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Effect of Different Salinity Levels on Expression of Three Plasma Membrane Associated Genes of *Penaeus monodon*

Song Jiang, Dong-liang Liu, Fa-lin Zhou, Xian-bin Mo, Qi-bin Yang, Jian-hua Huang, Li-shi Yang and Shi-gui Jiang*

Key Lab. of South China Sea Fishery Resources Exploitation and Utilization, The Ministry of Agriculture, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China 510300;
Shenzhen Base of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shenzhen 518108, P.R. China

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SGJ, SJ and XBM designed the study. HH took part in the execution of study. LSY, QBY and DLL implemented the study and were involved in sampling as well as testing. SJ, XBM and SGJ drafted the manuscript.

Key words
Salinity, *Penaeus monodon*, *PmATP synthase*, *PmNa$^+$-K$^+$-ATPase*, *PmToll receptor*

ABSTRACT

In this study, three genes, *PmATP synthase*, *PmNa$^+$-K$^+$-ATPase* and *PmToll receptor* gene, were selected to study their relative expression in different tissues of *Penaeus monodon*. The results showed that the mRNA level of *PmATP synthase* gene was not significantly up-regulated under low salt stimulation ($P>0.05$),while under high salinity stimulation, the transcription level of *PmATP synthase* mRNA reached the maximum at 16th h ($P<0.05$).The expression level of *PmNa$^+$-K$^+$-ATPase* mRNA increased at 4th h and 16th h, which was significantly higher than that in the beginning under low salt stimulation ($P<0.05$) and then decreased at 32nd h, ,while under high salinity stimulation, the expression level of *PmNa$^+$-K$^+$-ATPase* mRNA reached the highest at 8th h and 32nd h. The expression level of *PmToll receptor* mRNA reached the highest at 4th h after low salinity stimulation ($P<0.05$), and then down-regulated after 16th h, while under high salinity stress, the expression level was up-regulated and reached the maximum at 8th h, which was 2.4 times of that the in the control group ($P<0.05$) and decreased at 16th h after stress. *PmATP synthase* gene was relatively high expression, while *PmNa$^+$-K$^+$-ATPase* and *PmToll receptor* gene expression were low, so we guess that the large amounts of ATP of the body synthesis mainly use for the growth of the body. *PmNa$^+$-K$^+$-ATPase* and *PmToll receptor* gene expression are relatively high, which indicate that the body osmotic pressure regulation and the body's immune process consume a lot of energy.

INTRODUCTION

Salinity is one of the most important non-biological environmental factors in aquaculture, which can affect the metabolism, osmotic regulation and other functions of aquatic animals (Navarro, 1988; Kim *et al*., 1998). Whether in natural water or artificial breeding environment, some factors like evaporation, heavy rainfall, tides will cause dramatic changes in salinity. Coastal intertidal marine organisms need to adjust the physiological activities to adapt to changes in salinity constantly. Different aquatic organisms have different tolerance to salinity, and the optimum salinity is different (Ye *et al*., 2009; Péqueux, 1995).

Aquatic animals have their own set of osmotic adjustment mechanisms to adapt to different salinity environments (Romano *et al*., 2012). Studies have shown that *P. monodon* is broad salt animal, the optimum salinity is 10~35 PPT (Nikapitiya *et al*., 2014; Wang *et al*., 2006). In the outdoor breeding environment, changes in salinity are the main factors affecting the yield of shrimp farming, because the salinity changes beyond a certain range will affect the shrimp immune system, making it more vulnerable to pathogens (Nikapitiya *et al*., 2014). Moreover, the sudden change in salinity also affects the feeding and metabolism of cultured animals, and because of the need for higher energy for osmotic adjustment, which will eventually result in slow growth of cultured animals (Joseph *et al*., 2007).

P. monodon is not only one of the world's three major cultured shrimp, but also China's important marine aquaculture varieties. Due to the development of high density intensive culture and the impact of climate change, the new varieties of *P. monodon* have become the key to the sustainable development of the shrimp culture in recent years. At present, the impact of salinity on aquatic animals has been extensively studied both at home and abroad. Studies showed that when the *Acipenser sinensis* and

* Corresponding author: jiangsg@21cn.com
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Salmo salar were subjected to salinity stress, the activity of *PmNa⁺-K⁺-ATPase* in the gill was significantly fluctuated and the body's urinary incontinence or gill drainage was used to maintain the osmotic pressure balance (He *et al*., 2009; Handeland *et al*., 2002). The ability to tolerate the fluctuation of salinity in *P. monodon* could be an important index for the selection of desalination to cultivate juvenile shrimp.

With the continuous development of biomolecules, the information of biological organisms is being excavated step by step. The deep discovery of biological information is not only conducive to a more thorough understanding of a biological characteristics, but also provide more information for the biology itself and humans. At present, the evaluation of *P. monodon* salt tolerance at the molecular level has not been reported yet, so we studied the expression level of *PmATP synthase*, *PmNa⁺-K⁺-ATPase* and *PmToll receptor* gene in different tissues of shrimp under different salinity, which may provid basic data and theoretical support for the breeding of *P. monodon*.

MATERIALS AND METHODS

Experimental materials

Experiments were carried out at the Shenzhen Experimental Base of the South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. The shrimp used in the experiment was bred and preserved by the research group, and the body weight was 22.00 ± 1.00g. Before the start of the test, the shrimp were temporary kept in the cement pond and the water salinity was 33 and the temperature was 25 ± 1℃ for 3 days. During this period, commercial feed (Guangdong Shuanghu Feed Co., Ltd.) was fed to the shrimp and the shrimp was unfed 24 hs before the experiment. About 1/3 amount of water was replaced at 18:00 pm every day.

Salinity stress

The salinity of the seawater in Shenzhen base was 33, and the experimental gradient of salinity was 23 and 43. The salted seawater was mixed with the filtered tap water to adjust the experimental salinity, A high-precision salinity meter (ATAGO, Guangzhou, ATAGO Atago China branch) was used to detect seawater salinity. Before the start of the formal experiment, the experimental use of *P. monodon* were transferred from the outdoor large pond to the indoor cement pool, with natural sea water holding 3 days. The formal stress experiment was carried out in a foam box and three parallel wells were set up. Thirty shrimp were placed in each cement pond and injected with salted sea water at a temperature of (29.0 ± 1.5) °C and a pH of 7.0 ± 0.5. In the experiment, the hepatopancreas, gills and intestines were immediately stored in RNA later (Invitrogen, USA) at 0, 4, 8, 16, and 32 h, respectively, and placed at -80℃.

Total RNA and cDNA synthesis

Each sample was homogenized in liquid nitrogen and total RNAwas extracted by using TRIzol reagent (Invitrogen, Shanghai,China), following the manufacturer's directions. The extracted RNA was re-suspended in DEPC-treated sterile water and stored at -80℃ until needed. RNA quantity, purity, and integrity were verified by both native RNA electrophoresis on 1.2% agarose gel and the ratio of UV absorbance at 260 and 280 nm (Nano Drop Technologies, DE, USA). cDNA was synthesized from total RNA by using a Prime Script™ II Reverse Transcriptase kit (Ta Ka Ra, Dalian, China), following the manufacturer's protocol. The RACE cDNA templates were prepared using the SMARTer RACE cDNA Amplification Kit (Clontech, Dalian, China), the cDNA template for real-time quantitative PCR was synthesized using the Prime Script RT reagent Kit with gDNA Eraser (Takara, Japan). The cDNA was diluted one fold to use as the template of PCR reactions.

Primer design

Three pairs of primers which have been reported on the NCBI: *Toll receptor* (Gen Bank No: EF117252.1), *ATP synthase* (GenBeda: JF303646.1), *Na⁺-K⁺-ATPase* (GenBank: JX397998. 1), as the target gene for primer synthesis.

Tissue expression analysis of salinity-related genes

The mRNA expression profile of salinity-related Genes in different tissues from the shrimps at different time was detected by real-time quantitative PCR. Gene specific primers for *PmToll receptor*, *PmATP synthase*, *PmNa⁺-K⁺-ATPase* (Table I) was amplified and all reaction were run in three replicates in an Mastercycler ep realplex machine (Eppendorf, USA) with elongation factor 1a (EF-1α) as internal reference gene (forward primer EF-1α-F and reverse EF-1α-R, Table I). Fluorescence quantitative PCR was used to amplify the cDNA of the spermatophore at each time point. The PCR reaction system was 12.5 μL, including 4.25 μL of SYBR Premix Ex Taq (Tli RnaseH Plus) (Takara), 0.5 μL of primer (10 μmol / L), 1 μL of cDNA template, double distilled water to make up 12.5 μL, and instead of template instead of template, The reaction temperature is 95℃ pre-denaturation 30s; 95℃ denaturation 20s, 60℃ annealing 5s, 45 cycles; 65℃ extension 15s; dissolution temperature rose from 55℃ to 97℃; 37℃ cooling 5min. The experimental data were calculated using the relative CT method ($2^{-\Delta\Delta CT}$ method)

for the expression of glutamine synthetase mRNA.

Statistical analysis

Statistical analyses were performed using Excel 2013 and IBM SPSS statistics version 23.0, and Statistical differences were estimated by one-way ANOVA followed by Duncan ‘s multiple range test and the difference was considered significant if $P < 0.05$. Test data are shown as means ± standard deviation (X ± SD).

Table I. Oligonuleotide primers used for QPCR.

PmToll receptor -qF	5`CAGACAGTTTGAGTTTGAG3`
R	5`CTGAGGGACTTCACATC3`
PmATP synthase-qF	5`GCTGATGACTTGACTGA3`
R	5`CACGAGACAACACAGTA3`
PmNa⁺-K⁺-ATPase-qF	5`CGTGACATGACATCTGA3`
R	5`AGAGCAGGAGAATCATTC3`
EF-1α-qF	5`AAGCCAGGTATGGTTGTCAACTTT3`
R	5`CGTGGTGCATCTCCACAGACT3`

RESULTS

Observation on survival of P. monodon *by salinity stress*

Salinity stress experiment lasted for 32 h, and the number of shrimp became less. At 32 h after the experiment, the prawned shrimp got stabilized, the molting occurred, and no more death occurred. After the observation period, the experiment was stopped. The Figure 1 showed the difference expression of three different genes in different tissues, and the results were analyzed by differential expression and combined with different salinity survival rates.

Expression of three different mRNA genes in P. monodon *in various tissues under salinity stress*

Expression of PmNa⁺-K⁺-ATPase gene in gill tissue

The expression of *PmNa⁺-K⁺-ATPase* mRNA in *P. monodon* was regulated by two salinity stimuli (Fig. 1A). In the low salinity group, the expression level increased from 4th h (P>0.05), and then reached the maximum at the 16th h, which was 2.5 times than that in the control group (P<0.05), the expression level was down-regulated at 32nd h after stress. Under the condition of high salinity stress, the expression level was up-regulated from 4th h, and the slight decrease was observed at the 16th h and reached the maximum at the 32nd h, which was 2.3 times than that in the control group (P<0. 05).

Expression of PmToll receptor gene in gill tissue

The expression of *PmToll receptor* mRNA in *P. monodon* was regulated by two salinity stimuli (Fig. 1B).

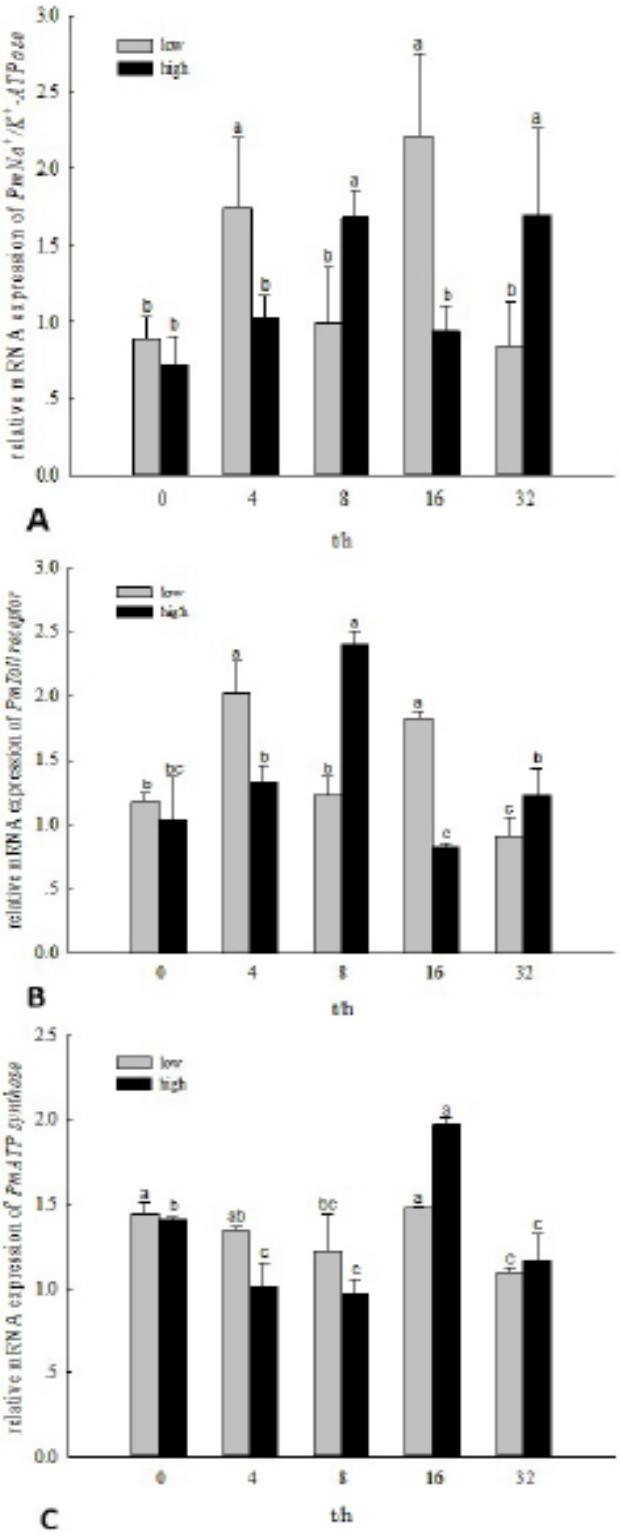

Fig. 1. Relative expression levels of *PmNa⁺-K⁺-ATPase* (A), *PmToll receptor* (B) and *PmATP synthase* (C) after salinity challenge.

In the low salinity group, the expression level reached the maximum at 4th h (P<0.05), and the expression level was down-regulated at 16th h. Under high salinity stress, the expression level was up-regulated from 4th h, reached the maximum at 8th h, which was 2.4 times (P<0. 05) than that in the control group and decreased at 16th h after stress.

Expression of PmATP synthase gene in hepatopancreas

The expression level of *PmATP synthase* mRNA in *P. monodon* was regulated by two salinity stimuli (Fig. 1C). In the low salinity group, the expression was not up-regulated (P>0.05), 8th h and 32nd h were lower than that in the control. Under high salinity stress, the expression level was up-regulated at 16th h and reached the maximum (P<0.05). The expression level was down-regulated at 32nd h after stress.

DISCUSSION

P. monodon was a broad salt aquatic organisms, and its osmotic regulation was strong (Cheng *et al*., 1986). Studies had shown that *P. monodon* were able to survive in the range of salinity 1 to 57 (Chen, 1990), and over low salinity for shrimps could have serious impact on the growth, osmotic adjustment and resistance (Li *et al*., 2000). Yang (2008) studied the effect of salinity on the growth of *P. monodon*, and the results showed that the specific growth rate and survival rate of *P. monodon* were significantly decreased in low salt environment. Studies have shown that within a certain range, with the reduction of salinity, ammonia nitrogen on the toxicity of *P. monodon* was also stronger, the weaker the disease resistance (Hu *et al*., 2009). Joseph and Philip (2007) had shown that low salt stress affects hemolymph metabolism and immunity in *P. monodon*, making it more susceptible to WSSV infection. Wang and Chen (2006) had shown that excessive high or low salinity increases susceptibility to *Photobacterium damselae* for *P. monodon*.

ATP was the main provider of energy required for living organisms, ATP synthesis was mainly catalyzed by ATP synthase (Wang *et al*., 2014). ATP synthase catalyze redox reaction was a mitochondria, which produced the most important enzyme in the ATP process. 95% of the organism was formed through this process, and ATP synthase played an important role in life activities. ATP synthetase activity showed the active performance of the body, and the ability to synthesize ATP is higher.

Na^+-K^+-ATPase played an important role in the body, which transported the hydronium and provides Na^+ transmembrane electrochemical gradient, especially played an important role on the irritating tissue organs, such as shrihcmp and crabs Gill tissue. So far, ATP synthase researched more in microbial and plant, however less in aquatic animals. Wang *et al*. (2014) had shown that water dissolved oxygen would affect the reduction of *silver carp* Fish ATP synthase activity, which affected the synthesis of ATP.

The results showed that the mRNA transcription level of ATP synthase was not significantly up-regulated under low salt stimulation (P>0.05). Under high salinity stimulation, the mRNA level of *PmATP synthase* was expressed at the 16th day maximum value (P<0.05). The expression of *PmATP synthase* gene was not upregulated by low salt stimulation, which may be related to the strong adaptability of low salt. Lin and Cao (2000) found that the lowest limit salinity of *P. monodon* larvae tolerance is 3-4. Joseph and Philip (2007) found that 35 high salt irritation could cause metabolic levels and immune changes in *P. monodon*. Endoplasmic reticulum as an important place for protein processing folding, when the body is stimulated so that the folding of the protein error occurs, which will send a signal to the nucleus, activation can modify these errors (Lu *et al*., 2014), ATP synthase, as an important endoplasmic reticulum stress protein, may accelerate the folding of the wrong protein to regulate the penetration of the body when the osmotic pressure of the body is too high.

Na^+-K^+-ATPase was the main ATPase which could maintain the permeability of the plasma membrane for shrimp and maintain the balance of the osmotic pressure of inside and outside the cells (Kumar *et al*., 2014). There was a direct correlation between the regulation of osmotic pressure and the activity of Na^+-K^+-ATPase in aquatic crustaceans. Many studies have found that the activity of Na^+-K^+-ATPase in aquatic crustaceans and the gene expression increased when the salinity of the outside water changes (Brooks *et al*., 2006; Hurtado *et al*., 2007). Zang *et al*. (2002) showed that the energy consumption of the shrimp in the low salinity is higher and the growth was slowed down. Shull and Clarke (1992) showed that aquatic animals have more than 1/4 of the ATP are consumed by Na^+-K^+-ATPase, indicating that the osmotic pressure regulation consumed the most of body's energy. According to osmotic pressure regulation principle, their additional metabolic energy can be paid at least and their conversion rate for growth is the highest when shrimps are at the isotonic point. Pan (2004) studied that the activity of Na^+-K^+-ATPase increased with in 3 days after salinity changes, and remained stable at 3-15 days, but was significantly higher than that in the control group, and the lower the salinity is, the greater the activity of the enzyme is.

In this experiment, the results showed that the expression level of *PmNa$^+$-K$^+$-ATPase* mRNA was up-regulated at 4 h after low salt stimulation (P<0.05), and reached the initial level after 32nd h. The highest level

of *PmNa*$^+$*-K*$^+$*-ATPase* mRNA was up-regulated at 8th h after high salinity stimulation (P<0.05). This indicated that the activity of Na$^+$-K$^+$-ATPase increased with the salinity stimulation, and the expression of *PmNa*$^+$*-K*$^+$*-ATPase* gene increased, which would consume more energy to promote the exchange of sodium and potassium hydroniums and to cope with the stimulation of external salinity. In addition, when the external environment changes, the gill cells respond quickly to adapt to external changes. Therefore *PmNa*$^+$*-K*$^+$*-ATPase* mRNA expression increased significantly in the adaptive period, and the excessive expression of the product also produced feedback suppression in the regulation of osmotic pressure balance and regulated the expression of *PmNa*$^+$*-K*$^+$*-ATPase* gene in the cells, so as to achieve the basic stability of osmotic pressure and hydronium concentration (Feng *et al*., 2012).

In addition, the *PmToll receptor* gene is one of the major pattern recognition receptors which involved in congenital immunity of prawns. The study found that ATP synthase also plays an important role in immune immunity. Wang *et al*. (2007) and Bourchookarn *et al*. (2008) showed that the expression of β subunit of ATP synthase increased significantly after shrimps infected with White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV), indicating that *PmToll receptor* gene needs to consume a certain amount of ATP to complete the immune activity when it was involved in the immune process. The various types of immune factors shrimp will be affected, and the body's stable state is easy to be broken when the external environmental factors change, than the body's susceptibility to pathogens increased, so that the body is in active immune response state (Guan *et al*., 2008).

The results showed that the expression level of *PmToll receptor* mRNA was the highest at 4th h after low salinity stimulation (P<0.05), and the expression level of *PmToll receptor* was down-regulated at 16th h after stress. Under high salinity stress, the expression level was up-regulated from 4th h and reached the maximum at 8th h, which was 2.4 times (P<0.05) than that in the control group, and decreased at 16th h after stress. The results indicated that larger the body affected by changes in environmental factors are, stronger the body's ability to withstand low salt will be.

CONCLUSIONS

Heavy rain and other bad weather often change the salinity of the water in the outdoor breeding process. *P. monodon* were more cultured in China's Pearl River Delta region. Salinity changes comparatively large in the estuary, shallow sea and coastal areas. So, the study about the molecular mechanism of salinity variety of *P. monodon* is great significance to the development of shrimp aquaculture. In addition, the expression levels of *PmATP synthase*, *PmNa*$^+$*-K*$^+$*-ATPase* and *PmToll receptor* gene were significantly changed at high and low salinity (P<0.05), indicating that the three genes were involved in salinity stimulation. The reaction process indicates that *PmATP synthase*, *PmNa*$^+$*-K*$^+$*-ATPase* and *PmToll receptor* gene can be used as a potential indicator of salinity changes during the culture of *P. monodon*. The results of this study provide a theoretical reference for the study of salinity tolerance of *P. monodon*, which can be validated and selected in the next breeding work, and also provide a good reference for further breeding of salinity tolerance of *P. monodon*.

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Statement of conflict of interest

The authors declare there is no conflict of interest.

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