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Changes in Physiological Homeostasis in the Gills of *Litopenaeus vannamei* Under Carbonate Alkalinity Stress and Recovery Conditions

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Abstract: Carbonate alkalinity (CA) is the major toxic factor that interferes with the survival and growth of shrimp in saline–alkaline water. Gills are the main entry organ for CA toxicity in shrimp. In this study, low-salinity cultured *Litopenaeus vannamei* were exposed to 5 mmol/L CA stress for 7 days and then recovered for 7 days to explore the physiological changes in the gills under CA stress and recovery conditions at multiple biological levels. The results showed that CA stress increased the activities of antioxidative biochemical indexes (T-AOC, T-SOD, and POD) and the relative expression levels of *romo1*, *nrf2*, and *gpx* genes, while it decreased the relative expression levels of the *sod* and *hsp70* genes. In addition, CA stress also increased the relative expression levels of genes involved in endoplasmic reticulum (ER) stress (*bip*, *ire1*, and *xbp1*), immunity (*alf*, *crus*, *pen-3* and *propo*), apoptosis (*casp-3*), detoxification metabolism (*cyp450* and *gst*), and osmotic adjustment (*ca*, *nka-α*, *nka-β*, *vatp*, *nhe*, *clc*, *aqp*, *tip4*, and *ccp*). Although changes in some of the physiological indexes were reversed after the CA stress was relieved, they still could not effectively recover to the control level. These results reveal that CA stress has a negative impact on physiological homeostasis in the shrimp gills by inducing oxidation and ER stress and by interfering with immunity, apoptosis, detoxification, and osmotic adjustment.

Keywords: shrimp; carbonate alkalinity; gills; physiological response

Key Contribution: This study explores physiological response characteristics in the gills of the Pacific white shrimp *Litopenaeus vannamei* after carbonate alkalinity exposure and recovery by integrating multiple biological indexes, which is beneficial for the development of anti-stress strategies for shrimp farming in saline–alkali water.

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1. Introduction

The Pacific white shrimp, also known as *Litopenaeus vannamei*, is an important aquatic economic species for global aquaculture. *L. vannamei* has the characteristics of fast growth, strong adaptability, and wide salt tolerance, allowing it to live from low-salinity to seawater environments [1]. In recent years, the saline–alkaline aquaculture of shrimp has garnered widespread attention. Saline–alkaline water is a type of non-marine saltwater resource that is widely distributed in the northwest, northeast, and north of China and along the northern bank of the Yangtze River. Saline–alkaline water is considered to be a low-yield water resource on a global scale. According to their different ion compositions, saline–alkaline water can be roughly divided into three types, namely carbonate, chloride, and sulfate

types. In general, carbonate saline–alkaline water is a common type in low-salinity areas [2]. With its own buffer system, carbonate saline–alkaline water can basically maintain relative stability of the water pH [3]. However, due to its high alkalinity, carbonate alkalinity (CA) has toxicological effects on the health of aquatic animals [4,5]. It has been found that CA stress can produce toxic effects on the survival, immunity, and metabolism of aquatic animals [4,6]. Therefore, exploring the physiological response mechanism of *L. vannamei* to CA stress is helpful for the healthy cultivation of shrimp in saline–alkaline water.

CA stress can affect the physiological homeostasis of *L. vannamei*. For example, CA stress affects the survival rate [7], induces acute stress responses in the hepatopancreas and gills [8,9], and decreases antioxidative and digestive functions in *L. vannamei* [10]. Gene transcription functions in the gills of *L. vannamei* are also changed under CA stress, involving immune response and circulatory function-related pathways [6]. Changes in immune-related genes in *L. vannamei* under acute CA stress can help it to cope with osmotic adjustment to CA stress [11]. In addition, CA stress can also cause changes in substance transport, immunity, digestion, and absorption functions in the hepatopancreas of *L. vannamei* [7]. CA stress also leads to a change in the acid–base balance of hemolymph and inhibit the ion transport function of *Exopalaemon carinicauda* [12].

Gills are the respiratory and osmotic regulatory organs of aquatic animals that are directly in contact with the water environment and are easily affected by environmental changes, which can cause a series of stress responses [8,12–15]. Acute high alkalinity stress could induce morphological changes and stress responses in the gills of *L. vannamei* (2.5–3 cm) and interfere with the gene transcription function [8,10]. However, the effect of CA stress on the physiological function of the gills of *L. vannamei* is still unclear. Therefore, in this study, we exposed low-salinity cultured *L. vannamei* to CA stress for 7 days, then released the stress and allowed them to recover for 7 days. Finally, the physiological changes in the gills of *L. vannamei* after CA stress and recovery were explored from multiple biological aspects, including redox and endoplasmic reticulum (ER) stress, immunity, apoptosis, detoxification, and osmoregulation. The results of this study can provide new insights for the study of gill toxicity of CA stress on shrimp and provide a theoretical basis for shrimp culture in saline–alkaline water.

2. Materials and Methods

2.1. Shrimp and Culture Conditions

Healthy *L. vannamei* used in this study were obtained from an indoor pond at the Shenzhen Base of the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (Shenzhen, China), and had an average weight of 9.6 ± 0.4 g. Before the stress experiment, the shrimp were temporarily reared for one week in an experimental tank filled with 300 L of rearing water at a water temperature of 25 ± 0.5 °C, pH of 8.2 ± 0.2, salinity of 3‰, and uninterrupted aeration for 24 h. The water was changed every day, and the shrimp were fed compound feed based on 5% of their body weight. The feed was adjusted according to the feeding situation, and the residual feed feces were cleaned in a timely manner.

2.2. CA Stress Experiment and Sample Collection

After 7 days of temporary culture, the shrimp were randomly divided into two groups: the control (CK) group and the CA stress group. Each group had three duplicate tanks, with 50 shrimp in each tank. The CK group was in normal 3‰ low-salinity water without the addition of sodium bicarbonate. The CA concentration of the CA group was 5 mmol/L, which was adjusted by adding sodium bicarbonate to the rearing water. Each tank's water was replaced with fresh water, with the CA concentration adjusted in advance every day. Except for the different concentration of CA in the water, other culture conditions in the stress period were consistent with those in the temporary culture period. Based on the research and experimental data of Song et al. [6], we chose 7 days to carry out the short-term reaction and recovery process experiment for the organisms. After 7 days of stress exposure,

the water of the CA group was replaced with normal 3‰ low-salinity water; that is, the CA stress recovery (RCA) group was set up, with 15 shrimp per tank, and normal culture was continued for 7 days.

The gills of the shrimp in each group were sampled on the 7th day of stress exposure and the 7th day of recovery, respectively. Specifically, the gills of five shrimp from each tank were collected, mixed, and stored at −80 °C for the determination of the biochemical indexes. The gills of three shrimp from each tank were collected, mixed, and placed in RNA protection solution (RNAFollow, New Saimei Biotech Co., Ltd., Suzhou, China) at 4 °C for 24 h and then stored at −80 °C until gene expression analysis.

2.3. Biochemical Index Determination

After thawing at low temperature, about 0.1 g of the gill tissue samples was accurately weighed, then a 9-fold volume of 0.9% physiological saline (Biosharp, Guangzhou, China) solution was added, and a tissue homogenizer (TissueLyser II, Germany Qiagen, Berlin, Germany) was used for low-temperature grinding to prepare 10% tissue homogenates. The homogenate was centrifuged at 4 °C and 3500 revolutions/min for 15 min in a centrifuge (Centrifuge JIDI-21R, Guangzhou, China), and the supernatant was collected and stored at −80 °C until biochemical analysis. All the biochemical indexes, including total antioxidative capacity (T-AOC), superoxide dismutase (T-SOD), catalase (CAT), and peroxidase (POD), were measured with the same set of kits manufactured by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All analyses were performed on the microplate reader.

2.4. Gene Expression Analysis

TRIzol reagent was used to extract total RNA from the gills, and RQ1 RNase-free DNase was used to remove excess genomic DNA from the RNA. Subsequently, Nanodrop 2000 (MPBIO, Irvine, CA, USA) was used to measure the concentration and purity of the RNA, and its integrity was evaluated by 1% agarose gel electrophoresis. Using the Servicebio® RT First Strand cDNA Synthesis Kit (Servicebio, Wuhan, China), the purified RNA was reverse transcribed into cDNA and stored at −80 °C.

The changes in gene expression were analyzed using real-time fluorescent qPCR. The nucleotide sequences of the target genes of *L. vannamei* were obtained from NCBI, and the *β-actin* gene was used as an internal reference. Primer Premier 5.0 was used to design the qPCR primers (Table S1), and the specificity and amplification efficiency of the primers were determined by amplification plots and melting curve analysis. The qPCR was performed using the SGExcel Fast SYBR qPCR mixture kit (Sangon Biotech, Shanghai, China) on a real-time quantitative PCR system (CG-05 Heal Force, Shanghai, China). The qPCR reaction system contained 7.5 μL of SYBR mixture, 1.0 μL cDNA, 0.6 μL preprimer (10 μmol/L), 0.6 μL reverse primer (10 μmol/L), and 5.3 μL sterile deionized water. The amplification procedure was set at 95 °C for 30 s, then 40 times at 95 °C for 5 s and 30 s at 60 °C. The relative levels of mRNA were calculated by the method of Livak and Schmittgen [16], and these were shown as the fold-change relative to the CK group.

2.5. Statistical Analysis

The data in the study were expressed as the mean ± standard error (SE). IBM SPSS Statistics 26.0 was used for statistical analysis, and a one-way ANOVA with Duncan's technique and LSD was carried out. Differences with $p < 0.05$ were regarded as significant, indicating that the differences in the data were non-accidental and statistically significant.

3. Results

3.1. Changes in the Biochemical Indicators of Oxidative Stress in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the T-AOC and the activities of T-SOD, and POD were increased significantly in the CA group ($p < 0.05$) (Figure 1A,B,D); the CAT activity was also increased slightly in the CA group,

but this difference was not significant ($p > 0.05$) (Figure 1C). After recovery, in comparison with the CA group, the T-AOC did not obviously change in the RCA group, but it was still higher than that in the CK group; T-SOD activity was decreased significantly to the level of the CK group; CAT activity was increased slightly in the RCA group and was higher than that in the CK group, but there was no significant difference ($p > 0.05$); POD activity was decreased, but it was still significantly higher than that in the CK group ($p < 0.05$).

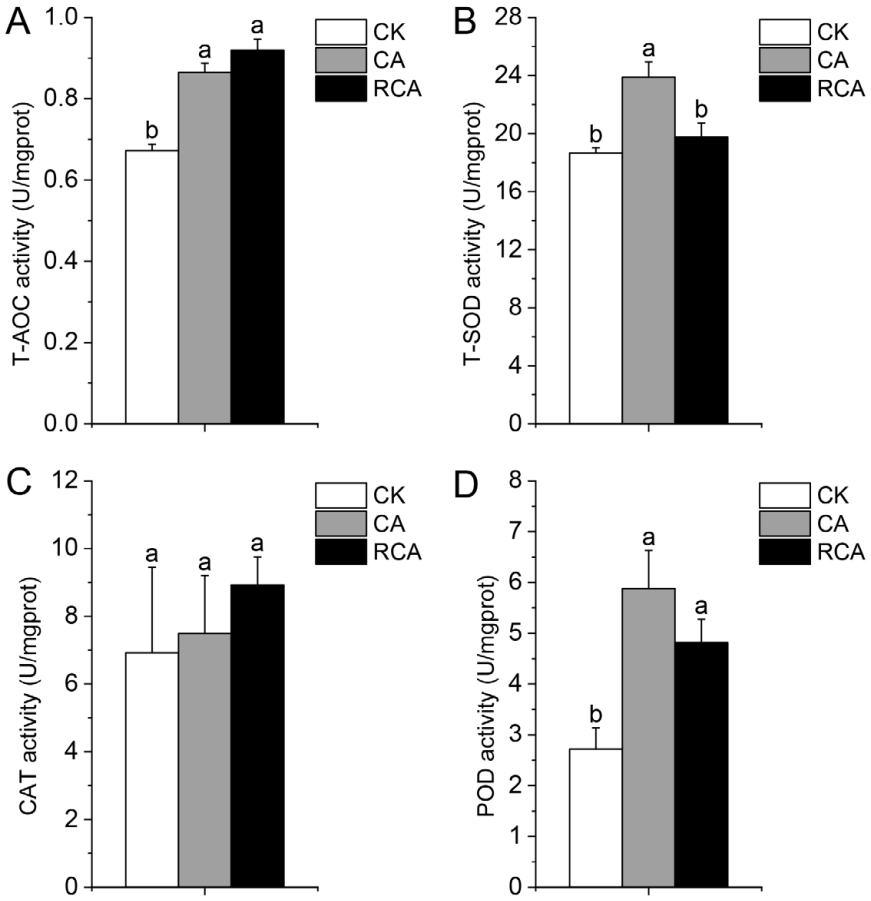

Figure 1. Changes in oxidative stress biochemistry indexes in the gills of *L. vannamei* after CA stress and recovery. (**A**) T-AOC activity; (**B**) T-SOD activity; (**C**) CAT activity; (**D**) POD activity. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

3.2. Changes in the Expression Levels of Antioxidant-Related Genes in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the relative mRNA expression levels of the oxygen regulatory factor 1 (*romo1*) and glutathione peroxidase (*gpx*) genes were increased significantly in the CA group ($p < 0.05$). The relative mRNA expression levels of the nuclear transcription factor 2 (*nrf2*) gene was increased slightly in the CA group, but difference was not significant ($p > 0.05$), while the levels of the copper zinc superoxide dismutase (*sod*) and heat-shock protein 70 (*hsp70*) genes were decreased significantly in the CA group ($p < 0.05$) (Figure 2). After recovery, in comparison with the CA group, the relative mRNA expression levels of the *nrf2*, *gpx*, and *hsp70* genes were significantly increased in the RCA group and were higher than those in the CK group ($p < 0.05$); the level of the *sod* gene was still decreased significantly in the RCA group and was lower than that in the CK group ($p < 0.05$); the level of the *romo1* gene did not change significantly in the RCA group, but it was still significantly higher than that in the CK group ($p < 0.05$).

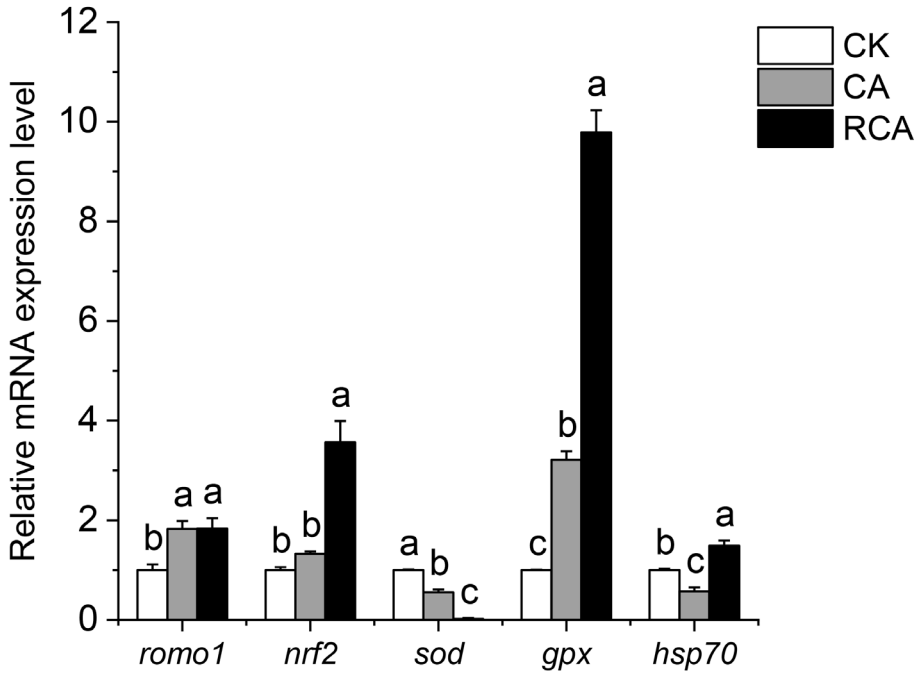

Figure 2. Changes in the relative mRNA expression levels of antioxidant-related genes in the gills of *L. vannamei* after CA stress and recovery. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

3.3. Changes in the Expression of ER Stress-Related Genes in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the relative mRNA expression level of the inositol demand enzyme-1 (*ire1*) gene was increased significantly in the CA group ($p < 0.05$). The relative mRNA expression levels of immunoglobulin heavy chain binding protein (*bip*) and X-box binding protein-1 (*xbp1*) were also increased in the CA group, but the differences were not significant ($p > 0.05$) (Figure 3). After recovery, in comparison with the CA group, the relative mRNA expression levels of the *bip*, *ire1*, and *xbp1* genes continued to increase significantly in the RCA group and were higher than those in the CK group ($p < 0.05$).

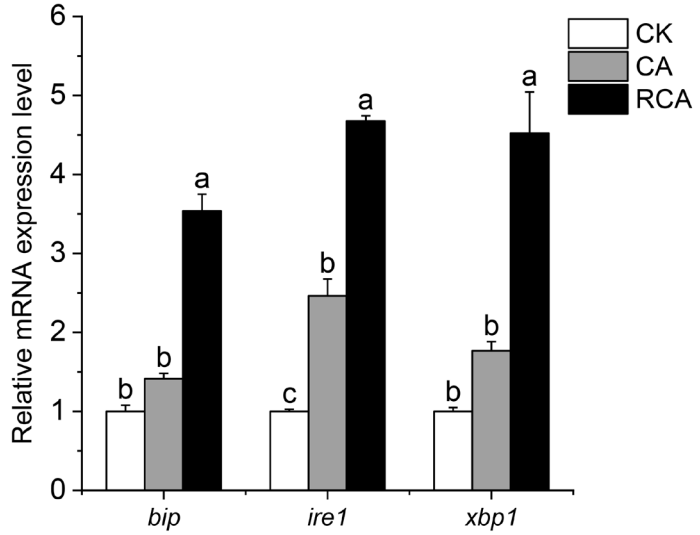

Figure 3. Changes in the relative mRNA expression levels of ER stress-related genes in the gills of *L. vannamei* after CA stress and recovery. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

3.4. Changes in the Expression Levels of Immune-Related Genes in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the relative mRNA expression levels of the anti-lipopolysaccharide factor (*alf*), crustin (*crus*), and phenoloxidase (*propo*) genes were increased significantly in the CA group ($p < 0.05$). The expression levels of the penaeidin 3a (*pen-3*) and lysozyme (*lys*) genes were increased slightly in the CA group but with no significant difference ($p > 0.05$) (Figure 4). After recovery, in comparison with the CA group, the relative mRNA expression levels of the *alf*, *pen-3*, and *lys* genes were increased significantly in the RCA group ($p < 0.05$), while the level of the *crus* gene did not change significantly in the RCA group, but they were significantly higher than those in the CK group; the level of the *propo* gene was decreased significantly in the RCA group ($p < 0.05$) and recovered to the level of the CK group.

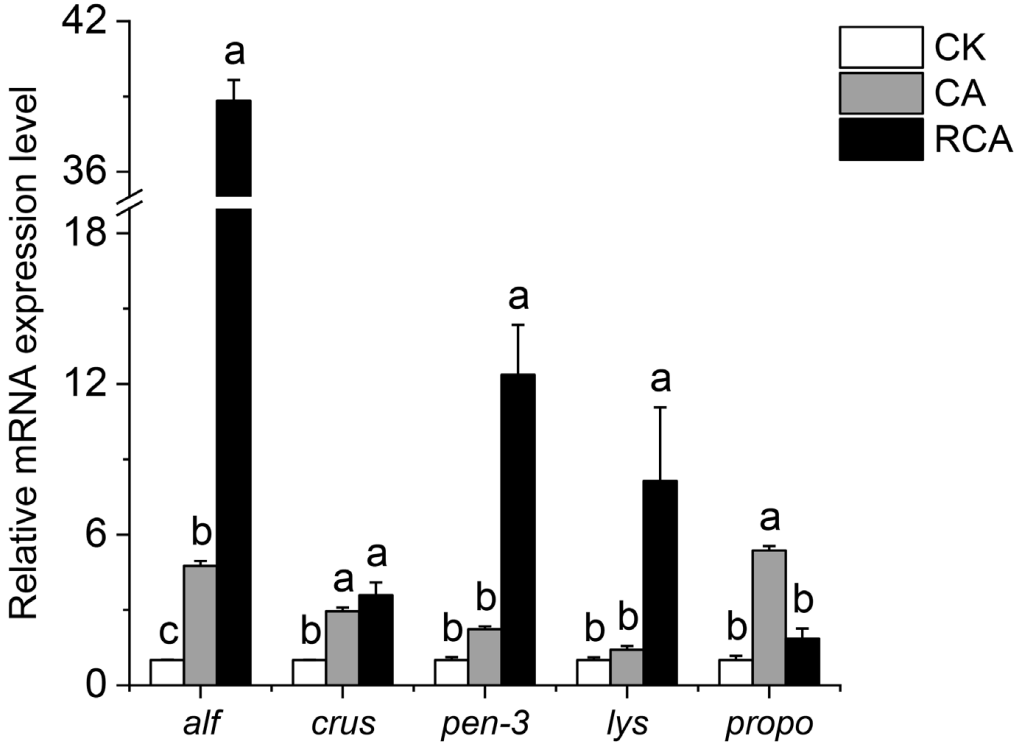

Figure 4. Changes in the relative mRNA expression levels of immune-related genes in the gills of *L. vannamei* after CA stress and recovery. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

3.5. Changes in the Expression Levels of Apoptosis-Related Genes in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the relative mRNA expression level of caspase-3 (*casp-3*) gene was increased significantly in the CA group ($p < 0.05$), but the level of caspase-9 (*casp-9*) gene did not change significantly ($p > 0.05$) (Figure 5). After recovery, in comparison with the CA group, the relative mRNA expression levels of *casp-3* and *casp-9* genes were increased significantly in the RCA group and were higher than those in the CK group ($p < 0.05$).

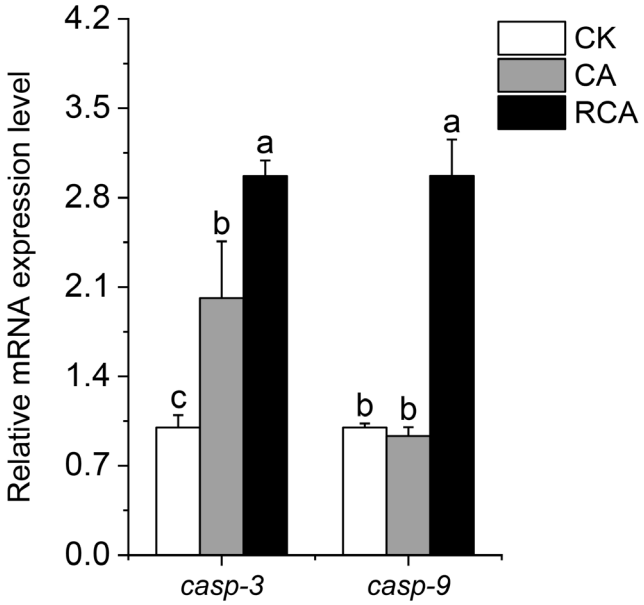

Figure 5. Changes in the relative mRNA expression levels of apoptosis-related genes in the gills of *L. vannamei* after CA stress and recovery. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

3.6. Changes in the Expression Levels of Detoxification-Metabolism-Related Genes in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the relative mRNA expression level of the cytochrome P450 (*cyp450*) gene was increased significantly in the CA group ($p < 0.05$); the level of the glutathione S-transferase (*gst*) gene was also increased slightly in the CA group, but this was not a significant difference ($p > 0.05$) (Figure 6). After recovery, in comparison with the CA group, the relative mRNA expression level of the *cyp450* gene was decreased significantly in the RCA group ($p < 0.05$) and recovered to the level of the CK group; in contrast, the level of the *gst* gene was increased significantly in the RCA group and was higher than that in the CK group ($p < 0.05$).

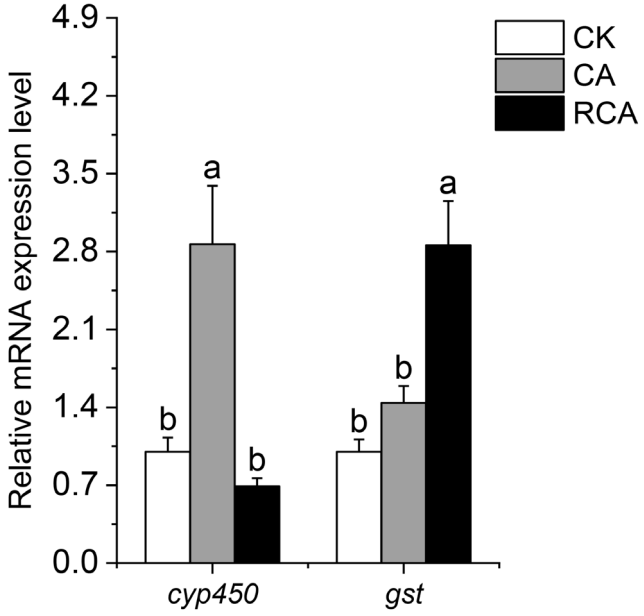

Figure 6. Changes in the relative mRNA expression levels of detoxification-related genes in the gills of *L. vannamei* after CA stress and recovery. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

3.7. Changes in the Expression Levels of Osmoregulation-Related Enzyme Genes in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the relative mRNA expression levels of the carbonic anhydrase (*ca*), sodium/potassium ATPase α subunit (*nka-α*), sodium/potassium transporter ATPase subunit (*nka-β*), and V-type proton ATPase subunit C (*vatp*) genes were all increased significantly in the CA group ($p < 0.05$) (Figure 7). After recovery, in comparison with the CA group, the relative mRNA expression levels of the *ca* gene was decreased significantly in the RCA group and lower than the CK group ($p < 0.05$); the levels of the *nka-α* and *vatp* genes were decreased significantly in the RCA group ($p < 0.05$) and recovered to the level of the CK group; the level of the *nka-β* gene was decreased significantly in the RCA group, but it was still significantly higher than that in the CK group ($p < 0.05$).

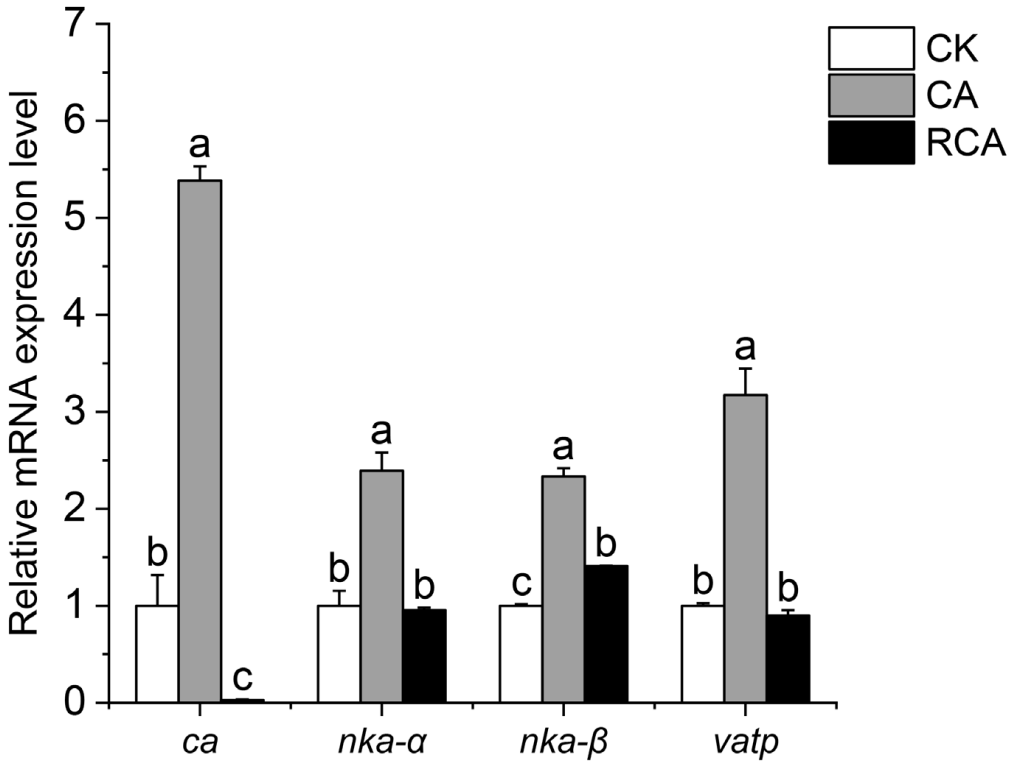

Figure 7. Changes in the relative mRNA expression levels of osmoregulation-related enzyme genes in the gills of *L. vannamei* after CA stress and recovery. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

3.8. Changes in the Expression Levels of Osmotic-Adjustment-Related Protein Genes in the Gills

After the shrimp were exposed to CA stress, in comparison with the CK group, the relative mRNA expression levels of the sodium/hydrogen exchanger (*nhe*), chloride channel protein 2 (*clc*), aquaporin (*aqp*), aquaporin tip4 (*tip4*), and two pore calcium channel protein 1 (*ccp*) genes were all increased significantly in the CA group ($p < 0.05$) (Figure 8). After recovery, in comparison with the CA group, the relative mRNA expression levels of the *nhe* and *clc* genes did not change significantly in the RCA group. The level of the *aqp* gene was increased significantly in the RCA group, while the levels of *tip4* and *ccp* genes were decreased significantly ($p < 0.05$). The levels of the *tip4* and *ccp* genes recovered to the levels of the CK group; however, levels of the *nhe*, *clc* and *aqp* genes were still significantly higher than those of the CK group ($p < 0.05$).

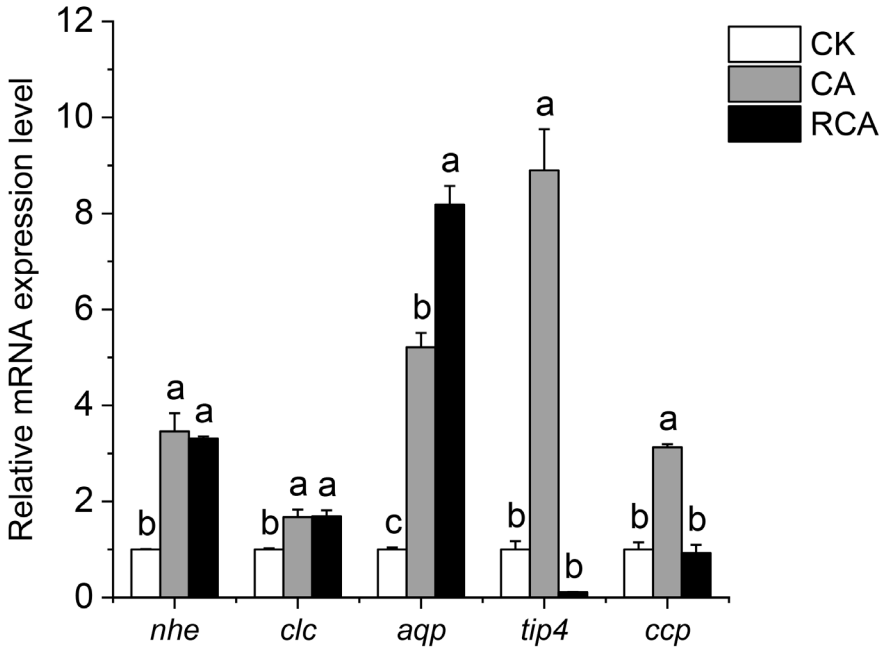

Figure 8. Changes in the relative mRNA expression levels of osmotic-adjustment-related proteins in the gills of *L. vannamei* after CA stress and recovery. Different letters above the bars indicate significant differences ($p < 0.05$) between the different groups.

4. Discussion

CA is a common environmental factor in saline–alkaline water [17,18], and CA stress can affect the survival, growth, and physiological function of shrimp. However, the effect of CA stress on the physiological response in the gills of *L. vannamei* is not clear. In this study, after being treated with CA stress, the survival rate of shrimp was 70.66%. After returning to normal conditions, the survival rate was 91.11%. Therefore, we comprehensively evaluated the physiological changes in the gills of *L. vannamei* under CA stress and recovery from the perspectives of oxidative stress, ER stress, immunity, apoptosis, detoxification, and osmoregulation.

Oxidative stress is one of the most common effects of environmental stress on shrimp [19]. Antioxidative enzymes such as SOD, CAT, GPx, and POD can protect organisms from oxidative stress [20,21]. In this study, after CA stress, the T-AOC and the activities of T-SOD and POD were up-regulated in the gills of the shrimp, which indicated that CA exposure led to oxidative stress in the gills of the shrimp and that the antioxidative system was activated to cope with CA stress. In the recovery stage, the activities of T-AOC and POD were still higher than those in the CK group, indicating that oxidative stress still existed in the gills and that the redox steady state could not be effectively recovered.

Environmental stress can induce the excessive production of reactive oxygen species (ROS) [22]. Romo1 plays an important role in redox-dependent regulation of mitochondrial dynamics and ROS-dependent signal transduction [23,24]. Nrf2 is a multifunctional transcription factor that is critical to the antioxidative system [25,26]. Hsp70 plays a key role in the depolymerization and reactivation of proteins [27]. It has been found that acute CA stress induces a decrease in the antioxidative capacity of *L. vannamei* [10]. In this study, after CA stress, expression levels of the *romo1*, *nrf2*, and *gpx* genes were up-regulated in the gills of the shrimp, while levels of the *sod* and *hsp70* genes were down-regulated, which indicated that CA exposure led to oxidative stress in the gills of the shrimp and interfered with redox homeostasis. In the recovery stage, the expression levels of the *romo1*, *nrf2*, *gpx*, and *hsp70* genes were still higher than those in the CK group, while the level of the *sod* gene was down-regulated, indicating that the homeostasis of antioxidant gene expression was still disturbed and did not return to normal completely.

Environmental stress can induce ER stress and, consequently, affect functional ER homeostasis [28,29]. In the early stages of ER stress, bip binds to improperly folded or misfolded proteins, and then ire1 dissociates from bip. With continuous stress, ire1 is activated, which further promotes the expression of xbp1 to regulate ER homeostasis [30]. In this study, after CA stress, the up-regulated expression levels of *bip*, *ire1*, and *xbp1* genes indicated that the ER stress occurred in the gills of the shrimp, the unfolded protein response was activated, and the organism adapted to the stress through the unfolded protein response mechanism. In the recovery stage, the relative expression levels of the *bip*, *ire1*, and *xbp1* genes were continuously up-regulated, indicating that ER stress persisted in the gills of the shrimp, which was not conducive to the homeostasis of ER function and which might further trigger tissue immunity and apoptosis procedures.

Shrimp lack a specific immune system and relies heavily on non-specific immune factors to defend against environmental stress. The alf and crus proteins are common antimicrobial peptides with a wide range of antimicrobial activities [31,32]. Lys is an innate immune molecule that plays an important role in the immune response [33]. Pen-3 is a small immune molecule necessary for host innate immunity, which can quickly identify and eliminate pathogens [34]. The propo system is an enzyme cascade system, similar to the complement system in vertebrates, with powerful immune regulation ability in crustaceans [35]. It was reported that acute CA stress could induce an excessive immune response in *L. vannamei* [6]. In this study, after CA stress, the expression levels of immune-related genes (*alf*, *crus*, *pen-3*, *lys*, and *propo*) were up-regulated, indicating that CA stress induced immune response in the gills of the shrimp, and the organism actively mobilized the immune system to defend against stress. In the recovery stage, the expression level of the *propo* gene recovered to the level of the CK group, while the expression levels of *crus*, *alf*, *pen-3*, and *lys* genes were still higher than the CK group. This phenomenon suggested that the immune system was continuously activated in the gills of the shrimp, but that it had not completely recovered to normal.

Prolonged ER stress will lead to the expression of pro-apoptosis signals and, ultimately, to apoptosis [20,29,30]. Apoptosis is a programmed death process produced by cells autonomously, and it is a physiological process in which cells actively respond to the injury process of the organism [36]. Casp-9 is activated in the intracellular mitochondrial pathway when cells are stimulated by internal or external apoptosis, with cytc activating casp-9 through the formation of an apoptosis complex and then activating casp-3, which eventually leads to apoptosis [37,38]. In this study, after CA stress, the expression level of the *casp-3* gene was up-regulated, which indicated that the executive stage of apoptosis was activated or enhanced in the gills of the shrimp under CA stress. In the recovery stage, the expression levels of *casp-3* and *casp-9* genes were still up-regulated, indicating that the apoptosis process in the gills was still in an activated state and the negative effects of stress-induced apoptosis could not be effectively eliminated.

Aquatic animals can employ their detoxification metabolic systems, including phase I (such as cyp450) and phase II (such as gst) enzymes, to cope with environmental stress such as converting harmful substances into water-soluble metabolites and excrete them from cells [39,40]. In this study, after CA stress, the expression levels of *cyp450* and *gst* genes were up-regulated, which indicated that the detoxification metabolism function in the gills of the shrimp was activated to remove harmful substances induced by stress. In the recovery stage, the expression level of the *cyp450* gene was down-regulated to the control level, while the expression level of the *gst* gene was still up-regulated, indicating that the detoxification metabolism function in the gills of the shrimp had not completely returned to normal.

Crustaceans achieve osmoregulation by regulating the transport of ions [41]. CA can regulate intracellular pH [42]. The nka is a membrane-bound protease composed of α and β subunits, which can provide power for the transmembrane transport of Na^+ and K^+ [43]. Vatp is related to acid–base regulation [44]. In this study, after CA stress, the relative expression levels of osmotic-adjustment-related enzymes (*ca*, *nka-α*, *nka-β*, and *vatp*) genes

were all up-regulated, which indicated that CA stress affected ion transport function in the gills of the shrimp, and that the organism regulated osmotic adjustment by inducing the gene expression of ion transport enzymes. In the recovery stage, the relative expression levels of the *nka-α* and *vatp* genes recovered to the levels in the CK group, while the *nka-β* gene was still at a high expression level, the *ca* gene was still at a low expression level, indicating that the gills of the shrimp might recover some osmotic adjustment functions to some extent.

Crustaceans balance the osmotic pressure between the external medium and their internal fluid through osmoregulation, which maintains the stability of the internal environment [45]. Nka can regulate the acid–tbase balance and he ion exchange with CA and nhe [46]. Aqp and tip4 are two important aquaporins that are involved in water transport and osmotic adjustment [47,48]. Clc and ccp participate in the transmembrane transport of Cl^- and Ca^+, respectively [19]. In this study, after CA stress, the relative expression levels of osmotic-adjustment-related proteins (*aqp*, *tip4*, *clc*, *nhe*, and *ccp*) are all up-regulated, which indicates that CA stress could affect the transmembrane transport of water and ions in the gills of the shrimp, which then affect the osmotic adjustment function. In the recovery stage, the relative expression level of the *ccp* and *tip4* genes recovered to normal, while levels of the *nhe*, *clc* and *aqp* genes were still in disorder, indicating that the function of osmotic adjustment related proteins in the gills of the shrimp did not recover to normal.

5. Conclusions

This study revealed that CA stress would negatively affect physiological homeostasis in the gills of shrimp. Specifically, CA stress induced oxidative stress and ER stress, which further interfered with its immunity, apoptosis, detoxification metabolism, and osmotic adjustment functions. Although some physiological indexes could be recovered to control levels after the CA stress was relieved, physiological homeostasis could not be completely recovered to the normal state within a short time. Therefore, we should pay attention to the toxic effect of CA on shrimp in saline–alkali aquacultures and develop corresponding anti-stress measures.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9110463/s1, Table S1: Primer sequences used in this study.

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