



# Transcriptome Analysis to Study the Molecular Response in the Gill and Hepatopancreas Tissues of *Macrobrachium nipponense* to Salinity Acclimation

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*Macrobrachium nipponense* is an economically important prawn species and common in Chinese inland capture fisheries. During aquaculture, *M. nipponense* can survive under freshwater and low salinity conditions. The molecular mechanism underlying the response to salinity acclimation remains unclear in this species; thus, in this study, we used the Illumina RNA sequencing platform for transcriptome analyses of the gill and hepatopancreas tissues of *M. nipponense* exposed to salinity stress [0.4‰ (S0, control group), 6‰ (S6, low salinity group), and 12‰ (S12, high salinity group)]. Differentially expressed genes were identified, and several important salinity adaptation-related terms and signaling pathways were found to be enriched, such as “ion transport,” “oxidative phosphorylation,” and “glycometabolism.” Quantitative real-time PCR demonstrated the participation of 12 key genes in osmotic pressure regulation in *M. nipponense* under acute salinity stress. Further, the role of carbonic anhydrase in response to salinity acclimation was investigated by subjecting the gill tissues of *M. nipponense* to *in situ* hybridization. Collectively, the results reported herein enhance our understanding of the mechanisms via which *M. nipponense* adapts to changes in salinity.

**Keywords:** *Macrobrachium nipponense*, transcriptome, salinity, crustaceans, carbonic anhydrase

## INTRODUCTION

The increase in inland salinity owing to human activities (Williams, 2001) has led to major economic, social, and environmental consequences. Inland saline water and saline–alkali land can be potentially used for aquaculture, serving as a source of revenue. Salinity is a key environmental factor in aquatic ecosystems and is known to influence the growth performance, survival, immune system, and respiratory and energy metabolism of crustaceans (Freire et al., 2011; Gao et al., 2016; Li et al., 2017; Koyama et al., 2018; Chen et al., 2019). Some aquatic crustaceans can tolerate a wide range of salinity levels; few previous studies have investigated the effects of salinity on the growth and development of *Macrobrachium nipponense* (Huang Y. H. et al., 2019), *M. rosenbergii* (Chand et al., 2015), *Penaeus monodon* (Le et al., 2009), and *Nephrops norvegicus* (Torres et al., 2011). It appears that aquatic

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crustaceans respond to changes in salinity via osmotic pressure regulation to maintain the balance of water and salt.

The adaptation of aquatic crustaceans to alterations in environmental salinity is a complex process and predominantly involves three types of regulatory pathways. First, osmotic pressure regulation is a pivotal process that controls the permeability and concentration of ions, such as  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Cl}^-$ , in plasma. Ion transporter proteins [ $\text{Na}^+/\text{K}^+$ -ATPase (NKA) and carbonic anhydrase (CA)] in the membranes of the epithelium and antennal glands are the main sites for regulating ion permeation in crustaceans in response to salinity acclimation (McNamara et al., 2015; Huang Y. et al., 2019). The CA enzyme appears to be a central molecular component in the adaptations to low salinity found in euryhaline crustaceans (Henry, 2001; Pongsomboon et al., 2009; Mitchell and Henry, 2014). Second, the energy channeled by crustaceans to respond to salinity acclimation leads to a significant reduction in growth, considering that the energy budget for osmotic adjustment is much higher (Ye et al., 2009; Ma et al., 2021). Therefore, fluctuations in water salinity seem to inhibit energy metabolism, which may cause oxidative damage (Choi et al., 2008). Finally, neuropeptides and their G protein-coupled receptors in the central nervous system regulate the physiological response of crustaceans to salinity acclimation (Sun et al., 2020; Réalis-Doyelle et al., 2021; Tong et al., 2022).

Transcriptome sequencing has been widely applied to develop molecular resources for non-model organisms with biological and economic importance (Riesgo et al., 2012; Sun et al., 2015; Liu et al., 2021). Transcriptome sequencing has been used to analyze the molecular mechanisms underlying the response to changes in salinity in many economically important species, such as *Scylla paramamosain*, *Litopenaeus vannamei*, *Eriocheir sinensis*, and *M. rosenbergii* (Zhang et al., 2015; Liu et al., 2016; Wang et al., 2018; Yang et al., 2019; Sun et al., 2020). To the best of our knowledge, although genomes information of the oriental freshwater prawn *M. nipponense* has been reported (Jin et al., 2021), salinity acclimation-related transcriptome data for gill and hepatopancreas tissues of the *M. nipponense* remain to be reported. As a member of euryhaline crustaceans, *M. nipponense* is primarily distributed in freshwater and low salt environments in Asia, and it is one of the most economically important aquaculture species in China, with annual production exceeding 200,000 tons and output reaching two billion RMB (Fu et al., 2012). Previous studies on *M. nipponense* have mainly focused on germplasm heredity, nutrition regulation, immunity performance, and resistance to hypoxia stress (Fu et al., 2004; Li et al., 2018; Sun et al., 2018; Zhao et al., 2018). To date, the molecular mechanisms via which *M. nipponense* responds to changes in salinity remain poorly understood.

Herein we performed transcriptome analysis of the hepatopancreas and gill tissues of *M. nipponense* cultivated at three different salinities using the Illumina platform to generate a *de novo* assembly. Our objectives were as follows: 1) construct hepatopancreas and gill tissue libraries of *M. nipponense* cultivated at different salinities; 2) identify differentially expressed genes (DEGs) and pathways that may play a key role in salinity acclimation in *M. nipponense*; and 3) validate

target transcripts involved in osmotic pressure regulation, energy metabolism, and antioxidant defense that might have critical functions under conditions of acute salinity stress. The results of this study could improve our understanding of the molecular mechanisms used by *M. nipponense* to adapt to salinity acclimation, which is bound to enhance the sustainability of aquaculture production of *M. nipponense*.

## MATERIALS AND METHODS

### Experimental Animals and Acclimation

Juvenile prawns (*M. nipponense*) were obtained from a farm in Shanghai (Qingpu) and acclimated to laboratory conditions for 14 days in freshwater (temperature,  $24 \pm 1^\circ\text{C}$ ; pH,  $7.7 \pm 0.6$ ; dissolved oxygen,  $6.5 \pm 0.5$  mg/L). Thereafter, 360 healthy prawns ( $2.15 \pm 0.20$  g wet weight) were randomly divided into 12 tanks (30 prawns/tank), and the tanks were randomly assigned to three groups (3 tanks/group). Salinity was gradually adjusted on the same day to reach target salinity levels for each group: S0 =  $\sim 0\text{‰}$  (control group), S6 =  $6\text{‰} \pm 0.2\text{‰}$  (low salinity), and S12 =  $12\text{‰} \pm 0.2\text{‰}$  (high salinity). Salinity and water quality were maintained as previously described (Huang Y. H. et al., 2019), and prawns were given commercial feed (Zhejiang Tongwei Feed Group CO, Ltd.) twice a day for 1 week at a ratio of 6%–8% of their body weight. After 4 weeks of acclimation, hepatopancreas and gill tissues were obtained from each treatment group in triplicates, rapidly frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until needed.

### RNA Extraction, Library Construction, and Sequencing

The gill and hepatopancreas tissues were used for total RNA extraction. Briefly, total RNA was extracted using TRIzol (Invitrogen, CA, United States), as per manufacturer instructions. RNA quality and quantity were determined with a NanoDrop 2000 (Thermo Fisher Scientific Inc, United States) spectrophotometer; all  $\text{OD}_{260}/\text{OD}_{280}$  values were in the range of 1.9–2.0. RNA integrity was assessed by 1% agarose gel electrophoresis, and 18 cDNA libraries were prepared using 2.5  $\mu\text{g}$  total RNA, following the protocol of the Illumina TruSeq™ RNA Sample Preparation Kit. The libraries thus obtained were sequenced on Illumina HiSeq 2500 (2  $\times$  150 bp read length; Illumina, Inc, San Diego, CA, United States) and paired-end reads were finally generated (Sun et al., 2020). The raw sequencing data have been deposited in the NCBI Sequence Read Archive (Accession no. SRP251206).

### De Novo Transcriptome Assembly and Gene Annotation

Raw data were processed to obtain clean reads, as follows: 1) joint sequences in reads were removed, 2) low-quality reads (bases with Q-value < 20) at the end of the sequence (3'-end) were eliminated, 3) reads with an N ratio of >10% were removed, and 4) reads containing adaptor

sequences and sequences <75 bp after quality trimming were discarded.

The clean reads thus obtained were used for sequence assembly with Trinity v2.3.2 (Plymouth, MA, United States); default parameters were used for assembly generation, and the minimum contig length was 200 bp (Grabherr et al., 2011). All unigenes were annotated based on the following databases with a cut-off E-value of  $10^{-5}$ : NCBI non-redundant (Nr) (<http://www.ncbi.nlm.nih.gov>), Swiss-Prot (<http://www.expasy.ch/sprot>), Clusters of Orthologous Groups (COG) (<http://www.ncbi.nlm.nih.gov/>), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg>). For Pfam domain/family annotation, predicted protein sequences were submitted to search against HMM profiles in the Pfam database v27.0 using HMMER v3.0 (Finn et al., 2017). Further, Blast2GO was used for gene ontology (GO) analysis (<http://www.geneontology.org/>), and COG classification and signal pathway annotation of unigenes were performed by conducting BLASTx searches against the COG and KEGG databases, respectively. Assembly metrics and annotation completeness were obtained using BUSCO 3.0.1 (Simão et al., 2015) with the arthropoda\_odb9 dataset.

## Identification of DEGs and Enrichment Analysis

The expression of all unigenes was estimated by calculating read density as fragments per kilobase of transcript per million reads using the RSEM program (Li and Dewey, 2011). To identify DEGs, false discovery rate (FDR)  $\leq 0.001$  and two-fold change ( $\log_2$  ratio)  $\geq 1$  or  $\leq -1$  defined significant differences in gene expression levels (Anders and Huber, 2010). Pearson correlation coefficient was determined as per the gene expression level of each sample, and hierarchical clustering was applied to classify samples with high similarity levels till all DEGs were clustered. Functional enrichment analysis of DEGs involved GO and KEGG pathway enrichment analyses, which were performed using Goatools (<https://github.com/tanghaibao/GOatools>) and KOBAS (<http://kobas.cbi.pku.edu.cn/home.do>), respectively ( $p \leq 0.05$ ).

## Identification of Simple Sequence Repeat Markers

The MicroSATellite search module (<http://pgrc.ipk-gatersleben.de/misa/>) was used to identify SSR markers and for primer design (Li et al., 2009). Mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs were designed using default parameters; the minimum repeat numbers for these SSRs were 10, 6, 5, 5, 5, and 5, respectively.

## Quantitative Real-Time PCR (qPCR)

In order to observe expression trends of the DEGs, 12 DEGs with high expression levels from transcriptome were selected to observe genes expression profile according to acute salinity stress (18‰) using qPCR. RNA samples were extracted from hepatopancreas and gill tissue samples, which were collected at 3,

**TABLE 1** | The specific primers used to in this study.

Primer Name	Sequence (5'→3')
Carbonic anhydrase	F:TGGGTGTTTGACGGAGTGTAAAGG R:CCTCTGCGGTGACGATGTTGAC
Heat shock protein 70	F:GCCTCTGCTCAAGCTAGTGT R:TGGTGAACCTCCAACAAGG
Catalase	F:TCGTGGCTTCGCTGTCAAGTTC R:GGTGTGTTGCTGATTCTCTCTTG
copper/zinc superoxide dismutase	F:GGCTCATTACAACCCAGACGGATTG R:AGTTCCATCCTCACCGCTCTCG
manganese superoxide dismutase	F:TGTGGGTGTGAAAGTTCTGGTTG R:GGGTCCCTGTTTGGCAAGTG
Glucose transporter 1	F:CCAACGGGTGTCTGACACCTCC R:GCACCTACTGAAAATAGAGACA
glutathione peroxidase 3	F:AGAGGTTAATGGCGAGAAGGAACAC R:AGGCGTGGATCAGCGAAAG
Hexokinase	F:CCACCCTCACTTCCACAATCTCATG R:GCAACAGCAGCAACCAAGCAG
lactate dehydrogenase	F:CCAGAGGAGTGTGTATGCGGTTTC R:GTTGGTCTTCTCGGCGTCTGTC
6-phosphofructokinase	F:GCTCACTTGCTGTGGATCAGTTAG R:ATCTTCGCCGCTCTCTCTCTG
pyruvate dehydrogenase	F:ACAACAAGAGTAGCAGCAGGTCAAC R:TTCATCCCGCTCCATTTCTTATCC
Na <sup>+</sup> /K <sup>+</sup> + ATPase	F:CAGCCCAAGACGACATTCCCATC R:GTCACCGCAAGCCAATTCACAC
$\beta$ -actin	F:TATGCACTTCTCATGCCAT R:AGGAGGCGGAGTGTGCAT

6, 12, 24, 48, and 96 h in *M. nipponense* responded to acute salinity stress. Specific primers were designed using Primer Premier 5.0. The  $\beta$ -actin gene served as an internal control for normalizing experimental results. **Table 1** lists all gene primers.

First-stranded cDNA was synthesized from 1  $\mu$ g RNA using the PrimeScript<sup>®</sup> RT reagent kit (Takara, DRR037A, Dalian, China). qPCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, C11744-500, CA, United States); the 20- $\mu$ L reaction mixture consisted of 10  $\mu$ L buffer (qPCR SYBR Green Master Mix), 0.4  $\mu$ L of forward and reverse primers each, and 9.2  $\mu$ L template cDNA. The amplification cycle comprised the following steps: 40 cycles of 95°C for 30 s, 95°C for 10 s, and 60°C for 30 s  $\beta$ -actin served as the internal reference. The efficiency of amplification for each primer was estimated by constructing a standard curve using serial dilutions of pure cDNA samples; the efficiency values ranged from 0.9 to 1.02. The  $2^{-\Delta\Delta CT}$  method was used to calculate relative expression levels (Livak and Schmittgen, 2001).

## In Situ Hybridization

*In situ* hybridization was performed to analyze the CA mRNA in the gill, which showed the highest expression level in those two tissues by qPCR analysis. *M. nipponense* gill tissue samples were obtained as described earlier, fixed in 4% paraformaldehyde in PBS, and incubated at 4°C overnight. The samples were then analyzed using *in situ* hybridization, according to our previous study (Sun et al., 2016). The anti-sense and sense probes of CISH (Chromogenic *in-situ* hybridization) study with DIG signal were designed by Primer 5 software, and synthesized by Shanghai Sangon Biotech Company. *In situ* hybridization experiments

**TABLE 2** | Basic statistics of RNA-seq reads in *M. nipponense*.

Sample	Raw Reads	Raw Bases	Clean Reads	Clean Bases	Q20% <sup>a</sup>	Q30% <sup>b</sup>	GC% <sup>c</sup>
LG-1	39,171,432	5,875,714,800	35,393,414	5,275,374,022	98.52	94.95	44.82
LG-2	46,201,618	6,930,242,700	40,744,942	6,065,221,795	98.46	94.76	44.77
LG-3	47,266,458	7,089,968,700	42,426,326	6,321,349,979	98.5	94.9	44.6
HG-1	40,253,752	6,038,062,800	37,071,372	5,531,352,170	98.57	95.01	42.04
HG-2	59,588,288	8,938,243,200	54,053,198	8,060,452,409	98.62	95.17	42.89
HG-3	41,314,824	6,197,223,600	38,706,680	5,778,650,912	98.68	95.38	44.7
FG-1	40,668,266	6,100,239,900	37,999,698	5,657,273,336	98	93.61	44.27
FG-2	53,989,276	8,098,391,400	50,753,610	7,557,280,709	98.06	93.76	44.43
FG-3	41,389,072	6,208,360,800	38,618,716	5,749,922,485	98.11	93.9	43.8
LH-1	41,580,366	6,237,054,900	38,764,410	5,786,335,185	98.35	94.48	46.32
LH-2	38,308,492	5,746,273,800	35,572,040	5,308,698,607	98.57	95.07	46.46
LH-3	40,942,340	6,141,351,000	37,801,184	5,639,162,660	98.54	94.99	46.1
HH-1	38,824,410	5,823,661,500	36,002,966	5,377,877,295	98.62	95.17	43.98
HH-2	45,160,524	6,774,078,600	42,106,088	6,290,257,258	98.7	95.41	44.46
HH-3	44,719,422	6,707,913,300	40,445,100	6,038,081,919	98.58	95.08	43.74
FH-1	37,313,474	5,597,021,100	35,177,300	5,241,056,393	98.13	93.96	44.2
FH-2	49,288,664	7,393,299,600	46,567,284	6,944,014,083	98.12	93.92	45.86
FH-3	44,445,096	6,666,764,400	42,169,950	6,288,958,016	98.28	94.36	45.09
Sum	790,425,774	118,563,866,100	730,374,278	108,911,319,233	/	/	/
Average	43,912,543	6,586,881,450	40,576,348	6,050,628,846	98.42	94.66	44.59

LG, low-salinity gill tissue; HG, high-salinity gill tissue; FG, freshwater gill tissue; LH: low-salinityhepatopancreas tissue; HH: high-salinity hepatopancrea tissue; FH, freshwater hepatopancrea tissue.

<sup>a</sup>Q20%, percentage of bases with Phred value > 20.

<sup>b</sup>Q30%, percent of bases with Phred value > 30.

<sup>c</sup>GC%, percentage of G and C bases among total bases.

were performed in triplicate for each tissue. Slides were examined under light microscope for evaluation.

## Statistical Analysis

All data are presented as means ± standard error (SM). One-way analysis of variance (ANOVA) and Student's t-tests were used to determine whether data were statistically significant ( $p < 0.05$ ) between the control and the treatment groups. And the Dunn-Bonferroni post hoc method following a significant Kruskal-Wallis test was used when the data distribution was skewed.

## RESULTS

### Transcriptome Sequencing and *De Novo* Assembly

On constructing and sequencing the 18 libraries from the three groups, we obtained approximately 790,425,774 raw reads and 118,563,866,100 raw bases, respectively. Analysis using the BUSCO pipeline indicated that >92% arthropoda orthologs were present in the assembled transcriptome [93.2% complete BUSCOs (C)]. After removing adaptor sequences, ambiguous 'N' nucleotides, and low-quality sequences, 730,374,278 clean reads, representing 108,911,319,233 clean nucleotides, were obtained. The average Q30 percentage and GC content were 94.66% and 44.59%, respectively, indicating the high accuracy of our transcriptome data (Table 2). In total, 162,250 unigenes were obtained from the combined transcripts, with the total length being 187,598,373 bp. Among these unigenes, 52,154 (32.14%) were 1–600 bp, 61,901 (38.14%) were 601–1200 bp, and 48,195

(29.72%) were >1200 bp. **Supplementary Tables S1, S2** provide an overview of assembly results. **Supplementary Figure S1** shows specific fragment distribution.

### Gene Annotation and COG Assignment

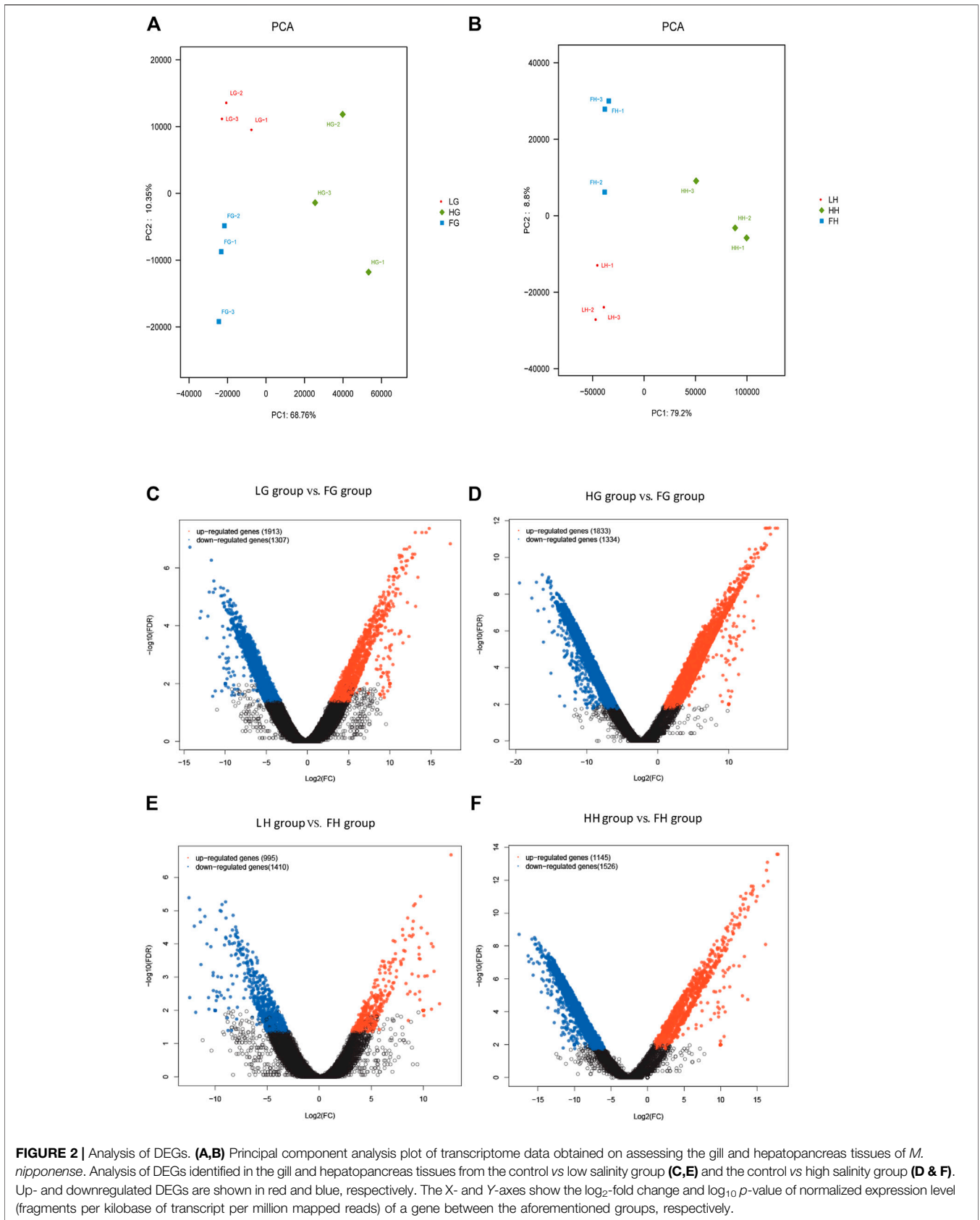
For the unigene sequences obtained by splicing, BLASTx comparison (BLAST+ 2.7.1, E-value <  $10^{-5}$ ) was used for annotation against the COG, GO, KEGG, Swiss-Prot, and NCBI NR databases. In total, 162,250 unigenes were searched; of them, 51,172 (31.54%), 34,276 (21.13%), 10,002 (6.16%), 27,554 (16.98%), and 16,078 (9.89%) were annotated using the Nr, Swiss-Prot, GO, KEGG, and COG databases, respectively (**Supplementary Table S3**). On GO analysis, 10,002 unigenes were enriched into 58 functional subgroups. Further, based on COG analysis, 8,755 unigenes were allocated to 25 COGs (Figure 1A, B).

### DEGs

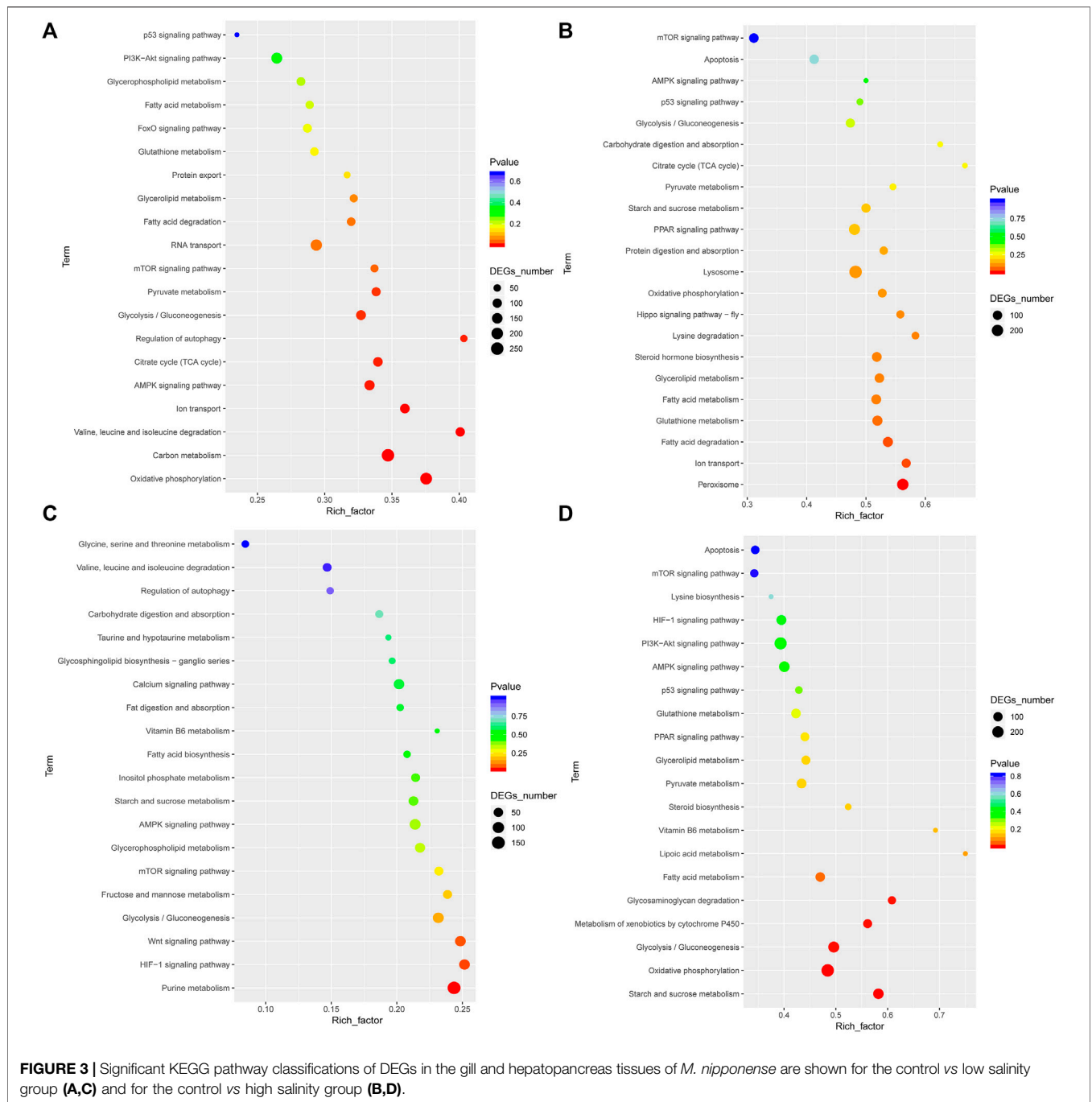
The expression levels of DEGs in the six groups were evaluated using FPKM values: low-salinity gill tissue (LG), high-salinity gill tissue (HG), freshwater gill tissue (FG), low-salinity hepatopancreas tissue (LH), high-salinity hepatopancreas tissue (HH), and freshwater hepatopancreas tissue (FH). All DEGs with the absolute value of  $\log_2$  ratio  $\geq 1$  and FDR  $\leq 0.001$  are shown in **Figure 2**. Overall, 3,220 and 3,167 DEGs were detected on comparing LG vs FG (1,913 up- and 1,307 down-regulated genes) and HG vs FG (1,833 up- and 1,334 down-regulated genes), respectively, and 2,405 and 2,671 DEGs were detected on comparing LH vs FH (955 up- and 1,410 down-regulated genes) and HH vs FH (1,145 up- and 1,526 down-regulated genes), respectively.







**FIGURE 2 |** Analysis of DEGs. **(A,B)** Principal component analysis plot of transcriptome data obtained on assessing the gill and hepatopancreas tissues of *M. nipponense*. Analysis of DEGs identified in the gill and hepatopancreas tissues from the control vs low salinity group **(C,E)** and the control vs high salinity group **(D & F)**. Up- and downregulated DEGs are shown in red and blue, respectively. The X- and Y-axes show the log<sub>2</sub>-fold change and log<sub>10</sub>-p-value of normalized expression level (fragments per kilobase of transcript per million mapped reads) of a gene between the aforementioned groups, respectively.



vs FH and HH vs FH, the significantly enriched pathways were associated with energy metabolism (Figure 3).

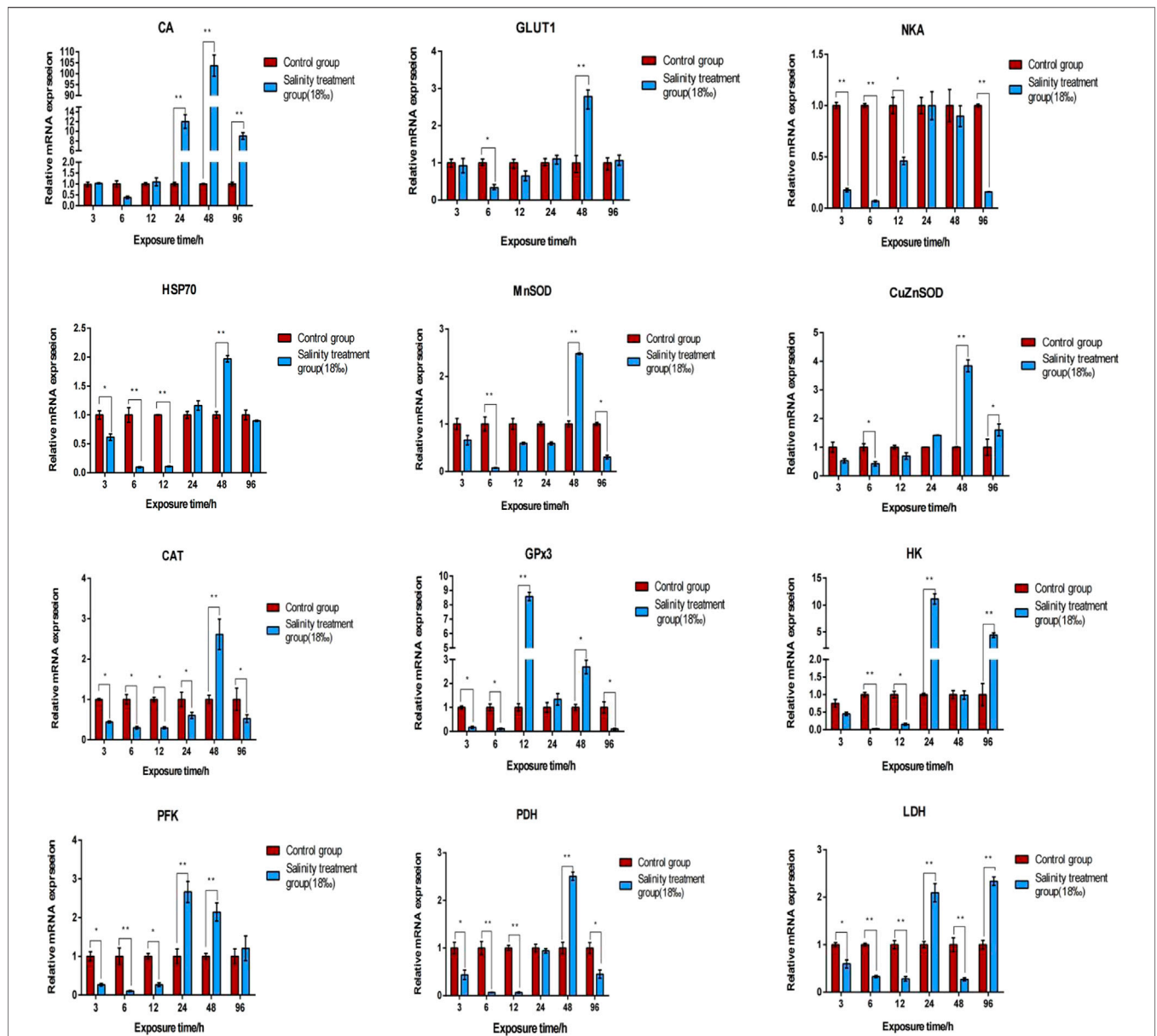
### Analysis of SSRs

In total, 73,804 SSRs were identified. All SSRs were identified in 194,085 unigenes, and 14,923 unigenes contained >1 SSR (Supplementary Table S5). Of the 73,804 SSR motifs identified in *M. nipponense*, the largest fraction consisted of mononucleotides (30,063), followed by di- (28,021) and trinucleotides (14,273). A/T (29,334) was the most common

type of mononucleotide repeat motif, followed by C/G (620), and AG/CT (18,107) was the most common type of dinucleotide repeat motif, followed by AT/AT (4,977) and AC/GT (4,819) (Supplementary Figure S3).

### qPCR Validation

Table S6 lists DEGs identified across all comparative groups. To investigate the gene expression profile in the gill and hepatopancreas tissues of *M. nipponense* in response to 96-h acute salinity stress (18‰), twelve salinity adaptation-



**FIGURE 4** | Validation of the expression profiles of 12 DEGs identified in the gill tissue of *M. nipponense* using qPCR. Log-fold changes are expressed as the ratio of gene expression after normalization to  $\beta$ -actin expression levels. Data are shown as mean  $\pm$  SE (standard error) of tissues in three separate individuals. \*indicates significant difference ( $p < 0.05$ ), \*\*indicates extremely significant difference ( $p < 0.01$ ).

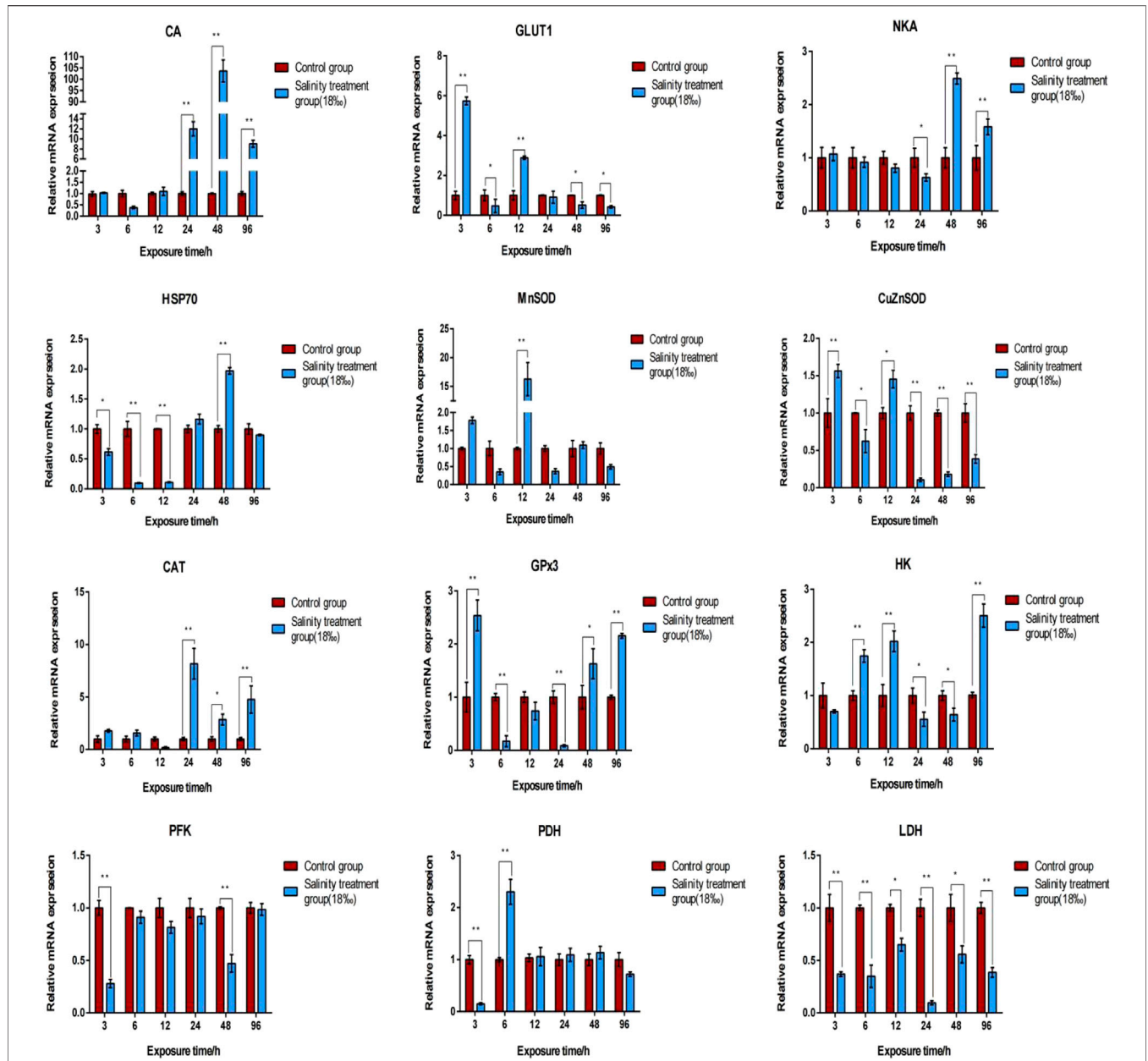
related genes were chosen: CA, NKA, catalase (CAT), Cu/Zn superoxide dismutase (Cu/ZnSOD), Mn superoxide dismutase (MnSOD), glucose transporter (GLUT1), glutathione peroxidase 3 (GPx3), hexokinase (HK), lactate dehydrogenase (LDH), phosphofructose kinase (PFK), pyruvate dehydrogenase (PDH), heat shock protein 70 (HSP70). qPCR results show the effects of salinity on the expression of these genes in the gill (Figure 4) and hepatopancreas (Figure 5) tissues of *M. nipponense*, respectively. We found that genes involved in antioxidant,

glycolysis and osmotic regulation play important role in response acute salinity stress, especially in CA gene.

### Localization of CA mRNA

To further confirm this finding, *in situ* hybridization was performed using frozen gill and hepatopancreas tissues. In case of the gill tissue, no signal was observed when the negative control sense strand probe (Figure 6A) was hybridized with the sense CA probe; however, in response to salinity acclimation, the antisense probe generated a positive signal in the epithelial cell nuclei, cytoplasm, and





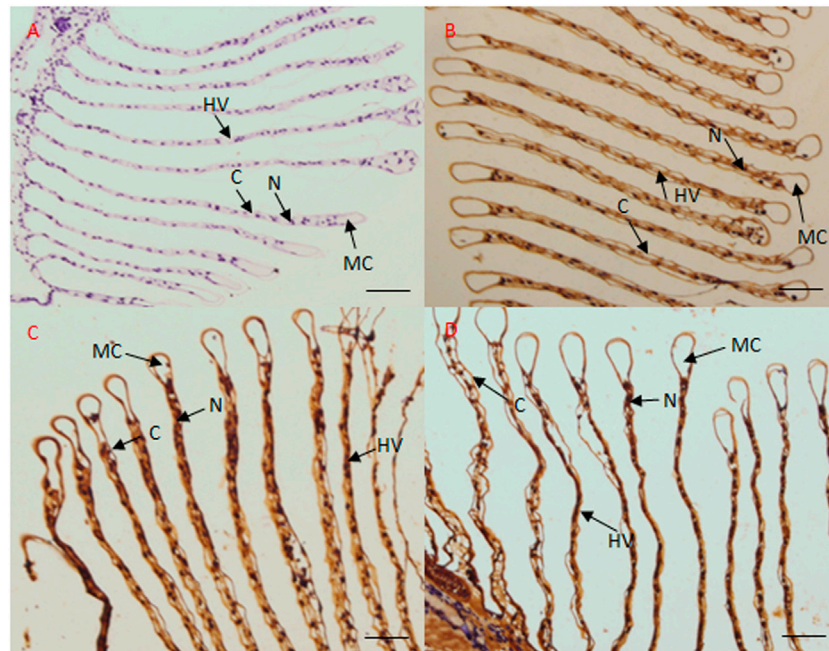
**FIGURE 5** | Validation of the expression profiles of 12 DEGs identified in the hepatopancreas tissue of *M. nipponense* using qPCR. Log-fold changes are expressed as the ratio of gene expression after normalization to  $\beta$ -actin expression levels. Data are shown as mean  $\pm$  SE (standard error) of tissues in three separate individuals. \*indicates significant difference ( $p < 0.05$ ), \*\*indicates extremely significant difference ( $p < 0.01$ ).

hemolymph vessel (**Figure 6B**). Further, in response to high salinity, the antisense probe yielded a signal in the epithelial cell nuclei (**Figures 6C,D**).

## DISCUSSION

Euryhaline crustaceans are a key model organism for investigating fundamental and evolutionarily conserved processes. The chromosome-level genome assembly of *M.*

*nipponense* was recently reported; the total assembled genome size was approximately 4.5 Gb, and a scaffold N50 of 86.8 Mb was produced using the Illumina platform (Jin et al., 2021). Our previous study used the Illumina platform to conduct gene expression profile analyses so as to identify neuropeptides and G protein-coupled receptors from eyestalk tissues of prawns exposed to salinity stress (Sun et al., 2020). Further, a recent study evaluated the effects of salinity, photoperiod, and light spectrum on larval survival, growth, and related enzyme activities in the giant freshwater



**FIGURE 6** | Localization of CA transcripts in the gill tissue of *M. nipponense* by *in situ* hybridization. Sense probes were used as negative controls, which generated no signal (A). In the gill tissue, CA transcripts mainly localized to the epithelial cell nuclei and hemolymph vessel under freshwater (B), low salinity (C), and high salinity (D) conditions. MC, marginal channel; (C) cuticle; N, epithelial cell nuclei; HV, hemolymph vessel. Scale bar: 50  $\mu\text{m}$ .

prawn *M. rosenbergii* (Wei et al., 2021). However, the molecular responses in the gill and hepatopancreas tissues of *M. nipponense* in response to salinity acclimation remain unknown. Few previous studies have attempted to investigate osmotic adaptive response of estuarine crustaceans by mimicking salinity stress in laboratory environments (Li et al., 2015; Hudson et al., 2018; Liu et al., 2022). In case of the oriental river prawn, 14‰ is the tolerable salinity level for normal physiological activities (Huang Y. H. et al., 2019), with the gills and hepatopancreas being the core metabolic tissues and the main sites of ion transport (Pierrot et al., 1995; Gao et al., 2012). Thus, in this study, we identified DEGs in the gill and hepatopancreas tissues of *M. nipponense* subjected to acute salinity stress.

## Enrichment Analysis to Explore Major Biological Associations

Crustaceans tackle changes in environmental salinity, for example, via osmotic pressure regulation, acid–base balance regulation, and respiratory metabolism (Chen and Lin, 1995; Long et al., 2017; Chen et al., 2019). To understand the molecular regulation mechanism of *M. nipponense* in response to salinity acclimation, transcriptome data were obtained by assessing the gill and hepatopancreas tissues of prawns cultured under S0 (freshwater), S6 (low salinity), and S12 (high salinity) conditions. GO and KEGG pathway enrichment analyses led to the identification of differentially enriched pathways, such as “glycolysis/gluconeogenesis,” “TCA cycle,”

and “fatty acid metabolism.” Crustaceans, such as Chinese shrimp *Fenneropenaeus chinensis* (Li et al., 2019) and Pacific white shrimp *L. vannamei* (Chen et al., 2015), evidently increase their dependence on aerobic metabolism for fueling osmoregulation. It appears that glycolipid metabolism reduces osmotic stress in crustaceans by supplying extra energy or adjusting the membrane structure to facilitate osmoregulation in organs such as the gills. Other important GO terms identified in this study included “oxidative phosphorylation” and “peroxisome.” These findings indicated that high salinity induces oxidative stress, maybe leading to oxidative damage and apoptosis in crustaceans (Rivera-Ingraham et al., 2016; Huang et al., 2020).

## Osmoregulation

Decapod crustaceans show variable degrees of euryhalinity and osmoregulatory capacity, and they respond to changes in salinity through anisomotic extracellular regulation and/or cell volume regulation (Cuenca et al., 2021). Osmotic pressure regulation is an important method for crustaceans to adapt to changes in salinity (Malik and Kim, 2021). NKA and CA are widely known ion transporters that play a key role in osmotic pressure balance in crustaceans. Different ions are transported through different ion transport channels (Huang Y. et al., 2019). Herein our transcriptome analysis revealed that in comparison to the control groups, the mRNA expression levels of NKA and CA were significantly up-regulated in the gill and hepatopancreas tissues of *M. nipponense* in the salinity acclimation groups (HG vs FG, LG vs FG, HH vs FH, and LH vs FH). qPCR results indicated that the expression levels of NKA and CA in the gill and

hepatopancreas tissues of *M. nipponense* were significantly upregulated under acute salinity stress conditions as compared to those under freshwater conditions. Similar expression profiles have been reported for other crustaceans, such as *E. sinensis* and *L. vannamei* (Mao et al., 2014; Liu et al., 2015). Collectively, these data suggest that NKA and CA play a key role in osmotic pressure regulation in *M. nipponense* under conditions of acute salinity stress and chronic salinity acclimation. In addition, we found that CA was mainly localized in the epithelial cell nuclei and hemolymph vessels in the gill tissue, suggesting that Carbonic anhydrase (CA) is a ubiquitous enzyme involved in acid-base regulation and osmoregulation in gill tissue of crustacean, which was similar with previous studies in fish (Gilmour, 2012; Ferreira-Martins et al., 2016; Kumar et al., 2020). Thus, we believe that the CA gene can serve as a molecular indicator of acute salinity stress in *M. nipponense* at specific time points.

## Energy Metabolism

Ion transport and ion channel proteins participate in osmotic pressure regulation, which requires abundant energy (Tseng and Hwang, 2008). Glucose metabolism, which involves glycolysis, followed by the TCA cycle and electron transport chain, plays a chief role in supplying energy for osmotic pressure regulation (De Boeck et al., 2000). Little is known of the properties and variation of anaerobic and aerobic metabolism in tissues during osmoregulation. In this study, transcriptome sequencing results indicated that the expression of HK, PFK, PK, PDH, LDH, and GLUT1 was upregulated in the gill and hepatopancreas tissues of *M. nipponense* under low salinity conditions. Under high salinity conditions, the expression of citrate synthase and cytochrome C oxidase was downregulated and that of other glycolysis-related enzymes was upregulated. Moreover, our results demonstrated that the expression of the rate-limiting enzymes PFK and LDH was markedly upregulated under high salinity conditions (Li et al., 2019). It appears that under conditions of acute high salinity stress, glycolysis is the most primitive method for organisms such as prawns and oysters to acquire energy (Chen et al., 2022). However, osmoregulation at high salinity levels might disrupt glucose metabolism. Therefore, exposing prawn to very high salinity conditions, such as during stock enhancement, should be avoided.

## Antioxidant System

Changes in the water environment can induce oxidative stress and antioxidant response in aquatic organisms (Wang et al., 2009; Wang et al., 2020). In response to salinity stress, shrimps produce reactive oxygen species (ROS), which can destroy cells, cause morphological changes in the hepatopancreas, and induce the expression of antioxidant enzymes and proteins (Paital and Chainy, 2010). ROS are thus continuously removed by the antioxidant enzyme defense system, which mainly comprises SOD, CAT, and GPx (Cheng and Chen, 2000; Pan et al., 2005). The process of removing ROS is as follows: SOD catalyzes  $O_2^-$  disproportionation into  $H_2O_2$  and  $H_2O$ , and CAT then decomposes  $H_2O_2$  into  $H_2O$  and  $O_2$ , resulting in effective detoxification (Zhang et al., 2007). In the antioxidant stress response, HSPs catalyze the conversion of ROS by activating endogenous peroxidase (Shi et al., 2016). In this study,

transcriptome analyses indicated that in comparison to the control groups, the expression of SODs (Cu/ZnSOD and MnSOD) was downregulated in the salinity acclimation groups, while that of CAT, GPx3, and HSP70 was upregulated. Further, we found that 96-h acute salinity stress inhibited the mRNA expression of MnSOD, CAT, and GPx3 in the gill tissue of *M. nipponense*; however, in the hepatopancreas tissue, an increase followed by a decrease was observed in the mRNA expression of CAT and GPx3. This could have been a mechanism to activate antioxidant and heat stress responses and to reduce oxidative damage. Altogether, these findings suggested that salinity stress modulates oxidative stress and antioxidant defenses in *M. nipponense* in a tissue-specific manner, which is similar to the results reported for *S. serrata* (Paital and Chainy, 2010).

To summarize, we performed transcriptome analyses to investigate the molecular response of the gill and hepatopancreas tissues of *M. nipponense* to salinity acclimation. Several DEGs and core pathways involved in, for example, osmotic pressure regulation and energy metabolism, were identified. Further, based on CA localization results obtained on performing *in situ* hybridization, we believe that the CA gene can be used as a molecular indicator of acute salinity stress in *M. nipponense*. Future studies are warranted to further understand pertinent mechanisms so as to develop new methods to enhance the survival rate of *M. nipponense* and improve brackish water aquaculture.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

SS conceived and designed the experiments. CX, KX, and YJ performed the experiments and analyzed the data. SS supervised the project. SS and CB wrote the manuscript. All authors reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2022.926885/full#supplementary-material>



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