Unraveling Molecular Mechanisms of Low-Salinity Adaptation in *Fenneropenaeus chinensis*: Insights from Gill Tissue Transcriptomics

Jun Liu [1,2†], Rongchen Liu [1,2†], Hui Jiang[3], Hai Zhang [2], Dianyuan Zheng [2], Daizhen Zhang[1*]

[1]Jiangsu Key Laboratory for Bioresources of Saline Soils, Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Jiangsu Synthetic Innovation Center for Coastal Bio-Agriculture, Yancheng Teachers University, Yancheng, China;

[2]Lianyungang Key Laboratory of Biotechnology, Jiangsu Engineering and Research Center for Advanced Materials, Lianyungang Normal College, China;

[3]Yancheng Milu Research Institute, Yancheng, China.

*Correspondence: zhangdzyc@126.com

†These authors contributed equally to this work.

Abstract: This study investigates the molecular responses of Chinese shrimp (*Fenneropenaeus chinensis*) to low salinity stress, employing RNA-seq and functional enrichment analyses. A total of 12,332 expressed genes were identified, with 227 differentially expressed genes (DEGs) detected, revealing intricate adaptive responses in gill tissues. Functional enrichment analysis highlighted DEGs associated with ion-transport regulation, bio-metabolism and detoxification (based on GO terms). The upregulation of *ATP1A1*, *SLC26A2*, and *STK39* suggested a pivotal role of ion-transport regulation, emphasizing the importance of maintaining osmotic balance. In terms of bio-metabolism, *ENPP6*, *GLS*, and *DMGDH* exhibited significant upregulation, indicating adjustments in energy metabolism for low-salinity adaptation. Notably, the downregulation of *GSTO1* and *CTH* raised questions about detoxification pathways, potentially reflecting adaptations or compromises in response to low salinity. Further correlation network analysis highlighted key genes like *GLS*, *STK39*, *SESN1*, and *SARDH*, revealing intricate relationships among DEGs. RT-qPCR validation confirmed the consistency of gene expression patterns with RNA-seq results. In conclusion, this study provides a comprehensive understanding of the molecular mechanisms underlying Chinese shrimp responses to low salinity stress. The identified genes lay the groundwork for targeted interventions and strategies to enhance shrimp aquaculture system resilience amid changing salinity levels.

Keywords: Aquaculture; Crustaceans; Differentially Expressed Genes; Osmoregulators

1. Introduction

The adaptation of commercial aquaculture crustaceans to low salinity has garnered significant attention [1-4]. It is necessary to understand their adaptation ability to low salinity and mechanisms used by these organisms to tolerate low-salinity environments on purpose of improving the low-salinity aquaculture of marine crustaceans. As osmoregulators, some euryhaline marin and brackish crustaceans have a strong ability to adapt to environments with varying salinities (from almost 0 ppt up to 40 ppt) [5-8]. Therefore, this ability to tolerate low salinity is a key factor affecting the distribution of such crustaceans in low-salinity environments [9-10].

Previous studies have explored the physiological mechanisms underlying low-salinity tolerance in mariculture crustaceans at the organismal, cellular, and molecular levels [8, 11-14]. In general, the most common adaptive strategies for hyperosmoregulation aim to maintain hemolymph osmolarity above that of the ambient medium, both via salt absorption and via permeability reduction (i.e., reducing or limiting water inflow) [6]. However, most euryhaline crustaceans produce isosmotic urine, and thus considerable salt is lost in low-salinity environments [15-16]. Therefore, osmoregulation should be the focal point of our considerations. Studies of low-salinity tolerance in crustaceans have shown that the gills participate in osmoregulation. In detailed reviews, Péqueux (1995) and Henry (2012) assessed the specialized functions of gills and gill parts in various crustaceans and showed that both cuticle permeability and the membrane characteristics of the salt-transporting gill epithelial cells were critical to osmoregulation [6, 8]. Some bio-molecules in the gill epithelial cells facilitate salt absorption, and these biomolecules include Na^+/K^+-ATPase, K^+ channels, Cl^- channels, carbonic anhydrase, aquaporins (AQPs) and various exchangers (Na^+/NH_4^+, Na^+/H^+, and Cl^-/HCO_3^-) [6, 9, 17-20]. With development of RNA-seq technology, high-throughput screening of genes in response to low salinity stress in crustaceans is currently feasible.

The euryhaline Chinese shrimp (*Fenneropenaeus chinensis*), which has an isosmotic point of 25 ppt, is naturally distributed primarily in the Chinese Yellow Sea, the Bohai Sea, and along the western coast of the Korean Peninsula [21-22]. It is an important commercial shrimp along the coasts of China and Korea [21]. As these shrimp are cultured in much lower salinity (under the isosmotic point), they must manage or tolerate substantial changes in water osmolality. The poorer ability limits the farming development of *F. chinensis* compared to *Penaeus vannamei* [22]. In our prior investigation, an

experimental design was implemented to assess the low-salinity tolerance of the target species. However, the experimental protocol involved a group-wise allocation of three prawns each, designated as the low-salinity group (LS), which were subjected to a salinity level of 5 ppt for a duration of 24 hours. To further investigate the molecular mechanisms underlying the low-salinity adaptation of the euryhaline Chinese shrimp, this study aims to fill a necessary gap in our understanding of how these key physiological processes are orchestrated at the molecular level, particularly within the gill tissue with a more meticulous design.

2. Materials and Methods

2.1 Sample collection and treatment

From July to August 2020, live shrimp were obtained from a farming pool in Lianyungang (N 34°48′ 52.47″, E 119°12′19.08″) and transported to our laboratory at Lianyungang Normal College. All shrimp were acclimated to a salinity of 25 ppt at the beginning. Shrimp acclimation and water-salinity control were reported in our latest research [23]. In short, gill tissues from a random subset of surviving individuals in each group (six from each) were used for transcriptome analysis and real-time quantitative PCR (RT-qPCR).

2.2 RNA isolation, library construction, and sequencing

The method here was consistent with that of our previous work[23]. Total RNA was extracted using TRIzol reagent (Invitrogen Corp., USA). RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and RNA integrity was assessed using 1.5% agarose gel electrophoresis. Magnetic oligo (dT) beads were used to isolate mRNA from total RNA. The mRNA was then fragmented into fragments approximately 200 bp long using fragmentation buffer (Tris-acetate, KOAc, and MgOAc) at 94°C for 35 min. The fragmented mRNA was used to construct the cDNA libraries. At least 5 μl of mRNA solution (≥ 200 ng/μl) was used to construct each library. Sequencing libraries for each sample were generated using the TruSeq RNA Sample Prep Kit (Illumina, USA). The read length was 300bp.

2.3 Transcriptome Assembly, Gene Functional Annotation, and Analysis of Differentially Expressed Genes

Prior to assembly, the raw reads were subjected to filtering using fastp (v0.23.4) [24]. Reads containing adapters, exceeding 10% of unknown nucleotides, exhibiting low quality, or originating

from rRNA were excluded from further analysis. This filtering step aimed to obtain a set of high-quality clean reads. Subsequently, the clean reads were mapped to the Penaeus chinensis reference genome (ASM1920278v2) using HISAT2.2.1 [25]. The mapped reads were then assembled using StringTie (v1.3.1) [26-27] in a reference-based approach. This process ultimately yielded the reads and TPM (transcripts per million) expression matrix.The identified unigenes were annotated against five databases: NCBI nonredundant protein sequences (NR), Protein Families (PFAM), KEGG (Kyoto Encyclopedia of Genes and Genomes), Gene Ontology (GO), and eggNOG [28-29].

Differential expression analysis of RNAs between the FCN10 and FCN25 groups was conducted using the DESeq2 (v1.6.3) software. Genes exhibiting the false discovery rate (FDR) parameter < 0.05 and $|\text{Log2 FC}| \geq 1$ were considered differentially expressed genes (DEGs). To evaluate functional annotation and pathway enrichment, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed on DEGs. Statistically significant GO terms and KEGG pathways with padj < 0.05 were determined via statistical enrichment analysis[23]. Z-score was also used to investigate the significance of GO terms[30]. Correlation analysis entails utilizing the stat package based on R (v4.2.3) and employing the Pearson correlation coefficient to assess the correlation between genes[31].

2.4 Verification of DEGs using RT-qPCR

We selected 8 RNA-Seq DEGs (7 upregulated and 1 downregulated) for RT-qPCR validation. We used the *TUBA* (Tubulin) as the internal reference gene, against which to normalize the expression levels of the target genes. It was stably expressed in this study. Gene-specific primers were designed based on sequences derived from the transcriptome assembly and annotation using Primer Premier 5.0 (Table 1). Each RT-qPCR (25 μl) contained 12.5 μl of 2 × SYBR qPCR Mix, 1 μl each of forward and reverse primers, 1 μl of cDNA, and 10.5 μl of RNase-free H2O. RT-qPCRs were performed on an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), with the following cycling conditions: an initial denaturation step of 3 min at 95°C; 40 cycles of 15 s at 94°C, 15 s at 55°C, and 25 s at 72°C; and a standard dissociation cycle. Three technical replicates were performed per gene, and the $2^{-\triangle\triangle CT}$ method was used to calculate relative expression levels[32].

Table 1. Primers used for real-time quantitative PCR

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
TUBA (Tubulin)	CTACGAGGAGGTCGGAGTGG	TGCTTCGGAGACGGTTGTT
ATP1A (Na^+/K^+-ATPase)	AGAAGGCCGATATTGGTG	CAGGGATGTTAGATGTCAGG
SLC26A2 (Diastrophic dysplasia sulfate transporter)	TCACAGTCAGCAAGTAACA	AATAACAACAGCAAGGAAGG
DMGDH (Dimethylglycine dehydrogenase)	ATGAATAACGCCGTCTCTT	CATAGTCCTCTAACCATCTGA
STK39 (Serine threonine kinase)	ATGTGCCATCAAGCGAAT	ATAACTTCAGGTGCCATCC
SARDH (Sarcosine dehydrogenase)	TCAACCACAGAGGAGGAAT	TCACCAACGAAGGACAAC
GLS (Glutaminase)	GGAACAACCTGATGGAAGT	ATTAAGAGCAATCGCATACG
ENPP6 (Ectonucleotide phosphodiesterase/pyrophosphatase 6)	GAATGGCATCACAACTCAG	GACTATCATACACCTTCTCAAC
GSTO1 (Glutathione S-transferase omega-1)	GAACTCGTCAACATCAACAC	GGAATCTCATAGCCGTCTC

3. Results

3.1 Sequencing and Annotation

After sequencing and raw data cleaning, this research yielded 65.05 Gb data with more than 38 million clean reads for per library. The value of Q30 (bases with quality scores > 30) was more than 90% in average, indicating a high accuracy in base recognition (Table 2). Totally 20, 076 unigenes were annotated against databases of NR, GO, KEGG, eggNOG and PFAM, accounting for 98.9% of unigenes (Table 3).

Table 2. Profile of sequencing data

Sample	Clean reads	Q30 bases	Alignment rate	Group ID
SRR14718710	38.32M (98.62%)	5.18G (91.68%)	87.81%	FC25_1
SRR14718711	40.45M (98.57%)	5.57G (93.07%)	87.82%	FC25_2
SRR14718712	38.32M (98.42%)	5.24G (92.65%)	87.24%	FC25_3
SRR17931570	56.67M (97.51%)	7.74G (91.66%)	88.88%	FC25_4
SRR17931571	46.43M (97.38%)	6.28G (90.89%)	89.64%	FC25_5
SRR17931572	49.36M(97.43%)	6.75G (91.91%)	88.40%	FC25_6
SRR14718716	40.27M (98.47%)	5.52G (93.07%)	85.79%	FC10_1
SRR14718719	43.52M (98.74%)	5.98G (93.00%)	87.85%	FC10_2
SRR14718720	40.17M (99.09%)	5.55G (93.15%)	88.56%	FC10_3
SRR17931576	52.22M (97.40%)	7.12G (91.77%)	86.52%	FC10_4
SRR17931579	45.64M (97.50%)	6.27G (92.14%)	87.90%	FC10_5
SRR17931580	53.78M (97.54%)	7.35G (91.70%)	82.92%	FC10_6

Table 3. Summary of unigenes' annotation

Database	Number of Unigenes	Rate(%)
NR	14, 810	72.9%
PFAM	7, 302	36.0%
eggNOG	7, 398	36.5%
GO	6, 279	30.9%
KEGG	5, 611	27.8%
Annotated in at least one database	2, 0076	98.9%

3.2 DEGs in gill of F. chinensis and their functional enrichment

According to the screening results by DESeq2, we detected 12, 242 expressed genes (Supplement 1) and identified 227 DEGs, including 109 up-regulated genes and 118 down-regulated genes (Figure 1，Supplement 2).

Further enrichment analysis based on GO showed 120 GO terms were significantly enriched by DEGs (p adjust ≤ 0.05). These GO terms are mainly classified into three categories (Figure 2): the first category is related to ion transport, including "negative regulation of ion transmembrane transport" (GO: 0034766), "negative regulation of ion transport" (GO: 0043271), and "positive regulation of transmembrane transport" (GO: 0034764) etc.; The second category is associated with bio-metabolism, including "serine family amino acid biosynthetic process" (GO: 0009070), "cellular amino acid biosynthetic process" (GO: 0008652) and "vitamin biosynthetic process" (GO: 0009110) etc.; The third category involves processes related to detoxification, including "oxidoreductase activity, acting on the CH-NH group of donors(GO: 0016491); cellular oxidant detoxification (GO: 0098869) ; cellular detoxification (GO: 1990748) etc. (Description of all GO terms were listed in Supplement 3).

Results of KEGG enrichment analysis showed DEGs enriched in pathways which are also associated with ion transport, bio-metabolism and detoxification (Figure 3), including "Proximal tubule bicarbonate reclamation", "Ascorbate and aldarate metabolism", "GABAergic synapse", "Alanine, aspartate and glutamate metabolism", "Protein digestion and absorption" and so on.

3.3 Correlationship among DEGs

Based on the results of DEGs functional enrichment, a total of 32 DEGs were selected to construct a correlation network, focusing on processes related to ion transport, bio-metabolism and detoxification (Figure 4). The findings revealed a notable correlation pattern among these genes. Specifically, genes

162 such as *GLS* (Glutaminase), *STK39* (Serine threonine kinase), *SESN1* (PA26 p53-induced protein), and
163 *SARDH* (Sarcosine dehydrogenase) exhibited a significantly positive correlation with most of the 32
164 DEGs, while *CTH* (Cystathionine gamma-lyase) displayed a marked negative correlation with the
165 majority of these DEGs.
166
167 **3.4 RT-qPCR verification**
168 To verify the reliability of the RNA-seq results, we selected 8 representative genes related to
169 low-salinity stress response in aspects of ion-transport regulation, bio-metabolism and detoxification
170 using RT-qPCR method. The results showed that the expression patterns of DEGs were consistent with
171 the RNA-seq results. Specifically, in the low-salinity treatment group, the expression of *ENPP6*,
172 *SARDH*, *SLC26A2*, *GLS*, *ATP1A1*, *STK39* and *DMGDH* genes were up-regulated, while the expression
173 of *GSTO1* gene was down-regulated (Figure 5).
174
175 **4. Discussion**
176 **4.1 Ion-transport Regulation**
177 During in response to low salinity stress, *F. chinensis* have been shown to be able to consume
178 more energy during osmoregulation [6, 8], and they will active the immune-related enzymes,
179 antioxidant system, especially the in hepatopancreas tissue [3-4]. This study reveals the intricate
180 molecular mechanisms employed by Chinese shrimp in response to low salinity stress, encompassing
181 key genes associated with ion-transport regulation, bio-metabolism and detoxification. Ion-transport
182 regulation is an important way for crustaceans to cope with low- salinity stress rapidly, and strategies
183 based on it are known as the "complementary process" of osmoregulation [33]. In the process, Na^+/K^+-
184 ATPase, accounting for more than 70% of the activity of ATPase, is the most important example of ion
185 transport enzymes in maintaining the body's sodium-potassium balance and regulating the lymph
186 osmotic pressure in crustaceans including Chinese shrimp [34]. Upregulation of *ATP1A1* in shrimp
187 exposed to low-salinity condition in the study indicated that they experienced a positive stress response.
188 In short, it is now most generally accepted as the "pump" in osmoregulation has been well reported in
189 previous studies.
190 Due to diffusive effluxes of cations, anions from the inside of branchial cells will be attracted to
191 the outside of the cells. The branchial epithelial cells of crustaceans have strong permeability for Cl^-,

192 and a large number of Cl^- flow outside into the hemolymph occurring at the basolateral membrane. In
193 order to maintain the intracellular balance of Cl^-, selective channels were activated [35]. In the study,
194 upregulation of *SCL26A2* should be closed related to the anion (Cl^-) regulation, as the production of
195 *SLC26A2* appears to function as a SO_4^{2-}/Cl^- exchanger, which could move Cl^- into cells [36].
196 According to the model of ion transport into epithelial cells across the apical membrane mentioned by
197 Pequeux (1995), upregulation of *SLC26A2* in this study implied that this gene's production should be
198 located in the apical membrane providing a channel for the chloride ion (Cl^-) entering the cell, and by
199 which Chinese shrimp could maintain intracellular chloride balance. In aspects of osmoregulation
200 mechanisms in crustacean, the function of *SLC26A2* may be a supplement point to the mechanism
201 HCO_3^-/Cl^- - exchange.

202 Otherwise, as to known, the Ste20 kinase is essential for acute volume recovery and survival after
203 hypertonic shrinkage in *Caenorhabditis elegans*, the founding member of serine/threonine kinases,
204 regulate a *Caenorhabditis elegans* ClC anion channel [37]. In this study, *STK39* as the member of
205 serine/threonine kinases, whose upregulation in response to low salinity stress in Chinese shrimp may
206 seem to make sense. As to date, however, there is no report on the research of the gene's participation
207 in osmoregulation of shrimp species, thus it is necessary to pay more attention on this gene in the
208 future.

209 **4.2 Ion-transport Regulation**

210 The upregulation of *ENPP6* and *GLS* sheds light on the importance of bio-metabolic processes in
211 Chinese shrimp adaptation to low-salinity stress. *ENPP6*, known for its hydrolytic efficiency of
212 glycerophosphorylcholine (GPC), may play a role in reducing organic osmolytes like GPC, aiding the
213 shrimp in adapting to hypoosmotic environments [38]. It was mentioned that in the renal medulla of
214 mammals, GPC plays an important role as an organic osmolyte to maintain intracellular osmotic
215 pressure, and accumulating of organic osmolytes, cells can adapt to environmental hypertonicity by
216 high osmolyte concentrations[39]. In other words, reduction of organic osmolytes in cells such as the
217 GPC may imply Chinese shrimp's adaptation to hypoosmotic environments, and the involvement of
218 *ENPP6* in the response to low salinity stress highlights the significance of bio-metabolic processes in
219 Chinese shrimp adaptation. However, there are no reports on the gene in relation to shrimp at present.
220 Moreover, the upregulation of *GLS* (glutaminase) in response to low-salinity conditions further

221 emphasizes the importance of bio-metabolic pathways in aspects of the energy supplement.
222 Glutaminase catalyzes the conversion of glutamine to glutamate, playing a key role in amino acid
223 metabolism as well as in energy production[40-41]. This finding suggests a potential shift in energy
224 metabolism to facilitate the adaptation of Chinese shrimp to low-salinity stress. Thus, the modulation
225 of amino acid metabolism might contribute to the synthesis of osmoregulatory molecules or act as a
226 compensatory mechanism for energy production under altered environmental conditions.

227 Meanwhile, the upregulation of *DMGDH* suggests its involvement in bio-metabolic adjustments,
228 which encodes dimethylglycine dehydrogenase involving in the metabolism of dimethylglycine[42]. It
229 was reported that *DMGDH* expression suppresses human hepatocellular carcinoma metastasis in vitro
230 and in vivo [43]. The role of *DMGDH* in low-salinity adaptation in organism is not well-established,
231 and further research is needed to elucidate its specific contributions. Nevertheless, the observed
232 upregulation suggests its potential involvement in bio-metabolic adjustments to cope with the
233 challenges imposed by low salinity.

234 **4.3 Detoxification**

235 The down-regulated expression of *GSTO1* (glutathione S-transferase omega-1) in the low-salinity
236 treatment group raises questions about its role in detoxification processes. *GSTO1* is known for its
237 participation in the detoxification of reactive oxygen species, facilitating the binding of glutathione to
238 oxidized substances and aiding in the efficient elimination of harmful compounds from the body
239 [44-45]. The reduced expression of *GSTO1* in response to low salinity might indicate a lower demand
240 for detoxification pathways under the condition (4 days domestication in 10 ppt water). Alternatively, it
241 could suggest a potential compromise in the detoxification capacity of Chinese shrimp, which warrants
242 further investigation, although it was reported that *GST* is also differently expressed in hepatopancreas
243 of Chinese shrimp in response to low salinity stress [3].

244 In addition to *GSTO1*, it is noteworthy to different expression of the *CTH* gene. *CTH*, encoding
245 cystathionine gamma-lyase, is a key enzyme in the sulfur metabolism pathway, involved in
246 biosynthesis of cysteine [46]. The negative correlation of *CTH* with the majority of differentially
247 expressed genes (DEGs) implies that the expression of *CTH* is inversely related to genes associated
248 with ion transport, bio-metabolism, and detoxification. Cysteine is an essential amino acid and a
249 precursor to glutathione, a crucial antioxidant molecule. Therefore, like *GSTO1*, the downregulation of

CTH may negatively impact antioxidant defense mechanisms, contributing to the overall adaptive response to low-salinity stress. This observation is consistent with a previous study and reinforces the importance of examining multiple genes and pathways to gain a comprehensive understanding of the molecular responses to environmental stressors in Chinese shrimp [47].

The different expression of sarcosine dehydrogenase (*SARDH*) under low-salt conditions also suggests its potential role in the adaptive response of shrimp to environmental challenges. Sarcosine can be degraded to glycine by sarcosine dehydrogenase [48]. Glycine is a significant amino acid and participates in the synthesis of glutathione, creatine and uric acid, and serves as an advance signal that activates thyroid function coincident with energy and nutrient intake [4]. As we mentioned above, reduced expression of *GSTO1* in response to low salinity might indicate a lower demand for detoxification pathways, thus the integrated analysis illustrated that upregulation of *SARDH* may play a limited role in detoxification mechanisms in gill tissue.

5. Conclusions

In conclusion, our study provides a comprehensive analysis of the molecular mechanisms employed by Chinese shrimp in response to low salinity stress. Through RNA-seq and functional enrichment analyses, we identified key genes associated with ion-transport regulation, bio-metabolism, and detoxification, shedding light on the intricate adaptive responses of Chinese shrimp to environmental challenges. The upregulation of *ATP1A1*, *SLC26A2*, and *STK39* suggests a crucial role of ion-transport regulation in rapidly coping with low-salinity stress, highlighting the significance of Na^+/K^+-ATPase and chloride ion regulation in maintaining osmotic balance. Furthermore, the involvement of *ENPP6*, *GLS*, and *DMGDH* in bio-metabolic processes underscores the importance of energy metabolism adjustments for shrimp adaptation to hypoosmotic environments. The differential expression of *GSTO1* and *CTH* raises questions about the nuanced interplay of detoxification pathways, suggesting potential compromises and adaptations in response to low salinity. Our findings contribute to a deeper understanding of the molecular responses of Chinese shrimp to low salinity stress, providing valuable insights for aquaculture practices and further research. Future investigations should delve into the specific roles of identified genes, their interactions, and the broader implications for the adaptive capacity of Chinese shrimp in fluctuating environmental conditions. Overall, this study lays

the foundation for exploring targeted interventions and strategies to enhance the resilience of shrimp aquaculture in the face of changing salinity levels.

Supplementary Materials: Supplement 1: Functional annotation of genes; Supplement 2: Results of difference on gene expression; Supplement 3: Results of GO enrichment analysis.

Author Contributions: Daizhen Zhang and Jun Liu were pivotal in conceptualizing and designing the study. Rongchen Liu and Hui Jiang took the lead in analyzing the sequencing results, providing critical insights into the data interpretation. Hai Zhang and Dianyuan Zheng were instrumental in conducting the experiments related to the project. Both Rongchen Liu and Jun Liu contributed significantly to the writing and drafting of the manuscript.

Acknowledgments: We thank Dan Liu, Zhen Wang for their assistance in our work. We thank Dachuan Dong for its linguistic assistance during the preparation of this manuscript. Reviewers provided constructive recommendations for improving our manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Funding: This work was funded by National Natural Science Foundation of China (32070526) and sponsored by “Qing Lan Project of Jiangsu Province of China”; Young Talents Support Program in Lianyungang Normal College (LSZQNXM201702); Open Foundation of Jiangsu Key Laboratory for Bioresources of Saline Soils (JKLBS2016008).

Data Availability Statement: The datasets utilized in this research endeavor are publicly accessible through online repositories. The datasets can be accessed via the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA734726.

References

[1] De Vos S, Van Stappen G, Sorgeloos P, Vuylsteke M, Rombauts S, Bossier P. Identification of salt stress response genes using the Artemia transcriptome. Aquaculture. 2019; 500: 305-314. doi: 10.1016/j.aquaculture.2018.09.067

[2] Yuan J, Zhang X, Wang M, Sun Y, Liu C, Li S, et al. Simple sequence repeats drive genome plasticity and promote adaptive evolution in penaeid shrimp. Communications Biology. 2021; 4: 186. doi: 10.1038/s42003-021-01716-y

[3] Tang D, Wu Y, Huang S, Wu L, Luo Y, Wang Z. Transcriptome reveals the mechanism of immunity in the low salinity stress of the chinese shrimp (*Fenneropenaeus chinensis*). An International Journal of Marine Sciences. 2022; 38: 977-987. doi: 10.1007/s41208-022-00440-6

[4] Fan Y, Wu X, Ling F, Wang Z, Ma K, Xie N, et al. Transcriptome, proteome, histology, and biochemistry analysis of oriental river prawn *Macrobrachium nipponense* under long-term salinity exposure. Frontiers in Marine Science. 2023; 9: 1104984. doi: 10.3389/fmars.2022.1104984

[5] Foster C, Amado EM, Souza MM, Freire CA. Do osmoregulators have lower capacity of muscle water regulation than osmoconformers? A study on decapod crustaceans. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology. 2010; 313A(2): 80-94. https://doi.org/10.1002/jez.575

[6] Henry RP, Lucu Č, Onken H, Weihrauch D. Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. Frontiers in Physiology. 2012; 3: 431. doi: 10.3389/fphys.2012.00431

[7] McNamara JC, Faria SC. Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: a review. Journal of Comparative Physiology B. 2012; 182: 997-1014. doi: 10.1007/s00360-012-0665-8

[8] Péqueux A. Osmotic regulation in crustaceans. Journal of Crustacean Biology. 1995; 15(1): 1-60. doi: 10.2307/1549010

[9] Henry RP. Environmentally mediated carbonic anhydrase induction in the gills of euryhaline crustaceans. Journal of Experimental Biology. 2001; 204(5): 991-1002. doi: 10.1242/jeb.204.5.991

[10] Sundell K, Wrange AL, Jonsson PR, Blomberg A. Osmoregulation in barnacles: An evolutionary perspective of potential mechanisms and future research directions. Frontiers in Physiology. 2019; 10: 877. doi: 10.3389/fphys.2019.00877

[11] Bonilla-Gómez JL, Chiappa-Carrara X, Galindo C, Jeronimo G, Cuzon G, Gabriela G. Physiological and biochemical changes of wild and cultivated juvenile pink shrimp *Farfantepenaeus duorarum* (Crustacea: Penaeidae) during molt cycle. Journal of Crustacean Biology. 2012; 32(4): 597-606. doi:10.1163/193724012X630679

342 [12] Havird JC, Mitchell RT, Henry RP, Santos SR. Salinity-induced changes in gene expression from anterior and
343 posterior gills of *Callinectes sapidus* (Crustacea: Portunidae) with implications for crustacean ecological
344 genomics. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 2016; 19(C): 34-44.
345 doi: 10.1016/j.cbd.2016.06.002
346 [13] Li E, Chen L, Zeng C, Yu N, Xiong Z, Chen X, et al. Comparison of digestive and antioxidant enzymes
347 activities, haemolymph oxyhemocyanin contents and hepatopancreas histology of white shrimp, *Litopenaeus*
348 *vannamei*, at various salinities. Aquaculture. 2008; 274(1): 80-86. doi: 10.1016/j.aquaculture.2007.11.001
349 [14] Li J, Li W, Zhang X. Effects of dissolved oxygen, starvation, temperature, and salinity on the locomotive
350 ability of juvenile Chinese shrimp *Fenneropenaeus chinensis*. Ethology Ecology & Evolution. 2019; 31(2):
351 155-172. doi: 10.1080/03949370.2018.1526215
352 [15] Cameron JN, Batterton CV. Antennal gland function in the freshwater blue crab, *Callinectes sapidus*: Water,
353 electrolyte, acid-base and ammonia excretion. Journal of Comparative Physiology. 1978a; 123(2): 143-148.
354 doi: 10.1007/BF00687842
355 [16] Cameron JN, Batterton CV. Temperature and blood acid-basse status in the blue crab, *Callinectes sapidus*.
356 Respiration physiology. 1978b; 35(2): 101-110. doi: 10.1016/0034-5687(78)90015-4 PMID: 33428.
357 [17] Chung JS, Maurer L, Bratcher M, Pitula JS, Ogburn MB. Cloning of aquaporin-1 of the blue crab, *Callinectes*
358 *sapidus*: its expression during the larval development in hyposalinity. Aquatic Biosystems. 2012; 8(1): 21. doi:
359 10.1186/2046-9063-8-21
360 [18] Henry RP, Cameron JN. The distribution and partial characterization of carbonic anhydrase in selected
361 aquatic and terrestrial decapod crustaceans. Journal of Experimental Zoology, 1982; 221(3): 309-321.doi:
362 10.1002/jez.1402210306.
363 [19] Neufeld GJ, Pritchard JB. Osmoregulation and gill Na, K-ATPase in the rock crab, Cancer irroratus:
364 Response to DDT. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology. 1979;
365 62c(2): 165-172. doi: 10.1016/0306-4492(79)90005-4
366 [20] Varley DG, Greenaway P. Nitrogenous excretion in the terrestrial carnivorous crab *Geograpsus grayi*: site
367 and mechanism of excretion. Journal of Experimental Biology. 1994; 190(1): 179-193. doi:
368 10.1242/jeb.190.1.179
369 [21] Chen JC, Lin JL. Osmolality and chloride concentration in the hemolymph of subadult *Penaeus chinensis*
370 subjected to different salinity levels. Aquaculture. 1994; 125(1-2): 167-174. doi:
371 10.1016/0044-8486(94)90293-3
372 [22] Li J, Xu X, Li W, Zhang X. Linking energy metabolism and locomotor variation to osmoregulation in
373 Chinese shrimp *Fenneropenaeus chinensis*. Comparative Biochemistry and Physiology Part B: Biochemistry
374 and Molecular Biology. 2019; 234: 58-67. doi:10.1016/j.cbpb.2019.05.006

375 [23] Liu J, Zhang D, Zhang L, Wang Z, Shen J. New insight on vitality differences for the Penaeid shrimp,
376 *Fenneropenaeus chinensis*, in low salinity environment through transcriptomics. Frontiers in Ecology and
377 Evolution. 2022; 10: 716018. doi:10.3389/fevo.2022.716018
378 [24] Chen S. Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. iMeta.
379 2023; 2(2): e107. doi: 10.1002/imt2.107
380 [25] Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with
381 HISAT2 and HISAT-genotype. Nature Biotechnology. 2019; 37(8): 907-915. doi:
382 10.1038/s41587-019-0201-4
383 [26] Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq
384 experiments with HISAT, StringTie and Ballgown. Nature Protocols. 2016; 11(9): 1650-1667. doi:
385 10.1038/nprot.2016.095
386 [27] Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved
387 reconstruction of a transcriptome from RNA-seq reads. Nature Biotechnology. 2015; 33(3): 290-295. doi:
388 10.1038/nbt.3122
389 [28] Cantalapiedra CP, Hernández-Plaza A, Letunic I, Bork P, Huerta-Cepas J. eggNOG-mapper v2: Functional
390 annotation, orthology assignments, and domain prediction at the metagenomic scale. Molecular biology and
391 evolution. 2021; 38(12): 5825-5829. doi: 10.1093/molbev/msab293
392 [29] Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. eggNOG 5.0: a
393 hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and
394 2502 viruses. Nucleic Acids Research. 2019; 47(D1): D309-D314. doi:10.1093/nar/gky1085
395 [30] Walter W, Sánchez-Cabo F, Ricote M. GOplot: an R package for visually combining expression data with
396 functional analysis. Bioinformatics. 2015; 31(17): 2912-2914. doi: 10.1093/bioinformatics/btv300
397 [31] Coefficient PC. Pearson's correlation coefficient. New Zealand Medical Journal. 1996; 109(1015): 38. doi:
398 10.1136/bmj.e4483
399 [32] Wang S , Wang Y , Wu H , Hu L. Rbp2 induces epithelial-mesenchymal transition in non-small cell lung
400 cancer. Plos One, 2013; 8(12): e84735. doi: 10.1371/journal.pone.0084735
401 [33] Rainbow PS, Black WH. Effects of changes in salinity on the apparent water permeability of three crab
402 species: *Carcinus maenas*, *Eriocheir sinensis* and *Necora puber*. Journal of Experimental Marine Biology and
403 Ecology. 2001; 264(1): 1-13. doi: 10.1016/S0022-0981(01)00289-1
404 [34] Furriel RPM, McNamara JC, Leone FA. Characterization of (Na^+, K^+)-ATPase in gill microsomes of the
405 freshwater shrimp *Macrobrachium olfersii*. Comparative Biochemistry and Physiology Part B: Biochemistry
406 and Molecular Biology. 2000; 126(3): 303-315. doi:10.1016/S0305-0491(00)00184-X

[35] Péqueux A. Osmotic regulation in crustaceans. Journal of Crustacean Biology. 1995; 15(1): 1-60. doi:10.1163/193724095X00578

[36] Dorwart MR, Shcheynikov N, Yang D, Muallem S. The solute carrier 26 family of proteins in epithelial ion transport. Physiology. 2008; 23(2): 104-114. doi:10.1152/physiol.00037.2007

[37] Choe KP, Strange K. Evolutionarily conserved WNK and Ste20 kinases are essential for acute volume recovery and survival after hypertonic shrinkage in *Caenorhabditis elegans*. American Journal of Physiology-Cell Physiology. 2007; 293(3): C915-C927. doi:10.1152/ajpcell.00126.2007

[38] Sakagami H, Aoki J, Natori Y, Nishikawa K, Kakehi Y, Natori Y, et al. Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. Journal of Biological Chemistry. 2005; 280(24): 23084-23093. doi: 10.1074/jbc.M413438200

[39] Beck FX, Burger-Kentischer A, Müller E. Cellular response to osmotic stress in the renal medulla. Pflügers Archiv: European journal of physiology. 1998; 436(6): 814-827. doi: 10.1007/s004240050710

[40] Márquez J, Matés JM, Campos-Sandoval JA. Glutaminases. In: Schousboe A, Sonnewald U, editors. The Glutamate/GABA-Glutamine Cycle. Cham: Springer International Publishing; 2016. pp. 133-171. doi: 10.1007/978-3-319-45096-4_6

[41] Kovacevic Z, McGivan JD. Mitochondrial metabolism of glutamine and glutamate and its physiological significance. Physiological Reviews. 1983; 63(2): 547-605. doi: 10.1152/physrev.1983.63.2.547

[42] Luka Z, Pakhomova S, Loukachevitch LV, Newcomer ME, Wagner C. Folate in demethylation: The crystal structure of the rat dimethylglycine dehydrogenase complexed with tetrahydrofolate. Biochemical and Biophysical Research Communications. 2014; 449(4): 392-398. doi: 10.1016/j.bbrc.2014.05.064

[43] Liu G, Hou G, Li L, Li Y, Zhou W, Liu L. Potential diagnostic and prognostic marker dimethylglycine dehydrogenase (DMGDH) suppresses hepatocellular carcinoma metastasis in vitro and in vivo. Oncotarget. 2016; 7(22): 32607-32616. doi: 10.18632/oncotarget.8927

[44] Homolya, Váradi A, Sarkadi B. Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate. Biofactors. 2003; 17(1-4): 103-114. doi: 10.1002/biof.5520170111

[45] Dasari S, Ganjayi MS, Yellanurkonda P, Basha S, Meriga B. Role of glutathione S-transferases in detoxification of a polycyclic aromatic hydrocarbon, methylcholanthrene. Chemico-Biological Interactions. 2018; 294(0): 81-90. doi:10.1016/j.cbi.2018.08.023

[46] Fernández-Rodríguez C, Conter C, Oyenarte I, Favretto F, Quintana I, Martinez-Chantar ML, et al. Structural basis of the inhibition of cystathionine γ-lyase from Toxoplasma gondii by propargylglycine and cysteine. Protein Science. 2023; 32(4): e4619. doi: 10.1002/pro.4619

[47] Jurkowska H, Kaczor-Kamińska M, Bronowicka-Adamska P, Wróbel M. Cystathionine γ-lyase. Advances in Hygiene and Experimental Medicine. 2014; 68: 1-9. doi:10.5604/17322693.1085372

[48] Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature. 2009; 457: 910-914. doi:10.1038/nature07762

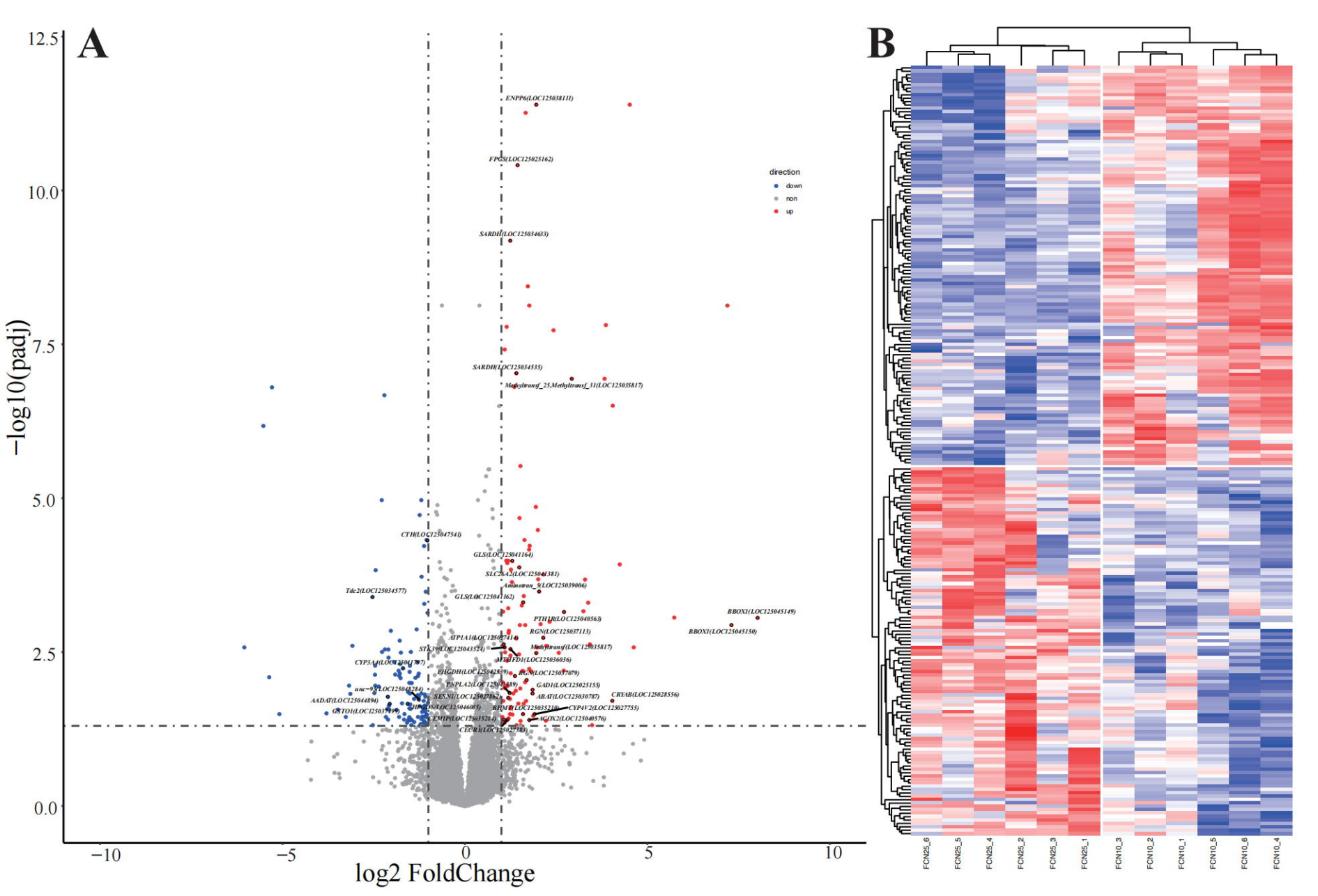
Figure 1. Profiles of gene expression. (**a**) Volcano plot depicting all genes under low salt stress, with up-regulated differently expressed genes represented in red (log2 Fold Change value ≥1) and down-regulated differently expressed genes represented in blue (log2 Fold Change value ≤ -1); (**b**) Selected 227 differently expressed genes in response to low salt stress to construct the heat map.

Figure 2. Profiles of gene expression. GO enrichment analysis on DEGs. Three concentric circles represent ion transport, detoxification and bio-metabolism. Each square in the outer circle represents specific GO terms associated with each functional category, with red dots indicating up-regulated genes and blue dots indicating down-regulated genes. The size and color intensity of each red square in the inner circle represent the magnitude of the Z-score.

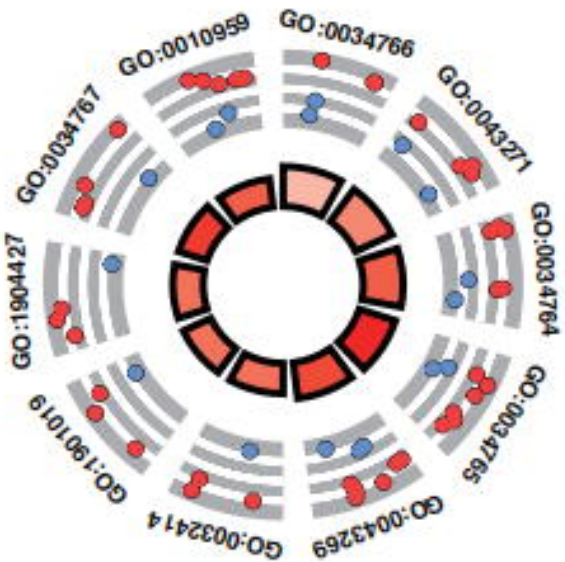
Figure 3. KEGG enrichment analysis on DEGs. This chart illustrates the gene count and the proportion it represents in relation to the total annotated genes for each KEGG metabolic pathway. The y-axis denotes the names of the KEGG metabolic pathways, while the x-axis represents the number of genes.

Figure 4. Illustration of pairwise correlation among key DEGs. Heatmap illustrating the pairwise correlation among 32 genes, represented by colors indicating the strength of correlation. Red color means positive relation, Blue color means negatively relation. "*" denotes genes with p-value less than 0.05, "**" represents p-value less than 0.01, and "***" indicates p-value less than 0.001.

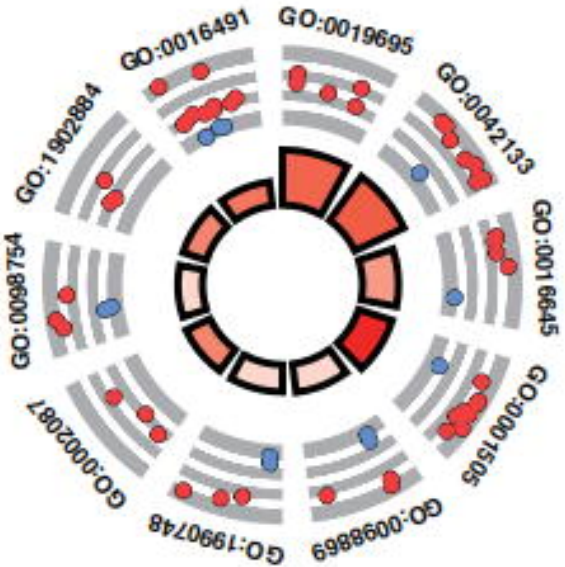
Figure 5. RT-qPCR verification of 8 differentially expressed genes. Between groups of *F. chinensis* in 10 ppt (FC10) and 25 ppt (FC25) salinity environments, 7 genes were upregulated (log2 FC ≥ 1) in shrimp from the FC10 group compared to those from the FC25 group, one gene was downregulated (log2 FC ≤ -1). "*" denotes genes with p-value less than 0.05, "**" represents p-value less than 0.01.


A
B
−log10(padj)
log2 FoldChange
direction
down
non
up
ENPP6(LOC125038111)
FPGS(LOC125025162)
SARDH(LOC125034633)
SARDH(LOC125034535)
Methyltransf_25,Methyltransf_31(LOC125035817)
CTH(LOC125047541)
GLS(LOC125041164)
SLC26A2(LOC125041381)
Aminotran_5(LOC125039006)
Tdc2(LOC125034577)
GLS(LOC125041162)
BBOX1(LOC125045149)
BBOX1(LOC125045150)
PTH1R(LOC125040563)
RGN(LOC125037113)
ATP1A1(LOC125027414)
STK39(LOC125043524)
Methyltransf(LOC125035817)
MTHFD1(LOC125036036)
CYP3A4(LOC125041797)
PHGDH(LOC125042539)
RGN(LOC125037079)
PNPLA2(LOC125041389)
GAD1(LOC125025155)
unc−93(LOC125048284)
ABAT(LOC125030787)
CRYAB(LOC125028556)
AADAT(LOC125044894)
SESN1(LOC125027862)
GSTO1(LOC125037499)
CYP4V2(LOC125027755)
CEMIP(LOC125035284)
ACOX2(LOC125040576)
CECR1(LOC125027383)
FCN25_6
FCN25_5
FCN25_4
FCN25_2
FCN25_3
FCN25_1
FCN10_3
FCN10_2
FCN10_1
FCN10_5
FCN10_6
FCN10_4

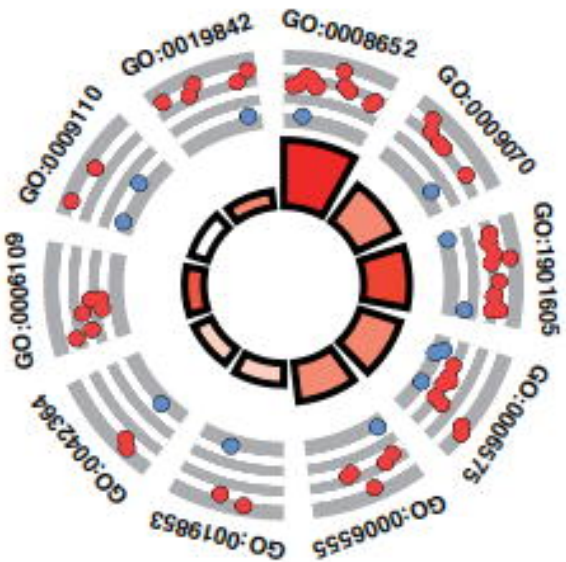
Ion transport

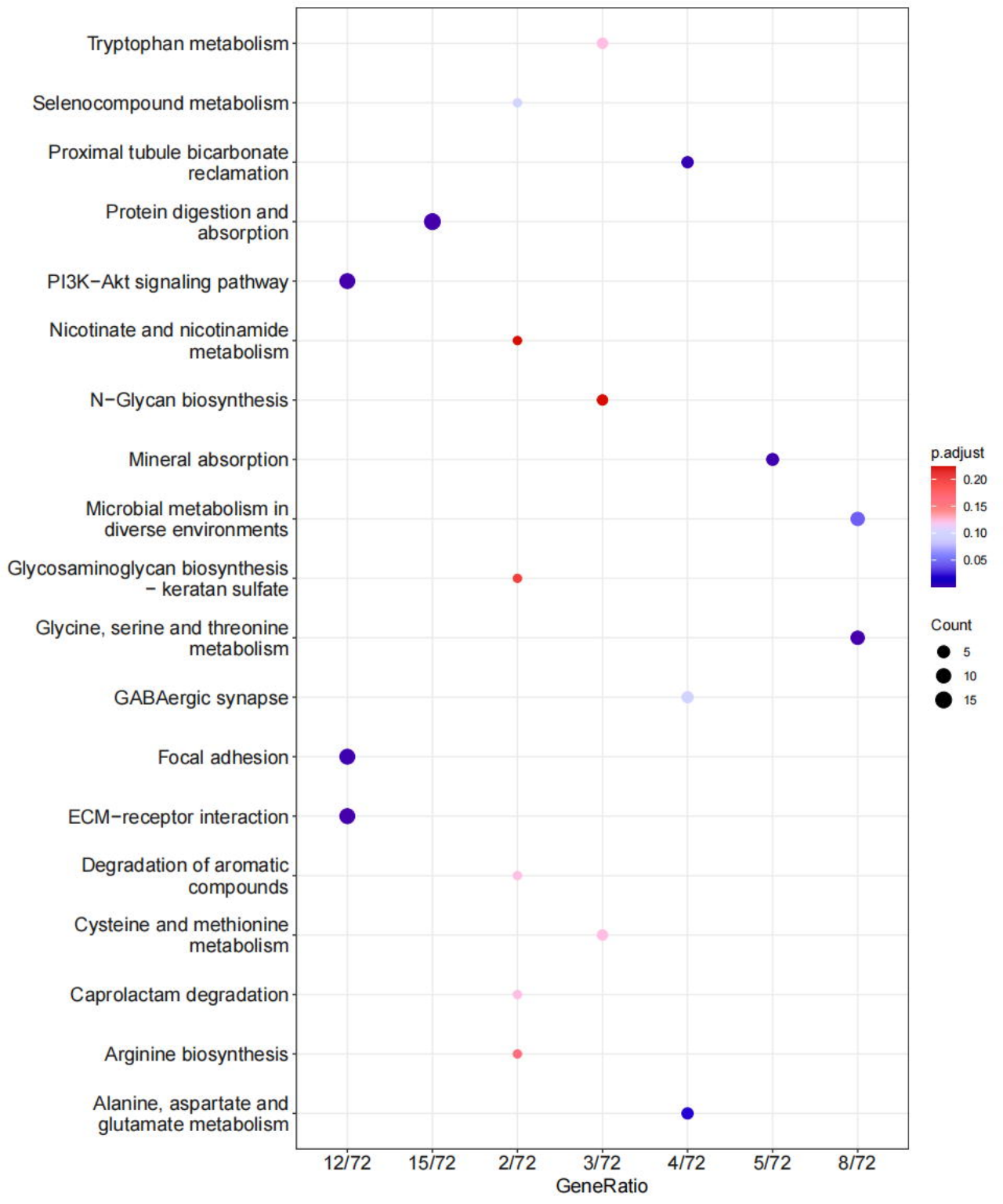


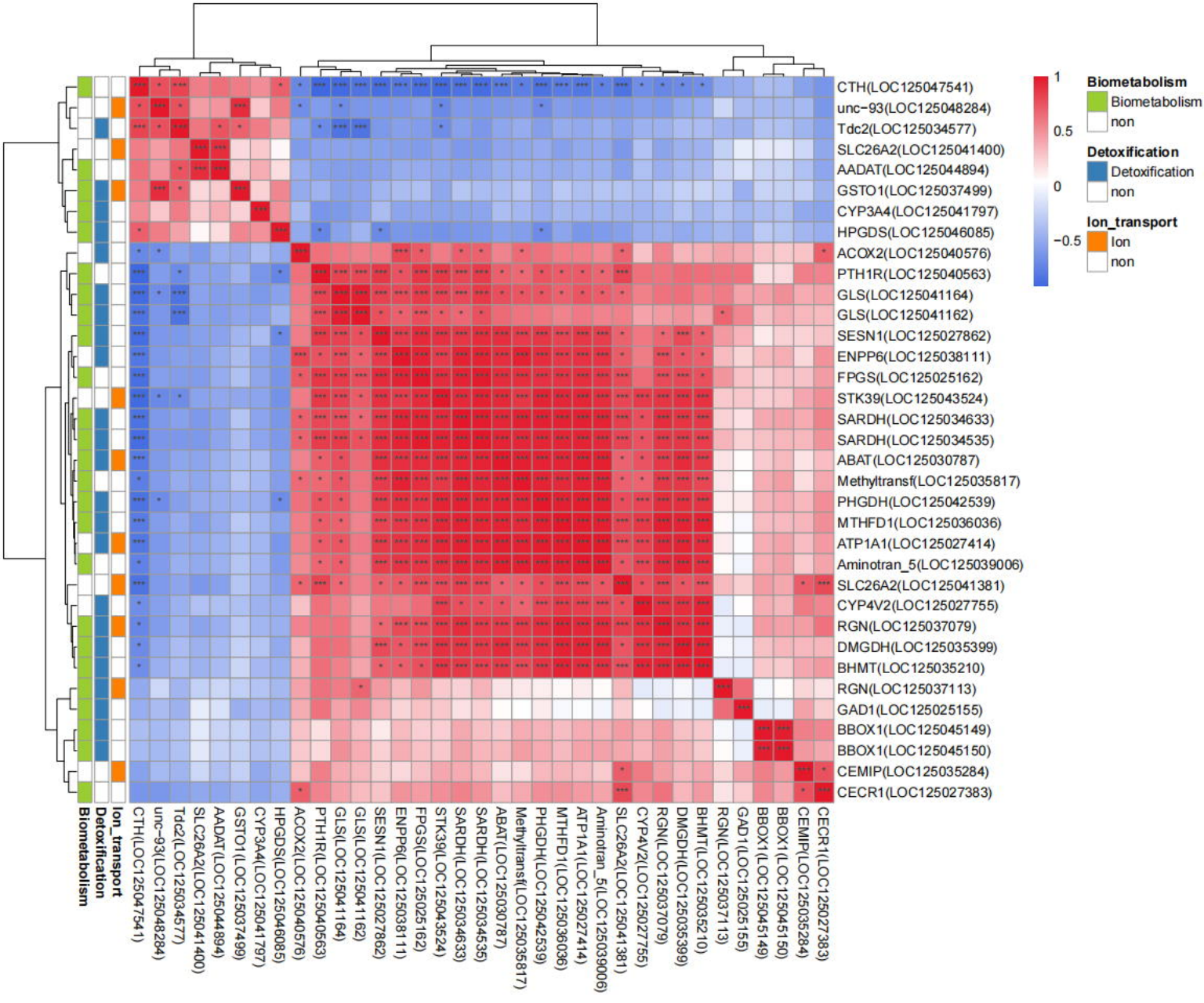
Detoxification

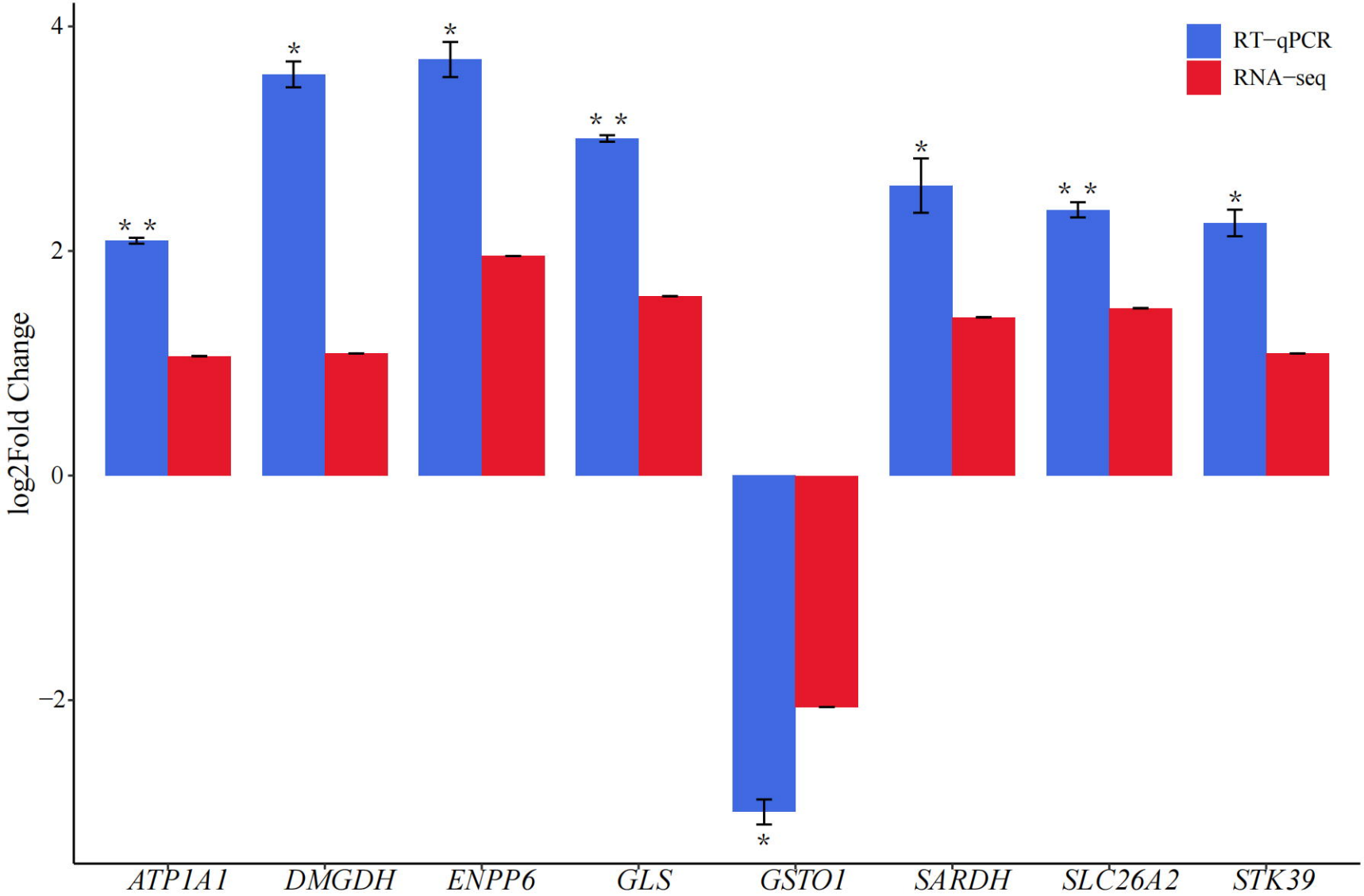


Bio-metabolism




Tryptophan metabolism
Selenocompound metabolism
Proximal tubule bicarbonate reclamation
Protein digestion and absorption
PI3K−Akt signaling pathway
Nicotinate and nicotinamide metabolism
N−Glycan biosynthesis
Mineral absorption
Microbial metabolism in diverse environments
Glycosaminoglycan biosynthesis − keratan sulfate
Glycine, serine and threonine metabolism
GABAergic synapse
Focal adhesion
ECM−receptor interaction
Degradation of aromatic compounds
Cysteine and methionine metabolism
Caprolactam degradation
Arginine biosynthesis
Alanine, aspartate and glutamate metabolism
12/72
15/72
2/72
3/72
4/72
5/72
8/72
GeneRatio
p.adjust
0.20
0.15
0.10
0.05
Count
5
10
15


CTH(LOC125047541)
unc−93(LOC125048284)
Tdc2(LOC125034577)
SLC26A2(LOC125041400)
AADAT(LOC125044894)
GSTO1(LOC125037499)
CYP3A4(LOC125041797)
HPGDS(LOC125046085)
ACOX2(LOC125040576)
PTH1R(LOC125040563)
GLS(LOC125041164)
GLS(LOC125041162)
SESN1(LOC125027862)
ENPP6(LOC125038111)
FPGS(LOC125025162)
STK39(LOC125043524)
SARDH(LOC125034633)
SARDH(LOC125034535)
ABAT(LOC125030787)
Methyltransf(LOC125035817)
PHGDH(LOC125042539)
MTHFD1(LOC125036036)
ATP1A1(LOC125027414)
Aminotran_5(LOC125039006)
SLC26A2(LOC125041381)
CYP4V2(LOC125027755)
RGN(LOC125037079)
DMGDH(LOC125035399)
BHMT(LOC125035210)
RGN(LOC125037113)
GAD1(LOC125025155)
BBOX1(LOC125045149)
BBOX1(LOC125045150)
CEMIP(LOC125035284)
CECR1(LOC125027383)
Biometabolism
Detoxification
Ion_transport
1
0.5
0
−0.5
Biometabolism
Biometabolism
non
Detoxification
Detoxification
non
Ion_transport
Ion
non


RT−qPCR
RNA−seq
log2Fold Change
4
2
0
−2
ATP1A1
DMGDH
ENPP6
GLS
GSTO1
SARDH
SLC26A2
STK39