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Effects of concentrated dephenolic cottonseed protein instead of fish meal on stress resistance of *Penaeus monodon*

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Abstract

To explore the effect of concentrated dephenolic cottonseed protein (CDCP) partly instead of fish meal on the non-specific immune of *Penaeus monodon*, we designed a single factor test to study the four types of non-specific immunity of *P. monodon* hepatopancreas after acute salinity stress. A total number of 5,400 *P. monodon* were selected with an initial body weight of 1.76 ± 0.15 g and randomly divide into 6 groups (3 replicates per group, 900 tails per replicate). Shrimp were feed with two protein level diets (Diet A and Diet B). After 8 weeks of feeding, *P. monodon* were randomly selected with an average weight of 14.71 ± 1.06 g in each feed group, and subjected to acute salinity stress of 10ppt and 20ppt after temporary rearing, and the heat shock protein 70 (HSP70), $\text{Na}^+\text{-K}^+\text{-ATPase}$, cortisol and the content of alkaline phosphatase (ALP) were measured. The results of the study showed that when the CDCP partially replaced fish meal of *P. monodon* diet, the hepatopancreas HSP70, and the contents of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and cortisol were significantly higher than those of the control group. In the acute salinity change, there was no significant difference in the survival rate of *P. monodon* at 48h under the two salinity stresses, and the four immune indexes showed different trends. The expression level of Cortisol was more prominent than that of HSP70, which could better reflect the stress degree of *P. monodon*. Most of the indicators showed a trend of rising and then falling and then rising compared with the original level, and the changes of the four indicators of hepatopancreas in the Diet B group were significantly higher than those in the Diet A group at most time point of the two salinities.

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Introduction

In shrimp culture, feed cost accounts for more than half of the total cost (Jiang *et al.*,2018). At present, shrimp feed mainly relies on fish meal as protein source and the resource of fish meal is limited and the demand is increasing rapidly (Zhang *et al.*,2018). Some scholars carried out the research on the replaceable of plant protein to reduce the demand of fish meal in shrimp feed and found out the most suitable amount of plant protein in diet (Yu *et al.*,2016; Riche *et al.*, 2011; Liu *et al.*,2012). However, some studies showed that when the animal protein content in feed was too low, it would have a certain impact on the growth and immune performance of cultured organisms (Niu *et al.*,2015; Gomez-Requeni *et al.*,2004). Therefore, the immune and anti-stress properties of cultured organisms fed with plant protein instead of fish meal protein need to be further studied.

Cortisol, HSP70 and ALP are important indicators involved in the body's stress immune response, and their levels and activities are closely related to the health and immunity of aquatic animals (Flik G *et al.*,2006; Yvette S *et al.*,2011). Na⁺-K⁺-ATPase is a typical marker of osmoregulation (Xiao,2018). Therefore, it is of great significance to study the change trend of the above four immune indexes for understanding the immune performance of cultured organisms. In this study, *P. monodon* was fed with normal fish meal protein level feed and CDCP partially replace fish meal feed for 8 weeks, and the changes of immune indexes were studied by acute salinity stress experiment. The experiment provides data reference for immune research of *P. monodon* fed with low fish meal protein, and provides theoretical reference for evaluation of environmental factors and health status of *P. monodon*.

Materials and Methods

Feed formula and preparation.

Fish meal, soybean meal, CDCP and peanut meal were used as the main protein sources, and the formula was designed according to the principle of equal energy and unequal protein. Two kinds of feeds (**Table 1**) were prepared: Diet A group (fish meal content in feed is 30%) and Diet B group (fish meal content in feed is 10%; CDCP content is 20%). All feed materials were crushed through 80-mesh sieve, mixed step by step, and then stirred with water to make 1.00- and 1.50-mm diameter pellet feed, baked in 90 °C oven (put some water in the oven) for 2 hours, dried in the air-conditioned room, stored in -20 °C refrigerator for standby.

Experimental shrimp and feeding management.

The experiment was carried out in the genetic and breeding center of *P. monodon* in the Ministry of Agriculture of the People's Republic of China. The *P. monodon* used in the experiment were selected as the core breeding family of the research group after several generations of continuous breeding. A total number of 5400 shrimp with the average body weight of 1.76±0.15g were selected and divided into 2 feed groups and each feed group was set up with 3 parallel groups. Shrimp were feed 3 times a day at 8:00, 17:00 and 22:00. The daily feeding amount was 7% - 8% of the shrimp weight. The aquaculture water was sand filtered seawater. The water was changed 1/3 every week, and the breeding period was 56 days. During the experiment, the micro tube aerator was used for 24 hours to aerate and increase oxygen. The water temperature was 27-32 °C, the salinity was 31-33, the pH was 7.8-8.2, and the dissolved oxygen was 6.6-7.0 mg / L.

Experimental design and sample processing

After the breeding experiment, *P. monodon* with an average weight of 14.71±1.06g were selected from Diet A and Diet B respectively and temporarily reared for three days. The experiment was set up two salinity treatment groups of 10ppt and 20ppt, and each treatment group had three parallel treatment groups. A number of 60 selected shrimp were placed in each black breeding bucket and no feed was given after the experiment started. At the time points of 0h, 8h, 16h, 24h, 32h, 40h, and 48h, the hepatopancreas samples of 5 shrimp were cut, mixed, and put into a 1.5mL cryopreservation tube. After sampling, immediately transfer to -80°C refrigerator for storage for testing.

An appropriate amount of hepatopancreas tissue sample was taken, and the upper tissue fluid was absorbed with filter paper, weigh it and place it in a centrifuge tube, add the homogenate to m (tissue, g): v (homogenate, ml) =1:9, and homogenize Select PBS (PH=7.2-7.4, concentration

of 0.01mol/L). Tissue homogenate was carried out by vortex shaker (G56E, Qite Analytical Instrument Company) and then centrifuged at 3000r.min⁻¹ 4°C for 10 minutes, and the supernatant was taken for subsequent operation. The activities of four immune indexes, Na⁺-K⁺-ATPase, HSP70, cortisol and ALP, were carried out according to the corresponding shrimp ELISA kit produced by Beijing Huabodeyi Biotechnology Co., Ltd., and the kit was determined by double antibody sandwich method.

Data processing

The experimental data were analyzed by One-Way ANOVA with SPSS25.0, and $P < 0.05$ showed significant difference.

Table 1 Composition and nutrient content of experimental diets

Ingredients (%)	Diet A	Diet B
Fish meal	30	10
Soybean meal	18	18
Concentrated dephenolization cottonseed protein	0	20
Peanut meal	10	10
Wheat flour	21.99	17.1
Beer yeast	3	3
Shrimp head meals	5	5
Soybean protein concentrate	4.8	6.2
Soybean lecithin	1	1
Fish oil	1	1
Soybean oil	0.5	2.1
CAscorbic Phosphate ester	0.1	0.1
Cholesterol	0.5	0.5
Vitamin premix ^a	1	1
Mineral premix ^b	1	1
Ca(H ₂ PO ₄) ₂	1	1
Lysine	0	0.97
Methionine	0.1	0.47
Threonine	0	0.55
Carboxymethylcellulose	1	1
Y ₂ O ₃	0.01	0.01
Sum	100	100
Proximate composition		
Moisture (%)	8.32	7.88
Crude protein (%)	39.65	39.29
Crude lipid (%)	5.70	6.09
Ash (%)	11.89	10.47

Note: 1. Vitamin premix (g·kg⁻¹): V_A 2.5; V_D 6.25; V_E 75; V_K 2.5; V_{B1} 0.25; V_{B2} 1.0; V_{B3} 5.0; V_{B6} 0.75; V_{B12} 2.5; folic acid 0.25; biotin 2.5; inositol 379; cellulose 500; 2. Premix Minerals (g·kg⁻¹), KCl, 90; KI 0.04, NaCl, 40g; CuSO₄·5H₂O; 3. ZnSO₄·7H₂O; 4. CoSO₄·7H₂O, 0.02; FeSO₄·7H₂O, 20; MnSO₄·H₂O, 3; MgSO₄·7H₂O, 124; Ca(H₂PO₄)₂·2H₂O, 500; CaCO₃, 215.

Results

Effect of CDCP instead of partial fish meal on immune indexes of *Penaeus monodon*.

The effects of CDCP instead of fish meal on activities of ALP, cortisol, HSP70 and Na⁺-K⁺-ATPase of *P. monodon* were shown in **Figure 1(A)**, **Figure 2(A)**, **Figure 3(A)** and **Figure 4(A)** for 0h. The result in **Figure 1(A)** showed that the ALP activity of *P. monodon* in Diet B group was significantly different from that in Diet A group after 56 days of culture experiment ($P < 0.05$). The

activities of cortisol, HSP70, Na⁺-K⁺-ATPase in *P. monodon* in Diet B group were significantly different from those in Diet A group ($P < 0.01$).

Effects of salinity of 10ppt on nonspecific immune indexes of Penaeus monodon.

Compared with the experimental group Diet B, after 8h of stress, the ALP activity of Diet A group reached the highest, which was significantly higher than that of Diet B group ($P < 0.01$), and then decreased at 16h. At 48h, the ALP activity of Diet B group was higher than that of Diet A group ($P < 0.05$). And Diet B group rose to the highest at 48h, while Diet A group rose to the highest at 8h (**Figure 1-A**). In (**Figure 2-A**), the HSP70 of the Diet A group reached the highest at 8h, which was significantly higher than that of the Diet B group ($P < 0.01$). The Diet B group rose to the highest at 24h, and then, became the same level with Diet A group, which presented a trend of decreasing at first and then increasing. In (**Figure 3-A**), the expression of cortisol in Diet B group reached the highest at 0h, and then showed a trend of decreasing and then increasing. At most time points, cortisol in Diet B group was significantly higher than that in Diet A group ($P < 0.01$). In (**Figure 4-A**), the activity of Na-K-ATPase in hepatopancreas of *P. monodon* showed a trend of increasing at first and then decreasing, while Na-K-ATPase reached the highest level in Diet A group at 16h and Diet B group at 32h.

Effects of salinity 20ppt stress on nonspecific immune indexes of Penaeus monodon.

The ALP in hepatopancreas of control group Diet A and experimental group Diet B showed a turning point from increasing to decreasing after 16 hours of stress. during 8-48 hours, the ALP expression in hepatopancreas of Diet B group was higher than that of Diet A group at most time points, and there were significant differences ($P < 0.01$) (fig. 1-B). The expression of HSP70 in Diet A of control group and Diet B of experimental group showed an inflection point from increasing to decreasing at 16h, and reached the highest at 32h in Diet A group and 8h in Diet B group (fig. 2-B). The expression of Cortisol in hepatopancreas of Diet A group and Diet B group increased at 8h, showed an inflection point from increasing to decreasing at 16h, and showed a trend of returning to 0h expression level at 48h, and there was a significant difference between Diet B group and Diet A group ($P < 0.01$) (**Figure 3-B**). In (**Figure 4-B**), the expression of Na⁺-K⁺-ATPase in Diet A group and Diet B group showed an increasing trend from 0 to 0-16h hours, and then both groups showed a decreasing and then increasing trend, and their expression levels at 48h were higher than those at 0 h.

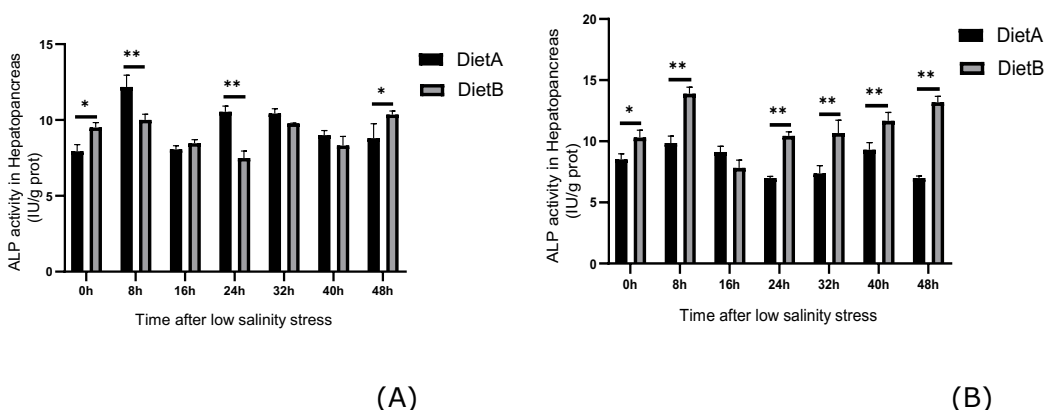


Figure 1 The effect of salinity stress on ALP activity of *Penaeus monodon*

* means that the difference between groups is significant at the same time point ($p < 0.05$); ** means that the difference between groups is extremely significant at the same time point ($p < 0.01$). (A) is the stress Figure of salinity 10ppt, and (B) is the stress Figure of salinity 20ppt. Same as below.

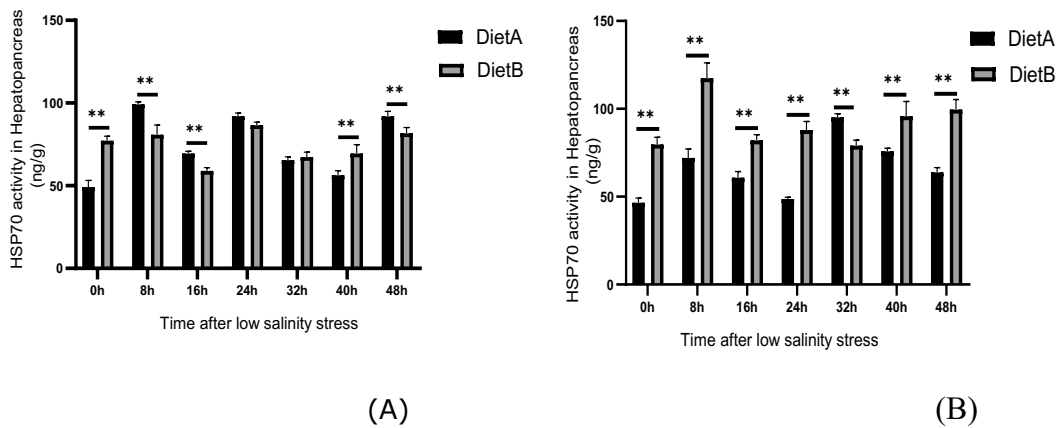


Figure 2 Effects of salinity stress on HSP70 of *Penaeus monodon*

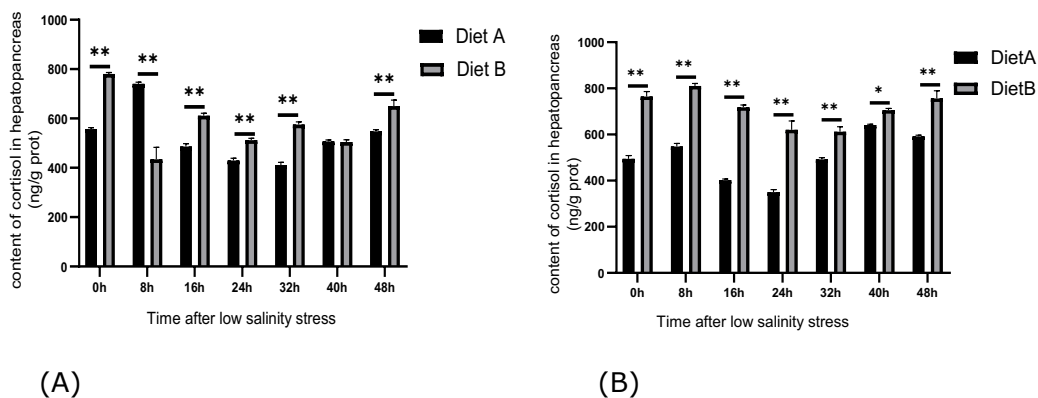


Figure 3 Effects of salinity stress on the cortisol content of *Penaeus monodon*

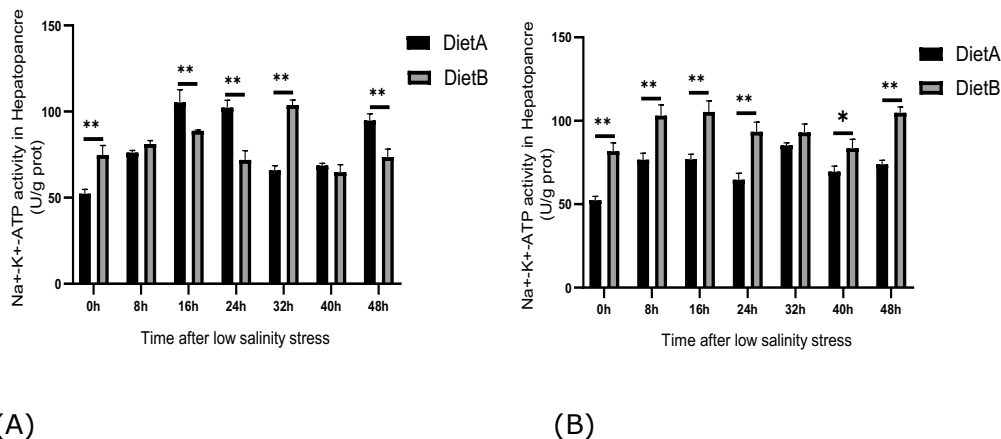


Figure 4 Effects of salinity stress on the activity of Na⁺-K⁺-ATPase in *Penaeus monodon*

Discussion

Effect of CDCP instead of fish meal on immune indexes of *P. monodon*.

Many studies showed that the plant protein added in feed would have an impact on the immune index of fish (Sun et al., 2013; Xu et al., 2012; Lin et al., 2011). Lin (Lin et al., 2011) found that the increase of the level of CDCP replacing fish meal could affect the total antioxidant capacity of hepatopancreas, the activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), superoxide dismutase (SOD) and liver glycogen content of tilapia. In the low fish meal feed of tilapia, the level of CDCP replacing fish meal should not exceed 50%. Excessive substitution level would have a negative impact on the immune response of tilapia. Lin (Lin et

al.,2010) used different plant protein sources to replace fish meal in tilapia feed, it was found that rapeseed meal and cottonseed meal had the greatest influence on GOT, GPT and SOD activities in hepatopancreas of tilapia, followed by peanut meal, and soybean meal had the least influence. Zhang (Zang et al.,2018) replaced fish meal with high-protein cottonseed meal, it was found that with the increase of the proportion of high-protein cottonseed meal, the contents of phenoloxidase, total superoxide dismutase, acid phosphatase, alkaline phosphatase in the serum of *Litopenaeus vannamei* and malondialdehyde in the hepatopancreas changed. When the substitution ratio did not exceed 50%, the immune system of *L. vannamei* was not negatively affected, while the substitution amount reached 40%, the alkaline phosphatase activity decreased significantly. In this experiment, 66.67% of fish meal was replaced by CDCP. After 56 days of culture experiment, it was found that the four nonspecific immune indexes of cortisol, Na⁺-K⁺-ATPase, HSP70 and ALP in hepatopancreas were significantly higher than those in the control group, which indicated that adding a certain amount of CDCP in *P. monodon* feed could stimulate the immunity of the organism itself.

Evaluation of stress degree of P. monodon under low salinity stress.

The adaptation of crustaceans to salinity was mainly through the adjustment of osmotic pressure of the body. When the salinity of water suddenly changed, the body needed to make dynamic osmotic adjustment through osmotic pressure adjusting organs to reach a new equilibrium state that can adapt to the external environment. If the salinity changed too much or exceeded the adjustment ability of the body, it would cause irreversible damage, and then caused corresponding changes in related physiological indexes such as feeding, respiratory metabolism, immune enzymes and hormones, and even affect the growth and survival of organisms (Guo et al.,2013). In this study, there was no significant difference in the mortality of *P. monodon* for 48 hours under the stress of different salinity groups. This indicates that *P. monodon* had strong salinity regulation and adaptability.

Some studies had shown that the hypothalamus-pituitary-adrenal axis (HPI) of fish was activated under the action of environmental pressure, which lead to the synthesis and release of stress hormones such as cortisol (Wendelaar et al.,1997). Cortisol played an important role in osmotic pressure regulation, which could stimulate proliferation and differentiation of gill chloride-secreting cells, regulate water-salt balance, activate Na⁺-K⁺-ATPase activity, enhance salinity tolerance and maintain osmotic pressure balance (McCormick et al.,2001; Pelis et al.,2001; Zhou et al.,2003). The results of salinity of 10ppt stress in this experiment showed that cortisol in Diet A feed group increased to the highest level at 8h, then decreased to the lowest level at 32h, and then increased and tended to return to the original level. However, under salinity stress of 20ppt, it increased at 8h and decreased to the lowest level at 24h, and the subsequent performance was consistent with the stress trend of salinity of 10ppt. Therefore, it was speculated that the acute change of salinity had strong stress on *P. monodon*, which led to the higher expression of cortisol at 8h than at 0h. The expression dropped to the lowest level at 32h and 24h respectively, which probably because the immune system was challenged during this period, and the material metabolism of its body cells was disordered, which was also an adaptive adjustment stage of the body to this salinity stress, and gradually adjusted and adapted in the following time, so the non-specific immune stress index rose again and had a tendency to return to the level before the initial stress. Cortisol in Diet B group decreased significantly at 8h, and then increased at first and then decreased, and tended to return to the original level around 48h. Under the stress of salinity of 10ppt, cortisol in Diet B group showed a completely opposite trend to that in Diet A group at 8h. It was speculated that cortisol was a sensitive indicator of stress, and the stress degree was 0-8 hours, and *P. monodon* was already in disorder, so it decreased significantly. The expression of cortisol in Diet B group was significantly higher than that in Diet A group at most time points in two stress salinities ($P<0.01$). The result may be that the expression of cortisol in Diet B group was significantly higher than that in Diet A group at the initial stage after 56 days of culture experiment. Therefore, the expression of cortisol in Diet B group was significantly higher than that in Diet A group after salinity stress.

Heat shock protein (HSP) was a family of polypeptide proteins existing in all organisms. It was a highly conserved family of protein molecules in the process of biological evolution. Among them, HSP70 family had the most members. It had been reported that HSP70 was not only extremely sensitive to external temperature, but also susceptible to dissolved oxygen, hunger,

acidity, crowding and other stimuli (Vinagre *et al.*,2012). A large number of studies had shown that HSP70 could maintain intracellular homeostasis, and its large expression in cells could significantly improve the viability of cells and improve their tolerance to environmental stress or injury (Wu *et al.*,2006). However, the current research on HSP70 had rarely been reported in invertebrates, especially *P. monodon*. This study found that HSP70 increased significantly at 8h during both salinity stresses, followed by a process of decrease and then increase, and the expression of HSP70 in the Diet B group was significantly higher than that of the Diet A group at most time points. This may be because the immune system of *P. monodon* had been challenged, and the material metabolism of the cells of the body had been disordered, and then gradually adjusted, and gradually adapted to the subsequent time. However, Guo (Guo *et al.*,2013) found that, HSP70 cultured in three different salinity environments showed a slow increase trend, but the salinity had no significant effect on HSP70 of the juvenile pomfret at this stage, which was inconsistent with the results of this study. It was speculated that the same stress was caused by different species. The indicator expression would be different, and it may also be caused by the different time interval between the sampling time points.

The effect of low salt stress on enzyme activity of P. monodon.

Alkaline phosphatase (ALP) was an important non-specific phosphohydrolase in the process of animal energy metabolism and an important component of lysosomal enzymes. It played an important role in the absorption and utilization of nutrients in fish and its immune response. The activity of ALP was usually used as an evaluation index for shrimp non-specific immunity (Xiao *et al.*, 2010; Du *et al.*,2013). From the results of this experiment, in the feed group with stress salinity of 10ppt and 20ppt, *P. monodon's* non-specific immunity indexes showed an increasing trend in the initial stage of stress, but as time went on, hepatopancreas ALP showed a significant decrease and significant upward trend. This indicates that with the development of time, this stress caused the damage of the immune system of *P. monodon*, the enzyme activity in the body appeared intermittently low activity, and then the body adjusted and recovered through its own, and the enzyme activity increased again. This is an adaptation process of *P. monodon* to drastic external environmental changes. Research of Qiao on *Procambarus clarkia* found that with the increase of the stress concentration, the ALP and acid phosphatase (ACP) of *P. clarkii* also increased significantly, and showed a decreasing trend as time passed (Qiao *et al.*,2020).The result suggested that with the increase of salinity, the body consumed a lot of ATP in order to carry out osmotic adjustment to maintain the body's steady state, and the inorganic phosphoric acid necessary for the synthesis of ATP requires ACP and ALP to jointly catalyze the production of phospholipids, which led to ALP and ACP increased activity, thereby enhancing the body's immune activity. Afterwards, it decreased significantly, and the activities of ALP and ACP in the body were significantly inhibited, indicating that with time, salinity stress will affect the body's immune system, thereby reducing the body's disease resistance.

Na⁺-K⁺-ATPase was the most important protease for osmotic adjustment of crustaceans, mainly located on the basal plasma membrane, and it participated in the active transport of Na⁺ and K⁺ ions across the membrane on both sides of the cell, thereby maintaining the ion gradient and membrane potential inside and outside the cell, regulating the volume of the cell, and driving the transport of sugar and amino acids in the cell (Fang *et al.*,2001). The change of Na⁺-K⁺-ATPase was an important detection index for the immune regulation of aquatic animals with euryhalinity. Steven studied *Gammarus pulex* and found that the Na⁺-K⁺-ATPase activity of the gills of aquatic crustaceans in high-salinity water environments increased after entering low-salinity water environments (Steven *et al.*,2006). In the studies of *L. vannamei* and *Marsupenaeus japonicus* respectively, it was found that the gill Na⁺-K⁺-ATPase activity increased by 35%-58% (Huetado *et al.*,2007; Pan *et al.*,2006). However, at present, the reports on low salt stress of crustaceans mainly focus on the gill of shrimp and crab, while the study on Na⁺-K⁺-ATPase activity of hepatopancreas of *P. monodon* had not been found yet. According to the results of this experiment, the low salinity stress of 10ppt and 20ppt had significant effect on the activity of Na⁺-K⁺-ATPase in hepatopancreas. Diet B and Diet A showed an increasing trend in 0-16h, then decreased and then increased. It was speculated that there may be two reasons: The first was that the increase of Na⁺-K⁺-ATPase provided energy for a large number of Na⁺ and K⁺ ions to be discharged. With the extension of salinity stress time, Na and K ions gradually balanced with the

external environment, inhibiting Na⁺-K⁺-ATPase activity, and then the body gradually adapted to the environment, making Na⁺-K⁺-ATPase activity gradually return to the original level. Secondly, the increase of cortisol in hepatopancreas of *P. monodon* activated the activity of Na⁺-K⁺-ATPase in a short period of stress, which led to the increase of Na⁺-K⁺-ATPase activity.

Acknowledgments

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