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Effects of low salinity on hemolymph osmolality and transcriptome of the Iwagaki oyster, *Crassostrea nippona*

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ABSTRACT

Crassostrea nippona is a kind of oysters with great development value as it can be edible in summer for its late reproductive period. Salinity is one of the important limiting abiotic factors to the survival and distribution of this stenohaline species. To better understand the physiological and immunological response of *C. nippona* to varying environmental salinities, the effects of low salinity on the hemolymph osmolality and gill transcriptome were investigated in this study. The osmolality of hemolymph in vivo and surrounding water were assessed regularly over one week at five test salinities ranging from 5 psμ to 30 psμ. They reached osmotic equilibrium within hours above 15 psμ but remained hyperosmotic at 10 and 5 psμ for the whole sampling period. Through comparative transcriptome analysis, there were less differentially expressed genes (DEGs) in pairwise comparison of S1 (10 psμ) vs S3 (30 psμ) than in S2 (20 psμ) vs S3. KEGG enrichment analysis identified ubiquitin-mediated proteolysis and mitochondrial apoptosis pathway specifically enriched at 10 psμ. This study gained comprehensive insights on the low salinity response of *C. nippona* at the molecular level, which provide a theoretical basis for understanding the immune mechanism under low salinity stress.

1. Introduction

Coastal ecosystems are the most ecologically and socio-economically vital on the planet [1]. Due to the influences of tides, freshwater inputs, evaporation, and increasingly anthropogenic contaminants, this area is thus more exposed to salinity fluctuations. The salinity of estuaries runs from just above 0 psμ to as high as 40 psμ [2,3]. Furthermore, it is believed that seawater salinity in the future will decrease gradually because of global warming, increased rainfall, and tides [4].

Crassostrea nippona is naturally distributed in shallow water along coasts of East Asia [5,6] and has great potential for large-scale oyster farming. It belongs to the genus *Crassostrea*. The leading molluscan species by the quantity produced is oysters [7,8], and species of *Crassostrea* contribute the most [8]. The commercial price of *C. nippona* is estimated at five-folds than that of the *C. gigas* in Japan [9] because of its unique flavor, delicious taste [10], and marketability in summer when *C. gigas* is inedible due to utilizing glycogen to develop gonads [11]. However, compared with other species in the family *Crassostrea* distributed in intertidal zones with large salinity fluctuation, *C. nippona* is a kind of stenohaline species. Previous studies have shown that low salinity affected the growth and survival of its larvae and juvenile [12,13]. It is necessary to learn more about the molecular response in the organism under low salinity stress.

It is known that RNA-Seq-based transcriptome analysis developed rapidly and has been widely used in building stress-responsive gene expression profiles of many aquatic animals. Regarding *Crassostrea* oysters, comparative transcriptomics has been applied to characterize the low-salinity responsive genes in *C. gigas* [14–16], *C. virginica* [17], *C. hongkongesis* [16], and *C. ariakensis* [18] to date. Recently, more elaborate and thorough research has been carried out in bivalves, especially in hemocytes of mussels [19,20].

In this study, the hemolymph osmolality over time and gill transcriptome of *C. nippona* exposed to variant salinities were examined. This could provide more comprehensive information on low salinity response in *C. nippona*.

2. Materials and methods

2.1. Experimental samples and design

Approximately 180 two-year-old *C. nippona* (shell height, 6.6 ± 1.4 cm; shell length, 5.3 ± 0.6 cm) were collected from Rushan Bay (36°

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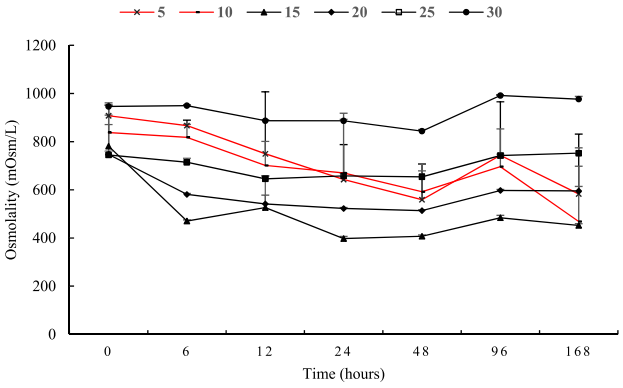

Fig. 1. Hemolymph osmolality in oysters at test salinities. Data expressed as mean values of three replicates for each sampling time and salinity. Bars represent a positive standard deviation.

Table 1
Summary of Illumina short reads from the gill transcriptome of *C. nippona*.

Sample	Raw reads	Q30(%)	Trim rate (%)	Map rate (%)
S1-1	43260476	94.3	99.76	86.40
S1-2	50559190	94.92	99.86	89.37
S1-3	38861800	94.24	99.47	90.52
S2-1	44464968	94.54	99.83	86.93
S2-2	45097466	94.52	99.77	90.04
S2-3	38854004	94.33	99.54	89.09
S3-1	46121292	94.39	99.83	87.47
S3-2	50587446	94.0	99.82	89.37
S3-3	39860584	94.43	99.52	91.06

43′–37° 36′ N and 121° 28′–121° 39′ E), Shandong, China and transported to the Haiyi Aquaculture Cooperation, Yantai, China for carrying out the experiment. For acclimatization, they were reared in the Haiyi hatchery and fed an algal diet of *Isochrysis galbana* and *Platymonas subcordigoramis* at daily rations of 3–4% (dry algal weight/dry oyster meat weight) in the morning per day for one week.

During experiment, they were randomly divided into 6 groups with salinities of 30, 25, 20, 15, 10, and 5 practical salinity unit (psu). Equally 30 individuals were cultivated in each 70 L tank. The low salinity water was prepared by diluting aerated and sand-filtered seawater with tap water. The feeding strategy is as usual as before. Half of the seawater was renewed at dusk every day.

2.2. Hemolymph osmolality

Paired hemolymph and water samples were taken from each tank at 0, 6, 12, 24, 48, 96, and 148 h. It was sampled by withdrawing 500 μL from the adductor muscle using a 1-mL syringe with a 27-gauge needle. Each sample was analyzed under a microscope to ensure hemocytes were present and no contamination with tissue debris. VaporPro® vapor 5520 pressure osmometer (Wescor Inc., USA) was used to determine the osmolality of the hemolymph and corresponding water.

2.3. RNA extraction and Illumina sequencing

Gill tissues of the oysters in 30, 20, and 10 psμ at 148 h were collected and immediately placed into liquid nitrogen to freeze and then stored at −80 °C for further transcriptome analysis.

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and integrity of total RNA were determined using Nanodrop 2000 spectrophotometer (Thermo, USA) and Agilent 2100 BioAnalyzer (Agilent Technologies, USA). A total of nine samples were used for library construction. After PCR amplification, the 150bp library was then sequenced via Illumina Hiseq™ 4000.

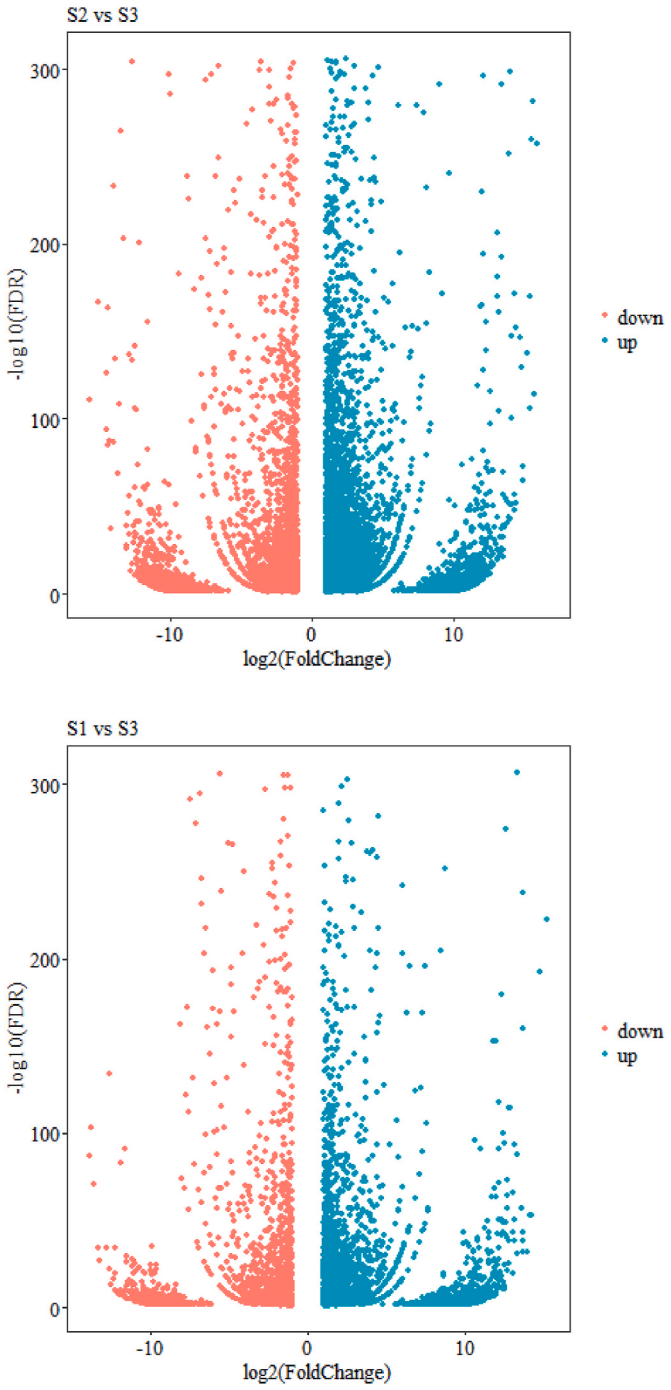

Fig. 2. Volcano plot for the differentially expressed genes shows the estimated log2(foldchange) (x-axis) against its statistical significance (y-axis) between low salinity groups (S1 and S2) and control group. Gene that are significantly up-regulated and down-regulated are in green and pink, respectively.

2.4. Functional annotation and enrichment analysis of differentially expressed genes (DEGs)

Clean reads were obtained by removing 'dirty' reads containing adapter sequences, sequences with more than 10% unknown bases, and low-quality reads containing more than 40% of low quality (q-value<10) bases. They were mapped against the reference transcriptome

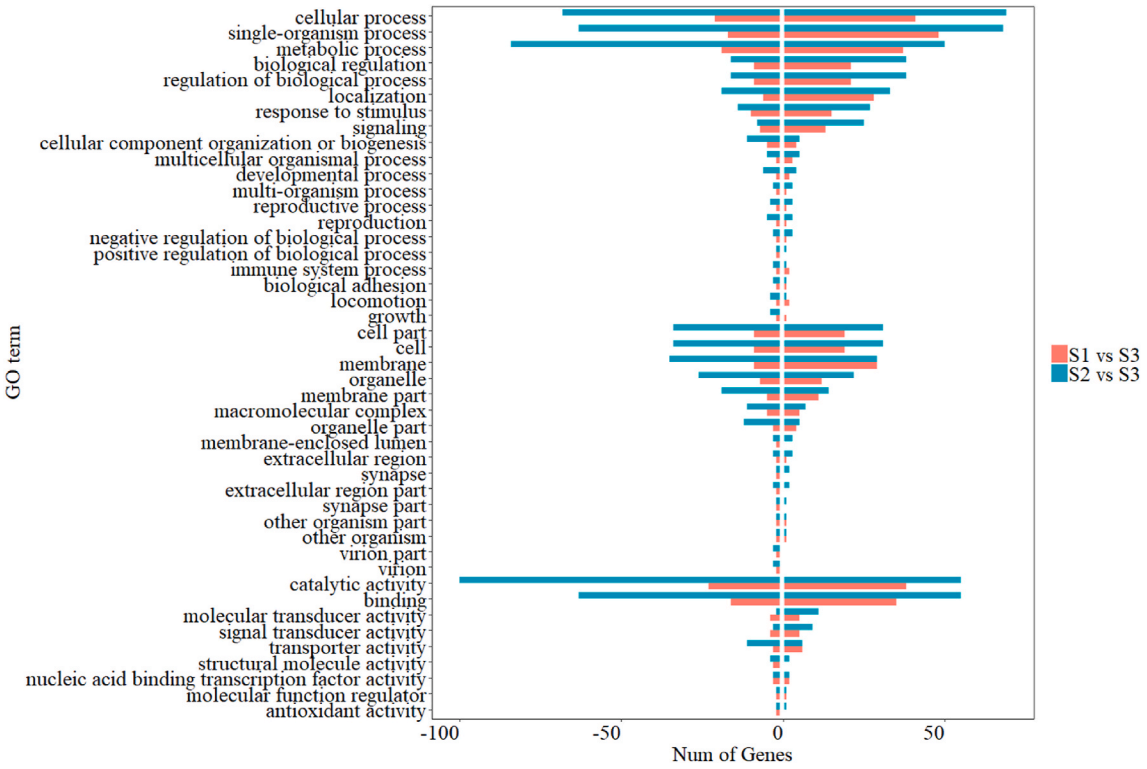

Fig. 3. GO enrichment analysis of the differentially expressed genes between the low salinity groups (S1 and S2) and control group (S3) in the gill of *C. nippona*. The y-axis shows the number of differentially expressed genes within each category, and the x-axis presents the second level GO terms for cellular biological process, cellular component, and molecular function.

(GenBank accession number: GGUV02000000) [21] using Bowtie2 [22] by default parameters afterwards. The reads number mapped to each gene was estimated by RSEM [23]. The expression level of each transcript was transformed using log2(FPKM+1). The expression fold change of a transcript was estimated by DESeq [24]. The standard for considering DEGs is $|\log_2(\text{Fold Change})| > 1$ and p-value < 0.05.

The National Center for Biotechnology Information (NCBI) non-redundant nucleotide sequences (Nt) and NCBI non-redundant protein sequences (Nr) were used to annotate the DEGs by BLAST v2.2.28. Gene ontology (GO) enrichment analysis was implemented with blast2go [25]. The ultra-geometric test was used to detect significantly enriched GO terms in DEGs compared with the transcriptome background. Pathways of DEGs were annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using KOBAS v2.0 [26]. Enriched KEGG pathways were identified according to the same formula used in the GO analysis.

2.5. Quantitative reverse-transcription PCR (qRT-PCR) verification

Total RNA used in the qRT-PCR analysis was isolated with the same method used in the RNA-seq. Specific primers were designed using primer 5.0. EF1A was used as the internal control. The analysis was performed using LightCycler® 480 instrument (Roche, Switzerland) with QuantiNova™ SYBR Green PCR Kit (Qiagen, Germany). The PCR mixture contained 2 μL cDNA, 5 μL 2x SYBR Green PCR Master Mix, 0.7 μL forward (reverse) primers, and 1.6 μL distilled water in a final volume of 10 μL. Cycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s, and then a melt curve stage after the cycling stage. The relative expression of selected genes based on cycle threshold (C_T) values was calculated using the "pcr" R package (http://CRAN.R-project.org/package=pcr) [27].

3. Results

3.1. Hemolymph osmolality under salinity stress

The hemolymph osmolality of *C. nippona* was hyperosmotic to the external water during the initial exposure and reached osmotic equilibrium within 12–24 h at 30, 25, 20, and 15 psμ. Below 15 psμ, hemolymph of oysters remained hyperosmotic relative to the external environment throughout the sampling period (Fig. 1). Additionally, oyster mortalities were observed in tanks at 5 and 10 psμ resulting in reduced numbers at the last sampling period.

3.2. Gill transcriptome of C. nippona under salinity stress

A total of 397,667,226 raw reads (150 bp) were obtained from nine gill tissues. After being filtered, 396,664,156 qualified clean reads were obtained. Q30 (%) varies from 94.0% to 94.92%. For the nine sequencing libraries, over 86.40% of the reads could be mapped to the reference sequences (Table 1).

3.3. Functional annotation and enrichment analysis of DEGs

A total of 1191 and 2984 genes were identified as differentially expressed in pairwise comparisons (S1 vs S3 and S2 vs S3), respectively. Among all DEGs, 747 genes up-regulated and 484 genes down-regulated were detected in S1 vs S3, together with 1253 genes up-regulated and 1731 genes down-regulated in S2 vs S3 (Fig. 2).

DEGs were enriched in 45 GO terms with the same distribution in two pairwise comparisons (Fig. 3). According to KEGG enrichment analysis, ubiquitin-mediated proteolysis and apoptosis pathway were particularly enriched in S1 vs S3 (Fig. 4).

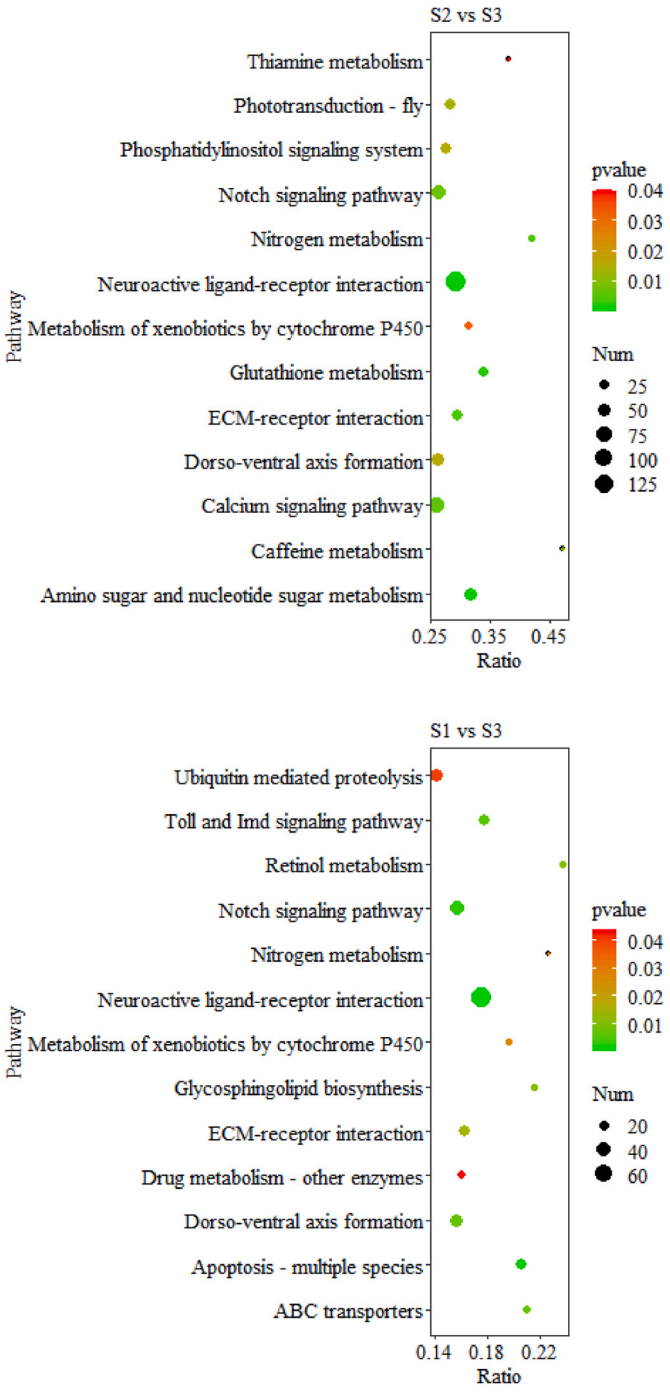

Fig. 4. KEGG enrichment analysis of the differentially expressed genes between the salinity groups (S1 and S2) and control group (S3) in the gill of *C. nippona*.

3.4. *C. nippona* mitochondrial apoptosis pathway analysis

The major mitochondrial apoptosis pathway-related genes in *C. nippona* included BCL-2, BAK, BAX, SMAC, HtrA2, IAP and Casp (Table S1). These genes perform similar expression trends using RPKM and qPCR calculation (Fig. 5). BH3, the "button" of the pathway in mammals, is yet to be discovered in the data.

4. Discussion

Oysters, like most marine animals, are typically considered to be osmoconformers. That means that their extracellular fluid is isosmotic

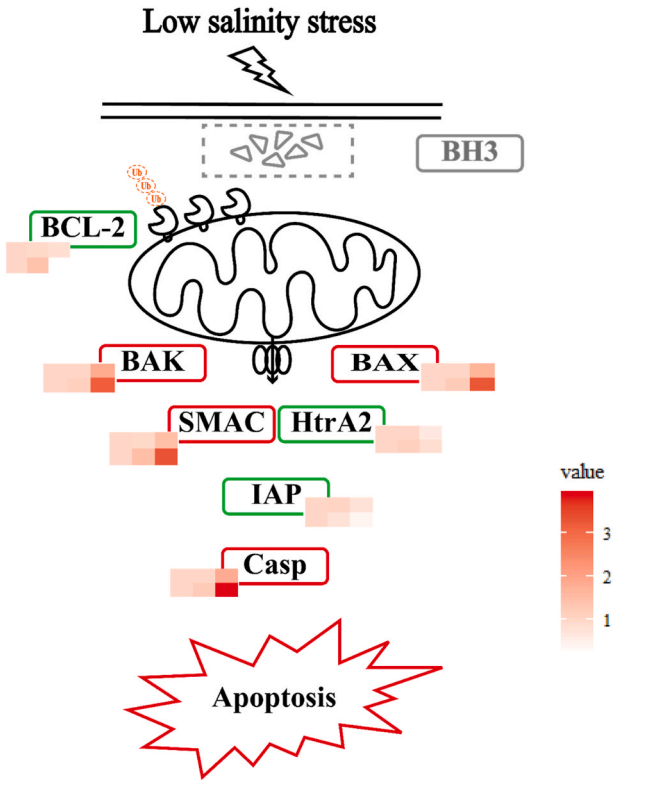

Fig. 5. Schematic of *C. nippona* DE transcripts involved in low salinity response. Mitochondrial apoptosis related transcripts differentially expressed in *C. nippona* in response to low salinity are shown, with red box indicating up-regulation, green box indicating down-regulation, gray box indicating missing and dashed box indicating speculation based on transcriptome data only. The filled colors represent the relative expression of these DE transcripts using RPKM (up) and qPCR (down).

with the surrounding seawater. When the exposed salinity near or beyond their tolerance limits, they chose to close their valves to isolate the organism from the external environment [28,29]. As sessile organisms thriving in estuaries and intertidal zones, oysters generally can tolerate wide fluctuations in salinity [14]. In the previous study, *C. virginica* could track all treatment salinities (down to 5 psμ) within 1 week of exposure, even though they consume longer to reach isosmotic conditions at the lower salinities [30]. However, the hemolymph osmolality of *C. nippona* has little power to adapt to an environment below 15 psμ. Considering *C. nippona* can only live in the shallow water where the salinity is relatively stable [5,6], the difference in their tolerance of salinity can be explained by variant biotypes they inhabit [31].

The following transcriptome expression analysis further proved the previous result. There are less DEGs in S1 vs S3. It implies that when *C. nippona* is exposed to the environment with a salinity of 10 psμ for a period, the organism may enter a statement of reduced metabolism and induced immune process [32]. It has been reported that when oysters suffered abiotic stresses, the highly enriched GO terms are mainly associated with protein binding and apoptosis, indicating the over-representation of some host-defense genes [33].

In bivalves, gills have large surface area and constant contact with the ambient seawater. Except for the normal life activities, several "environment related" KEGG pathways were found both at 20 and 10 psμ, such as "neuroactive ligand-receptor interaction", "calcium signaling", "metabolism of xenobiotics by cytochrome P 450" and "ECM-receptor interaction" [34]. Immune related terms were specifically enriched in the gill of *C. nippona* at 10 psμ, such as "Toll and Imd signaling pathway", "Glycosphingolipid biosynthesis pathway" and

"apoptosis pathway". Previous experiments have shown that aquatic animals usually undergo changes in immune status under environmental stresses [35]. Unlike vertebrates, oysters lack an adaptive immune system and thus must rely heavily on the innate immune system as a protection from biotic and abiotic stresses [36,37]. Apoptosis is the important immune mechanism of oyster in adaptive responses to environmental stress, which have been noticed to play important roles in *C. gigas* [15], and the clam *Cyclina sinensis* [38]. It is initiated and transduced via the intrinsic (mitochondrial) or the extrinsic (receptor mediated) pathway [39]. Environmental stressors such as low salinity stress has been confirmed to activate the mitochondrial pathway in molluscs [15]. The pro-apoptotic and pro-survival members of the Bcl-2 family proteins are the central regulators. The absence of BH3-only homolog is already confirmed in pacific oyster, *C. gigas* [40], the manila clam, Ruditapes philippinarum [41], and the marine mussel, *Mytilus edulis* [42]. However, it has been identified in vitro that the ubiquitin-mediated proteolysis could not only destruct misfolded or damaged proteins, but also suppress the expression of Bcl-2 and thereby activate the apoptosis [43,44]. Taken collectively, more relevant studies about the stimulation of intrinsic apoptosis in invertebrates need carrying out.

5. Conclusion

In this study, the transcriptome sequencing on *C. nippona* exposed to hyposalinity environment was performed using the Illumina Hiseq platform. Together with measuring hemolymph osmolality over time, we can get a better understand of the salinity capacity for *C. nippona*. This is the first report about the molecular mechanism involved in environmental stress immune response of the organism. The physiological and molecular level analysis together can overcome the interference of the valve movements, which could provide a novel approach in the relevant research.

CRediT authorship contribution statement

Jianwen Gong: Methodology, Data curation, Writing – original draft. **Qi Li:** Writing – review & editing. **Hong Yu:** Resources. **Shikai Liu:** Visualization, Investigation. **Lingfeng Kong:** Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.05.051.

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