HAEMATOLOGY OF *FENNEROPENAEUS INDICUS* IN RESPONSE TO ENVIRONMENTAL ALTERATIONS AND MICROBIAL INFECTIONS

Thesis submitted to

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

by

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MAY 2008

.....to my beloved brother

Late Sri. Sivan Subramanian

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Certificate

This is to certify that the thesis entitled **"HAEMATOLOGY OF *FENNEROPENAEUS INDICUS* IN RESPONSE TO ENVIRONMENTAL ALTERATIONS AND MICROBIAL INFECTIONS"** *is an authentic record of research work carried out by* ***Mr. Selven S.*** *under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Ocean Science and Technology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of* ***Doctor of Philosophy*** *in Microbiology and no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.*

Kochi -16
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Declaration

I hereby do declare that the thesis entitled "**HAEMATOLOGY OF *FENNEROPENAEUS INDICUS* IN RESPONSE TO ENVIRONMENTAL ALTERATIONS AND MICROBIAL INFECTIONS**" is an authentic record of research work done by me under the supervision and guidance of **Dr. Rosamma Philip**, Senior Lecturer, Department of Marine Biology, Microbiology and Biochemistry, School of Ocean Science and Technology, Cochin University of Science and Technology for the degree of **Doctor of Philosophy** in Microbiology and that no part thereof has been presented before for the award of any other degree in any university.

Kochi- 16
May, 2008

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ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude and profound indebtedness to my supervising guide, Dr. Rosamma Philip, Senior Lecturer, Department of Marine Biology, Microbiology and Biochemistry, School of Ocean science and technology, Cochin University of Science and Technology for her inspiring guidance, valuable suggestions, intellectual criticisms and unfailing help and moral support. I believe that it is a privilege indeed to work under her guidance and I have been truly rewarded.

It is my pleasure to express my sincere thanks to Dr. A.V. Saramma, Head, Department of Marine Biology, Microbiology and Biochemistry for all her valuable help and support throughout the period.

My thanks are due to Prof. (Dr.) K.T. Damodaran, Director, School of Ocean Science and Technology, CUSAT for allowing me to utilise the facilities. Thanks are due to Dr. K. Mohankumar, Dean, Faculty of Marine Sciences for all the valuable suggestions and motivation.

I wish to record my sincere thanks to Prof. (Dr.) I.S. Bright Singh, Dean and Director, School of Environmental Studies for his valuable suggestions, guidance and also for the facilities provided.

I thank Prof. (Dr.) Babu Philip, Prof. (Dr.) Aneykutty Joseph, Dr. A. A. Mohammed Hatha and Dr. Bijoy Nandan, Faculty members of the Department of Marine Biology, Microbiology and Biochemistry for all their help, suggestions and support.

Unfailing support and blessings received from my teachers Dr. N.R. Menon (Emeritus Professor), Dr. R. Damodaran, Dr. K.Y. Mohammed Salih, Dr. V.J. Kuttiyamma, Dr. K. J. Joseph and Dr. C.K. Radhakrishnan helped me in achieving the goals throughout the period of my study.

I would like to express my deep sense of gratitude to Dr. Winny Varghese, Principal, Mar Athanasius College of Arts and Science, Kothamangalam for his encouragement and moral support.

My deep sense of gratitude to Prof. (Dr.) A. Mohandas, Emeritus Professor, School of Environmental Studies for his timely help and valuable suggestions.

I thank Dr. P. A. Lokabharathi, Scientist F, NIO, Goa for her encouragement and valuable suggestions in this endeavour.

I express my sincere thanks to Dr. Nandini Menon for her timely help and moral support in this endeavour. Thanks are due to Dr. Najmudeen T. M. and Dr. Soja Louis for their valuable suggestions and advice.

Dr. T. P. Sajeevan, Mr. Harikrishnan E., Mr. Harish R., Dr. Pramod P. K., Mr. Sunesh Thampy and Dr.Sincy Joseph always rendered help and moral support in each and every step of this research programme.

I am also thankful to Dr. Valsamma Joseph and Dr. Maya Paul for their sisterly affection and moral support.

I express my heart-felt thanks to all my friends Mr. Nilof S. Pasha, Mr. Abdul Jaleel, Ms.Remya Varadarajan, Ms. Prabha Pillai, Ms. Jisha V.K., Ms. Simi Joseph P., Ms. Smitha C.K., Mrs. Sini P.J., Ms. Jisha Jose, Ms. Jini Jacob, Ms. Pavitha K.R., Ms. Beena S John, Ms. Bindya Bhargavan, Mr. Kesavan,K., Ms. Seena Jose, Ms. Priyaja P., Ms. Vrinda S., Mrs. Annies Joseph, Mrs. Smitha S.L., Mrs. Smitha V. Bhanu, Ms. Manjusha K, Mrs. Lakshmi G. Nair, Ms. Swapna P. Antony, Mrs. Sreedevi N.Kutty, Mrs. Anila T.N., Mr. Hari Sankar H.S., Mr. Abhilash K.R., Mr. Manoj, Mr. Anit, Mr. Padmakumar, Mr. Sanilkumar, Mr. Akhilesh K.V. Mr. Prabhakaran M.P. and all the research scholars of the Dept. of Marine Biology and National Centre for Aquatic Animal Health for giving me all the support throughout the work.

I am thankful to my colleagues Mr. Paul George, Dr. Aby P. Varghese, Mrs. Breezy Jacob, Ms.Jitha Joseph, Ms.Deepa G, Mrs.Asha G, Mrs. Jaya V.L., Ms. Sreedevi C.D.,Ms. Tinu Ebraham, Dr. Aji C. Panikkar and Mrs. Nikitha Divakaran for their personal encouragement and help.

I gratefully acknowledge the untiring support, timely help and brotherly affection rendered by Mr. Neil Scolastin Correya and Mr. Anilkumar P.R. throughout the period.

I thank my classmates Mr. Rejishkumar V. J., Mr. Sreedharan K., Mr. Nousherkhan, Mrs. Anupama Nair, Mrs. Roja Sebastian and Ms. Sreeja Mol K.L. for their care and moral support.

I take this opportunity to thank my senior colleagues Dr. Beatrice Amar, Dr.Biji Mathew, Dr. Sarlin P.J. and Dr. Meera Venugopal for their advices and support.

Thanks are due to all the people at Njarakkal and Puthuvypu shrimp farms for providing me with specimens.

I express my sincere thanks to all the administrative staff of the Dept. of Marine Biology, Microbiology and Biochemistry, CUSAT.

I acknowledge with deep sense of gratitude, the love and blessings of my father, mother, sisters and brother-in-laws that has played a great role in the successful completion of this work.

My sincere thanks to all the inmates of CUMS hostel for their inspiring friendship and care.

I express my sincere gratitude to University Grants Commission, India for providing me research fellowship through CSIR-UGC examination.

Above all I thank God Almighty for leading me through the trials towards this accomplishment.

Selven S.

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Chapter 1

GENERAL INTRODUCTION

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1.1 Aquaculture

Aquaculture has developed to become one of the fastest growing food producing sectors in the world. The Food and Agricultural Organisation (FAO) of the United Nations (UN) acknowledge that global fishery out put must be increased by at least 50% to offset projected shortfalls in dietary protein by 2030. Developing countries contribute a substantial amount (over 87%) of the total production, out of which Asian countries alone contributed 90.1%. Presently, traditional fisheries and aquaculture jointly produce approximately 150 million tonnes.

According to FAO, the annual world production is over 8 million metric tonnes. Today India is one among the major shrimp producing countries in the world. The export of marine products from India set an ever time record of 6,12,641 tonnes of value of Rs. 8363.53 Crores and USD1852.93 million during 2006-07(http://www.mpeda.com). In terms of export earnings, frozen shrimp continued to be the largest export item (54% in value and 22% total export quantity)(http://www.mpeda.com).

1.2 Shrimp culture practices

There are extensive and intensive shrimp culture practices. In extensive shrimp culture, shrimps are stocked at low densities (< 25 PLs m^{-2}) in large ponds or tidal enclosures in which little or no management is exercised or possible. Farmers depend almost entirely on natural conditions in extensive cultures. Intensive shrimp culture is carried out in high densities (>200 PLs m^{-2}). Much of the world shrimp production still comes from extensive culture.

1.2.1 Shrimp diseases

There is a growing demand for fish and marine products for human and animal consumption. This demand has led to rapid growth of aquaculture, which some times has been accompanied by ecological

impacts and economic loss due to diseases. The expansion of shrimp culture always accompanies local environmental degradation and occurrence of diseases. Disease out breaks is recognised as a significant constraint to aquaculture production. Environmental factors, water quality, pollution due to effluent discharge and pathogenic invasion due to vertical and horizontal transmission are the main causes of shrimp disease out breaks. Nutritional imbalance, toxicant and other pollutants also account for the onset of diseases.

Shrimp pathogens include viruses, bacteria, fungi and parasites. Viruses are the most economically significant pathogens of the cultured shrimps world wide. Among the 20 known shrimp viral pathogens, white spot syndrome virus (WSSV) is one of the most important and it causes massive production loss in all shrimp growing countries. Diagnostic methods for the rapid detection of aquatic diseases have improved to a higher extent with the aid of recent biotechnological tools, but at the same time treatment of the infected stock is still lagging behind, especially the viral diseases. Therefore proactive disease management measures are to be taken to reduce the risk factors in aquaculture.

1.2.2 Disease control in shrimp aquaculture

Disease control in shrimp aquaculture should focus first on preventive measures for eliminating disease promoting factors. In order to design prophylactic and proactive measures against shrimp diseases, it is mandatory to understand the immune make up of the cultivable species, its optimum culture conditions and the physico chemical parameters of the rearing environment. It has been proven beyond doubt that disease is an end result of complex interaction of environment, pathogen and the host animal. The aquatic environment is abounded with infectious microbes. The transmission of disease in this environment is extremely easy, especially under dense, culture conditions. Therefore, a better

understanding of the immune responses of the cultured animal in relation to its environmental alterations and microbial invasions is essential in devising strategic measures against aquaculture loss due to diseases.

1.3 Shrimp immune system

Shrimps have a semi-open circulatory system. Haemolymph moves through a series of arteries to various organs and from there into the interstitial spaces of the haemocoel (body cavity) and subsequently to the gills (for oxygenation) and back to the heart for distribution (Cameron and Magnum, 1983). The fluid within the system is called haemolymph because there is no separation between the circulatory and lymphatic system in crustacea (Martin and Hose, 1992). The hard cuticle forms structural and chemical barrier to parasites. Cellular and humoral factors play a key role in the defence reactions in shrimp. The important immune responses including clotting, non-self recognition, phagocytosis, melanisation, cytotoxicity and cell-to-cell communication has been brought about by haemocytes (Soderhall, 1999).

1.4 Cellular Immunity

1.4.1 Cellular components

The circulating haemocytes play an extremely important role not only by direct sequestration and killing of infectious agents, but also by synthesis and exocytosis of an array of bioactive molecules. Essentially the heamocytes execute an important role in cellular responses, including clotting, non-self recognition, phagocytosis, melanisation, encapsulation, cytotoxicity, cell-to-cell communication, production of reactive oxygen metabolites and the release of microbicidal proteins.

1.4.1.1 Haemocytes

Three different types of shrimp haemocytes have been distinguished based on the quantity and sizes of the granules contained within; Hyalinocytes (agranulocytes), semigranulocytes and granulocytes

(Martin and Graves, 1985). In decapods, haemocytes are produced within specialised haematopoietic tissue (HPT), the location and architecture varies greatly, even within close taxonomic groups. In penaeid shrimps, haematopoiesis is believed to occur in paired epigastric nodules consisting of an extensive network of vessels derived from ophthalmic artery. Morphological variations of cells in the haematopoietic tissue of penaeid shrimps have been shown by van de Braak *et al.*, (2002). The mechanism by which maturing haemocytes are released into circulation is not clear. In shrimps, haemocytes migrate into the lumen of the haematopoietic tubule, which is continuous with the ophthalmic artery (Martin *et al.*, 1987).

Hyaline cells in most decapod crustaceans are characterized by the absence of granules, although some cytoplasmic inclusion bodies have been reported by electron microscopic observations (Martin and Graves, 1985) and are capable of phagocytosis (Smith and Soderhall, 1983). In penaeid shrimp *Penaeus paulensis* it accounts for 41% of the total circulating haemocytes (Gargioni and Baracco, 1998).

The semigranular cells, which contain small granules and display some phagocytic capacities, are specialized in particle encapsulation (Persson *et al.*, 1987). Semigranular cells can respond to microbial polysaccharides such as lipopolysaccharides and β-1,3-glucan by degranulation process (Johansson and Soderhall ,1985). The same cell has the capacity to encapsulate foreign particles (Persson *et al.*, 1987).

The granular haemocytes are filled with large granules. They do not show phagocytic activity and they will not respond to the microbial polysaccharides directly unless they are pre-treated with some haemolymph proteins called pattern recognising proteins (PRP). The main function of these granular haemocytes is to store prophenoloxidase activating system (proPO system), which plays a key role in the defence reaction of crustaceans. The granular cells can be triggered to undergo

exocytosis and subsequent release of proPO system from the granules (Barracco *et al.,* 1991).

1.4.2 Cellular immune mechanisms

1.4.2.1 Phagocytosis

Phagocytosis is the most common of the cellular defence reactions and together with humoral components constitutes the first line of defence. Phagocytosis is comparatively inefficient in the absence of opsonins, the co-factors that coat microorganisms and enhance the ability of phagocytes to engulf them (opsonisation). Studies in fresh water crayfish and lobster have revealed the presence of some opsonins in the haemolymph, which enhances phagocytosis (Tyson and Jenkin, 1974).

1.4.2.2 Nodule formation

When a large number of microorganisms enter the body, nodule formation or cell clumping occurs. These microorganisms entrapped in several layers of haemocytes, get melanised heavily. Such aggregates have been observed in the gill vasculature of penaeid shrimp *Sicyonia ingentis* (Martin *et al.*,1993). However, in other crustaceans haemocyte agglutinations (nodule) have been reported to be dispersed throughout the body as well as in the antennal gland, the heart and the gill (Bauchau, 1981; Johnson *et al.*, 1981). Nodule formation is not an isolated event but occurs in conjunction with phagocytosis and other immune responses to affect a highly efficient clearance mechanism capable of dealing with pathogens. Mode of killing within the nodules is unknown but may involve melanin production and its toxic precursors, lysozyme or release of other enzymes.

1.4.2.3 Encapsulation

In addition to nodule formation and phagocytosis, invertebrate blood cells are capable of immobilizing parasites, which are too large to be ingested by a single blood cell by surrounding them with multi cellular

sheaths. In crustaceans the only cells to react to foreign molecules like β-1,3-glucan from fungi or lipo-polysaccharides (LPS) from bacteria are the semi granular cells. This cell is also the first one to react to foreign particles and to encapsulate any invading pathogens. Some opsonin factors present in the haemolymph can also mediate the encapsulation process. The exact process of encapsulation is yet to be understood.

1.5 Humoral immunity

Humoral factors are primarily non-self recognition factors that include a variety of defensive enzymes, lectins, lipoproteins, antimicrobial peptides and reactive oxygen intermediates.

1.5.1 Lectins

Lectins have been regarded as potential molecules involved in immune recognition and phagocytosis of microorganisms through opsonisation. They are non-enzyme proteins or glycoproteins without catalytic activity that binds to specific carbohydrates expressed on different cell surfaces. These types of carbohydrate binding proteins, which recognize surface structures common for different pathogens, represent a primitive immune response and are called pattern recognition proteins (PRP). Some lectins act as opsonins and bind to foreign particles that facilitate their removal by phagocytosis (Marques and Barracco, 2000). The PRPs recognize targets such as lipopolysaccharides (LPS) or peptidoglycan from bacteria, and β-1,3-glucans or mannans from fungi. Several PRPs recognizing β-1,3-glucans have been found in arthropods.

1.5.2 The pro Phenoloxidase system

The best-studied enzymatic system of crustaceans is phenoloxidase cascade (Sritunyalucksana and Soderhall, 2000). This enzyme is a part of complex system of proteinases, pattern recognition proteins and proteinase inhibitors constituting the so called prophenoloxidase (proPO)

activating system. It is proposed to be non-self recognition system because conversion of prophenoloxidase to active enzyme can be brought about by miniscule amounts of molecules such as LPS, peptidoglycan and β-1,3-glucan of microbial cell wall. Several components of this system have been isolated and their structures determined. Phenoloxidase (monophenyl L-dopa: oxygen oxidoreductase; EC1.14.18.1) catalyses the oxidation of phenols to quinones followed by several intermediate steps that lead to the production of melanin, a brown pigment. During the formation of melanin, toxic metabolites are formed which have microbicidal activities (Soderhall *et al.*, 1990).

Microbial polysaccharides, like LPS or β-1,3-glucan can mediate the activation of these inactive serine protease to active form, which in turn activate the inactive proPO into active phenoloxidase. Phenoloxidase then oxidises the phenolic group containing amino acids (tyrosine) into semiquinones, which have microbicidal action, and these semiquinones are polymerized into melanin (Cerenius and Soderhall, 2004). Melanisation is involved in the process of tanning of cuticle during the post-moult period in wound healing and in defence reactions (encapsulation of invading microorganisms). This pigment can be recognized as dark brown spots in the cuticle of shrimps that have been injured.

Together with the activation of proPO, another important component of proPO system gets activated. That is a 76 KDa protein that mediate and enhance cell adhesion and degranulation (Johansson and Soderhall, 1989). This is a multifunctional immune factor, which also promotes encapsulation and function as a phagocytosis stimulating opsonin (when released together with the molecules of the proPO system). Molecular characterisations of this 76 KDa protein were done and it revealed that they belong to the family of peroxidases (Johansson *et al.*, 1995).

1.5.3 Antimicrobial peptides

Antimicrobial peptides are widespread in the living kingdom, and a large number of these molecules have been isolated from vertebrates and invertebrates. Three antimicrobial peptides have been isolated and characterized from *Penaeus vannamei* and named penaeidins (Destoumieux *et al.*, 1997, 2000) and recent results show that these peptides are ubiquitous in crustaceans. These peptides are often broad spectrum in nature and probably act against many infectious agents. They showed activity against the shrimp fungal pathogen, *Fusarium oxysporum* and also to some Gram-positive bacteria (Destoumieux *et al.*, 1997). They are classified into three distinct groups based on amino acid sequences, secondary structure and functional similarities (Bachere, 2003). The first and large group is composed of peptides stabilised by intramolecular disulphide bonds, and the other two groups are linear peptides and polypeptides characterized by (1) α-helical structure or (2) a high content of proline residues and/or a high percentage of glycine residues. The haemocytes are found to be the site of production and storage of these peptides. Degranulation of the haemocytes by stress or pathogenic invasion can lead to the release of these peptides into the haemolymph. In most cases, anti-microbial peptides were shown to disrupt microbial membrane by a pore forming action or by a detergent effect.

1.5.4 Reactive Oxygen Intermediates (ROI)

This response termed as the respiratory burst, is an aerobic process, which generates highly reactive oxygen species such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl ions (OH^-) and singlet oxygen (O_2^1) (Reactive Oxygen Intermediates or ROI). In *P. monodon*, production of ROI has been induced by immunostimulants like β-glucan and zymosan, which confers enhanced protection against bacterial or viral infections (Song and Hsieh, 1994).

1.6 Antioxidant defence system of shrimps

Recently it has been reported that WSSV infection affects the antioxidant defence system in shrimps with an increase in lipid peroxidation and a reduced activity of antioxidant enzymes in *Fenneropenaeus indicus* (Mohankumar and Ramasamy, 2006) and in *Penaeus monodon* (Rameshthangam and Ramasamy (2006) and Mathew *et al.*, 2007).

In a normal cell, there is an appropriate prooxidant : antioxidant balance (Winston and Di Giulio, 1991). However, this balance can be shifted towards the prooxidant when production of oxygen species is increased under stress conditions or when levels of antioxidants are diminished. This state is called oxidative stress and can result in serious cell damage if the stress is massive or prolonged. Antioxidant defence mechanism is operated to detoxify or scavenge highly reactive oxygen intermediates (ROI). It comprises of different types of functional components classified as first line, second line and third line of defences.

Cells convene substantial resources to protect themselves from the potentially damaging effects of reactive oxygen species. Several vitamins and micronutrients, which are active at quenching these free radical intermediates or required for their enzymic detoxification, as well as the enzymes, such as superoxide dismutases (SOD), glutathione peroxidases (GPx) and catalases (CAT), constitute a first line of defence against ROI, and are generally referred to as primary antioxidants.

Superoxide dismutase mainly acts by quenching superoxide (O_2^-) and active oxygen radicals, produced in different aerobic metabolism. Catalase is a tetrameric enzyme, present in most of the cells, and act by catalysing the decomposition of H_2O_2 to water and oxygen. Glutathione peroxidase (GPx) is a selenium containing enzyme which catalyses the reduction of H_2O_2 and lipid

hydroperoxide (LO_2H), generated during lipid peroxidation, to water using reduced glutathione as substrate. In cytosolic and mitochondrial compartments the oxidised glutathione (GSSG) is reduced at the expense of NADPH by the ubiquitous flavin containing enzyme glutathione reductase.

The antioxidants belonging to second line of defence include glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E (mainly α-tocopherol), carotenoids, flavanoid and ubiquinol. Glutathione (GSH) is the most abundant non-protein thiol, synthesised in the liver and acts as a substrate for glutathione peroxidase enzyme. This also serves as a scavenger of different free radicals. Similarly β-carotene (Pro-vitamin A), vitamin C and vitamin E are some important scavenging antioxidant vitamins, which cannot be synthesised by most mammals including human beings, and therefore, are required from diet.

Third line antioxidants are a complex group of enzymes for repair of damaged DNA, damaged protein, oxidised lipids and peroxides and also to stop chain propagation of peroxyl lipid radical. e.g. Lipases, proteases, DNA repair enzymes, transferases, methionine sulphoxide reductases etc. The detection of oxidative stress has relied largely on the quantification of compounds such as conjugated dienes, hydroperoxides as well as malondialdehyde (MDA), which are formed by the degradation of initial products of free radical attack.

Most of the studies on oxidative stress in crustaceans are restricted to their presence as well as their action during exposure to xenobiotics (Le Moullac and Haffner, 2000; Song and Hsieh, 1994; Arun *et al.*, 1999). However, there are reports emphasizing the alterations in the antioxidative defence profile of crustaceans in response to environmental parameters like temperature (Parihar *et al.* (1996, 1997 and Roche and Boge, 1996) ammonia-N concentration (Wang *et al.*, 2006) and salinity (Roche and Boge, 1996). Very Recently, Liu *et al.* (2007) have shown that a wide

change in salinity (from 30‰to 5‰and 50‰) is found capable of generating severe oxidative stress in shrimp, *Litopenaeus vannamei*

1.7 Rationale of haematology in shrimp disease management

Study on the haemolymph parameters of penaeid prawns is of paramount importance in understanding the physiology and immune status of the animals. Haemolymph proteins play an important role in the immune mechanism of penaeid prawns. There are diverse types of proteins in the haemolymph of shrimps, which are located in the haemocytes and plasma. Various studies have shown that these proteins are of great significance in reflecting environmental alterations and microbial infections of the shrimps. They can also be considered as the supreme indicators of alterations prevailing in the ambient environment of the organisms. It is known that disease outbreaks are not just a matter of pathogenic organisms being present. They are the result of complex interactions between the pathogen, the host and the environment. Host must be susceptible to a pathogen and this is affected by genetics, nutritional status, age and the presence of the pathogen at the earlier life stages, the physiological state of the host, the presence and effect of stressors on the host and the quality of the environment that the animals are being reared in. In this context, the present study was undertaken with the following objectives

a) To understand the effect of alterations in salinity on haematological responses and susceptibility of *Fenneropenaeus indicus* to microbial infections

b) To assess the modulatory effect of heavy metal stress on haematological responses and susceptibility of *F.indicus* to infection

c) To understand the effect of an immunostimulant on the haematological responses and susceptibility of *F.indicus* to infections.

d) To identify the most reliable haematological health indicators in *F. indicus*

First chapter presents a general introduction of the topic. The results of the present study are presented in eight chapters. Haematological profile of *Fenneropenaeus indicus* acclimated to varying salinity levels is presented in chapter 2. Third chapter deals with the haematological responses and susceptibility of *Fenneropenaeus indicus* held at different salinity levels to *Vibrio harveyi* under experimental conditions. Immunological profile of *Fenneropenaeus indicus* under acute salinity stress and its susceptibility to white spot syndrome virus (WSSV) infection is presented in chapter 4. The fifth chapter deals with the antioxidative defence profile of *Fenneropenaeus indicus* subjected to salinity stress and challenged with WSSV. In chapter 6, the modulatory effect of Copper on the haematological responses of *Fenneropenaeus indicus* is presented. Chapter 7 deals with the immunological variations of *Fenneropenaeus indicus* in response to glucan administration and its susceptibility to WSSV. In chapter 8, the multiple regression and correlation analysis for identifying reliable health markers for *Fenneropenaeus indicus* is presented. This is followed by summary, list of references and appendix.

Chapter 2

EFFECT OF AMBIENT SALINITY ON THE HAEMATOLOGICAL PROFILE OF *FENNEROPENAEUS INDICUS*

Contents

2.1 Introduction

Salinity is an important water quality parameter in aquaculture and is one of the main environmental factors that wield selective pressure on aquatic organism (Charmantier and Charmantier-Daures, 2001). A considerable change in the salinity may bring significant physiological responses among aquatic species and it stands alone as the most intensively studied environmental parameter in penaeid shrimp biology. Salinity affects the growth and survival of penaeids, (Raj and Raj, 1982) food consumption, conversion efficiency, growth and survival (Venkataramaiah *et al.*, 1972; Staples and Heales, 1991). Studies with juvenile Indian white shrimp, *F. indicus,* revealed that this species is capable of osmoregulating at salinities of between 3 and 40‰ and *F. indicus* grows well at 25‰ (780mOsm/kg) (Parado-Estepa *et al.*, 1987; Diwan and Laximinarayana, 1989). It has been suggested that the maximum growth of an organism occurs in iso osmotic media, since the animal would be expending the minimal amount of energy in osmotic regulation (Panikkar, 1968).

Mass mortality of the stock may occur quite frequently, due to adverse environmental conditions. Euryhaline shrimps adapt physiologically to the alteration in the surrounding medium. These changes are most obviously manifested in the composition of the haemolymph (Pequeux, 1995).

In shrimps the internal medium; the haemolymph reflect its physiological status as well as environmental fluctuations through the up and down regulation of its components. Thus, haemolymph composition of shrimps will provide an indication about the physiological modifications associated with molting process, developmental stages, defence mechanism and environmental stress. Haemocyanin and other haemolymph proteins and the acid base balance of crustaceans were

found to be altered by exchanges in ambient salinity, temperature, dissolved oxygen (Ferraris *et al.*, 1986), ammonia-N (Chen and Cheng, 1993), nitrite-N (Chen and Cheng, 1995) and saponin (Chen and Chen, 1996).

Comprehensive data denoting the immune status of *F. indicus* with respect to the ambient salinity is scanty. Therefore, the present study was aimed to establish a base line information on the haematological profile of *F. indicus* acclimatized to different salinity levels (0,5,10,15,20,25,30 and 35‰) considering 25‰ as the optimal salinity for *F. incdicus.* It was also aimed to understand the salinity tolerance of the organism in terms of the haematological variations at different levels of salinity.

This chapter deals with the haematology of *F. indicus* acclimated to different salinity levels at experimental rearing conditions.

2.2 Materials and Methods

2.2.1 Experimental animals and rearing conditions

Adult *Fenneropenaeus indicus* (Fig.2.1) were brought to the Laboratory within one hour of capture from a commercial shrimp farm located at Panangad, Kochi, India. The average wet weight of the shrimp was 15.24±1.68g (Mean±SD). Shrimps were reared in rectangular concrete tanks containing 25‰ clean seawater and allowed to acclimate for a period of seven days. Continuous aeration was provided using air pumps and was maintained on a commercial shrimp diet (Higashimaru, Pvt. Ltd., Kochi). Water quality parameters *viz.* temperature, dissolved oxygen, NH_3-N, NO_2-N and NO_3-N were monitored daily following standard procedures (APHA, 1995) and maintained at optimal levels as per Table 2.1. Unused feed and faecal matter was siphoned out daily and 30% of water exchanged every alternate day. A biological filter was set up to maintain the appropriate levels of water quality parameters.

Fig. 2.1 *Fenneropenaeus indicus*

2.2.2 Experimental Design

The shrimps were distributed in the experimental tanks (Fig. 2.2) containing 500L of sea water (n=35). Shrimps in the intermolt stage alone were used for the experiment (Robertson *et al.*, 1987). There were two groups (Group1 and Group 2) and the experiment was conducted in triplicate *i.e.*, 3 tanks per treatment. Salinity of the Group 1 was maintained at 25‰ and that of the Group 2 was adjusted to 35‰.

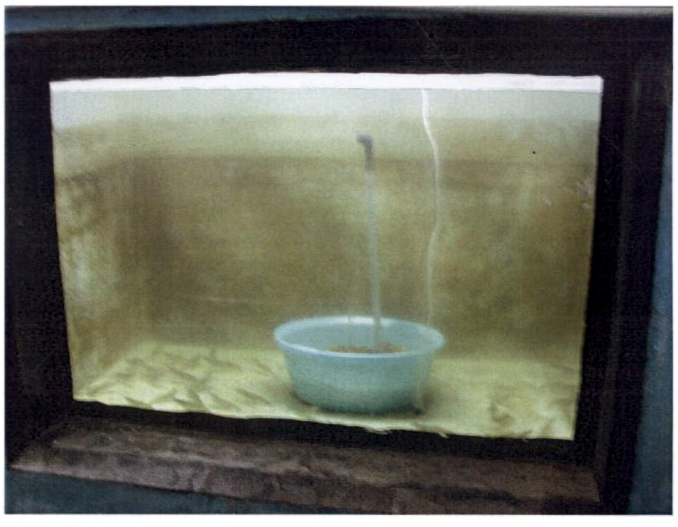


Fig. 2.2 Experimental set up used to study the haematology of *F. indicus*

Table. 2.1. Rearing conditions and water quality.

Animal used	*Fenneropenaeus indicus*
Size of animal	15±1.68 gm
Stocking density (per tank)	35
Tank Capacity	500L
Feeding level	5-10% body weight.
Feeding frequency	Twice daily.
Feeding period	7 days.
Water temperature	25-27°C
pH	7.5- 8.0
NH_3-N	0.01-0.02 ppm
NO_3-N	below detectable
NO_2-N	0.00-0.01 ppm
Dissolved Oxygen	6-7 mg/L

Group 2 shrimps maintained at 35‰ were used for salinity treatment and the Group 1 shrimps held at 25‰ were treated as the control group. After one-week acclimation at these salinity levels, six shrimps were randomly sampled from the control (G1) and the salinity treatment (G2) groups. In order to avoid any effects due to food material, the shrimps were starved before twelve hours of each sampling (Hall and van Ham, 1998). Subsequently the salinity of the Group 2 shrimps was reduced to 30, 25, 20, 15, 10, 5 and 0‰ giving one-week time for acclimation to each salinity level and six shrimps were sampled at each time. The control group remained at 25‰ throughout the experimental period and shrimps were sampled from the control group as well.

2.2.3 Extraction of haemolymph

Haemolymph was withdrawn aseptically from the rostral sinus of the shrimps (Fig. 2.3.) using a specially designed sterile capillary tube having a diameter of 0.5mm. The samples were transferred to sterile microcentrifuge tubes containing measured quantity of pre-cooled

anticoagulant (0.02M sucrose, 0.01M tri-sodium citrate in 0.01M Tris-HCl, pH 7.6) (Song and Hsieh, 1994). Serum was obtained by keeping the haemolymph at room temperature without anticoagulant. This is allowed to clot and then centrifuged at 1700 x g for 10minutes in a cooling centrifuge to get the serum. A fraction of haemolymph (0.1ml) was immediately centrifuged at 600 x g for 15minutes to separate plasma and haemocytes. Samples from six shrimps (n=6) were analyzed separately.

Fig. 2.3 Extraction of haemolymph from the rostral sinus of *F. indicus* for haematological analysis

2.2.4 Haematolgical analysis

Haemolymph biochemical parameters *viz.*, total haemolymph protein (THP), plasma protein (PLP), serum protein (SRP), haemocyte protein (HCP) and total free amino acids (TFAA) were determined spectrophotometrically employing standard techniques and expressed as

mg ml^{-1} haemolymph. The Immune assays *viz.* phenol oxidase, NBT assay, alkaline phosphatase and acid phosphatase were also estimated spectrophotometrically.

2.2.4.1 Estimation of Total Haemolymph Protein

Bradford Method (1976) was used for the estimation of haemolymph proteins. Bovine Serum Albumin was used as the standard. To a volume of 0.1ml haemolymph 1.9 ml of 80% ethanol was added and centrifuged at 1500-x g for 10 minutes. The pellet was dissolved in 1ml of 1N NaOH and used for total haemolymph protein estimation.

2.2.4.2 Estimation of Plasma Protein

Haemolymph (0.1ml) was centrifuged at 350 x g for 15 minutes. The supernatant was treated as plasma and the protein was estimated as per the above procedure.

2.2.4.3 Estimation of Haemocyte Protein

The pellet received after removing the plasma was washed with Phosphate Buffed Saline twice, dissolved in 1 ml of 0.1N NaOH, and used for protein estimation.

2.2.4.4 Estimation of Serum Protein

Haemolymph withdrawn from the shrimps was allowed to clot in vials for 30 minutes. Then the clotted haemolymph was centrifuged at 2200 x g for 15 minutes. The clot free liquid was treated as serum for estimation.

2.2.4.5 Estimation of Total Free Amino Acids

The Ninhydrin method (Yemm and Cocking, 1955) was adopted for the estimation of total free amino acids in the haemolymph. A combined standard of glycine and glutamic acid was prepared, 1ml each contained 0.006mg amino nitrogen, which is equivalent to 0.04758mg amino acid per ml. Haemolymph (50µl) was taken in a clean micro centrifuge tube and

950µl deproteinising agent (80% ethyl alcohol) was added. The precipitate was subjected to centrifugation at 2200 x g for 5min. and the supernatant was decanted. To 1ml supernatant 0.5ml, 0.2M Citrate buffer (pH 5) was added. Then 1.2 ml of freshly prepared 1% ninhydrin solution in methyl cellosolve was added. After that, the reaction mixture was heated in a boiling water bath for 15 min and cooled in running tap water for 15min. Then 2.3 ml of 60% ethyl alcohol was added. The optical density of the purple colour developed was measured at 570nm against the blank.

2.2.4.6 Phenoloxidase (PO) Activity

Phenoloxidase activity of haemolymph was measured spectrophotometrically by using L-3, 4-dihydroxyphenylalanine (L-DOPA) as substrate according to Soderhall (1981). Briefly, 100µl of haemolymph was mixed with 100µl of 1% SDS for three minutes at 25°C. Then 1 ml of L-DOPA was added to the haemolymph. Increase in absorbance at 495 nm was measured for 3 min with an interval of 30 sec. using a UV-Visible Spectrophotometer (Hitachi. U-2001). L-DOPA with distilled water was used as blank. Enzyme activity was expressed as increase in absorbance per minute per ml haemolymph.

2.2.4.7 Superoxide anion (NBT reduction) assay

Respiratory burst activity of haemocytes was measured spectrophotometrically as per the method described by Song and Hsieh (1994) with minor modifications. Nitroblue Tetrazoluim Chloride (SRL Chemicals, India) was used as substrate that gives a blue formazan colour due to its reduction by superoxide anion produced during phagocytosis of heamocytes. Haemolymph (100µl) was taken into a micro centrifuge tube and 100µl NBT solution (2mg/ml) prepared in Tris-HCl buffer (pH 7.6) were added to it and incubated at room temperature for 30min. Tubes were centrifuged at 300 x g for 10 min. in a cooling centrifuge. The supernatant was discarded and the reaction was stopped by adding 1ml

absolute methanol followed by incubation for 10min. Spun down the tubes again, discarded the supernatant, and left the tubes for air-drying for 30min. The tubes were washed with 50% methanol for three times and a final washing was done using PBS of pH 7.6. 2M KOH (120µl) followed by 140µl dimethylsulphoxide (DMSO, SRL Chemicals) were added to the tubes. Finally, 2ml distilled water was added. The optical density at 620nm was recorded by using UV-Visible Spectrophotometer against a blank consisting of reagents and 2 ml distilled water and expressed as NBT activity per ml haemolymph.

2.2.4.8 Alkaline phosphatase

Alkaline phosphatase catalyses the hydrolytic cleavage of phosphoric acid esters and their pH optima lie in the alkaline pH range 9.0. Absorbance at 405nm was measured spectrophotometrically (Gonzalez *et al.*, 1994). Haemolymph (100µl) was added to 2 ml of 4- nitrophenyl phosphate substrate solution (0.5% in Glycine- NaOH buffer pH 9.0). The mixture was incubated at 37°C for 30min. at the end of the incubation period, the enzyme reaction was terminated by adding 2.9ml of 0.1N NaOH. The yellow coloured solution was read against a blank at 405nm. The blank was prepared by incubating a mixture of 2.9ml 0.1N NaOH and 2ml of substrate solution to which finally 100µl of haemolymph was added.

2.2.4.9 Acid phosphatase

ACP was estimated using the substrate, p- nitro phenyl phosphate as described in section 2.2.4.8. The buffer used was Citrate buffer (pH 5.0). Optical density measurement was done at 405nm in a UV-Visible Spectrophotometer (Gonzalez *et al.*, 1994).

2.2.5 Statistical analysis

One-way ANOVA was done followed by Duncan's multiple range test in order to determine the significant differences between means. All

the data were presented as mean±SD and differences were regarded as statistically significant when $p<0.01$.

2.3 Results

2.3.1 Total Haemolymph Protein (THP)

ANOVA showed that there was significant difference ($p<0.01$) in the concentration of total haemolymph protein of shrimps held at varying salinity levels as illustrated in the Fig. 2.4 and Table 2.2. of Appendix. Total haemolymph protein in 25‰ (control) salinity was found to be 90.16±11.03 mg/ml and showed variations in different salinity. An increase in THP was found in shrimps maintained at all other salinity. Maximum THP was obtained for shrimps maintained at 5‰ (141.19±6.35mg/ml) followed by THP in shrimps held at 10‰ (133.49±6.06mg/ml). At high salinity also (35‰) the shrimps showed comparatively higher THP than the control groups.

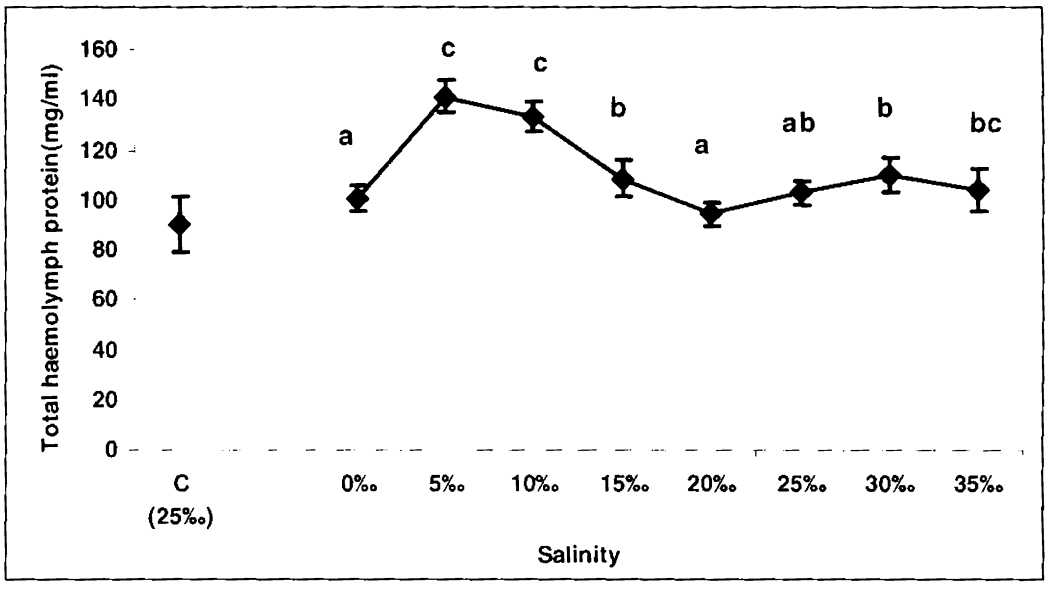


Fig. 2.4 Total haemolymph protein concentration of *F. indicus* held at different salinity levels. Each data represent mean±SD of six separate determinations. The lowercase letters shows significant variations in the THP concentration among different salinity levels at $p<.0.01$. Data with same lowercase letter do not vary significantly. C- Control.

2.3.2 Plasma Protein (PLP)

Significant variations of plasma protein concentration in shrimps held at different salinity levels were observed (Fig. 2.5 and Table 2.2. of Appendix). PLP ranged from 82.23±10.61mg/ml (control) to 130.52±6.24mg/ml (5‰). Considerable elevation in the plasma protein concentration was noted in shrimps held at lower salinity range *i.e.*, at 10‰ (122.19±5.4mg/ml) and at 5‰ (130.52±6.24mg/ml).

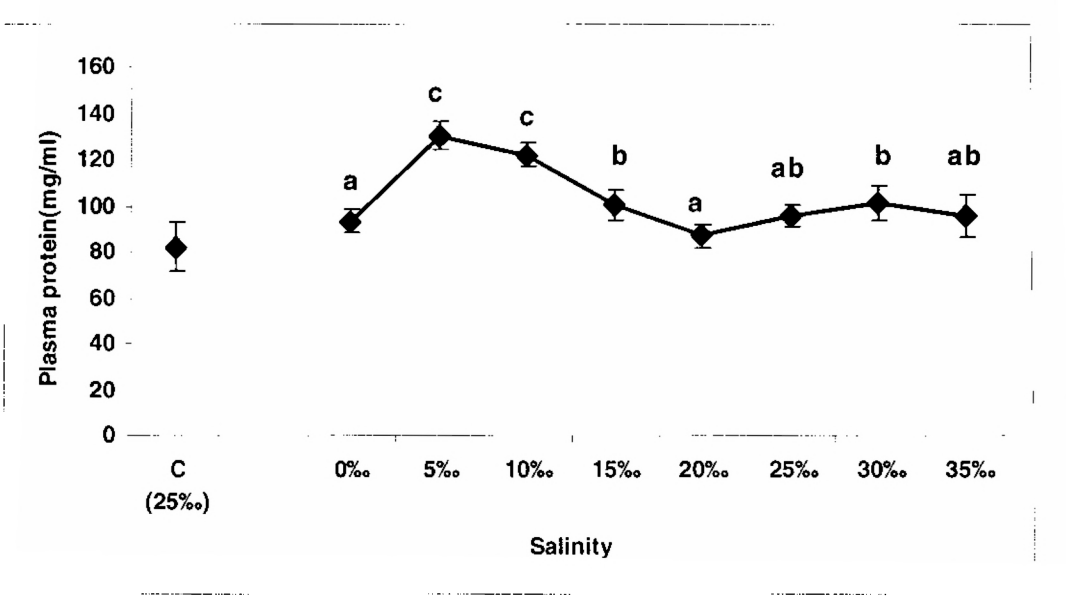


Fig.2.5 Plasma protein concentration (mean ± SD) of *F. indicus* held at different salinity levels (See Fig. 2.4 for statistical details).

2.3.3 Serum Protein (SRP)

The serum protein concentration of shrimps held at different salinities showed a similar trend as that of plasma protein concentration. Maximum SRP concentration was obtained in shrimps maintained at 10‰ salinity (96.18± 8.96mg/ml). Significant increase ($p<0.01$) in SRP was obtained in shrimps held at lower salinities when compared to control groups (Fig. 2.6 and Table 2.2. of Appendix).

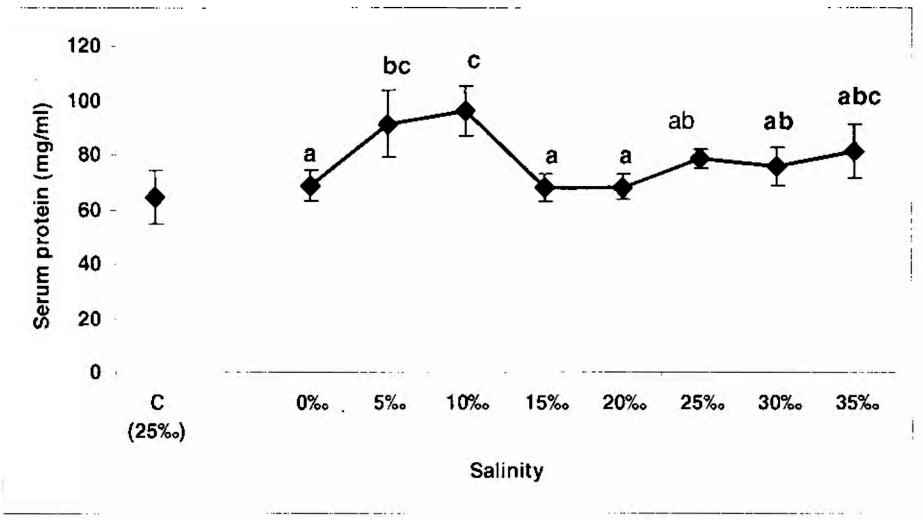


Fig. 2.6 Serum protein concentration (mean ± SD) of *F. indicus* held at different salinity levels (See Fig.2.4 for statistical details).

2.3.4 Haemocyte Protein (HCP)

Haemocyte protein concentration in the control group of shrimps was 8.94±0.99mg/ml and highest HCP was found in shrimps held at 5‰ (10.10±0.75mg/ml). There was remarkable difference ($p<0.01$) in the haemocyte protein concentrations of shrimps held at different salinity levels compared to control. At 0‰ HCP (6.25±0.33mg/ml) was lower than the control. Higher HCP concentration was obtained at 35‰ salinity (9.24± 1.14mg/ml) (Fig. 2.7 and Table 2.2. of Appendix).

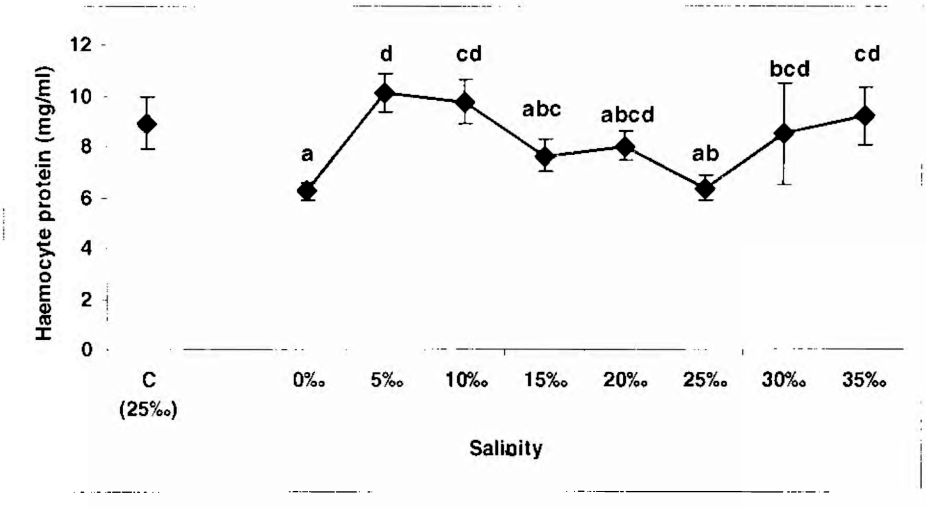


Fig. 2.7 Haemocyte protein concentration (mean ± SD) of *F. indicus* held at different salinity levels (See Fig.2.4 for statistical details).

2.3.5 Total Free Amino Acids (TFAA)

A significant variation ($p<0.01$) in the total free amino acids could be noted in shrimps held at different salinity levels compared to control. The control group of shrimps showed TFAA concentration of 5.64± 0.93mg/ml. TFAA in the haemolymph of shrimps was higher at lower salinity levels. Highest TFAA concentration was for shrimps at 0‰ salinity (17.36±1.63mg/ml). At higher salinity levels (30 and 35‰), the shrimps showed a slight increase in TFAA concentration (6.91±1.06mg/ml, 7.74±1.18mg/ml respectively) (Fig. 2.8 and Table .2.2).

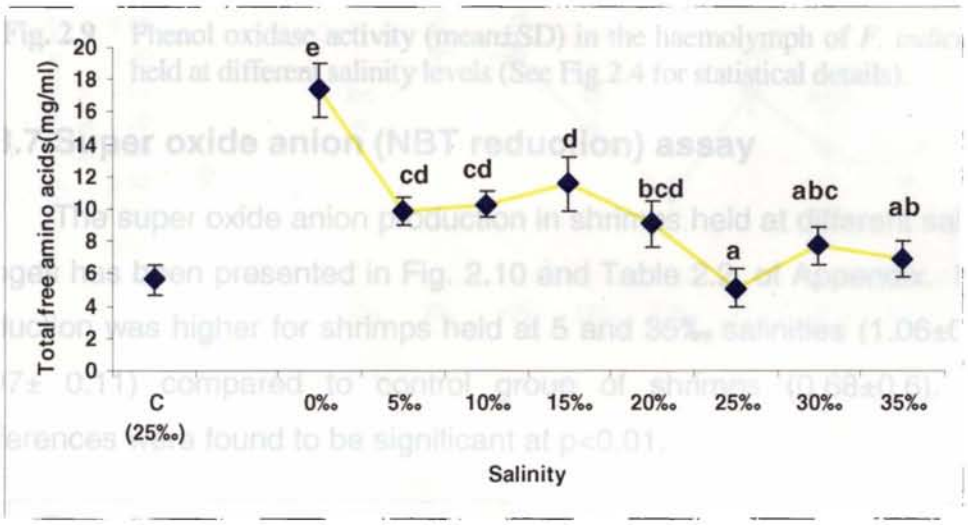


Fig.2.8 Total free amino acids concentration (mean ± SD) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.2.4 for statistical details).

2.3.6 Phenoloxidase (PO) Activity

Phenol oxidase activity of shrimps held at different salinity levels was significantly ($p<0.01$) different when compared to control group. The control group showed an activity of 0.33±0.02 OD/min. A considerable increase in the PO activity was observed in shrimps held at 0, 5, 10 and 15‰ salinity levels. Highest PO activity was found in shrimps held at 5‰ (0.86±0.11 OD/min). Generally there was an elevation in the phenol oxidase activity in shrimps held at lower levels of salinity (Fig. 2.9 and Table 2.2. of Appendix).

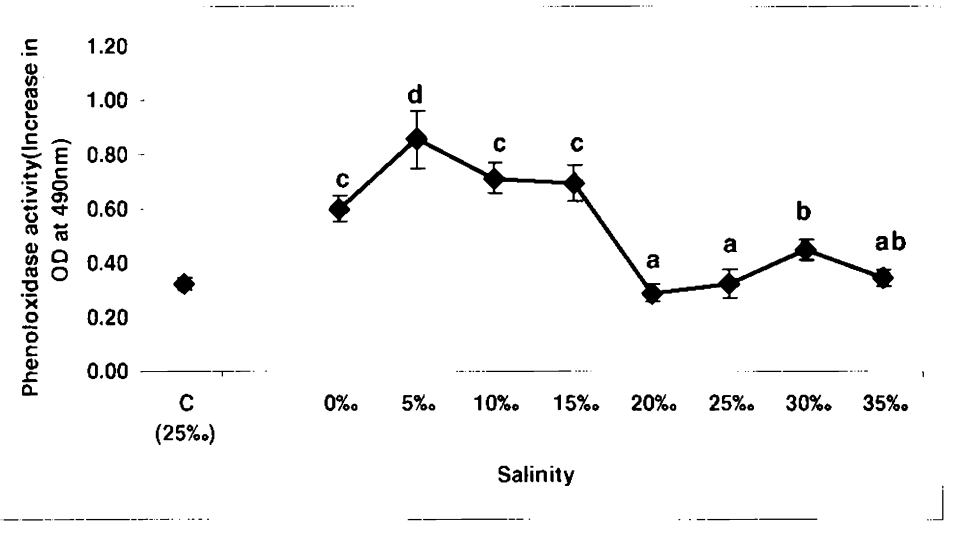


Fig. 2.9 Phenol oxidase activity (mean±SD) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.2.4 for statistical details).

2.3.7 Super oxide anion (NBT reduction) assay

The super oxide anion production in shrimps held at different salinity ranges has been presented in Fig. 2.10 and Table 2.2. of Appendix. NBT reduction was higher for shrimps held at 5 and 35‰ salinities (1.06±0.16, 0.97± 0.11) compared to control group of shrimps (0.68±0.6). The differences were found to be significant at $p<0.01$.

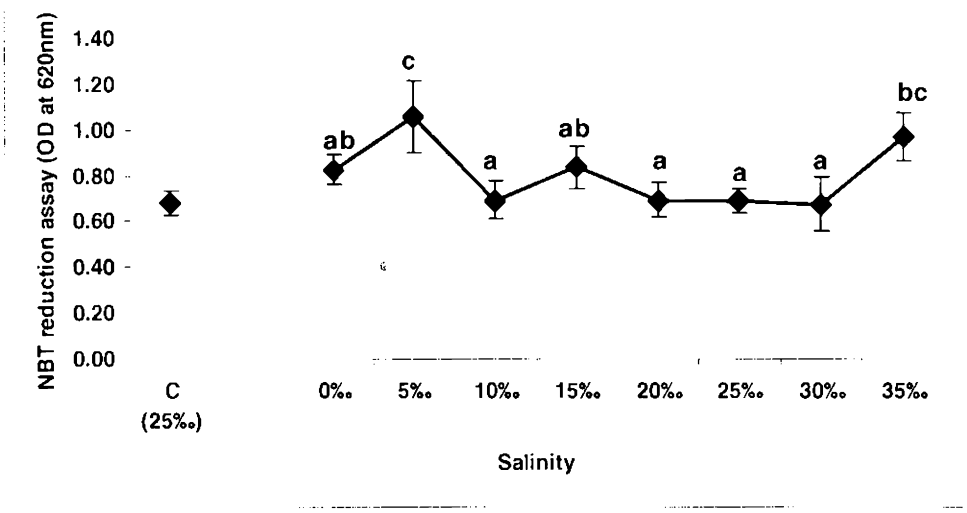


Fig.2.10 Intracellular super anion production (mean ± SD) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.2.4 for statistical details).

2.3.8 Alkaline phoshphatase (ALP) activity

Shrimps held at control salinity showed an alkaline phosphatase activity of (0.33±0.04mg/ml). An elevation in the ALP activity could be observed in shrimps held at lower salinity range (0‰ to 10‰). Significantly higher ($p<0.01$) ALP activity was found in shrimps held at 5‰ (0.72±0.14) followed by shrimps at 10‰ (0.71±0.07 mg/ml) (Fig. 2.11 and Table 2.2. of Appendix).

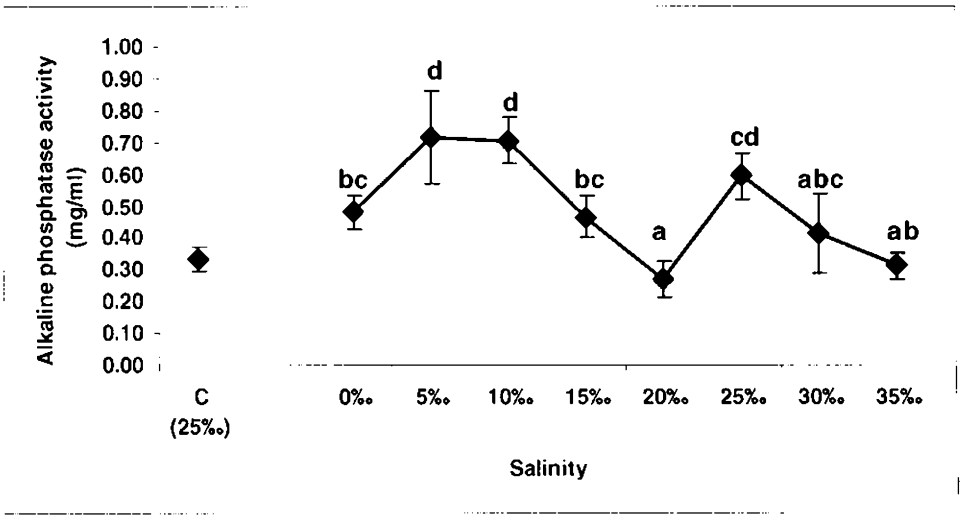


Fig. 2.11 Alkaline phosphatase activity (mean ± SD) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.2.4 for statistical details).

2.3.9 Acid phoshphatase (ACP) activity

Acid phosphatase activity was found to show an increasing trend from shrimps held at 5‰ to higher levels of salinity. Highest ACP activity was found in shrimps held at 35‰ (0.64±0.05mg/ml) compared to the control group at 25‰ (0.37±0.05mg/ml) ($p<0.01$). The variations in ACP activity was found to be significant at $p<0.05$ (Fig. 2.12 and Table 2.2. of Appendix).

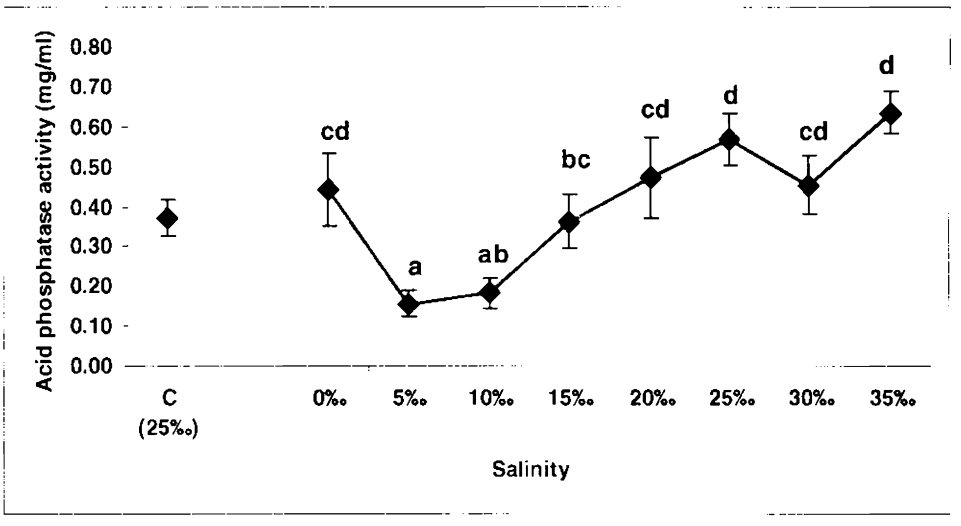


Fig.2.12 Acid phosphatase activity (mean ± S.D) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.2.4 for statistical details).

2.4 Discussion

It has been well demonstrated that *Fenneropenaeus indicus* is able to withstand a wide change in salinity (3-40‰) though its optimum salinity level is 25‰. However, how this organism adjusts its metabolism in terms of haematological parameters has not been studied in detail. The present investigation has demonstrated that *F. indicus* acclimatized at different salinity levels exhibited measurable amount of variations in the haemolymph protein components *viz.* total haemolymph protein, plasma protein and serum protein concentrations compared to those obtained for control group of shrimps. A considerable increase in these haematological parameters was found at the lower salinity ranges (5 and 10‰) and at higher salinity level (35‰). Post hoc tests have showed that the elevation in the haemolymph variables is significant at $p<0.01$. The increase in the haemolymph protein components may be attributed to the increased production of haemolymph proteins such as haemocyanin and other stress proteins in response to the hypo osmotic and hyper osmotic environment. Villarreal and Hewitt (1993) showed that the metabolic rate of yellow leg

shrimp has been increased at high salinities as a physiological response to stress compared to its optimum salinity level where weight gain and lower mortality was recorded. The plasma protein is a very important immunological tool of penaeid shrimp. It correlates with the pathogenic infection (Vogan and Rowley, 2002; Song *et al.,* 2003) as well as with environmental stress (Chen *et al.,* 1994). Hypo osmotic condition has triggered the increased production of haemocyanin, the respiratory pigment in decapod crustaceans which represent 80-95% of the total protein concentration in the shore crab, *Carcinus maenas* (Boone and Schoffeniels, 1979) and in the tiger shrimp, *Penaeus monodon* (Chen *et al.,* 1994). The observations in the present investigation concur with the above results in the case of haematological variable such as total haemolymph protein, plasma protein and serum protein in shrimps held at hypo osmotic conditions.

Prominent variations in total haemocytes in terms haemocyte protein concentration of shrimps at different salinity levels could be observed in the present study. A considerable increase in the haemocyte protein concentration was obtained for shrimps held at 5 and 10‰ and the HCP concentration was lower in shrimps held at all other salinities compared to the control group. Many authors have demonstrated that hyper osmotic conditions can increase the number of circulating haemocytes (Lee Moullac and Haffner, 2000; Cheng, *et al.*, 2004). However, in contradictory to the above we observed an increase in the HCP at lower salinity indicating that hypo osmotic conditions may augment the haemocytes in *F. indicus.* The circulating haemocyte number can be a stress indicator but this parameter varies non-specifically according to the natural rhythms of the environment, and chemical and physico-chemical stress. The observation in the present study highlights the importance of further studies to reveal the impact of salinity stress on haemocytes of shrimps.

Free amino acids (FAA) are known to play a major role in osmoregulation of marine invertebrates (Deaton *et al.*, 1984; Dalla Via, 1986). In cray fish and crab, more than 40 to 60% of the intracellular osmolarity has been contributed by FAA (Shaw, 1958; Robertson, 1961). In the present study, increased concentration of amino acids was noted in the shrimps at lower salinity, highest concentration of FAA being at 0‰, which may be a physiological adaptation for osmoregulation. However, this observation in no way agrees with the findings of the previous authors. It was demonstrated that hyper osmotic condition was directly correlated with high amino acid concentration in crustaceans (Okuma and Abe, 1994; Huong *et al.*, 2001). As such, the prolonged effect of lower salinity than an acute osmotic shock on the haemolymph free amino acid concentration necessitates further examination.

We found that phenoloxidase activity was high for shrimps held at lower salinity levels maximum being at 5‰ salinity when compared with control and higher salinity levels. The phenoloxidase enzyme has a number of important roles in crustacean immune defence, including nonself recognition, melanin formation, and haemocyte degranulation and adhesion (Soderhall, 1992; Soderhall *et al.,* 1994). More recently, investigations have focused on the response of the phenoloxidase system to various environmental forces such as tidal cycle entrainment (Hauton *et al.,* 1995). Previous reports indicated that perturbations in the ambient salinity had profound influence on the phenoloxidase system of crustaceans. Lu Qing *et al.*, (2005) have shown that salinities between 5‰ to 30‰ have been found to peak the phenol oxidase activity in *Litopenaeus vannamei* at the 12^{th} hr of exposure. Similarly, in yellow leg shrimp, proPO was found to increase directly proportional to salinity and the increase was independent of the plasma total protein concentration, which did not show variation within the different salinity treatments (Vargas-Albores *et al.*, 1998). The significant increase in phenol oxidase

activity observed in our study may be attributed to the hyper immune response of the organism under hypo osmotic stress. Cheng and Chen (2000) have made a similar observation that phenoloxidase activity of *M. rosenbergii* was significantly higher for animals reared in 5 and 10‰ than those reared in fresh water and 15‰. Lamela *et al.* (2005) has reported that phenoloxidase activity was decreased in *Litopenaeus schmitti* exposed to low salinity for 48hrs. The present results imply the relevance of further investigation on acute and chronic osmotic stress in response to this enzyme.

Intracellular super oxide anions production increases because of elevated activity of NADPH oxidase and by the decreased activity of super oxide dismutase. In the present study, it was found that super oxide anion production was high at lower salinity (5 and 10‰) and at higher salinity 35‰) levels. Recent reports have demonstrated that production of intracellular super oxide anions have been influenced by the fluctuations in the ambient salinity. According to Cheng *et al.* (2004), the super oxide anion production has been found to decrease in *Haliotis diversicolor supertexta* when transferred to 20, 25 and 35‰ from 30‰.

Lysosomal responses are widely accepted as cellular biomarkers of general stress. Cheng and Rodrick (1975) have reported that alkaline phosphatase is an important component of lysosomal enzymes that originate from haemocytes to destroy extracellular "invaders". In crustacean cells, phosphatase is the most important element of lysosomal enzymes, which perform the double function of digestion and defence (Jiang and Mu, 1999). In the present study, alkaline and acid phosphatase activities have shown marked differences in different salinities. Alkaline phosphatase was found to be significantly higher at lower salinity levels (5 and 10‰) and acid phosphatase was positively correlated with higher salinity levels. The results suggest the possible implication of the

phosphatases in acute osmotic crisis in shrimps. Zhang *et al.* (2005) have reported that ACP and ALP in serum and haemocytes of *Chlamys farreri* were more important than other enzymes in immune defence. Previous studies showed that the ACP activity in serum can reflect the immune state of the scallop (Zhang *et al.,* 2005). It has been showed that environmental factors and other xenobiotics can cause structural and physiological changes such as lysosomal fragility and subsequent release of ACP (Cheng and Dougherty, 1989; Pampanin *et al.,* 2002). There is no available information about changes of ALP activity under osmotic stress. Lovett *et al.* (1994) reported the presence of an alkaline phosphatase at pH 9.1 in the posterior gills of *Carcinus sapidus* and the activity was sensitive to environmental salinity. The specific activity of this alkaline phosphatase was greater in *C. sapidus* acclimated to 35‰ than crabs acclimated to 10‰. Since Lovett *et al.* (1994) postulated the role of alkaline phosphatase in modulating the osmoregulatory response of *C. sapidus* by varying in expression in response to acclimation salinity, our results also support the possible role of phosphatases in osmoregulation in crustaceans.

It has been already well demonstrated that 25‰ salinity is the best salinity for Indian *F. indicus* strains for culture (Raj and Raj, 1982; Kumlu and Jones, 1993 and 1995). The alterations of the salinity beyond the optimum level have evoked stress responses in the haematological variables analysed. According to Charmantier (1987), *F. indicus* adults are not as capable of withstanding lower salinities as post larvae and juveniles do. Dall (1981) suggested that the tolerance of shrimp to euryhaline conditions may be lost gradually with development under stable conditions. Kumlu and Jones (1995) has reported that post larvae of *F. indicus* cultured at 10‰ salinity showed poor survival after PL40 indicating that shrimps (PL35-40) become more stenohaline and prefer higher salinities. The unsuitability of *F. indicus* as a candidate species for culture

at salinities lower than 10‰ has been discussed before (Parado-Estapa *et al.*, 1987; Kumlu and Jones., 1995). Our results indicated that the adult *F. indicus* acclimated to extreme salinities were under stress. These stress responses have been reflected in the haematological profile emphasizing the unsuitability of lower salinity (below 10‰) for the culture of *F. indicus.*

The haematological alterations were prominent at lower salinity levels (5 and 10‰) showing maximum stress on the animals at these salinity levels. Significant increase of haemolymph protein components and majority of the haemolymph enzymes in shrimps maintained at lower salinities establish the immunophysiological responses to cope with the ambient stressful condition. Salinity of 15-35‰ was found to be the desirable range for culture of *Fenneropenaeus indicus* to avoid stress and related physiological malfunctions. These haematological profiles can be used as an index for monitoring the health conditions of shrimps, which are susceptible to a variety of environmental stressors and thereby preventing reduced growth often associated with stressors.

Chapter 3

HAEMATOLOGICAL RESPONSES AND SUSCEPTIBILITY OF *F. INDICUS TO VIBRIO HARVEYI* AT DIFFERENT SALINITY LEVELS

Contents

3.1 Introduction

The shrimp aquaculture industry is beset by disease mostly due to bacteria and viruses. This is because of the intensification of farming style to increase profit (Karunasagar *et al.*, 1994). Due to this intensification, pathogen problem have become very serious. *Vibrio* sp., especially the luminous *Vibrio harveyi* have been implicated as the most notorious bacterial pathogens of shrimps (Alavandi *et al.,* 2006) and is a scourge of aquaculture industry throughout the world.

Environmental factors that affect the immune system of shrimp leading to the increased susceptibility to opportunistic pathogens have been documented by many researchers. Effects of ammonia (Cheng and Chen, 2002; Liu and Chen, 2004), salinity (Prayitno and Latchford, 1995); Hauton *et al.,* 2000; Wang and Chen, 2005; Wang and Chen, 2006), temperature (Riquelme *et al.*, 1995) and pH (Prayitno and Latchford, 1995) have been demonstrated for their role in facilitating pathogenic invasion. These parameters can also affect the growth of pathogens and their production of toxins (Weinberg, 1985; Arp, 1998; Ramesh *et al.,* 1989; Alavandi *et al.,* 2006). In decapod crustaceans, it is well known that life cycle, food intake, disease outbreaks, pollutants and environmental stress (temperature, pH, salinity, dissolved oxygen and ammonia) affect the circulating haemocyte count both in quantity and quality (Le Moullac *et al.,* 1997; Le Moullac and Haffner, 2000; Persson *et al.,* 1987; Smith and Johnston, 1992; Truscott and White, 1990; Cheng and Chen, 2002; Joseph and Philip, 2007).

It has been reported that opportunistic bacterial pathogens of shrimps prevalent in seawater may take advantage of ecological changes introduced when the water is used in aquaculture (Moriarty, 1998). Hauton *et al.* (2000) have demonstrated that the initial pathogenicity of a bacterium in the marine environment is dependent upon the virulence of

the pathogen, the environmental conditions like temperature and salinity as well as the immunocompetence of the host at the same environment and have described the scope for the management of potential diseases through the pertinent use of environmental conditions. Prevention and control of diseases are now the priority for the durability of aquaculture industry. Therefore, other methods of disease control are urgently needed, and an understanding of virulence mechanisms and environmental factors controlling pathogens is of primary importance in developing such alternative proactive measures.

A comprehensive data denoting the immune status of *Fenneropenaeus indicus* with respect to the environmental changes leading to disease susceptibility is lacking. Further, scientific information on the role of physico-chemical conditions of rearing on the onset of vibriosis caused by *V. harveyi* is also scanty.

Against this background the present study was aimed at evaluating

a) the haematological responses of *F. indicus* adults to alterations in ambient salinity.

b) the haematological variations in shrimp after *V. harveyi* infection at different salinity levels.

c) the susceptibility of *F. indicus* adults to *V. harveyi* infection when subjected to variations in ambient salinity.

3.2 Materials and methods

3.2.1 Shrimp

Adult *F. indicus* were brought to the Laboratory within one hour of capture from a commercial shrimp farm located at Panangad, Kochi, India. The average wet weight of the shrimp was 16.45±2.12gm (Mean±SD). Shrimps were reared in rectangular concrete tanks containing 25‰ clean seawater and allowed to acclimate for a period of seven days. Continuous

aeration was provided using air pumps and was maintained on a commercial shrimp diet (Higashimaru, Pvt. Ltd., Kochi). Water quality parameters *viz.*, temperature, dissolved oxygen, NH_3-N, NO_2-N and NO_3-N were monitored daily following standard procedures (APHA, 1995) and maintained at optimal levels as per Table 2.1. Unused feed and faecal matter was siphoned out daily and 30% of water exchanged every alternate day. A biological filter was set up to maintain the appropriate levels of water quality parameters. After acclimating to 25‰ for seven days, six (n=6) shrimps were sampled for baseline data.

3.2.2 Bacterial pathogen

Bacterial pathogen, *V. harveyi* strain MCCB111 isolated from diseased larvae from a prawn hatchery in Kochi and maintained in the Microbiology Laboratory of the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi was used for the study. *V. harveyi* was grown on Thio sulfate Citrate Bile Salts Sucrose Agar (Lightner, 1983) and restreaked on Prawn flesh agar medium (Singh and Philip, 1993) to improve its virulence.

3.2.3 Determination of LD_{50} for *V. harveyi*

LD_{50} was determined by injecting known concentration of *V. harveyi* in physiological saline. 10^3 to 10^8 cells were injected in a volume of 20µl and a control with physiological saline was also injected. Based on the results LD_{50} was calculated as (10^6cells) following probit analysis as per Finney (1971).

3.2.4 Experimental setup and salinity adjustments

Shrimps of apparently uniform size were distributed in the experimental tanks containing 500L of seawater (n=35/tank). Shrimps in the intermoult stage only were used (Robertson *et al.*, 1987). There were four treatment Groups (G-I, G-II, G-III and G-IV) and the experiment was conducted in triplicate *i.e.*, 3 tanks per treatment. Salinity of all the tanks

was adjusted to 25‰ prior to the experiment. After 12 hours of starvation, the salinity of G-I shrimps was lowered from 25‰ to 5‰ by diluting with fresh water. Whereas, the salinity of G-II was raised from 25‰ to 35‰ by adding seawater. The desired salinity was adjusted over a period of six hours. Shrimps of G-III and G-IV were maintained at 25‰ itself with no salinity change. All four groups were maintained on commercial diet. After seven days of acclimation to each salinity level, six shrimps from each group (n=6) were sampled (Post Salinity change Day 7(PSD7).

3.2.5 Experimental infection with *V. harveyi*

The three groups of shrimps (G-I, G-II and G-III) were injected with *V. harveyi* (10^6cells/animal) and the fourth group (G-IV) was injected with physiological saline (0.89% NaCl) alone to serve as the untreated group. Random sampling of six animals (n=6) was done on Post Challenge Day 1 (PCD1), 3 (PCD3) 5 (PCD5), 7 (PCD7) and 10 (PCD10). Before each sampling, the shrimps were starved for 12 hours to eliminate variations caused by the ingested food (Hall and van Ham, 1998). Survival in each group was recorded daily for a period of 10 days with dead animals removed promptly.

3.2.6 Haematological analysis

Haemolymph was withdrawn aseptically from rostral sinus using specially designed sterile capillary tubes of diameter 0.5mm, rinsed thoroughly with pre-cooled anticoagulant. The samples were transferred to sterile microcentrifuge tubes containing measured quantity of pre-cooled anticoagulant (0.02M sucrose, 0.01M tri-sodium citrate in 0.01M Tris-Cl, pH 7.6) (Song and Hsieh, 1994). Serum was obtained by keeping the haemolymph at room temperature without anticoagulant. This is allowed to clot and then centrifuged at 1700x g for 10 minutes in a cooling centrifuge to get the serum. A fraction of haemolymph (0.1ml) was immediately centrifuged at 600x g for 15 minutes to separate plasma and haemocytes. The immune

variables *viz.* phenoloxidase, NBT reduction, alkaline phosphatase and acid phosphatase and haemolymph metabolic variables *viz.*, total protein, plasma protein, serum protein, haemocyte protein and total free amino acids were estimated. Refer section 2.2.4 of Chapter 2 for details.

3.2.7 Pathogenicity study

Virulence of *V. harveyi* in terms of its proliferation in the infected shrimp haemolymph in three different salinities was noted.100µL diluted haemolymph aseptically drawn from bacteria challenged prawns maintained at 5, 25 and 35‰ salinities were plated in triplicate on nutrient agar plates prepared in 30‰ seawater. Typical luminescent colonies were counted after 24hrs.

3.2.8 Statistical analysis

The statistical analysis was carried out using the software SPSS 10. All the data were presented as mean±SD and subjected to ANOVA. Differences were regarded as statistically significant when $p<0.05$.

3.3 Results

3.3.1 Effect of salinity changes on the haematological parameters of *F. indicus*

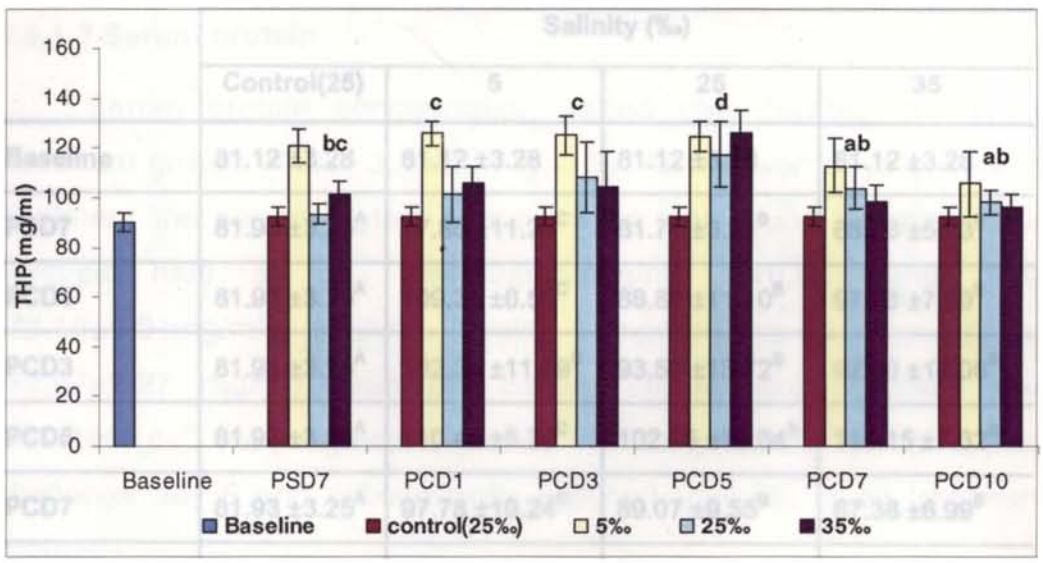
3.3.1.1 Total Haemolymph Protein

Table 3.1 and Fig. 3.1 present the results obtained for total haemolymph proteins in shrimps subjected to salinity alterations and challenged with *V. harveyi.* Remarkable variation was observed for THP among different treatment groups. THP in the haemolymph of shrimps held at 5‰ (120.85±6.55mg/ml) and 35‰ (101.68±5.05mg/ml) showed an elevation ($p < 0.05$) after 7 days of salinity treatment (PSD7) and prior to *V. harveyi* injection compared to the control group (92.56±3.85mg/ml). Highest concentration was noted in shrimps held at 5‰ compared to other groups. After *V. harveyi* challenge, there was a progressive increase in the THP of shrimps held at all salinity levels compared to the unchallenged group up to PCD5. However, during the later stage of *V. harveyi* infection

(PCD7 and PCD10) THP of shrimps showed a declining trend least being in those held at 35‰.

Table.3.1 Total haemolymph protein concentration of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Total Haemolymph protein (mg/ml)			
	Salinity (‰)			
	Control(25)	5	25	35
Baseline	90.16±3.75	90.16±3.75	90.16±3.75	90.16±3.75
PSD7	92.56±3.85^{A}	120.85±6.55^{C}	93.29±4.15^{B}	101.68±5.05^{B}
PCD1	92.56±3.85^{A}	125.95±5.05^{C}	101.42±11.01^{B}	97.23±6.34^{B}
PCD3	92.56±3.85^{A}	125.17±8.07^{C}	108.10±14.19^{B}	104.65±14.03^{B}
PCD5	92.56±3.85^{A}	124.71±6.37^{C}	117.42±13.27^{B}	125.96±9.06^{B}
PCD7	92.56±3.85^{A}	112.99±10.62^{C}	103.72±8.63^{B}	98.44±6.95^{B}
PCD10	92.56±3.85^{A}	105.63±13.30^{C}	98.14±4.98^{B}	96.27±5.43^{B}



THP- Total Haemolymph Protein
PSD7- Post Salinity change Day 7, PCD- Post Challenge Day

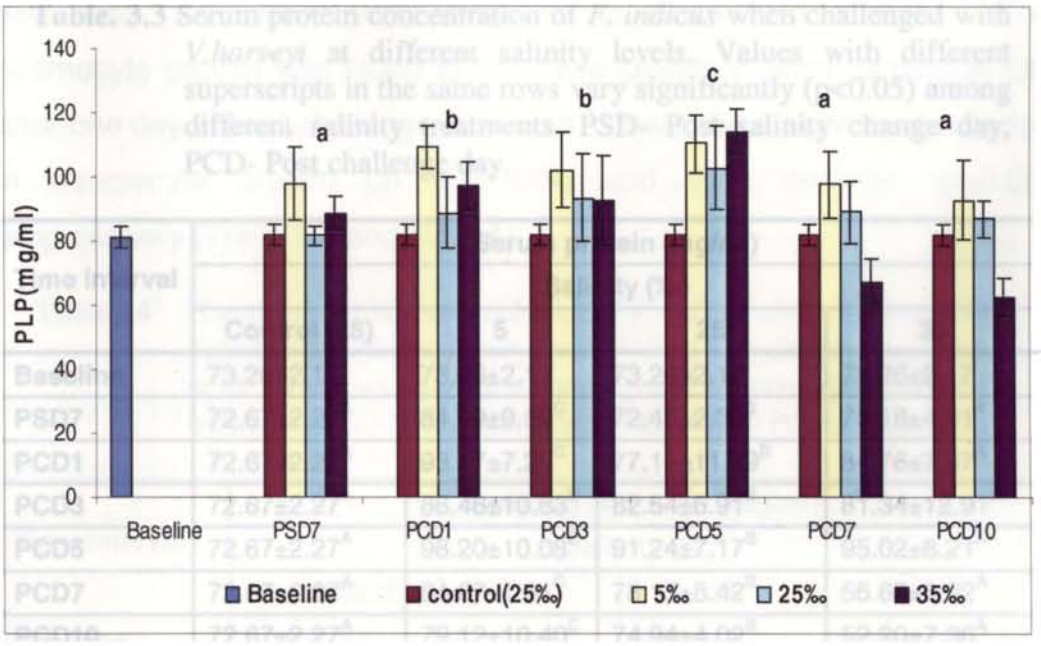
Fig. 3.1 Total haemolymph protein concentration (Mean ± SD) of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Bars with different lower case letters vary significantly among different exposure times.

3.3.1.2 Plasma protein

Plasma protein concentration of shrimps held at different salinity ranges varied greatly among the different treatment groups compared to control groups. PLP concentration was found to elevate significantly ($p<0.05$) after seven days of salinity treatment in shrimps held at 5‰ (97.85±11.20 mg/ml) and 35‰ (88.86±5.23 mg/ml) salinity levels compared to those held at 25‰ (81.73±3.21 mg/ml) salinity. PLP showed an increasing trend ($p<0.05$) after *V. harveyi* challenge up to PCD5. A decline ($p<0.05$) in PLP concentration could be found on PCD7 and PCD10 in all treatment groups. (Table 3.2. and Fig.3.2)

Table. 3.2 Plasma protein concentration of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Plasma protein (mg/ml)			
	Salinity (‰)			
	Control(25)	5	25	35
Baseline	81.12 ±3.28	81.12 ±3.28	81.12 ±3.28	81.12 ±3.28
PSD7	81.93 ±3.25^{A}	97.85 ±11.20^{C}	81.73 ±3.21^{B}	88.86 ±5.23^{B}
PCD1	81.93 ±3.25^{A}	109.35 ±6.55^{C}	88.80 ±11.10^{B}	97.23 ±7.59^{B}
PCD3	81.93 ±3.25^{A}	102.33 ±11.69^{C}	93.59 ±13.72^{B}	92.88 ±14.06^{B}
PCD5	81.93 ±3.25^{A}	110.42 ±8.78^{C}	102.95 ±12.84^{B}	114.15 ±7.32^{B}
PCD7	81.93 ±3.25^{A}	97.78 ±10.24^{C}	89.07 ±9.55^{B}	67.38 ±6.99^{B}
PCD10	81.93 ±3.25^{A}	92.97 ±12.46^{C}	87.00 ±5.60^{B}	62.48 ±6.10^{B}



PLP- Plasma protein,

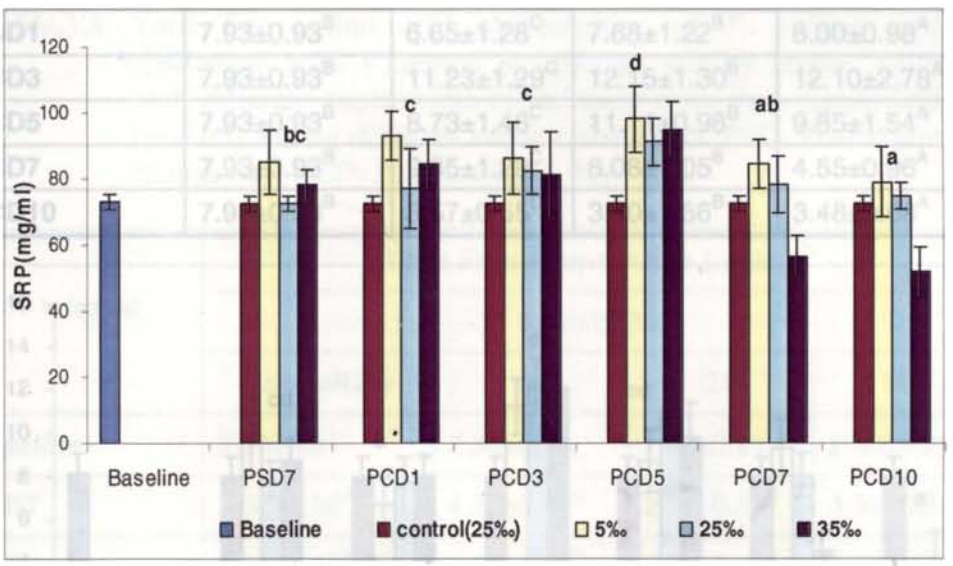
Fig.3.2 Plasma protein concentration (Mean ± S.D) of *F. indicus* when challenged with *V.harveyi* at different salinity levels (See Fig.3.1 for statistical details).

3.3.1.3 Serum protein

Serum protein concentration varied considerably in all the treatment groups (Table 3.3 and Fig. 3.3). After seven days of salinity treatment the serum protein concentration was found to increase in shrimps held at 5‰ (84.99±9.59mg/ml, $p<0.05$) and 35‰ (78.18±4.91mg/ml) salinity levels compared to control group (72.67±2.27 mg/ml) salinities. Serum protein concentration was elevated ($p<0.05$) progressively after *V. harveyi* challenge up to post challenge day 5 and subsequently reduced ($p<0.05$) in all treatment groups.

Table. 3.3 Serum protein concentration of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Serum protein (mg/ml)			
	Salinity (‰)			
	Control (25)	5	25	35
Baseline	73.26±2.17	73.26±2.17	73.26±2.17	73.26±2.17
PSD7	72.67 ± 2.27^{A}	84.99 ± 9.59^{C}	72.45 ± 2.32^{B}	78.18 ± 4.91^{A}
PCD1	72.67 ± 2.27^{A}	93.17 ± 7.20^{C}	77.11 ± 11.99^{B}	84.76 ± 7.37^{A}
PCD3	72.67 ± 2.27^{A}	86.46 ± 10.83^{C}	82.54 ± 6.91^{B}	81.34 ± 12.91^{A}
PCD5	72.67 ± 2.27^{A}	98.20 ± 10.08^{C}	91.24 ± 7.17^{B}	95.02 ± 8.21^{A}
PCD7	72.67 ± 2.27^{A}	84.67 ± 7.61^{C}	78.15 ± 8.42^{B}	56.67 ± 5.92^{A}
PCD10	72.67 ± 2.27^{A}	79.12 ± 10.40^{C}	74.94 ± 4.02^{B}	52.20 ± 7.36^{A}



SRP- Serum protein

Fig. 3.3 Serum protein concentration (Mean ± SD) of *F. indicus* when challenged with *V.harveyi* at different salinity levels (See Fig.3.1 for statistical details).

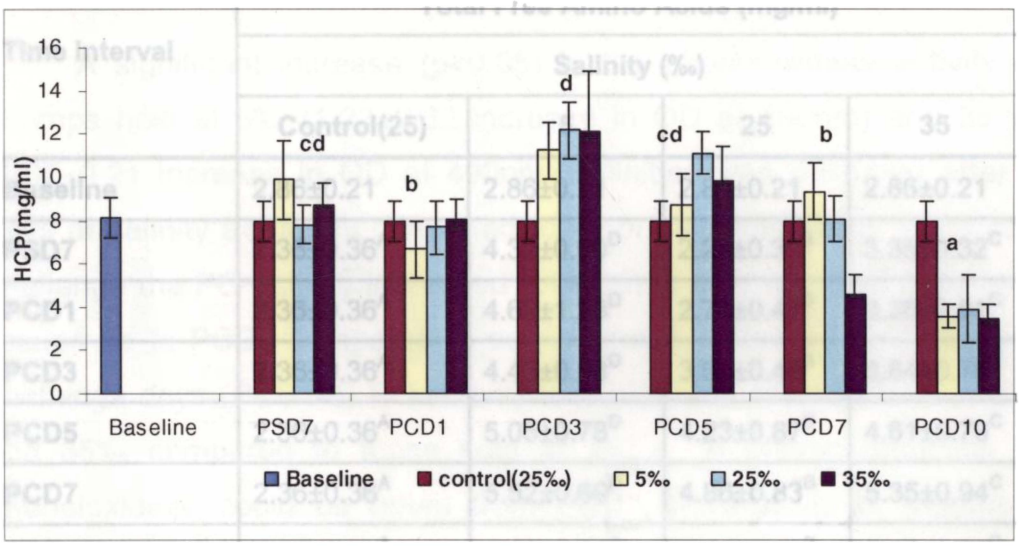
3.3.1.4 Haemocyte protein

Salinity wise variation in the haemocyte protein concentration was statistically significant ($p<0.05$). Haemocyte protein concentration enhanced after salinity treatment in shrimps held at 5‰ (9.86±1.78 mg/ml)

and 35‰ (8.4±0.96 mg/ml) compared to those held at 25‰. The haemocyte protein was found to decrease slightly in shrimps held at 5‰ after one day of post challenge. This was followed by a significant increase in haemocyte protein up to PCD3 and then declined ($p<0.05$) progressively (Table 3.4 and Fig.3.4).

Table.3.4 Haemocyte protein concentration of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Haemocyte protein (mg/ml)			
	Salinity (‰)			
	Control(25)	5	25	35
Baseline	8.12±0.87	8.12±0.87	8.12±0.87	8.12±0.87
PSD7	7.93 ± 0.93^{B}	9.86 ± 1.78^{C}	7.82 ± 0.90^{B}	8.74 ± 0.96^{A}
PCD1	7.93 ± 0.93^{B}	6.65 ± 1.28^{C}	7.68 ± 1.22^{B}	8.00 ± 0.98^{A}
PCD3	7.93 ± 0.93^{B}	11.23 ± 1.29^{C}	12.15 ± 1.30^{B}	12.10 ± 2.78^{A}
PCD5	7.93 ± 0.93^{B}	8.73 ± 1.46^{C}	11.11 ± 0.98^{B}	9.85 ± 1.54^{A}
PCD7	7.93 ± 0.93^{B}	9.35 ± 1.29^{C}	8.08 ± 1.05^{B}	4.55 ± 0.96^{A}
PCD10	7.93 ± 0.93^{B}	3.57 ± 0.55^{C}	3.90 ± 1.56^{B}	3.48 ± 0.68^{A}



HCP- Haemocyte protein

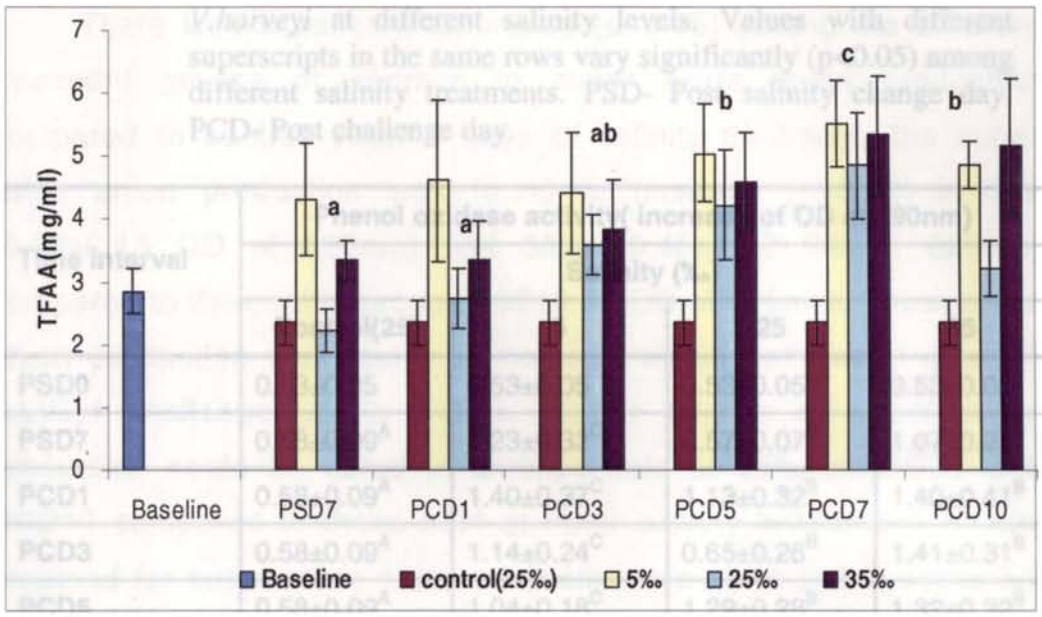
Fig.3.4 Haemocyte protein concentration (Mean ± S.D) of *F. indicus* when challenged with *V.harveyi* at different salinity levels (See Fig.3.1 for statistical details).

3.3.1.5 Total free amino acids

Total free amino acids in the haemolymph of shrimps held at different salinity ranges showed considerable variations among the treated and untreated groups. An elevation in TFAA was obtained on post salinity change day 7 (PCD7) in shrimps held at 5‰ (4.32±0.90 mg/ml) and 35 ‰ (3.35±0.32 mg/ml) compared to control group (2.36±0.36 mg/ml). Total free amino acid concentration elevated progressively after *V. harveyi* challenge in shrimps held at 5, 25 and 35‰ salinity up to PCD7. *F. indicus* held at 5‰ salinity showed comparatively higher amount of TFAA concentration throughout the experimental period except for post challenge day 10 (Table 3.5. and Fig.3.5).

Table.3.5 Total free amino acid concentration of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Total Free Amino Acids (mg/ml)			
	Salinity (‰)			
	Control(25)	5	25	35
Baseline	2.86±0.21	2.86±0.21	2.86±0.21	2.86±0.21
PSD7	2.36 ± 0.36^{A}	4.32 ± 0.90^{D}	2.23 ± 0.33^{B}	3.35 ± 0.32^{C}
PCD1	2.36 ± 0.36^{A}	4.62 ± 1.28^{D}	2.74 ± 0.47^{B}	3.36 ± 0.61^{C}
PCD3	2.36 ± 0.36^{A}	4.43 ± 0.98^{D}	3.62 ± 0.48^{B}	3.84 ± 0.79^{C}
PCD5	2.36 ± 0.36^{A}	5.05 ± 0.78^{D}	4.23 ± 0.87^{B}	4.61 ± 0.76^{C}
PCD7	2.36 ± 0.36^{A}	5.52 ± 0.69^{D}	4.86 ± 0.83^{B}	5.35 ± 0.94^{C}
PCD10	2.36 ± 0.36^{A}	4.88 ± 0.37^{D}	3.24 ± 0.45^{B}	5.17 ± 1.08^{C}



TFAA- Total free amino acids

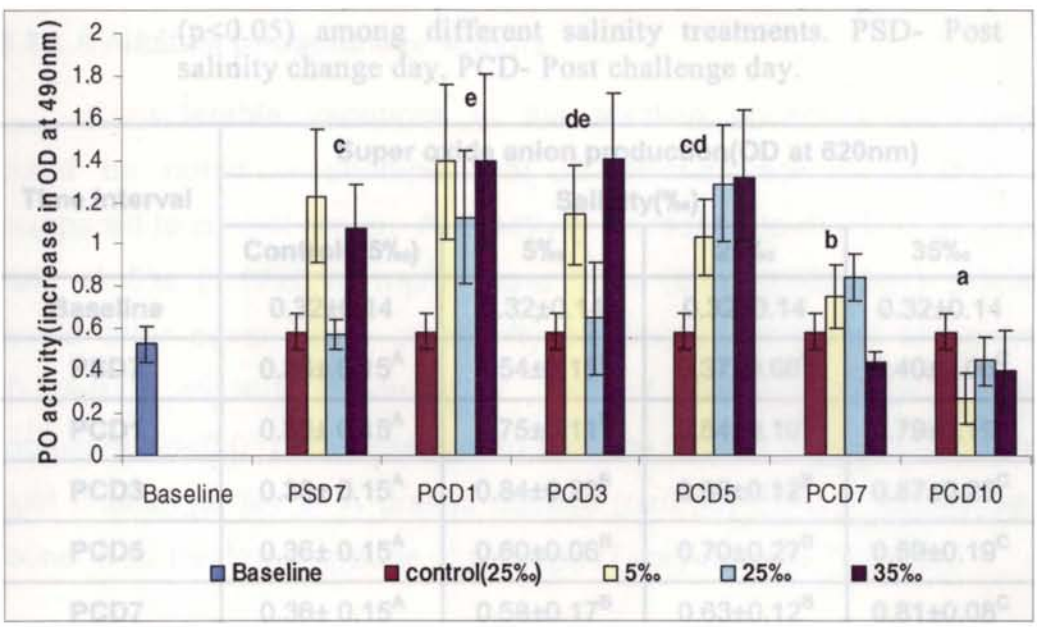
Fig.3.5 Total free amino acid concentration (Mean ± S.D) of *F. indicus* when challenged with *V.harveyi* at different salinity levels (See Fig.3.1 for statistical details).

3.3.1.6 Phenoloxidase activity

A significant increase ($p<0.05$) in the phenoloxidase activity of shrimps held at 5‰ (1.23±0.32 increase in OD at 490nm) and 35 ‰ (1.07±0.21 increase in OD at 490nm) salinities was observed after 7 days of salinity treatment compared to other salinities. After *V. harveyi* challenge the PO activity increased ($p<0.05$) in shrimps in all treatment groups up to PCD5 compared to control group. During the initial post challenge days (PCD1-5) PO activity was higher in shrimps held at 5‰ and 35‰ compared to those held at 25‰. A drastic reduction in phenoloxidase could be noted from PCD7 onwards in all treatment groups (Table 3.6 and Fig.3.6).

Table.3.6 Phenol oxidase activity of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Phenol oxidase activity(increase of OD at 490nm)			
	Salinity (‰)			
	Control(25)	5	25	35
PSD0	0.53±0.05	0.53±0.05	0.53±0.05	0.53±0.05
PSD7	0.58 ± 0.09^{A}	1.23 ± 0.32^{C}	0.57 ± 0.07^{B}	1.07 ± 0.21^{B}
PCD1	0.58 ± 0.09^{A}	1.40 ± 0.37^{C}	1.13 ± 0.32^{B}	1.40 ± 0.41^{B}
PCD3	0.58 ± 0.09^{A}	1.14 ± 0.24^{C}	0.65 ± 0.26^{B}	1.41 ± 0.31^{B}
PCD5	0.58 ± 0.09^{A}	1.04 ± 0.18^{C}	1.29 ± 0.28^{B}	1.32 ± 0.32^{B}
PCD7	0.58 ± 0.09^{A}	0.75 ± 0.15^{C}	0.85 ± 0.11^{B}	0.44 ± 0.05^{B}
PCD10	0.58 ± 0.09^{A}	0.27 ± 0.12^{C}	0.45 ± 0.11^{B}	0.41 ± 0.18^{B}



PO activity - Phenol oxidase activity

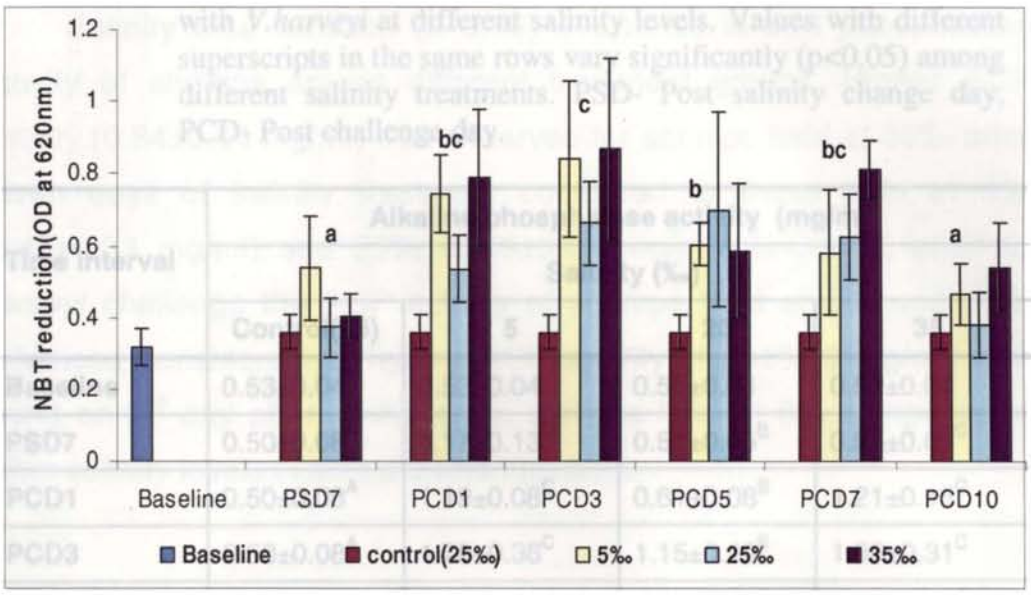
Fig.3.6 Phenol oxidase activity (Mean ± SD) of *F. indicus* when challenged with *V.harveyi* at different salinity levels (See Fig.3.1 for statistical details).

3.3.1.7 Super oxide anion production

There was significant difference ($p<0.05$) among the different treatment groups of shrimps in super oxide anion production compared to control. After 7 days of salinity treatment, the super oxide anion production was found to increase ($p<0.05$) in 5‰ (0.54±0.15 OD at 620nm) and 35‰ (0.40±0.06 OD at 620nm) compared to the control group (0.36±0.15 OD at 620nm). Super oxide anion production was found to increase significantly even after *V. harveyi* challenge up to PCD3. Higher degree of super oxide production could be noted in shrimps held at 35‰ on PCD7 and PCD10 compared to those held at other salinity levels. The results obtained for super oxide anion production are given in the Table 3.7 and Fig. 3.7.

Table.3.7 Superoxide anion production in *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Super oxide anion production(OD at 620nm)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	0.32±0.14	0.32±0.14	0.32±0.14	0.32±0.14
PSD7	0.36 ± 0.15^{A}	0.54 ± 0.15^{B}	0.37 ± 0.08^{B}	0.40 ± 0.06^{C}
PCD1	0.36 ± 0.15^{A}	0.75 ± 0.11^{B}	0.54 ± 0.10^{B}	0.79 ± 0.19^{C}
PCD3	0.36 ± 0.15^{A}	0.84 ± 0.22^{B}	0.66 ± 0.12^{B}	0.87 ± 0.25^{C}
PCD5	0.36 ± 0.15^{A}	0.60 ± 0.06^{B}	0.70 ± 0.27^{B}	0.59 ± 0.19^{C}
PCD7	0.36 ± 0.15^{A}	0.58 ± 0.17^{B}	0.63 ± 0.12^{B}	0.81 ± 0.08^{C}
PCD10	0.36 ± 0.15^{A}	0.47 ± 0.08^{B}	0.38 ± 0.09^{B}	0.54 ± 0.13^{C}



NBT reduction- Nitroblue tetrazolium reduction

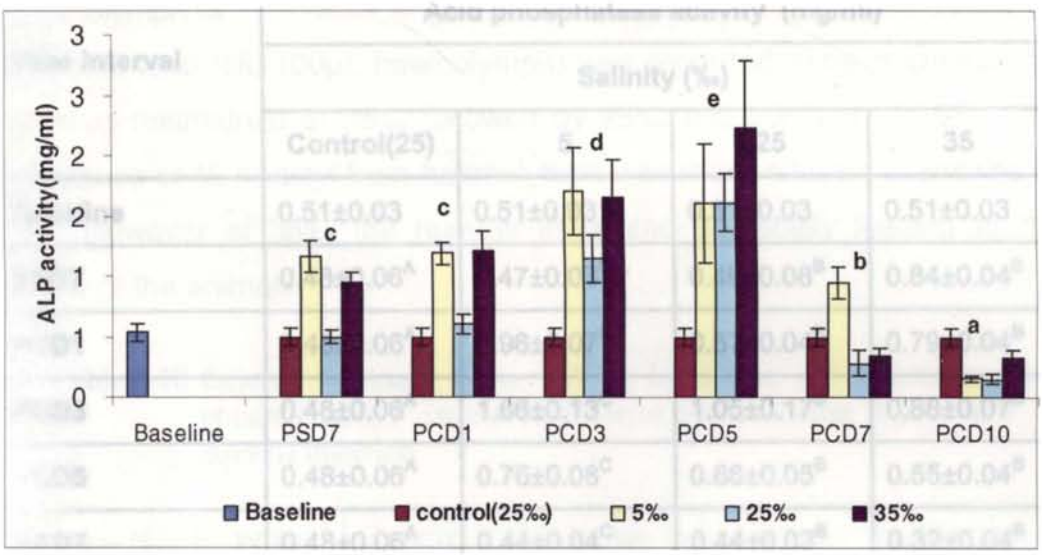
Fig.3.7 Superoxide anion production (Mean ± SD) in *F. indicus* when challenged with *V. harveyi* at different salinity levels (See Fig.3.1 for statistical details).

3.3.1.8 Alkaline phosphatase activity

Considerable variations in the alkaline phosphatase activity could be noted in shrimps held at different salinity treatments compared to control group. ALP activity increased ($p<0.05$) in shrimps held at 5‰ (1.17±0.13 mg/ml) and 35‰ (0.95±0.08mg/ml) salinity levels after seven days of salinity treatment compared to control (0.50±0.08 mg/ml). Following *V. harveyi* challenge, ALP activity increased ($p<0.05$) progressively in shrimps held at all salinities up to post challenge day 5. A drastic decline ($p<0.05$) in ALP activity was found in all treatment groups of shrimps (Table 3.8 and Fig. 3.8).

Table.3.8 Alkaline phosphatase activity of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Alkaline phosphatase activity (mg/ml)			
	Salinity (‰)			
	Control(25)	5	25	35
Baseline	0.53±0.04	0.53±0.04	0.53±0.04	0.53±0.04
PSD7	0.50 ± 0.08^{A}	1.17 ± 0.13^{C}	0.51 ± 0.05^{B}	0.95 ± 0.08^{C}
PCD1	0.50 ± 0.08^{A}	1.19 ± 0.08^{C}	0.61 ± 0.08^{B}	1.21 ± 0.15^{C}
PCD3	0.50 ± 0.08^{A}	1.70 ± 0.36^{C}	1.15 ± 0.19^{B}	1.66 ± 0.31^{C}
PCD5	0.50 ± 0.08^{A}	1.60 ± 0.49^{C}	1.61 ± 0.24^{B}	2.23 ± 0.55^{C}
PCD7	0.50 ± 0.08^{A}	0.95 ± 0.13^{C}	0.28 ± 0.10^{B}	0.35 ± 0.06^{C}
PCD10	0.50 ± 0.08^{A}	0.16 ± 0.03^{C}	0.16 ± 0.04^{B}	0.37 ± 0.08^{C}



ALP activity - Alkaline phosphatase activity

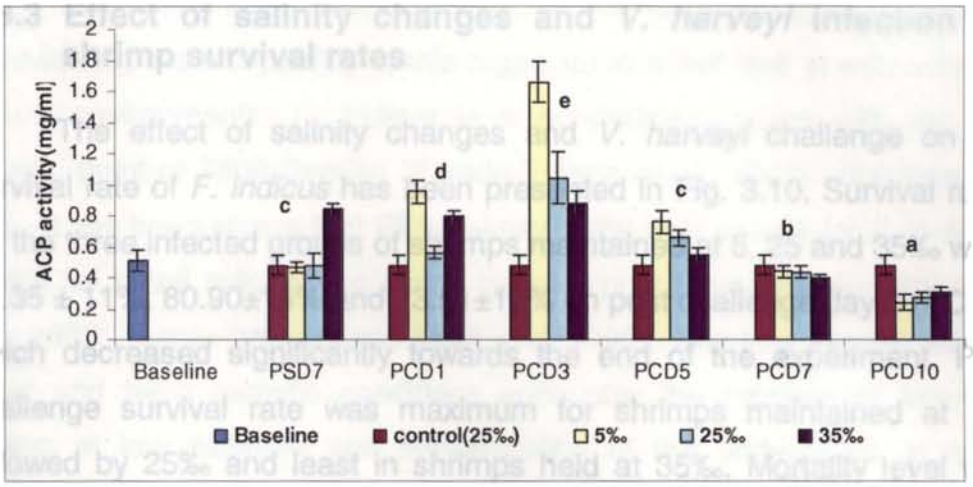
Fig. 3.8 Alkaline phosphatase activity (Mean ± S.D) of *F. indicus* when challenged with *V.harveyi* at different salinity levels (See Fig.3.1 for statistical details).

3.3.1.9 Acid phosphatase activity

Salinity wise variation ($p<0.05$) was noted in acid phosphatase activity of shrimps among different treatment groups. Higher ACP activity (0.84±0.04 mg/ml) was observed for shrimps held at 35‰ after seven days of salinity treatment compared to those held at 5‰ (0.47±0.03 mg/ml) and 25‰ (0.49±0.08 mg/ml). However, after *V. harveyi* challenge the ACP activity of shrimps held at 5‰ and 35‰ enhanced considerably. Highest ACP activity (1.66±0.13 mg/ml) was found on 3rd day after challenge in shrimps held at 5‰ compared to other salinity levels (Table 3.9 and Fig. 3.9).

Table.3.9 Acid phosphatase activity of *F. indicus* when challenged with *V.harveyi* at different salinity levels. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. PSD- Post salinity change day, PCD- Post challenge day.

Time interval	Acid phosphatase activity (mg/ml)			
	Salinity (‰)			
	Control(25)	5	25	35
Baseline	0.51±0.03	0.51±0.03	0.51±0.03	0.51±0.03
PSD7	0.48 ± 0.06^{A}	0.47 ± 0.03^{C}	0.49 ± 0.08^{B}	0.84 ± 0.04^{B}
PCD1	0.48 ± 0.06^{A}	0.96 ± 0.07^{C}	0.57 ± 0.04^{B}	0.79 ± 0.04^{B}
PCD3	0.48 ± 0.06^{A}	1.66 ± 0.13^{C}	1.05 ± 0.17^{B}	0.88 ± 0.07^{B}
PCD5	0.48 ± 0.06^{A}	0.76 ± 0.08^{C}	0.66 ± 0.05^{B}	0.55 ± 0.04^{B}
PCD7	0.48 ± 0.06^{A}	0.44 ± 0.04^{C}	0.44 ± 0.03^{B}	0.32 ± 0.04^{B}
PCD10	0.48 ± 0.06^{A}	0.24 ± 0.05^{C}	0.28 ± 0.03^{B}	0.31 ± 0.03^{B}



ACP activity- Acid phosphatase activity

Fig. 3.9 Acid phosphatase activity (Mean ± S D) of *F. indicus* when challenged with *V.harveyi* at different salinity levels (See Fig.3.1 for statistical details).

3.3.2 Effect of salinity change on *V. harveyi* proliferation (Virulence of *V. harveyi*)

The effect of salinity change on *V. harveyi* proliferation in the haemolymph of *F. indicus* is given in the Table.3.10. The post challenge bacterial load (cfu/100µL haemolymph) was recorded to be maximum for shrimps maintained at 35‰ followed by 25‰ and minimum in 5‰. The clearance of *V. harveyi* from haemolymph was more efficient in prawns at 5‰. However at 35‰ the number increased drastically leading to the death of the animals.

Table.3.10 Colony Forming Units (CFU) in 100µL of haemolymph obtained from *F. indicus* in different post challenge days after *V. harveyi* injection

Salinity (‰)	PCD1	PCD3	PCD5	PCD7	PCD10
5	9±2	38±6	40±8	75±11	95±12
25	14±3	60±7	82±11	128±19	163±14
35	25±2	102±9	172±16	***	***

*** Uncountable PCD- Post Challenge Day

3.3.3 Effect of salinity changes and *V. harveyi* infection on shrimp survival rates

The effect of salinity changes and *V. harveyi* challenge on the survival rate of *F. indicus* has been presented in Fig. 3.10. Survival rates for the three infected groups of shrimps maintained at 5, 25 and 35‰ were 84.35 ± 11%, 80.90±14% and 73.51±12% on post challenge day 3 (PCD3) which decreased significantly towards the end of the experiment. Post challenge survival rate was maximum for shrimps maintained at 5‰ followed by 25‰ and least in shrimps held at 35‰. Mortality level was higher at 35‰ throughout the experimental period. It could be observed that high salinity resulted in significantly ($p<0.05$) higher shrimp. Control group showed 100% survival till the end of the experiment.

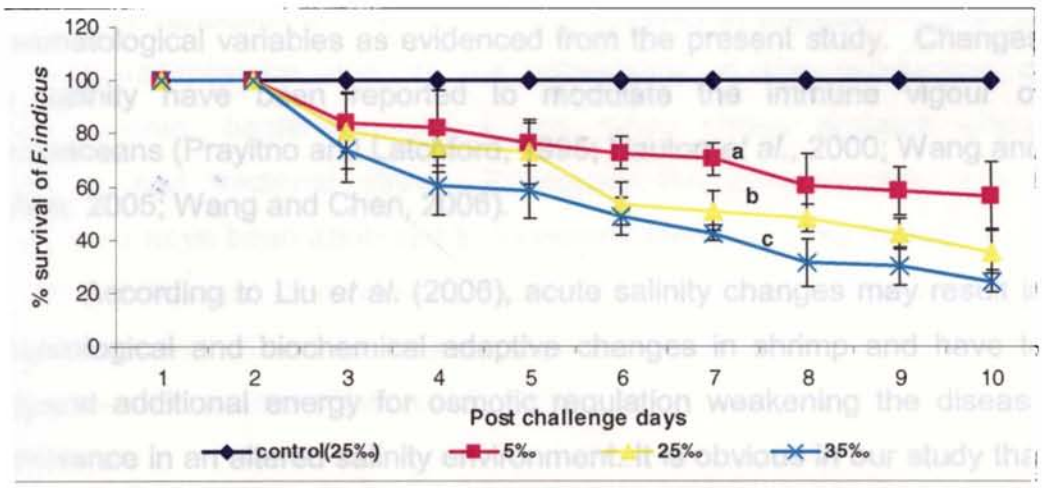


Fig. 3.10 Survival of *F. indicus* in different salinities when challenged with *V.harveyi* (See Fig.3.1 for statistical details).

3.4 Discussion

The present study demonstrates that *F. indicus* acclimated to different salinity levels has shown statistically significant differences in the haematological variables in response to salinity alterations and *V. harveyi* infection. Our results indicate that the adult *F. indicus* acclimated to hypo osmotic (5‰) and hyper osmotic (35‰) salinities are under stress. These

stress responses have been reflected in the haematological variables emphasizing the incapability of this organism to thrive well at sub optimal culture environments. *F. indicus* is a euryhaline species with an iso osmotic point of 780mOsm/kg (Parado-Estepa *et al.*, 1987) equivalent to 26‰. It has been shown that 25‰ salinity is the best salinity for *F. indicus* strains for larval culture and during nursery post larval rearing (Raj and Raj, 1982; Kumlu and Jones, 1993 and 1995). Shrimps transferred to hyper and hypo osmotic conditions encounter the problem of internal dilution at low salinities and concentration of the body fluids at high salinities (Parado- Estapa *et al.*, 1987). The salinity adjustments require extra energy allocation and therefore the immunocompetence at suboptimal level is much reduced than optimal level. The alterations in salinity beyond the optimum level have evoked stress responses in the haematological variables as evidenced from the present study. Changes in salinity have been reported to modulate the immune vigour of crustaceans (Prayitno and Latchford, 1995; Hauton *et al.*, 2000; Wang and Chen, 2005; Wang and Chen, 2006).

According to Liu *et al.* (2006), acute salinity changes may result in physiological and biochemical adaptive changes in shrimp and have to expend additional energy for osmotic regulation weakening the disease resistance in an altered salinity environment. It is obvious in our study that predominant changes in the ambient salinity not only altered the virulence of the pathogen but also affected the defence mechanism of the hosts. It is clearly depicted that salinity alterations increased the virulence of *V. harveyi* to *F. indicus* with a concurrent reduction in immune parameters resulting in heavy mortality of shrimps after seventh day of challenge (PCD7). Cheng *et al.* (2004) reported that the change in salinity triggered disease outbreaks in *Haliotis diversicolor supertexta* by affecting the defence mechanism of the host and rendering susceptibility to *V. parahaemolyticus* when the animals were transferred to 20, 25 and 35‰

salinity from 30‰ salinity. In addition, Wang and Chen (2005) documented the susceptibility of *L. vannamei* to *V. alginolyticus* and has correlated with reductions in immune parameters including phenol oxidase activity, respiratory burst, SOD activity, phagocytic activity and clearance efficiency when the shrimps were transferred to low salinity levels (5‰ and 15‰) from 25‰. Our study reveals that *F. indicus* is more susceptible to *V. harveyi* when transferred to lower (5‰) and higher (35‰) salinities from 25‰ well correlating the reductions in PLP and SRP concentration, PO activity, alkaline phosphatase and acid phosphatase activities on seventh (PCD7) and tenth days (PCD10) of post challenge. The immune reduction on seventh day was higher at 35‰ indicating increased susceptibility and immune fatigue of shrimps at hyper osmotic condition.

The variations in total protein components in the haemolymph of *F. indicus* may be due to the alterations in the production of haemocyanin, bacterial proteins and other stress proteins under osmotic and bacterial stress. Previously the haemolymph protein variations have been attributed to increased metabolic rate in yellow leg shrimp (Villarreal and Hewitt, 1993) and increased production of haemocyanin in *Carcinus maenas* (Boone and Schoffeniels, 1979) and in *Penaeus monodon* (Chen *et al.*, 1994).

In the present study, total haemocyte protein concentration was found to increase on 3rd and 5th day of post challenge at 5, 25 and 35‰ and then tend to decrease on 7th day at 35‰. The results indicated that osmotic stress alters the haemocyte proliferation in shrimps. Similar observations have been made by Lee Moullac, (2000) in *Farfantepenaeus paulensis*, Cheng *et al.* (2000) in *M. rosenbergii,* Cheng *et al.*, (2004) in *Haliotis diversicolar supertexta,* Wang and Chen (2005) in *Litopenaeus vannamei* and by Wang and Chen (2006) in *Penaeus monodon*. However, further research is needed to clarify

whether this variations in THC results from proliferation of the cells, or movement of cells from tissues into the circulation (Pipe and Coles, 1995), or osmosis of the water between haemolymph and medium for osmotic regulation.

Free amino acids are utilized for osmoregulation in aquatic organisms. The high concentration of free amino acids in lower saline conditions in the present study may be a physiological adaptation to maintain the osmotic balance and ionic strength of the organism. Metabolic pools of free amino acids (FAA) are also known to play a major role in osmoregulation of marine invertebrates contributing more than 40 to 60% of the intracellular osmolarity in certain species of cray fish and crab (Chaplin *et al.,* 1967; Clark, 1968; Boone and Claybrooh, 1977; Deaton *et al.*, 1984; Dalla Via, 1986; Shaw, 1958 and Robertson, 1961). Though we have reported high levels of free amino acids in hyper saline conditions more studies have to be done to explain the exact physiology behind this. This may probably be due to the protein catabolism under stress conditions. Chen *et al.* (1993) reported that exposure of *Penaeus chinensis* to elevated ambient ammonia caused accumulation of ammonia and decrease of protein and suggested that ammonia may cause catabolism of protein to balance osmoregulation.

Significant variations in phenoloxidase and other enzymes may be attributed to the hyper immune response of the organism under salinity and bacterial stress. Prophenoloxidase activity increased directly with salinity for the yellow leg shrimp, *Farfantepenaeus californiensis* reared in salinity levels of 28‰, 32‰, 36‰, 40‰ and 44‰ (Vargas-Albores *et al.,* 1998), for the white shrimp, *L. vannamei* reared in salinity levels of 5‰, 15‰, 25‰ and 35‰ (Wang and Chen, 2005), and for the tiger shrimp, *P. monodon* reared in salinity levels of 5‰, 15‰ and 25‰ (Wang and Chen, 2006). Cheng *et al.* (2000, 2003) have reported that phenoloxidase activity

of *M. rosenbergii* was significantly higher for animals reared at 5 and 10‰ than those reared in fresh water and 15‰. In the present study, PO activity has enhanced at lower (5‰) and higher (35‰) salinities after *V. harveyi* challenge when compared to control (25‰).

Production of intracellular superoxide anion has increased in hyper and hypo-osmotic conditions when challenged with bacteria compared to control. Cheng *et al.* (2004) reported that *H. diversicolar supertexta* when transferred to 20, 25 and 35‰ decreased the release of superoxide anion as compared to the abalone, reared in 30‰. White shrimp *L. vannamei* when transferred to 5‰ and 15‰ after 24h decreased the release of superoxide anion as compared to the shrimp reared in 25‰ and 35‰ (Le Moullac *et al.,* 1998). Wang and Chen (2006) found that tiger shrimp *P. monodon* when transferred to 5‰, 15‰ and 35‰ decreased the release of superoxide anion and SOD activity after 12hrs, as compared to the shrimp reared in 25‰. In contrast to this, the present results showed that release of super oxide anion was high in shrimps reared at hypo (5‰) and hyper osmotic (35‰) salinities, as compared with iso osmotic (25‰) salinity. The results suggest that the activities of NADPH oxidase responsible for the release of superoxide anion and superoxide dismutase (SOD) responsible for scavenging superoxide anion are to be examined in detail.

The enhanced production of ALP and ACP in response to *V. harveyi* in lower as well as higher salinities describes immune response under bacterial threat in unhealthy ambient environment. Cheng and Rodrick (1975) observed that alkaline phosphatase is an important component of lysosomal enzymes that originate from haemocytes to destroy extracellular "invaders". It has been documented that alkaline phosphatase activity of crabs, *Cryptograpsus angulatus* (Pinoni and Lopez Mananes, 2004) and *Chasmagnathus granulatus* (Pinoni *et al.*, 2005) acclimated to low salinity (10‰) was lower than in high 35‰ salinity. Lovett *et al.* (1994) reported

the presence of an alkaline phosphatase at pH 9.1 in the posterior gills of *Callinectes sapidus* and the activity was sensitive to environmental salinity and that specific activity of the alkaline phosphatase was greater in *C. sapidus* acclimated to 35‰ than crabs acclimated to 10‰. In the present study, ALP was high in shrimps held at 5 and 35‰ compared to 25‰ till post challenge day 5 and a decrease in ALP activity was noted in shrimps held at 35‰ on post challenge day 7 onwards compared to other groups. The changes of alkaline phosphatase in response to acclimation salinity suggested the role of this enzyme in modulating the osmoregulatory response of *C. sapidus* (Lovett *et al.*, 1994).

It is known that environmental parameters affect the growth of pathogens and their production of toxins (Weinberg, 1985; Arp, 1988). The present study has shown that *F. indicus* adults acclimated to high salinity (35‰) has shown increased susceptibility to *V. harveyi* infection with highest mortality of the shrimps held at hyper osmotic condition. A significant reduction in many of the analysed haematological parameters are well correlated with the increased susceptibility of shrimps to the pathogen. The suppressed immuno-physiological responses displayed by the shrimps maintained at 35‰ in the later stages of infection indicated that the shrimps are more vulnerable to this pathogen under hyper osmotic condition. It was observed that the opportunistic pathogen, *V. harveyi* preferred high saline condition and has proliferated maximum in shrimps held at 35‰ salinity indicating the opportunistic nature and high saline preference of the pathogenic *V. harveyi.* Wang and Chen (2006) reported that concentration of NaCl at 2.5% significantly increased the growth rate of *Photobacterium damselae* subsp. *damselae* and an addition of 2.5% NaCl in TSB medium resulted in an increased virulence of *P. damselae* to *P. monodon*. It is known that fluctuations in normal environmental conditions such as temperature, salinity and oxygen

have a significant effect on the virulence of *V. harveyi*, and according to Kautsky (2000) higher salinity increases virulence to shrimp more than at higher temperatures. In our study, *V. harveyi* showed higher infectivity in high saline condition (35‰) indicating that pathology and virulence of the halophilic *V. harveyi* is maximal at higher salinity and that lower salinity is not congenial for pathogenicity of the isolate. However, the ambient salinity affected the organism's immune status at a faster rate at 5‰. The study showed a direct relation between the pathogen and salinity. Similar observations have been reported previously. Farghay (1950) demonstrated that the environmental factors such as low salinity and changeable pH had reduced the growth rate of luminous bacteria. Cheng and Chen (1999) reported that incubation of *L. gravieae* under optimal conditions in BHIP containing 0.5% to 1.0% NaCl significantly enhanced its virulence for *M. rosenbergii*. Later, Hauton *et al.* (2000) documented that high salinity (32‰ promoted the growth of the bacterial inoculate, *Listionella anguillarum* and its proliferation was higher in *Ostrea edulis* at high salinity. In contrast to our results, Prayitno and Latchford (1995) reported that exposure of *V. harveyi* to low salinities (10‰ and 15‰) significantly increased its virulence in *P. monodon* larvae resulting in higher rate of mortalities. But, recent report of Alavandi *et al.* (2006) echoes our conclusion that physico-chemical conditions of larval rearing influenced the pathogenicity of *V. harveyi* and that higher temperature and salinity appeared to play a role on the mortality of larval shrimp upon bacterial challenge.

In conclusion, the significant decrease of haemolymph protein components and majority of the haemolymph enzymes in *F. indicus* maintained in 35‰ salinity has been directly correlated with mortality and inversely correlated with proliferation of bacterial pathogen. The study indicates that the opportunistic *V. harveyi* become highly virulent in

shrimps held at hyper osmotic conditions and the reduction in the immune parameters have resulted in higher mortality of *F. indicus* at high salinity. In other salinities (5 and 25‰) V. harveyi proliferation was comparatively less causing lower rate of mortality and immune suppression. These haematological profiles can be used as an index for monitoring the health conditions of shrimps, which are susceptible to a variety of environmental stressors.

Chapter 4

HAEMATOLOGICAL RESPONSES AND SUSCEPTIBILITY OF *F. INDICUS* TO WHITE SPOT SYNDROME VIRUS UNDER ACUTE SALINITY STRESS

Contents

4.1 Introduction

Viruses are the most economically significant pathogens of cultured shrimps. More than 20 viral species have been identified from diseased shrimp (Lightner and Redman, 1998). Among them, white spot syndrome virus (WSSV) is one of the most important and it causes massive production loss in all shrimp-growing countries where rapid expansion and intensification of shrimp farming practices are common. Within years after its first discovery in Asia in the early 1990s, white spot syndrome (WSS) has developed into an epizootic disease.

WSSV is extremely virulent and infects vital organs of mesodermal and ectodermal origin, as evidenced by the presence of degenerate cells with hypertrophied nuclei in the infected tissues (Ramasamy, 2000 and Rajan *et al.*, 2000). The clinical signs of the syndrome include lethargy, anorexia, the presence of white spots on the cuticle and, often, a generalized reddish to pink discoloration (Durand *et al.*, 1997). Infection in *P. monodon* was readily recognized by the presence of numerous white spots on the carapace, while in *P. indicus*, it was necessary to remove the carapace to confirm infection, usually accompanied by a reddish body colouration, (Wang *et al.*, 1995; Chang *et al.*, 1996; Wongteerasupaya *et al.*, 1996; Lo *et al.*, 1996 b).

White spot syndrome virus (WSSV) is the type species of the genus Whispovirus of the new family Nimaviridae (Mayo, 2002). The ultra structure of WSSV has been studied by many researchers. The intact virus was enveloped and elliptical in shape measuring 266 x 112 nm, and the nucleocapsid of WSSV was cylindrical in shape (420 x 68 nm) with one end flat and the other pointed, and had a pattern of opaque and transparent striations arranged perpendicularly to the long axis of the nucleocapsid (Sahul Hameed *et al.*, 1998). Negative staining could be used to reveal the tail-like appendages. WSSV virions are enveloped,

have a bacilliform to ovoid shape, are about 275 nm in length by 120 nm in width and have a tail-like appendage at one end (Rajan *et al.*, 2000).

The virions can be found throughout the body of infected animals, infecting most tissues and circulating ubiquitously in the haemolymph. The enveloped virions contain a single nucleocapsid with a distinctive striated appearance. The WSSV virion consists of 5 major and about 13 minor proteins (Van Hulten *et al.*, 2000a & b; Huang *et al.*, 2002). Sequencing of the WSSV genome revealed a circular sequence of 292, 967 base pairs (bp) (Van Hulten *et al.*, 2001). But there is variation in size among geographic isolates of WSSV (Yang *et al.*, 2001).

Lightner and Redman (1998) demonstrated that the disease is the end result of a complex interaction between the shrimp, its environment and the pathogen itself. Under some conditions, the host (or host populations) and its potential pathogen co-exist with little or no adverse effect. Experience has also shown that shrimps infected with WSSV could survive the whole culture period without clinical signs by manipulating the environmental factors. Therefore, studies about the effects of the environmental factors on WSSV outbreak are of major importance to shrimp aquaculture.

WSSV outbreak seems to be triggered or aggravated by changes in seawater quality including hardness, temperature, and dissolved oxygen (Kautsky *et al.*, 2000). Cheng and Chen (2000) found that the total haemocyte count (THC) of freshwater prawn *Macrobrachium rosenbergii* (de Man) was directly proportional to salinity and that phenoloxidase activity was significantly higher at 5 and 10‰ than at 0 and 15‰, respectively. A relationship between salinity and infectious hypodermal and haematopoietic necrosis virus (IHHNV) has been observed in *Litopenaeus vannamei* (Bray *et al.*, 1994).

WSSV prevention and control are priorities and shrimp immunology has become a prime area of research. Furthermore, it is very important to notice that environmental stressors can affect shrimp immunity. Elevated ammonia has been reported to reduce growth and enhance molting (Wickens, 1976; Chen and Kou, 1992), suppress immune parameters such as total haemocyte count (THC), prophenoloxidase (proPO), superoxide dismutase (SOD) and peroxidase (POD) (Sun and Ding, 1999) leading to the death of penaeid shrimp (Kou and Chen, 1991). Liu *et al.* (2006) reported that salinity changes over a particular range could result in a decrease of immunocompetence and obvious WSSV proliferation in the shrimps leading to white spot syndrome developing from a latent infection to an acute out break.

The impact of WSSV infection in *Fenneropenaeus indicus* has been already studied in terms of immunological parameters (Yoganadhan *et al.*, 2003) and antioxidant parameters (Mohankumar and Ramasamy, 2006). However, the susceptibility of *F.indicus* to WSSV under salinity stress has not been investigated so far. Hence, the present study was aimed at assessing the haemolymph parameters viz total haemolymph protein (THP), plasma protein (PLP), serum protein (SRP), total free amino acids (TFAA) phenol oxidase, superoxide anion production, alkaline phosphatase and acid phosphatase in the haemolymph of shrimp under acute salinity stress and WSSV challenge to understand the effects of salinity on the immunocompetence of *F. indicus* to WSSV.

4.2 Materials and methods

4.2.1 Experimental animals and rearing conditions

Adult *Fenneropenaeus indicus* were brought to the Laboratory within one hour of capture from a commercial shrimp farm located at Panangad, Kochi, India. The average wet weight of the shrimp was 15.86 ± 2.32g (Mean ± S.D.). Shrimps were reared in rectangular concrete

tanks containing 25 ‰ clean sea water and allowed to acclimate for a period of seven days. Continuous aeration was provided using air pumps and the animals were maintained on a commercial shrimp diet (Higashimaru, Pvt.Ltd. Kochi). Water quality parameters *viz.* temperature, dissolved oxygen, NH_3-N, NO_2-N and NO_3-N were monitored daily following standard procedures (APHA, 1995) and maintained at optimal levels as per Table.2.1. Unused feed and faecal matter was siphoned out daily and 30 % water exchanged every alternate day. A biological filter was set up to maintain the appropriate levels of water quality parameters. After acclimating to 25‰ for seven days six (n=6) shrimps were sampled for baseline data.

4.2.2 Experimental setup and salinity adjustments.

Shrimps of apparently uniform size were distributed in the experimental tanks containing 500L of seawater (n=35/tank). Shrimps in the intermoult stage only were used (Robertson *et al.*, 1987). There were four treatment Groups (G-I, G-II, G-III and G-IV) and the experiment was conducted in triplicate i.e., 3 tanks per treatment. Salinity of all the tanks was adjusted to 25‰ prior to the experiment. After 12 hours of starvation, the salinity of G-I shrimps was lowered from 25‰ to 5‰ by diluting with fresh water. Whereas, the salinity of G-II was raised from 25‰ to 35‰ by adding sea water. The desired salinity was adjusted over a period of six hours. Shrimps of G-III and G-IV was maintained at 25‰ itself with no salinity change. All four groups were maintained on commercial diet. Ten minutes after the desired salinity level was reached six shrimps from each group (n=6) were sampled (post salinity day 0, PSD0). Water quality parameters were maintained at optimal levels as in section 4.2.1.

4.2.3 WSSV challenge

The shrimps of G-II, G-III and G-IV were then challenged with White Spot Syndrome Virus. Challenge was performed through oral administration

i.e., by feeding white spot virus infected frozen tissue at the rate of 1g/shrimp. Group- I was maintained as the unchallenged control. Shrimps were sampled (n=6) after 24 h (post challenge day1, PCD 1), 48 h (post challenge day 2, PCD 2), 72 h (post challenge day 3, PCD 3) and 120 h of challenge (post challenge day 5, PCD 5). Before each sampling, the shrimps were starved for 12 hours to eliminate physiological variations caused by the ingested food (Hall and van Ham, 1998). Survival in each group was recorded daily for a period of 10 days with dead animals removed promptly. Mortality by WSSV infection was identified by checking the characteristic white spots on the carapace of infected shrimps and further confirmed by PCR detection.

4.2.4 PCR confirmation of WSSV infection

Gill samples (30 mg) from WSSV challenged and unchallenged prawns were used for DNA extraction and the presence of WSSV was confirmed by diagnostic PCR following Lo *et al.* (1996 a). The primers were synthesized by M/s. Bangalore Genei, Bangalore, India. (Fig.4.1)

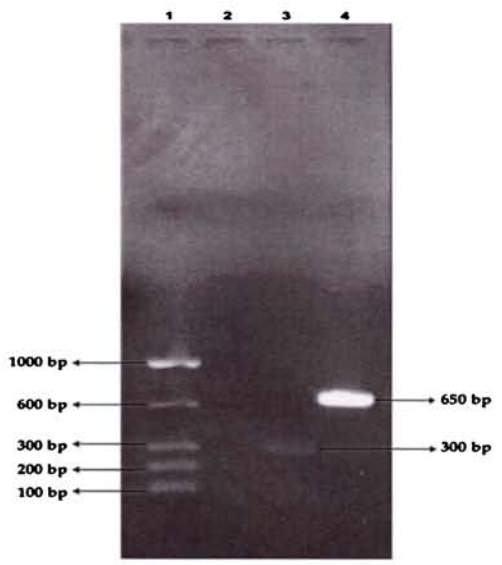


Fig.4.1 PCR amplified DNA products of WSSV templates. DNA from infected gill tissues of *F. Indicus.*

Lane 1. Molecular weight markers.
Lane 2. Absence of PCR amplified products (WSSV-ve)
Lane 3. Nested PCR +ve
Lane 4. Presence of PCR amplified products (WSSV +ve)

4.2.5 Extraction of haemolymph and haematological analysis.

Extraction of haemolymph and haematological analysis (THP, PLP, SRP, TFAA, PO activity, NBT reduction, ALP activity and ACP activity were as given in section.2.2.4.

An aliquot of haemolymph was placed in an Improved Neubauer Chamber and the haemocyte count (THC) was done using Light microscope. THC was expressed as number of cells per ml of haemolymph.

4.2.6 Statistical analysis

A multiple comparison (Tukey) test was conducted to compare the significant differences among treatment groups using the software SPSS 10.00 package. All data are presented as mean ± S.D. and the differences were regarded as statistically significant when $p < 0.05$.

4.3 Results

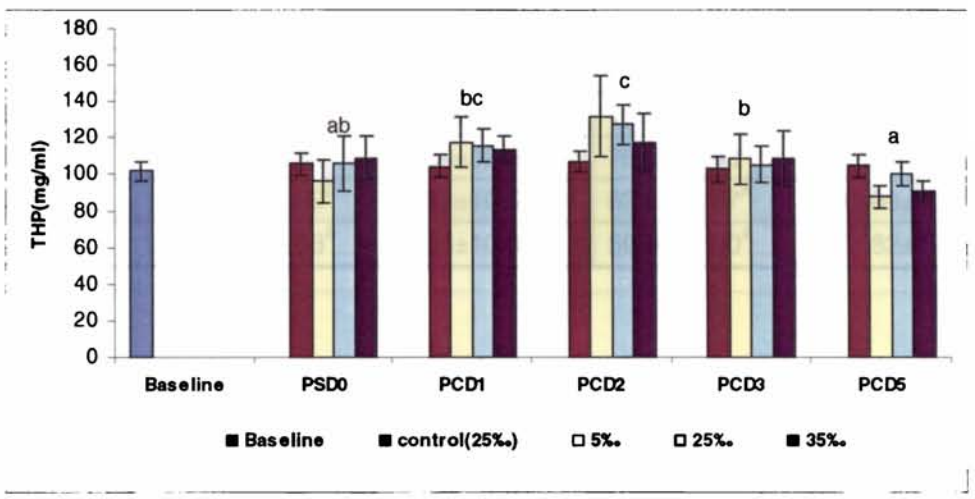
4.3.1 Effect of salinity changes on the haematological parameters of *F. indicus*

4.3.1.1 Total haemolymph protein

The acute salinity stress has reduced the total haemolymph protein concentration in shrimps held at 5‰. After virus challenge, THP of shrimps held at different salinity levels were enhanced compared to those of control groups. Maximum THP concentration was observed in shrimps held at 5‰ compared to those held at 25 and 35‰. There was no significant difference in the THP among the shrimps held at 5, 25 and 35‰ salinity. Considerable increase ($p<0.05$) in the THP of shrimps could be noted after two days of WSSV challenge. The THP concentration in the haemolymph of shrimps held at different salinity (5, 25 and 35‰) and challenged with WSSV were found to decrease from post challenge day three. (Table 4.1 and Fig.4.2).

Table.4.1 Total haemolymph protein of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Each value represents the mean ± SD. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Total haemolymph protein(mg/ml)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	102.15±5.36			
PSD0	105.81±6.17^{A}	96.26±11.82^{A}	106.12±14.74^{A}	109.28±9.08^{A}
PCD1	104.38±6.23^{A}	117.91±11.93^{A}	115.88±14.87^{A}	113.93±11.88^{A}
PCD2	107.12±5.69^{A}	131.97±14.14^{A}	127.47±9.05^{A}	117.63±7.21^{A}
PCD3	103.22±7.12^{A}	108.53±22.19^{A}	105.26±11.18^{A}	109.00±15.49^{A}
PCD5	105.02±6.23^{A}	87.71±13.87^{A}	100.46±9.88^{A}	91.12±14.80^{A}



THP- Total haemolymph protein
PSD- Post salinity change, PCD- Post challenge day

Fig. 4.2. Total haemolymph protein of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Each bar represents the mean value from six separate determinations (Mean ± SD). Bars with different lowercase letters vary significantly ($p<0.05$) among different exposure times. Control - unchallenged.

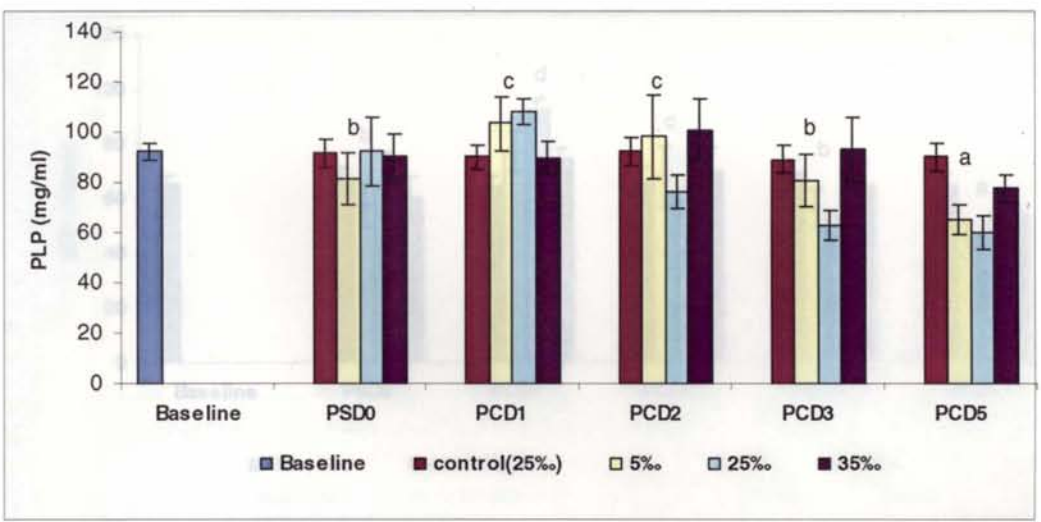
4.3.1.2 Plasma protein

Acute salinity change slightly reduced the plasma protein concentration of shrimps held at hypoosmotic condition. There was salinity wise variation in the plasma protein of shrimps held at 5 and 35‰ salinity levels compared to

PLP of shrimps held at 25‰. Comparatively higher ($p<.0.05$) plasma protein concentration was obtained during PCD1 and PCD2. A reduction in the plasma protein concentration compared to the unchallenged group was observed from 3rd day of post challenge onwards. Differences were found to be significant at $p < 0.05$. (Table 4.2 and Fig. 4.3)

Table.4.2 Plasma protein (mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Plasma protein (mg/ml)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	92.26±3.14			
PSD0	91.58± 5.76^{B}	81.28± 3.17^{B}	92.34± 14.06^{A}	90.14±9.31^{B}
PCD1	90.16±5.79^{B}	103.36±10.45^{B}	108.02±13.86^{A}	89.96±9.38^{B}
PCD2	92.33±4.98^{B}	98.34±10.54^{B}	76.08±5.40^{A}	100.45±6.15^{B}
PCD3	89.12±5.39^{B}	80.88±10.54^{B}	62.82±6.67^{A}	93.09±13.23^{B}
PCD5	90.07±5.68^{B}	65.36±10.34^{B}	59.96±5.90^{A}	77.82±12.64^{B}



PLP- Plasma protein
PSD- Post salinity change, PCD- Post challenge day

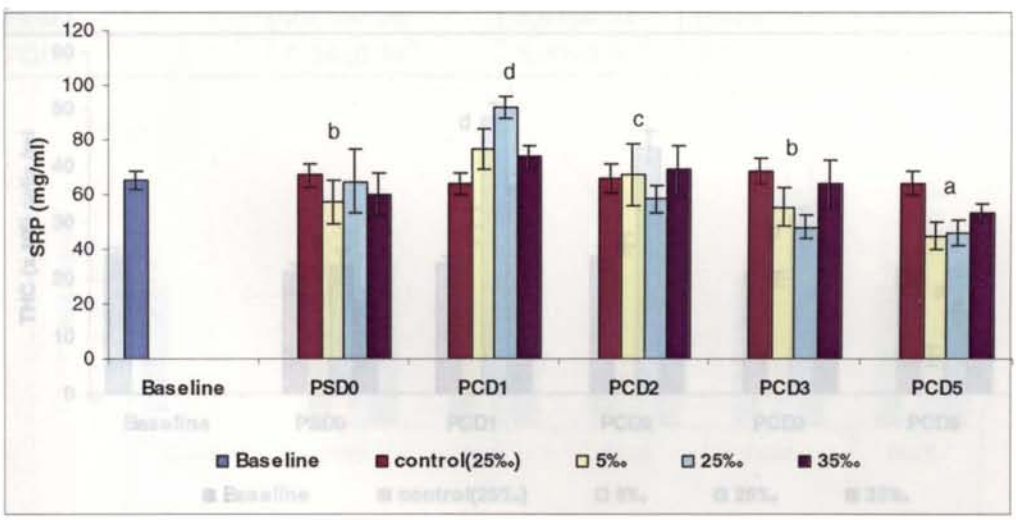
Fig. 4.3. Plasma protein (Mean ±SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV (See Fig.4.2. for statistical details).

4.3.1.3 Serum protein

Post salinity variation in SRP was prominent in shrimps maintained at 5‰ salinity. Serum protein concentration showed lesser variations with changing salinities. After WSSV challenge the serum protein concentration has shown an increase ($p<.0.05$) on PCD1 and then showed a gradual decline (Table 4.3 and Fig.4.4).

Table. 4.3 Serum protein (mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Serum protein (mg/ml)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	65.14±3.2			
PSD0	$67.03±4.24^{B}$	$57.28±7.18^{A}$	$64.82±11.54^{A}$	$60.25±7.39^{AB}$
PCD1	$64.01±4.11^{B}$	$76.59±7.75^{A}$	$92.03±11.81^{A}$	$73.68±7.68^{AB}$
PCD2	$66.11±5.22^{B}$	$67.56±7.24^{A}$	$58.43±4.15^{A}$	$69.21±4.24^{AB}$
PCD3	$68.56±4.52^{B}$	$55.56±11.36^{A}$	$48.25±5.12^{A}$	$64.14±9.12^{AB}$
PCD5	$64.28±4.29^{B}$	$44.91±7.10^{A}$	$46.05±4.53^{A}$	$53.62±8.71^{AB}$



SRP- Serum protein
PSD- Post salinity change, PCD- Post challenge day

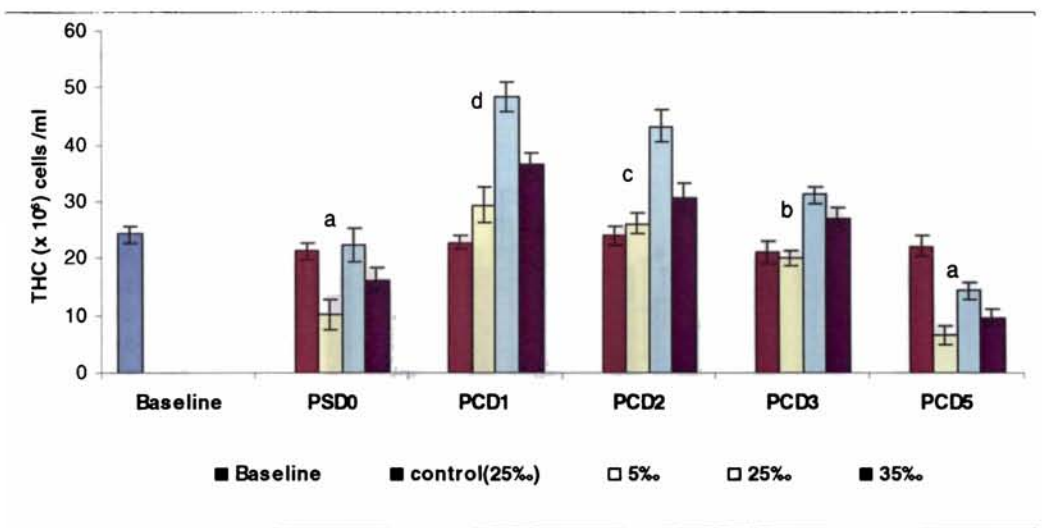
Fig. 4.4. Serum protein (Mean ±SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV (See Fig.4.2. for statistical details).

4.3.1.4 Total haemocyte count

Total haemocyte count of *F. indicus* has significantly reduced ($p < 0.05$) in shrimps subjected to acute salinity stress. After WSSV challenge there was a slight increase in the haemocyte count on PCD 1 and 2. This was followed by a reduction ($p<0.05$) in THC from PCD 3 onwards in all challenged groups. THC of shrimps held at 5 and 35‰ salinities were lowering ($p<0.05$) than that of those held at 25 ‰ salinity (Table 4.4 and Fig.4.5).

Table. 4.4 Total haemocyte Count (mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Total haemocyte count (THC x 10^6 cells/ml)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	24.29±1.44			
PSD0	21.35±1.45^{A}	10.26±2.61^{A}	22.38±2.78^{C}	16.18±2.27^{B}
PCD1	22.86±1.23^{A}	29.50±2.59^{A}	48.54±2.86^{C}	36.50±2.26^{B}
PCD2	24.12±1.71^{A}	26.17±3.16^{A}	43.33±2.58^{C}	30.50±2.17^{B}
PCD3	20.95±1.97^{A}	20.00±1.90^{A}	31.17±2.71^{C}	27.17±2.83^{B}
PCD5	22.24±1.80^{A}	6.67±1.37^{A}	14.38±1.60^{C}	9.67±1.87^{B}



THC- Total haemocyte count
PSD- Post salinity change, PCD- Post challenge day

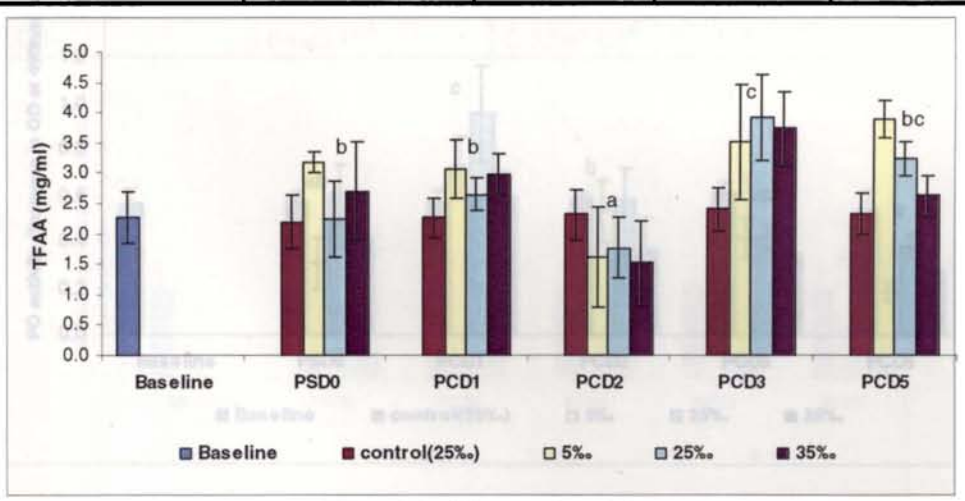
Fig. 4.5. Total haemocyte Count (Mean ±SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV (See Fig.4.2. for statistical details).

4.3.1.5 Total free amino acids

Total free amino acids in the haemolymph of shrimps held at all three salinity ranges showed variations among the treated and untreated groups. An increase ($p<0.05$) in the TFAA content could be noted on PSD 0 and PCD 1. TFAA concentration reduced significantly ($p < 0.05$) on PCD 2 in all salinity ranges. Highest amount ($p<0.05$) of TFAA was found in shrimps at PCD 3 (Table 4.5 and Fig.4.6).

Table .4.5 Total free amino acids (mean ± SD) in the haemolymph of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Total Free Amino acids (mg/ml)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	2.27±0.42			
PSD0	2.19 ± 0.44^{A}	3.18 ± 0.43^{B}	2.24 ± 0.18^{B}	2.71 ± 0.81^{B}
PCD1	2.26 ± 0.34^{A}	3.06 ± 0.18^{B}	2.65 ± 0.63^{B}	2.99 ± 0.82^{B}
PCD2	2.32 ± 0.42^{A}	1.62 ± 0.48^{B}	1.77 ± 0.27^{B}	1.53 ± 0.33^{B}
PCD3	2.41 ± 0.36^{A}	3.51 ± 0.83^{B}	3.92 ± 0.51^{B}	3.75 ± 0.69^{B}
PCD5	2.34 ± 0.34^{A}	3.90 ± 0.96^{B}	3.25 ± 0.71^{B}	2.63 ± 0.61^{B}



TFAA- Total free aminoacids
PSD- Post salinity change, PCD- Post challenge day

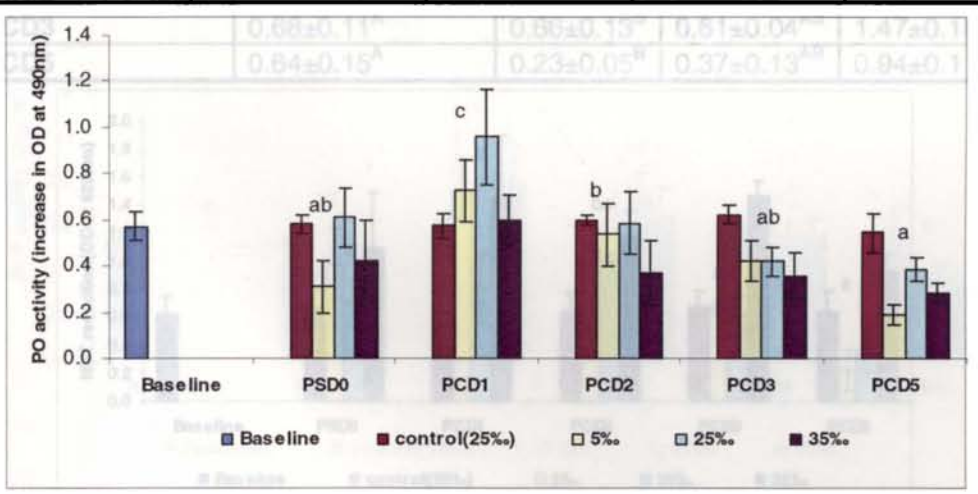
Fig. 4.6 Total free amino acids (Mean ±SD) in the haemolymph of *F. indicus* subjected to acute salinity stress and then challenged with WSSV (See Fig.4.2. for statistical details).

4.3.1.6 Phenol oxidase activity

Significant reduction ($p < 0.05$) in phenol oxidase activity was obtained after acute salinity stress. A profound increase ($p < 0.05$) in phenol oxidase activity of shrimps held at 5 and 25‰ salinity was found after PCD 1 of WSSV challenge when compared to control group. A gradual reduction ($p < 0.05$) in the phenol oxidase activity was observed from PCD 2 onwards in all challenged groups. (Table 4.6 and Fig.4.7).

Table .4.6. Phenol oxidase activity (mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Phenol oxidase activity (Increase in OD at 490nm)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	0.57±0.06			
PSD0	0.58 ± 0.04^{A}	0.31 ± 0.07^{A}	0.61 ± 0.12^{A}	0.54 ± 0.14^{A}
PCD1	0.57 ± 05^{A}	0.73 ± 0.11^{B}	0.96 ± 0.13^{B}	0.60 ± 0.18^{B}
PCD2	0.60 ± 02^{A}	0.54 ± 0.14^{B}	0.59 ± 0.21^{B}	0.37 ± 0.11^{B}
PCD3	0.62 ± 0.04^{A}	0.42 ± 0.14^{B}	0.42 ± 0.14^{B}	0.36 ± 0.14^{B}
PCD5	0.54 ± 0.08^{A}	0.19 ± 0.09^{B}	0.38 ± 0.06^{B}	0.28 ± 0.10^{B}



PO – Phenol oxidase
PSD- Post salinity change, PCD- Post challenge day

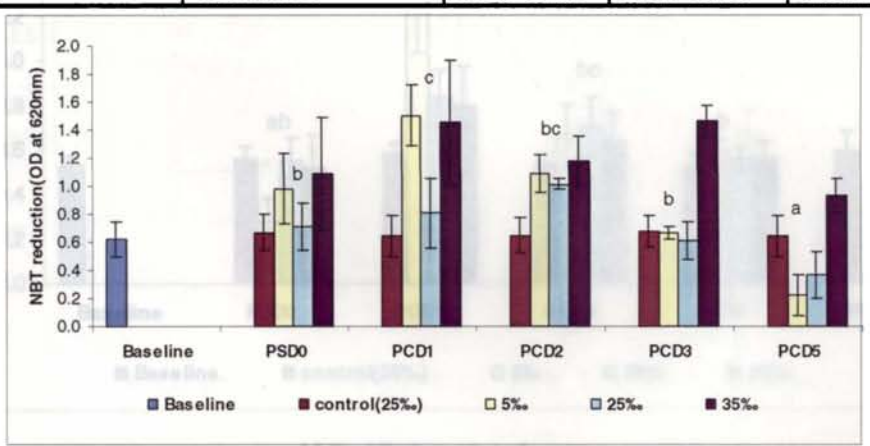
Fig. 4.7 Phenol oxidase activity (Mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV(See Fig.4.2. for statistical details).

4.3.1.7 Super oxide anion production

The results obtained for super oxide anion production is given in the Table 4.7 and Fig. 4.8. Super oxide anion production was higher ($p < 0.05$) in shrimps held at 35‰ after sudden salinity change compared to other groups. There was significant difference ($p < 0.05$) in the super oxide anion production among the control and challenged groups of shrimps. Super oxide anion production was found to increase significantly on PCD 1 and 2 in shrimps held at 5 and 35‰. Considerable increase in super oxide production could be noted in shrimps held at 35‰ compared to those held at other salinity levels on all sampling days.

Table . 4.7 Super oxide anion production (mean ± SD) in the haemolymph of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Super oxide anion production (OD at 620nm)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	0.62±0.12			
PSD0	0.67 ± 0.12^{A}	0.98 ± 0.26^{B}	0.71 ± 0.18^{AB}	1.09 ± 39^{C}
PCD1	0.64 ± 0.12^{A}	1.50 ± 0.25^{B}	0.81 ± 0.17^{AB}	1.45 ± 40^{C}
PCD2	0.65 ± 0.15^{A}	1.09 ± 0.22^{B}	1.02 ± 2.25^{AB}	1.18 ± 0.45^{C}
PCD3	0.68 ± 0.11^{A}	0.66 ± 0.13^{B}	0.61 ± 0.04^{AB}	1.47 ± 0.18^{C}
PCD5	0.64 ± 0.15^{A}	0.23 ± 0.05^{B}	0.37 ± 0.13^{AB}	0.94 ± 0.11^{C}



NBT reduction- Nitroblue tetrazolium reduction
PSD- Post salinity change, PCD- Post challenge day

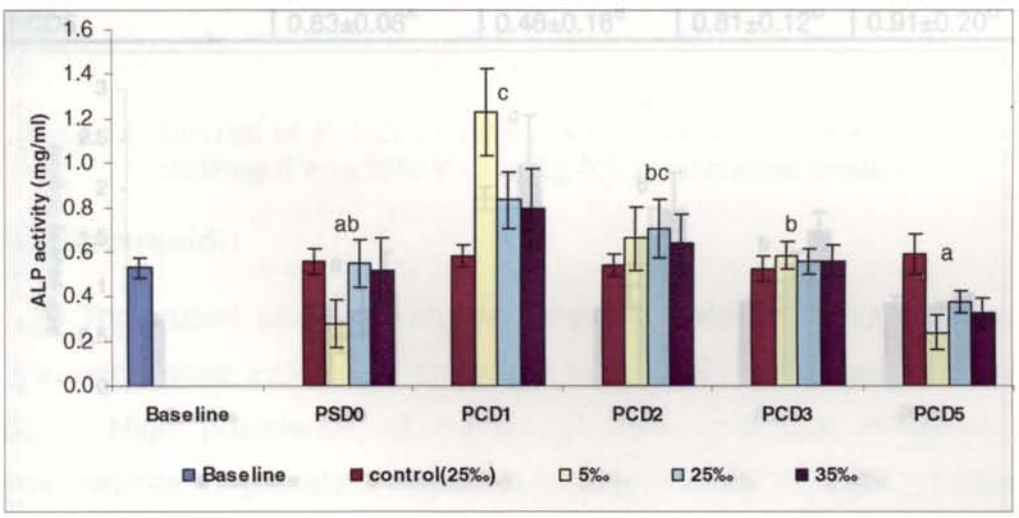
Fig. 4.8 Super oxide anion production (NBT reduction) (Mean ± SD) in the haemolymph of *F. indicus* subjected to acute salinity stress and then challenged with WSSV (See Fig.4.2. for statistical details).

4.3.1.8 Alkaline phosphatase activity

Alkaline phosphatase activity showed a significant decrease ($p < 0.05$) in shrimps held at 5‰ salinity after acute salinity stress. After WSSV challenge ALP activity of shrimps held at different salinity levels showed a gradual increase in all three salinities ($p < 0.05$) compared to the control group. Higher activity ($p < 0.05$) of ALP was found at PCD 3 in shrimps held at 5‰ salinity compared to those held at other salinity ranges (Table 4.8 and Fig.4.9).

Table .4.8 Alkaline phosphatase activity (mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Alkaline phosphatase (mg/ml)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	0.53±04			
PSD0	0.56 ± 06^{A}	0.28 ± 0.12^{B}	0.55 ± 0.10^{A}	0.52 ± 16^{A}
PCD1	0.58 ± 0.05^{A}	1.23 ± 0.11^{B}	0.83 ± 0.11^{A}	0.80 ± 0.15^{A}
PCD2	0.54 ± 0.05^{A}	0.66 ± 0.20^{B}	0.71 ± 0.13^{A}	0.64 ± 0.17^{A}
PCD3	0.52 ± 0.06^{A}	0.59 ± 0.14^{B}	0.56 ± 0.13^{A}	0.56 ± 0.13^{A}
PCD5	0.59 ± 0.09^{A}	0.24 ± 0.06^{B}	0.38 ± 0.06^{A}	0.33 ± 0.07^{A}



ALP- Alkaline phosphatase
PSD- Post salinity change, PCD- Post challenge day

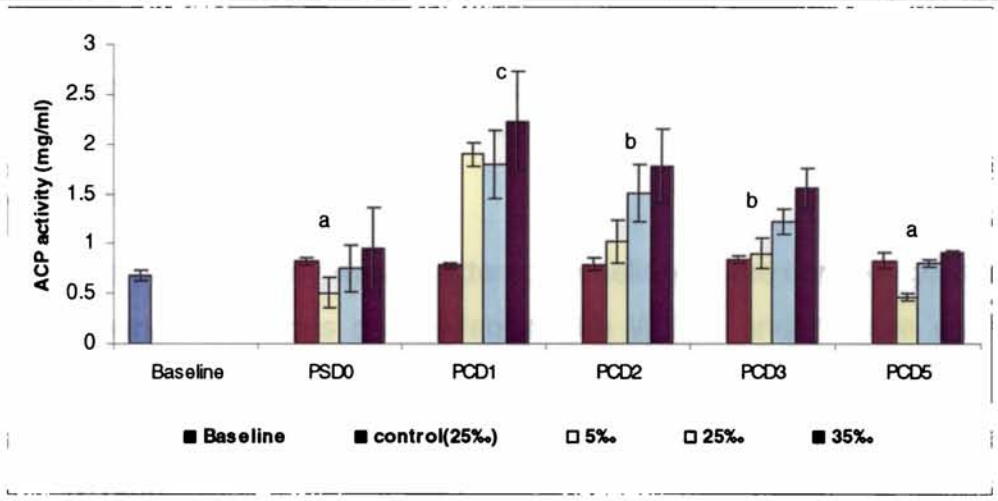
Fig. 4.9 Alkaline phosphatase activity (Mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV(See Fig.4.2. for statistical details).

4.3.1.9 Acid phosphatase activity

Acid phosphatase activity lowered after salinity stress in shrimps held at 5 and 25‰ compared to control. However, a slight increase in ACP activity could be observed after acute salinity change at 35‰ salinity level. ACP activity of shrimps held at 5, 25 and 35‰ salinities showed a gradual increase from PCD 1 to PCD 3 compared to the acid phosphatase activity of control group of shrimps ($p < 0.05$). Higher activity of ACP was noted in shrimps held at 35‰ compared to those held at other salinity levels (Table 4.9 and Fig.4.10).

Table .4.9 Acid phosphatase activity (mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV. Values with different superscripts in the same rows vary significantly ($p<0.05$) among different salinity treatments. Control - unchallenged.

Time interval	Acid phosphatase (mg/ml)			
	Salinity(‰)			
	Control(25‰)	5‰	25‰	35‰
Baseline	0.68±0.05			
PSD0	0.82 ± 0.04^{A}	0.51 ± 0.18^{B}	0.76 ± 21^{C}	0.95 ± 39^{D}
PCD1	0.78 ± 0.03^{A}	1.90 ± 0.45^{B}	1.79 ± 0.23^{C}	2.23 ± 0.41^{D}
PCD2	0.80 ± 0.06^{A}	1.02 ± 0.31^{B}	1.50 ± 0.34^{C}	1.78 ± 0.49^{D}
PCD3	0.85 ± 0.04^{A}	0.90 ± 0.22^{B}	1.21 ± 0.29^{C}	1.57 ± 0.37^{D}
PCD5	0.83 ± 0.08^{A}	0.46 ± 0.16^{B}	0.81 ± 0.12^{C}	0.91 ± 0.20^{D}



ACP- Acid phosphatase
PSD- Post salinity change, PCD- Post challenge day

Fig. 4.10. Acid phosphatase activity (Mean ± SD) of *F. indicus* subjected to acute salinity stress and then challenged with WSSV(See Fig.4.2. for statistical details).

4.3.2 Effect of salinity on WSSV infectivity and mortality *in F. indicus*

Post challenge survival rates showed significant variation ($p < 0.05$) in all treatment groups. Maximum survival was observed for shrimps maintained at 25‰ followed by 35‰ and least in shrimps held at 5‰. Shrimps held at 5‰ salinity showed early mortality on PCD3 onwards compared to other groups. Throughout the experiment the survival rate was least in shrimps maintained at 5‰. Comparatively higher survival has been shown by shrimps held at 25‰. Animals in the control group (unchallenged) showed 100% survival. (Fig.4.11).

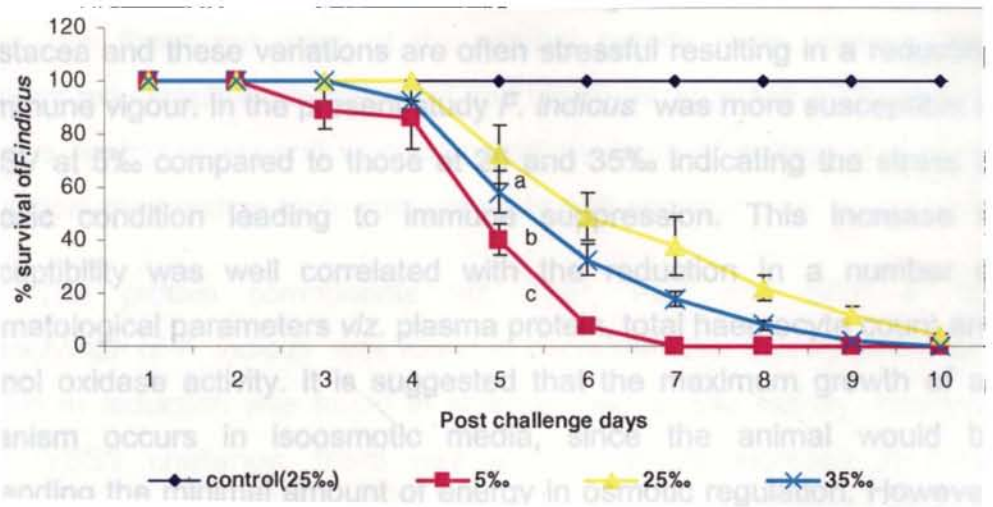


Fig.4.11 Survival of *F. indicus* subjected to acute salinity stress and then challenged with WSSV (See Fig.4.2. for statistical details).

4.4 Discussion

The present study was an attempt to delineate the immune profile of *Fenneropenaeus indicus* on challenge with WSSV under acute salinity stress. High prevalence of certain diseases may be indicative of environmental degradation or poor management and disease symptoms can be crude indicators of stress. Hence, characterising the immune responses of healthy shrimp in response to environmental alterations and pathogenic invasion has been the focus of much research to detect deleterious effects before stress results in disease that harms the

population. An array of biomarkers that could be used to evaluate shrimp health was measured. The suitability of certain immunological parameters to be used as health indicators in shrimp was considered in detail.

The present study could document the immune profile of *Fenneropenaeus indicus* on challenge with WSSV under different salinity. There were measurable variations ($p<0.05$) in the haematological parameters of shrimps held at 5, 25 and 35‰ salinity and challenged with WSSV compared to the unchallenged group.

Environmental variations induce changes in immune status of Crustacea and these variations are often stressful resulting in a reduction of immune vigour. In the present study *F. indicus* was more susceptible to WSSV at 5‰ compared to those at 25 and 35‰ indicating the stress at hypoxic condition leading to immune suppression. This increase in susceptibility was well correlated with the reduction in a number of haematological parameters *viz.* plasma protein, total haemocyte count and phenol oxidase activity. It is suggested that the maximum growth of an organism occurs in isoosmotic media, since the animal would be expending the minimal amount of energy in osmotic regulation. However, salinity itself has little effect on the metabolic rate of euryhaline shrimp, indicating that the energy required for osmotic regulation may be relatively small. On the other hand, under unhealthy conditions such as in viral infection, the stress provoked by high salinity further augments growth retardation produced by the infection (Bray *et al.*, 1990). Moreover, Kautsky *et al.* (2000) have reported that changes in seawater quality including hardness, temperature, and dissolved oxygen can trigger and aggravate WSSV infection. Similarly, in the present study hypoosmotic condition severely triggered the WSSV infection in *F. indicus* making it more susceptible to the pathogen.

F. indicus was found to be more susceptible to WSSV when the animals were transferred to 5‰ and 35‰ from 25‰ in 96 h. In addition, *F. indicus* was more susceptible at 5 ‰compared to 35 ‰. Wang and Chen (2005) observed that *L.vannamei* was more susceptible to *V.alginolyticus* when the animals were transferred to 5‰ and 15‰ from 25‰ in 24 h. Liu *et al.* (2006) reported that acute salinity change from 22 to 14‰ resulted in rapid proliferation of WSSV in *Fenneropenaeus chinensis*, after 10 h of acute salinity change and the shrimps were more susceptible to WSSV following salinity changes. These results indicate that disease resistance of shrimps in a reduced salinity environment would be expected to be lower than those remaining at the original salinity since acute salinity changes may result in physiological and biochemical adaptive changes in shrimps having to expend corresponding amounts of energy in osmotic regulation and become more susceptible to pathogens.

The protein components *viz.* THP, PLP and SRP in the haemolymph of *F. indicus* was found to decrease after salinity stress and maximum reduction was found in shrimps held at 5‰ salinity. However after WSSV challenge, there was a considerable increase in these parameters in the haemolymph. Increase in haemolymph proteins owing to the increase in the amount of virus in haemolymph has been previously reported in *Manduca sexta* (Harwood *et al.*, 1994) and in *F. indicus* (Sahul Hameed *et al.*, 1998, Yoganadhan *et al.*, 2003). The plasma protein plays a vital role in the immunity of crustaceans; it not only correlates with the infection of pathogen (Vogan and Rowley, 2002; Song *et al.*, 2003) but also with environmental stress (Chen *et al.*, 1994)

In the present study, the total free amino acid levels of *F. indicus* were found to increase after salinity and WSSV stress indicating the metabolic adjustments to prevent the osmotic stress at suboptimal salinities.

It appears that a low circulating haemocyte number in crustaceans is strongly correlated with a greater sensitivity to pathogens as well as a low THC can be an indication of higher susceptibility to the infectious disease. In our study there was significant difference in the total haemocyte count of shrimps examined during the experiment at same sampling time in different salinity levels. THC was highest in shrimps maintained at 25‰ and was lowest in shrimps at 5‰ compared to those at 35‰. Haemocytes participate in defence reactions by being phagocytic and by playing an important role in wound closure and clotting in crustaceans (Bauchau, 1981). Maeda *et al.* (1997) have observed a decline in total haemocyte count in shrimp infected with penaeid rod-shaped DNA virus. The decrease in THC in infected animals was probably caused by hemocytic accumulation at the site of injection for wound healing and phagocytosis of foreign bodies (Bauchau and Mengeot, 1978; Ratcliffe and Rowley, 1979; Sahul Hameed, 1989). Another possibility is that the THC decline would be due to cell burst resulting from budding of the virus, or by virus induced apoptosis, since this type of cell "suicide" may be induced or repressed during some viral infections (Cohen, 1993). The THC of *P. monodon* infected with WSSV also decreased significantly (Chang *et al.*, 1999). Haemocyte lysis (Omori *et al.*, 1989), cell recruitment towards infected tissues, nodule formation (Martin *et al.*, 1998) or interference with haematopoiesis could contribute to lower THC. In addition, hypoxia significantly decreased the THC in *P. stylirostris* and stressed shrimp became susceptible to *Vibrio alginolyticus* infection (Le Moullac *et al.*, 1998).

Prophenol oxidase, the key enzyme in the synthesis of melanin, occurs in haemolymph as an inactive pro-enzyme prophenoloxidase (proPO). proPO is activated to form PO when it reacts with zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ions, trypsin, or heat. This PO enzyme binds to infected cells

via a probable thiol-ester-like motif, leading to production of toxic intermediates of phenol, which might aid in preventing the virus from gaining entry into the body cavity. Following salinity stress a reduction in PO activity could be observed in the present study. It has been previously reported that prophenoloxidase activity increased directly with salinity for the yellowleg shrimp *Farfantepenaeus californiensis* reared in salinity levels of 28‰, 32‰, 36‰, 40‰ and 44‰ (Vargas-Albores *et al.*, 1998), for the white shrimp *L. vannamei* reared in salinity levels of 5‰ , 15‰, 25‰ and 35‰ (Wang and Chen 2005), and for the tiger shrimp *P. monodon* reared in salinity levels of 5 ‰ , 15 ‰ and 25 ‰(Wang and Chen, 2006). Cheng *et al.* (2003) have reported that phenol oxidase activity of *M.rosenbergii* was significantly higher for animals reared at 5 and 10‰ than those reared in fresh water and 15‰. The apparent reduction in phenol oxidase activity in shrimps held at hypoosmotic condition indicates that the prophenol oxidase cascade has been affected by osmotic stress and the animal's immune vigour is suppressed under hypoosmotic condition in conjunction with WSSV proliferation. After that, PO activity was increased on PCD 1 in all salinities after WSSV challenge when compared to control (25‰). Significant difference in the phenol oxidase activity could also be observed at same exposure time in different salinities, the lowest being at 35‰followed by 5‰and 25‰

Salinity stress has induced an increase in super oxide anion production in shrimps held at 5 and 35‰. Intracellular superoxide anion production in shrimps maintained at 5‰ was maximum at PCD 1 and gradually reduced towards the end of the experiment. Similar pattern was shown by animals at 25 and 35‰. Cheng *et al.* (2004) reported that *H.diversicolar supertexta* when transferred to 20, 25 and 35‰ decreased the release of superoxide anion as compared to the abalone reared in 30‰. They could not distinguish whether the decrease in superoxide anion resulted from decreased NADPH oxidase activity or from increased

activity of superoxide dismutase (SOD) responsible for scavenging superoxide anions. The present study shows that there was significant difference in the intracellular anion production of shrimps held in different salinities at same exposure time. There was a significant increase in the superoxide anion production in shrimps maintained at 35‰ compared to those held at 5 and 25‰ salinities. However, at 5‰ the superoxide anion production in shrimps decreased drastically towards the end of the experiment.

The current study reports a reduction in the activity of alkaline and acid phosphatase enzymes in shrimps subjected to acute salinity stress. Lovett *et al.* (1994) reported the presence of an alkaline phosphatase at pH 9.1 in the posterior gills of *C. sapidus* and its alterations with respect to environmental salinity. The specific activity of this alkaline phosphatase was greater in *C. sapidus* acclimated to 35‰ than crabs acclimated to 10‰. The changes in the activity of alkaline phosphatase in response to salinity suggested the role of this enzyme in modulating the osmoregulatory response of *C. sapidus*. WSSV challenge, however, enhanced the activity of these enzymes. Many researches had showed that two phosphates played important role in the immune system as a key compound of lysozomal enzymes Wang *et al.* (2005). It has been known that phosphatases play an important role in acute energy crisis in aquatic organisms and involve in cytolysis and differentiation processes. In addition, phosphatase is the most important element of lysozomal enzymes, in crustacean cells, they perform the double function of digestion and defense (Jiang and Mu, 1999). Liu *et al.* (1999) reported that ACP was a sign of lysozome activity to digest the invading organisms in shrimps and ACP and ALP activity could reflect directly the nonspecific immunological state in them.

Cheng and Rodrick (1975) observed that alkaline phosphatase was an important component of lysozomal enzymes that originate from haemocytes to destroy extracellular "invaders". In the present study there was significant difference in the alkaline phosphatase activity of shrimps held at different salinities for the same sampling time. ALP activity of shrimps held at 5‰ salinity was significantly higher compared to those at 25‰ and 35‰ whereas ACP was maximum at 35‰ and lowest at 5‰.

Acute salinity changes result in physiological and biochemical adaptive changes in shrimps that have to expend corresponding amounts of energy in osmotic regulation. Therefore, disease resistance of shrimps will be lower in case of alterations in salinity of the culture environment. Environmental parameters profoundly influence the health and defense mechanisms of the host besides the virulence and prevalence of pathogens. These environmental variations are often stressful for crustaceans, resulting in a reduction of immunocompetence. It is concluded that under lower salinity, the *F. indicus* are at stress and more susceptible to infection compared to higher salinity levels as has been evidenced in the present study by reduced survival rate and immunological parameters of shrimps held at hypoosmotic conditions after WSSV infection. This study would be helpful in adopting prophylactic measures in case of salinity alterations of the culture environment through proper husbandry and management practices.

Chapter 5

ANTIOXIDANT DEFENCE PROFILE OF *F. INDICUS* CHALLENGED WITH WHITE SPOT SYNDROME VIRUS UNDER ACUTE SALINITY STRESS

Contents

5.1 Introduction

Exposure to environmental stressors is an inescapable aspect of an aquatic organism's life. Among the stressors oxygen is very toxic to aquatic animals and cause damage. Though oxygen is inherently dangerous to the existence of eukaryotic aerobic organisms, it is irony to note that they cannot exist without oxygen. In a normal cell, there is an appropriate prooxidant : antioxidant balance (Winston and Di Giulio, 1991). However, this balance can be shifted towards the prooxidant when production of oxygen species is increased under stress conditions or when levels of antioxidants are diminished. This state is called oxidative stress and can result in serious cell damage if the stress is massive or prolonged. Antioxidant defence mechanism is operated to detoxify or scavenge highly reactive oxygen species (ROS). It comprises of different types of functional components classified as first line, second line and third line defences.

ROS are known to be the most important components of the defense responses of crustaceans to invading microorganisms like bacteria, fungi and viruses (Munoz *et al.*, 2000). ROS such as super oxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen and the hydroxyl radical (OH^-) are highly microbicidal (Bachere *et al.*, 1995 and Munoz *et al.*, 2000). However, when an organism is under the influence of chemical, physical or biological stress (including infection by a pathogen) rapid shortage of oxygen causes irregular metabolic pathways, resulting in the formation of excessive amounts of singlet oxygen (Ranby and Rabek, 1978) and free radicals. Free radicals or reactive oxygen species (ROS) can damage lipids, proteins, carbohydrates and nucleotides (Yu, 1994), which are important constituents of membranes, enzymes and DNA respectively.

Antioxidants are potential indicators of oxidative stress in marine organisms (Agius *et al.*, 1998, Neves *et al.*, 2000 and Downs *et al.*, 2001). Virus (WSSV) has been causing havoc by producing devastating epidemics in Asia since 1988 (Primavera, 1997). Recently it has been reported that the WSSV infection affects the antioxidant defence system in shrimps with an increase in lipid peroxidation and a reduced activity of antioxidant enzymes in *Fenneropenaeus indicus* (Mohankumar and Ramasamy, 2006) and in *Penaeus monodon* (Rameshthangam and Ramasamy, 2006; Mathew *et al.*, 2007).

Recently, several investigators reported the effect of a variety of putative stressors on antioxidant defense systems in an attempt to obtain simple and reliable biomarkers of oxidative stress for aquatic organisms (Chien *et al.*, 2003; Wang *et al.*, 2005). Most of the studies on oxidative stress in crustaceans are restricted to their presence as well as their action during exposure to xenobiotics (Le Moullac and Haffner, 2000; Song and Hsieh, 1994; Arun *et al.*, 1999). However, there are reports emphasizing the alterations in the antioxidant defence profile of crustaceans in response to environmental parameters like temperature (Parihar *et al.*, 1996, 1997; Roche and Boge, 1996) and salinity (Roche and Boge, 1996). Recently, Liu *et al.* (2007) have shown that a wide change in salinity (from 30‰to 5‰ and 50‰) caused severe oxidative stress in shrimp, *Litopenaeus vannamei.*

The experimental investigations on the alterations in the tissue antioxidant defence system under pathogenic infection are relatively meager. It is important to study the changes occurring in the prophenol oxidase system and tissue antioxidant defence systems during pathogenesis to derive a conclusion to improve the disease management practices. In the present study, level of lipid peroxidation of in *Fenneropenaeus indicus* tissues (hepatopancreas, gill, muscler and

haemolymph) by estimating the hydroperoxides, conjugated dienes and malondialdehyde, besides the antioxidative enzymes (catalase, super oxide dismutase, glutathione peroxidase, glutathione reductase and glutathione –S- transferase) and the non enzymatic antioxidant reduced glutathione were estimated in order to understand the roles that the above parameters play in the defence mechanism of *F. indicus* to WSSV and to evaluate the effects of salinity on the immunocompetence of *F. indicus* to WSSV.

5.2 Materials and Methods

5.2.1 Tissue samples

Tissue samples of animals subjected to acute salinity stress and challenged with WSSV (Chapter 4) were used for the study. After extracting the haemolymph, shrimps were dissected out to collect hepatopancreas, gills and muscle tissues. These tissues were rinsed in ice cold 0.1M Tris –HCl buffer (pH 7.4), weighed and homogenized in Tris–HCl buffer using whole glass homogenizer. The homogenate was subjected to centrifugation at 2250 x g and the supernatant was used for the analysis. Antioxidant and lipid peroxidation products were analysed for haemolymph, hepatopancreas, muscle and gill tissues of *F. indicus* .

5.2.2 Assessment of antioxidant status and lipid peroxidation in *F. indicus* subjected to acute salinity stress and then challenged with WSSV

Antioxidant enzymes such as super oxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-s-transferase and the non-enzymic antioxidant substance, glutathione in various tissues were determined. The concentrations of enzymes and glutathione were estimated and expressed per milligram of protein in the corresponding tissues and therefore protein content of the tissues were also determined. Lipid peroxidation was assessed in terms of (MDA) thiobarbituric acid reactive substances (TBARS), hydroperoxides (HP) and

conjugated dienes (CD). The following methods are used for the biochemical analysis.

5.2.2.1 Estimation of super oxide dismutase (SOD)

Super oxide dismutase in different tissues was determined using the method of Kakkar *et al.* (1984). The tissue homogenate was subjected to differential centrifugation under cold conditions to obtain the cytosol fraction. Before estimating the activity, an initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulphate and this fraction was then dialysed against 0.0025 M Tris-HCl buffer (pH 7.4). The supernatant was used as the enzyme source. Assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052μM), 0.1 ml of PMS (186 μM), 0.3 ml of NBT (300 μM), 1.3 ml of distilled water and 0.1 ml of the enzyme source in 0.33m Sucrose buffer. The tubes were kept at 30^0C for 1 minute and then 0.2ml NADH (780 μM) was added and incubated at 30^0C for 90 sec and the reaction was stopped by the addition of 1 ml glacial acetic acid. Reaction mixture was shaken vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. The upper butanol layer was removed. Absorbance of the chromogen in butanol was measured at 560nm against n-butanol blank. A reagent blank without the enzyme source served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in one minute under the assay conditions and specific activity is expressed as units/mg protein.

5.2.2.2 Estimation of Catalase (CAT)

Catalase level in different tissues was determined using the method of Machly and Chance (1955). The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 30 mM hydrogen peroxide and the enzyme extract prepared by homogenising the tissue in phosphate buffer and centrifuging at 5000 rpm. Specific activity

was expressed as International Units (IU) / mg protein. 1 IU = change in absorbance/ min / extinction coefficient.

5.2.2.3 Estimation of Glutathione peroxidase (GPx)

Glutathione peroxidase in different tissues was estimated by the method of Rotruck (1973). To 0.2 ml of 0.4 M Tris buffer (pH 7.0), 0.2 ml EDTA (0.4mM), 0.1 ml sodium azide (10mM) and 0.5 ml tissue homogenate were added and mixed well. To this mixture 0.2 ml of GSH (2 mM) followed by 0.1 ml H_2O_2 (0.2mM) solution were added. The contents were mixed and incubated at 37°C for 10 minutes along with a control containing all reagents except tissue homogenate. After 10 minutes the reaction was arrested by the addition of 0.5 ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH as per Ellman's method (Ellman, 1959). The values were expressed as µg of GSH / min / mg protein.

5.2.2.4 Estimation of Glutathione reductase (GR)

Glutathione reductase was estimated by the method of Bergmeyer *et al.* (1974).The decrease in absorbance of the solution containing glutathione oxidized (GSSG) (1.15%), NADPH (0.06%), EDTA (15mM) and 0.067 M phosphate buffer (pH 6.6), was noted for 3-5 minutes at 340nm using a UV-visible spectrophotometer. The controls were run with distilled water instead of GSSG. Enzyme activity was expressed as units / mg protein. One unit is defined as the change in absorbance /minute.

5.2.2.5 Estimation of Glutathione-S-Transferase (GST)

Glutathione-S-transferase in different tissue was determined using the method of Beutler (1986). The reaction mixture containing 1ml of 0.5 M phosphate buffer (pH 6.5), 0.1ml of 30mM 1-chloro-2, 4- dinitro benzene (CDNB) in 95% ethanol,, and 1.8ml distilled water was taken in the control tube and 1ml phosphate buffer, 0.1ml CDNB, 0.1ml tissue extract and 1.7ml distilled water were taken in the sample test tubes. Then

the tubes were incubated at 37^{0}C for 5 minutes. After the incubation, 0.1ml of 30 mM reduced glutathione was added to all the tubes. Increase in absorbance was noted at 340 nm for 5 minutes in a quartz cuvette of 1 cm path length in a UV-visible spectrophotometer. Values were expressed in µmoles of CDNB complexed / min/ mg protein. The extinction coefficient between CDNB-GSH conjugate and CDNB is 9.6 mM^{-1} cm^{-1}.

5.2.2.6 Estimation of total reduced Glutathione (GSH)

Glutathione was measured by its reaction with DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) to give a compound that absorbs at 412 nm as per Ellman's method (Ellman,1959) .1ml tissue homogenate was mixed with 4ml of metaphosphoric acid(1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30g NaCl in 100ml water) . The precipitate was removed by centrifugation. To 2ml of the supernatant, 2ml of 0.4M disodium hydrogen phosphate and 1ml of DTNB reagent (40mg DTNB in 100 ml of 1% trisodium citrate) were added. The absorbance was read within 2min at 412nm against a reagent blank (without tissue homogenate). A set of standards (20mg reduced glutathione was dissolved in 100ml water) was also treated in the above manner. The amount of glutathione was expressed as µg/mg protein.

5.2.2.7 Estimation of Hydroperoxides (HP)

Hydroperoxides were estimated by the method of Mair and Hall (1977). 1 ml of the tissue homogenate was mixed thoroughly with 5 ml of chloroform: methanol (2:1) followed by centrifugation at 1000g for 5 minutes to separate the phases. 3ml of the lower chloroform layer was recovered using a syringe and placed in a test tube and dried in a 45 ^{0}C water bath under a stream of Nitrogen. 1 ml of acetic acid: chloroform (3:2) mixture followed by 0.05 ml of potassium iodide (KI) (0.80 g/ml) was quickly added and the test tubes were stoppered and mixed. The tubes

were placed in dark at room temperature for exactly 5 minutes followed by the addition of 3 ml of 0.5% cadmium acetate. The solution was mixed and centrifuged at 1000 g for 10 minutes. The absorbance of the upper phase was read at 353 nm against a blank containing the complete assay mixture except the tissue homogenate. Molar extinction coefficient of Hydroperoxides is $1.73 \times 10^4 M^{-1} cm^{-1}$. The values were expressed as milli moles /g wet wt. of tissue.

5.2.2.8 Estimation of Conjugated Dienes (CD)

The concentration of conjugated dienes was estimated according to the method of Retnagal and Ghoshal (1966). Membrane lipids were extracted and evaporated to dryness as described for the iodometric assay for hydroperoxides. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance at 233nm was determined against a cyclohexane blank. Molar extinction coefficient of conjugated dienes is $2.52 \times 10^4 M^{-1} cm^{-1}$. The values were expressed as milli moles / g wet wt. tissue.

5.2.2.9 Estimation of Malondialdehyde (MDA)

Malondialdehyde was estimated by the method of Nihaeus and Samuelson (1958). The tissue homogenate of different tissues were prepared in 0.1 M Tris-HCl buffer (pH 7.5) buffer and was combined with TCA-TBA-HCl reagents(15% (w/v) Trichloroacetic acid. 0.375 % (w/v) Thiobarbituric acid (TBA) in 0.25 N HCl) and mixed thoroughly and heated for 15 minutes in a boiling water bath. It was then cooled and centrifuged for 10 minutes at 600 g. The absorbance of the sample was read spectrophotometrically at 535 nm against a reagent blank that did not contain tissue extract. The extinction coefficient for malondialdehyde is $1.56 \times 10^5 M^{-1} cm^{-1}$. The values were expressed as milli moles / g wet wt. tissue.

5.2.2.10 Estimation of protein

Total protein of tissues was estimated by the method of Bradford *et al.* (1976), using Bovine Serum Albumin (BSA) as standard protein.

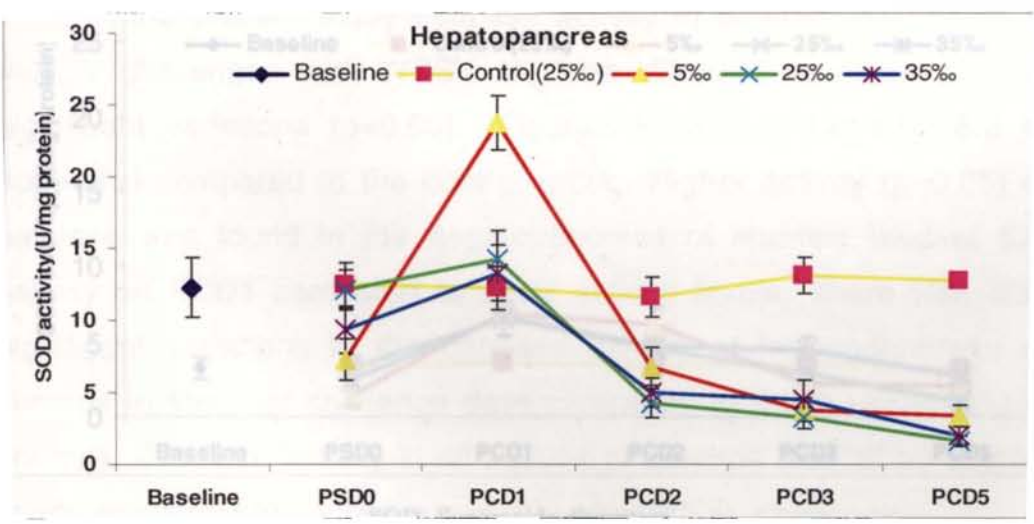
5.2.3 Statistical analysis

Statistical evaluation was done using two-way analysis of variance (ANOVA) followed by Tukey multiple comparison test using the SPSS statistical software package version 10.0. The level of significance was set at $p< 0.05$.

5.3 Results

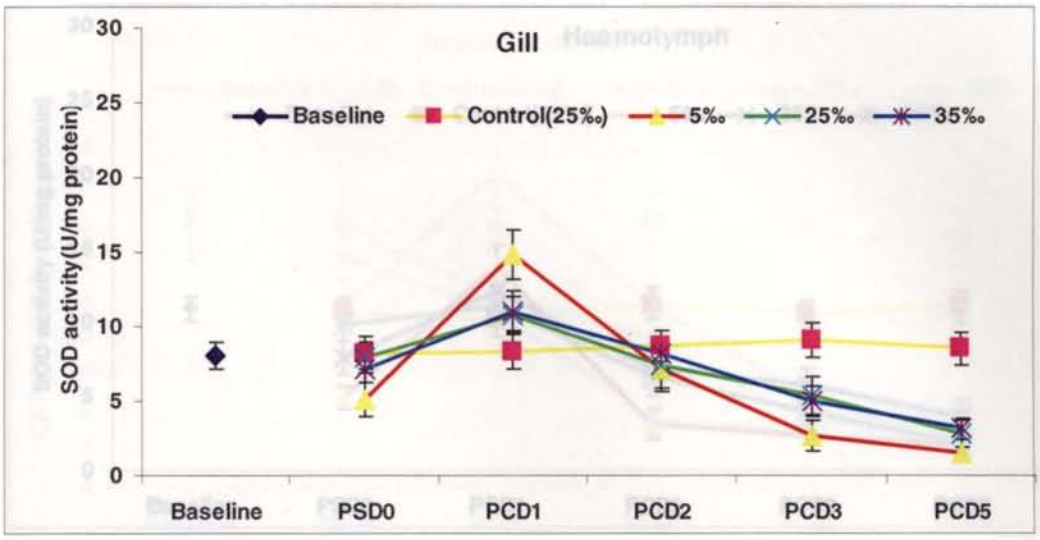
5.3.1 Super Oxide Dismutase (SOD)

Superoxide dismutase activity was found to be significantly high in the hepatopancreas of WSSV challenged *F. indicus* compared to other tissues. After salinity stress a decline in SOD activity could be noted in all tissues and this was prominent in shrimps held at 5‰ compared to other salinity ranges. White Spot Syndrome Virus challenge evoked an enhanced production of SOD at PCD1 in shrimps held at 5‰. SOD activity of all tissues (hepatopancreas, gills, muscle and haemolymph) was found to decrease ($p<0.05$) from post challenge day 1 to post challenge day 5 compared to the unchallenged group. (Fig.5.1- 5.4 and Table.5.1- 5.4 of appendix)



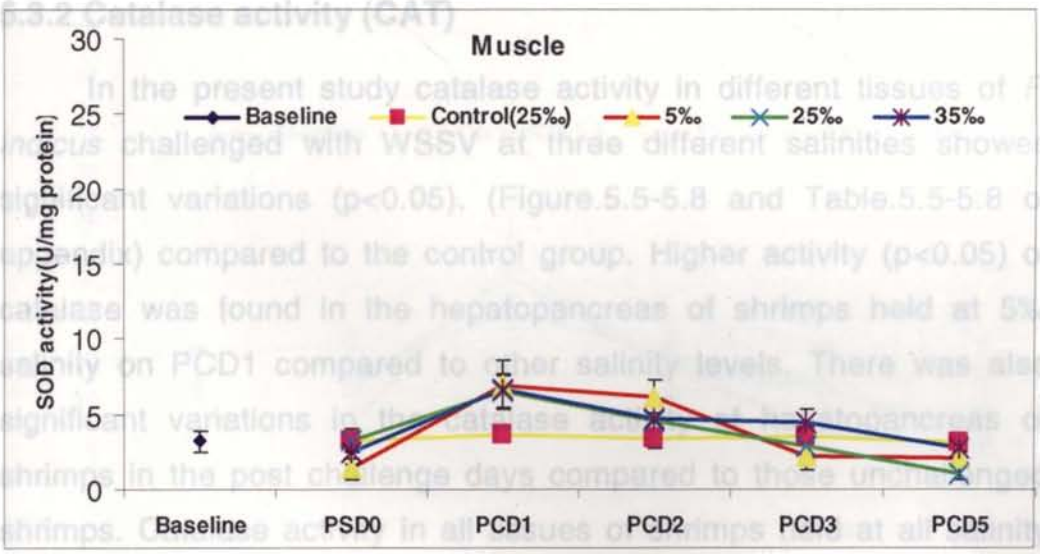
SOD- Superoxide dismutase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.1 Superoxide dismutase (SOD) activity in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Values represent mean±S.D of six separate determinations. Control- unchallenged.



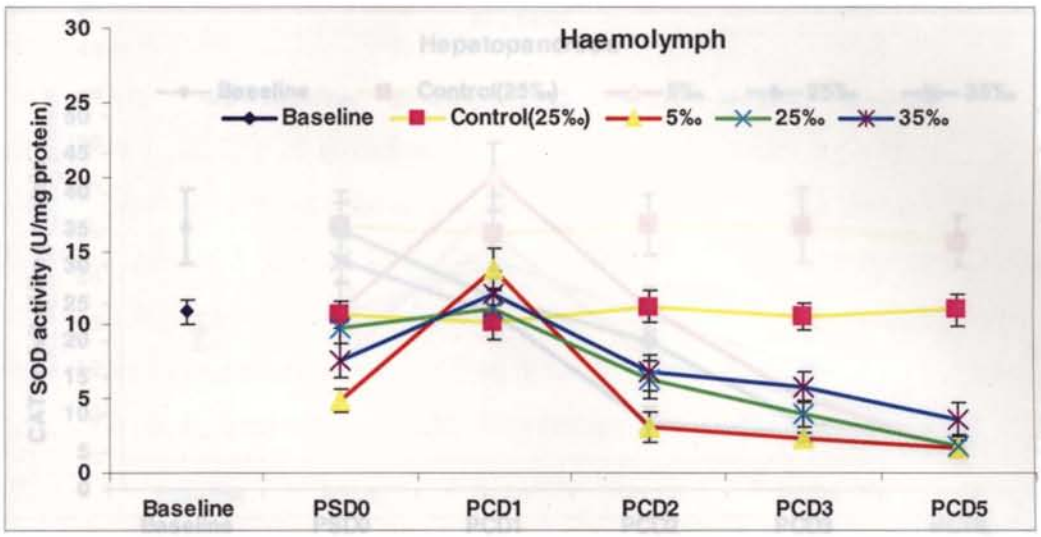
SOD- Superoxide dismutase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.2 SOD activity (Mean±S.D.) in the gills of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Values represent mean±SD of six separate determinations. Control- unchallenged.



SOD- Superoxide dismutase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.3 SOD activity (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Values represent mean±SD of six separate determinations. Control- unchallenged.

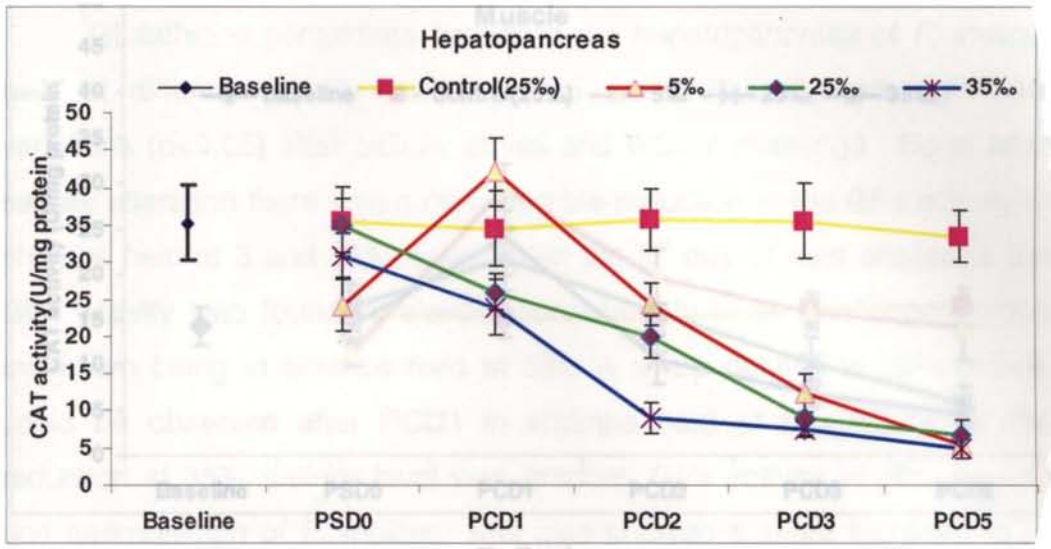


SOD- Superoxide dismutase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.4 SOD activity (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Values represent mean±SD of six separate determinations. Control- unchallenged.

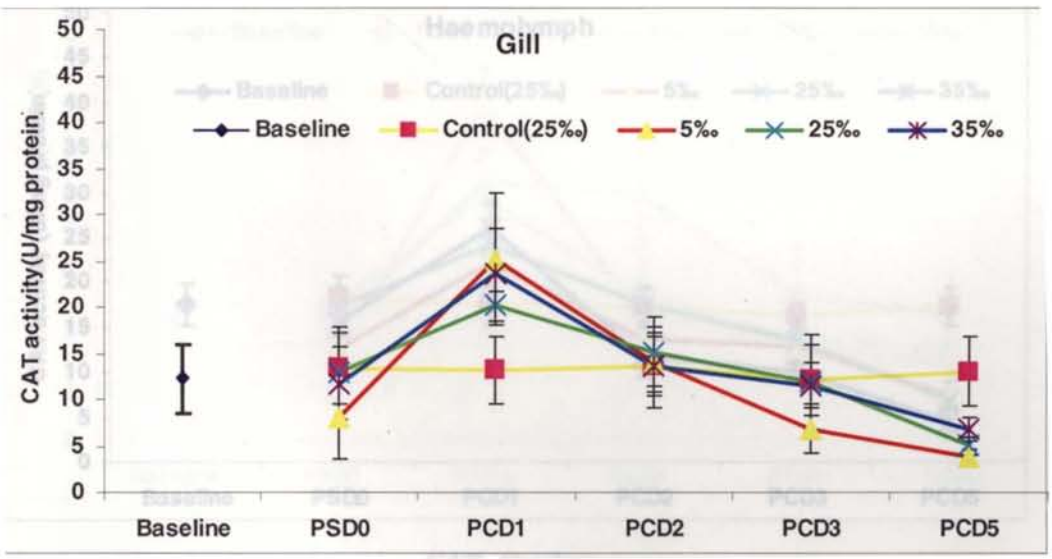
5.3.2 Catalase activity (CAT)

In the present study catalase activity in different tissues of *F. indicus* challenged with WSSV at three different salinities showed significant variations ($p<0.05$). (Figure.5.5-5.8 and Table.5.5-5.8 of appendix) compared to the control group. Higher activity ($p<0.05$) of catalase was found in the hepatopancreas of shrimps held at 5‰ salinity on PCD1 compared to other salinity levels. There was also significant variations in the catalase activity of hepatopancreas of shrimps in the post challenge days compared to those unchallenged shrimps. Catalase activity in all tissues of shrimps held at all salinity levels was found to decrease ($p<0.05$) after WSSV challenge.



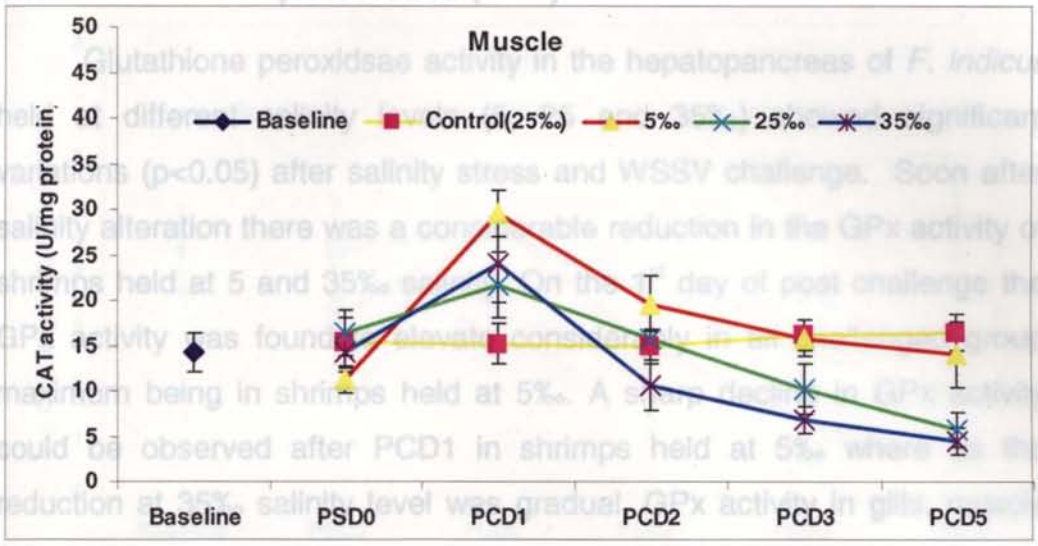
CAT- Catalase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.5 Catalase (CAT) activity (Mean±S.D.) in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



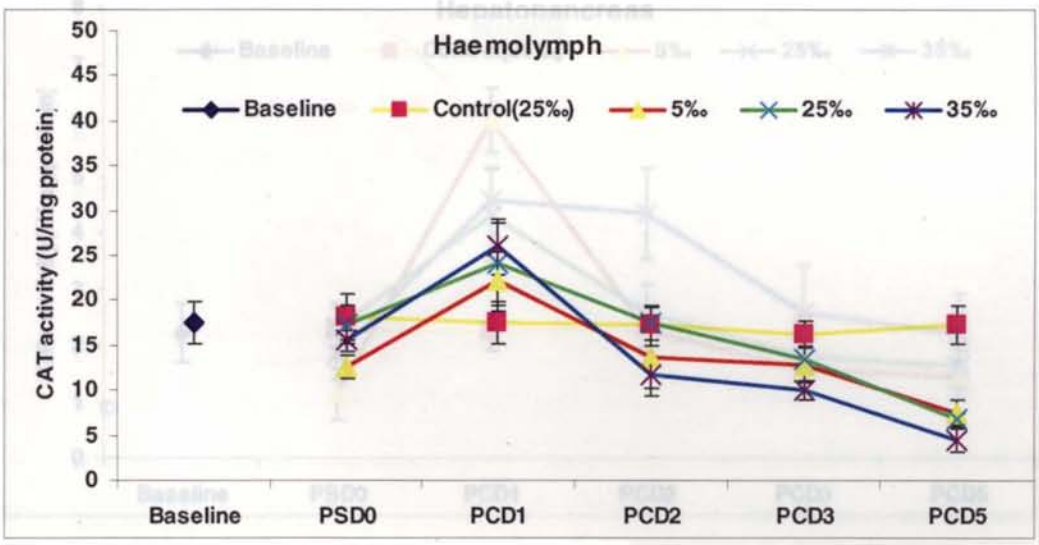
CAT- Catalase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.6 Catalase activity (Mean±S.D.) in the gills of *F. indicus* subjected to acute salinity stress and challenged with WSSV Control- unchallenged.



CAT- Catalase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.7 Catalase activity (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control-unchallenged.

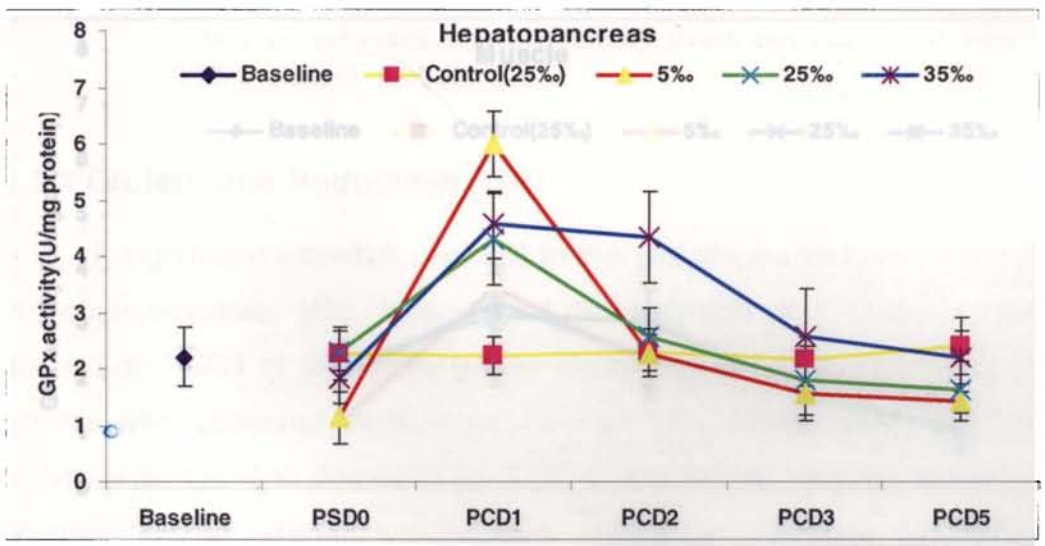


CAT- Catalase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.8 Catalase activity (Mean±S.D.) haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control-unchallenged.

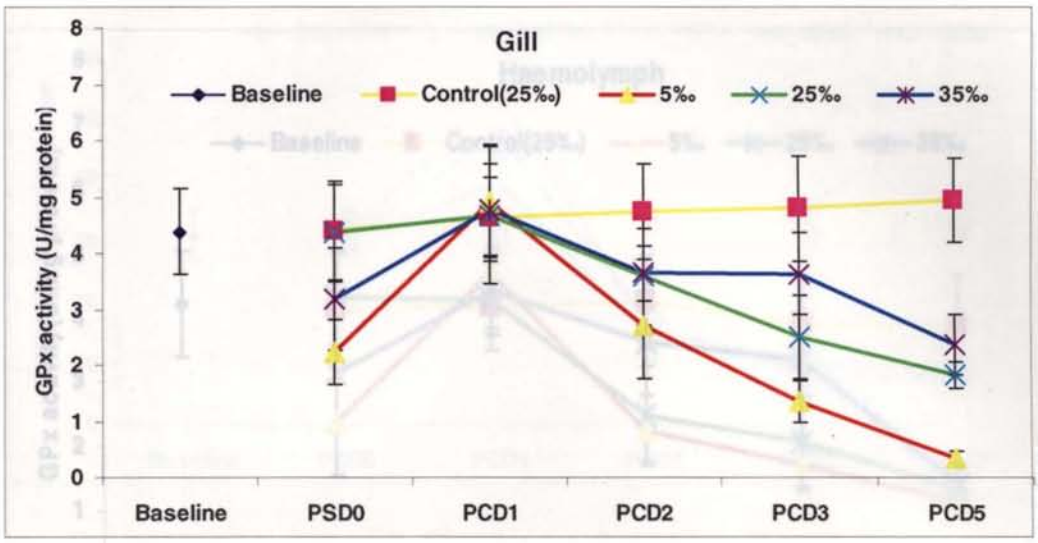
5.3.3 Glutathione peroxidase (GPx)

Glutathione peroxidsae activity in the hepatopancreas of *F. indicus* held at different salinity levels (5, 25 and 35‰) showed significant variations ($p<0.05$) after salinity stress and WSSV challenge. Soon after salinity alteration there was a considerable reduction in the GPx activity of shrimps held at 5 and 35‰ salinity. On the 1st day of post challenge the GPx activity was found to elevate considerably in all challenged group maximum being in shrimps held at 5‰. A sharp decline in GPx activity could be observed after PCD1 in shrimps held at 5‰ where as the reduction at 35‰ salinity level was gradual. GPx activity in gills, muscle and haemolymph of *F. indicus* was also showed a slight increase at all salinity levels compared to the control group (unchallenged) on PCD1. The GPx activity was found to decrease significantly from post challenge day1 to 5 over the experimental period. (Fig.5.9-5.12 and Table 5.9-5.12 of appendix)



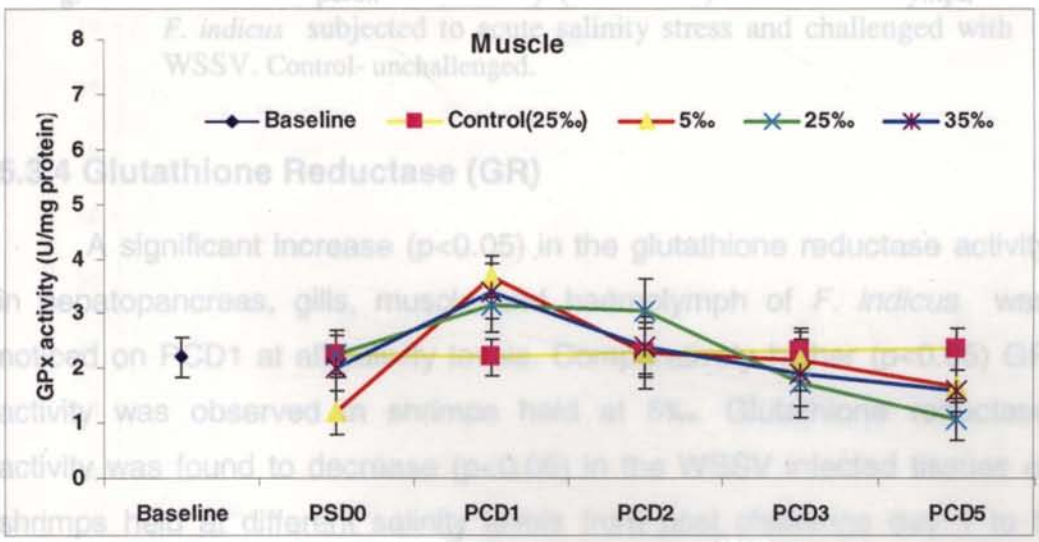
GPx- Glutathione peroxidase
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.9 Glutathione peroxidase (GPx) activity (Mean±S.D.) in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



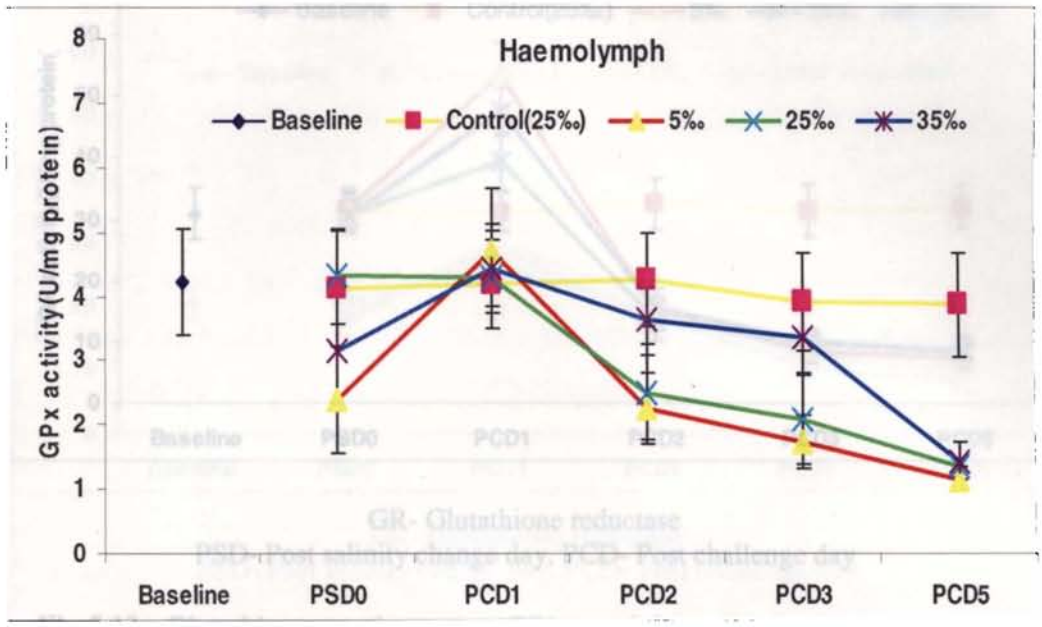
GPx- Glutathione peroxidase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.10 Glutathione peroxidase activity (Mean±S.D.) in the gills of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



GPx- Glutathione peroxidase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.11 Glutathione peroxidase activity (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

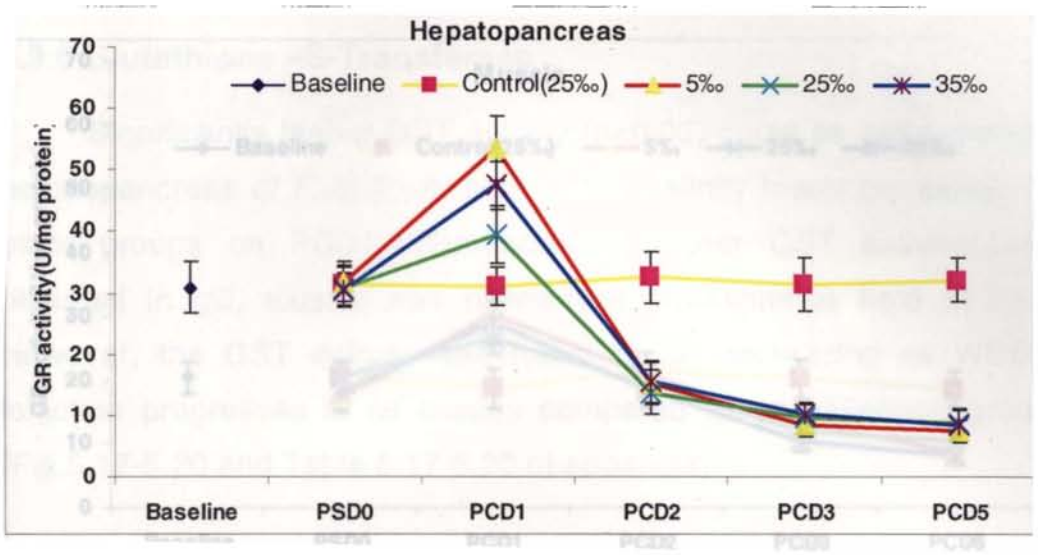


GPx- Glutathione peroxidase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.12 Glutathione peroxidase activity (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

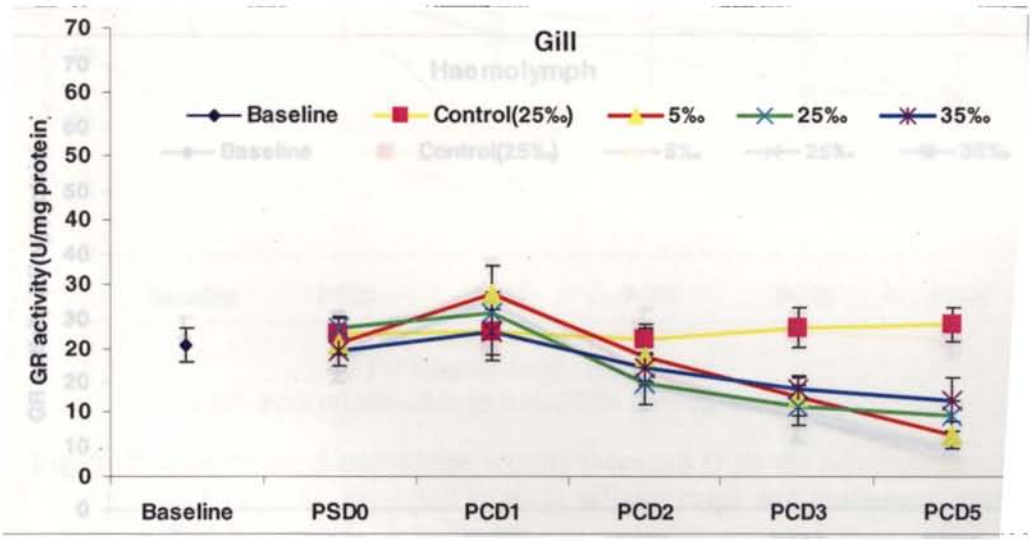
5.3.4 Glutathione Reductase (GR)

A significant increase ($p<0.05$) in the glutathione reductase activity in hepatopancreas, gills, muscle and haemolymph of *F. indicus* was noticed on PCD1 at all salinity levels. Comparatively higher ($p<0.05$) GR activity was observed in shrimps held at 5‰. Glutathione reductase activity was found to decrease ($p<0.05$) in the WSSV infected tissues of shrimps held at different salinity levels from post challenge day 1 to 5 (Fig.5.13-5.16 and Table.5.13-5.16 of appendix).



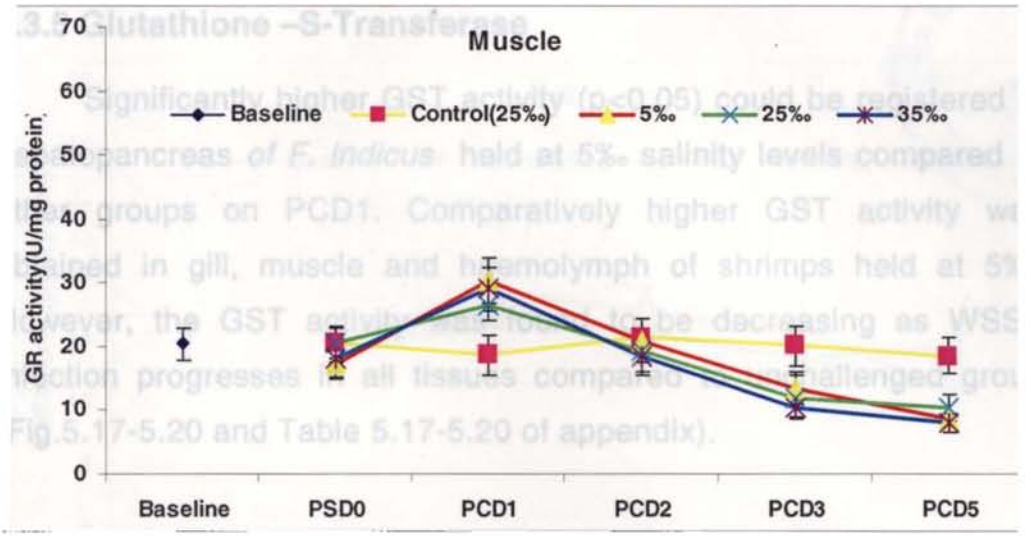
GR- Glutathione reductase
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.13 Glutathione reductase (GR) activity (Mean±S.D.) in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



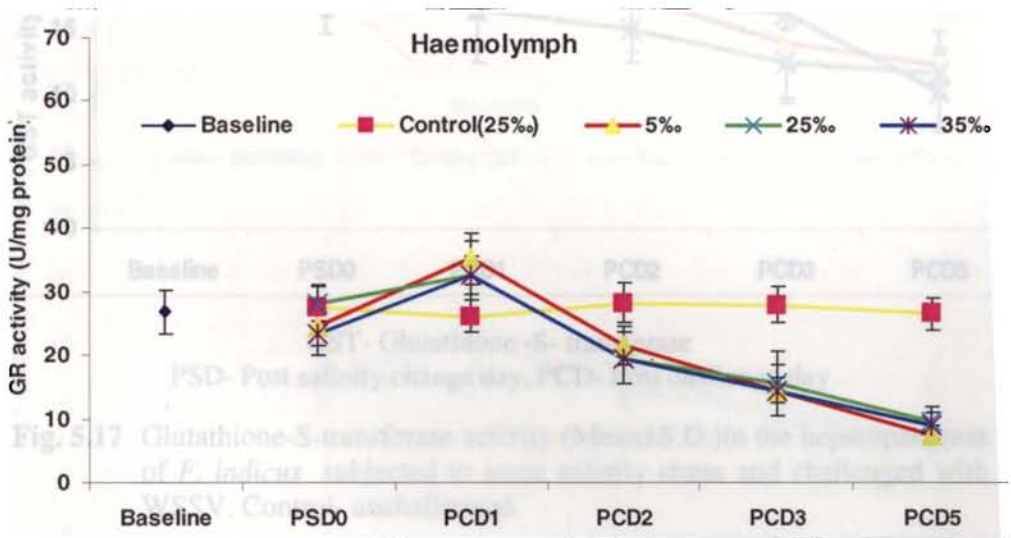
GR- Glutathione reductase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.14 Glutathione reductase (GR) activity (Mean±S.D.) in the gill of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



GR- Glutathione reductase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.15 Glutathione reductase activity (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

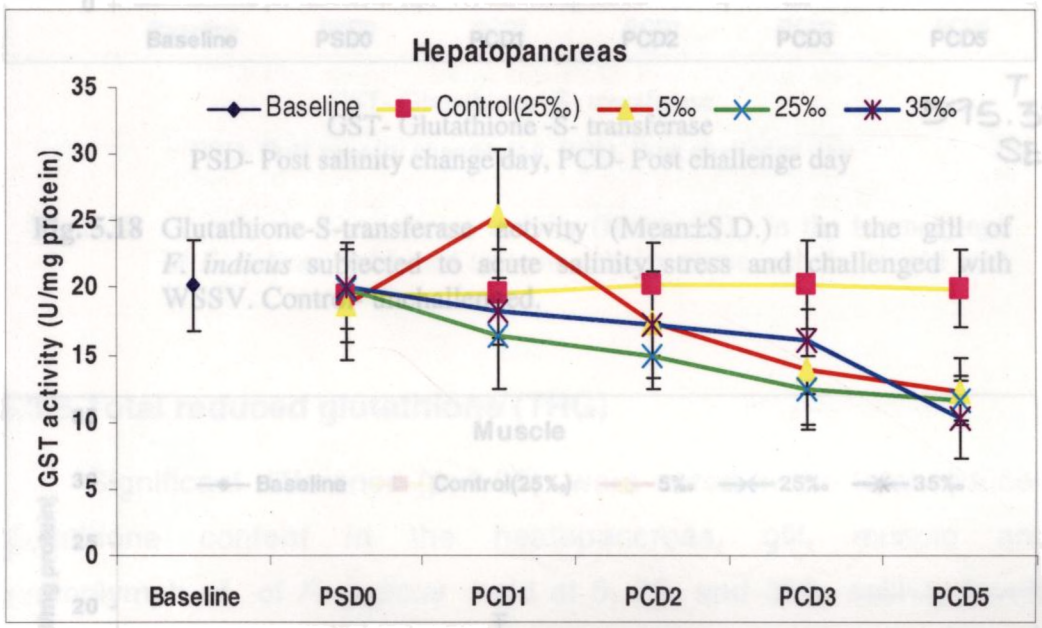


GR- Glutathione reductase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.16 Glutathione reductase activity (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

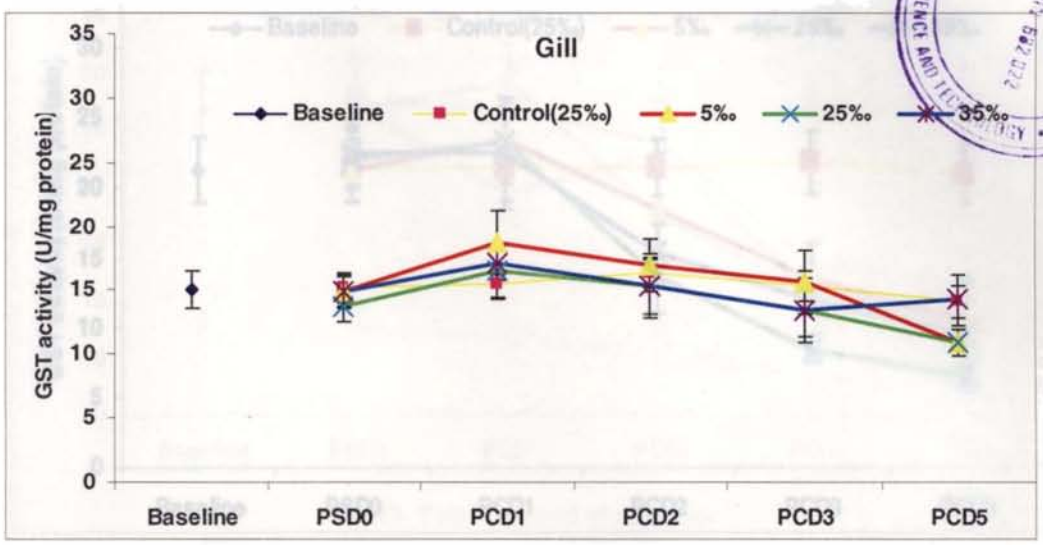
5.3.5 Glutathione –S-Transferase

Significantly higher GST activity ($p<0.05$) could be registered in hepatopancreas *of F. indicus* held at 5‰ salinity levels compared to other groups on PCD1. Comparatively higher GST activity was obtained in gill, muscle and haemolymph of shrimps held at 5‰. However, the GST activity was found to be decreasing as WSSV infection progresses in all tissues compared to unchallenged group (Fig.5.17-5.20 and Table 5.17-5.20 of appendix).



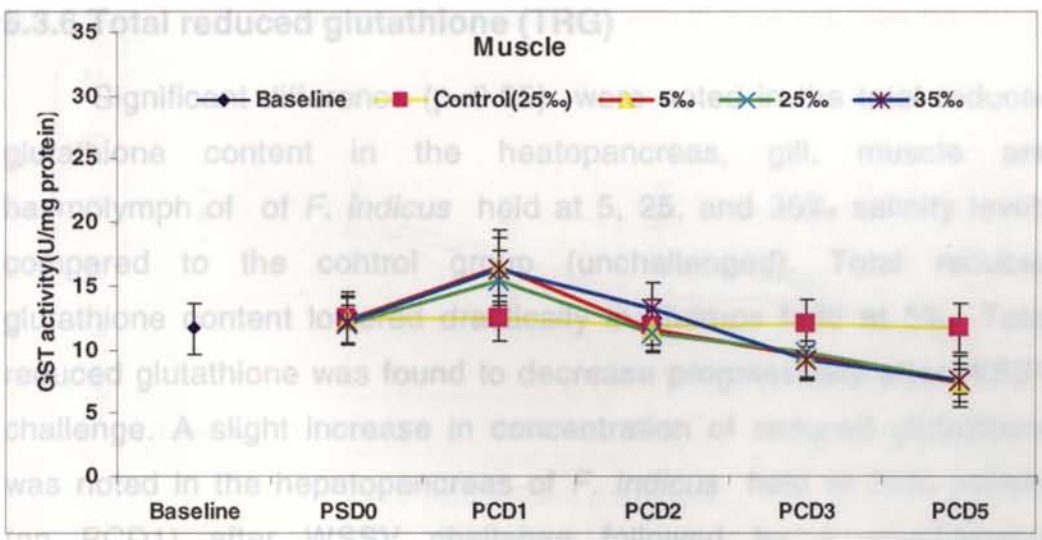
GST- Glutathione -S- transferase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.17 Glutathione-S-transferase activity (Mean±S.D.)in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



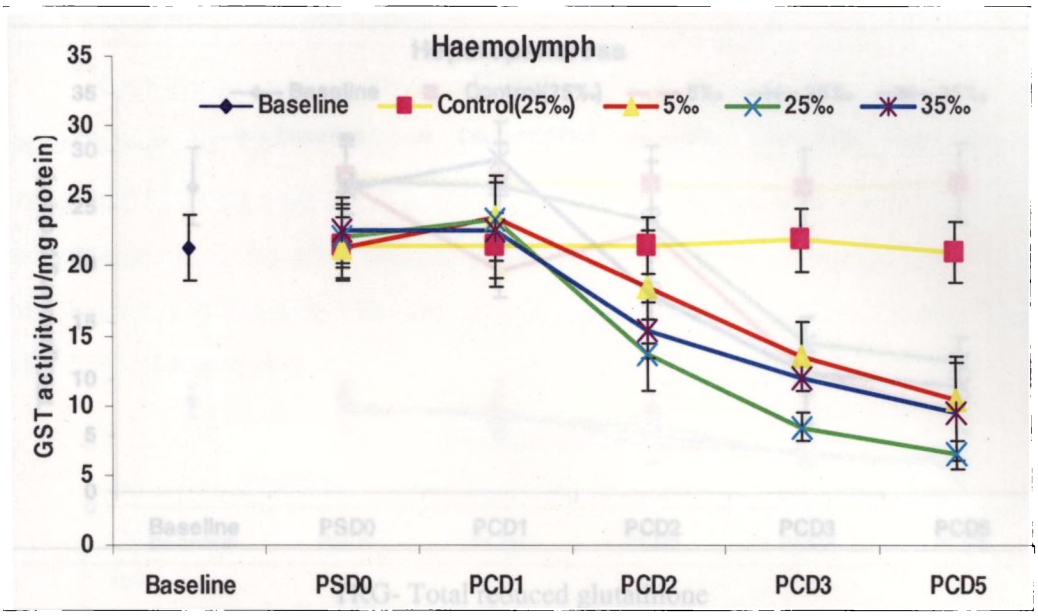
GST- Glutathione -S- transferase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.18 Glutathione-S-transferase activity (Mean±S.D.) in the gill of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



GST- Glutathione -S- transferase
PSD- Post salinity change day, PCD- Post challenge day

Fig. 5.19 Glutathione-S-transferase activity (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



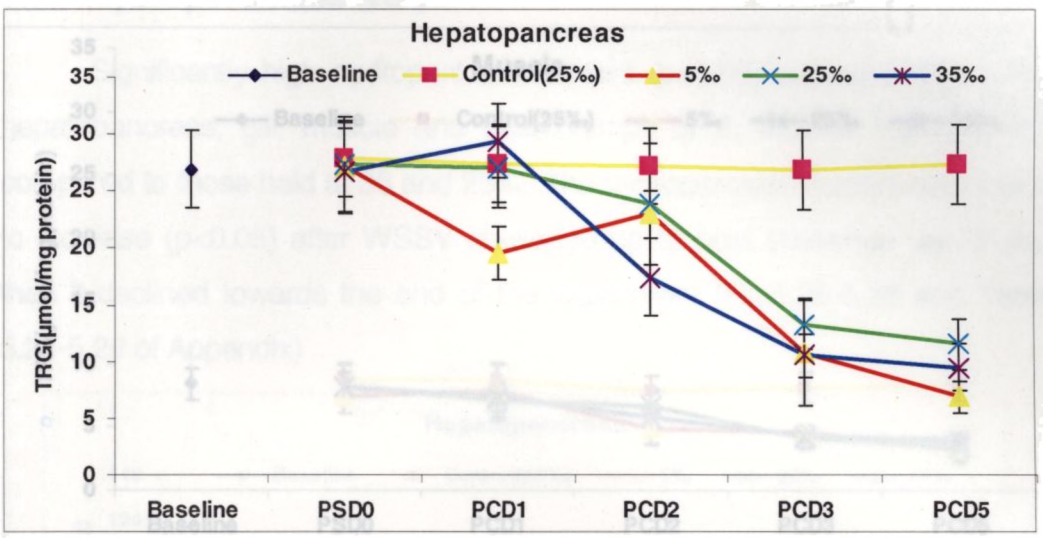
GST- Glutathione -S- transferase

PSD- Post salinity change day, PCD- Post challenge day

Fig.5.20 Glutathione-S-transferase activity (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

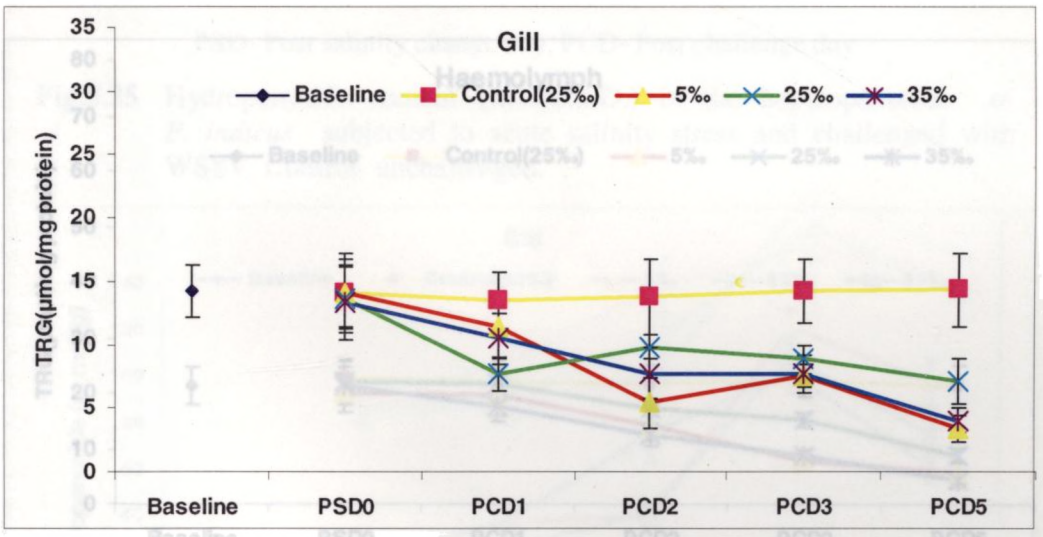
5.3.6 Total reduced glutathione (TRG)

Significant difference ($p<0.05$) were noted in the total reduced glutathione content in the heatopancreas, gill, muscle and haemolymph of of *F. indicus* held at 5, 25, and 35‰ salinity levels compared to the control group (unchallenged). Total reduced glutathione content lowered drastically in shrimps held at 5‰. Total reduced glutathione was found to decrease progressively after WSSV challenge. A slight increase in concentration of reduced glutathione was noted in the hepatopancreas of *F. indicus* held at 35‰ salinity (on PCD1) after WSSV challenge followed by a considerable decrease.(Fig.5.21-5.24 and Table 5.21-5.24 of Appendix).



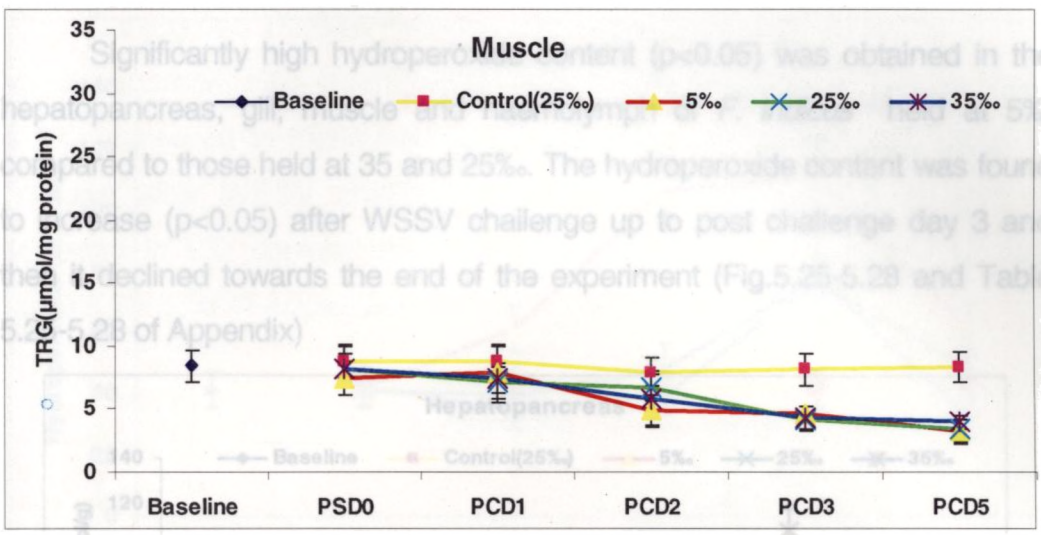
TRG- Total reduced glutathione
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.21 Total reduced glutathione (TRG) content (Mean±S.D.) in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



TRG- Total reduced glutathione
PSD- Post salinity change day, PCD- Post challenge day

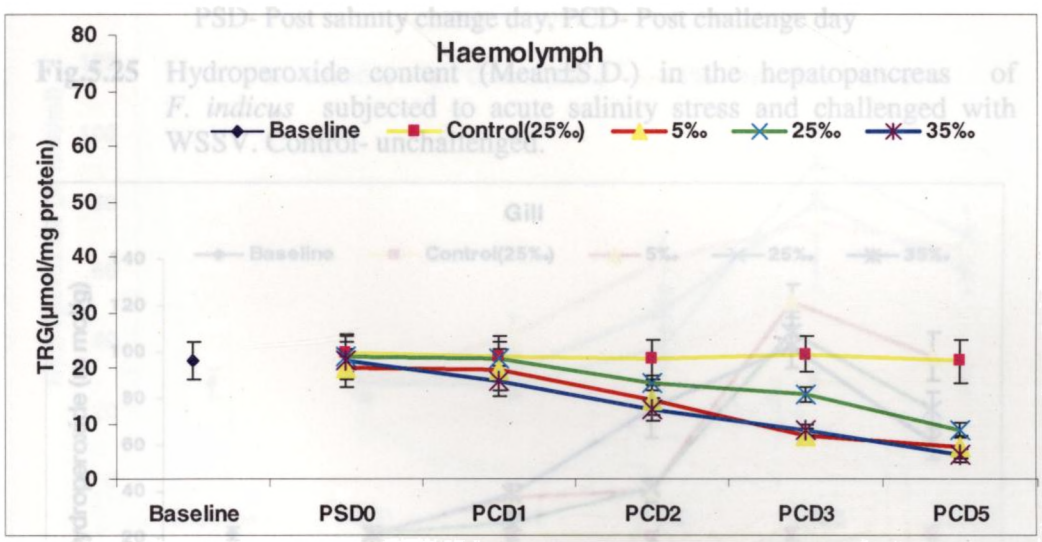
Fig.5.22 Total reduced glutathione content (Mean±S.D.) in the gills of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



TRG- Total reduced glutathione

PSD- Post salinity change day, PCD- Post challenge day

Fig.5.23 Total reduced glutathione content (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



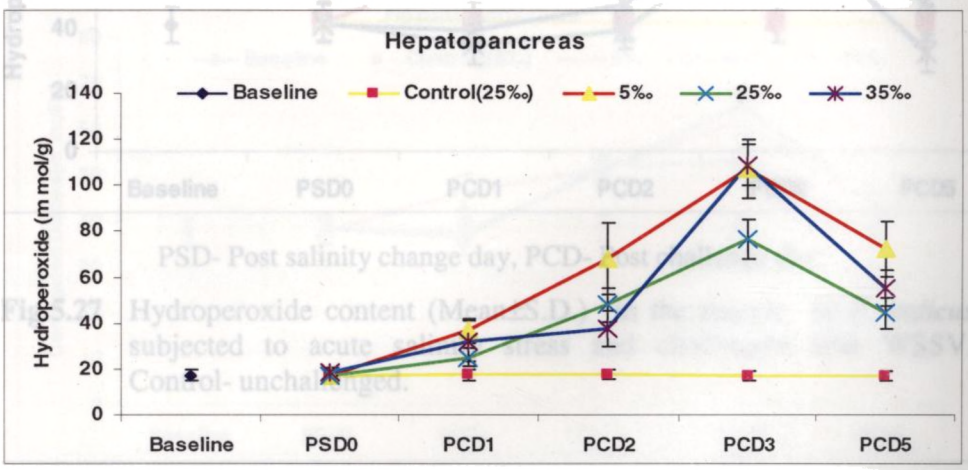
TRG- Total reduced glutathione

PSD- Post salinity change day, PCD- Post challenge day

Fig.5.24 Total reduced glutathione content (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

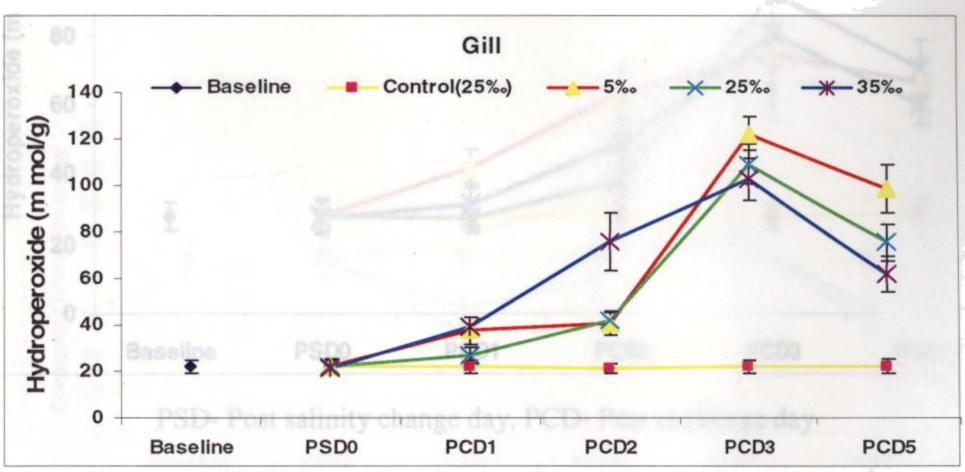
5.3.7 Hydroperoxides (HP)

Significantly high hydroperoxide content ($p<0.05$) was obtained in the hepatopancreas, gill, muscle and haemolymph of *F indicus* held at 5‰ compared to those held at 35 and 25‰. The hydroperoxide content was found to increase ($p<0.05$) after WSSV challenge up to post challenge day 3 and then it declined towards the end of the experiment (Fig.5.25-5.28 and Table 5.25-5.28 of Appendix)



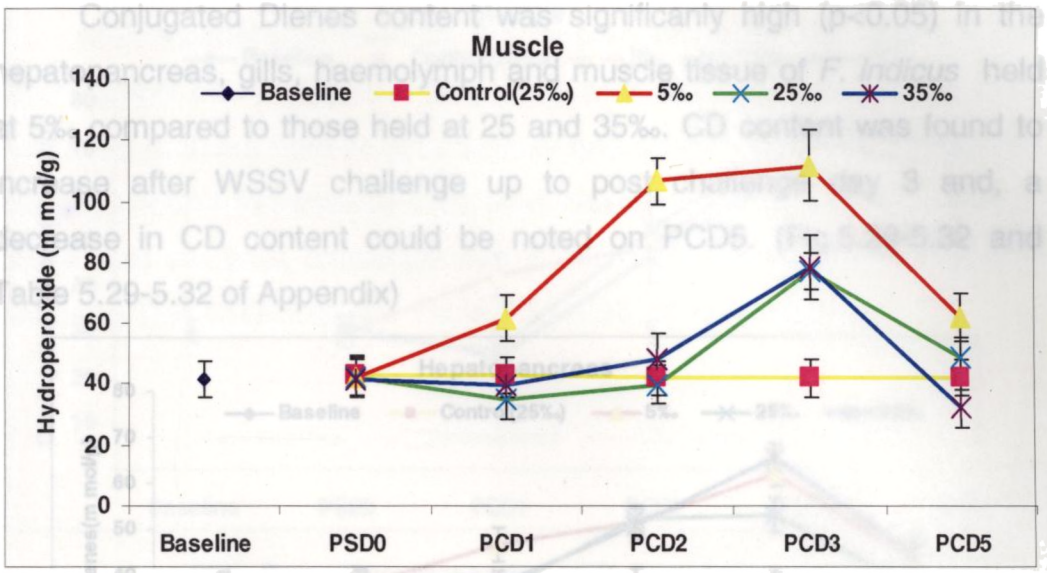
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.25 Hydroperoxide content (Mean±S.D.) in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



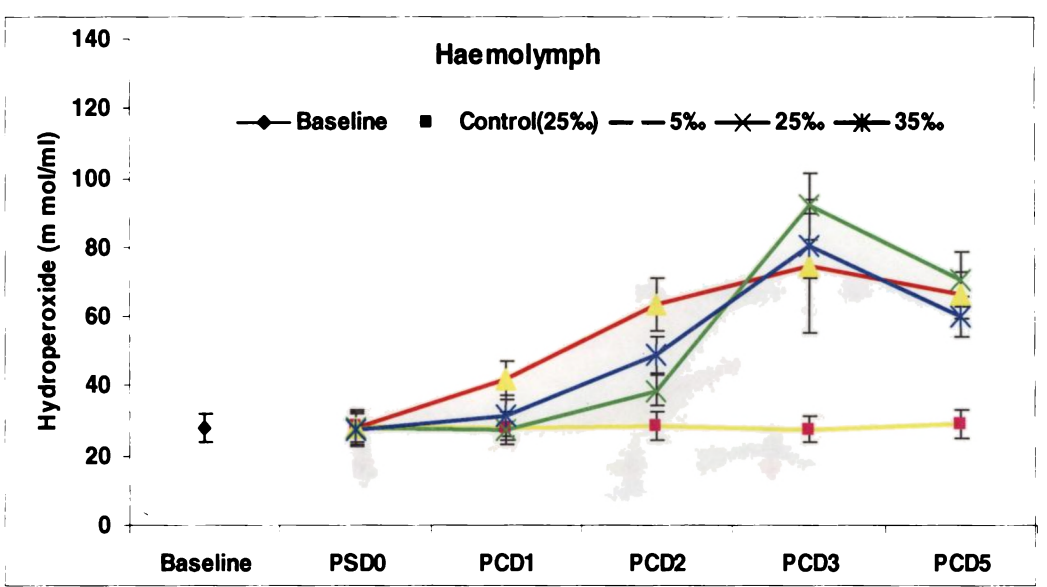
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.26 Hydroperoxide content (Mean±S.D.) in the gills of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



PSD- Post salinity change day, PCD- Post challenge day

Fig.5.27 Hydroperoxide content (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

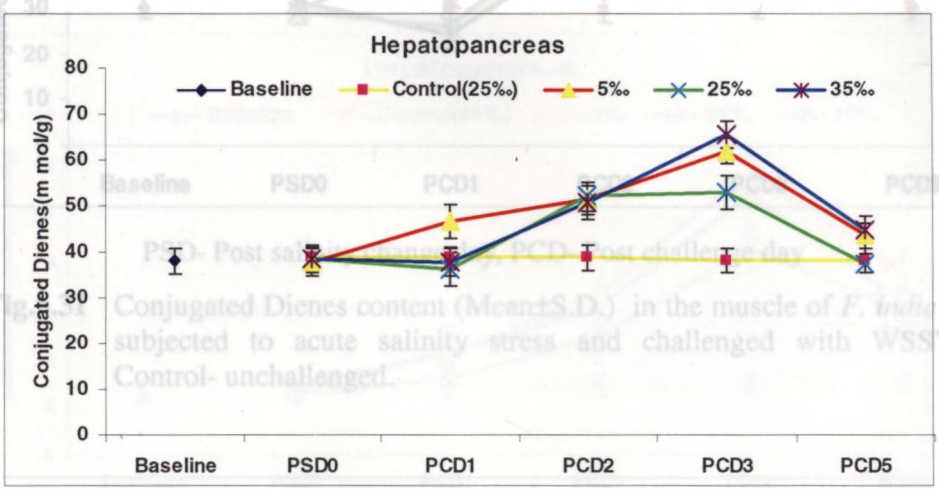


PSD- Post salinity change day, PCD- Post challenge day

Fig.5.28 Hydroperoxide content (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

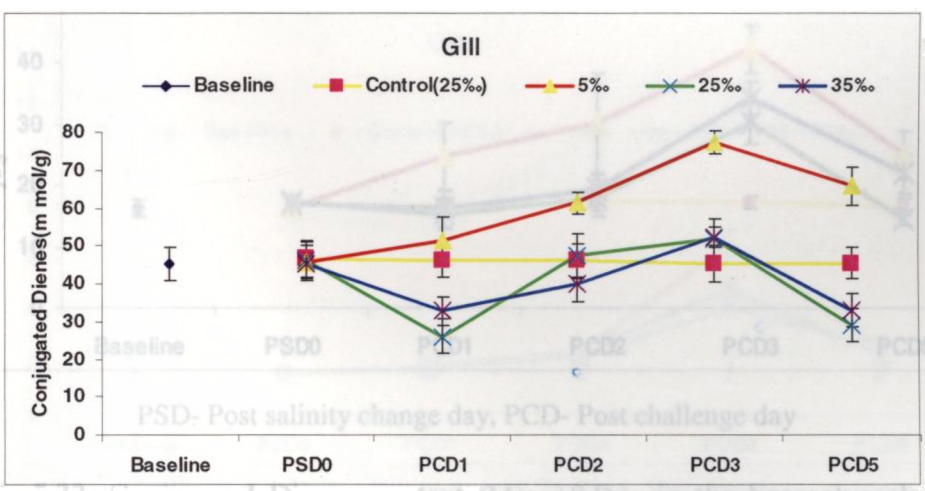
5.3.8 Conjugated Dienes (CD)

Conjugated Dienes content was significanly high ($p<0.05$) in the hepatopancreas, gills, haemolymph and muscle tissue of *F. indicus* held at 5‰ compared to those held at 25 and 35‰. CD content was found to increase after WSSV challenge up to post challenge day 3 and, a decrease in CD content could be noted on PCD5. (Fig.5.29-5.32 and Table 5.29-5.32 of Appendix)



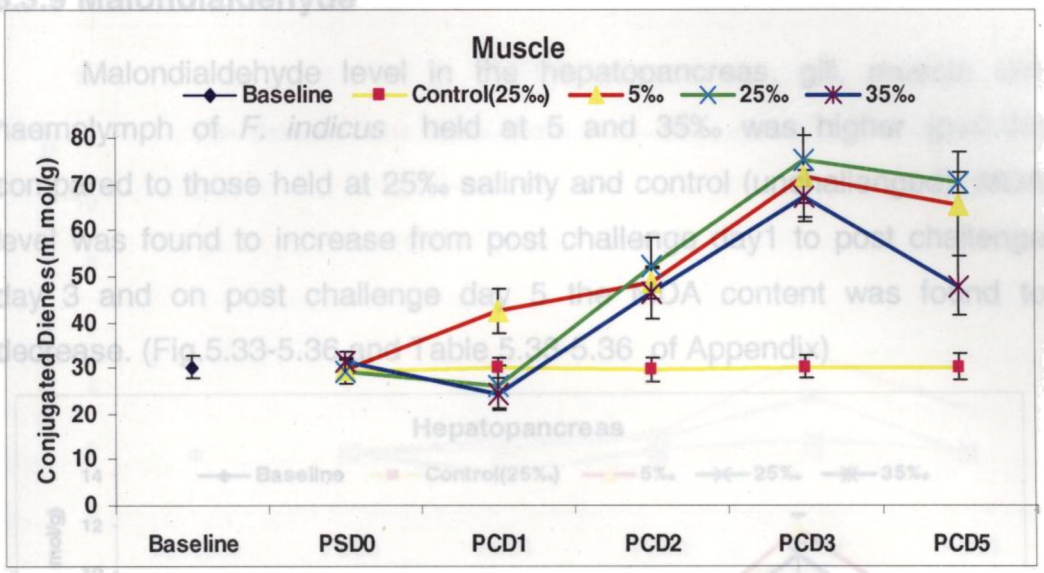
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.29 Conjugated Dienes content (Mean±S.D.) in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



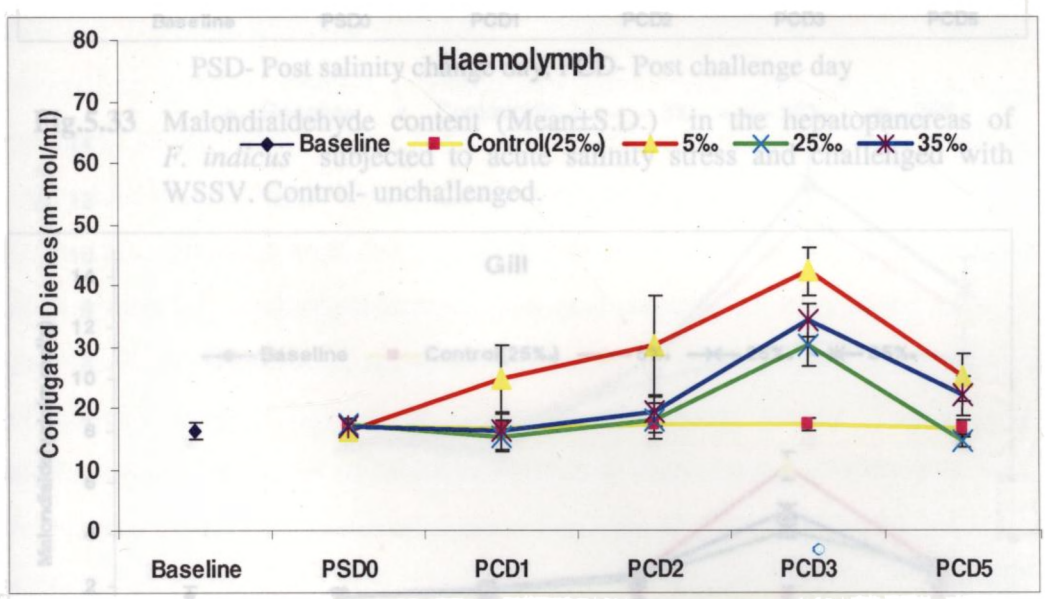
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.30 Conjugated Dienes content (Mean±S.D.) in the gills of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



PSD- Post salinity change day, PCD- Post challenge day

Fig.5.31 Conjugated Dienes content (Mean±S.D.) in the muscle of *F indicus* subjected to acute salinity stress and challenged with WSSV Control- unchallenged.

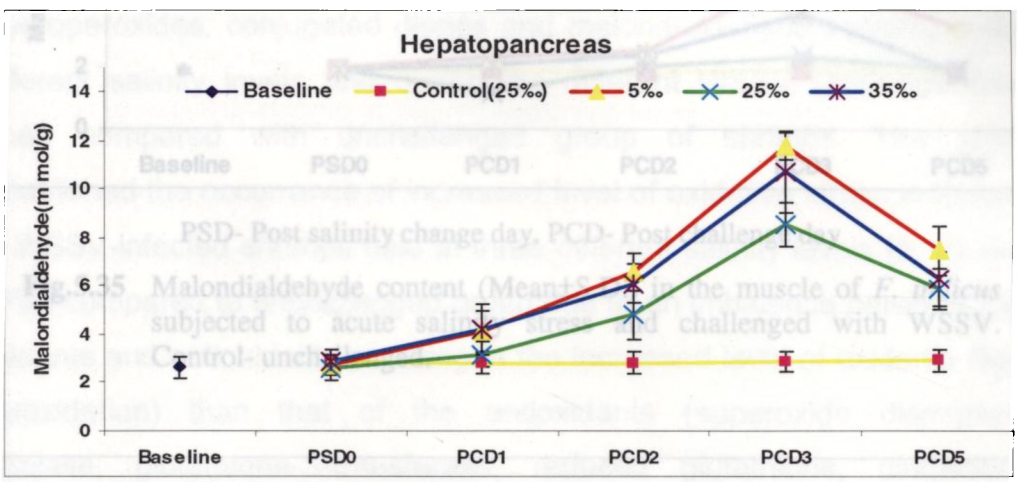


PSD- Post salinity change day, PCD- Post challenge day

Fig.5.32 Conjugated Dienes content (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.

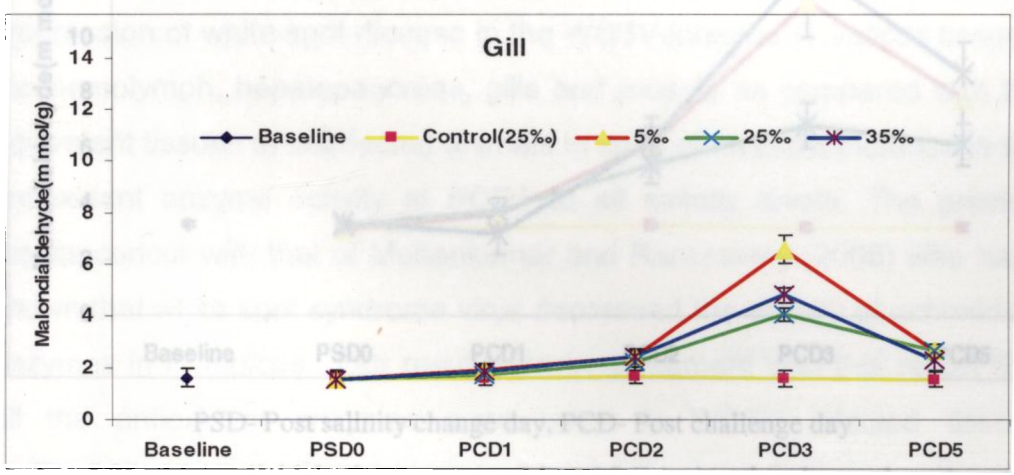
5.3.9 Malondialdehyde

Malondialdehyde level in the hepatopancreas, gill, muscle and haemolymph of *F. indicus* held at 5 and 35‰ was higher ($p<0.05$) compared to those held at 25‰ salinity and control (unchallenged). MDA level was found to increase from post challenge day1 to post challenge day 3 and on post challenge day 5 the MDA content was found to decrease. (Fig.5.33-5.36 and Table 5.33-5.36 of Appendix)



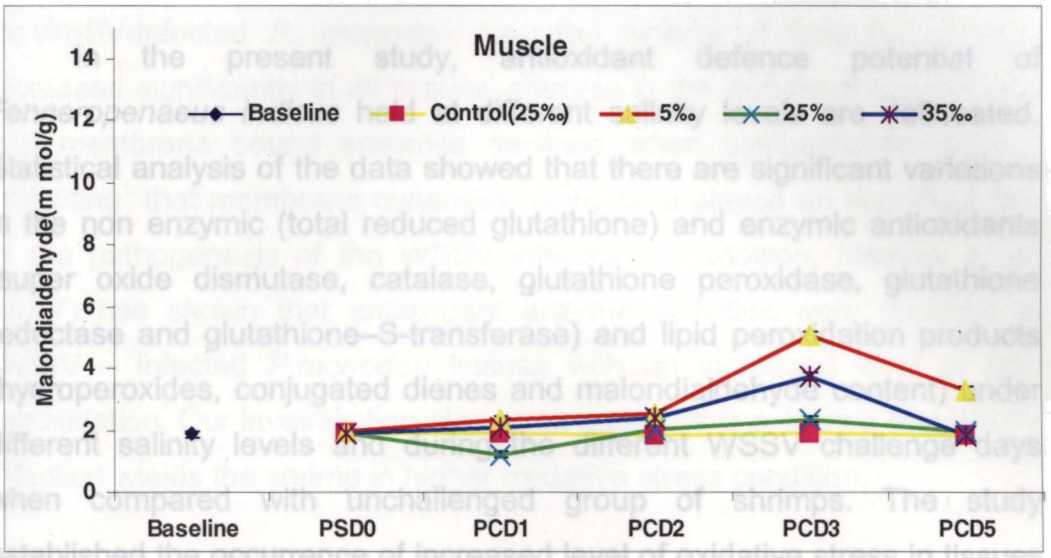
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.33 Malondialdehyde content (Mean±S.D.) in the hepatopancreas of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



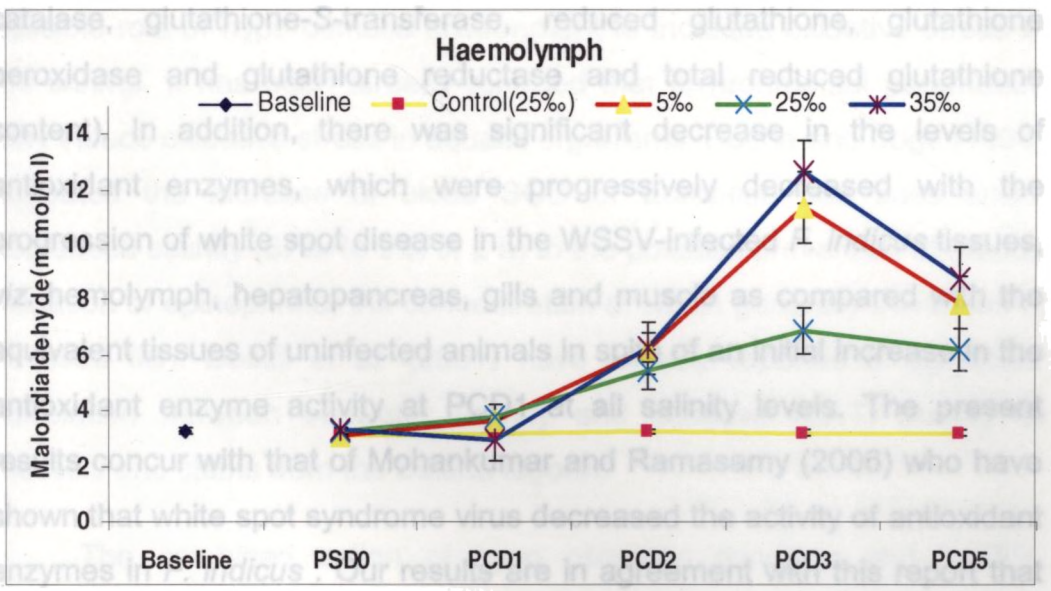
PSD- Post salinity change day, PCD- Post challenge day

Fig.5.34 Malondialdehyde content (Mean±S.D.) in the gills of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



PSD- Post salinity change day, PCD- Post challenge day

Fig.5.35 Malondialdehyde content (Mean±S.D.) in the muscle of *F. indicus* subjected to acute salinity stress and challenged with WSSV. Control- unchallenged.



PSD- Post salinity change day, PCD- Post challenge day

Fig.5.36 Malondialdehyde content (Mean±S.D.) in the haemolymph of *F. indicus* subjected to acute salinity stress and challenged with WSSV Control- unchallenged.

5.4 Discussion

In the present study, antioxidant defence potential of *Fenneropenaeus indicus* held at different salinity levels are delineated. Statistical analysis of the data showed that there are significant variations in the non enzymic (total reduced glutathione) and enzymic antioxidants (super oxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione–S-transferase) and lipid peroxidation products (hydroperoxides, conjugated dienes and malondialdehyde content) under different salinity levels and during the different WSSV challenge days when compared with unchallenged group of shrimps. The study established the occurrence of increased level of oxidative stress in tissues of WSSV-infected shrimps held at three different salinity levels (5, 25 and 35‰) compared to unchallenged group due to an imbalance between pro-oxidants and antioxidants resulting in the increased level of oxidants (lipid peroxidation) than that of the antioxidants (superoxide dismutase, catalase, glutathione-S-transferase, reduced glutathione, glutathione peroxidase and glutathione reductase and total reduced glutathione content). In addition, there was significant decrease in the levels of antioxidant enzymes, which were progressively decreased with the progression of white spot disease in the WSSV-infected *F. indicus* tissues, *viz.* hemolymph, hepatopancreas, gills and muscle as compared with the equivalent tissues of uninfected animals in spite of an initial increase in the antioxidant enzyme activity at PCD1 at all salinity levels. The present results concur with that of Mohankumar and Ramasamy (2006) who have shown that white spot syndrome virus decreased the activity of antioxidant enzymes in *F. indicus* . Our results are in agreement with this report that all the antioxidant enzymes were lower in WSSV infected tissues compared with that of control shrimps. Likewise, the lipid peroxidaton in terms of TBARS has been elevated after severe WSSV infection in all the treated groups as already shown by Mohankumar and Ramasamy (2006).

Similar results were reported by Rameshthangam and Ramasamy (2006) in WSSV-infected *P. monodon*, i.e., the activity of lipid peroxidation increased significantly in all tissues whereas in the activity of antioxidants and membrane bound enzymes declined when compared to controls indicating that membrane dynamics might have played an important role in the pathogenesis of the WSSV infection. In addition, Mathew *et al.* (2007) has shown that antioxidant enzyme activities were declined in WSSV – infected *P.monodon* tissues with an increased level of lipid peroxidation. Our investigation also gives quantitative evidence that WSSV infection wields the shrimp in higher oxidative stress condition.

The present study has shown that the level of lipid peroxidation was significantly increased in WSSV-infected hemolymph, hepatopancreas, gills and muscle of *F. indicus* held at 5, 25 and 35‰ salinity levels when compared with control animals (unchallenged). Highest level of lipid peroxidation was observed in shrimps held at 5‰ salinity implying the possible role of hypo osmotic environment to increase oxidative stress in the shrimp. It has been already reported that environmental parameters may induce oxidative stress in aquatic organisms. Roche and Boge (1996) attributed the increase of blood SOD of European sea bass under decreased salinity (37‰ to 5‰ in 2 h) to the possible prevention of natural oxidation of epinephrine, the concentration of which generally increases in stressed fish. Dellali *et al.* (2001) have already reported a significant correlation between seawater salinity and catalase (CAT) activity in mussels and clams from the Bizerta lagoon.

The combined action of hypo osomotic condition and WSSV-infection are attributed to the increased level of lipid peroxidation resulting in severe oxidative stress and depletion of antioxidant scavenger systems in *F. indicus*. Liu *et al.* (2007) has shown that salinity stress was linked to over production of reactive oxygen species and a wide change in salinity

(from 30‰ to 5‰ and 50‰) was found capable of generating severe oxidative stress in *Litopenaeus vannamei.* It could be assumed that the combined action of WSSV and salinity made the animal succumb to disease due to over production of reactive oxygen species with a concomitant reduction in antioxidant enzymes.

In the present study the activities of super oxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione –S-transferase and the concentration of non-enzymatic antioxidant, reduced glutathione were elevated at PCD1 and then reduced significantly in all tissues (hepatopancreas, gills, muscle and haemolymph) of *F. indicus* held at different salinity levels except for reduced glutathione which showed a gradual reduction throughout the experimental period.

Superoxide dismutase (SOD) is one of the main antioxidant defence enzymes generated in response to oxidative stress. In the present study, the activity of SOD was significantly lowered in hemolymph, hepatopancreas, gills and muscle of WSSV-infected *F. indicus* held at 5, 25 and 35‰ salinity levels when compared with control animals. However an increase in the SOD activity in all tissues at all salinity levels after 24hr WSSV challenge was also noted. The abundantly available singlet oxygen and hydroxyl radicals in the immediate environment may be responsible for the declined activity of SOD observed in the post challenge days 2 to 5 of the present study. These results are in agreement with the findings of Lin (1998) and Chang *et al.* (2003) who have found that SOD decrease in WSSV infected *P. monodon* than that of the uninfected control animals. Moreover, Mohankuamr and Ramasamy (2006) and Mathew *et al.* (2007) have reported a similar decrease in SOD activity of WSSV infected shrimp tissues compared to the uninfected tissues. Superoxide dismutase has been reported to contain arginine and histidine residues at its active site (Mallinowski and Fridovich, 1979). Free radicals attack these highly

reactive amino acids resulting in chemical modification of the protein structure and loss of enzyme activity. Escobar *et al.* (1996) also reported that singlet oxygen and hydroxyl radicals were reported to inactivate SOD with resultant loss of enzyme activity. This might have been the cause of low SOD activity observed in the present investigation.

The reduction in CAT activity in WSSV infected shrimp tissues has been previously reported by Mohankumar and Ramasamy (2006) and Mathew *et al.* (2007). The present investigation also demonstrated a similar decrease in the CAT activity of WSSV infected *F. indicus* tissues at different salinity levels. Mathew *et al.* (2007) has reported an increase in CAT activity during the initial period of WSSV infection as observed in our study attempting to counteract or neutralize the harmful free radicals generated due to WSSV infection. This may have been due to progressive increase in damage to cells of ectodermal and mesodermal tissues. Rajan *et al.* (2000) found that the level of WSSV infection and pathogenic change increased progressively in ectodermal and mesodermal tissues of shrimp with increase in the number of days of survival after WSSV infection. The lower activity of catalase in infected animals may be due to inactivation of these enzymes by the oxidative stress generated singlet oxygen.

Glutathione peroxidase catalyses the reduction of hydroperoxides, with the conversion of reduced glutathione (GSH) to glutathione disulfide (GSSG) (Meister and Anderson, 1983). It has been reported that GPx is inactivated with increased levels of hydrogen peroxide, which in turn is due to the lower activity of catalase (Searle and Wilson, 1980). Since GSH acts as the substrate for GPx, the decreased availability of GSH in the tissues of WSSV infected *F. indicus* might have resulted in a decline in the activity of GPx. (Mathew *et al.*, 2007) noted a significant reduction in the activities of glutathione-dependent antioxidant enzymes in WSSV infected shrimp

compared to control animals. Also, the level of GSH was significantly reduced. Decline in the activity of GPX makes cellular and subcellular membranes more sensitive to oxidative damage. A decrease in reduced glutathione (GSH) content as obtained in the present study may decrease the activities of antioxidant enzymes and so aggravate the effects of oxidative stress (Garg *et al.*, 1996).

Glutathione reductase was essential for the regeneration of reduced glutathione (GSH) (Miller *et al.*, 1993). Glutathione is regenerated from oxidised glutathione by NADPH, requiring glutathione redutase (GR). However, the present study has shown that the activity of glutathione reductase (GR) was significantly lowered in the haemolymph, hepatopancreas, gills and muscle of *F. indicus* infected with WSSV as compared with the control animals. The decreased activity of GSH and glutathione peroxidase (GPx) leads to a reduction in the level of oxidized glutathione (GSSG). Since GSSG acts as the substrate for GR, the decreased availability of GSSG might in turn cause a decline in the activity of GR. If potentially toxic H_2O_2 is present in a tissue, GSH is oxidized by glutathione peroxidase(GPX) to glutathione disulfide (GSSG) and GSSG is reduced back to GSH by glutathione reductase (GR) (Meister and Anderson, 1983).

Glutathione-S-transferase is another important enzyme known to catalyse antioxidant metabolism of thiol compounds, and this in turn protects cells from electrophiles, free radical induced damage and oxidative stress (Dixon *et al.*, 1998). Significant reduction in the activities of GPX and GST might lead to the formation of O_2^- and H_2O_2, which in turn form hydroxyl radical (OH•) and bring about a number of reactions harmful to cell membranes. (Mathew *et al.*, 2007). In the present study, the activity of glutathione-S-transferase was also significantly low in WSSV-infected shrimp hemolymph, hepatopancreas, gills and muscle as

compared with control animals. The decreased activity of glutathione-S-transferase in WSSV-infected tissues might be due to the decreased availability of reduced glutathione (GSH). Elevated levels of GSH protect cellular proteins against oxidation through the glutathione-redox cycle and also directly detoxify reactive oxygen species. The decreased activity of GPx, GST and GR in WSSV-infected shrimp may be due to reduced availability of GSH.

The present study has shown an increased level of oxidative stress in terms of hydrpperoxides, conjugated dienes and malondialdehyde concnentartion in the various tissues of *F.indcus* held at diffeent salinity levels. MDA reflected membrane degradation in a variety of pathological conditions (Shirali *et al.*, 1994). Viral infection and salinity alterations have been implicated in the manifestation of increased oxidative stress in aquatic animals. There fore in the present study the increase in MDA levels can be related to pathological invasion under environmental stress.

The activities of antioxidant enzymes in hemolymph, hepatopancreas, gills and muscle of WSSV-infected *F. indicus* was found to be gradually reduced with the increase in the days of survival of WSSV-infected shrimp and these may have been due to the increase in the number of viruses in the infected ectodermal and mesodermal tissues. Rajan *et al.* (2000) found that the WSSV infectivity and pathogenicity increased in ectodermal and mesodermal tissues with the increase in the number of days of survival of WSSV infected shrimp. Mathew *et al.* (2007) found evidence of oxidative damage to tissues of *P.monodon* as indicated by increased lipid peroxidation in comparison to control. Dandapat *et al.* (2003) reported that high levels of TBARS were indicative of high oxidative assault on cellular and subcellular membranes.

The present study gives information on the antioxidant defence of *F. indicus* under salinity stress and WSSV infection. Understanding of the antioxidant defence profile in response to the environmental alterations may help to control the environmentally induced shrimp diseases by adopting proper prophylactic measures.

Chapter 6

MODULATORY EFFECT OF AMBIENT COPPER ON THE HAEMATOLOGICAL RESPONSES AND SUSCEPTIBILITY OF *F. INDICUS* TO WHITE SPOT SYNDROME VIRUS

Contents

6.1 Introduction

Environmental pollution by metals has become one of the most important problems in the world (Chandran *et al.*, 2005). Environmental poisoning by metals has increased in the last decades due to extensive use of metals in agricultural, chemical and industrial processes that are becoming threats to living organisms (Cheung *et al.*, 2003). Environmental degradation due to heavy metal contamination has been posing considerable impact on aquaculture sector also. It has been demonstrated that there exists a clear linkage between environmental conditions and disease, although the precise nature of the relationship is complex and has to be established (Snieszko, 1973).

Copper is essential in small quantities by organisms for metabolic functions, but it is potentially very toxic if the internal available concentration exceeds the capacity of physiological and biochemical detoxification processes (Sunda and Hanson, 1987; Rainbow, 1992). Copper sulfate is commonly applied to shrimp ponds to eradicate filamentous algae and blue-green algae like *Oscillatoria* that synthesize and excrete aversive flavored compounds, such as geosmin. According to Boyd (1990) the application rate of copper sulfate can be varied from 0.025 to 2 mg l^{-1} and was directly related to water total alkalinity. The concentration of copper sulfate remaining in the water and its effect on the resistance of cultured shrimps are of primary concern since farmers often apply excess amounts of copper sulphate in pond management. Cu functions in haematopoiesis and in numerous Cu-dependent enzymes including lysyl oxidase, cytochrome c oxidase (CCO), ferroxidase, tyrosinase and superoxide dismutase (SOD) (O'Dell, 1976). Most crustaceans possess haemocyanin which contains Cu as their main oxygen-carrying blood pigment (Dallinger, 1977).

There are at least 12 major proteins that require copper as an integral part of their structure, including the respiratory enzyme cytochrome oxidase (Hassall and Dangerfield, 1990) and most crustaceans possess haemocyanin containing much copper as their main oxygen-carrying blood protein (Dallinger, 1977). Penaeid shrimps are among the most economically important crustaceans and are abundant in tropical and subtropical coasts. The limit between the requirement and toxicity of copper is delicate and dependent on a variety of endogenous and exogenous factors (Weber *et al.*, 1992). Tolerance of animals to toxicants varies with certain endogenous (i.e., stage, species, physiology) and exogenous (i.e., temperature, salinity, alkalinity) factors. The mechanism of acute toxicity of metals on aquatic animals is generally synergistic, caused by salinity, temperature, presence of ligands, oxygen, alkalinity, etc. (Jones, 1975; Depledge, 1987; Voyer and Modica, 1990; Weber *at al.*, 1992).

The toxicity of copper sulfate on penaeid shrimps has been studied on *P. japonicus* (Bambang *et al.*, 1995) and *P. monodon* (Guo and Liao, 1992; Chen and Lin, 2001) and *Metapenaeus ensis* (Wong *et al.*, 1995).

Heavy metal may reduce the immune vigour of the organisms in many ways. It may be through a direct toxic effect on the haemocytes themselves, perhaps inducing lysis or degranulation, as is the case with LPS or other non-self agents (Soderhall and Smith, 1983) or, alternatively it may occur as a generalised stress response to physiological disturbance, as proposed by Smith and Johnston (1992) and Smith *et al.* (1995). Crustaceans regulate the concentrations of these in their bodies; small amounts of these metals may be maintained in a metabolically available form to play essential biochemical roles, but excess amounts are detoxified into metabolically inert chemical forms and then stored temporally or permanently in the body (Bryan, 1984; Rainbow, 1988). The

accumulation/detoxification strategies of Crustacea vary depending on species and metals (Rainbow, 1988, 1997).

However, the effect of copper sulfate on the resistance of penaeid shrimp to WSSV is scanty. The purpose of the present study is aimed at determining (1) the susceptibility of *Fenneropenaeus indicus* to WSSV at different Cu^{2+} concentrations (2) the immune and antioxidant response of *F. indicus* under ambient Cu^{2+} stress and (3) the heavy metal accumulation in the haemolymph of shrimp.

6.2 Materials and methods

6.2.1 Experimental animals

Adult *Fenneropenaeus indicus* were brought to the Laboratory within one hour of capture from a commercial shrimp farm located at Njarakkal, Kochi, India. The average wet weight of the shrimp was 14.17±2.24g (Mean±SD). Shrimps were reared in rectangular concrete tanks containing 25‰ clean sea water and allowed to acclimate for a period of seven days. Continuous aeration was provided using air pumps and was maintained on a commercial shrimp diet (Higashimaru, Pvt. Ltd. Kochi). Water quality parameters *viz.*, temperature, dissolved oxygen, NH_3-N, NO_2-N and NO_3-N were monitored daily following standard procedures (APHA, 1995) and maintained at optimal levels as per Table 2.1. Unused feed and faecal matter was siphoned out daily and 30% water exchanged every alternate day. A biological filter was set up to maintain the appropriate levels of water quality parameters. After acclimating to 25‰ for seven days six (n=6) shrimps were sampled for baseline data.

6.2.2 Experimental setup and dosing with Copper (Cu^{2+})

Shrimps of apparently uniform size were distributed in the experimental tanks containing 500L of seawater (*n*=35/tank). Shrimps in the intermoult stage only were used (Robertson *et al.*, 1987). There were six treatment Groups (G-I, G-II, G-III, G-IV, G-V and G-VI) and the

experiment was conducted in triplicate i.e., 3 tanks per treatment. Salinity of all the tanks were adjusted to 25‰ prior to the experiment. After 12 hours of starvation, G-I, G-II, G-III, G-IV shrimps were dosed with 0.075, 0.150, 0.225 and 0.30ppm Cu^{2+} respectively by adding copper sulphate solution. The G-V and G-VI groups were maintained as the control with no Cu^{2+} treatment. All six groups were maintained on commercial diet. Six shrimps from each group (n=6) were sampled after seven days of metal exposure (PMD7) and 14 days of metal exposure (PMD14).

6.2.3 WSSV challenge

The shrimps of Group-I, Group-II, Group-III and Group-IV and Group-V were then challenged with White Spot Syndrome Virus. Challenge was performed through oral administration i.e., by feeding white spot virus infected frozen tissue at the rate of 1g/shrimp. Group- VI was maintained as the unchallenged control. Shrimps were sampled (*n*=6) after 48 h (post challenge day2, PCD2) and 120 h of challenge (post challenge day 5, PCD5). Before each sampling the shrimps were starved for 12 hours to eliminate variations caused by the ingested food (Hall and van Ham, 1998). Survival in each group was recorded daily for a period of 10 days with dead animals removed promptly. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of infected shrimps.

6.2.4 Extraction of haemolymph and haematolgical analysis.

Haemolymph was extracted and haematological parameters *viz.*, total haemolymph protein (THP), plasma protein (PLP), serum protein(SRP), haemocyte protein (HCP), Total free amino acids (TFAA), phenol oxidase activity(PO), super oxide anion production (NBT reduction), alkaline phosphatae (ALP) activity and acid phosphatase activity(ACP) were analyzed as described in section.2.2.

6.2.5 Antioxidant parameters in haemolymph

Antioxidant parameters (super oxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and malondialdehyde (MDA)) in the haemolymph were also assessed. See section 5.2. for details.

6.2.6 Estimation tissue level Cu^{2+}

The Cu^{2+} concentration was estimated in haemolymph, of *F. indicus* sampled periodically. Haemolymph (100µL) was taken in clean dry digestion tubes. Extra pure Conc. Nitric acid (2ml) and 1 ml of perchloric acid was added and digested for two hours at 150°C in digestion chamber, until clear solution was obtained. The samples were diluted appropriately with de ionised water and analysed in Atomic Absorption Spectrophotometer (Ecil Corporation of India) using air acetylene flame at 324.5nm for copper against blank and standard stock solutions of Cu^{2+} were also assayed in the same manner.

6.2.7 Statistical analysis

A multiple comparison (Tukey) test was conducted to compare the significant differences among treatments using the software SPSS 10.00 package. All data are presented as mean ± SD and the differences were regarded as statistically significant when $p<0.05$.

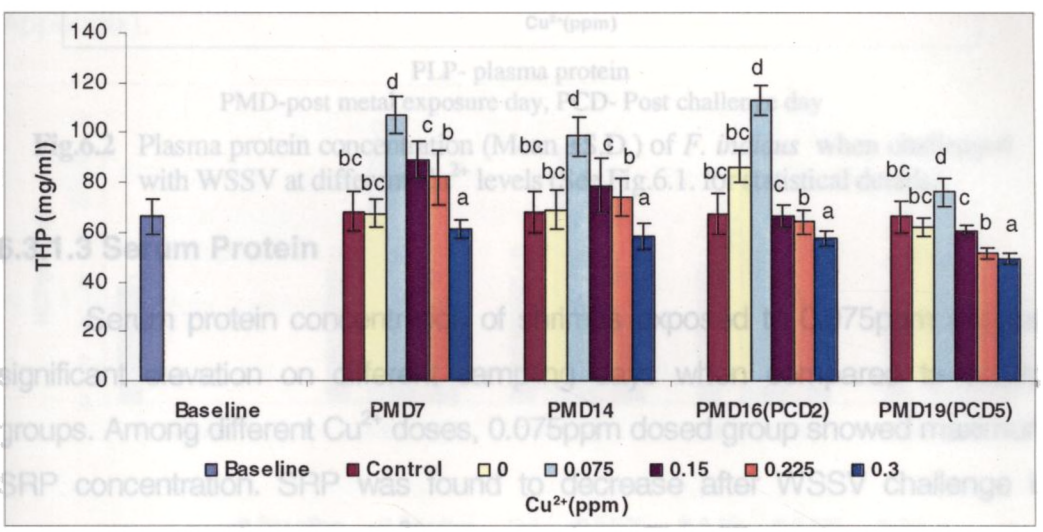
6.3 Results

6.3.1 Haematological alterations in *F. indicus* when exposed to Cu^{2+} and challenged with WSSV.

6.3.1.1 Total Haemolymph Protein(THP)

The variations in the total haemolymph proteins of shrimps exposed to different sub lethal concentrations of Cu^{2+} followed by WSSV challenge has been represented in the Fig.6.1 and Table 6.1 of Appendix. On the 7^{th} day of Cu^{2+} exposure THP was maximum in shrimps treated with 0.075ppm. This hike was maintained throughout the sampling period. An increase in THP could be noticed in the control group after 2days of WSSV challenge.

Shrimps dosed with 0.15 and 0.225ppm showed high THP on 7th day followed by a gradual decline from fourteenth day and after WSSV challenge. In shrimps with highest concentration of Cu^{2+} the THP was lower on all sampling days compared to control. High concentration of THP was obtained on 7th day compared to other WSSV exposure timings. The maximum difference in the THP was observed in shrimps dosed with 0.075ppm Cu^{2+} The differences were found to be statistically significant at $p<0.05$.

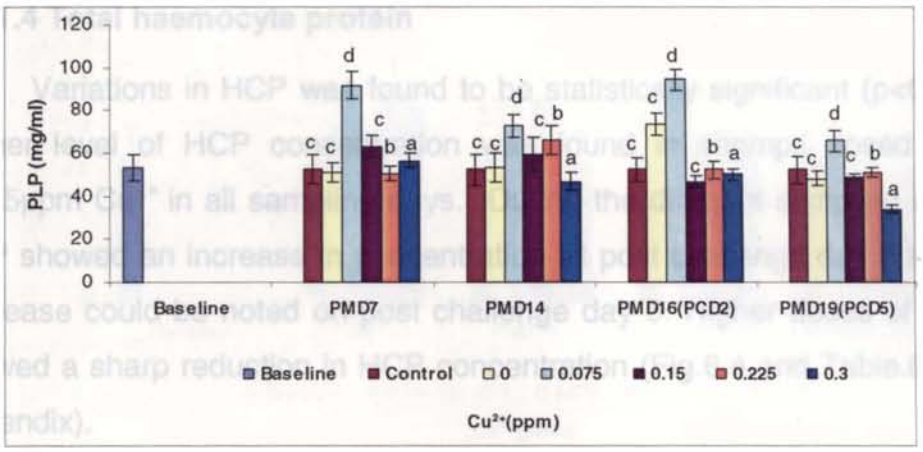


THP- Total haemolymph protein
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.1 Total haemolymph protein concentration of *F. indicus* when challenged with WSSV at different Cu^{2+} levels. Each bar diagram represents mean ± S.D. of six separate determinations. Data with same lowercase letters do not vary significantly ($p>0.05$) among different treatment groups.

6.3.1.2 Plasma protein

Plasma protein concentration of shrimps exposed to 0.075ppm showed a similar increase as that of THP. Plasma protein was maximum during the 2nd day of WSSV challenge. Statistically significant variations ($p<0.05$) were observed in PLP of shrimps dosed with 0.075, 0.15 and 0.225ppm. Shrimps dosed with 0.3ppm showed a reduction in PLP concentration when compared with control (Fig.6.2 and Table 6.2 of Appendix).

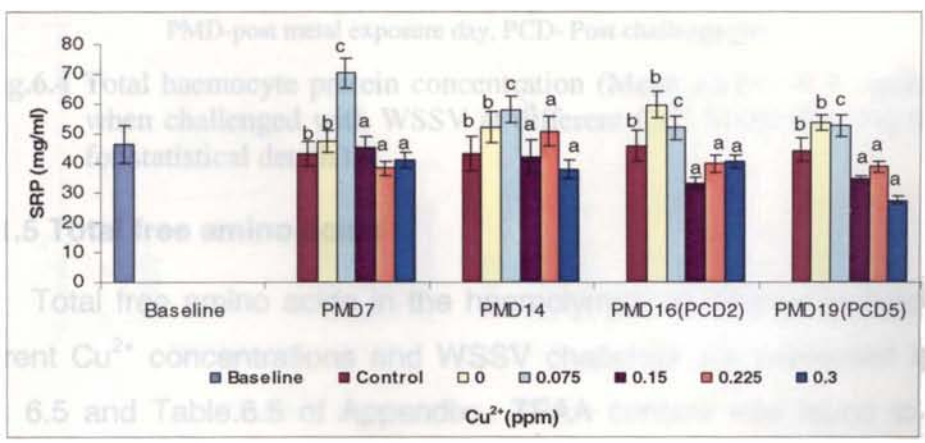


PLP- plasma protein
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.2 Plasma protein concentration (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.1.3 Serum Protein

Serum protein concentration of shrimps exposed to 0.075ppm showed significant elevation on different sampling days when compared to control groups. Among different Cu^{2+} doses, 0.075ppm dosed group showed maximum SRP concentration. SRP was found to decrease after WSSV challenge in shrimps dosed with higher Cu^{2+} concentrations. The differences were found to be statistically significant when $p<0.05$ (Fige.6.3 and Table 6.3 of Appendix).

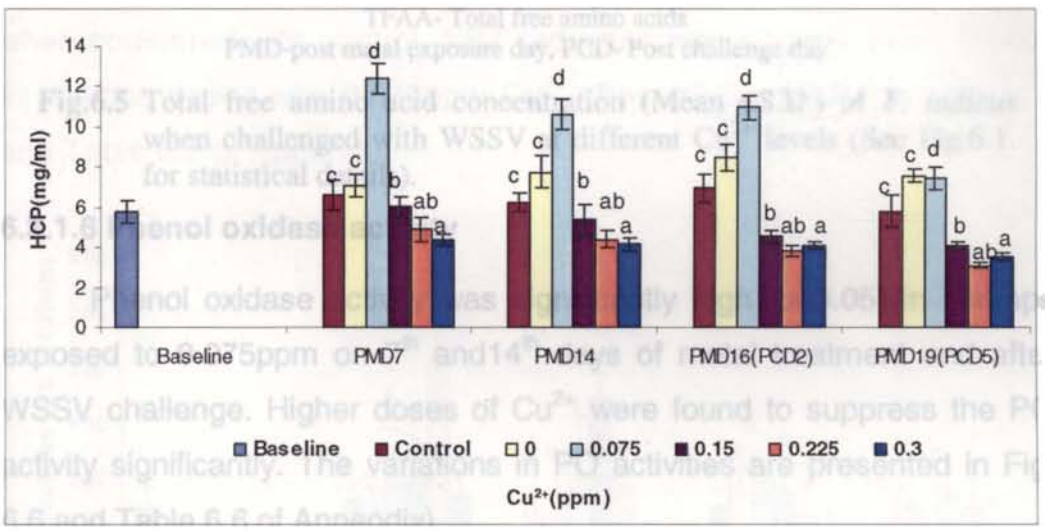


SRP- serum protein
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.3 Serum protein concentration (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.1.4 Total haemocyte protein

Variations in HCP was found to be statistically significant ($p<0.05$). Higher level of HCP concentration was found in shrimps dosed with 0.075ppm Cu^{2+} in all sampling days. During the different sampling days, HCP showed an increase in concentration till post challenge day 2 and a decrease could be noted on post challenge day 5. Higher doses of Cu^{2+} showed a sharp reduction in HCP concentration (Fig.6.4 and Table.6.4 of Appendix).

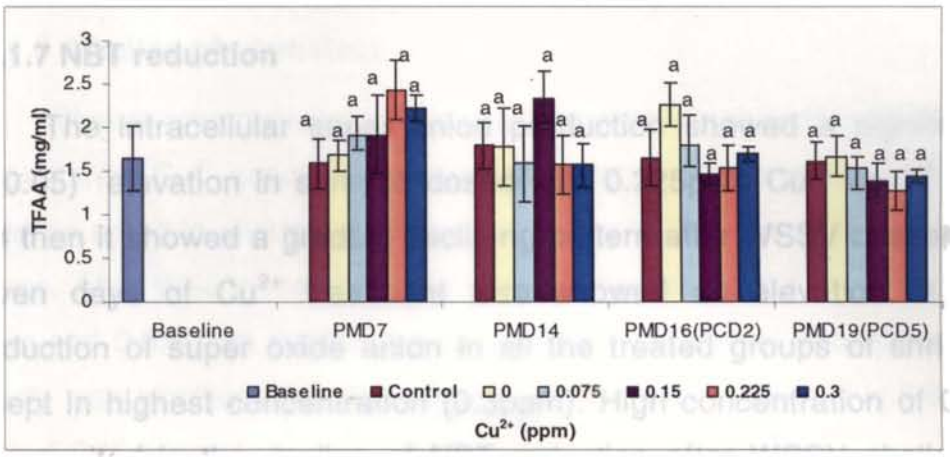


HCP- Haemocyte protein
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.4 Total haemocyte protein concentration (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.1.5 Total free amino acids

Total free amino acids in the haemolymph of shrimps treated with different Cu^{2+} concentrations and WSSV challenge are presented in the Fig. 6.5 and Table.6.5 of Appendix . TFAA content was found to vary slightly during different sampling days. TFAA was particularly high after seven days of metal exposure in higher doses. Copper treatment was not found to significantly affect the TFAA in shrimps during the experimental periods.

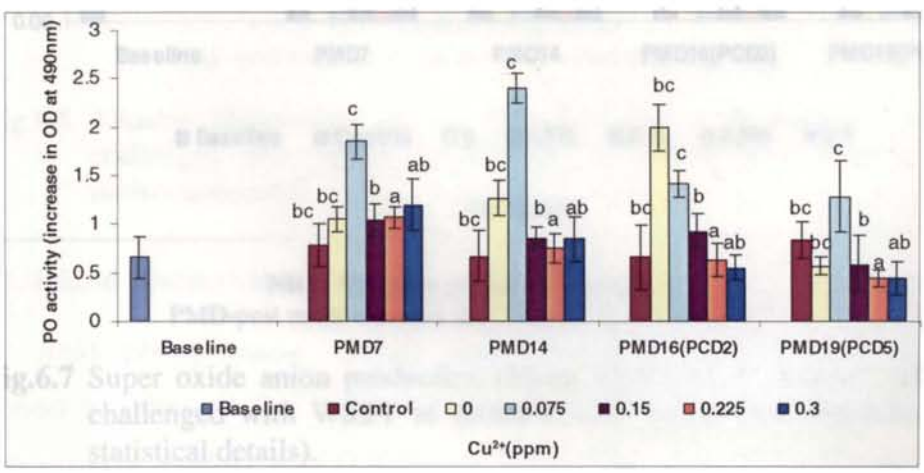


TFAA- Total free amino acids
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.5 Total free amino acid concentration (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.1.6 Phenol oxidase activity

Phenol oxidase activity was significantly high ($p<0.05$) in shrimps exposed to 0.075ppm on 7th and14th days of metal treatment and after WSSV challenge. Higher doses of Cu^{2+} were found to suppress the PO activity significantly. The variations in PO activities are presented in Fig. 6.6 and Table 6.6 of Appendix).

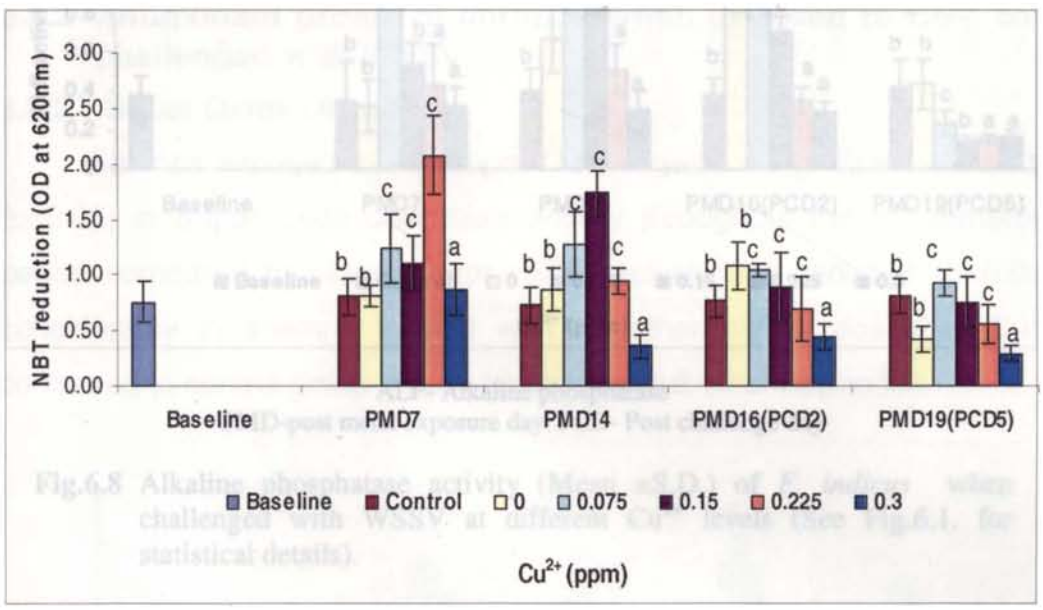


PO- Phenol oxidase
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.6 Pheol oxidase activity (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.1.7 NBT reduction

The intracellular super anion production showed a significant ($p<0.05$) elevation in shrimps dosed with 0.225ppm Cu^{2+} on 7th day and then it showed a gradual declining pattern after WSSV challenge. Seven days of Cu^{2+} treatment also showed an elevation in the production of super oxide anion in all the treated groups of shrimps except in highest concentration (0.3ppm). High concentration of Cu^{2+} has resulted in the decline of NBT reduction after WSSV challenge when compared with control. NBT reduction was comparatively higher in shrimps dosed with 0.075ppm Cu^{2+} after WSSV challenge (Fig. 6.7 and Table 6.7 of Appendix).

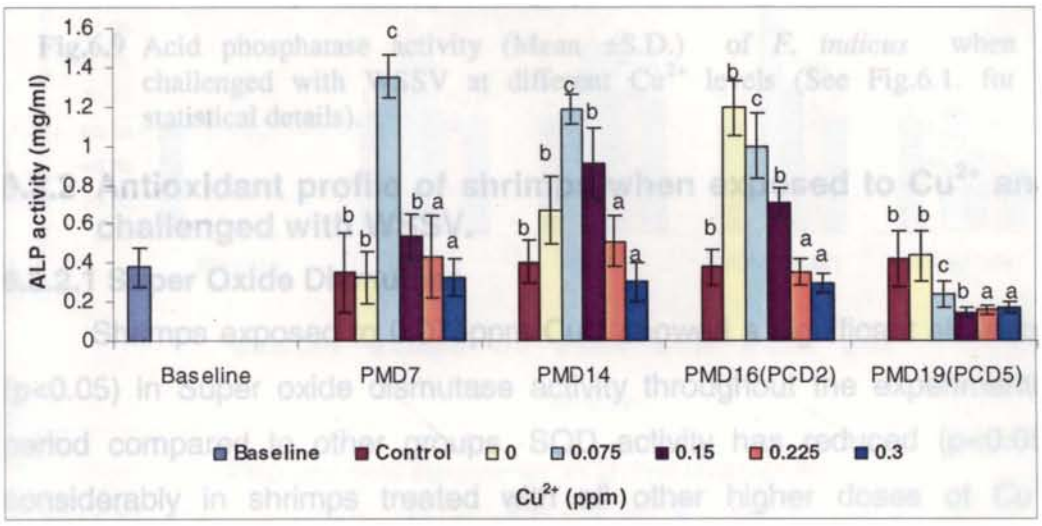


NBT- Nitroblue tetrazolium reduction
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.7 Super oxide anion production (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.1.8 Alkaline phosphatase

Alkaline phosphatase activity was remarkably high in shrimps dosed with different Cu^{2+} concentrations except 0.3ppm. Maximum ALP activity could be noted in shrimps dosed with 0.075ppm Cu^{2+} compared to other groups. After WSSV challenge the ALP activity was found to increase in 0ppm and 0.075ppm treated groups of shrimp compared to control. ALP activity was significantly low in shrimps dosed with 0.3ppm (Fig.6.8 and Table 6.8 of Appendix).

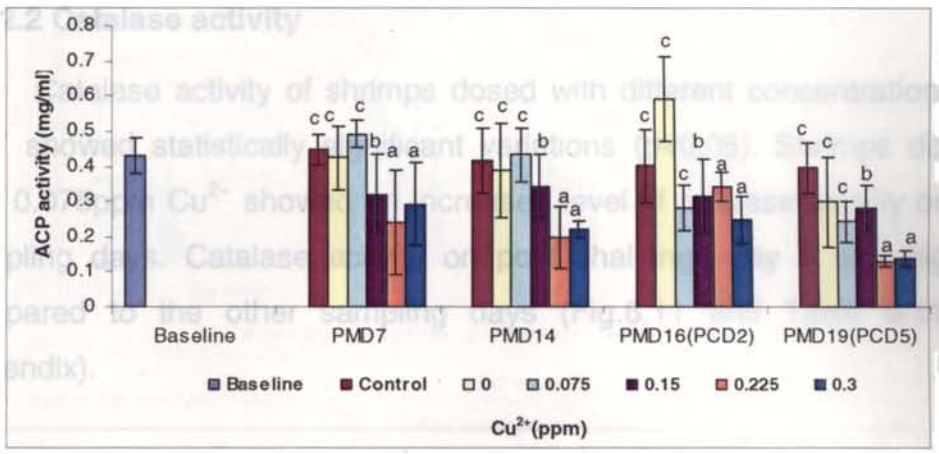


ALP- Alkaline phosphatase
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.8 Alkaline phosphatase activity (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.1.9 Acid phosphatase

Acid phosphatase activity was found to decrease in shrimps exposed to different Cu^{2+} concentrations on 7th and 14th days except in those groups treated with 0.075ppm Cu^{2+}. After WSSV challenge, ACP activity was found to decrease in all doses of Cu^{2+} when compared to control (Fig.6.9 and Table 6.9 of Appendix).



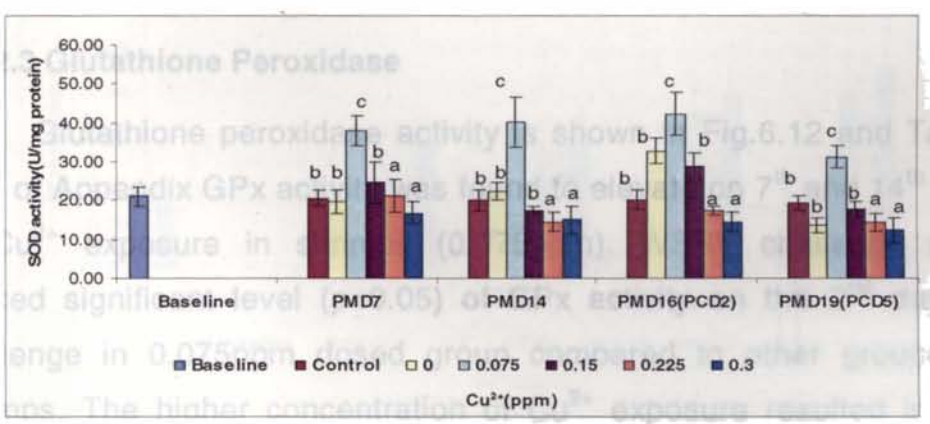
ACP- Acid phosphatase
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.9 Acid phosphatase activity (Mean ±S.D.) of *F. indicus* when challenged with WSSV at different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.2 Antioxidant profile of shrimps when exposed to Cu^{2+} and challenged with WSSV.

6.3.2.1 Super Oxide Dismutase

Shrimps exposed to 0.075ppm Cu^{2+} showed a significant elevation ($p<0.05$) in Super oxide dismutase activity throughout the experimental period compared to other groups. SOD activity has reduced ($p<0.05$) considerably in shrimps treated with all other higher doses of Cu^{2+} compared to control group (Fig. 6.10 and Table 6.10 of Appendix).

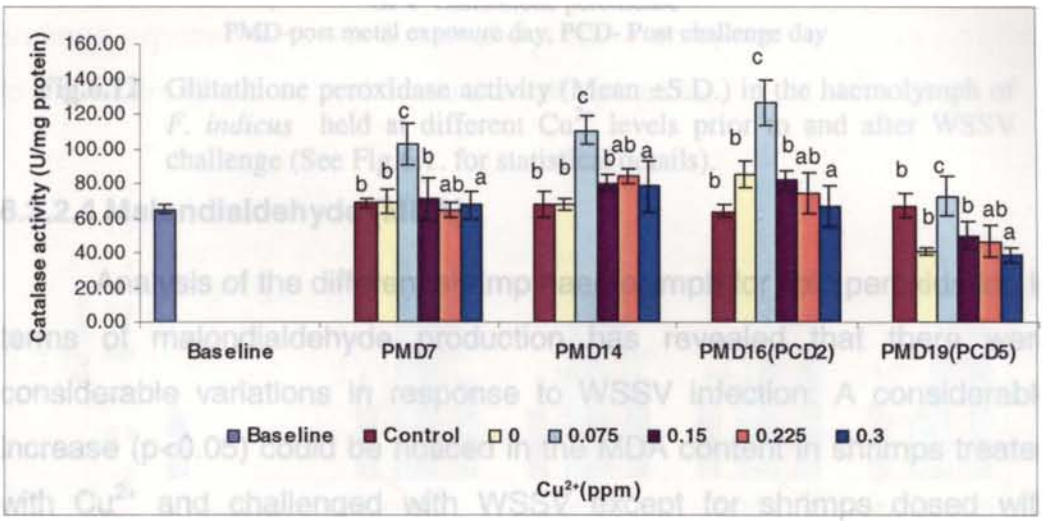


SOD- Superoxide dismutase
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.10 Superoxide dismutase activity (Mean ±S.D.) in the haemolymph of *F. indicus* held at different Cu^{2+} levels prior to and after WSSV challenge (See Fig.6.1. for statistical details).

6.3.2.2 Catalase activity

Catalase activity of shrimps dosed with different concentrations of Cu^{2+} showed statistically significant variations ($p<0.05$). Shrimps dosed with 0.075ppm Cu^{2+} showed an increased level of catalase activity on all sampling days. Catalase activity on post challenge day 2 was higher compared to the other sampling days (Fig.6.11 and Table 6.11 of Appendix).

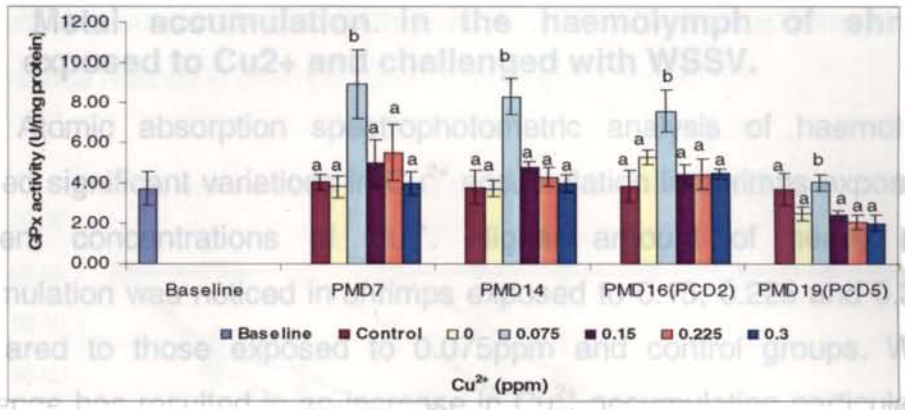


PMD-post metal exposure day, PCD- Post challenge day

Fig.6.11 Catalase activity (Mean ±S.D.) in the haemolymph of *F. indicus* held at different Cu^{2+} levels prior to and after WSSV challenge (See Fig.6.1. for statistical details).

6.3.2.3 Glutathione Peroxidase

Glutathione peroxidase activity is shown in Fig.6.12 and Table 6.12 of Appendix GPx activity was found to elevate on 7th and 14th day of Cu^{2+} exposure in shrimps (0.075ppm). WSSV challenge also evoked significant level ($p<0.05$) of GPx activity on the 2nd day of challenge in 0.075ppm dosed group compared to other groups of shrimps. The higher concentration of Cu^{2+} exposure resulted in the reduction of GPx activity when compared to the control group.

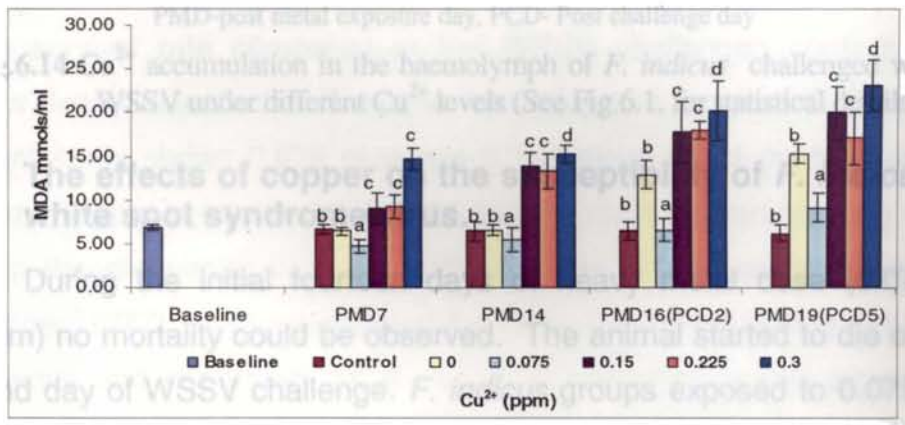


GPx- Glutathione peroxidase
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.12 Glutathione peroxidase activity (Mean ±S.D.) in the haemolymph of *F. indicus* held at different Cu^{2+} levels prior to and after WSSV challenge (See Fig.6.1. for statistical details).

6.3.2.4 Malondialdehyde (MDA)

Analysis of the different shrimp haemolymph for lipid peroxidation in terms of malondialdehyde production has revealed that there were considerable variations in response to WSSV infection. A considerable increase ($p<0.05$) could be noticed in the MDA content in shrimps treated with Cu^{2+} and challenged with WSSV except for shrimps dosed with 0.075ppm where the MDA concentration was comparatively lower (Fig.6.13 and Table 6.13 of Appendix).

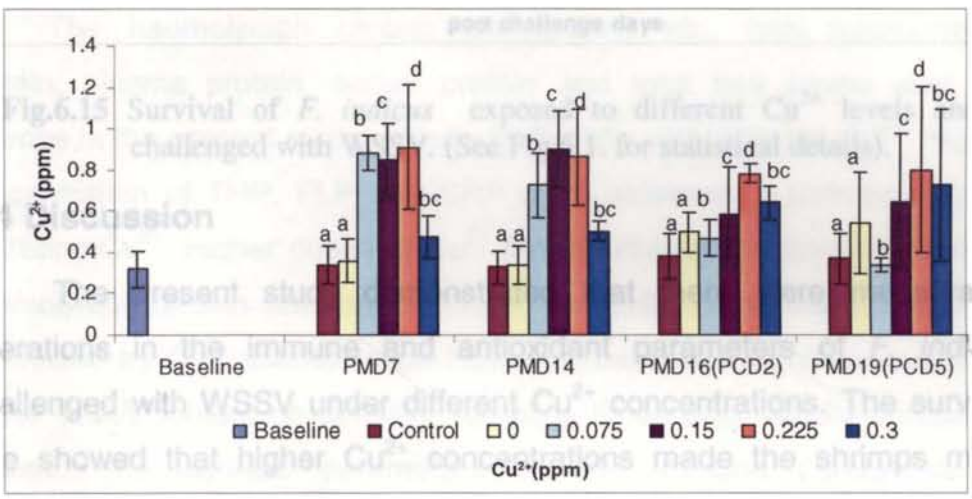


MDA- Malondialdehyde
PMD-post metal exposure day, PCD- Post challenge day

Fig.6.13 Malondialdehyde content (Mean ±S.D.) in the haemolymph of *F. indicus* held at different Cu^{2+} levels prior to and after WSSV challenge (See Fig.6.1. for statistical details).

6.3.3 Metal accumulation in the haemolymph of shrimps exposed to Cu2+ and challenged with WSSV.

Atomic absorption spectrophotometric analysis of haemolymph showed significant variations in Cu^{2+} accumulation in shrimps exposed to different concentrations of Cu^{2+}. Higher amount of heavy metal accumulation was noticed in shrimps exposed to 0.15, 0.225 and 0.3ppm compared to those exposed to 0.075ppm and control groups. WSSV challenge has resulted in an increase in Cu^{2+} accumulation particularly in shrimps exposed to 0, 15, 0.225 and 0.3ppm compared to those exposed to 0.075ppm (Fig.6.14 and Table 6.14 of Appendix).



PMD-post metal exposure day, PCD- Post challenge day

Fig.6.14 Cu^{2+} accumulation in the haemolymph of *F. indicus* challenged with WSSV under different Cu^{2+} levels (See Fig.6.1. for statistical details).

6.3.4 The effects of copper on the susceptibility of *F. indicus* to white spot syndrome virus.

During the initial fourteen days of heavy metal dose (0.075 to 0.3ppm) no mortality could be observed. The animal started to die on the second day of WSSV challenge. *F. indicus* groups exposed to 0.075ppm Cu^{2+} showed a better survival rate compared to the WSSV challenged controls. Animals maintained at higher concentrations of Cu^{2+} showed lower survival percentage compared to the challenged control. The shrimps

progressively succumbed to death towards the end of the experiment. Least survival was noticed in shrimps dosed with 0.3ppm (Fig. 6.15)

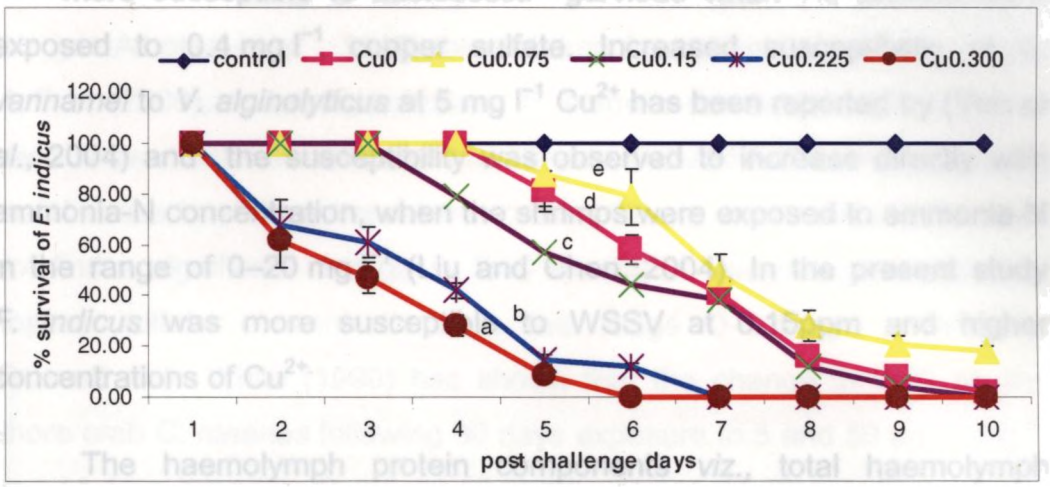


Fig.6.15 Survival of *F. indicus* exposed to different Cu^{2+} levels and challenged with WSSV. (See Fig.6.1. for statistical details).

6.4 Discussion

The present study demonstrated that there were measurable alterations in the immune and antioxidant parameters of *F. indicus* challenged with WSSV under different Cu^{2+} concentrations. The survival rate showed that higher Cu^{2+} concentrations made the shrimps more susceptible to WSSV. *F. indicus* exposed to 0.075ppm Cu^{2+} showed a better survival rate compared to the WSSV challenged controls. The results clearly indicate that *F. indicus* can tolerate a range of Cu^{2+} and the present study shows 0.075 ppm has a beneficial effect on the immune system. Copper is an essential metal and the main oxygen carrying blood protein (haemocyanin) in crustaceans possess copper as main element (Dallinger, 1977). The limit between the requirement and toxicity of copper is delicate and dependent on a variety of endogenous and exogenous factors (Weber *et al.*, 1992). Tolerance of animals to toxicants varies with certain endogenous (i.e., stage, species, physiology) and exogenous (i.e., temperature, salinity, alkalinity) factors. It has been already reported that

environmental toxicants increases the susceptibility of aquatic organisms to pathogens. Cheng and Wang (2001) have shown that *M. rosenbergii* was more susceptible to *Lactococcus garvieae* when the prawns were exposed to 0.4 mg l^{-1} copper sulfate. Increased susceptibility of *L. vannamei* to *V. alginolyticus* at 5 mg l^{-1} Cu^{2+} has been reported by (Yeh *et al.*, 2004) and the susceptibility was observed to increase directly with ammonia-N concentration, when the shrimps were exposed to ammonia-N in the range of 0–20 mg l^{-1} (Liu and Chen, 2004). In the present study *F. indicus* was more susceptible to WSSV at 0.15ppm and higher concentrations of Cu^{2+}.

The haemolymph protein components *viz.*, total haemolymph protein, plasma protein, serum protein and total free amino acid sof shrimps in the present study varied significantly with different Cu^{2+}. Higher concentration of THP, PLP and SRP were obtained in shrimps held at 0.075ppmCu^{2+}. Higher doses of Cu^{2+} tend to reduce the concentration of haemolymph protein components. Total free amino acids was found to be unaffected by Cu^{2+} concentration However, there was significant varation in the TFAA content on different sampling times after WSSV challenge. Influence of extrinsic parameters on the immune parameters of crustaceans has been reported by many researchers Vargas-Albores *et al.*, 1998; Le Moullac and Haffner, 2000; Lorenzon , 2001. Here, high doses of Cu^{2+} decreased the protein components due to reduction in the metabolic activities of shrimp. Interestingly the lowest Cu^{2+} (0.075ppm) concentration in the study was found to be immune stimulating for shrimps. Immunostimulatory property of copper has been observed by Cheng and Wang (2001) and Yeh *et al.* (2004).

In the current study total circulating haemocytes were estimated as total haemocyte protein (HCP). HCP was significantly higher in shrimps dosed with 0.075ppm Cu^{2+} and lower at other Cu^{2+} concentrations through

out the period of study. Extrinsic factors like temperature, salinity, pH, and heavy metals affect circulating haemocytes in several species of decapod crustaceans including yellowleg shrimp *Farfantepenaeus californiensis* (Vargas-Albores *et al.* , 1998), blue shrimp *L. stylirostris* (Le Moullac and Haffner (2000), and rockpool prawn *P. elegans* (Lorenzon , 2001). It has been shown that different responses are considered for THC under heavy metal stress for different species and time. Cheng and Wang (2001) could not find a significant change in THC for the *M. rosenbergii* exposed to copper sulfate concentrations in the range 0–0.4 mg l^{-1}. Similarly, Truscott and White (1990) has shown that the change in THC for the shore crab *C. maenas* following 30 days exposure to 5 and 50 µg l^{-1} Hg^{2+}, and 14 days exposure to 0.5 mg l^{-1} Cd^{2+}) was not significant. According to Yeh *et al.* (2004) an exposure of *L. vannamei* to 1.0 mg l^{-1} Cu^{2+} for 48 did not affect its THC, but to 5 mg l^{-1} Cu^{2+} decreased THC by 28% significantly following 24 h exposure.

After metal exposure the phenol oxidase activity was higher in shrimps treated with 0.075ppm Cu^{2+} and higher doses of Cu^{2+} suppressed the phenol oxidase activity. Our results are in agreement with that of Cheng and Wang (2001) who reported that the phenoloxidase activity of *M. rosenbergii* was significantly lower for the prawns exposed to copper sulfate at 0.1, 0.2, 0.3 and 0.4 mg l−1 after 48 h. Yeh *et al* (2004) has also reported a decrease in phenoloxidase activity in *L. vannamei* following 24 h exposure to Cu^{2+} as low as 5 mg l^{-1} together with a decrease in THC was due to reduced THC and probably reduced granular cells.

Super oxide anion production was higher in shrimps exposed to 0.225ppm and 0.15ppm compared to 0.075ppm. Stimulation of production of superoxide anion in *L. vannamei* following 48 and 168 h exposure to 11.10 and 21.60 mg l^{-1} ammonia-N has been reported by Liu and Chen (2004). Cheng and Wang (2001) also reported that exposure of

M. rosenbergii to 0.2, 0.3 and 0.4 mg l^{-1} copper sulfate for 48 and 96 h stimulated the production of superoxide anion. Yeh *et al* .(2004) has shown that the production of superoxide anion in *L. vannamei* following 24 h exposure to Cu^{2+} as low as 5 mg l^{-1} has been stimulated. A small increase in the superoxide anion is considered to be beneficial with respect to increased immunity (Munoz *et al.* , 2000). However, too great an increase may be toxic to the host Cheng and Wang (2001). In our study shrimps exposed to 0.075ppm have shown a small increase in super oxide anion production.

Alkaline phosphatase is an intrinsic plasma membrane enzyme of almost all animal cells. ALP is sensitive to metals and the effects of metals on ALP have been reported (Mazorra *et al.*, 2002). In the present study alkaline phosphatase of shrimps exposed to 0.075ppm was higher after 7days of treatment and remained high during 14^{th} day and post challenge days 2 and 5 compared to control groups. ALP activity of shrimps treated with higher concentrations showed lesser activity.

Super oxide dismutase activity of shrimp exposed to 0.075ppm was found to be increasing after Cu^{2+} treatment and higher activity was obtained on the 2^{nd} day of WSSV challenge followed by a decrease in post challenge day5. The results indicate that an adequate and optimum level of ambient Cu^{2+} has an immuno stimulating effect on the shrimp. Lee and Shiau (2003) have shown that about 10– 30 mg Cu kg^{-1} diet has increased the non-specific immune responses in *P. monodon* under experimental conditions.

Superoxide dismutase (SOD) is one of the main antioxidant defence enzymes generated in response to oxidative stress. Superoxide dismutase (SOD) converts superoxide anions into hydrogen peroxide and oxygen. In the present study, the activity of SOD was significantly lower in the hemolymph of WSSV-infected *F. indicus* held at different Cu^{2+} levels when

compared with control animals. However an increase in the SOD activity at 0.075ppm Cu^{2+} level on 7th day of metal exposure onwards and after 24h of WSSV challenge was also noted. The abundantly available singlet oxygen and hydroxyl radicals in the immediate environment may be responsible for the declined activity of SOD observed in shrimps exposed to higher doses of Cu^{2+} and during the post challenge days 2 to 5 of the present study. These results are in agreement with the findings of Lin (1998) and Chang *et al.* (2003) who found that SOD decreased in WSSV infected *P. monodon* than that of the uninfected control animals. Moreover, Mohankuamr and Ramasamy (2006) and Mathew *et al.* (2007) have reported a similar decrease in SOD activity of WSSV infected shrimp tissues compared to the uninfected tissues. Superoxide dismutase has been reported to contain arginine and histidine residues at its active site (Mallinowski and Fridovich, 1979). Cu^{2+} might have exacerbated the oxidative stress in shrimps. Free radicals attack these highly reactive amino acids resulting in chemical modification of the protein structure and loss of enzyme activity. Escobar *et al.* (1996) also reported that singlet oxygen and hydroxyl radicals were reported to inactivate SOD with resultant loss of enzyme activity. This may have been the cause of low SOD activity in shrimps dosed with higher concentration of Cu^{2+} observed in the present investigation. It is interesting to note that the lowest concentration (0.075ppm Cu^{2+}) in the present investigation has shown an increase in the SOD in response to WSSV. The results indicate the possible role of Cu^{2+} as an immunostimulant at optimum concentration for invertebrate species. More work has to be carried out to elucidate the physiology behind this.

The reduction in CAT activity in WSSV infected shrimp tissues has been previously reported by Mohankumar and Ramasamy, 2006; Mathew *et al.*, 2007). The present investigation also demonstrated a similar decrease in the CAT activity of WSSV challenged *F. indicus* haemolymph

at different Cu^{2+}levels. The lower activity of catalase in infected animals may be due to inactivation of these enzymes by the oxidative stress generated singlet oxygen. The higher concentration of Cu^{2+} has decreased the immune vigour of shrimps by acting as immunotoxic and the situation has been exacerbated by viral infection. However, the catalase activity of shrimps exposed to 0.075ppm Cu^{2+} showed a higher rate throughout the experimental period.

Glutathione peroxidase has also been reported to play a pivotal role in cell division and differentiation. Evidence suggests that it is an important factor in regulating the onset of mitosis (Cotgreave and Gerdes, 1998). Glutathione peroxidase catalyses the reduction of hydroperoxides, with the conversion of reduced glutathione (GSH) to glutathione disulfide (GSSG) (Meister and Anderson, 1983). It has been reported that GPx is inactivated with increased levels of hydrogen peroxide, which in turn is due to the lower activity of catalase (Searle and Wilson, 1980). Since GSH acts as the substrate for GPx, the decreased availability of GSH in the tissues of WSSV-infected *F. indicus* might have resulted in a decline in the activity of GPx. Mathew *et al.* (2007) noted a significant reduction in the activities of glutathione-dependent antioxidant enzymes in WSSV infected shrimp maintained at different Cu^{2+} levels compared to control animals. Decline in the activity of GPX makes cellular and subcellular membranes more sensitive to oxidative damage. GPx activity of shrimps dosed with 0.075ppm Cu^{2+} has been elevated considerably in the present study.

Reactive oxygen species (ROS) are reported to be released by oxidative stress generated in crustaceans in response to invading microorganisms (Munoz *et al.*, 2000). It has been reported that viral infection has been associated with dramatic increase in the level of free radical mediated oxidative damage of cellular and subcellular membranes (Skulachev, 1998; Mathew *et al.*, 2007). Bell and Smith (1993) also reported the *in vitro*

production of superoxide by virus inoculated hyaline cells of the shore crab *Carcinus maenas* (L). Similar increase of lipid peroxidation due to oxidative stress caused by influenza virus infection in the liver tissues and in serum (Brown *et al.*, 1982). The present study has shown that the level of lipid peroxidation significantly increased in WSSV-infected hemolymph of *F. indicus* held at different Cu^{2+} levels when compared with control animals. Highest level of lipid peroxidation was observed in shrimps held at 0.300ppm implying the possible role of high concentration of Cu^{2+} to increase oxidative stress in the shrimp. It has been already reported that environmental parameters may induce oxidative stress in aquatic organisms. Roche and Boge (1996) attributed the increase of blood SOD of European sea bass under decreased salinity to the possible prevention of natural oxidation of epinephrine, the concentration of which generally increases in stressed fish.

Atomic absorption spectrophotometric analysis of haemolymph showed significant variations in Cu^{2+} accumulation in shrimps exposed to different concentrations of Cu^{2+}. Higher amount of heavy metal accumulation was noticed in shrimps exposed to 0.15, 0.225 and 0.3ppm compared to those exposed to 0.075ppm and control groups. WSSV challenge has resulted in an increase in Cu^{2+} accumulation particularly in shrimps exposed to 0.15, 0.225 and 0.3ppm compared to those exposed to 0.075ppm.

In conclusion high levels of Cu^{2+} enhanced the mortality of *F. indicus* and with concurrent reduction in haematological and antioxidant parameters together with an increased accumulation of Cu^{2+}. High levels of Cu^{2+} depressed growth and impaired feed conversion in channel catfish (Murai *et al.*, 1981), rainbow trout (Lanno *et al.*, 1985) and shrimp (Lee and Shiau, 2003). A concentration of 0.075ppm Cu^{2+} in the rearing water was found to have beneficial effect in shrimps in terms of immunostimulation and higher survival against WSSV infection.

Chapter 7

EFFECT OF β1, 3-GLUCAN INCORPORATED DIET ON THE HAEMATOLOGICAL RESPONSES AND SURVIVAL OF *F. INDICUS* WHEN CHALLENGED WITH WHITE SPOT SYNDROME VIRUS

Contents

7.1 Introduction

Immunostimulants are substances, which enhance the non-specific defence mechanism and provide resistance against pathogenic organism. Immunostimulants are effective in crustaceans since their immune system is of mainly non-specific and relies on phagocytosis, encapsulation and agglutination alongside the phenoloxidase-mediated production of melanin through the pro-phenoloxidase cascade (Smith and Soderhall, 1983). Protective efficacies of various immunostimulants have been reported against WSSV infection, for example oral administration of immunostimulants like Peptidoglucan, Lipopolysaccharides, etc. (Namikoshi *et al.*, 2004). β-1, 3 -glucan has been found to be one of the most important immunostimulants used in aquaculture. It belongs to the class of drugs known as Biological Response Modifiers.

Glucan is an oligosaccharide of glucose units linked together by β-1, 3 linkages with 1, 6 branching, where the branches may have one or more glucose units. The β-1,3-glucans of certain fungi and yeasts have been successfully used as immunostimulants to enhance the defence potential of fish and shellfish against bacterial and viral infection. β-Glucan has been reported to be a potent immunostimulant for fish and crustaceans against bacterial and viral infections (Itami *et al.*, 1994; Sung *et al.*, 1994; Su *et al.*, 1995; Liao *et al.*, 1996; Yadomae and Ohno, 1996; Song *et al.*, 1997; Chang *et al.*, 2000, 2003; Sajeevan *et al.*, 2006).

Reports on marine yeast as a source of immunostimulants in penaeids has been scanty. The immunostimulatory effect of a marine isolate of *Candida sake* in *Fenneropenaeus indicus* against WSSV infection has been reported by Sajeevan *et al.* (2006). Immunity in crustaceans has not been studied in detail, particularly the capacity to generate oxidative and antioxidant responses (Roch, 1999).

Against this background, the present study has been undertaken with the following objectives. 1) to assess the haematoogical responses of *Fenneropenaeus indicus* fed on glucan diet and challenged with WSSV. 2) to evaluate the antioxidant defence status of *F. indicus* under this experimental conditions and 3) to examine the survival rate of shrimps under experimental treatments.

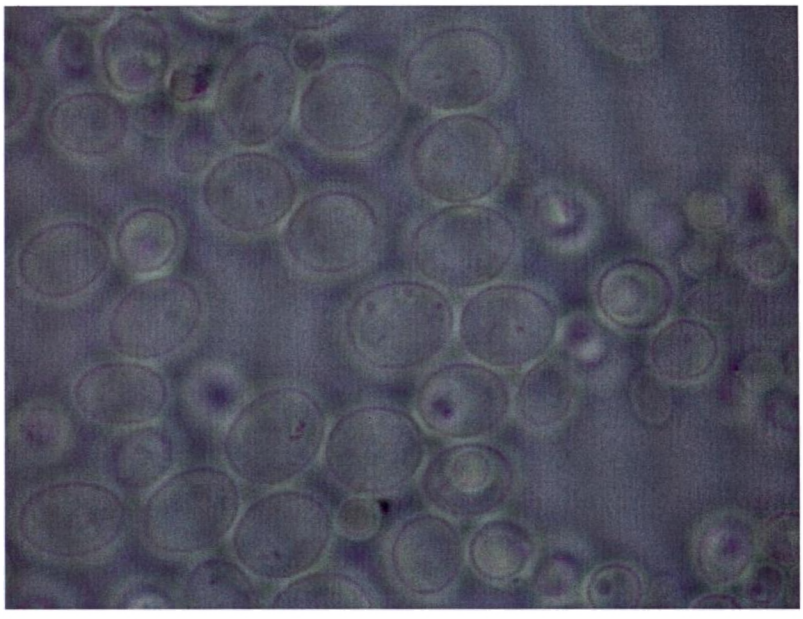
7.2 Materials and methods

7.2.1 Shrimps

Adult *Fenneropenaeus indicus* were brought to the Laboratory within one hour of capture from a commercial shrimp farm located at Panangad, Kochi, India. The average wet weight of the shrimp was 16.45±2.12g (Mean±S.D.). Shrimps were reared in rectangular concrete tanks containing 25‰ clean sea water and allowed to acclimate for a period of seven days. Continuous aeration was provided using air pumps and was maintained on a commercial shrimp diet (Higashimaru, Pvt.Ltd. Kochi). Water quality parameters *viz.* temperature, dissolved oxygen, NH_3-N, NO_2-N and NO_3-N were monitored daily following standard procedures (APHA, 1995) and maintained at optimal levels as per Table.2.1. Unused feed and faecal matter was siphoned out daily and 30% water exchanged every alternate day. A biological filter was set up to maintain the appropriate levels of water quality parameters. After acclimating to 25‰ for seven days six (n=6) shrimps were sampled for baseline data.

7.2.2 Yeast strain

Marine yeast, *Debaryomyces hansenii* S169 (Fig.7.1) was used for the study. The yeast used was isolated from the seawater sample collected from the west coast of India and maintained in the Microbiology laboratory of the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology.



S 169 ***Debaryomyces hansenii***

Fig. 7.1 Light microscopic Photograph of the marine yeast used for the study (400x)

7.2.3 Production of yeast biomass

The media used for the production of yeast biomass was malt extract agar medium. The composition of malt extract agar medium is given in table 7.1

Table 7.1 Media composition of Malt extract agar for the production of yeast, *Debaryomyces hansenii* S169 biomass.

Malt extract powder	20g
Mycological peptone	5g
Agar	20g
Seawater	1000ml
pH	5.5

Malt extract agar medium was prepared and sterilized in an autoclave at 121°C for 15 minutes. Plates were prepared and lawn culture of the marine yeast was prepared. Incubation was done for 7-8 days at room temperature (28±2°C). After the incubation period, the cells were harvested using sterile seawater. The suspension was centrifuged at 5000 x g for 15 minutes in a cooling centrifuge. Supernatant was removed

and the cell mass spread on an aluminium sheet and dried in a hot air oven at 80°C for approximately 36 hours.

7.2.4 Glucan extraction

The dried yeast cell mass was finely powdered using a mortar and pestle and used for extraction as per Williams *et al.* (1991).

Alkali treatment

One gram of dried yeast biomass was taken in a conical flask and 3% NaOH (3g NaOH in 100 ml water) was added and boiled for three hours in a water bath. Centrifuged the residue at 8000 rpm for 10 minutes, at 15°C.the supernatant was discarded and the residue was given a second alkali treatment. The residue was used for acid treatment.

Acid treatment

To the residue, 20 ml 0.5 N acetic acid was added and boiled for 3 hours in a water bath. Centrifuged (5000x g, 10 minutes, 15°C) and collected the residue. The acid treatment was repeated and collected the residue.

Alcohol treatment

After treating the residue with 100 ml distilled water (in boiling water bath for 30 minutes) twice and centrifuging, the residue was subjected to alcohol treatment. The residue was suspended in 100 ml ethanol (95%) and heated to boiling and centrifuged to collect the residue. This treatment was repeated once again.

Glucan and distilled water treatment

The residue after the second alcohol treatment was treated with distilled water (100 ml distilled water in a boiling water bath for 10 minutes). Centrifuged and repeated the procedure. The residue was dried overnight and the dry mass obtained was glucan. The weight of the residue was taken.

7.2.5 Preparation of glucan incorporated feed

Glucan (0.2%) was taken in a 50 ml beaker and about 20-25 ml distilled water was added to it. The solution was then sonicated by using an ultrasonicator (SONIC VIBRA, USA) at 20 KHz at 100 Watt for 15 minutes. The sonicated solution was then added to 100 g of Higashimaru commercial feed pellets in a plastic basin and mixed well and dried for 10 minutes in a hot air oven. To this, 5 ml of binder (Bindex gel, Matrix Pvt. Limited) was then added, mixed well and dried for 10 minutes in a hot air oven. It was then stored in an airtight container in deep freezer for further use (Fig.7.2).

Fig. 7.2 *Debaryomyces hansenii* S 169 glucan incorporated feed used for the study

7.2.6 Experimental set up

Shrimps of apparently uniform size were distributed in the experimental tanks containing 500L of seawater (*n*=35/tank). Shrimps in the intermoult stage only were used (Robertson *et al.*, 1987). There were two treatment Groups (G-I and G-II) and the experiment was conducted in triplicate i.e., 3 tanks per treatment. Salinity of all the tanks was adjusted to 25‰ prior to the experiment. Individuals were

reared with the experimental diets for 28 days. Individuals in the control tank(G-1) were fed with control diet with binder and the other(G-2) with glucan incorporated feed. The prawns were fed daily twice at the rate of 10% of the body weight. Glucan incorporated feed were given twice to the particular shrimp groups on day 1, 7, 14, 21, and 28th day. On all other days they were fed with the ordinary Higashimaru feed (with bindex). Shrimps from the two groups were sampled randomly (n=6) on 1st 15th and 28th day of the experiment for haematological analysis.

7.2.7 Challenge with WSSV

After 28 days of the feeding experiment (28 days), the glucan treated group (G-2) and the control group (G-1) were challenged with white spot virus (WSSV). Challenge was performed through oral administration i.e., by feeding white spot virus infected frozen tissue at the rate of 1g/shrimp. Shrimps were sampled (*n*=6) after 2 days (post challenge day 2, PCD2) and 7 days (post challenge day 7, PCD7). Before each sampling, the shrimps were starved for 12 hours to eliminate variations caused by the ingested food (Hall and van Ham, 1998). Survival in each group was recorded daily for a period of 10 days with dead animals removed promptly.

7.2.8 Haemolymph extraction and haematological analysis

Haematological parameters like plasma protein, total haemocyte count, phenoloxidase activity, superoxide anion production, alkaline phosphatase and acid phosphatase activities were estimated in the haemolymph of *F. indicus* as per standard protocols. Refer section.2.2.3 and 2.2.4 for details.

7.2.9 Analysis of antioxidant parameters

Antioxidative parameters like superoxide dismutase, catalase, glutathione peroxidase, hydroperoxide, conjugated dienes and

malondialdehyde concentrations in the haemolymph of shrimps were assessed as per standard procedures. Refer section 5.2.2. for details.

7.2.10 Statistical analysis

Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test using the SPSS statistical software package version 10.0. The level of significance was set at $p< 0.05$.

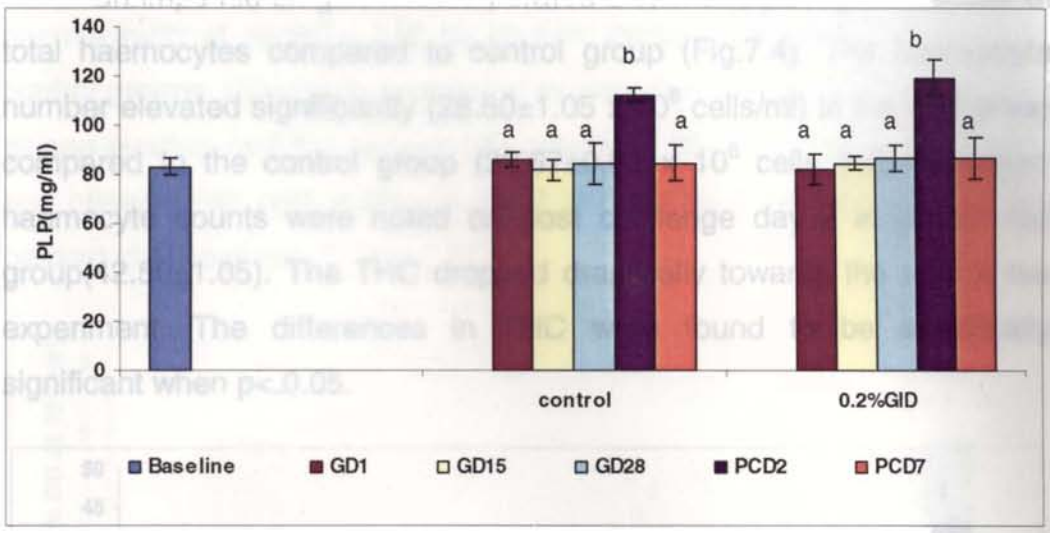
7.3 Results

7.3.1 Haematological profile of *F. indicus* fed on glucan incorporated diet

Haematological parameters like plasma protein, total haemocyte count, phenol oxidase, superoxide anion production assay, alkaline phosphatase and acid phosphatase activities have been assessed in the haemolymph of *F. indicus* fed on glucan incorporated diet and control diet.

7.3.1.1 Plasma protein

Plasma protein concentration of shrimps fed on glucan incorporated feed has been represented in Fig.7.3. Significant increase in PLP concentration (119.45±7.17mg/ml) was observed on post challenge day 2 in shrimps fed on glucan incorporated feed compared to the control group (112.37±3.31 mg/ml). No significant change could be noted in the plasma protein concentration of shrimps between 0.2% glucan incorporated diet and control feed group on other sampling periods.



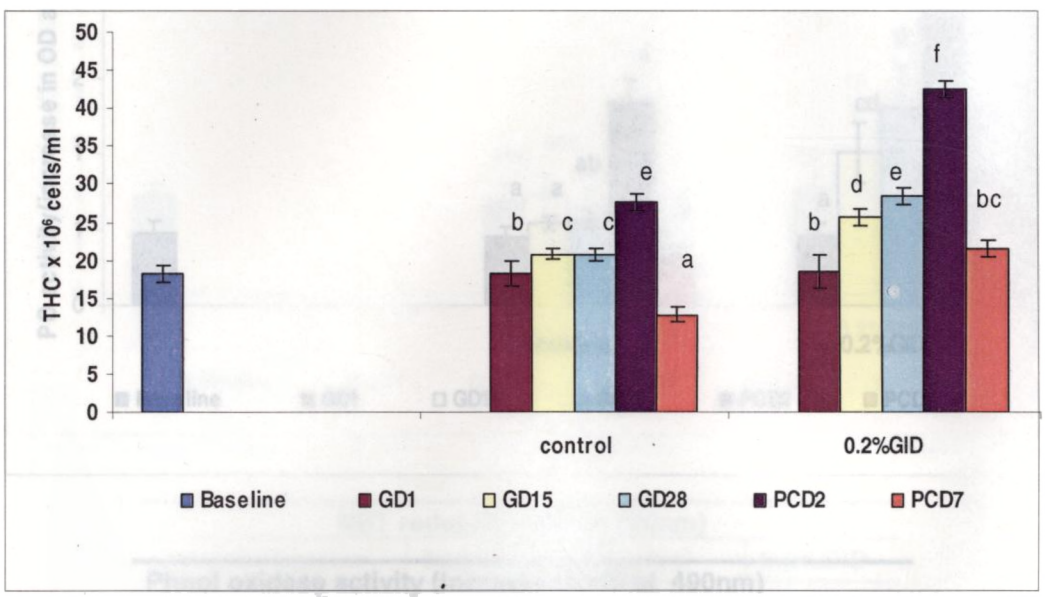
Plasma Protein(mg/ml)			
	Baseline	Control	0.2%GID
Time interval	83.26±3.36		
GD1		86.21±2.76	82.18±6.37
GD15		82.02±4.31	84.95±2.44
GD28		84.63±8.29	87.17±5.51
GD30(PCD2)		112.37±3.31	119.45±7.17
GD35(PCD7)		84.98±7.48	86.91±8.13

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.3 Plasma protein concentration of *F. indicus* fed on glucan incorporated diet. Each bar diagram is average value (Mean±S.D.) of six independent determinations. Bar diagrams with same lowercase letters do not vary significantly ($p<0.05$) among different exposure time.

7.3.1.2 Total haemocyte count

Shrimps fed on glucan incorporated diet showed a higher number of total haemocytes compared to control group (Fig.7.4). The haemocyte number elevated significantly (28.50±1.05 x 10^6 cells/ml) in the test group compared to the control group (20.67±0.82 x 10^6 cells /ml). Maximum haemocyte counts were noted on post challenge day 2 in glucan fed group(42.50±1.05). The THC dropped drastically towards the end of the experiment. The differences in THC were found to be statistically significant when p<.0.05.



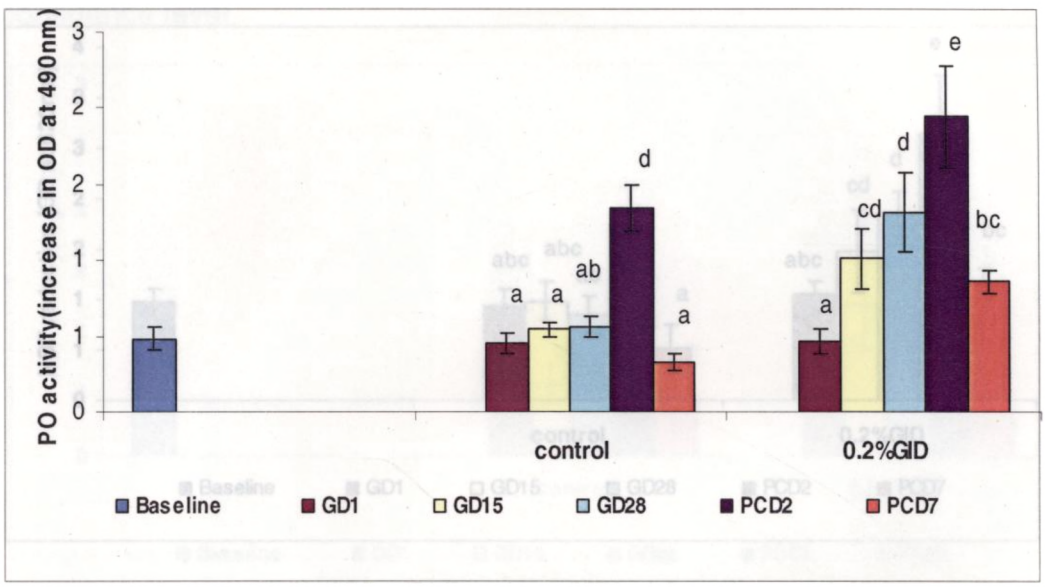
(THC x 10^6 cells/ml)			
	Baseline	Control	0.2%GID
Time interval	18.17±1.17		
GD1		18.12±1.67	18.38±2.21
GD15		20.83±0.75	25.67±1.03
GD28		20.67±0.82	28.50±1.05
GD30(PCD2)		27.67±1.03	42.50±1.05
GD35(PCD7)		12.83±0.98	21.50±1.05

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.4 Total haemocyte count (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.1.3 Phenol oxidase activity

Phenoloxidase activity was significantly higher ($p<0.05$) in the haemolymph of shrimps fed on glucan incorporated diet compared to control groups. PO activity was found to increase on 15th and 28th days of glucan feeding and the highest PO activity (1.95±0.33 increase OD 490) was observed on post challenge day 2 followed by a decline in PO activity on post challenge day 7. (Fig.7.5).



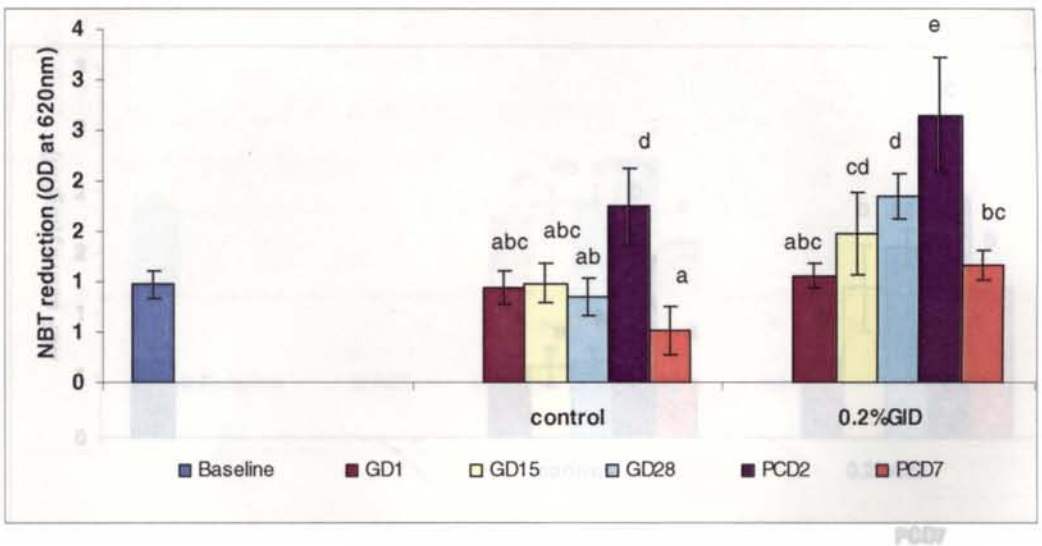
Phnol oxidase activity (Increase in OD at 490nm)			
	Baseline	Control	0.2%GID
Duration	0.48±0.07		
PGD1		0.45±0.07	0.47±0.08
PGD15		0.54±0.05	1.01±0.20
PGD28		0.56±0.07	1.31±0.26
PGD30(PCD2)		1.35±0.15	1.95±0.33
PGD35(PCD7)		0.33±0.05	0.86±0.07

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.5 Phenol oxidase activity (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.1.4 Superoxide anion assay (NBT reduction)

The intracellular superoxide anion production was found to vary significantly in the different treatment groups (Fig.7.6). NBT reduction was higher ($p<0.05$) in shrimps fed on glucan incorporated diet compared to control groups on 15, 28 and post challenge day 2 of the experiment.



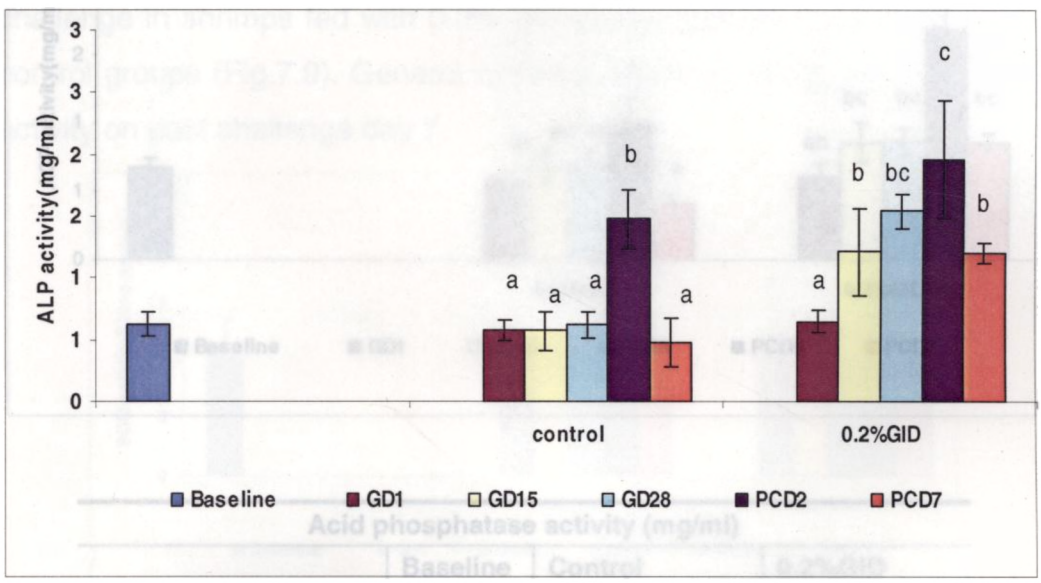
NBT reduction (OD at 620nm)			
	Baseline	**Control**	**0.2%GID**
Time interval	0.97±0.13		
GD1		0.95±0.17	1.06±0.12
GD15		0.99±0.19	1.49±0.41
GD28		0.85±0.18	1.85±0.22
GD30(PCD2)		1.75±0.37	2.66±0.57
GD35(PCD7)		0.52±0.25	1.17±0.15

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.6 Superoxide anion production (NBT reduction) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.1.5 Alkaline phosphatase activity

The alkaline phosphatase activity of shrimps fed on glucan incorporated feed and control feed is given in Fig.7.7. The ALP activity was found to increase in shrimps with 0.2% glucan incorporated diet from 15th day to 28th day and the ALP activity reached its highest on post challenge day 2 followed by a decrease on post challenge day 7. The differences in ALP activity were found to be statistically significant at 95% confidence level.



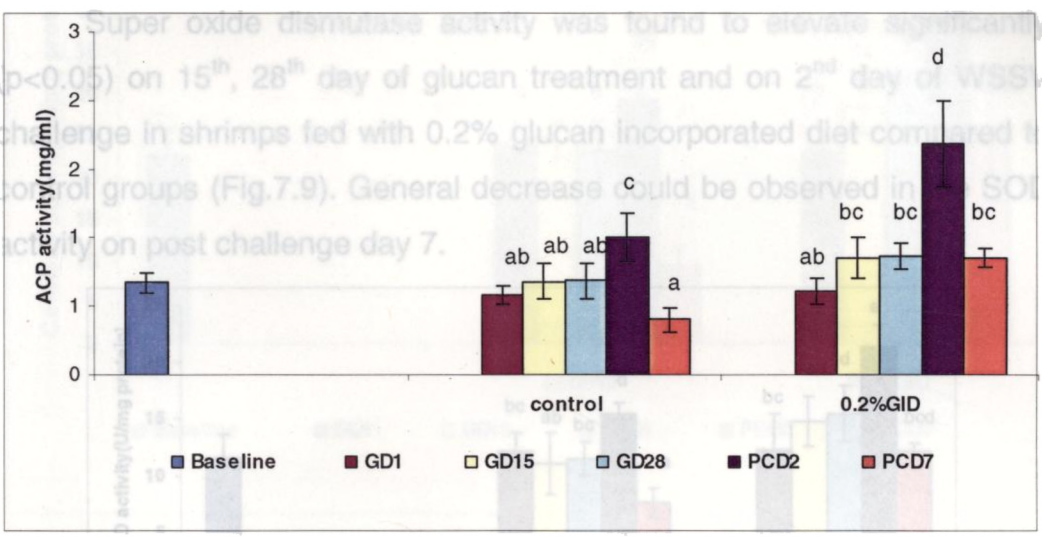
Alkaline phosphatase activity (mg/ml)			
	Baseline	Control	0.2%GID
Time interval	0.62±0.09		
GD1		0.58±0.08	0.65±0.09
GD15		0.57±0.15	1.21±0.35
GD28		0.62±0.11	1.54±0.14
GD30(PCD2)		1.48±0.24	1.96±0.48
GD35(PCD7)		0.48±0.19	1.20±0.08

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.7 Alkaline phosphatase activity (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.1.6 Acid phosphatase activity

Acid phosphatase activity also showed a similar pattern as that of ALP. Highest ACP activity was noted on the 2nd day of post challenge in shrimps fed with glucan extract incorporated feed. The differences were statistically significant at 95% confidence level (Fig.7.8).



Acid phosphatase activity (mg/ml)			
	Baseline	**Control**	**0.2%GID**
Time interval	0.67±0.08		
GD1		0.58±0.07	0.61±0.09
GD15		0.68±0.13	0.85±0.15
GD28		0.69±0.14	0.87±0.09
GD30(PCD2)		1.01±0.17	1.68±0.31
GD35(PCD7)		0.40±0.09	0.85±0.07

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

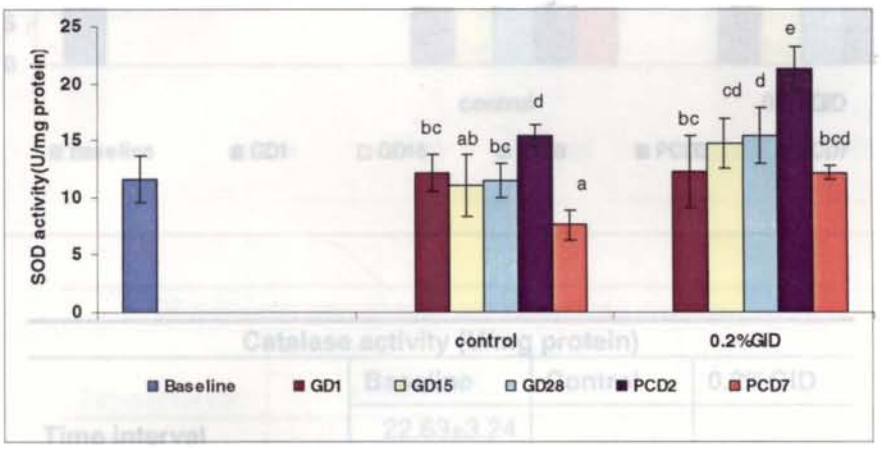
Fig.7.8 Acid phosphatase activity (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.2 Antioxidant profile of *F. indicus* maintained on glucan diet.

Antioxidant enzymes i.e. superoxide dismutase, catalase, glutathione peroxidase and lipid peroxidation products like hydroperoxides, conjugated dienes and malondialdehyde have been estimated in the haemolymph of *F. indicus*.

7.3.2.1 Super oxide dismutase activity

Super oxide dismutase activity was found to elevate significantly ($p<0.05$) on 15th, 28th day of glucan treatment and on 2nd day of WSSV challenge in shrimps fed with 0.2% glucan incorporated diet compared to control groups (Fig.7.9). General decrease could be observed in the SOD activity on post challenge day 7.



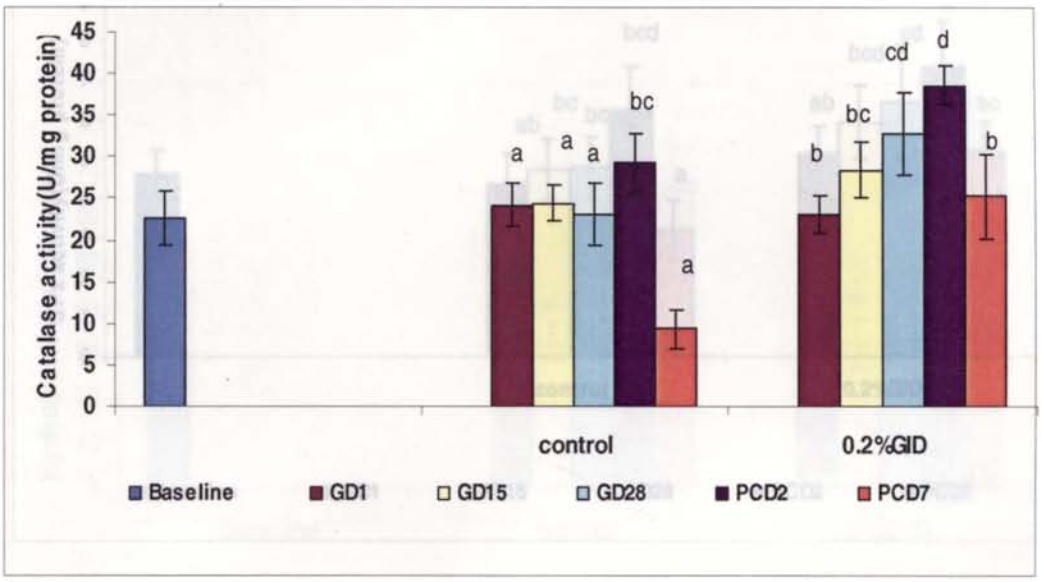
Super Oxide dismutase activity (U/mg protein)			
	Baseline	**Control**	**0.2%GID**
Time interval	11.64±2.03		
GD1		12.14±1.62	12.28±3.12
GD15		11.03±2.73	14.73±2.17
GD28		11.46±1.47	15.44±2.52
GD30(PCD2)		15.45±0.96	21.35±1.88
GD35(PCD7)		7.62±1.28	12.21±0.61

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.9 Superoxide dismutase activity (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.2.2 Catalase activity

Catalase activity of shrimps fed on glucan incorporated diet was considerably higher ($p<0.05$) on 15th, 28th and post challenge day 2 compared to control group (Fig.7.10).



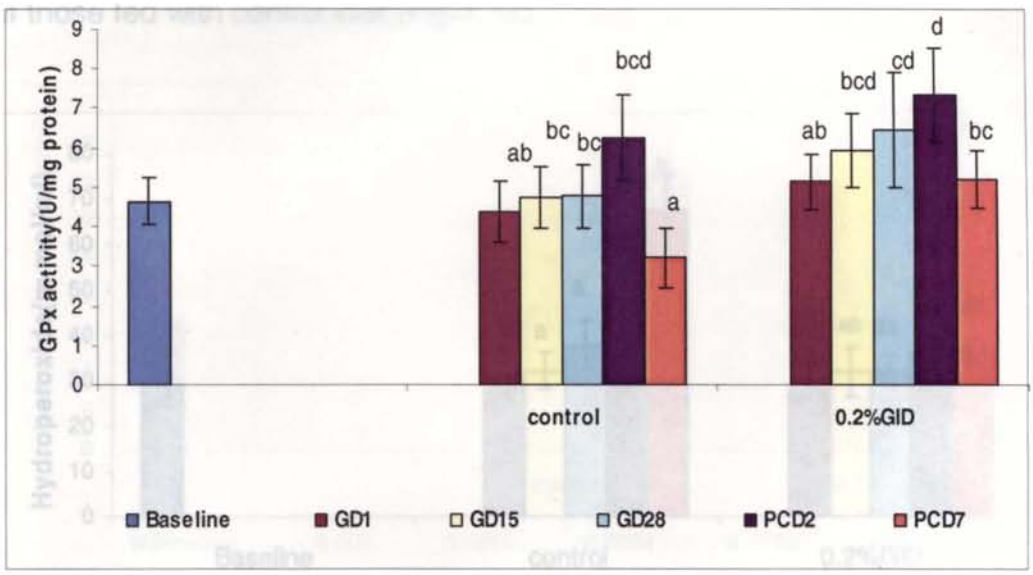
Catalase activity (U/mg protein)			
	Baseline	**Control**	**0.2%GID**
Time interval	22.63±3.24		
GD1		24.18±2.65	**23.15±2.18**
GD15		24.48±2.22	28.46±3.29
GD28		23.13±3.62	32.86±4.97
GD30(PCD2)		29.26±3.62	38.62±2.38
GD35(PCD7)		9.34±2.36	25.24±5.08

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.10 Catalase activity (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.2.3 Glutathione peroxidase activity

Glutathione peroxidase activity of shrimps fed on glucan showed a similar pattern as that of catalase activity. Highest GPx activity was noted on post challenge day 2 of the experiment (Fig. 7.11).



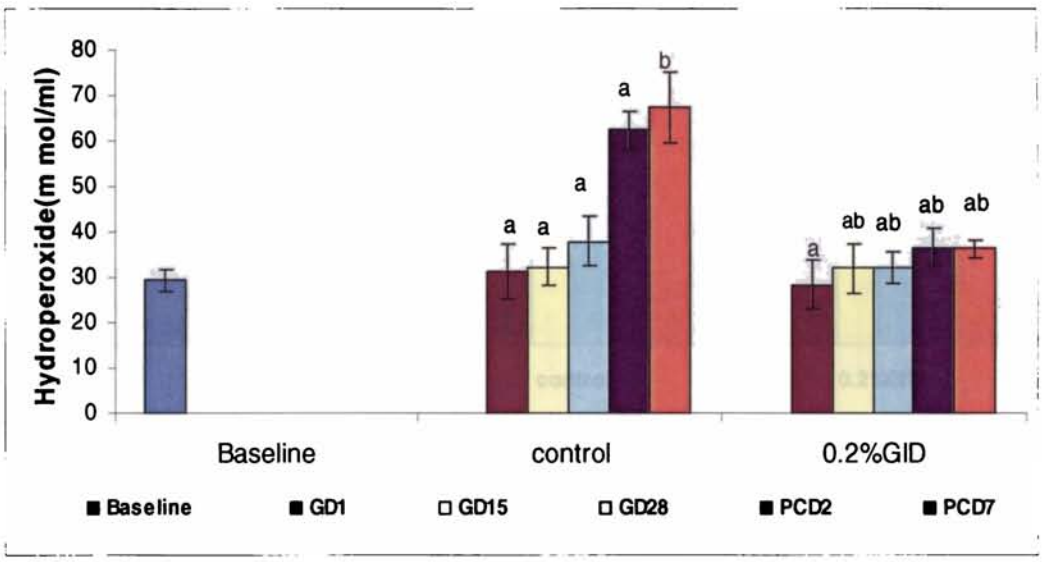
Glutathione peroxidase activity (U/mg protein)			
	Baseline	**Control**	**0.2%GID**
Time interval	4.66±0.60		
GD1		4.38±0.78	5.13±0.71
GD15		4.74±0.80	5.93±0.93
GD28		4.77±0.81	6.45±1.44
GD30(PCD2)		6.26±1.10	7.35±1.19
GD35(PCD7)		3.22±0.75	5.21±0.74

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.11 Glutathione peroxidase activity (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.2.4 Hydroperoxide concentration

Hydroperoxide content of shrimps showed significant variations after WSSV challenge. Hydroperoxide concnentration in the haemolymph of shrimps fed on glucan incorporated diet was comparatively lower than that of those fed with control diet (Fig.7.12).



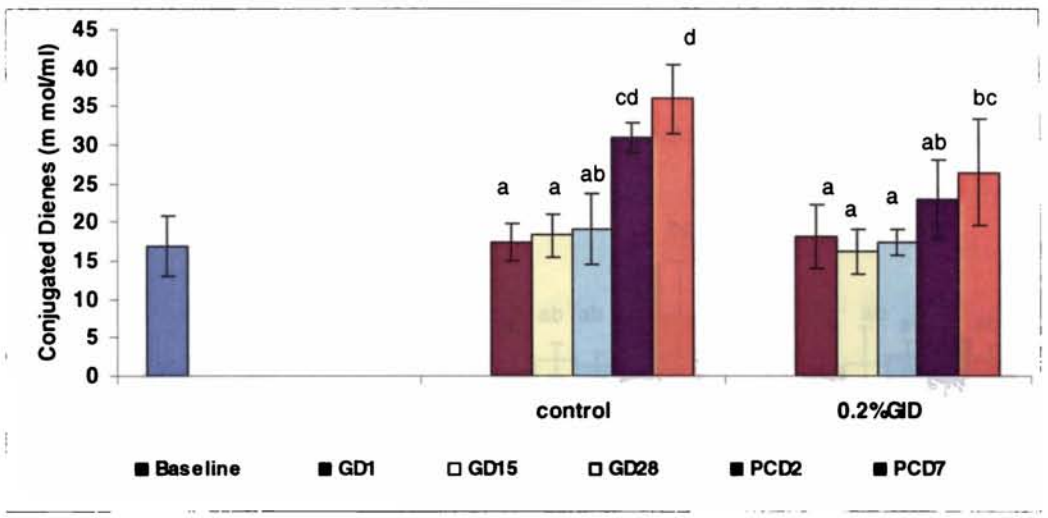
Hydroperoxide (m mol/ml)			
	Baseline	**Control**	**0.2%GID**
Time interval	*29.41±9.43		
GD1		31.38±6.19	28.43±5.28
GD15		32.34±4.04	31.96±5.32
GD28		38.02±5.35	32.16±3.37
GD30(PCD2)		62.43±4.24	36.68±4.10
GD35(PCD7)		67.29±7.87	36.33±1.92

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.12 Hydroperoxide concentration (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.2.5 Conjugated dienes concentration

Conjugated dienes was found to increase after WSSV infection in all treatment groups. However, the CD content in shrimps fed with 0.2% glucan incorporated diet was lower compared to the control group. Highest CD concentration was obtained on post challenge day 7(Fig. 7.13).



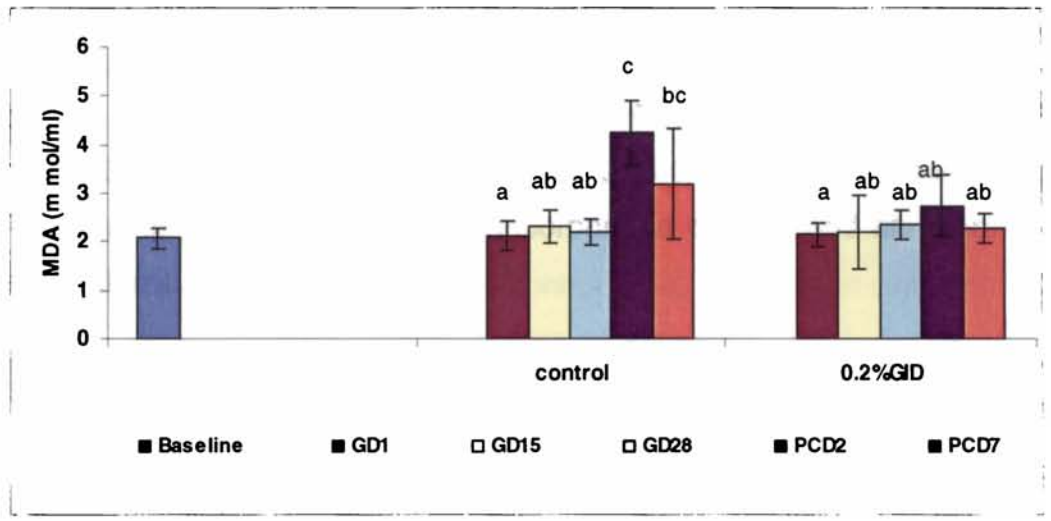
Conjugated Dienes (m mol/ml)			
	Baseline	**Control**	**0.2%GID**
Duration	17.01±3.92		
PGD1		17.45±2.39	18.21±4.12
PGD15		18.35±2.81	16.21±2.88
PGD28		19.11±4.63	17.46±1.74
PGD30(PCD2)		30.98±2.01	22.94±5.02
PGD35(PCD7)		35.96±4.39	26.46±6.91

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.13 Conjugated dienes concentration (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.2.6 Malondialdehyde concentration

Malondialdehyde concentration in the haemolymph of shrimps fed with glucan incorporated and control diets are given in Fig.7.14. Statistically significant variation in the MDA concentration could be observed on post challenge days 2 and 7. Maximum MDA concentration was found in the haemolymph of control shrimps after WSSV infection. Shrimps fed with glucan incorporated diet have shown comparatively lower concentration of MDA content.



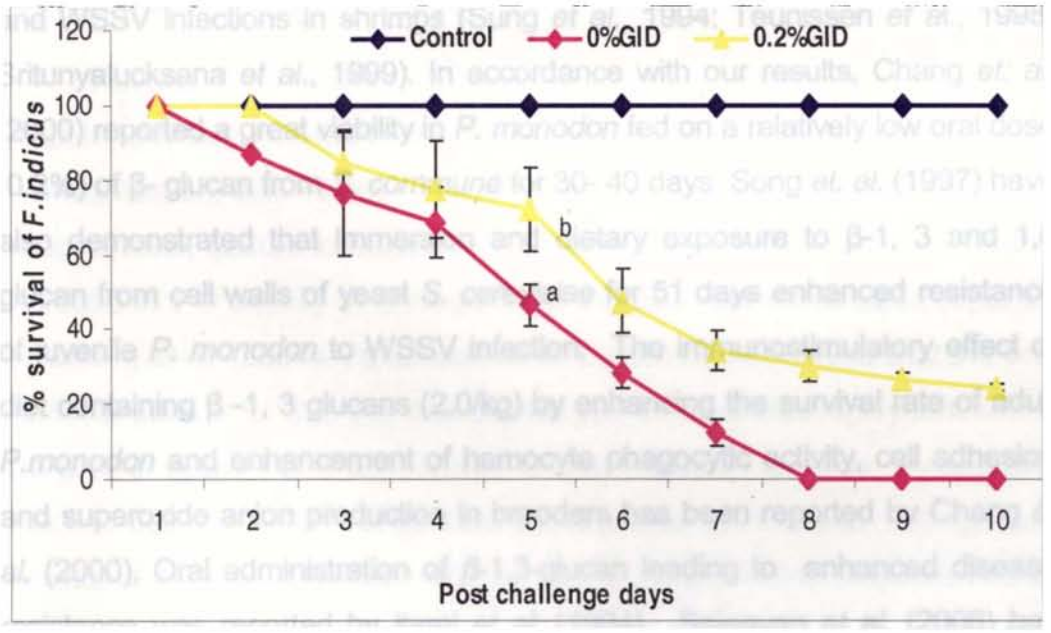
Malondialdehyde (m mol/ml)			
	Baseline	Control	0.2%GID
Time interval	2.08±0.21		
GD1		2.13±0.3	2.15±0.26
GD15		2.30±0.34	2.19±0.77
GD28		2.20±0.26	2.35±0.32
GD30(PCD2)		4.24±0.67	2.75±0.62
GD35(PCD7)		3.18±1.14	2.27±0.30

GD- Glucan administration Day, PCD- Post Challenge Day,
GID- Glucan Incorporated Diet

Fig.7.14 Malondialdehyde concentration (Mean±S.D.) of *F. indicus* fed on glucan incorporated diet. See Fig.7.3 for statistical details.

7.3.3 Survival of *F. indicus* fed on glucan diet and challenged with WSSV

Survival rate of *F. indicus* fed on glucan diet and challenged with WSSV has been presented in the Fig.7.15. The shrimps fed on control diet for 28 days and challenged with WSSV exhibited lower post challenge survival rate compared to those fed on glucan incorporated diet. Mortality of shrimps fed on control diet has begun on the second day of post challenge. The shrimps fed on glucan diet for 28 days followed by WSSV challenge have shown survival rates of, 100, 84.38±8.18, 77.19 ± 12.98, 72.16±11.26, 47.34±8.54, 34.48±5.28 and 28.16± 4.26 on post challenge days 2,3,4,5,6, 7 and 8 respectively. The % survival of shrimps fed on control diet was 87±6, 76.34±16.84, 68.62±9.29, 46.48±5.48, 20.38±4.38, 12.46±3.36 and 0 on post challenge day 2, 3,4,5,6, 7 and 8 respectively. The shrimp groups fed on glucan incorporated diet showed an enhanced survival rate.



GID- Glucan Incorporated Diet

Fig.7.15 Post challenge survival of *F. indicus* fed on glucan incorporated diet.

7.4 Discussion

The present work was undertaken to delineate the immune and antioxidant responses of *F. indicus* fed on 0.2% glucan diet and then challenged with WSSV. The various immune indices were assessed and correlated with survival data of shrimps fed on glucan incorporated diet as well as those with control diet. Analysis of variance has shown that there are measurable variations in the different indices assessed in response to glucan administration and WSSV challenge.

In the present investigation, *F. indicus* fed yeast glucan incorporated diet showed an enhanced performance in terms of post challenge survival along with an elevation in immune and antioxidant parameters. The shrimps fed on 0.2% glucan extract incorporated diet for 28 days and followed by WSSV challenge have shown considerable increase in the survival rates on all post challenge days compared to the control group. It has been demonstrated that glucan or inactivated *Vibrio* have immune stimulatory effect against *Vibrio* and WSSV infections in shrimps (Sung *et al.*, 1994; Teunissen *et al.*, 1998; Sritunyalucksana *et al.*, 1999). In accordance with our results, Chang *et. al.* (2000) reported a great viability in *P. monodon* fed on a relatively low oral dose (0.2%) of β- glucan from *S. commune* for 30- 40 days. Song *et. al.* (1997) have also demonstrated that immersion and dietary exposure to β-1, 3 and 1,6 glucan from cell walls of yeast *S. cerevisiae* for 51 days enhanced resistance of juvenile *P. monodon* to WSSV infection. The immunostimulatory effect of diet containing β -1, 3 glucans (2.0/kg) by enhancing the survival rate of adult *P.monodon* and enhancement of hemocyte phagocytic activity, cell adhesion and superoxide anion production in brooders has been reported by Chang *et al.* (2000). Oral administration of *β*-1,3-glucan leading to enhanced disease resistance was reported by Itami *et al.* (1994). Sajeevan *et al.* (2006) has reported that *Candida sake*, a marine isolate has been found to increase the survival rate of *F. indicus* in response to WSSV. The present study also

demonstrate the efficacy of marine yeast glucan in enhancing the immune vigour of *F. indicus* to combat WSSV.

The current study could clearly demonstrate the boost in the haematological parameters like plasma protein, haemocyte count, phenol oxidase activity, superoxide anion production, alkaline phosphatase and acid phosphatase activites etc. in the shrimps fed on glucan incorporated feed compared to the control group. Plasma protein was found to increase considerably after WSSV challenge on 2nd day. High concentration of haemolymph proteins has been reported for crustaceans after WSSV challenge by Lo *et al.* (1997). This may be due to WSSV heavy load and the over expression of defence protein to counteract the effects of WSSV. Beckage (1996) has shown that the increase in protein concentration in the virus infected shrimps may also be due to the fact that baculoviruses encode a variety of proteases and other enzymes that 'melt' the tissues and that the proteins of the 'melted' cells (muscle and hepatopancreas) would be incorporated into the shrimp haemolymph.

Results from several experiments have implied that prophenol oxidase, the key enzyme in the synthesis of melanin apart from their role in melanisation, also stimulate several cellular defence reactions, including phagocytosis, nodule formation and encapsulation (Soderhall *et al.*, 1986). Increase in PO activity in shrimps after β-glucan diets has been reported by Chang *et al.* (2003). Takahashi *et al.* (2000) has observed that the oral administration of LPS to the viral challenged shrimps was highly influenced the proPO system and contributed to the exclusion of virus. *P.monodon* that had been immersed in aerated yeast glucan (β -1, 3 and 1, 6- glucan) increased its phenol oxidase activity (Sung *et. al.,* 1994). Sajeevan *et al.* (2006) has shown that PO activity of *F. indicus* fed on marine yeast as a source of glucan and feed supplement has been increased in response to WSSV challenge. In the present study the phenol oxidase activity was

significantly higher in shrimps fed on glucan incorporated diet compared to control.

Being the first product released during the respiratory burst, O_2^- concentration is widely accepted as an accurate parameter quantifying the intensity of a respiratory burst (Secombes, (1990) and Secombes and Olivier, 1997). *P.monodon*, which had been immersed in viable cell suspension of *V.vulnificus*, yeast glucan (β-1, 3 and 1, 6- glucan) or zymosan, all stimulated the release of superoxide anion (Sung *et. al.,* 1994). *L.vanammei*, which had been immersed in aerated β -1, 6 glucan from *S.cerevisiae*, sulphated polysaccharide from cyanobacteria strain *Cyanothece* sp. and laminarin from *Laminaria digitata*, all increased its release of superoxide anion in 6 hr (Campa-Cordora *et. al.,* 2000). In the present study, *F. indicus* administered with glucan incorporated diet showed a very high activity after WSSV challenge.

Haemocytes play an important role in cellular defence. A lower-than-normal number of circulating haemocytes in crustaceans correlate well with a reduced resistance to pathogens (Le Moullac *et al.*,1998; Le Moullac and Haffner, 2000). THC is a useful indicator of shrimp health (Chang *et al.*,1999). Sajeevan *et al.* (2006) has shown an increase in THC of *F. indicus* fed on yeast incorporated diet after WSSV challenge. Similar results were obtained in the present study where THC was found to increase in shrimps fed on glucan incorporated diet when compared to control.

Alkaline and acid phosphatases are important defence enzymes in crustaceans. In the present study both alkaline and acid phosphatases were found to be higher in shrimps fed with glucan incorporated diet. The concurrent over production of phosphatases shows that these enzymes are being activated by glucan diet. ACP and ALP were composed of many kinds of phosphomonoesterases, which were very important to the

crustacean immune system. Liu *et al.* (1999) reported that ACP was a sign of lysosome activity to digest the invading organisms in shrimps. ACP had been released during phagocytosis, nodule and capsule formation to hydrolyse phosphate groups of the invaders. ALP had taken part in the transfer of phosphate groups and metabolism of Ca and P as the key regulatory enzymes. It has been shown that two phosphates played important role in the immune system as a key compound of lysosomal enzymes (Wang *et al.*, 2005).

The present study also investigated the *F. indicus* fed on glucan incorporated diet for antioxidant parameters like superoxide dismutase, catalase and glutathione peroxidase,and lipid peroxidation products like hydroperoxides. conjugated dienes and malondialdehydes. It could be inferred from the results that the antioxidant enzymes *viz.* SOD, CAT and GPx and lipid peroxidation products *viz.* hydroperoxides, conjugated dienes and malondialdehyde) concentrations of shrimps fed on glucan incorporated diet has considerably varied from those of control shrimps. The antioxidative enzyme activities have been significantly increased in shrimps fed on glucan diet with a concurrent reduction in lipid peroxidation products conferring more protection to the shrimps against WSSV.

The study clearly documented that the glucan incorporated diet induced the antioxidant enzymes in shrimps. SOD is an oxidoreductase that accelerates the conversion of O_2^- to H_2O_2 and O_2 ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) and thereby negates the direct toxic effects of the ROS (Fridovich. 1989). The increase in respiratory burst capacity and antioxidant levels in stimulated haemocytes is considered to be a response to changes in the lipid composition of cell membranes, and to enhance the production of cell-activating factors (cytokines or chaperonins) that may improve the phagocytic capability of haemocytes (Itami *et al.*, 1998). The catalase and glutathione peroxidase activity of shrimps were also elevated after WSSV challenge in 0.2% glucan

incorporated diet in order to scavenge the excessive hydrogen peroxide produced as a result of increased SOD activity.

The present study has shown a reduced level of WSSV induced oxidative stress in terms of hydrpperoxides, conjugated dienes and malondialdehyde concnentartion in the haemolymph of *F. indicus* held on 0.2% glucan diet. MDA reflected membrane degradation in a variety of pathological conditions (Shirali *et al.*, 1994). Rajan *et al.* (2000) found that the WSSV infectivity and pathogenicity increased in ectodermal and mesodermal tissues with the increase in the number of days of survival of WSSV infected shrimp. Mathew *et al.* (2007) found evidence of oxidative damage to tissues of *P.monodon* as indicated by increased lipid peroxidation in comparison to control. Dandapat *et al.* (2003) reported that high levels of TBARS were indicative of high oxidative assault on cellular and sub cellular membranes.

In conclusion, shrimps fed on glucan incorporated diet have shown a better survival rate with concurrent increment in haematological as well as antioxidative parameters. The oxidative stress was lower in shrimps fed on glucan diet as evidenced by low lipid peroxidation products. The immune vigour of the shrimps fed on glucan diet has been improved considerably compared to those fed on control diet. Therefore, glucan extract (0.2%) from the marine yeast isolate *Debaromyces hansenii* S169 can be utilised for shrimp aquaculture systems as an effective immunostimulant against WSSV.

Chapter 8

IDENTIFICATION OF POTENTIAL HAEMATOLOGICAL BIOMARKERS AS HEALTH INDICATORS IN *F. INDICUS*

Contents

8.1 Introduction

In aquaculture, therapy is often difficult, since we have to treat the whole ecosystem i.e. the culture environment, which will result in the disturbance of microbial balance in the ecosystem and deterioration of the culture environment and water quality. Therefore prophylactic approach is of prime importance. This necessitates frequent health monitoring of the cultured animals and appropriate measures to avoid disease out break and crop loss. In this chapter an attempt is made to identify biomarkers for shrimp health assessment through regression analysis. Correlation matrix of haematological parameters and survival was also worked out.

Correlation and regression analysis are effective statistical tools to analyse the data with two types of variables. In the present study, data on the immune and antioxidant parameters of *F. indicus* was generated in response to different environmental conditions and microbial infections. The survival rates of *F. indicus* under experimental conditions were found to vary according to the degree of severity of stress. Correlation studies reveal how evenly the different haematological parameters are correlated to the survival rate of shrimps. Multivariate regression analysis is employed to analyse one dependent variable also known as response variable (here survival rate) and one or more independent variables also known as explanatory variables (here immune and antioxidant parameters). The aim of this study is to delineate the correlation matrix between the survival rate and haematological parameters and to employ the multiple regression analysis of survival rate on haematological parameters to elucidate the suitable health predictors for *F. indicus* for an effective health monitoring of the stock.

8.2 Materials and methods

8.2.1 Data used for the analysis

a) Haematological responses and susceptibility of *F. indicus to Vibrio harveyi* at different salinity levels (Refer chapter 3).

b) Haematological responses and susceptibility of *F. indicus* to White spot syndrome virus under acute salinity stress (Refer chapter 4).

c) Antioxidant defence profile of *F. indicus* challenged with White spot syndrome virus under acute salinity stress (Refer chapter 5).

d) Modulatory effect of ambient Cu on the haematological responses and susceptibility of *F. indicus* to White spot syndrome virus (Refer chapter 6).

e) Effect of β-1,3-glucan incorporated diet on the haematological responses and survival of *F. indicus* when challenged with White spot syndrome virus (Refer chapter 7).

8.2.2 Statistical analysis

8.2.2.1 Correlation analysis

Pearson correlation analysis was done using SPSS 10.00 version software. This was done to understand the correlation between the immune and antioxidant responses with the survival of shrimp.

8.2.2.2 Multiple regression analysis

Multiple regression analysis was done using the SPSS 10.00 version software. In multiple regression analysis, the two kinds of variables were considered for the analysis i.e. response variable and the explanatory variables. Survival rate of *F. indicus* was considered as the dependent variable and immune and antioxidant responses of the shrimps were considered as the independent variables. The percentage variability in the survival rate of *F. indicus* attributed by the

independent variables was determined. Significant regression coefficients were found out to identify most reliable health parameters in *F. indicus*

8.3 Results

8.3.1 Haematological responses and susceptibility of *F. indicus to Vibrio harveyi* at different salinity levels (Refer chapter 3).

a) Correlation matrix between survival rate and immune parameters

Pearson correlation coefficients showed that all variables except superoxide anion production (NBT) and total free amino acids (TFAA) exhibited positive correlation with the survival rate. TFAA showed a negative correlation ($p<0.05$) with survival of shrimps. The immune variables exhibited a greater degree of correlation with each other (Table 8.1).

Table 8.1. Correlation matrix between survival rate and immune parameters of *V. harveyi* infected *F. indicus* maintained at different salinity levels.

Variables	THP	PLP	SRP	HCP	PO	NBT	TFAA	ALP	ACP	SURVL
THP	1.000									
PLP	.867**	1.000								
SRP	.779**	.957**	1.000							
HCP	.437**	.505**	.548**	1.000						
PO	.344*	.428**	.449**	.681**	1.000					
NBT	.032	.040	.013	-.215	-.093	1.000				
TFAA	-.033	-.160	-.150	-.048	-.281*	-.302*	1.000			
ALP	.465**	.520**	.559**	.469**	.559**	.076	-.301*	1.000		
ACP	.280*	.218	.227	.595**	.656*	.141	-.319*	588**	1.000	
SURVL	.364**	.385**	.432**	.821**	.802**	-.068	-.300*	623**	.856**	1.000

* $p< 0.05$, ** $p< 0.01$

b) Multiple regression of survival rate and immune parameters

When multiple regression of survival rate on all immune parameters were considered, the amount of variability was 92% (R Square=0.924). When only variables with significant regression coefficients (ACP, HCP and PO) were taken into account, it was found that ACP ($p<0.000$), HCP ($p<0.000$) and PO ($p<0.05$) together are explaining the 91% (R Square=0.906) of variability, indicating that these three are better indicators of health/ survival in *F. indicus* (Table 8.2).

Table. 8.2 Multiple regression of survival rate and haematological parameters of *V.harveyi* infected *F. indicus* held at different salinity levels.

R Square- **0.924**
Predictors- **ACP, NBT, PLP, TFAA, ALP, PO, HCP, THP, SRP**
Dependent Variable: **SURVIVAL**

Variable	THP	PLP	SRP	HCP	PO	NBT	TFAA	ALP	ACP
Significance	0.522	0.091	0.130	0.000**	0.022*	0.227	0.053	0.531	0.000**

* p< 0.05 , ** p< 0.01

R Square- **0.906**
Predictors- **ACP, HCP, PO**
Dependent Variable: **SURVIVAL**

8.3.2 Haematological responses and susceptibility of *F. indicus* to White spot syndrome virus under acute salinity stress (Refer chapter 4).

a) Correlation matrix between survival rate and immune parameters

Correlation coefficients showed that all variables except total free amino acids exhibited a positive correlation with the survival rate (Table 8.3)

Table.8.3 Correlation matrix between survival rate and immune parameters of WSSV infected *F. indicus* under acute salinity stress.

Variables	THP	PLP	SRP	THC	TFAA	PO	NBT	ALP	ACP	SURVL
THP	1.000									
PLP	.851**	1.000								
SRP	.848**	.968**	1.000							
THC	.600**	.731**	.861**	1.000						
TFAA	-.070	-.229	-.268	-.328	1.000					
PO	.588**	.727**	.807**	.852**	-.124	1.000				
NBT	.540**	.631**	.581**	.489**	-.273	.349*	1.000			
ALP	.663**	.755**	.728**	.657**	-.087	.712**	.680**	1.000		
ACP	.714**	.755**	.796**	.766**	-.185	.659**	.748**	.818**	1.000	
SURVL	.635**	.737**	.871**	.984**	-.311	.824**	.499**	.636**	.799**	1.000

*** p<0.05, ** p<0.01**

b) Multiple regression of survival rate and immune parameters

When multiple regression of survival rate on all immune parameters were considered, the amount of variability explained was 98% (R Square=0.978). When significant regression coefficients among the haematological parameters were taken into account, it was found that THC ($p<0.001$) and ACP ($p<0.05$) together are explaining the 97% (R Square=0.972) of variability, indicating that these two are mainly responsible for the survival rate (Table 8.4).

Table.8.4 Multiple regression of survival rate and immune parameters of WSSV infected *F. indicus* subjected to acute salinity stress.

	R Square- **0.978**
	Predictors- **ACP, TFAA, PO, THP, NBT, ALP, THC, PLP, SRP**
	Dependent Variable: SURVIVAL

Variables	THP	PLP	SRP	THC	PO	NBT	TFAA	ALP	ACP
Significance	0.614	0.079	0.065	0.000***	0.689	0.817	0.624	0.396	0.032*

	R Square- **0.972**
	Predictors- **THC, ACP**
	Dependent Variable: SURVIVAL

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

8.3.3 Antioxidant defence profile of *F. indicus* challenged with White spot syndrome virus under acute salinity stress (Refer chapter 5).

a) Correlation matrix between survival rate and antioxidant parameters

Among antioxidant parameters hydroperoxides and malondialdehyde showed negative correlation with survival (Table 8.5).

Table 8.5 Correlation matrix between survival rate and antioxidant parameters of WSSV infected *F. indicus* under acute salinity stress.

Variables	SOD	CAT	GPX	GR	GST	TRG	MDA	CD	HP	SURVL
SOD	1.000									
CAT	.859**	1.000								
GPX	.946**	.849**	1.000							
GR	.953**	.850**	.949**	1.000						
GST	.890**	.783**	.869**	.915**	1.000					
TRG	.938**	.852**	.892**	.905**	.863**	1.000				
MDA	-.817**	-.814**	-.827**	-.862**	-.792**	-.826**	1.000			
CD	-.084	-.245	-.156	-.133	.027	-.097	.298	1.000		
HP	-.839**	-.840**	-.855**	-.858**	-.806**	-.809**	.747**	.257**	1.000	
SURVL	.807**	.863**	.831**	.843**	.807**	.792**	-.832**	-.400	-.898*	1.000

* p<0.05, ** p<0.01

b) Multiple regression of survival rate and antioxidant parameters

The amount of variability explained by the antioxidant parameters of haemolymph was 91% (R Square=0.911). When significant regression coefficients were taken into account, it was found that HP ($p<0.001$), CD ($p<0.001$) and GST ($p<0.05$) together are explaining the 89% (R Square=0.891) of variability, indicating that these three haemolymph parameters are mainly responsible for shrimp survival (Table 8.6)

Table 8.6 Multiple regression of survival rate and antioxidant parameters of WSSV infected *F. indicus* subjected to acute salinity stress.

R Square- **0.911**	
Predictors-	**HP, CD, MDA, CAT, GST, TRG, GPX, SOD, GR**
Dependent Variable: SURVIVAL	

Variables	SOD	CAT	GPX	GR	GST	TRG	MDA	CD	HP
Significance	0.763	0.108	0.934	0.622	0.028*	0.673	0.211	0.007**	0.004*

R Square- **0.891**	
Predictors-	**HP, CD, GST**
Dependent Variable: SURVIVAL	

* p< 0.05 , ** p< 0.01 *** p< 0.001

8.3.4 Modulatory effect of ambient Cu on the haematological responses and susceptibility of *F. indicus* to White spot syndrome virus (Refer chapter 6).

a) Correlation matrix between survival rate and haematological parameters

Correlation co-efficients showed that all variables among immune parameters and all antioxidant parameters except malondialdehyde exhibited positive correlation with the survival rate. The immune variables and antioxidant parameters exhibited a greater degree of correlation with each other (Tables 8.7 and 8.8).

Table. 8.7 Correlation matrix between survival rate and immune parameters of WSSV infected *F. indicus* under ambient Cu

Variables	THP	PLP	SRP	HCP	PO	TFAA	NBT	ALP	ACP	SURVL
THP	1.000									
PLP	.698**	1.000								
SRP	.610**	.593**	1.000							
HCP	.847**	.732**	.715**	1.000						
PO	.767**	.394**	.685**	.738**	1.000					
TFAA	.416**	.298**	.507**	.476**	.627**	1.000				
NBT	.721**	.397**	.550**	.565**	.789**	.343**	1.000			
ALP	.765**	.570**	.601**	.699**	.826**	.640**	.683**	1.000		
ACP	.477**	.279**	.509**	.523**	.762**	.625**	.514**	.729**	1.000	
SURVL	.939**	.649**	.632**	.806**	.830**	.471**	.804**	.828**	.530**	1.000

* p< 0.05 , ** p< 0.01 *** p< 0.001

Table 8.8 Correlation matrix between survival rate and antioxidant parameters of WSSV infected *F. indicus* under ambient Cu

Variables	SOD	CAT	GPX	MDA	SURVL
SOD	1.000				
CAT	.818**	1.000			
GPX	.783**	.914**	1.000		
MDA	-.735**	-.629	-.582	1.000	
SURVL	.923**	.892**	.888**	-.775**	1.000

* p< 0.05 , ** p< 0.01 *** p< 0.001

b) Multiple regression of survival rate and haematological parameters

When multiple regression of survival rate on all immune parameters of WSSV infected *F. indicus* under 0, 0.075, 0.15, 0.225 and 0.300ppm Cu^{2+} were considered, the amount of variability explained was 94% (R Square=0.936). When significant regression coefficients were taken into account, it was found that THP (p<0.001), NBT (p<0.05) and ALP (p<0.05) together are explaining the 93% (R Square=0.930) of variability, indicating that these three are mainly responsible for the survival rate (Table 8.9). The amount of variability explained by the antioxidant parameters was 94% (R Square=0.941). When significant regression coefficients were taken into account, it was found that SOD (p<0.001), GPx (p<0.001) and MDA (p<0.001) together are explaining the 94% (R Square=0.938) of variability, indicating that these three are mainly responsible for the survival rate (Table 8.10).

Table.8.9 Multiple regression of survival rate and haematological parameters of WSSV infected *F. indicus* under ambient Cu

R Square- **0.936**
Predictors- **ACP, PLP, NBT, TFAA, SRP, HCP, ALP, THP, PO**
Dependent Variable: SURVIVAL

Variables	THP	PLP	SRP	HCP	PO	NBT	TFAA	ALP	ACP
Significance	0.000***	0.428	0.694	0.939	0.125	0.012*	0.878	0.020*	0.069

R Square- **0.930**
Predictors- **THP,NBT, ALP**
Dependent Variable: SURVIVAL

* p< 0.05 , ** p< 0.01 *** p< 0.001

Table 8.10 Multiple regression of survival rate and antioxidant parameters of WSSV infected *F. indicus* under ambient Cu

R Square- **0.941**
Predictors- **MDA GPX, SOD, CAT**
Dependent Variable: SURVIVAL

Variables	SOD	CAT	GPX	MDA
Significance	0.000**	0.217	0.000**	0.000**

R Square- **0.938**
Predictors- SOD, GPX, MDA
Dependent Variable: SURVIVAL

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

8.3.5 Effect of β-1, 3-glucan incorporated diet on the haematological responses and survival of *F. indicus* when challenged with White spot syndrome virus (Refer chapter 7).

a) Correlation matrix between survival rate and haematological parameters

Correlation coefficients showed that all haematological variables except conjugated dienes exhibited positive correlation with the survival rate. The immune variables and antioxidant parameters exhibited a greater degree of correlation with each other (Tables 8.11 and 8.12).

Table.8.11 Correlation matrix between survival rate and immune parameters of WSSV infected *F. indicus* maintained on β-1,3-glucan incorporated diet

Variables	PP	THC	PO	NBT	ALP	ACP	SURVL
PP	1.000						
THC	.851**	1.000					
PO	.833**	.949**	1.000				
NBT	.860**	.902**	.830**	1.000			
ALP	.693**	.870**	.848**	.788**	1.000		
ACP	.770**	.932**	.915**	.813**	.779**	1.000	
SURVL	.902**	.911**	.924**	.863**	.851**	.834**	1.000

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

Table.8.12 Correlation matrix between survival rate and antioxidant parameters of WSSV infected *F. indicus* maintained on β-1,3-glucan incorporated diet

Variables	HP	MDA	CD	CAT	GPX	SOD	SURVL
HP	1.000						
MDA	.469*	1.000					
CD	.593**	.248	1.000				
CAT	-.602**	.004	-.576**	1.000			
GPX	-.535**	.010	-.561**	.847**	1.000		
SOD	-.519**	.015	-.659**	.899**	.826**	1.000	
SURVL	-.365	.209	-.509*	.902**	.849**	.919**	1.000

* p< 0.05 , ** p< 0.01 *** p< 0.001

b) Multiple regression of survival rate and haematological parameters

When multiple regression of survival rate on all immune parameters were considered, the amount of variability explained was 93% (R Square=0.932). When significant regression coefficients among the immune parameters were taken into account, it was found that THC ($p<0.001$), PO ($p<0.05$), ALP ($p<0.05$) and PLP ($p<0.05$) together are explaining the 84% (R Square=0.842) of variability, indicating that these four are mainly responsible for the survival rate (Table 8.13). The amount of variability explained by the antioxidant parameters of was 94% (R Square=0.938. When significant regression coefficients among the antioxidant parameters were taken into account, it was found that SOD ($p<0.01$) and CAT ($p<0.05$) together are explaining the 87.4% (R Square=0.874) of variability, indicating that these four are mainly responsible for the survival rate (Table 8.14).

Table. 8.13 Multiple regression of survival rate and immune parameters of WSSV infected *F. indicus* maintained on β-1,3-glucan incorporated diet.

R Square- **0.932**
Predictors- **ACP, PP, ALP, NBT, PO, THC**
Dependent Variable: SURVIVAL

Variables	PLP	THC	PO	NBT	ALP	ACP
Significance	0.024*	0.000*	0.015*	0.071	0.016*	0.817

R Square- **0.842**
Predictors- **THC ,PO , ALP,PLP**
Dependent Variable: SURVIVAL

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

Table. 8.14 Multiple regression of survival rate and antioxidant parameters of WSSV infected *F. indicus* maintained on β-1,3-glucan incorporated diet.

R Square- **0.938**
Predictors- **SOD, MDA, HP, CD, GPX, CAT**
Dependent Variable: SURVIVAL

Variables	SOD	CAT	GPX	CD	HP	MDA
Significance	0.007**	0.021*	0.091	0.682	0.090	0.178

R Square- **0.874**
Predictors- **SOD, CAT**
Dependent Variable: SURVIVAL

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

8.3.6 Multiple regression analysis of haematological variables on survival rate of *F. indicus* with combined data of various bioassays.

When multiple regression of survival rate on all immune parameters in all bioassays were considered, the amount of variability explained was 66% (R Square=0.662). When significant regression coefficients among the immune parameters were taken into account, it was found that PO

(p<0.001), NBT (p<0.05) and THC (p<0.05) together are explaining the 65% (R Square=0.652) of variability, indicating that these three are mainly responsible for the survival rate (Table 8.15).

Table 8.15 Multiple regression analysis of immune variables on survival rate of *F. indicus* with combined data of various bioassays.

R Square- 0.662
Predictors- ACP, NBT, PO, PLP, ALP, THC
Dependent Variable: SURVIVAL

Variables	PLP	THC	PO	NBT	ALP	ACP
Significance	0.292	0.048*	0.000**	0.020*	0.104	0.086

R Square- 0.652
Predictors- NBT, PO, THC
Dependent Variable: SURVIVAL

The amount of variability explained by the antioxidant parameters was 84% (R Square=0.839. When significant regression coefficients among the antioxidant parameters were taken into account, it was found that and CAT (p<0.001) CD (p<0.01) together are explaining the 81% (R Square=0.806) of variability, indicating that these two are mainly responsible for the survival rate (Table 8.16). A comparative representation of significant regression coefficients has been presented in Table 8.17 and Table 8.18.

Table 8.16 Multiple regression analysis of antioxidant variables on survival rate of *F. indicus* with combined data of various bioassays.

R Square- 0.839
Predictors- CD, CAT, MDA, HP, GPX, SOD
Dependent Variable: SURVIVAL

Variables	SOD	CAT	GPX	MDA	CD	HP
Significance	0.101	0.001**	0.176	0.284	0.003**	0.918

R Square- 0.806
Predictors- CAT, CD
Dependent Variable: SURVIVAL

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

Table 8.17 Significant predictors obtained on multiple regression analysis of immune variables with survival rate of *F. indicus* in various bioassays.

Multiple regression analysis of immune variables on survival rate of *F. indicus*				
V.harveyi infected under salinity alterrations	WSSV infected under salinity alterations	WSSV Infected under Cu dose	WSSV Infected under Glucan diet	Bioassays 1,2,3, and 4 combined
HCP (0.000***) ACP (0.000***) PO (0.022*)	THC (0.000***) ACP (0.032*)	THP (0.000***) NBT (0.012*) ALP (0.020*)	THC (0.000***) PO (0.015*) ALP (0.016*)	**PO (0.000***) NBT (0.020*) THC (0.048*)**

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

Table 8.18 Significant predictors obtained on multiple regression analysis of antioxidant variables with survival rate of *F. indicus* in various bioassays.

Multiple regression analysis of antioxidant variables on survival rate of *F. indicus*			
Bioassays			
WSSV infected under salinity alterations	WSSV infected under Cu dose	WSSV infected under Glucan diet	Bioassays 1,2,3, and 4 combined
HP (0.004**) CD (0.007**) GST (0.028*)	SOD 0.000***) GPx (0.000***) MDA (0.000***)	SOD (0.007**) CAT (0.021*)	**CAT (0.001***) CD (0.003**)**

*** p< 0.05 , ** p< 0.01 *** p< 0.001**

8.4 Discussion

Efforts have been taken world wide towards better understanding of the shrimp health parameters under various physiological conditions. Both immune and antioxidant parameters can be good indicators of shrimp health under defined conditions. They may be correlated with the survival of shrimps under stress to predict the variability of survival under specific

conditions. The aim of the present study was to find out suitable shrimp health predictors for *F. indicus* under a variety of stressors including bacterial and viral pathogens besides salinity, heavy metal and immunostimulant administration.

In the present study Pearson correlation analysis revealed that all immune and antioxidant variables showed positive correlation with shrimp survival rate with a few exceptions. Immune variables of the *F. indicus* acclimated to different salinity levels and then challenged with *V. harveyi* were positively correlated with shrimp survival rate except total free amino acid concentration which showed a significant negative correlation. Progressive accumulation of TFAA could be noticed during post challenge days. In the case of *F. indicus* infected with WSSV under acute salinity stress also the same observation could be made where a negative correlation of TFAA with survival rate could be noticed. However, Joseph and Philip (2007) noticed a positive correlation in the case of TFAA and survival in *P.monodon* on challenge with WSSV under acute salinity stress. In the case of antioxidants the lipid peroxidation products *viz.* hydroperoxides, Conjugated dienes and malondialdehyde exhibited negative correlation with the survival rate implying high level of oxidative stress in shrimps under salinity stress and microbial infection.

On administration of immunostimulant, β-1,3-glucan all the immune parameters assessed showed a positive correlation with the shrimp survival. Among the antioxidant parameters the lipid peroxidation products *viz.* hydroperoxides and Conjugated dienes showed a negative correlation. However, the MDA did not exhibit significant correlation with survival.

Multiple regression analysis of the haematological parameters with the survival was done in order to obtain reliable predictors of shrimp health. The haematological data generated through application of various stressors (salinity, Cu^{2+}) and through microbial challenge (*Vibrio* and

WSSV) in *F. indicus* was used for the analysis. It was interesting to note that biomarkers of shrimp health varied with the type of environmental stress as well as the source (microorganism) of infections.

When the shrimps were infected with *V.harveyi* at different salinity levels, acid phosphatase, haemocyte protein and phenol oxidase influenced the survival rate of *F. indicus* largely. In the case of WSSV infected shrimps under acute salinity stress, total haemocyte count and acid phosphatase could explain the variability in survival to a greater extent. With antioxidant parameters hydroperoxides, conjugated dienes and glutathione-S-transferase were found to influence the variability in shrimp survival. Joseph and Philip (2007) have reported that THC, ALP and PO are the major responsible parameters for *P. monodon* survival rate under acute salinity stress and these factors were proposed as the most potential biomarkers of health in haemolymph for periodic assessment of health in shrimp.

However, on copper exposure and challenge with WSSV, *F. indicus* showed remarkable difference in response. Here, total haemolymph protein, superoxide anion production and alkaline phosphatase were the predictors of shrimp survival. Among antioxidants super oxide dismutase, glutathione peroxidase and malondialdehyde were most suitable indicators of shrimp health status.

Similarly when assessed for reliable health indicators of *F. indicus* on administration of glucan incorporated diet and WSSV challenge, the immune parameters identified with significant regression coefficients were total haemocyte count, phenol oxidase, alkaline phosphatase and plasma protein concentration. Among antioxidants super oxide dismutase and catalase were found to indicate the survival of shrimps maintained under glucan diet.

When the data generated through all the assays with *F. indicus* were considered for regression analysis, the biomarkers of health obtained were PO, NBT and THC. Among the antioxidants the most reliable health indicators were catalase and conjugated dienes. However, further investigations are required with other shrimps and varied environmental conditions for confirmation of these parameters as biomarkers of health in shrimps.

In aquaculture animals are always under the threat of varied environmental stress and microbial infections. Proper husbandry and management is of utmost importance in aquaculture health management. Health monitoring include observation of gross clinical signs, internal abnormalities, histopathology, immunological assays and microbiological investigations including DNA based (PCR) detection of pathogens. Since the haemolymph responses are a true reflection of environmental alterations in culture system and microbial infection, haemolymph based biomarker would be a better predictor of shrimp health which can be used for routine shrimp health monitoring and implementation of appropriate remedial measures to avoid huge economic loss in the aquaculture sector.

Chapter 9

SUMMARY AND CONCLUSION

Shrimp culture has been gaining increased attention as a highly profitable industry world over during the last three decades. However, currently the frequent out break of diseases has caused a major set back to the industry. These epidemics, especially of viral origin often result in cent percent mortality of the animals incurring huge economic loss. Diseases are always the outcome of complex interactions between pathogens, environmental alterations and the host animals. Mere the presence of a pathogen need not cause disease in animals but are often linked to stressful environment in culture systems. This stress will be reflected in the internal body fluid of the shrimps and perturbs the immunocompetence of animal making it susceptible to pathogens. There is dearth of scientific data on the haematological profile of shrimps in response to varying stressors. Since, haematological responses are true reflectors of stressful conditions, a better understanding of the haematology with respect to stressors is of paramount importance in the present scenario of aquaculture. Immune and antioxidant components in the shrimp haemolymph may serve as potential health indicators. Therefore, the present study on haematology of the candidate species *Fenneropenaeus indicus* was conducted with the following objectives.

a) To understand the effect of alterations in salinity on haematological responses and susceptibility of *Fenneropenaeus indicus* to microbial infections

b) To assess the modulatory effect of heavy metal (copper) on haematological responses and susceptibility of *F. indicus* to infection

c) To understand the effect of immunostimulants on the haematological responses and susceptibility of *F. indicus* to infection

d) To identify the most reliable haematological health indicators in *F. indicus*

The salient findings of the study are summarised as follows

- Ambient salinity play an important role on the immune responses in *F. indicus*.A general increase in haematological variables could be noticed in shrimps acclimated to 5 and 10‰ salinity
- Haemolymph protein components in *F. indicus* were high when acclimated to 5 and 10‰ compared to 25‰. Total free amino acids were high at 0‰ compared to other salinities.
- Phenol oxidase activity, superoxide anion production and alkaline phosphatase activity of *F. indicus* were high at 5 and 10‰ compared to 25‰.
- Acid phosphatase activity were high in shrimps maintained at 35‰ and notably low at 5 and 10‰.
- Higher salinity favoured the infectivity of *V. harveyi* causing maximum mortality in shrimps held at 35‰ followed by 25‰. Generally the haematological responses were high in shrimps held at 5 and 35‰ both before and after challenge with *V. harveyi* showing the importance of salinity as a stress factor.
- A progressive increase in total free amino acids could be noticed after challenge with *V. harveyi* and WSSV at various salinity.
- Generally an increase in immune responses could be noticed PCD1 to PCD3 in the case of *V. harveyi* challenge.
- When *F. indicus* were challenged with WSSV under salinity stress the haematological responses were higher at 5 and 35‰ compared to 25‰.
- On WSSV challenge, the immune responses in *F. indicus* were maximum on PCD1 and there after a reduction was noticed.

- *F. indicus* was more susceptible to white spot syndrome virus infection at lower salinity (5‰) showing maximum mortality at this salinity.
- Significant increase in antioxidant enzymes in *F. indicus* challenged with WSSV on post challenge day1 followed by a reduction there after. These enzyme levels were maximum in animals maintained at 5‰.
- The non-enzymic antioxidant, reduced glutathione decreased after WSSV challenge at all salinity levels.
- Lipid peroxidation products *viz.* conjugated dienes, hydroperoxides and malondialdehyde concentration were elevated after WSSV challenge showing high degree of oxidative stress (maximum at 5‰).
- Salinity stress was found to increase oxidative stress in *F. indicus* most prominently at 5‰. Oxidative stress was found to be maximum in hepatopancreas followed by haemolymph, muscle and gill.
- Exposure to Cu^{2+} at 0.075mg/l was found to have immunostimulatory effect in *F. indicus* as being evidenced from the immune profile and post challenge survival.
- Lipid peroxidation induced oxidative stress was minimum in *F. indicus* dosed with 0.075mg/l Cu^{2+}.
- The immune and antioxidant responses were lower in *F. indicus* exposed to higher doses of Cu^{2+} indicating immunosuppression and increased susceptibility to WSSV challenge.
- Copper accumulation was found in shrimps on exposure to Cu^{2+} at sub lethal levels. This accumulation increased in relation to the concentration of copper in the rearing water up to 0.225 ppm and there after a reduction could be observed.
- Significant increase could be noticed in the haematological responses of *F. indicus* fed on 0.2% glucan diet imparting better survival on challenge with WSSV

- Lipid peroxidation was comparatively low in shrimps fed on 0.2% glucan incorporated feed indicating lesser oxidative stress.
- β-1,3 glucan from marine yeast *Debaryomyces hansenii* S169 improved the immune responses of *F. indicus* rendering the animals more resistant to WSSV infection.
- Phenol oxidase, superoxide anion production and catalase were identified as potential health indicators in *F. indicus* by multiple regression analysis.

The present study emphasizes the fact that haemolymph responses are a true reflection of the environmental conditions and health status of shrimps. Eventhough *F. indicus* is euryhaline and tolerates wide range of salinity their defence potential is very much influenced by salinity. Especially the sudden alterations in salinity cause severe stress in the animals making them susceptible to microbial infections. Shrimps were found to be more susceptible to diseases both at lower and higher level salinities. Prevalence of heavy metals at ppb levels (Cu) was found to have beneficial effects on the animals whereas high levels were toxic with deleterious effect. So the study clearly indicates the need for adequate husbandry and management practices to thwart infection under environmental stress. Application of immunostimulants evoked immune responses in the animals conferring protection against microbial infection. The multiple regression analysis has shown that the health indicators varied with the type of stressors and the type of pathogens. Overall analysis has shown that Phenol oxidase, NBT and Catalase are best indicators of health in shrimps. This study accentuate the importance of proper and regular health monitoring in shrimps employing the most appropriate haematological biomarkers for application of suitable prophylactic measures in order to avoid serious health hazards in shrimp culture systems.

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APPENDICES

Appendix 1

Table.2.2 Haematological parameters of *F.indicus* held at different salinity levels.

Haematological parameter	Salinity(‰)								
	Control(25)	0	5	10	15	20	25	30	35
THP (mg/ml)	90.16±11.03	100.64±5.00	141.19±6.35	133.49±6.06	108.68±7.03	94.20±4.75	103.03±4.80	109.82±6.98	104.28±8.83
PLP(mg/ml)	82.23±10.61	93.28±5.33	130.52±6.24	122.19±5.40	100.30±6.44	87.14±4.82	96.01±4.65	101.34±7.33	95.84±9.35
SRP(mg/ml)	64.64±9.82	68.80±5.65	91.33±12.35	96.18±8.96	67.87±5.04	68.25±4.45	78.29±3.51	75.93±6.95	81.52±9.82
HCP(mg/ml)	8.94±0.99	6.25±0.33	10.10±0.75	9.77±0.85	7.65±0.64	8.02±0.57	6.36±0.50	8.50±2.00	9.24±1.14
TFAA(mg/ml)	5.64±0.93	17.36±1.63	9.93±0.86	10.30±0.81	11.59±1.66	9.10±1.43	5.12±1.13	7.74±1.18	6.91±1.06
PO (OD 490)	0.33±0.02	0.60±0.05	0.86±0.11	0.71±0.06	0.70±0.07	0.29±0.03	0.32±0.05	0.45±0.04	0.35±0.03
NBT(OD 620)	0.68±0.06	0.83±0.06	1.06±0.16	0.70±0.08	0.84±0.09	0.69±0.07	0.69±0.05	0.68±0.12	0.97±0.11
ALP(mg/ml)	0.33±0.04	0.48±0.05	0.72±0.14	0.71±0.07	0.47±0.07	0.27±0.06	0.60±0.07	0.42±0.13	0.31±0.04
ACP(mg/ml)	0.37±0.05	0.44±0.17	0.16±0.03	0.19±0.04	0.36±0.07	0.47±0.14	0.57±0.06	0.46±0.07	0.64±0.05

THP- Total haemolymph protein, PLP- Plasma protein, SRP- Serum protein, HCP- Haemocyte protein, TFAA- Total free amino acids, PO – Phenol oxidase activity, NBT- Nitroblue tetrazolium reduction assay, ALP- alkaline phosphatase, ACP- Acid phosphatase

Each value represents mean ± SD of six separate determinations.

Table.5.1 Super oxide dismutase (SOD) activity (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Each value represents the mean ± SD. Values with different superscripts in the same column vary significantly ($p<0.05$) among different salinity treatments and values with different subscripts in the same row vary significantly ($p<0.05$) among different exposure time. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day.

SOD activity (U/mg protein) in the hepatopancreas						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	12.28±2.04					
Control(25)		$^{A}12.46\pm1.57_{b}$	$^{A}12.24\pm1.54_{e}$	$^{A}11.68\pm1.5_{c}$	$^{A}13.17\pm1.37_{b}$	$^{A}12.77\pm1.25_{a}$
5		$^{B}7.29\pm1.44_{b}$	$^{B}23.76\pm1.86_{e}$	$^{B}6.79\pm1.31_{c}$	$^{B}3.79\pm0.85_{b}$	$^{B}3.35\pm0.77_{a}$
25		$^{A}12.14\pm1.37_{b}$	$^{A}14.19\pm2_{e}$	$^{A}4.23\pm0.99_{c}$	$^{A}3.24\pm0.79_{b}$	$^{A}1.67\pm0.39_{a}$
35		$^{A}9.42\pm1.61_{b}$	$^{A}13.27\pm1.93_{e}$	$^{A}4.98\pm0.99_{c}$	$^{A}4.53\pm1.36_{b}$	$^{A}1.96\pm0.63_{a}$

Table.5.2 Super oxide dismutase (SOD) activity (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

SOD activity (U/mg protein) in the gill						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	8.04±0.88					
Control(25)		$^{A}8.25\pm1.07_{c}$	$^{A}8.34\pm1.12_{d}$	$^{A}8.76\pm1.02_{c}$	$^{A}9.08\pm1.18_{b}$	$^{A}8.55\pm1.09_{a}$
5		$^{A}5.14\pm1.17_{c}$	$^{A}14.89\pm1.63_{d}$	$^{A}7.21\pm1.62_{c}$	$^{A}2.73\pm1_{b}$	$^{A}1.5\pm0.42_{a}$
25		$^{A}7.94\pm1.02_{c}$	$^{A}10.83\pm1.23_{d}$	$^{A}7.43\pm1.47_{c}$	$^{A}5.34\pm1.3_{b}$	$^{A}2.83\pm0.88_{a}$
35		$^{A}7.12\pm0.9_{c}$	$^{A}11.04\pm1.36_{d}$	$^{A}8.22\pm1.06_{c}$	$^{A}4.98\pm0.99_{b}$	$^{A}3.16\pm0.71_{a}$

Table.5.3 Super oxide dismutase (SOD) activity (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

SOD activity (U/mg protein) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	3.22±0.75					
Control(25)		$^{A}3.31\pm0.79_{b}$	$^{A}3.63\pm0.49_{d}$	$^{A}3.36\pm0.57_{c}$	$^{A}3.53\pm0.55_{b}$	$^{A}3.18\pm0.64_{a}$
5		$^{AB}1.38\pm0.72_{b}$	$^{AB}6.96\pm1.63_{d}$	$^{AB}6.18\pm1.15_{c}$	$^{AB}2.23\pm0.87_{b}$	$^{AB}2.06\pm0.68_{a}$
25		$^{A}3.24\pm0.57_{b}$	$^{A}6.54\pm1.02_{d}$	$^{A}4.59\pm1.12_{c}$	$^{A}2.87\pm0.88_{b}$	$^{A}1.07\pm0.43_{a}$
35		$^{B}2.42\pm0.69_{b}$	$^{B}6.6\pm1.15_{d}$	$^{B}4.7\pm1.26_{c}$	$^{B}4.44\pm0.88_{b}$	$^{B}2.74\pm0.93_{a}$

Table.5.4 Super oxide dismutase (SOD) activity (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

SOD activity (U/mg protein) in the haemolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	10.88±0.82					
Control(25)		$^{A}10.67\pm0.92_{d}$	$^{A}10.18\pm1.15_{e}$	$^{A}11.26\pm1.11_{c}$	$^{A}10.61\pm0.89_{b}$	$^{A}11.05\pm1.1_{a}$
5		$^{A}4.86\pm0.78_{d}$	$^{A}13.83\pm1.38_{e}$	$^{A}3.11\pm1.05_{c}$	$^{A}2.33\pm0.33_{b}$	$^{A}1.73\pm0.41_{a}$
25		$^{A}9.78\pm1.03_{d}$	$^{A}11.07\pm1.29_{e}$	$^{A}6.33\pm1.26_{c}$	$^{A}3.98\pm0.95_{b}$	$^{A}1.85\pm0.68_{a}$
35		$^{B}7.62\pm1.12_{d}$	$^{B}12.15\pm1.45_{e}$	$^{B}6.77\pm1.17_{c}$	$^{B}5.8\pm1.01_{b}$	$^{B}3.57\pm1.14_{a}$

Table.5.5 Catalase (CAT) activity (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Catalase activity (U/mg protein) in the hepatopancreas						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	35.30±5.02					
Control(25)		$^{A}35.39\pm4.67_{d}$	$^{A}34.55\pm5.05_{d}$	$^{A}35.71\pm4.07_{c}$	$^{A}35.56\pm5.07_{b}$	$^{A}33.54\pm3.47_{a}$
5		$^{B}24.21\pm3.46_{d}$	$^{B}42.15\pm4.46_{d}$	$^{B}24.29\pm2.85_{c}$	$^{B}12.51\pm2.56_{b}$	$^{B}5.55\pm1.21_{a}$
25		$^{A}34.99\pm3.70_{d}$	$^{A}26.01\pm2.46_{d}$	$^{A}19.94\pm2.76_{c}$	$^{A}9.15\pm2.32_{b}$	$^{A}6.62\pm2.31_{a}$
35		$^{A}30.91\pm2.99_{d}$	$^{A}23.92\pm3.76_{d}$	$^{A}9.11\pm2.07_{c}$	$^{A}7.52\pm1.01_{b}$	$^{A}4.90\pm1.39_{a}$

Table.5.6 Catalase (CAT) activity (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Catalase activity (U/mg protein) in the gill						
	Duration					
Salinity(‰)	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	12.33±3.72					
Control(25)		$^{A}13.40\pm3.74_{b}$	$^{A}13.20\pm3.60_{d}$	$^{A}13.58\pm4.35_{c}$	$^{A}12.14\pm3.74_{b}$	$^{A}13.05\pm3.74_{a}$
5		$^{A}8.07\pm4.42_{b}$	$^{A}25.19\pm7.05_{d}$	$^{A}14.03\pm3.27_{c}$	$^{A}6.89\pm2.65_{b}$	$^{A}3.76\pm0.90_{a}$
25		$^{A}12.89\pm5.00_{b}$	$^{A}20.13\pm1.58_{d}$	$^{A}15.15\pm3.75_{c}$	$^{A}11.88\pm5.10_{b}$	$^{A}5.05\pm0.99_{a}$
35		$^{A}11.61\pm4.16_{b}$	$^{A}23.54\pm5.03_{d}$	$^{A}13.60\pm3.18_{c}$	$^{A}11.56\pm2.45_{b}$	$^{A}6.76\pm1.23_{a}$

Table.5.7 Catalase (CAT) activity (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Catalase activity (U/mg protein) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	14.27±2.12					
Control(25)		$^{A}15.32\pm2.85_{a}$	$^{A}15.2\pm2.22_{c}$	$^{A}14.88\pm1.85_{b}$	$^{A}16.19\pm1.75_{a}$	$^{A}16.44\pm2.05_{a}$
5		$^{B}11.26\pm1.45_{a}$	$^{B}29.56\pm2.64_{c}$	$^{B}19.66\pm3.11_{b}$	$^{B}15.85\pm2.09_{a}$	$^{B}14.08\pm3.63_{a}$
25		$^{A}16.38\pm2.51_{a}$	$^{A}21.68\pm3.63_{c}$	$^{A}15.77\pm2.72_{b}$	$^{A}10.21\pm2.71_{a}$	$^{A}5.8\pm1.94_{a}$
35		$^{A}14.33\pm2.15_{a}$	$^{A}24.14\pm4.31_{c}$	$^{A}10.65\pm2.82_{b}$	$^{A}6.83\pm1.42_{a}$	$^{A}4.44\pm1.5_{a}$

Table.5.8 Catalase (CAT) activity (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Catalase activity (U/mg protein) in the haemolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	17.58±2.32					
Control(25)		$^{A}18.15\pm2.52_{c}$	$^{A}17.57\pm2.32_{d}$	$^{A}17.24\pm2.21_{b}$	$^{A}16.18\pm1.46_{b}$	$^{A}17.36\pm2.14_{a}$
5		$^{AB}12.57\pm1.35_{c}$	$^{AB}22.16\pm3.36_{d}$	$^{AB}13.68\pm4.32_{b}$	$^{AB}12.92\pm2.01_{b}$	$^{AB}7.4\pm1.49_{a}$
25		$^{B}17.22\pm2.12_{c}$	$^{B}24.07\pm4.63_{d}$	$^{B}17.45\pm1.88_{b}$	$^{B}13.49\pm1.8_{b}$	$^{B}6.88\pm0.59_{a}$
35		$^{A}15.54\pm1.15_{c}$	$^{A}26.02\pm3.05_{d}$	$^{A}11.66\pm1.37_{b}$	$^{A}10.09\pm1.12_{b}$	$^{A}4.42\pm1.26_{a}$

Table.5.9 Glutathione peroxidase (GPx) activity (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GPx activity (U/mg protein) in the hepatopancreas						
Salinity(‰)						Duration
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	2.22±0.52					
Control(25)		$^{A}2.27\pm0.48_{a}$	$^{A}2.23\pm0.34_{c}$	$^{A}2.29\pm0.43_{b}$	$^{A}2.18\pm0.4_{a}$	$^{A}2.41\pm0.51_{a}$
5		$^{A}1.14\pm0.46_{a}$	$^{A}6\pm1.2_{c}$	$^{A}2.28\pm0.33_{b}$	$^{A}1.57\pm0.48_{a}$	$^{A}1.41\pm0.18_{a}$
25		$^{A}2.32\pm0.37_{a}$	$^{A}4.31\pm0.82_{c}$	$^{A}2.58\pm0.49_{b}$	$^{A}1.81\pm0.61_{a}$	$^{A}1.62\pm0.53_{a}$
35		$^{B}1.82\pm0.42_{a}$	$^{B}4.57\pm0.59_{c}$	$^{B}4.34\pm0.81_{b}$	$^{B}2.56\pm0.86_{a}$	$^{B}2.21\pm0.48_{a}$

Table.5.10 Glutathione peroxidase (GPx) activity (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GPx activity (U/mg protein) in the gill						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	4.38±0.76					
Control(25)		$^{A}4.41\pm0.88_{c}$	$^{A}4.66\pm0.68_{d}$	$^{A}4.75\pm0.84_{c}$	$^{A}4.81\pm0.93_{b}$	$^{A}4.94\pm0.75_{a}$
5		$^{A}2.24\pm0.58_{c}$	$^{A}4.9\pm1.03_{d}$	$^{A}2.72\pm0.95_{c}$	$^{A}1.36\pm0.38_{b}$	$^{A}0.35\pm0.12_{a}$
25		$^{B}4.36\pm0.86_{c}$	$^{B}4.69\pm1.22_{d}$	$^{B}3.58\pm0.87_{c}$	$^{B}2.52\pm0.75_{b}$	$^{B}1.84\pm0.23_{a}$
35		$^{B}3.19\pm0.91_{c}$	$^{B}4.77\pm0.84_{d}$	$^{B}3.65\pm0.49_{c}$	$^{B}3.64\pm0.72_{b}$	$^{B}2.38\pm0.55_{a}$

Table.5.11 Glutathione peroxidase (GPx) activity (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GPx activity (U/mg protein) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	2.2±0.38					
Control(25)		$^{A}2.26\pm0.43_{c}$	$^{A}2.21\pm0.33_{d}$	$^{A}2.28\pm0.45_{c}$	$^{A}2.32\pm0.42_{b}$	$^{A}2.36\pm0.37_{a}$
5		$^{A}1.21\pm0.4_{c}$	$^{A}3.67\pm0.37_{d}$	$^{A}2.28\pm0.65_{c}$	$^{A}2.14\pm0.53_{b}$	$^{A}1.67\pm0.3_{a}$
25		$^{A}2.25\pm0.35_{c}$	$^{A}3.14\pm0.49_{d}$	$^{A}3.04\pm0.59_{c}$	$^{A}1.73\pm0.63_{b}$	$^{A}1.07\pm0.38_{a}$
35		$^{A}2.02\pm0.41_{c}$	$^{A}3.41\pm0.5_{d}$	$^{A}2.38\pm0.47_{c}$	$^{A}1.91\pm0.61_{b}$	$^{A}1.6\pm0.38_{a}$

Table.5.12 Glutathione peroxidase (GPx) activity (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GPx activity (U/mg protein) in the haemolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	4.23±0.82					
Control(25)		$^{A}4.12\pm0.89_{c}$	$^{A}4.19\pm0.68_{c}$	$^{A}4.25\pm0.72_{b}$	$^{A}3.91\pm0.77_{b}$	$^{A}3.87\pm0.81_{a}$
5		$^{A}2.39\pm0.83_{c}$	$^{A}4.7\pm0.97_{c}$	$^{A}2.25\pm0.56_{b}$	$^{A}1.73\pm0.43_{b}$	$^{A}1.16\pm0.2_{a}$
25		$^{A}4.32\pm0.74_{c}$	$^{A}4.31\pm0.82_{c}$	$^{A}2.5\pm0.74_{b}$	$^{A}2.08\pm0.68_{b}$	$^{A}1.36\pm0.38_{a}$
35		$^{B}3.16\pm0.9_{c}$	$^{B}4.44\pm0.59_{c}$	$^{B}3.64\pm0.56_{b}$	$^{B}3.37\pm0.56_{b}$	$^{B}1.43\pm0.29_{a}$

Table.5.13 Glutathione Reductase (GR) activity (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GR activity (U/mg protein) in the hepatopancreas						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	30.85±4.2					
Control(25)		$^{A}31.52\pm3.53_{c}$	$^{A}31.14\pm3.18_{d}$	$^{A}32.54\pm4.24_{b}$	$^{A}31.44\pm4.33_{a}$	$^{A}32.15\pm3.65_{a}$
5		$^{B}31.63\pm2.92_{c}$	$^{B}53.5\pm5.3_{d}$	$^{B}15.24\pm3.5_{b}$	$^{B}8.5\pm1.84_{a}$	$^{B}7.5\pm1.79_{a}$
25		$^{A}30.89\pm3.28_{c}$	$^{A}39.5\pm4.6_{d}$	$^{A}13.82\pm3.68_{c}$	$^{A}9.72\pm2.5_{a}$	$^{A}8.67\pm2.5_{a}$
35		$^{A}30.45\pm2.89_{c}$	$^{A}47.5\pm3.95_{d}$	$^{A}15.5\pm3.5_{b}$	$^{A}10.38\pm1.5_{a}$	$^{A}8.5\pm2.5_{a}$

Table.5.14 Glutathione Reductase (GR) activity (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GR activity (U/mg protein) in the gill						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	20.34±2.65					
Control(25)		$^{A}22.31\pm2.3_{c}$	$^{A}22.49\pm3.56_{d}$	$^{A}21.29\pm2.36_{b}$	$^{A}23.16\pm3.11_{a}$	$^{A}23.72\pm2.59_{a}$
5		$^{A}20.68\pm1.83_{c}$	$^{A}28.44\pm4.5_{d}$	$^{A}18.78\pm4.5_{b}$	$^{A}12.5\pm3_{a}$	$^{A}6.5\pm2_{a}$
25		$^{A}23.11\pm1.77_{c}$	$^{A}25.51\pm3.4_{d}$	$^{A}14.42\pm3.24_{b}$	$^{A}11\pm3.12_{a}$	$^{A}9.5\pm2.5_{a}$
35		$^{A}19.45\pm2.14_{c}$	$^{A}22.5\pm4.5_{d}$	$^{A}17\pm2.31_{b}$	$^{A}13.5\pm2.16_{a}$	$^{A}12\pm3.5_{a}$

Table.5.15 Glutathione Reductase (GR) activity (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GR activity (U/mg protein) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	20.41±2.67					
Control(25)		$^{A}20.54\pm2.4_{b}$	$^{A}18.75\pm3.12_{c}$	$^{A}21.52\pm2.99_{b}$	$^{A}20.18\pm3.14_{a}$	$^{A}18.5\pm2.81_{a}$
5		$^{A}17.35\pm2.34_{b}$	$^{A}30.33\pm3.5_{c}$	$^{A}21\pm3.5_{b}$	$^{A}13.48\pm2.5_{a}$	$^{A}8.5\pm1_{a}$
25		$^{A}20.5\pm2.83_{b}$	$^{A}26.59\pm2.5_{c}$	$^{A}19.5\pm3.5_{b}$	$^{A}12\pm3.44_{a}$	$^{A}10.5\pm2.15_{a}$
35		$^{A}18.15\pm2.91_{b}$	$^{A}29.1\pm3.5_{c}$	$^{A}18.5\pm3_{b}$	$^{A}10.5\pm1.5_{a}$	$^{A}8\pm1.5_{a}$

Table.5.16 Glutathione Reductase (GR) activity (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

GR activity (U/mg protein) in the haemolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	26.86±3.45					
Control(25)		$^{A}27.5\pm3.5_{d}$	$^{A}26.15\pm2.55_{e}$	$^{A}28.14\pm3.15_{c}$	$^{A}27.94\pm2.79_{b}$	$^{A}26.58\pm2.52_{a}$
5		$^{A}24.54\pm2.89_{d}$	$^{A}35.21\pm4_{e}$	$^{A}21.5\pm3_{c}$	$^{A}14.5\pm4_{b}$	$^{A}7.5\pm1.5_{a}$
25		$^{A}28.1\pm2.75_{d}$	$^{A}32.5\pm5.5_{e}$	$^{A}19.5\pm4_{c}$	$^{A}15.5\pm5_{b}$	$^{A}9.5\pm2.5_{a}$
35		$^{A}23.25\pm3.12_{d}$	$^{A}32.52\pm3_{e}$	$^{A}19.5\pm4_{c}$	$^{A}14.5\pm2_{b}$	$^{A}9\pm2_{a}$

Table.5.17 Glutathione-S-Transferase (GST) activity (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

G-S- Transferase activity (U/mg protein) in the hepatopancreas						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	20.17±3.46					
Control(25)		$^{A}19.43\pm3.54_{c}$	$^{A}19.58\pm3.28_{d}$	$^{A}20.18\pm3.18_{b}$	$^{A}20.28\pm3.34_{b}$	$^{A}19.96\pm2.91_{a}$
5		$^{B}18.82\pm4.14_{c}$	$^{B}25.42\pm5_{d}$	$^{B}17.5\pm2.5_{b}$	$^{B}13.88\pm4.5_{b}$	$^{B}12.27\pm2.5_{a}$
25		$^{A}19.89\pm2.97_{c}$	$^{A}16.38\pm4_{d}$	$^{A}14.95\pm2.5_{b}$	$^{A}12.36\pm2.5_{b}$	$^{A}11.62\pm1.5_{a}$
35		$^{B}20.14\pm3.22_{c}$	$^{B}18.19\pm2.5_{d}$	$^{B}17.28\pm4_{b}$	$^{B}16.1\pm4_{b}$	$^{B}10.36\pm3_{a}$

Table.5.18 Glutathione-S-Transferase (GST) activity (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

G-S- Transferase activity (U/mg protein) in the gill						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	15.08±1.43					
Control(25)		$^{A}15.21\pm1.2_{b}$	$^{A}15.46\pm1.14_{c}$	$^{A}16.42\pm1.19_{b}$	$^{A}15.35\pm1.12_{b}$	$^{A}14.13\pm1.23_{a}$
5		$^{A}14.85\pm1.32_{b}$	$^{A}18.70\pm2.5_{c}$	$^{A}16.99\pm2.04_{b}$	$^{A}15.70\pm2.5_{b}$	$^{A}10.92\pm1.04_{a}$
25		$^{A}13.77\pm1.28_{b}$	$^{A}16.52\pm2.12_{c}$	$^{A}15.30\pm2.11_{b}$	$^{A}13.50\pm2.5_{b}$	$^{A}10.92\pm1.02_{a}$
35		$^{A}14.98\pm1.25_{b}$	$^{A}17.10\pm1.3_{c}$	$^{A}15.30\pm2.5_{b}$	$^{A}13.50\pm2.06_{b}$	$^{A}14.28\pm2.03_{a}$

Table.5.19 Glutathione-S-Transferase (GST) activity (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

G-S- Transferase activity (U/mg protein) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	11.68±1.98					
Control(25)		$^{A}12.53\pm2.01_{b}$	$^{A}12.4\pm1.78_{c}$	$^{A}11.88\pm2.04_{b}$	$^{A}12.05\pm1.88_{a}$	$^{A}11.75\pm1.92_{a}$
5		$^{A}12.25\pm1.92_{b}$	$^{A}16.5\pm3_{c}$	$^{A}11.5\pm1.5_{b}$	$^{A}9.5\pm2_{a}$	$^{A}7.44\pm1.5_{a}$
25		$^{A}11.95\pm1.59_{b}$	$^{A}15.37\pm2.5_{c}$	$^{A}11.33\pm1_{b}$	$^{A}9.82\pm2_{a}$	$^{A}7.5\pm2_{a}$
35		$^{A}12.18\pm1.84_{b}$	$^{A}16.31\pm2.5_{c}$	$^{A}13.21\pm2_{b}$	$^{A}9.22\pm1.5_{a}$	$^{A}7.5\pm1_{a}$

Table.5.20 Glutathione-S-Transferase (GST) activity (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

G-S- Transferase activity (U/mg protein) in the haemolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	21.27±2.31					
Control(25)		$^{A}21.54\pm2.5_{c}$	$^{A}21.52\pm2.34_{c}$	$^{A}21.45\pm2.08_{b}$	$^{A}21.88\pm2.22_{a}$	$^{A}20.98\pm2.18_{a}$
5		$^{B}21.35\pm2.15_{c}$	$^{B}23.5\pm2.5_{c}$	$^{B}18.5\pm4_{b}$	$^{B}13.5\pm2.5_{a}$	$^{B}10.5\pm3_{a}$
25		$^{A}22.12\pm2.23_{c}$	$^{A}23.41\pm3_{c}$	$^{A}13.62\pm2.5_{b}$	$^{A}8.42\pm1_{a}$	$^{A}6.5\pm1_{a}$
35		$^{AB}22.58\pm2.31_{c}$	$^{A}22.5\pm4_{c}$	$^{A}15.43\pm2_{b}$	$^{A}11.98\pm1_{a}$	$^{A}9.5\pm3.5_{a}$

Table.5.21 Total Reduced Glutathione (TRG) (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Total Reduced Glutathione (µg/mg protein) in the hepatopancreas						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	26.94±3.45					
Control(25)		$^{AB}27.86\pm3.24_{d}$	$^{AB}27.44\pm3.42_{c}$	$^{AB}27.18\pm3.34_{b}$	$^{AB}26.82\pm3.51_{a}$	$^{AB}27.31\pm3.49_{a}$
5		$^{A}27.14\pm4.04_{d}$	$^{A}19.53\pm2.3_{c}$	$^{A}23.02\pm2.08_{b}$	$^{A}10.86\pm4.66_{a}$	$^{A}6.90\pm1.37_{a}$
25		$^{A}27.36\pm4.12_{d}$	$^{A}27.08\pm3.57_{c}$	$^{A}23.85\pm5.34_{b}$	$^{A}13.26\pm2.16_{a}$	$^{A}11.54\pm2.3_{a}$
35		$^{A}26.75\pm3.74_{d}$	$^{A}29.42\pm3.28_{c}$	$^{A}17.42\pm3.4_{b}$	$^{A}10.60\pm1.91_{a}$	$^{A}9.50\pm2.04_{a}$

Table.5.22 Total Reduced Glutathione (TRG) (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Total Reduced Glutathione (µg/mg protein) in the gill						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	14.23±2.11					
Control(25)		$^{B}14.08\pm3.05_{c}$	$^{B}13.56\pm2.23_{b}$	$^{B}13.77\pm3.01_{a}$	$^{B}14.21\pm2.55_{a}$	$^{B}14.34\pm2.85_{a}$
5		$^{A}14.1\pm2.68_{c}$	$^{A}11.44\pm2.48_{b}$	$^{A}5.46\pm2.02_{a}$	$^{A}7.55\pm0.88_{a}$	$^{A}3.43\pm1.06_{a}$
25		$^{A}13.68\pm2.45_{c}$	$^{A}7.78\pm1.33_{b}$	$^{A}9.84\pm0.96_{a}$	$^{A}8.84\pm1.17_{a}$	$^{A}7.12\pm1.78_{a}$
35		$^{A}13.39\pm2.95_{c}$	$^{A}10.49\pm2.01_{b}$	$^{A}7.76\pm2.05_{a}$	$^{A}7.67\pm1.46_{a}$	$^{A}4.04\pm0.97_{a}$

Table.5.23 Total Reduced Glutathione (TRG) (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Total Reduced Glutathione (µg/mg protein) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	8.46±1.28					
Control(25)		$^{B}8.75\pm1.24_{c}$	$^{B}8.74\pm1.25_{c}$	$^{B}7.95\pm1.15_{bc}$	$^{B}8.17\pm1.28_{b}$	$^{B}8.34\pm1.21_{a}$
5		$^{A}7.45\pm1.32_{c}$	$^{A}7.83\pm2.3_{c}$	$^{A}4.09\pm1.25_{bc}$	$^{A}4.55\pm0.63_{b}$	$^{A}3.29\pm0.93_{a}$
25		$^{A}8.22\pm1.18_{c}$	$^{A}7.13\pm1.25_{c}$	$^{A}6.73\pm1.04_{bc}$	$^{A}4.24\pm1.03_{b}$	$^{A}3.38\pm1.14_{a}$
35		$^{A}8.15\pm2.01_{c}$	$^{A}7.44\pm1.16_{c}$	$^{A}5.78\pm2.26_{bc}$	$^{A}4.31\pm0.87_{b}$	$^{A}3.97\pm0.5_{a}$

Table.5.24 Total Reduced Glutathione (TRG) (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Total Reduced Glutathione (µg/mg protein) in the hamolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	21.38±3.26					
Control(25)		$^{B}22.75\pm3.43_{d}$	$^{B}22.1\pm3.63_{d}$	$^{B}21.64\pm3.54_{c}$	$^{B}22.49\pm3.29_{b}$	$^{B}21.22\pm3.77_{a}$
5		$^{A}20.12\pm3.35_{d}$	$^{A}19.89\pm3.72_{d}$	$^{A}14.38\pm2.7_{c}$	$^{A}7.86\pm1.32_{b}$	$^{A}5.61\pm1.89_{a}$
25		$^{B}22.15\pm3.45_{d}$	$^{B}21.81\pm2.84_{d}$	$^{B}17.42\pm1.39_{c}$	$^{B}15.30\pm1.23_{b}$	$^{B}8.97\pm1.05_{a}$
35		$^{A}21.35\pm3.25_{d}$	$^{A}17.64\pm2.63_{d}$	$^{A}12.52\pm2.04_{c}$	$^{A}8.81\pm0.91_{b}$	$^{A}4.28\pm1.11_{a}$

Table.5.25 Hydroperoxide (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Hydroperoxide (m mol/g) in the hepatopancreas						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	17.16±2.43					
Control(25)		$^{A}17.23\pm2.60_{a}$	$^{A}17.42\pm2.23_{b}$	$^{A}17.52\pm2.34_{c}$	$^{A}17.12\pm1.86_{d}$	$^{A}16.92\pm1.98_{c}$
5		$^{D}17.41\pm1.65_{a}$	$^{D}36.63\pm5.50_{b}$	$^{D}68.17\pm15.93_{c}$	$^{D}107.19\pm12.63_{d}$	$^{D}72.52\pm11.77_{c}$
25		$^{B}17.11\pm2.25_{a}$	$^{B}25.09\pm3.73_{b}$	$^{B}48.04\pm7.47_{c}$	$^{B}76.74\pm8.75_{d}$	$^{B}44.48\pm6.84_{c}$
35		$^{C}18.80\pm2.04_{a}$	$^{C}32.03\pm8.92_{b}$	$^{C}37.83\pm7.97_{c}$	$^{C}108.82\pm8.93_{d}$	$^{C}55.61\pm7.82_{c}$

Table.5.26 Hydroperoxide (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Hydroperoxide (m mol/g) in the gill						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	21.84±2.84					
Control(25)		$^{A}22.29\pm2.56_{a}$	$^{A}22.14\pm2.62_{b}$	$^{A}21.53\pm2.18_{c}$	$^{A}22.10\pm3.02_{d}$	$^{A}22.26\pm2.97_{c}$
5		$^{D}22.21\pm2.42_{a}$	$^{D}37.71\pm5.74_{b}$	$^{D}40.47\pm4.94_{c}$	$^{D}122.27\pm7.23_{d}$	$^{D}98.78\pm10.47_{c}$
25		$^{B}22.12\pm3.15_{a}$	$^{B}26.78\pm3.27_{b}$	$^{B}41.98\pm4.02_{c}$	$^{B}109.26\pm8.67_{d}$	$^{B}75.68\pm7.77_{c}$
35		$^{C}21.63\pm3.03_{a}$	$^{C}39.56\pm3.85_{b}$	$^{C}75.96\pm12.30_{c}$	$^{C}102.73\pm8.69_{d}$	$^{C}62.24\pm7.60_{c}$

Table.5.27 Hydroperoxide (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Hydroperoxide (m mol/g) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	41.58±5.82					
Control(25)		$^{A}42.91\pm6.32_{a}$	$^{A}42.69\pm5.87_{a}$	$^{A}41.98\pm5.56_{b}$	$^{A}42.11\pm6.12_{c}$	$^{A}42.41\pm6.04_{a}$
5		$^{C}42.23\pm6.16_{a}$	$^{C}61.56\pm7.63_{a}$	$^{C}106.78\pm7.47_{b}$	$^{C}111.81\pm11.45_{c}$	$^{C}62.24\pm8.08_{a}$
25		$^{B}42.32\pm6.41_{a}$	$^{B}35.17\pm6.72_{a}$	$^{B}39.91\pm6.00_{b}$	$^{B}76.94\pm5.94_{c}$	$^{B}48.66\pm7.04_{a}$
35		$^{B}41.90\pm6.37_{a}$	$^{B}39.69\pm4.17_{a}$	$^{B}48.47\pm8.20_{b}$	$^{B}78.45\pm10.51_{c}$	$^{B}32.04\pm6.42_{a}$

Table.5.28 Hydroperoxide (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Hydroperoxide (m mol/ml) in the haemolymph

Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	28.15±4.14					
Control(25)		$^{A}28.34\pm4.18_{a}$	$^{A}28.15\pm4.54_{a}$	$^{A}28.64\pm4.29_{c}$	$^{A}27.63\pm3.88_{d}$	$^{A}29.12\pm4.12_{c}$
5		$^{C}28.11\pm5.02_{a}$	$^{C}41.73\pm5.71_{a}$	$^{C}63.60\pm7.42_{b}$	$^{C}74.67\pm19.01_{d}$	$^{C}66.36\pm6.84_{c}$
25		$^{BC}27.86\pm4.41_{a}$	$^{BC}27.17\pm2.79_{a}$	$^{BC}38.54\pm4.38_{b}$	$^{BC}91.91\pm9.63_{d}$	$^{BC}70.82\pm7.99_{c}$
35		$^{B}27.34\pm4.72_{a}$	$^{B}31.31\pm5.89_{a}$	$^{B}49.24\pm5.22_{b}$	$^{B}80.74\pm9.37_{d}$	$^{B}60.26\pm5.91_{c}$

Table.5.29 Conjugated Dienes (CD) content (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Conjugated Dienes (m mol/g) in the hepatopancreas

Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	37.97±2.81					
Control(25)		$^{A}38.76\pm2.56_{a}$	$^{A}38.78\pm2.37_{ab}$	$^{A}38.74\pm2.77_{c}$	$^{A}38.17\pm2.75_{d}$	$^{A}38.08\pm2.51_{b}$
5		$^{D}37.85\pm2.96_{a}$	$^{D}46.52\pm3.69_{ab}$	$^{D}51.66\pm3.37_{c}$	$^{D}62.01\pm2.90_{d}$	$^{D}43.53\pm2.78_{b}$
25		$^{B}38.55\pm2.39_{a}$	$^{B}36.25\pm3.57_{ab}$	$^{B}52.14\pm3.18_{c}$	$^{B}52.99\pm3.80_{d}$	$^{B}37.56\pm2.14_{b}$
35		$^{D}38.54\pm2.88_{a}$	$^{D}37.77\pm3.10_{ab}$	$^{D}50.71\pm3.79_{c}$	$^{D}65.58\pm2.89_{d}$	$^{D}44.74\pm3.03_{b}$

Table.5.30 Conjugated Dienes (CD) content (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Conjugated Dienes (m mol/g) in the gill

Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	45.18±4.31					
Control(25)		$^{B}46.45\pm4.93_{bc}$	$^{B}46.07\pm4.19_{a}$	$^{B}46.09\pm4.61_{c}$	$^{B}45.41\pm4.92_{b}$	$^{B}45.47\pm3.99_{b}$
5		$^{C}45.73\pm4.25_{bc}$	$^{C}51.55\pm6.22_{a}$	$^{C}61.36\pm3.00_{c}$	$^{C}77.44\pm2.99_{b}$	$^{C}65.81\pm5.14_{b}$
25		$^{A}46.23\pm4.69_{bc}$	$^{A}26.05\pm4.51_{a}$	$^{A}47.44\pm5.69_{c}$	$^{A}51.90\pm5.09_{b}$	$^{A}28.84\pm4.35_{b}$
35		$^{A}45.36\pm4.59_{bc}$	$^{A}32.94\pm3.71_{a}$	$^{A}40.04\pm4.78_{c}$	$^{A}52.42\pm2.72_{b}$	$^{A}33.06\pm4.28_{b}$

Table.5.31 Conjugated Dienes (CD) content (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Conjugated Dienes (m mol/g) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	30.16±2.48					
Control(25)		$^{A}29.25\pm2.77_{a}$	$^{A}30.12\pm2.20_{a}$	$^{A}29.57\pm2.71_{b}$	$^{A}30.18\pm2.48_{d}$	$^{A}30.11\pm2.90_{c}$
5		$^{D}29.72\pm2.16_{a}$	$^{D}42.35\pm4.72_{a}$	$^{D}48.62\pm3.49_{b}$	$^{C}71.63\pm8.66_{d}$	$^{D}65.59\pm11.36_{c}$
25		$^{C}29.33\pm2.81_{a}$	$^{C}25.89\pm4.48_{a}$	$^{C}52.35\pm5.84_{b}$	$^{C}75.26\pm9.26_{d}$	$^{C}70.32\pm2.33_{c}$
35		$^{B}31.25\pm2.46_{a}$	$^{B}24.18\pm3.62_{a}$	$^{B}46.34\pm5.47_{b}$	$^{B}67.40\pm5.55_{d}$	$^{B}47.76\pm6.41_{c}$

Table.5.32 Conjugated Dienes (CD) content (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Conjugated Dienes (m mol/ml) in the haemolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	16.28±1.22					
Control(25)		$^{A}16.95\pm1.30_{a}$	$^{A}16.48\pm1.31_{ab}$	$^{A}17.19\pm1.18_{c}$	$^{A}17.21\pm1.06_{d}$	$^{A}16.74\pm1.35_{bc}$
5		$^{C}16.12\pm1.15_{a}$	$^{C}24.64\pm5.62_{ab}$	$^{C}30.12\pm8.03_{c}$	$^{C}42.25\pm4.02_{d}$	$^{C}24.92\pm3.98_{bc}$
25		$^{A}17.14\pm1.26_{a}$	$^{A}15.42\pm2.54_{ab}$	$^{A}17.82\pm2.75_{c}$	$^{A}30.21\pm3.53_{d}$	$^{A}14.51\pm1.01_{bc}$
35		$^{B}16.87\pm1.23_{a}$	$^{B}16.20\pm2.98_{ab}$	$^{B}19.27\pm2.34_{c}$	$^{B}34.26\pm2.71_{d}$	$^{B}21.94\pm3.17_{bc}$

Table.5.33 Malondialdehyde (MDA) content (Mean±S.D.) in the hepatopancreas of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Malondialdehyde (m mol/g) in the hepatopancreas						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	2.65±0.44					
Control(25)		$^{A}2.88\pm0.47_{a}$	$^{A}2.82\pm0.46_{b}$	$^{A}2.83\pm0.47_{c}$	$^{A}2.86\pm0.42_{d}$	$^{A}2.91\pm0.44_{c}$
5		$^{C}2.72\pm0.44_{a}$	$^{C}4.11\pm0.86_{b}$	$^{C}6.60\pm0.79_{c}$	$^{C}11.77\pm0.55_{d}$	$^{C}7.49\pm1.01_{c}$
25		$^{B}2.58\pm0.46_{a}$	$^{B}3.17\pm0.52_{b}$	$^{B}4.81\pm1.00_{c}$	$^{B}8.56\pm0.92_{d}$	$^{B}5.89\pm0.85_{c}$
35		$^{C}2.91\pm0.43_{a}$	$^{C}4.21\pm1.06_{b}$	$^{C}6.12\pm0.80_{c}$	$^{C}10.73\pm1.67_{d}$	$^{C}6.32\pm1.13_{c}$

Table.5.34 Malondialdehyde (MDA) content (Mean±S.D.) in the gill of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Malondialdehyde (m mol/g) in the gill						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	1.62±0.36					
Control(25)		$^{A}1.60\pm0.34_{a}$	$^{A}1.62\pm0.27_{a}$	$^{A}1.65\pm0.24_{b}$	$^{A}1.60\pm0.34_{c}$	$^{A}1.56\pm0.31_{b}$
5		$^{C}1.56\pm0.36_{a}$	$^{C}1.93\pm0.22_{a}$	$^{C}2.45\pm0.24_{b}$	$^{C}6.60\pm0.53_{c}$	$^{C}2.68\pm0.26_{b}$
25		$^{B}1.54\pm0.30_{a}$	$^{B}1.70\pm0.36_{a}$	$^{B}2.22\pm0.28_{b}$	$^{B}4.09\pm0.26_{c}$	$^{B}2.65\pm0.30_{b}$
35		$^{B}1.56\pm0.34_{a}$	$^{B}1.87\pm0.21_{a}$	$^{B}2.39\pm0.38_{b}$	$^{B}4.83\pm0.29_{c}$	$^{B}2.29\pm0.37_{b}$

Table.5.35 Malondialdehyde (MDA) content (Mean±S.D.) in the muscle of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Malondialdehyde (m mol/g) in the muscle						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	1.88±0.11					
Control(25)		$^{A}1.93\pm0.10_{ab}$	$^{A}1.90\pm0.10_{a}$	$^{A}1.87\pm0.08_{c}$	$^{A}1.93\pm0.11_{d}$	$^{A}1.87\pm0.09_{b}$
5		$^{C}1.98\pm0.11_{ab}$	$^{C}2.34\pm0.13_{a}$	$^{C}2.57\pm0.11_{c}$	$^{C}5.07\pm0.14_{d}$	$^{C}3.30\pm0.10_{b}$
25		$^{A}1.91\pm0.10_{ab}$	$^{A}1.22\pm0.09_{a}$	$^{A}2.06\pm0.12_{c}$	$^{A}2.36\pm0.09_{d}$	$^{A}1.94\pm0.12_{b}$
35		$^{B}1.92\pm0.09_{ab}$	$^{B}2.11\pm0.09_{a}$	$^{B}2.44\pm0.07_{c}$	$^{B}3.75\pm0.11_{d}$	$^{B}1.81\pm0.10_{b}$

Table.5.36 Malondialdehyde (MDA) content (Mean±S.D.) in the haemolymph of *F.indicus* subjected to acute salinity stress and challenged with WSSV. Control - unchallenged. PSD- Post salinity change day, PCD- Post challenge day (See Table.5.1. for statistical details).

Malondialdehyde (m mol/ml) in the haemolymph						
Salinity(‰)	Duration					
	Baseline	PSD0	PCD1	PCD2	PCD3	PCD5
Baseline(25)	3.28±0.12					
Control(25)		$^{A}3.09\pm0.12_{a}$	$^{A}3.17\pm0.11_{a}$	$^{A}3.25\pm0.10_{b}$	$^{A}3.19\pm0.11_{d}$	$^{A}3.21\pm0.12_{c}$
5		$^{C}3.14\pm0.11_{a}$	$^{C}3.61\pm0.16_{a}$	$^{C}6.23\pm0.93_{b}$	$^{C}11.37\pm1.27_{d}$	$^{C}7.91\pm0.97_{c}$
25		$^{B}3.26\pm0.13_{a}$	$^{B}3.80\pm0.43_{a}$	$^{B}5.48\pm0.70_{b}$	$^{B}9.89\pm0.85_{d}$	$^{B}6.21\pm0.74_{c}$
35		$^{C}3.31\pm0.10_{a}$	$^{C}2.96\pm0.74_{a}$	$^{C}6.28\pm0.55_{b}$	$^{C}12.68\pm1.13_{d}$	$^{C}8.79\pm1.16_{c}$

Appendix III

Table.6.1 Total haemolymph protein concentration (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. Each data represents mean ± S.D. of six separate determinations. Values with same lowercase superscripts in the same column do not vary significantly ($p>0.05$) among different exposure time and data with same uppercase superscripts in the same row do not vary significantly ($p>0.05$) among different (Cu^{2+} dose) treatment groups. PMD- Post metal exposure day, PCD- post challenge day.

Total haemolymph protein(mg/ml)

Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	66.28±7.29						
PMD7		$^{d}68.48±7.98^{BC}$	$^{d}67.72±5.43^{BC}$	$^{d}106.76±7.49^{D}$	$^{d}88.98±7.40^{C}$	$^{d}82.16±10.69^{B}$	$^{d}61.60±3.86^{A}$
PMD14		$^{b}68.08±8.02^{BC}$	$^{b}69.15±8.02^{BC}$	$^{b}98.48±7.54^{D}$	$^{b}78.96±11.08^{C}$	$^{b}74.25±7.36^{B}$	$^{b}58.57±5.09^{A}$
PMD16 (PCD2)		$^{c}67.18±8.15^{BC}$	$^{c}86.21±6.36^{BC}$	$^{c}112.79±6.06^{D}$	$^{c}66.59±4.44^{C}$	$^{c}64.16±4.80^{B}$	$^{c}57.89±2.98^{A}$
PMD19 (PCD5)		$^{a}66.38±6.38^{BC}$	$^{a}62.06±3.71^{BC}$	$^{a}76.23±5.65^{D}$	$^{a}60.89±1.77^{C}$	$^{a}51.79±2.47^{B}$	$^{a}49.44±2.54^{A}$

Table.6.2 Plasma protein concentration (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Plasma protein(mg/ml)

Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	53.41±6.18						
PMD7		$^{bc}52.53±6.83^{C}$	$^{bc}51.07±4.55^{C}$	$^{bc}91.70±6.44^{D}$	$^{bc}63.35±5.27^{C}$	$^{bc}50.51±3.16^{B}$	$^{bc}56.55±3.54^{A}$
PMD14		$^{b}52.31±7.18^{C}$	$^{b}53.19±6.50^{C}$	$^{b}72.78±5.57^{D}$	$^{b}58.90±8.27^{C}$	$^{b}66.08±6.55^{B}$	$^{b}46.74±4.06^{A}$
PMD16 (PCD2)		$^{c}52.14±5.81^{C}$	$^{c}73.45±5.42^{C}$	$^{c}94.28±4.83^{D}$	$^{c}46.53±3.10^{C}$	$^{c}52.13±3.90^{B}$	$^{c}50.19±2.58^{A}$
PMD19 (PCD5)		$^{a}52.22±6.33^{C}$	$^{a}48.27±3.17^{C}$	$^{a}65.96±4.89^{D}$	$^{a}48.62±1.41^{C}$	$^{a}50.86±2.43^{B}$	$^{a}33.97±1.75^{A}$

Table.6.3 Serum protein concentration (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Serum protein(mg/ml)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	46.28±6.12						
PMD7		$^{b}42.96\pm4.19^{B}$	$^{b}47.39\pm3.59^{B}$	$^{b}70.43\pm4.94^{C}$	$^{b}45.04\pm3.74^{A}$	$^{b}38.29\pm2.40^{A}$	$^{b}41.22\pm2.58^{A}$
PMD14		$^{b}43.16\pm5.87^{B}$	$^{b}51.99\pm5.29^{B}$	$^{b}58.09\pm4.45^{C}$	$^{b}42.01\pm5.90^{A}$	$^{b}50.57\pm5.01^{A}$	$^{b}37.96\pm3.30^{A}$
PMD16 (PCD2)		$^{a}45.76\pm5.18^{B}$	$^{a}59.70\pm4.40^{B}$	$^{a}52.00\pm3.85^{C}$	$^{a}33.18\pm2.21^{A}$	$^{a}39.89\pm2.98^{A}$	$^{a}40.77\pm2.10^{A}$
PMD19 (PCD5)		$^{a}44.30\pm3.87^{B}$	$^{a}53.48\pm2.58^{B}$	$^{a}52.65\pm3.90^{C}$	$^{a}34.67\pm1.01^{A}$	$^{a}38.92\pm1.86^{A}$	$^{a}27.59\pm1.42^{A}$

Table.6.4 Haemocyte protein concentration (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Haemocyte protein(mg/ml)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	5.81±0.56						
PMD7		$^{c}6.60\pm0.76^{C}$	$^{c}7.08\pm0.54^{C}$	$^{c}12.44\pm0.73^{D}$	$^{c}6.05\pm0.50^{B}$	$^{c}4.90\pm0.64^{AB}$	$^{c}4.37\pm0.27^{A}$
PMD14		$^{c}6.26\pm0.49^{C}$	$^{c}7.78\pm0.79^{C}$	$^{c}10.63\pm0.74^{D}$	$^{c}5.37\pm0.75^{B}$	$^{c}4.43\pm0.44^{AB}$	$^{c}4.16\pm0.36^{A}$
PMD16(PCD2)		$^{b}6.96\pm0.69^{C}$	$^{b}8.51\pm0.63^{C}$	$^{b}11.00\pm0.59^{D}$	$^{b}4.53\pm0.30^{B}$	$^{b}3.83\pm0.29^{AB}$	$^{b}4.11\pm0.21^{A}$
PMD19(PCD5)		$^{a}5.80\pm0.81^{C}$	$^{a}7.61\pm0.37^{C}$	$^{a}7.46\pm0.55^{D}$	$^{a}4.14\pm0.12^{B}$	$^{a}3.09\pm0.15^{AB}$	$^{a}3.51\pm0.18^{A}$

Table.6.5 Total free amino acids concentration (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Total Free Amino acids(mg/ml)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	1.65±0.37						
PMD7		$^{c}1.59\pm0.28^{A}$	$^{c}1.68\pm0.17^{A}$	$^{c}1.91\pm0.21^{A}$	$^{c}1.92\pm0.45^{A}$	$^{c}2.43\pm0.35^{A}$	$^{c}2.22\pm0.15^{A}$
PMD14		$^{b}1.79\pm0.26^{A}$	$^{b}1.78\pm0.44^{A}$	$^{b}1.59\pm0.43^{A}$	$^{b}2.34\pm0.30^{A}$	$^{b}1.58\pm0.34^{A}$	$^{b}1.57\pm0.25^{A}$
PMD16 (PCD2)		$^{b}1.65\pm0.32^{A}$	$^{b}2.26\pm0.25^{A}$	$^{b}1.80\pm0.34^{A}$	$^{b}1.46\pm0.12^{A}$	$^{b}1.55\pm0.26^{A}$	$^{b}1.70\pm0.08^{A}$
PMD19 (PCD5)		$^{a}1.61\pm0.21^{A}$	$^{a}1.67\pm0.23^{A}$	$^{a}1.53\pm0.14^{A}$	$^{a}1.39\pm0.20^{A}$	$^{a}1.27\pm0.22^{A}$	$^{a}1.45\pm0.07^{A}$

Table.6.6 Phenol oxidase activity (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Phenol oxidase activity (Increase in OD at 490nm)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	0.66±0.21						
PMD7		$^{c}0.78\pm0.23^{BC}$	$^{c}1.05\pm0.13^{BC}$	$^{c}1.85\pm0.17^{C}$	$^{c}1.04\pm0.17^{B}$	$^{c}1.07\pm0.11^{A}$	$^{c}1.20\pm0.26^{AB}$
PMD14		$^{c}0.67\pm0.27^{BC}$	$^{c}1.26\pm0.18^{BC}$	$^{c}2.40\pm0.16^{C}$	$^{c}0.86\pm0.12^{B}$	$^{c}0.75\pm0.15^{A}$	$^{c}0.85\pm0.23^{AB}$
PMD16 (PCD2)		$^{b}0.66\pm0.33^{BC}$	$^{b}1.99\pm0.24^{BC}$	$^{b}1.42\pm0.14^{C}$	$^{b}0.93\pm0.18^{B}$	$^{b}0.63\pm0.16^{A}$	$^{b}0.55\pm0.13^{AB}$
PMD19 (PCD5)		$^{a}0.83\pm0.19^{BC}$	$^{a}0.57\pm0.09^{BC}$	$^{a}1.28\pm0.37^{C}$	$^{a}0.59\pm0.30^{B}$	$^{a}0.44\pm0.09^{A}$	$^{a}0.45\pm0.17^{AB}$

Table.6.7 Super oxide anion production (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Supper oxide anion production (OD at 620nm)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	0.76±0.18						
PMD7		$^{d}0.81\pm0.16^{B}$	$^{d}0.81\pm0.09^{B}$	$^{d}1.23\pm0.31^{C}$	$^{d}1.11\pm0.24^{C}$	$^{d}2.08\pm0.36^{C}$	$^{d}0.87\pm0.23^{A}$
PMD14		$^{c}0.73\pm0.17^{B}$	$^{c}0.87\pm0.19^{B}$	$^{c}1.28\pm0.28^{C}$	$^{c}1.74\pm0.20^{C}$	$^{c}0.95\pm0.12^{C}$	$^{c}0.37\pm0.10^{A}$
PMD16 (PCD2)		$^{b}0.77\pm0.14^{B}$	$^{b}1.08\pm0.21^{B}$	$^{b}1.04\pm0.06^{C}$	$^{b}0.89\pm0.30^{C}$	$^{b}0.70\pm0.29^{C}$	$^{b}0.44\pm0.11^{A}$
PMD19 (PCD5)		$^{a}0.82\pm0.16^{B}$	$^{a}0.42\pm0.12^{B}$	$^{a}0.93\pm0.12^{C}$	$^{a}0.76\pm0.22^{C}$	$^{a}0.56\pm0.17^{C}$	$^{a}0.30\pm0.07^{A}$

Table.6.8 Alkaline phosphatase activity (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Alkaline phosphatase activity (mg/ml)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	0.38±0.10						
PMD7		$^{b}0.35\pm0.21^{B}$	$^{b}0.33\pm0.13^{B}$	$^{b}1.36\pm0.11^{C}$	$^{b}0.53\pm0.12^{B}$	$^{b}0.43\pm0.21^{A}$	$^{b}0.33\pm0.10^{A}$
PMD14		$^{b}0.41\pm0.11^{B}$	$^{b}0.67\pm0.18^{B}$	$^{b}1.19\pm0.08^{C}$	$^{b}0.91\pm0.19^{B}$	$^{b}0.51\pm0.13^{A}$	$^{b}0.30\pm0.10^{A}$
PMD16 (PCD2)		$^{b}0.38\pm0.09^{B}$	$^{b}1.19\pm0.14^{B}$	$^{b}1.00\pm0.17^{C}$	$^{b}0.71\pm0.08^{B}$	$^{b}0.36\pm0.07^{A}$	$^{b}0.30\pm0.05^{A}$
PMD19 (PCD5)		$^{a}0.42\pm0.14^{B}$	$^{a}0.44\pm0.13^{B}$	$^{a}0.24\pm0.06^{C}$	$^{a}0.15\pm0.02^{B}$	$^{a}0.16\pm0.02^{A}$	$^{a}0.18\pm0.03^{A}$

Table.6.9 Acid phosphatase activity (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Acid phosphatase activity (mg/ml)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	0.43±0.05						
PMD7		$^{c}0.45\pm0.04^{C}$	$^{c}0.42\pm0.09^{C}$	$^{c}0.49\pm0.04^{C}$	$^{c}0.32\pm0.11^{B}$	$^{c}0.24\pm0.15^{A}$	$^{c}0.29\pm0.12^{A}$
PMD14		$^{b}0.42\pm0.09^{C}$	$^{b}0.39\pm0.14^{C}$	$^{b}0.43\pm0.08^{C}$	$^{b}0.34\pm0.09^{B}$	$^{b}0.20\pm0.09^{A}$	$^{b}0.22\pm0.02^{A}$
PMD16 (PCD2)		$^{bc}0.40\pm0.10^{C}$	$^{b}0.59\pm0.12^{C}$	$^{b}0.28\pm0.07^{C}$	$^{b}0.31\pm0.11^{B}$	$^{b}0.34\pm0.04^{A}$	$^{b}0.25\pm0.07^{A}$
PMD19 (PCD5)		$^{a}0.40\pm0.07^{C}$	$^{a}0.30\pm0.13^{C}$	$^{a}0.24\pm0.06^{C}$	$^{a}0.28\pm0.06^{B}$	$^{a}0.13\pm0.02^{A}$	$^{a}0.14\pm0.02^{A}$

Table.6.10 Super oxide dismutase activity (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Super oxide dismutase activity (U/mg protein)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	21.15±2.57						
PMD7		$^{c}20.69\pm1.91^{B}$	$^{c}19.83\pm3.12^{B}$	$^{c}38.09\pm3.82^{C}$	$^{c}24.75\pm5.33^{B}$	$^{c}21.37\pm4.21^{A}$	$^{c}16.74\pm2.86^{}$
PMD14		$^{b}20.13\pm2.57^{B}$	$^{b}22.43\pm2.43^{B}$	$^{b}40.29\pm6.48^{C}$	$^{b}17.40\pm1.16^{B}$	$^{b}14.57\pm2.39^{A}$	$^{b}15.03\pm3.70^{}$
PMD16 (PCD2)		$^{d}20.31\pm2.50^{B}$	$^{d}32.80\pm3.15^{B}$	$^{d}42.33\pm5.39^{C}$	$^{d}29.03\pm3.18^{B}$	$^{d}17.40\pm1.16^{A}$	$^{d}14.57\pm2.39^{}$
PMD19 (PCD5)		$^{a}19.50\pm1.90^{B}$	$^{a}13.53\pm1.92^{B}$	$^{a}31.26\pm2.84^{C}$	$^{a}17.69\pm2.21^{B}$	$^{a}14.51\pm2.24^{A}$	$^{a}12.34\pm3.27^{}$

Table.6.11 Catalase activity (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Catalase activity (U/mg protein)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	65.31±2.94						
PMD7		$^{b}68.81\pm2.79^{B}$	$^{b}69.61\pm6.72^{B}$	$^{b}102.35\pm11.90^{C}$	$^{b}71.08\pm12.59^{B}$	$^{b}64.89\pm4.04^{AB}$	$^{b}67.98\pm8.17^{A}$
PMD14		$^{c}67.87\pm7.57^{B}$	$^{c}68.12\pm3.76^{B}$	$^{c}110.63\pm8.25^{C}$	$^{c}80.32\pm4.80^{B}$	$^{c}84.63\pm4.33^{AB}$	$^{c}78.65\pm14.33^{A}$
PMD16 (PCD2)		$^{c}64.04\pm3.89^{B}$	$^{c}85.23\pm7.46^{B}$	$^{c}126.54\pm12.89^{C}$	$^{c}81.85\pm5.73^{B}$	$^{c}74.41\pm11.90^{AB}$	$^{c}66.95\pm11.79^{AA}$
PMD19 (PCD5)		$^{a}67.46\pm7.04^{B}$	$^{a}41.20\pm2.46^{B}$	$^{a}72.93\pm11.44^{C}$	$^{a}50.10\pm7.98^{B}$	$^{a}46.90\pm8.81^{AB}$	$^{a}38.91\pm4.56^{A}$

Table.6.12 Glutathione peroxidase activity (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

GPx activity (U/mg protein)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	3.74±0.80						
PMD7		$^{b}4.04\pm0.41^{A}$	$^{b}3.83\pm0.55^{A}$	$^{b}8.92\pm1.69^{B}$	$^{b}5.01\pm1.13^{A}$	$^{b}5.52\pm1.40^{A}$	$^{b}4.03\pm0.57^{A}$
PMD14		$^{b}3.82\pm0.80^{A}$	$^{b}3.72\pm0.39^{A}$	$^{b}8.31\pm0.89^{B}$	$^{b}4.79\pm0.28^{A}$	$^{b}4.31\pm0.47^{A}$	$^{b}4.02\pm0.43^{A}$
PMD16 (PCD2)		$^{b}3.84\pm0.80^{A}$	$^{b}5.29\pm0.37^{A}$	$^{b}7.60\pm1.01^{B}$	$^{b}4.40\pm0.52^{A}$	$^{b}4.46\pm0.76^{A}$	$^{b}4.49\pm0.25^{A}$
PMD19 (PCD5)		$^{a}3.72\pm0.76^{A}$	$^{a}2.51\pm0.37^{A}$	$^{a}4.06\pm0.39^{B}$	$^{a}2.41\pm0.26^{A}$	$^{a}2.06\pm0.37^{A}$	$^{a}2.03\pm0.$

Table.6.13 Malondialdehyde content (Mean±S.D.) of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

MDA (m mol/ml)							
Time interval	Copper concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	6.90±0.33						
PMD7		$^{a}6.72\pm0.56^{B}$	$^{a}6.44\pm0.48^{B}$	$^{a}4.74\pm0.78^{A}$	$^{a}9.09\pm1.65^{C}$	$^{a}9.22\pm1.32^{C}$	$^{a}14.72\pm1.25^{D}$
PMD14		$^{b}6.44\pm1.05^{B}$	$^{b}6.57\pm0.65^{B}$	$^{b}5.53\pm1.34^{A}$	$^{b}13.85\pm1.66^{C}$	$^{b}13.34\pm1.98^{C}$	$^{b}15.31\pm1.05^{D}$
PMD16 (PCD2)		$^{c}6.48\pm1.03^{B}$	$^{c}12.88\pm1.61^{B}$	$^{c}6.55\pm1.26^{A}$	$^{c}17.75\pm3.42^{C}$	$^{c}17.98\pm1.05^{C}$	$^{c}20.23\pm3.41^{D}$
PMD19 (PCD5)		$^{d}6.25\pm0.94^{B}$	$^{d}15.34\pm1.05^{B}$	$^{d}9.15\pm1.58^{A}$	$^{d}20.00\pm3.00^{C}$	$^{d}17.08\pm3.01^{C}$	$^{d}23.16\pm2.96^{D}$

Table.6.14 Copper accumulation (Mean±S.D.) in the haemolymph of *F.indicus* when challenged with WSSV at different Cu^{2+} levels. PMD- Post metal exposure day, PCD- post challenge day (See Table.6.1. for statistical details).

Copper accumulation (ppm)							
Time interval	Cu^{2+} concentration(ppm)						
	Baseline	Control	0	0.075	0.15	0.225	0.3
Baseline	0.32±0.09						
PMD7		$^{a}0.34\pm0.07^{A}$	$^{a}0.36\pm0.10^{A}$	$^{a}0.88\pm0.08^{B}$	$^{a}0.85\pm0.18^{C}$	$^{a}0.91\pm0.30^{D}$	$^{a}0.48\pm0.10^{BC}$
PMD14		$^{a}0.33\pm0.08^{A}$	$^{a}0.34\pm0.09^{A}$	$^{a}0.73\pm0.16^{B}$	$^{a}0.90\pm0.19^{C}$	$^{a}0.86\pm0.23^{D}$	$^{a}0.50\pm0.05^{BC}$
PMD16 (PCD2)		$^{a}0.38\pm0.11^{A}$	$^{a}0.50\pm0.10^{A}$	$^{a}0.47\pm0.09^{B}$	$^{a}0.58\pm0.23^{C}$	$^{a}0.78\pm0.04^{D}$	$^{a}0.64\pm0.08^{BC}$
PMD19 (PCD5)		$^{a}0.37\pm0.12^{A}$	$^{a}0.54\pm0.24^{A}$	$^{a}0.34\pm0.04^{B}$	$^{a}0.65\pm0.33^{C}$	$^{a}0.80\pm0.41^{D}$	$^{a}0.73\pm0.37^{BC}$