Physiological Genomic Response to Variable Salinity Environments by the Tiger Shrimp (*Penaeus monodon*)

A Thesis

By

Khairun Naher Azad

Examination Roll: MS-190628

Session: 2019-2020

A thesis submitted to the Fisheries and Marine Resource Technology Discipline in partial fulfilment of the requirements for the degree of

Master of Science

In

Fish Genetics and Biotechnology

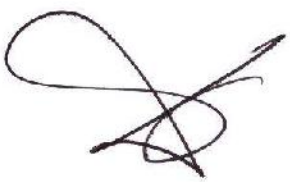
Khulna University, Khulna

Bangladesh.

December, 2019

Physiological and Genomic Response to Variable Salinity Environments by the Tiger Shrimp (*Penaeus monodon*)

Approved as to style and content by



(Signature of the Head)

Dr. Muhammad Abdur Rouf

Professor and Head

Fisheries and Marine Resource Technology Discipline

Khulna University, Khulna

Bangladesh.

December, 2019

Declaration

Physiological Genomic Response to Variable Salinity Environments by the Tiger Shrimp (*Penaeus monodon*)

The results submitted in this thesis are entirely of the candidate's own investigations and no part of the results has been accepted for any degree, nor is it being concurrently submitted for any degree.

Candidate

Khairun Naher

(Signature of the candidate)

Khairun Naher Azad

Examination Roll No: MS-190628

Session: 2019-2020

Supervisor

(Signature of the Supervisor)

Dr. Md. Lifat Rahi

Associate Professor

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh

December, 2019

Dedicated to

My Beloved Parents

Acknowledgement

First of all, my admiration goes to the supreme creator and Ruler of the universe, the Almighty Allah, for great mercy to keep me alive and enables me to pursue my education in Fisheries and Marine Resource Technology Discipline as well as to complete my thesis for the degree of Master of Science in 'Fish Genetics and Biotechnology' from Khulna University.

I would like to express my sincere gratitude and profound appreciation to my supervisor **Dr. Md. Lifat Rahi,** Associate Professor, Fisheries and Marine Resource Technology Discipline for his continuous guidance, valuable suggestions and sustained support throughout the experiment. I am really grateful to him for introducing me to the field of molecular genetics; his skills and compassion as a teacher are unsurpassed.

I would like to express my profound gratitude to Dr. Muhammad Abdur Rouf, Professor and Head; Dr. Md. Moshiur Rahman and Dr. Muhammad Yousuf Ali, Professors, Fisheries and Marine Resource Technology Discipline, Khulna University for their valuable suggestions and providence of all necessary facilities.

I recall with warm appreciation and great indebtedness or the support, guidance and suggestions extended to me by all of my respected teachers in Fisheries and Marine Resource technology discipline, Khulna University.

I am especially thankful to Biddut M. Iqbal, CEO of Gazi Shrimp culture Ltd. and my senior Shujon Mahmud for their cordial co-operation and suggestion in my research work.

I wish to express my sincere thanks to the lab section officer Sk. Omar Ali for helping me to use necessary equipment.

Finally, I would like to express my earnest appreciation to my parents, for their immeasurable blessing, continuous inspiration throughout the study.

Khairun Naher Azad
Khulna University
December, 2019

Abstract

Osmoregulation is considered as the principal mechanisms for survival and adaptation to different salinity conditions. The present study was conducted to evaluate mRNA expression levels of six osmoregulatory (two hemolymph regulatory and four ion regulatory) genes that play important role in osmoregulation in a Penaeid shrimp, *Penaeus monodon*, exposed to three different experimental salinities (0‰, 10‰ and 20‰) for a period of 30 days, using an RT-qPCR approach. Relative mRNA expression patterns revealed comparatively higher expression levels of Crustacean Hyper-glycaemic Hormone (CHH) and $Na^+/K^+/2Cl^-$ Co-transporter (NKCC) genes at raised salinities (10‰ and 20‰). At 0‰ vs 20‰, expression levels of these two genes were significantly higher ($p < 0.05$) and showed 1.5–2 fold and 2–3 fold higher (higher in 10‰ than 0‰) expression levels for CHH and NKCC, respectively. Significantly different expression levels were observed for these two genes among salinities ($p < 0.05$) throughout the experiment. Significantly higher expression levels ($p < 0.05$) were observed at 0‰ for the Diuretic Hormone (DH), Na^+/K^+-ATPase (NKA), Na^+/H^+ exchanger (NHE) and V-type H^+-ATPase (VTA) genes over 10‰ and 20‰ throughout the experimental period. But initially higher expression levels of VTA were found at 0‰ up to 24 h and then dropped towards the level at 10 day. The ion regulatory gene V-type H^+-ATPase (VTA) and Na^+/H^+ exchanger (NHE) showed very high expression levels at reduced salinity (0‰). At 0‰ vs 20‰, expression levels of these two genes were significantly higher ($p < 0.05$) and showed 4–6 fold and 3–5 fold higher (higher in 0‰ than 20‰) expression levels for VTA and NHE, respectively. In general, CHH, NKA, NHE and VTA were up-regulated in freshwater while DH and NKCC were up-regulated under raised salinities. Results of this study revealed that the genes examined here played key roles in adaptive responses to variable environmental salinity conditions broadly in aquatic crustaceans.

Table of Contents

List of Tables

List of Figures

LIST OF ABBRIVIATIONS

cDNA	Complimentary Deoxyribonuclic Acid
‰	PPT / Parts per thousand
C	Control group
CHH	Crustacean Hyper-glycaemic Hormone
DH	Diuretic Hormone
DoF	Department of Fisheries
et al.	Et alia (L) and others
etc.	Etcetra
FAO	Food and Agriculture Organization
Fig.	Figure
mRNA	Messenger Ribo Nucleic Acid
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/2Cl^-$ Co-transporter
°C	Degree Celsius
PCR	Polymerase Chain Reaction
PL	Post larvae
PPT	Parts per thousand
QUT	Queensland University of Technology
RNA	Ribo Nucleic Acid
rpm	Rotation Per Minute
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
RVD	Regulatory cell Volume Decrease
RVI	Regulatory cell Volume Increase
SE	Standard Error
VTA	V-type (H^+) ATPase

Chapter One

INTRODUCTION

1. Introduction

1.1 Background of the study

The black tiger shrimp (*Penaeus monodon*), locally known as "Bagda or "Bagda chingry" is one of the most important coastal aquatic species in Bangladesh (as well as throughout the world) due to its high demand in both local and international markets. It has become the world's second most prevalent cultured crustacean species (after *Litopenaeus vannamei*) because of its fast growth, adaptability to a wide range of salt and temperature conditions (Chen et al., 2016; Mudagandur et al., 2016). Bangladesh exports frozen tiger shrimp and other fish and fisheries products to more than 50 countries (DOF, 2017). Currently, *P. monodon* is in the dominant position as a coastal aquaculture species in Bangladesh and contributing significantly to the economy of the country. The availability of its both natural and hatchery produced seed, feed and favorable climatic conditions for farming have attracted the coastal communities to culture this species.

Tiger shrimp is a marine species but larval stage of this species passes in the coastal brackish waters (particularly in the mangrove regions). Moreover, farming of this species at low salinity environments is a common phenomenon all over the world. In fact, *P. monodon* is a euryhaline species that can tolerate a wide range of environmental salinities ranging from 3 ppt to 35 ppt (Zhang et al., 1989; Solis, 1988; Tantulo and Fotedar, 2007). Thus, an interesting research question could be how the tiger shrimp (*P. monodon*) can tolerate large salinity fluctuations (what are the underlying physiological and genetic mechanisms?).

A large number of crustacean taxa regularly migrate from freshwater to brackish or sea water and vice-versa to complete their larval development, and are able to osmoregulate (perform ionic balance) successfully under a wide range of environmental salinities (Vogt, 2013; Rahi et al., 2017). Recent studies on some crustacean taxa revealed that evolution of some specialized attributes of their physiology and morphology allow successful acclimation and adaptation to variable salinity conditions (Freire et al., 2008; Mykles and Hui, 2015; McNamara et al., 2015). Specialized gill structures have evolved in crustaceans in response to associated selection pressures (e.g. lack or excess of ions in the external

aquatic medium) that allow active transport and ion exchange against any osmotic gradient (Lee et al., 2011). The antennal gland (main excretory organ) is also considered as a crucial organ in aquatic crustaceans that plays a secondary role in osmoregulation (Freire et al., 2008).

Changes in environmental salinity levels can impose severe pressure or stress (osmotic stress) on organisms. Main challenge with salinity level changes involves ionic balance between organism's body fluid and surrounding environment. The principal mechanism through which aquatic organisms deal with any change in environmental salinity levels is "osmoregulation". The morphological aspect of osmoregulatory function due to salinity change involve: changes in gill ultra-structure, extension or contraction of gill lamellae and perform regulatory cell volume increase (RVI) or decrease (RVD) depending on external salinity. Main physiological mechanism include: changes in the ionic content (uptake or release of ions based on external environmental salinity) of body fluid (hemolymph). While genetic or genomic response may be include: changes in expression pattern of certain major candidate genes by modifying amount of mRNA production for protein synthesis (Nadal et al., 2011; Barman et al., 2012).

To efficiently deal with environmental salinity change, organisms must need to regulate ionic content of their body fluid (known as hemolymph in crustaceans) immediately for maximum survival (Lee et al., 2011; Rahi et al., 2017). Crustacean species are well known to rapidly regulate their body fluid concentration and ionic content with salinity change (Sang and Fotedar, 2004; McNamara et al., 2015). Thus, crustaceans can be considered as an interesting group to investigate salinity induced changes in body physiology and expression pattern of underlying candidate genes regulating body fluids. *Penaeus monodon* in this regard can provide an excellent model species for investigating the physiological genomic basis of hemolymph regulation with salinity change, that can directly help successful osmoregulation via ionic balance. To date, 32 candidate genes have been identified (using modern genetic particularly genomic technologies) that have a functional role in osmoregulation for successful ionic balance (Moshtaghi et al., 2016; Moshtaghi et al., 2017; Rahi et al., 2017). Out of these 32 genes, 3 are known to support the process of body fluid (hemolymph) regulation including: Crustacean Hyperglycemic Hormone (CHH), Crustacean

Cardio-vascular Peptide (CCP) and Diuretic Hormone (DH). Investigating the expression profiling of these candidate genes can provide important insights in understanding genomic mechanisms controlling body fluid (hemolymph) regulation with regard to environmental salinity change. The CHH gene is involved with hemolymph production, helps to produce higher levels of hemolymph production with salinity increase but this gene has less important functional role in freshwater (Rahi et al., 2017). CCP gene is involved with hemolymph regulation under any aquatic condition (freshwater or saltwater) with salinity change (Havird et al., 2014). While the DH gene is highly active in freshwater condition, directly involved with water balance between body fluid and surrounding aquatic environment (Rahi et al., 2017, Rahi et. Al., 2018). Although these 3 genes are very important in maintaining body physiology of crustaceans, very little studies have been conducted on this aspect.

The most important ion regulatory genes in crustaceans that are reported previously: Na^+/K^+-ATPase (NKA), V-type H^+-ATPase (VTA), Na^+/H^+ exchanger (NHE), $Na^+/K^+/2Cl^-$ Co-transporter (NKCC), Aquaporin (AQP), Na^+/HCO_3^- Co-transporter (NHC), Carbonic Anhydrase (CA), Calreticulin and Claudin (Barman et al., 2012; Ali et al., 2015; Boudour-Boucheker et al., 2016; Moshtaghi et al., 2016). In general, the functional roles of these genes involve active ion transport and exchange, tolerance of osmotic stress, water channel maintenance and regulatory cell volume control. There are also a number of additional osmoregulatory genes but they are considered to play minor roles in adaptive responses to salinity changes in crustaceans (Nadal et al., 2011; Moshtaghi et al., 2016; Rahi et al., 2017).

Hemolymph, is a fluid, analogous to the blood in vertebrates that circulates within the interior of the invertebrate body having direct contact with the animal's tissues. It is composed of a fluid called plasma in which hemolymph cells are called hemocytes (Wang and Wang, 2015). Crustaceans lack adaptive immune system and they rely exclusively on their innate immune mechanisms that include both cellular and humoral responses. Hemolymph contributes significant roles in the immune system and in transport of hormones, nutrients, and metabolites (Yepiz-Plascencia et al., 2000; Fredrick and Ravichandran, 2012; Terwilliger, 1999).

A salinity range of 15–25 ppt is considered to be the optimal range for giant tiger shrimp (*Penaeus monodon*) farming (Ferraris et al., 1987; Chen et al., 1995). Hemolymph osmolality linearly increases with increasing salinity of the rearing environment (Cheng and Liao, 1986; Ferraris et al., 1987; Diwan et al., 1989; Allan and Maguire, 1992; Chen et al., 1995; Chen and Lin, 1994).

Previous studies have explained that various inorganic ions, particularly sodium and chloride, are the major contributors to hemolymph osmolality of crustaceans. Salinity affects other physiological processes of crustaceans, and there are various exponents for evaluating their physiological responses and metabolism (Chen et al., 1997; Jia et al., 2012). *P. monodon* is known as a euryhaline species and a strong osmoregulator as they can function uniformly well in low salinity to brackish water environments and also in marine water. This transition from low salinity to high salinity environments can impose severe stress on this species and therefore, this species needs well-developed osmoregulatory capacity for survival. Hence, it is expected that *P. monodon* has special ability to perform osmoregulatory functions to cope with the environmental salinity fluctuation (Long et al., 2017; Zhao et al., 2016).

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) is currently considered as a cost effective and reliable approach for investigating changes in gene expression pattern of target genes under varying environmental (or treatment) conditions. If a suitable species is maintained under appropriate experimental condition, studying RT-PCR based gene expression pattern can provide a powerful insight to infer the role of specific gene under a certain experimental condition. As *P. monodon* is a euryhaline species, it provides ideal opportunities to investigate the salinity induced changes in expression pattern of the hemolymph and ion regulatory genes under different experimental salinity conditions. This approach will ultimately help us to infer the ability of this commercially important species to deal with salinity change in farming condition if salinity levels drop from its suitable salinity conditions. Studying only gene expression pattern cannot provide very powerful insights to infer adaptive mechanisms to salinity change because other biological mechanisms (e.g., physiological, biochemical, cellular etc.) are also involved with the process. Therefore, combining gene expression results with at least one more biological aspect (preferably

physiological mechanism that involves body fluid regulation) can provide a very powerful insight in understanding the process of adaptive response. Thus, the present study will adopt both RT-qPCR based gene expression pattern with body fluid (hemolymph) regulation to investigate the physiological genomic responses in *Penaeus monodon* with regard to salinity change.

1.2 Objectives of the study

- To investigate the changes in relative gene expression patterns of six candidate genes in tiger shrimp (*P. monodon*) exposed to three different salinity levels (0‰, 10‰ and 20‰).
- To investigate the changes in body fluid (hemolymph) osmolality with regard to salinity change in the tiger shrimp (*P. monodon*).

Chapter Two

LITERATURE REVIEW

2. Literature Review

2.1 Brief summary about *P. monodon*

P. monodon is commonly known as the black tiger shrimp and locally as Bagda chingry, occupies the 1st position among the frozen foods exported from Bangladesh (DOF, 2017). Commercially it is one of the most important species among the edible crustaceans and has attained a high price for larger sizes (Motoh and Kuronuma, 1980). Members of the genus *Penaeus* principally inhabit in the marine environments and are cultured under varying environmental salinity conditions in many tropical and sub-tropical countries across the world (Zhang et al., 1989; Tantulo and Fotedar, 2007). Most of the euryhaline penaeid shrimp species exhibit a common migratory behavior of returning to high saline conditions for maturation and spawning (Castille and Lawrence, 1981) and *Penaeus monodon* is a notable follower of this pattern (Diwan and Kakati, 1989).

P. monodon farming has been practiced for more than a century as an important food and the livelihood option for coastal people in many Asian countries, including Indonesia, the Philippines, Taiwan Province of China, Thailand, Viet Nam etc. The introduction or importation of wild broodstock is commonly practiced among the major producing countries because local supplies are in many instances insufficient and domestication technology for broodstock development has not yet been commercially started. However, disease-free broodstock are highly desirable and some countries require health certification of imported stock (Hatai et al., 1980). Recently remarkable improvements have been occurred through production of specific pathogen free (SPF) lines of *P. monodon* that helped to have higher production successes for the last few years.

2.2 Economic importance of *P. monodon*

The tiger shrimp, *Penaeus monodon* Fabricius, is one of the largest penaeid shrimp in the world (Motoh, 1985). To date, it is the most important coastal aquaculture species in Bangladesh due to its high demand in both local and international markets. In total, 36

marine shrimp species are available in Bangladesh. Bangladesh exports frozen shrimp (both as cooked and raw) and other fish and fisheries products to more than 50 countries, including Belgium, UK, Netherlands, Germany, USA, China, France, Russian Federation, Japan and Saudi Arabia. Main exported countries are EU countries, USA and Japan (DOF, 2017).

According to July 2019, the total shrimp production of 2016-2017 is 198,366 metric ton in closed water and 48,847 metric ton in open water. So, the total amount of Shrimp Production is 247,213 metric ton. The percentage of the increasing rate of total cultured shrimp production is 52.96% (DOF, 2019).

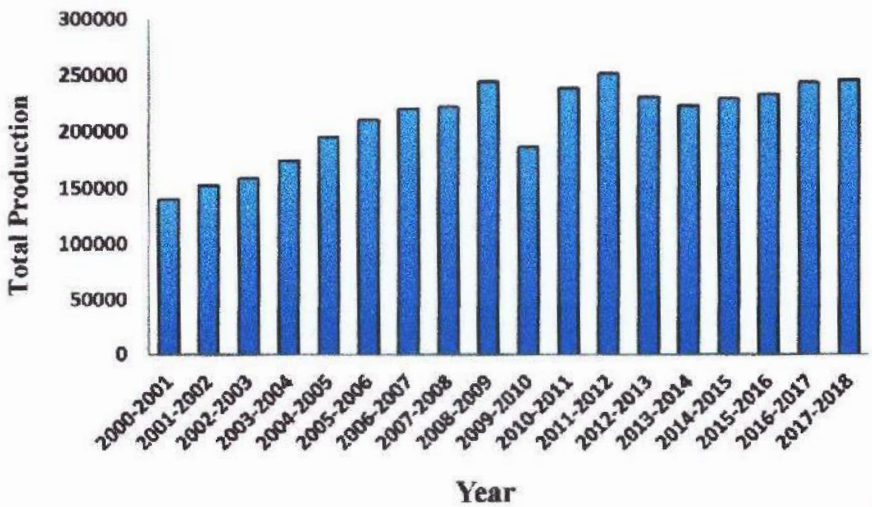


Fig 01: Yearly Shrimp Production in Metric Ton (2000-2018)

2.3 Geographical distribution and habitat preference

Generally, the distribution of *P. monodon* is from 30°E to 155°E in longitude and from 35°N to 25°S in latitude. The tiger shrimp (*P. monodon*) is distributed widely throughout the greater part of the Indo-Pacific region, South Africa, Tanzania, Kenya, Somalia, Madagascar, Saudi Arabia, Oman, Pakistan, India, Bangladesh, Sri Lanka, Indonesia, Thailand, Malaysia, Singapore, Philippines, Hongkong, Taiwan, Korea, Japan, Australia, and Papua New Guinea (Racek 1955; Holthuis and Rosa 1965; Motoh 1981, 1985).

The main fishing grounds are mostly located in tropical countries, particularly in Indonesia, Malaysia and the Philippines. The fry, juveniles and PL inhabit on surface waters such as shore areas and mangrove estuaries, while most of the adults live in waters down to almost 160 m (Motoh, 1985; Dall, 1957; Racek, 1959).

The vertical distribution was found to be from surface in brackish water rivers down to about 70 m offshore, where the range of water temperature and salinity was between 22 to 34 °C, and 4 to 35 ‰, respectively (Lightner et al., 1983).

Fig 02: Worldwide distribution pattern of *P. monodon*

2.4 Salinity tolerance in *Penaeus monodon*

P. monodon is a euryhaline species that is able to tolerate a wide range of salinities ranging from 3 ppt to 35 ppt (experimentally it was found to be able to tolerate up to 71 ppt) (Solis, 1988; Zhang et al., 1989; Tantulo and Fotedar, 2007). But the optimal salinity for the culture or farming of *P. monodon* is reported to be 15-25 ppt (Ferraris et al., 1987; Sang and Fotedar, 2004). Both salinity and temperature have greater impact on growth and survival of euryhaline penaeids. The optimal salinity conditions for penaeid shrimp ranges differently for different species (Sang and Fotedar, 2004).

2.5 Body fluid (hemolymph) and its function in crustaceans

Hemolymph is a fluid in the circulatory system of crustaceans (and widely in arthropods). Hemolymph fills all of the interior (the hemocoel) part of the animal's body and surrounds all cells. Crustaceans do not have adaptive immune system and they are totally depended exclusively on their innate immune system that include both cellular and humoral responses (Fredrick and Ravichandran, 2012; Wang and Wang, 2015).

Blood and lymph are two fluids in the bodies of vertebrates that do related but perform separate functions (Sowers et al., 2006; Sang and Fotedar, 2004). They help to remove waste materials, transport nutrients, and through the system removes/cleans pathogens and debris. (Yepiz-Plascencia et al., 2000). The major distinction between insect blood and the blood of vertebrates, including humans, is that vertebrate blood contains red blood cells. Hemolymph is mostly water, but it also contains ions, carbohydrates, lipids, glycerol, amino acids, hormones, some cells and pigments (Fredrick and Ravichandran, 2012). The key component of hemolymph is water, which functions as a solvent for a variety of molecules. Water in hemolymph makes up to 20–50% of the total water in insect bodies, with larval stages typically having a larger relative hemolymph volume than adults. Hemolymph serves as a water storage pool to be used by tissues during desiccation and as a storage depot for other types of chemicals. It also contains circulating cells called hemocytes. Hemolymph serves important roles in the immune system and in transport of hormones, nutrients, and metabolites (Terwilliger, 1999; Wang and Wang, 2015).

Amplitude of hemolymph osmolality and ion fluctuation has direct relationship with environmental salinity variations (increases with rising salinity levels and vice versa). The rate of hemolymph osmolality and ion exchange was found to be directly associated with the rate of ambient salinity change (Havird et al., 2014; Ferraris et al., 1997; Chen et al., 1995). Dilution of seawater with artificial river water instead of deionized water resulted in a reduced amplitude of fluctuation of hemolymph osmolality and ion concentration (Kallen et al., 1990; Chen and Lin, 1994). Most of the hemolymph osmolality fluctuation was occurs due to the solute movement and not to shifts in body water. Rapid changes in hemolymph osmolality with regard to low-salinity and high salinity environments is mandatory for

survival of migratory species and this ability is of specific (Sowers et al., 2006; Terwilliger, 1999; Chen and Lin, 1994).

2.6 Changes in crustacean body physiology with salinity fluctuations

Osmotic and ionic regulation is an important mechanism under variable environmental conditions by crustaceans due to salinity change. However, drastic changes in abiotic and biotic factors in aquatic environments impose severe stress to the shrimps during the culture period. Temperature and salinity are the two main environmental factors that have a large impact on culture of shrimp, that affect their physiological and metabolic and parameters, which in turn affect shrimp growth, molting, and survival (Staples and Heales, 1991; Chen et al., 1995; Mudagandur et al., 2016). Salinity changes can also result in altered biochemical and physiological parameters of the shrimps due to imposed stress (Chen and Lin, 1994; Perazzolo et al., 2002). Osmoregulation is an important mechanism by which euryhaline crustaceans or any aquatic organisms are able to regulate and maintain ionic balance between internal body fluid and external surrounding environment. Ionic compositions of the crustacean body fluid (hemolymph) change immediately with environmental salinity alterations. If environmental salinity levels increase, crustaceans produce more hemolymph and absorb more ions from the surrounding medium for ionic balance to ensure survivability in high salinity environment (Freire et al., 2008; McNamara et al., 2015). In contrast, crustaceans perform a reverse pattern in low saline or freshwater environments, reduce hemolymph production and release ions from body fluid for maintaining an equilibrium state with the external aquatic environment (Moshtaghi et al., 2017; Rahi et al., 2017). Different species normally adopt different physiological techniques to cope with a dilute external medium (low salinity environment). The main physiological adaptive mechanism consists of reduced hemolymph concentration that is crucial for acclimation in freshwater environments (Rahi et al., 2017). Crustacean osmoregulation predominantly involves ion balance through active uptake or secretion, as well as maintaining composition and concentration of hemolymph and urine (Barman et al., 2012; McNamara and Faria, 2012).

2.7 Saline tolerant genes already identified in *P. monodon* and other crustaceans

So far, 32 different genes have been identified that are involved with ionic balance or osmoregulation in a wide range of crustacean species (Ali et al., 2015; Moshtaghi et al., 2016; Rahi et al., 2017). The main functional roles of these genes involve: ion transport, ion exchange, ion binding, osmotic stress tolerance, body fluid (hemolymph) regulation, cell volume regulation, maintaining cellular junctions and water channel regulation. The most important genes to date are: Na^+/K^+-ATPase (NKA), V-type H^+-ATPase (VTA), Na^+/ H^+-exchanger (NHE), $Na^+/ K^+/2Cl^-$ Co-transporter (NKCC), Aquaporin (AQP), Integrin (ITG), Claudins (CLA), Ca^{+2}-ATPase, Carbonic Anhydrase (CA), Crustacean Cardiovascular Peptide (CCP), Crustacean Hyperglycaemic Hormone (CHH) and Diuretic Hormone (DH). Of the 32 genes, only 3 genes are known to have functional roles in body fluid (hemolymph) regulation in crustaceans with regard to salinity change (Rahi et al., 2017). These three genes are involved with hemolymph production and water balance to maintain ionic balance between body fluid and surrounding environment.

2.8 Gene expression and osmoregulation studies in *P. monodon* and other crustaceans

Although *P. monodon* is a prime aquaculture species globally (and farmed at different salinity levels), there is not enough information on osmoregulation or body fluid regulation with regard to salinity stress. Sang and Fotedar (2004) conducted an experiment on *P. monodon* at different levels of salinities and found that hemolymph osmolality increases at higher levels of salinities. Crustaceans inhabiting at higher salinity levels maintain higher levels of hemolymph osmolality and decreases with salinity reduction (McNamara et al., 2015). A few studies have already been published in a number of crustacean species that investigated salinity induced changes in expression pattern of some candidate genes including: *Halocaridina rubra* (Havird et al., 2014); *Macrobrachium rosenbergii* (Barman et al., 2012); *M. australiense* (Moshtaghi et al., 2017); *M. amazonicus* (Faleiros et al., 2010); Crab (*Dilocarcinus pagei*) (Furriel et al., 2010); *Carcinus maenas* (Henry et al., 2003); *P. monodon* (Sang and Fotedat, 2004) etc. Unfortunately, none of the previous studies considered salinity induced changes in both hemolymph regulation and expression pattern of hemolymph regulatory genes. Each of the previous studies considered the expression pattern of 2 to 3 important osmoregulatory (ion regulatory) genes at different salinity levels.

Table 01: Candidate genes involved with a functional role in osmoregulation in aquatic decapod crustaceans (Rahi et al., 2017; Rahi et al., 2018).

Name of candidate genes	Functional role of the genes
Alkaline phosphatase (ALP)	Phosphatase activity; ion binding; metabolic process; precipitation of ions
Aquaporin (AQP)	Cell volume regulation, water channel regulation, osmotic stress tolerance
Arginine kinase (ARK)	ATP binding; kinase activity; ion transport; salinity stress tolerance
ATP-binding cassette 12 (ABC12)	Protein binding; regulate osmoregulatory pathway; response to osmotic stress; ATP binding
Bestrophin (BSP)	Chloride transport; cellular water homeostasis
Carbonic anhydrase (CA)	Ion transport & binding, response to salinity stress, protein binding, pH balance, ion exchange
Ca^{+2}/Cl^- channel regulator (CCR)	Ion channel activity, ion transport, protein binding, cellular response to hypoxia
Ca^{+2}-ATPase (CAP)	Calcification, Ca^{+2} regulation, ion binding and transport, ATP binding, metabolic process
Calreticulin (CAL)	Osmotic stress response, signal transduction, protein folding, molecular chaperon, Ca^{+2} homeostasis
Claudins (CLA)	Osmotic stress tolerance, regulate cellular volume and junctions, maintain epithelial permeability
Crustacean cardiovascular peptide (CCP)	Body fluid regulation, stress tolerance, regulate signaling pathway
Crustacean hyperglycemic hormone (CHH)	Hemolymph production, body fluid regulation, stress signaling pathway
Diuretic hormone (DH)	Body fluid regulation, water balance
Heat shock protein (HSP)	Stress response, protein folding, chaperon function
H^+/Cl^- exchanger (HCE)	Cell volume and Cl^- channel regulation, anion transport, transfer signals

Integrin (ITG)	Cell volume and cellular junction regulation, osmotic stress response, mediate signal transduction pathway
Interleukin factor 2 (ILF2)	Osmotic stress response, ATP binding, transcriptional regulation
K^+/Cl^- symporter (KCS)	Transport K^+ and Cl^-; electromechanical balance
Mitogen activated protein kinase 38 (MAPK38)	Stress response; signal transfer for osmotic stress, ATP binding; protein phosphorylation
Mitochondrial carrier protein (MCP)	Signal transduction for osmotic stress; transmembrane transport; integral membrane component
Mg^{+2} transporter (MGT)	Integral membrane component; ion transport; signal transduction
Na^+/Ca^{+2} exchanger (NCE)	Na^+/Ca^{+2} anti-porter activity; ion transport; transmembrane transport; cell communication
Na^+/H^+ exchanger (NHE)	$Na^+:H^+$ anti-porter activity, ion transport & exchange; integral membrane component
Na^+/HCO3-transporter (NHT)	ATP binding, transport and exchange of ions
Na^+/K^+-ATPase (NKA)	Osmoregulation; ion transport, ion exchange; ion binding; ATP binding; response to stimulus
$Na^+/K^+/2Cl^-$ cotransporter (NKCC)	$Na^+:K^+:2Cl^-$ symporter activity, ion transport and exchange, integral membrane component
Potassium voltagegated channel protein (KCP)	Potassium ion transport; transmembrane transport; signal transduction; phosphorylation
Selenophosphate (SLP)	Salinity stress tolerance, ATP binding
Sodium myo-inositol co-transporter (NMIC)	Ion transporter, transmembrane transport
USP6	Stress tolerance; regulation of GTPase activity
V-type proton (H^+)-ATPase (VTA)	Osmoregulation, ion transport, ion exchange, cell volume regulation, cellular homeostasis

2.9 RT-qPCR technique for gene expression

Real time quantitative Polymerase Chain Reaction (RT-qPCR) is currently considered as the gold standard for precise, sensitive and fast measurement of gene expression (Derveaux et al., 2010). Recent studies showed that this method is strongly influenced by total RNA (in fact mRNA) integrity. Total RNA integrity is thought to have an effect on the performance of RT-qPCR as well as the quantitative results in mRNA expression profiling. Using a suitable normalization method could partly reduce the impairing effect of total RNA integrity (Becker et al., 2010).

A suitable reference gene is required for RT-qPCR based gene expression studies. To date, 18S and β-actin are considered to be the most suitable reference genes for gene expression studies in crustaceans (Havird et al., 2014; Ali et al., 2015; Moshtaghi et al., 2017; Rahi et al., 2017). For gene expression studies, total RNA is extracted from target tissue which is then converted to cDNA (Furriel et al., 2010). The cDNA stocks are then used for gene expression measurements using suitable sets of primers from the target candidate genes (Havird et al., 2014). Gene expression studies under different salinity levels can provide powerful insights in deciphering specific functional roles of target genes for hemolymph and ion regulation in *Penaeus monodon*.

2.10 Research Gap

Although *P. monodon* is an important and one of the most promising coastal aquaculture species in Bangladesh (and globally), no research have been conducted yet at the molecular level to understand the salinity tolerance issue including both gene expression pattern and body physiology (hemolymph and ion). This provides an ideal opportunity to investigate changes in gene expression profiling of some important candidate genes under different salinities at different time intervals in a finer detail.

Chapter Three

MATERIALS AND METHODS

3. Materials and Methods

3.1 Specimen collection and maintenance

Adult individuals of the target species (*P. monodon*) (6-8 g) were collected from 'Panna SPF Hatchery' (Botiaghata, Khulna) for this study. Water salinity, conductivity and temperature of the hatchery were 10‰ (ppt), 448 µS/cm and 23°C, respectively at time of collection. In total, 200 live adult shrimp were collected from the hatchery in plastic bags with supplied O_2 and then brought to the Khulna University Campus. Individuals were then transferred in plastic containers with aeration to the "Water Quality Laboratory" under Fisheries and Marine Resource Technology Discipline, Khulna University. In total, 15 rectangular 25L plastic tanks were used for this experiment. Experimental tanks were filled with water (10‰) collected from the hatchery. Tanks were initially filled with 25L of hatchery water (10‰) and kept under continuous aeration (Appendix 3). In total, 13-14 shrimps were allocated randomly to each experimental tank. Shrimps were then acclimated to tank conditions for 7 days prior to the commencement of the salinity stress experiment. The experimental tanks were cleaned by siphoning everyday and individuals in each tank were fed twice a day (morning and late afternoon) with commercially available aquarium fine grains (Sano S-PAK 8/2).

3.2 Salinity stress experiment and sample collection

Three different salinities (0‰, 10‰ and 20‰) were maintained for this experiment with five replicate tanks for each salinity treatment. Salinity levels in five experimental tank were reduced by 2‰ per day by mixing pond water in the tanks. Pond water was added in each tank slowly to reduce salinity levels in order to establish experimental salinity levels at 0‰. Salinity levels in five other experimental tanks were increased by 2‰ per day by mixing saline water in the tanks. Sea salt water was added in each tank slowly to increase salinity levels in order to establish experimental salinity level at 20‰. It took five days to achieve the target salinity levels at 0‰ and 20‰ (Appendix 3). The remaining 5 tanks reflected

normal condition or control for this study (10‰). Salinity reduction and increase in the tanks started at the same time to achieve target salinities in all tanks simultaneously.

Table 02: Experimental design showing treatment and control groups with replications and sampling time (here, T= Tank).

Time duration	Number of Samples Collected														
	0ppt					10ppt					20ppt				
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15
1 hr	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6 hr	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24 hr	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2nd day	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3rd day	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5th day	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10th day	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20th day	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30th day	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Gill tissue was collected from the individuals at different time intervals after exposure to different salinity levels. There were nine sampling times (1hr, 6hr, 24hr, 2 day, 3 day, 5 day, 10 day, 20 day and 30 day) over a 30 day experimental period. Five individuals (5 biological replicates) were sampled randomly from each salinity treatment (one from each tank). Gill tissue was collected and preserved separately in eppendorf tubes filled with 500 µL of RNAlater® solution (Applied Biosystems, Warrington, UK) immediately after sampling. Preserved samples were kept at room temperature for 12 hours and then kept in -80°C for long term storage (sample collection table in appendix 1 and photo gallery in appendix 3). Samples preserved in RNAlater were then sent to the Molecular Genetics Research Facility (MGRF) at Queensland University of Technology (QUT) for subsequent analysis.

Table 03: Specific primers for RT-qPCR assay of different genes.

Gene name	Primer type	Sequence	T_m (^{0}C)	Product size (bp)
Crustacean Hyper-Glycaemic Hormone (CHH)	Forward	TTCTGCAGTCTCTCCAACGA	60	156
	Reverse	GCGACGTATGGTTTCCTGTA	58	
Diuretic Hormone (DH)	Forward	ACAACTCGGCTCTGGTGTTC	60	224
	Reverse	CTCGGCCTAGACCCAAGTC	59	
Na+/K+-ATPase (NKA)	Forward	ACTGCTCTTGTGGATTGGTG	55.3	215
	Reverse	GTCTTCTGCCTGCACATTCT	53.3	
Na+/K+/2Cl- Co-transporter (NKCC)	Forward	GGGTCACCAGGGTCCAGAT	59.1	180
	Reverse	TAGCACCAGCAACAATTCCA	54.7	
Na+/H+ exchanger (NHE)	Forward	GTGGATCTTCTGCGCTTGTT	55.7	204
	Reverse	CCAGGTGGTCGAAGAAGAGT	56.4	
V-type H+-ATPase (VTA)	Forward	GGCAACTTTTGAGAAACTTGAAA	52.5	197
	Reverse	CCAGCAACAAATCCAATGTG	52.6	
18S	Forward	GCGGTAATTCCAGCTCCA	55	200
	Reverse	AGCCTGCTTTGAGCACTCTC	58	

The RT-qPCR reactions were performed in a 20 µl reaction mixture that included: 3 µl ultra-pure distilled water (Invitrogen, USA), 5 µl cDNA (template), 1 µl forward primer, 1 µl reverse primer and 10 µl 2x SensiFAST SYBR No-ROX Mix (Bioline, UK). After that, Reaction mixtures were transferred to the thermal cycler R-Corbett (RG-6000, Australia). Initially, conditions of the reaction included polymerase inactivation at 95°C for 2 minutes and then 40 cycles of: denaturation at 95°C for 5 minutes, annealing at 54-60°C (depending on temperature of each gene primer) for 20 seconds and extension at 72°C for 20 seconds. A standard melt curve analysis was performed to ensure the amplification of a single qPCR product according to Velotta et al. (2015). Quantitative gene expression values for each gene from each sample were obtained in terms of C_t (cycle threshold) values. C_t values were then used to obtain relative gene expression values by using the delta-delta C_t method (Pfaffli,

2001) that applies the equation: $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$, where control group represents expression values of 18S reference gene. For comparing relative gene expression patterns at different salinities with time, gene expression values were analyzed using SPSS software (Version 23) to test for statistical differences in gene expression pattern in three different salinity treatments for each gene. A two-way Analysis of Variance (ANOVA) test was used by applying the 0.05 level of significance using experimental salinities and sampling times as variables.

3.5 Assay of Hemolymph osmolality

Immediately following tissue dissection, gill tissue were preserved in glutaraldehyde and sodium cacodilate (3:1) solution (anticoagulant) at each sampling time. Osmolality of the hemolymph was then measured using a Cryoscopic Osmometer (Osmomet 030). The following equation was applied to calculate hemolymph osmolality according to Sang and Fotedar, (2004):

Hemolymph osmolality = 3 x osmolality of mix – 2 x osmolality of anticoagulant
Changes in hemolymph osmolality over the experimental time frame were analyzed using MS Excel (version 3.1.2) to produce bar graph. SPSS software (version 23) was used to test two-way ANOVA.

3.6 Data analysis

Qualitative and quantitative analysis of all kinds of gene expression data (CHH, DH, NKA, NKCC, NHE, and VTA) and hemolymph osmolality data according to salinity and duration were carried out. MS Excel was used to store all the data and presentation of the table and graphs from different types of data. SPSS software (version 23) was used to test level of significance (at 95% confidence interval) for a two-way ANOVA, multiple Comparisons among salinities and testing the relationship between-Subjects effects for all the genes and hemolymph osmolality.

Chapter Four

RESULTS

4. Results

In the present study, salinity induced changes in relative gene expression patterns were observed for six selected osmoregulatory genes in the tiger shrimp (*Penaeus monodon*) including two hemolymph regulatory genes (CHH and DH) and four ion regulatory genes (NKA, NKCC, VTA, NHE) using an RT-qPCR approach . In addition, changes in hemolymph osmolality were also observed under three different salinity levels (0‰, 10‰ and 20‰) for a period of 30 days.

4.1 Expression pattern of CHH gene

A significant interaction between salinity, sampling time and gene expression was obtained for the CHH gene ($F_{(2, 26)} = 97.878$, $p = 0.000$) from a 2-way ANOVA testing. There was a strong impact of salinity on the expression pattern of CHH gene. Significant differences were observed between all three salinities across the whole experimental timeframe (Fig. 03 and in Appendix 2). However, a generalized decreasing trend in expression pattern of CHH gene was observed with salinity reduction (Fig. 03). There was no significant difference in expression pattern of CHH gene at different sampling times for 20‰ salinity. (Multiple comparisons in Appendix 2). A gradual increase from 1 to 6 hours and then a decreasing trend was observed at 10‰ salinity from 6 hours to 2 day, then there was a stable pattern for the remaining timeframe. A mild decline was observed at 0‰ salinity from 1 to 24 hours, followed by a stable pattern to the end (Fig. 03).

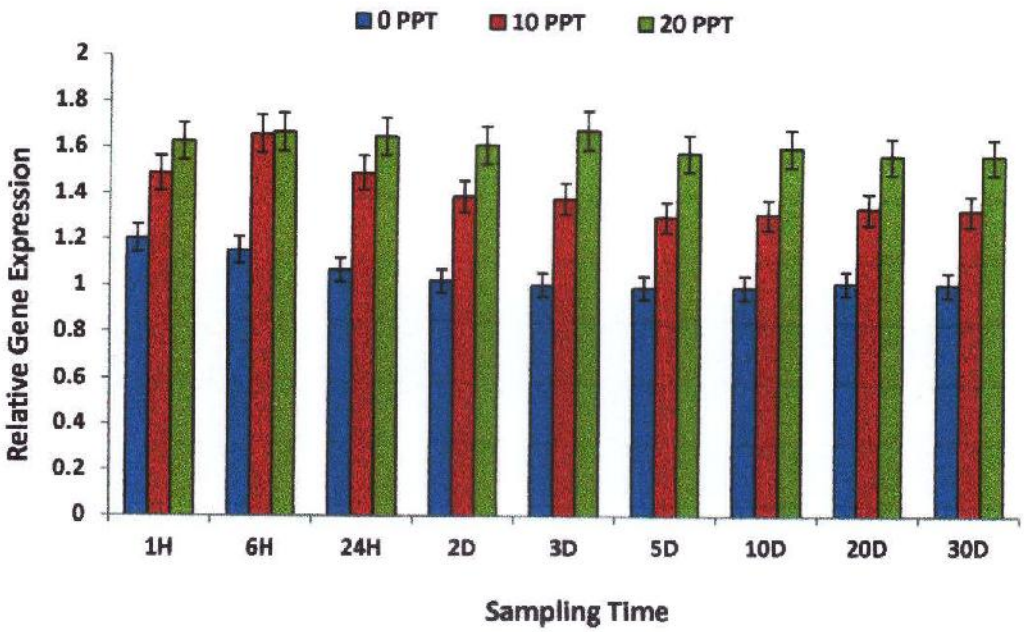


Fig 03: CHH (Crustacean Hyper- glycaemic Hormone) mRNA expression levels (relative to 18S expression) in *P. monodon* for the three salinity treatments for 30 days. Error bars represent +/- 1 SE.

4.2 Expression pattern of DH gene

There was a strong impact of salinity and sampling time on the expression pattern of DH gene ($F_{(2, 26)} = 8.139$, $p = 0.002$) in the current study. Significantly higher ($p < 0.05$) expression levels of the DH gene were observed at 0‰ compared with 10‰ and 20‰ across the experimental period. In contrast, significant differences were observed between 10‰ and 20‰ salinities only from 1 to 24 hours. For the remaining sampling times, there were no significant differences in expression pattern of DH gene between 10‰ and 20‰ salinities (Fig. 04). But significant differences in expression pattern were observed between sampling times (from 1 hour to 3 day) at 0‰ salinity (Multiple comparisons in Appendix 2). Almost constant expression patterns were observed from 3 day to the end of this experiment at 0‰

salinity level. The generalized expression pattern of DH gene at 0‰ treatment involved rapid increase in expression pattern from 1 to 24 hours, followed by a decreasing trend from 24 hours to 3 day, and then a stable trend towards the end of this experiment (Fig. 04). Relatively stable expression pattern was observed for DH at 10‰ salinity (control) throughout the experimental period. At 20‰, relatively higher expression levels were observed from 1 hour to 24 hour, then there was a gradual decline from 24 hour to 3 day following which there was a stable expression pattern.

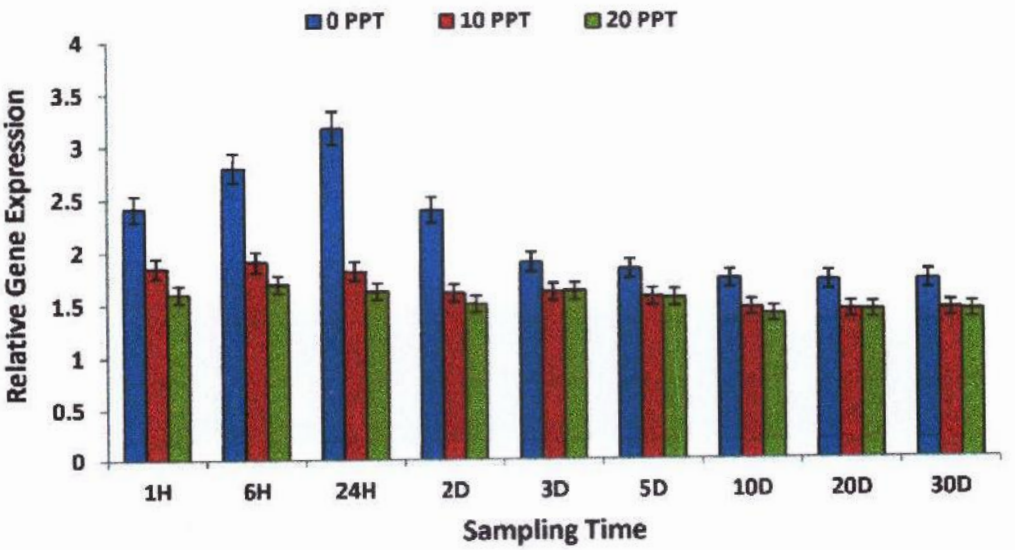


Fig 04: DH (Diuretic hormone) mRNA expression levels (relative to 18S expression) in *P. monodon* at three different salinity treatments over 30 days. Error bars represent +/- 1 SE.

4.3 Expression pattern of NKA gene

The two-way ANOVA indicates that expression levels of NKA showed a significant interaction between salinity level and sampling times ($F_{(2, 26)} = 15.145$, $p = 0.000$). Levels of NKA gene expression were relatively high under reduced salinity condition (0‰) from 1 hour to 5 day. At 0‰ salinity, expression levels continued to increase sharply until 24 hours and then there was a sharp decline upto 2 day. A gradual decline in expression pattern was observed from day 2 to day 3 (Fig. 5), following which expression of NKA remained fairly stable. In contrast, there were no significant differences in expression pattern of NKA gene between 10‰ and 20‰ salinities (Multiple comparisons in Appendix 2). At higher salinity levels (10 ‰ and 20‰), no impact on the expression pattern (there was no significant difference in expression pattern between sampling times) of NKA gene was observed across the experimental period (expression remained fairly stable at these two salinities).

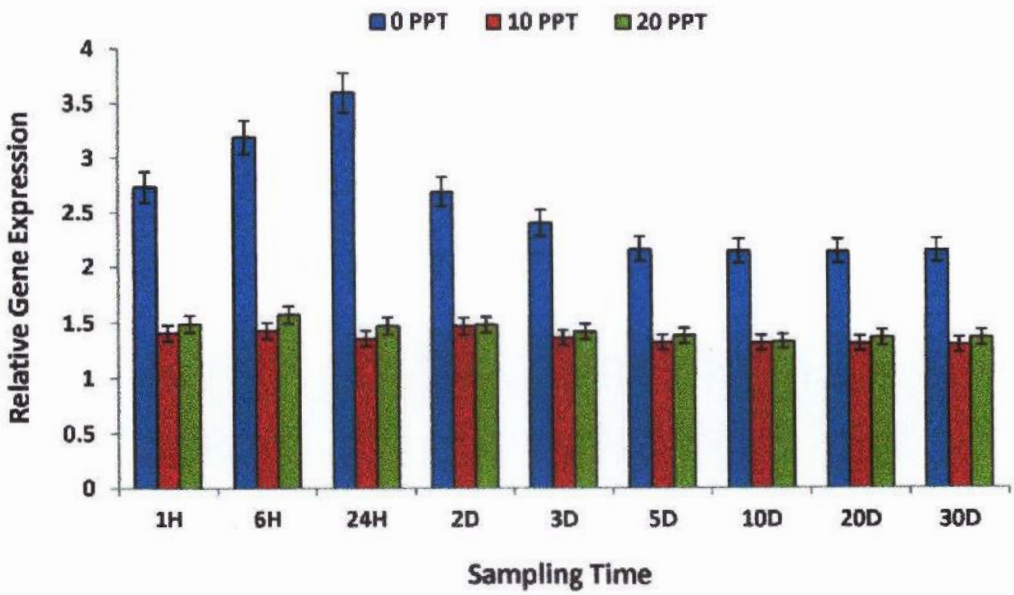


Fig 05: NKA (Na^+/K^+-ATPase) mRNA expression levels (relative to 18S expression) of *P. monodon* under three different salinity treatments for 30 days. Error bars represent +/- 1 SE.

4.4 Expression pattern of NKCC gene

There was a significant interaction between salinity treatments and sampling time for the NKCC gene ($F_{(2, 26)} = 116.510$, $p = 0.000$). There was a strong impact of salinity on the expression pattern of NKCC. Significant differences were observed between all three salinities across the experimental timeframe (Multiple comparisons among salinities in Appendix 2). However, a generalized decreasing trend in expression pattern of NKCC gene was observed at 0‰ (Fig. 06). There was no significant difference in expression pattern of NKCC gene at different sampling times for 20‰ salinity (Multiple comparisons in Appendix 2). A gradual decrease at 10‰ from 1 to 6 hours and then a up down trend upto 10 day but the differences were not significant (appendix 2), then there was a stable pattern for the remaining timeframe. A gradual decline was observed at 0‰ salinity from 1 hour to 2 day, followed by a stable pattern to the end (Fig. 06). Similarly to CHH, the 20‰ treatment led to a significantly higher expression level for NKCC gene.

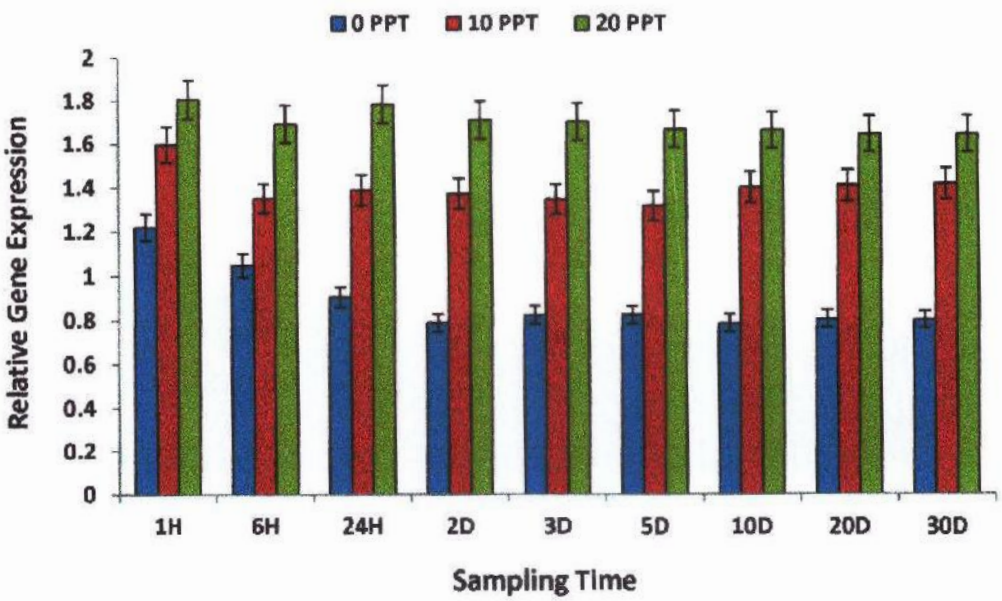


Fig 06: NKCC ($Na^+/K^+/2Cl^-$ Co-transporter) mRNA expression levels (relative to 18S expression) of *P. monodon* under three different salinity treatments for 30 days. Error bars represent +/- 1 SE.

4.5 Expression pattern of NHE gene

Two-way ANOVA results revealed that there was a significant interaction between salinity treatments and sampling time for the NHE gene ($F_{(2, 26)} = 31.516$, $p = 0.001$). Expression levels of the NHE gene were in relative terms, quite low at higher salinity levels (10‰ and 20‰) (Fig. 07). Levels of NHE expression were relatively high under reduced salinity condition (0‰) from 1 hour to 2 day. At 0‰ salinity, expression levels continued to increase until 2 day and then there was a sharp decline upto 3 day followed by a stable pattern upto the end (Fig. 07). In contrast, there were no significant differences in expression pattern of NHE gene at 10‰ and 20‰ salinities (Multiple Comparisons in appendix 2). Little impact was observed of high salinity (10‰ and 20‰) on the expression pattern of NHE gene across the experimental period (expression remained fairly stable at these two salinities).

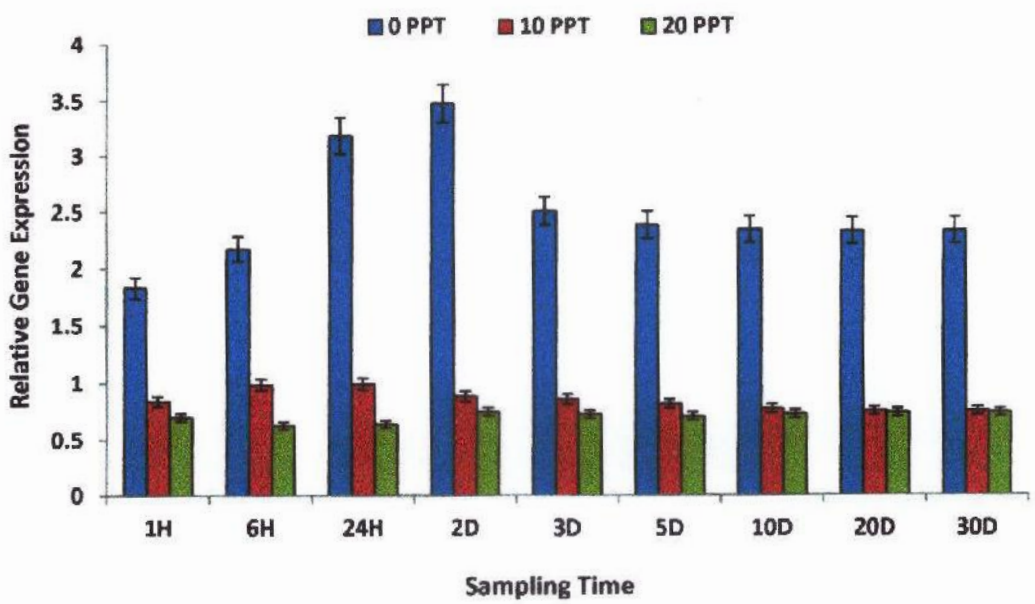


Fig 07: NHE (Na^+/ H^+ exchanger) mRNA expression levels (relative to 18S expression) of *P. monodon* at three different salinity treatments over 30 days. Error bars represent +/- 1 SE.

4.6 Expression pattern of VTA gene

Significant interaction was found between salinity treatments and sampling time for the VTA gene ($F_{(2, 26)} = 28.848$, $p = 0.000$). Expression results showed that VTA expression levels were stable at 10‰ and 20‰ salinities over the whole experimental time duration ($p < 0.05$). Levels of VTA gene expression were relatively high under the freshwater condition (0‰). At the 0‰ salinity, gradual increase was observed from 1 to 24 hour followed by a decreasing trend towards 10 day and then all remained essentially constant at the same level to the end of the experiment (Fig. 08). Significant differences ($p < 0.05$) were observed in VTA expression levels between the 0‰ vs. 10‰ and 0‰ vs. 20‰ salinities across the whole experimental time (multiple comparisons in appendix 2).

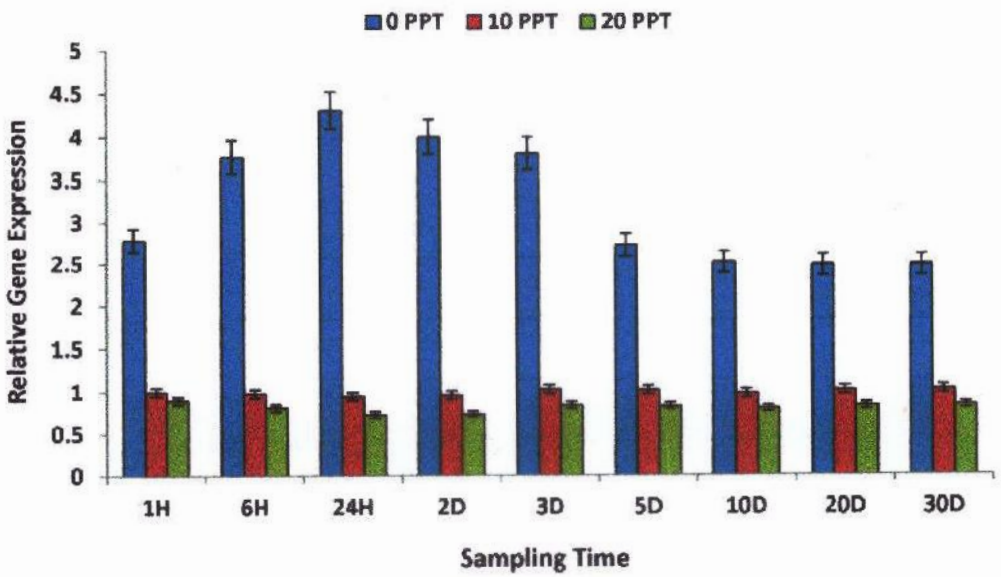


Fig 08: VTA (V-type H^+-ATPase) mRNA expression levels (relative to 18S expression) of *P. monodon* for three different salinity treatments over 30 days. Error bars represent +/- 1 SE.

4.7 Expression pattern of Hemolymph osmolality

Significant differences were found between salinities and sampling time ($F_{(2, 26)} = 39.792$, $p = 0.000$). Hemolymph osmolality ($mOsmkg^{-1}$ H_2O) was found to be highly dependent on the experimental salinity levels in the order of 20‰>10‰>0‰ (Fig. 09). Significant differences ($p < 0.05$) were observed in hemolymph osmolality among salinities across the whole experimental time (multiple comparisons in appendix 2). At 20‰, the hemolymph concentration (osmolality) was stable across the whole experimental time (Fig. 09). At 10‰, a decreasing trend was observed towards 3 day and then all remained essentially constant at the same level upto the end of the experiment (Fig. 09). At the 0‰ salinity, gradual decrease was observed from 1 hour to 24 hours followed by a decreasing trend towards 5 day and then all remained almost constant at the same level upto the end of the experiment.

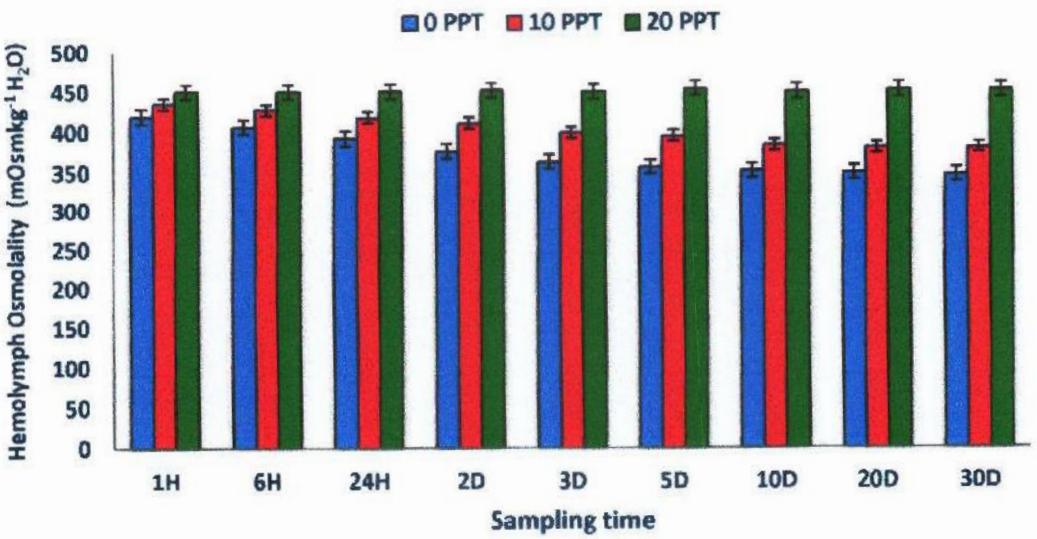


Fig 09: Changes in hemolymph osmolality ($mOsmkg^{-1}$ H_2O) in *P. monodon* at three different salinity treatments over 30 days. Error bars represent +/- 1 SE.

Chapter Five

DISCUSSION

5. Discussion

Expression levels of the six target genes tested here (CHH, DH, NKA, NKCC, NHE, and VTE) showed variations in expression patterns and were affected differently by both sampling time and salinity treatments. Few studies have previously been conducted on these six genes to characterize and understand the functional roles of these genes under different environmental salinity conditions for different crustaceans including prawn (Moshtaghi et al., 2017; Rahi et al., 2017), lobster (Havird et al., 2014), marine shrimp (Chung and Lin, 2006) etc. Results also demonstrate that exposure to various salinity environments significantly changed hemolymph osmolality and expression patterns of hemolymph and ion regulatory genes.

5.1 Expression pattern of ion regulatory genes

Expression patterns of the NKA gene were found to vary significantly among treatments across the whole experimental period with relative expression levels ranked as 0‰ > 20‰ > 10‰, showing a clear role of this gene in dealing with salinity changes (Fig. 05). The principle functional role of NKA is to establish a firm electromechanical gradient that support active ion transport and exchange between body fluids (hemolymph) and surrounding aquatic environments (McNamara et al., 2015; Freire et al., 2008; Henry et al., 2012). As *P. monodon* is a marine species and thus, reduced salinity conditions must have imposed severe osmotic stress at freshwater condition (0‰). So, Na^+/K^+-ATPase can be considered as an important gene for ionic balance in both fresh, brackish and sea water conditions in *P. monodon.*

NKA is well known as a master gene for its functional roles in maintaining ionic balance in both freshwater and salt water conditions; highly active under any salinity level for ionic balance via osmoregulation (Faleiros et al., 2010; Ali et al., 2015; Furriel et al., 2010; Chung and Lin, 2006; Havird et al., 2013; Liu et al., 2008; Moshtaghi et al., 2016). Here, relatively higher expression levels were observed for NKA at 0‰ salinity in *P. monodon*, supporting the idea that this gene has a critical role in ion regulation over a wide range of salinities.

NHE and NKCC both play important roles in absorbing Na^+ from the surrounding environment but they play important roles in contrasting environments: NHE plays an essential role in freshwater while NKCC is critical in salt water conditions (Genovese et al., 2000; Havird et al., 2014). Here, we observed salinity specific contrasting expression patterns for NHE and NKCC that reflect their differential roles in low and high ionic environments, respectively.

A gradually increased expression level of NKCC was observed with elevated salinity level in *P. monodon* (Fig. 06). Significant differences ($p < 0.05$) were observed among different salinities in expression level for this gene. Results clearly indicate a very important role of this gene under raised salinity conditions (may be involved with uptake or secretion of ions via basolateral membrane) while in contrast, it may function only a very minor or secondary functional role (i.e., cell volume control or transporter activity) in freshwater (Genovese et al., 2000; Barman et al., 2012; Moshtaghi et al., 2016.). Barman et al. (2012) observed higher expression of NKCC in *M. rosenbergii* when individuals experienced higher environmental salinity compared with low saline conditions. The pattern for *P. monodon* was similar, indicating an important role of NKCC in elevated environmental salinity conditions compared with low ionic conditions. Raised salinity (10‰ and 20‰) induced higher NKCC expression levels probably to facilitate ion transport and exchange under the changed osmotic conditions (Fig. 06). At 10‰, acclimatization time was 1 hour to 10 day to obtain a stable condition towards the end of the experiment. McNamara and Faria (2012) stated very alike functional roles of NKCC in their review.

NHE expression levels under saltwater conditions (10‰ and 20‰) were very low, while it was always higher in freshwater (Fig. 07). Significant differences were observed for the relative expression of NHE among salinities initially but this pattern decreased with time. This gene is known to have an important role in maintaining osmotic balance in low ionic conditions (Brett et al., 2005). Reported functional roles of NHE include a diverse pattern of physiological processes, including control of the cell cycle and cell proliferation, trans-epithelial Na^+ movement, ion transport, exchange of mono-valent ions, and ion absorption in low ionic environments (Boudour-Boucheker et al., 2016; Moshatghi et al., 2016). Towle et al. (1997) conducted an experiment on a crab species (*Carcinus maenas*) and identified

potential role(s) for this gene as an electrogenic and electroneutral exchanger. In the current study, NHE expression level at 0‰ salinity was found as continuous increasing pattern initially (1 hr to 2 day) followed by a sharp decrease (2 to 3 day) and then no differences were evident among treatments by 30 day (Fig. 07). This suggests that the gene may be important for dealing with initial exposure to a reduced salinity but after acclimation over a 3 day period, expression levels declined to conserve energy. Previous studies on aquatic crustaceans showed that NHE remains passive or inactive under high saline environments while this gene is highly expressed at lower salinity levels for brackish water or marine species (Havird et al., 2013; Havird et al., 2014; Rahi, 2017; Rahi et al., 2018). Exactly the same pattern of results was found in this study with higher expression levels of NHE at 0‰ while very lower levels of expression under raised salinities.

The functional role of VTA is to induce a negative electromechanical gradient across the gill membranes that is essential for the uptake of monovalent ions in freshwater. So, VTA is usually highly expressed under low ionic conditions (Rahi et al., 2018; Rahi et al., 2017). Higher expression of VTA at 0‰ compared with 10‰ and 20‰ (Figure 08) suggests that this gene has a more important functional role than NKA and NHE in freshwater.

Comparatively high VTA (V-type H^+-ATPase) expression levels was observed in the 0‰ and 10‰ treatments over the first 24 hours, after which expression levels declined gradually between 24 hours to 3 day followed by a decreasing trends and towards 10 day and then established a relatively constant expression to the end of the experiment (Fig. 08). VTA is reported to be a key gene involved with osmoregulation that has been well studied in many organisms (Faleiros et al., 2010; Lee et al., 2011; Ali et al., 2015; Moshtaghi et al., 2016). VTA is functionally involved in ion uptake and ion regulation, the control of cellular volume and also in maintaining ionic balance. Generally, it is expressed at higher levels in freshwater compared with raised salinity conditions (Faleiros et al., 2010; Genovese et al., 2000; BoudourBoucheker et al., 2016). This is because VTA is a specialized gene in absorbing critical monovalent ions in low ionic freshwater environments while this gene is less active in high salinity conditions (Rahi, 2017). Thus, in high ionic environments absorption of ions is not required that much as in freshwater (Rahi et al., 2018).

5.2 Expression pattern of hemolymph regulatory genes

Crustacean Hyperglycemic Hormone (CHH) gene is a neurohormone gene which is known to regulate numerous vital functions in crustaceans like ionic and energetic metabolism, molting and reproduction (Kallen et al., 1990). CHH genes were searched in taxa outside decapods, where they have been, to date, poorly investigated (Havird et al., 2014; Kallen et al., 1990). Diuretic Hormone gene secondarily promote sodium and water retention in the body by stimulation of adreno-cortical hormone secretion. It express more in freshwater condition and a reverse pattern is found for CHH gene.

CHH and DH genes are known to be the key genes for regulating hemolymph concentration as well as ionic balance in crustaceans (Chen and Lin., 1994; Moshtaghi et al., 2017; Rahi et al., 2017). In the current study, expression patterns of these two genes showed rapid responses with salinity change. Thus, it is likely that both CHH and DH genes play very important roles for rapid acclimation to salinity change in *Penaeus monodon* by ensuring efficient body fluid (hemolymph) regulation.

Expression patterns of the CHH gene were consistent among treatments across the whole experimental period with relative expression levels ranked as 20‰>10‰>0‰, indicating a clear functional role of this gene in dealing with salinity changes (Fig. 03). Although 10‰ salinity was the control condition, consistent higher expression levels was observed for CHH gene at 20‰ salinity condition (Fig. 03). At 10‰ salinity, expression pattern of CHH showed a gradual increase from 1 to 6 hours followed by a gradual declining trend up towards 2 day, then a stable expression pattern was maintained until the end of this experiment. A trend was observed at 0‰ salinity where expression levels showed a mild declining trend from 1 hour to 2 day followed by a fairly stable trend from 2 day to the end of this experiment. There were no significant differences ($p>0.05$) in expression pattern for this gene at 20‰ salinity among sampling times (Fig. 03).

A reverse pattern was observed for the DH gene that showed the highest expression levels at 0‰ and the lowest expression levels at 20‰. Significant differences were observed in expression levels between 10‰ and 20‰ salinities as well as among sampling times from 1 to 24 hours for the entire experimental time frame (Fig. 04). But expression levels of DH

gene at 0‰ salinity varied greatly among sampling times and as such there were significant differences ($p<0.05$) in expression levels between sampling times up to 3 day; but after that there were no significant differences. There was a rapid increase in expression of DH gene at 0‰ from 1 to 24 hours followed by a rapid decline from 24 hours to 3 day (Fig. 04).

CHH showed higher levels of gene expression at raised salinities (10‰ and 20‰) and DH showed higher levels of expression at the lowest salinity condition (0‰) (Fig 03 and Fig. 04). These results simply imply that both of the target genes have salinity specific important functional roles in maintaining body fluid regulation. High salinity specific (10‰ and 20‰) higher expression of CHH indicates more important role of this gene in salt water condition while low salinity specific higher expression of the DH gene indicates its important functional role in body fluid regulation at lower ionic condition.

While saltwater is the normal habitat for *P. monodon*, still a lot of regulatory activities are required at 20‰ to maintain ionic balance between body fluid and surrounding environment. This might explain why CHH gene expression level was higher at 20‰ treatment compared with 10‰ and 0‰ salinities in the current study. In parallel, the probable reason for higher expression levels of DH gene at the lowest salinity treatment (0‰) could be due to higher differences in the osmotic gradient between the environment and the body fluid. Major challenges for a shrimp under this condition involve loss of excessive ions from body fluid/hemolymph and excessive water gain in the hemolymph. These challenges may induce higher expression levels of DH to regulate body fluid in *P. monodon* in the opposite direction.

5.3 Changes in hemolymph Osmolality

In freshwater, ionic concentration of *P. monodon* hemolymph is higher than the surrounding water, and thus, it is crucial to conserve internal ions by maintaining tighter cellular junctions that create an impermeable gill membrane. Brackish or marine water species are characterized by more leaky cellular junctions in their gill epithelia, resulting in high rates of diffusive ion loss when they are exposed to low salinity environments (Havird et al., 2014).

In the current study, Hemolymph osmolality ($mOsmkg^{-1}$ H_2O) was found to be highly dependent on the experimental salinity levels in the order of increasing salinity (20‰>10‰>0‰) (Fig. 09). Highest levels of hemolymph osmolality were observed at 20‰ at a constant rate across the whole experimental timeframe. Relatively gradual decreasing trend (with expression stabilizing after 3 day) in hemolymph osmolality was observed at 0‰ and 10‰ (Fig. 09). Maintaining hemolymph osmolality is one of the principle physiological responses for survival and acclimation to salinity change (Rahi et al., 2017; Rahi et al., 2018). Freshwater crustaceans generally act as osmoregulators and maintain their hemolymph osmolality range between ≈350 to 450 $mOsmkg^{-1}$ H_2O (above the ambient medium) while brackish water inhabitants maintain their hemolymph osmolality as almost iso-osmotic (≈500-700 $mOsmkg^{-1}$ H_2O) to the surrounding medium (Sang and Fotedar, 2004; Havird et al., 2014). Thus, maintaining ionic balance is easier and requires less energy in brackish water compared with both fresh and sea water. The highest level of hemolymph osmolality was recorded in the current study at 20‰ salinity, because ionic content of the surrounding medium is also high. In contrast, experiences in low external ionic content in freshwater and therefore, acts to maintain relatively low levels of hemolymph osmolality.

Chapter Six

CONCLUSION

6. Conclusion

Osmoregulatory performance is regarded as the principal mechanism for maintaining ionic balance when environmental salinity levels fluctuate. Body fluid (hemolymph) regulation in crustaceans plays the central role for successful osmoregulatory performance with salinity change. A study of relative gene expression patterns (based on RT-qPCR) of six important candidate genes (including hemolymph and ion regulatory) in *P. monodon* revealed remarkable differences in expression patterns under three different experimental salinity conditions. Different genes showed different levels of expression patterns depending on the salinity treatment and timing (or duration) of exposure to salinity stress. As a master gene for osmoregulation, NKA showed higher expression levels both at low (0‰) and high (20‰) salinity levels. NHE and NKCC showed contrasting expression patterns where NHE expressed highly at 0‰ but NKCC expression was at its peak at 20‰. VTA showed very higher levels of expression pattern at 0‰. Hemolymph osmolality changes were also strongly affected by the salinity levels. Higher levels of hemolymph osmolality were observed at 20‰ while the lowest levels were at 0‰. Hemolymph regulatory genes, CHH and DH also showed highly variable expression patterns with regard to salinity change. Overall results clearly indicate important functional role of these six genes for body fluid and ion regulation for effective ionic balance via osmoregulation with environmental salinity change.

Chapter Seven

REFERENCES

7. References

Ali, M. Y., Pavasovic, A., Mather, P. B. and Prentis, P. J., 2015. Analysis, characterization and expression of gill-expressed carbonic anhydrase genes in freshwater crayfish *Cherax quadricarinatus*. *Gene 564*(2): 176-187.

Allan, G. L. and Maguire, G. B., 1992. Effects of pH and salinity on survival, growth and osmoregulation in *Penaeus monodon* Fabricius. *Aquaculture, 107*: 33– 47.

Barman, H. K., Patra, S. K., Das, V., Mohapatra, S. D., Jayasankar, P., Mohapatra, C. and Rath, S. N., 2012. Identification and characterization of differentially expressed transcripts in the gills of freshwater prawn (*Macrobrachium rosenbergii*) under salt stress. *The Scientific World Journal*, 1-11.

Becker, C., Hammerle-Fickinger, A., Riedmaier, I. and Pfaffl, M. W., 2010. mRNA and microRNA quality control for RT-qPCR analysis. *Methods, 50*(4): 237-243.

Boudour-Boucheker, N., Boulo, V., Charmantier-Daures, M., Anger, K., Charmantier, G. and Lorin- Nebel, C., 2016. Osmoregulation in larvae and juveniles of two recently separated *Macrobrachium* species: Expression patterns of ion transporter genes. *Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology, 195*: 39-45.

Brett, C. L., Donowitz, M. and Rao, R., 2005. Evolutionary origins of eukaryotic sodium/proton exchangers. *American Journal of Physiology-Cell Physiology, 288*(2): C223-C239.

Castille Jr, F. L. and Lawrence, A. L., 1981. The effect of salinity on the osmotic, sodium and chloride concentrations in the hemolymph of euryhaline shrimp of the genus *Penaeus*. *Comparative Biochemistry and Physiology Part A: Physiology, 68*(1): 75-80.

Chanratchakool, P., 2003. Advice on aquatic animal health care: Problems in *Penaeus monodon* culture in low salinity areas. *Aquaculture Asia, 8*(1): 54-56.

Chen, J. C. and Chia, P. G., 1997. Osmotic and ionic concentrations of *Scylla serrata* (Forskål) subjected to different salinity levels. *Comparative Biochemistry and Physiology Part A: Physiology, 117*(2): 239-244.

Chen, J. C. and Lin, J. L., 1994. Osmolality and chloride concentration in the hemolymph of subadult *Penaeus chinensis* subjected to different salinity levels. *Aquaculture, 125* (1-2): 167-174.

Chen, J. C., Lin, M. N., Ting, Y. Y. and Lin, J. N., 1995. Survival, hemolymph osmolality and tissue water of *Penaeus chinensis* juveniles acclimated to different salinity and temperature levels. *Comparative Biochemistry and Physiology Part A: Physiology, 110*(3): 253-258.

Chen, T., Ren, C., Wang, Y., Gao, Y., Wong, N.K., Zhang, L. and Hu, C., 2016. Crustacean cardiovascular peptide (CCAP) of the white pacific shrimp (*Litopenaeus vannamei*): molecular characterization and its potential roles in osmoregulation and freshwater tolerance. *Aquaculture, 451*: 405-412

Chomczynski, P. and Mackey, K., 1995. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. *Biotechniques, 19*: 942-945.

Chung, K. F. and Lin, H. C., 2006. Osmoregulation and Na, K-ATPase expression in osmoregulatory organs of *Scylla paramamosain. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 144*(1): 48-57.

Dall, W., 1957. A revision of the Australian species of Penaeidae (Crustacea Decapoda: Penaeidae). *Marine and Freshwater Research, 8*(2): 136-232.

Derveaux, S., Vandesompele, J. and Hellemans, J., 2010. How to do successful gene expression analysis using real-time PCR. *Methods, 50*(4): 227-230.

Diwan, A. D., Laxminarayana, A. and Kakati, V. S., 1989. Osmoregulatory ability of *Penaeus monodon* (Fabricius) in relation to varying salinities. *Indian Journal of Fisheries, 36*(1): 53-57.

DOF, 2017. Yearbook of Fisheries Statistics of Bangladesh. Fisheries Resources Survey System (FRSS). Department of Fisheries, Bangladesh, 33: 124pp.

DOF, 2019. Annual Report of Department of Fisheries. Matsya Pakha, 143pp.

Faleiros, R. O., Goldman, M. H., Furriel, R. P. and McNamara, J. C., 2010. Differential adjustment in gill Na^+/K^+- and V-ATPase activities and transporter mRNA expression during osmoregulatory acclimation in the cinnamon shrimp *Macrobrachium amazonicum* (Decapoda, Palaeomonidae). *Journal of Experimental Biology, 213*: 3894-3905.

Ferraris, R. P., Parado-Estepa, E. D., de Jesus, E. G. and Ladja, J. M., 1987. Osmotic and chloride regulation in the hemolymph of the tiger prawn *Penaeus monodon* during molting in various salinities. *Marine Biology, 95*(3): 377-385.

Fredrick, W. S. and Ravichandran, S., 2012. Hemolymph proteins in marine crustaceans. *Asian Pacific journal of tropical biomedicine, 2*(6): 496-502.

Freire, C. A., Onken, H. and McNamara, J. C., 2008. A structure-function analysis of ion transport in crustacean gills and excretory organs. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 151*(3): 272-304.

Furriel, R. P. M., Firmino, K. C. S., Masui, D. C., Faleiros, R. O., Torres, A. H. and McNamara, J. C., 2010. Structural and biochemical correlates of Na^+, K^+-ATPase driven ion uptake across the posterior gill epithelium of the true freshwater crab, *Dilocarcinus pagei* (Branchyura, Trichodactylidae). *Journal of Experimental Zoology, 313A*: 508-523.

Genovese, G., Ortiz, N., Urcolan, M. R. and Luquet, C. M., 2005. Possible role of carbonic anhydrase, V-H^+-ATPase, and Cl^-/HCO_3- exchanger in electrogenic ion transport across the gills of the euryhaline crab Chasmagnathus granulates. *Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology, 142*(3): 362-369.

Hatai, K., Bian, B. Z., Baticados, M. C. L. and Egusa, S., 1980. Studies on the fungal diseases in crustaceans. II. Haliphthoros philippinensis sp. nov. isolated from cultivated larvae

of the jumbo tiger prawn (*Penaeus monodon*). *Transactions of the Mycological Society of Japan, 21*(1): 47-55.

Havird, J. C., Henry, R. P. and Wilson, A. E., 2013. Altered expression of Na^+/K^+-ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: A meta analysis of 59 quantitative PCR studies over 10 years. *Comparative Biochemistry and Physiology D*, 8(2): 131-140.

Havird, J. C., Santos, S. R. and Henry, R. P., 2014. Osmoregulation in the Hawaiian anchialine shrimp *Halocaridina rubra* (Crustacea: Atyidae): expression of ion transporters, mitochondria-rich cell proliferation and hemolymph osmolality during salinity transfers. *Journal of Experimental Biology, 217*(13): 2309-2320.

Henry, R. P., Gehnrich, S., Weihrauch, D. and Towle, D. W., 2003. Salinity-mediated carbonic anhydrase induction in the gills of the euryhaline green crab, *Carcinus maenas*. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 136*(2): 243-258.

Henry, R. P., Lucu, C., Onken, H. and Weihrauch, D., 2012. Multiple functions of the crustacean gill: osmotic/ionic regulation, acid-base balance, ammonia excretion, and bioaccumulation of toxic metals. *Frontiers in Physiology, 3*: 1-33.

Holthuis, L. B. and Rosa H. Jr., 1965. List of species of shrimps and prawns of economic value. Rome: Food and Agriculture Organization of the United Nations, 21pp.

Holthuis, L. B., 1949. The identity of *Penaeus monodon* Fabr. *Proceedings van de Koninklijke Nederlandsche Akademie van Wetenschappen, 52*: 1051-1057.

Jia, X., Zhuang, P., Feng, G., Wang, R., Lu, J. and Huang, X., 2012. The relationship between hemolymph biochemical parameters and salinity in female parent Chinese mitten crab (*Eriocheir sinensis*). *Journal of Fisheries of China, 36*(1): 91-97.

Kallen, J. L., Abrahamse, S. L. and Van Herp, F., 1990. Circadian rhythmicity of the crustacean hyperglycemic hormone (CHH) in the hemolymph of the crayfish. *The Biological Bulletin, 179*(3): 351-357.

Lee, C. E., Kiergaard, M., Gelembiuk, G. W., Eads, B. D. and Posavi, M., 2011. Pumping ions: rapid parallel evolution of ionic regulation following habitat invasions. *Evolution, 65*: 2229-2244.

Lightner, D. V., Redman, R. M. and Bell, T. A., 1983. Observations on the geographic distribution, pathogenesis and morphology of the baculovirus from *Penaeus monodon* Fabricius. *Aquaculture, 32*(3-4): 209-233.

Liu, H., Pan, L. and Fu, L., 2008. Effect of salinity on hemolymph osmotic pressure, sodium concentration and Na^+-K^+-ATPase activity of gill of Chinese crab, *Eriocheir sinensis*. *Journal of Ocean University of China, 7*(1): 77-82.

Long, X., Wu, X., Zhao, L., Ye, H., Cheng, Y. and Zeng, C., 2017. Effects of salinity on gonadal development, osmoregulation and metabolism of adult male Chinese mitten crab, *Eriocheir sinensis*. *PloS one, 12*(6): e0179036.

McNamara, J. C. and Faria, S. C., 2012. Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod crustacea: A review. *Journal of Comparative Physiology B, 182*(8): 997-1014.

McNamara, J. C., Freire, C. A., Torres Jr, A. H. and Faria, S. C., 2015. The conquest of fresh water by the palaemonid shrimps: an evolutionary history scripted in the osmoregulatory epithelia of the gills and antennal glands. *Biological Journal of the Linnean Society, 114*(3): 673-688.

Moshtaghi, A., Rahi, M. L., Mather, P. B. and Hurwood, D. A., 2017. Understanding the genomic basis of adaptive response to variable osmotic niches in freshwater prawns: a comparative intraspecific RNA-seq analysis of *Macrobrachium australiense*. *Journal of Heredity, 108*(5): 544-552.

Moshtaghi, A., Rahi, M. L., Nguyen, V. T., Mather, P. B. and Hurwood, D. A., 2016. A transcriptomic scan for potential candidate genes involved in osmoregulation in an obligate freshwater palaemonid prawn (*Macrobrachium australiense*). *PeerJ* 4, e2520. doi: 10.7717/peerj.2520.

Motoh, H. and Kuronuma, K., 1980. Field guide for the edible Crustacea of the Philippines. SEAFDEC. *Aquaculture Department Iloila, Philippines*, 96pp.

Motoh, H., 1985. Biology and ecology of *Penaeus monodon*. In *Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps, 4-7 December 1984, Iloilo City, Philippines*, 27-36pp. Aquaculture Department, Southeast Asian Fisheries Development Center.

Motoh, H., and Buri. P., 1981. Identification of Postlarvae of the Genus *Penaeus* Appearing in Shore Waters. *Researches on Crustasea*, *11*: 86-94.

Mudagandur, S. S., Gopalapillay, G. and Vijayan, K. K., 2016. Effect of Salinity Stress on Gene Expression in Black Tiger Shrimp *Penaeus monodon*. *Abiotic and Biotic Stress in Plants-Recent Advances and Future Perspectives*, 101-120.

Mykles, D. L. and Hui, J. H. L., 2015. *Neocaridina denticulate*: A decapod crustacean model for functional genomics. *Integrative and comparative biology*, *55*(5): 891-897.

Nadal, E. D., Ammerer, G. and Posas, F., 2011. Controlling gene expression in response to stress. *Nature Reviews Genetics*, *12*: 833-844.

Perazzolo, L. M., Gargioni, R., Ogliari, P. and Barracco, M. A., 2002. Evaluation of some hemato-immunological parameters in the shrimp *Femtopenaeus paulensis* submitted to environmental and physiological stress. *Aquaculture*, *214*(1-4): 19-33.

Pfaffi, M. W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*, *29*(9): 45.

Racek, A. A., 1955. Littoral penaeinae from New South Wales and adjacent Queensland waters. *Marine and Freshwater Research*, *6*(2): 209-241.

Racek, A. A., 1959. *Prawn investigations in eastern Australia*. Government Printer, South Africa, 6: 57pp.

Rahi, M. L., Amin, S., Mather, P. B. and Hurwood, D. A., 2017. Candidate genes that have facilitated freshwater adaptation by palaemonid prawns in the genus *Macrobrachium*:

identification and expression validation in a model species (*M. koombooloomba*). PeerJ 5, e2977. doi: 10.7717/peerj.2977.

Rahi, M. L., Moshtaghi, A., Mather, P. B. and Hurwood, D. A., 2018. Osmoregulation in decapod crustaceans: physiological and genomic perspectives. *Hydrobiologia, 825*(1): 177-188.

Sang, H. M. and Fotedar, R., 2004. Growth, survival, hemolymph osmolality and organosomatic indices of the western king prawn (*Penaeus latisulcatus* Kishinouye, 1896) reared at different salinities. *Aquaculture, 234*(1-4): 601-614.

Shekhar, M., Kiruthika, J. and Ponniah, A., 2013. Identification and analysis of differentially expressed genes from shrimp (*Penaeus monodon*) in response to low salinity stress. *Fish and Shellfish Immunology 35*: 1957-1968.

Solis, N. B., 1988. Brackishwater Aquaculture Information System, Biology and Culture of *Penaeus monodon*. Southeast Asian Fisheries Development, Tigbauan, Iloilo, Philippines, 3-36pp.

Sowers, A. D., Young, S. P., Grosell, M., Browdy, C. L. and Tomasso, J. R., 2006. Hemolymph osmolality and cation concentrations in *Litopenaeus vannamei* during exposure to artificial sea salt or a mixed-ion solution: relationship to potassium flux. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 145*(2): 176-180.

Staples, D. J. and Heales, D. S., 1991. Temperature and salinity optima for growth and survival of juvenile banana prawns *Penaeus merguiensis. Journal of Experimental Marine Biology and Ecology, 154*(2): 251-274.

Tantulo, U., and Fotedar, R., 2007. Osmo and ionic regulation of black tiger prawn (*Penaeus monodon* Fabricius 1798) juveniles exposed to K+ deficient inland saline water at different salinities. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 146*(2): 208-214.

Terwilliger, N. B., 1999. Hemolymph proteins and molting in crustaceans and insects. American Zoologist, 39(3): 589-599.

Towle, D. W., Rushton, M. E., Heidysch, D., Magnani, J. J., Rose, M. J., Amstutz, A., Jordan, M. K., Shearer, D. W. and Wu, W. S., 1997. Sodium/proton antiporter in the euryhaline crab *Carcinus maenas*: molecular cloning, expression and tissue distribution. *Journal of Experimental Biology, 200*(6): 1003-1014.

Untergaser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. and Rosen, S. G., 2012. Primer3- new capabilities and interfaces. *Nucleic Acids Research, 40*(15): 115.

Velotta, J. P., McCormick, S. D. and Schultz, E. T., 2015. Trade-off in osmoregulation and parallel shifts in molecular function follow ecological transitions to freshwater in the Alewife. *Evolution, 69*(10): 2676-2688.

Vogt, G., 2013. Abbreviation of larval development and extension of brood care as key features of the evolution of freshwater Decapoda. *Biological Reviews, 88*(1): 81-116.

Wang, X. W. and Wang, J. X., 2015. Crustacean hemolymph microbiota: Endemic, tightly controlled, and utilization expectable. *Molecular immunology, 68*(2): 404-411.

Wray, G. A., 2013. Genomics and the evolution of phenotypic traits. *Annual review of ecology, evolution, and systematics, 44*: 51-72.

Yepiz-Plascencia, G., Vargas-Albores, F. and Higuera-Ciapara, I., 2000. Penaeid shrimp hemolymph lipoproteins. *Aquaculture, 191*(1-3): 177-189.

Zhang, S., Wang, J. and Huang, N., 1989. The effect of low salinity on growth and survival of juveniles *Penaeus monodon* Fabricius. *Transections of the Ocean and Limnology, 2*: 66–70.

Zhao, L., Long, X. W., Wu, X. G., He, J., Shi, Y. H., Zhang, G. Y. and Cheng, Y. X., 2016. Effects of water salinity on osmoregulation and physiological metabolism of adult male Chinese mitten crab *Eriocheir sinensis*. *Acta Hydrobiologica Sinica, 40*(1): 27-34.

Chapter Eight

Appendix

8. Appendix

Appendix-1

Sampling sheet for salinity tolerance level measurement

Place: "Water Quality Laboratory" in Khulna University

Time and Date of sampling:

There were 9 different time intervals for sample collection (1 hr, 6 hr, 24 hr, 2 day, 3 day, 5 day, 10 day, 20 day, 30 day) during the experiment. The sampling time and date are given below –

Time and date:

Number of Sampling	Time	Date
1, 2	12pm (1hr) & 6pm (6 hr)	11. 09. 2019
3	12pm (24 hr)	12. 09. 2019
4	12pm (2 day)	13. 09. 2019
5	12pm (3 day)	14. 09. 2019
6	12pm (5 day)	16. 09. 2019
7	12pm (10 day)	21. 09. 2019
8	12pm (20 day)	1. 10. 2019
9	12pm (30 day)	11. 10. 2019

Total RNA Isolation

Homogenize sample (30 mg)

With liquid N2 (making fine powder)

Cell lysis

Transfer powder in a tube with 350 µm Lysis buffer RLY and 3.5 µL b-ME

200 ml CHCl3 mix and centrifuged at 1500 rpm for 12 minutes and Collection of supernatant

Filter lysate

11000 rpm, 1 min

Adjust RNA binding conditions

350 µL 70% ethanol mix

Bind total RNA

Load sample

11000 rpm, 30s

Desalt silica membrane

350 µL MEM (Ethanol and salt remove)

11000 rpm, 1 min

Digest DNA: 95 µL DNase \\ reaction mixture RT 15 min

Wash and dry silica membrane

1st wash 200 350 µL wash buffer RW1

2nd wash and 600 µL wash Buffer W2

1st and 2nd: 11000 rpm, 30s

3rd: 11000 rpm, 2 min

Elute RNA

60 µL RNase-free water

11000 rpm, 2 min

Isolation RNA

Appendix – 2

Some statistical analysis for CHH gene expression:

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1.482	2	.741	97.878	.000
	Residual	.182	24	.008		
	Total	1.664	26			

a. Dependent Variable: Gene expression

b. Predictors: (Constant), Salinity, Duration

Tests of Between-Subjects Effects

Dependent Variable: Gene expression

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.614[a]	10	.161	51.396	.000
Intercept	50.457	1	50.457	16070.163	.000
Duration	.105	8	.013	4.200	.007
salinity	1.508	2	.754	240.178	.000
Error	.050	16	.003		
Total	52.121	27			
Corrected Total	1.664	26			

a. R Squared = .970 (Adjusted R Squared = .951)

Multiple Comparisons (Among Salinities)

Dependent Variable: Gene expression

Tukey HSD

(I) salinity	(J) salinity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.00	10.00	-.3622*	.02641	.000	-.4304	-.2941
	20.00	-.5722*	.02641	.000	-.6404	-.5041
10.00	.00	.3622*	.02641	.000	.2941	.4304
	20.00	-.2100*	.02641	.000	-.2782	-.1418
20.00	.00	.5722*	.02641	.000	.5041	.6404
	10.00	.2100*	.02641	.000	.1418	.2782

Based on observed means.

The error term is Mean Square (Error) = .003.

*. The mean difference is significant at the .05 level.

Some statistical analysis for NHE gene expression:

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	14.636	2	7.318	31.516	.001
	Residual	5.573	24	.232		
	Total	20.209	26			

a. Dependent Variable: Gene expression

b. Predictors: (Constant), Salinity, Duration

Tests of Between-Subjects Effects

Dependent Variable: Gene expression

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	18.870[a]	10	1.887	22.547	.000
Intercept	49.262	1	49.262	588.610	.000
Duration	.805	8	.101	1.203	.007
salinity	18.064	2	9.032	107.922	.000
Error	1.339	16	.084		
Total	69.470	27			
Corrected Total	20.209	26			

a. R Squared = .934 (Adjusted R Squared = .892)

Multiple Comparisons (Among Salinities)

Dependent Variable: Gene expression

Tukey HSD

(I) salinity	(J) salinity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.00	10.00	1.6578*	.13637	.000	1.3059	2.0097
	20.00	1.8033*	.13637	.000	1.4514	2.1552
10.00	.00	-1.6578*	.13637	.000	-2.0097	-1.3059
	20.00	.1456	.13637	.547	-.2063	.4974
20.00	.00	-1.8033*	.13637	.000	-2.1552	-1.4514
	10.00	-.1456	.13637	.547	-.4974	.2063

Based on observed means.

The error term is Mean Square (Error) = .084.

*. The mean difference is significant at the .05 level.

Some statistical analysis for Hemolymph osmolality:

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	28980.657	2	14490.329	39.792	.000
	Residual	8739.549	24	364.148		
	Total	37720.207	26			

a. Dependent Variable: Hemolymph osmolality

b. Predictors: (Constant), salinity, Duration

Tests of Between-Subjects Effects

Dependent Variable: Hemolymph osmolality

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	34144.229[a]	10	3414.423	15.277	.000
Intercept	4526654.003	1	4526654.003	20253.611	.000
Duration	6051.827	8	756.478	3.385	.018
salinity	28092.402	2	14046.201	62.847	.000
Error	3575.978	16	223.499		
Total	4564374.210	27			
Corrected Total	37720.207	26			

a. R Squared = .905 (Adjusted R Squared = .846)

Multiple Comparisons (Among Salinities)

Dependent Variable: Hemolymph osmolality

Tukey HSD

(I) salinity	(J) salinity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.00	10.00	-30.7889*	7.04744	.001	-48.9736	-12.6042
	20.00	-78.4111*	7.04744	.000	-96.5958	-60.2264
10.00	.00	30.7889*	7.04744	.001	12.6042	48.9736
	20.00	-47.6222*	7.04744	.000	-65.8069	-29.4375
20.00	.00	78.4111*	7.04744	.000	60.2264	96.5958
	10.00	47.6222*	7.04744	.000	29.4375	65.8069

Based on observed means.

The error term is Mean Square (Error) = 223.499.

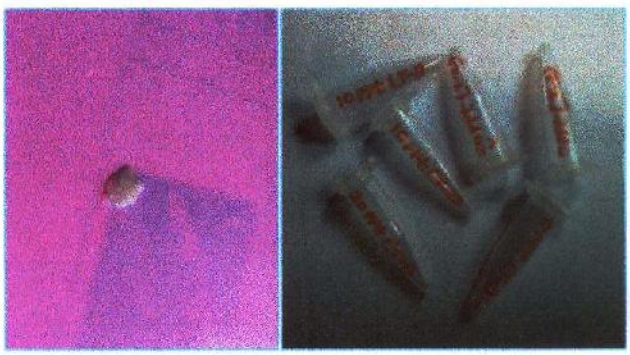
*. The mean difference is significant at the 0.05 level.

Appendix – 3 (Photo Gallery)

Shrimp rearing tank

Changing salinity

Collecting samples (gill)



Freezing (-80° C) of samples after labeling