Metabolomics and biochemical assays reveal the metabolic responses to hypo-salinity stress and osmoregulatory role of cAMP-PKA pathway in *Mercenaria mercenaria*

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A B S T R A C T

Hypo-salinity events frequently occur in marine ecosystem due to persistent rainfall and freshwater inflow, reducing the cytosol osmolarity and triggering cellular stress responses in aquatic organisms. Euryhaline bivalves have developed sophisticated regulatory mechanisms to adapt to salinity fluctuations over a long period of evolution. In this study, we performed multiple biochemical assays, widely targeted metabolomics, and gene expression analysis to investigate the comprehensive metabolic responses to hypo-salinity stress and osmoregulation mechanisms in hard clam *Mercenaria mercenaria*, which is a euryhaline bivalve species widely cultured in China. During hypo-salinity stress, increased vacuoles appeared in gill filaments. The Na^+ and Cl^- concentrations in gills significantly decreased because of the up-regulation of Na^+/K^+-ATPase (NKA) activity. The cAMP content dramatically decreased at 5 d post hypo-salinity stress. Meanwhile, the gene expression levels of adenylate cyclase, proteinkinase A, and sodium and calcium channel proteins were evidently down-regulated, suggesting that cAMP-PKA pathway was inhibited to prevent ambient inorganic ions from entering the gill cells. Antioxidant metabolites, such as serine and Tyr-containing dipeptides, were significantly up-regulated to resist oxidative stress. Glycerolipid metabolism was strengthened to stabilize membrane structure when hypo-salinity stress was prolonged to 5 days. At 1 d post hypo-salinity stress, an increase in alanine and lactate contents marked the initiation of anaerobic metabolism. Acylcarnitines accumulation indicated that fatty acids β-oxidation was promoted to provide energy for osmoregulation. The potential biomarkers of hypo-salinity stress were identified in hard clams. This study provides novel insights into the metabolic regulatory mechanisms to hypo-salinity stress in euryhaline bivalves.

1. Introduction

Salinity is a significant environmental factor affecting the distribution, survival, and physiological processes of aquatic organisms [1]. Owing to global climate change, a decrease in seawater salinity has been observed over the past decades [2,3]. Bivalves inhabiting coastal and estuarine areas are frequently exposed to hypo-salinity stress due to persistent rainfall and terrestrial runoff during the rainy season [4,5]. Euryhaline bivalves have developed sophisticated regulatory mechanisms to adapt to salinity fluctuations over a long period of evolution. Bivalves can regulate their cardiac activity, oxygen consumption, nitrogen metabolism, and immunological parameters at physiological and metabolic level to tolerate acute hypo-salinity stress [6,7]. Additionally, transcriptome studies have reported that several genes involved in carbon metabolism and

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fatty acid degradation were significantly up-regulated in *Cyclina sinensis* during acute hypo-salinity stress [8]. Bivalves are osmo-conformers whose haemolymph osmolality drops rapidly upon hypo-salinity exposure [9]. Gills of bivalves are important tissues that directly interact with the external environment [10]. The osmotic gradient between haemolymph and gills causes water influx and subsequent cell swelling [11,12]. Therefore, the osmoregulation ability is essential for bivalves to restore the normal structures and functions of osmotically disturbed gill cells [13,14].

Osmoregulation is closely related to the regulations of various metabolic pathways. For bivalves, osmoregulation and cell volume regulation depend on the rapid release or accumulation of intracellular free amino acids (FAAs) and inorganic ions (i.e., Na^+, K^+, and Cl^-) [15,16]. FAAs account for approximately 30 % of the total osmotically active substances in bivalves [7]. Variations in FAAs composition and concentration have been reported in various hypo-salinity stressed bivalves species, such as *Crassostrea gigas* [10], *Mytilus edulis* [6], and *Rangia cuneata* [17]. Bivalves that utilize a larger FAAs pool are considered to have greater tolerance to salinity stress [18]. In addition to the osmoregulatory role, several FAAs, such as alanine, glutamate, glycine, and taurine, are important building blocks of hormones and enzymes [7], and play an vital role in antioxidant responses [19]. Moreover, glutamate can enter the glycolysis and tricarboxylic acid cycle through a transamination reaction, thereby participating in the regulation of energy metabolism [7,20]. Na^+/K^+-ATPase (NKA) is a sodium pump that can break down adenosine triphosphate (ATP) to provide the driving force for the *trans*-membrane transport of Na^+ and K^+. The osmoregulatory role of NKA has been well studied in fishes and crustaceans [21,22]. When exposed to hypo-salinity conditions, NKA activity in bivalves exhibited species-specific variations, such as significant up-regulation in *Pinctada fucata* [23] and *Cyclina sinensis* [24], and almost no change in *Meretrix lusoria* [25]. Other ion channel proteins in the cell membranes, such as K^+ and Cl^- channels and K-Cl cotransporters, also play important roles in osmoregulation, promoting the release of intracellular KCl when external osmolarity decreases [7]. Some ion channel proteins can be activated by phosphorylation of protein kinase A, whose activity is normally activated by excess cAMP. Since the *trans*-membrane transport of FAAs and inorganic ion consumes lots of energy, bivalves tend to reduce the respiration rates and increase anaerobic metabolism to reduce energy expense on respiration [26,27]. To date, knowledge of the critical metabolic pathways involved in osmoregulation in bivalves is still limited.

Metabolites are the intermediate or end products of cellular regulatory processes that can reflect the stress responses of biological systems to environmental changes [28]. Metabolomics is a powerful tool for quantitative measurements of multiple small-molecular metabolites, and can be used to investigate the metabolic responses of organisms to ambient fluctuations [29,30]. Traditional liquid chromatography-mass spectrometry (LC-MS)-based metabolomics includes untargeted and targeted metabolomics. Widely targeted metabolomics combines the advantages of both and is characterized by universality and accuracy [31]. To date, multiple metabolomics techniques have been applied to explore the metabolic responses to environmental stressors in several marine bivalves, including *Perna canaliculus*, *Ostrea edulis*, and *Scapharca subcrenata* under heat stress [32–34]; *Ruditapes philippinarum*, *Mytilus edulis*, and *C. gigas* under hypoxia stress [35,36]; and *C. gigas* and *Pecten maximus* under ocean acidification stress [37,38]. Most previous research focused on certain aspects of the metabolic response to hypo-salinity stress, such as adjustments of FAAs concentration and energy metabolism, and pointed out that these metabolism alterations greatly contribute to hypo-salinity tolerance in bivalves [6,7,39]. A comprehensive study using widely targeted metabolomics is essential to investigate the overall metabolite alterations and metabolic pathway regulation in hypo-salinity stressed bivalves, which will facilitate a better understanding of the relationship between the metabolic patterns and osmoregulation.

The hard clam *Mercenaria mercenaria* is a euryhaline bivalve species native to the east coast of the United States of America (USA) and Canada [40]. Since its introduction to China for commercial exploitation in 1997, hard clams have become an important bivalve species in pond farming owing to their high economic value. Farming areas of hard clams cover nearly 0.67 km^2, distributed in coastal areas from Liaoning Province to Guangdong Province in China. During persistent rainfall and flood discharge, the salinity of cultured pond water may drop to 10 psu (practical salinity units). Hence, hard clams are constantly exposed to hypo-salinity stress for several days in the process of natural growth and artificial farming. In our previous aquaculture experiments, hard clams exhibited remarkable tolerance to hypo-salinity stress. Most adult individuals can survive for more than ten days in 10 psu seawater. Consequently, hard clam may serve as a suitable model for exploring the osmoregulation and hypo-salinity tolerance mechanisms in euryhaline bivalves.

In the present study, we integrated multiple experimental methods, including histological observation, determinations of Na^+ and Cl^-ion concentration, enzyme activity assays of NKA and protein kinase A, widely targeted metabolomics, and quantitative real-time PCR (qRT-PCR) of genes in the cAMP-PKA pathway, on the gills of hard clams exposed to 10 psu seawater for 8 h, 1 d, and 5 d. Variation in the composition and quantity of the differential metabolites among different samples reflected the major metabolic responses of hard clams to different durations of hypo-salinity stress. Kyoto encyclopedia of genes and genomes (KEGG) annotation and enrichment analysis were performed on these differential metabolites to better understand their functions and uncover the critical osmoregulatory pathway in hard clams. Additionally, the potential biomarkers of short-term (up to 5 days) hypo-salinity stress were also identified in this organism. The results of this study provide novel insights into the metabolic regulatory mechanisms to hypo-salinity stress in marine euryhaline bivalves.

2. Materials and methods

2.1. Sample collection and experimental design

The adult hard clams (shell length 43.13 ± 2.07 mm) were collected from a farming pond in Tianjin, China on June 25, 2021. The clams were acclimated in a laboratory aquarium with aerated seawater (salinity: 30 ± 0.5 psu, pH: 8.0 ± 0.2, dissolved oxygen: 8.0 ± 1.0 mg/L, temperature: 15 ± 1 ℃), and fed with *Spirulina* spp. powder for two weeks before the hypo-salinity treatment. Half of the water volume was exchanged daily to remove the metabolic waste and residual food during acclimation period. Considering the range of natural salinity variations and the salinity tolerance of hard clams, 10 psu is regarded as low salinity in this study [41]. A total of 100 clams were removed from the aquarium and immediately placed into another aquarium containing 10 psu seawater (mixed with specific proportion of distilled water and artificial seawater salt). The remaining clams were maintained in 30 psu seawater. During low salinity treatment, the hypo-salinity seawater was exchanged daily, and no additional *Spirulina spp* was feed to the hard clams. 18 live clams were randomly sampled from the 30 psu seawater (S30, control group), and 10 psu seawater at 8 h (S10_8h), 1 d (S10_1d), and 5 d (S10_5d), respectively. Once sampled, the gill of each clam was aseptically dissected and

washed with phosphate-buffered saline. For each group, gills from three clams were pooled into one sample, and six biological replicates were set up. The samples were frozen immediately in liquid nitrogen and then stored at −80 ℃ for ion concentration and enzyme activity determination, and metabolite and RNA extractions. Additionally, three live clams were randomly sampled from the S30, S10_1d, and S10_5d groups, respectively. These gill samples were aseptically dissected and fixed with 4 % paraformaldehyde for histological observation.

2.2. Histological observation

The fixed gill samples were washed and dehydrated with different concentrations of alcohol. Then the samples were cleaned with xylene and embedded in paraffin. After cooling and solidification, paraffin blocks were cut into slices at a thickness of 5 μm. Then the paraffin slices were dewaxed with xylene and hydrated with different concentrations of alcohol. Finally, samples were stained with haematoxylin and eosin, and observed under the light microscope (Nanjing jiangnan Novel Optis Co., ltd, China).

2.3. Na$^+$, Cl$^-$ concentration and enzyme activity assay

For each sample, 30 mg of gill tissue was added to 300 μL of deionized water, then homogenized at high speed for 5 min. After centrifugation at 2500 rpm for 10 min, 40 μL supernatant was used to determine the Na$^+$ and Cl$^-$ concentrations using using sodium and chlorine assay kits (Nanjing Jiancheng Bioengineering Institute, China). The optical density (OD) value of each well was measured at 630 nm using a spectrophotometer (UNICO, USA). The Na$^+$ and Cl$^-$ concentrations of each sample were calculated as per the manufacturer's instructions. Another 50 μL supernatant was used to establish the enzymatic assay system by using a Na$^+$/K$^+$-ATPase assay kit (Nanjing Jiancheng Bioengineering Institute, China). The OD value was measured at 636 nm using a microplate reader (ThermoFisher Scientific, USA). Additionally, 100 mg of gill tissue from each sample was used to determine the activity of protein kinase A following the instructions of Shellfish Protein kinase A ELISA Kit (Shanghai Enzyme-linked Biotechnology, China). Before the experiment, exogenous cAMP (Solarbio, China) and H-89 (specific inhibitor of protein kinase A, Beyotime, China) were used to validate the suitability of this kit for hard clams. In the positive control group, nine clams were treated with 10 μM cAMP in a beaker containing 2 L aerated seawater (30 psu). In the negative control group, nine clams were treated with 20 μM H-89 in a beaker containing 2 L aerated seawater (30 psu). After 12 h treatment, the gills of each hard clams were aseptically dissected and washed with phosphate-buffered saline. For each group, gills from three clams were pooled into one sample, and then stored at −80 ℃ for protein kinase A activity determination. Three biological replicates were set up for these experiments.

2.4. HPLC conditions and ESI-QTRAP-MS/MS data acquisition

All gill samples were taken out from the −80 °C and thawed on ice. Then the samples were thoroughly ground under liquid nitrogen. Approximately 20 mg sample was added into 400 μL of 70 % methanol water internal standard extractant, and homogenized at 1500 rpm for 5 min. Mixture was placed on ice for 15 min, and then centrifuged at 12000 rpm, 4 °C for 10 min. 300 μL supernatant was removed from the EP tube and stood still at −20 °C for 30 min. After final centrifugation (12000 rpm, 4 °C, 3 min), the supernatant of each sample were analyzed through the LC-ESI-MS/MS system (UPLC: ExionLC AD, https://sciex.com.cn/; MS: QTRAP® System, https://sciex.com/). Analytical conditions were as follows: UPLC column: Waters ACQUITY UPLC HSS T3 C18

(1.8 μm, 2.1 mm*100 mm); column temperature: 40 ℃; flow rate: 0.4 mL/min; injection volume: 2 μL; solvent system: water (0.1 % formic acid), and acetonitrile (0.1 % formic acid); gradient program: 95:5 V/V at 0 min, 10:90 V/V at 11.0 min, 10:90 V/V at 12.0 min, 95:5 V/V at 12.1 min, and 95:5 V/V at 14.0 min. LIT and triple quadrupole (QQQ) scans were acquired on a QTRAP® LC-MS/MS System. This system were equipped with an ESI Turbo Ion-Spray interface, and operated in positive and negative ion mode and controlled by Analyst 1.6.3 software (Sciex). ESI source operation parameters in this study were same as the previous study [42]. Instrument tuning and mass calibration were performed with 10 and 100 μmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. A specific set of MRM transitions were monitored for each period according to the eluted metabolites.

2.5. Differential metabolites identification

Analyst 1.6.3 software was used to perform MS data analysis based on the self-built MWDB database (Metware Biotechnology Co., ltd. Wuhan, China), and Z-score was used to normalize the metabolite content data [43]. In order to identify the clustering pattern of all samples, unsupervised principal component analysis (PCA) was performed by using statistics function prcomp in R (https://www.r-project.org) based on the metabolite content data. Since PCA was insensitive to variables with low correlation, the supervised orthogonal projections for latent structures-discriminant analysis (OPLS-DA) was performed to enhance understanding of factors responsible for classification and obtain effective group separations by using R package MetaboAnalystR (v1.0.1). Models generated by OPLS-DA with classification parameters R^2Y and Q^2 values > 0.5 were considered robust and had adequate goodness-of-fit. Variable important in projection (VIP) values of each metabolite was extracted from OPLS-DA. Differential metabolites between groups were determined by both absolute Log2FC (fold change) $\geq$ 1 and VIP $\geq$ 1.

2.6. Heatmap analysis

Heatmap analysis of the contents of key differential metabolites were conducted by using the OmicShare heatmap tool with default parameters (https://www.omicshare.com/tools/home/report/reportheatmap.html).

2.7. KEGG annotation and enrichment analysis

All identified metabolites were annotated using KEGG compound database (https://www.kegg.jp/kegg/compound/), and the differential metabolites were mapped to KEGG pathway database to further understand their functions (https://www.kegg.jp/kegg/pathway.html). Significantly enriched pathways were identified with a hypergeometric test, and $P < 0.05$ was considered statistically significant.

2.8. RNA extraction, cDNA synthesis and qRT-PCR analysis

RNA was extracted from all gill samples using miniBEST Universal RNA Extraction kit (TaKaRa Bio, Japan) according to the manufacturer's instructions. RNA degradation and contamination were monitored using 1 % agarose gels electrophoresis (buffer: 1 × TAE, voltage: 300 V, time: 15 min). Concentrations and purity of RNA were detected using Nanodrop device (ThermoFisher Scientific, USA). Then the RNA was used to synthesize double-stranded cDNA using TaKaRa Prime Script™RT reagent Kit (TaKaRa Bio, Japan). Expression variations of seven genes in cAMP-PKA signal transduction pathway (ko04024) were detected by qRT-PCR analysis (Table 1). cDNA sequence of these genes were obtained from the

Table 1
The seven critical genes in cAMP-PKA pathway selected for qRT-PCR analysis.

Gene ID	Gene Length	Swiss-Prot Description (E-value < 1E-10)
evm.model. Hic_asm_8.2998	825	Adenosine receptor A2a (*Canis familiaris*)
evm.model. Hic_asm_7.687	852	guanine nucleotide-binding protein G(s) subunit alpha (*Anopheles gambiae*)
evm.model. Hic_asm_12.1687	2062	guanine nucleotide-binding protein G(i) subunit alpha
evm.model. Hic_asm_13.1467	6852 5185	(*Canis familiaris*) Adenylate cyclase type 1
evm.model. Hic_asm_10.1991		(*Mus musculus*) cAMP-dependent protein kinase catalytic subunit (*Drosophila melanogaster*)
evm.model. Hic_asm_9.1757	1940	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (*Homo sapiens*)
evm.model. Hic_asm_0.408	5184	Voltage-dependent ʟ-type calcium channel subunit alpha-1C (*Rattus norvegicus*)

*M. mercenari*a genome [44], and their primers were designed using primer5 v5.5.0 (Table S1). Transcription elongation factor 1 alpha (EF1α) was used as a reference gene to normalize the expression levels between samples [45]. All primers were validated by 1.5 % agarose gels electrophoresis (buffer: $1 \times$ TAE, voltage: 100 V, time: 20 min). TB Green Premix Ex Taq (TaKaRa Bio, Japan) was used for qRT-PCR analysis. PCR reaction was as follows: 0.5 μL forward primer (5 μmol/L), 0.5 μL reverse primer (5 μmol/L), 10 μL TB Green Prime Ex Taq, 1 μL cDNA template, and 8 μL ddH$_2$O. qRT-PCR was conducted on the Eppendorf RealPlex Mastercycler, and the PCR conditions were as follows: 95 ℃ for 2 min; 40 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, and a melting curve was generated. PCR products were detected by 1 % agarose gel electrophoresis. Relative expression level was calculated through the $2^{-\Delta\Delta Ct}$ method [46].

2.9. Receiver operating characteristic curve (ROC) analysis

10 differential metabolites with the largest absolute log2 (Fold change) value between the stressed groups (S10_8h, S10_1d, and S10_5d) and control group (S30) were selected for ROC analysis using the OmicShare ROC tool with default parameters (https://www.omicshare.com/tools/home/report/reportroc.html). Metabolites with area under the curve (AUC) values > 0.9 were considered as biomarkers.

2.10. Statistical analysis

The statistical analysis of results of Na$^+$, Cl$^-$ concentration, NKA enzyme activity, key metabolite content, and gene expression were performed using IBM SPSS Statistics 25 (IBM Corp, US). Data were tested for homogeneity of variances (*F* text), and tested by one-way analysis of variance (ANOVA) with Tukey's test. *P* < 0.05 was considered statistically significant.

3. Results

3.1. Histological observation

As shown in Fig. 1A, the gill filaments in S30 group were neatly arranged and exhibited normal structure. As the hypo-salinity stress duration increased, more intracellular and extracellular vacuoles appeared in the gill filaments. The arrangement of gill filaments was gradually disordered, and their lengths seemed to decrease during stress (Fig. 1B and 1C).
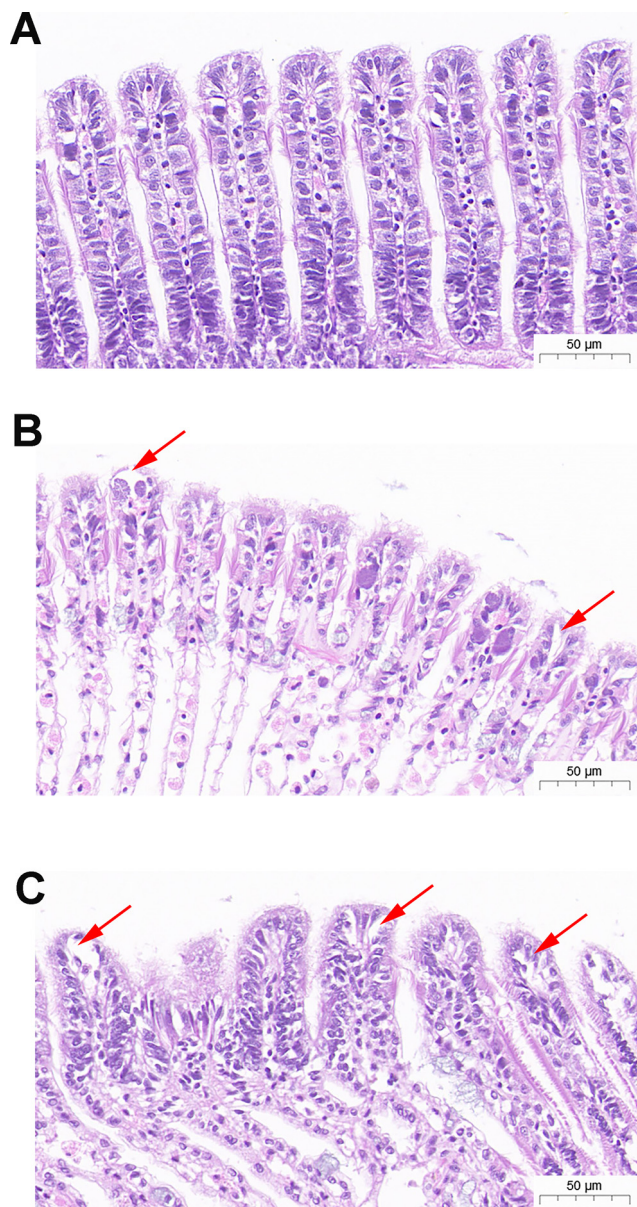

Fig. 1. Histological observation of *M. mercenaria* gills exposed to different durations of hypo-salinity stress. (A) S30; (B) S10_1d; (C) S10_5d. Intracellular and extracellular vacuoles are marked by red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Ion concentration and NKA enzyme activity assay

During hypo-salinity stress, the Na$^+$ and Cl$^-$ concentrations in gills exhibited similar variation trends, which remained stable within 1 d, and decreased significantly at 5 d (0.72- and 0.79-fold compared to that of the control, respectively) (Fig. 2A and 2B). NKA enzyme activity in gills remained nearly unchanged within 1d, while it increased dramatically and reached the highest level in S10_5d group (1.29-fold compared to that of the control). This suggests that NKA activity is up-regulated to pump Na$^+$ out of gill cells to maintain osmotic equilibrium.

3.3. Overview of metabolomics profiles

Based on the self-built MWDB database, 979 metabolites from 24 samples (4 groups $\times$ 6 replicates) were identified using widely targeted metabolomics. These metabolites were divided into 16
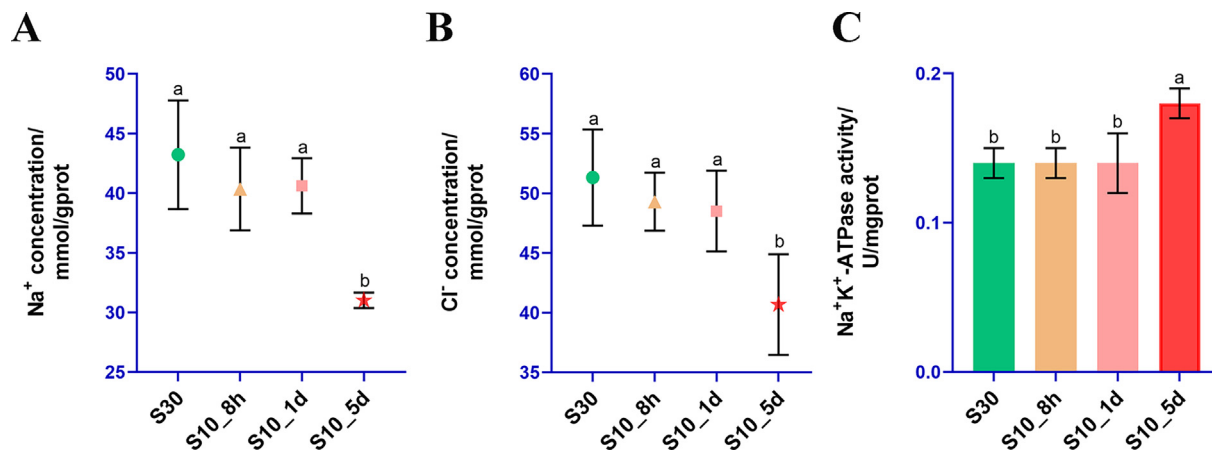
Fig. 2. Ion concentration and NKA enzyme activity variations in *M. mercenaria* gills during hypo-salinity stress. (A) Na$^+$ concentration; (B) Cl$^-$ concentration; (C) NKA enzyme activity.

classes, such as amino acid and its metabolomics (265, 27.07 %), nucleotide and its metabolomics (85, 8.86 %), carbohydrates and its metabolites (32, 3.27 %), fatty acyl (125, 12.77 %), glycerophospholipids (95, 9.70 %), and coenzyme and vitamins (16, 1.63 %). PCA results illustrated that the group separations were not distinct among S30, S10_8h, and S10_1d groups but differed between S30 and S10_5d (Fig. S1). This indicates that 5-d hypo-salinity stress has the most significant influences on the physiological processes in hard clams. More effective group separations were obtained using supervised OPLS-DA. Three pairs of pairwise comparisons (S10_8h vs S30, S10_1d vs S30, and S10_5d vs S30) in each OPLS-DA score plot were evidently separated into two sides, and all their R^{2}Y and Q^2 values exceeded 0.5 (Fig. 3).

3.4. Differential metabolite screening

The differential metabolites between the stressed groups (S10_8h, S10_1d, and S10_5d) and the control group (S30) were screened out by pairwise comparison. The number of differential

metabolites gradually increased with the duration of stress. In detail, 66 metabolites were significantly up-regulated, and 3 were significantly down-regulated in S10_8h vs S30. In S10_1d vs S30, 61 metabolites were significantly up-regulated, and 7 were significantly down-regulated. 168 metabolites were significantly altered (including 153 up-regulated and 15 down-regulated) in S10_5d vs S30. The venn diagram analysis shows that S10_8h vs S30 and S10_1d vs S30 share 48 (approximately 70 %) common differential metabolites (Fig. S2). This suggests that 8-h and 1-d hypo-salinity stress cause similar metabolic alterations in hard clams. Moreover, the compositions of the differential metabolites in S10_8h vs S30 and S10_1d vs S30 were similar (Fig. 4). Proportions of amino acid and its metabolomics, and nucleotide and its metabolomics to total differential metabolites were highest in S10_8h vs S30 (20.29 %), and S10_1d vs S30 (20.59 %), respectively. In S10_5d vs S30, 46 (27.38 %) types of glycerophospholipids and 26 (15.48 %) types of fatty acyls were identified as differential metabolites, which were remarkably greater than that in S10_8h vs S30 and S10_1d vs S30.
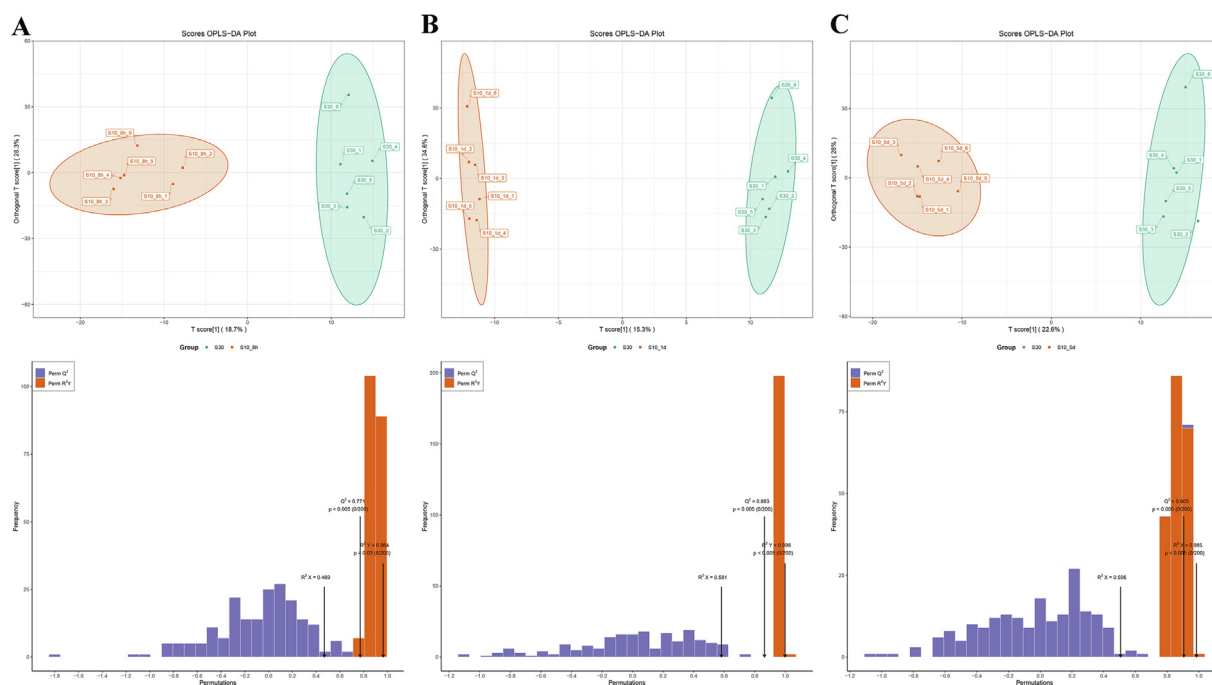

Fig. 3. OPLS-DA of the metabolites in *M. mercenaria* gill samples. (A) S10_8h vs S30; (B) S10_1d vs S30; (C) S10_5d vs S30.
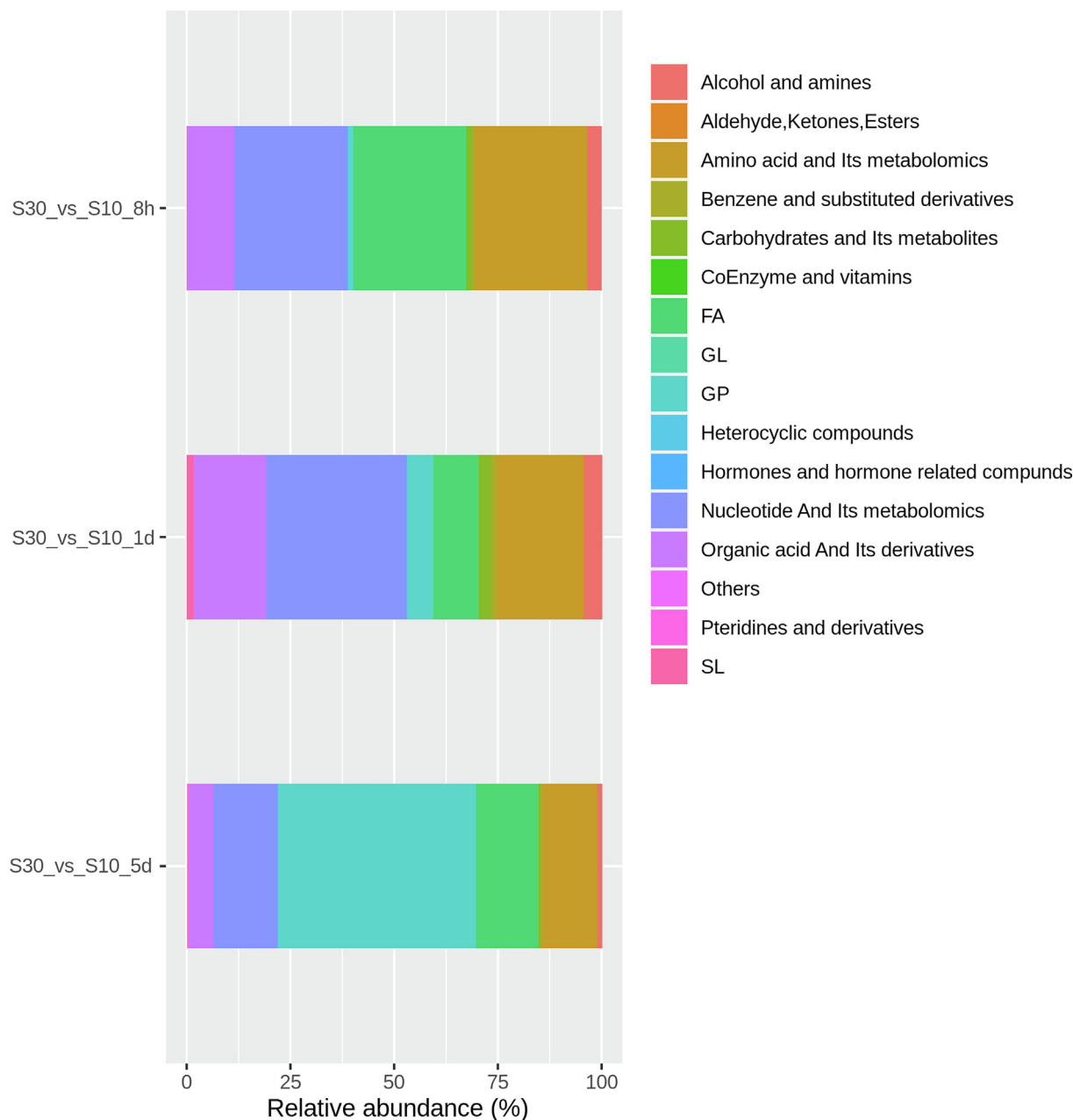
Fig. 4. Variations in the composition of differential metabolites in S10_8h vs S30, S10_1d vs S30, and S10_5d vs S30. The differential metabolites belonging to different classes are marked by different colors.

3.5. KEGG enrichment analysis of differential metabolites

As shown in Fig. S3, "purine metabolism (ko00230)", "morphine addiction (ko05032)", and "cAMP signaling pathway (ko04024)" were significantly enriched ($P < 0.05$) by the differential metabolites in S10_8h vs S30. The pathway enriched by the largest number of differential metabolites was "metabolic pathways (ko01100)", followed by "purine metabolism (ko00230)" and "ABC transporters (ko02010)". In S10_1d vs S30, "Galactose metabolism (ko00052)" and "cAMP signaling pathway (ko04024)" were significantly enriched ($P < 0.05$). The most enriched pathway was "metabolic pathways (ko01100)", followed by "purine metabolism (ko00230)" and "cAMP signaling pathway (ko04024)". In S10_5d vs S30, the most significantly enriched pathways were "purine metabolism (ko00230)", "glycerolipid metabolism (ko00561)", and "cAMP signaling pathway (ko04024)" ($P < 0.05$).

3.6. Variations in gene expression and metabolite contents in cAMP-PKA pathway and protein kinase a activity

Since the "cAMP signaling pathway" was the only significantly enriched pathway in all pairs of pairwise comparisons, the expression variations during hypo-salinity stress of seven critical genes in this pathway were investigated. The primers used for qRT-PCR were validated through 1.5 % agarose gels electrophoresis (Fig. S4). As shown in Fig. 5, the expression level of adenosine receptor A2a (*ADOR*) was significantly inhibited during hypo-salinity stress. Guanine nucleotide-binding protein G(s) subunit alpha (*GNAS*) expression remained stable within 1 d, while it decreased evidently at 5 d (0.70-fold compared to that in S30 group). In contrast, guanine nucleotide-binding protein G(i) sub-unit alpha (*GNAI*) expression was significantly up-regulated, and reached the highest level in S10_5d group (8.25-fold compared to
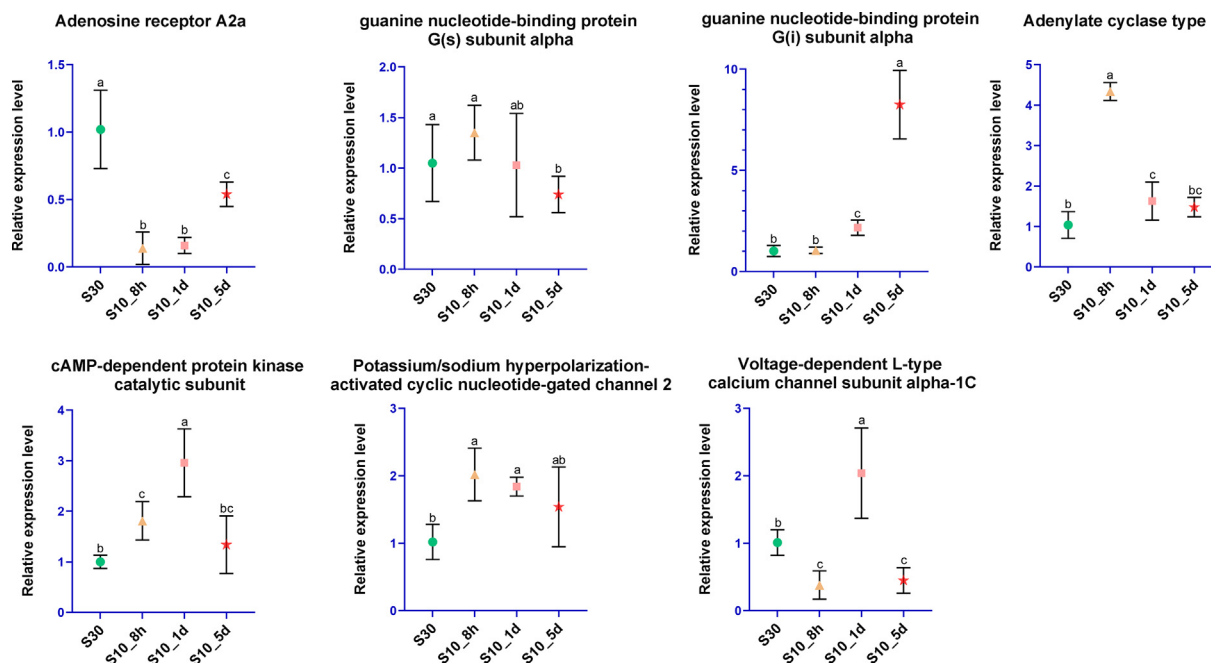
Fig. 5. Expression variations in seven critical genes in cAMP-PKA pathway during hypo-salinity stress. Expression levels of the selected genes were normalized to *EF1α* expression level. Data are means (±SE) of three replicates. Different letters above the bars indicate significant difference in gene expression between groups ($P < 0.05$).

that of the control). Adenylate cyclase type 1 (*ADCY1*) expression was rapidly up-regulated at 8 h, followed by an evident decline at 5 d (1.48-fold compared to that of the control). The variations in expression patterns of cAMP-dependent protein kinase catalytic subunit (*PKA*), potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (*HCN2*), and voltage-dependent L-type calcium channel subunit alpha-1C (*VDCC1C*) were similar. Their expression levels reached the highest levels in S10_8h or S10_1d groups, and were dramatically decreased in S10_5d group. The down-regulation of *HCN2* and *VDCC1C* facilitated to prevent ambient Na^+, K^+, and Ca^{2+} from entering cells.

The variations in relative content of four metabolites in cAMP-PKA pathway during hypo-salinity stress were shown in Fig. S5. Adenosine content significantly increased by 2.24-fold in S10_1d group compared to that of the control, while it decreased in S10_5d group. Succinate content continued to increase during stress, and sharply accumulated at 5 d (4.36-fold compared to that of the control). cAMP content increased slightly within 1 d, whereas decreased significantly at 5 d (0.56-fold compared to that of the control).

As shown in Fig. S6A, the protein kinase A activity in gills of hard clams significantly increased after exogenous cAMP treatment and significantly decreased after H-89 treatment. This suggests that protein kinase A was functional in hard clams and the kit could be applied to this organism. During hypo-salinity stress, the protein kinase A activity remained stable within 1 d, while it was down-regulated dramatically at 5 d (0.69-fold compared to that in the control group) (Fig. S6B). A schematic diagram of the role of the cAMP-PKA pathway in osmoregulation in *M. mercenaria* was shown in Fig. 6.

3.7. Biomarkers identification

The top 10 differential metabolites in each pair of pairwise comparisons were used in ROC analysis to identify the potential biomarkers of hypo-salinity stress in hard clams. In S10_8h vs S30, all screened metabolites except 5′AMP and deoxyguanosine 5′-monophosphate were potential biomarkers of 8-h hypo-

salinity stress (AUC values > 0.9) (Fig. S7). In S10_1d vs S30, all top 10 differential metabolites were biomarkers of 1-d hypo-salinity stress (Fig. S8). These two pairs of pairwise comparisons share eight identical biomarkers, which can be explained by the fact that 8-h and 1-d hypo-salinity stress cause similar degrees of metabolic changes in hard clams. In S10_5d vs S30, the identified biomarkers obviously differed from those in other pairs of pairwise comparisons. Except for 5′AMP, all screened metabolites were identified as biomarkers of 5-d hypo-salinity stress (AUC values = 1) (Fig. S9). N-(Phosphonomethyl)glycine, carnitine C16:0, and carnitine C9:2-OH were the common biomarkers in three pairs of pairwise comparisons. Hence, they were potential biomarkers in hard clams when exposed to short-term (up to 5 days) hypo-salinity stress.

3.8. Major metabolic responses to hypo-salinity stress

Ten oxidized lipids were identified as differential metabolites in S10_5d vs S30 (not found in other pairs of pairwise comparisons), and the content of seven increased significantly. This suggests that hard clams suffered serious oxidative stress at 5 d post hypo-salinity stress. To resist oxidative stress, the content of metabolites with reactive oxygen species (ROS) scavenging properties or antioxidant abilities, such as serine (Fig. S10), Tyr-containing dipeptides (Fig. 7A), and myoinositol, were significantly up-regulated. Moreover, 3, 6, and 46 glycerophospholipids were significantly accumulated in S10_8h, S10_1d, and S10_5d group, respectively, suggesting that glycerophospholipid metabolism and cell membrane structure in hard clams were affected by prolonged hypo-salinity stress. Of the 46 glycerophospholipids accumulated in S10_5d group, 39 were identified as lysophosphatides, including 3 lysophosphatidic acids (LPA), 17 lysophosphatidylethanolamines (LPE), and 19 lysophosphatidylcholines (LPC). The heatmap analysis results of these lysophosphatides (excluding isomers) contents are shown in Fig. 7B. Lysophospholipids at high concentrations might function to rupture the severely damaged cell membranes caused by oxidative stress, thus maintaining the overall stability and integrity of cell
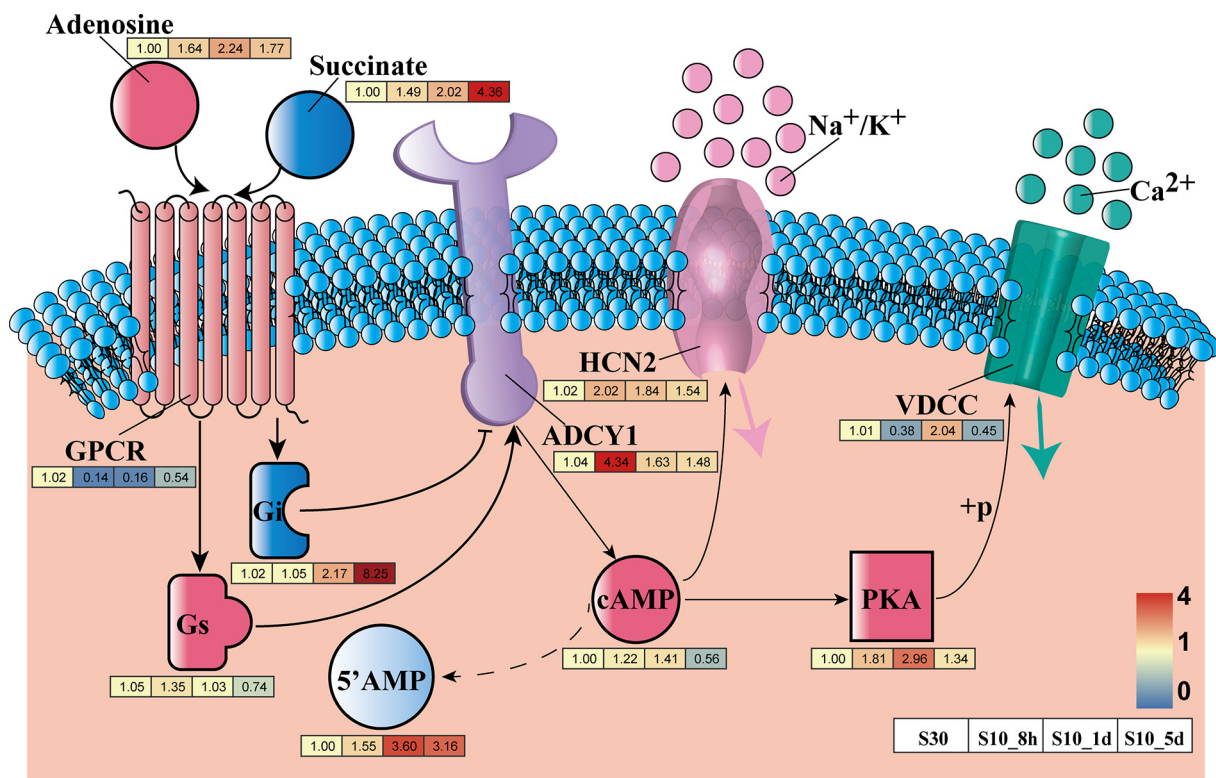
Fig. 6. Schematic diagram of *M. mercenaria* cAMP-PKA pathway involved in osmoregulation. The four boxes in a row below each metabolite or gene represent different groups: S30, S10_8h, S10_1d, and S10_5d. Colors of each box indicate the relative content or expression level of each metabolite or gene. Red indicates a high level, and blue indicates low. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
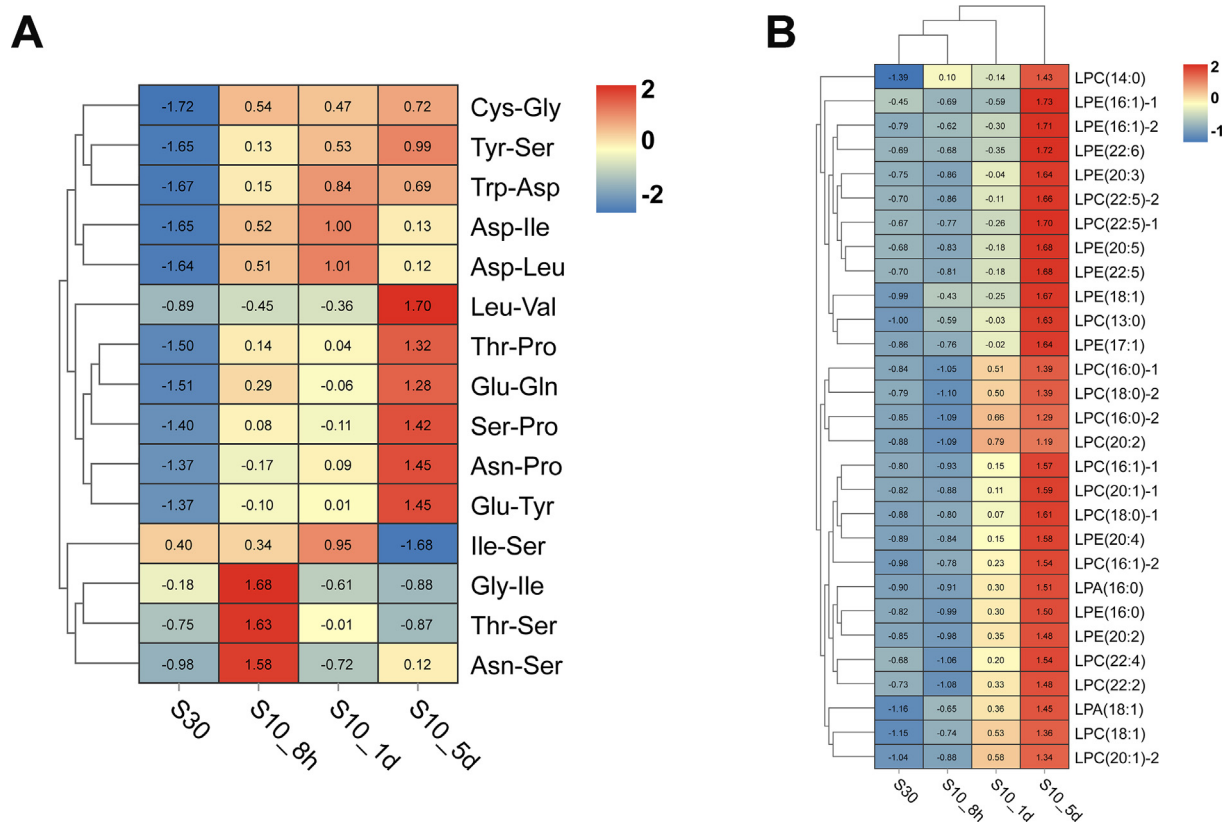

Fig. 7. Heatmap analysis depicts the content variations in dipeptides (A), and lysophosphatide (B) in hard clams during hypo-salinity stress.

membranes. Hypo-salinity stress also affected energy metabolism because huge energy was consumed for osmoregulation. Lactate content significantly increased in S10_1d group (Fig. S11A). Alanine content continued to increase, while aspartate content decreased continuously during stress. This marks the initiation of anaerobic metabolism, which is an important alternative or supplementary energy source. Additionally, 9, 8, and 12 kinds of acylcarnitines were identified as the differential metabolites in S10_8h vs S30, S10_1d vs S30, and S10_5d vs S30, respectively. The heatmap analysis results of these acylcarnitines contents are shown in Fig. S11B. This suggests that fatty acids β-oxidation in hard clams is promoted to provide sufficient energy for osmoregulation and other cellular stress responses. Based on the results of metabolomics analysis and biochemical assays, the major metabolic responses of hard clams to hypo-salinity stress are shown in Fig. 8.

4. Discussion

When exposed to hypo-salinity stress, euryhaline bivalves can initiate a cascade of physiological responses to maintain osmotic equilibrium and normal metabolic functions [14]. However, studies on the comprehensive metabolic responses to hypo-salinity stress and osmoregulation mechanisms are still limited [47,48]. In the present study, we performed multiple biochemical assays, widely targeted metabolomics, and gene expression analysis on gills of hard clams to investigate the metabolic responses to different durations of hypo-salinity stress. Results showed that the gill filaments were disordered and filled with intracellular and extracellular vacuoles. This indicates that prolonged hypo-salinity stress changes the permeability and fluidity of gill filaments, thereby influencing the water and ion exchange between gills and haemolymph. Additionally, the metabolic responses, including osmoregulation, ROS scavenging, membrane lipids reorganization, and energy metabolism adjustment, are essential for hard clams to tolerate hypo-salinity stress.

4.1. Osmoregulation

As osmoconformers, osmolality and ionic concentrations in the haemolymph of bivalves decrease when exposed to hypo-salinity conditions. Intracellular organic osmolytes and inorganic ions are degraded or excreted outside the cell to maintain osmotic equilibrium and prevent extreme cell swelling [11]. Na$^+$ and Cl$^-$ are the most important inorganic ions in osmoregulation [49]. In hard clams, the Na$^+$ and Cl$^-$ concentrations in the gills remained constant at the initial stage of hypo-salinity stress, possibly because the water and ion exchange are hindered by shell closure, which is a protective behavioral response to salinity stress [16,50]. As stress time increased to 5 d, Na$^+$ and Cl$^-$ concentrations decreased significantly. Meanwhile, NKA activity was significantly upregulated. This suggests that NKA can actively pump Na$^+$ out of gills to achieve osmotic equilibrium. Previous studies on NKA in marine organisms mainly focused on fishes and crustaceans [21,22]. Results of this study provide direct evidence that NKA plays a osmoregulatory role in hard clams.

FAAs are the main components of organic osmolytes involved in cell volume adjustments in osmoconformers [51]. In the gills of hard clams, lysine, methionine, phenylalanine, histidine, and aspartic acid contents decreased evidently during hypo-salinity stress (Fig. S10), suggesting that they are predominant FAAs involved in osmoregulation. Previous studies have reported that the predominant FAAs used as osmolytes in *C. gigas* and *Crassostrea hongkongensis* are taurine, proline, alanine, glycine and glutamate [10,52]. This is not contradictory with our results because different bivalves may employ different combinations of FAAs and their derivatives to achieve osmotic equilibrium [53]. In addition to FAAs, trigonelline and creatinine are important organic osmolytes, which were significantly down-regulated in hard clams when exposed to hypo-salinity stress.

KEGG analyses identified the cAMP signaling pathway as the only significantly enriched pathway in three pairs of pairwise com-
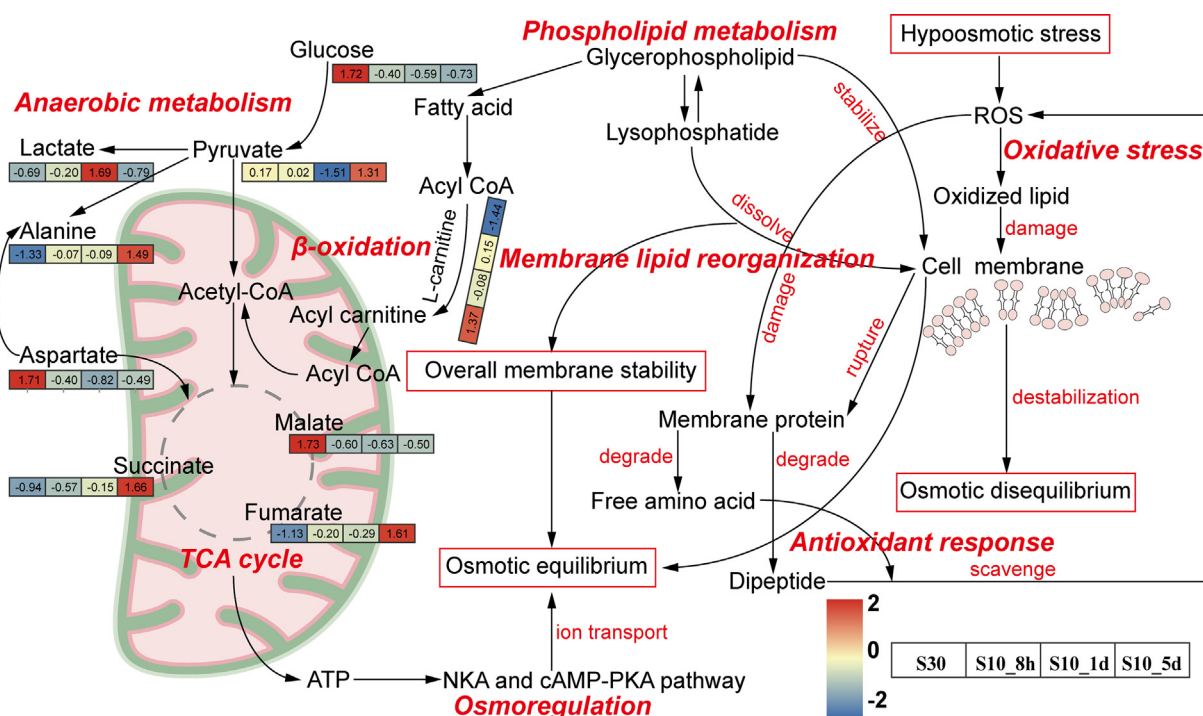

Fig. 8. Schematic diagram of the metabolic responses of hard clams to hypo-salinity stress. The four boxes in a row near each metabolite represent different groups: S30, S10_8h, S10_1d, and S10_5d. Colors of each box indicate the relative content of each metabolite. Red indicates high content, and blue indicates low. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

parisons, suggesting its vital role in osmoregulation in hard clams. In mammalian cells, extracellular signal molecules (i.e., adenosine) can bind to guanine nucleotide-binding protein (G protein)-coupled receptors (*GPCR*), leading to activation of *GNAS*. *GNAS* then stimulates *ADCY1* to catalyze the production of cAMP, which is an important second messenger regulating the intracellular signal transduction process [54]. The cAMP accumulation results in up-regulation of *HCN2*, promoting the entry of Na$^+$ and K$^+$ into cells. Moreover, accumulated cAMP activates *PKA*, which can phosphorylate *VDCC1C*, resulting in the entry of Ca^{2+} into cells. The cAMP-PKA pathway is inhibited by antagonistic signal molecules, such as succinate. Succinate can bind to *GPCR*, leading to the production of *GNAI*, which then inhibits *ADCY1* activity and cAMP generation. In hard clams, adenosine and cAMP contents in gills reached the highest levels in S10_1d group, indicating the temporary activation of cAMP-PKA pathway after short-term hypo-salinity stress. Consistent with this, the expression levels of *GNAS*, *ADCY1*, *PKA*, *HCN2* and *VDCC1C* peaked in S10_8h or S10_1d groups. We speculate that the temporary cAMP-PKA activation functions to regulate glycogen metabolism [55]. However, activation of cAMP-PKA pathway will increase the entry of Na$^+$, K$^+$, and Ca^{2+} into cells, thus aggravating osmotic disequilibrium. To restore osmotic equilibrium, succinate content in gills dramatically increased in S10_5d group (4.36-fold compared to that of the control) to suppress cAMP-PKA pathway. Meanwhile, the expression of *GNAI* reached the highest level, and *ADCY1*, *PKA*, *HCN2*, and *VDCC1C* expression levels were inhibited. Moreover, the activity of protein kinase A dramatically decreased at 5 d post hypo-salinity stress, which was consistent with the down-regulation of the gene expression of its catalytic subunit. This suggests that cAMP-PKA pathway is inhibited to prevent Na$^+$, K$^+$, and Ca^{2+} from entering the gill cells of hard clams when exposed to prolonged hypo-salinity stress ($\geq$5 days). cAMP-dependent ion transport has been reported in crustaceans, such as *Portunus trituberculatus* [56] and *Litopenaeus vannamei* [57]. In bivalves, Evans and Somero reported that the phosphorylation of protein kinase A was involved in cellular metabolism adjustment to cope with osmotic stress [58]. However, no studies have detailed the osmoregulatory role of cAMP-PKA pathway in bivalves. Our results suggest that up-regulation of NKA activity to pump Na$^+$ out of gill cells and inhibition of cAMP-PKA pathway to prevent extracellular Na$^+$, K$^+$, and Ca^{2+} from entering cells may be important osmoregulation mechanisms in euryhaline bivalves.

4.2. ROS scavenging and membrane lipids reorganization

Cell membranes are highly sensitive to ambient perturbations. Membranes stability is essential to sustain tissue functions and power metabolic activities [59]. Lipid peroxidation is an indicator of significant cell membrane damage triggered by oxidative stress [60]. We report that several oxidized lipids were significantly accumulated in S10_5d group, implying that prolonged hypo-salinity stress induced severe oxidative stress and membrane damage in hard clams. Organisms can initiate a cascade of antioxidant responses to alleviate oxidative damage, such as up-regulation of antioxidant enzyme activities [61]. Moreover, many non-enzyme metabolites also exhibit powerful ROS scavenging abilities. Increased FAAs concentrations have been reported to alleviate oxidative stress [19,62]. For instance, serine accumulation facilitates the maintenance of membrane stability in mammalian cells [63]. We speculate that the aim of most FAAs (including serine) up-regulations at the late stage of hypo-salinity stress is to scavenge excessive ROS in gills (Fig. S10). Some dipeptides, including Tyr-containing dipeptides which exhibit the highest ROS scavenging activities, have been reported to protect cells from oxidative damage [64,65]. In this study, 11 of the 15 identified dipeptides

in hard clams, including Tyr-Ser and Glu-Tyr, were continuously up-regulated during hypo-salinity stress. Moreover, an evident accumulation of myoinositol, which has been reported to scavenge free radicals generated by ambient stress [19], appeared after 1-d hypo-salinity stress. These up-regulated metabolites with ROS scavenging properties help hard clams to resist oxidative stress and alleviate membrane damage during hypo-salinity stress.

Glycerophospholipids are the main components in cell membranes and are closely related to cell membrane permeability and NKA activity [66,67]. We report that numerous glycerophospholipids were significantly up-regulated in S10_5d group. Similarly, glycerophospholipid metabolism in *C. hongkongensis* was affected by hypo-salinity [68]. In *Echinolittorina malaccana*, enhanced glycerophospholipid metabolism helps to maintain cell membrane structure under heat stress [69]. Notably, 84.78 % of glycerophospholipids accumulated in S10_5d group were identified as lysophosphatides, including 3 LPA, 17 LPE, and 19 LPC. LPC and LPE can be transformed into LPA catalyzed by lysophospholipase D. LPA is an important signal transduction molecule involved in cell morphology changes and cell migration. Lysophosphatides are the main precursors of phospholipid biosynthesis that aid proteins embedded in cell membranes [70,71]. Additionally, lysophosphatides will cause cell membrane rupture (i.e., mammalian red blood cells) if their concentrations are too high. We speculate that lysophosphatides contents may increase in hypo-salinity stressed hard clams to synthesize more phospholipids for cell structure stabilization and membrane permeability recovery. Those cell membranes severely damaged by oxidative stress may be ruptured by high concentrations of lysophosphatides to maintain the homeostasis of the internal environment.

4.3. Energy metabolism adjustment

Salinity fluctuations increase energy expenditure for osmoregulation in bivalves [15]. Adequate energy supply is essential to maintain normal metabolic functions and support cellular stress responses [72]. Under stress conditions, bivalves tend to initiate anaerobic metabolism because tissues are deprived of oxygen caused by shell closure. Alanine is a biomarker of anaerobic metabolism generated by aspartate [33]. An increase in alanine content accompanied by decreased aspartate content occurred in gills of *M. edulis* and *C. gigas* under hypoxia stress [36]. In this study, similar results were observed in hard clams during hypo-salinity stress. Anaerobic metabolism will induce the accumulation of organic acids (i.e., lactate, succinate) in tissues [73]. In hard clams, lactate and succinate contents significantly increased in S10_1d group. These results suggests that anaerobic metabolism was activated in gills of hard clams after 1-d hypo-salinity stress. Additionally, succinate accumulation suggests that the hard clams were in a state of metabolic depression [74], which might be caused by the large energy expenditure for osmoregulation.

The mitochondrial inner membrane is impermeable to polar molecules, such as CoA. Transport of long-chain fatty acids into mitochondria requires the involvement of acylcarnitines. Acylcarnitines have been reported to play an important role in cellular stress responses [75]. A significant accumulation of acetylcarnitines was observed in *Mytilus californianus* exposed to low tide [76]. In hard clams, many acylcarnitines were identified as the differential metabolites between the stress groups and the control group. Interestingly, the relative content of acylcarnitines exhibited chain length-specific variation during hypo-salinity stress (Fig. 7B). Acylcarnitines with long chains (C $\geq$ 12) were continuously up-regulated, and reached the highest levels in S10_5d group. In contrast, short-chain acylcarnitines (C $\leq$ 11) were evidently down-regulated during stress. Increased strength of hydrophobic interactions resulting from hypo-salinity may stimu-

late the binding of fatty acyl carnitine to its transferase, thereby promoting the transport of fatty acids into the mitochondria [77]. The accumulation of large amounts of acylcarnitines in hypo-salinity stressed hard clams indicates that fatty acids β-oxidation was promoted to provide energy for osmoregulation and other energy-consuming cellular stress responses.

5. Conclusions

In the present study, we performed multiple biochemical assays, widely targeted metabolomics, and gene expression analysis to investigate the metabolic responses of hard clams to different durations of hypo-salinity stress. Results of this study showed that increased intracellular and extracellular vacuoles appeared in the gill filaments with the duration of stress time. The Na^+ and Cl^- concentrations remained constant at the initial stage of stress, and decreased significantly at 5 d, indicating that the osmotic pressure between gills and haemolymph tends to balance. PCA results suggest that the degree of metabolic alterations is consistent with the stress duration. KEGG enrichment and gene expression analyses revealed the critical role of cAMP-PKA pathway in osmoregulation. Up-regulation of NKA activity to pump Na^+ out of gill cells and inhibition of cAMP-PKA pathway to prevent ambient inorganic ions from entering cells are important osmoregulation mechanisms in hard clams. During hypo-salinity stress, large amounts of metabolites with ROS scavenging properties were significantly up-regulated to alleviate oxidative stress. Membrane lipids were reorganized to stabilize cell structure and maintain the homeostasis of the internal environment at 5 d. Anaerobic metabolism was initiated after 1 d, and fatty acids β-oxidation was promoted to provide energy for osmoregulation and other energy-consuming metabolic responses. These metabolic responses may be important aspects of hypo-salinity tolerance mechanisms in euryhaline bivalves. Additionally, N-(Phosphonomethyl)glycine, carnitine C16:0, and carnitine C9:2-OH were identified as the potential biomarkers of short-term (up to 5 days) hypo-salinity stress in hard clams. The results of this study will facilitate the assessment of the physiological status of hard clams in aquaculture and shed novel insights into the osmoregulation and hypo-salinity tolerance mechanisms in euryhaline bivalves.

CRediT authorship contribution statement

Cong Zhou: Investigation, Data curation, Methodology, Writing – original draft. **Hao Song:** Data curation, Writing – review & editing. **Jie Feng:** Methodology, Investigation. **Zhi Hu:** Methodology. **Mei-jie Yang:** Methodology. **Pu Shi:** Investigation, Data curation. **Yong-ren Li:** Investigation. **Yong-jun Guo:** Investigation, Methodology. **Hai-zhou Li:** Investigation. **Tao Zhang:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.08.004.

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