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Research Article

Effect of Acute Salinity Stress on Metabolism, Antioxidant Status, and Histological Structure of *Procambarus clarkii*

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Procambarus clarkii is a well-known invasive species with a strong environmental tolerance that has spread worldwide. Crayfish were exposed to five different salinities (0, 6, 12, 18, and 24 parts per thousand (ppt)) to determine how salinity affects physiological and histological responses. The metabolism-related enzymes pyruvate kinase (PK) and fatty acid synthase (FAS) activities of the hepatopancreas and gill tissues significantly decreased with an increase of salinity (6, 12, 18, 24 ppt), except the increase of FAS activity at 6 ppt. Salinity caused an immune disorder, as displayed by the decreased activities of lysozyme (LSZ) and superoxide dismutase (SOD). In contrast, catalase (CAT) activities showed increased activity. The heat shock protein 90 (HSP90) concentration significantly increased at 6 ppt and then significantly decreased at 24 ppt in the hepatopancreas and gill tissues. A HE section revealed that salinity stress influenced the tissue structures. High salinity (24 ppt) damaged the hepatopancreas and deformed the gills. In conclusion, *P. clarkii* is resistant to acute exposure to low salinities and suffers physiological damage when exposed to hyperosmotic salinities. Our study provides a valuable reference to analyse the adaptation mechanisms of crayfish in response to salinity.

1. Introduction

Salinity is a key ecological factor in the life of many crustaceans and affects the principal biological aspects of animals, including survival, development, growth, metabolism, the molting cycle, and even behavior [1, 2]. Crustacean cultures in a suitable salinity range have the best production yield. Unsuitable range affects growth, reproduction, and other processes. The Pacific white shrimp (*Penaeus vannamei*) had the fastest growth rate at 18 parts per thousand (ppt), and the optimal growth salinity ranged from 14–22 ppt [3]. A hatching rate of more than 87% could be attained at salinities of 27–30 ppt in the Chinese pearl oyster (*Pinctada martensii*) [4]. However, the survival rate of *Penaeus japonicus* decreased significantly with decreasing salinity [5].

Procambarus clarkii is a well-known invasive species widely distributed worldwide. Because of its unique taste, crayfish have become a popular freshwater species in China, widely cultured along the Yangtze River in China, and the culture region was gradually expanded in recent years [6]. Crayfish have strong environmental tolerance and are reported to tolerate salinity [7]. They can survive and grow in water with salinity below 14 ppt, and have strong

adaptability to salinity below 10 ppt [8]. Wild *P. clarkii* in Italy, exposed to increasing salt concentrations, reaching 35.3% after 65 days, had a survival rate of 93.5% [9]. The safe salinity value of crayfish is about 6.00 ppt [10]; above that, the survival rate and growth decrease [11].

Changes in salinity affect the growth of crustaceans and increase the rate of physiological metabolism and energy consumption. Prolonged salinity stimulation reduces immunity and antioxidant capacities [12]. Acute salinity stress activates the antioxidant system and nonspecific immune system, including hemolymph phenoloxidase (POX) of *P. clarkii* [13], but long-term salinity stress seems to lead to a decrease in immunity, that immune-related DEGs were downregulated [14]. However, increased salinity decreases aquatic pathogenic bacteria abundance by gut microbiota analysis in the freshwater species red claw crayfish, *Cherax quadricarinatus* [15]. In addition, there are many reports on the effect of salinity on the physiological and biochemical indicators of crustaceans [16, 17]. Changes in salinity can lead to an imbalance between salt ions in the water environment and the cells of organisms. Therefore, the body requires ion exchange across epithelial cell membranes in various tissues to ensure that the body is protected from hypertonic or hypotonic effects and maintains the original structure [18].

China has more than 30% of the world's saline soil, with more than 3 million hectares of saline-alkali soil is not suitable for cultivation and is close to water sources [19]. Full exploitation and utilization of these lands can make up for the shortage of freshwater resources. Crayfish have a strong environmental tolerance. Therefore, it is important to analyse the possibility of raising *P. clarkii* in saline soil. First of all, investigate the effect of salinity on the crayfish's physiological and biochemical changes is necessary. In this study, we determined the activities of immune-, metabolism-related enzymes, and antioxidase under four acute salinity stress concentrations up to 24 ppt. This will provide basic data for the molecular mechanisms of crayfish in salinity tolerance.

2. Material and Methods

2.1. Experimental Animals. Crayfish (weight 15.0 ± 2.0 g) were purchased from the local aquatic market in Shenzhen city. They were temporarily raised in the aquaculture system of the Shenzhen Experimental Base of the South China Sea Fisheries Research Institute and fed a commercial diet at a ratio of 3% body weight daily. The water temperature was $25 \pm 1^{\circ}$C, the pH was 7.5, and the dissolved oxygen was 6.5 ± 0.5 mg/L.

2.2. Experimental Design. Adjust salinity (6, 12, 18, and 24 ppt) by diluted natural seawater treated with sand filtration with pre-prepared nonchlorinated fresh water in different proportions. The crayfish were divided into five groups with different salinity levels (i.e., 0 (control group), 6, 12, 18, and 24 ppt groups), with 30 crayfish per group. The tissues, including the hepatopancreas and gills, of three crayfish from each group exposed for 36 h were dissected and was cut into two equal parts from the midline, one part was frozen in liquid nitrogen, and the other was stored in 4% paraformaldehyde (Sigma-Aldrich).

2.3. Enzyme Activities and Protein Concentration Measurement. The hepatopancreas and gill tissue (0.1 g) were thawed, and quickly homogenized in 1 mL PBS (PH = 7.4). The supernatant was collected after homogenates were centrifuged for about 10 minutes (3000–3500 r/min).

The enzyme activities, including superoxide dismutase (SOD), lysozyme (LSZ), catalase (CAT), pyruvate kinase (PK), and fatty acid synthase (FAS), were measured using commercial ELISA kits (Beijing Huabo Deyi Biotechnology Co., Ltd.) according to the manufacturer's instructions. The heat shock protein 90 (HSP90) concentration was determined using the shrimp heat shock 90 kits (Beijing Huabo Deyi Biotechnology Co., Ltd.).

2.4. Histological Observation. The hepatopancreatic and gill tissues were removed from the 4% paraformaldehyde fixative and sliced according to our previous experimental method. The samples were dehydrated and embedded in paraffin (Sigma). Sections with $5\,\mu m \pm 1\,\mu m$ thickness were cut using a pathology slicer (Leica), dewaxed with xylene, rehydrated through a series of ethanol washes, and then stained with haematoxylin-eosin (HE). The images were taken using a camera of Olympus microscope.

2.5. Statistical Analysis. All data were performed using SPSS 17.0 and GraphPad Prism 8 software. One-way analysis of variance (ANOVA) was used to assess between groups. Differences were considered statistically significant at $P < 0.05$. The results were expressed as mean ± SD.

3. Results

3.1. Effects of Acute Salinity Stress on Metabolic Enzyme Activities in Hepatopancreas and Gill Tissues. The value of enzyme activates was analysed by the statistical software and found a difference in different salinity stress. PK and FAS activities displayed similar trends in the hepatopancreas and gill tissues. PK activities were significantly decreased in crayfish treated with 6, 12, 18, and 24 ppt at 36 h ($P < 0.05$) (Figures 1(a) and 1(c)). FAS activities were significantly increased in 6 ppt group at 36 h, but decreased significantly accompanied with the increase of salinity from 12 to 24 ppt ($P < 0.05$) (Figures 1(b) and 1(d)).

3.2. Effects of Acute Salinity Stress on Immune-Related Enzymes and Antioxidase Activities in Hepatopancreas and Gill Tissues. As shown in Figure 2, the lowest LSZ activity was at 12 ppt, and the highest level was at 24 ppt ($P < 0.05$) (Figures 2(a) and 2(d)). LSZ activity in the hepatopancreas and gill tissues first decreased and then increased with an increase in salinity.

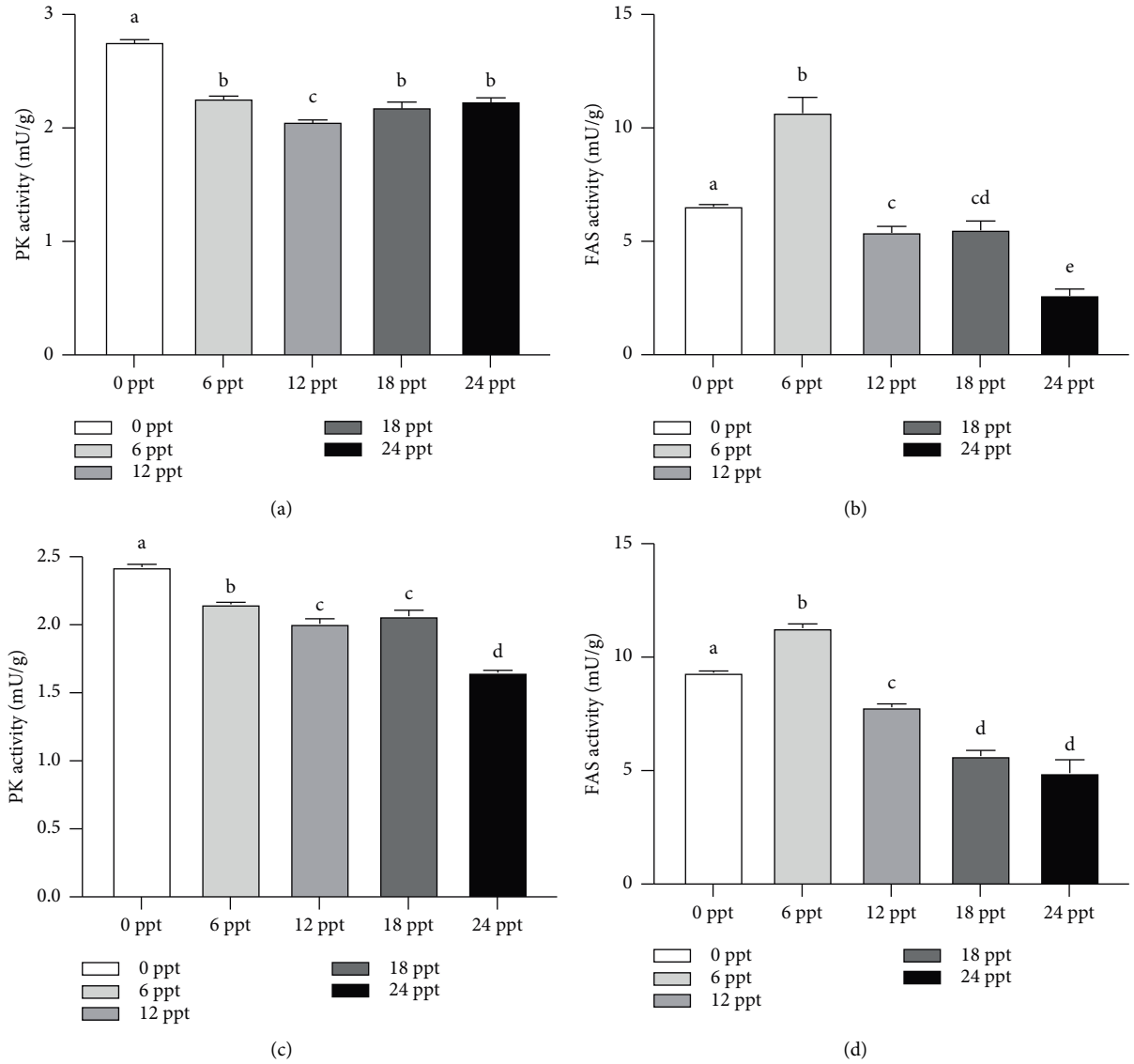

Figure 1: The activity of metabolism-related enzymes of *Procambarus clarkii* under acute salinity conditions. PK and FAS activity in the hepatopancreas (a), (b). PK and FAS activity in the gill tissue (c), (d). Different letters indicated a statistical difference between groups ($P < 0.05$).

The SOD activity in the hepatopancreas and gill tissues was significantly lower when treated with 12, 18, and 24 ppt, but significantly higher at 6 ppt ($P < 0.05$) (Figures 2(b)and 2(e)). In contrast, CAT activity was higher when treated with 12, 18, and 24 ppt and significantly lower at 6 ppt ($P < 0.05$) (Figures 2(c)and 2(f)).

3.3. Effects of Acute Salinity Stress on HSP90 Concentration in Hepatopancreas and Gill Tissues. The highest concentration of HSP90 was at 6 ppt, and the lowest was at 24 ppt ($P < 0.05$), which showed first increased and then decreased with the salinity increased in the hepatopancreas and gill tissues (Figure 3).

3.4. Effects of Acute Salinity Stress on the Hepatopancreas Tissue Structure. Acute salinity stress after 36 h significantly affects the hepatopancreas tissue structure of crayfish (Figure 4). The basement membrane of hepatic tubules was intact, and the structure of hepatocytes was normal and formerly distributed without salinity stress (0 ppt, control group). At 6 ppt, the boundary of the basement membrane became unclear, the volumes of *B* and *R* cells increased, and the lumen space increased slightly compared with the control group. At 12 ppt and 18 ppt, the basement membrane was widely folded, *B* cell transport vesicles were ruptured, the lumen was deformed, and severe deformation of *R* cells was observed. The hepatic tubules were severely deformed and became fluid, the basement membrane was ruptured, and the necrotic contents of hepatocytes flowed in high salinity of 24 ppt.

3.5. Effects of Acute Salinity Stress on the Structure of the Gills. After 36 hours of different salinity stress, the structure of the gill tissue of crayfish also changed (Figure 5). In the control group, the structure of the gill membrane was complete and

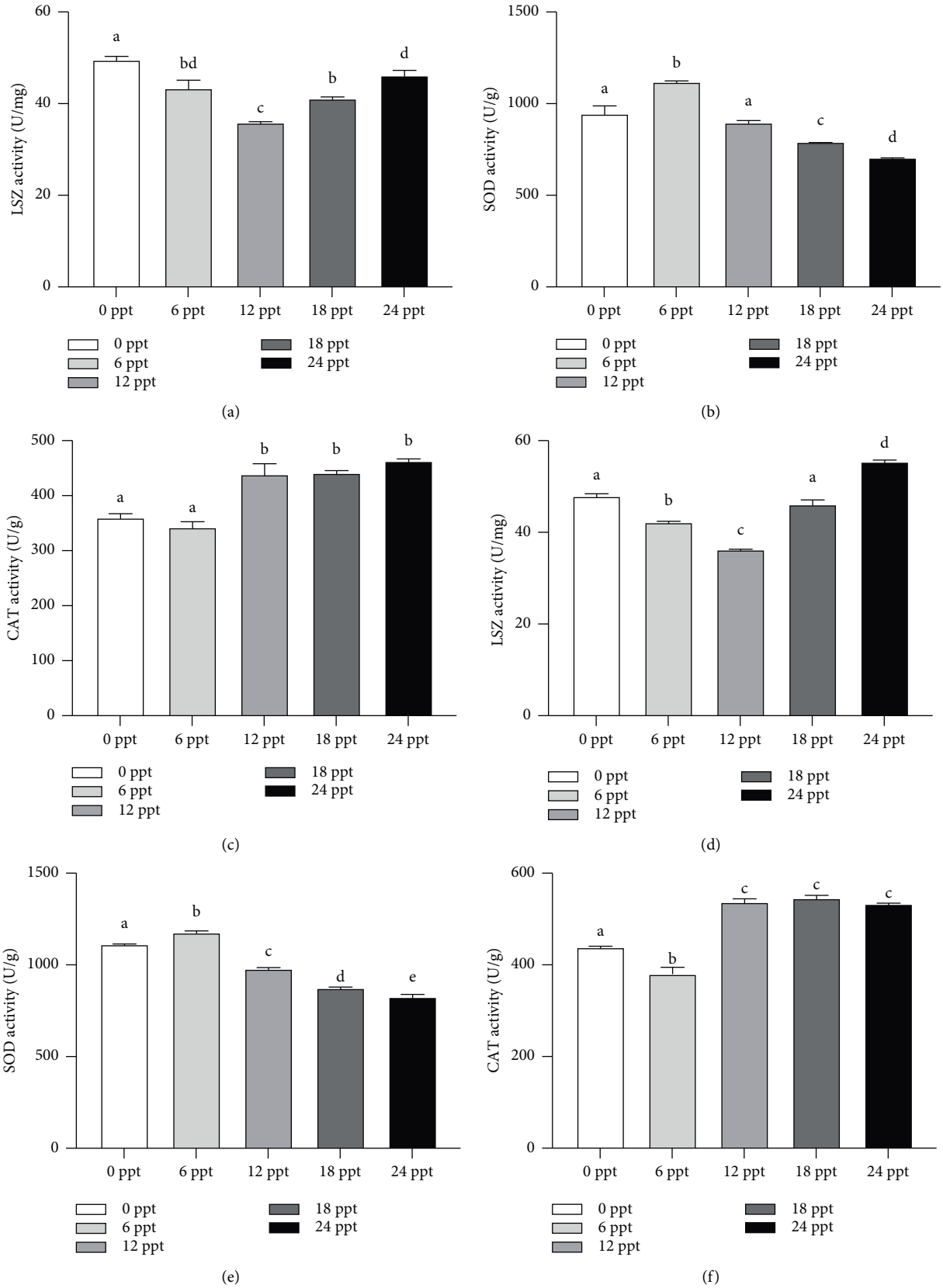

Figure 2: The activity of immune-related enzymes and antioxidase of *Procambarus clarkii* under acute salinity conditions. LSZ, SOD, and CAT activity in the hepatopancreas (a), (b), and (c). LSZ, SOD, and CAT activity in the gill tissue (d), (e), and (f). Different letters indicated a statistical difference between groups ($P < 0.05$).

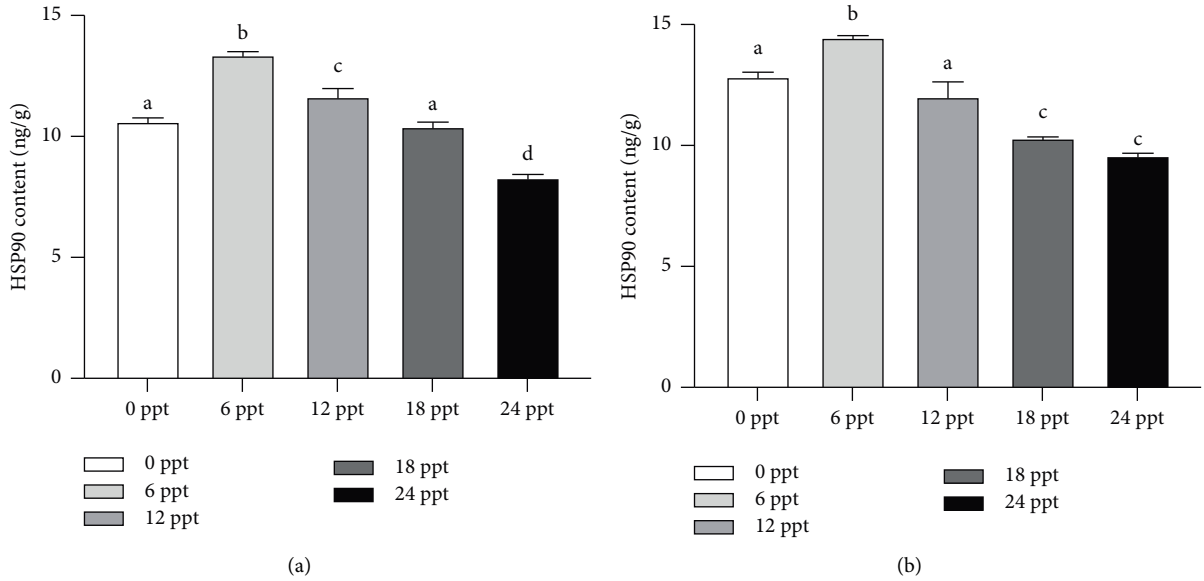

FIGURE 3: Heat shock protein 90 concentration of *Procambarus clarkii* under acute salinity conditions in the hepatopancreas (a) and the gill tissue (b). Different letters indicated a statistical difference between groups ($P < 0.05$).

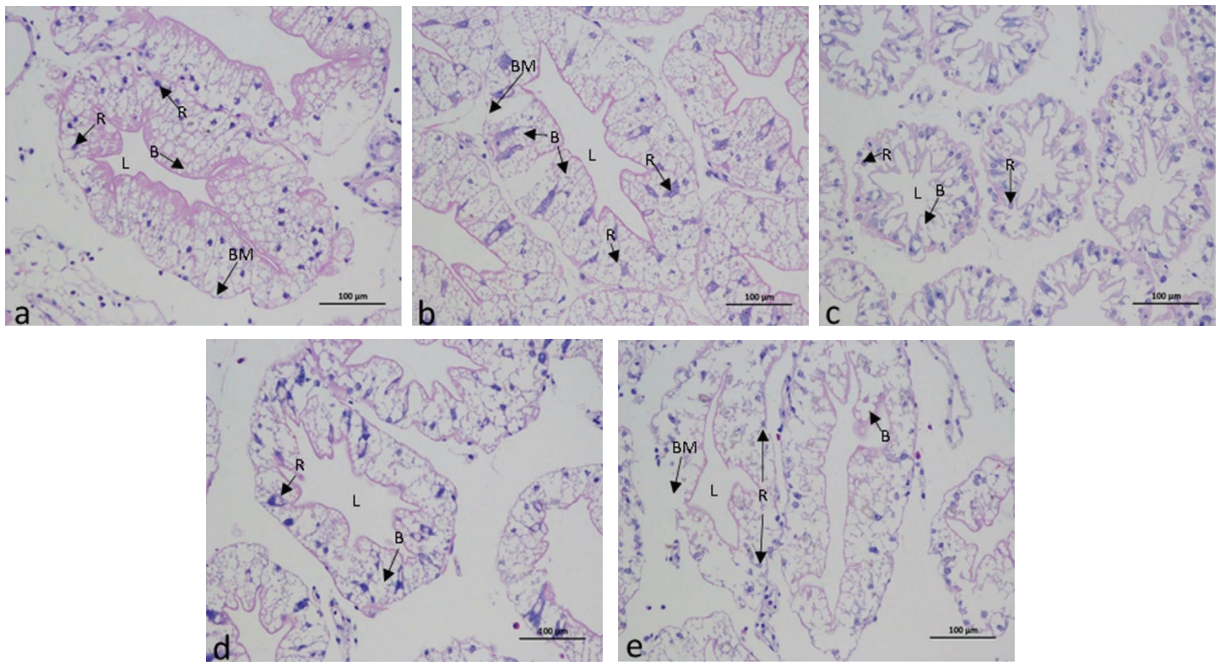

FIGURE 4: The effect of acute salinity stress ((a) 0 ppt, (b) 6 ppt, (c) 12 ppt, (d) 18 ppt, and (e) 24 ppt) on the microstructure of the hepatopancreas of *Procambarus clarkii* (*B*, secretory cells; *R*, storage cells; *L*, hepatopancreas tubules lumen; BM: basement membrane. 400× magnification).

smooth, and the respiratory epithelial cells and blood cells in the microcavity were normal in shape and evenly distributed (Figure 5(a)). At 6 ppt, the respiratory epithelial cells were partially shed, and the number of microcavity blood cells were decreased (Figure 5(b)). At 12 ppt, the number of respiratory epithelial cells decreased, vacuolization was severe, and microcavity blood cells aggregated (Figure 5(c)). At 24 ppt, the gill membrane was ruptured, the gill filaments were deformed, the respiratory epithelial cells were severely sloughed off, and the contents were lost (Figure 5(e)).

4. Discussion

The crayfish industry is widely and rapidly developing in China due to the high economic benefits. China became the largest aquaculture country for crayfish production in 2015 [20]. However, the industry faced problems such as improving farming performance in the face of adversity [6, 21, 22].

Reports showed that *P. clarkii* exposed to increasing salt concentrations reaching 35 ppt, still have a high survival rate [9]. We did not detect dead animals when exposed to

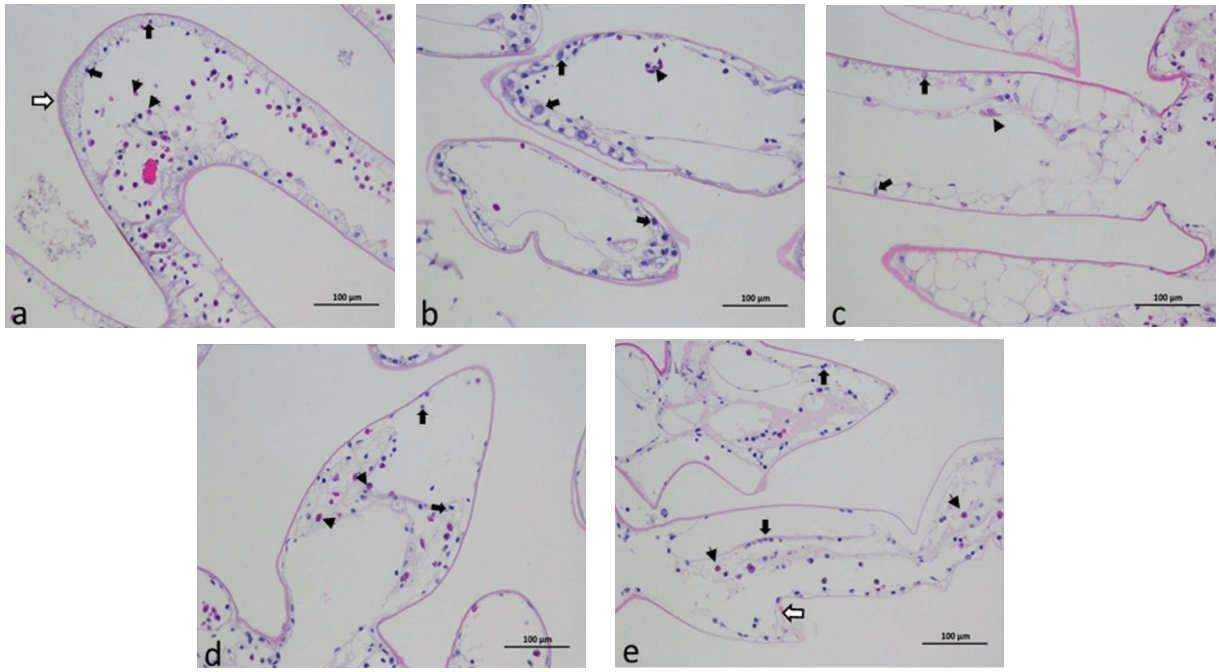

FIGURE 5: The effect of acute salinity stress ((a) 0 ppt, (b) 6 ppt, (c) 12 ppt, (d) 18 ppt, and (e) 24 ppt) on the microstructure of the gill tissue of *Procambarus clarkii* (⇨, gill membrane; ➡, respiratory epithelial cells; ▶, microcavity blood cells. 400× magnification).

6–24 ppt for 36 h in our study, revealing strong body homeostasis. The activities of key enzymes in glucose and lipid metabolism changed. PK is a key rate-limiting enzyme that catalyses the conversion of phosphoenolpyruvate and adenosine diphosphate (ADP) to pyruvate and adenosine triphosphate (ATP) in glycolysis. FAS is a key enzyme in fatty acid synthesis, catalysing acetyl-CoA and malonyl-CoA to generate long-chain fatty acids. PK activities of the hepatopancreas and gill tissues decreased slightly at low salinity and decreased significantly at 24 ppt. FAS activity showed higher decreases in salinity stress; expect an increase at 6 ppt. The crayfish need to accelerate sugar decomposition and restrain fat synthesis to generate energy for various ion balance reactions. However, this process requires a certain adaptation time. This is consistent with the metabolic rates of stenohaline marine species showing a metabolic depression at enhanced salt concentrations, reflecting osmotic stress [1].

P. clarkii responded to acute changes in environmental salinity by adjusting their immune function [12]. LSZ activities showed a decrease in most treatments during our study. However, SOD showed a decrease and CAT an increase at 12, 18, and 24 ppt, SOD slightly increased at low salinity of 6 ppt. Decreased SOD activity at high salinity and increased CAT activity suggest a decrease of H_2O_2 concentration, possibly due to oxidative damage to the enzymatic structure by superoxide anion (O_2^-) and malondialdehyde (MDA) overload. It suggested that the immune system adapted to a low salinity but was damaged at high salinity. This is consistent with the research that survivors of elevated salinity concentration showed increased levels of SOD, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) [9]. Studies have also shown that although SOD and CAT perform the function of protecting cells from free radical damage simultaneously, they are not completely synergistic, and their activity levels have a dynamic balance mechanism [23].

The heat shock proteins are classic molecular chaperones or stress proteins distributed ubiquitously in tissues. They were proven to protect cellular functions and structures from many types of stress and maintain cellular homeostasis [24–26]. HSP90 can prevent the aggregation of heat-denatured proteins and unstable proteins in *vitro*, usually combined with HSP70 and HSP40 [27–29]. HSP90 concentration in this study showed a slight increase at low salinity (6 ppt) and decreased remarkably when the crayfish were treated with high salinity (24 ppt). It was similar to the HSP90 concentration in the gills of *Crassostrea hongkongensis*, where mRNA first increased and then gradually reduced under hyper-osmotic treatment but returned to the initial level after long-term stress [30]. HSP90 content was differentially altered in the hepatopancreas and gills of crayfish, suggesting that salinity stress may disrupt the normal structure of intracellular proteins in the hepatopancreas and gills of crayfish. The body reduces the misfolding of damaged proteins by regulating the HSP90 concentration and protects cells from hypertonic injury [31].

HE staining of sections was conducted, and the microscopic changes of the gills and hepatopancreas were analysed to verify changes in the tissue structure. A balance of ions and charges inside and outside the cell membrane of various tissues is key to maintaining a stable internal environment [32]. Hyperosmotic treatment clearly influenced the two tissue structures, and the effect increased with rising

salinity. At 6 ppt, the boundary of the hepatopancreas basement membrane was unclear. When the salinity reached 24 ppt, the basement membrane of the hepatopancreas and gills was visibly ruptured, consistent with the above results of weakened antioxidant levels that resulted in damaged lipids and proteins of cellular compartments, leading to the loss of cellular membrane integrity and cell death. High salinity (9–11 ppt) causes the gill epithelial cells of freshwater fish *Chalcalburnus chalcoides aralensis* to thicken and bulge, and necrosis, exfoliation, hepatocyte enlargement, and severe tissue vacuolization occur [33]. The haemolymph osmolality of red claw crayfish was unchanged at salinities ranging from 0 to 10 ppt. However, an increase was observed at 12 ppt, revealing strong osmoregulation at the proper salinity range 0–12 ppt, whereas decreased growth rate and increased sodium-potassium ATPase activity, and sodium potassium chloride cotransporter expression were reported under high salinity [34]. In conclusion, our results confirmed that although low salinity causes minor tissue damage of tissue, high salinity destroys the tissue cell structure, and the damages are irreversible. It is recommended to conduct long-term breeding experiments at 0–6 ppt to increase its tolerance to salinity. No more than 6 ppt was recommended to raise the crayfish for long-term domestication of salinity for *Procambarus clarkii* aquaculture.

Data Availability

All data used to support the findings of this study are available in the article.

Ethical Approval

All international, national, and institutional guidelines applicable for the care and use of animals were followed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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