Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 27(3): 131 – 141 (2023)
www.ejabf.journals.ekb.eg

Relative Expression of *Rab7* Gene in the Penaeid Shrimp Post-Larvae Reared in Different Salinity Conditions

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ARTICLE INFO

Article History:
Received: Nov. 13, 2022
Accepted: March 27, 2023
Online: May 17, 2023

Keywords:
Shrimp post-larvae,
Salinity,
Rab7,
Expression

ABSTRACT

The black tiger shrimp, *Penaeus monodon*, is the most economically important penaeid shrimp in Bangladesh for both culture and capture fishery. The culture of these penaeid shrimps is always risky due to different diseases and environmental stressors. This study aimed to assess the relative expression of the *Penaeus monodon Rab7* gene in the post larvae (PL) of the *P. monodon* cultured under different salinity conditions. We challenged the PL of the *P. monodon* in aquariums with two different salinity and dissolved oxygen conditions during a 7-day test. The relative expression of *Rab7* was upregulated during the experimental period of 36 hours. Average differences in the relative expression of *Rab7* were 0.3-folds and 0.5-folds, and 0.4-folds and 0.6-folds after 18 and 36 hours of rearing in 2 and 54 ppt salinity treated shrimp PL, respectively, when β-actin was used as an internal control. On the other hand, when elongation factor 1 alpha (EF1α) was used for normalization, the average relative expression of *Rab7*'s fold differences were 0.2-folds and 0.5-folds, and 0.3-folds and 0.6-folds after 18 and 36 hours of rearing in 2 and 54 ppt salinity treated shrimp PL, respectively. Therefore, our results suggested that the relative expression of the *Rab7* was positively correlated with an increased level of salinity and challenge duration. The significant increase in the expression level of *Rab7* in the PL of *P. monodon* exposed to high salinity stress (54 ppt) conditions indicated its functional role in osmotic stress in shrimps. However, further studies are necessary to elucidate the mechanisms of how *Rab7* is upregulated in shrimp cells after applying different levels of salinity using a larger sample group.

INTRODUCTION

Penaeus monodon is an eminent coastal aquaculture species in many countries of the world including Bangladesh (**Nisar *et al*., 2021**). Many people depend on this crustacean to maintain their livelihood. Bangladesh earns a good amount of foreign currency by producing this shrimp species along the coastal area, which is known as the black tiger shrimp (**Rahman & Hossain, 2009**). This penaeid shrimp has played a very significant

role in poverty alleviation as a requirement to achieve sustainable development goals (SDGs) in the developing and least developed countries. The crustaceans of the traditional and improved traditional farms faced different types of externalities. Among those, the most common are changes in physico-chemical parameters of culture water due to environmental trends, shocks and vulnerabilities (**Tendencia & Verreth, 2011**). However, changes in climatic conditions and impact on salinity caused many problems for the shrimp culture.

Salinity intrusion due to storm surges and the sea level rise, and a decrease in salinity in ponds as a result of heavy rainfall had shown adverse effects on the culture system. Many researchers observed the relation of changes in the physico-chemical parameters with the growth of shrimps in ponds (**Davis & Arnold, 1993; Tendencia & Verreth, 2011**). Studies have found that changes in the environmental parameters can affect these penaeid shrimps (**Le Moullac & Haffner, 2000**). Differentially expressed genes including ones responsible for coding apoptosis proteins, cellular and membrane proteins, structural proteins, hypothetical proteins and proteins involved in reproduction, growth, signal transduction, energy and metabolism, cellular process, ion transport, osmoregulation, immune response, nucleic acid regulation and replication were observed with high and low salinity levels (**Gao *et al*., 2016**).

Rab7 is a small GTPase found in eukaryotes known mainly to play its role in the endocytosis, helping the cell to internalize protein or any non-particulate matter through engulfing (**Agola *et al*., 2011**). Implications on signal transduction and the role in cellular pathways; they have been extensively studied besides its known function in endocytosis (**Kong *et al*., 2007; Wang *et al*., 2011**). The current study targeted to observe its relative expression in shrimps challenged with adverse salinity conditions, as previous studies suggested that signalling events induced by salinity lead to changes in the gene expression (**Kosova *et al*., 2013**). Moreover, there is a great relationship of binding of Rab7 protein with the structural protein VP28 or the structural protein complex of white spot syndrome virus (**Sritunyalucksana *et al*., 2006**). It is also assumed that, shrimps are more vulnerable to diseases when they face adverse environmental conditions (**Tendencia & Verreth, 2011**). The current study considered different salinity conditions for experiments as it is the utmost imperative abiotic factor, which affects penaeid shrimps' growth and survival (**Kumlu *et al*., 2000**).

MATERIALS AND METHODS

Shrimp post-larvae (PL) collection

For the current study, the PL of shrimps were collected from a hatchery in Cox's Bazar that produces disease-free PL and supply the shrimp-producing farms in the area nearby (**FRSS, 2017**). The hatchery collected the mother shrimps from the bay of Bengal.

Acclimatization of shrimp post-larvae (PL) in the experimental tanks

Shrimp PL were acclimatized in the small glass jars at the Department of Fisheries and Marine Science prior to the experimental assay. During acclimatization, they were given commercially available supplementary feed (**Chen *et al*., 2000; Gunasekaran *et al*., 2018**). The water temperature between 27–28 °C, salinity 18 ppt and dissolved oxygen 5–6 ppm were maintained throughout the acclimatization period.

Preparation of experimental aquarium water with desired salinity for post-larvae (PL) rearing

Sterile water was collected from a shrimp hatchery in Cox's Bazar. Aquarium salts were used to prepare water with a salinity of 2 and 54 ppt. Salinity was strictly monitored and maintained so that no gradual or sudden changes could occur. Salinity meter was used to measure the salinity. Important physico-chemical parameters like dissolved oxygen (DO) concentration ranged between 5-6 ppm, and temperature was maintained within 27- 28°C. These parameters were monitored regularly using the standard methods (**APHA, 1998**).

***In-vivo* experimental challenge**

There were three groups of shrimp PL, one reared in 2 ppt, the second reared in 54 ppt, and the third was the control group reared in 15 ppt salinity. Each group contained 720 shrimp PL and was divided into three treatment groups, each containing 240 shrimp PL. Treatments were run for 36 hours. Within these 36 hours, shrimp PL health condition was observed in every six hours. Aeration was stopped for a few minutes to observe rheotaxis. Observation of hepatopancreas under a light microscope was also conducted in the sample PL, which showed weakness in locomotion after being exposed to both conditions of salinity. Shrimps PL at the beginning of the treatments with salinity stressed conditions were observed under a light microscope to observe their health. The PL from the control treatment group were under visual observation every six hours.

RNA extraction and cDNA synthesis

New England Biolab's RNA extraction kit was used for the extraction of RNA from the PL samples that were reared in two different conditions of salinity and the control group. cDNA synthesis was done using New England Biolab's kit (PhotoScript II First Strand cDNA Synthesis Kit, New England Biolabs, Ipswich, MA, USA), where two reverse transcriptase reactions were conducted using conventional PCR (**Hasan *et al*., 2022**).

Primers used

β-actin and EF1α were used as house-keeping genes. *Rab7* primer for real-time PCR was designed considering the sequences found reported in the Genbank database (Accession no. DQ231062.1). The targeted product length was 124. The primers used are listed in Table (1).

Table 1. List of primers used in this study

Name of gene	Primer (5-3)	Expected PCR product size (bp)	Accession no.	Reference
β-actin	F: CCCTGTTCCAGCCCTCATT R: GGATGTCCACGTCGCACTT	90	JN808449	(**Shekhar *et al*., 2015**)
EF1α	F: GGTGCTGGACAAGCTGAAGGC R: CGTTCCGGTGATCATGTTCTTGATG	125	Group of sequences	(**Jeena *et al*., 2012**)
Rab7	F: TAGGGCGGTATCAACGAAGC R: ATTGCGAGCAATGGTCTGGA	124	DQ231062	-

Real-time PCR and relative gene expression analysis

Real-time PCR conditions followed a cycle of initial denaturation at 95°C temperature for 60 seconds, 45 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 30 seconds. New England Biolab's Luna Universal qPCR master mix was used according to the protocol of the manufacturer (**Hasan *et al*., 2022**). For the analysis of average-fold difference in gene expression, the comparative delta C_T method was used (**Jeena *et al*., 2012**). Amplification in the experiments of control group was deemed for the laboratory analysis of gene expression. Computation of Delta C_T was made by deducting C_T value of housekeeping gene (endogenous control) from the objective gene, and in the last part, mean delta C_T was valued from the standardised estimates of delta C_T. Delta-delta C_T was processed with reference to control by subtracting average delta C_T of control from mean delta C_T of the objective gene. Variations in average-fold gene expression in the shrimp PL reared in stressed conditions were calculated to $2^{-\text{delta delta CT}}$ values (**Jeena *et al*., 2012**). For comparison between different treatments at time intervals to see the differences in the transcript levels, one-way ANOVA test was conducted, and the p values beneath 0.05 were regarded as statistically significant.

RESULTS

1. Visual observation of shrimp post-larvae health

Shrimp PL samples were in excellent health condition at the beginning of the experimental trials. In all three treatments, they showed instinctive behavior during swimming at the very beginning of the experiments. After the first 6 hours, the crustaceans showed similar spontaneous behavior in swimming. Changes in swimming patterns were observed after 18 hours in both types of salinity stressed groups.

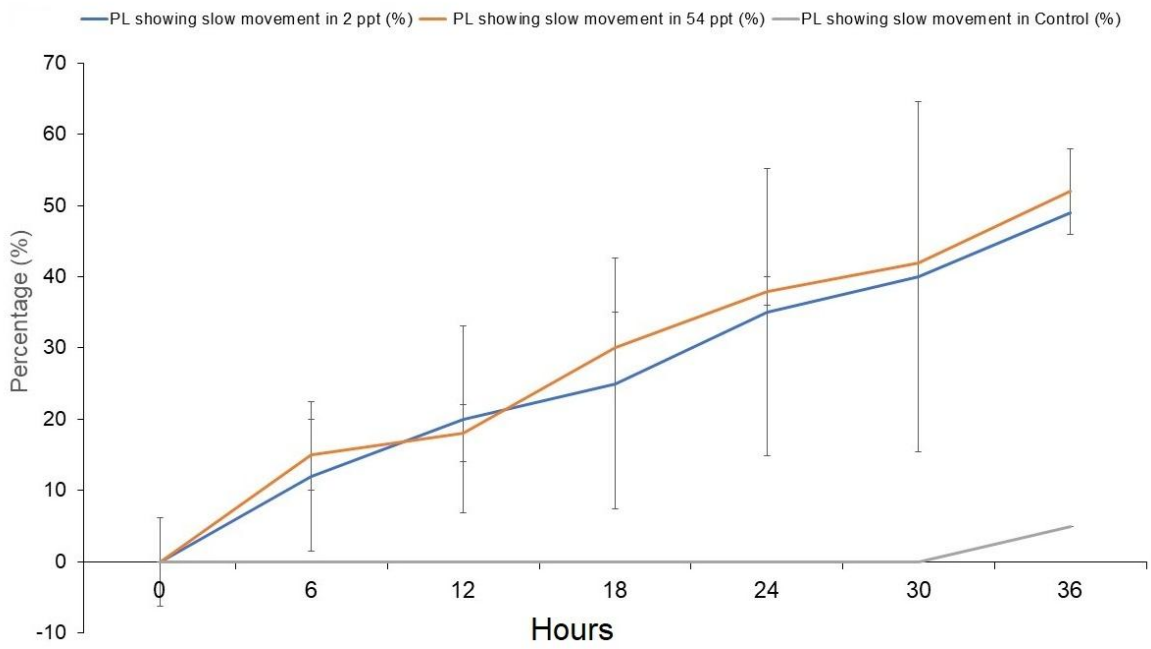

Fig. 1. Percentage of cumulative slow movement of shrimp post-larvae (PL) after two salinity treatments. The Y-axis represents the activity (in percentage), while the X-axis shows the hours of treatment. Error bars represent standard deviation.

However, the no. of PL showing abnormal motility was relatively higher in the treatments with higher salinity (54 ppt) (Fig. 1) though the visual observation of motility did not show significant difference. Till the 36 hours, the swimming behavior was relatively weak in the PL treated with 54 ppt salinity. There was a significant difference in the swimming behavior of both the salinity stressed groups with the control group (Fig. 1). Stress in the crustaceans was also confirmed by microscopic observation of the hepatopancreas. Comparatively large dark colored hepatopancreas was observed in the shrimp PL of the control group rather than the pale one in the PL stressed with salinity.

2. Gene expression analysis using real-time PCR

The created cDNA from the RNA collected from the tissue of treated shrimp PL along with the control group were examined for gene expression analysis of *Rab7* in real-time PCR. Fig. (2) shows the melt curves of *Rab7* having single peaks at 81°C. In this study, *Rab7* expression was observed in all treatment groups of PL samples i.e., PL samples treated with 2 ppt, 54 ppt salinity and control group. Fig. (3) shows differences in average relative expression of *Rab7* in 2 ppt (Fig. 3A) and 54 ppt (Fig. 3B) treated PL, respectively, after 18 and 36 hours of rearing where β-actin was used for normalization. Fig. (4) demonstrates an average relative expression of the *Rab7* in the crustaceans after 18 and 36 hours of rearing in 2 ppt (Fig. 4A) and 54 ppt (Fig. 4B), where elongation factor 1 alpha (EF1α) was used as an internal control. Average differences in the relative expression of *Rab7* were 0.3-folds and 0.5-folds (Fig. 3A) and 0.4-folds and 0.6-folds

(Fig. 3B) after 18 and 36 hours of rearing in 2 and 54 ppt treated shrimp PL, respectively, when β-actin was used as an internal control. On the other hand, when EF1α was used for normalization, the average relative expression of *Rab7* gene's fold differences were 0.2-folds and 0.5-folds (Fig. 4A) and 0.3-folds and 0.6-folds (Fig. 4B) after 18 and 36 hours of rearing in 2 and 54 ppt treated shrimp PL, respectively. These fold-differences of average relative expression after 18 hours and 36 hours (Figs. 3, 4) where shrimp PL were reared in 2 and 54 ppt salinity were statistically significant ($P< 0.05$). While, comparing the expression in the challenged groups, it was found that average relative expressions were lower in 18-hours treated PL than in 36-hours treated PL. Therefore, an upregulation of gene expression was detected in every experiment till the 36 hours of exposure (Figs. 3, 4).

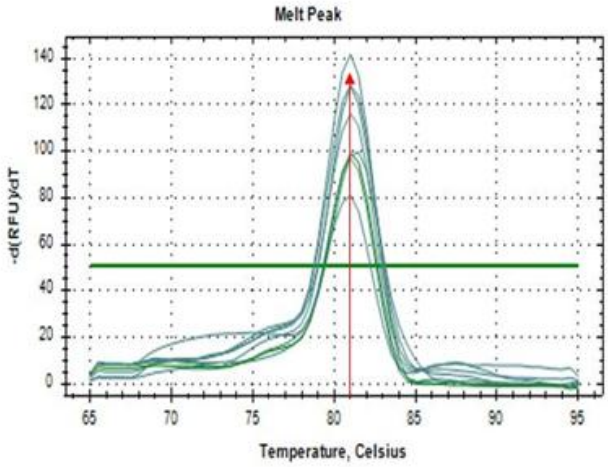

Fig. 2. Melt curves showing a single amplification peak at 81°C to ensure assay specificity, and different PCR products can be differentiated by their melting features.

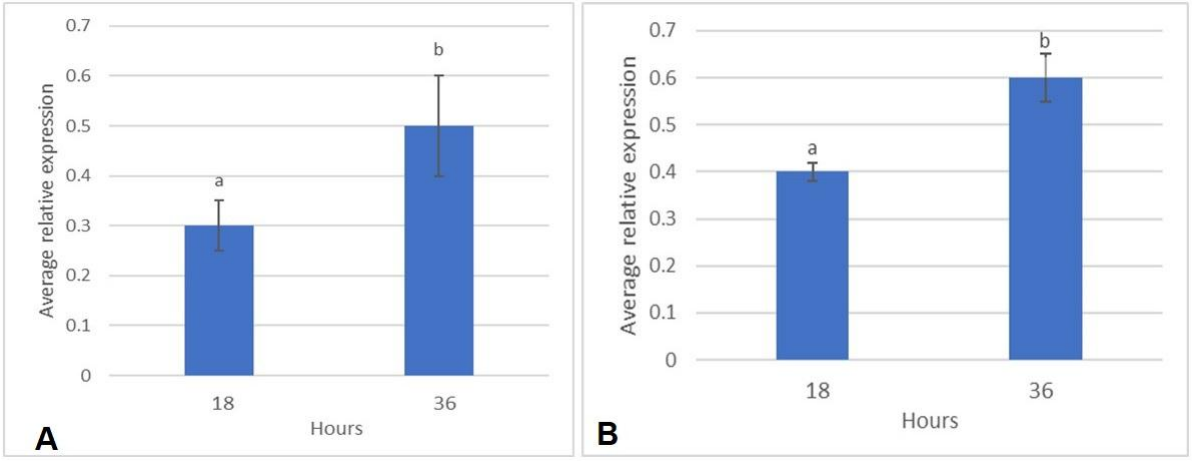

Fig. 3. Relative expression of *Rab7* in shrimp post-larvae after treatment with two salinity conditions. **(A)** Average relative expression of *Rab7* in 18 and 36 hours in shrimp PL reared in 2 ppt salinity, while β-actin acted as an internal control. **(B)** Average relative expression of *Rab7* in 18 and 36 hours in shrimp PL reared in 54 ppt salinity, when *β-actin* was used as an internal control. Alphabets (a, b) represent statistically significant differences ($P < 0.05$) in gene expression in different time intervals.

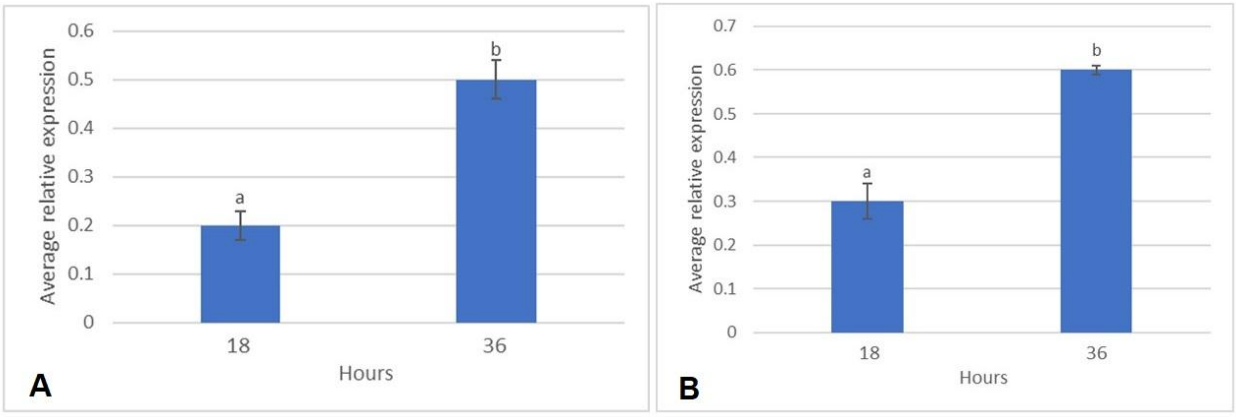

Fig. 4. Relative expression of *Rab7* in shrimp post-larvae after treatment with two salinity conditions. **(A)** Average relative expression of *Rab7* in 18 and 36 hours in shrimp PL reared in 2 ppt salinity condition. **(B)** Average relative expression of *Rab7* in 18 and 36 hours in shrimp PL reared in 54 ppt salinity condition. In both cases, elongation factor 1 alpha (EF1α) was used as an internal control. Alphabets (a, b) represent statistically significant differences ($P < 0.05$) in gene expression.

DISCUSSION

White spot syndrome virus (WSSV) is one of the most devastating pathogen causing mass mortalities in the aquaculture of shrimps worldwide. Water quality management is an important criterion in shrimp farming for the survival and growth of the shrimp. The optimal salinity conditions for penaeid shrimp ranges differently for different species. Osmotic and ionic regulation is an important mechanism of environmental adaptation in crustaceans. However, drastic changes in abiotic and biotic conditions result in stress for the shrimps during the culture period.

Upregulation of *Rab7* was found to be associated with stressed or diseased conditions in humans (**Agola *et al*., 2011**). In the current study, upregulation of the *Rab7* was observed in shrimp PL treatments with salinity stressed conditions. The Rab7 was found to be involved in controlling the innate immunity of crustaceans by regulating the phagocytosis and expression of antimicrobial peptides by cloning and characterizing the Rab7 from Chinese mitten crab, which was designated as EsRab7 (**Wang *et al*., 2019**). Significant upregulation of this EsRab7 was observed in haemocytes after Gram-positive and Gram-negative bacterial stimulation. Transcription of this small GTPase might be for protection against the stressed conditions in shrimps; however, the Rab7 was found to show variance in its pattern of expression (**Li *et al*., 2010; Song & Li, 2014**). *Rab* gene was upregulated in *Penaeus japonicus* when infected with virus (**Wu & Zhang, 2007**). **Ongvarrasopone *et al.* (2008)** had an observation of inhibition of viral infections when the *Rab* was suppressed with dsRNA in *Penaeus monodon*. A study with an experiment of 24 hours of challenge with salinity of 5, 10 and 20 ppt was conducted using the *Rab*

and the beta-actin gene to observe the gene expression pattern of the *Rab* in shrimps, where upregulation was observed in the stressed condition till 12 hours and returned to the preliminary conditions after 12 hours (**Wang *et al*., 2015**). In our study, upregulation was observed for the complete 36 hours of the experiment.

An association of the swimming behavior of shrimp PL was observed with the relative expression of *Rab7* in the current study, as shrimp PL samples were getting weaker with the passing of rearing hours in the stressed conditions, and the expression was upregulated. However, diseased conditions are linked to the stressed conditions. Notably, exposure to a long period of stressed or unusual conditions may lead to the mortality of shrimps. The expression pattern of *Rab* gene may change with the exposure time to salinity stressed conditions. It is well known that *P. monodon* is euryhaline-type and can tolerate a huge range of salinity. Rab7; the small GTPases encompass products of the Rab/YPT, Ras and Rho families of genes and are tangled in varied cellular tasks, incorporating the development of cells, variation and vesicular trafficking (**Zerial & MacBride, 2001**). The role of Rab7 seems to be necessary for effective phagocytosis as it panels the biogenesis of lysosomes (**Bucci *et al*., 2000**). *Rab7*'s overexpression in rice improved tolerance to stressed conditions and modulated the expression of other genes responsive to abiotic stress (**Ahuja *et al*., 2010; El-Esawi & Alayafi, 2019)**. We tested the PL with very low and high amounts of salinity and observed that, in both conditions the gene expression was upregulated in the PL tissue. Salinity is one of the major environmental factors that has huge impact on cultured shrimp, affecting their physiological and metabolic parameters, which in turn affect shrimp growth, moulting and survival (**Mudagandur *et al*., 2016**). Moreover, changes in the abiotic factors, chemical and biotic factors result in reduced immunity of shrimp and vulnerability to bacterial and viral diseases.

In summary, it is extremely crucial to consider that, sudden and drastic changes in the salinity of water of aquaculture might cause stressed conditions for shrimp PL, which could critically upregulate the expression of *Rab7* gene in crustaceans. Hence, our results suggest that relative expression of *Rab7* gene is positively correlated with extremely higher or lower level of salinity and challenge duration. The significant increase in expression level of *Rab7* in the PL of *P. monodon* exposed to high salinity stress (2 ppt and 54 ppt) conditions indicates its possible potential functional role in osmotic stress in shrimps. Nevertheless, additional studies are required to explain the mechanisms of how *Rab7* gene is upregulated in this type of crustacean cells after employing different levels of salinity, considering a larger sample set and a longer period of exposure.

ACKNOWLEDGMENTS

Authors would like to express their gratitude to the Committee of the Research Cell of Noakhali Science and Technology University as the fund from the Research Cell was used for this research work.

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