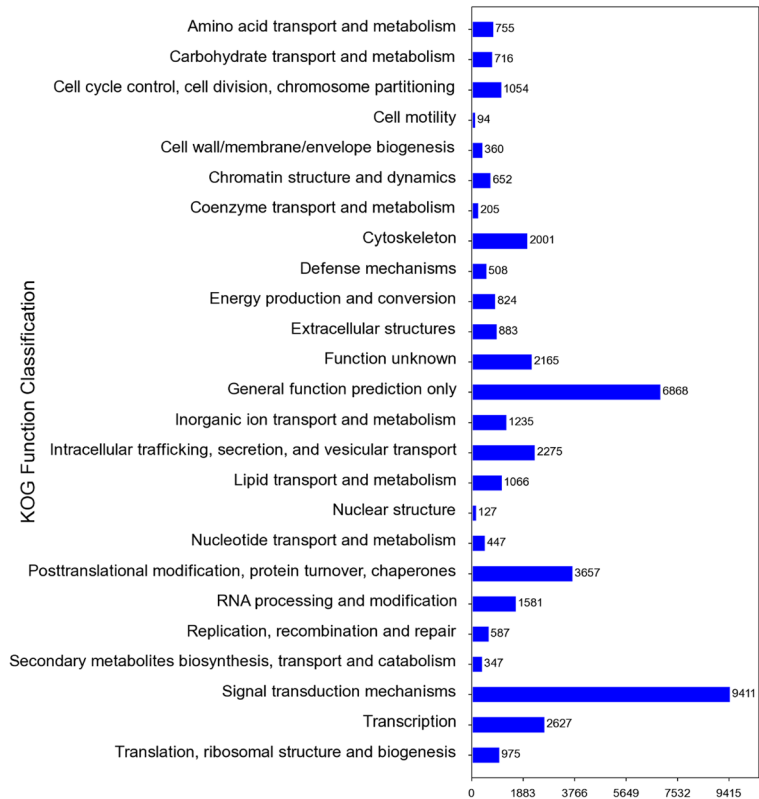
According to the results from real-time PCR assay presented in Fig. 6, the expression profiles of all ten genes were essentially consistent with those determined by Illumina sequencing analysis in terms of fold change and significance of differential expression. Although there was little difference between fold changes, the variation trend was identical. The results also indicated the gene expression analysis based on high-throughput sequencing data is reliable.

Discussion

With advancements in transcriptome sequencing, RNA-seq is being increasingly used in a wide spectrum of

model and non-model animal species (Yau-Chung et al. 2015). In this study, we performed a transcriptome analysis for cobia, a non-model species, to investigate the changes in the gene expression profiles of cobia when responding to salinity changes. Approximately 40,623,5674 raw reads with a Q20% higher than 98.10% and GC% ranging from 48.81 to 50.52% were found, indicative of good sequencing quality (Du et al. 2014). After the removal of low-quality sequences and assembly, 65,318 unigenes were generated with an average unigene length of 1255 bp and an N50 of 2758 bp. Higher N50 length and average unigene length are generally considered to be the basis for setting up de novo transcript assembly. Furthermore, 8805 DEGs and 11,866 DEGs were identified during acclimation to hypo- and hyper-salinity conditions, respectively. The varied expression of genes indicated that many

**Fig. 3** The cluster of orthologous groups (KOG) classification. In total, 18,950 unigenes were successfully annotated to 25 categories



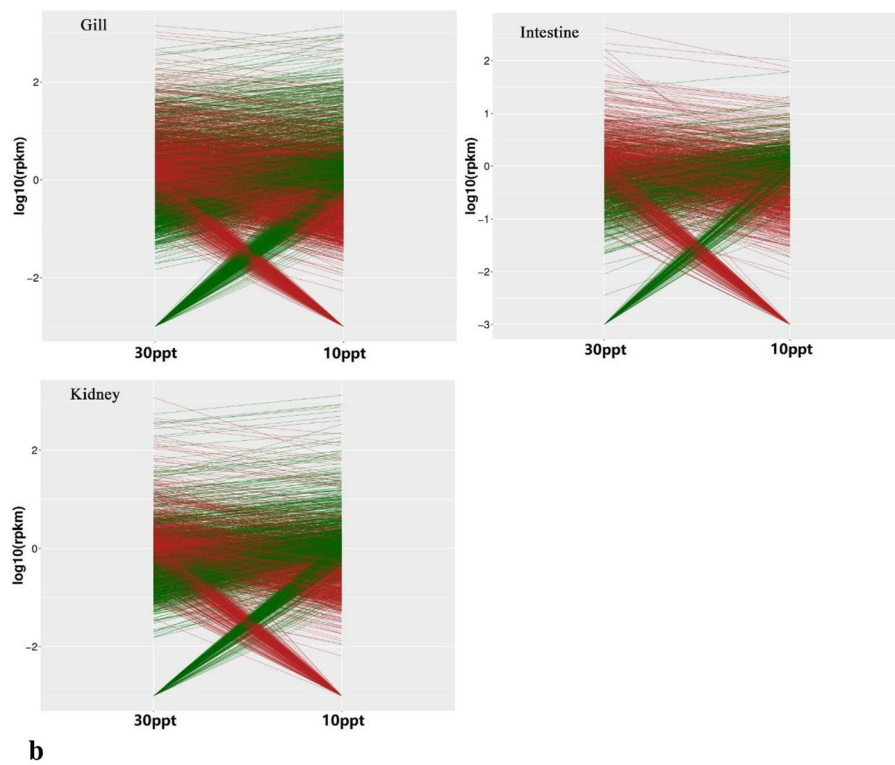
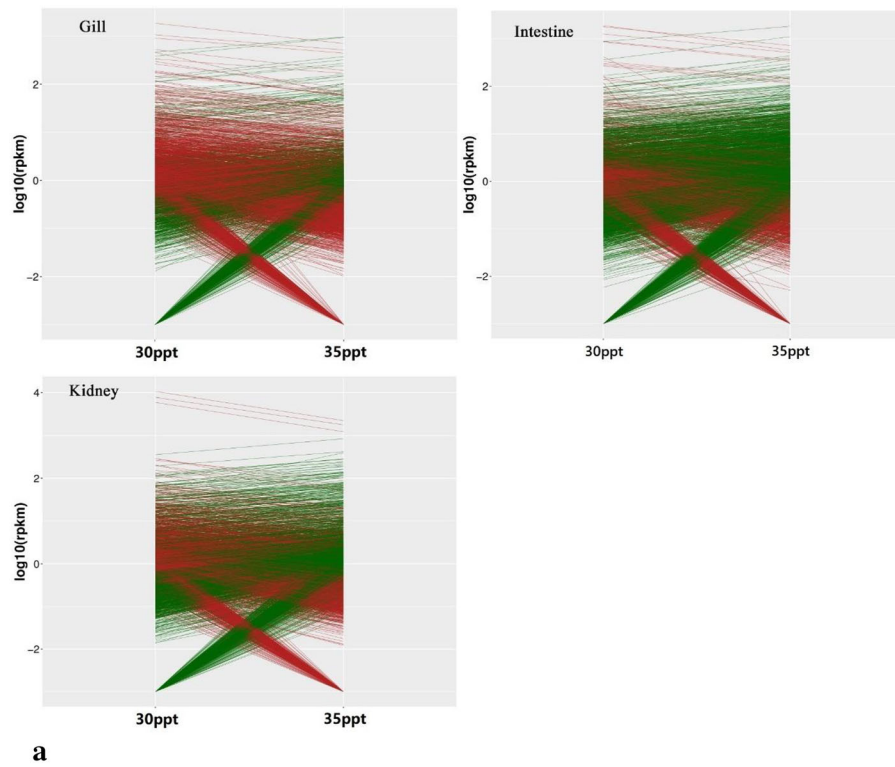
transcripts are differentially regulated in response to salinity acclimation. These DEGs are enriched in certain biological processes, comprising steroid biosynthesis, unsaturated fatty acids metabolism, glutathione metabolism, energy metabolism, osmoregulation, and immune response.

#### Genes involved in steroid biosynthesis

Reports suggested that euryhaline fish can use lipids as energy source under osmotic stress (Lai et al. 2015; Gibbons et al. 2017; Ricardo et al. 2003). Hence, it was not surprising that many cobia genes with major roles in lipid metabolism were upregulated during long-term exposure to elevated and reduced salinity conditions. The KEGG pathway analysis of DEGs also showed that steroid biosynthesis pathways related to lipid metabolism were enriched. Several studies have reported that steroid biosynthesis plays a key role in responding to salinity changes in aquatic animals (Xu et al. 2015). We identified certain steroid biosynthesis-related genes such as *ebp*, *Hsd17b7*, *lss*, *SQLE*, *dher7*, *dher24*, *Fdfl1*, and *cyp51A1* to be upregulated under the hypo-salinity environment. Among these, *cyp51A1*,

*dher24*, *dher7*, and *SQLE* were also upregulated under the hyper-salinity environment. Sterols are important compounds in many biofilms; they act not only as cell membrane components but also as transporters (Si et al. 2018). Emopamil binding protein (*ebp*), also called sterol isomerase, is an essential enzyme in the sterol biosynthesis pathway in eukaryotes (Silve et al. 1996). Lanosterol is the upstream precursor of sterol biosynthesis in all animal and fungal sterols, especially cholesterol (Yoshida et al. 2000). In the present study, *ebp* and *lss*, encoding lanosterol synthase, were upregulated in the gills after adaptation to hypo-salinity, indicating that the level of cholesterol in the gills increased under hypo-salinity conditions. Post-synthesis, lanosterol undergoes 14-demethylation by CYP51 to ultimately produce cholesterol via a series of complex reactions (Si et al. 2018). CYP51 belongs to a family of cytochromes P450 and catalyzes the three-step 14 $\alpha$ -demethylation of lanosterol to produce 4,4-dimethylcholesta-8(9),14,24-trien-3 $\beta$ -ol, a key step in steroid biosynthesis (Koteliensky et al. 2012; Pilloff et al. 2003; Masamrekh et al. 2018). Previous studies have shown that *cyp51* is upregulated to adapt to hypo-salinity conditions in the *Cynoglossus semilaevis* (Si et al. 2018). In





**Fig. 4** **a** The differentially expressed genes in three osmoregulatory organs between 30 and 35 ppt. **b** The differentially expressed genes in three osmoregulatory organs between 30 and 10 ppt

our study, *cyp51A1* was upregulated during acclimation to hypo- and hyper-salinity conditions, indicating that lipid metabolism and lipid levels of cobia increased in both hypo- and hyper-salinity environments. Furthermore, we observed that *lss* and *dhcr24* of the steroid biosynthesis pathway were upregulated in *O. niloticus* and *Cynoglossus semilaevis* (Si et al. 2018; Xu et al. 2015), which is consistent with our current findings.

#### Genes involved in unsaturated fatty acid metabolism

In this study, the metabolic pathways of unsaturated fatty acids such as linoleic acid, alpha-linolenic acid, and arachidonic acid were significantly enriched under high salinity conditions. PLA2G16, a member of the phospholipase A2 family in adipose tissue, can catalyze the hydrolysis of phospholipid diacyl groups to produce free fatty acids such as arachidonic acid (Duncan et al. 2008; Toru et al. 2012). Our studies showed that PLA2G16 was upregulated in the kidneys when adapted to hyper-salinity, indicative of active synthesis of unsaturated fatty acids. After the synthesis of unsaturated fatty acids, the EETs, produced by CYP2J-mediated catalysis of arachidonic acid, play an important role in protecting vascular endothelial cells and regulating the metabolism of water and salt in the kidney. The CYP2J enzymes are mainly involved in the metabolism of endogenous substances, especially arachidonic acid and linoleic acid metabolism (Scarborough et al. 1999). In our study, CYP2J6 was upregulated in the intestine, suggesting that linoleic acid and arachidonic acid metabolism increased in hyper-salinity environment.

#### Genes involved in glutathione metabolism

Enrichment of glutathione metabolism was observed under both hypo- and hyper-salinity conditions. GST2, GPx4, and other antioxidant genes were identified as DEGs. GST is an important detoxifying enzyme in organisms. It combines glutathione with various electrophilic compounds and increases their solubility, thereby promoting their excretion from cells. GST also possesses glutathione-dependent peroxidase activity and protects the body from endogenous peroxide damage (Yang et al. 2001). GPx4 is a GSH-Px family isoenzyme, which can bind to lipid peroxides in biofilms and its lipoproteins, and protects the lipoproteins in cells and cell membranes from peroxide-

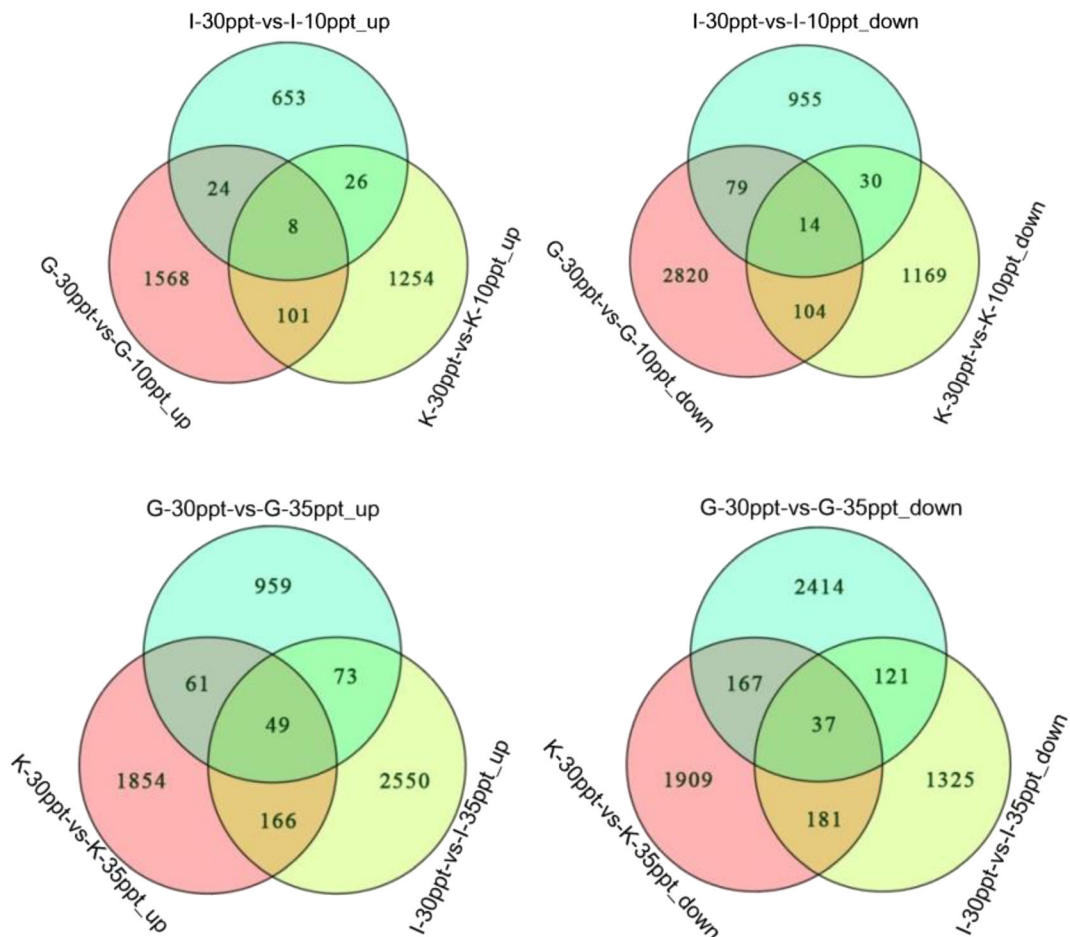
mediated damage. In this study, as GST2 and GPx4 were upregulated by changes in salinity, we conjectured that hyper-salinity stress may cause oxidative stress, and GST2 and GPx4 may play an important role in avoiding oxidative damage in cobia.

#### Genes involved in energy metabolism

For maintaining homeostatic and osmotic balance, ions and osmoregulatory processes are regulated by several transport proteins and enzymes that require large amounts of energy for their synthesis and operation (Tseng and Hwang 2008). Citrate synthase (CS) and isocitrate dehydrogenase (IDH) are important elements in the tricarboxylic acid cycle (TCA) (Nguyen et al. 2016). In our study, these genes were upregulated during salinity exposure, which indicated that energy is required during osmoregulation, as pyruvate is converted to acetyl-CoA by CS and IDH, and then to citrate/isocitrate in the mitochondria (Nguyen et al. 2016). Our results are similar to the study on *O. mossambicus*, in which CS activity was enhanced in isolated gill epithelial cells 2 weeks after transfer to seawater (Nguyen et al. 2016). Furthermore, we also observed that nitrogen metabolism of cobia was significantly enriched and that *Cps1* was upregulated during salinity changes. We speculated that the decrease in ammonia excretion rate during salinity change may lead to an increase in plasma ammonia concentration. Ammonia is converted to urea by the urea cycle in cobia and then excreted from the body. CPS1 is a key rate-limiting enzyme in the urea cycle, and increased expression of CPS1 may reduce the toxicity of high ammonia concentration in cobia when salinity changes.

#### Genes involved in osmoregulation

Osmoregulation is required to actively regulate osmotic pressure by maintaining the balance of intracellular solute concentration when adapting to the surrounding environment (Si et al. 2018). Aquaporins (AQP) constitute a small hydrophobic family that allows osmotic driving water and small solutes to pass through biofilms and is found in all living organisms (Calvanese et al. 2013). The upregulation of AQP1 in the kidney of cobia after adaptation to hypo-salinity indicated that AQP1 may be a regulator in teleost fish after salinity challenge (Wong et al. 2014). Aqp3 was downregulated in the



**Fig. 5** Up- and downregulated genes in different tissues of cobia under salinity challenge

intestine when adapted to hypo-salinity, which may be required to prevent loss of glycerol from the cell (Campbell et al. 2008). Another gene, *slc5a8* (SMCT), belongs to the  $\text{Na}^+$ /glucose co-transporter gene family and mediates the transport of various mono-carboxylates, including nicotinate, short-chain fatty acids, and lactate (Halestrap 2013). Its downregulation in the intestine when adapted to hypo-salinity and upregulation in the kidney when adapted to hyper-salinity suggested that *slc5a8* was involved in osmotic regulation during salinity adaptation.

Transporters transporting ions through the plasma membrane are essential for osmoregulation (Si et al. 2018). During changes in salinity, cells rely on transporters and accumulate intracellular permeants, including taurine, myo-inositol, betaine, and sorbitol, to balance any osmotic change (Alfieri and Petronini 2007; Ho 2010). The sodium/chloride-dependent taurine

transporter (SLC6A6) was specific for taurine (Hosoi et al. 2010). Previous studies have shown that the taurine transporter mRNA was highly expressed in the gill of seawater-acclimated Japanese eel (*Anguilla japonica*), and that the taurine transporter mRNA level increased significantly with environmental osmotic pressure of the carp cell line (*Cyprinus carpio*) (Nguyen et al. 2016). In cobia, we observed an increase in the expression of SLC6A6 in the intestine when adapted to hyper-salinity, indicated the role of the taurine pumping mechanisms in response to changes in the external osmotic environment. In addition to taurine, the accumulation of myo-inositol in the cell is maintained by the sodium/myo-inositol co-transporter (SMIT/SLC5A3), a plasma membrane protein (Coady et al. 2002). This ion transporter has been extensively studied in humans, yeast, and some mammalian species (Nguyen et al. 2016). Previous studies have shown that SMIT can act

**Table 3** Summary of the top 15 enriched KEGG pathways between 30 and 10 ppt

Pathway	Candidate genes with pathway annotation (1032)	All genes with pathway annotation (7875)	<i>P</i> value
Steroid biosynthesis	18 (1.74%)	29 (0.37%)	0.000000
Cytokine-cytokine receptor interaction	72 (6.98%)	278 (3.53%)	0.000000
Nitrogen metabolism	13 (1.26%)	23 (0.29%)	0.000001
Glutathione metabolism	22 (2.13%)	66 (0.84%)	0.000018
Cell adhesion molecules (CAMs)	59 (5.72%)	270 (3.43%)	0.000037
Biosynthesis of amino acids	34 (3.29%)	137 (1.74%)	0.000134
Metabolic pathways	288 (27.91%)	1837 (23.33%)	0.000140
Insulin secretion	5 (0.48%)	6 (0.08%)	0.000205
Pancreatic secretion	4 (0.39%)	4 (0.05%)	0.000293
Antigen processing and presentation	6 (0.58%)	9 (0.11%)	0.000295
Biosynthesis of secondary metabolites	102 (9.88%)	567 (7.2%)	0.000361
Biosynthesis of antibiotics	63 (6.1%)	318 (4.04%)	0.000400
Estrogen signaling pathway	7 (0.68%)	13 (0.17%)	0.000543
Arginine biosynthesis	14 (1.36%)	44 (0.56%)	0.001019
Metabolism of xenobiotics by cytochrome P450	15 (1.45%)	52 (0.66%)	0.002078

as a controller to maintain intracellular concentration of myo-inositol and is upregulated as a hyperosmotic stress response (Nguyen et al. 2016). In our study, the cobia *SLC5A3* was significantly upregulated in the gill and kidney at 35 ppt. This indicated that the gene is involved in the salinity acclimation of cobia.

#### Genes involved in immune response

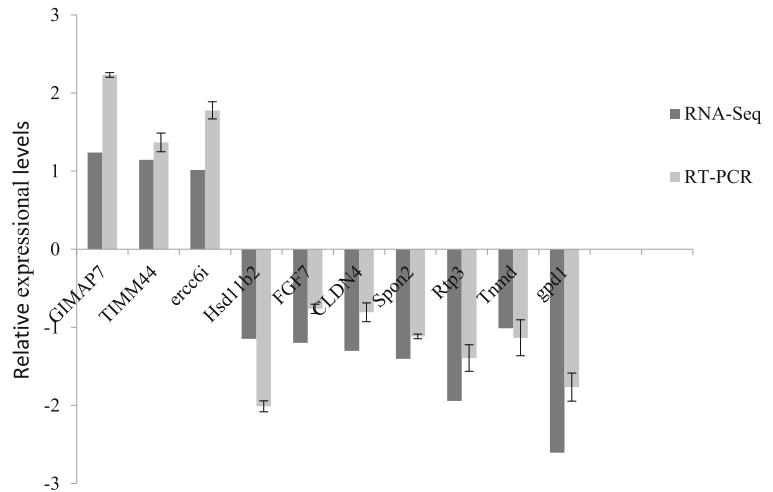
Previous studies have shown that juvenile cobias reared at 5 ppt salinities are more susceptible to stressors or diseases (Denson et al. 2003). We observed that several immune pathways were affected

**Table 4** Summary of the top 15 enriched KEGG pathways between 30 and 35 ppt

Pathway	Candidate genes with pathway annotation (1405)	All genes with pathway annotation (7875)	<i>P</i> value
Nitrogen metabolism	13 (0.93%)	23 (0.29%)	0.000034
Arginine biosynthesis	19 (1.35%)	44 (0.56%)	0.000080
Biosynthesis of amino acids	41 (2.92%)	137 (1.74%)	0.000337
Ribosome biogenesis in eukaryotes	29 (2.06%)	89 (1.13%)	0.000532
Biosynthesis of secondary metabolites	131 (9.32%)	567 (7.2%)	0.000582
Linoleic acid metabolism	12 (0.85%)	27 (0.34%)	0.001224
Biosynthesis of antibiotics	78 (5.55%)	318 (4.04%)	0.001365
Microbial metabolism in diverse environments	67 (4.77%)	266 (3.38%)	0.001419
Glutathione metabolism	22 (1.57%)	66 (0.84%)	0.001733
Metabolic pathways	369 (26.26%)	1837 (23.33%)	0.002475
Alpha-linolenic acid metabolism	11 (0.78%)	26 (0.33%)	0.003140
Galactose metabolism	19 (1.35%)	58 (0.74%)	0.004332
Arachidonic acid metabolism	21 (1.49%)	68 (0.86%)	0.006048
Neuroactive ligand--receptor interaction	90 (6.41%)	395 (5.02%)	0.006180
Steroid biosynthesis	11 (0.78%)	29 (0.37%)	0.008378

when cobia encountered hypo-salinity but not hyper-salinity. Chemokines are an important part of innate immunity in fish, which mainly mediate the chemotactic migration and activation of monocytes, phagocytes, lymphocytes, and granulocytes, and induce all types of white blood cells to reach the infected area and complete the repair of damaged tissue. We observed that a large number of chemokines were upregulated at low salinity, such as CCR4, XCR1, IL1R2, IL1B, IL21R, IL10, and CXCL8. As many chemokines were upregulated at hypo-salinity, we speculated that hypo-salinity may lead to the

**Fig. 6** Validation of differentially expressed genes in RNA-seq by real-time PCR



enhancement of immune response in cobia. Therefore, we can further speculate that the adaptive response, rather than the negative response, may occur at 10 ppt.

## Conclusions

RNA-seq is a rapid and revolutionary technology for transcriptome analysis relative to traditional methods. In this paper, transcriptomic response of cobia to different salinity concentrations (10, 30, and 35 ppt) was investigated following the application of RNA-seq, and abundant differentially expressed genes and signaling pathways related to salinity adaptation were detected. Among the top 15 enriched KEGG pathways, metabolism was the most affected pathway by salinity changes. Furthermore, the bioinformation of transcriptome in this study will be of great value in understanding the mechanisms of salinity adaptation in cobia and will provide genomic resource for further investigations regarding fish biology.

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**Availability of data and materials** The RNA sequence data supporting the conclusions of this article are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA): SRP202920, [https://submit.ncbi.nlm.nih.gov/?logout\\_from=%2Fsubs%2F](https://submit.ncbi.nlm.nih.gov/?logout_from=%2Fsubs%2F).

**Authors' contributions** ZW, GC, JZ, CP, JH, HZ, and QM designed the experiments. DC, JL, and BH conducted the

experiments. DC and ZW prepared the manuscript. All authors read and approved the final manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that there are no conflicts of interest.

**Ethics approval and consent to participate** All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Guangdong Ocean University. The field studies did not involve endangered or protected species.

**Abbreviations** *bp*, base pairs (measuring unit); *DEGs*, differentially expressed genes; *RPKM*, reads per kb per million reads; *FDR*, false discovery rate; *GO*, gene ontology; *KEGG*, Kyoto Encyclopedia of Genes and Genomes; *NCBI*, National Center for Biotechnology Information; *RPKM*, reads per kilobase per million reads; *RNA-seq*, RNA-sequencing; *Ebp*, emopamil binding protein;; *CS*, citrate synthase; *IDH*, isocitrate dehydrogenase; *LDH*, lactate dehydrogenase

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