HAEMATOLOGICAL RESPONSES OF *PENAEUS MONODON* TO ENVIRONMENTAL ALTERATIONS AND PATHOGENIC INVASION

THESIS SUBMITTED TO THE

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In partial fulfillment of the requirements for the degree of

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IN

MICROBIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

BY

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Dedicated to

My Beloved Parents,

My Guide,

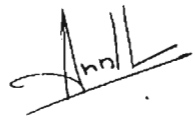
My Husband,

&

My Kids

DECLARATION

I hereby do declare that the thesis entitled **"Haematological responses of *Penaeus monodon* to environmental alterations and pathogenic invasion"** 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, diploma or associateship in any University.



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CERTIFICATE

This is to certify that the thesis entitled **"Haematological responses of *Penaeus monodon* to environmental alterations and pathogenic invasion"** is an authentic record of research work carried out by Mrs. Annies Joseph 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.

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Annies Joseph

CONTENTS

ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometer
ACP	Acid phosphatase activity
ALP	Alkaline phosphatase activity
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BL	Baseline
BMN	Baculoviral midgutgland necrosis
CAT	Catalase
cDNA	Complementary DNA
CDNB	di-Chloro di-nitro Benzene
Ch	Cholesterol
CHH	Crustacean hyperglycemic hormone
DNA	Deoxyribonucleic acid
DOPA	Dihydroxy phenyl alanine
DTNB	Dithio dinitro benzene
EDTA	Ethylene diamine tetraacetic acid
Gl	Glucose
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Oxidized glutathione
GST	Glutathione *S*-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
hp	Horse power
HPV	Hepatopancreatic parvovirus
HSP	Heat shock proteins
IHHN	Infectious hypodermal and haematopoietic necrosis
kDa	Kilo Dalton
LC_{50}	50% Lethal concentration
LPO	Lipid peroxidation
LPs	Lipoproteins
MDA	Malondialdehyde
MT	Metallothioneins
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium salt reduction
OD	Optical density

Abbreviations continued...

PAH	Poly aromatic hydrocarbons
PCB	Polychlorinated biphenyls
PCD	Post challenge day
PCR	Polymerase chain reaction
PO	Phenol oxidase activity
PMD	Post metal exposure day
PMS	Phenazine methosulphate
PL	Post larvae
proPO	proPhenol oxidase
ppA	prophenol oxidase activating enzyme
PSD	Post salinity change day
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
rpm	Revolutions per minute
SD	Standard deviation
SPSS	Statistical package for the social sciences
SOD	Superoxide dismutase
TSV	Taura syndrome virus
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TCBS	Thiosulphate citrate bilesalt sucrose agar
TC	Total carbohydrates
TFAA	Total free amino acids
THC	Total haemocyte count
TL	Total lipids
TP	Total protein
GSH	Total reduced glutathione
WSSV	White spot syndrome virus
YHV	Yellow head virus

CHAPTER 1

General Introduction

1.1 Introduction

Commercial shrimp farming is rapidly expanding due to its high value and demand in the international market. The production of farmed shrimp increased six-fold from 1984 to 1999. The culture of penaeid shrimp that accounted for 20% of the total penaeid production in 1984, increased to almost 50% in the year 1999 (FAO, 2001a). In the Eastern Hemisphere, China, Indonesia, Thailand and Vietnam produce around 300,000 metric tons of farm-raised shrimp a year; Bangladesh, Malaysia and India too have big industries (Rosenberry, 2006). More than 85% of the cultured shrimp production was realized by farmers of Eastern hemisphere in the year 2000 (Rosenberry, 2001). In the Western Hemisphere, Brazil, Ecuador, Peru, Panama and Mexico are the leading producers with the production increasing substantially during recent years. Good statistics on world shrimp farming do not exist. Nevertheless, shrimp farming that started more than a century ago in Southeast Asia is expanding almost everywhere (Rosenberry, 2006).

1.2 Shrimp culture strategies

Extensive, semi-intensive and intensive culture are the three main strategies of shrimp culture being practiced which represent low, medium, and high stocking densities respectively. In extensive culture, the shrimps are stocked at low densities not exceeding 2 m^{-2} in large ponds or tidal enclosures and farmers depend entirely on natural conditions for water exchange and feed with little management. Yields are quite low from extensive ponds. In semi-intensive culture, the stocking densities are higher (5 to 20 post larvae {PL} m^{-2}) and the farmer adds supplemental feeds, and takes management effort to maintain water quality, including pumping to exchange water. Sometimes, aeration and water mixing are also necessary (Fig.1.1). The most technologically advanced culture systems are intensive that is carried out in very high densities (>200 PLs m^{-2}) in intensively managed pens, ponds, tanks and raceways. A high level of investment is required in intensive culture for providing the shrimps with a nutritionally complete ration and for installing pumps and mechanical devices to allow for high rates of water exchange and to circulate and aerate the water (Lee and Wickins, 1992; Rosenberry, 2001). With increase in production costs, the yield from semi-intensive and intensive culture systems is proportionately higher.

Fig.1.1 A semi-intensive shrimp culture pond

1.3 Cultured shrimp species

The commonly cultured penaeid shrimp species include the giant black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*Litopenaeus vannamei*), kuruma shrimp (*Metapenaeus japonicus*), blue shrimp (*P. stylirostris*) and Chinese white shrimp (*Fenneropenaeus chinensis*). *P. monodon* stands out as the most important species cultured.

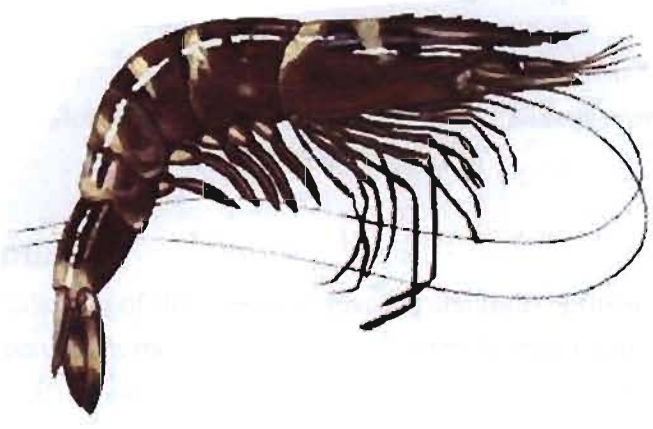


Fig.1.2 *Penaeus monodon*

1.3.1 Penaeus monodon

The Giant tiger shrimp, *P. monodon*, (Fig. 1.2) named for its huge size and banded tail, providing a tiger-striped appearance, accounted for more than 50% of the production in 1999 (FAO, 2001b). The major producers of *P. monodon* include Thailand, Vietnam, Indonesia, India, Philippines, Malaysia and Myanmar. Reaching a maximum length of 330 mm or more in body length and 45 g in weight, *P. monodon* is the largest and fastest growing of the farm-raised shrimp (Lee and Wickins, 1992). *P. monodon* can reach a market size of up to 25-30 g within 3-4 months after the stocking of post larvae (PL) in culture ponds and tolerates a wide range of salinities (Rosenberry, 1997). *P. monodon* mature and breed only in tropical marine habitats, and the larvae develop in sea water where the salinity does not vary greatly. The post larvae and juveniles migrate to shallow water and inhabit inshore area until the pre-adults return to sea (Chen, 1990). Total aquaculture production of *P. monodon* sharply increased from 200, 000 tonnes in 1988 to nearly 500, 000 tonnes with a value of US$ 3.2 billion in 1993. Since then, the production has been quite variable (FAO, 2007) (Fig.1.3).

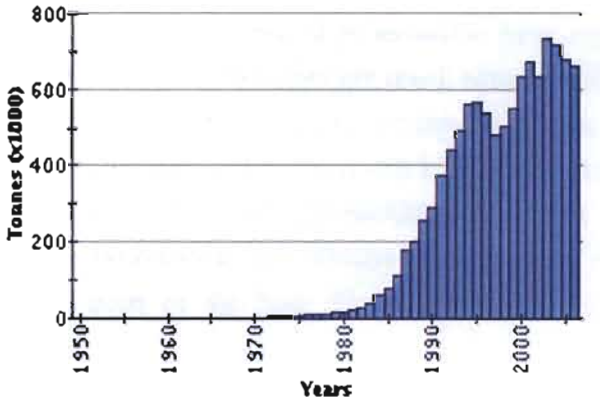


Fig.1.3 Global aquaculture production of *Penaeus monodon*
(FAO Fishery Statistics)

1.4 Major constraints

Frequent outbreaks of diseases that have substantially influenced the profitability of shrimp culture constitute the main barrier to the development and continuation of this industry. Although *P. monodon* was normally considered as exceptionally tough, the rapid growth and intensification of its culture industry generated crowding and increased environmental degradation, which made the animals more susceptible to diseases

(Lightner, 1983; Johnson, 1989). White spot syndrome virus (WSSV) has been causing great havoc by producing highly devastating epidemics in shrimps. Other viral threats include infectious hypodermal and haematopoietic necrosis (IHHN) virus, hepatopancreatic parvovirus (HPV), baculoviral midgutgland necrosis (BMN) virus, Taura syndrome virus (TSV), yellow head virus (YHV) etc. (Lightner, 1996; Briggs *et al.*, 2004). Penaeid shrimp culture has also suffered problems linked to infectious diseases caused by bacteria such as *Vibrio harveyi*, *V. damsela*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. splendidus*, and *V. anguillarum* (Lightner, 1988; Lavilla-Pitogo *et al.*, 1990; Song *et al.*, 1990; Ruangpan and Kitao, 1991; Song *et al.*, 1993; Karunasagar *et al.*, 1994; Lee *et al.*, 1996;) Luminous vibriosis often caused by *V. harveyi* is a major constraint in shrimp production (Lightner *et al.*, 1992). Occurrence of diseases is mostly unpredictable, leading to 100% mortality and significant economic loss. The prevention and control of diseases have hence gained priority for an economically viable shrimp production.

The production of *P. monodon* has been unofficially reported to have declined since 2002 mainly due to the problems caused by viral disease outbreaks apart from other causes like shortages of broodstock, market competition and trade barriers (FAO, 2007). In addition, many farmers that originally reared *P. monodon* have replaced this species with *L. vannamei*, for which culture technologies are much simpler and disease problems are less severe, particularly for culture in inland freshwater ponds. The price for *P. monodon* in the export market has also fallen but is still higher than that for *L. vannamei*. Promoting domestic consumption to avoid the problematic export markets, efficient development of disease-free broodstock and effective treatment of shrimp pathogens, particularly viruses are the need of the hour for improving the sustainability of *P. monodon* production.

1.5 Prevention and Control of Diseases

Diseases are often linked to the environmental deterioration and stress associated with the intensification of shrimp farming. Poor water quality, high animal densities, low oxygen content, rapid changes in temperature, pH and salinity, inadequate nutrition etc. are some of the major stressors contributing to the spread of diseases in shrimp aquaculture. Maintaining a healthy stock therefore requires a multidisciplinary approach and should include stress reduction and disease control. Minimizing stress in shrimp culture systems by reducing the stocking density, by providing adequate nutrition and through better water quality management can certainly reduce the risk of occurrence of

infectious diseases. Disease control in fact, depends on a complex of three factors: diagnosis, treatment and preventive measures (Sindermann and Lightner, 1988).

Immunostimulants and probiotics are two promising options to aid in disease control. Immunostimulants increase disease resistance by causing up-regulation of the non-specific defense mechanisms of the host against pathogenic microorganisms. Immunostimulants that have received maximum attention are the glucan of yeast cell wall, peptidoglycans and lipopolysaccharides of certain bacteria. Probiotics are live microbial or cultured product feed supplements, which beneficially affect the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating the immune function and improving the microbial balance (Fuller, 1989; Verchuere *et al.*, 2000). Several alternative elicitors such as vitamins and minerals have also been applied successfully in aquaculture for disease control (Robertson *et al.*, 1994).

In addition, periodic assessment of the health and immune status of shrimps would be an extremely valuable tool in managing shrimp culture ponds. This simple technique can provide essential information on the physiological status of animals and therefore help the aquaculturists to take proper prophylactic measures. Apart from the classical techniques and DNA based technologies, haematology, one of the principal diagnostic tools of human and veterinary medicine has potential to be used as a diagnostic tool in penaeid shrimp pathology.

1.6 Significance of Haemolymph

Many fundamental features of class Crustacea are reflected in the nature of their internal medium, the haemolymph. Haemolymph is an important component involved in the respiration, digestion and defense mechanism of shrimps. It is an important medium for the transportation of ions and molecules involved in energy metabolism. Haemolymph composition of shrimps provides an insight into the physiological modifications associated with molting process, reproductive cycles, developmental changes and environmental stress. Haemolymph is infact a carrier of every kind of biochemical constituent from one part of the body to the other. Any alteration in the physico-chemical characteristics of the environment will be reflected in the composition of haemolymph.

1.6.1 Metabolic indices in Shrimp Haemolymph

Potential molecular and biochemical indicators in haemolymph are suitable for evaluating stress, as stress classically leads to the rapid onset of a cascade of molecular and physiological responses. Stress is defined as a condition in which the dynamic equilibrium of an organism (homeostasis) is threatened or disturbed as a result of intrinsic or extrinsic stimuli (stressors) (Chrousos and Gold, 1992). However, scarce information is available regarding the physiological responses of shrimps in the event of environmental variations and how it could affect the immune status of shrimps. Several metabolic constituents in haemolymph including protein, glucose, cholesterol, triacylglycerols, oxyhaemocyanin, lactate etc. have been suggested as suitable for evaluating the physiological status in shrimps (Palacios, 2000; Sanchez *et al.*, 2001; Pascual *et al.*, 2003a, b).

Proteins play an important role in ensuring better disease resistance in shrimps as the shrimp immune system has a solid protein base. Proteins are involved in recognizing foreign glucans through the lipopolysaccharide binding protein and ß-1, 3-glucan binding protein (Vargas-Albores and Yepiz-Plascencia, 2000). A clotting protein is involved in engulfing foreign invading organisms and prevents blood loss upon wounding (Hall *et al.*, 1999). proPhenol oxidase (proPO) activating system (Sritunyalucksana and Soderhall, 2000) is regulated by a number of proteins. Antimicrobial peptides are produced against Gram-positive bacteria (Destoumieux *et al.*, 2000) and haemocyanin is a multifunctional protein with both nutritional and immunological roles (Chen and Cheng, 1995; Rosas *et al.*, 2002) and a precursor of proPO like enzyme (Adachi *et al.*, 2003)

Carbohydrates are important sources of energy but simple sugars like glucose are poorly utilized by shrimps compared to complex ones (Andrews *et al.*, 1972). Metabolic pools of free amino acids are known to play a major role in osmoregulation of marine invertebrtates. Glycine, proline and alanine are considered to function as specific osmotic effectors in a number of crustacean species (Dalla Via, 1986, Lang, 1987; Dalla Via, 1989; Huong *et al.*, 2001). Lipids are very important macromolecules for all living organisms including crustaceans since they are the major sources of energy and provide essential components for membranes. In addition, some lipids are essential nutrients to be provided in the diet. These essential lipids include cholesterol and polyunsaturated fatty acids that are important for proper growth and maturation. Cholesterol has a crucial role in moulting process and is important in maintaining the integrity and chemical permeability of cell walls. Lipids are also found in the haemolymph as water-soluble

molecules formed by apoproteins and lipid moieties constituting the lipoproteins (LPs). LPs transport lipids from sites of absorption, storage or synthesis to sites of utilization.

1.6.2 Immune Effectors in Shrimp Haemolymph

Primary immune response in crustaceans is non-specific (Anderson, 1992) that is chiefly related to their haemolymph and to its circulating cells or haemocytes. Shrimps lack an adaptive immune response and rely on innate immune responses against microbial invasion. Haemocytes play a crucial role in immune response by their participation in various cellular defense functions such as phagocytosis, encapsulation, nodule formation, formation of reactive oxygen intermediates etc. (Johansson and Soderhall, 1989; Bachere *et al.*, 1995). Three types of circulating haemocytes have been recognized in Decapod crustaceans: hyaline cells, semigranular cells and large granular cells (Martin and Graves, 1985; Tsing *et al.*, 1989) each with distinct morphological features and physiological functions (Johansson *et al.*, 2000). The smallest are the hyaline cells that lack distinctive granules (Soderhall *et al.*, 1986). Semigranular cells contain small granules whereas the granular cells contain large granules.

Phagocytosis is the most common cellular defense reaction and in combination with humoral components, constitutes the first line of defense against parasites or other intruders that evade the physico-chemical barrier of the cuticle. Hyaline cells carry out phagocytosis. Phagocytes produce lysosomal enzymes which efficiently degrade and remove foreign material. Alpha-naphthyl acetate esterase, ß-glucuronidase and acid and alkaline phosphatases are some of the phagocytosis-related lysosomal enzymes (Sung and Sun, 1999). When invaded by a large number of microorganisms, nodule formation occurs, whereby the microorganisms become entrapped in several layers of haemocytes and normally the nodule becomes heavily melanised. Haemocytes are also capable of immobilizing parasites that are too large to be removed by a single cell by the process of encapsulation wherein several haemocytes form multicellular sheaths, sealing off the parasite from circulation (Soderhall and Cerenius, 1992). Semigranular cells that contain small granules display some phagocytic activity but are the principal cells involved in encapsulation reactions (Persson *et al.*, 1987).

Several reactive oxygen species are produced during phagocytosis. Beginning this process, the membrane-bound enzyme complex, NADPH oxidase gets activated on binding of the cell to a foreign particle, which in turn increases oxygen consumption and reduces molecular oxygen to superoxide anion (O_2^-), subsequently leading to the production of hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radicals (OH^-)

and numerous other reactive compounds (Munoz *et al.*, 2000). These short-lived compounds can be directly toxic to pathogens, act in concert with hypohalides and halidamines generated by peroxidases, or exert synergistic effects with several lysosomal enzymes (Roch, 1999). This phenomenon is known as respiratory burst and the associated oxidative killing plays an important role in microbicidal activity.

Although reactive oxygen intermediates (ROIs) play an especially important role in host defense, host cells can be damaged by the over-expression of ROIs. Most cells have acquired the relevant antioxidant protective mechanisms to maintain the lowest possible levels of ROIs inside the cell, including superoxide dismutase, catalase and glutathione peroxidase.

Haemocytes are also involved in the carriage and release of the prophenol oxidase (proPO) activating system (Bayne, 1990; Soderhall and Cerenius, 1998) that is responsible, for the non-self-recognition process of the defense mechanism (Vargas-Albores, 1995; Hernandez-lopez *et al.*, 1996). Activation of proPO system results in the production of various proteins including phenol oxidase, which participates in melanisation around the parasite, coagulation, opsonisation of foreign materials and direct microbial killing (Soderhall and Hall, 1984). Both semigranular and granular cells carry out the functions of proPO system by a degranulation process (Johansson and Soderhall, 1985). proPO activating system is initiated by minute amounts of lipopolysaccharides or peptidoglycans from bacteria and ß- 1, 3- glucans from fungi through pattern-recognition proteins. proPO, the inactive proenzyme located inside the haemocytes is converted to phenoloxidase by an endogenous trypsin-like serine protease, the so-called prophenol oxidase activating enzyme (ppA). Phenol oxidase catalyses hydroxylation of monophenol to diphenol and oxidation of diphenol to quinones, subsequently leading to melanin synthesis (Lee and Soderhall, 2002; Cerenius and Soderhall, 2004). Regulation of the proPO system in crustaceans is subject to certain protease inhibitors (Johansson and Soderhall, 1989; Aspan *et al.*, 1990), such as α_2-macroglobulin (Hergenhahn and Soderhall, 1985; Hergenhahn *et al.*, 1988) and a 155 kDa trypsin inhibitor named pacifastin detected and purified in crayfish (Hergenhahn *et al.*, 1987).

Humoral factors such as plasma lectins (Marques and Barracco, 2000), and proteins involved in haemolymph coagulation (Sritunyalucksana and Soderhall, 2000) have also been characterized in various crustaceans. The innate immune response of shrimp also relies upon the production, in haemocytes, of antimicrobial peptides called

penaeidins that are active against a large number of pathogens essentially directed against Gram-positive bacteria (Destoumieux *et al.*, 2000).

1.7 Relevance of the present study

For further development of improved disease prevention and management practices, more information has to be acquired regarding the defense responses of shrimps and other physiological and biochemical alterations induced by environmental stress conditions and pathogens. Though there is a developing awareness that diseases are linked to environmental changes (Le Moullac and Haffner, 2000), scientific data supporting the link between environmental stress and increased susceptibility to diseases in shrimps are scarce. Since stress responses are well reflected in the composition of haemolymph, analysis of haematological responses may provide a better understanding in this regard. Purpose of the present study was to evaluate the effects of various environmental alterations on *P. monodon* especially in the event of an infection, by analyzing the haematological responses. Several metabolic variables in haemolymph *viz.*, total protein, total carbohydrates, total free amino acids (TFAA), total lipids, glucose and cholesterol and several immune variables *viz.*, total haemocyte count (THC), phenol oxidase activity (PO), nitroblue tetrazolium salt reduction (NBT), alkaline phosphatase activity (ALP) and acid phosphatase activity (ACP) were determined to study the haematological responses. The study aimed to elaborate the knowledge of the roles played by these haematological parameters in determining the physiological and immune condition of the shrimp. The potential use of these parameters as health status indicators is also discussed.

1.8 Objectives

The present study on the haematological responses of *P. monodon* was undertaken with the following objectives.

- To acquire information on the metabolic and immune responses of *P. monodon* to *V. harveyi* and WSSV infection.

- To assess the influence of salinity on the metabolic and immune responses and susceptibility of *P. monodon* to *Vibrio harveyi* and WSSV infection.

- To assess the modulatory effects of ambient copper and zinc on the metabolic and immune responses and susceptibility of *P. monodon* to WSSV infection.

- To assess the modulatory effects of ambient copper and zinc on the antioxidative defense responses of *P. monodon*.

- To identify the most potential haematological biomarkers in *P. monodon*.

1.9 Outline of the thesis

The thesis is presented in 8 chapters. Chapter 2 elucidates the haematological responses of *P. monodon* in the event of *V. harveyi* infection and how acute salinity variations influence the responses and susceptibility. The haematological responses of *P. monodon* to white spot syndrome virus (WSSV) infection is dealt with in chapter 3 with special emphasis on the influence of acute salinity stress on the responses and susceptibility. Chapter 3 and 4 elucidates the haematological responses and susceptibility of *P. monodon* to WSSV infection under the influence of increasing ambient concentrations of essential minerals copper and zinc respectively. The antioxidative defense responses of *P. monodon* in the event of WSSV infection and the modulatory effects of ambient copper and zinc are detailed in chapter 6. In chapter 7, an attempt to identify the most potential haematological biomarkers of health in *P. monodon* is done by elucidating the relationships between survival rate and haematological parameters through correlation and regression analysis. The present research work is summarized in chapter 8 with special emphasis on salient findings of the study. Future prospects of the work are also discussed. This is followed by a list of references and appendices.

CHAPTER 2

Effect of Acute Salinity Stress on the Haematological Responses and Susceptibility of Penaeus monodon to Vibrio harveyi Infection

2.1 Introduction

Penaeid shrimp culture during the past fifteen years has been badly hit by an epidemic of infectious diseases caused by bacteria such as *Vibrio harveyi* (Karunasagar *et al.*, 1994), *V. damsela* (Song *et al.*, 1993), *V. parahaemolyticus* (Ruangpan and Kitao, 1991) and *V. alginolyticus* (Lee *et al.*, 1996) and that caused by viruses such as white spot syndrome virus (WSSV), monodon baculovirus (MBV), yellow head virus (YHV), infectious hypodermal and haematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) (Chang *et al.*, 2003). Susceptibitlity is often intensified by the highly stressful environment in culture systems.

Vibrio spp. are by far the major bacterial pathogens of shrimps, responsible for a group of diseases such as shell disease, luminous bacterial disease and bacterial septicemia. *Vibrio* spp. are Gram-negative, motile, and straight or curved, rod-shaped, facultative anaerobes. They form part of the indigenous microflora of aquatic habitats of varying salinity (Colwell, 1984). They have been isolated from all major shrimp culture systems. *V. harveyi,* the species commonly present in various marine and brackish water habitats is the major causative agent of luminous vibriosis. It is the predominant species found in coastal waters though several species of luminous bacteria are common in the open sea (Reichelt and Baumann, 1973). *V. harveyi* was isolated as the dominant luminous species from commercial penaeid shrimp hatcheries in Tamil Nadu (Abraham and Palaniappan, 2004).

Occurrence of vibriosis has been associated with a large increase in the number of pathogenic *Vibrio* spp. in the cultured pond waters (Sung *et al.*, 2001). Sung *et al.* (1999) observed a tremendous increase in the number of *V. harveyi* in the hepatopancreas of diseased shrimps and in pond water where there had been an outbreak of vibriosis. Vibriosis is often considered to be a secondary infection as most vibrios act as opportunistic pathogens to shrimps causing mortalities when other physiological or environmental factors become adverse (Lightner *et al.*, 1992). Harish *et al.* (2003) could observe a high prevalence of opportunistic pathogens such as *Vibrio*, *Aeromonas* and *Pseudomonas* in the paddy cum shrimp farms adjoining the Vembanad Lake with no

noticeable disease outbreaks probably due to the stable nature of the physico-chemical parameters in the system.

Virulence of pathogenic microbes is another important factor leading to disease outbreaks. Variations in the inoculum dosages required to cause mortality from experimental *Vibrio* infections as reported by several authors (Lavilla-Pitogo *et al.*, 1990; Sae-Qui *et al.*, 1987) may be due to difference in the virulence of the strains. Advanced post larvae of *Fenneropenaeus indicus* challenged with *V. harveyi* inoculum levels of 10^3, 10^4 and 10^5 cfu ml^{-1} for 96 h did not show luminescence or other clinical signs of luminous vibriosis (Pillai and Jayabalan, 1993). On the other hand, significant mortalities of zoea, mysis, and post larvae of *P. monodon* within 48 h of challenge with an inoculum level as low as 10^2 cfu ml^{-1} has been reported by Lavilla-Pitogo *et al.* (1990). Fluctuations in environmental parameters such as dissolved oxygen, salinity, temperature and pH have significant effects on the virulence of *V. harveyi* to penaeid shrimp. At high salinities, *V. harveyi* is more lethal to penaeid shrimp than at high temperatures (Kautsky *et al.*, 2000). Exposure to *V. harveyi* at pH 5.5 for 12 h before use in immersion challenge tests with *P. monodon* larvae resulted in lower mortality than that at pH 6.0, 7.2 and 9.0 (Prayitno and Latchford, 1995). Williams and LaRock (1985) showed that in marine environment, *V. parahaemolyticus* and *V. vulnificus* predominated in spring and summer, while *V. cholerae* and *V. alginolyticus* predominated in the late summer and fall. There are reports of luminous bacterial diseases during rainy seasons (Sunaryanto and Mariam, 1986).

Salinity is one of the fundamental environmental factors affecting marine shrimp culture. It modifies physiological responses of aquatic organisms such as the metabolism, growth, tolerance, life cycle, nutrition and the intra- and inter-specific relationships. (Fry, 1971; Kinne, 1971; Venkataramiah *et al.*, 1974). Marine organisms that regulate the osmolality of body fluids encounter the dual problems of internal dilution at low salinities and concentration of body fluids at high salinities (Castille and Lawrence, 1981). Maximum growth of an organism occurs in the isosmotic media, since the animal would be expending minimum amount of energy in osmoregulation (Panikkar, 1968). The capacity of shrimps to adapt to varying salinity is a major factor determining survival (Ferraris *et al.*, 1987). According to Diwan *et al.* (1989) adult *P. monodon* could osmoregulate well between salinities 3‰ and 45‰ for 24 and 48 h duration with isosmotic points around 18.5‰ and 23.5‰ respectively and a duration of 48 h is essential for the prawns to adjust to the new medium. Under high salinities free amino acid concentrations increased dramatically in the fresh water prawn *Macrobrachium*

rosenbergii (Huong *et al.*, 2001). Haemolymph urea and uric acid level increased significantly in hyposmotic conditions in *Marsupenaeus japonicus* (Lee and Chen, 2003). THC and PO markedly decreased in *Farfantepenaeus paulensis* at low salinity (Perazzolo *et al.*, 2002).

Establishment of an infection may not only be due to an increase in the number of pathogens but also may be highly dependent on the physiological state of the animals (Roque *et al.*, 2005). Recent evidences of immune depression and metabolic changes in response to environmental changes in shrimps rendering them more susceptible to pathogens are available. Concentration of oxyhaemocyanin, acylglycerol and cholesterol decreased significantly when *Litopenaeus vannamei* were exposed to high levels of ambient ammonia (Racotta and Hernandez-Herrera, 2000). *P. monodon* transferred from 26°C to 22°C and 34°C suffered a decrease in the THC, PO activity, respiratory burst activity and super oxide dismutase activity (Wang and Chen, 2006a). Hypoxia induced a significant decrease in THC and NBT reduction and increased the susceptibility of *P. stylirostris* to *V. alginolyticus* (Le Moullac *et al.*, 1998). The immune ability of *L. vannamei* was reduced by high levels of nitrite (Tseng and Chen, 2004) in water, with increased mortality from *V. alginolyticus* infection.

Tiger shrimp *P. monodon*, the most important shrimp species currently being cultured in many countries is a marine euryhaline form having a wide salinity tolerance ranging from 1 ‰ to 57 ‰ (Chen, 1990). *P. monodon* with an iso-osmotic point of 750 mOsM kg^{-1} (equivalent to 25‰) exhibits hyper-osmotic regulation in low salinity levels, and hypo-osmotic regulation in high salinity levels (Cheng and Liao, 1986). Salinity level suitable for the growth of this species is in the range of 10‰ to 35‰ (Liao, 1986). However, it is very often cultured at a salinity range of 10‰ to 20‰, as they are believed to exhibit better growth in brackish water than in pure seawater under culture conditions (Fang *et al.*, 1992). Although penaeids are very potent hyper-hypo osmoregulators and they maintain relatively constant internal osmolality when exposed to varying salinities, salinity changes may cause stress to the animals. It is possible that acute salinity changes over a particular range weaken the immune system of shrimp and make them highly vulnerable to opportunistic pathogens like *V. harveyi*. Drastic salinity changes may also affect the feed intake, metabolism and higher energy utilization for osmoregulation resulting in poor growth. There are however, no reports on the effects of salinity on the susceptibility of shrimps to *V. harveyi* infection.

The present study on *P. monodon* was therefore aimed at determining the:

- Effect of acute salinity change on the metabolic and immune variables of haemolymph
- Effect of acute salinity stress on the susceptibility to *V. harveyi* infection
- Effect of *V. harveyi* infection on the haemolymph metabolic variables and immune response of shrimps maintained at optimal salinity and those subjected to acute salinity stress.

2.2 Materials and methods

2.2.1 Experimental animals

Adult *P. monodon* (PCR negative for WSSV) obtained from a commercial farm in Panangad, Kochi were the experimental shrimps used in the present study. They were transported to the laboratory within one hour of capture. Average wet weight of the shrimp was 18.4 ± 2.6g (Mean ± S.D.).

Table2.1 Rearing conditions and water quality

Stocking density	30 shrimps/tank
Tank capacity	500 L
Feeding level	10-15% body weight
Feeding frequency	twice daily
Water temperature	24-27°C
pH	7.5-8.0
Salinity	15‰-18‰
NH_3-N	0.01-0.02 mgl^{-1}
NO_3-N	below detectable level
NO_2-N	0.00-0.01 mgl^{-1}
Dissolved oxygen	6-7 mgl^{-1}

2.2.2 Acclimation and maintenance

The shrimps were reared in rectangular concrete tanks containing 15‰ sea water and allowed to acclimate for a week. Continuous aeration was provided from a 1 h. p. compressor through air stones. They were fed on a commercial shrimp diet (Higashimaru, Kochi). Water quality parameters *viz.*, salinity, 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. The unused feed and faecal matter was siphoned out daily and 25% water exchanged every second day. A biological filter was set up to maintain the appropriate levels of water quality parameters. After acclimation

for a period of seven days, the metabolic and immunological profile was obtained from a group of shrimps (n=6) as the baseline (BL) data.

2.2.3 Experimental design

Shrimps were distributed in the experimental tanks containing 500L of seawater with 30 individuals per tank (n=30/tank) (Fig.2.1, 2.2). There were 4 treatments (Group-I, Group-II, Group-III and Group-IV) and the experiment was conducted in triplicate i.e., 3 tanks per treatment. Salinity of all the tanks was adjusted to 15‰ prior to the experiment. Shrimps in the intermoult stage only were used. The moult stage was recognized by the observation of uropoda in which partial retraction of the epidermis can be distinguished (Robertson *et al.*, 1987).

2.2.4 Salinity stress

Shrimps were maintained in the experimental tanks at 15‰ for two days. The Group-II and Group-IV shrimps were then subjected to sudden salinity changes. Shrimps were starved for 12 hours prior to salinity change. The salinity of Group-II was lowered from 15‰ to 5‰ by diluting with fresh water. Whereas, the salinity of Group-IV was raised from 15‰ to 35‰ by adding sea water. The desired salinity was adjusted over a period of seven hours. Shrimps of Group-I and Group-III was maintained at 15‰ itself with no salinity change. Ten minutes after the desired salinity level was reached, 6 prawns from each group (n=6) were sampled (post salinity change day 0, PSD0).

2.2.5 Challenge with Vibrio harveyi

V. harveyi strain MCCB111 isolated from diseased larvae obtained from a prawn hatchery in Kochi and maintained at the National Center for Aquatic Animal Health, CUSAT, Kochi was used for the study. *V. harveyi* was cultured on thiosulphate citrate bilesalt sucrose (TCBS) agar medium (Lightner, 1983) prepared in 25‰ sea water and then restreaked on prawn flesh agar medium (Singh and Philip, 1993) to improve its virulence. Bacterial suspension containing 5×10^8 cfu ml^{-1} (colony forming units ml^{-1}) was used for the study.

In order to assess the influence of salinity stress on susceptibility to *V. harveyi* infection, the shrimps of Group-II, Group-III and Group-IV were challenged with *V. harveyi* ten minutes after the desired salinity level was reached. The challenge was performed through intramuscular injection. The shrimps were injected with 25µl (1.25×10^7 cfu/shrimp) of *V. harveyi* suspension. Group-I was maintained as the unchallenged control. Shrimps were sampled (n=6) after 48 h (post challenge day 2, PCD2), 7 days

(post challenge day 7, PCD7), and 10 days of challenge (post challenge day 10, PCD10). Sampling days were fixed based on the rate of mortality that occurred. 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.

2.2.6 Extraction of haemolymph

Anticoagulant for haemolymph extraction was prepared by adding 10mM EDTA-Na_2 salt to the Shrimp Salt Solution (45 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3, 850 mOsM kg^{-1}, Vargas-Albores *et al.*, 1993). Haemolymph was withdrawn aseptically from the rostral sinus using specially designed sterile capillary tubes of diameter 0.5 mm, rinsed thoroughly with pre-cooled anticoagulant. The region near the rostrum was cleaned with sterile distilled water and wiped with sterile cotton swabs before inserting the capillary tube. The samples were transferred to sterile eppendorf vials containing pre-cooled anticoagulant. The haemolymph collected from six shrimps (n=6) of each treatment group was analysed separately. Sampling was carried out at the beginning of the experiment (baseline), on post salinity change day 0 (PSD0) and post challenge day 2, 7 and 10 from the four experimental groups (Group-I, Group-II, Group-III and Group-IV). The immune parameters were analysed immediately and the samples were stored at –20°C for the analysis of metabolic variables.

2.2.7 Analysis of haematological parameters

The following haematological parameters were analysed:

Metabolic variables

- Total protein
- Total carbohydrates
- Total free amino acids (TFAA)
- Total lipids
- Glucose
- Cholesterol

Immune variables

- Total haemocyte count (THC)
- Phenol oxidase activity (PO)
- Nitroblue tetrazolium salt reduction (NBT)
- Alkaline phosphatase activity (ALP)
- Acid phosphatase activity (ACP)

2.2.7a Biochemical assays

2.2.7a.1 Total protein

Protein determination was done employing the Bradford (1976) method using Coomassie Brilliant Blue G-250. Red form of the dye is converted to the blue form upon binding to protein. Binding of the protein is a very rapid process and the protein-dye complex has a high extinction co-efficient.

Reagents

1. Protein reagent- 100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml of 95% ethanol. Added 100 ml orthophosphoric acid and diluted to 1 litre with distilled water.
2. 1 N NaOH
3. Bovine Serum Albumin- 10 mg in 10 ml of 1 N NaOH

Assay

A sample of 100 µl haemolymph was centrifuged with 900 µl of 80% ethanol. The protein precipitated out was dissolved in 1ml of 1 N NaOH and diluted further (1: 100). To 100 µl of the diluted sample was then added 5 ml of protein reagent and the absorbance at 595 nm was read after 2 minutes and before 1 hour in a U-V Visible spectrophotometer (Hitachi, U 2001). A series of standards were also run using bovine serum albumin and concentration of the sample was determined. The concentration of total protein was expressed in mg ml^{-1} haemolymph.

2.2.7a.2 Total carbohydrates

Anthrone method was used for the determination of total carbohydrates. It is based on the reaction of anthrone with furfural or furfural derivatives (formed by the hydrolysis of oligo and polysaccharides into monosaccharides and subsequent dehydration) to produce a complex blue-green coloured product with absorption maxima at 620 nm (Hedge and Hofreiter, 1962).

Reagents

1. Anthrone reagent- 0.2 g anthrone dissolved in 100 ml Conc. H_2SO_4.
2. 80% Ethanol
3. Glucose standard- 10 mg in 10 ml of double distilled water.

Assay

To a sample of 100 µl haemolymph was added 900 µl of 80% ethanol and centrifuged. 100 µl of the protein-free supernatant was taken in a tube and 5 ml of anthrone reagent was added and mixed. The tubes were placed in boiling water bath for 15 minutes and cooled in dark. The colour was read at 620 nm against a blank. A series

of standards using different concentrations of glucose was also run to determine the concentration of the sample. The value was expressed in mg ml^{-1} haemolymph.

2.2.7a.3 Total free amino acids

Total free amino acids were determined using the ninhydrin method by Yemm and Cocking (1955).

Reagents

1. Citrate buffer- pH 5.0
2. 70% Ethanol
3. Ninhydrin- 1% in methyl cellosolve

Assay

A sample of 100 µl haemolymph was centrifuged with 900 µl of 80% ethanol and the protein precipitated out. 100 µl of the supernatant was taken in a tube and 0.5 ml of citrate buffer and 1.2 ml of ninhydrin reagent was added. The reaction mixture was boiled at 100°C for 15 minutes. After cooling in running tap water for 10 minutes, 2.3 ml of 70% ethanol was added and the absorbance read at 570 nm against a blank. A mixture of Glycine and Glutamic acid was used as the standard. The concentration was then expressed in mg ml^{-1} haemolymph.

2.2.7a.4 Total lipids

Lipids were determined using the sulphophosphovanillin method by Barnes and Blackstock (1973).

Reagents

1. 2:1 Chloroform-methanol solution
2. 0.9% NaCl
3. Phosphovanillin reagent- added 400 ml of orthophosphoric acid to 100 ml of distilled water and 1 g vanillin dissolved in that solution.
4. Cholesterol standard- 10 mg in 10 ml of 2:1 chloroform-methanol

Assay

To a sample of 100 µl haemolymph was added 1 ml of Chloroform-methanol solution and mixed well. After adding 0.2 ml of NaCl solution it was allowed to stand overnight at 4°C. The upper layer was carefully removed from the bottom layer formed. Volume of the lower phase that contained all the lipids was adjusted to 1 ml by adding chloroform. 0.5 ml of the extract was taken into a clean tube and allowed to dry in vaccum dessicator over silica gel. Then it was dissolved in 0.5 ml conc. H_2SO_4 and mixed well. The tube was then placed in boiling water for 10 minutes. After cooling the tubes to room temperature, 0.2 ml of the acid digest was taken in a separate tube and 5 ml of

vanillin reagent added, mixed and allowed to stand for half an hour. The colour developed was measured at 520 nm. A series of standards were also run using cholesterol as standard and the concentration of samples were determined. The values were expressed in mg ml^{-1} haemolymph.

2.2.7a.5 Glucose

Glucose was determined by the method of Marks (1959).

Reagents

1. 0.9% NaCl
2. $ZnSO_4.7H_2O$ solution- 5%
3. 0.3 N NaOH
4. Fermcozyme- a stable liquid preparation of glucose oxidase containing 750 units ml^{-1}.
5. Glucose oxidase reagent- added 0.5 ml of Fermcozyme to 80 ml of acetate buffer (pH 5) and to that was added 5 ml of peroxidase solution (20 mg $100ml^{-1}$ of acetate buffer), mixed and added 1 ml of toluidine (1% in absolute ethanol). This was made up to 100 ml with buffer and stored in refrigerator in a dark bottle.

Assay

A sample of 100 µl haemolymph was added to 0.4 ml of 5% $ZnSO_4.7H_2O$ solution, 0.4 ml of 0.3 N NaOH and 1.1 ml of 0.9% NaCl and mixed well. Supernatant was separated after centrifuging and 1.0 ml was transferred to a test tube. 1.0 ml of water and 1.0 ml of standard glucose solution was measured into 2 other test tubes. 3.0 ml of glucose oxidase reagent was added to each at half-minute intervals, mixed gently for ten seconds and read the colour developed after 10 minutes at 625 nm. The values were expressed as mg ml^{-1} haemolymph.

2.2.7a.6 Cholesterol

Cholesterol was estimated according to the method of Zak (1957).

Reagents

1. Stock ferric chloride reagent- 840 mg pure ferric chloride dissolved in 100 ml of glacial acetic acid.
2. Ferric chloride precipitating reagent- 10 ml of stock ferric chloride made up to 100 ml with pure glacial acetic acid.
3. Ferric chloride diluting reagent- 8.5 ml of the stock ferric chloride diluted to 100 ml with pure glacial acetic acid.

4. Cholesterol standard- 100 mg cholesterol dissolved and made up to 100 ml in pure glacial acetic acid. 10 ml of that was diluted to 100 ml with 0.85 ml of ferric chloride stock reagent and pure glacial acetic acid (100 $\mu g\ ml^{-1}$).

Assay

To a sample of 100 µl haemolymph was added ferric chloride (precipitating reagent), mixed well, allowed to stand for a while and centrifuged. 2.5 ml of the clear supernatant was transferred to a test tube, 2.5 ml of ferric chloride (diluting reagent) was added and mixed well. 0.5 to 2.5 ml of working standard made up to 5.0 ml with ferric chloride diluting reagent and a blank with 5.0 ml ferric chloride diluting regent was used. Tubes were kept in cold water, 4.0 ml of conc. H_2SO_4 was added drop by drop and mixed well. After 30 minutes the intensity of the colour developed was read at 540 nm against the blank. The values were expressed as mg ml^{-1} haemolymph.

2.2.7b Immune assays

2.2.7b.1 Total haemocyte count

A drop of anticoagulant-haemolymph mixture was placed on an improved Neubaeur's haemocytometer immediately after extraction. The haemocytes were then counted under a phase-contrast microscope and the values expressed in THC ml^{-1} hemolymph.

2.2.7b.2 Phenol oxidase activity

Phenol oxidase activity was measured spectrophotometrically using L-DOPA as substrate (Soderhall, 1981). The dopachrome formed from L-DOPA when oxidized by phenol oxidase was measured at 490 nm.

Reagents

1. L-DOPA- 0.01 M DOPA in Tris-HCl buffer (0.05 M, pH 7.6)
2. SDS- 10 mg dissolved in 10ml distilled water

Assay

A sample of 100 µl haemolymph was incubated for 10 minutes at 20°C with 100 µl SDS. Then 2 ml of substrate was added. Increase in OD at 490 nm every 30 seconds for a period of 3 minutes was recorded. The activity was then expressed as increase in OD $minute^{-1}100\mu l^{-1}$ haemolymph.

2.2.7b.3 NBT reduction (Respiratory burst activity)

Respiratory burst activity of haemocytes was measured spectrophotometrically using the reduction of nitroblue tetrazolium salt to blue formazan as a measure of superoxide anion production (Song and Hsieh, 1994).

Reagents

1. NBT- 0.02 g in Tris-HCl buffer (0.05 M) saline (containing 2% NaCl, pH 7.6)
2. Dimethyl sulphoxide (DMSO)
3. 2 M KOH
4. Methanol

Assay

A sample of 100 µl haemolymph was incubated with 100 µl of NBT for 1 hour at 20°C. The cells were then centrifuged and fixed in 100% methanol for 10 minutes. It was again centrifuged, the supernatant removed and the cells were allowed to dry. Dried cells were then rinsed in 50% methanol and solubilised in 140 µl of DMSO and 120 µl of KOH. The absorbance was read at 620nm and the activity expressed as OD $100\mu l^{-1}$ haemolymph.

2.2.7b.4 Alkaline and acid phosphatase activity

Alkaline and acid phosphatase activity was measured by the method of Gonzalez *et al.* (1994). The amount of p-nitrophenol released in alkaline medium as a result of hydrolysis of the substrate p-nitrophenyl phosphate was measured spectrophotometrically at 405 nm as a measure of phosphatase activity.

Reagents

1. p-nitophenyl phosphate- 2 g in 100 ml citrate buffer (pH 5) for ACP and 2 g in 100 ml Glycine-NaOH buffer (pH 9) for ALP
2. NaOH- 0.1 N
3. p-nitrophenol standard- 1 mg ml^{-1}

Assay

To a sample of 100 µl haemolymph, 2.0 ml of p-nitrophenyl phosphate was added and incubated at 37°C in a water bath for 30 minutes. The reaction was stopped by adding 2.9 ml of NaOH and the absorbance read at 405 nm. The activity was expressed as mg p-nitrophenol released ml^{-1} haemolymph. A series of standards were also run using p-nitrophenol and the activity was determined.

2.2.8 Statistical analysis

The experimental data was analysed by means of one-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means. Significance level for the

analysis was set to $P<0.05$. Statistical analyses were carried out using the software SPSS 10.0.

2.3 Results

Significant variations in the haemolymph metabolic and immune variables could be observed when shrimps were subjected to salinity stress. In the case of metabolic variables, an increase could be observed in shrimps at 35‰ except TFAA, which were maximum in shrimps at 5‰. Whereas, in the case of immune variables a decrease could be observed both at 35‰ and 5‰ except for PO and ALP and the reduction was maximum at 5‰.

Following *V. harveyi* challenge, there was a significant enhancement in metabolic variables in general on PCD2 and immune variables on PCD7. Compared to those subjected to salinity stress, a pronounced increase in the metabolic and immune variables could be observed in shrimps maintained at 15‰ on PCD2 and PCD7. Maximum immune response could be observed only on PCD7 in the case of shrimps subjected to salinity stress, except for ACP at 5‰. Post challenge survival of shrimps was significantly higher at 15‰ and acute salinity stress reduced the survival rate. Pathogenicity of *V. harveyi* was slightly higher at 35‰ than at 5‰ (Figs.2.1 - 12).

2.3a Haemolymph metabolic variables

Total protein

A significant increase in the total protein concentration was noted in shrimps at 35‰ after salinity change (91.87 ± 7.9 mg ml^{-1}) ($P<0.05$). When challenged with *V. harveyi*, an increase was observed in the total protein concentration in shrimps at all salinities. Shrimps maintained at 15‰ registered comparatively higher protein levels on PCD7 as well as on PCD10. Mean (± S.E.) protein levels recorded were 115.71 ± 10.9, 100.6 ± 11.0 and 103.19 ± 11.3 mg ml^{-1} on PCD2, PCD7 and PCD10 respectively in shrimps maintained at 15‰. However, the total protein concentration of shrimps under salinity stress was found to decline progressively (Fig.2.1).

Total carbohydrates

The total carbohydrate concentration in haemolymph significantly increased after acute salinity change to 5‰ and 35‰, the variation being higher at 5‰ ($P<0.05$). The concentration increased from 4.34 ± 0.7 to 6.25 ± 0.71 and 5.41 ± 0.75 mg ml^{-1} at 5‰

and 35‰ respectively. The challenged shrimps at 15‰ registered a significant increase in total carbohydrates compared to those at 5‰ and 35‰ ($P<0.05$) (Fig.2.2).

Total free amino acids

The concentration of TFAA increased significantly in shrimps subjected to 5‰ stress (3.46 ± 0.36 mg ml^{-1}) ($P<0.05$). Following *V. harveyi* challenge, the TFAA concentration increased significantly in shrimps at 15‰ (3.86 ± 0.32 mg ml^{-1}) and 35‰ (3.65 ± 0.39 mg ml^{-1}) on PCD2. TFAA levels were significantly higher at all salinities on PCD10, compared to the control (Fig.2.3).

Total lipids

Shrimps subjected to 5‰ stress showed a significant decrease in total lipid level on PSD0 (1.4 ± 0.25 mg ml^{-1}) ($P<0.05$). PCD2 registered an increase in the total lipids at 5‰ (2.18 ± 0.2 mg ml^{-1}) and 15‰ (2.43 ± 0.24 mg ml^{-1}). The total lipids progressively declined from PCD2 to PCD10 in shrimps at all salinities (Fig.2.4).

Glucose

There was a slight elevation in the glucose level of shrimps at 35‰ (0.427 ± 0.05 mg ml^{-1}) after salinity change and a slight decrease at 5‰ (0.246 ± 0.05 mg ml^{-1}) ($P<0.05$). Following *V. harveyi* challenge, a steady increase was noticed in the haemolymph glucose concentration in shrimps that was more pronounced at 15‰. Glucose levels increased to 0.5 ± 0.06, 0.557 ± 0.06 and 0.581 ± 0.07 mg ml^{-1} on PCD2, PCD7 and PCD10 respectively in shrimps maintained at 15‰. The glucose concentration decreased on PCD2 in shrimps held at 35‰. Further the concentration increased on PCD7 and PCD10 at 35‰. Comparatively low glucose concentrations were obtained in shrimps held at 5‰ except on PCD7 (Fig.2.5).

Cholesterol

A significant increase in cholesterol was noticed in shrimps subjected to 35‰ stress (0.849 ± 0.07 mg ml^{-1}) ($P<0.05$). Comparatively higher cholesterol levels were obtained for shrimps held at 35‰ compared to those held at 5‰ and 15‰ except on PCD2. On PCD2, shrimps at 5‰ and 15‰ showed significantly higher levels *viz.*, 0.899 ± 0.12 and 0.818 ± 0.1 mg ml^{-1} respectively. Thereafter the total lipid levels decreased in shrimps at 5‰ and 15‰ (Fig.2.6).

2.3b Immune response

Total haemocyte count

THC significantly decreased by 23.9% and 21.1% at 5‰ and 35‰ on PSD0 compared to the baseline ($P<0.05$). There was a significant reduction in THC on PCD2 following *V. harveyi* challenge at all salinities. THC decreased by 57.3%, 25.4% and 56.9% at 5‰, 15‰ and 35‰ respectively on PCD2. THC further increased on PCD7, which again declined on PCD10. Shrimps held at 15‰ showed significantly higher THC *viz.*, 23.0 ± 2.41, 17.47 ± 2.19 × 10^6 cells ml^{-1} on PCD7 and PCD10 compared to those under salinity stress. THC of 15‰ Group on PCD7 was significantly greater than the other treatment groups (Fig.2.7).

Phenol oxidase activity

Phenol oxidase activity registered a slight increase (0.153 ± 0.03 increase in OD min^{-1} 100μl^{-1}) in the shrimps subjected to a salinity change to 35‰ and a slight decrease (0.05 ± 0.01) in those subjected to a change to 5‰ ($P<0.05$). The activity significantly increased following *V. harveyi* challenge. Maximum PO activity was seen on PCD7. PO activity on PCD2 was significantly higher (0.406 ± 0.05, $P<0.05$) for the shrimps held at 15‰ compared to those at 35‰ and 5‰ stress. Whereas, shrimps held at 35‰ showed the maximum PO activity on PCD7. The activity increased to 0.365 ± 0.04, 0.479 ± 0.05 and 0.573 ± 0.05 on PCD7 at 5‰, 15‰ and 35‰ respectively (Fig.2.8).

NBT reduction

The NBT reduction was significantly low in *P. monodon* subjected to salinity stress of 5‰ and 35‰ ($P<0.05$). The activity increased on *V. harveyi* challenge. After the initial decrease on PSD0, the shrimps subjected to salinity stress also showed an increase in the activity on PCD2. Shrimps held at 15‰ showed comparatively higher activity on PCD2 and PCD7 than those at 35‰ and 5‰ stress. Mean NBT activity (± S.E.) increased to 1.209 ± 0.1 in shrimps at 15‰ on PCD7 whereas it increased to 0.922 ± 0.07 and 0.743 ± 0.08 OD 100μl^{-1} at 5‰ and 35‰ respectively. PCD10 showed a general decline in NBT reduction at all salinities and no significant difference was observed among the NBT values of shrimps held at 5‰, 15‰ and 35‰ (Fig.2.9).

Alkaline phosphatase activity

Alkaline phosphatase activity increased after the sudden increase in salinity to 35‰ and decreased with the sudden decrease to 5‰ ($P<0.05$). Following *V. harveyi*

challenge, ALP activity significantly reduced in shrimps subjected to salinity stress. Challenged shrimps at 15‰ registered a significant increase in ALP activity on PCD2 (0.679 ± 0.05 mg p-nitrophenol released ml^{-1}). However, for shrimps at 5‰ and 35‰ a reduction in ALP activity was observed on PCD2 followed by an increase on PCD7. The activity decreased on PCD10 at all salinities (Fig.2.10).

Acid phosphatase activity

Acid phosphatase activity significantly reduced after salinity stress, being lower at 5‰ than at 35‰ (Mean value of 0.438 ± 0.05 and 0.573 ± 0.05 mg p-nitrophenol released ml^{-1} at 5‰ and 35‰ respectively) ($P<0.05$). The activity increased on *V. harveyi* challenge in shrimps at 5‰ to 0.943 ± 0.07 on PCD2. The shrimps at 15‰ also showed an increase in ACP activity on PCD2 (0.838 ± 0.06). Whereas, the shrimps at 35‰ showed maximum activity on PCD7 (0.756 ± 0.05 mg p-nitrophenol released ml^{-1}) (Fig.2.11).

2.3c Percentage survival

Fig.2.12 shows the percentage survival of *P. monodon* subjected to acute salinity stress and challenged with *V. harveyi*. Percentage survival of the challenged shrimps held at 15‰ was comparatively high. Acute salinity stress reduced the survival rate. Shrimps at 35‰ stress showed least survival followed by the 5‰ Group. Maximum mortality occurred on PCD1 when the survival rate decreased by 22.2% and 15.5% in groups held at 35‰ and 5‰ respectively. Further, the mortality rate reduced, but was comparatively higher in the 35‰ Group than in the 5‰ Group. No significant differences were observed between the percentage survival values on post challenge days in the 15‰ Group ($P<0.05$), which recorded a mean survival of 94.4% on PCD10. On the other hand, shrimps at 35‰ and 5‰ stress showed comparatively lower survival of 64.5 ± 3 % and 75.6 ± 2 % respectively on PCD10 (Fig.2.12).

2.4 Discussion

Stress produced by acute salinity change affected the haemolymph metabolic variables of *P. monodon* as revealed by the present study. Acute salinity change to 35‰ induced an increase in the haemolymph total protein, total carbohydrates, glucose and cholesterol immediately after the salinity change. A reduction in haemolymph glucose and total lipids in addition to an increase in total carbohydrates and TFAA were measured in shrimps subjected to 5‰ stress. Variations in environmental factors have previously been reported to induce variations in shrimp haemolymph metabolites. A reduction in

protein, triacylglycerol and cholesterol in captivity stress were measured in *Litopenaeus setiferus* males acclimated at both 27°C and 31°C (Sanchez *et al.*, 2001). In *Litopenaeus schmitti*, reduction in salinity up to 8% for 48 h did not induce alteration in total protein concentration but reduced glucose concentration accompanied by oedema in gills (Lamela *et al.*, 2005). In *Farfantepenaeus californiensis*, plasma protein did not show variations within the different salinity treatments (Vargas-Albores *et al.*, 1998).

Initial response to cope with the hypo-saline environment (5‰) was probably an increase in the level of free amino acids as they function as osmotic effectors in haemolymph. According to Smith and Dall (1991) free amino acid may contribute to more than 40-60% of the intracellular osmolality in Penaeid shrimps. Increased free amino acid level is considered to play an important role in the regulation of intracellular osmolality of crustaceans during acclimation to hypo-osmotic environments (Boone and Claybrook, 1977). Significantly high TFAA were found in *Fenneropenaeus indicus* from less saline mud-bank area compared to the non-mud bank sample (Jayasree and Selvam, 2000). Haemolymph proteins probably assisted in adjusting to the hyper-saline environment (35‰) that showed a significant increase. A similar elevation in heat shock protein (HSP) or stress protein mRNA expression after 0.5 h of osmotic stress has been observed in American lobster (Chang, 2005). An immediate variation in proteins and free amino acids could be a consequence of cellular release (Jury *et al.*, 1994) that has been suggested as a passive mechanism to maintain internal osmolality in crustaceans. Some authors have reported increase in amino acid pool as a consequence of protein catabolism. However in the present study the protein levels were not affected at 5‰ even though the TFAA level increased. TFAA concentration seemed to decrease at 35‰ but was not statistically significant ($P>0.05$).

Generally, glucose and total carbohydrates in haemolymph increase in stressed shrimps to meet the energy demands to ward off stress. Though an increase in total carbohydrates was observed both at lower and higher salinity stress, increase in glucose was observed only at 35‰, which is indicative of a rapid and selective consumption of the simple sugar at 5‰. Lamela *et al.* (2005) has observed a reduction in haemolymph glucose of *L. schmitti* exposed to lower salinities.

Lipid mobilization and involvement of these compounds in osmotic acclimation process after an osmotic shock has been verified in the euryhaline crab *Chasmognathus granulata* (Luvizotto-santos *et al.*, 2003). A preferential usage of lipids as an energy source was evident at a lower salinity stress. However cholesterol seemed to be spared

and presumably retained as an osmolyte at 5‰. Higher cholesterol level noticed at both salinities was a clear indication of lipid transport that occurred since shrimps cannot synthesise cholesterol *de novo* (Teshima and Kanazawa, 1971).

Following *V. harveyi* challenge, there was an overall increase in the levels of haemolymph metabolic variables in *P. monodon* maintained at 15‰ that showed maximum post challenge survival. Most probably, metabolites were transported to the haemolymph from hepatopancreas and muscle to meet the energy requirements to ward off infection. Yoganandhan *et al.* (2003) could observe a reduction in the total carbohydrate and glucose levels in muscle and hepatopancreas of WSSV-infected *F. indicus* and a corresponding increase in the haemolymph. Compared to those at 15‰, a general reduction in the metabolic variables could be observed in shrimps under salinity stress following bacterial challenge. Osmoregulatory stress and pathological stress could have resulted in a more rapid usage of the metabolites in shrimps under salinity stress.

Pronounced increase in total protein levels at 15‰ on challenge compared to those under salinity stress could be possibly due to increase in the proportion of specific immune proteins. In shrimps under salinity stress proteins might have also been used as an energy source for the production of ATP necessary for osmoregulation as suggested by Noga (2000). An increase in total carbohydrates on *V. harveyi* challenge was evident only at 15‰. A further elevation in carbohydrate levels that increased prior to challenge did not occur in shrimps under osmotic stress. Hyperglycemia as a stress response has been reported in several crustaceans. Hall and van Ham (1998) showed a significant elevation of blood glucose in *P. monodon* after a depleted dissolved oxygen and increased CO_2 levels. Hyperglycemia was quite evidently seen in shrimps maintained at 15‰ upon bacterial challenge. Active consumption of glucose as an energy source for osmoregulation might have brought down the glucose levels in infected shrimps under salinity stress. Occasionally, hypoglycemia has also been found in shrimps under stress. Glucose levels decreased in *L. setiferus* males under captivity stress (Sanchez *et al.*, 2001). In the present study, glucose levels were comparatively lower in *P. monodon* held at 5‰.

Total free amino acids increased on *V. harveyi* challenge in shrimps maintained at 15‰ and 35‰. Although salinity changes induced an increase in TFAA at 5‰ and a decrease at 35‰, following challenge, the levels decreased at 5‰ and increased at 35‰. Further studies on the osmoregulatory role of free amino acids in infected shrimps under osmotic stress may be required for a plausible explanation. Increasing energy demand to

meet the pathological stress was also met by lipids as the lipid concentration showed a depleting trend in the present study. Increase of fatty acid level in the hepatopancreas and decrease in the haemolymph of infected shrimps have earlier been reported (Bowser *et al.*, 1981; Hameed, 1989). Zoea 1 larvae of *Cancer pagurus* and *Homarus gammarus* exposed to low salinities exhibited very low lipid and protein contents at the end of the experiment (Torres *et al.*, 2002). The very low level of protein and lipids in shrimps under salinity stress on PCD10 is hinting at a preferential degradation of these metabolic constituents as an energy source for osmoregulation at a later stage. Cholesterol levels decreased after challenge except in shrimps held at 35‰. However, an increase was noted on PCD2 at 15‰ and 5‰. Cholesterol may be used to repair membranes in case of severe osmotic stress that may result in the alteration of membrane permeability.

Immunological analysis has shown that the immediate effect of an acute salinity change in *P. monodon* is the depression of immune response at 0‰ stress as shown by the decreased THC, PO activity, respiratory burst activity, ALP and ACP activity. The immune system of *P. monodon* subjected to 35‰ stress was also affected as is evident from the decreased THC, respiratory burst activity and ACP activity and slightly increased PO activity and ALP activity. A similar reduction in THC, PO activity and respiratory burst activity has been reported in *P. monodon* (Wang and Chen, 2006b) transferred to 5‰, 15‰ and 35‰ from 25‰ after 12 h. In *L. vannamei* transferred to 35‰ from 25‰, an increase in PO activity and respiratory burst activity was observed after 12 h (Wang and Chen, 2005). Following challenge, the shrimps maintained at 15‰ exhibited maximum immune response on PCD2, which could be correlated with the post challenge survival. Osmoregulatory stress suppressed the immune system of shrimps as is evident from the changes in immune variables immediately after salinity change.

A significant decrease could be observed in the total haemocyte count of *P. monodon* on injection with *V. harveyi*. Previous workers have shown that larger particles or clumps of bacteria are efficiently encapsulated in Crustaceans. According to Johnson (1987), cells responsible for the removal of foreign material include circulating haemocytes and fixed phagocytes. In *P. monodon*, the injected live *V. anguillarum* caused a rapid decrease of live circulating bacteria in the haemolymph (Van de Braak *et al.*, 2002). Smith and Ratcliffe (1980) showed in *Carcinus maenas* a dramatic reduction in haemocytes after a bacterial infection. Haemocytic migration to the site of bacterial injection and strong phagocytic activity could be suggested as the possible reasons behind the reduction in THC on *V. harveyi* challenge. Marked haemocytopenia in shrimps subjected to salinity stress presumably affected the immediate phagocytic efficiency to

rapidly clear off bacteria from circulation. This may be pointed out as a prime reason for the increased sensitivity of shrimps under salinity stress to *V. harveyi*.

Injection of *V. harveyi* in the present study could stimulate the phenol oxidase activity of shrimps at all salinities. According to Smith *et al* (1984), phenol oxidase, the terminal enzyme of the proPO system is specifically activated by ß-1,3-glucan, lipopolysaccharides or peptidoglycan from microorganisms through pattern recognition proteins. Hence, proPO activating system is of great importance in the non-self recognition process of the defense mechanism of shrimps (Vargas-Albores, 1995; Hernandez-Lopaz *et al.*, 1996). An activation of the NADPH-oxidase also occurred as is evident from the increased NBT activity, a measure of superoxide anion production. It may also be noted that a significant decrease in NBT reduction occurred on sudden salinity change.

Though phenol oxidase activity and respiratory burst activity increased significantly following *V. harveyi* infection, a relative decrease was noticed in shrimps under salinity stress. Cheng *et al.* (2004) have reported a significant reduction in phenol oxidase activity, respiratory burst activity and phagocytic efficiency of *Haliotis diversicolor supertexta* transferred to high and low salinities. The reduction in phenol oxidase and respiratory burst activity has been strongly correlated to an increased susceptibility to *V. alginolyticus* infection in *Marsupenaeus japonicus* (Cheng *et al.*, 2007). Further research on variations in the amount of transcript encoding phenol oxidase and the activities of antioxidant enzymes at different salinities may provide better clarification for the observation in the present study. Significant variations in the activity of alkaline and acid phosphatases at different salinities may be related to the alterations in the phagocytic ability of haemocytes. The percentages of haemocytes positive for the three lysosomal enzymes (acid phosphatase, ß-glucuronidase and a-naphthyl acetate esterase) increased in *P. monodon* and *Macrobrachium rosenbergii* following treatment with *V. vulnificus* (Sung and Sun, 1999).

The susceptibility of *P. monodon* to *V. harveyi* was found to increase on acute salinity change to 5‰ and 35‰. Shrimps subjected to salinity stress showed significantly higher mortality rate compared to those maintained at 15‰, which showed significantly higher survival. Higher mortality rate on PCD1 in the 5‰ Group and 35‰ Group shows that a small percentage of shrimps under salinity stress could not effectively fight against the pathogen injected directly to the tissue and succumbed to early death. Sudden changes in salinity therefore enhanced the susceptibility of *P. monodon* to *V. harveyi* infection.

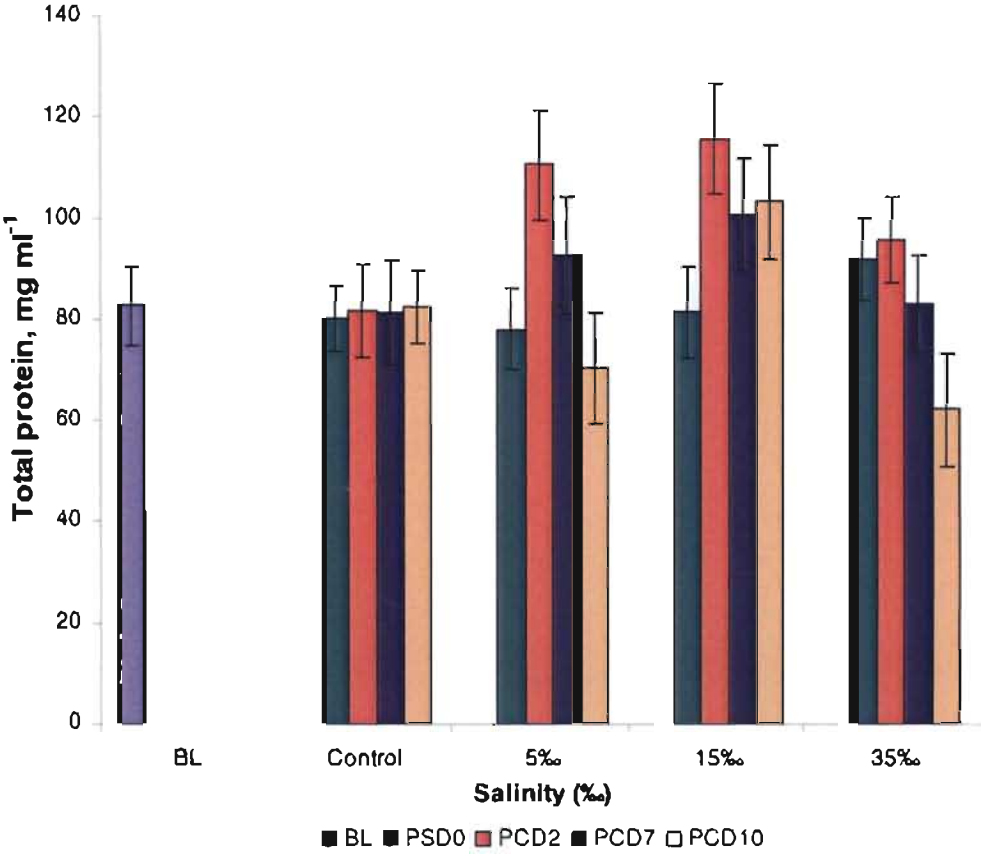
Similar phenomenon has been reported previously. *L. vannamei* were more susceptible to *V. alginolyticus* when transferred from 25‰ to 5‰ and 15‰ (Wang and Chen, 2005). In the present study, susceptibility was enhanced by both low and high salinity, which was in agreement with the observation in Taiwan abalone, *Haliotis diversicolor supertexta*. Susceptibility to *V. parahaemolyticus* increased in abalone when transferred from 30‰ to 20‰, 25‰ and 35‰ in 72 h (Cheng *et al.*, 2004). It can therefore be concluded from the study that fluctuations in environmental salinity is involved in triggering outbreaks of Luminous vibriosis.

In spite of the greater immune suppression, mortality rate was slightly lower at 5‰ compared to those at 35‰. This may perhaps be due to the less growth rate and virulence of *V. harveyi* at a lower salinity. According to Farghaly (1950), environmental factors such as low salinity and changeable pH reduced the growth rate of luminous bacteria. Increased growth rate and virulence at a higher salinity might have enhanced the pathogenicity of *V. harveyi* at 35‰. *V. parahaemolyticus* incubated in TSB medium containing 2.5 –3.5% NaCl significantly enhanced its virulence for *Haliotis diversicolor supertexta* (Cheng *et al.*, 2004). However, Prayitno and Latchford (1995) have shown that exposure of *V. harveyi* to low salinity significantly increased its virulence and resulted in higher mortality rate when used in immersion challenge experiments with *P. monodon* larvae reared in sea water.

Overall, it may be generalized from the present investigation that sudden salinity changes trigger vibriosis outbreaks not only by weakening the physiological and immune response of the shrimps, but also by enhancing the pathogenicity of *V. harveyi*. Hence, the study documents the importance of avoiding drastic salinity changes that arise from seasonal variations by maintaining appropriate salinity levels towards effective control of luminous vibriosis and successful shrimp farming.

Fig. 2.1 Shrimp culture bioassay system

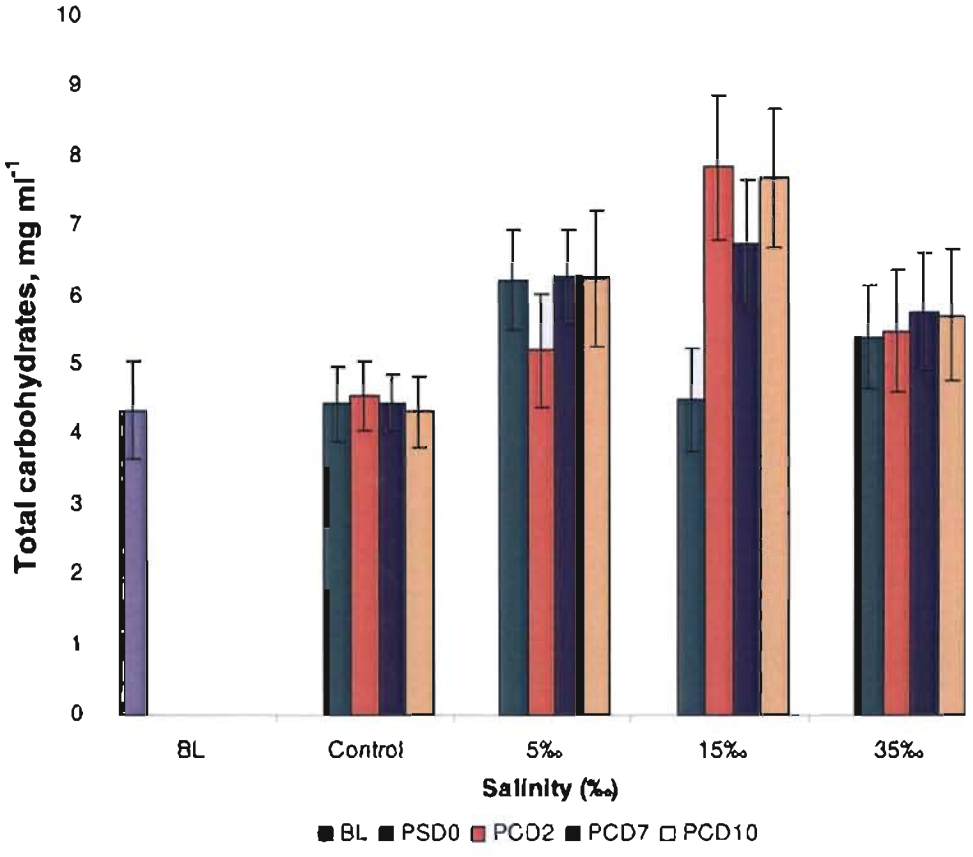
Fig. 2.2 *Penaeus monodon* in the culture system



	Total protein (mg ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	82.63 ± 7.8	$_{b}80.01 \pm 6.4^{A}$	$_{c}81.76 \pm 9.2^{A}$	$_{c}81.35 \pm 10.2^{A}$	$_{b}82.26 \pm 7.2^{A}$
5‰		$_{b}77.91 \pm 8.3^{C}$	$_{d}110.45 \pm 10.7^{A}$	$_{ab}92.66 \pm 11.3^{B}$	$_{bc}70.25 \pm 11.1^{C}$
15‰		$_{b}81.46 \pm 8.8^{C}$	$_{a}115.71 \pm 10.9^{A}$	$_{a}100.60 \pm 11.0^{B}$	$_{a}103.19 \pm 11.3^{BC}$
35‰		$_{a}91.87 \pm 7.9^{AB}$	$_{b}95.65 \pm 8.5^{A}$	$_{c}83.26 \pm 9.5^{B}$	$_{c}62.16 \pm 11.2^{C}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

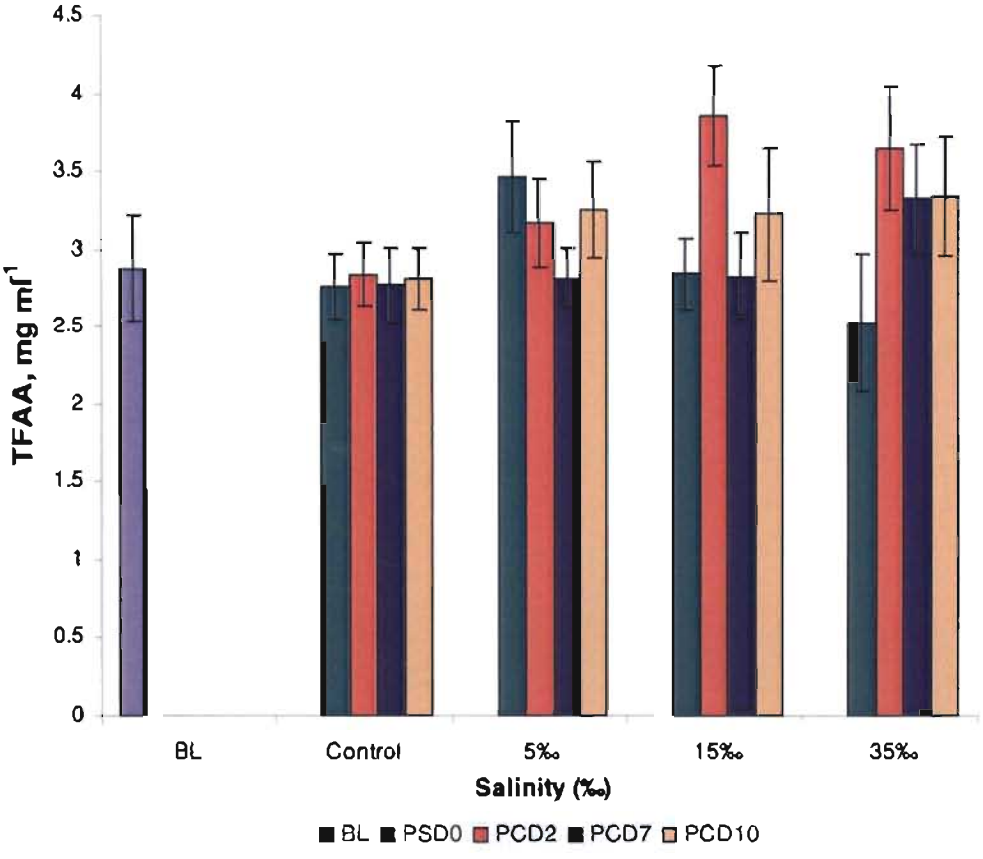
Fig.2.1 Total protein in the haemolymph of *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



Total carbohydrates (mg ml^{-1})					
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	4.34 ± 0.7	$_{c}4.45 \pm 0.54^{A}$	$_{b}4.57 \pm 0.5^{A}$	$_{c}4.46 \pm 0.41^{A}$	$_{c}4.33 \pm 0.51^{A}$
5‰		$_{a}6.25 \pm 0.71^{A}$	$_{b}5.23 \pm 0.82^{B}$	$_{ab}6.29 \pm 0.67^{A}$	$_{b}6.25 \pm 0.99^{A}$
15‰		$_{c}4.50 \pm 0.74^{B}$	$_{a}7.85 \pm 1.02^{A}$	$_{a}6.76 \pm 0.91^{A}$	$_{a}7.69 \pm 0.99^{A}$
35‰		$_{b}5.41 \pm 0.75^{A}$	$_{b}5.50 \pm 0.87^{A}$	$_{b}5.77 \pm 0.85^{A}$	$_{b}5.72 \pm 0.95^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

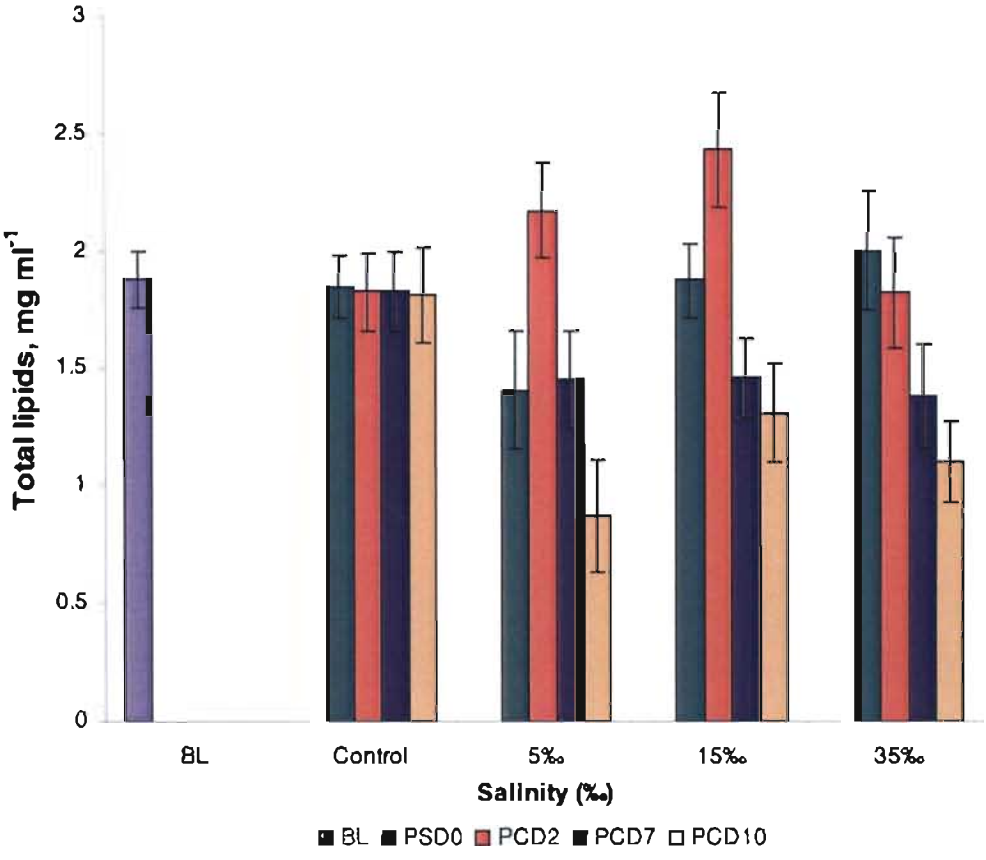
Fig.2.2 Total carbohydrates in the haemolymph of *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



Total free amino acids (mg ml^{-1})					
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	2.88 ± 0.34	$_{b}2.77 \pm 0.21^{A}$	$_{b}2.84 \pm 0.21^{A}$	$_{b}2.77 \pm 0.24^{A}$	$_{b}2.81 \pm 0.2^{A}$
5‰		$_{a}3.46 \pm 0.36^{A}$	$_{b}3.17 \pm 0.29^{A}$	$_{b}2.81 \pm 0.19^{B}$	$_{a}3.26 \pm 0.31^{A}$
15‰		$_{b}2.84 \pm 0.23^{B}$	$_{a}3.86 \pm 0.32^{A}$	$_{b}2.83 \pm 0.28^{B}$	$_{a}3.23 \pm 0.43^{B}$
35‰		$_{b}2.53 \pm 0.44^{B}$	$_{a}3.65 \pm 0.39^{A}$	$_{a}3.33 \pm 0.35^{A}$	$_{a}3.34 \pm 0.38^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PSD -Post salinity change day, PCD -Post challenge day

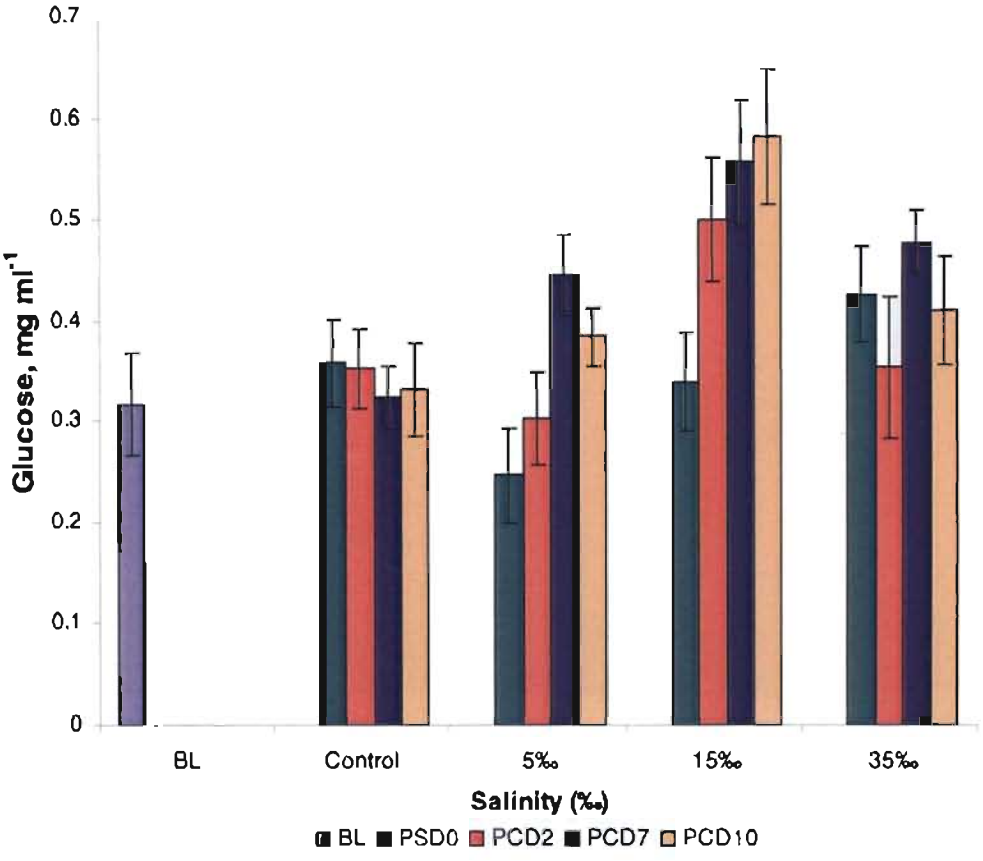
Fig.2.3 Total free amino acids (TFAA) in the haemolymph of *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	Total lipids (mg ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	1.88 ± 0.12	$_{a}1.85 \pm 0.14^{A}$	$_{b}1.83 \pm 0.17^{A}$	$_{a}1.83 \pm 0.17^{A}$	$_{a}1.81 \pm 0.21^{A}$
5‰		$_{b}1.40 \pm 0.25^{B}$	$_{a}2.18 \pm 0.2^{A}$	$_{b}1.45 \pm 0.2^{B}$	$_{c}0.87 \pm 0.24^{C}$
15‰		$_{a}1.87 \pm 0.16^{B}$	$_{a}2.43 \pm 0.24^{A}$	$_{b}1.46 \pm 0.17^{C}$	$_{b}1.30 \pm 0.21^{C}$
35‰		$_{a}2.0 \pm 0.25^{A}$	$_{b}1.82 \pm 0.24^{A}$	$_{b}1.38 \pm 0.22^{B}$	$_{bc}1.10 \pm 0.17^{C}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PSD -Post salinity change day, PCD -Post challenge day

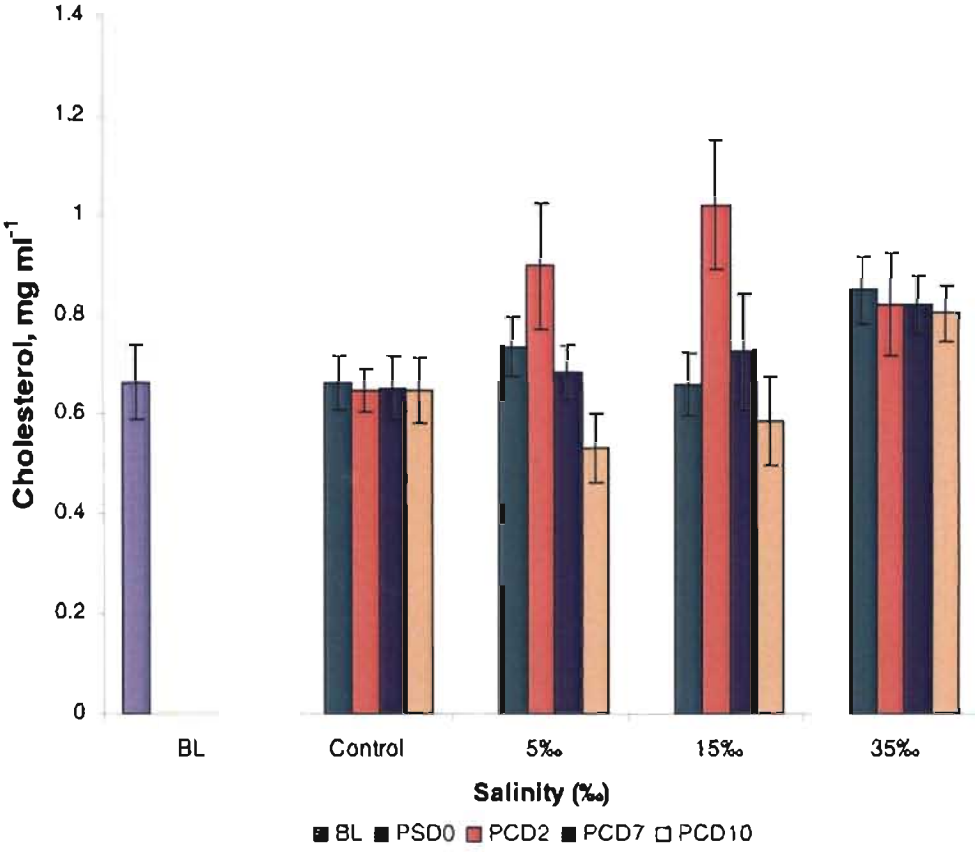
Fig.2.4 Total lipids in the haemolymph of *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	Glucose (mg ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	0.316 ± 0.05	$_{b}0.357 \pm 0.04_{A}$	$_{b}0.352 \pm 0.04_{A}$	$_{c}0.323 \pm 0.03_{A}$	$_{c}0.330 \pm 0.05_{A}$
5‰		$_{c}0.246 \pm 0.05_{C}$	$_{b}0.303 \pm 0.05_{C}$	$_{b}0.445 \pm 0.04_{A}$	$_{bc}0.383 \pm 0.03_{B}$
15‰		$_{b}0.340 \pm 0.05_{C}$	$_{a}0.500 \pm 0.06_{B}$	$_{a}0.557 \pm 0.06_{AB}$	$_{a}0.581 \pm 0.07_{A}$
35‰		$_{a}0.427 \pm 0.05_{AB}$	$_{b}0.353 \pm 0.07_{C}$	$_{b}0.478 \pm 0.03_{A}$	$_{b}0.410 \pm 0.05_{BC}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

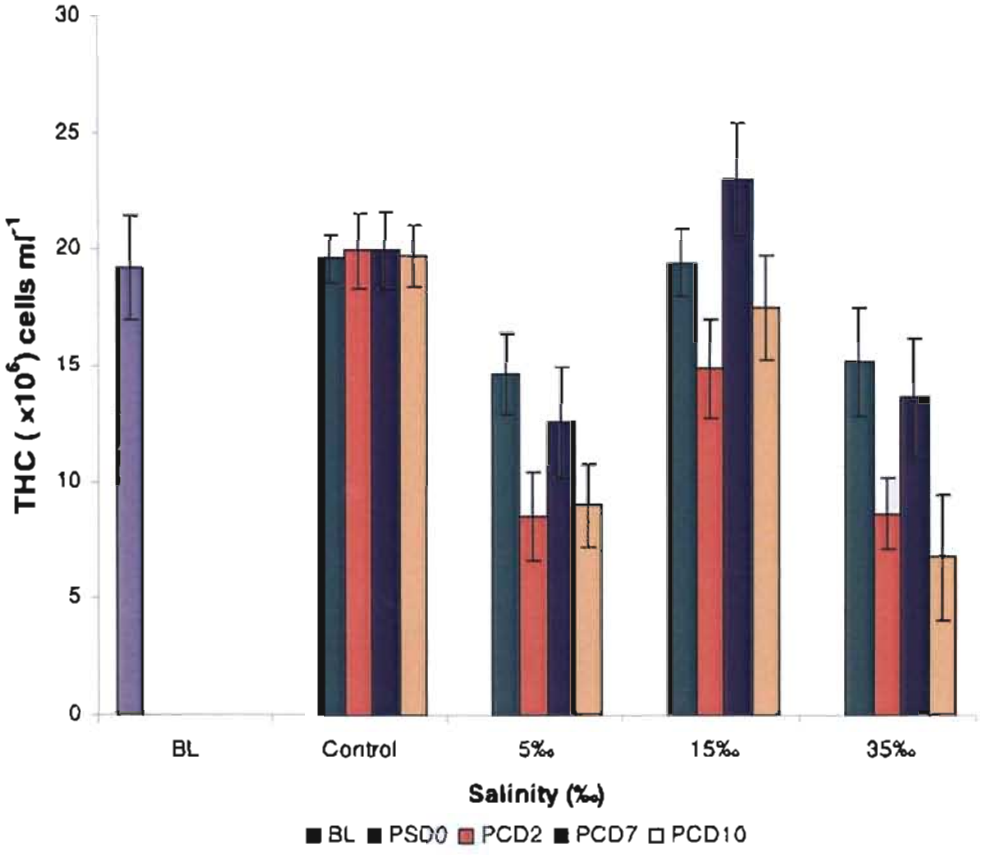
Fig.2.5 Glucose levels in the haemolymph of *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	Cholesterol(mg ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	0.663 ± 0.08	$_{b}0.662 \pm 0.05^{A}$	$_{c}0.646 \pm 0.04^{A}$	$_{b}0.651 \pm 0.06^{A}$	$_{b}0.647 \pm 0.06^{A}$
5‰		$_{b}0.735 \pm 0.06^{B}$	$_{ab}0.899 \pm 0.12^{A}$	$_{b}0.684 \pm 0.05^{B}$	$_{c}0.532 \pm 0.07^{C}$
15‰		$_{b}0.659 \pm 0.06^{BC}$	$_{a}1.021 \pm 0.13^{A}$	$_{b}0.726 \pm 0.12^{B}$	$_{bc}0.586 \pm 0.09^{C}$
35‰		$_{a}0.849 \pm 0.07^{A}$	$_{b}0.818 \pm 0.1^{A}$	$_{a}0.820 \pm 0.06^{A}$	$_{a}0.803 \pm 0.05^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PSD –Post salinity change day, PCD –Post challenge day

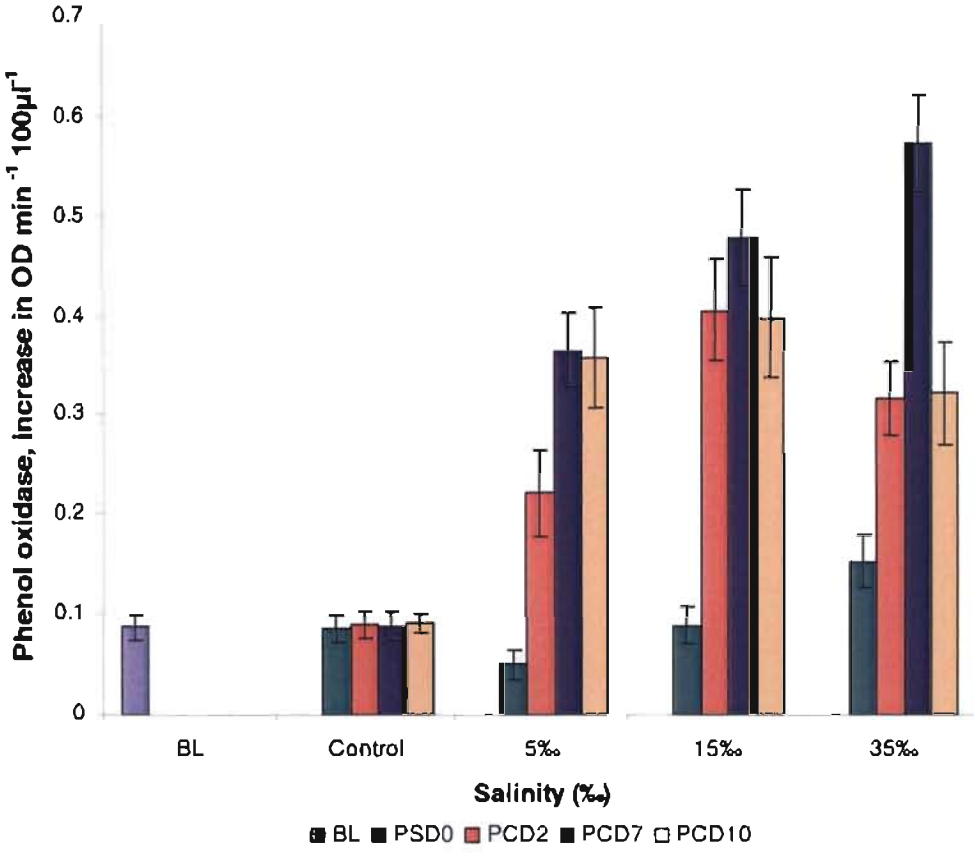
Fig.2.6 Cholesterol levels in the haemolymph of *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	Total Haemocyte Count (×10^6 cells ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	19.18 ± 221.2	$_a$19.59 ± 1.0^A	$_a$19.93 ± 1.6^A	$_b$19.94 ± 1.6^A	$_a$19.67 ± 1.3^A
5‰		$_b$14.61 ± 1.8^A	$_c$8.51 ± 1.9^B	$_c$12.51 ± 2.4^A	$_b$8.95 ± 1.8^B
15‰		$_a$19.39 ± 1.4^B	$_b$14.86 ± 2.1^C	$_a$23.01 ± 2.4^A	$_a$17.48 ± 2.2^B
35‰		$_b$15.15 ± 2.3^A	$_c$8.59 ± 1.5^B	$_c$13.59 ± 2.6^A	$_b$6.75 ± 2.7^B

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

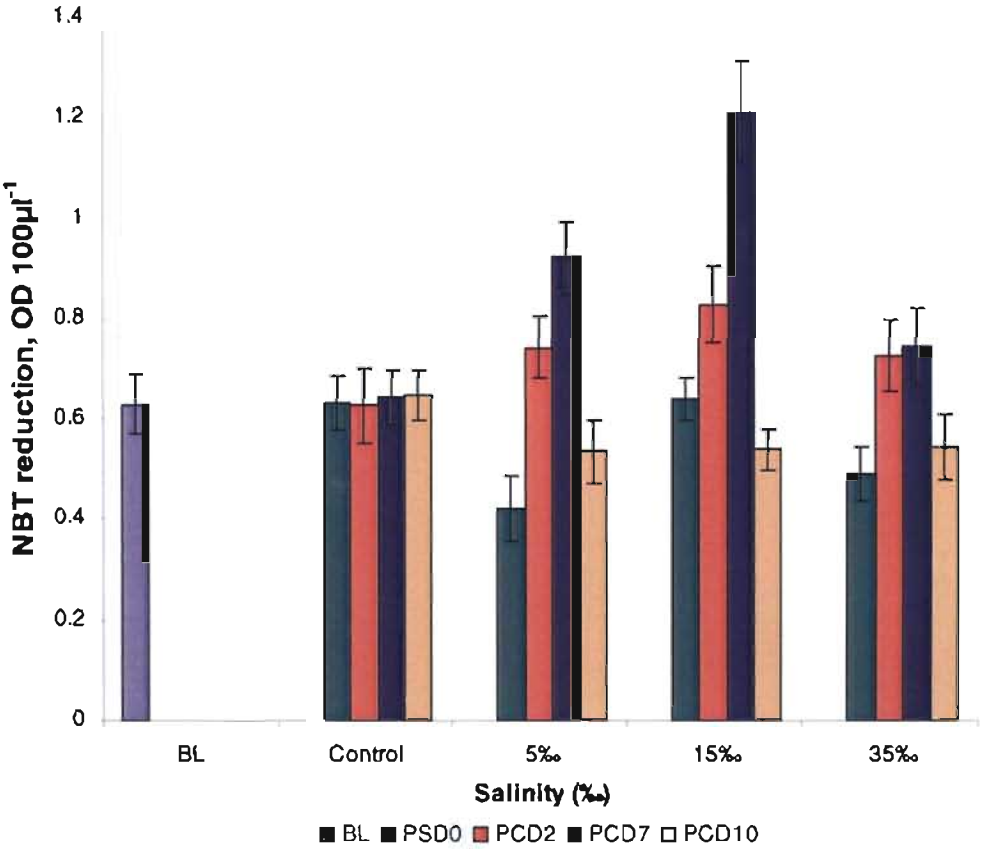
Fig.2.7 Total haemocyte count (THC) in *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	Phenol oxidase activity (increase in OD min^{-1} $100\mu l^{-1}$)				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	0.086 ± 0.01	$_{b}0.085 \pm 0.01^{A}$	$_{d}0.089 \pm 0.01^{A}$	$_{d}0.087 \pm 0.01^{A}$	$_{c}0.09 \pm 0.01^{A}$
5‰		$_{c}0.05 \pm 0.01^{C}$	$_{c}0.221 \pm 0.04^{B}$	$_{c}0.365 \pm 0.04^{A}$	$_{ab}0.358 \pm 0.05^{A}$
15‰		$_{b}0.09 \pm 0.02^{C}$	$_{a}0.406 \pm 0.05^{B}$	$_{b}0.479 \pm 0.05^{A}$	$_{a}0.398 \pm 0.06^{B}$
35‰		$_{a}0.153 \pm 0.03^{C}$	$_{b}0.317 \pm 0.04^{B}$	$_{a}0.573 \pm 0.05^{A}$	$_{b}0.322 \pm 0.05^{B}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

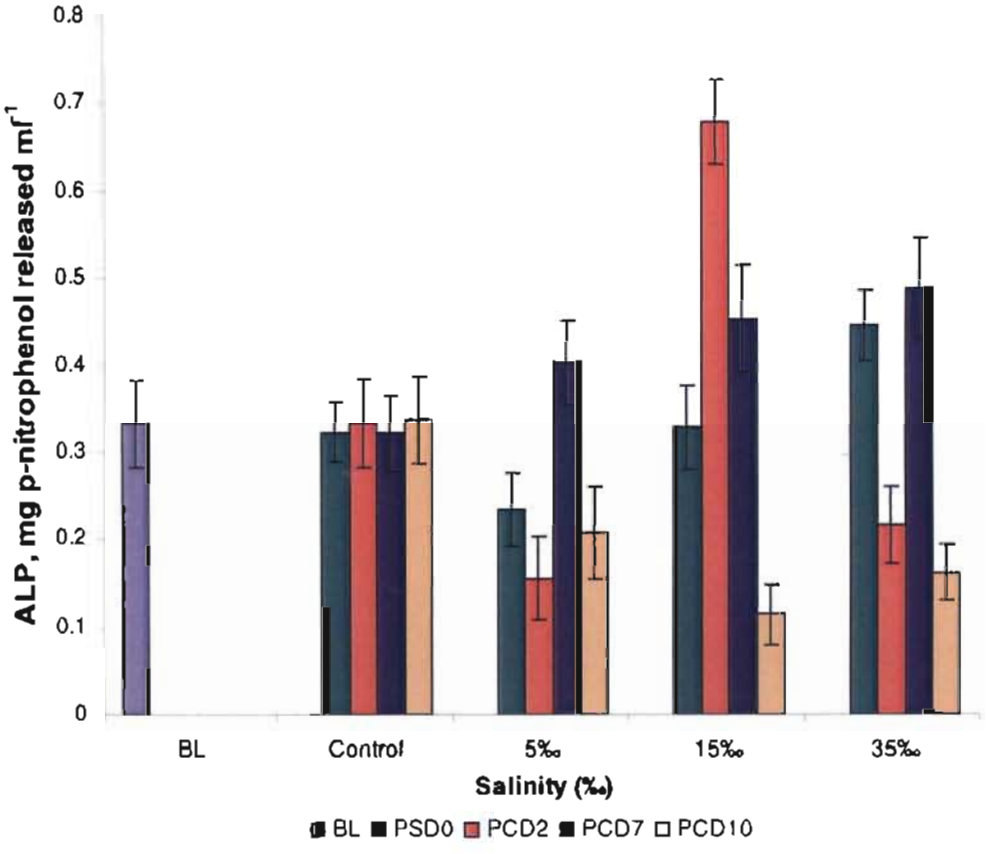
Fig.2.8 Phenol oxidase activity in *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	NBT Reduction (OD $100\mu l^{-1}$)				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	0.627 ± 0.06	$_{a}0.629 \pm 0.05^{A}$	$_{c}0.624 \pm 0.07^{A}$	$_{d}0.641 \pm 0.05^{A}$	$_{a}0.646 \pm 0.05^{A}$
5‰		$_{c}0.42 \pm 0.07^{D}$	$_{b}0.741 \pm 0.06^{B}$	$_{b}0.922 \pm 0.07^{A}$	$_{b}0.533 \pm 0.06^{C}$
15‰		$_{a}0.637 \pm 0.04^{C}$	$_{a}0.829 \pm 0.08^{B}$	$_{a}1.209 \pm 0.1^{A}$	$_{b}0.536 \pm 0.04^{D}$
35‰		$_{b}0.488 \pm 0.05^{B}$	$_{b}0.725 \pm 0.07^{A}$	$_{c}0.743 \pm 0.08^{A}$	$_{b}0.542 \pm 0.06^{B}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PSD –Post salinity change day, PCD –Post challenge day

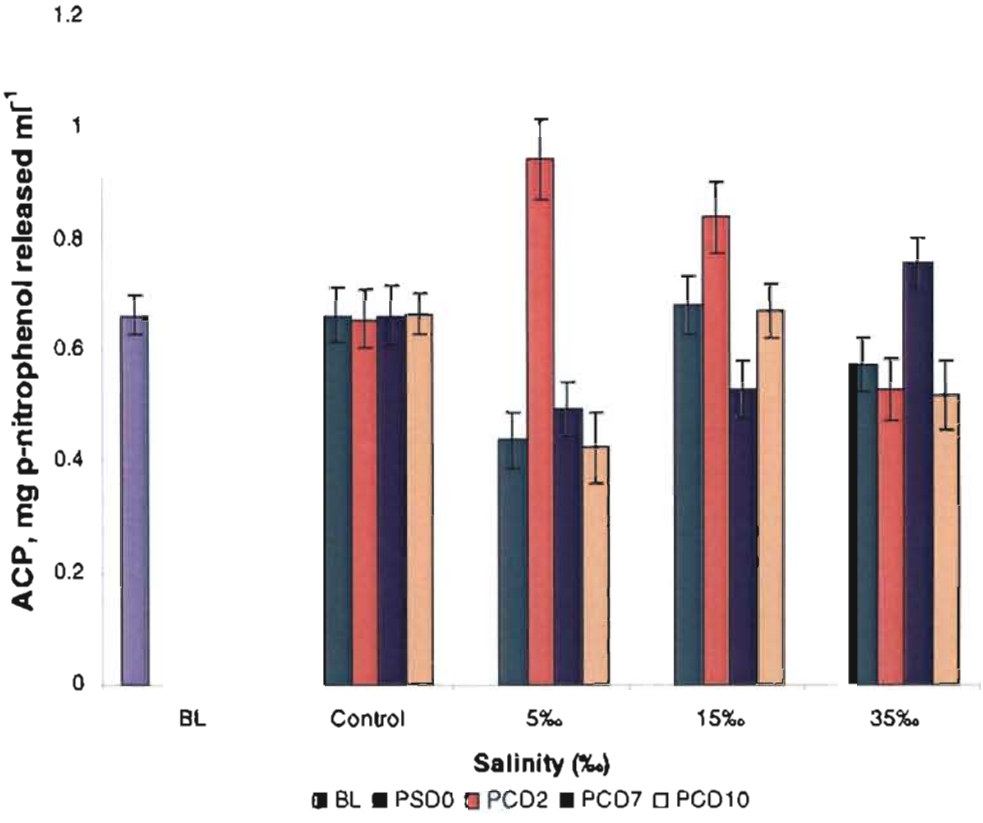
Fig.2.9 NBT reduction in *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	Alkaline Phosphatase activity (mg p-nitrophenol released ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	0.332 ± 0.05	$_{b}0.322 \pm 0.03^{A}$	$_{b}0.333 \pm 0.05^{A}$	$_{c}0.321 \pm 0.04^{A}$	$_{a}0.336 \pm 0.05^{A}$
5‰		$_{c}0.234 \pm 0.04^{B}$	$_{d}0.155 \pm 0.05^{C}$	$_{b}0.404 \pm 0.05^{A}$	$_{b}0.207 \pm 0.05^{BC}$
15‰		$_{b}0.328 \pm 0.05^{C}$	$_{a}0.679 \pm 0.05^{A}$	$_{ab}0.453 \pm 0.06^{B}$	$_{c}0.114 \pm 0.03^{D}$
35‰		$_{a}0.447 \pm 0.04^{A}$	$_{c}0.216 \pm 0.04^{B}$	$_{a}0.489 \pm 0.06^{A}$	$_{bc}0.162 \pm 0.03^{C}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL-Baseline, PSD -Post salinity change day, PCD -Post challenge day

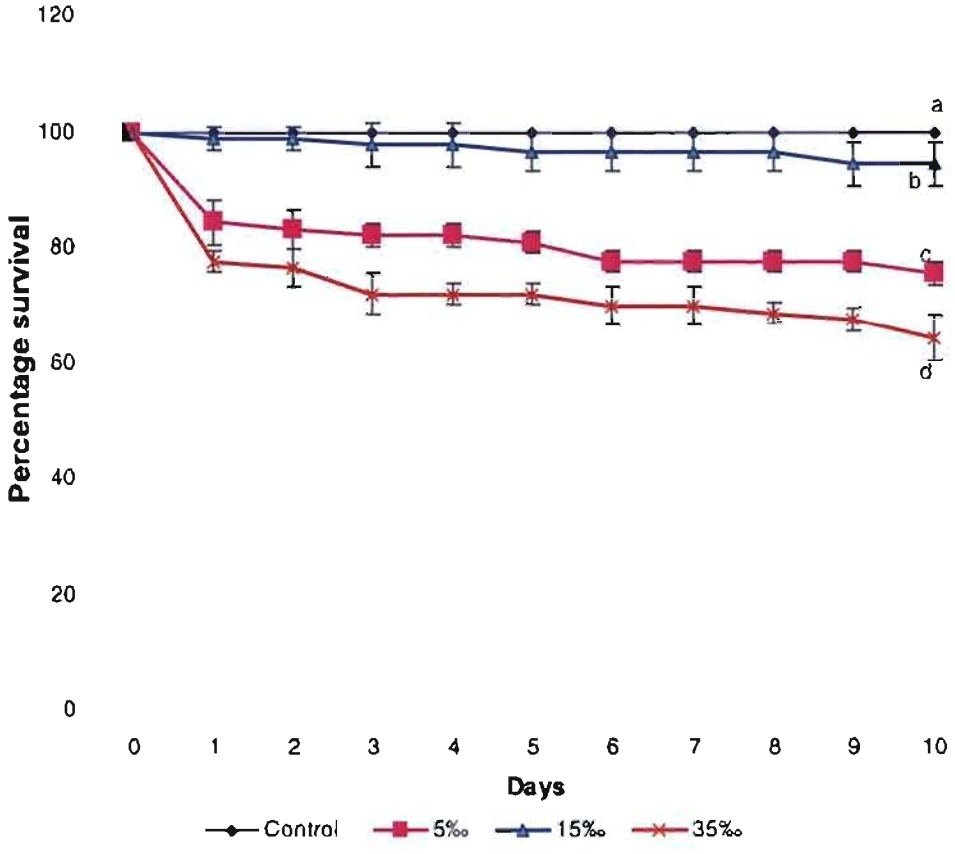
Fig.2.10 Alkaline phosphatase activity (ALP) in *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



	Acid Phosphatase activity (mg p-nitrophenol released ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD7	PCD10
Control	0.663 ± 0.04	$_{a}0.663 \pm 0.05^{A}$	$_{c}0.656 \pm 0.05^{A}$	$_{b}0.663 \pm 0.05^{A}$	$_{a}0.665 \pm 0.04^{A}$
5‰		$_{c}0.438 \pm 0.05^{B}$	$_{a}0.943 \pm 0.07^{A}$	$_{c}0.493 \pm 0.05^{B}$	$_{c}0.424 \pm 0.06^{B}$
15‰		$_{a}0.681 \pm 0.05^{B}$	$_{b}0.838 \pm 0.06^{A}$	$_{c}0.529 \pm 0.05^{C}$	$_{a}0.671 \pm 0.05^{B}$
35‰		$_{b}0.573 \pm 0.05^{B}$	$_{d}0.527 \pm 0.06^{B}$	$_{a}0.756 \pm 0.05^{A}$	$_{b}0.518 \pm 0.06^{B}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PSD -Post salinity change day, PCD -Post challenge day

Fig.2.11 Acid phosphatase activity (ACP) in *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.



*Different letters indicate statistical difference among different treatments

Fig.2.12 Post challenge survival of *P. monodon* subjected to salinity stress and challenged with *V. harveyi*.

CHAPTER 3

Effect of Acute Salinity Stress on the Haematological Responses and Susceptibility of Penaeus monodon to White Spot Syndrome Virus (WSSV) Infection

3.1 Introduction

White Spot Syndrome has emerged as the most serious threat to commercial shrimp farming causing high mortalities and severe damage to shrimp cultures. White spot syndrome virus (WSSV), the causative agent of white spot syndrome is the most virulent virus hitherto reported from the farmed shrimps (Flegel and Alday-Sanz, 1998; Huang *et al.*, 2002). It can cause 100% mortalities within 3-10 days of the onset of the symptoms. The virus has a broad host range and has been observed not only in shrimps but also in other crustaceans including crabs and crayfishes.

Fig.3.1 White spot syndrome virus

WSSV is a large, circular double stranded DNA virus (Fig.3.1). Since its first discovery in East Asia in 1992, WSSV has spread rapidly to shrimp-farming areas in Southeast-Asia and Central and Latin America, causing major economic damage to shrimp culture. Based on the morphology, the genomic structure and composition as well as phylogenetic analyses, WSSV has been identified as a member of the genus Whispovirus within a new virus family called Nimaviridae, referring to the thread-like polar extension on the virus particle (Vlak *et al.*, 2005). Obvious white spots on the carapace, appendages and other exoskeletal surfaces of the body are the characteristic features of white spot syndrome (Fig.3.2). Other signs include lethargy, red body colouration, reduced feeding and preening activities, empty gut and 'marching' at the pond margins (Momoyama *et al.*, 1994; Takahashi *et al.*, 1994). Progressive tissue disintegration and mortality of shrimp with the onset of WSSV infection has been reported (Rameshthangam and Ramasamy, 2005).

Fig.3.2 Whitespot syndrome

Temperature, salinity, dissolved oxygen and pH are the major physico-chemical parameters that have a greater impact on shrimps influencing their metabolism, growth, immune function and survival. Low oxygen tension reduces the growth and moulting frequency of shrimps and hampers the metabolic performances (Allan and Maguire, 1991) and immune response (Le Moullac and Haffner, 2000). Osmoregulatory capacity (Charmantier *et al.*, 1994), THC and antibacterial activites decreased and PO activity increased when *L. vannamei* was exposed to hypoxic conditions (Jiang *et al.*, 2005). Water temperature directly affects the oxygen consumption, metabolism, growth, moulting and survival. A higher temperature, pH and a very low salinity reduced the phagocytic activity, clearance efficiency, (Cheng *et al.*, 2003) THC and phenol oxidase activity of *Macrobrachium rosenbergii* (Cheng and Chen, 2000). High temperature evoked a loss of osmoregulatory capacity, a reduction in proPhenol oxidase activity and a reduction in the blood metabolites at day 5 (Pascual *et al.*, 2003b). From the above mentioned works it is clearly evident that the stress caused by variations in environmental factors lead to the onset of a cascade of immunological and biochemical responses well reflected in the composition of haemolymph. Marked fluctuations are brought about in the immune response and metabolic performance of shrimps.

There are very recent evidences to support links between physico-chemical changes and increased vulnerability to invading pathogens in shrimps. Wang and Chen (2006b) reported a reduction in the total haemocyte count, phenol oxidase activity, respiratory burst, superoxide dismutase activity, phagocytic activity and clearance efficiency when *P. monodon* were transferred to very high and low salinities which in

turn reduced the resistance against *Photobacterium damselae* infection. Hypoxic conditions caused a depression of the immune system of *M. rosenbergii* and increased susceptibility to *Enterococcus* infection (Cheng *et al.*, 2002). The immune ability of *L. vannamei* was reduced by high levels of ammonia (Liu and Chen, 2004) in water, which increased mortality from *V. alginolyticus* infection.

Very few studies on the effects of environmental parameters on WSSV outbreak have been reported recently. Jiang *et al.* (2004) found that ammonia level at 5 mgl^{-1} decreased the virulence of WSSV even though it reduced the immunocompetence of *P. japonicus*. Temperature was found to influence the WSSV pathogenicity in crayfish; which may act as a carrier at low temperature and could develop the disease when the water temperature is increased (Jiravanichpaisal *et al.*, 2004). A very high WSSV proliferation was found in *F. chinensis* when shrimps with latent WSSV were subjected to acute salinity change (Liu *et al.*, 2006).

P. monodon, being a euryhaline form having wide salinity tolerance ranging from 1‰ to 57‰ (Chen, 1990), salinity changes are usually neglected in the culture ponds. The salinity of culture ponds may decrease suddenly to as low as 0‰ after a heavy rainfall. Mass mortality due to WSSV infection or failure to attain the expected market size is a usual phenomenon seen after the outbreak of monsoon in the shrimp farms of Kerala. There are reports of WSSV outbreaks with the onset of monsoon in Malaysia when intense rainfall decreased the salinity of aquaculture areas (Oseko, 2006). In the previous experiment, acute salinity stress was found to induce alterations in the haemolymph metabolic variables of *P. monodon* and reduce the immunocompetence to *V. harveyi* infection. It is also possible that acute changes in salinity make them highly vulnerable to WSSV infection.

Therefore, the present study on *P. monodon* was aimed at determining the:

- Effect of WSSV infection on the haemolymph biochemical variables and immune response of shrimps maintained at optimal salinity and those subjected to acute salinity stress.
- Effect of acute salinity stress on the susceptibility to WSSV infection.

3.2 Materials and methods

3.2.1 Experimental animals

Adult *P. monodon* obtained from a commercial farm in Panangad, Kochi were used as experimental shrimps in the present study. They were transported to the laboratory within one hour of capture. Average wet weight of the shrimp was 19.8 ± 2.2 g (Mean ± S.D.). The shrimps were reared in concrete rectangular tanks containing 15‰ sea water and allowed to acclimate for a week. Rearing conditions and water quality were maintained as that for the first experiment (Refer section 2.2.2). After acclimation for a period of seven days, the metabolic and immunological profile was obtained from a group of shrimps (n=6) as the baseline (BL) data.

3.2.2 Experimental design

Shrimps were distributed in the experimental tanks containing 500L of seawater with 30 individuals per tank (n=30/tank). There were 4 treatments (Group-I, Group-II, Group-III and Group-IV) and the experiment was conducted in triplicate i.e., 3 tanks per treatment. Salinity of all the tanks was adjusted to 15‰ prior to the experiment. Shrimps in the intermoult stage only were used. The moult stage was recognized by the observation of uropoda in which partial retraction of the epidermis can be distinguished (Robertson *et al.*, 1987).

3.2.3 Salinity stress

Shrimps were maintained in the experimental tanks at 15‰ for two days. The Group-II and Group-IV shrimps were then subjected to sudden salinity changes. Shrimps were starved for 12 hours prior to salinity change. The salinity of Group-II was lowered from 15‰ to 0‰ by diluting with fresh water. Whereas, the salinity of Group-IV was raised from 15‰ to 35‰ by adding sea water. The desired salinity was adjusted over a period of seven hours. Shrimps of Group-I and Group-III was maintained at 15‰ itself with no salinity change. Ten minutes after the desired salinity level was reached, 6 prawns from each group (n=6) were sampled (post salinity change day 0, PSD0).

3.2.4 WSSV challenge

In order to assess the influence of salinity stress on susceptibility to WSSV infection, the shrimps of Group-II, Group-III and Group-IV were challenged with white spot syndrome virus ten minutes after the desired salinity level was reached. The 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 48 h (post challenge day-2, PCD2) and 120 h (post challenge day-5, PCD5) of challenge. Sampling days were fixed based on the rate of mortality that occurred. Before each sampling the shrimps were fasted 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.

3.2.5 Extraction of haemolymph

Haemolymph was extracted according to the procedure described earlier (Refer section 2.2.6). Sampling was carried out at the beginning of the experiment (baseline), on post salinity change day 0 (PSD0) and post challenge day 2 and 5 from the four experimental groups (Group-I, Group-II, Group-III and Group-IV). The immune parameters were analysed immediately and the samples stored at –20°C for the analysis of metabolic variables.

3.2.6 Analysis of haematological parameters

Metabolic variables in the haemolyph *viz.*, total protein, total carbohydrates, total free amino acids, total lipids, glucose, cholesterol and immune variables *viz.*, total haemocyte count, phenol oxidase activity, NBT reduction, alkaline phosphatase activity and acid phosphatase activity was determined according to the methods described previously (Refer Section 2.2.7).

3.2.7 Statistical analysis

Data obtained from the experiment was analysed by means of one-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means. Significance level for the analysis was set to $P<0.05$. Statistical analyses were carried out using the software SPSS 10.0.

3.3 Results

Haemolymph metabolic and immune variables showed a general increase following WSSV infection on PCD2 in shrimps maintained at 15‰ and the immune activities showed a declining trend on PCD5. The metabolic and immune variables in general were maximum at 15‰ compared to those at 35‰ and 0‰. This could be

correlated with the survival also. Immune responses of the shrimps under salinity stress were better at 35‰ compared to those held at 0‰, showing that salinity change to a lower level is more stressful and the shrimps are highly susceptible to WSSV infection.

3.3a Haemolymph metabolic variables

Total protein

Acute salinity change to 35‰ induced a significant increase in the total protein concentration of shrimps (110.48 ± 13.9 mg ml^{-1}) ($P<0.05$). Post challenge total protein levels were significantly higher in shrimps at all salinities compared to the control. Significantly higher protein levels of 109.81 ± 11.9 and 121.51 ± 16.4 mg ml^{-1} were recorded on PCD2 in shrimps at 0‰ and 15‰ respectively ($P<0.05$). Post challenge protein levels were lower for shrimps at 35‰ (Fig. 3.1).

Total carbohydrates

Total carbohydrates in haemolymph significantly increased on PSD0 in shrimps at 0‰ (6.5 ± 0.75 mg ml^{-1}) and 35‰ (5.56 ± 0.67 mg ml^{-1}). No significant difference was noted between post challenge carbohydrate levels for the shrimps held at 0‰ and 35‰. Haemolymph total carbohydrates were found to increase in WSSV challenged *P. monodon* maintained at 15‰ ($P<0.05$). Compared to the 0‰ and 35‰ Group, 15‰ Group registered significantly higher total carbohydrate levels following infection. A mean concentration of 7.13 ± 0.59 and 6.46 ± 0.61 mg ml^{-1} was recorded on PCD2 and PCD5 respectively in shrimps held at 15‰ (Fig. 3.2).

Total free amino acids

TFAA was found to increase significantly in response to acute salinity change to 0‰ (3.88 ± 0.52 mg ml^{-1}, $P<0.05$). The concentration decreased after challenge in shrimps held at 0‰. Shrimps held at 35‰ showed an increase in TFAA on PCD5. A progressive elevation in the haemolymph TFAA concentration was observed in shrimps maintained at 15‰ following WSSV challenge (mean value of 3.43 ± 0.46 and 3.79 ± 0.39 mg ml^{-1} on PCD2 and PCD5 respectively) ($P<0.05$) (Fig. 3.3).

Total lipids

Significantly lower lipid levels were observed in shrimps at 0‰ after salinity change and on post challenge days compared to the 15‰ and 35‰ Group ($P<0.05$). Haemolymph total lipid concentration decreased in the challenged shrimp compared to

the control. The total lipid levels reduced to 1.29 ± 0.19, 1.62 ± 0.17 and 1.71 ± 0.2 mg ml^{-1} on PCD5 in shrimps held at 0‰, 15‰ and 35‰ respectively (Fig. 3.4).

Glucose

On acute salinity change the glucose levels of shrimps were found to increase slightly at 35‰ (0.386 ± 0.06 mg ml^{-1}) and decrease at 0‰ (0.227 ± 0.04 mg ml^{-1}) ($P<0.05$). An elevation in the haemolymph glucose concentration was noted in shrimps maintained at 15‰ following challenge. The glucose levels increased from 0.322 ± 0.06 to 0.397 ± 0.04 and 0.423 ± 0.06 mg ml^{-1} on PCD2 and PCD5 respectively. However, a significant reduction could be noticed in the glucose level of shrimps held at 35‰ ($P<0.05$). Significantly lower glucose levels were recorded on post challenge days in shrimps subjected to salinity stress compared to those maintained at 15‰ ($P<0.05$) (Fig. 3.5).

Cholesterol

The cholesterol concentration significantly increased after salinity change to 0.619 ± 0.06 and 0.739 ± 0.08 mg ml^{-1} in shrimps at 0‰ and 35‰ respectively ($P<0.05$). Following challenge, the cholesterol concentration showed a declining trend on PCD5. Comparatively higher cholesterol concentration was recorded in shrimps held at 35‰ ($P<0.05$) (Fig. 3.6).

3.3b Immune response

Total haemocyte count

Significantly lower THC was recorded in shrimps held at 0‰ and 35‰ stress immediately after salinity change and on post challenge days compared to the control shrimps and those held at 15‰ ($P<0.05$). A general decline in THC was observed on PCD2 at all salinities. Thereafter the THC slightly improved on PCD5 for shrimps held at 15‰ and 35‰, being significantly higher at 15‰ ($P<0.05$). THC decreased by 34% and 48% for shrimps at 0‰ post salinity change and on PCD2 respectively. A decrease by 22% and 46% was observed in shrimps at 35‰ after salinity change and PCD2 respectively compared to that of the control. THC of shrimps maintained at 15‰ registered a decrease of 19% on PCD2 (Fig. 3.7).

Phenol oxidase activity

Phenol oxidase activity showed a slight increase in shrimps at 35‰ (0.172 ± 0.03 increase in OD min^{-1} $100\mu l^{-1}$) and a slight decrease at 0‰ (0.063 ± 0.02) ($P<0.05$).

Following WSSV challenge, the PO activity increased significantly in shrimps held at 15‰ and 0‰, being higher at 15‰ (0.336 ± 0.05) (P<0.05). Though the activity declined on PCD5, it remained higher compared to the control in the 15‰ Group and it went down that of the control in 0‰ Group. In the case of shrimps held at 35‰ an increased PO activity of 0.197 ± 0.02 was seen on PCD5 compared to the lower activity on PCD2 (Fig. 3.8).

NBT reduction

The NBT reduction decreased by 31.5% and 22.4% in shrimps at 0‰ and 35‰ respectively (P<0.05). Following challenge, significantly higher activities i.e., 1.01 ± 0.09, 1.037 ± 0.08 and 0.82 ± 0.07 OD 100µl^{-1} were recorded at 0‰, 15‰ and 35‰ respectively on PCD2 (P<0.05). The activity significantly declined on PCD5 in shrimps held at 0‰. Whereas, the NBT reduction was significantly higher in shrimps at 15‰ and 35‰ on PCD5 compared to the control and those at 0‰ (Fig. 3.9).

Alkaline phosphatase activity

Alkaline phosphatase activity decreased to 0.353 ± 0.04 and increased to 0.606 ± 0.05 mg p-nitrophenol released ml^{-1} at 0‰ and 35‰ respectively compared to the baseline. A significant elevation in the activity was noticed in shrimps at 15‰ on PCD2 (0.8 ± 0.06 mg p-nitrophenol released ml^{-1}) (P<0.05). The activity declined on PCD5 at all salinities. Post challenge ALP activity was significantly lower for the shrimps held at 0‰ compared to those at 15‰ and 35‰ (P<0.05) (Fig. 3.10).

Acid phosphatase activity

Following salinity stress, the acid phosphatase activity significantly reduced at 0‰ (0.432 ±0.07) and 35‰ (0.581 ± 0.05 mg p-nitrophenol released ml^{-1}) (P<0.05). PCD2 showed a significant elevation in the activity at all salinities (P<0.05). The acid phosphatse activity was considerably low on PCD5 at 0‰ compared to the 15‰ and 35‰ Group (Fig. 3.11).

3.3c Post challenge survival

The percentage survival rates of *P. monodon* maintained at 15‰ were significantly higher than for those held at 35‰ and 0‰. The least survival rate was recorded for shrimps subjected to 0‰ stress, which succumbed to death (100%) within 6 days of challenge. On the same day, 15‰ and 35‰ Groups recorded significantly higher survival rates of 88.9% and 52.8% respectively. The onset of mortality occurred early on PCD2 and PCD3 respectively in the challenged shrimps held at 0‰ and 35‰ compared

to those held at 15‰ where the death began only on PCD6. The percentage survival of 35‰ group reached 0 by PCD10 when the 15‰ Group showed a relatively higher survival (41.2%). One-way ANOVA has revealed that percentage survival from PCD3 to PCD10 is significantly different in the 3 treatment groups ($P<0.05$) (Fig. 3.12).

3.4 Discussion

Haemolymph metabolites have been used as a tool to identify the nutritional and physiological state of the shrimp, as haemolymph together with muscle and digestive gland is a reserve tissue (Gibson and Barker, 1979). Changes in the levels of haemolymph metabolic variables have been described in shrimps in response to captivity stress (Sanchez *et al.*, 2001), temperature alterations (Pascual et al., 2003b), depleted dissolved oxygen (Hall and van Ham, 1998) and high ambient ammonia (Racotta and Hernandez-Herrera, 2000).

A prominent increase was observed in the haemolymph metabolic variables except lipids in shrimps maintained at 15‰ following WSSV infection. Yoganandhan *et al.*, (2003) reported similar increase in haemolymph metabolites in WSSV-infected *F. indicus*. A similar enhancement in metabolic variables in *P. monodon* was also observed in the previous study after *V. harveyi* challenge. Increase in haemolymph metabolites at the initial stages of infection may be attributed to the mobilization of energy reserves from the reserve tissues- hepatopancreas and muscle to meet the energy requirements to ward off infection.

Concentration of haemlymph metabolites in shrimps subjected to salinity stress was less compared to that in the infected shrimps at 15‰ following challenge. However, there was no striking difference in the performance of haemolymph metabolic variables between the shrimps at 0‰ and 35‰ stress except for the very low total lipid level at 0‰. This significant reduction of haemolymph metabolites in shrimps under salinity stress could be explained as a deviation in the energy flow to support osmotic work as they were under a dual stress (salinity stress and pathogenic stress). A metabolic stress probably resulted as they were spending more energy for osmoregulation and thereby not able to function effectively against infection. According to previous workers salinity itself has very little effect on the metabolic rate of euryhaline shrimp (Bishop *et al.*, 1980; Gaudy and Sloane, 1981). Since *P. monodon* were subjected to acute salinity changes in the present investigation, a rapid change in the osmolal concentration of the haemolymph caused osmotic stress and consequent metabolic adjustments. An increase in

haemolymph metabolites was seen at 35‰ with the exception of TFAA. Sudden change to 5‰ evoked an increase in total carbohydrates, TFAA and cholesterol as well as a decrease in glucose and total lipids. Metabolic rate also might have altered slightly compared to those maintained at optimal salinity. The disturbed animal naturally required time to reach a steady state of equilibrium. *L. setiferus* required 3-4 days to stabilize the hemolymph as reported by Castille and Lawrence (1981). The entry of virus placed an additional burden on the metabolic requirements of the animal contributing to a relative reduction in the levels of metabolic constituents in the haemolymph.

An increase in the protein content in *P. monodon* has been related to an increase in the haemocyanin content and protein reserves (Chen and Cheng, 1995). According to Yoganandhan *et al.* (2003), sharp increase in the total protein of WSSV-infected shrimp might owe to increase in the amount of virus. Taking into account the significantly higher total protein in shrimps held at 15‰ it may be suggested that enzymes involved in immune function that display elevated transcription during pathogenic stress are also contributing to the increase. Rameshthangam and Ramaswamy (2005) detected new and intensely expressed protein patters in WSSV-infected *P. monodon*. Further research on protein profile in shrimps under salinity stress may provide better clarification.

During stress shrimps use carbohydrates as a source of energy. Haemolymph glucose and the total carbohydrates are reported to increase in the infected shrimp to ward off infection (Yoganandhan *et al.*, 2003). Hyperglycemia on WSSV infection was evident only in shrimps maintained at 15‰. Increased secretion of CHH (Crustacean Hyperglycemic Hormone) may cause hyperglycemia. An increase in plasma CHH concentration was reported in Norway lobsters infected with *Hemtodinium* (Steniford *et al.*, 2001). As the pathogenic burden increases, a steadily increasing demand is placed upon the hosts' haemolymph glucose. Significant reduction in haemolymph glucose in the infected shrimps under salinity stress could be due to the dual stress suffered by the shrimps. More energy might be produced in the form of ATP for the active functioning of branchial pumping mechanisms. Na^+/K^+ ATPase pump drives the major part of osmotic regulation in crustaceans across the gills (Lamela *et al.* 2005). Hyperglycemia may also be an indication of the stimulation of other compensatory mechanisms. Eventhough an increase was noted at 0‰, the glucose levels were seen to decrease on PCD5. Further research is needed to clarify whether the decrease in glucose was due to less release of CHH as the total carbohydrate level increased.

A progressive increase could be observed in the TFAA level in shrimps maintained at 15‰ on WSSV challenge. Lo *et al.* (1997) reported the increase in haemolymph amino acids to be due to WSSV load. On *V. harveyi* challenge, a similar increase in TFAA was observed both at 15‰ and 35‰, though the increase was not progressive. Free amino acids are better known to be involved in the active adjustment of intracellular osmoregulation in marine invertebrates and the major free amino acids involved are glycine, alanine, proline, glutamic acid, taurine and aspartic acid (Gilles, 1979; Claybrook, 1983). The increase in TFAA level that occurred soon after acute salinity change could be attributed to its osmoregulatory role. However, further studies may be required to find a reasonable explanation for the increase of TFAA in infected shrimps.

The decrease of fatty acid level in haemolymph is a usual phenomenon in the infected shrimp (Hameed, 1989), the reason of which is yet to be defined. However, an increase in lipid concentration occurred after the sudden osmotic shock to 35‰, supposedly related to the osmotic acclimation process (Luvizotto-Santos *et al.*, 2003). Lipids have the advantage of producing more energy than carbohydrates. Energy rich lipid compounds are capable of meeting the energy expenditure for active ion transport associated with extracellular osmoregulation.

Haemocytes, along with the proPhenol oxidase activity and respiratory burst activity, has been used as an index of the capability of the immune system in Penaeid shrimps (Le Moullac *et al.*, 1998; Tseng and Chen, 2004). Circulating haemocytes are affected by extrinsic factors like temperature (Pascual *et al.*, 2003b; Wang and Chen, 2006a), salinity (Vargas-Albores *et al.*, 1998), pH (Cheng and Chen, 2000) and dissolved oxygen (Jiang *et al.*, 2005) in several species of decapod crustaceans.

Immediately after acute salinity change there occurred a depression in the immune response, which was maximum at a lower salinity stress than at a higher level. All the immune variables reduced at 0‰ and THC, NBT and ACP reduced at 35‰. The immune response following WSSV infection was surprisingly high in case of shrimps maintained at optimal salinity (15‰) compared to those under salinity stress. An enhancement in all the immune parameters analysed was observed after 48 hours of WSSV challenge at 15‰ as is evident from the significantly higher phenol oxidase activity, respiratory burst activity, ALP and ACP activity on PCD2. The activities showed a declining trend on PCD5 but were higher compared to the unchallenged control and those under salinity stress. A similar upward trend in percentage phagocytosis, ALP

and phenol oxidase activity after 6 h and a declining trend after 54 h was reported in WSSV challenged *M. japonicus* (Jiang *et al.*, 2004). Such an enhanced phenol oxidase activity and respiratory burst activity was also observed in *F. indicus* following WSSV challenge on PCD3 and a declining trend on PCD5 (Sajeevan *et al.*, 2006). In spite of the enhanced phenol oxidase activity, respiratory burst activity and acid phosphatase activity on PCD2, shrimps at 0‰ succumbed to death on PCD6. Presumably the shrimps suffered an immune fatigue after the enhanced response on PCD2, as the immune system was weak at the time of WSSV challenge due to acute salinity stress. Significant reduction in the immune response was noted on PCD5 in shrimps at 0‰ stress. Shrimps with 35‰ showed responses similar to that of the challenged shrimps at 15‰ on PCD5, exhibiting a comparatively better resistance to WSSV than those at 0‰.

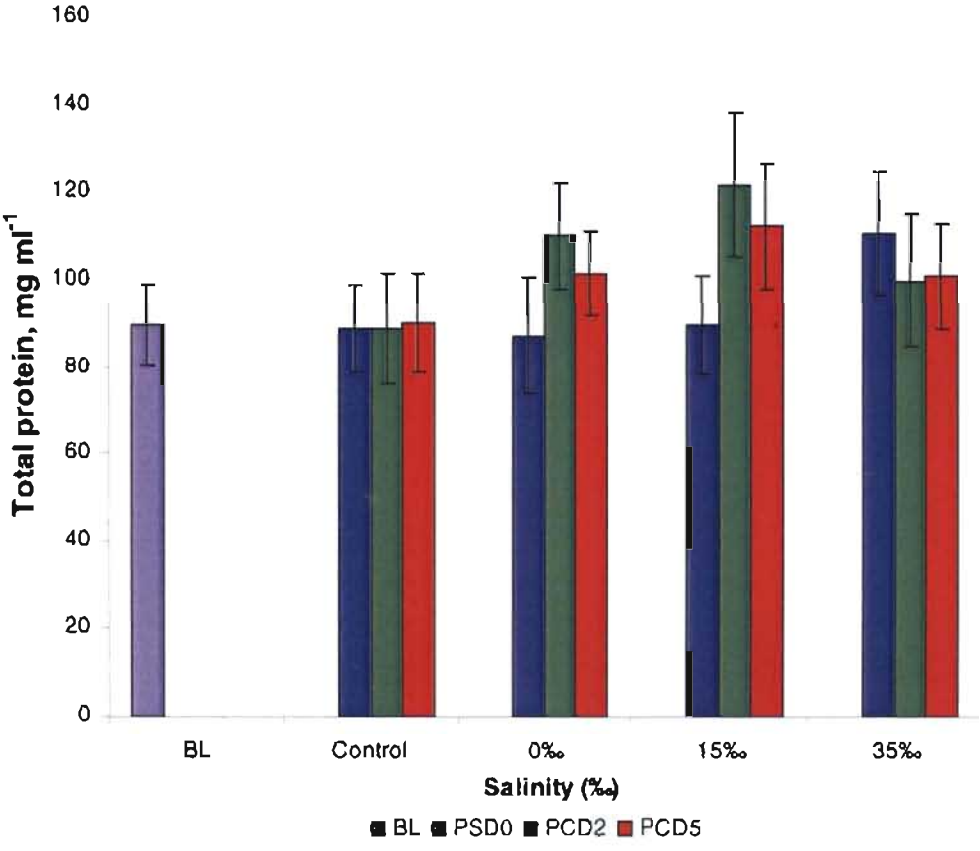
THC was found to decrease following infection in *P. monodon* maintained at all salinities, but the count was comparatively higher for the shrimps at 15‰ than those under salinity stress. Decrease in THC exhibited by WSSV-infected shrimps at all salinities is most likely caused by haemocytic accumulation at the site of injection for wound healing and phagocytosis of foreign bodies (Ratcliffe and Rowley, 1979). A similar decrease in THC has been reported by Song *et al.* (2003) in Taura syndrome virus infected *L. vannamei* to 21% of untreated control values. The initial haemocyte values were reduced by more than 43% in WSSV-infected *F. indicus* at moribund stage (Yoganandhan *et al.*, 2003). The significant reduction in THC at the time of infection may be interpreted as a major factor behind the decreased immunocompetence of shrimps under salinity stress. The reduction in haemocyte count that occurred soon after salinity stress might be a consequence of cell lysis, diapedesis or movement of cells from circulation to tissues or osmosis of the water between haemolymph and medium for osmotic regulation (Pipe and Coles, 1995). A low circulating haemocyte count is strongly correlated with a greater sensitivity to pathogens (Persson *et al.*, 1987) Van de Braak *et al.* (2002) has reported an increase in the young and immature haemocytes just after infection indicating an intense proliferation of haematopoietic tissue. A similar increase in THC was observed in the present study on PCD5 in the case of shrimps maintained at 15‰ and 35‰ after an initial decrease.

A positive correlation could be established between THC and PO activity from the present study. Previous workers have found both negative (Hauton *et al.*, 1995; Le Moullac *et al.*, 1998) and positive (Cheng et al., 2004) correlation between THC and PO activity. Variation in phenol oxidase activity could also be a consequence of alterations in the regulatory mechanisms of proPO system. Variations in respiratory burst activity may

be attributed to the disparity in NADPH oxidase activity, phagocytic rate and/or the number of hyaline cells (Holmblad and Soderhall, 1999; Sajeevan *et al.*, 2006). Phosphatases are the most important components of lysosomal enzymes, which perform a dual function of digestion and defense (Jiang and Mu, 1999). Alkaline phosphatase and acid phosphatase that originate from haemocytes play a key role in destroying the extracellular invaders (Cheng and Rodirick, 1975). Hence their activities are related to the phagocytic ability of haemocytes.

Sudden salinity changes were found to reduce the survival rate of *P. monodon* in the present study. Fluctuations in environmental salinity over a particular range have recently been proved to influence the susceptibility of shrimps to infection. *P. monodon* were more susceptible to *Photobacterium damselae* subsp. *damselae* when the animals were transferred from 25‰ to 5‰, 15‰ and 35‰ after 96 h (Wang and Chen, 2006b). WSSV-challenged *F. chinensis* subjected to salinity change from 22‰ to 14‰ had nearly 3 times viral load compared to the control group (Liu *et al.*, 2006). Shrimps were highly susceptible to WSSV infection at 0‰ stress compared to 35‰. Whereas, *P. monodon* maintained at optimal salinity (15‰) with no salinity change showed maximum survival after WSSV infection. Therefore it can be concluded that acute salinity stress increases the susceptibility of *P. monodon* to WSSV infection, being significantly more at a lower salinity stress (0‰). Chang *et al.* (1998) in their studies on the virucidal effects of salinity on white spot syndrome baculovirus (WSBV) could prove that salinity has little effect on the infectivity of WSBV. Hence the higher susceptibility of *P. monodon* to WSSV infection at 0‰ cannot be related to the virulence of WSSV.

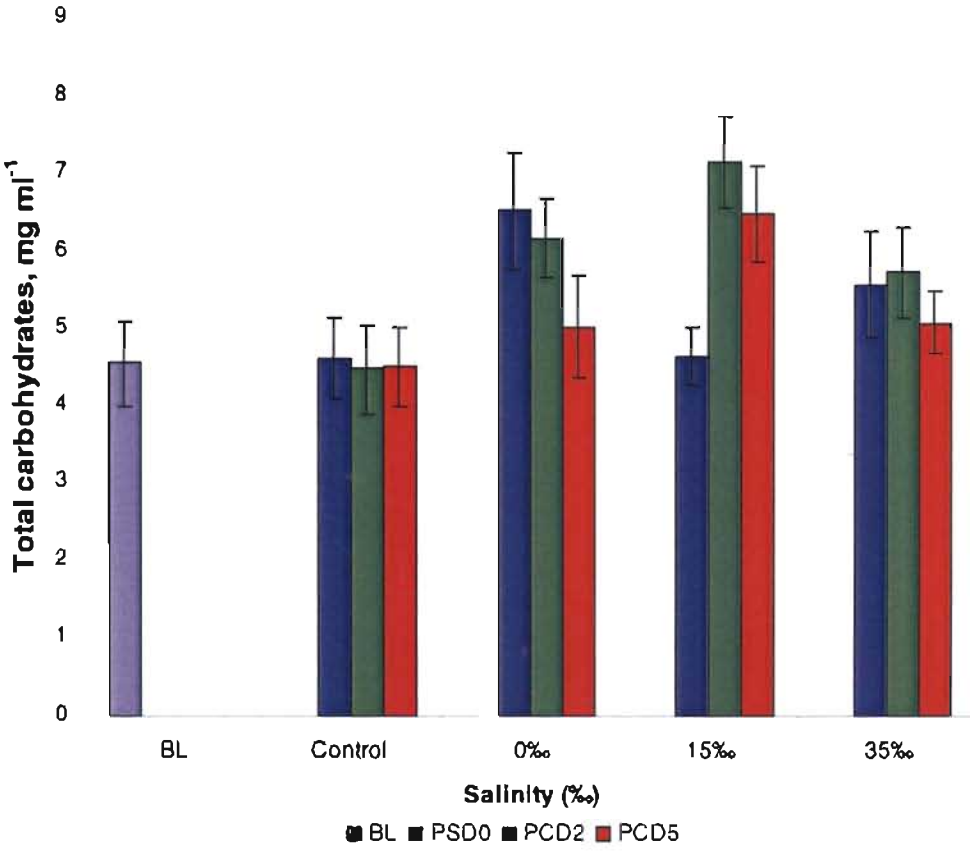
In accordance with the results obtained from the present study it can be concluded that acute salinity stress induces alterations in haemolymph metabolic variables and affects the immunocompetence of *P. monodon* resulting in increased susceptibility to WSSV infection, being significantly more at a lower salinity stress. Shrimps maintained at optimal salinity (15‰) though could not completely eliminate the virus particles from circulation and thwart an infection, their powerful immune defense and metabolic response could overwhelm the pathogen during early stages of infection that delayed the onset and pace of mortality. The study hence points to the significance of appropriate management measures to be adopted to minimize acute salinity stress in *P. monodon* culture ponds, which in turn will help to achieve the expected market size of shrimps and minimize loss from WSSV infection.



	Total protein (mg ml^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	89.28 ± 9.2	$_{b}$88.35 ± 10.1^{A}	$_{c}$88.58 ± 12.9^{A}	$_{b}$90.01 ± 11.4^{A}
0‰		$_{b}$86.96 ± 13.3^{C}	$_{ab}$109.81 ± 11.9^{A}	$_{ab}$101.32 ± 9.8^{AB}
15‰		$_{b}$89.38 ± 11.3^{B}	$_{a}$121.51 ± 16.4^{A}	$_{a}$112.04 ± 14.2^{A}
35‰		$_{a}$110.48 ± 13.9^{A}	$_{bc}$99.7 ± 15.3^{A}	$_{ab}$94.06 ± 11.1^{A}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

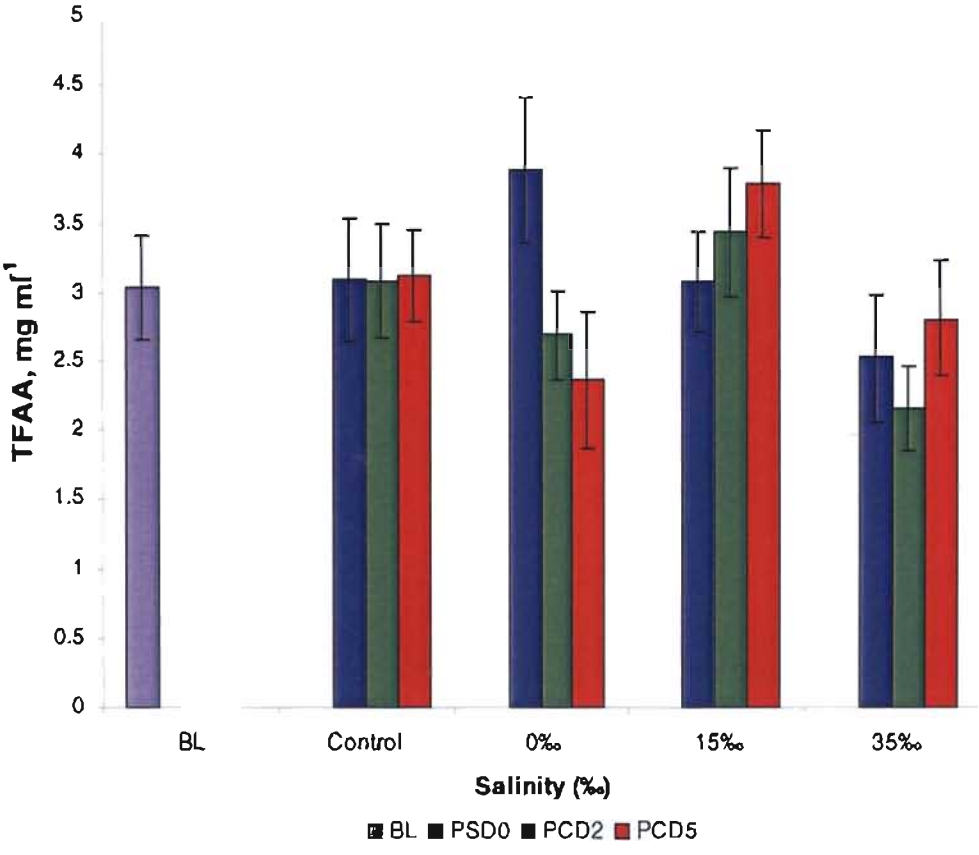
Fig.3.1 Total protein in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.



	Total carbohydrates (mg ml^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	4.53 ± 0.55	$_{c}4.59 \pm 0.53^{A}$	$_{c}4.46 \pm 0.58^{A}$	$_{b}4.49 \pm 0.52^{A}$
0‰		$_{a}6.5 \pm 0.75^{A}$	$_{b}6.15 \pm 0.51^{A}$	$_{b}5.0 \pm 0.66^{B}$
15‰		$_{c}4.62 \pm 0.38^{C}$	$_{a}7.13 \pm 0.59^{A}$	$_{a}6.46 \pm 0.61^{B}$
35‰		$_{b}5.56 \pm 0.67^{A}$	$_{b}5.71 \pm 0.58^{A}$	$_{b}5.06 \pm 0.41^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

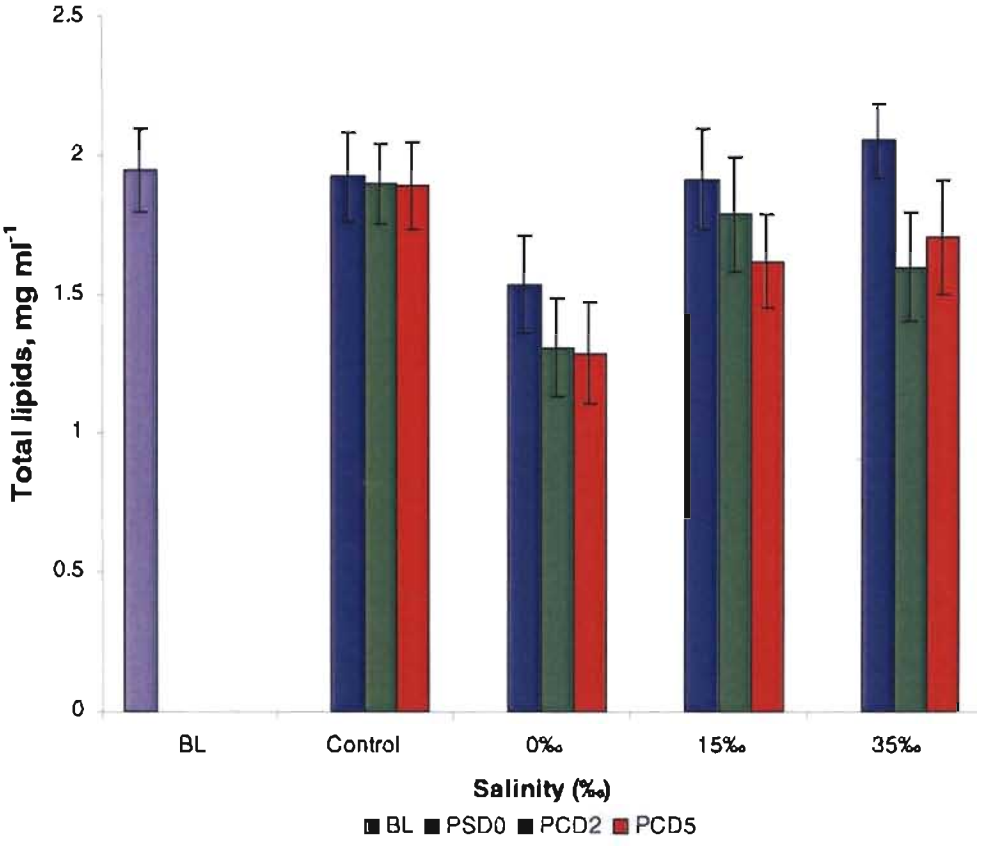
Fig.3.2 Total carbohydrates in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.



Total free amino acids (mg ml^{-1})				
Salinity	BL	PSD0	PCD2	PCD5
Control	3.03 ± 0.38	$_{b}3.09 \pm 0.45^{A}$	$_{ab}3.08 \pm 0.42^{A}$	$_{b}3.12 \pm 0.33^{A}$
0‰		$_{a}3.88 \pm 0.52^{A}$	$_{b}2.69 \pm 0.33^{B}$	$_{c}2.36 \pm 0.5^{B}$
15‰		$_{b}3.09 \pm 0.36^{B}$	$_{a}3.43 \pm 0.46^{AB}$	$_{a}3.79 \pm 0.39^{A}$
35‰		$_{b}2.52 \pm 0.46^{AB}$	$_{c}2.16 \pm 0.3^{B}$	$_{b}2.81 \pm 0.42^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

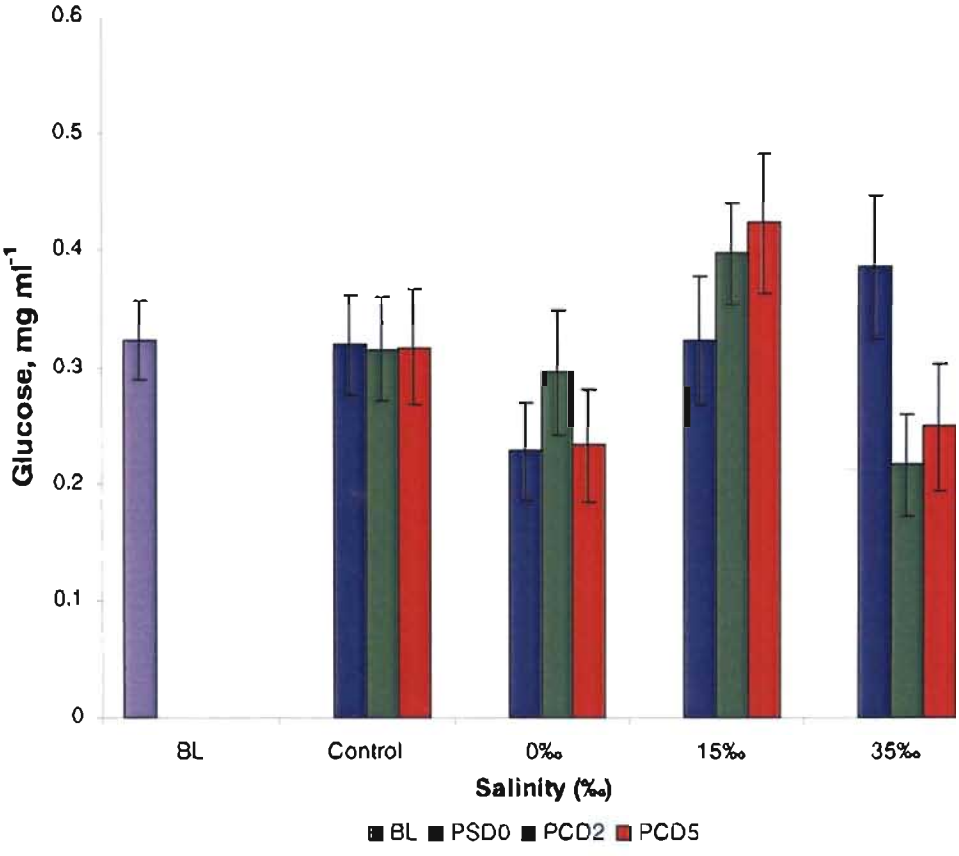
Fig.3.3 Total free amino acids (TFAA) in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.



	Total lipids (mg ml^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	1.94 ± 0.15	$_{a}$1.92 ± 0.16^{A}	$_{a}$1.90 ± 0.14^{A}	$_{a}$1.89 ± 0.16^{A}
0‰		$_{b}$1.54 ± 0.18^{A}	$_{c}$1.31 ± 0.18^{B}	$_{c}$1.29 ± 0.19^{B}
15‰		$_{a}$1.91 ± 0.18^{A}	$_{ab}$1.79 ± 0.21^{AB}	$_{b}$1.62 ± 0.17^{B}
35‰		$_{a}$2.05 ± 0.13^{A}	$_{b}$1.6 ± 0.2^{B}	$_{ab}$1.71 ± 0.2^{B}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

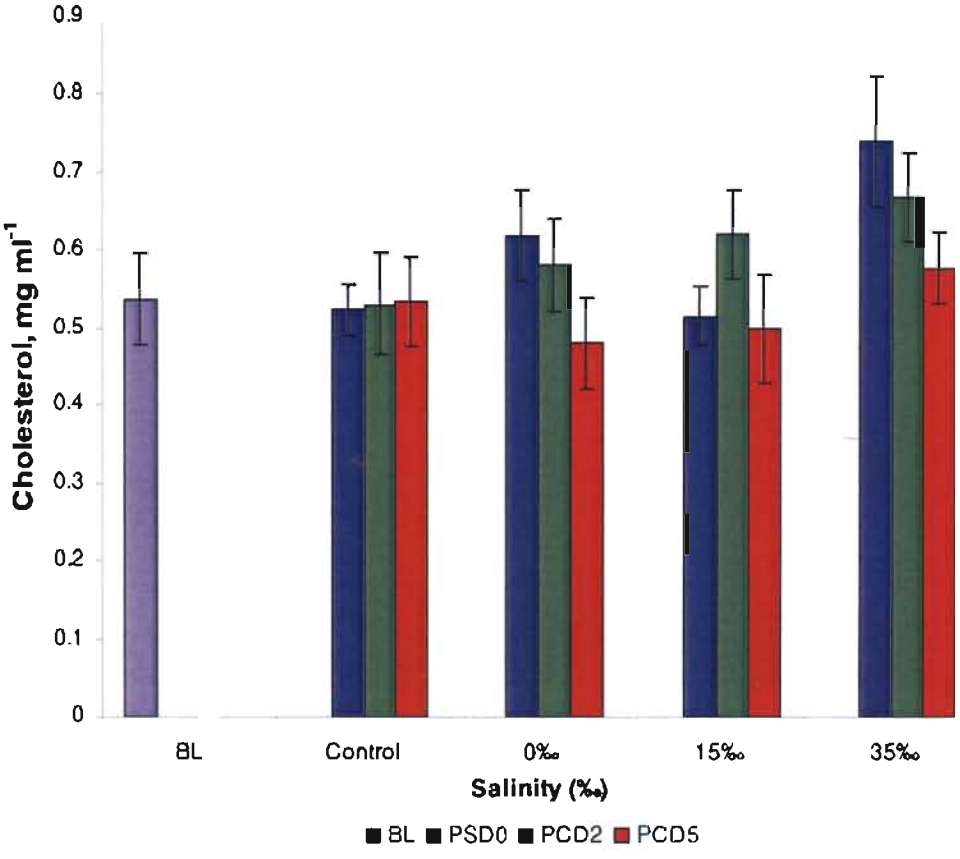
Fig.3.4 Total lipids in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.



	Glucose (mg ml^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	0.322 ± 0.03	$_{b}0.318 \pm 0.04^{A}$	$_{b}0.315 \pm 0.04^{A}$	$_{b}0.316 \pm 0.05^{A}$
0‰		$_{c}0.227 \pm 0.04^{B}$	$_{b}0.295 \pm 0.05^{A}$	$_{c}0.232 \pm 0.05^{B}$
15‰		$_{b}0.322 \pm 0.06^{B}$	$_{a}0.397 \pm 0.04^{A}$	$_{a}0.423 \pm 0.06^{A}$
35‰		$_{a}0.385 \pm 0.06^{A}$	$_{c}0.216 \pm 0.04^{B}$	$_{c}0.248 \pm 0.05^{B}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

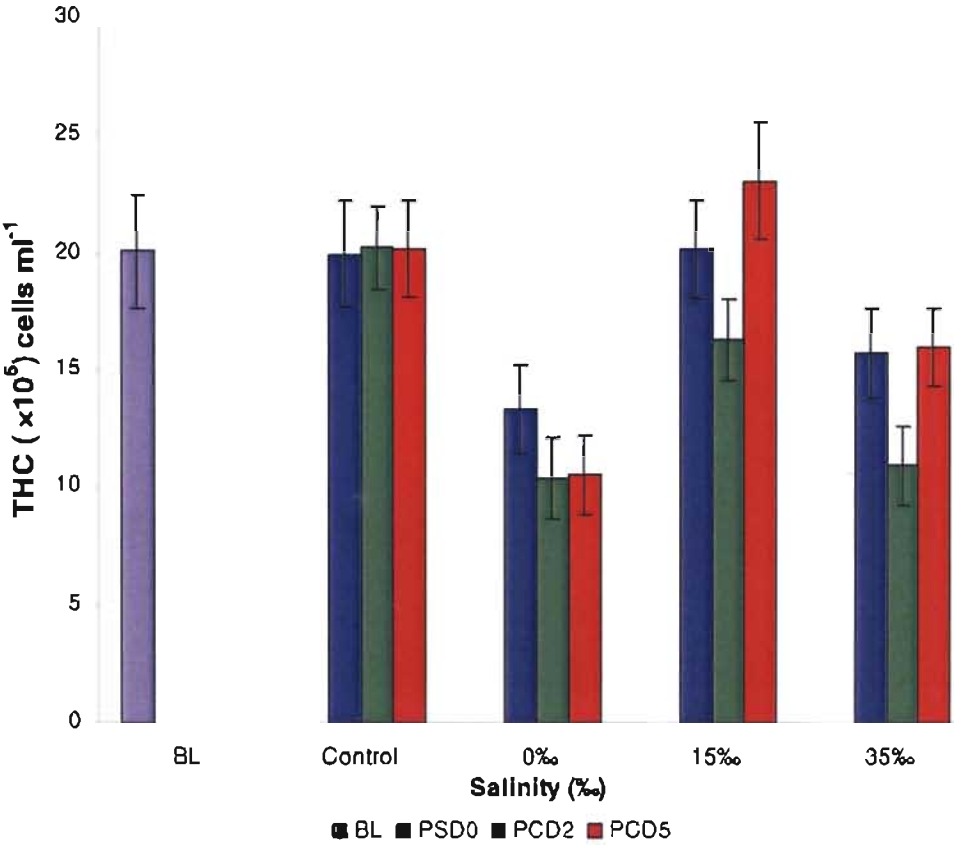
Fig.3.5 Glucose levels in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.



	Cholesterol (mg ml^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	0.537 ± 0.06	$_{c}$0.524 ± 0.03^{A}	$_{c}$0.530 ± 0.07^{A}	$_{ab}$0.533 ± 0.06^{A}
0‰		$_{b}$0.619 ± 0.06^{A}	$_{b}$0.581 ± 0.06^{A}	$_{b}$0.479 ± 0.06^{B}
15‰		$_{c}$0.515 ± 0.04^{B}	$_{ab}$0.62 ± 0.06^{A}	$_{b}$0.499 ± 0.07^{B}
35‰		$_{a}$0.739 ± 0.08^{A}	$_{a}$0.668 ± 0.06^{A}	$_{a}$0.577 ± 0.05^{B}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

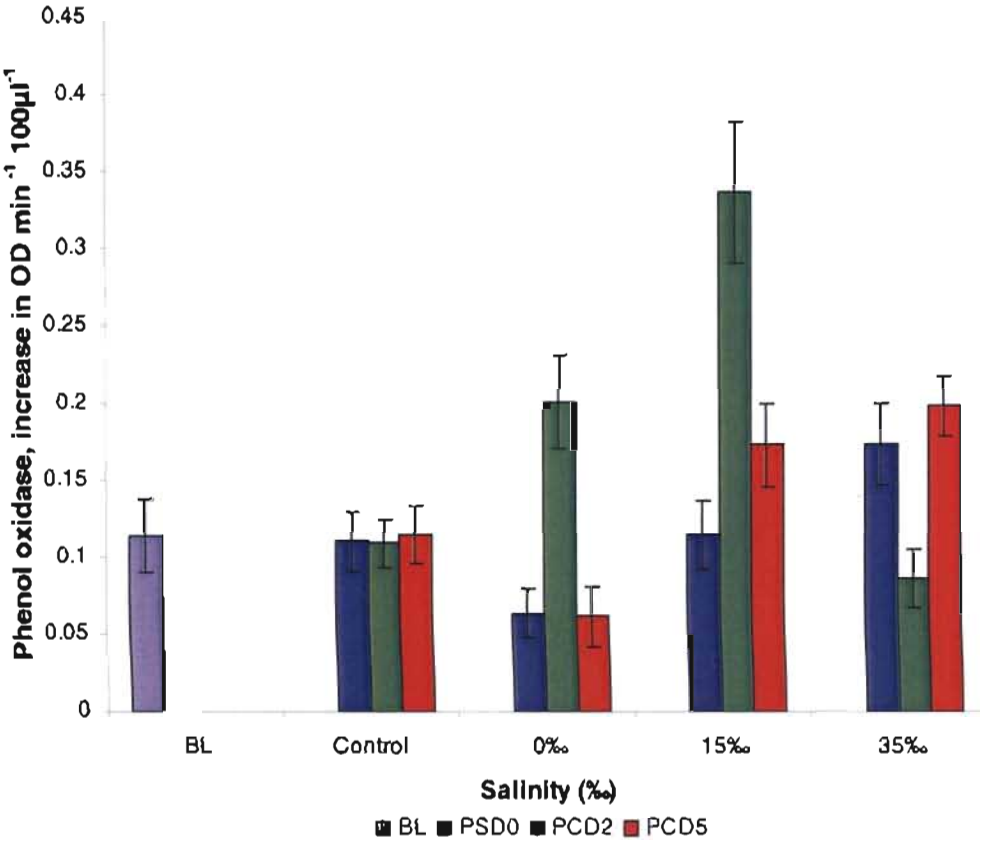
Fig.3.6 Cholesterol levels in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.



	Total Haemocyte Count (×10⁶ cells ml⁻¹)			
Salinity	BL	PSD0	PCD2	PCD5
Control	20.04 ± 2.4	$_{a}19.90 \pm 2.2^{A}$	$_{a}20.19 \pm 1.8^{A}$	$_{b}20.10 \pm 2.0^{A}$
0‰		$_{b}13.29 \pm 1.9^{A}$	$_{c}10.38 \pm 1.7^{B}$	$_{d}10.50 \pm 1.7^{B}$
15‰		$_{a}20.10 \pm 2.01^{B}$	$_{b}16.28 \pm 1.8^{C}$	$_{a}23.03 \pm 2.5^{A}$
35‰		$_{b}15.70 \pm 1.9^{A}$	$_{c}10.89 \pm 1.7^{B}$	$_{c}15.95 \pm 1.7^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

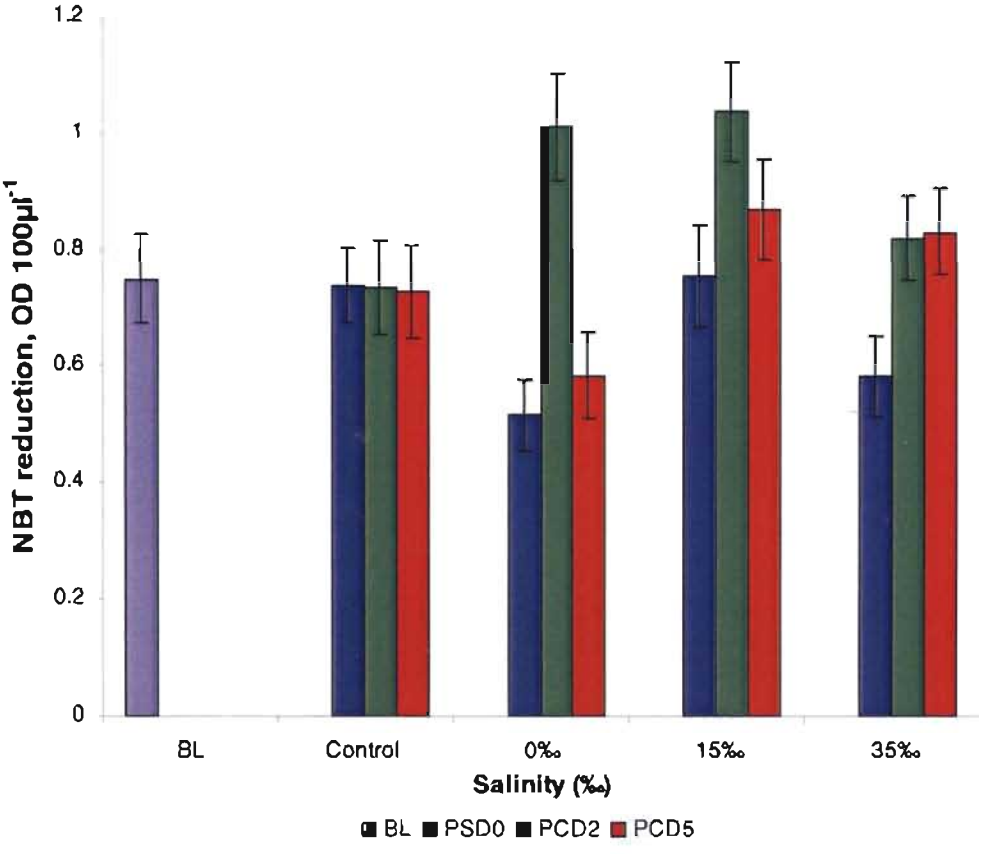
Fig.3.7 Total haemocyte count (THC) in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.



	Phenol oxidase activity (increase in OD min^{-1} $100\mu l^{-1}$)			
Salinity	BL	PSD0	PCD2	PCD5
Control	0.113 ± 0.02	$_{h}0.109 \pm 0.02^{A}$	$_{c}0.108 \pm 0.02^{A}$	$_{c}0.114 \pm 0.02^{A}$
0‰		$_{c}0.063 \pm 0.02^{B}$	$_{h}0.2 \pm 0.03^{A}$	$_{d}0.061 \pm 0.02^{B}$
15‰		$_{h}0.114 \pm 0.02^{C}$	$_{a}0.336 \pm 0.05^{A}$	$_{b}0.172 \pm 0.03^{B}$
35‰		$_{a}0.172 \pm 0.03^{A}$	$_{c}0.085 \pm 0.02^{C}$	$_{a}0.197 \pm 0.02^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

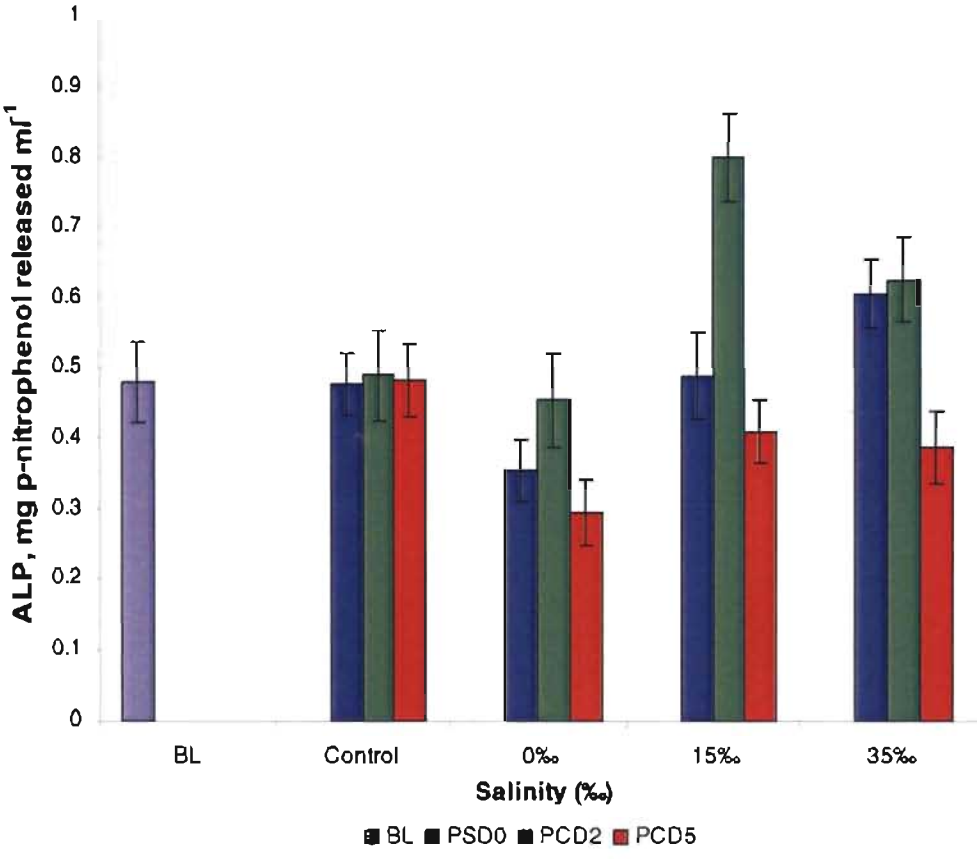
Fig.3.8 Phenol oxidase activity in *P. monodon* subjected to salinity stress and challenged with WSSV.



	NBT Reduction (OD 100µl^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	0.749 ± 0.08	$_{a}$0.738 ± 0.07^{A}	$_{b}$0.736 ± 0.08^{A}	$_{b}$0.727 ± 0.08^{A}
0‰		$_{b}$0.513 ± 0.06^{B}	$_{a}$1.01 ± 0.09^{A}	$_{c}$0.582 ± 0.07^{B}
15‰		$_{a}$0.755 ± 0.09^{C}	$_{a}$1.037 ± 0.08^{A}	$_{a}$0.869 ± 0.08^{B}
35‰		$_{b}$0.581 ± 0.07^{B}	$_{b}$0.82 ± 0.07^{A}	$_{a}$0.831 ± 0.07^{A}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

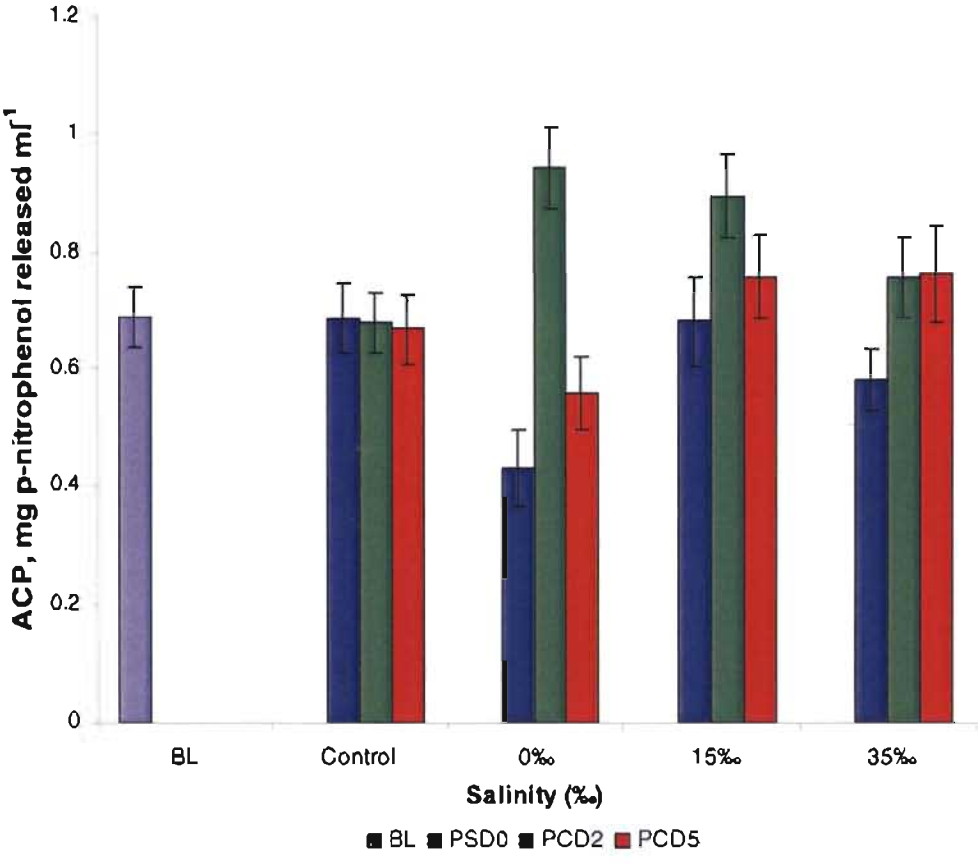
Fig.3.9 NBT reduction in *P. monodon* subjected to salinity stress and challenged with WSSV.



	Alkaline Phosphatase activity (mg p-nitrophenol released ml^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	0.478 ± 0.06	$_{b}0.476 \pm 0.04^{A}$	$_{c}0.488 \pm 0.06^{A}$	$_{a}0.48 \pm 0.05^{A}$
0‰		$_{c}0.353 \pm 0.04^{B}$	$_{c}0.453 \pm 0.07^{A}$	$_{c}0.294 \pm 0.05^{B}$
15‰		$_{b}0.488 \pm 0.06^{B}$	$_{a}0.8 \pm 0.06^{A}$	$_{b}0.409 \pm 0.05^{C}$
35‰		$_{a}0.606 \pm 0.05^{A}$	$_{b}0.624 \pm 0.06^{A}$	$_{b}0.387 \pm 0.05^{B}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

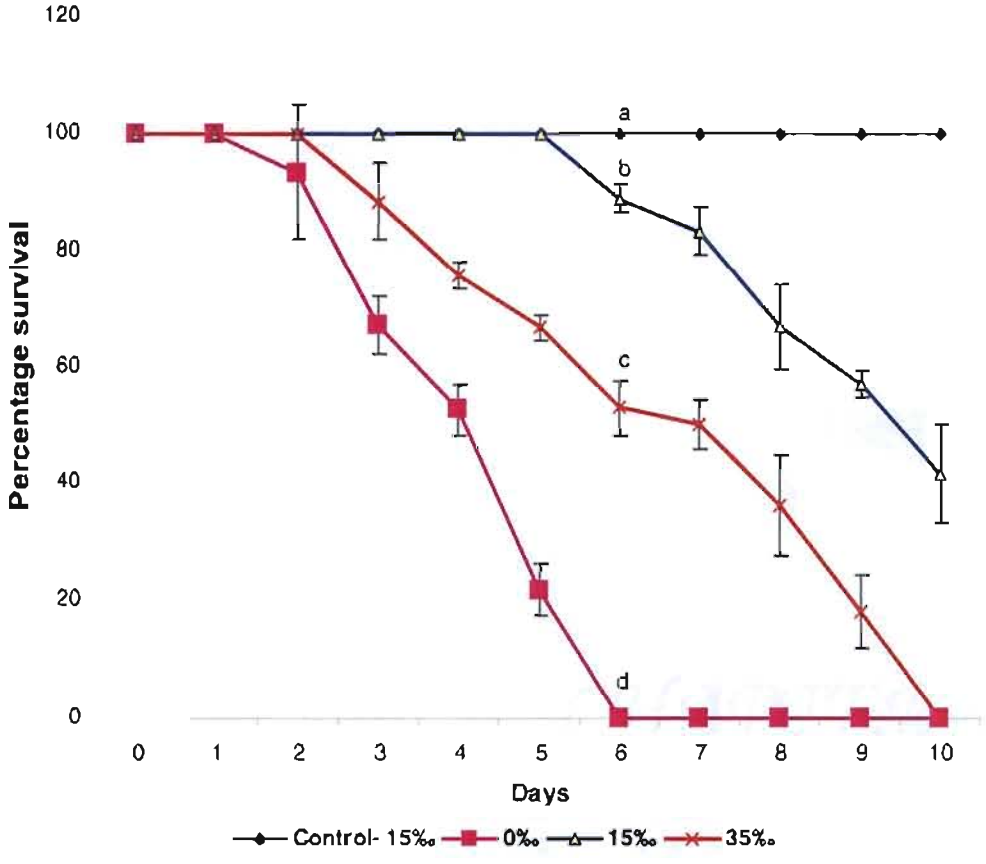
Fig.3.10 Alkaline phosphatase activity (ALP) in *P. monodon* subjected to salinity stress and challenged with WSSV.



	Acid Phosphatase activity (mg p-nitrophenol released ml^{-1})			
Salinity	BL	PSD0	PCD2	PCD5
Control	0.689 ± 0.05	$_{a}0.686 \pm 0.06^{A}$	$_{b}0.679 \pm 0.05^{A}$	$_{b}0.667 \pm 0.06^{A}$
0‰		$_{c}0.432 \pm 0.07^{C}$	$_{d}0.943 \pm 0.07^{A}$	$_{c}0.556 \pm 0.06^{B}$
15‰		$_{a}0.681 \pm 0.08^{B}$	$_{d}0.895 \pm 0.07^{A}$	$_{d}0.758 \pm 0.07^{B}$
35‰		$_{b}0.581 \pm 0.05^{B}$	$_{b}0.757 \pm 0.07^{A}$	$_{a}0.762 \pm 0.08^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PSD –Post salinity change day, PCD –Post challenge day

Fig.3.11 Acid phosphatase activity (ACP) in *P. monodon* subjected to salinity stress and challenged with WSSV.



*Different letters indicate statistical difference among different treatments

Fig.3.12 Post challenge survival of *P. monodon* subjected to salinity stress and challenged with WSSV.

CHAPTER 4

Modulatory Effect of Ambient Copper on the Haematological Responses and Susceptibility of Penaeus monodon to WSSV infection

4.1 Introduction

Metals such as copper, zinc, iron, selenium, manganese, chromium etc. are essential trace elements or micro minerals required in relatively lower amounts to support various structural and metabolic functions. They are particularly essential by virtue of their association with the functioning of many enzymatic proteins. Crustaceans and fishes generally require the same essential minerals as terrestrial animals (National Research Council, 1993) and may obtain dissolved minerals from the ambient water. However, if present in elevated concentrations, these micro-essential metals may act as pollutants or environmental contaminants producing toxic effects similar to that caused by other heavy metals such as cadmium, mercury, lead etc., which are non-essential.

Crustaceans take up trace metals from solution in proportion to the dissolved concentration of the metal (Weeks and Rainbow, 1991; Chan and Rainbow, 1993) through permeable body surfaces and from the gut (Rainbow, 2006). The free (hydrated) metal ion is commonly considered to be the form of dissolved metal available for uptake from solution (the bioavailable form) (De Lisle and Roberts, 1988; O'Brien *et al.*, 1990; Blust *et al.*, 1992). When an essential metal enters the body, it will initially be in a metabolically available form *i.e.* it is available to bind to sites where it can play an essential role (Rainbow, 2006). Excess metal is subjected to detoxification strategies and/or excreted. Detoxification through metallothioneins is known to regulate the sequestration and metabolism of variety of metals such as cadmium and copper (Viarengo, 1985). Decapod crustaceans notably have the ability to regulate their internal concentrations of copper and zinc over a range of external metal concentrations (White and Rainbow, 1982; Rainbow and White, 1989). On the other hand, if the rate of uptake is faster than the rate of its excretion and detoxification combined, the metal will accumulate and toxicity will ensue when it starts binding at sites where it will interfere with the normal metabolic functioning (Rainbow, 2006). Gills are reported to be the main target organ for all metals, followed by digestive gland and to a lesser extent by abdominal muscle (Geret *et al.*, 2002). Large quantities of stored copper are found in the hepatopancreas of crustaceans (Bryan, 1968). Manganese accumulates primarily in the nervous tissue but also in the haemolymph of *Nephrops norvegicus* (Baden and Neil, 1998; Baden *et al.*, 1999). Environmental variables such as salinity and temperature also

have influence on the solubility and toxicity of a metal as well as on the level of accumulation inside marine organisms.

Among the many trace metals, Copper (Cu) is especially important with regard to crustaceans, as it is a main component of haemocyanin, the respiratory pigment found in most crustaceans (Lall, 1989). Haemocyanin is a multifunctional protein. Apart from its prime role as oxygen transporter, it is also a storage protein, osmolite, ecdysone transporter, precursor of antifungal-peptides, and a phenol-oxidase like enzyme and is an important blood component (Fielder *et al.*, 1971; Gellisen *et al.*, 1991; Chen and Cheng, 1993a, b; Destoumieux *et al.*, 2001; Adachi *et al.*, 2003; Pascual *et al.*, 2003a). 40% of the whole body copper load in shrimp is found in haemocyanin (Depledge, 1989). This suggests a considerable increase in the physiological demand for copper by crustaceans above that required by vertebrates. Apart from haemocyanin, Cu is also bound to many other metalloproteins and enzymes which include various oxidases, and blue proteins. Lysyl oxidase, cytochrome c-oxidase, ferroxidase, amine oxidase, ascorbate oxidase, galactose oxidase, dopamine hydroxylase, tyrosinase, ceruloplasmin, superoxide dismutase etc are some of the major Cu-dependent enzymes (Lloyd *et al.*, 1978; Spiro, 1981). Copper is also involved in the absorption and metabolism of iron and functions in the formation of haemoglobin in vertebrates (Lall, 1989). Cu is the third most abundant metallic element following iron and zinc.

Copper also plays an important immunological role in organisms. Recent studies suggest that copper deficiency causes immunological impairment in humans with a decrease in circulating haemocyte numbers (Kikuchi *et al.*, 2005). Studies in animals have also clearly shown that copper deficiency lowers disease resistance (Percival, 1998). Excessive serum copper is however, immunosuppressive (Massie *et al.*, 1993). According to Lee and Shiau (2002), Cu content normally present in rearing water is not sufficient enough for shrimps to meet their physiological requirement and that dietary supplementation of 10-30 mg Cu kg^{-1} diet is required for eliciting effective non-specific immune responses.

Though an essential metal with significant biological role, when present at higher concentrations, Cu becomes toxic to living organisms, including crustaceans and fishes (Ahsanullah *et al.*, 1981; Migliore and de Nicola Giudici, 1990; Pelgrom *et al.*, 1995) Toxic doses of Cu that cause environmental contamination are produced either by natural or anthropogenic activities, mainly industrial and agricultural activities (IPCS, 1998). The primary effect of copper in fish is in the gills (Evans, 1987), which are structurally

damaged (Wilson and Taylor, 1993). A variety of metabolic disturbances, which may cause physiological stress also occur at sublethal doses and they are reflected in the composition of haemolymph. Plasma Na^+, protein and osmolality decreased and glucose and cortisol levels increased in *Oreochromis niloticus* when exposed to Cu at 400 μg l^{-1} (Monteiro *et al.*, 2005). Copper elevated oxygen consumption in brown trout (*Salmo trutta*) (Beaumont *et al.*, 1995) and bluegill (*Lepomis macrochirus*) (O'Hara, 1971) and decreased oxygen consumption in common carp (*Cyprinus carpio*) (DeBoeck *et al.*, 1995). Information on the influence of Cu on the blood metabolites is meagre with regard to crustaceans, especially shrimps. Following 48 h exposure to Cu in the range of 0-40 mg l^{-1}, the shore crab *Carcinus maenas* decreased its haemolymph osmolality (Thurberg *et al.*, 1973). Hypo and hyper-osmoregulatory capacity significantly reduced in *P. japonicus* exposed to Cu at 0.5, 1.0 and 1.5 mg l^{-1} (Bambang *et al.*, 1995).

Environmental contaminants may also result in more subtle alterations in homeostatic mechanisms such as the immune system. Modulatory effects on immune system influence the susceptibility of organisms to infection. Several studies have been conducted on the immunomodulatory effects of Cu in fishes and shellfishes, particularly molluscs (Cheng and Sullivan, 1984; Pickwell and Steinert, 1984; Suresh and Mohandas, 1990; Fagotti *et al.*, 1996). Exposure to Cu at a concentration of 0.05, 0.10, 0.15 mg l^{-1} caused a dose-dependent decrease in the number of kidney leucocytes and phagocytic response in zebra fish, *Brachydanio rerio* (Rougier *et al.*, 1994). Percentage of monocytes and neutrophils consistently elevated and respiratory burst activity reduced in rainbow trout, *Oncorhynchus mykiss* on exposure to a sub lethal dose of Cu at 26.9 μg l^{-1} (Dethloff *et al.*, 1998). When challenged with *V. tubiashi*, a significant decrease in the percentage of circulating eosinophils and intracellular release of superoxide was observed in *Mytilus edulis* pre-xposed to Cu (Pipe and Coles, 1995). Javanese carp, *Puntius gonionotus* exposed to higher doses of Cu and then challenged with *Aeromonas hydrophila* was reported to be immunosuppressed (Shariff *et al.*, 2001).

However, only very few studies have been reported on metal-induced immunomodulation in crustaceans. Cu has been reported to decrease the haemocyte count in rockpool prawn, *Palemon elegans* (Lorenzon *et al.*, 2001). Phenol oxidase activity and respiratory burst activity reduced in *Macrobrachium rosenbergii* exposed to Cu (Cheng and Wang, 2001). According to a study by Truscott and White (1990), immune system of *Carcinus maenas* is relatively tolerant to metal stress including Cu. Evans *et al.* (1999) observed a progressive reduction in haemocyte titre in crayfish, *Cherax tenuimanus* exposed to Cu at 0.2 or 0.8 mg l^{-1} after 2 weeks.

Very recently, the higher susceptibility under Cu stress was proved in *M. rosenbergii* (Cheng and Wang, 2001) and *L. vannamei* (Yeh *et al.*, 2004). Copper sulphate was used as the Cu source and use of $CuSO_4$ in evaluating the effects of copper in shrimps has added significance, as $CuSO_4$ is commonly applied in shrimp ponds to eradicate filamentous algae and is very effective in reducing the abundance of phytoplankton, including *Microcystis* and other blue-green algae (Chen and Lin, 2001). The application rate of copper sulfate varies from 0.025 to 2 mg l^{-1}; directly related to total alkalinity (Boyd, 1990). Shrimp farmers often apply excess amounts of copper sulfate in pond management. The concentration of copper sulfate remaining in water is therefore of prime concern. The 96 h LC_{50} of copper (applied as copper sulphate) for *P. monodon* juveniles was found to be 3.13 and 7.73 mgl^{-1} Cu at 15‰ and 25‰ respectively (Chen and Lin, 2001).

Stimulatory effects of metals at certain lower doses have also been reported, though focus has been given to the suppressive effects at higher doses. Cu causes stimulatory effects on fish macrophage activity (Khangarot and Tripathi, 1991). According to Bryan (1984), small amounts of metals such as Cu may be maintained in a metabolically available form to play essential biochemical roles. Acute Cu exposure of 0.2 mg l^{-1} resulted in a significant stimulation of phagocytic activity in mussel, *Mytilus edulis* (Pipe *et al.*, 1999). Also, there is evidence that small increases in metal levels can have a stimulatory effect on the growth of marine organisms (Stebbing, 1976).

The influence of ambient Cu on the haematology of *P. monodon* has not gained much attention though it is a widely cultured species, and an important part of human food. Literally, no studies have been conducted on the influence of increasing ambient Cu on the immunocompetence of *P. monodon* to WSSV, the highly devastating shrimp disease. It may be possible that Cu at higher doses makes them more susceptible to WSSV infection. Toxic effects of Cu not only affect the profit of the industry, but also can become hazardous to human health. It may also be possible that small increase in ambient Cu has beneficial stimulatory effects.

The present study on *P. monodon* was therefore aimed at determining the:

- Effects of increasing ambient Cu on the metabolic and immune variables of haemolymph
- Effects of Cu exposure on susceptibility to WSSV infection

- Effects of Cu exposure on the haemolymph metabolic variables and immune response of shrimps to WSSV infection.

4.2 Materials and methods

4.2.1 Experimental animals

Adult *P. monodon* obtained from a commercial farm in Olathala, Chertalai were used as experimental shrimps in the present study. Average wet weight of the shrimp was 22.8 ± 2.7 g (Mean ± S.D.). Shrimps were reared in concrete rectangular tanks and allowed to acclimate for a week. Rearing conditions and water quality were maintained as that for the first experiment (Refer Section 2.2.2). Metabolic and immunological profile was obtained from a group of shrimps (n=6) after acclimation for a period of seven days, as the baseline (BL) data.

4.2.2 Experimental design

Shrimps were distributed in the experimental tanks containing 500L of seawater with 30 individuals per tank (n=30/tank). There were 6 treatments (Group-I, Group-II, Group-III, Group-IV, Group-V and Group-VI) and the experiment was conducted in triplicate i.e., 3 tanks per treatment. Shrimps in the intermoult stage only were used.

4.2.3 Exposure to copper

Shrimps were maintained in the experimental tanks for 2 days, after which, the shrimps were exposed to Cu ($CuSO_4$) at various concentrations of 0.05, 0.1, 0.2 and 0.3 mg l^{-1}. During the period of exposure, water was exchanged daily in order to maintain the metal concentration and to decrease the toxicity caused by potentially poisonous metabolites excreted by animals themselves (Mc Mahon, 2001). Group-I and Group-II were maintained without Cu. After 14 days of exposure to Cu, the exposed and the unexposed shrimps (n=6) were randomly sampled (PMD14, post metal exposure day 14).

4.2.4 WSSV challenge

The shrimps of Group-II (Cu at 0 mg l^{-1}), Group-III (Cu at 0.05 mg l^{-1}), Group-IV (Cu at 0.1 mg l^{-1}) Group-V (Cu at 0.2 mg l^{-1}) and Group-VI (Cu at 0.3 mg l^{-1}) were challenged with white spot syndrome virus after 14 days of exposure to Cu. The 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 48 h (post challenge day 2,

PCD2), 120 h (post challenge day 5, PCD5) and after 10 days (post challenge day 10, PCD10) of challenge. 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 with dead animals removed promptly. Sampling days were fixed based on the rate of mortality that occurred. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of infected shrimps.

4.2.5 Extraction of haemolymph

Haemolymph was extracted according to the procedure described earlier (Refer section 2.2.6). Sampling was carried out at the beginning of the experiment (baseline), on post metal exposure day 14 (PMD14) and post challenge day 2, 5 and 10 from the six experimental groups (Group-I, Group-II, Group-III, Group-IV, Group-V and Group-VI). The immune parameters were analysed immediately and the samples stored at –20°C for the analysis of metabolic variables.

4.2.6 Analysis of haematological parameters

Metabolic variables in the haemolymph *viz.*, total protein, total carbohydrates, total free amino acids, total lipids, glucose, cholesterol and immune variables *viz.*, total haemocyte count, phenol oxidase activity, NBT reduction, alkaline phosphatase activity and acid phosphatase activity was determined according to the methods described previously (Refer Section 2.2.7).

4.2.7 Analysis of Copper

Shrimps were dissected after the extraction of haemolymph and a portion of the muscle was removed and stored in 0.1 M Tris-HCl buffer, pH 7.6, at –20°C, to analyse the extent of metal accumulation. Haemolymph was also analysed for Cu.

Tissues were acid digested prior to analysis. 5 ml of Conc. HNO_3 was added to 100 mg of muscle /100μl^{-1} of haemolymph samples, covered and left overnight at 60°C in a microwave oven. The completely digested samples were allowed to cool to room temperature, filtered and made up to 50 ml using ultra-pure (Milli-Q) water.

The processed samples were analysed for Cu as per standard conditions employing an Atomic Absorption Spectrophotometer (EC Electronic Corporation of India Ltd. AAS 4141). Air-acetylene flame was used for estimation and the absorbance was noted by adjusting the cathode lamp at a specific wavelength of 324.8 nm. Working standards for calibration were prepared by diluting commercially available standard Cu

(1000 mg l^{-1}) solution in ultra-pure water (Milli-Q). Blanks and standards were also analyzed the same way as for the samples. The concentration of Cu was then expressed in $\mu g\ ml^{-1}$ haemolymph and $\mu g\ g^{-1}$ muscle.

4.2.8 Statistical analysis

The experimental data was analysed by means of one-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means. Significance level for the analysis was set to $P<0.05$. Statistical analyses were carried out using the software SPSS 10.0.

4.3 Results

Exposure to copper at different concentrations resulted in significant variations in the haemolymph metabolic and immune variables of *P. monodon*. With regard to metabolic variables, a significant increase could be observed in total carbohydrates and total lipids in Cu-exposed shrimps. In addition, total protein and glucose significantly increased in shrimps with Cu at 0.1 mg l^{-1} and total protein markedly reduced at 0.3 mgl^{-1}. A significantly higher THC, PO and NBT were observed in shrimps exposed to Cu at 0.3 mg l^{-1} whereas, a moderate increase in all the immune variables was noted in shrimps with Cu at 0.1 mg l^{-1} except for NBT. There was a general reduction in all the metabolic variables except TFAA in shrimps with Cu at 0.2 and 0.3 mg l^{-1} on PCD2. Exposure to Cu at 0.1 mg l^{-1} was found to have an immunostimulatory effect on shrimps, which showed maximum increase in all the immune variables following infection, and enhanced post challenge survival. Exposure to higher concentrations of Cu (0.2 and 0.3 mg l^{-1}) affected the immune response of shrimps and increased the susceptibility to WSSV infection.

4.3a Copper content in shrimp tissues

A significant increase in haemolymph Cu concentration was observed at 0.3 mgl^{-1} (36.09 ± 5.81 $\mu g\ ml^{-1}$, $P<0.05$). Though the haemolymph Cu concentration tend to increase at 0.1 and 0.2 mg l^{-1} on PMD14, it was not statistically significant. A slight increase in muscle was noted in shrimps with Cu at 0.2 and 0.3 mg l^{-1}, compared to the unexposed. Following challenge, Cu levels in haemolymph tend to increase on PCD5, with the highest levels at 0.3 and 0.2 mg l^{-1} (45.43 ± 6.4 and 39.65 ± 4.51 $\mu g\ ml^{-1}$ respectively). On the other hand, Cu levels in muscle tend to decrease after challenge (Table. 4.1a and b).

Table 4.1a Copper content in the haemolymph of *P. monodon* exposed to copper and then challenged with WSSV.

	Cu (μg ml^{-1})			
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	29.90 ± 4.84	$_{ab}$30.19 ± 2.93^{A}	$_{a}$29.54 ± 4.12^{A}	$_{c}$30.06 ± 4.06^{A}
0		$_{ab}$30.54 ± 2.95^{A}	$_{a}$31.64 ± 4.59^{A}	$_{bc}$34.55 ± 5.67^{A}
0.05		$_{b}$28.93 ± 4.77^{AB}	$_{a}$28.35 ± 4.42^{B}	$_{bc}$35.61 ± 6.97^{A}
0.1		$_{ab}$32.86 ± 5.53^{A}	$_{a}$31.55 ± 6.04^{A}	$_{b}$37.75 ± 6.92^{A}
0.2		$_{ab}$34.42 ± 4.94^{A}	$_{a}$27.38 ± 4.39^{B}	$_{ab}$39.65 ± 4.51^{A}
0.3		$_{a}$36.09 ± 5.81^{B}	$_{a}$25.74 ± 4.67^{C}	$_{a}$45.43 ± 6.40^{A}

Table 4.1b Copper content in the muscle of *P. monodon* exposed to copper and then challenged with WSSV.

	Cu (μg g^{-1})			
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	4.10 ± 0.39	$_{ab}$4.06 ± 0.35^{A}	$_{ab}$3.98 ± 0.41^{A}	$_{ab}$4.04 ± 0.31^{A}
0		$_{ab}$4.07 ± 0.30^{A}	$_{b}$3.85 ± 0.36^{A}	$_{a}$4.27 ± 0.38^{A}
0.05		$_{b}$3.89 ± 0.33^{AB}	$_{b}$3.76 ± 0.21^{B}	$_{ab}$4.12 ± 0.21^{A}
0.1		$_{ab}$4.18 ± 0.38^{A}	$_{ab}$3.99 ± 0.19^{A}	$_{ab}$4.04 ± 0.51^{A}
0.2		$_{a}$4.43 ± 0.53^{A}	$_{ab}$4.2 ± 0.49^{AB}	$_{bc}$3.67 ± 0.31^{B}
0.3		$_{a}$4.55 ± 0.43^{A}	$_{a}$4.45 ± 0.46^{A}	$_{c}$3.53 ± 0.40^{B}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

4.3b Haemolymph metabolic variables

Total protein

A significant increase (P<0.05) in haemolymph total protein was noted in shrimps exposed to Cu at 0.1 and 0.2 mg l^{-1}, whereas a significant decrease (P<0.05) was obtained with Cu at 0.3 mg l^{-1}. Even though total protein levels increased following WSSV infection in all groups, post challenge protein levels were significantly lower in shrimps with Cu at 0.3 mg l^{-1}. Significantly higher protein concentrations *viz.*, 136.85 ± 17.9, 117.56 ± 15.4 and 133.12 ± 18.8 mg ml^{-1} were recorded on PCD2 in unexposed and shrimps exposed to Cu at 0.05 and 0.1 mg l^{-1} respectively. Maximum protein concentration on PCD10 was recorded in shrimps with Cu at 0.1 mg l^{-1} (104.3 ± 13.4 mg ml^{-1}) (Fig.4.1).

Total carbohydrates

Exposure to Cu resulted in a significant increase in total carbohydrate concentration of shrimp haemolymph ($P<0.05$). Carbohydrate levels recorded on PMD14 were 4.56 ± 0.55, 4.1 ± 0.57, 4.36 ± 0.49 and 4.09 ± 0.22 mg ml^{-1} in shrimps exposed to Cu at 0.05, 0.1, 0.2 and 0.3 mg l^{-1} respectively. Following challenge, carbohydrate levels reduced in shrimps with Cu at 0.2 and 0.3 mg l^{-1}. However, the concentration increased on PCD5. No significant difference was noted in the carbohydrate concentration following challenge in shrimps exposed to Cu at 0.1 and 0.05 mg l^{-1}. PCD10 showed a declining trend in total carbohydrate concentration in the surviving shrimps (Fig.4.2).

Total free amino acids

Cu exposure resulted in a significant increase in TFAA concentration in shrimp haemolymph ($P<0.05$). Significantly lower TFAA levels of 2.61 ± 0.39, 2.51 ± 0.33, 2.58 ±0.28 and 2.53 ± 0.18 mg ml^{-1} were recorded in shrimps with Cu at 0.05, 0.1, 0.2 and 0.3 mg l^{-1} respectively compared to that in unexposed shrimps (3.02 ± 0.23 mg ml^{-1}). Following challenge, TFAA increased in both Cu-exposed and unexposed groups, with maximum increase in shrimps with Cu at 0.3 (4.59 ± 0.64 mg ml^{-1}) and 0.2 mg l^{-1} (4.05 ± 0.46 mg ml^{-1}) on PCD2 ($P<0.05$) (Fig.4.3).

Total lipids

Total lipid levels increased significantly in shrimps exposed to Cu at 0.1, 0.2 and 0.3 mg l^{-1}, being significantly higher at 0.3 and 0.2 mg l^{-1} ($P<0.05$). Total lipid concentration recorded in haemolymph was 2.04 ± 0.14, 2.27 ± 0.16 and 2.37 ± 0.24 mg ml^{-1} in shrimps with Cu at 0.1, 0.2 and 0.3 mg l^{-1} respectively on PMD14. Following challenge, lipid levels reduced in all groups and significantly lower levels *viz.*, 1.41 ± 0.11, 1.58 ± 0.2, 1.48 ± 0.2, 1.56 ± 0.2 and 1.48 ± 0.17 mg ml^{-1} were recorded on PCD5 in unexposed shrimps and shrimps exposed to Cu at 0.05, 0.1, 0.2 and 0.3 mg l^{-1} respectively ($P<0.05$) (Fig.4.4).

Glucose

Glucose concentration significantly increased in shrimps exposed to Cu at 0.05, 0.1 and 0.2 mg l^{-1}, with maximum increase at 0.1 mg l^{-1} ($P<0.05$). Haemolymph glucose concentration recorded on PMD14 was 0.469 ± 0.04, 0.668 ± 0.04 and 0.535 ± 0.07 mg ml^{-1} in shrimps exposed to Cu at 0.05, 0.1 and 0.2 mg l^{-1} respectively. Comparatively higher glucose concentration was obtained in shrimps exposed to Cu at 0.1 mg l^{-1} on PCD2. Cu-exposed shrimps showed significantly higher glucose levels on PCD5

compared to the unexposed (P<0.05). Shrimps with Cu at 0.3 mg l^{-1} showed a tremendous increase in glucose concentration on PCD5 (1.277 ± 0.11 mg ml^{-1}) (Fig.4.5).

Cholesterol

A slight decrease in cholesterol concentration was noted in shrimps exposed to Cu at 0.05 and 0.3 mg l^{-1} on PMD14 (P<0.05). Cholesterol concentration following WSSV-challenge was significantly lower in shrimps exposed to Cu at 0.05, 0.2 and 0.3 mg l^{-1} (P<0.05). Significantly higher cholesterol concentrations were recorded in the surviving shrimps (Fig.4.6).

4.3c Immune response

Total haemocyte count

A significantly higher THC was observed in shrimps exposed to Cu at 0.1, 0.2 and 0.3 mg l^{-1}, with a remarkable increase at 0.3 mg l^{-1} (P<0.05). THC recorded were 24.34 ± 2.7, 25.38 ± 2.5 and 28.41 ± 3.0 × 10^6 cells ml^{-1} in shrimps exposed to Cu at 0.1, 0.2 and 0.3 mg l^{-1} respectively on PMD14. Following challenge, maximum decrease was observed in shrimps with Cu at 0.2 and 0.3 mg l^{-1}. THC on PCD2 decreased by 28.3%, 41.1%, 69.3% and 71.4% on PCD2 in shrimps exposed to Cu at 0.05, 0.1, 0.2 and 0.3 mgl^{-1} respectively. Shrimps with Cu at 0.1 mg l^{-1} showed maximum THC on PCD10 (Fig.4.7).

Phenol oxidase activity

Cu exposure resulted in a significant increase in the PO activity of shrimps with a remarkably higher increase at 0.3 mg l^{-1} (P<0.05). PO activity in shrimps following exposure to Cu at 0.05, 0.1, 0.2 and 0.3 mg l^{-1} were 0.136 ± 0.01, 0.133 ± 0.02, 0.14 ± 0.02 and 0.163 ± 0.02 increase in OD min^{-1} 100µl^{-1} respectively. Following challenge, though the activity increased in all groups, Cu-exposed shrimps showed lesser activity compared to the unexposed with maximum reduction at 0.2 and 0.3 mg l^{-1} (P<0.05). PO activity on PCD2 in shrimps exposed to Cu at 0.05, 0.1, 0.2 and 0.3 mg l^{-1} were 0.148 ± 0.02, 0.174 ± 0.02, 0.123 ± 0.02 and 0.115 ± 0.02 respectively (Fig.4.8).

NBT reduction

Exposure to Cu at 0.2 and 0.3 mg l^{-1} induced a significant increase in NBT reduction (P<0.05). Following WSSV-challenge the activity significantly reduced in shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} with maximum reduction at 0.3 mg l^{-1} (0.526 ± 0.09 OD 100µl^{-1}). Maximum NBT activity on PCD2 was obtained for unexposed

shrimps (1.076 ± 0.09) and those exposed to Cu at 0.1 mg l^{-1} (1.054 ± 0.09 OD 100µl^{-1}). A significantly higher activity was recorded on PCD5 in shrimps with Cu at 0.1 mg l^{-1} (0.929 ± 0.08 OD 100µl^{-1}) (P<0.05) (Fig 4.9).

Alkaline phosphatase activity

Exposure to Cu at 0.1 mg l^{-1} induced a significant increase in ALP activity (0.541 ± 0.07), whereas, Cu at 0.3 mg l^{-1} reduced the activity significantly (0.277 ± 0.05 mg p-nitrophenol released ml^{-1}). Following challenge, the activity increased in unexposed and exposed shrimps with Cu at 0.05 and 0.1 mg l^{-1}. Significantly higher activity compared to the unexposed was obtained in shrimps with Cu at 0.1 mg l^{-1} on PCD2 (1.16 ± 0.09) and PCD5 (0.713 ± 0.06 mg p-nitrophenol released ml^{-1}). Shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} showed a significantly lower post challenge ALP activity (Fig.4.10).

Acid phosphatase activity

ACP activity significantly increased in shrimps following exposure to Cu at 0.1 mg l^{-1} (0.965 ± 0.07 mg p-nitrophenol released ml^{-1}) (P<0.05). Maximum activity following challenge was also observed in shrimps exposed to Cu at 0.1 mg l^{-1} (1.159 ± 0.11). Comparatively lower activities were found in shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} on post challenge days (P<0.05). ACP activity in shrimps with Cu at 0.2 and 0.3 mg l^{-1} were 0.859 ± 0.06 and 0.85 ± 0.07 mg p-nitrophenol released ml^{-1}on PCD2 (Fig.4.11).

4.3d Post challenge survival

Fig.4.12 shows the percentage survival rates of *P. monodon* exposed to sublethal concentrations of Cu and then challenged with WSSV. Shrimps exposed to Cu at 0.1 mgl^{-1} showed significantly higher post challenge survival compared to the unexposed and other Cu-exposed shrimps (P<0.05). Exposure to Cu at 0.2 and 0.3 mg l^{-1} significantly reduced the survival rate. Shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} succumbed to death on PCD9 when the unexposed and exposed shrimps at 0.05 and 0.1 mg l^{-1} showed significantly higher percentage survival of 59.72 ± 2.4%, 66.67 ± 4.2% and 77.78 ± 4.8% respectively (P<0.05). Shrimps with Cu at 0.1 mg l^{-1} showed 46.97 ± 2.6% survival on PCD12 when the unexposed shrimps and those with Cu at 0.05 mg l^{-1} succumbed to death.

4.4 Discussion

Nutritional status is considered one of the important factors that determine the ability of animals to withstand infections; hence, nutritional status together with the immune condition is considered a good health indicator of cultured shrimp (Bachere 2000; Lopez *et al.*, 2003). Results of the present study have revealed that Cu in ambient water significantly influences the metabolic variables of shrimp haemolymph. Slightly improved levels of haemolymph total protein, total carbohydrates and total lipids were observed in shrimps with Cu at 0.1 mg l^{-1} indicating a better nutritional condition.

The pronounced reduction of haemolymph total protein in shrimps exposed to Cu at 0.3 mg l^{-1} is mostly due to a decrease of haemocyanin. The respiratory pigment haemocyanin represents near to 90% of blood proteins in crustaceans (Rochu and Fine, 1978; Depledge and Bjeregaard, 1989). An increase in protein content in haemolymph related to haemocyanin has been seen in *P. monodon* (Chen and Cheng, 1995) and *P. japonicus* (Chen and Cheng, 1993b). A decrease in haemocyanin can have serious reflexes on the oxygen transport to the shrimp tissues and results in a general debilitation of the animals. Possibly, there was also a preferential utilization of proteins as an energy source to meet the increasing energy demands under stress, as suggested by Racotta and Palacios (1998). Decrease in total proteins has been reported repeatedly during several stressful conditions in shrimp (Chen *et al.*, 1994; Sanchez *et al.*, 2001; Perazzolo *et al.*, 2002). The physiological and immune condition of shrimps depends mainly on proteins. Hence the decrease in proteins can also be related to a depletion of the immune proteins contributing the immune response. On the contrary, there was an increase in haemolymph total protein in shrimps exposed to Cu at 0.1 and 0.2 mg l^{-1}. However, under pathological stress, the protein levels in shrimps exposed to Cu at 0.2 mg l^{-1} were comparatively lower. In effect, shrimps exposed to Cu at 0.1 mg l^{-1} showed a better physiological and immune status when both the pre-challenge and post-challenge protein levels were taken into consideration.

Slightly elevated total carbohydrate concentration was observed in Cu-exposed shrimps. However, no substantial changes were noticed at different doses. On the other hand, the hike in glucose levels under Cu-exposure varied with the dosage and at the highest concentration of 0.3 mg l^{-1}, glucose levels did not vary. When the degree of glucose elevation at 0.1 mg l^{-1} is taken into consideration, it may be assumed that the increase in total carbohydrates at 0.1 mg l^{-1} is predominantly a result of an increase in glucose. Whereas, in other exposed groups, complex carbohydrates also accounts for the

increase and at 0.3 mg l^{-1}, the increase is solely due to complex forms. This fact throws light on the inhibitory effects of Cu at higher doses on tissue uptake and biochemical processes related to carbohydrate metabolism. Shrimps otherwise utilize complex carbohydrates more efficiently than simple carbohydrates (Shiau and Peng, 1991). However, following pathogenic stress a delayed increase in total carbohydrates was observed in shrimps with Cu at 0.2 and 0.3 mg l^{-1}. According to Reddy *et al.* (1996), heavy metals may cause hyperglycemia *via* increased secretion of CHH. Surprisingly, in the present study, hyperglycemia was not observed with Cu at 0.3 mg l^{-1}. However, following challenge a four-fold increase was noted on PCD5 in shrimps with Cu at 0.3 mg l^{-1}. Dhanapakiam and Ramasamy (2001) reported a decrease in serum glucose after 30 days of exposure to Cu and Zn mixtures. These observations stimulate considerable research into the mechanisms by which glucose levels are increased. Hyperglycemia is usually indicative of a short-term stress. Hyperglycemia observed after 14 days of Cu-exposure may be a mechanism to activate several other compensatory responses. Although glucose levels elevated almost two-fold (0.36 to 0.66 mg ml^{-1} with Cu at 0.1 mg l^{-1}) in haemolymph of shrimps exposed to Cu at 0.1 mg l^{-1}, it did not reach the absolute level of 30 mg dl^{-1} generally reported for shrimp submitted to short-term stressors (Hall and van Ham, 1998; Racotta and Palacios, 1998).

A variation in the level of free amino acids in shrimp haemolymph following Cu exposure was probably a mechanism to cope with the osmoregulatory disturbances. Free amino acid level slightly reduced in Cu-exposed shrimps. There are reports of osmoregulatory disturbances in fishes and crustaceans induced under Cu exposure. Acute exposure to ambient Cu significantly reduced plasma sodium and chloride concentration in different freshwater fish species (Grosell *et al.*, 2002). In *Opsanus beta*, a marine teleost fish, iono and osmoregulatory imbalances occurred after Cu exposure (Grosell *et al.*, 2004). Free amino acids play a prime role in the osmoregulation of several crustaceans (Cobb *et al.*, 1975; Abe *et al.*, 1999). Change in amino acid level in the present case may hence be considered an indication of osmotic imbalance. Amino acids might have been used to maintain the osmotic pressure. According to Graney and Giesy (1968), the mechanisms, which may account for changes in free amino acids, may include interference with protein metabolism, increased oxidation of amino acids and changes in protein synthesis rates. However, following challenge the increasing trend usually seen in unexposed challenged shrimps was seen in Cu-exposed shrimps too. Presumably, the temporary regulatory mechanisms hampered after the entry of pathogen and free amino acid level increased with a maximum at higher doses of Cu. Further research may be required in this regard for better explanation.

An improved appetite leading to an increased feed intake may be a plausible explanation for the moderate increase in haemolymph metabolites including total lipids with Cu at 0.1 mg l^{-1}. Role of Zinc in the regulation of food intake and impaired appetite under Zn deficiency has been proved (Flier and Flier, 1998). However, too high an increase with 0.2 and 0.3 mg l^{-1} suggests the involvement of other factors. Cu has a great capacity to alter membrane structural lipids and could provoke membranous disruption (Roncero *et al.*, 1992). Due to constant contact with the external environment, gills are the first target and the thin and extensive gill epithelium may be damaged by ambient Cu at higher doses (De Boeck *et al.*, 2001; Mazon *et al.*, 2002). Hence, it may be possible that lipids were required for the repair of structural damage caused to gills and mobilization of lipid reserves from hepatopancreas and muscle occurred to enhance the repair. This self-repair process probably hindered following challenge and the lipid levels reduced as in unexposed. Plasma cholesterol levels increased for the first 15 days in rainbow trout on exposure to sublethal levels of Cu and decreased at day 21 (Munoz *et al.*, 1991). In the present study, a small decrease in haemolymph total cholesterol was noted in shrimps with Cu at 0.2 and 0.3 mg l^{-1} on day 14. Total cholesterol level was found to be associated with disease resistance in fish (Maita *et al.*, 1998). Following challenge, increased cholesterol level was noticed on PCD10 in unexposed shrimps and those exposed to Cu at 0.05 and 0.1 mg l^{-1}, the groups which showed comparatively better post challenge survival.

THC increased in shrimp haemolymph following Cu exposure in the present study. Increase in the number of total circulating haemocytes is more commonly reported in bivalves on exposure to environmental toxicants. Cu concentrations of 0.02 and 0.05 mg l^{-1} increased the total number of circulating haemocytes in *Mytilus edulis* (Pipe and Coles, 1995). Reports on the effects of metals on haemocyte number in crustaceans are however, conflicting. Short-term exposure (96 h) to sub lethal levels of dissolved Cu, Zn, Hg, Cr and Cd caused a decrease in haemocyte count during the first 8 h exposure but returned to the initial levels after 16 h in the shrimp *Palaemon elegans* (Lorenzon *et al.*, 2001). Truscott and White (1990) noticed no changes in haemocyte number in shore crab, *Carcinus maenas* following exposure to Cu, Hg or Cd for 30 days. According to Coles *et al.* (1995) increase in total numbers of circulating haemocytes following contaminant exposure, occurs either by migration of cells from tissues or by blood cell proliferation. In the present case, increase in THC following Cu exposure was probably due to the migration of cells from tissues. Though Cu exposure stimulated an increase in haemocyte numbers of shrimps, a reversible decrease was observed after 48 h of WSSV challenge.

Maximum decrease was observed in shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} that showed a remarkably higher THC before challenge. This in turn suggests a possibility that the metal at higher dose actually reduced the whole body cell counts, as against the higher haemocyte count observed in haemolymph. Further increase on PCD5 occurred probably as a result of blood cell proliferation following infection, which may be noticed only in unexposed shrimps, and those exposed to Cu at 0.05 and 0.1 mg l^{-1}.

Exposure to Cu significantly increased phenol oxidase activity in the present study. An earlier work on *L. vannamei* showed a significant reduction in phenol oxidase activity following 24 h exposure to Cu at 5mg l^{-1} or greater (Yeh *et al.*, 2004). However, the exposure time and Cu dose differed in the two studies. Shrimps were exposed to Cu for 14 days and to relatively lower dosages (Cu at 0.05, 0.1, 0.2 and 0.3 mg l^{-1}) in the present experiment. Cu at nominal doses presumably stimulated the proPhenol oxidase system. Although phenol oxidase activity increased at higher doses, following pathogenic stress the activity was found to decline, presumably due to the inability to withstand the dual stress. Increase in phenol oxidase activity induced from the administration of immunostimulants by immersion or through diet has earlier been reported in *P. monodon*, *M. japonicus* and *L. vannamei* (Itami *et al.*, 1989; Boonyaratpalin *et al.*, 1993; Cheng *et al.*, 2005). Very recently, Vitamins C and E were found to increase the PO activity when treated with haemocyte lysate fraction of *M. rosenbergii* (Sahoo *et al.*, 2005). Further research on mRNA transcription of proPO and serine protease activity of Cu-exposed *P. monodon* may be required for a reliable explanation on the stimulatory effects of Cu on proPO system.

An increase in respiratory burst activity following WSSV-challenge was observed only in shrimps exposed to Cu at 0.05 and 0.1 mg l^{-1} similar to the trend in the unexposed. On the other hand, respiratory burst activity was found to reduce in shrimps with Cu at 0.2 and 0.3 mg l^{-1} upon infection. Superoxide anions with microbicidal activity produced by phagocytes by the activation of the membrane-bound NADPH-oxidase plays an important role in the host defense. Hence, increased superoxide anion production in the event of a pathogenic invasion is beneficial to the host. However, in shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1}, a significant increase in superoxide anion production was observed on the 14^{th} day of exposure, prior to challenge. This increased generation of reactive oxygen species probably occurred under the influence of higher Cu doses. The inhibition of antioxidant enzyme activities may have aggravated the stress. Environmental contaminants can influence oxyradical generation as well as the antioxidant enzyme levels (Pipe and Coles, 1995). According to Estevez *et al.* (2002)

accumulation of heavy metals may enhance superoxide anion production. A very high increase in respiratory burst activity was also seen in *M. rosenbergii* on exposure to copper sulfate at 0.2, 0.3 and 0.4 mg l^{-1} (Cheng and Wang, 2001). A small increase in superoxide anion is beneficial with respect to increased immunity (Munoz *et al.*, 2000), but too great an increase may be toxic to the host. An imbalance of oxidants and antioxidants in favour of the former, leads to oxidative stress and is capable of inflicting injury on membrane lipids, proteins and nucleic acids (Zimmerman, 1998). Determination of antioxidant enzyme activities and lipid peroxidation levels in haemolymph as well as hepatopancreas and gills of Cu-exposed shrimps in chapter 6 provides better clarification in this regard.

Cu at 0.1 mg l^{-1} enhanced activities of the important degradative lysosomal enzymes *viz.*, acid and alkaline phosphatase in the present study. The increase in activity persisted even after challenge, higher than that in the unexposed shrimps. However, Cu at 0.3 mg l^{-1} significantly reduced the alkaline phosphatase activity. Pickwell and Stenert (1984) could find an increase in the release of degradative enzymes into haemolymph under cupric ion stress. ß-glucuronidase activity in *Crassostrea virginica* was also found to be stimulated on Cu exposure (Cheng, 1989). Few other works on the contrasting effects produced by lower and higher levels of contaminants supporting the observation in present study has also been reported. Phagocytic indices for bivalve molluscs were enhanced by low levels of heavy metal exposure (Cheng and Sullivan, 1984), but tend to decline with higher concentrations (Fries and Tripp, 1980). These contrasting effects hence suggest that defining the limits for a particular stress factor may be an important concept in immunomodulation.

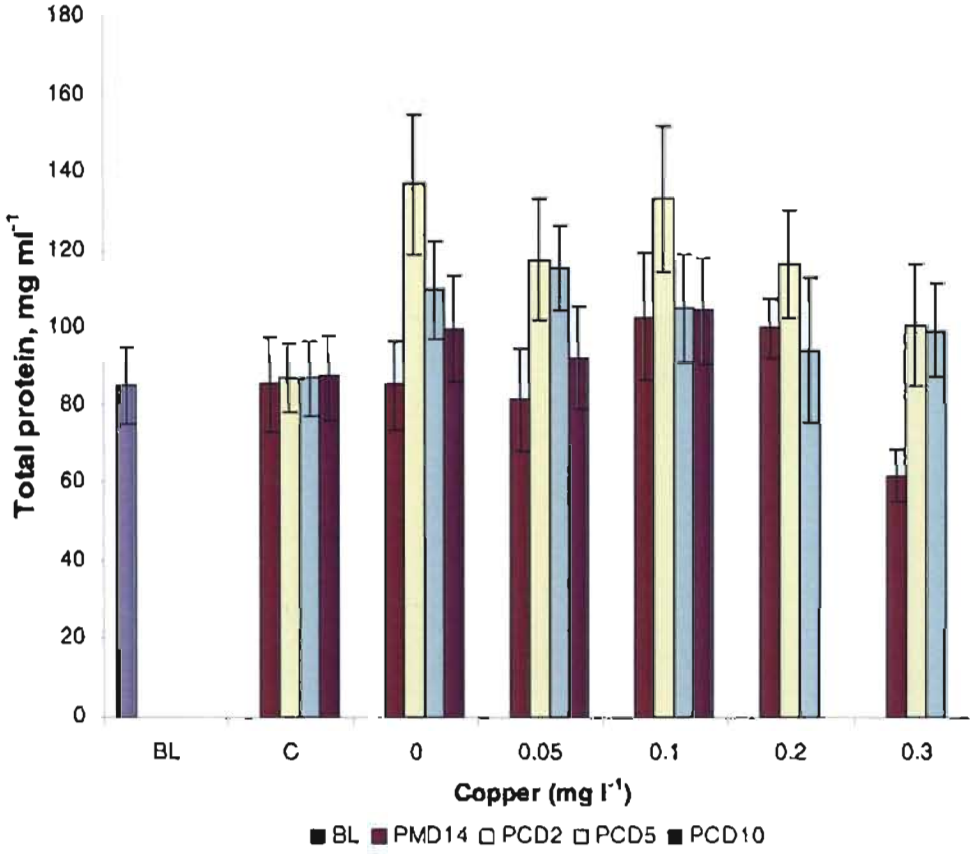
Increased metal uptake with increasing ambient Cu is evident from the haemolymph Cu concentration of the various Cu-exposed groups, though a statistically significant increase was noted only at 0.3 mg l^{-1}. Most aquatic animals absorb heavy metals *via* the gills and are then transferred to blood and other parts of the body (Soegianto *et al.*, 1999). Cu turnover into the plasma from gill is known to increase with elevated dissolved Cu levels in fishes (Pelgrom *et al.*, 1995; Grosell *et al.*, 1997). However, the accumulation pattern depends on both uptake and elimination rates (Hakanson, 1984). The slight increase seen in the present study shows the effective detoxification of the metal and storage in hepatopancreas or excretion. *Palaemon elegans* is able to regulate the body concentration of Cu over a wide range of dissolved Cu exposures (Rainbow and White, 1989). Compared to the elimination rate, the uptake rate was probably more at higher doses of Cu as slight accumulation was noted in muscle. A

post challenge elevation in haemolymph Cu concentration and a reduction in muscle are probably due to the mobilization of stored Cu.

Exposure to Cu in the present study, at relatively higher doses was found to reduce the survival rate of *P. monodon*. Shrimps were more susceptible to WSSV infection when exposed to Cu at 0.2 and 0.3 mg l^{-1}. Higher susceptibility to infection at higher doses of Cu is in agreement with the earlier observations. Mortality increased with increasing Cu concentration in *V. tubiashi* challenged mussel, *Mytilus edulis* (Pipe and Coles, 1995). Concentration of Cu^{2+} at 1 mg l^{-1} or greater increased the susceptibility of *L. vannamei* to *V. alginolyticus* infection (Yeh *et al.*, 2004). However, shrimps had better resistance against infection when a Cu dose of 0.1 mg l^{-1} was present in the ambient water. Compared to the unexposed, shrimps exposed to Cu at 0.1 mg l^{-1} showed maximum post challenge survival. Previous work by Cheng and Wang (2001) has reported the decreased susceptibility of *M. rosenbergii* against *Lactococcus garvieae* infection when exposed to 0.1 mg l^{-1} copper sulfate, and the increased susceptibility when reared in water containing copper sulfate at 0.4 mg l^{-1}. A similar phenomenon was observed in the present study. Exposure to Cu at 0.1 mg l^{-1} greatly enhanced the resistance of shrimps to WSSV infection whereas; relatively higher Cu doses of 0.2 and 0.3 mg l^{-1} lowered the resistance.

Overall, the analysis of haematological indices has showed that exposure to Cu significantly influenced various aspects of metabolic and immune function in shrimps. A better physiological and immune status at the time of challenge could be well correlated with the better post challenge survival in shrimps with Cu at 0.1 mg l^{-1}. Poor metabolic and immune response in shrimps with Cu at 0.2 and 0.3 mg l^{-1} after challenge resulted in the poor post challenge survival. Although Cu induced immunomodulation in *P. monodon*, both at nominal doses of 0.05 and 0.1 mg l^{-1}, and at relatively higher doses of 0.2 and 0.3 mg l^{-1}, immunostimulatory effects provoked by Cu at 0.1 mg l^{-1} Cu increased the immunocompetence of *P. monodon* to WSSV. Higher doses had a suppressive effect on the shrimp immune system as the immunocompetence was reduced. The role of Cu (at a lower dose) in eliciting effective immune response in shrimps is also clear from the present study. It is possible that the interaction of metal with the host is generating a generalized response rather than certain specific effects. Further research is recommended to elucidate the actual mechanism through which the metal stimulates the shrimp immune system and the more harmful effects produced by much higher concentrations of Cu.

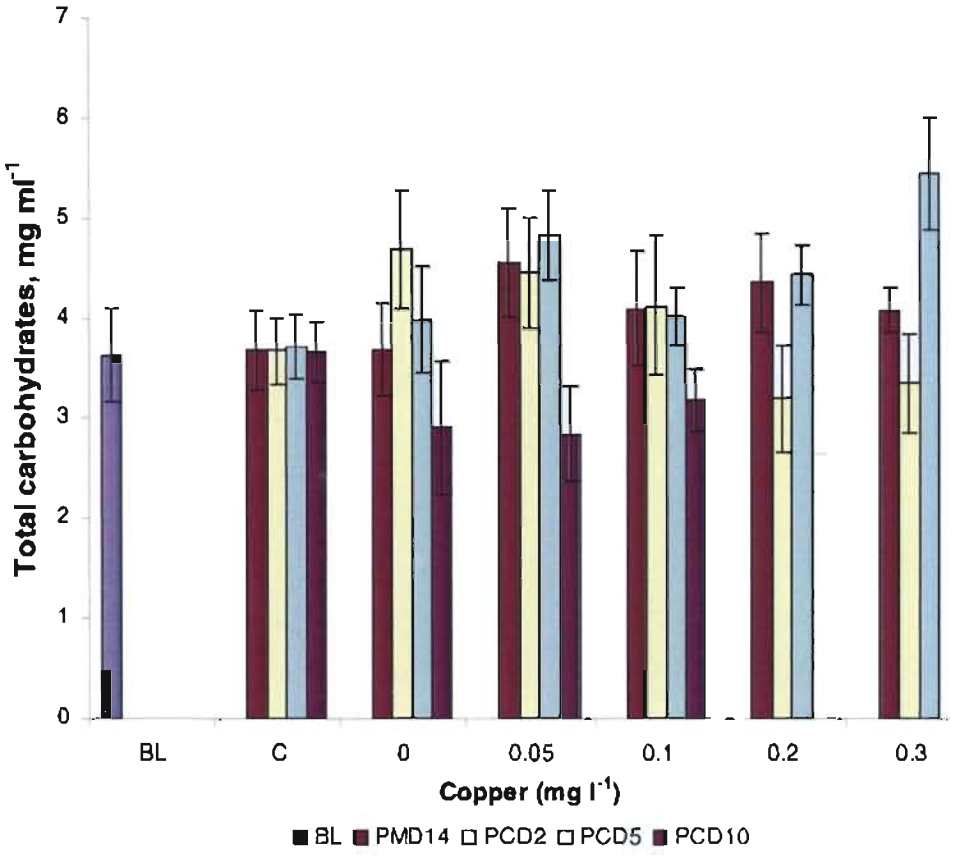
It can therefore be concluded from the present study that concentration of Cu in ambient water greatly influences the immune ability and susceptibility of *P. monodon* to WSSV infection. Since shrimps can absorb Cu from salt water (Djangmah and Grove, 1970), addition of Cu in water may serve as a means of mineral supplementation. A slighter increase of Cu at 0.1 mg l^{-1} is beneficial to the shrimps in improving the general physiological condition and as a form of immunostimulation. No metal accumulation in the muscle at this concentration makes it a safe level too, when the health aspects related to human consumption is considered. However, an increase above that level was proved harmful in exerting negative effects on the metabolic response and immunocompetence. Knowledge regarding the effects of increasing ambient Cu on the physiological and immune status of shrimps may be considered in pond management during shrimp farming towards the effective control of WSSV infection.



	Total protein (mg ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	85.07 ± 10.1	$_{b}$85.27 ± 12.5^{A}	$_{a}$87.01 ± 8.9^{A}	$_{c}$86.87 ± 10.0^{A}	$_{b}$87.16 ± 11.2^{A}
0		$_{b}$85.23 ± 11.6^{C}	$_{a}$136.85 ± 17.9^{A}	$_{ab}$109.86 ± 12.7^{B}	$_{ab}$99.67 ± 13.7^{BC}
0.05		$_{b}$81.44 ± 13.2^{B}	$_{bc}$117.56 ± 15.4^{A}	$_{a}$115.46 ± 10.8^{A}	$_{ab}$92.18 ± 13.4^{B}
0.1		$_{a}$102.66 ± 16.5^{B}	$_{ab}$133.12 ± 18.8^{A}	$_{ab}$105.06 ± 13.8^{B}	$_{a}$104.30 ± 13.4^{B}
0.2		$_{a}$99.91 ± 7.6^{AB}	$_{bc}$116.46 ± 13.6^{A}	$_{bc}$94.22 ± 18.6^{B}	
0.3		$_{c}$61.85 ± 6.5^{B}	$_{c}$100.70 ± 15.8^{A}	$_{abc}$99.36 ± 12.1^{A}	

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PMD -Post metal exposure day, PCD -Post challenge day

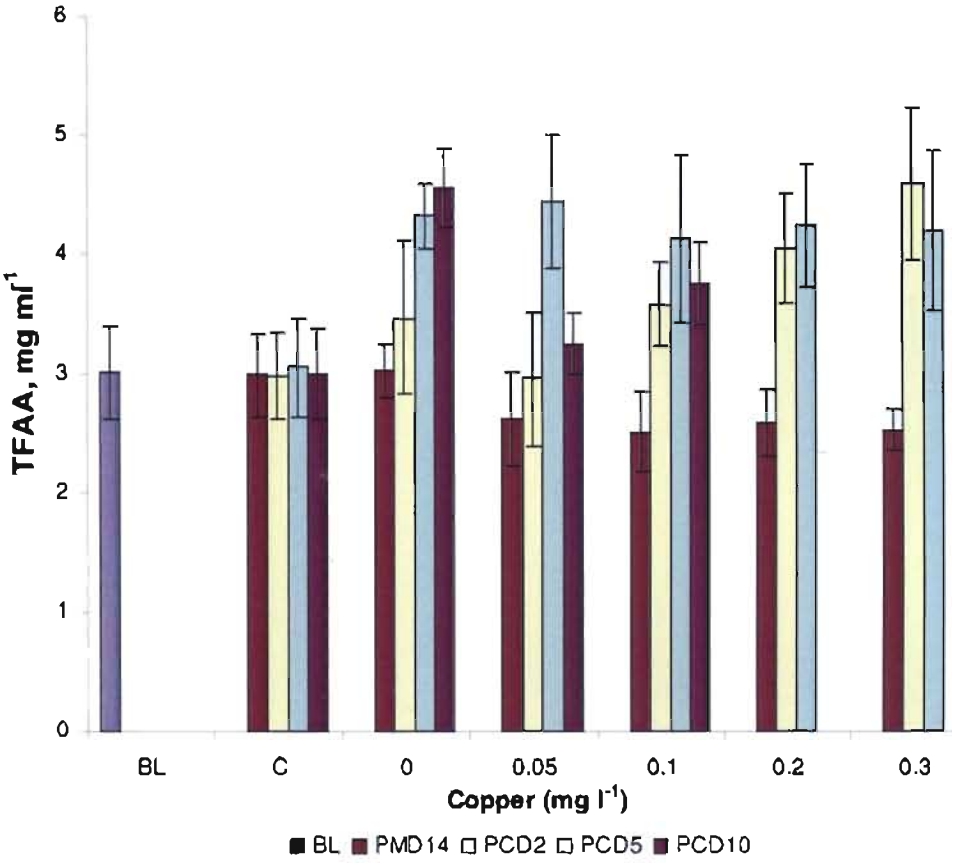
Fig.4.1 Total protein in the haemolymph of *P. monodon* exposed to copper and challenged with WSSV.



	Total carbohydrates (mg ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	3.62 ± 0.47	$_{b}3.67 \pm 0.41^{A}$	$_{bc}3.67 \pm 0.33^{A}$	$_{a}3.71 \pm 0.32^{A}$	$_{a}3.66 \pm 0.3^{A}$
0		$_{b}3.68 \pm 0.47^{B}$	$_{a}4.68 \pm 0.58^{A}$	$_{cd}3.99 \pm 0.53^{B}$	$_{b}2.9 \pm 0.66^{C}$
0.05		$_{a}4.56 \pm 0.55^{A}$	$_{a}4.46 \pm 0.55^{A}$	$_{b}4.83 \pm 0.44^{A}$	$_{b}2.83 \pm 0.47^{B}$
0.1		$_{ab}4.10 \pm 0.57^{A}$	$_{ab}4.12 \pm 0.7^{A}$	$_{cd}4.02 \pm 0.29^{A}$	$_{ab}3.18 \pm 0.31^{B}$
0.2		$_{a}4.36 \pm 0.49^{A}$	$_{c}3.2 \pm 0.54^{B}$	$_{bc}4.44 \pm 0.3^{A}$	-
0.3		$_{ab}4.09 \pm 0.22^{B}$	$_{c}3.34 \pm 0.5^{C}$	$_{a}5.44 \pm 0.56^{A}$	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

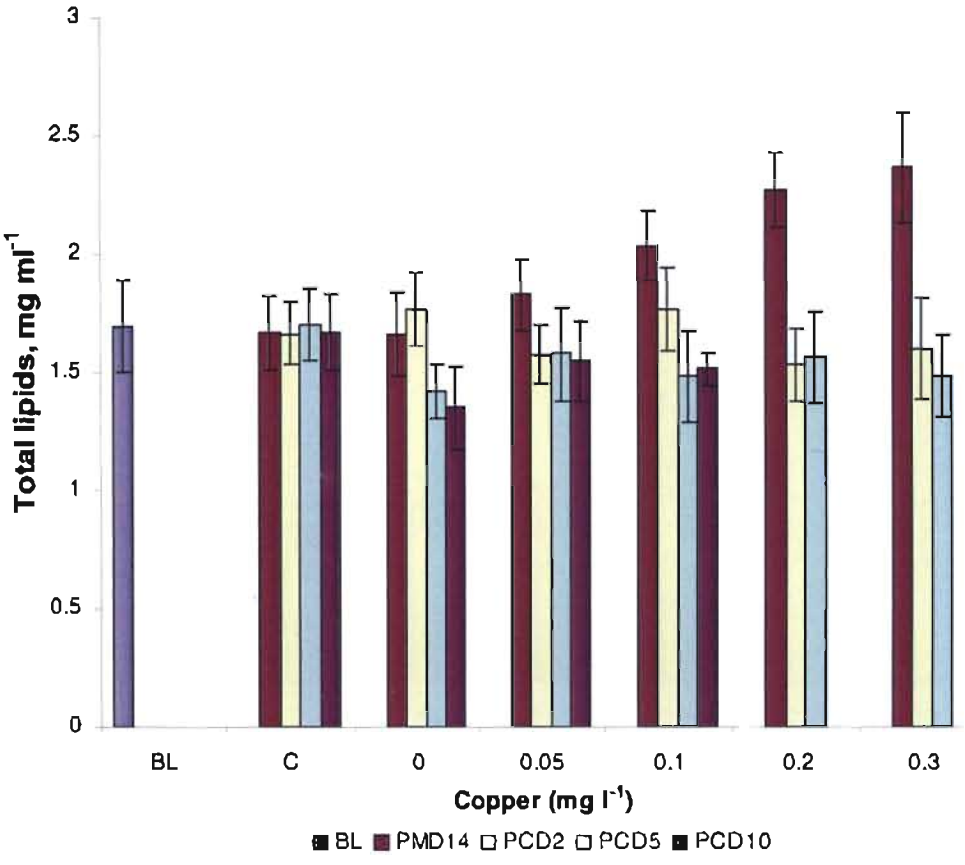
Fig.4.2 Total carbohydrates in the haemolymph of *P. monodon* exposed to copper and challenged with WSSV.



	Total free amino acids (mg ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	3.01 ± 0.39	$_{a}2.99 \pm 0.35^{A}$	$_{c}2.98 \pm 0.37^{A}$	$_{b}3.05 \pm 0.41^{A}$	$_{c}2.99 \pm 0.38^{A}$
0		$_{a}3.02 \pm 0.23^{B}$	$_{bc}3.47 \pm 0.64^{B}$	$_{a}4.32 \pm 0.26^{A}$	$_{a}4.56 \pm 0.32^{A}$
0.05		$_{b}2.61 \pm 0.39^{C}$	$_{c}2.95 \pm 0.56^{BC}$	$_{a}4.45 \pm 0.56^{A}$	$_{c}3.25 \pm 0.26^{B}$
0.1		$_{b}2.51 \pm 0.33^{B}$	$_{bc}3.58 \pm 0.35^{A}$	$_{a}4.14 \pm 0.7^{A}$	$_{b}3.76 \pm 0.35^{A}$
0.2		$_{b}2.58 \pm 0.28^{B}$	$_{ab}4.05 \pm 0.46^{A}$	$_{a}4.24 \pm 0.51^{A}$	-
0.3		$_{b}2.53 \pm 0.18^{B}$	$_{a}4.59 \pm 0.64^{A}$	$_{a}4.20 \pm 0.67^{A}$	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

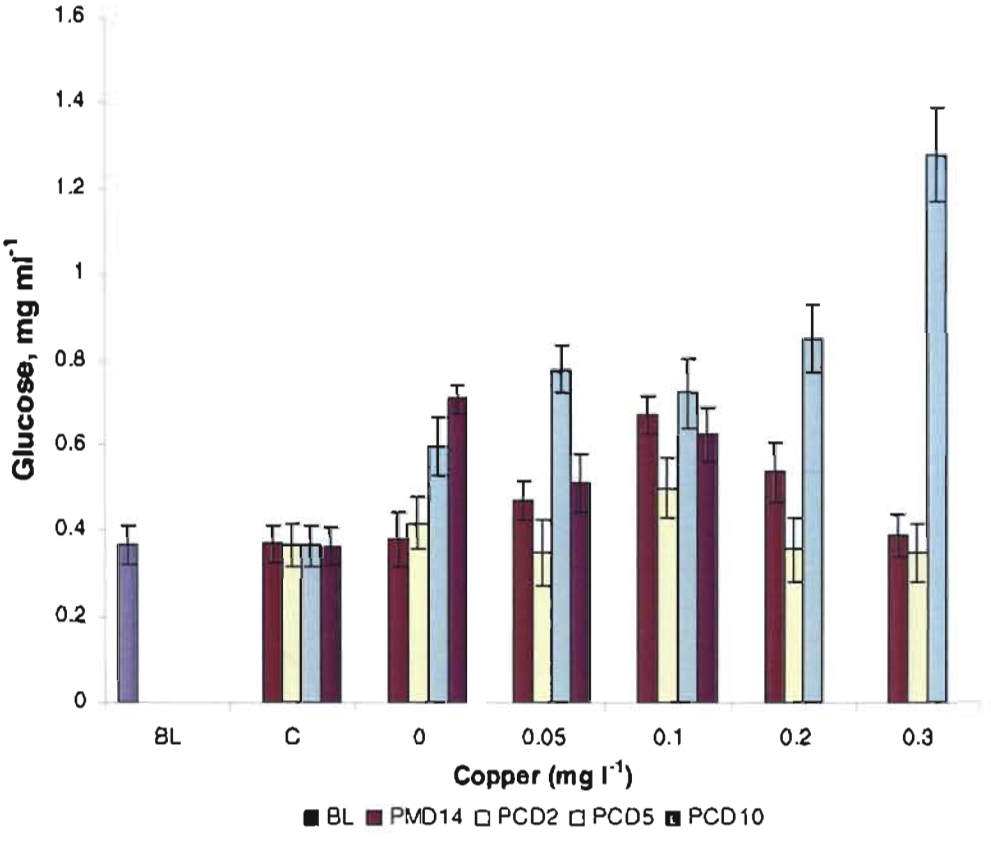
Fig.4.3 Total free amino acids (TFAA) in the haemolymph of *P. monodon* exposed to copper and challenged with WSSV.



	Total lipids (mg ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	1.69 ± 0.2	$_{c}$1.66 ± 0.16^{A}	$_{ab}$1.66 ± 0.13^{A}	$_{a}$1.70 ± 0.16^{A}	$_{a}$1.67 ± 0.16^{A}
0		$_{c}$1.66 ± 0.18^{A}	$_{a}$1.77 ± 0.15^{A}	$_{b}$1.41 ± 0.11^{B}	$_{b}$1.35 ± 0.17^{B}
0.05		$_{c}$1.83 ± 0.15^{A}	$_{ab}$1.57 ± 0.132^{B}	$_{ab}$1.58 ± 0.2^{B}	$_{a}$1.55 ± 0.17^{B}
0.1		$_{b}$2.04 ± 0.14^{A}	$_{a}$1.76 ± 0.18^{B}	$_{ab}$1.48 ± 0.2^{C}	$_{ab}$1.51 ± 0.07^{C}
0.2		$_{a}$2.27 ± 0.16^{A}	$_{b}$1.53 ± 0.16^{B}	$_{ab}$1.56 ± 0.2^{B}	-
0.3		$_{a}$2.37 ± 0.24^{A}	$_{ab}$1.60 ± 0.22^{B}	$_{ab}$1.48 ± 0.17^{B}	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

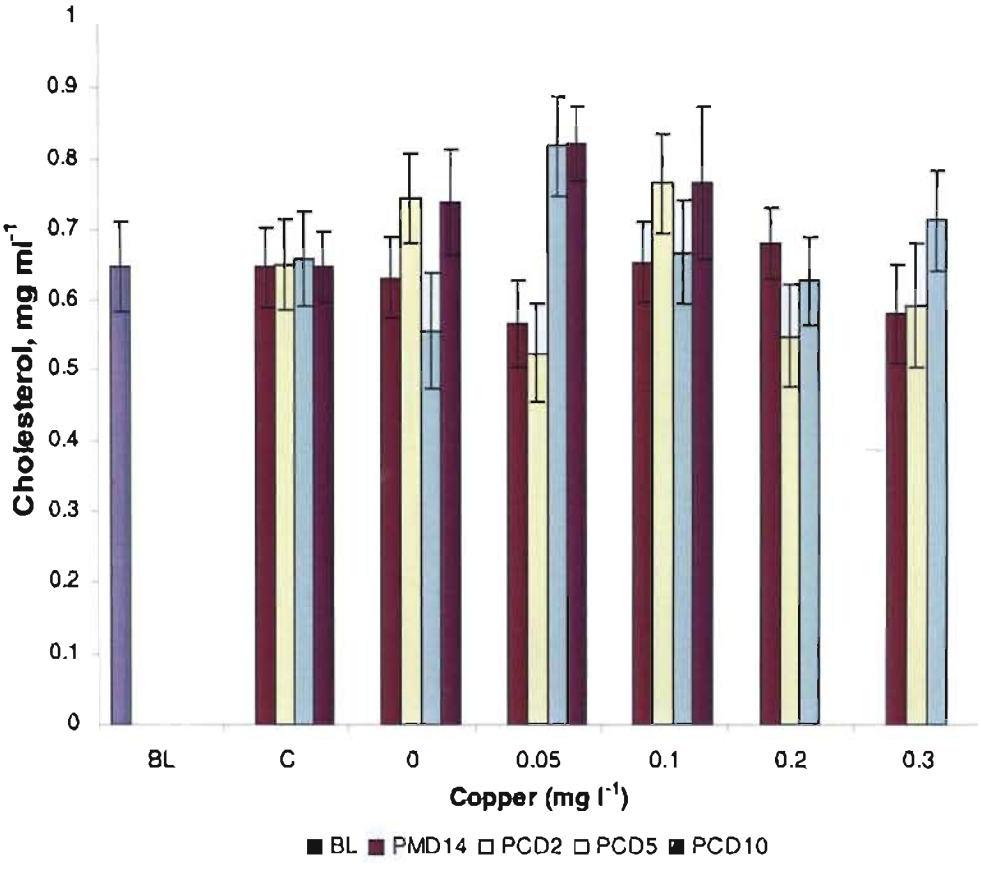
Fig.4.4 Total lipids in the haemolymph of *P. monodon* exposed to copper and challenged with WSSV.



	Glucose (mg ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	0.365 ± 0.05	$_{d}$0.369 ± 0.04^{A}	$_{b}$0.365 ± 0.05^{A}	$_{c}$0.364 ± 0.05^{A}	$_{d}$0.362 ± 0.04^{A}
0		$_{d}$0.380 ± 0.06^{C}	$_{b}$0.417 ± 0.06^{C}	$_{d}$0.594 ± 0.07^{B}	$_{a}$0.707 ± 0.03^{A}
0.05		$_{c}$0.469 ± 0.04^{B}	$_{b}$0.349 ± 0.08^{C}	$_{bc}$0.776 ± 0.06^{A}	$_{c}$0.509 ± 0.07^{B}
0.1		$_{a}$0.668 ± 0.04^{AB}	$_{d}$0.497 ± 0.07^{C}	c0.719 ± 0.08^{A}	$_{b}$0.622 ± 0.06^{B}
0.2		$_{b}$0.535 ± 0.07^{B}	$_{b}$0.355 ± 0.08^{C}	$_{b}$0.848 ± 0.08^{A}	-
0.3		$_{d}$0.388 ± 0.05^{B}	$_{b}$0.348 ± 0.07^{B}	$_{a}$1.277 ± 0.11^{A}	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

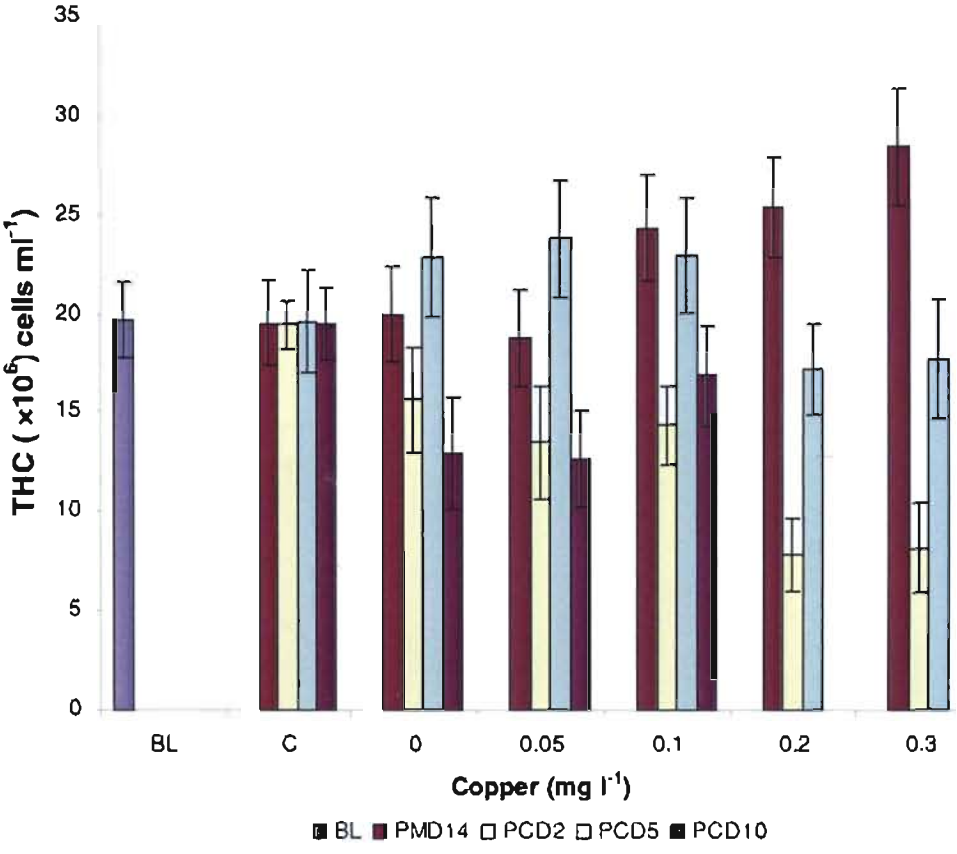
Fig.4.5 Glucose levels in the haemolymph of *P. monodon* exposed to copper and challenged with WSSV.



	Cholesterol(mg ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	0.648 ± 0.06	$_{ab}$0.647 ± 0.06^{A}	$_{b}$0.650 ± 0.06^{A}	$_{b}$0.657 ± 0.07^{A}	$_{b}$0.648 ± 0.05^{A}
0		$_{abc}$0.632 ± 0.06^{B}	$_{a}$0.743 ± 0.06^{A}	$_{c}$0.556 ± 0.08^{B}	$_{a}$0.738 ± 0.07^{A}
0.05		$_{c}$0.567 ± 0.06^{B}	$_{c}$0.524 ± 0.07^{B}	$_{a}$0.817 ± 0.07^{A}	$_{a}$0.821 ± 0.05^{A}
0.1		$_{ab}$0.654 ± 0.06^{B}	$_{a}$0.765 ± 0.07^{A}	$_{b}$0.668 ± 0.07^{AB}	$_{a}$0.766 ± 0.11^{A}
0.2		$_{a}$0.680 ± 0.05^{A}	$_{c}$0.549 ± 0.07^{B}	$_{bc}$0.627 ± 0.06^{A}	-
0.3		$_{bc}$0.581 ± 0.07^{B}	$_{bc}$0.592 ± 0.09^{B}	$_{b}$0.713 ± 0.07^{A}	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PMD -Post metal exposure day, PCD -Post challenge day

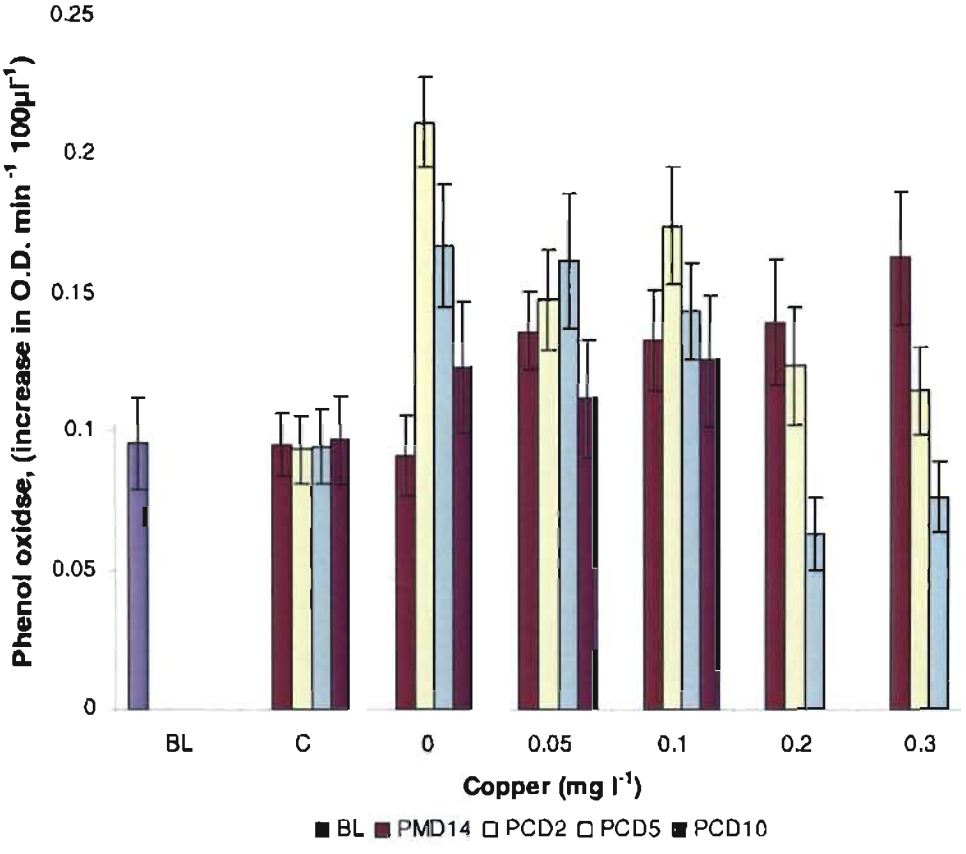
Fig.4.6 Cholesterol levels in the haemolymph of *P. monodon* exposed to copper and challenged with WSSV.



	Total Haemocyte Count (×10^6 cells ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	19.68 ± 2.0	$_{c}$19.52 ± 2.2^{A}	$_{a}$19.45 ± 1.2^{A}	$_{bc}$19.56 ± 2.6^{A}	$_{a}$19.45 ± 1.8^{A}
0		$_{c}$19.94 ± 2.4^{A}	$_{b}$15.63 ± 2.7^{B}	$_{ab}$22.85 ± 3.0^{A}	$_{b}$12.92 ± 2.8^{B}
0.05		$_{c}$18.79 ± 2.5^{B}	$_{b}$13.48 ± 2.9^{C}	$_{a}$23.79 ± 2.9^{A}	$_{b}$12.67 ± 2.4^{C}
0.1		$_{b}$24.34 ± 2.7^{A}	$_{b}$14.32 ± 2.0^{B}	$_{ab}$22.97 ± 2.9^{A}	$_{a}$16.83 ± 2.6^{B}
0.2		$_{b}$25.38 ± 2.5^{A}	$_{c}$7.79 ± 1.8^{C}	$_{c}$17.16 ± 2.3^{B}	-
0.3		$_{a}$28.41 ± 3.0^{A}	$_{c}$8.13 ± 2.3^{C}	$_{c}$17.67 ± 3.0^{B}	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PMD -Post metal exposure day, PCD -Post challenge day

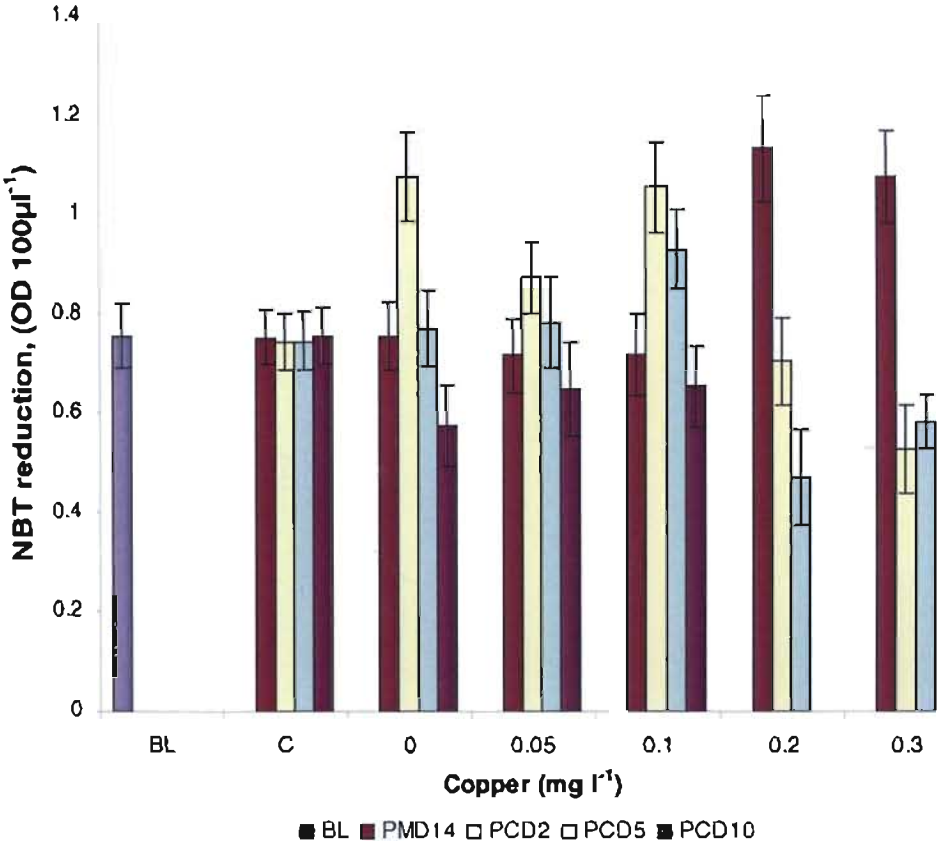
Fig.4.7 Total haemocyte count (THC) in *P. monodon* exposed to copper and challenged with WSSV.



	Phenol oxidase activity (increase in OD min^{-1} 100μl^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	0.095 ± 0.02	$_{c}0.095 \pm 0.01^{A}$	$_{c}0.093 \pm 0.01^{A}$	$_{c}0.094 \pm 0.01^{A}$	$_{b}0.097 \pm 0.02^{A}$
0		$_{c}0.091 \pm 0.01^{B}$	$_{a}0.212 \pm 0.02^{A}$	$_{a}0.167 \pm 0.02^{B}$	$_{ab}0.123 \pm 0.02^{C}$
0.05		$_{b}0.136 \pm 0.01^{B}$	$_{c}0.148 \pm 0.02^{AB}$	$_{ab}0.162 \pm 0.02^{A}$	$_{ab}0.112 \pm 0.02^{C}$
0.1		$_{b}0.133 \pm 0.02^{B}$	$_{b}0.174 \pm 0.02^{A}$	$_{b}0.144 \pm 0.02^{B}$	$_{a}0.126 \pm 0.02^{B}$
0.2		$_{b}0.140 \pm 0.02^{A}$	$_{d}0.123 \pm 0.02^{A}$	$_{d}0.063 \pm 0.01^{B}$	-
0.3		$_{a}0.163 \pm 0.02^{A}$	$_{d}0.115 \pm 0.02^{B}$	$_{d}0.076 \pm 0.01^{C}$	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

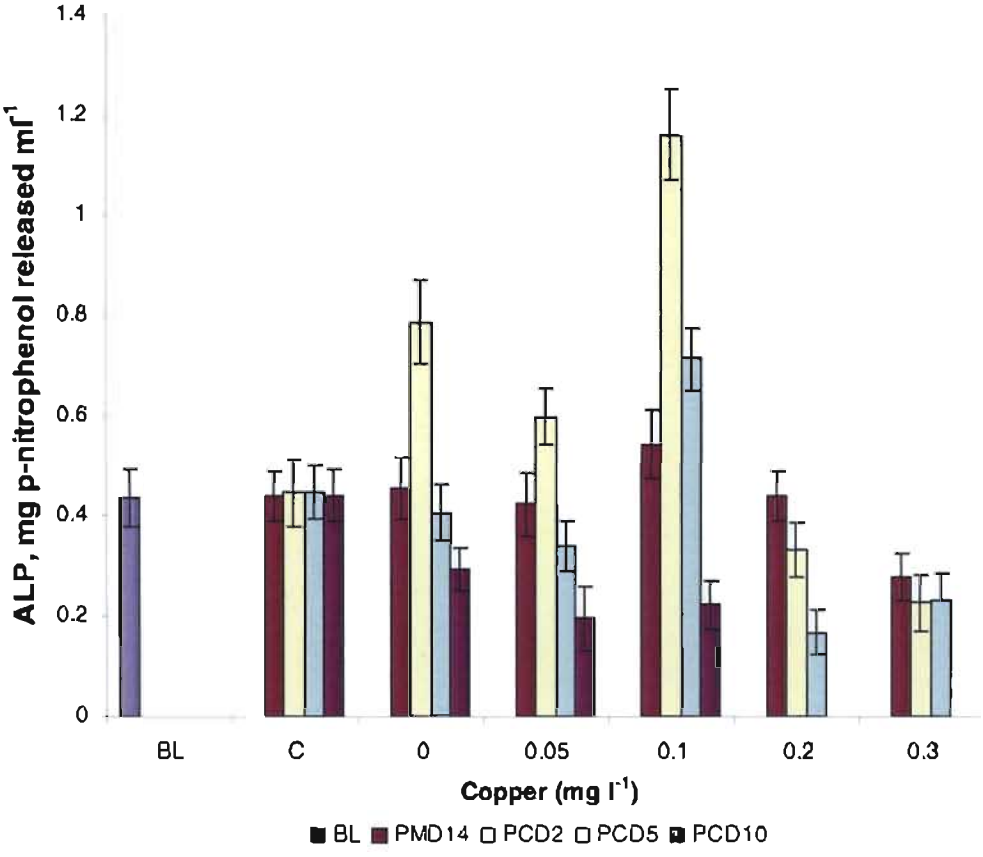
Fig.4.8 Phenol oxidase activity in *P. monodon* exposed to copper and challenged with WSSV.



	NBT Reduction (OD 100µl^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	0.754 ± 0.07	$_{b}0.752 \pm 0.05^{A}$	$_{c}0.744 \pm 0.06^{A}$	$_{b}0.744 \pm 0.06^{A}$	$_{a}0.754 \pm 0.06^{A}$
0		$_{b}0.754 \pm 0.07^{B}$	$_{a}1.076 \pm 0.09^{A}$	$_{b}0.77 \pm 0.08^{B}$	$_{b}0.574 \pm 0.08^{C}$
0.05		$_{b}0.714 \pm 0.07^{BC}$	$_{b}0.873 \pm 0.07^{A}$	$_{b}0.781 \pm 0.09^{AB}$	$_{b}0.648 \pm 0.10^{C}$
0.1		$_{b}0.719 \pm 0.08^{C}$	$_{a}1.054 \pm 0.09^{A}$	$_{a}0.929 \pm 0.08^{B}$	$_{b}0.653 \pm 0.08^{C}$
0.2		$_{a}1.131 \pm 0.11^{A}$	$_{c}0.704 \pm 0.09^{B}$	$_{d}0.468 \pm 0.10^{C}$	-
0.3		$_{a}1.074 \pm 0.09^{A}$	$_{d}0.526 \pm 0.09^{B}$	$_{c}0.579 \pm 0.05^{B}$	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline. PMD -Post metal exposure day, PCD -Post challenge day

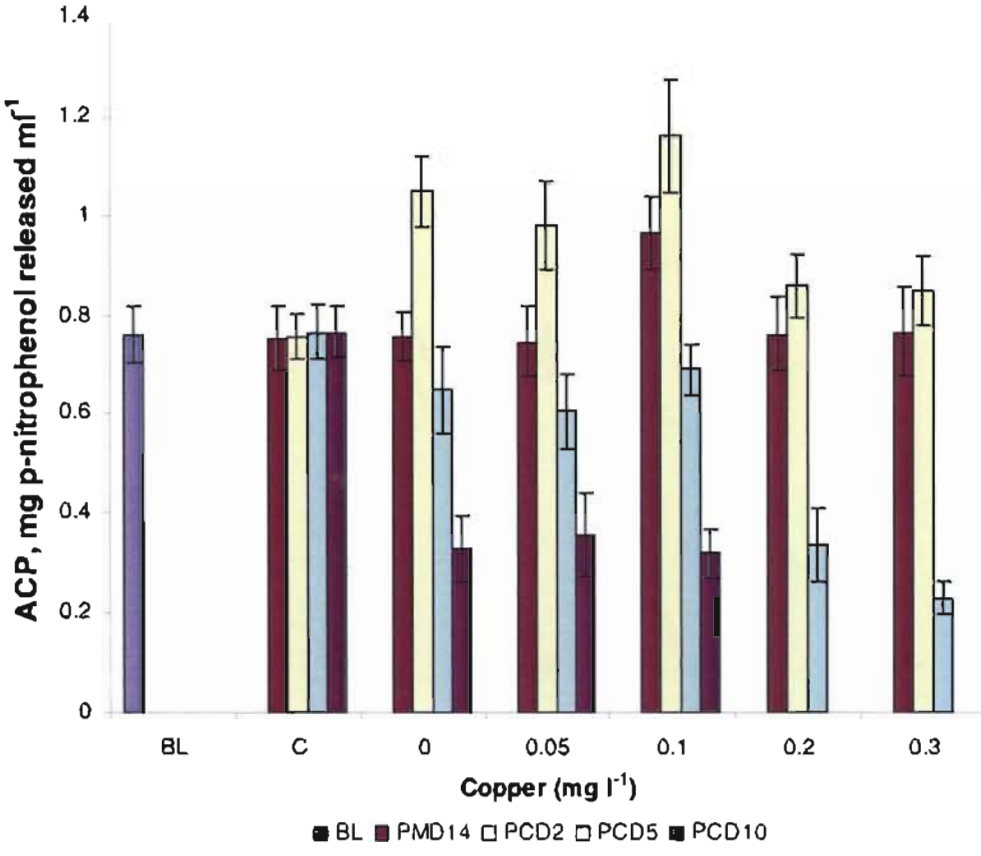
Fig.4.9 NBT reduction in *P. monodon* exposed to copper and challenged with WSSV.



	Alkaline Phosphatase activity (mg p-nitrophenol released ml^{-1})				
Cu ($mg\ l^{-1}$)	BL	PMD14	PCD2	PCD5	PCD10
Control	0.435 ± 0.06	$_{b}0.438 \pm 0.05^{A}$	$_{d}0.444 \pm 0.07^{A}$	$_{b}0.445 \pm 0.06^{A}$	$_{d}0.439 \pm 0.05^{A}$
0		$_{b}0.454 \pm 0.06^{B}$	$_{b}0.787 \pm 0.08^{A}$	$_{b}0.404 \pm 0.06^{B}$	$_{b}0.292 \pm 0.04^{C}$
0.05		$_{b}0.421 \pm 0.06^{B}$	$_{c}0.596 \pm 0.06^{A}$	$_{c}0.339 \pm 0.05^{C}$	$_{c}0.194 \pm 0.06^{D}$
0.1		$_{d}0.541 \pm 0.07^{C}$	$_{a}1.160 \pm 0.09^{A}$	$_{d}0.713 \pm 0.06^{B}$	$_{c}0.221 \pm 0.05^{D}$
0.2		$_{b}0.438 \pm 0.05^{A}$	$_{c}0.329 \pm 0.05^{B}$	$_{c}0.166 \pm 0.04^{C}$	-
0.3		$_{c}0.277 \pm 0.05^{A}$	$_{f}0.225 \pm 0.06^{A}$	$_{d}0.232 \pm 0.05^{A}$	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

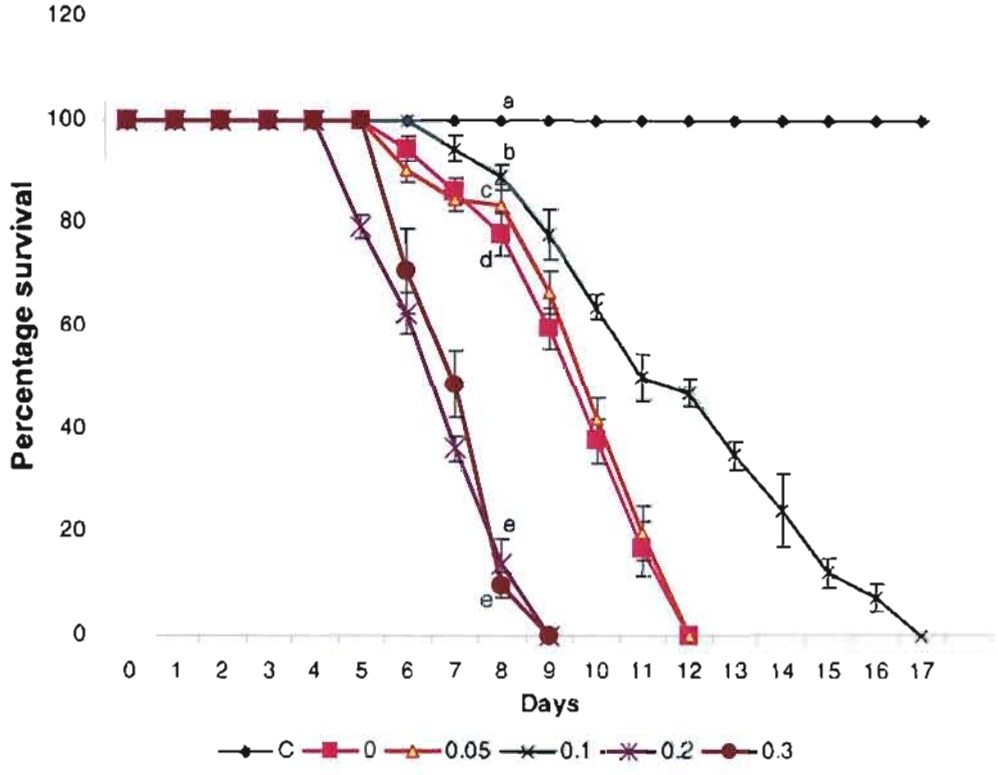
Fig.4.10 Alkaline phosphatase activity (ALP) in *P. monodon* exposed to copper and challenged with WSSV.



	Acid Phosphatase activity (mg p-nitrophenol released ml^{-1})				
Cu (mg l^{-1})	BL	PMD14	PCD2	PCD5	PCD10
Control	0.761 ± 0.06	$_{b}0.751 \pm 0.07^{A}$	$_{d}0.757 \pm 0.05^{A}$	$_{a}0.765 \pm 0.06^{A}$	$_{a}0.765 \pm 0.05^{A}$
0		$_{b}0.756 \pm 0.05^{B}$	$_{b}1.048 \pm 0.07^{A}$	$_{bc}0.649 \pm 0.09^{C}$	$_{b}0.327 \pm 0.07^{D}$
0.05		$_{b}0.745 \pm 0.07^{B}$	$_{b}0.980 \pm 0.09^{A}$	$_{c}0.605 \pm 0.08^{C}$	$_{b}0.357 \pm 0.08^{D}$
0.1		$_{a}0.965 \pm 0.07^{B}$	$_{a}1.159 \pm 0.11^{A}$	$_{ab}0.689 \pm 0.05^{C}$	$_{b}0.319 \pm 0.05^{D}$
0.2		$_{b}0.761 \pm 0.07^{B}$	$_{c}0.859 \pm 0.06^{A}$	$_{d}0.334 \pm 0.07^{C}$	-
0.3		$_{b}0.765 \pm 0.09^{B}$	$_{c}0.850 \pm 0.07^{A}$	$_{c}0.229 \pm 0.03^{C}$	-

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods.
BL -Baseline, PMD -Post metal exposure day, PCD -Post challenge day

Fig.4.11 Acid phosphatase activity (ACP) in *P. monodon* exposed to copper and challenged with WSSV.



*Different letters indicate statistical difference among different treatments.

Fig.4.12 Post challenge survival of *P. monodon* exposed to copper and challenged with WSSV.

CHAPTER 5

Modulatory Effect of Ambient Zinc on the Haematological Responses and Susceptibility of Penaeus monodon to WSSV infection

5.1 Introduction

Zinc is a ubiquitous essential trace metal required for the normal growth, development and functioning of animal species. Numerous proteins, crucial enzymes, and transcription factors depend on Zn for their functions. Zn is in fact, the most important intracellular trace element that plays a crucial role in a wide range of cellular processes, including cellular respiration, cell proliferation and reproduction (Powell, 2000; Ho, 2004). Zinc is also essential for the maintenance of immunity (Shankar and Prasad, 1998).

Zn plays the role of cofactor in more than 200 metalloenzymes including carbonic anhydrase, carboxypeptidases A and B, alcohol dehydrogenase, glutamic dehydrogenase, D-glyceraldehyde 3-PO_4 dehydrogenase, lactate dehydrogenase, malic dehydrogenase, alkaline phosphatase, aldolase, superoxide dismutase, ribonuclease and DNA polymerase (Lloyd *et al.*, 1978; National Research Council, 1980). Several proteases, transphosphorylases and transcarbamylases are also Zn-containing enzymes (Wu and Wu, 1981). Carbonic anhydrase, a major Zn-containing enzyme also plays a major role in the laying down of calcium carbonate in crustacean calcified cuticle and mollusc shells (Bundy, 1977; Giraud, 1980). Zn is a functional component of transcription factor proteins contributing to gene expression and regulation. Zn exerts significant influence on the nervous system and in the optimal metabolism of vitamin A (Klassen, 2001). Zn is involved in the maintenance of gut structure and function and is importantly related to gut immune function (Scott and Koski, 2000). Zn is also important in the maintenance of cell membrane integrity, sequestration of free radicals and protection against lipid peroxidation (Hendy *et al.*, 2001). Although there is no non-enzymatic zinc protein equivalent to haemocyanin, the Cu-containing protein, Martin *et al.* (1977) has suggested that Zn may serve to stabilize the quarternary structure of the haemocyanin molecule. However, there is little supportive evidence for this particular role of zinc.

Like Cu, crustaceans absorb Zn from ambient water (Bryan, 1968; Renfro *et al.*, 1975) and decapods regulate the whole body Zn levels over a range of concentrations (Rainbow and White, 1989). Zn has the ability to induce the synthesis of metallothioneins, which is a factor in regulating the metabolism of Zn, including

absorption and storage (Klaassen, 2001). Hepatopancreas is the main accumulatory organ for Zn as well as the site of their detoxified storage (Nunez-Noguiera and Rainbow, 2005; Nunez-Noguiera *et al.*, 2006). Gills are indeed, a major site of entry of the metal and act as transient stores for accumulated metal (Soegianto *et al.*, 1999). In *Carcinus maenas*, majority of the Zn in haemolymph was seen bound to haemocyanin (Zatta, 1984, 1985; Martin and Rainbow, 1998). Unlike Cu, there were not a specific number of Zn atoms for every molar subunit of haemocyanin (Martin and Rainbow, 1998). Zn appears to have a protective effect against the toxicities of both Cd (Calabrese *et al.*, 1985) and Pb (Sanstead, 1976).

Like many other trace metals Zn can be toxic if present in high concentrations in aquatic ecosystems (Eisler and Wapner, 1975). Zn enters water bodies mainly from industrial and domestic wastes (Bat *et al.*, 2000). Because of the importance of Zn in the industrialized world, large quantities of the metal are discharged annually into marine waters near densely populated regions (Young *et al.*, 1973). Chronic exposure to waterborne Zn has been shown to cause a variety of physiological and behavioural changes including loss of appetite, reduced growth and ion loss in fish (Chapman, 1978; Hogstrand and Wood, 1996). An increase in serum protein, aspartate aminotransferase and alanine aminotransferase was recorded in freshwater fish *Labeo rohita* on exposure to Zn (Vankhede *et al.*, 2004). In *L. vannamei*, exposure to Zn caused an inhibition in oxygen consumption (Wu and Chen, 2004).

Although an essential element significant to the immune status, reports on the modulatory effects of Zn on the immune system are very few. Both suppressive and stimulatory effects of Zn have been reported in studies of humoral antibody response (O' Neil, 1981) and cell-mediated immunity (Ghanmi *et al.* 1989) in fishes. Zebrafish, *Brachydanio rerio* (Rougier *et al.*, 1994) exposed to Zn had a lower cytotoxic activity, and an enhanced macrophage response. In fact, most of the studies related to the immunomodulatory effects of metals on fishes and shellfishes are concentrated on Cu. Cd, Mn, Se, Pb, Cr etc. are some of the other metals dealt with in the immunomodulatory studies. The total haemocyte count, degranulating activity of granular haemocytes and the expression of a gene coding for Runt-domain protein (involved in maturation of immune active haemocytes) reduced in Norway lobster, *Nephrops norvegicus* on exposure to Mn at 20 mgl^{-1} for 10 days (Hernroth *et al.*, 2004). Cd slightly suppressed the phagocytosis of bacteria *in-vitro* in *Carcinus maenas* (Truscott and White, 1990). Short-term exposure (96 h) to sub lethal levels of dissolved heavy metals such as Hg, Cd, Cr, Zn and Pb caused a decrease in haemocyte count during the first 8 h exposure, and returned to the

initial levels after 16 h in the shrimp *Palaemon elegans* with the greatest decrease induced by Pb (Lorenzon *et al.*, 2001). Suppressive and stimulatory effects of Mn and Cd on fish macrophage activity have been proved in *Cyprinus carpio* and *Salmo gairdneri*.

In shrimps, the haematological studies related to Zn is mainly dealt with the uptake, accumulation and related toxicity. Effects of Zn on the physiological well-being and immune status of shrimps, with respect to the health indicators in haemolymph have not received much attention. Very recently, Shiau and Jiang (2006) could prove that *P. monodon* has a requirement for Zn that cannot be met by Zn in the rearing water and that dietary supplementation is essential for better non-specific immune responses. In the previous experiment with Cu, the stimulatory and suppressive effects of increasing ambient Cu on *P. monodon* could be proved which could also be correlated with the immunocompetence to WSSV infection. The same hypothesis is applied here with Zn.

The present study on *P. monodon* was therefore aimed at determining the potential:

- Effects of increasing ambient Zn on the metabolic and immune variables of haemolymph
- Effects of Zn exposure on the haemolymph metabolic variables and immune response of shrimps to WSSV infection.
- Effects of Zn exposure on the susceptibility to WSSV infection

5.2 Materials and methods

5.2.1 Experimental animals

Adult *P. monodon* obtained from a commercial farm in Olathala, Chertalai were used as experimental shrimps in the present study. Average wet weight of the shrimp was 15.8 ± 2.9 g (Mean ± S.D.). Shrimps were reared in concrete rectangular tanks and allowed to acclimate for a week. Rearing conditions and water quality were maintained as that for the first experiment (Refer Section 2.2.2). Biochemical and immunological profile was obtained from a group of shrimps (n=6) after acclimation for a period of seven days, as the baseline (BL) data.

5.2.2 Experimental design

Shrimps were distributed in the experimental tanks containing 500L of seawater with 30 individuals per tank (n=30/tank). There were 6 treatments (Group-I, Group-II,

Group-III, Group-IV, Group-V and Group-VI) and the experiment was conducted in triplicate i.e., 3 tanks per treatment. Shrimps in the intermoult stage only were used.

5.2.3 Exposure to zinc

Shrimps were maintained in the experimental tanks for 2 days, after which, the shrimps were exposed to Zn ($ZnSO_4$) at various concentrations of 0. 5, 1.0, 1.5 and 2.5 mg l^{-1}. During the period of exposure, water was exchanged daily in order to maintain the metal concentration and decrease the toxicity caused by potentially poisonous metabolites excreted by animals themselves (Mc Mahon, 2001). Group-I and Group-II were maintained without Zn. After 14 days of exposure, Zn-exposed and the unexposed shrimps (n=6) were randomly sampled (PMD14, post metal exposure day 14).

5.2.4 WSSV challenge

The shrimps of Group-II (0 mg l^{-1} Zn), Group-III (0. 5 mg l^{-1} Zn), Group-IV (1.0 mg l^{-1} Zn) Group-V (1.5 mg l^{-1} Zn) and Group-VI (2.5 mg l^{-1} Zn), were challenged with white spot syndrome virus after 14 days of exposure to Zn. The 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 48 h (post challenge day 2, PCD2), and 120 h (post challenge day 5, PCD5) of challenge. Before each sampling the shrimps were fasted for 12 hours to eliminate variations caused by the ingested food (Hall and van Ham, 1998). Survival in each group was recorded daily with dead animals removed promptly. Sampling was done only on PCD2 and PCD5 as the parameters were found to exhibit a depleting trend with the progress of infection as observed in the previous studies. Mortality by WSSV infection was confirmed by checking the characteristic white spots on the carapace of infected shrimps.

5.2.5 Extraction of haemolymph

Haemolymph was extracted according to the procedure described earlier (Refer section 2.2.6). Sampling was carried out at the beginning of the experiment (baseline), on post metal exposure day 14 (PMD14) and post challenge day 2 and 5 from the six experimental groups (Group-I, Group-II, Group-III, Group-IV, Group-V and Group-VI). The immune parameters were analysed immediately and the samples stored at –20°C for the analysis of biochemical variables.

5.2.6 Analysis of haematological parameters

Metabolic variables in the haemolyph *viz.*, total protein, total carbohydrates, total free amino acids, total lipids, glucose, cholesterol and immune variables *viz.*, total haemocyte count, phenol oxidase activity, NBT reduction, alkaline phosphatase activity and acid phosphatase activity was determined according to the methods described previously (Refer Section 2.2.7).

5.2.7 Analysis of Zinc

Muscle tissue for the analysis of Zn was collected from shrimps after the extraction of haemolymph following the methods described earlier. Haemolymph and muscle samples were processed as per the standard protocol (Refer section 4.2.7) and the analysis was done in an Atomic Absorption Spectrophotometer (AAS). Air-acetylene flame was used for the estimation and absorbance was noted by adjusting the cathode lamp at a specific wavelength of 213.9 nm. Working standards for calibration were prepared by diluting standard Zn (1000 mg l^{-1}) solution in ultra-pure water (Milli-Q). Blanks and standards were also analyzed the same way as for the samples. The concentration of Zn was then expressed in μg ml^{-1} haemolymph and μg g^{-1} muscle.

5.2.8 Statistical analysis

The experimental data was analysed by means of one-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means. Significance level for the analysis was set to $P<0.05$. Statistical analyses were carried out using the software SPSS 10.0.

5.3 Results

Exposure to Zn could not induce any significant alterations in haemolymph total protein and total carbohydrates of *P. monodon*. Glucose and cholesterol levels increased in Zn-exposed shrimps except at 1.5 mg l^{-1} where an increase in cholesterol was not observed. TFAA increased and total lipids decreased in shrimps with 1.5 and 2.5 mg l^{-1} Zn. With regard to the immune variables, a significant increase in THC and PO was observed in Zn-exposed shrimps with a maximum at 1.0 mg l^{-1}. A significant increase in NBT reduction was seen in shrimps with Zn at 2.5 mg l^{-1}. Metabolic variables (except TFAA) following infection were significantly higher in shrimps exposed to Zn at 1.0 mg l^{-1} and least with 2.5 mg l^{-1}. The immune response following infection was also best with

Zn at 1.0 mg l^{-1} and least with 2.5 mg l^{-1}, which could be correlated with the survival also.

5.3a Zinc content in shrimp tissues

Haemolymph Zn concentration increased slightly in shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1}. However, on post challenge days, shrimps exposed to 1.0 mg l^{-1} showed elevated levels of Zn in haemolymph. An increase in haemolymph Zn level was also noted in unexposed shrimps after challenge. Zn concentration in muscle was not altered by ambient Zn. A sharp depletion in Zn concentration was observed in muscle on post challenge days (Table 5.1a and b).

Table 5.1a Zinc content in the haemolymph of *P. monodon* exposed to zinc and then challenged with WSSV.

	Zn (μg ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	41.68 ± 4.09	$_{ab}$41.94 ± 4.6^{A}	$_{b}$40.92 ± 3.84^{A}	$_{b}$41.05 ± 3.11^{A}
0		$_{ab}$40.86 ± 4.23^{B}	$_{ab}$45.49 ± 5.52^{AB}	$_{a}$49.28 ± 5.51^{A}
0.5		$_{b}$40.50 ± 7.57^{A}	$_{b}$42.55 ± 6.01^{A}	$_{ab}$44.35 ± 5.98^{A}
1		$_{ab}$42.13 ± 6.02^{B}	$_{a}$49.92 ± 4.71^{A}	$_{a}$51.17 ± 6.29^{A}
1.5		$_{ab}$46.32 ± 6.74^{A}	$_{ab}$44.66 ± 5.60^{A}	$_{ab}$47.62 ± 5.48^{A}
2.5		$_{a}$47.65 ± 7.0^{A}	$_{ab}$43.39 ± 5.59^{A}	$_{ab}$45.43 ± 5.60^{A}

Table 5.1b Zinc content in the muscle of *P. monodon* exposed to zinc and then challenged with WSSV.

	Zn (μg g^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	15.49 ± 1.91	$_{a}$16.15 ± 1.82^{A}	$_{a}$15.62 ± 1.83^{A}	$_{a}$16.22 ± 1.86^{A}
0		$_{a}$15.70 ± 1.72^{A}	$_{ab}$13.50 ± 2.16^{A}	$_{b}$7.78 ± 1.58^{B}
0.5		$_{a}$15.81 ± 1.63^{A}	$_{bc}$13.13 ± 1.96^{B}	$_{b}$8.10 ± 1.69^{C}
1		$_{a}$16.38 ± 2.05^{A}	$_{bc}$13.20 ± 2.08^{B}	$_{b}$8.37 ± 1.52^{C}
1.5		$_{a}$16.47 ± 2.02^{A}	$_{c}$11.05 ± 1.34^{B}	$_{b}$7.61 ± 1.78^{C}
2.5		$_{a}$15.66 ± 2.16^{A}	$_{bc}$11.72 ± 1.73^{B}	$_{b}$6.79 ± 0.61^{C}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the in the same row with different superscripts are statistically different among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

5.3b Haemolymph metabolic variables

Total protein

Total protein concentration in shrimp haemolymph was not altered by Zn (P<0.05). However, following challenge, total protein increased in Zn-exposed shrimps

as seen with the unexposed. Compared to the unexposed, shrimps with Zn at 1.0 mg l^{-1} showed higher protein levels on post challenge days ($P<0.05$). Whereas, shrimps with Zn at 1.5 and 2.5 mg l^{-1} showed significantly lower levels, with the least at 2.5 mg l^{-1}. Total protein concentrations recorded on PCD2 were 141.25 ± 17.3, 130.04 ± 16.1, 123.48 ± 14.6, 114.79 ± 16.0 and 97.79 ± 12.1 mg ml^{-1} in shrimps with Zn at 1.0, 0.5, 0, 1.5 and 2.5 mg l^{-1} respectively (Fig. 5.1).

Total carbohydrates

No significant variations were seen between the haemolymph total carbohydrate concentrations in Zn-exposed shrimps, compared to the unexposed. Carbohydrate levels increased after challenge, with the maximum increase in shrimps exposed to Zn at 1.0 mgl^{-1} on PCD2 (6.32 ± 0.79 mg ml^{-1}) ($P<0.05$) (Fig. 5.2).

Total free amino acids

Total free amino acids were found to increase in shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1}, with the highest increase at 2.5 mg l^{-1} (4.09 ± 0.34 mg ml^{-1}) ($P<0.05$). A slight decrease in TFAA was noted in shrimps with Zn at 1.0 mg l^{-1} (2.87 ± 0.28 mg ml^{-1}). A progressive increase in TFAA following challenge (usual trend seen with the unexposed) was observed only in shrimps exposed to Zn at 0.5 mg l^{-1}. No significant variations were seen in shrimps with Zn at 1.0 mg l^{-1} before and after challenge and the levels remained considerably lower compared to the other groups ($P<0.05$) (Fig. 5.3).

Total lipids

Haemolymph total lipids significantly reduced in shrimps when exposed to higher doses of Zn ($P<0.05$). Total lipid levels recorded were 1.66 ± 0.16 and 1.75 ± 0.14 mg ml^{-1} in shrimps with Zn at 1.5 and 2.5 mg l^{-1} respectively on PMD14. Comparative reduction was also noticed on post challenge days in shrimps with Zn at 1.5 and 2.5 mg l^{-1} . However, the significant reduction seen with the unexposed on PCD5 was not seen in shrimps with Zn at 1.0 mg l^{-1} ($P<0.05$) (Fig. 5.4).

Glucose

Shrimps exposed to Zn exhibited a significant increase in glucose levels, with the maximum increase at 1.0 mg l^{-1} (0.608 ± 0.07 mg ml^{-1}) ($P<0.05$). A significant reduction of glucose in shrimps with Zn at 2.5 mg l^{-1}, 48 h after challenge was a notable feature. Shrimps with Zn at 1.0 mg l^{-1} showed the highest glucose levels on PCD2, whereas on PCD5, the unexposed shrimps showed the highest level (0.801 ± 0.11 mg ml^{-1}) and those

with Zn at 1.5 (0.41 ±0.07 mg ml^{-1}) and 2.5 mg l^{-1} (0.479 ± 0.06 mg ml^{-1}) showed the least (P<0.05) (Fig. 5.5).

Cholesterol

A significant increase in haemolymph cholesterol concentration was observed in shrimps exposed to Zn at 0.5 (0.78 ± 0.06 mg ml^{-1}), 1.0 (0.717 ± 0.08 mg ml^{-1}) and 2.5 mg l^{-1} (0.716 ± 0.08 mg ml^{-1}) (P<0.05). Post challenge variations in cholesterol were not so pronounced in Zn-exposed groups compared to the unexposed. However, the unexposed and those exposed to Zn at 1.0 mg l^{-1} showed comparatively higher cholesterol levels on PCD2 (P<0.05) (Fig. 5.6).

5.3c Immune response

Total haemocyte count

A significantly higher increase was observed in the haemolymph THC following Zn exposure. 1.0 mg l^{-1} (56.77 ± 6.6 $\times 10^6$ cells ml^{-1}) induced maximum elevation, followed by 0.5 mg l^{-1} (50.43 ± 5.5 $\times 10^6$ cells ml^{-1}) (P<0.05). On PCD2, compared to the reduced THC in control, shrimps exposed to Zn showed significantly high cell counts with maximum at 0.5 and 1.0 mg l^{-1}. However, on PCD5 the unexposed and those exposed to Zn at 1.0 mg l^{-1} showed higher THC and the cell counts were found to reduce in shrimps with Zn at 0.5, 1.5 and 2.5 mg l^{-1} (P<0.05) (Fig. 5.7).

Phenol oxidase activity

Phenol oxidase activity of shrimps significantly increased on exposure to Zn (P<0.05). Maximum activity was noted in shrimps with Zn at 1.0 mg l^{-1} before and after challenge. PO activity of shrimps exposed to Zn at 1.0 and 0.5 mg l^{-1} progressively increased after challenge, compared to the decreasing tendency on PCD5 in the unexposed and other Zn-exposed groups. Least post challenge activity was seen in shrimps with Zn at 2.5 mg l^{-1} (P<0.05) (Fig. 5.8).

NBT reduction

Shrimps exposed to Zn at 2.5 mg l^{-1} exhibited a significant increase in NBT reduction, compared to the unexposed and other Zn-exposed groups (1.079 ± 0.16 OD 100μl^{-1}) (P<0.05). However, following challenge, the activity was found to reduce in shrimps with Zn at 2.5 mg l^{-1}. Shrimps exposed to Zn at 1.0 mg l^{-1} recorded significantly higher activity (1.262 ± 0.14) on PCD2 and showed a declining trend on PCD5 similar to

that seen in the unexposed. Least activity on PCD5 was seen in shrimps with Zn at 1.5 and 2.5 mg l^{-1} ($P<0.05$) (Fig. 5.9).

Alkaline phosphatase activity

ALP activity was not altered in any of the treatments receiving additional Zn ($P<0.05$). Following challenge, the shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1} failed to register an increase in the ALP activity. Maximum increase in ALP activity on PCD2 was exhibited by the unexposed shrimps (0.923 ± 0.13 mg p-nitrophenol released ml^{-1}), followed by those exposed to Zn at 0.5 (0.854 ± 0.11) and 1.0 mg l^{-1} (0.778 ± 0.1). Shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1} showed very low activity on PCD5 ($P<0.05$) (Fig. 5.10).

Acid phosphatase activity

No significant variations were seen in ACP activity of shrimps exposed to Zn, compared to the control ($P<0.05$). Following challenge, the activity increased in all Zn-exposed groups like in the unexposed, except at 2.5 mg l^{-1}. The activity on the contrary, was found to reduce in shrimps with Zn at 2.5 mg l^{-1}. Maximum activity on PCD2 was recorded in the control shrimps (1.143 ± 0.08 mg p-nitrophenol released ml^{-1}). Whereas, shrimps exposed to 1.0 mg l^{-1} showed significantly high activity on PCD5 (0.809 ± 0.08) ($P<0.05$) (Fig. 5.11).

5.3d Post challenge survival

Fig.5.12 shows the percentage survival rates of *P. monodon* exposed to Zn and then challenged with WSSV. The percentage survival rates of *P. monodon* exposed to Zn at 1.0 mg l^{-1} were significantly high, compared to the control and other Zn-exposed groups ($P<0.05$). Least survival rate was recorded with the shrimps exposed to Zn at 2.5 mg l^{-1}, which succumbed to early death ($P<0.05$). Compared to the unexposed, survival rate was also lower in shrimps with Zn at 1.5 mg l^{-1}, whereas shrimps exposed to Zn at 0.5 mg l^{-1} showed a comparatively better survival ($P<0.05$). The onset of mortality was much delayed in shrimps exposed to Zn at 1.0 mg l^{-1} and occurred only on PCD10, by which time the survival rates of shrimps with Zn at 1.5 and 2.5 mg l^{-1} reached 0.

5.4 Discussion

No significant improvements in the concentration of haemolymph metabolic variables (total protein, total carbohydrates, total lipids) could be observed in shrimps exposed to Zn at 1.0 mg l^{-1} that showed an enhanced metabolic and immune response and

a better post challenge survival following WSSV infection. In the previous study with Cu, a moderate increase in total protein, total carbohydrates and total lipids could be observed in shrimps exposed to Cu at 0.1 mg l^{-1}. According to Flier and Flier (1998) Zn played a central role in the activation of numerous enzyme systems that synthesize and degrade bioactive peptides; some of which called neuropeptides have been proposed to be involved in the regulation of food intake. However, in the light of the present results no conclusion could be made supporting the above statement. Unfortunately, the weight gain or the biochemical composition of other tissues, *viz.*, muscle or hepatopancreas was not analysed. With Zn at 0.5 mg l^{-1} also, except for the increase in glucose and cholesterol, no other pronounced variations were observed. And at higher doses total lipids were found to reduce. However, the results of the present study might indicate that lower doses of ambient Zn did not induce many metabolic disturbances in shrimps. On the other hand, at higher doses, except for the total protein and total carbohydrates, all other variables were found to vary, which in turn implies that comparatively more metabolic adjustments were required at higher doses.

Zn at relatively higher doses did not induce any proteolysis or reduction in protein content in the present study. Malik *et al.* (1998) have reported a decrease in the protein content and calorific value of muscle and liver of Murrel, *Channa punctatus* exposed to sublethal concentration of Zn at 2.4 mg l^{-1}. When shrimps were exposed to Cu at a higher dose in the previous study, total protein was found to decrease. However, Vankhede *et al.* (2004) reported an increase in total serum protein content in *Labeo rohita* on treatment with $ZnCl_2$. Total carbohydrate levels in shrimp haemolymph was also not altered by the presence of excess Zn in ambient water suggesting that Zn did not alter the carbohydrate metabolism and utilization. But glucose levels increased that showed a maximum in shrimps with Zn at 1.0 mg l^{-1}. According to Bonga (1997), a rise in cortisol levels that have some protective functions (Flik and Perry, 1989; Goss *et al.*, 1992; Bury *et al.*, 1998; Kelly and Wood, 2002) in fish during stress is frequently followed by hyperglycemia. Hyperglycemia observed on Zn exposure, which was also seen with Cu might be an indication of the stimulation of other compensatory mechanisms. Though Vankhede *et al.* (2004) could notice an increase in blood glucose in fish, *Labeo rohita* after 10 days of exposure to sublethal doses of $ZnCl_2$, the levels decreased after 20 and 30 days of treatment.

The variation in free amino acids is indirectly indicating the osmoregulatory disturbances in shrimps associated with Zn exposure. According to previous workers, in addition to being utilized as energy substrates and components of body structures, amino

acids can be more important than ions in the maintenance of osmotic pressure in shrimps (McFarland and Lee, 1963; Rosas *et al.*, 1999). Hence, the increase in TFAA at higher doses may be a mechanism to rectify the osmoregulatory imbalance. An increase in free amino acids in liver, kidney, stomach, intestine, testis, ovary and muscle has been reported in freshwater fish *Clarias batrachus* after treatment with Hg, As and Pb (Jana *et al.*, 1986). Osmoregulatory disturbances on treatment with Zn have been previously reported. Zn exposure produced a decline in plasma chloride in bluegill *Lepomis macrochirus* (Alan, 1987). Hogstrand *et al.* (1995) have shown a Zn-induced depression of plasma ions, particularly Ca^{2+}. However, it may be noted that a decrease in TFAA was observed in shrimps when treated with Cu. Such contrasting results stimulate further research into the osmoregulatory mechanisms adopted by shrimps under the influence of various metals.

A reduction in haemolymph total lipids was obtained in shrimps with Zn at 1.5 and 2.5 mg l^{-1}. Lipids were supposedly used in the repair of disrupted gill epithelial membranes. In Zn-exposed rainbow trout, *Salmo gairdneri*, gill structural alterations, including destruction of gill epithelium was reported (Skidmore, 1970). No mobilization of the reserve lipids to make up the loss probably resulted in the depletion. Lipids might have also been preferentially used as an energy source to tide over the metal stress. Chinni and Yallapragada (2000) have reported depletion in total lipids in Pb-exposed post larvae of *F. indicus*. Cholesterol levels were found to increase in shrimps on exposure to Zn except with 1.5 mg l^{-1}. Cholesterol is important in maintaining the integrity and chemical permeability of cell walls (Kanazawa *et al.*, 1971). Since shrimps cannot synthesise cholesterol *de novo* (Teshima and Kanazawa, 1971), the increase in cholesterol might be a stress response. Somehow, the cholesterol levels recorded were slightly lower at 1.5 mg l^{-1}. Further studies may be required for more explanation.

Following infection, the enhancement in metabolic variables (except TFAA) was maximum in shrimps with Zn at 1.0 mg l^{-1} compared to the unexposed and other exposed groups. This in turn implies that shrimps with Zn at 1.0 mg l^{-1} had more of the metabolites as reserve. Hence, the maximum increase may be attributed to the higher degree of mobilization of the energy reserves to ward off the invading pathogen. The relative proportion of immune proteins might also have been higher in shrimps with Zn at 1.0 mg l^{-1}. The usual haemolymph lipid depletion noted in infected shrimps was not observed in shrimps with Zn at 1.0 mg l^{-1}. On the contrary, the lipid content did not vary and showed levels similar to that of the control. However, with regard to total free amino acids, the usual increasing trend in infected shrimps was not seen with Zn at 1.0 mg l^{-1}

and was significantly lower than in the other groups. When the enhanced post challenge survival in shrimps with Zn at 1.0 mg l^{-1} is taken into consideration the constant TFAA level may be presumed as a positive indication of the reduced impact of pathogen. Shrimps exposed to higher Zn levels showed the least levels of haemolymph total protein, total lipid, glucose and cholesterol following WSSV challenge. This comparative reduction may be correlated to the decreased availability of energy reserves already been used up to combat the metal stress. Pathogenic stress further increased the energy demands. The decreased concentration of haemolymph metabolites might have harmfully affected the shrimp's resistance to pathogen, which is partly responsible for the decreased immunocompetence of shrimps exposed to higher Zn doses. On the other hand, the better metabolic response in shrimps exposed to Zn at 1.0 mg l^{-1} could be correlated to the enhanced post challenge survival.

THC was found to increase in shrimps on treatment with Zn, as seen with Cu. Increase in the number of total circulating haemocytes on exposure to metals has been recorded for a wide range of molluscan species including *Mytilus edulis*, on exposure to Cd (Coles *et al.*, 1995) and Cu (Pipe and Coles, 1995) and *Crassostrea gigas* and *C. virginica*, on exposure to Cd (Cheng, 1988; Auffret and Oubella, 1994). The stimulation of cell proliferation from the haematopoeitic nodules may be a plausible explanation for the increase in the present case. The reversible decrease that was noted following infection in shrimps exposed to Cu was not observed with Zn. Hence, the increase in THC could not be a consequence of cell migration. The slight decrease on PCD2 might be due to degranulation or cell migration from circulation to tissues. The increase in cell count was also observed at higher doses, but WSSV challenge induced reduction to a greater degree. Shiau and Jiang (2006) have reported an increase in THC in shrimps fed diets with 35 and 48 mg Zn kg^{-1}.

Treatment with Zn lead to an increase of PO activity in shrimps, which was maximum with Zn at 1.0 mg l^{-1} and least at 2.5 mg l^{-1}. proPO system is particularly involved in the non-self recognition of shrimps (Vargas-Albores, 1995; Hernandez-Lopez *et al.*, 1996). Apart from being activated by various microbial polysaccharides (Soderhall and Unestam, 1979; Soderhall *et al.*, 1990), additional factors such as calcium, sodium dodecyl sulfate, trypsin, high temperature (Ashida *et al.*, 1983; Ashida and Soderhall, 1984; Dulary and Lackie, 1985; Leonard *et al.*, 1985; Sugumaran and Nellaiappan, 1991) and vitamins (Sahoo *et al.*, 2005) have also been found to elicit the proPO system. The present study has proved that metals can also act as elicitors of proPO system. Exposure to both Cu and Zn resulted in an increase of phenol oxidase activity in shrimps. The

enhanced PO activity before and after challenge seen in shrimps with Zn at 1.0 mg l^{-1} may be considered as a major factor contributing to the enhanced disease resistance. However, it is crucial to understand the action of metal elicitors on the proPO function.

No significant differences were observed in NBT activity on day 14 in shrimps exposed to Zn except at 2.5 mg l^{-1}. According to Hendy *et al.* (2001) Zn plays an important role in sequestering free radical, but it is evident from the present study that Zn at higher doses induced the production of more superoxide anions. This may also be due to the inhibitory effect of higher doses of Zn on the antioxidant enzyme activity, which is dealt with in detail in chapter 6. However, it may be noted that superoxide anion production was highest in shrimps exposed to Zn at 1.0 mg l^{-1} following challenge. This observation implies that the enzyme NADPH-oxidase was more activated at an exposure level of Zn at 1.0 mg l^{-1} on entry of the virus, which helped in effective oxidative killing. Though Zn is an integral component of the enzyme alkaline phosphatase, no significant elevation was seen in its activity. Quantification of the enzyme may be required to determine the actual effect. Increased plasma ALP activity has been reported in growing rats (Sun *et al.*, 2006) and pigs (Xu and Wang, 2001) fed diet containing high Zn levels. No significant increase in phosphatase activity, especially ALP was found in infected shrimps exposed to higher doses of Zn. Instead inhibition was more during the later stage of infection. Release of degradative enzymes during phagocytosis was found to be significantly suppressed in *Mytilus edulis* exposed to Cd at 40 μg l^{-1}.

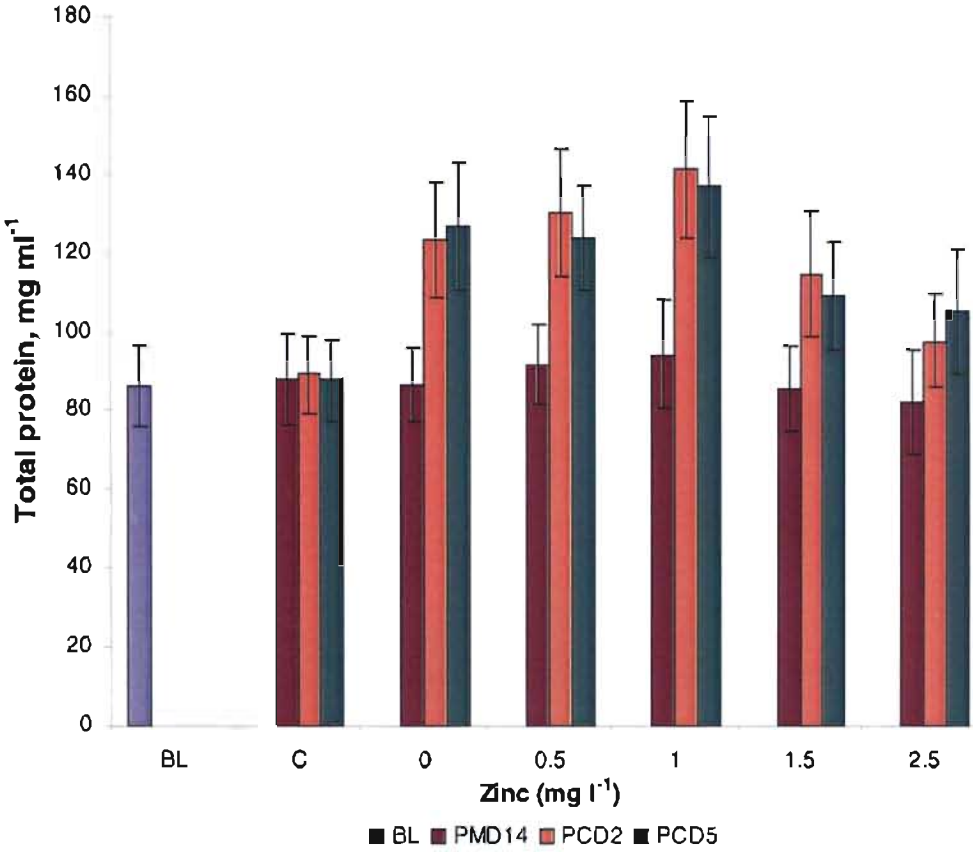
A significant increase in haemolymph Zn concentration was only seen with 2.5 mg l^{-1}. In rainbow trout, plasma Zn concentration has been found to increase with increasing Zn concentration in water, suggesting that Zn may enter the gill tissue and pass through it (Spry *et al.*, 1988; Zia and McDonald, 1994). Though an increase could be observed in shrimps with Zn at 1.5 mg l^{-1} it was not statistically significant. Zn concentration in shrimp muscle was not elevated in any of the treatments receiving additional dissolved Zn. It is likely that Zn induced the synthesis of Metallothionein (Klaassen, 2001), which helped in the regulation of body levels of Zn. According to Nunez-Nogueira *et al.* (2006), hepatopancreas is the main accumulatory organ for Zn and Cd taken up from solution in *F. indicus*. However, the Zn content in hepatopancreas was not analysed in the present study. Two excretion routes of Zn have been suggested for crustaceans by previous workers, *via* urine production in the antennal gland and *via* the loss of detoxified Zn-rich granules from hepatopancreas (Rainbow, 1998; Vogt and Quinitio, 1994). A comparison of the post challenge Zn content showed significantly lower level in muscle on PCD2 in shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1} and an

increase in haemolymph Zn in shrimps exposed to Zn at 1.0 mg l^{-1}. It is likely that Zn is retained in haemolymph post infection for increased metabolic availability, as Zn is an integral component of many enzymes. For an accurate explanation, further studies on are required.

The survival data clearly showed that shrimps treated with Zn at 1.0 mg l^{-1} thrived without significant mortality till PCD12. In contrast, shrimps treated with higher doses of Zn suffered continuous mortality and the whole population declined to zero well before. Even the shrimps under controlled conditions perished on PCD12. This strongly suggested that exposure to Zn at 1.0 mg l^{-1} significantly enhanced the immunity of shrimps. The better metabolic and immune response could be correlated to the enhanced post challenge survival. Compared to the immunostimulatory effect with Cu at 0.1 mg l^{-1}, Zn at 1.0 mg l^{-1} had an amazingly higher effect. Possibly, the better post challenge survival could also be related to the role played by Zn in the gut immune function as reported by Scott and Koski (2000). Upon infection, WSSV infected cells are observed first in the stomach, gill and cuticular epidermis of the shrimp and then subsequently spreads to other tissues of mesodermal and ectodermal origin (Chang *et al.*, 1996). On the other hand, the suppressive effects with higher doses of Zn that reduced the immunocompetence of shrimps upon WSSV challenge were also clearly evident from the survival data. The poor metabolic and immune response could be correlated to the least post challenge survival seen; especially in shrimps with Zn at 2.5 mg l^{-1}. Although increased susceptibility to infection under metal stress has been proved with Cu and Cd (Baker *et al.*, 1983; Pipe and Coles, 1995; Shariff *et al.*, 2001) there is a scarcity of data with respect to Zn.

In conclusion, the present study on *P. monodon* has documented the stimulatory and suppressive effects of Zn in ambient water. The ambient Zn concentration up to 1.0 mg l^{-1} was found to be immunostumulatory. Enhanced metabolic and immune response offered better resistance and protection from mortality due to WSSV infection. Though *P. monodon* was found to be relatively tolerant to higher doses of Zn in ambient water, physiological response to WSSV infection was weaker and the lowered disease resistance under metal stress led to enhanced susceptibility. Therefore, the present information on the physiological and immunomodulatory effects of ambient Zn needs to be considered during shrimp pond management, especially while adopting disease management strategies. It could be particularly proposed that application of Zn at a concentration of 1.0 mg l^{-1} in culture pond water may act as an alternative measure for disease control,

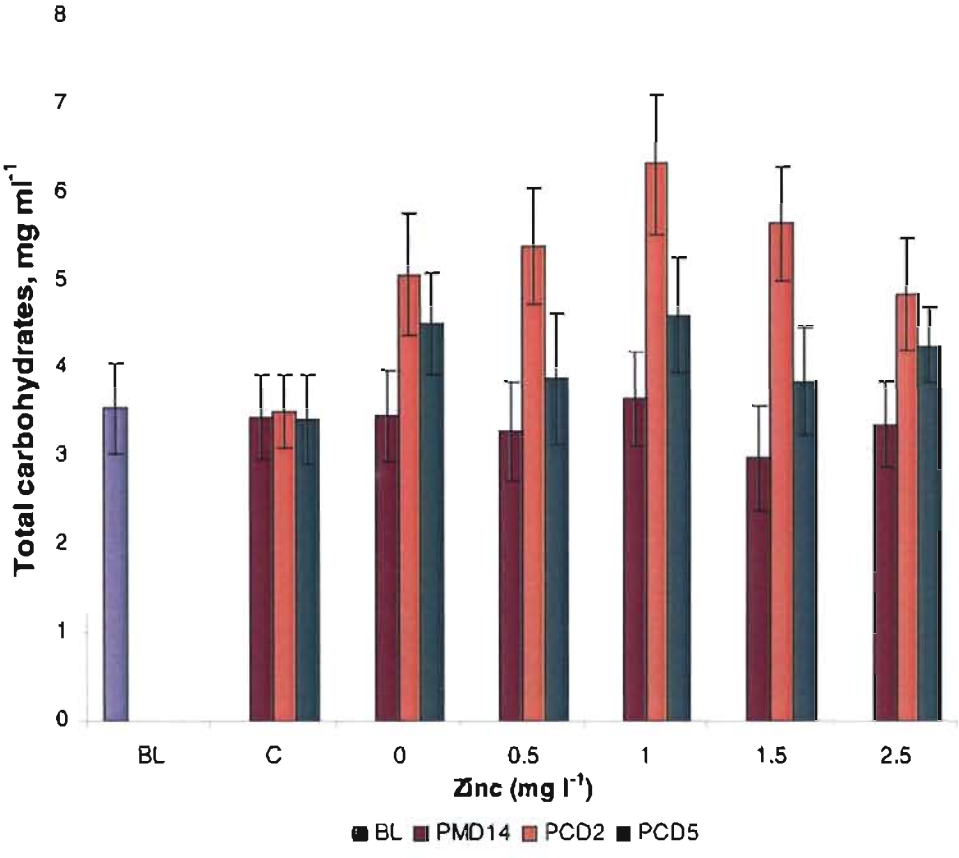
especially WSSV. However, further studies on the long-term effects of Zn and the effects on the weight gain and growth rate may be required prior to field application.



	Total protein (mg ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	86.0 ± 10.4	$_{a}87.78 \pm 11.9^{A}$	$_{a}89.03 \pm 9.8^{A}$	$_{a}87.77 \pm 10.5^{A}$
0		$_{a}86.55 \pm 9.7^{B}$	$_{ab}123.48 \pm 14.6^{A}$	$_{ab}126.83 \pm 16.0^{A}$
0.5		$_{a}91.87 \pm 10.3^{B}$	$_{ab}130.04 \pm 16.1^{A}$	$_{ab}123.89 \pm 13.2^{A}$
1		$_{a}94.26 \pm 13.9^{B}$	$_{a}141.25 \pm 17.3^{A}$	$_{a}136.91 \pm 17.7^{A}$
1.5		$_{a}85.51 \pm 11.0^{B}$	$_{bc}114.79 \pm 16.0^{A}$	$_{bc}109.25 \pm 13.8^{A}$
2.5		$_{a}82.17 \pm 13.4^{B}$	$_{c}97.79 \pm 12.1^{AB}$	$_{c}105.30 \pm 15.9^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

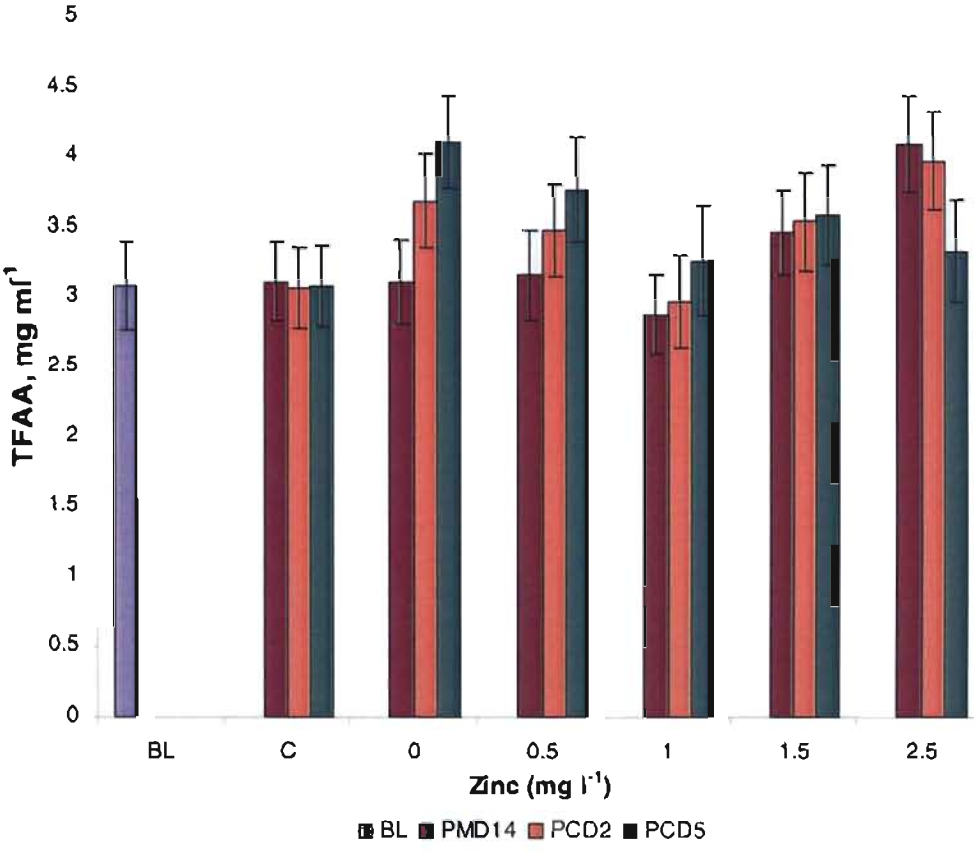
Fig.5.1 Total protein in the haemolymph of *P. monodon* exposed to zinc and challenged with WSSV.



	Total carbohydrates (mg ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	3.53 ± 0.51	$_{a}3.43 \pm 0.47^{A}$	$_{c}3.50 \pm 0.42^{A}$	$_{b}3.41 \pm 0.50^{A}$
0		$_{ab}3.46 \pm 0.52^{B}$	$_{b}5.07 \pm 0.69^{A}$	$_{a}4.51 \pm 0.57^{A}$
0.5		$_{a}3.28 \pm 0.58^{B}$	$_{b}5.39 \pm 0.65^{A}$	$_{ab}3.88 \pm 0.74^{B}$
1		$_{a}3.66 \pm 0.54^{C}$	$_{a}6.32 \pm 0.79^{A}$	$_{a}4.61 \pm 0.65^{B}$
1.5		$_{a}2.98 \pm 0.60^{C}$	$_{ab}5.65 \pm 0.64^{A}$	$_{ab}3.85 \pm 0.62^{B}$
2.5		$_{a}3.36 \pm 0.49^{B}$	$_{b}4.84 \pm 0.63^{A}$	$_{a}4.27 \pm 0.43^{A}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

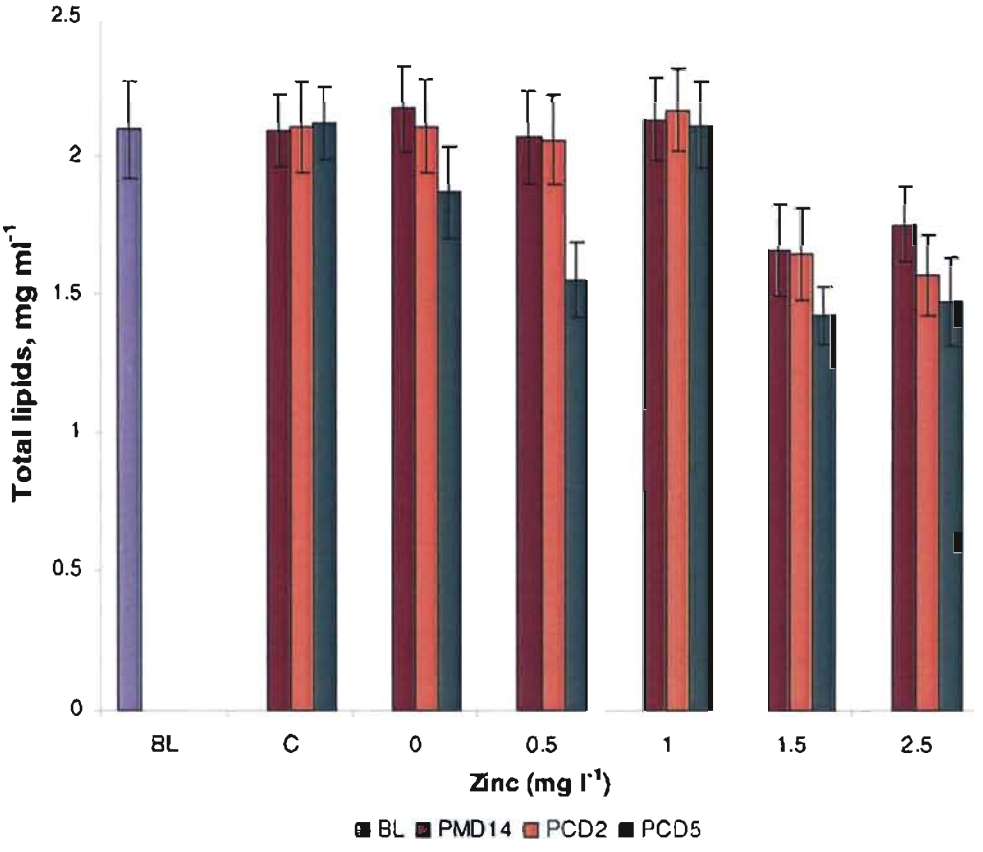
Fig.5.2 Total carbohydrates in the haemolymph of *P. monodon* exposed to zinc and challenged with WSSV.



	Total free amino acids (mg ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	3.07 ± 0.32	$_{bc}3.10 \pm 0.28^{A}$	$_{c}3.06 \pm 0.29^{A}$	$_{d}3.08 \pm 0.29^{A}$
0		$_{bc}3.10 \pm 0.30^{C}$	$_{ab}3.68 \pm 0.33^{B}$	$_{a}4.09 \pm 0.33^{A}$
0.5		$_{bc}3.15 \pm 0.32^{B}$	$_{b}3.47 \pm 0.33^{AB}$	$_{ab}3.76 \pm 0.37^{A}$
1		$_{c}2.87 \pm 0.28^{A}$	$_{c}2.96 \pm 0.33^{A}$	$_{cd}3.26 \pm 0.39^{A}$
1.5		$_{b}3.46 \pm 0.30^{A}$	$_{b}3.53 \pm 0.35^{A}$	$_{bc}3.58 \pm 0.36^{A}$
2.5		$_{a}4.09 \pm 0.34^{A}$	$_{a}3.97 \pm 0.34^{A}$	$_{bcd}3.33 \pm 0.36^{B}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

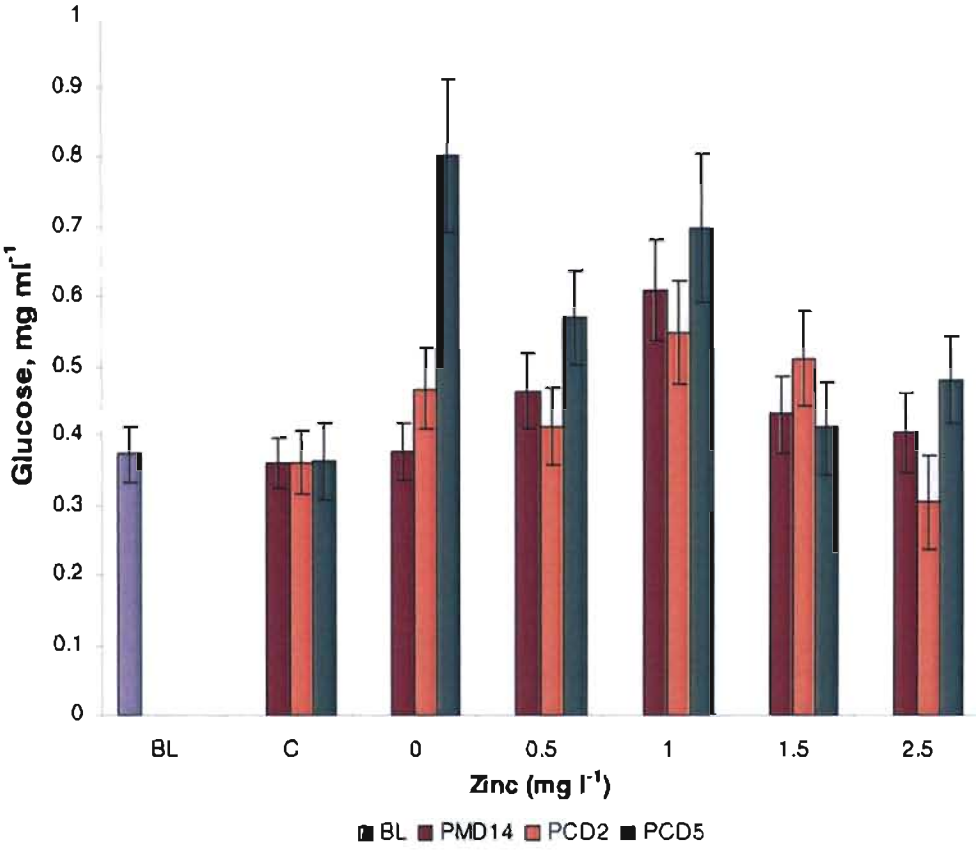
Fig.5.3 Total free amino acids (TFAA) in the haemolymph of *P. monodon* exposed to zinc and challenged with WSSV.



	Total lipids (mg ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	2.09 ± 0.17	$_{a}$2.09 ± 0.13^{A}	$_{a}$2.10 ± 0.17^{A}	$_{a}$2.11 ± 0.13^{A}
0		$_{a}$2.17 ± 0.16^{A}	$_{a}$2.10 ± 0.17^{A}	$_{b}$1.86 ± 0.16^{B}
0.5		$_{a}$2.06 ± 0.17^{A}	$_{a}$2.05 ± 0.16^{A}	$_{c}$1.55 ± 0.13^{B}
1		$_{a}$2.13 ± 0.15^{A}	$_{a}$2.17 ± 0.15^{A}	$_{a}$2.11 ± 0.15^{A}
1.5		$_{b}$1.66 ± 0.16^{A}	$_{b}$1.65 ± 0.16^{A}	$_{c}$1.43 ± 0.11^{B}
2.5		$_{b}$1.75 ± 0.14^{A}	$_{b}$1.57 ± 0.14^{B}	$_{c}$1.48 ± 0.16^{B}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline. PMD –Post metal exposure day, PCD –Post challenge day

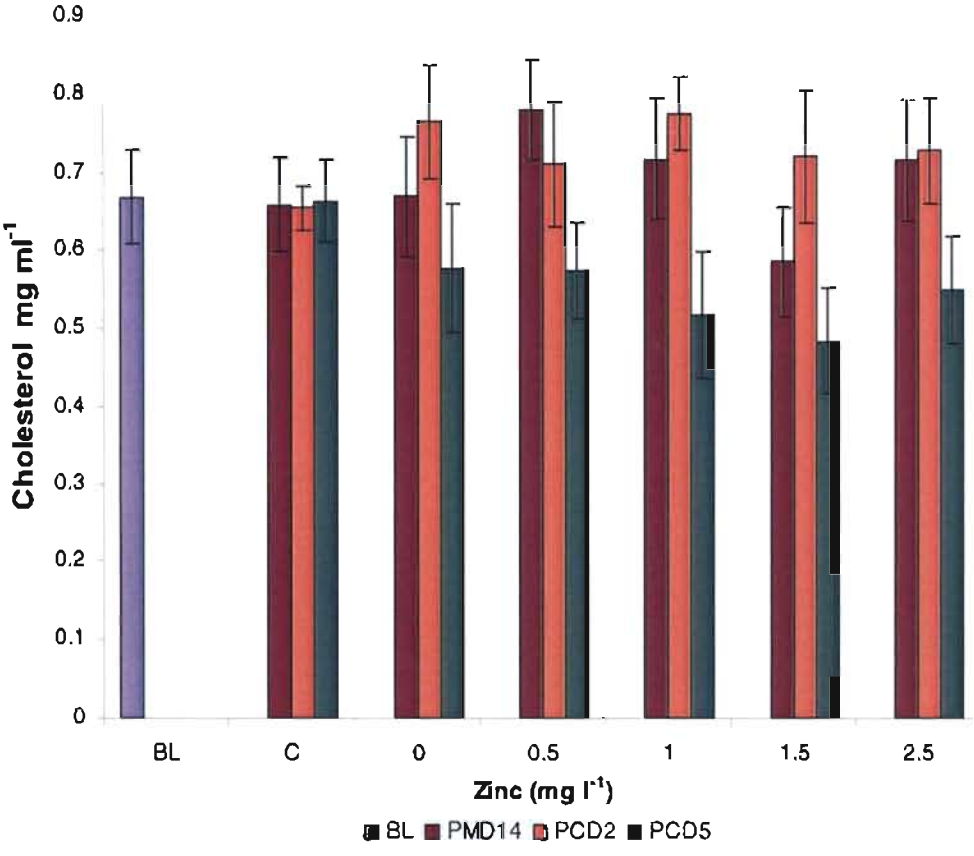
Fig.5.4 Total lipids in the haemolymph of *P. monodon* exposed to zinc and challenged with WSSV.



	Glucose (mg ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	0.372 ± 0.04	$_{c}$0.360 ± 0.04^{A}	$_{dc}$0.359 ± 0.05^{A}	$_{c}$0.362 ± 0.06^{A}
0		$_{c}$0.376 ± 0.04^{B}	$_{bc}$0.467 ± 0.06^{B}	$_{a}$0.801 ± 0.11^{A}
0.5		$_{b}$0.464 ± 0.05^{B}	$_{cd}$0.412 ± 0.06^{B}	$_{c}$0.569 ± 0.07^{A}
1		$_{a}$0.608 ± 0.07^{AB}	$_{a}$0.549 ± 0.07^{B}	$_{b}$0.697 ± 0.10^{A}
1.5		$_{bc}$0.429 ± 0.06^{B}	$_{ab}$0.511 ± 0.07^{A}	$_{dc}$0.410 ± 0.07^{B}
2.5		$_{bc}$0.404 ± 0.06^{A}	$_{c}$0.304 ± 0.07^{B}	$_{cd}$0.479 ± 0.06^{A}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

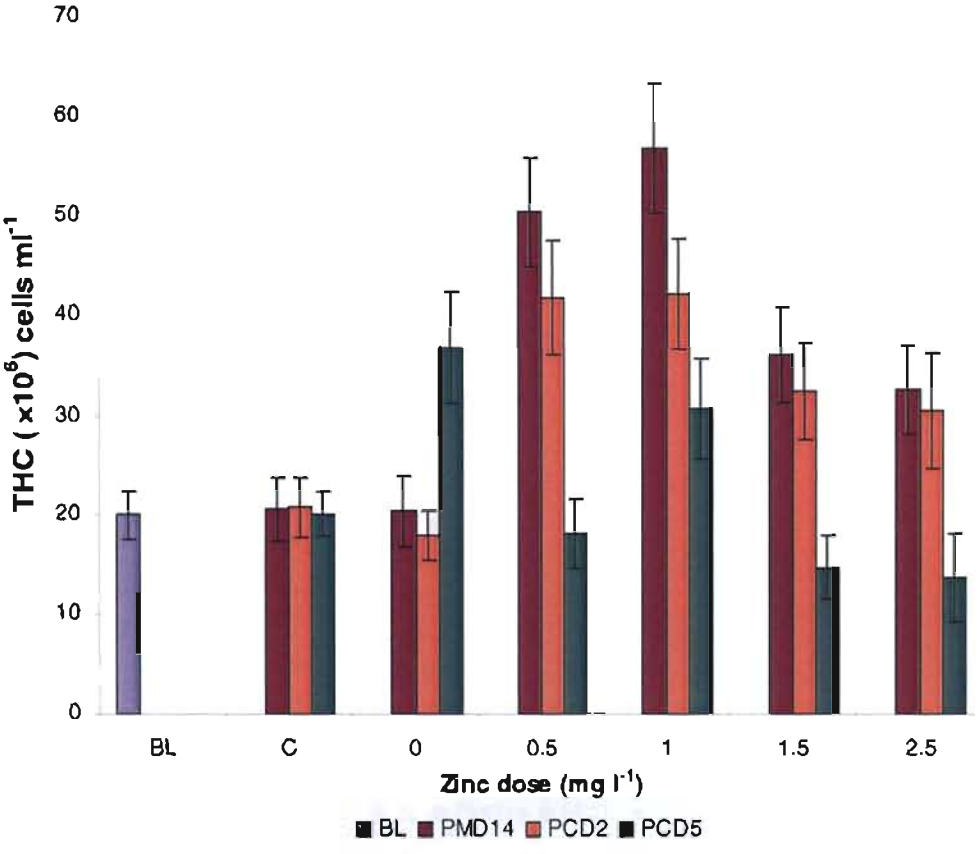
Fig.5.5 Glucose levels in the haemolymph of *P. monodon* exposed to zinc and challenged with WSSV.



	Cholesterol (mg ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	0.668 ± 0.06	$_{bc}$0.658 ± 0.06^{A}	$_{b}$0.654 ± 0.03^{A}	$_{a}$0.663 ± 0.05^{A}
0		$_{bc}$0.669 ± 0.08^{B}	$_{a}$0.764 ± 0.07^{A}	$_{b}$0.577 ± 0.08^{B}
0.5		$_{a}$0.780 ± 0.06^{A}	$_{ab}$0.710 ± 0.08^{A}	$_{b}$0.575 ± 0.06^{B}
1		$_{ab}$0.717 ± 0.08^{A}	$_{a}$0.776 ± 0.05^{A}	$_{bc}$0.518 ± 0.08^{B}
1.5		$_{c}$0.586 ± 0.07^{B}	$_{ab}$0.720 ± 0.08^{A}	$_{c}$0.484 ± 0.07^{C}
2.5		$_{ab}$0.716 ± 0.08^{A}	$_{ab}$0.727 ± 0.07^{A}	$_{bc}$0.549 ± 0.07^{B}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

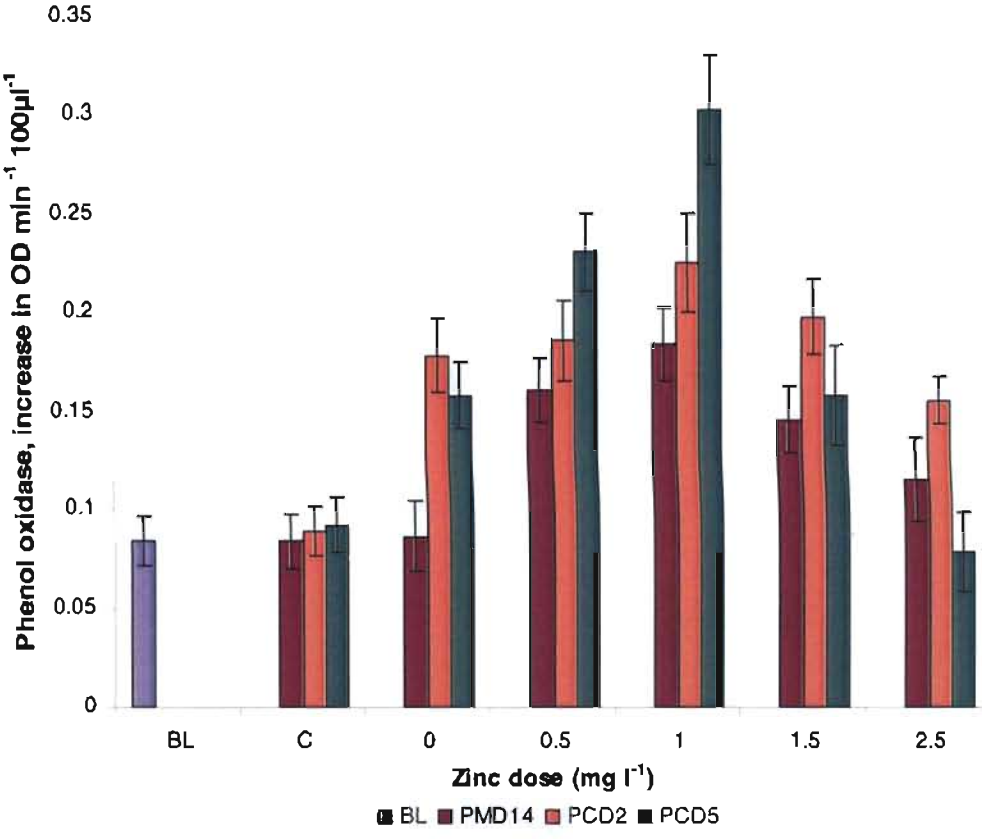
Fig.5.6 Cholesterol levels in the haemolymph of *P. monodon* exposed to zinc and challenged with WSSV.



	Total Haemocyte Count (×10^6 cells ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	19.85 ± 2.3	$_{d}$20.44 ± 3.2^{A}	$_{c}$20.65 ± 2.9^{A}	$_{c}$20.03 ± 2.3^{A}
0		$_{d}$20.30 ± 3.5^{B}	$_{c}$17.85 ± 2.5^{B}	$_{a}$36.81 ± 5.6^{A}
0.5		$_{b}$50.43 ± 5.5^{A}	$_{d}$41.78 ± 5.8^{B}	$_{cd}$18.09 ± 3.5^{C}
1		$_{a}$56.77 ± 6.6^{A}	$_{a}$42.20 ± 5.5^{B}	$_{b}$30.68 ± 5.1^{C}
1.5		$_{c}$36.14 ± 4.8^{A}	$_{b}$32.34 ± 4.9^{A}	$_{d}$14.64 ± 3.2^{B}
2.5		$_{c}$32.55 ± 4.5^{A}	$_{b}$30.46 ± 5.9^{A}	$_{d}$13.57 ± 4.4^{B}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

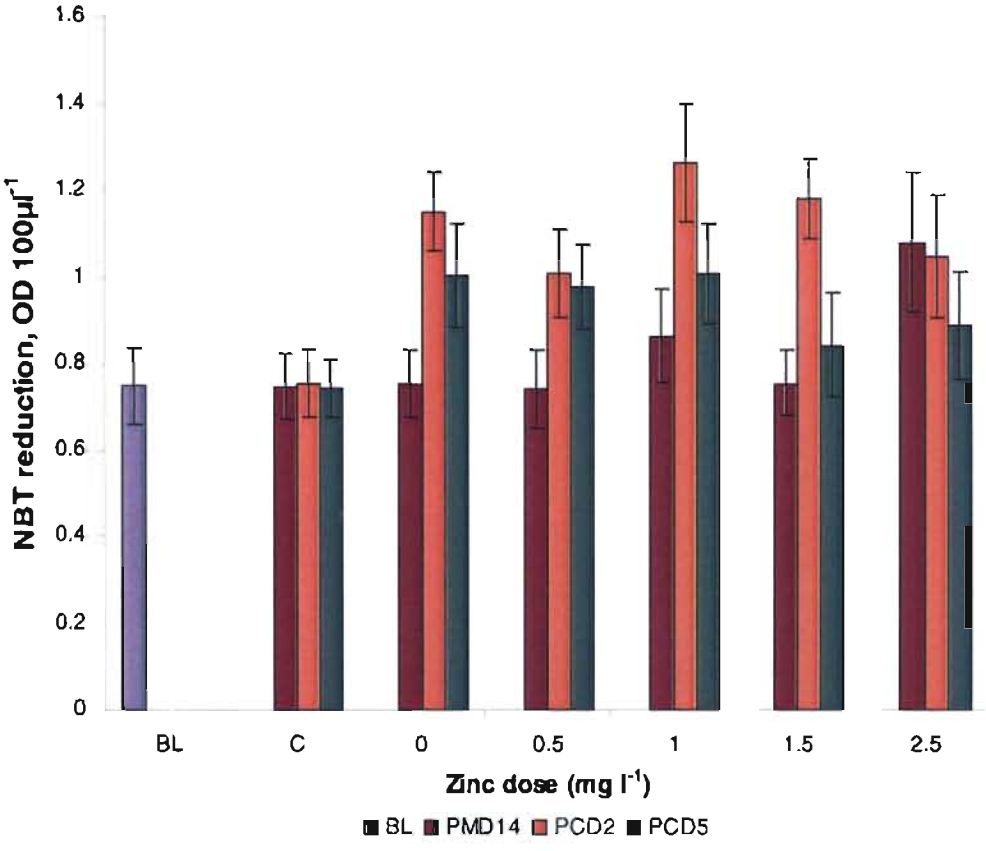
Fig.5.7 Total haemocyte count (THC) in the haemolymph of *P. monodon* exposed to zinc and challenged with WSSV.



	Phenol oxidase activity (increase in O.D. min^{-1} $100\mu l^{-1}$)			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	0.084 ± 0.01	$_{d}0.084 \pm 0.01^{A}$	$_{d}0.089 \pm 0.01^{A}$	$_{d}0.092 \pm 0.01^{A}$
0		$_{d}0.086 \pm 0.02^{B}$	$_{b}0.178 \pm 0.02^{A}$	$_{c}0.158 \pm 0.02^{A}$
0.5		$_{b}0.160 \pm 0.02^{C}$	$_{b}0.186 \pm 0.02^{B}$	$_{b}0.230 \pm 0.02^{A}$
1		$_{a}0.184 \pm 0.02^{C}$	$_{a}0.225 \pm 0.02^{B}$	$_{a}0.302 \pm 0.03^{A}$
1.5		$_{b}0.145 \pm 0.02^{B}$	$_{b}0.198 \pm 0.02^{A}$	$_{c}0.157 \pm 0.03^{B}$
2.5		$_{c}0.115 \pm 0.02^{B}$	$_{c}0.155 \pm 0.01^{A}$	$_{d}0.078 \pm 0.02^{C}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

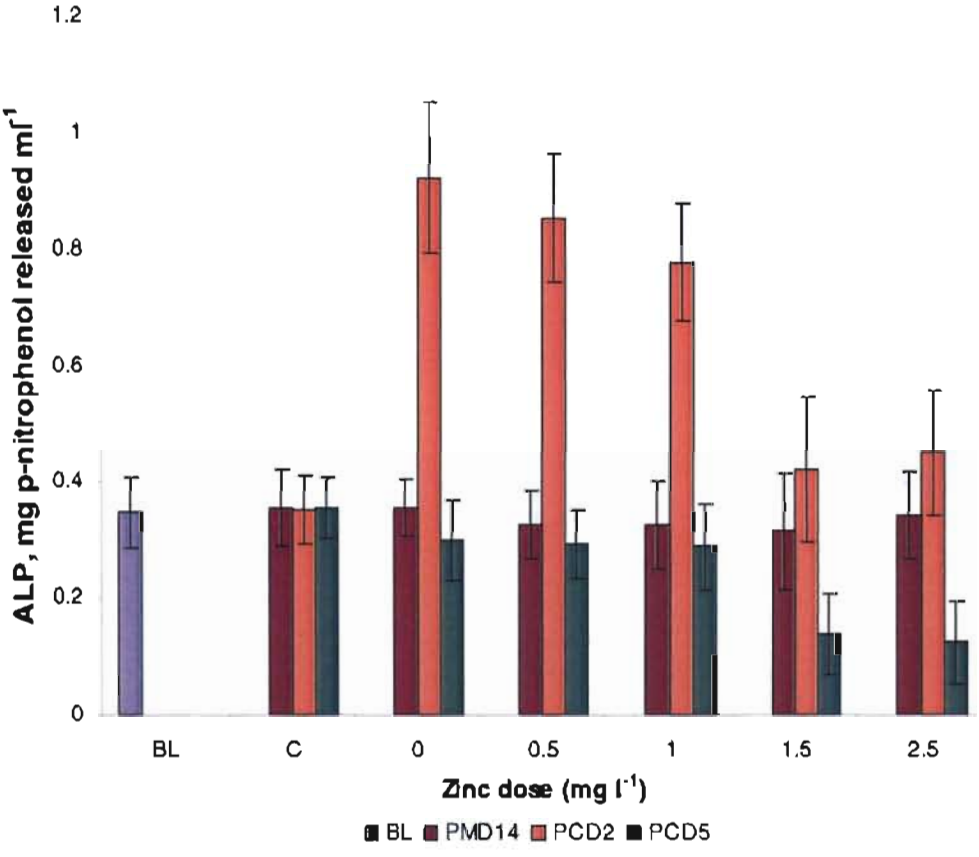
Fig.5.8 Phenol oxidase activity in *P. monodon* exposed to zinc and challenged with WSSV.



	NBT Reduction (O.D. at 620nm 100µl^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	0.745 ± 0.09	$_{b}$0.745 ± 0.08^{A}	$_{d}$0.751 ± 0.08^{A}	$_{c}$0.741 ± 0.07^{A}
0		$_{b}$0.750 ± 0.08^{C}	$_{ab}$1.149 ± 0.09^{A}	$_{a}$1.003 ± 0.12^{B}
0.5		$_{b}$0.740 ± 0.09^{B}	$_{c}$1.008 ± 0.10^{A}	$_{a}$0.976 ± 0.10^{A}
1		$_{b}$0.863 ± 0.11^{B}	$_{a}$1.262 ± 0.14^{A}	$_{a}$1.008 ± 0.11^{B}
1.5		$_{b}$0.753 ± 0.08^{B}	$_{ab}$1.177 ± 0.09^{A}	$_{bc}$0.840 ± 0.12^{B}
2.5		$_{a}$1.079 ± 0.16^{A}	$_{bc}$1.048 ± 0.14^{AB}	$_{ab}$0.889 ± 0.12^{C}

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

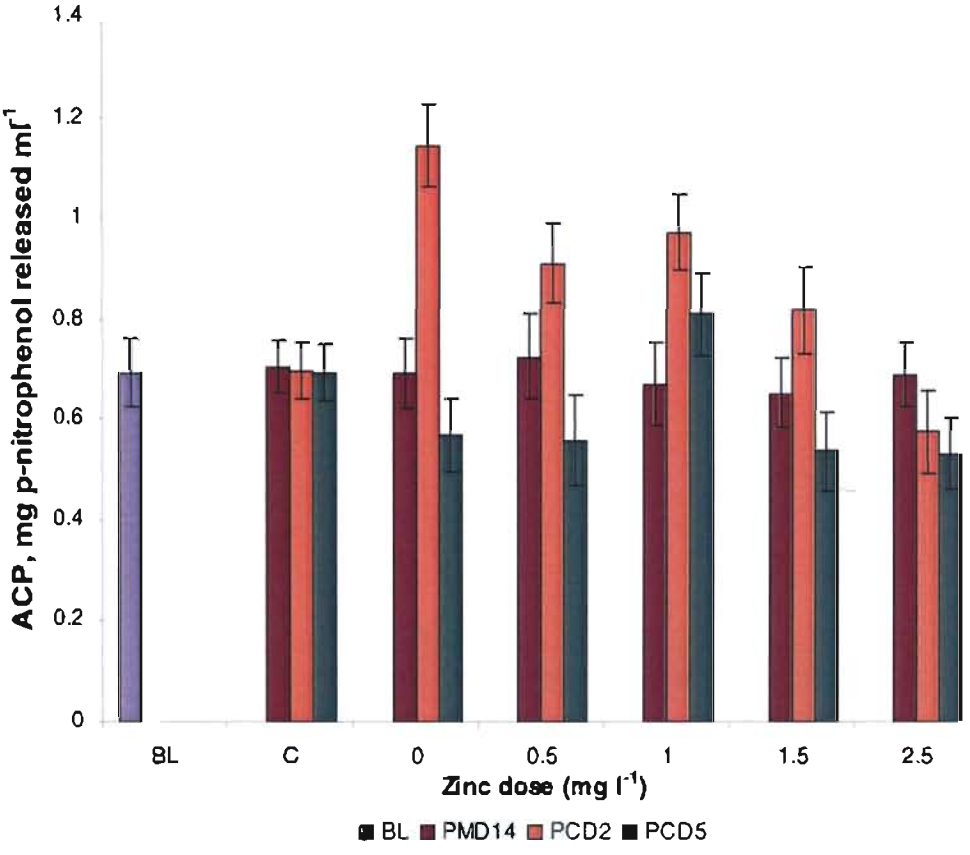
Fig.5.9 NBT reduction in *P. monodon* exposed to zinc and challenged with WSSV.



	Alkaline Phosphatase activity (mg p-nitrophenol released ml^{-1})			
Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Control	0.347 ± 0.06	$_{d}0.354 \pm 0.07^{A}$	$_{c}0.352 \pm 0.06^{A}$	$_{a}0.354 \pm 0.05^{A}$
0		$_{a}0.355 \pm 0.05^{B}$	$_{a}0.923 \pm 0.13^{A}$	$_{a}0.30 \pm 0.07^{B}$
0.5		$_{a}0.325 \pm 0.06^{B}$	$_{ab}0.854 \pm 0.11^{A}$	$_{d}0.292 \pm 0.06^{B}$
1		$_{a}0.325 \pm 0.08^{B}$	$_{b}0.778 \pm 0.10^{A}$	$_{a}0.287 \pm 0.07^{B}$
1.5		$_{a}0.315 \pm 0.10^{A}$	$_{c}0.420 \pm 0.13^{A}$	$_{b}0.137 \pm 0.07^{B}$
2.5		$_{a}0.341 \pm 0.07^{B}$	$_{c}0.450 \pm 0.11^{A}$	$_{b}0.123 \pm 0.07^{C}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL -Baseline, PMD -Post metal exposure day, PCD -Post challenge day

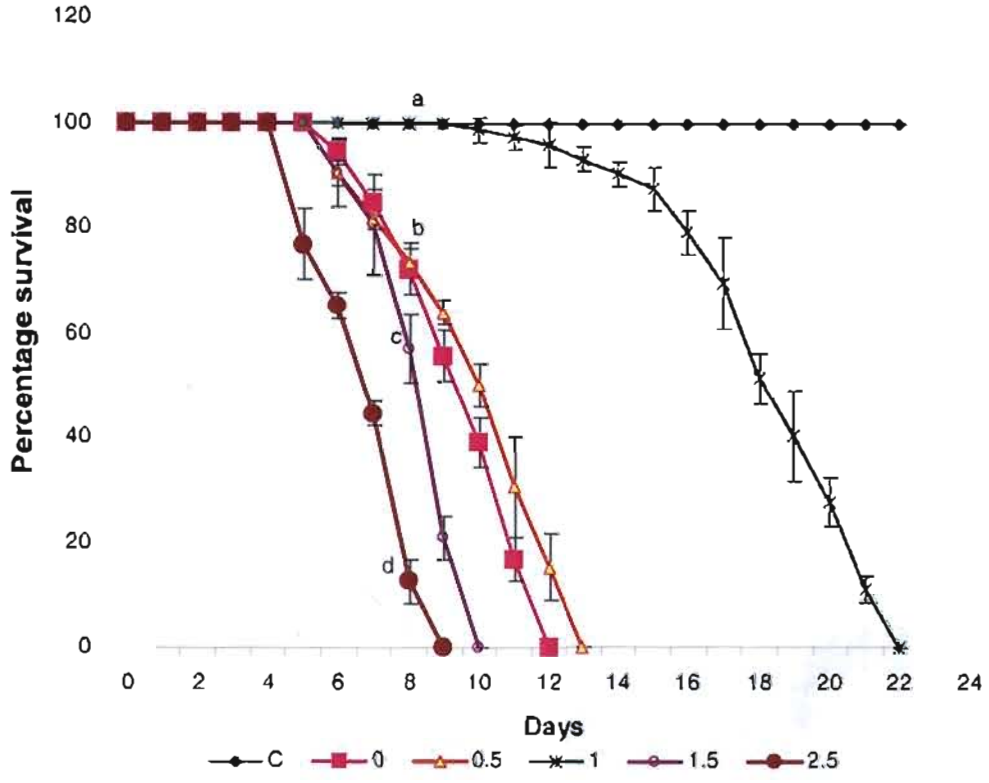
Fig.5.10 Alkaline phosphatase activity (ALP) in *P. monodon* exposed to zinc and challenged with WSSV.



	Acid Phosphatase activity (mg p-nitrophenol released ml^{-1})			
Zn ($mg\ l^{-1}$)	BL	PMD14	PCD2	PCD5
Control	0.691 ± 0.07	$_{a}0.704 \pm 0.05^{A}$	$_{a}0.698 \pm 0.06^{A}$	$_{b}0.693 \pm 0.06^{A}$
0		$_{a}0.692 \pm 0.07^{B}$	$_{a}1.143 \pm 0.08^{A}$	$_{c}0.567 \pm 0.07^{C}$
0.5		$_{a}0.724 \pm 0.09^{B}$	$_{b}0.911 \pm 0.08^{A}$	$_{c}0.557 \pm 0.09^{C}$
1		$_{a}0.669 \pm 0.08^{C}$	$_{b}0.971 \pm 0.07^{A}$	$_{a}0.809 \pm 0.08^{B}$
1.5		$_{a}0.652 \pm 0.07^{B}$	$_{c}0.817 \pm 0.09^{A}$	$_{c}0.535 \pm 0.08^{C}$
2.5		$_{a}0.688 \pm 0.06^{A}$	$_{c}0.574 \pm 0.08^{B}$	$_{c}0.530 \pm 0.07^{B}$

*Data in the same column with different subscripts are statistically different among treatments at the same exposure time and data in the same row with different superscripts are statistically different among different time periods. BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

Fig.5.11 Acid phosphatase activity (ACP) in *P. monodon* exposed to zinc and challenged with WSSV.



*Different letters indicate statistical difference among different treatments.

Fig.5.12 Post challenge survival of *P. monodon* exposed to zinc and challenged with WSSV.

CHAPTER 6

Antioxidative Defense Responses in WSSV Infected Penaeus monodon –Modulatory Effect of Ambient Copper and Zinc

6.1 Introduction

Respiratory burst or generation of reactive oxygen species with powerful microbicidal activity is an important defense mechanism in crustaceans that relies on non-specific and non-adaptive immune response. The stimulation of phagocytes leading to increased consumption and reduction of O_2, catalysed by a membrane-bound NADPH oxidase, give rise to superoxide anions (O_2^-). Further, an array of highly reactive oxygen species with microbicidal activity are formed, either directly or indirectly from superoxide (Bachere *et al.*, 1995; Munoz *et al.*, 2000). Apart from pathological stress, other biological, physical and chemical stress may also result in the formation of excessive amounts of free radicals in organisms (Ranby and Rabek, 1978). Excess reactive oxygen species (ROS) production is potentially harmful as it can lead to significant oxidative damage including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation. The efficiency of antioxidant defense mechanism to detoxify excess ROS and alleviate the anticipated oxidative stress is therefore of prime significance.

Reactive oxygen species (ROS) or free radicals are produced in aerobic organisms under normal conditions as an unavoidable byproduct of metabolism. A free radical can be defined as any chemical species that contains unpaired electrons in their outer orbit and thus can react virtually with all cell components (Slater, 1984; Collier *et al.*, 1992; Przekwas *et al.*, 2003). The univalent reduction of oxygen generates reactive oxygen intermediates. Common examples of free radicals include the hydroxyl radical (OH^-), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), nitric oxide (NO) and peroxynitrite ($ONOO^-$) (Dormandy, 1983). Cellular processes such as mitochondrial and microsomal electron transport, active phagocytosis and the activity of several enzymes, like, xanthine oxidase, produce ROS as intermediates (Halliwell and Gutteridge, 1984; Winston and Di Giulio, 1991). ROS, commonly referred to as prooxidants (Mates *et al.*, 1999) can cause direct cellular injury by inducing lipid and protein peroxidation and damage to nucleic acid (Takeda *et al.*, 1984; Richard *et al.*, 1990).

Key components of the antioxidant defense system include the free radical scavenging enzymes, which form the first line of defense against oxidative injury. Three major antioxidant enzymes are the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Hebbel, 1986; Sies, 1993). Glutathione *S*-transferase (GST) and glutathione reductase (GR) are the major secondary enzymatic antioxidants. An array of low molecular weight non-enzymatic antioxidants that provides protection against ROS forms the second line of antioxidant defense. Total reduced glutathione (GSH), vitamin C (ascorbic acid), vitamin E (α -tocopherol), vitamin A and ceruloplasmin are the major non-enzymatic antioxidants. α-lipoic acid, carotenoids, coenzyme Q_{10}, bioflavanoids and minerals like Cu, Zn, Mn and Se are also potent antioxidants. They work in synergy with each other against various types of free radicals (Asayama et al., 1989). DNA repair enzymes, proteases, lipases etc. capable of repairing the damage inflicted by ROS to nucleic acids, proteins and lipids (Warner, 1994) form the third line of defense.

Superoxide dismutase (SOD), the enzyme specific for scavenging superoxide radicals converts superoxide anions (O_2^-) into less toxic H_2O_2.

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

SOD occurs in three forms, which differ with regard to the metal-ions present in the active center of the enzyme. CuZnSOD is the major form found in eukaryotes and is described as being largely a cytosolic enzyme (Fridovich, 1978). The other two forms are MnSOD and FeSOD (Abele-Oeschger, 1996).

Catalase (CAT) is an oxidoreductase enzyme that promotes the degradation of H_2O_2 to H_2O and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

If H_2O_2 is not scavenged in time, it could turn to more dangerous hydroxyl radicals. Hydroxyl radical (OH^-) is the most reactive oxygen species, frequently proposed to initiate lipid peroxidation and DNA damage (Stohs and Bagchi, 1995).

The enzyme Glutathione peroxidase (GPx) is also involved in the detoxification of H_2O_2. However, organic hydroperoxides are the preferred substrate for GPx. GPx catalyses the reduction of hydroperoxides, with the conversion of reduced glutathione (GSH) to glutathione disulfide (GSSG; the oxidized form) (Meister and Anderson, 1983).

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$$

Two different GPx have been identified in animal tissues, Selenium-dependent and Selenium-independent (Aceto *et al.*, 1994).

Glutathione *S*-transferases (GSTs) are a group of multifunctional enzymes that catalyse the conjugation of reduced glutathione with a large variety of electrophilic compounds or cellular components damaged by ROS attack (Pang *et al.*, 2000). Thus GSTs have evolved for cellular protection against a range of xenobiotics and oxidative metabolic byproducts (Boutet *et al.*, 2004).

The flavoenzyme Glutathione reductase (GR) is the enzyme responsible for the reversion of oxidized glutathione (GSSG) to the reduced form GSH. NADPH is used as electron donor in the process (Hasspieler *et al.*, 1994).

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

Thus GR regenerates GSH, which has been converted to the oxidized form (GSSG) by oxidation and thiol-transfer reactions.

Reduced Glutathione is thought to be one of the most important antioxidant agents involved in the protection of cell membranes from lipid peroxidation (Meister, 1989). The cellular tripeptide, glutathione (L-glutamyl cysteinyl glycine) exerts protective antioxidant functions through a complex enzyme system including glutathione peroxidase, glutathione S-transferase and glutathione reductase (de Talamoni *et al.*, 1996; Otto *et al.*, 1997). Reduced glutathione also has a direct antioxidant function by reaction with superoxide, hydroxyl radical and singlet oxygen, leading to the formation of oxidized glutathione and other disulfides (Kosower and Kosower, 1978).

Generally, in normal cells, there exists a delicate balance between the pro-oxidant forces and antioxidant defenses known as the redox balance. When the antioxidant defenses are overwhelmed by pro-oxidants, oxidative stress occurs (Halliwell and Gutteridge, 1986). Excess ROS leads to significant oxidative damage. Lipid peroxidation (LPO) is considered to be a major mechanism, by which oxyradicals can cause tissue damage, leading to impaired cellular function and alterations in physico-chemical properties of cell membranes, which in turn disrupt vital functions (Rikans and Hornbrook, 1997). Polyunsaturated fatty acids are easy targets for ROS driven oxidation owing to their conjugated double bond structures (Storey, 1996). Conjugated dienes are the initial product of free radical attack on lipids and lipid hydroperoxides form an intermediate product. Lipid peroxides decompose and produce a variety of alkanes, alkenes, ketones and aldehydes, the most important of which is malondialdehyde (MDA) (Leibovitz and Siegel, 1980). LPO determination in terms of malondialdehyde (MDA),

the terminal product of lipid peroxidation is the most commonly used criteria of oxidative stress.

A close relationship exists between environmental stress and oxidative stress in an organism as the rate of ROS generation is greatly influenced by environmental conditions (Storey, 1996). Studies carried out on marine species have demonstrated that antioxidants and lipid peroxidation level could serve as potential indicators of oxidative stress (Rodriguez-Ariza *et al.*, 1992; Pedrajas *et al.*, 1995; Aigus *et al.*, 1998; Neves *et al.*, 2000; Downs *et al.*, 2001). Variations in antioxidant enzyme activities induced by various environmental pro-oxidant conditions (i.e., increased ROS generation) such as elevated temperature (Buchner *et al.*, 1996; Abele *et al.*, 1998), hypoxia, hyperoxia (Abele-Oeschger *et al.*, 1994; Abele-Oeschger and Oeschger, 1995), exposure to various types of pollution (Dandapat *et al.*, 1999; Romeo *et al.*, 2000) etc. have been reported. Antioxidant enzymes are also affected by various exogenous and endogenous factors such as age (Viarengo *et al.*, 1991b; Arun and Subramanian, 1998), diet (Peters *et al.*, 1994), seasonality, reproductive cycle (Viarengo *et al.*, 1991a; Ringwood and Conners, 2000), sex etc.

Metals constitute an important set of elements that significantly influence the antioxidant status of aquatic organisms. Metals such as Cu, Mn, Se, and Zn are antioxidant minerals and function primarily in the metalloenzymes (e.g., CuZnSOD, Se-GPx). Expression of antioxidant metalloenzyme activities may be limited by the availability of their trace metal co-factors (Ahmad, 2005). Metals are also powerful pro-oxidants. Cu is both an essential trace element and a potential source of toxicity by multiple mechanisms, including the generation of reactive oxygen species (Livingstone, 2001). The latter can result in various types of oxidative damage, including lipid peroxidation (Viarengo *et al.*, 1987; Ringwood *et al.*, 1998). In the presence of transition metals like Fe and Cu, O_2 and H_2O_2 can generate OH^- through Fenton reaction. Although an antioxidant that can assure protection against free radicals, Zn can produce cellular damage at higher concentrations (Powell, 2000; Ho, 2004).

Regarding the effects of metals, various mechanism of action and contradictory effects have been reported, with some authors reporting their induction (Regoli and Principato, 1995; Canesi *et al.*, 1999) and others inhibition (Tang *et al.*, 1996; Moreira and Guilhermino, 2005). In molluscs, induction of antioxidant enzyme defences and lipid peroxidation has been observed on exposure to metals such as Cu (Livingstone *et al.*, 1990; Livingstone, 2001). Metallothioneins (MT), low molecular weight cysteine rich

proteins that bind metals converting them to non-toxic forms (Viarengo *et al.*, 1997); also have antioxidant properties (Viarengo *et al.*, 1999). The amelioration of cellular stress responses by increasing MT production has been observed in Cu-exposure studies with mussels (Viarengo *et al.*, 1987; Ringwood *et al.*, 1998). Modulatory effects of Cu pre-exposure that afforded protection against endosulfan and deltamethrin has been reported in freshwater fish, *Channa punctatus* (Pandey *et al.*, 2001; Parvez and Raisuddin, 2006).

Very recently, few studies related to antioxidant enzyme activities in crustaceans were reported. Some studies have dealt with the changes during embryogenesis and larval development. A gradual increase of antioxidant enzyme activities during development has been documented in *Macrobrachium malcolmsonii* (Arun and Subramanian, 1998), *M. rosenbergii* (Dandapat *et al.*, 2003) and grass shrimp, *Palaemonetes pugio* (Winston *et al.*, 2004) that provided protection from oxidative assault during larval progression and metamorphosis. Dietary supplementation of antioxidants like vitamin C (Wang *et al.*, 2006), and astaxanthin (Pan *et al.*, 2003) has been proved to enhance the antioxidant defense capability and resistance to ammonia stress in *L. vannamei* and *P.monodon*. Dietary vitamin E could modulate the antioxidant defense system in *M. rosenbergii* (Dandapat *et al.*, 2000). cDNA sequencing of antioxidant enzymes like catalase and GPx in *L. vannamei* has been done (Tavares-Sanchez *et al.*, 2004; Liu *et al.*, 2007). In crustaceans, in addition to GPx and CAT, peroxinectin, a multifunctional protein, containing biological activity of peroxidase, is also proposed to play a critical role in the antioxidant defense by preventing oxidative damage from H_2O_2 (Johansson *et al.*, 1995; Liu *et al.*, 2004). Reoxygenation following hypoxia was found to increase superoxide anion production and decrease the antioxidant capacity in cultured shrimp, *L. vannamei* (Zenteno-Savin *et al.*, 2006). However, there are meagre reports on the effects of metals on the antioxidant defense of crustaceans, especially shrimps.

The generation of highly toxic metabolites is greatly increased in pathological conditions when the flux of ROS exceeds the capability of antioxidant mechanism (McCord, 1988). In humans, increase of lipid peroxidation due to oxidative stress caused by influenza virus infection in the liver tissues and serum (Brown *et al.*, 1982) and in patients with chronic hepatitis and hepatitis C was reported (Suematsu *et al.*, 1977; Higueras *et al.*, 1994). ROS may be generated in crustaceans in response to invading microorganisms like viruses, bacteria and fungi (Bachere *et al.*, 1995; Munoz *et al.*, 2000). Although, microbicidal ROS is generated inside the phagocytic vacuoles during pathogenic invasion, an important quantity crosses into the extravacuolar and extracellular environment and may cause damage to the cells (Warner, 1994). However,

there are few data on the relation of antioxidant systems to microbial infection especially in invertebrates. With regard to WSSV infection in shrimps, the virus has been causing havoc by producing devastating epidemics since 1988 (Primavera, 1997). Even then, the exact pathophysiologic and biochemical alterations occurring in infection are still not clear. The increased oxidative stress generated during infection might play an important role in the pathogenesis of WSSV infection.

In the previous study, pre-exposure to Cu and Zn were proved to have immunostimulatory and suppressive effects on the immunocompetence of *P. monodon* to WSSV infection at lower and higher doses respectively. In view of the significant role of metals in modifying the antioxidant status of an organism, an attempt was made in the present study to investigate the modulatory effects induced by ambient Cu and Zn on the antioxidant defense of shrimps, especially after WSSV infection. Since the antioxidant defense system of hepatopancreas and gills is known to be highly responsive, these tissues were also chosen as target organs for the analysis of antioxidant potential in addition to haemolymph. Hepatopancreas is the primary organ that deals with insults to organism from environmental alterations such as pollutants, toxins and metals (Livingstone *et al.*, 1992) and gills experience direct exposure to environmental alterations (Cheung *et al.*, 2001).

In brief, the present study on *P. monodon* aimed at determining the potential:

- Effects of Cu and Zn-exposure on the extent of lipid peroxidation
- Effects of Cu and Zn-exposure on the antioxidant defense against WSSV infection.

6.2 Materials and methods

6.2.1 Experimental animals

Shrimps exposed to nominal doses of Cu (0.05, 0.1, 0.2 and 0.3 mg l^{-1}) and Zn (0.5, 1.0, 1.5 and 2.5 mg l^{-1}) and challenged with WSSV after 14 days (Refer section 4.2 and 5.2) were examined for the lipid peroxidation level and antioxidant enzyme activities. The treated shrimps (n=6) were sampled after 14 days of metal exposure (PMD14), on post challenge day 2 (PCD2) and post challenge day 5 (PCD5).

6.2.2 Haemolymph extraction and tissue separation

The procedure adopted for haemolymph extraction has been described previously (Refer section 2.2.6). For separating the tissues, the shrimps were dissected after extracting the haemolymph. Hepatopancreas and gills were then carefully removed. The separated tissues were rinsed in ice-cold saline, weighed and suspended in Tris-HCl (0.1 M, pH 7.4) and stored at –20°C. Prior to analysis, the tissues suspended in Tris-HCl buffer were homogenized using a glass homogeniser and centrifuged at 8000 rpm for 10 minutes at 4°C. The resultant supernatants were used for the assays.

6.2.3 Analysis of lipid peroxidation and antioxidants

The following parameters in haemolymph and tissue homogenates (supernatants) were analysed:

- Malondialdehyde (MDA)
- Superoxide dismutase (SOD)
- Catalase (CAT)
- Glutathione peroxidase (GPx)
- Glutathione *S*-transferase (GST)
- Glutathione reductase (GR)
- Total reduced glutathione (GSH)

The antioxidant enzyme activities being expressed in units mg^{-1} protein; total protein was determined according to the method of Bradford (1976) (Refer section 2.2.7a.1).

6.2.3.1 Malondialdehyde (MDA)

The extent of lipid peroxidation was evaluated by determining the MDA concentration. The thiobarbituric acid assay method of Neihaus and Samuelson (1968) was employed for the estimation of MDA.

Reagents

1. 15% TCA
2. 0.375% TBA in 0.25 N HCl.
3. 0.25 N HCl.
4. TCA-TBA-HCl mixture- mixed 15% TCA, 0.375% TBA and 0.25 N HCl in the ratio 1:1:1.

Assay

A sample of 1.0 ml was taken in a tube and 2 ml of TCA-TBA-HCl mixture was added and mixed thoroughly. The contents were heated in a boiling water bath for 15 minutes. After cooling, the flocculated precipitate was removed by centrifugation at 1000 g for 10 minutes. Absorbance of the pink-coloured supernatant was read at 535 nm in a

U-V Visible Spectrophotometer (Hitachi, U 2001). The concentration of MDA was calculated using the molar extinction co-efficient of 1.56×10^5 μ^{-1} cm^{-1} and expressed as n M ml^{-1} haemolymph and mM $100g^{-1}$ wet tissue.

6.2.3.2 Superoxide dismutase activity

SOD activity was determined according to the method of Kakkar *et al.* (1984).

Reagents

1. 0.052 M Sodium pyrophosphate buffer, pH 8.3
2. 186 µM Phenazine methosulphate (PMS).
3. 300 µM Nitroblue Tetrazolium salt (NBT).
4. 78 µM NADH (Na_2).
5. n-Butanol.

Assay

A sample of 100 µl was added to the tubes containing 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate and 0.3 ml of NBT. A control that contained 0.1 ml of distilled water instead of the enzyme source was also taken. 1.3 ml of distilled water was also added into all the tubes and kept at 30°C in a water bath. After 1 minute, 0.2 ml of NADH was added and incubated for 90 seconds. Then 1 ml of glacial acetic acid was added, stirred and added 4 ml of n-butanol. Shaken well and allowed to stand for 10 minutes and centrifuged at 1000 rpm for 5 minutes. Chromogen in the upper butanol layer was measured at 560 nm in a U-V Visible Spectrophotometer against n-butanol as blank. The specific activity was expressed as units mg^{-1} protein and one unit is defined as the enzyme concentration required to inhibit formazan formation by 50% in one minute under the assay conditions.

6.2.3.3 Catalase activity

Catalase activity in different tissues was determined using the method of Maehly and Chance (1955).

Reagents

1. 0.01 M Phosphate buffer, pH 7.0.
2. 30 mM H_2O_2 in phosphate buffer.

Assay

A sample of 20 µl was added to 3 ml of buffered H_2O_2 and the decrease in absorbance at 230 nm was monitored every 30 seconds up to 3 minutes in a U-V Visible Spectrophotometer. The specific activity was expressed as International units (IU) mg^{-1} protein. 1 IU is defined as the change in absorbance $minute^{-1}$ extinction $co\text{-}efficient^{-1}$ (0.021).

6.2.3.4 Glutathione peroxidase activity

GPx activity was assayed according to the method of Rotruck *et al.* (1973). The technique was adapted to a microplate method enabling the absorbance to be read using a microplate reader.

Reagents

1. 0.4 M Tris buffer, pH 7.0.
2. 10 mM Sodium azide solution.
3. 10% TCA.
4. 0.4 mM EDTA.
5. 0.2 mM H_2O_2 solution.
6. 2 mM GSH.
7. 0.4 M Na_2HPO_4.
8. DTNB reagent- 40 mg of DTNB dissolved in 100 ml of 1% trisodium citrate.

Assay

A sample of 0.5 ml was added to tubes containing 0.2 ml of tris buffer, 0.2 ml of EDTA and 0.1 ml of sodium azide and mixed well. To that mixture was added 0.2 ml of GSH followed by 0.1 ml of H_2O_2 solution. The contents were mixed and incubated at 37°C for 10 minutes. A control containing all reagents except the enzyme source was also run. Reaction was arrested after 10 minutes by the addition of 0.5 ml of TCA. The tubes were centrifuged and the supernatants were assayed for GSH. To 100 µl of supernatant taken in microplate wells was added 100 µl of Na_2HPO_4 and 50 µl of DTNB reagent and the absorbance at 412 nm was recorded on a microplate reader (Tecan Infinite M-200). The activity was expressed as µg of GSH oxidized $minute^{-1}$ mg^{-1} protein.

6.2.3.5 Glutathione S-transferase activity

GST was determined by the method of Beutler (1986) adapted to a microplate method.

Reagents

1. 0.5 M Phosphate buffer, pH 6.5.
2. 30 mM CDNB in 95% ethanol.
3. 30 mM GSH.

Assay

A sample of 0.1 ml was taken in a tube and 1 ml of buffer, 1.7 ml of water and 0.1 ml of CDNB was added. The mixture was incubated for 5 minutes at 37°C. 180 µl of the incubated mixture was taken into microplate wells and 20 µl of GSH was added. The increase in O.D. at 340 nm was recorded every 30 seconds up to 5 minutes on a

microplate reader. The enzyme activity was calculated using the extinction co-efficient (9.6, the difference in the millimolar extinction co-efficient between CDNB-GSH conjugate and CDNB) and expressed as μmoles of CDNB-GSH conjugate formed $minute^{-1}$ mg^{-1} protein.

6.2.3.6 Glutathione reductase activity

GR activity was assayed according to the method of Bergmeyer (1974).

Reagents

1. 0.067 M K_2HPO_4 buffer, pH-6.6.
2. 15 mm EDTA
3. 0.06% NADPH
4. 1.15% Oxidized glutathione (GSSG).

Assay

A sample of 0.1 ml was added to the reaction mixture containing 1.6 ml of K_2HPO_4, 0.1 ml of EDTA, 0.12 ml of NADPH and 0.12 ml of GSSH and mixed well. The decrease in absorbance at 340 nm was recorded every 30 seconds for 3-5 minutes in a U-V Visible spectrophotometer. The enzyme activity was calculated using the molar extinction co-efficient (6.22×10^3) and expressed as μmoles of NADPH oxidized $min.^{-1}$ mg^{-1} protein.

6.2.3.7 Total reduced glutathione

GSH was determined employing the method of Ellman (1959), adapted to a microplate method.

Reagents

1. Metaphosphoric acid- 1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl dissolved in 100 ml of distilled water.
2. 0.4 M Na_2HPO_4.
3. DTNB reagent- 40 mg DTNB dissolved in 100 ml of 1% trisodium citrate.
4. GSH- 2 mg dissolved in 10 ml of distilled water.

Assay

A sample of 0.25 ml was mixed with 1 ml of metaphosphoric acid and centrifuged. 100 μl of supernatant was taken in microplate wells and 100 μl of Na_2HPO_4 was added followed by 50 μl of DTNB reagent. The absorbance at 412 nm was recorded on a microplate reader. A set of standards were also treated in the same manner and the amount of glutathione determined was expressed as μg mg^{-1} protein.

6.2.4 Statistical analysis

The experimental data was analysed by means of two-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means. Significance level for the analysis was set to $P<0.05$. Statistical analyses were carried out using the software SPSS 10.0.

6.3 Results

6.3a Effects of copper exposure

Significant variations could be observed in the antioxidant profiles of Cu-exposed shrimps, compared to the unexposed. Cu at 0.05 and 0.1 mg l^{-1} significantly enhanced the antioxidants in haemolymph and gills. With Cu at 0.1 mg l^{-1}, enhancement was also seen in hepatopancreas. Alternatively, Cu at 0.2 and 0.3 mg l^{-1} greatly inhibited the shrimp antioxidants except SOD. A corresponding increase was observed in MDA levels in shrimps with Cu at 0.2 and 0.3 mg l^{-1}. Antioxidant activity significantly declined on WSSV challenge in both unexposed and Cu-exposed shrimps. Minimum activity was observed after 48 h in most cases and activity tend to increase on PCD5. Shrimps with Cu at 0.1 mg l^{-1} that showed lower MDA levels after challenge showed higher antioxidant activity, particularly on PCD5. Shrimps with Cu at 0.2 and 0.3 mg l^{-1} that showed higher MDA levels post challenge, showed lower antioxidant activity especially in hepatopancreas (Fig.6.1- 6.7; Appendix table 6.1 - 6.7).

Malondialdehyde

The extent of lipid peroxidation was significantly higher in haemolymph as well as in hepatopancreas and gills of shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} ($P<0.05$). Following challenge, MDA levels tend to increase in all tissues in general with a maximum in shrimps with Cu at 0.2 and 0.3 mg l^{-1}. Shrimps with Cu at 0.1 mg l^{-1} showed significantly lower MDA levels on PCD5 in all tissues and a significant increase after challenge was noted only in hepatopancreas ($P<0.05$). (Fig. 6. 1, Appendix table 6.1).

Superoxide dismutase activity

Exposure to Cu induced an elevation of SOD activity in shrimp haemolymph, with a maximum at 0.3 mg l^{-1} ($P<0.05$). However, a decrease was noted in hepatopancreas and gills with Cu at 0.3 mg l^{-1}. SOD activity in haemolymph after 48 h of challenge was least in shrimps with Cu at 0.2 and 0.3 mg l^{-1} and maximum with 0.1 mgl^{-1} ($P<0.05$). Compared to the unexposed, an increase was noted with Cu at 0.05 mg l^{-1} in

haemolymph also. In hepatopancreas and gills, shrimps exposed to Cu at 0.1 mg l^{-1} and the unexposed showed higher activities on PCD5. (Fig. 6. 2, Appendix table 6.2).

Catalase activity

CAT activity significantly increased in the haemolymph of shrimps exposed to Cu at 0.05 and 0.1 mg l^{-1} ($P<0.05$). Increase was also noted in the gills with Cu at 0.05 and 0.1 mg l^{-1}. Shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} markedly reduced their CAT activity in hepatopancreas and gills ($P<0.05$). In general, the activity significantly reduced in all tissues 48 h post challenge. In hepatopancreas and gills, shrimps with Cu at 0.1 mg l^{-1} showed higher activity. Comparatively reduced activity was observed in shrimps with Cu at 0.2 and 0.3 mg l^{-1} in all tissues on PCD5. (Fig. 6. 3, Appendix table 6.3).

Glutathione peroxidase activity

GPx activity of shrimps reduced in all tissues on exposure to Cu at 0.2 and 0.3 mg l^{-1} ($P<0.05$), with a highly pronounced reduction in hepatopancreas and gills. GPx activity of shrimps with Cu at 0.05 and 0.1 mg l^{-1} was found to increase in the gills. Following challenge, the activity significantly reduced in all groups and in all tissues examined ($P<0.05$). However, the activity increased in haemolymph on PCD5 in shrimps exposed to Cu at 0.1 mg l^{-1} and unexposed, with the highest at 0.1 mg l^{-1}. In shrimps with Cu at 0.2 and 0.3 mg l^{-1}, GPx activity declined to a greater degree in hepatopancreas on post challenge days. (Fig. 6. 4, Appendix table 6.4).

Glutathione S-transferase activity

Exposure to Cu at 0.2 and 0.3 mg l^{-1} induced a significant reduction in the GST activity of shrimps in all tissues ($P<0.05$). A slight decrease was also noted in shrimps with Cu at 0.1 mg l^{-1} in haemolymph whereas, in gills and hepatopancreas, the activity was found to increase. The activity increased with Cu at 0.05 mg l^{-1} in haemolymph and gills. Following challenge, the activity mostly decreased but significantly higher activity was exhibited by shrimps exposed to Cu at 0.1 mg l^{-1} in hepatopancreas on PCD2 and in all tissues on PCD5. (Fig. 6. 5, Appendix table 6.5).

Glutathione reductase activity

GR activity significantly increased in shrimps exposed to Cu at 0.1 mg l^{-1} and reduced with Cu at 0.3 mg l^{-1} in all tissues ($P<0.05$). An increase was noted with Cu at 0.05 mg l^{-1} in haemolymph and gills. The reduction in hepatopancreas and gills was also seen with Cu at 0.2 mg l^{-1}. Shrimps with Cu at 0.3 mg l^{-1} showed minimum activity on

post challenge days in all tissues and those with Cu at 0.2 mg l^{-1} showed lower activity on PCD5. Shrimps exposed to Cu at 0.1 mg l^{-1} showed better activity in gills, on PCD2 in haemolymph and on PCD5 in hepatopancreas ($P<0.05$). An enhancement was also seen in shrimps with Cu at 0.05 mg l^{-1} in hepatopancreas on PCD2 and gills on PCD5. (Fig. 6. 6, Appendix table 6.6).

Total reduced glutathione

Significantly lower GSH levels were observed in shrimps exposed to Cu at 0.2 and 0.3 mg l^{-1} in all tissues ($P<0.05$). Significantly elevated GSH levels were observed in hepatopancreas and gills with Cu at 0.1 mg l^{-1}. Increase was also noted with Cu at 0.05 mg l^{-1} in gills. GSH levels were found to reduce significantly after WSSV challenge ($P<0.05$). On PCD5, shrimps with Cu at 0.1 mg l^{-1} showed elevated GSH levels in all tissues and comparatively lower levels were recorded in shrimps with Cu at 0.3 mg l^{-1}. (Fig. 6. 7, Appendix table 6.7).

6.3b Effects of zinc exposure

Zn at 1.5 and 2.5 mg l^{-1} greatly inhibited the shrimp antioxidants. A corresponding increase in MDA was observed with Zn at 1.5 and 2.5 mg l^{-1}. Enhancement in some of the antioxidants was noticed in shrimps with Zn at 1.0 mg l^{-1}, which showed slightly lower levels of MDA, and to a lesser extent in shrimps with 0.5 mg l^{-1}. Antioxidant activity declined upon WSSV-challenge, similar to the trend observed in unexposed; with a few exceptions. Shrimps with Zn at 1.0 mg l^{-1} and to a lesser extent by those with 0.5 mg l^{-1} exhibited higher activity following challenge particularly in gills and hepatopancreas. A decrease in the MDA was noted in shrimps with Zn at 0.5 and 1.0 mg l^{-1} post challenge, with a minimum at 1.0 mg l^{-1}. Shrimps with higher doses that showed higher MDA levels on infection, showed lower activity with most of the antioxidants, particularly in gills and hepatopancreas.

Malondialdehyde

MDA levels significantly increased on exposure to Zn at 1.5 and 2.5 mg l^{-1} in haemolymph and other tissues examined ($P<0.05$). Compared to the unexposed, a significant decrease was observed in shrimps exposed to Zn at 1.0 mg l^{-1} in haemolymph and gills. In gills, a slight decrease was noted with Zn at 0.5 mg l^{-1} also. Generally, shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1} showed a higher extent of lipid peroxidation on post challenge days in all tissues. Significantly lower levels were observed in shrimps with Zn at 1.0 and 0.5 mg l^{-1} in haemolymph and gills, with a minimum at 1.0 mg l^{-1} ($P<0.05$). (Fig. 6. 8, Appendix table 6.8).

Superoxide dismutase activity

Zn at 1.5 and 2.5 mg l^{-1} reduced the SOD activity in all tissues ($P<0.05$). Whereas, Zn at 1.0 mg l^{-1} increased the activity in haemolymph and hepatopancreas. In hepatopancreas, an increase was noted with 0.05 mg l^{-1} also. Shrimps with Zn at 1.0 mgl^{-1} showed higher SOD activity on post challenge days in all tissues in general. Compared to the unexposed, an increase was also noted with Zn at 0.5 mg l^{-1}. Shrimps with Zn at 2.5 and 1.5 mg l^{-1} showed the least activity in hepatopancreas and gills respectively ($P<0.05$). (Fig. 6. 9, Appendix table 6.9).

Catalase activity

CAT activity significantly reduced in all tissues on exposure to Zn at 1.5 and 2.5 mg l^{-1}, with maximum reduction in gills ($P<0.05$). In hepatopancreas, an increase was noted in shrimps with Zn at 1.0 mg l^{-1}. CAT activity significantly reduced in haemolymph in all groups after challenge ($P<0.05$). In hepatopancreas and gills, the activity following challenge was least in shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1} and maximum at 1.0 mg l^{-1}. Shrimps with Zn at 0.5 mg l^{-1} also showed higher CAT activity on PCD2 in hepatopancreas and gills. (Fig. 6. 10, Appendix table 6.10).

Glutathione peroxidase activity

Shrimps on exposure to Zn at 1.5 and 2.5 mg l^{-1} reduced their GPx activity in haemolymph ($P<0.05$) and to a greater degree in gills. In shrmps with Zn at 2.5 mg l^{-1}, GPx activity reduced in hepatopancreas also. An increase in the activity was noted in shrimps exposed to Zn at 1.0 mg l^{-1} in haemolymph ($P<0.05$). Following challenge, shrimps exposed to Zn at 1.0 mg l^{-1} showed the highest GPx activity in all tissues examined in general. In hepatopancreas and gills, an increase was also noted with Zn at 0.5 mg l^{-1} on PCD5. (Fig. 6. 11, Appendix table 6.11).

Glutathione S-transferase activity

Exposure of shrimps to Zn at 1.5 and 2.5 mg l^{-1} significantly lowered the GST activity in all tissues ($P<0.05$). Although a slight reduction was noted in shrimps with Zn at 1.0 mg l^{-1} in haemolymph and gills, the activity was found to increase in hepatopancreas. Shrimps with Zn at 1.5 and 2.5 mg l^{-1} showed the least activity after challenge in all tissues except on PCD2 in haemolymph ($P<0.05$). Shrimps exposed to Zn at 1.0 mg l^{-1} showed the highest activity in gills on post challenge days. (Fig. 6. 12, Appendix table 6.12).

Glutathione reductase activity

GR activity of haemolymph increased in shrimps exposed to Zn at 1.0 mg l^{-1}. A reduction was observed in shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1} in all tissues (P<0.05). In gills, a reduction was also noted in shrimps with Zn at 0.5 and 1.0 mg l^{-1}. GR activity in haemolymph on PCD2 was significantly lower in Zn-exposed groups (P<0.05). Shrimps exposed to Zn at 1.0 mg l^{-1} showed the highest activity on post challenge days in gills and on PCD5 in haemolymph and hepatopancreas when an enhancement was also seen with Zn at 0.5 mg l^{-1}. (Fig. 6. 13, Appendix table 6.13).

Total reduced glutathione

GSH levels significantly reduced in shrimp haemolymph and to a greater degree in gills on exposure to Zn at 1.5 and 2.5 mg l^{-1}. With 0.5 and 1.0 mg l^{-1}, GSH levels were found to increase significantly in hepatopancreas and gills (P<0.05). Shrimps exposed to Zn at 0.5 and 1.0 mg l^{-1} showed higher GSH levels on post challenge days in hepatopancreas and on PCD2 in gills (P<0.05). Shrimps exposed to Zn at 1.5 and 2.5 mg l^{-1} showed the least levels except on PCD2 in haemolymph. (Fig. 6. 14, Appendix table 6.14).

6.4 Discussion

The present investigation has shown that Cu and Zn could modify the antioxidant activity in haemolymph and other tissues (hepatopancreas and gills) of *P. monodon*. An enhancement in antioxidants could be observed with lower doses and an inhibition was seen at higher doses. A greater degree of enhancement in the antioxidant activity could be observed in shrimps exposed to Cu at 0.1 mg l^{-1} and Zn at 1.0 mg l^{-1}. To a lesser extent, enhancement was also seen in shrimps with Cu at 0.05 mg l^{-1} and Zn at 0.5 mg l^{-1}. An induction in antioxidant defenses, preventing cell damage could be regarded as an adaptive response to the altered environment; in contrast, an inhibition could mean cell damage and toxicity (Vassuer and Cossu-Leguille, 2003).

According to Bailey (1995), when tissue antioxidant system is affected, the rate of free radical formation exceeds the rate of their removal and oxidative deterioration of cell membrane such as lipid peroxidation occurs. A corresponding increase in the extent of lipid peroxidation could be observed in shrimps exposed to higher doses of Cu and Zn, which showed reduced antioxidant activities. Contradictory effects are also reported. Higher levels of antioxidant activities and lipid peroxidation were found in Cu-exposed freshwater cladoceran, *Daphnia magna* (Barata *et al.*, 2005). Exposure to lower doses of

Cu and Zn did not induce lipid peroxidation in any of the tissues examined. A previous study on Cu pre-exposure at 10 ppb reported no significant changes in lipid peroxidation levels in freshwater fish, *Channa punctata* (Parvez and Raisuddin, 2006). In the present study, a notable reduction in lipid peroxidation level in shrimps exposed to Zn at 1.0 mg l^{-1} was also observed, which showed that tissue damage, was considerably reduced. Since lipid peroxidation is mediated through radical chain propagation, a significant decrease in the levels could be attributed to metal and free radical interaction.

SOD that converts highly toxic superoxide anions to H_2O_2 is one of the main antioxidant defense enzymes. Increase in SOD activity was observed in shrimps exposed to lower doses of both Cu (0.05 and 0.1 mg l^{-1}) and Zn (1.0 mg l^{-1}) in haemolymph. A simultaneous activation of CAT, that decomposes H_2O_2 very efficiently, occurred in shrimps exposed to Cu. In shrimps with Zn at 1.0 mg l^{-1}, GPx activity that consumes GSH increased to detoxify H_2O_2. GSH gets oxidized upon reaction with GPx and must be recycled by the NADPH-consuming enzyme, GR (Hasspieler *et al.*, 1994). Increase in GR activity that assisted in the regeneration of GSH occurred in shrimps with Cu and Zn. The increased GR activity is hinting at the increased levels of GSSG. The increase in GSSG could be due to an increase in GPx activity or GST activity that is involved in the removal of toxic metabolites by glutathione conjugation reactions. The direct scavenging action of GSH may also lead to an increased GSSG (Kosower and Kosower, 1978). Increase in GST activity was observed in shrimps with Cu at 0.05 mg l^{-1}. In shrimps with Cu at 0.1 mg l^{-1}, the direct scavenging action of GSH probably led to the activation of GR. Similar enhancement in antioxidants has been reported in shrimps on administration of immunostimulants. GPx activity in haemocytes of *L. vannamei* was significantly increased after administration of sodium alginate (Cheng *et al.*, 2005). Immunostimulation with ß-glucan and sulfated polysaccharide generated an increase in SOD activity in *L. vannamei* (Campa-Cordova *et al.*, 2002).

In hepatopancreas, an increase in SOD activity was accompanied by an increase in CAT activity in shrimps with Zn at 1.0 mg l^{-1}. With Cu, neither SOD nor CAT activity was altered. Increase in GR activity was observed in shrimps with Cu at 0.1 mg l^{-1}, probably to replenish the GSH used up during the increased activity of GST. However, in shrimps with Zn at 1.0 mg l^{-1}, even though GST activity increased, simultaneous increase in GR activity was not found. Even then, the GSH levels were significantly higher with Zn at 1.0 mg l^{-1}, which is an indication of the increased generation of GSH in hepatopancreas. Such an increase of GSH in hepatopancreas was also observed in shrimps with Zn at 0.5 mg l^{-1} and Cu at 0.1 mg l^{-1}. Previous studies on fish Cu exposure

have shown the ability of GSH to inhibit free radical formation through its ability to stabilize Cu in its oxidative state, preventing redox cycling and free radical generation (Pandey *et al.*, 2001; Parvez *et al.*, 2003). For a complete understanding, further studies concerning the activities of the enzymes involved in GSH synthesis (γ-glutamylcysteine synthetase and GSH synthetase) and GSH transport should be performed (γ -glutamyl transpepetidase). Similar increase in GSH content and GST activity were observed in the liver of cyprinid fish, *Brachydanio rerio* on exposure to sublethal concentrations of copper sulfate for 14 days (Paris-Palacios *et al.*, 2000). Exposure to polyphenolic compounds could also induce a similar enhancement of antioxidative capacity in the digestive gland cells of *Unio tumidus* (Labieniec and Gabryelak, 2007).

In gills, all antioxidants were activated in shrimps with lower doses of Cu. Increase in all the antioxidants ensures better defense, as they can act jointly, in concert with each other to destroy the harmful ROS effectively. Gills are considered to be the major entry site of metals. They also act as transient stores of metals (Soegianto *et al.*, 1999). This might have been the reason for increased sensitivity of antioxidants in gills with Cu. However, it may be noted that, in shrimps with Zn such a response was not seen. On the other hand, with lower doses of Zn, some of the antioxidants were slightly reduced (SOD, CAT, GST and GR at 1.0 mg l^{-1}; CAT and GR at 0.5 mg l^{-1}) and only GSH were increased (GST also at 0.5 mg l^{-1}). Lipid peroxidation level in the gills was however, slightly lower than the unexposed in shrimps with Zn at 0.5 and 1.0 mg l^{-1}. Most probably, Zn provided some protection against ROS, leading to a lesser induction of antioxidant enzyme activity. Zn is known to be a powerful antioxidant involved in the sequestration of free radicals (Hendy *et al.*, 2001). A similar decrease in the antioxidant enzyme activities was noted in characins (ornamental fish), *Hyphessobrycon callistus* fed pigmented diet, owing to the antioxidant capacity of the supplemented carotenoids (Wang *et al.*, 2006). A slight decrease at lower doses was also noted with some of the antioxidants in hepatopancreas and haemolymph with both Cu and Zn, especially with Cu at 0.05 mg l^{-1} and Zn at 0.5 mg l^{-1}. However, the MDA levels being same as that of the unexposed, it may be assumed that the stimulated antioxidant activities compensated for the effects of mild inhibition.

Differences may be noted in the types of antioxidants that were enhanced with lower doses of Cu and Zn. Even in case of the same metal, variations could be noticed with the different tissues examined. The scavenging enzymes, SOD and CAT were activated in haemolymph of shrimps with Cu. Activation of almost all the enzymes probably provided better protection against oxidative stress in gills of shrimps with Cu.

The increased GR activity in all tissues ensured the regeneration of used up GSH in shrimps with Cu. In shrimps with Zn at 1.0 mg l^{-1}, the scavenging enzymes, SOD and GPx were stimulated in haemolymph whereas, in hepatopancreas, SOD and CAT were stimulated. In addition, GSH-related enzymes, GR was stimulated in haemolymph and GST was stimulated in hepatopancreas. In gills, only the GSH levels were increased with Zn at 1.0 mg l^{-1}. Activation was also seen in shrimps with Zn at 0.5 mg l^{-1}. It may be noted that SOD was the common enzyme stimulated with lower doses of both Cu (0.05 and 0.1 mg l^{-1}) and Zn (1.0 mg l^{-1}) in haemolymph. This is certainly an indication of a slight increase in the generation of free radicals on metal exposure. In hepatopancreas and gills, GSH was the common antioxidant that increased with both Cu (1.0 mg l^{-1} and only in gills with 0.5 mg l^{-1}) and Zn (0.5 and 1.0 mg l^{-1}). Increase in GSH, anticipatory of oxidative stress probably helped to maintain the redox balance. Hence, it may be concluded that measurement of a single antioxidant is not enough to define a complete antioxidant defense under experimental or natural conditions.

Inhibition of the antioxidants at higher doses was clearly evident with both the metals under study. All the antioxidants in hepatopancreas and gills were severely affected in shrimps with higher doses of Cu. A few of them in haemolymph (GSH, GPx, GST) were also affected in shrimps with Cu at 0.2 and 0.3 mg l^{-1}. With higher doses of Zn, the antioxidants in haemolymph and gills were more affected. SOD, CAT, GST and GR activity of hepatopancreas were also inhibited in shrimps with Zn at 1.5 and 2.5 mgl^{-1} Zn. It may be noted that gills were the common organ affected with higher doses of both Cu and Zn. Gills may hence be considered as a target organ for the metals as the antioxidants in gills were highly sensitive. Decrease in SOD could be related to an increased generation of ROS. Singlet oxygen and hydroxyl radicals are reported to inactivate SOD with resultant loss of enzymatic activity (Escobar *et al.*, 1996). SOD contains arginine and histidine residues at its active site (Mallinowski and Fridovich, 1979) and free radicals may attack these highly reactive amino acids resulting in chemical modification of the protein structure and loss of enzyme activity. GPx activity has been reported to be inactivated with increased levels of H_2O_2, which in turn is due to the lower activity of CAT (Searle and Wilson, 1980). Previous authors have reported CAT inhibition to be due to superoxide anion (Kono and Fridovich, 1982; Romeo *et al.*, 2000). Reduced availability of GSH probably reduced the activity of GST. Metals such as Cd, Fe, Cu and Pb at LC_{50} levels and under acute pressure were able to decrease GPx activity and GSH levels in *P. perna* (Almeida *et al.*, 2004; Dafre *et al.*, 2004). GSH depletion was also observed in the bivalves *Unio tumidus* and *M. galloprovincialis* exposed to Cu (Doyotte *et al.*, 1997; Canesi *et al.*, 1998). The decreased activity of GSH,

GPx and GST led to a reduction in the level of GSSG that acts as the substrate for GR. The decreased availability of GSSG caused a decline in the activity of GR, which in turn resulted in GSH depletion. Compensatory synthesis of GSH neither seemed to occur. It is quite clear from the results that an equilibrium between the free radical scavenging enzymes that form the first line of defense against oxidative injury is important for the effective removal of oxygen stress (Andrew and Mathew, 1989). Similar decrease in antioxidant enzyme activities (SOD, CAT and GPx) has been reported in white shrimp, *L. vannamei* exposed to increasing levels of nitrite (Wang *et al.*, 2006).

Inhibition in majority of the antioxidant enzymes led to a greater oxidative stress in shrimps with higher doses of Cu and Zn. An increase in the extent of lipid peroxidation was evident from the higher MDA levels with both Cu and Zn. A similar increase in the level of MDA and a decrease in the concentration of glutathione were reported in the gills and digestive gland of mussel, *Mytilus galloprovincialis* exposed to Cu^{2+} or Zn^{2+} at the rate of 40 $\mu g\ l^{-1}animal^{-1}$ for six days (Viarengo *et al.*, 1990). Increases in oxidative damage were also seen with fishes for single and mixed contaminants, including Cu, Fe, Cd, PAHs and PCBs (Livingstone, 2001). Although stimulation in SOD was noted with higher doses of Cu in haemolymph, a simulataneous increase in CAT or GPx activity was not found. Reduction in the activities of antiperoxidative enzymes involved in the conversion of H_2O_2 to water, may lead to increased generation of H_2O_2 that could react with Fe^{2+} to form harmful hydroxyl radicals, which in turn could aggravate the oxidative stress. According to Charissou *et al.* (2004), oxidative stress leading to lipid peroxidation accompanied an increase in oxidative DNA damage. The 8-oxodGuo is a ubiquitous DNA oxidation product generated by ROS (Cadet *et al.*, 1997). Further studies may be conducted on 8-oxodGuo to determine the extent of DNA damage that could have occurred in metal-exposed shrimps.

Two probable mechanisms may be suggested by which the metals, Cu and Zn caused a modulation of the shrimp antioxidant activity. One is through direct action of metal on the enzyme, stimulating or inhibiting at a lower and higher dose respectively. Downregulation or upregulation of the corresponding genes may have also occurred. Direct metal-mediated structural alteration of the enzyme leading to the depression of enzymes has been suggested earlier (Palace *et al.*, 1992). In particular, metals were shown to have high affinity for –SH groups that might result in irreversible inhibition of several enzymes (Cousins, 1985; Jannaschik *et al.*, 1999). Variations in antioxidant activity could also be attributed to an indirect cause i.e., through an increase in the generation of reactive oxygen species. It is well known that metals can enhance the

production of ROS in living organisms (Stohs and Bagchi, 1995). As the reactive oxygen species increases, the natural tendency of the body is to raise the antioxidant activity. However, it may be noted that such an increase in the antioxidant activity was seen only in shrimps exposed to lower doses of the metals. Since there was no evidence of increased lipid peroxidation at these doses, it may be assumed that the increase in ROS was only slight and the compensatory response induced was adequate to stave off oxidative damage. Quantification of superoxide anions in shrimp haemolymph showed no significant difference on exposure to lower doses of Cu and Zn (Refer fig.4.9 and 5.9). Hence, it may be suggested that a slight increase in the generation of ROS under the influence of lower doses of Cu and Zn led to antioxidant stimulation. Alternatively, excess generation of ROS under the influence of higher doses of metals led to the inhibition of antioxidants. In other words, increase in oxidative stress led to the decrease and exhaustion of antioxidant defense system at higher doses. According to Gallego *et al.* (2007), the nature and concentration of metal are important factors in reactive oxygen species generation. A significant increase in superoxide anion production in haemolymph could be observed in shrimps with Cu at 0.3 mg l^{-1} and Zn at 1.5 and 2.5 mg l^{-1} (Refer fig.4.9 and 5.9). From the data on lipid peroxidation level, it is clearly understood that in shrimps exposed to higher doses, the pro-oxidants are exceeding the capacity of antioxidants. An inhibition in the activity of antioxidants as well as an excess generation of ROS would have been responsible for the higher MDA levels seen in shrimps with higher doses.

Following WSSV infection, a general decrease in antioxidant activity was seen in both the unexposed and metal exposed *P. monodon* despite the increased requirement of antioxidant defenses to counteract the increased generation of free radicals. A minimum antioxidant activity was observed after 48 h in most cases in the present study. According to Schwartz (1996), viruses can affect the host cell pro-oxidant/antioxidant balance by inhibiting the synthesis of antioxidant enzymes. A concomitant increase in lipid peroxidation level was also observed in most cases after WSSV infection. Failure of the antioxidant system to counteract the harmful free radicals generated might have caused the tissue damage. Lipid peroxides are themselves free radicals with large reaction constants and can therefore lead to cell death (Kidd, 1991). The high concentration of peroxides, coupled with the failure of antioxidant system must have contributed to the damage caused by viral infection. These results are in agreement with that of the previous workers who have reported a similar reduction in antioxidant enzyme activity and an increase in lipid peroxidation level induced by WSSV infection in haemolymph and other tissues of *P. monodon* (Rameshthangam and Ramasamy, 2006; Mathew *et al.*, 2007) and

F. indicus (Mohankumar and Ramasamy, 2006). But in contrast to the progressive decrease in antioxidant activity observed in their study, a tendency for improvement was observed on PCD5 in the present study. This difference noticed might be due to variations in the degree of viral infection that occurred in each case. It may be noted that Mathew *et al.* (2007) could observe an increase in CAT activity after 24 h of infection before the activity declined. An increase in antioxidant enzyme activities, SOD and GPx, following infection by *V. alginolyticus* was reported in *L. vannamei* (Liu *et al.*, 2007).

Compared to the unexposed, a comparative increase in antioxidant activity and a reduction in lipid peroxidation level could be observed in shrimps exposed to lower doses of Cu and Zn, especially with Cu at 0.1 mg l^{-1} and Zn at 1.0 mg l^{-1}. The antioxidants, particularly in hepatopancreas and gills of shrimps with Zn at 1.0 mg l^{-1} and to lesser extent with 0.5 mg l^{-1} were significantly higher on both post challenge days sampled. Shrimps with Cu at 0.1 mg l^{-1} also exhibited higher antioxidant activity in hepatopancreas and gills, particularly on PCD5. It may be noted that post challenge survival was significantly higher in these group of shrimps (Refer fig.4.13 and 5.13). The greater protection offered to hepatopancreas and gills against oxidative stress generated during WSSV infection also would have acted as determinant factors in increasing the post challenge survival. It may be noted that the activity of SOD, CAT and GPx, the three primary enzymes and GSH levels of hepatopancreas and gills were significantly higher on PCD2 in shrimps exposed to Zn at 1.0 mg l^{-1} Zn. In gills, significantly higher activities of all antioxidants could be noticed on PCD2 with Zn at 1.0 mg l^{-1}. The comparatively higher activity of scavenging enzymes on PCD2 was probably an added advantage in minimizing oxidative stress as respiratory burst activity initiated by the membrane-bound enzyme NADPH-oxidase is seen at its maximum on PCD2 (Refer Fig.5.9). CAT, GST, GR and GSH were the common enzymes that showed enhanced activity on PCD5 in hepatopancreas and gills of shrimps with Cu at 0.1 mg l^{-1} Cu.

On the other hand, a comparative reduction in the activity of most of the antioxidants and an increase in lipid peroxidation level were observed in shrimps with higher doses of Cu and Zn. SOD, CAT and GPx activity were significantly lower than the unexposed on PCD5 in the hepatopancreas and gills of shrimps with higher doses of Cu. In hepatopancreas, all the antioxidants were lower at higher doses of Cu. GSH was the common antioxidant inhibited in all tissues with higher doses of Zn, on PCD5. CAT activity was lower in hepatopancreas and gills on all post challenge days with Zn.

The present study has documented that the antioxidant defense system in *P. monodon* is operating at a lower rate after WSSV infection. The comparatively better antioxidant defense ensured by appropriate doses of Cu (0.1 mg l^{-1}) and Zn (1.0 mg l^{-1}) in shrimps may hence be considered as another important mechanism by which the shrimps were offered greater protection from mortality due to WSSV infection. Or in other words, the antioxidant enhancing property of Cu and Zn also contributed to their immunostimulatory effect in shrimps. The antioxidant property of Zn sustained to a greater degree after WSSV infection, compared to Cu, which must have been an added advantage in enhancing the post challenge survival that was significantly higher in shrimps with Zn at 1.0 mg l^{-1} compared to that with Cu at 0.1 mg l^{-1} (Fig.4.12 and 5.12). On the other hand, the oxidative stress was greater in shrimps exposed to higher doses of Cu and Zn as the antioxidant defense was poor and the MDA levels were found to be more, prior to and after WSSV infection. Poor antioxidant defense favoring the pro-oxidants may hence be proposed as one of the many mechanisms by which higher doses of Cu and Zn cause physiological stress to shrimps resulting in an increased susceptibility to WSSV infection.

Findings of the present study have not only added to the understanding of alterations in antioxidative defense induced by WSSV infection in *P. monodon*, but also have unraveled the modulation effected by Cu and Zn. Higher doses of Cu and Zn were proved to inhibit the antioxidants resulting in a deterioration of the oxidative stress generated during WSSV-infection. On the other hand, exposure to Cu and Zn at appropriate lower doses (Cu at 0.1 mg l^{-1} and Zn at 1.0 mg l^{-1}) was found to be effective in modulating the antioxidants towards offering better protection against oxidative stress generated by WSSV infection. Hence, the results of present study may be employed in the disease-management strategies adopted during pond management towards the control of WSSV infection. However, before extending to fields, further research is recommended to understand the cellular mechanisms involved in the protection offered by Cu and Zn against WSSV infection in *P. monodon*.

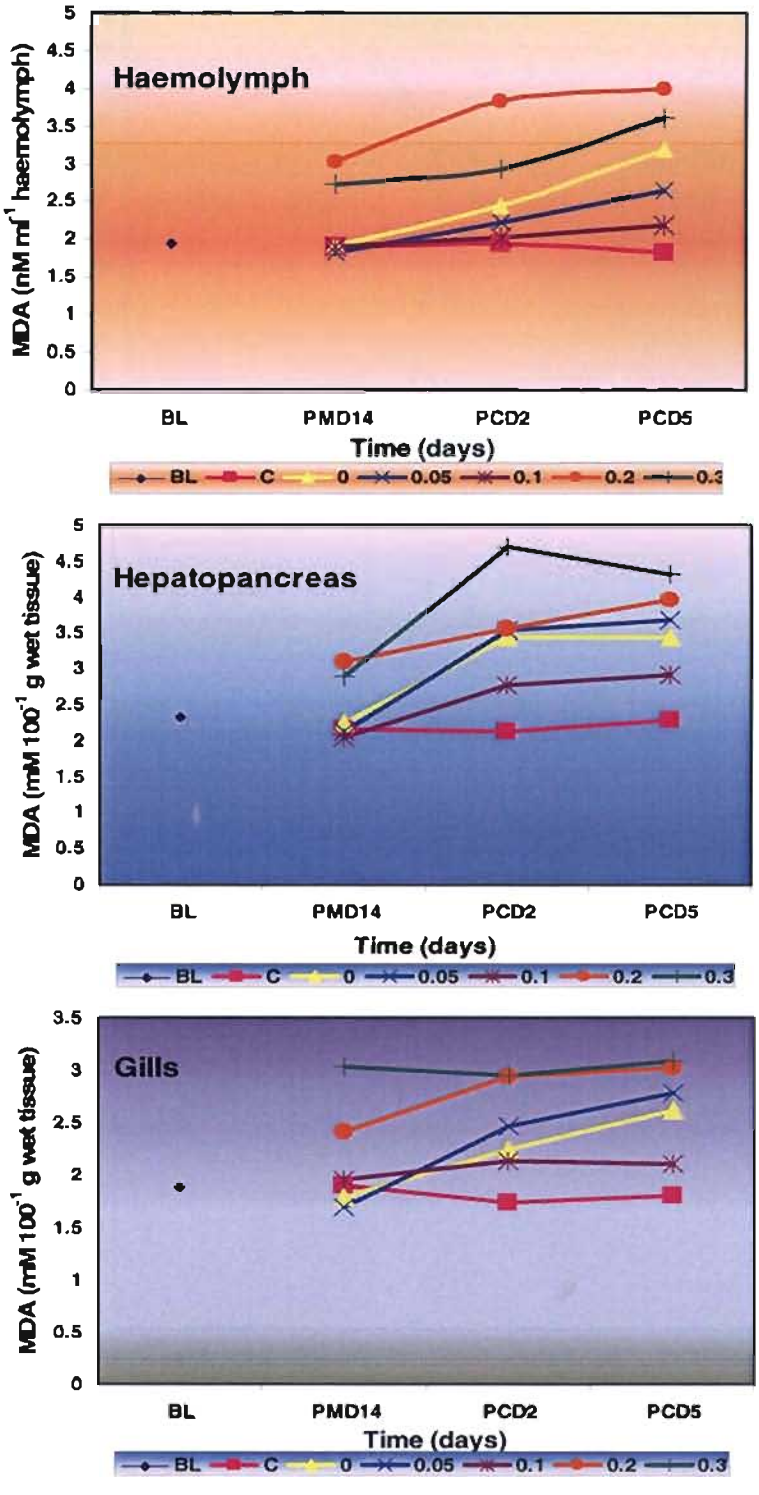


Fig.6.1 Malondialdehyde levels in *P. monodon* exposed to copper and challenged with WSSV.

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

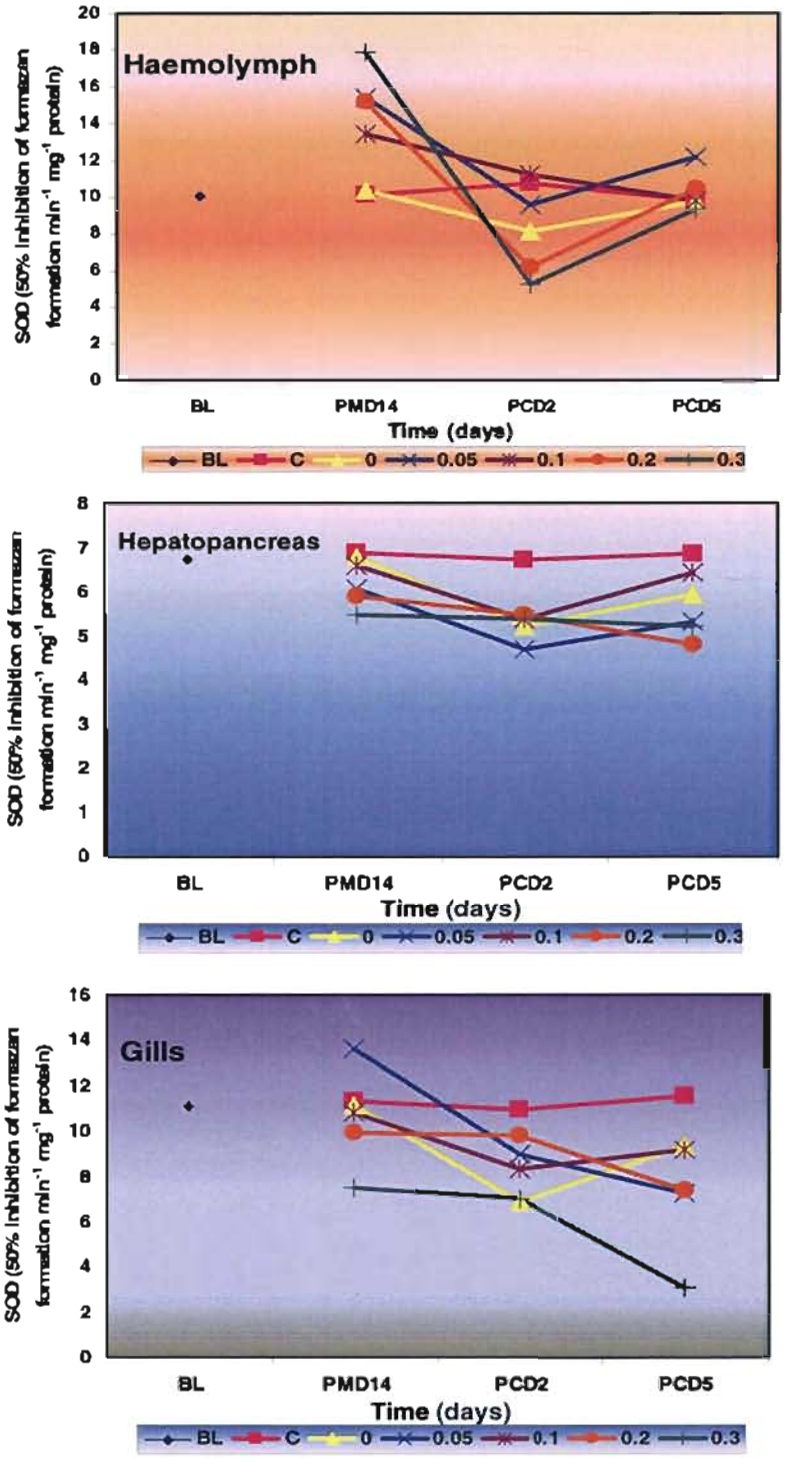


Fig.6.2 Superoxide dismutase activity in *P. monodon* exposed to copper and challenged with WSSV

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

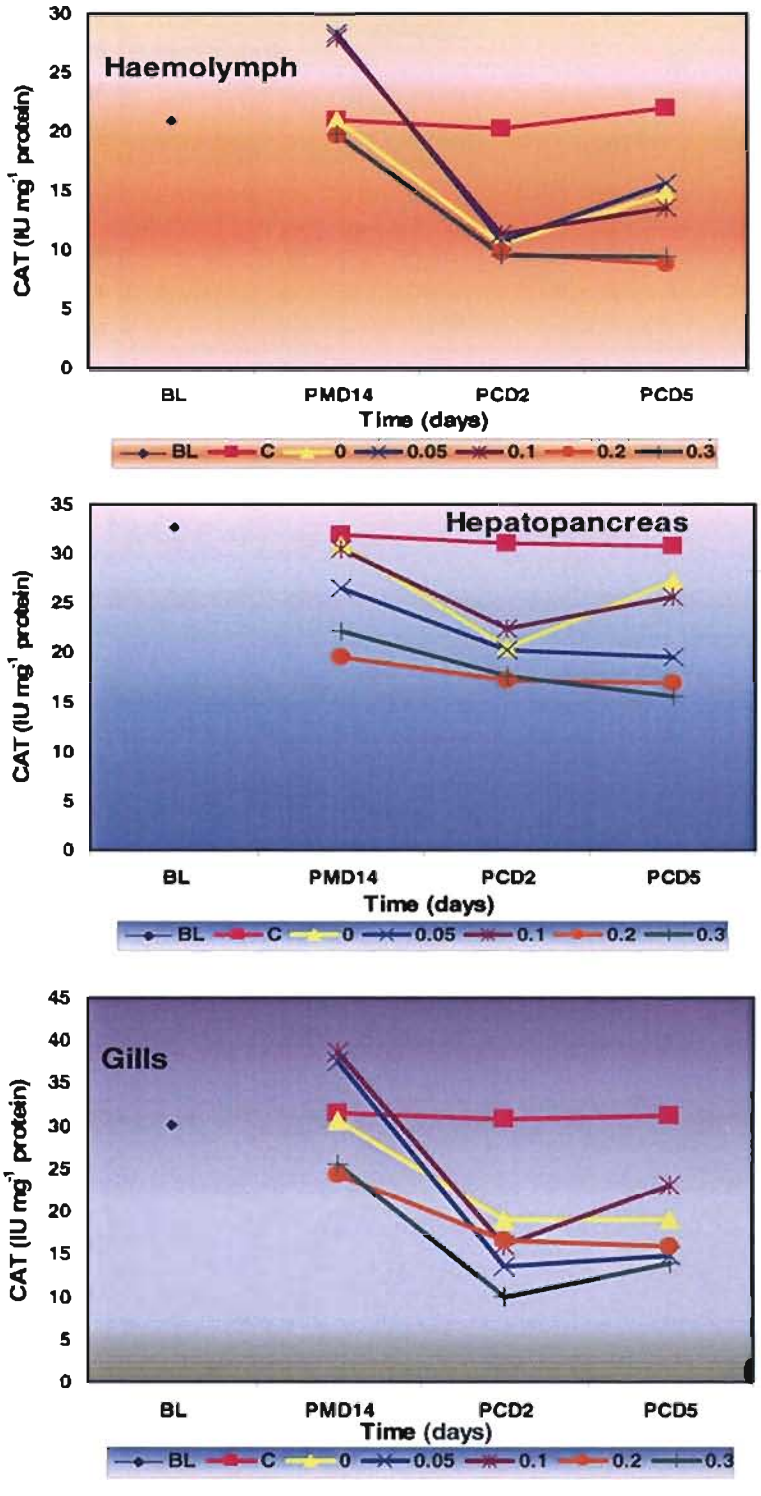


Fig.6.3 Catalase activity in *P. monodon* exposed to copper and challenged with WSSV

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

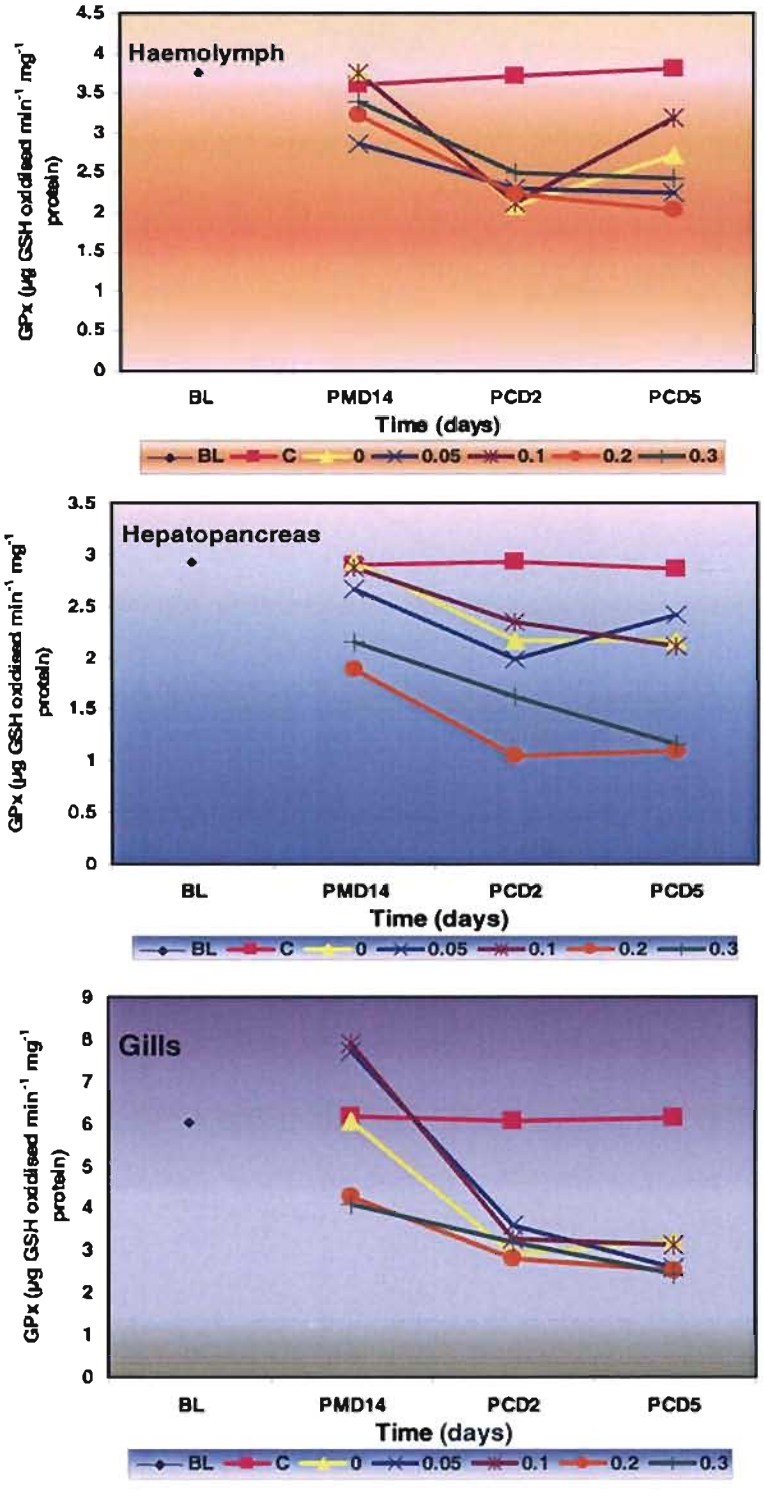


Fig.6.4 Glutathione peroxidase activity in *P. monodon* exposed to copper and challenged with WSSV

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

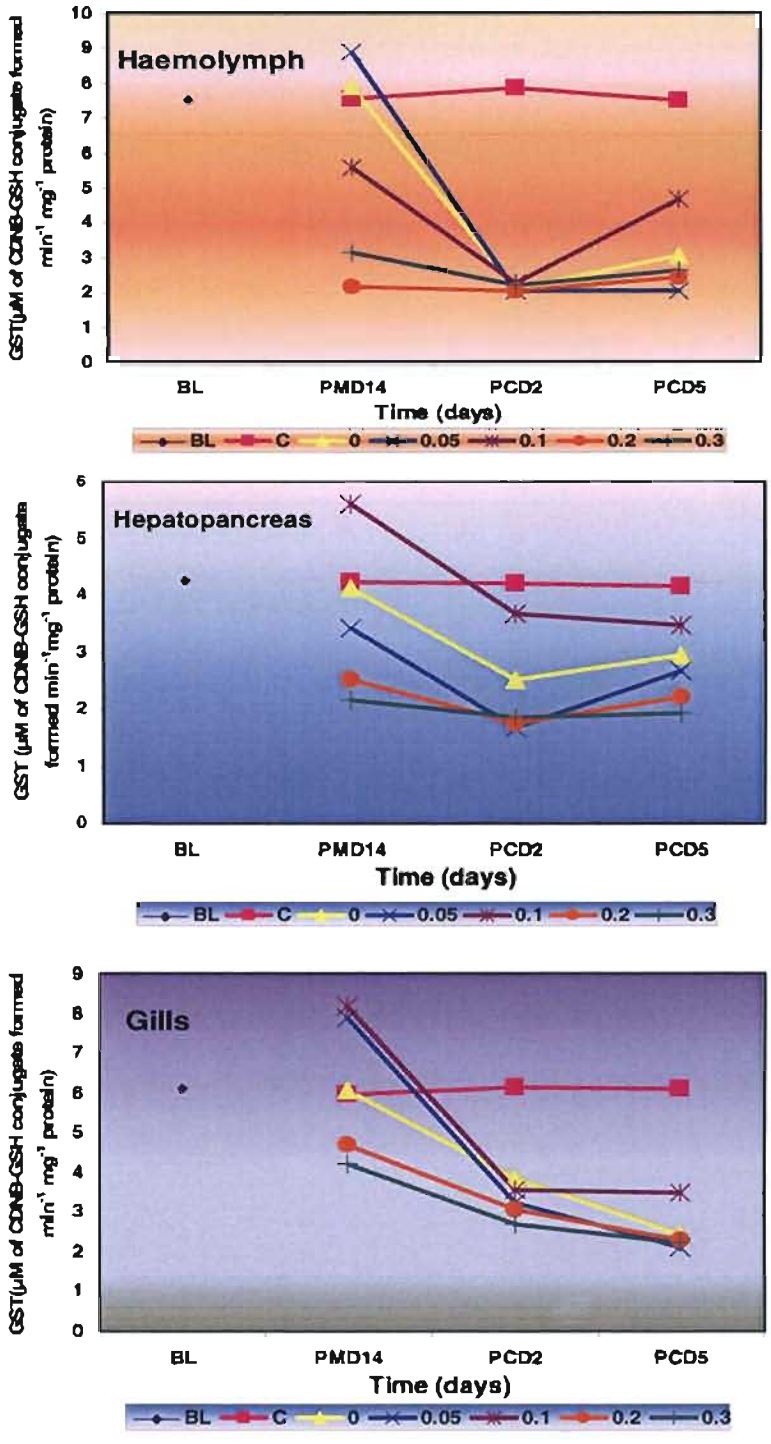


Fig.6.5 Glutathione *S*-transferase activity in *P. monodon* exposed to copper and challenged with WSSV

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

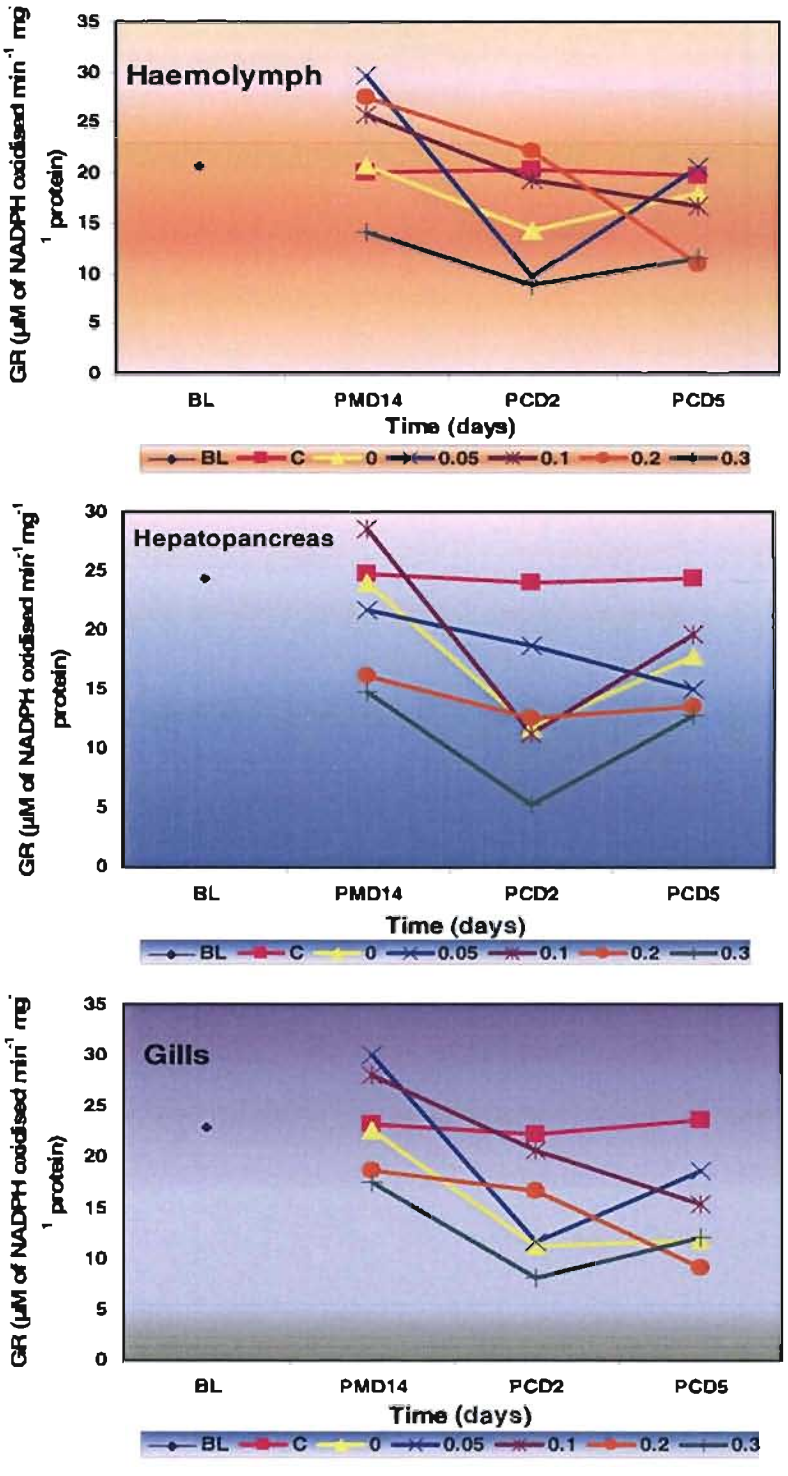


Fig. 6.6 Glutathione reductase activity in *P. monodon* exposed to copper and challenged with WSSV

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

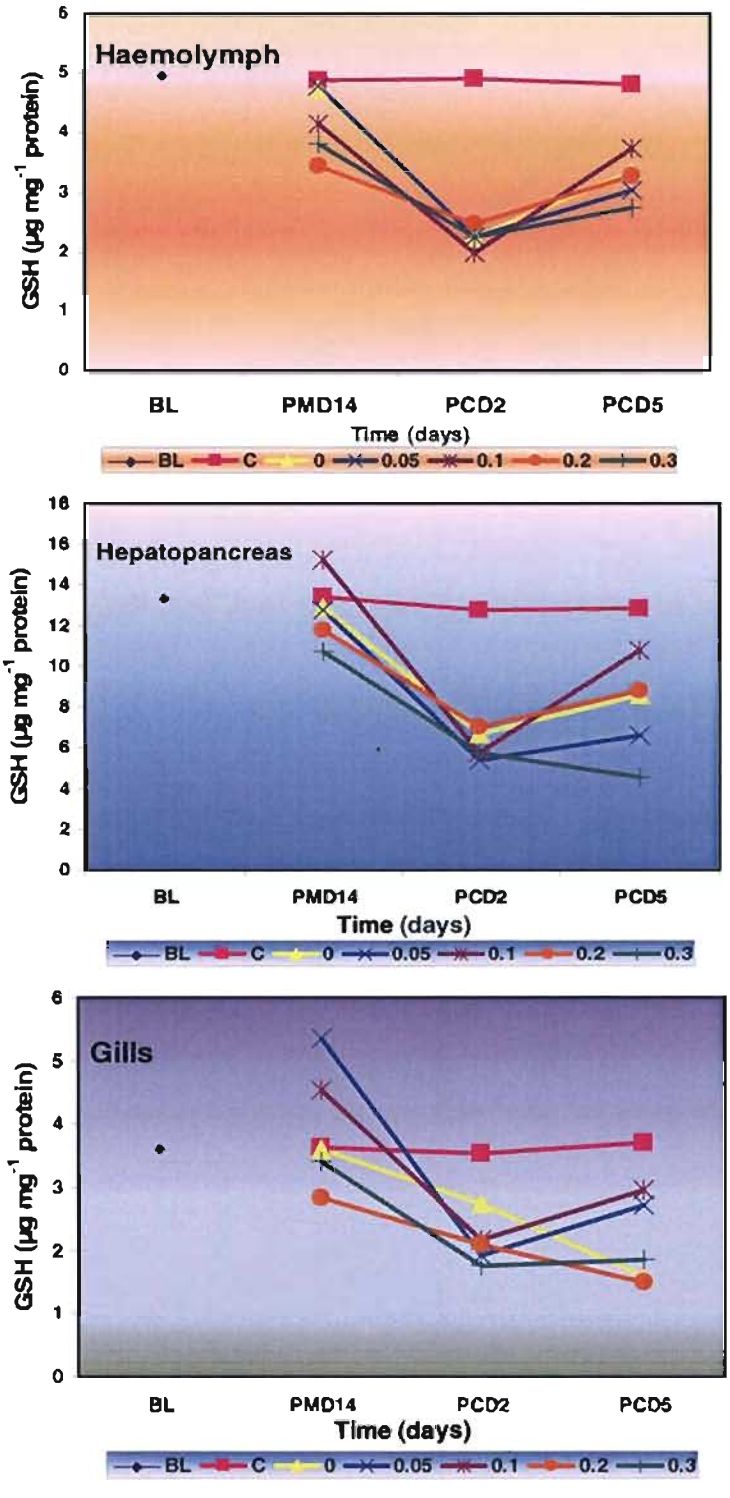


Fig.6.7 Total reduced glutathione levels in *P. monodon* exposed to copper and challenged with WSSV

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

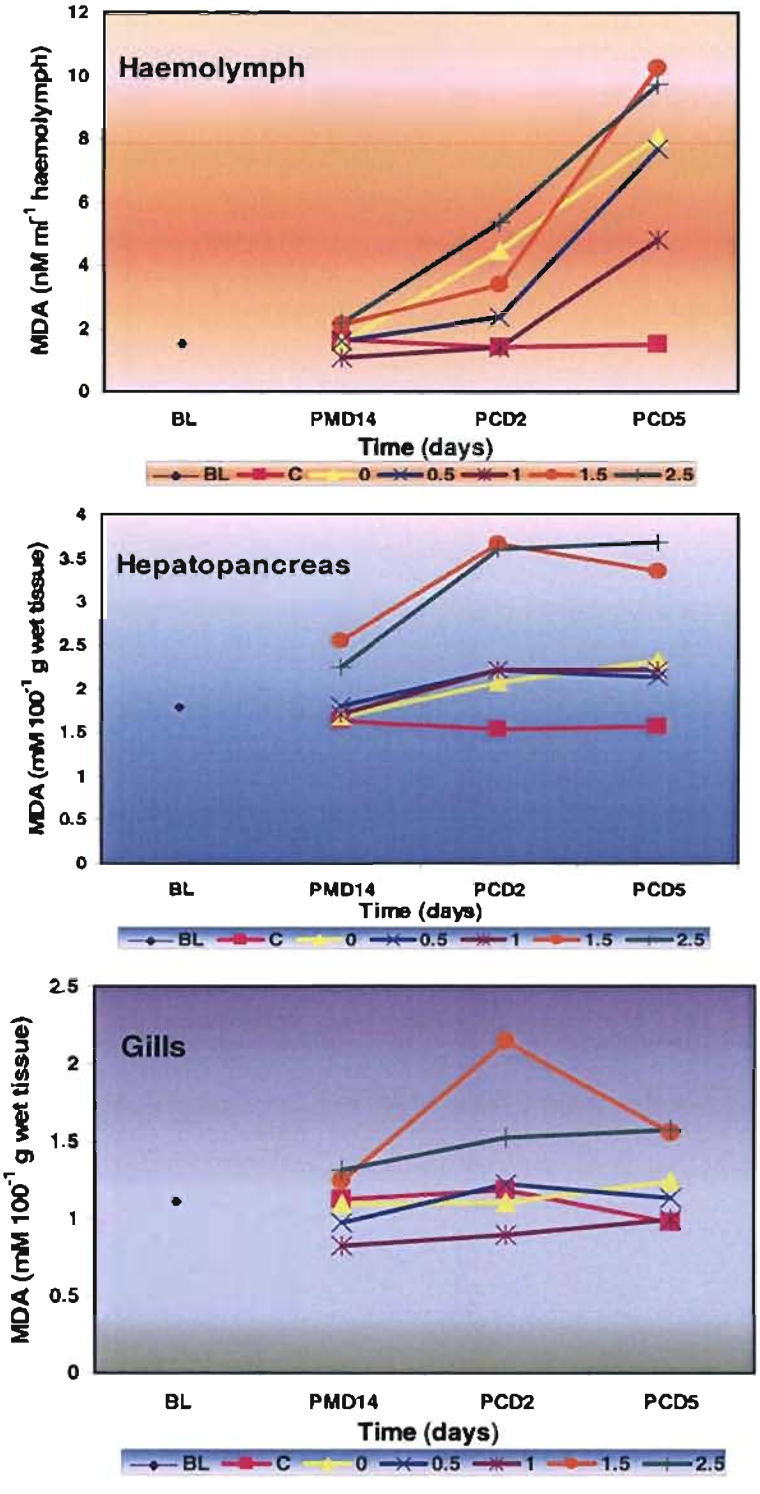


Fig.6.8 Malondialdehyde levels in *P. monodon* exposed to zinc and challenged with WSSV

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

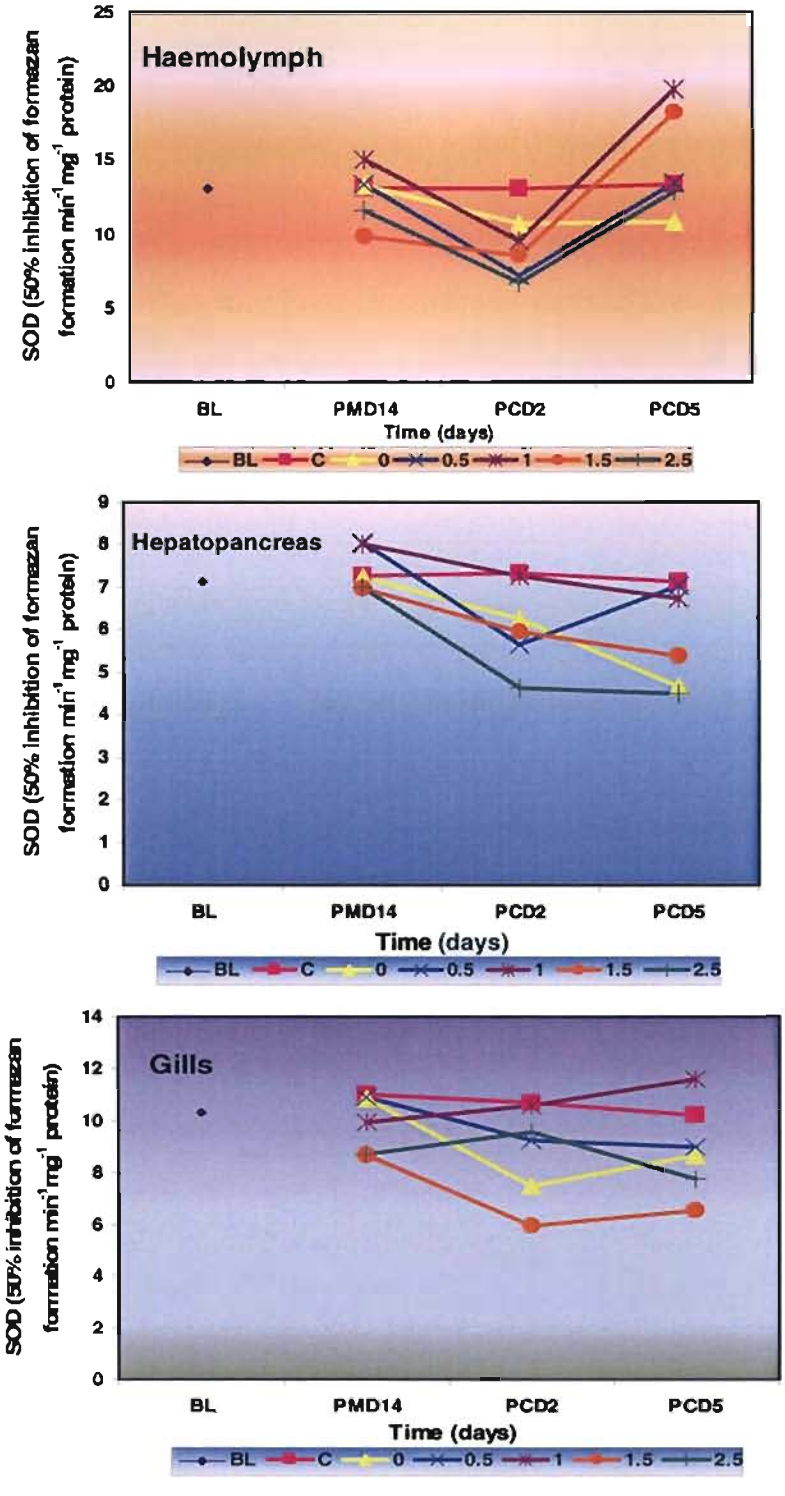


Fig.6.9 Superoxide dismutase activity in *P. monodon* exposed to zinc and challenged with WSSV

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

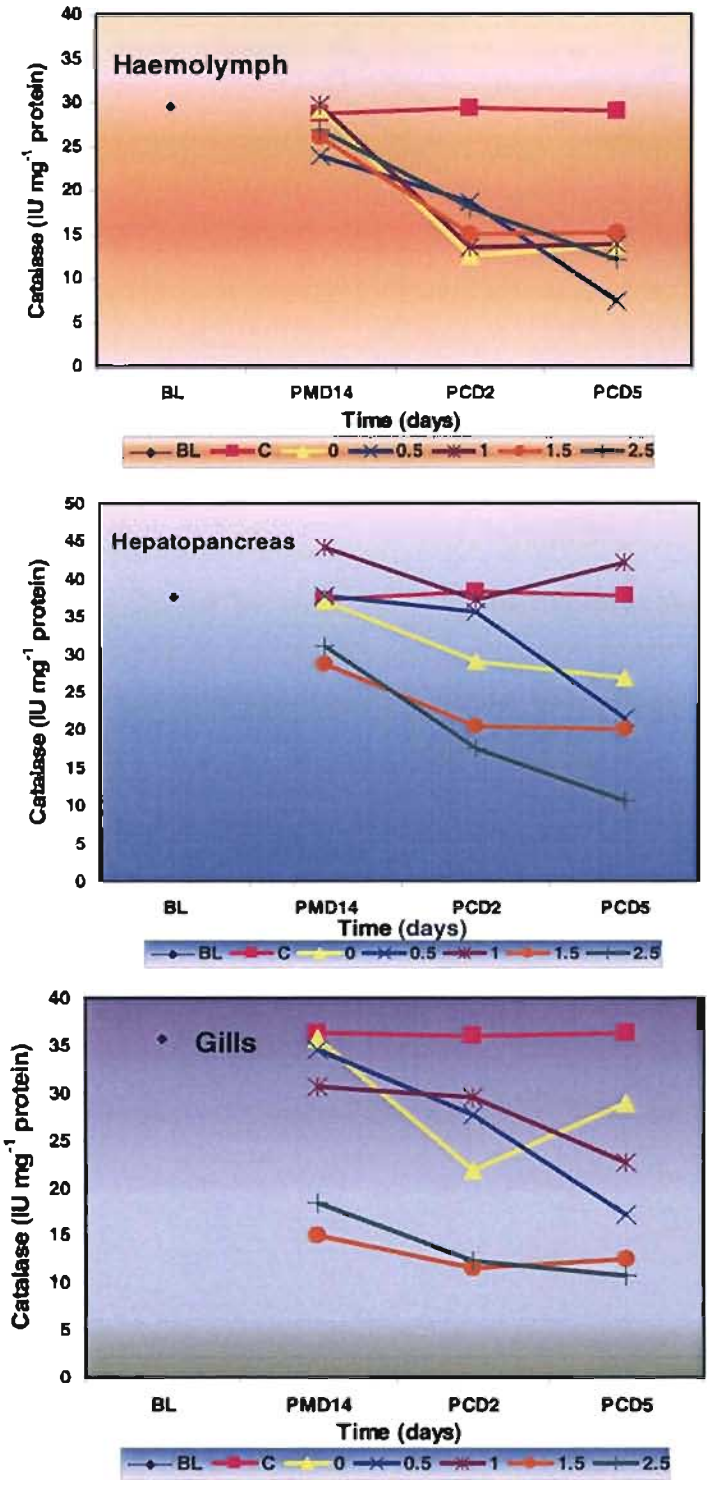


Fig.6.10 Catalase activity in *P. monodon* exposed to zinc and challenged with WSSV

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

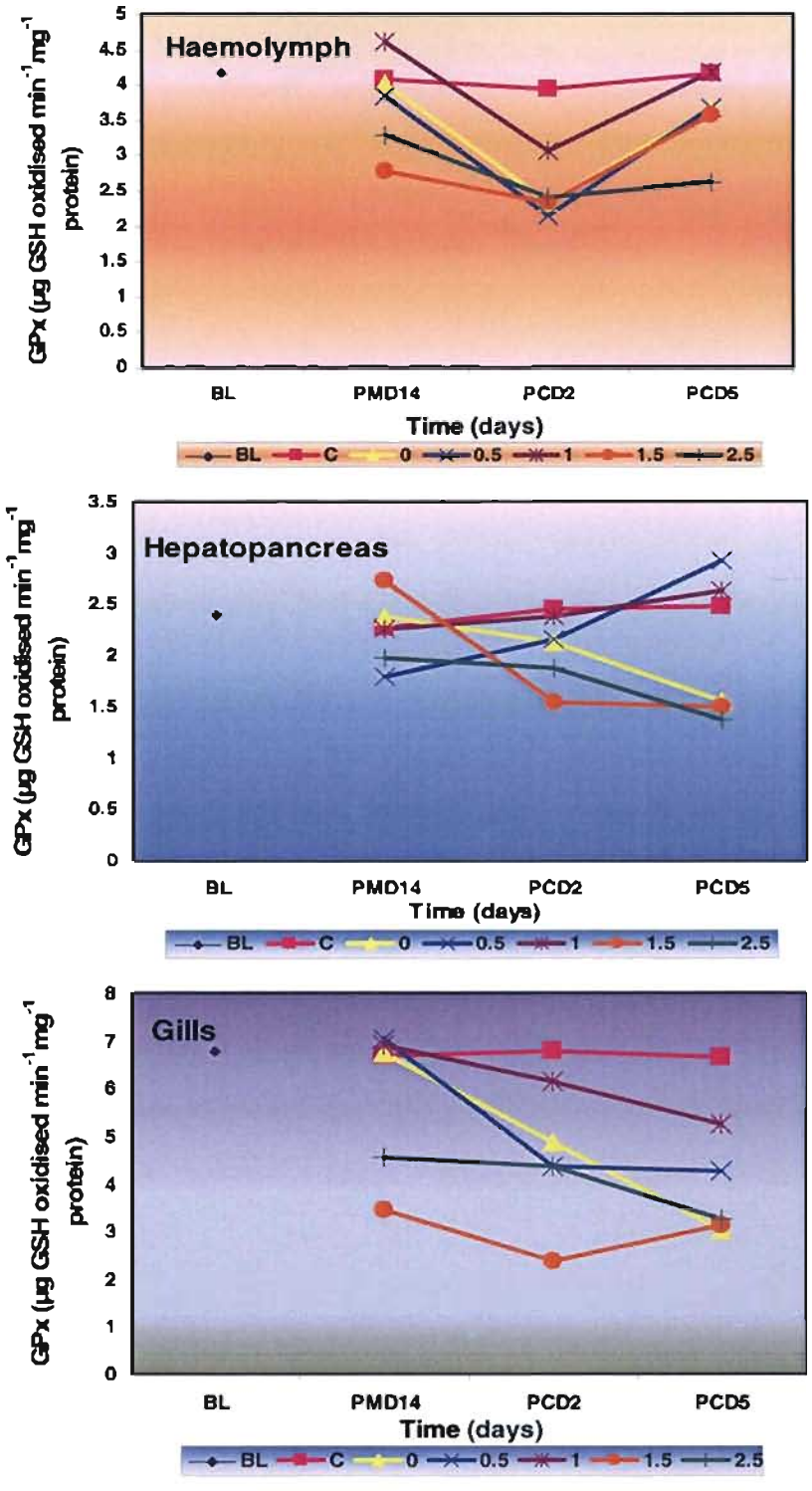


Fig.6.11 Glutathione peroxidase activity in *P. monodon* exposed to zinc and challenged with WSSV

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

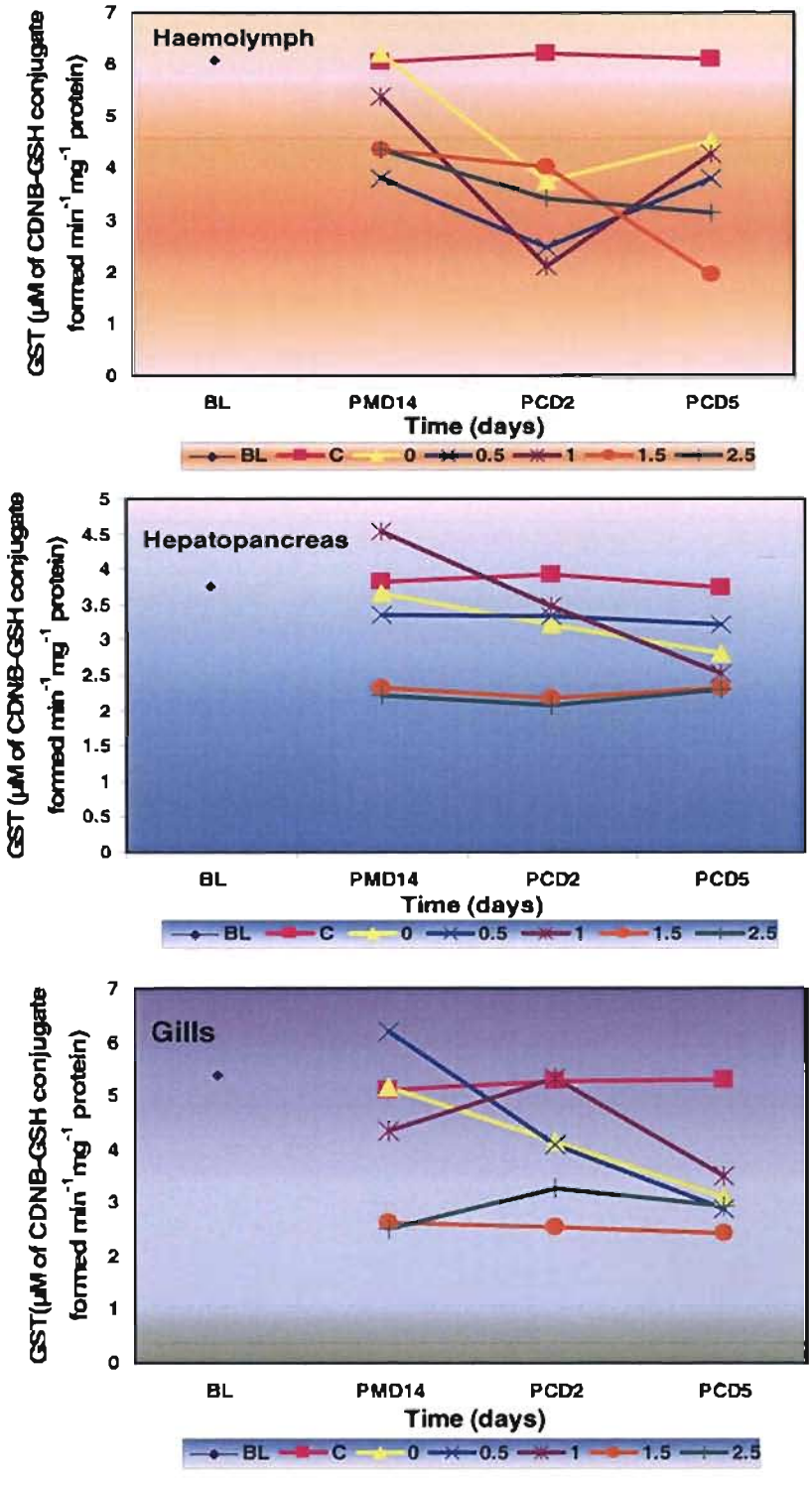


Fig.6.12 Glutathione *S*-transferase activity in *P. monodon* exposed to zinc and challenged with WSSV

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

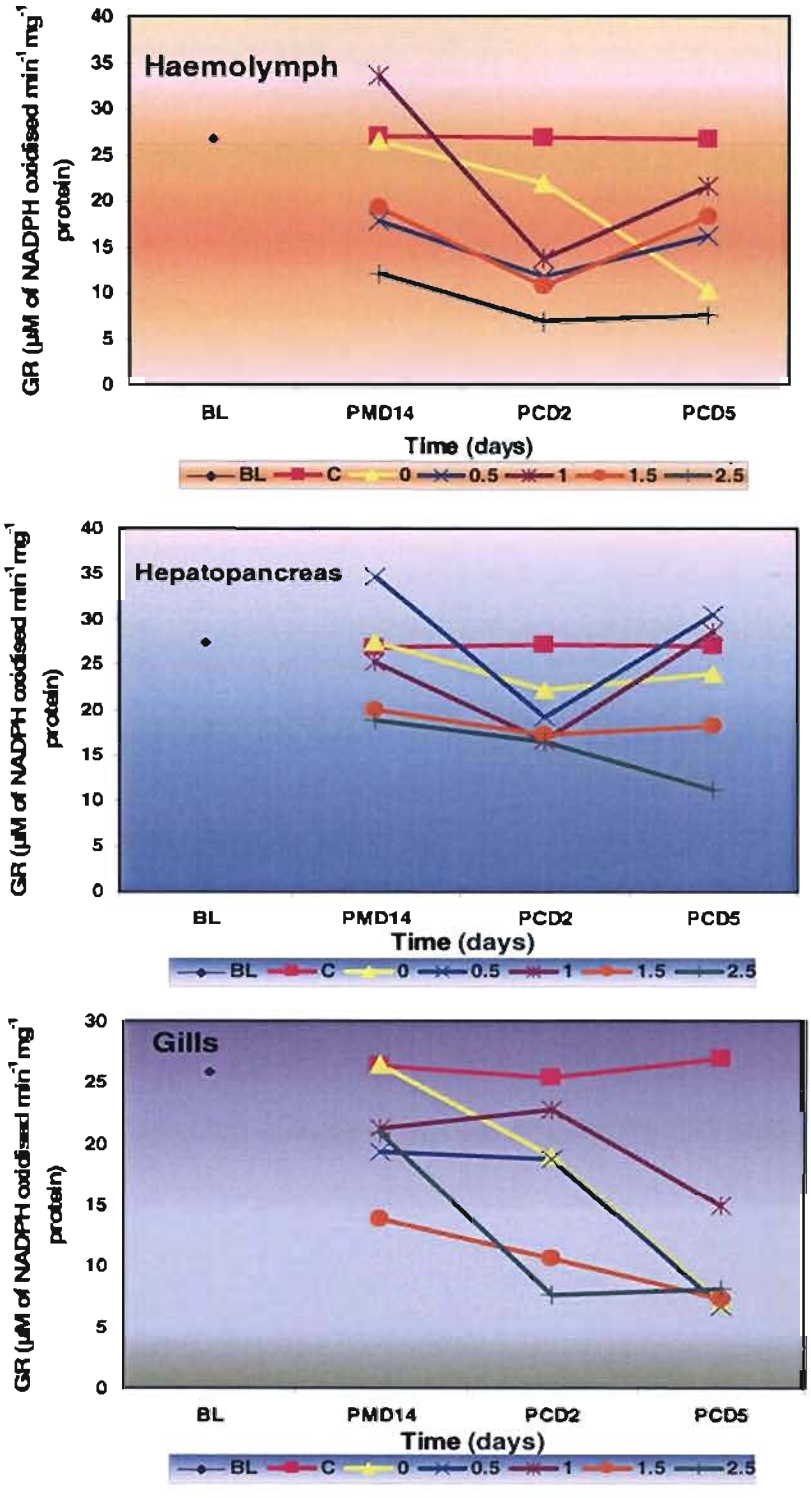


Fig.6.13 Glutathione reductase activity in *P. monodon* exposed to zinc and challenged with WSSV

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

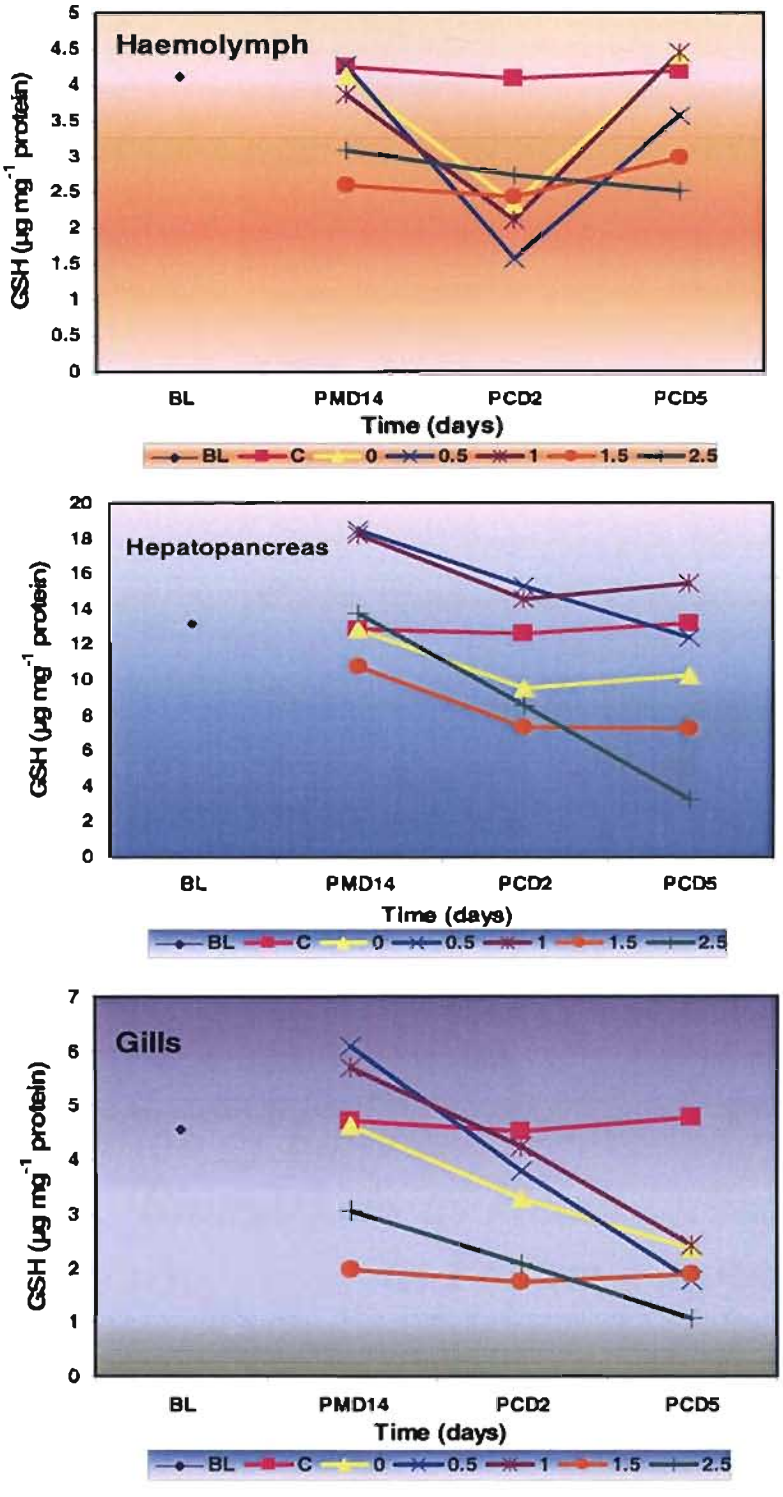


Fig.6.14 Total reduced Glutathione levels in *P. monodon* exposed to zinc and challenged with WSSV

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

CHAPTER 7

Identification of Potential Haematological Biomarkers as Heath indicators in Penaeus monodon

7.1 Introduction

Application of practical and reliable methods for a rapid analysis of shrimp health status is still uncommon among shrimp farmers. Health problems are usually detected at an advanced stage in cultivation systems through reduced shrimp growth, abnormal behaviour or even widespread mortalities. It is crucial to detect the deleterious effects before stress results in disease. Therefore, there is an urgent need for reliable, accurate and effective indicators of the physiological status of shrimp population.

Stress responses occur in animals when regulated physiological systems are extended beyond their normal range by external stressors. Indicators of such stress responses are useful in assessing the short-term well being or long-term health status of an animal. Hence, the development of field-friendly methods for accurately assessing the health status would be a highly valuable tool in managing shrimp populations. Periodic assessment of the health and immune status of shrimps will not only help the aqua culturists to monitor animal health, but will also enable them to predict disease outbreak and take prophylactic measures to combat stressful conditions in the culture environment.

Haemolymph is the most accessible internal tissue in shrimp that supplies important information on their physiological and pathological state. Taking haemolymph samples from shrimp does not increase mortality and has only short-term effects, if any on haemolymph parameters, although this depends on the size of the shrimp and the amount of haemolymph collected. The evaluation of blood chemistry parameters is a routine and important tool in vertebrate pathology that helps to make proper medical decisions. However, in comparison with the vertebrates in commercial animal production, there are practically no criteria for specific evaluation of the health status of shrimp, and invertebrates in general (Bachere, 2000). An ideal health parameter should be a sensitive indicator of the animal's health condition and immune capability and also should be easy to quantify. Very little is known regarding the haematological parameters that can be used as reliable indicators in shrimp.

Wide variations in hemato-immunological parameters related with moult cycle, viral infection, environmental stresses like hypoxia, low salinity and after the

administration of immunostimulants have been reported in some penaeids (Persson et al., 1987; Le Moullac *et al.*, 1998; Le Moullac *et al.*, 1997; Hennig *et al.*, 1998; Sajeevan *et al.*, 2006). And more recently, the influence of captivity stress on various blood metabolites and immune parameters was described in males of *L. setiferus* (Sanchez *et al.*, 2001). However, the predictive value of these parameters in the assessment of health status has not been properly explored so far. The present study which is based on a statistical analysis of the haematological data obtained with *P. monodon* subjected to diverse experimental conditions was primarily an attempt to assess the predictive value of various metabolic and immune variables in the haemolymph.

The assorted experimental data was further analysed with the following aims:

- To draw a haematological profile for normal/healthy *P. monodon*
- To find the degree of relationship between haematological parameters and survival rate.
- To identify the most reliable biomarkers of health in *P. monodon*

7.2 Materials and methods

7.2.1 Haematological profile of normal/healthy P. monodon

Baseline haematological data and the data obtained for the control shrimps from each bioassay (Refer chapter 2, 3, 4, 5, 6) were compiled and a haematological profile was drawn for normal/healthy *P. monodon* of average size 19.2 ± 3.6 g.

7.2.2 Statistical analysis

7.2.2.1 Experimental data used for the analysis

- Haematological parameters and survival rate of *Vibrio* infected *P. monodon* subjected to salinity stress. (Refer chapter 2).
- Haematological parameters and survival rate of WSSV infected *P. monodon* subjected to salinity stress. (Refer chapter 3).
- Haematological parameters and survival rate of WSSV infected *P. monodon* exposed to copper. (Refer chapter 4).
- Haematological parameters and survival rate of WSSV infected *P. monodon* exposed to zinc. (Refer chapter 5).

Data was analysed separately for each bioassay and collectively by assorting data from all the four bioassays. Analysis was done by means of the statistical software SPSS 10.0.

7.2.2.2 Correlation

Correlation analyses were done to elucidate the degree of relationship between survival rate and haematological parameters and among various haematological parameters. Significant correlations were identified by evaluating Pearson correlation co-efficients. Matrix of correlation gave the co-efficients of correlation between all the variables analysed. When the correlation co-efficient was positive and significant ($P<0.05$) it indicated that as one variable increases, the other variable also increases. When the correlation co-efficient was negative and significant ($P<0.05$) it indicated that as one variable increases, the other variable decreases (Shukla and Gulshan, 1986; Gurumani, 2004).

7.2.2.3 Regression

Further, the multiple regression analyses of the data were done to find out the most ideal haematological parameters that can act as predictors of the survival rate. Regression is a statistical method with the help of which it is possible to find out how much is one variable dependent on the other and whether it is possible to predict the unknown values of one variable from the known values of another variable. Regression analyses were done with survival as the dependent variable and haematological parameters as the predictors. *R* Square value gave the amount of variability explained by the predictors. The key variables that contributed to the variability was evaluated from the significant regression co-efficients ($P<0.05$) and identified as the most potential predictors of survival rate (Shukla and Gulshan, 1986; Gurumani, 2004).

7.3 Results

Table 7.1 shows the haematological profile obtained for normal/healthy *P. monodon* of average size 19.2 ± 3.6 g.

Pearson correlation co-efficients showed that all immune variables *viz.*, THC, PO, NBT, ALP and ACP exhibited a positive correlation ($P<0.05$) with the survival rate and with each other in almost all cases analysed. Metabolic and immune variables showed a greater degree of correlation with each other. Total protein, total carbohydrates and total

lipids were the key metabolic variables that exhibited a positive correlation with the survival rate and immune variables. MDA exhibited a significant negative correlation ($P<0.05$) with the survival rate as well as with all the immune variables and with the metabolic variables *viz.*, total protein, total lipids and cholesterol. The antioxidants, GPx and GST exhibited a positive correlation with GSH. GR was the only antioxidant enzyme that showed a positive correlation with the survival rate in both cases analysed (Table 7.2a – e).

When multiple regression of survival rate on all parameters was considered, a greater amount of variability was found to be explained. From the charts it was clear that the distribution of the survival rate is almost normal. The theoretical and observed values were very closer indicating that the fitted regression is a good fit to the data (Fig.7.3a – e). When significant regression co-efficients were taken into account, few of the variables were found to contribute significantly to the variability. When the combined data was analysed the amount of variability explained was 65.8% (R Square=0.658). THC ($P<0.01$), PO ($P<0.001$), ACP ($P<0.001$) and ALP ($P<0.01$) together were explaining the 63.6% of the variability (R Square=0.636), indicating that these four parameters are mainly responsible for the survival rate. THC, PO, ALP and ACP were hence deduced as the most potential haematological biomarkers of health in *P. monodon* (Table 7.3a – e).

7.4 Discussion

Haematological parameters for normal/healthy *P. monodon* of average size, 19.2 ± 3.6 g is defined in the present study. However, it apparently seems that creation of a reliable database to be used as a reference guideline requires sampling of more number of shrimps. Further field studies based on sampling of large numbers of shrimps may be required to set up a reliable reference scale.

Correlation analyses could reveal valuable information on the relationship that existed between various haematological parameters and survival rate. Among the examined haematological parameters, some of the metabolic variables *viz.*, total protein, total carbohydrates and total lipids and all immune variables *viz.*, THC, PO, NBT, ALP and ACP exhibited positive correlation with the survival rate in almost all cases analysed. Though recently, several investigations on changes in hematological parameters of penaeids subjected to stress conditions have been reported (Le Moullac and Haffner, 2000; Sanchez *et al.*, 2001; Pascual *et al.*, 2003b), statistical information regarding correlation with the survival rate is negligible. The present result is apparently a statistical

evidence to the intimate relationship of survival rate with various blood metabolites and immune components in shrimps.

From the greater correlation that was found to exist between metabolic variables and immune responses it could be expected that variations in haemolymph metabolites of shrimps result in changes in the quality and quantity of its immune components. Total protein, total carbohydrates and total lipids positively correlated in most cases with the survival rate and immune variables. Since the shrimp immune components are mostly proteins (Perazzolo and Barracco, 1997; Destoumieux *et al.*, 2000), a lowered immune capability and thereby a reduced survival rate in the event of a pathogenic infection may be aptly attributed to a reduction in the total protein levels. However, the levels of total carbohydrates and total lipids have not received much attention. Carbohydrates, the immediate energy yielders during stress and lipids that provide essential structural components also have a special role to play in enhancing the immunocompetence of shrimps resulting in better survival. Recent investigations on haemolymph metabolic components like total protein, glucose, triacylglycerol, cholesterol and lactate as indicators of the physiological status of shrimps are available (Palacios, 2000; Sanchez *et al.*, 2001). A general conclusion could not be obtained with glucose in the present study as it was found to exhibit both positive and negative correlations with the survival rate and immune variables. Cholesterol exhibited positive correlation with the immune variables to some extent, but failed to correlate with survival rate in majority of the cases analysed.

As expected, all the immune variables analysed showed a positive correlation with the survival rate which underlines the fact that an enhancement in the survival rate of shrimps is associated with an enhancement in various immune components and immune activities. Being so intimately related to the survival rate, the immune variables serve as sensitive stress indicators in shrimps. They also serve as sensitive indicators for checking the efficacy of immunostimulants. Total haemocyte count, phenol oxidase activity and superoxide anion production have earlier been considered as indicators of stress by many though their predictive value was not measured (Le Moullac *et al.*, 1998; Rodriguez and Le Moullac, 2000; Song *et al.*, 2003). However, it may be noted that other variables such as clotting time, phagocytic index, antibacterial activity, etc. also have been considered as potential stress indicators (Lightner, 1996; Wang and Chen, 2005; Cheng *et al.*, 2007).

To a great extent, all the immune variables were positively correlated with each other. Therefore, it could be expected that an increase in one immune activity invariably results in an enhancement in other activities as well. For example, an increase in THC will be coupled with an increase in the phenol oxidase activity, superoxide anion production and alkaline and acid phosphatase activities. On the other hand, a prolonged decrease of THC in shrimps exposed to some physiological or environmental stress could lead to an immune depletion and increased mortality from infection. It was recently shown that shrimp haemocytes, besides their role in cellular immune reactions, are the principal site of expression of genes encoding immune effectors (Gross *et al.*, 2001). This may be considered a plausible explanation for the intimate relation of THC with the other immune activities. An increase in phenol oxidase activity, superoxide anion production and ALP and ACP activities under stress could be attributed to an increase in THC in most cases. A positive correlation of phenol oxidase activity with THC was suggested by Cheng *et al.* (2004). By boosting one or more of these immune variables in shrimps, the immunity in general can be enhanced and thereby the survival rate.

MDA, the terminal byproduct of lipid peroxidation, showed a negative correlation with the survival rate and with all the immune variables. Higher MDA signifies that the redox balance of the organism has been hampered. Increased lipid peroxidation is one of the major factors leading to the loss of cell function under situations of oxidative stress (Hermes-Lima *et al.*, 1995). A greater extent of lipid peroxidation may therefore be presumed to contribute to the reduction of immune capability by affecting the functioning of haemocytes, thereby reducing the survival rate. However, MDA was found to exhibit both negative and positive correlations with the antioxidants that were mostly insignificant. Lipid peroxidation levels that reflects oxidative damage and antioxidant defense components have earlier been proposed as warning biomarkers of stress (Reid and MacFarlane, 2003; Bianchini and Monserrat, 2007). Among the antioxidants analysed, GR was the only enzyme that showed significant positive correlation with the survival rate in both cases analysed. Very recently, in a correlation analysis done by Pan *et al.* (2003), total antioxidant status in *P. monodon* juveniles fed diets supplemented with or without astaxanthin was found to have a significant positive correlation with survival rate. Reliable conclusion regarding the relation of antioxidants with superoxide anion production could not be established from the present analysis. The antioxidant enzymes, glutathione peroxidase and glutathione *S*-transferase, both of which uses GSH as the substrate showed a positive correlation with GSH. Another marked positive correlation was that found between THC and CAT.

Regression analysis was done particularly to assess the predictive value of various haematological parameters since a good number of the parameters were correlated with survival rate and seemed to serve as effective indicators of physiological and immune status of shrimps,. The analysis could prove that THC, PO, ACP and ALP activity that were mainly responsible for the survival rate possess a superior predictive value, signifying their validity as useful biomarkers of health. Apart from THC and PO, the classically used parameters, lysosomal enzyme activities, ACP and ALP also topped the list of predictors. Other variables like NBT, TP, TL, GI, MDA, CAT and GR were also found to have comparatively better predictive value, signifying their application as biomarkers of health in shrimps. However, they could be detected as key predictors only under one set of experimental conditions. THC, PO, ALP and ACP repeatedly happened to be prime predictors when the data from each experimental set up was treated separately and also when the assorted data under various experimental conditions was analysed. Therefore, in general, it may be suggested that immune variables have more predictive value compared to the metabolic variables and antioxidants. And it could be reliably proposed that THC, PO, ALP and ACP are the most important factors controlling survival rate and thereby the more appropriate parameters to evaluate shrimp health status.

In brief, it may be concluded from the study that THC, PO, ALP and ACP are good predictors of the survival rate in *P. monodon* and using these selected parameters, a presumptive prediction can be made on the health status and the probable problem of environmental stress or infection. It is also evident from the study that a poor metabolic response may lead to a lower level of immunocompetence. Predictive value of the selected biomarkers of health in *P. monodon* is however compromised by the lack of reliable baseline databases and available reference laboratories to properly analyse the samples. Therefore, there is an urgent need for developing reliable databases of shrimp haematological profile under healthy, stressed and diseased conditions and dissemination of this information at field level for better health management in shrimp aquaculture.

Table 7.1 Haematological profile of normal/healthy *P. monodon* (average size 19.2 ± 3.6 g)

Metabolic variables	
Total protein (mg ml^{-1})	69.35 - 112.21
Total carbohydrates (mg ml^{-1})	2.77 - 5.52
Total free amino acids (mg ml^{-1})	2.42 - 3.82
Total lipids (mg ml^{-1})	1.365 - 2.368
Glucose (mg ml^{-1})	0.247 - 0.485
Cholesterol (mg ml^{-1})	0.432 - 0.789
Immune variables	
Total haemocyte count ($\times 10^6$ cells ml^{-1})	15.49 - 26.31
Phenol oxidase activity (increase in OD min^{-1} 100μl^{-1})	0.064 - 0.154
Nitroblue tetrazolium salt reduction (OD 100μl^{-1})	0.502 - 0.896
Alkaline phosphatase activity (mg p-nitrophenol released ml^{-1})	0.242 - 0.582
Acid phosphatase activity (mg p-nitrophenol released ml^{-1})	0.566 - 0.848
Lipid peroxidation	
Malondialdehyde (nM ml^{-1})	0.619 - 2.586
Antioxidants	
Superoxide dismutase activity (50% inhibition of formazan formation min^{-1} mg^{-1} protein)	8.204 - 16.351
Catalase activity (IU mg^{-1} protein)	17.524 - 32.894
Glutathione peroxidase activity (µg GSH oxidised min^{-1} mg^{-1} protein)	3.158 - 5.039
Glutathione *S*-transferase activity (µM of CDNB-GSH conjugate formed min^{-1} mg^{-1} protein)	5.119 - 8.993
Glutathione reductase activity (µM of NADPH oxidised min^{-1} mg^{-1} protein)	15.63 - 31.246
Total reduced glutathione (µg mg^{-1} protein)	3.512 - 5.652

Table 7.2a Correlation matrix between survival rate and haematological parameters of *Vibrio*-infected *P. monodon* subjected to salinity stress.

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	GI	Ch	Survi
THC	1.000											
PO	0.563**	1.000										
NBT	0.692**	0.263	1.000									
ALP	0.549**	0.630**	0.232	1.000								
ACP	0.171	-0.405*	0.385*	-0.009	1.000							
TP	0.047	-0.352*	-0.046	0.122	0.399*	1.000						
TC	0.416*	0.262	0.307	0.655**	0.294	0.313	1.000					
TFAA	-0.260	-0.032	-0.449**	0.195	-0.351*	0.323	0.318	1.000				
TL	-0.266	-0.435*	-0.303	0.043	0.285	0.561**	0.408*	0.543**	1.000			
GI	0.723**	0.698**	0.599**	0.655**	0.067	-0.150	0.575**	-0.118	-0.223	1.000		
Ch	-0.153	-0.156	-0.391*	0.207	0.020	0.445**	0.398*	0.557**	0.581**	-0.178	1.000	
Survi	0.595**	0.036	0.587**	0.493**	0.550**	0.582**	0.607**	-0.039	0.360*	0.437**	0.144	1.000

Table 7.2b Correlation matrix between survival rate and haematological parameters of WSSV-infected *P. monodon* subjected to salinity stress.

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	GI	Ch	Survi
THC	1.000											
PO	0.350*	1.000										
NBT	0.211	0.722**	1.000									
ALP	0.056	0.628**	0.567**	1.000								
ACP	0.097	0.635**	0.814**	0.493**	1.000							
TP	0.270	0.407*	0.404*	0.337*	0.274	1.000						
TC	0.334*	0.533**	0.566**	0.512**	0.513**	0.390*	1.000					
TFAA	0.708**	0.519**	0.363*	0.113	0.310	0.256	0.552**	1.000				
TL	0.387*	0.455**	0.296	0.486**	0.124	0.028	0.296	0.230	1.000			
GI	0.665**	0.525**	0.451**	0.288	0.367*	0.410*	0.641**	0.707**	0.354*	1.000		
Ch	-0.227	0.202	0.306	0.572**	0.263	0.039	0.246	-0.312	0.461**	-0.158	1.000	
Survi	0.680**	0.589**	0.591**	0.621**	0.470**	0.331*	0.627**	0.608**	0.609**	0.630**	0.295	1.000

**$P<0.01$、*$P<0.05$

Table 7.2c Correlation matrix between survival rate and haematological parameters of WSSV-infected *P. monodon* exposed to Cu.

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	GI	Ch	SOD	CAT	GPx	GST	GR	GSH	MDA	Survi
THC	1.000																		
PO	0.190	1.000																	
NBT	0.184	0.795**	1.000																
ALP	0.037	0.633**	0.837**	1.000															
ACP	-0.373*	0.666**	0.673**	0.701**	1.000														
TP	-0.104	0.528**	0.593**	0.521**	0.579**	1.000													
TC	0.378**	0.007	0.134	-0.021	-0.329*	0.089	1.000												
TFAA	0.162	-0.248	-0.395**	-0.420**	-0.421**	-0.402**	-0.004	1.000											
TL	-0.225	0.183	0.243	0.342**	0.419**	0.441*	0.093	-0.264*	1.000										
GI	0.479**	-0.493**	-0.338**	-0.345**	-0.808**	-0.355**	0.595**	0.322*	-0.241	1.000									
Ch	0.277*	0.197	0.258*	0.280*	-0.064	0.203	0.388**	0.158	0.239	0.341**	1.000								
SOD	0.522**	0.106	0.233	0.235	-0.132	-0.034	0.342**	0.045	0.055	0.350**	0.318*	1.000							
CAT	0.431**	0.300*	0.300	0.138	0.008	0.134	-0.029	0.032	-0.170	0.026	0.286*	0.178	1.000						
GPX	0.263*	0.054	0.077	-0.027	-0.141	-0.304*	-0.129	0.160	-0.298*	0.079	-0.160	0.019	0.223	1.000					
GST	0.39**	-0.017	0.067	0.131	-0.150	-0.221	-0.071	0.103	-0.158	0.190	-0.017	0.141	0.211	0.369**	1.000				
GR	0.213	0.385**	0.337**	0.269*	0.182	0.235	-0.175	-0.013	0.007	-0.099	0.129	0.212	0.341**	0.076	-0.007	1.000			
GSH	0.473**	-0.166	-0.267	-0.263*	-0.462**	-0.274*	-0.095	0.169	-0.268*	0.358**	-0.024	0.229	0.291*	0.433**	0.506**	0.1	1.000		
MDA	-0.160	-0.591**	-0.664**	-0.679**	-0.566**	-0.324*	-0.013	0.312*	-0.261*	0.340**	-0.270*	-0.099	-0.289*	-0.094	-0.079	-0.092	0.112	1.000	
Survi	0.285*	0.832**	0.799**	0.738**	0.691**	0.482**	-0.066	-0.297*	0.202	-0.493**	0.181	0.23	0.441**	0.148	0.128	0.301*	-0.076	-0.775**	1.000

Table 7.2d Correlation matrix between survival rate and haematological parameters of WSSV-infected *P. monodon* exposed to zinc.

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	GI	Ch	SOD	CAT	GPx	GST	GR	GSH	MDA	Survi
THC	1.000																		
PO	0.286*	1.000																	
NBT	0.439**	0.283*	1.000																
ALP	0.455**	0.180	0.521**	1.000															
ACP	0.286*	0.355**	0.525**	0.787**	1.000														
TP	0.343	0.478**	0.386**	0.304*	0.379**	1.000													
TC	0.554**	0.183	0.641**	0.539**	0.540**	0.315*	1.000												
TFAA	-0.129	-0.211	-0.102	-0.077	-0.250	-0.141	-0.225	1.000											
TL	0.508**	0.488**	0.400**	0.662**	0.694**	0.486**	0.439**	-0.091	1.000										
GI	0.168	0.320*	0.056	-0.220	-0.028	0.481**	0.015	0.085	0.294*	1.000									
Ch	0.361**	0.004	0.605**	0.689**	0.549**	0.138	0.675**	0.007	0.375**	-0.192	1.000								
SOD	-0.391**	0.289*	-0.315*	-0.484**	-0.265*	0.065	-0.430**	-0.218	-0.138	0.261*	-0.662**	1.000							
CAT	0.258*	-0.020	-0.030	0.206	0.135	-0.100	0.244	0.016	0.104	-0.282*	0.231	-0.215	1.000						
GPX	-0.127	0.403**	-0.222	-0.435**	-0.260*	0.264*	-0.274*	0.023	-0.012	0.518**	-0.524**	0.501**	-0.23	1.000					
GST	-0.071	0.204	-0.012	-0.267	-0.048	-0.049	-0.161	0.327*	0.058	0.421**	-0.048	0.002	-0.285	0.165	1.000				
GR	-0.161	0.469**	-0.014	0.183	0.381**	0.273*	-0.063	-0.216	0.309*	0.149	-0.112	0.467**	-0.251	0.295*	-0.012	1.000			
GSH	-0.115	0.315*	-0.281*	-0.503**	-0.376**	0.01	-0.378**	0.202	-0.048	0.570**	-0.534**	0.507**	-0.174	0.557**	0.494**	0.23	1.000		
MDA	-0.652**	-0.446**	-0.615**	-0.704**	-0.661**	-0.283*	-0.614**	0.308*	-0.590**	0.306	-0.560**	0.390**	-0.204	0.292*	0.095	0.017	0.365**	1.000	
Survi	0.526**	0.796**	0.473**	0.516**	0.636**	0.588**	0.403**	-0.192	0.695**	0.353**	0.275*	0.047	-0.04	0.188	0.102	0.516**	0.084	-0.633**	1.000

**$P<0.01$, *$P<0.05$

Table 7.2e Correlation matrix between survival rate and haematological parameters of (WSSV or Vibrio) infected *P. monodon* (untreated or treated).

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	Gl	Ch	Survi
THC	1.000											
PO	0.007	1.000										
NBT	0.495**	0.284**	1.000									
ALP	0.231**	0.167*	0.503**	1.000								
ACP	0.002	0.179*	0.509**	0.623**	1.000							
TP	0.341**	-0.073	0.434**	0.362**	0.394**	1.000						
TC	0.096	0.515**	0.273**	0.215**	0.170*	-0.009	1.000					
TFAA	0.120	-0.158*	-0.202**	-0.115	-0.251**	0.014	-0.231**	1.000				
TL	0.270**	0.182*	0.239**	0.313**	0.369**	0.368**	0.312**	0.045	1.000			
Gl	0.304**	-0.087	-0.094	-0.151*	-0.437**	0.067	-0.031	0.468**	0.003	1.000		
Ch	0.021	0.383**	0.115	0.287**	0.145*	0.023	0.444**	0.100	0.425**	0.091	1.000	
Survi	0.335**	0.479**	0.580**	0.567**	0.608**	0.399**	0.334**	-0.111	0.436**	-0.154*	0.247	1.000

**$P<0.01$, *$P<0.05$

THC -total haemocyte count, PO -phenol oxidase, activity, NBT -NBT reduction, ALP -alkaline phosphatase activity, ACP -acid phosphatase activity, TP -total protein, TC -total carbohydrates, TFAA -total free amino acids, TL -total lipids, Gl -glucose, Ch -cholesterol, SOD -superoxide dismutase activity, CAT -catalase activity, GPx -glutathione peroxidase activity, GST -glutathione *S*-transferase activity, GR -glutathione reductase activity, GSH -total reduced glutathione, MDA -malondialdehyde, Survi -Survival.

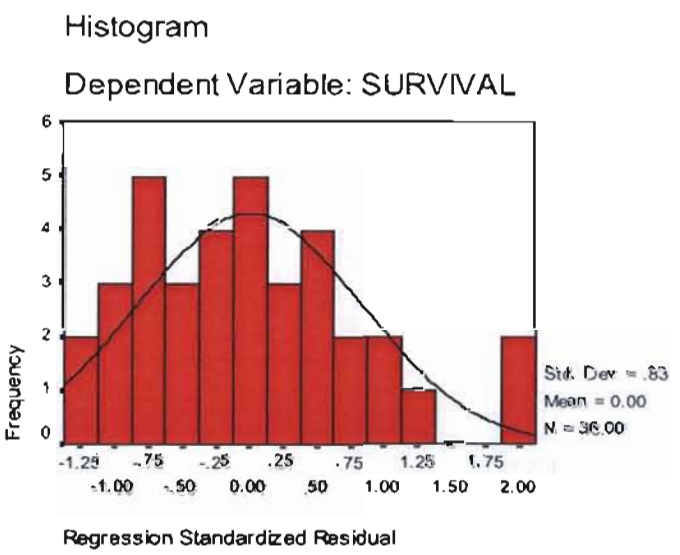
Table 7.3a Multiple regression of survival rate on haematological parameters of *Vibrio*-infected *P. monodon* subjected to salinity stress.

R Square- 0.897
Predictors-THC, PO, NBT, ALP, ACP, TP, TC, TFAA, TL, Gl, Ch
Dependent variable -Survival

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	Gl	Ch
Significance	0.093	0.296	0.001**	0.009**	0.732	0.001**	0.850	0.140	0.002**	0.839	0.819

R Square- 0.860
Predictors -NBT, TP, TL, ALP
Dependent variable -Survival

**$P \leq 0.01$, *$P \leq 0.05$



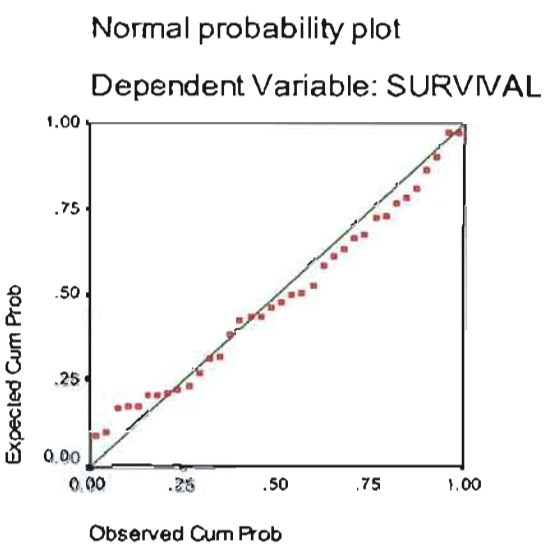


Fig.7.3a Regression plots for the multiple regression of survival rate on haematological parameters of *Vibrio*-infected *P. monodon* subjected to salinity stress.

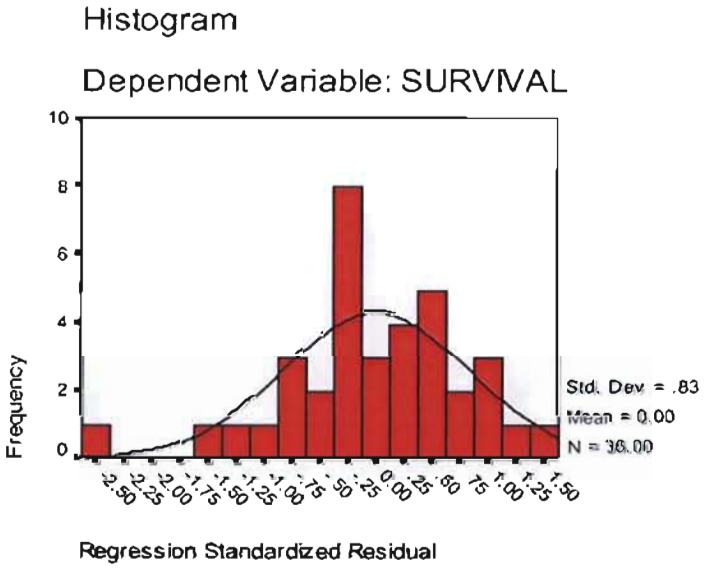
Table 7.3b Multiple regression of survival rate on haematological parameters of WSSV-infected *P. monodon* subjected to salinity stress.

R Square- 0.890
Predictors-THC, PO, NBT, ALP, ACP, TP, TC, TFAA, TL, GI, Ch
Dependent variable -Survival

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	GI	Ch
Significance	0.000**	0.042*	0.212	0.001**	0.456	0.841	0.78	0.058	0.256	0.777	0.234

R Square- 0.804
Predictors -THC, ALP, PO
Dependent variable -Survival

**$P<0.01$, *$P<0.05$



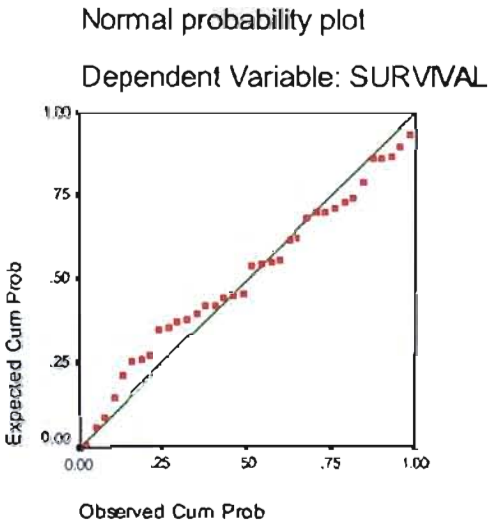


Fig.7.3b Regression plots for the multiple regression of survival rate on haematological parameters of WSSV-infected *P. monodon* subjected to salinity stress.

Table 7.3c Multiple regression of survival rate on haematological parameters of WSSV-infected *P. monodon* exposed to copper.

R Square- 0.929

Predictors-THC, PO, NBT, ALP, ACP, TP, TC, TFAA, TL, GI, Ch, SOD, CAT, GPX, GST, GR, GSH, MDA

Dependent variable -Survival

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	GI	Ch	SOD	CAT	GPX	GST	GR	GSH	MDA
Significance	0.001**	0.042*	0.906	0.237	0.047*	0.376	0.589	0.712	0.609	0.035*	0.901	0.063	0.012*	0.161	0.864	0.375	0.948	0.003**

R Square- 0.851

Predictors -THC, PO, ACP, GI, CAT, MDA

Dependent variable -Survival

**$P<0.01$, *$P<0.05$

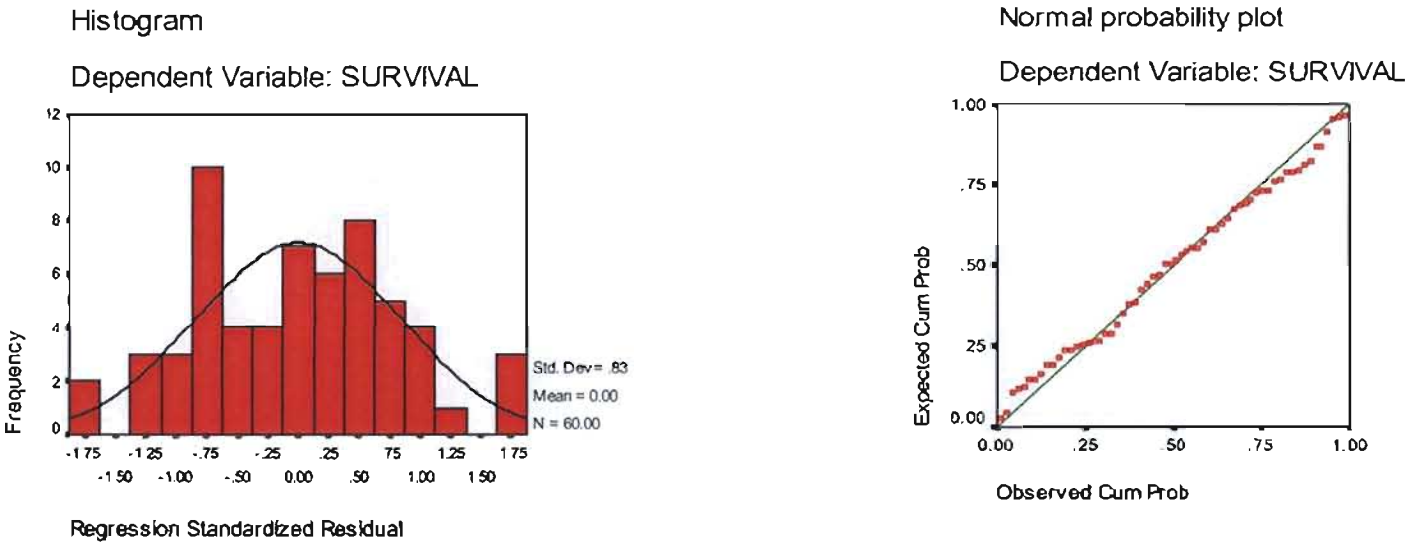


Fig.7.3c Regression plots for the multiple regression of survival rate on haematological parameters of WSSV-infected *P. monodon* exposed to copper.

Table 7.3e Multiple regression of survival rate on haematological parameters of (WSSV or *Vibrio*) infected *P. monodon* (untreated or treated).

R Square 0.658
Predictors-THC, PO, NBT, ALP, ACP, TP, TC, TFAA, TL, GI, Ch
Dependent variable -Survival

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	GI	Ch
Significance	0.001**	0.000**	0.396	0.008**	0.000**	0.055	0.731	0.246	0.076	0.529	0.262

R Square 0.636
Predictors -PO, ACP, THC, ALP
Dependent variable -Survival

**$P<0.01$, *$P<0.05$

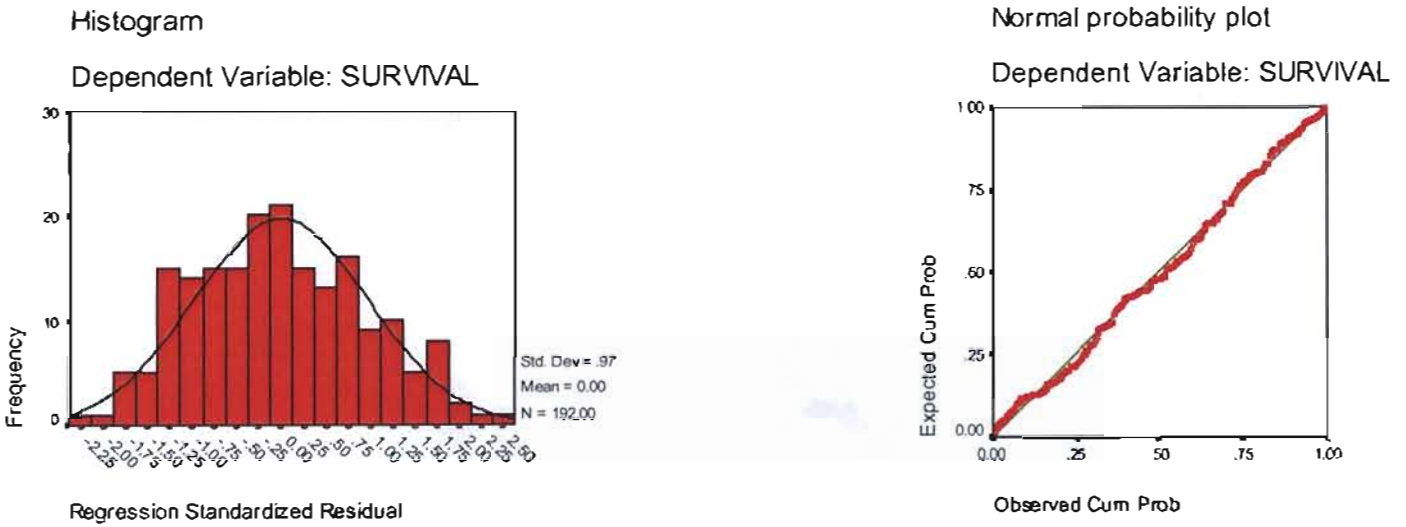


Fig.7.3e Regression plots for the multiple regression of survival rate on haematological parameters of (WSSV or *Vibrio*) infected *P. monodon* (untreated or treated).

CHAPTER 8

Summary and Conclusion

8.1 Summary

Tiger shrimp, *Penaeus mondon*, the widely cultured penaeid shrimp species is highly susceptible to infectious diseases. White spot syndrome caused by WSSV and very often, luminous vibriosis caused by *Vibrio harveyi* has emerged as the major constraints in shrimp production. The prevention and control of diseases has hence gained priority. Though diseases are often linked to the stressful environment in culture systems, there is a lack of scientific evidence in this regard. Further, the information regarding the biochemical and physiological alterations and immune responses of shrimps associated with various stress conditions including pathogenic invasion is limited. These in turn, has become a major barrier towards the development of better management practices and disease prevention strategies. Under this purview, a haematological analysis of *P. monodon* was conducted, the stress responses being well reflected in the composition of haemolymph. Metabolic variables *viz.*, total protein, total carbohydrates, total free amino acids (TFAA), total lipids, glucose and cholesterol and immune variables *viz.*, total haemocyte count (THC), phenol oxidase activity (PO), nitroblue tetrazolium salt reduction (NBT), alkaline phosphatase activity (ALP) and acid phosphatase activity (ACP) were the haematological parameters analysed. Objectives of the study were:

- Acquiring information on the metabolic and immune responses of *P. monodon* to *V. harveyi* and WSSV infection.
- Assessing the effects of acute salinity stress on the metabolic and immune responses and susceptibility of *P. monodon* to *V. harveyi* and WSSV infection.
- Assessing the modulatory effects of ambient copper and zinc on the metabolic and immune responses and susceptibility of *P. monodon* to WSSV infection.
- Assessing the modulatory effects of ambient copper and zinc on the antioxidative defense responses of *P. monodon* against WSSV infection.
- Identifying the most potential haematological biomarkers of health in *P. monodon*.

8.1.1 Salient findings

Salient findings of the study may be summarized as follows:

- Acute salinity change to 35‰ induced an increase in the haemolymph biochemical variables except TFAA, which was found to increase in shrimps at 5 and 0‰.

- A reduction in immune variables could be observed in shrimps subjected to acute salinity stress except for PO and ALP. The suppression was maximum at 5 and 0‰.

- An enhancement in metabolic and immune variables could be observed in shrimps following *V. harveyi* challenge that was maximum on PCD7 in case of immune variables and on PCD2 in case of metabolic variables.

- Significantly higher metabolic and immune variables were exhibited by *Vibrio*-challenged shrimps maintained at 15‰ whereas, a comparative reduction observed in *Vibrio*-challenged shrimps under salinity stress.

- Maximum post challenge survival following *V. harveyi* challenge was observed in shrimps maintained at 15‰. Acute salinity stress enhanced the susceptibility of *P. monodon* to *V. harveyi* infection and reduced the survival rate.

- Pathogenicity of *V. harveyi* was found to be higher at 35‰ than at 5‰.

- Haemolymph metabolic and immune variables showed a general increase following WSSV infection on PCD2 in shrimps maintained at 15‰ and the immune activities showed a declining trend on PCD5.

- A comparative reduction in metabolic variables and weaker immune response was observed in WSSV-infected shrimps under salinity stress.

- Maximum post challenge survival following WSSV challenge was observed in shrimps maintained at 15‰. Acute salinity stress reduced the immunocompetence of *P. monodon* and enhanced the susceptibility to WSSV infection.

- Immune responses of shrimps against WSSV infection were better at 35‰, compared to 0‰, the susceptibility being significantly more at a lower salinity (0‰) stress.

- Slightly improved levels of haemolymph total protein, total carbohydrates and total lipids could be observed in shrimps exposed to Cu at 0.1 mg l^{-1} indicating a better physiological condition.

- Cu was found to induce immunomodulation in *P. monodon*. A moderate increase in immune variables was noted in shrimps exposed to Cu at 0.1 mg l^{-1} except for NBT. A significant increase in NBT and a decrease in ALP were observed in shrimps with Cu at 0.3 mg l^{-1}, though THC and PO increased.

- Enhanced immune response and post challenge survival was exhibited by shrimps with Cu at 0.1 mg l^{-1} indicating the immunostimulatory effects of Cu at 0.1 mg l^{-1}.

- Higher doses of Cu had a suppressive effect on the shrimp metabolic and immune response that reduced the post challenge survival.

- A slight increase of ambient Cu at 0.1 mg l^{-1} was proved beneficial to shrimps in improving the physiological condition and in terms of immunostimulation, increasing the immunocompetence to WSSV.

- An increase of ambient Cu to higher levels was proved harmful by exerting negative effects on the metabolic response and immunocompetence to WSSV.

- Zn induced immunomodulation in *P. monodon*. A significant increase in THC and PO was observed in Zn-exposed shrimps with a maximum at 1.0 mg l^{-1}. With Zn at 2.5 mg l^{-1}, a significant increase in NBT was also noted.

- Better metabolic and immune response following WSSV infection in shrimps with Zn at 1.0 mg l^{-1}could be correlated to the enhanced post challenge survival.

- Ambient Zn at 1.0 mg l^{-1} proved immunostimulatory, reducing the susceptibility of *P. monodon* to WSSV infection.

- Immune response to WSSV infection was found weaker in shrimps with higher doses of Zn in ambient water that increased the susceptibility to WSSV infection.

- Extent of lipid peroxidation was found to be comparatively lower in shrimps with Cu at 0.1 mg l^{-1} after WSSV infection.

- In shrimps exposed to Zn at 1.0 mg l^{-1} the extent of lipid peroxidation was found to be lower prior to and after WSSV infection.

- Greater inhibition of antioxidants and a corresponding increase in MDA levels were observed in shrimps with higher doses of Cu and Zn, prior to and after WSSV infection.

- Significant enhancement of antioxidants could be observed in shrimps exposed to Cu at 0.1 mg l^{-1} in haemolymph, hepatopancreas and gills. In shrimps with Zn at 1.0 mg l^{-1} an enhancement of antioxidants could be observed in haemolymph and hepatopancreas, whereas a slight decrease was noticed in gills.

- In general, the antioxidant activity of *P. monodon* significantly declined on WSSV challenge with a minimum on PCD2.

- Comparatively higher antioxidant activity was recorded in shrimps exposed to Cu at 0.1 mg l^{-1} following WSSV infection particularly on PCD5. A higher post challenge antioxidant activity was also observed in WSSV-infected shrimps with Zn at 1.0 mg l^{-1}, particularly in hepatopancreas and gills.

- Greater antioxidant protection was offered by Zn at 1.0 mg l^{-1}compared to Cu at 0.1 mg l^{-1} in WSSV-infected *P. monodon*.

- Metabolic variables could be correlated with some or all of the immune variables. The key metabolic variables that exhibited a positive correlation with the survival rate and immune variables were total protein, total carbohydrates and total lipids.

- Malondialdehyde exhibited a negative correlation with the survival rate, immune variables and the metabolic variables *viz.*, total protein, total lipids and cholesterol.

- THC, PO, ALP and ACP that greatly correlated with the survival rate identified as the most potential biomarkers of health in *P. monodon*.

8.2 Future prospects

A normal/healthy range for the haematological biomarkers screened from the present study may be formulated for *P. monodon* of various sizes, moult stages and developmental stages that could serve as a reference scale for the periodic assessment of shrimp health status.

Further bioassays to harness the immunostimulatory effects of Cu at 0.1 mg l^{-1} and Zn at 1.0 mg l^{-1} for standardising the time of exposure and to investigate any influence on the market size achieved. The effects of cumulative application of both Cu and Zn may be investigated.

8.3 Conclusion

It may be concluded from the present study that stress is accompanied by alterations in haemolymph metabolic variables and immune responses that influences the susceptibility of *P. monodon* to infection. Acute salinity variations were proved to be a stress condition that enhances the susceptibility of *P. monodon* to *V. harveyi* and WSSV infection. Ambient Cu at 0.1 mg l^{-1} and ambient Zn at 1.0 mg l^{-1} proved immunostimulatory in increasing the immunocompetence of *P. monodon* to WSSV infection and higher concentrations of Cu and Zn proved immunosuppressive. Haemolymph total protein, total carbohydrates and total lipids showed the highest relation with immune responses. THC, PO, ACP and ALP that greatly correlated with the survival rate proposed as reliable biomarkers of health in *P. monodon*. The study highlights the need for proper management practices and regular health monitoring to be adopted to avoid mass mortality in shrimp culture ponds.

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Appendices

Appendix 1

Table 6.1 Malondialdehyde levels in *P. monodon* exposed to copper and challenged with WSSV.

		MDA (nM ml^{-1} haemolymph; mM 100^{-1} g wet tissue)			
Shrimp tissue	**Cu (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	1.94 ± 0.28	$_{b}1.92 \pm 0.28^{A}$	$_{c}1.96 \pm 0.23^{A}$	$_{e}1.83 \pm 0.37^{A}$
	0		$_{b}1.93 \pm 0.28^{C}$	$_{c}2.45 \pm 0.43^{B}$	$_{b}3.19 \pm 0.34^{A}$
	0.05		$_{b}1.83 \pm 0.36^{B}$	$_{c}2.22 \pm 0.38^{AB}$	$_{c}2.66 \pm 0.59^{A}$
	0.1		$_{b}1.91 \pm 0.28^{A}$	$_{c}2.03 \pm 0.38^{A}$	$_{de}2.18 \pm 0.38^{A}$
	0.2		$_{a}3.04 \pm 0.45^{B}$	$_{a}3.84 \pm 0.40^{A}$	$_{a}3.99 \pm 0.35^{A}$
	0.3		$_{a}2.73 \pm 0.34^{B}$	$_{b}2.93 \pm 0.49^{B}$	$_{ab}3.62 \pm 0.40^{A}$
Hepatopancreas	**Control**	2.31 ± 0.47	$_{b}2.16 \pm 0.43^{A}$	$_{d}2.11 \pm 0.50^{A}$	$_{d}2.28 \pm 0.40^{A}$
	0		$_{b}2.24 \pm 0.40^{B}$	$_{b}3.43 \pm 0.64^{A}$	$_{bc}3.42 \pm 0.56^{A}$
	0.05		$_{b}2.12 \pm 0.51^{B}$	$_{b}3.52 \pm 0.59^{A}$	$_{ab}3.66 \pm 0.59^{A}$
	0.1		$_{b}2.04 \pm 0.35^{B}$	$_{c}2.77 \pm 0.55^{A}$	$_{cd}2.90 \pm 0.47^{A}$
	0.2		$_{a}3.08 \pm 0.39^{B}$	$_{b}3.56 \pm 0.53^{AB}$	$_{ab}3.94 \pm 0.51^{A}$
	0.3		$_{a}2.88 \pm 0.37^{B}$	$_{a}4.70 \pm 0.60^{A}$	$_{a}4.31 \pm 0.43^{A}$
Gills	**Control**	1.88 ± 0.22	$_{c}1.90 \pm 0.26^{A}$	$_{c}1.73 \pm 0.26^{A}$	$_{c}1.80 \pm 0.24^{A}$
	0		$_{c}1.78 \pm 0.24^{B}$	$_{b}2.24 \pm 0.33^{A}$	$_{b}2.61 \pm 0.38^{A}$
	0.05		$_{c}1.68 \pm 0.44^{B}$	$_{b}2.46 \pm 0.42^{A}$	$_{ab}2.77 \pm 0.26^{A}$
	0.1		$_{c}1.95 \pm 0.36^{A}$	$_{b}2.13 \pm 0.25^{A}$	$_{c}2.11 \pm 0.30^{A}$
	0.2		$_{b}2.40 \pm 0.39^{B}$	$_{a}2.94 \pm 0.34^{A}$	$_{a}3.02 \pm 0.27^{A}$
	0.3		$_{a}3.03 \pm 0.37^{A}$	$_{a}2.95 \pm 0.38^{A}$	$_{a}3.08 \pm 0.24^{A}$

Table 6.2 Superoxide dismutase activity in *P. monodon* exposed to copper and challenged with WSSV.

		SOD (50% inhibition of formazan formation min^{-1} mg^{-1} protein)			
Shrimp tissue	**Cu (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	10.09 ± 1.78	$_{c}10.15 \pm 1.68^{A}$	$_{a}10.82 \pm 1.76^{A}$	$_{ab}9.86 \pm 1.25^{A}$
	0		$_{c}10.40 \pm 1.28^{A}$	$_{bc}8.17 \pm 1.97^{A}$	$_{ab}9.76 \pm 2.23^{A}$
	0.05		$_{ab}15.40 \pm 2.59^{A}$	$_{ab}9.58 \pm 2.46^{B}$	$_{a}12.18 \pm 2.71^{B}$
	0.1		$_{b}13.49 \pm 2.09^{A}$	$_{a}11.28 \pm 2.05^{AB}$	$_{ab}9.83 \pm 1.79^{B}$
	0.2		$_{ab}15.19 \pm 2.99^{A}$	$_{cd}6.26 \pm 1.70^{C}$	$_{ab}10.43 \pm 2.07^{B}$
	0.3		$_{a}17.90 \pm 2.89^{A}$	$_{d}5.25 \pm 1.29^{C}$	$_{b}9.33 \pm 2.46^{B}$
Hepatopancreas	**Control**	6.72 ± 0.51	$_{a}6.87 \pm 0.63^{A}$	$_{a}6.73 \pm 0.44^{A}$	$_{a}6.86 \pm 0.48^{A}$
	0		$_{a}6.77 \pm 0.57^{A}$	$_{bc}5.21 \pm 0.66^{B}$	$_{bc}5.94 \pm 0.68^{B}$
	0.05		$_{bc}6.06 \pm 0.50^{A}$	$_{c}4.69 \pm 0.52^{C}$	$_{cd}5.32 \pm 0.43^{B}$
	0.1		$_{ab}6.60 \pm 0.50^{A}$	$_{bc}5.39 \pm 0.53^{B}$	$_{ab}6.43 \pm 0.75^{A}$
	0.2		$_{c}5.89 \pm 0.61^{A}$	$_{b}5.47 \pm 0.47^{A}$	$_{d}4.78 \pm 0.44^{B}$
	0.3		$_{c}5.47 \pm 0.45^{A}$	$_{bc}5.37 \pm 0.64^{A}$	$_{cd}5.20 \pm 0.71^{A}$
Gills	**Control**	11.08 ± 1.35	$_{b}11.33 \pm 1.30A$	$_{a}10.94 \pm 1.13A$	$_{a}11.52 \pm 1.23A$
	0		$_{b}11.18 \pm 1.25A$	$_{e}6.87 \pm 0.93C$	$_{b}9.36 \pm 1.0B$
	0.05		$_{a}13.62 \pm 1.68A$	$_{bc}8.96 \pm 1.01B$	$_{c}7.25 \pm 0.72C$
	0.1		$_{b}10.84 \pm 1.40A$	$_{cd}8.38 \pm 1.57B$	$_{b}9.20 \pm 1.41AB$
	0.2		$_{b}9.96 \pm 1.71A$	$_{ab}9.84 \pm 1.40A$	$_{c}7.41 \pm 0.81B$
	0.3		$_{c}7.49 \pm 0.67A$	$_{de}7.04 \pm 0.92A$	$_{d}3.05 \pm 0.95B$

Appendix 2

Table 6.3 Catalase activity in *P. monodon* exposed to copper and challenged with WSSV.

		Catalase (IU mg^{-1} protein)			
Shrimp tissue	**Cu (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	20.95 ± 2.64	$_{b}21.13 \pm 2.97^{A}$	$_{a}20.39 \pm 2.89^{A}$	$_{a}22.06 \pm 2.84^{A}$
	0		$_{b}21.15 \pm 2.33^{A}$	$_{b}10.59 \pm 2.94^{C}$	$_{b}14.85 \pm 3.06^{B}$
	0.05		$_{a}28.40 \pm 4.03^{A}$	$_{b}10.86 \pm 1.83^{C}$	$_{b}15.75 \pm 2.50^{B}$
	0.1		$_{a}27.99 \pm 4.41^{A}$	$_{b}11.52 \pm 3.24^{B}$	$_{b}13.71 \pm 3.62^{B}$
	0.2		$_{b}19.76 \pm 3.18^{A}$	$_{b}9.85 \pm 2.37^{B}$	$_{c}8.88 \pm 2.42^{B}$
	0.3		b19.93 ± 3.19^{A}	$_{b}9.67 \pm 1.71^{B}$	$_{c}9.56 \pm 2.77^{B}$
Hepatopancreas	**Control**	32.65 ± 2.10	$_{a}31.87 \pm 3.59^{A}$	$_{a}31.02 \pm 2.52^{A}$	$_{a}30.82 \pm 3.01^{A}$
	0		$_{a}30.94 \pm 2.52^{A}$	$_{bc}20.50 \pm 3.43^{B}$	$_{ab}27.23 \pm 3.47^{A}$
	0.05		$_{b}26.49 \pm 1.43^{A}$	$_{bc}20.26 \pm 3.41^{B}$	$_{c}19.47 \pm 3.12^{B}$
	0.1		$_{a}30.51 \pm 3.37^{A}$	$_{b}22.47 \pm 4.26^{B}$	$_{b}25.62 \pm 3.87^{B}$
	0.2		$_{c}19.44 \pm 3.83^{A}$	$_{c}17.15 \pm 3.53^{A}$	$_{c}16.78 \pm 2.49^{A}$
	0.3		$_{c}22.13 \pm 4.09^{A}$	$_{c}17.51 \pm 2.53^{B}$	$_{c}15.51 \pm 3.27^{B}$
Gills	**Control**	30.14 ± 3.21	$_{b}31.51 \pm 2.87^{A}$	$_{a}30.85 \pm 3.54^{A}$	$_{a}31.15 \pm 2.89^{A}$
	0		$_{b}30.61 \pm 2.63^{A}$	$_{b}18.98 \pm 3.58^{B}$	$_{c}18.87 \pm 3.07^{B}$
	0.05		$_{a}37.58 \pm 4.11^{A}$	$_{cd}13.38 \pm 2.65^{B}$	$_{d}14.68 \pm 2.82^{B}$
	0.1		$_{a}38.67 \pm 4.25^{A}$	$_{bc}15.91 \pm 3.73^{C}$	$_{b}22.99 \pm 3.58^{B}$
	0.2		$_{c}24.33 \pm 3.95^{A}$	$_{bc}16.42 \pm 3.39^{B}$	$_{cd}15.80 \pm 2.92^{B}$
	0.3		$_{c}25.45 \pm 4.11^{A}$	$_{d}9.95 \pm 1.85^{B}$	$_{d}13.75 \pm 3.64^{B}$

Table 6.4Glutathione peroxidase activity in *P. monodon* exposed to copper and challenged with WSSV.

		GPx (µg GSH oxidised min^{-1} mg^{-1} protein)			
Shrimp tissue	**Cu (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	3.75 ± 0.42	$_{ab}3.60 \pm 0.40^{A}$	$_{a}3.72 \pm 0.40^{A}$	$_{a}3.81 \pm 0.34^{A}$
	0		$_{a}3.77 \pm 0.34^{A}$	$_{b}2.09 \pm 0.36^{C}$	$_{bc}2.73 \pm 0.29^{B}$
	0.05		$_{d}2.86 \pm 0.31^{A}$	$_{b}2.30 \pm 0.28^{B}$	$_{cd}2.26 \pm 0.57^{B}$
	0.1		$_{a}3.75 \pm 0.49^{A}$	$_{b}2.12 \pm 0.31^{C}$	$_{b}3.2 \pm 0.44^{B}$
	0.2		$_{cd}3.23 \pm 0.44^{A}$	$_{b}2.23 \pm 0.56^{B}$	$_{d}2.03 \pm 0.34^{B}$
	0.3		$_{bc}3.39 \pm 0.35^{A}$	$_{b}2.51 \pm 0.43^{B}$	$_{cd}2.43 \pm 0.47^{B}$
Hepatopancreas	**Control**	2.93 ± 0.33	$_{a}2.90 \pm 0.24^{A}$	$_{a}2.92 \pm 0.25^{A}$	$_{a}2.86 \pm 0.25^{A}$
	0		$_{a}2.93 \pm 0.32^{A}$	$_{b}2.16 \pm 0.31^{B}$	$_{b}2.16 \pm 0.31^{B}$
	0.05		$_{a}2.67 \pm 0.40^{A}$	$_{bc}1.99 \pm 0.29^{B}$	$_{b}2.41 \pm 0.40^{AB}$
	0.1		$_{a}2.88 \pm 0.28^{A}$	$_{b}2.34 \pm 0.36^{B}$	$_{b}2.11 \pm 0.24^{B}$
	0.2		$_{b}1.88 \pm 0.41^{A}$	$_{d}1.04 \pm 0.39^{B}$	$_{c}1.09 \pm 0.25^{B}$
	0.3		$_{b}2.14 \pm 0.42^{A}$	$_{c}1.62 \pm 0.35^{B}$	$_{c}1.16 \pm 0.38^{B}$
Gills	**Control**	6.01 ± 0.68	$_{b}6.16 \pm 0.57^{A}$	$_{a}6.05 \pm 0.61^{A}$	$_{a}6.12 \pm 0.45^{A}$
	0		$_{b}6.07 \pm 0.58^{A}$	$_{c}2.89 \pm 0.43^{B}$	$_{b}3.24 \pm 0.59^{B}$
	0.05		$_{a}7.70 \pm 0.77^{A}$	$_{b}3.59 \pm 0.50^{B}$	$_{cd}2.59 \pm 0.45^{C}$
	0.1		$_{a}7.87 \pm 0.62^{A}$	$_{bc}3.26 \pm 0.46^{B}$	$_{bc}3.13 \pm 0.55^{B}$
	0.2		$_{c}4.26 \pm 0.50^{A}$	$_{c}2.80 \pm 0.53^{B}$	$_{d}2.49 \pm 0.56^{B}$
	0.3		$_{c}4.08 \pm 0.55^{A}$	$_{bc}3.18 \pm 0.41^{B}$	$_{d}2.45 \pm 0.39^{C}$

Appendix 3

Table 6.5 Glutathione *S*-transferase activity in *P. monodon* exposed to copper and challenged with WSSV.

		GST(μM of CDNB-GSH conjugate formed min^{-1} mg^{-1} protein)			
Shrimp tissue	**Cu (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	7.51 ± 0.65	$_{b}7.55 \pm 0.71^{A}$	$_{a}7.86 \pm 0.71^{A}$	$_{a}7.51 \pm 0.78^{A}$
	0		$_{b}7.91 \pm 0.77^{A}$	$_{b}2.14 \pm 0.65^{C}$	$_{c}3.07 \pm 0.61^{B}$
	0.05		$_{a}8.91 \pm 1.03^{A}$	$_{b}2.04 \pm 0.65^{B}$	$_{c}2.04 \pm 0.74^{B}$
	0.1		$_{c}5.60 \pm 0.8^{A}$	$_{b}2.29 \pm 0.90^{B}$	$_{b}4.69 \pm 0.99^{A}$
	0.2		$_{c}2.15 \pm 0.59^{A}$	$_{b}2.06 \pm 0.57^{A}$	$_{c}2.44 \pm 1.10^{A}$
	0.3		$_{d}3.16 \pm 0.51^{A}$	$_{b}2.22 \pm 0.75^{B}$	$_{c}2.63 \pm 0.83^{AB}$
Hepatopancreas	**Control**	4.26 ± 0.55	$_{b}4.24 \pm 0.45^{A}$	$_{a}4.21 \pm 0.43^{A}$	$_{a}4.15 \pm 0.44^{A}$
	0		$_{b}4.13 \pm 0.45^{A}$	$_{b}2.50 \pm 0.69^{B}$	$_{bc}2.94 \pm 0.57^{B}$
	0.05		$_{c}3.42 \pm 0.51^{A}$	$_{c}1.68 \pm 0.55^{C}$	$_{cd}2.65 \pm 0.41^{B}$
	0.1		$_{a}5.59 \pm 0.56^{A}$	$_{a}3.67 \pm 0.62^{B}$	$_{b}3.46 \pm 0.22^{B}$
	0.2		$_{d}2.51 \pm 0.41^{A}$	$_{c}1.76 \pm 0.31^{B}$	$_{de}2.21 \pm 0.62^{AB}$
	0.3		$_{d}2.15 \pm 0.47^{A}$	$_{c}1.86 \pm 0.49^{A}$	$_{e}1.93 \pm 0.36^{A}$
Gills	**Control**	6.1 ± 0.69	$_{b}5.95 \pm 0.37^{A}$	$_{a}6.15 \pm 0.55^{A}$	$_{a}6.08 \pm 0.60^{A}$
	0		$_{b}6.05 \pm 0.53^{A}$	$_{b}3.83 \pm 0.67^{B}$	$_{c}2.43 \pm 0.74^{C}$
	0.05		$_{a}7.86 \pm 0.86^{A}$	$_{bcd}3.24 \pm 0.63^{B}$	$_{c}2.12 \pm 0.37^{C}$
	0.1		$_{a}8.17 \pm 0.99^{A}$	$_{bc}3.55 \pm 0.42^{B}$	$_{b}3.49 \pm 0.70^{B}$
	0.2		$_{c}4.68 \pm 0.87^{A}$	$_{cd}3.04 \pm 0.59^{B}$	$_{c}2.30 \pm 0.47^{B}$
	0.3		$_{c}4.21 \pm 0.68^{A}$	$_{d}2.69 \pm 0.63^{B}$	$_{c}2.20 \pm 0.45^{B}$

Table 6.6 Glutathione reductase activity in *P. monodon* exposed to Copper and challenged with WSSV

		GR (μM of NADPH oxidised min^{-1} mg^{-1} protein)			
Shrimp tissue	**Cu (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	20.61 ± 3.20	$_{b}19.98 \pm 2.74^{A}$	$_{a}20.33 \pm 2.7^{A}$	$_{a}19.74 \pm 2.37^{A}$
	0		$_{b}20.68 \pm 2.86^{A}$	$_{b}14.29 \pm 2.71^{B}$	$_{a}18.07 \pm 3.76^{AB}$
	0.05		$_{a}29.69 \pm 3.32^{A}$	$_{c}9.61 \pm 2.51^{C}$	$_{a}20.61 \pm 4.0^{B}$
	0.1		$_{a}25.77 \pm 3.34^{A}$	$_{a}19.33 \pm 3.7^{B}$	$_{a}16.78 \pm 2.40^{B}$
	0.2		$_{a}27.46 \pm 3.72^{A}$	$_{a}22.18 \pm 3.46^{B}$	$_{b}10.91 \pm 2.43^{C}$
	0.3		$_{c}14.17 \pm 3.29^{A}$	$_{c}8.75 \pm 2.15^{B}$	$_{b}11.47 \pm 3.33^{AB}$
Hepatopancreas	**Control**	24.35 ± 2.65	$_{b}24.80 \pm 2.64^{A}$	$_{a}23.99 \pm 2.80^{A}$	$_{a}24.44 \pm 2.54^{A}$
	0		$_{b}24.04 \pm 2.70^{A}$	$_{c}11.75 \pm 2.89^{C}$	$_{bc}17.86 \pm 3.61^{B}$
	0.05		$_{b}21.74 \pm 3.46^{A}$	$_{b}18.62 \pm 2.59^{AB}$	$_{cd}15.05 \pm 3.67^{B}$
	0.1		$_{a}28.54 \pm 3.82^{A}$	$_{c}11.24 \pm 2.41^{C}$	$_{b}19.63 \pm 3.94^{B}$
	0.2		$_{c}16.13 \pm 2.71^{A}$	$_{c}12.59 \pm 3.09^{B}$	$_{d}13.51 \pm 2.33^{AB}$
	0.3		$_{c}14.76 \pm 3.15^{A}$	$_{d}5.21 \pm 1.01^{B}$	$_{d}12.82 \pm 2.09^{A}$
Gills	**Control**	22.85 ± 2.38	$_{b}23.18 \pm 2.44^{A}$	$_{a}22.24 \pm 2.76^{A}$	$_{a}23.63 \pm 2.24^{A}$
	0		$_{b}22.56 \pm 2.68^{A}$	$_{c}11.29 \pm 2.48^{B}$	$_{d}11.87 \pm 2.42^{B}$
	0.05		$_{a}30.01 \pm 3.39^{A}$	$_{c}11.73 \pm 1.72^{C}$	$_{b}18.66 \pm 2.48^{B}$
	0.1		$_{a}27.99 \pm 3.14^{A}$	$_{a}20.65 \pm 2.55^{B}$	$_{c}15.34 \pm 2.68^{C}$
	0.2		$_{c}18.64 \pm 2.90^{A}$	$_{b}16.58 \pm 3.12^{A}$	$_{c}9.11 \pm 1.88^{B}$
	0.3		$_{c}17.54 \pm 2.98^{A}$	$_{d}8.17 \pm 2.08^{C}$	$_{d}12.08 \pm 2.0^{B}$

Appendix 4

Table 6.7 Total reduced Glutathione levels in *P. monodon* exposed to Cu and challenged with WSSV.

		GSH (μg mg^{-1} protein)			
Shrimp tissue	**Cu (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	4.96 ± 0.43	$_{a}4.88 \pm 0.57^{A}$	$_{a}4.91 \pm 0.48^{A}$	$_{a}4.81 \pm 0.60^{A}$
	0		$_{ab}4.75 \pm 0.51^{A}$	$_{b}2.27 \pm 0.45^{C}$	$_{bc}3.35 \pm 0.54^{B}$
	0.05		$_{ab}4.78 \pm 0.66^{A}$	$_{b}2.26 \pm 0.77^{B}$	$_{bc}3.04 \pm 0.57^{B}$
	0.1		$_{bc}4.15 \pm 0.45^{A}$	$_{b}2.0 \pm 0.48^{B}$	$_{b}3.74 \pm 0.65^{A}$
	0.2		$_{d}3.45 \pm 0.58^{A}$	$_{b}2.47 \pm 0.57^{B}$	$_{bc}3.28 \pm 0.52^{A}$
	0.3		$_{cd}3.82 \pm 0.47^{A}$	$_{b}2.26 \pm 0.47^{B}$	$_{c}2.74 \pm 0.57^{B}$
Hepatopancreas	**Control**	13.31 ± 1.63	$_{ab}13.43 \pm 1.74^{A}$	$_{a}12.75 \pm 1.48^{A}$	$_{a}12.84 \pm 1.5^{A}$
	0		$_{bc}12.87 \pm 1.49^{A}$	$_{b}6.63 \pm 1.62^{B}$	$_{c}8.61 \pm 1.73^{B}$
	0.05		$_{bc}12.76 \pm 1.42^{A}$	$_{b}5.40 \pm 0.98^{B}$	$_{d}6.57 \pm 1.0^{B}$
	0.1		$_{a}15.26 \pm 1.99^{A}$	$_{b}5.71 \pm 1.23^{C}$	$_{b}10.75 \pm 2.40^{B}$
	0.2		$_{bc}11.79 \pm 1.91^{A}$	$_{b}7.05 \pm 1.32^{B}$	$_{c}8.78 \pm 1.20^{B}$
	0.3		$_{c}10.68 \pm 2.09^{A}$	$_{b}5.72 \pm 1.87^{B}$	$_{c}4.55 \pm 0.81^{B}$
Gills	**Control**	3.59 ± 0.48	$_{c}3.63 \pm 0.49^{A}$	$_{a}3.54 \pm 0.50^{A}$	$_{a}3.70 \pm 0.56^{A}$
	0		$_{c}3.60 \pm 0.41^{A}$	$_{b}2.74 \pm 0.41^{B}$	$_{c}1.57 \pm 0.49^{C}$
	0.05		$_{a}5.34 \pm 0.65^{A}$	$_{c}1.92 \pm 0.27^{C}$	$_{b}2.72 \pm 0.48^{B}$
	0.1		$_{b}4.54 \pm 0.63^{A}$	$_{c}2.16 \pm 0.36^{C}$	$_{b}2.95 \pm 0.44^{B}$
	0.2		$_{d}2.83 \pm 0.37^{A}$	$_{c}2.08 \pm 0.41^{B}$	$_{c}1.49 \pm 0.29^{C}$
	0.3		$_{cd}3.42 \pm 0.50^{A}$	$_{c}1.74 \pm 0.41^{B}$	$_{c}1.85 \pm 0.31^{B}$

*Data (Mean ± S.D.) in the same column (for each tissue) with different subscripts are statistically different ($P<0.05$) among treatments at the same exposure time and data (Mean ± S.D.) in the same row (for each tissue) with different superscripts are statistically different ($P<0.05$) among different time periods.
BI –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

Appendix 5

Table 6.8 Malondialdehyde levels in *P. monodon* exposed to zinc and then challenged with WSSV.

		MDA (nM ml^{-1} haemolymph; mM 100^{-1} g wet tissue)			
Shrimp tissue	**Zn (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	1.50 ± 0.41	$_{ab}1.65 \pm 0.50_{A}$	$_{d}1.39 \pm 0.56^{A}$	$_{e}1.50 \pm 0.52^{A}$
	0		$_{ab}1.54 \pm 0.65^{C}$	$_{ab}4.47 \pm 0.72^{B}$	$_{bc}8.07 \pm 1.44^{A}$
	0.5		$_{ab}1.62 \pm 0.70^{B}$	$_{cd}2.36 \pm 1.33^{B}$	$_{c}7.67 \pm 1.85^{A}$
	1.0		$_{b}1.07 \pm 0.39^{B}$	$_{d}1.41 \pm 0.52^{B}$	$_{d}4.80 \pm 0.97^{A}$
	1.5		$_{a}2.12 \pm 0.31^{B}$	$_{bc}3.37 \pm 1.09^{B}$	$_{a}10.24 \pm 1.67^{A}$
	2.5		$_{a}2.17 \pm 0.93^{C}$	$_{a}5.38 \pm 1.59^{B}$	$_{ab}9.73 \pm 2.17^{A}$
Hepatopancreas	**Control**	1.78 ± 0.31	$_{b}1.63 \pm 0.25^{A}$	$_{c}1.53 \pm 0.29^{A}$	$_{c}1.56 \pm 0.28^{A}$
	0		$_{b}1.67 \pm 0.31^{B}$	$_{b}2.07 \pm 0.36^{AB}$	$_{b}2.32 \pm 0.37^{A}$
	0.5		$_{b}1.80 \pm 0.28^{B}$	$_{b}2.21 \pm 0.26^{A}$	$_{b}2.14 \pm 0.33^{AB}$
	1.0		$_{b}1.70 \pm 0.31^{B}$	$_{b}2.21 \pm 0.36^{A}$	$_{b}2.22 \pm 0.37^{A}$
	1.5		$_{b}2.54 \pm 0.45^{B}$	$_{a}3.66 \pm 0.38^{A}$	$_{a}3.36 \pm 0.49^{A}$
	2.5		$_{b}2.25 \pm 0.28^{B}$	$_{a}3.60 \pm 0.48^{A}$	$_{a}3.68 \pm 0.31^{A}$
Gills	**Control**	1.11 ± 0.22	$_{abc}1.12 \pm 0.27^{A}$	$_{c}1.18 \pm 0.28^{A}$	$_{b}0.97 \pm 0.16^{A}$
	0		$_{abc}1.09 \pm 0.28^{A}$	$_{c}1.10 \pm 0.19^{A}$	$_{ab}1.24 \pm 0.28^{A}$
	0.5		$_{bc}0.97 \pm 0.24^{A}$	$_{bc}1.22 \pm 0.30^{A}$	$_{b}1.13 \pm 0.31^{A}$
	1.0		$_{c}0.82 \pm 0.15^{A}$	$_{c}0.89 \pm 0.20^{A}$	$_{b}0.99 \pm 0.26^{A}$
	1.5		$_{ab}1.24 \pm 0.28^{B}$	$_{a}2.14 \pm 0.38^{A}$	$_{a}1.55 \pm 0.30^{B}$
	2.5		$_{a}1.31 \pm 0.23^{A}$	$_{b}1.52 \pm 0.25^{A}$	$_{a}1.57 \pm 0.30^{A}$

Table 6.9 Superoxide dismutase activity in *P. monodon* exposed to zinc and then challenged with WSSV.

		SOD (50% inhibition of formazan formation min^{-1} mg^{-1} protein)			
Shrimp tissue	**Zn (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	13.05 ± 1.51	$_{ab}13.06 \pm 1.76^{A}$	$_{a}13.11 \pm 1.47^{A}$	$_{b}13.36 \pm 1.52^{A}$
	0		$_{ab}13.28 \pm 2.40^{A}$	$_{b}10.71 \pm 1.88^{A}$	$_{b}10.87 \pm 1.85^{A}$
	0.5		$_{ab}13.34 \pm 2.62^{A}$	$_{c}7.22 \pm 1.56^{B}$	$_{b}13.52 \pm 2.75^{A}$
	1.0		$_{a}15.03 \pm 1.66^{B}$	$_{b}9.58 \pm 2.03^{C}$	$_{a}19.79 \pm 3.60^{A}$
	1.5		$_{c}9.88 \pm 2.06^{B}$	$_{bc}8.58 \pm 2.18^{B}$	$_{a}18.27 \pm 2.55^{A}$
	2.5		$_{bc}11.65 \pm 2.52^{A}$	$_{c}6.77 \pm 1.80^{B}$	$_{b}12.90 \pm 2.78^{A}$
Hepatopancreas	**Control**	7.14 ± 0.68	$_{ab}7.28 \pm 0.62^{A}$	$_{a}7.34 \pm 0.47^{A}$	$_{a}7.12 \pm 0.59^{A}$
	0		$_{ab}7.24 \pm 0.56^{A}$	$_{b}6.26 \pm 0.57^{B}$	$_{bc}4.65 \pm 0.60^{C}$
	0.5		$_{a}8.06 \pm 0.64^{A}$	$_{b}5.63 \pm 0.70^{C}$	$_{a}7.05 \pm 0.62^{B}$
	1.0		$_{a}8.0 \pm 0.70^{A}$	$_{a}7.27 \pm 0.69^{AB}$	$_{a}6.72 \pm 0.76^{B}$
	1.5		$_{b}6.96 \pm 0.95^{A}$	$_{b}5.96 \pm 0.76^{AB}$	$_{b}5.36 \pm 0.75^{B}$
	2.5		$_{b}7.0 \pm 0.86^{A}$	$_{c}4.61 \pm 0.79^{B}$	$_{c}4.49 \pm 0.79^{B}$
Gills	**Control**	10.33 ± 1.46	$_{a}11.01 \pm 1.51^{A}$	$_{a}10.71 \pm 1.65^{A}$	$_{ab}10.23 \pm 1.20^{A}$
	0		$_{a}10.84 \pm 1.86^{A}$	$_{bc}7.50 \pm 1.91^{B}$	$_{bc}8.68 \pm 1.23^{B}$
	0.5		$_{a}10.91 \pm 1.72^{A}$	$_{ab}9.23 \pm 1.92^{A}$	$_{bc}9.0 \pm 1.05^{A}$
	1.0		$_{ab}9.96 \pm 1.38^{A}$	$_{a}10.56 \pm 1.69^{A}$	$_{a}11.58 \pm 2.03^{A}$
	1.5		$_{b}8.67 \pm 1.65^{A}$	$_{c}5.93 \pm 1.16^{B}$	$_{d}6.52 \pm 1.83^{B}$
	2.5		$_{b}8.72 \pm 1.49^{A}$	$_{ab}9.57 \pm 2.28^{A}$	$_{cd}7.72 \pm 1.29^{A}$

Appendix 6

Table 6.10 Catalase activity in *P. monodon* exposed to zinc and then challenged with WSSV.

		Catalase (IU mg^{-1} protein)			
Shrimp tissue	Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Haemolymph	Control	29.45 ± 1.99	$_{a}28.72 \pm 2.67^{A}$	$_{a}29.33 \pm 1.97^{A}$	$_{a}28.98 \pm 1.95^{A}$
	0		$_{a}29.12 \pm 3.14^{A}$	$_{c}12.65 \pm 2.73^{B}$	$_{b}13.74 \pm 2.83^{B}$
	0.5		$_{b}23.91 \pm 3.26^{A}$	$_{b}18.74 \pm 3.61^{B}$	$_{c}7.39 \pm 2.79^{C}$
	1.0		$_{a}29.63 \pm 3.53^{A}$	$_{c}13.63 \pm 3.37^{B}$	$_{b}13.9 \pm 3.35^{B}$
	1.5		$_{ab}26.01 \pm 4.31^{A}$	$_{bc}15.14 \pm 3.99^{B}$	$_{b}15.26 \pm 4.38^{B}$
	2.5		$_{ab}26.92 \pm 2.53^{A}$	$_{b}17.97 \pm 3.27^{B}$	$_{b}12.13 \pm 2.16^{C}$
Hepatopancreas	Control	37.53 ± 4.13	$_{bc}37.23 \pm 4.22^{A}$	$_{a}38.24 \pm 2.51^{A}$	$_{a}37.74 \pm 3.68^{A}$
	0		$_{bc}36.97 \pm 4.23^{A}$	$_{b}29.06 \pm 3.46^{B}$	$_{b}26.82 \pm 6.29^{B}$
	0.5		$_{b}37.83 \pm 5.72^{A}$	$_{a}35.55 \pm 5.38^{A}$	$_{bc}21.50 \pm 4.48^{B}$
	1.0		$_{a}44.12 \pm 6.57^{A}$	$_{a}37.25 \pm 5.39^{A}$	$_{a}42.21 \pm 4.97^{A}$
	1.5		$_{d}28.64 \pm 4.69^{A}$	$_{c}20.38 \pm 4.16^{B}$	$_{c}20.13 \pm 4.54^{B}$
	2.5		$_{cd}30.97 \pm 4.67^{A}$	$_{c}17.49 \pm 5.56^{B}$	$_{d}10.54 \pm 4.13^{C}$
Gills	Control	35.62 ± 3.64	$_{a}36.31 \pm 3.79^{A}$	$_{a}35.97 \pm 3.34^{A}$	$_{a}36.22 \pm 3.62^{A}$
	0		$_{a}35.54 \pm 3.30^{A}$	$_{c}21.75 \pm 2.99^{C}$	$_{b}28.80 \pm 3.59^{B}$
	0.5		$_{ab}34.56 \pm 3.14^{A}$	$_{b}27.61 \pm 4.2^{B}$	$_{d}17.08 \pm 2.39^{C}$
	1.0		$_{b}30.54 \pm 4.45^{A}$	$_{b}29.44 \pm 2.71^{A}$	$_{c}22.58 \pm 3.98^{B}$
	1.5		$_{c}14.88 \pm 4.14^{A}$	$_{d}11.42 \pm 2.13^{A}$	$_{e}12.43 \pm 2.59^{A}$
	2.5		$_{c}18.42 \pm 2.57^{A}$	$_{d}12.23 \pm 2.49^{B}$	$_{c}10.67 \pm 2.81^{B}$

Table 6.11 Glutathione peroxidase activity in *P. monodon* exposed to zinc and then challenged with WSSV.

		GPx (µg GSH oxidised $min^{-1}mg^{-1}$ protein)			
Shrimp tissue	Zn (mg l^{-1})	BL	PMD14	PCD2	PCD5
Haemolymph	Control	4.16 ± 0.51	$_{ab}4.09 \pm 0.52^{A}$	$_{a}3.95 \pm 0.52^{A}$	$_{a}4.16 \pm 0.54^{A}$
	0		$_{ab}4.03 \pm 0.44^{A}$	$_{bc}2.33 \pm 0.58^{B}$	$_{a}3.66 \pm 0.65^{A}$
	0.5		$_{bc}3.84 \pm 0.62^{A}$	$_{c}2.15 \pm 0.50^{B}$	$_{a}3.68 \pm 0.52^{A}$
	1.0		$_{a}4.60 \pm 0.52^{A}$	$_{b}3.08 \pm 0.63^{B}$	$_{a}4.18 \pm 0.39^{A}$
	1.5		$_{d}2.78 \pm 0.63^{B}$	$_{bc}2.34 \pm 0.61^{B}$	$_{a}3.58 \pm 0.54^{A}$
	2.5		$_{cd}3.30 \pm 0.61^{A}$	$_{bc}2.41 \pm 0.76^{B}$	$_{b}2.61 \pm 0.52^{AB}$
Hepatopancreas	Control	2.40 ± 0.28	$_{bc}2.27 \pm 0.20^{A}$	$_{a}2.46 \pm 0.26^{A}$	$_{b}2.48 \pm 0.24^{A}$
	0		$_{b}2.38 \pm 0.22^{A}$	$_{ab}2.13 \pm 0.28^{A}$	$_{c}1.55 \pm 0.29^{B}$
	0.5		$_{d}1.79 \pm 0.15^{C}$	$_{ab}2.15 \pm 0.28^{B}$	$_{a}2.92 \pm 0.32^{A}$
	1.0		$_{bc}2.25 \pm 0.34^{A}$	$_{a}2.38 \pm 0.39^{A}$	$_{ab}2.63 \pm 0.38^{A}$
	1.5		$_{a}2.72 \pm 0.23^{A}$	$_{c}1.53 \pm 0.29^{B}$	$_{c}1.50 \pm 0.20^{B}$
	2.5		$_{cd}1.98 \pm 0.30^{A}$	$_{bc}1.87 \pm 0.34^{A}$	$_{c}1.37 \pm 0.34^{B}$
Gills	Control	6.76 ± 0.51	$_{a}6.68 \pm 0.59^{A}$	$_{a}6.75 \pm 0.62^{A}$	$_{a}6.63 \pm 0.56^{A}$
	0		$_{a}6.74 \pm 0.64^{A}$	$_{b}4.86 \pm 0.59^{B}$	$_{d}3.03 \pm 0.48^{C}$
	0.5		$_{a}7.01 \pm 0.47^{A}$	$_{b}4.38 \pm 0.45^{B}$	$_{c}4.26 \pm 0.72^{B}$
	1.0		$_{a}6.89 \pm 0.62^{A}$	$_{a}6.13 \pm 0.80^{A}$	$_{b}5.26 \pm 0.44^{B}$
	1.5		$_{a}3.44 \pm 0.33^{A}$	$_{c}2.36 \pm 0.39^{B}$	$_{d}3.13 \pm 0.78^{A}$
	2.5		$_{b}4.57 \pm 0.80^{A}$	$_{b}4.35 \pm 0.41^{A}$	$_{d}3.25 \pm 0.52^{B}$

Appendix 7

Table 6.12 Glutathione *S*-transferase activity in *P. monodon* exposed to zinc and then challenged with WSSV.

		GST(μM of CDNB-GSH conjugate formed $min^{-1}mg^{-1}$ protein)			
Shrimp tissue	**Zn (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	6.09 ± 0.58	$_{ab}6.06 \pm 0.64^{A}$	$_{a}6.23 \pm 0.72^{A}$	$_{a}6.11 \pm 0.53^{A}$
	0		$_{a}6.22 \pm 0.68^{A}$	$_{b}3.77 \pm 0.51^{C}$	$_{b}4.54 \pm 0.62^{B}$
	0.5		$_{c}3.83 \pm 0.47^{A}$	$_{c}2.49 \pm 0.44^{B}$	$_{cd}3.82 \pm 0.48^{A}$
	1.0		$_{b}5.41 \pm 0.60^{A}$	$_{c}2.13 \pm 0.59^{C}$	$_{bc}4.29 \pm 0.60^{B}$
	1.5		$_{c}4.38 \pm 0.73^{A}$	$_{b}4.05 \pm 0.65^{A}$	$_{e}1.97 \pm 0.62^{B}$
	2.5		$_{c}4.36 \pm 0.66^{A}$	$_{b}3.44 \pm 0.76^{B}$	$_{d}3.17 \pm 0.55^{B}$
Hepatopancreas	**Control**	3.76 ± 0.33	$_{b}3.83 \pm 0.33^{A}$	$_{a}3.92 \pm 0.28^{A}$	$_{a}3.74 \pm 0.41^{A}$
	0		$_{bc}3.67 \pm 0.29^{A}$	$_{b}3.21 \pm 0.34^{B}$	$_{bc}2.81 \pm 0.42^{B}$
	0.5		$_{c}3.36 \pm 0.41^{A}$	$_{b}3.33 \pm 0.32^{A}$	$_{b}3.21 \pm 0.36^{A}$
	1.0		$_{a}4.54 \pm 0.36^{A}$	$_{b}3.48 \pm 0.41^{B}$	$_{cd}2.53 \pm 0.25^{C}$
	1.5		$_{d}2.33 \pm 0.27^{A}$	$_{c}2.18 \pm 0.35^{A}$	$_{d}2.32 \pm 0.35^{A}$
	2.5		$_{d}2.21 \pm 0.43^{A}$	$_{c}2.07 \pm 0.25^{A}$	$_{d}2.30 \pm 0.32^{A}$
Gills	**Control**	5.37 ± 0.40	$_{b}5.08 \pm 0.43^{A}$	$_{a}5.25 \pm 0.51^{A}$	$_{a}5.27 \pm 0.54^{A}$
	0		$_{b}5.14 \pm 0.57^{A}$	$_{b}4.13 \pm 0.66^{B}$	$_{ab}3.09 \pm 0.63^{C}$
	0.5		$_{a}6.19 \pm 0.80^{A}$	$_{b}4.08 \pm 0.49^{B}$	$_{ab}2.86 \pm 0.45^{C}$
	1.0		$_{c}4.32 \pm 0.49^{B}$	$_{a}5.30 \pm 0.55^{A}$	$_{b}3.50 \pm 0.77^{C}$
	1.5		$_{d}2.63 \pm 0.55^{A}$	$_{d}2.52 \pm 0.6^{A}$	$_{c}2.42 \pm 0.42^{A}$
	2.5		$_{d}2.50 \pm 0.65^{A}$	$_{c}3.27 \pm 0.59^{A}$	$_{ab}2.93 \pm 0.68^{A}$

Table 6.13 Glutathione reductase activity in *P. monodon* exposed to zinc and challenged with WSSV

		GR (μM of NADPH oxidised $min^{-1}mg^{-1}$ protein)			
Shrimp tissue	**Zn (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	26.75 ± 3.29	$_{b}27.04 \pm 2.77^{A}$	$_{a}26.93 \pm 3.17^{A}$	$_{a}26.78 \pm 2.31^{A}$
	0		$_{b}26.58 \pm 2.92^{A}$	$_{b}22.01 \pm 3.28^{B}$	$_{d}10.37 \pm 3.57^{C}$
	0.5		$_{c}17.91 \pm 3.17^{A}$	$_{c}11.79 \pm 3.64^{B}$	$_{c}16.21 \pm 3.43^{A}$
	1.0		$_{a}33.54 \pm 4.29^{A}$	$_{c}13.74 \pm 3.40^{C}$	$_{b}21.67 \pm 3.29^{B}$
	1.5		$_{c}19.31 \pm 2.89^{A}$	$_{cd}10.74 \pm 4.33^{B}$	$_{bc}18.43 \pm 3.39^{A}$
	2.5		$_{d}12.18 \pm 2.37^{A}$	$_{d}6.91 \pm 2.07^{B}$	$_{d}7.54 \pm 2.13^{B}$
Hepatopancreas	**Control**	27.37 ± 3.09	$_{b}26.82 \pm 3.07^{A}$	$_{a}27.03 \pm 3.52^{A}$	$_{ab}27.0 \pm 3.56^{A}$
	0		$_{b}27.34 \pm 2.64^{A}$	$_{b}22.14 \pm 4.88^{B}$	$_{b}23.86 \pm 3.97^{AB}$
	0.5		$_{a}34.67 \pm 3.74^{A}$	$_{bc}19.07 \pm 3.46^{B}$	$_{a}30.46 \pm 4.10^{A}$
	1.0		$_{b}25.18 \pm 3.07^{A}$	$_{c}16.54 \pm 3.55^{B}$	$_{ab}28.54 \pm 5.64^{A}$
	1.5		$_{c}19.86 \pm 3.91^{A}$	$_{c}17.15 \pm 2.78^{A}$	$_{c}18.14 \pm 2.66^{A}$
	2.5		$_{c}18.75 \pm 3.14^{A}$	$_{c}16.49 \pm 3.38^{A}$	$_{d}11.16 \pm 2.42^{B}$
Gills	**Control**	25.92 ± 2.36	$_{a}26.34 \pm 2.49^{A}$	$_{a}25.40 \pm 2.45^{A}$	$_{a}26.87 \pm 2.49^{A}$
	0		$_{a}26.49 \pm 1.86^{A}$	b19.02 ± 2.49^{B}	$_{c}7.11 \pm 1.89^{C}$
	0.5		$_{b}19.31 \pm 2.32^{A}$	b18.78 ± 2.22^{A}	$_{c}6.79 \pm 1.56^{B}$
	1.0		$_{b}21.21 \pm 3.30^{A}$	$_{a}22.78 \pm 2.66^{A}$	$_{b}14.87 \pm 2.68^{B}$
	1.5		$_{c}13.85 \pm 3.02^{A}$	$_{c}10.63 \pm 2.45^{A}$	$_{c}7.28 \pm 2.44^{B}$
	2.5		$_{b}20.87 \pm 2.66^{A}$	$_{d}7.68 \pm 1.97^{B}$	$_{c}8.09 \pm 2.30^{B}$

Appendix 8

Table 6.14 Total reduced glutathione levels in *P. monodon* exposed to zinc and then challenged with WSSV.

		GSH (μg mg^{-1} protein)			
Shrimp tissue	**Zn (mg l^{-1})**	**BL**	**PMD14**	**PCD2**	**PCD5**
Haemolymph	**Control**	4.12 ± 0.50	$_{a}4.26 \pm 0.58^{A}$	$_{a}4.10 \pm 0.34^{A}$	$_{a}4.20 \pm 0.54^{A}$
	0		$_{a}4.14 \pm 0.51^{A}$	$_{bc}2.35 \pm 0.48^{B}$	$_{a}4.42 \pm 0.52^{A}$
	0.5		$_{a}4.28 \pm 0.59^{A}$	$_{d}1.58 \pm 0.38^{C}$	$_{b}3.58 \pm 0.49^{B}$
	1.0		$_{a}3.87 \pm 0.38^{A}$	$_{c}2.13 \pm 0.47^{B}$	$_{a}4.46 \pm 0.59^{A}$
	1.5		$_{b}2.61 \pm 0.42^{AB}$	$_{bc}2.46 \pm 0.38^{B}$	$_{bc}2.98 \pm 0.41^{A}$
	2.5		$_{b}3.09 \pm 0.39^{A}$	$_{b}2.75 \pm 0.45^{A}$	$_{c}2.53 \pm 0.48^{A}$
Hepatopancreas	**Control**	13.18 ± 1.22	$_{bc}12.83 \pm 1.83^{A}$	$_{b}12.62 \pm 1.42^{A}$	$_{b}13.17 \pm 1.38^{A}$
	0		$_{bc}12.83 \pm 1.11^{A}$	$_{c}9.53 \pm 1.54^{B}$	$_{c}10.23 \pm 1.25^{B}$
	0.5		$_{a}18.42 \pm 2.45^{A}$	$_{a}15.31 \pm 2.49^{B}$	$_{b}12.39 \pm 1.68^{C}$
	1.0		$_{a}18.24 \pm 2.09^{A}$	$_{ab}14.54 \pm 2.41^{B}$	$_{a}15.46 \pm 1.59^{B}$
	1.5		$_{c}10.74 \pm 1.71^{A}$	$_{c}7.31 \pm 1.50^{B}$	$_{d}7.27 \pm 2.09^{B}$
	2.5		$_{b}13.77 \pm 2.47^{A}$	$_{c}8.50 \pm 1.48^{B}$	$_{c}3.29 \pm 0.69^{C}$
Gills	**Control**	4.55 ± 0.41	$_{b}4.70 \pm 0.50^{A}$	$_{a}4.50 \pm 0.47^{A}$	$_{a}4.77 \pm 0.48^{A}$
	0		$_{b}4.62 \pm 0.51^{A}$	$_{c}3.26 \pm 0.58^{B}$	$_{b}2.35 \pm 0.60^{C}$
	0.5		$_{a}6.08 \pm 0.73^{A}$	$_{bc}3.78 \pm 0.61^{B}$	$_{c}1.77 \pm 0.42^{C}$
	1.0		$_{a}5.69 \pm 0.73^{A}$	$_{ab}4.24 \pm 0.66^{B}$	$_{b}2.41 \pm 0.38^{C}$
	1.5		$_{d}1.97 \pm 0.27^{A}$	$_{d}1.73 \pm 0.47^{A}$	$_{bc}1.87 \pm 0.58^{A}$
	2.5		$_{c}3.06 \pm 0.53^{A}$	$_{d}2.06 \pm 0.61^{B}$	$_{d}1.05 \pm 0.26^{C}$

*Data (Mean ± S.D.) in the same column (for each tissue) with different subscripts are statistically different ($P<0.05$) among treatments at the same exposure time and data (Mean ± S.D.) in the same row (for each tissue) with different superscripts are statistically different ($P<0.05$) among different time periods.
BL –Baseline, PMD –Post metal exposure day, PCD –Post challenge day

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Acute salinity stress alters the haemolymph metabolic profile of *Penaeus monodon* and reduces immunocompetence to white spot syndrome virus infection

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Abstract

Influence of acute salinity stress on the immunological and physiological response of *Penaeus monodon* to white spot syndrome virus (WSSV) infection was analysed. *P. monodon* maintained at 15‰ were subjected to acute salinity changes to 0‰ and 35‰ in 7 h and then challenged orally with WSSV. Immune variables viz., total haemocyte count, phenol oxidase activity (PO), nitroblue tetrazolium salt (NBT) reduction, alkaline phosphatase activity (ALP), acid phosphatase activity (ACP) and metabolic variables viz., total protein, total carbohydrates, total free amino acids (TFAA), total lipids, glucose and cholesterol were determined soon after salinity change and on post challenge days 2 (PCD2) and 5 (PCD5). Acute salinity change induced an increase in metabolic variables in shrimps at 35‰ except TFAA. Immune variables reduced significantly ($P<0.05$) in shrimps subjected to salinity stress with the exception of ALP and PO at 35‰ and the reduction was found to be more at 0‰. Better performance of metabolic and immune variables in general could be observed in shrimps maintained at 15‰ that showed significantly higher post challenge survival following infection compared to those under salinity stress. Stress was found to be higher in shrimps subjected to salinity change to lower level (0‰) than to higher level (35‰) as being evidenced by the better immune response and survival at 35‰. THC ($P<0.001$), ALP ($P<0.01$) and PO ($P<0.05$) that together explained a greater percentage of variability in survival rate, could be proposed as the most potential health indicators in shrimp haemolymph. It can be concluded from the study that acute salinity stress induces alterations in the haemolymph metabolic and immune variables of *P. monodon* affecting the immunocompetence and increasing susceptibility to WSSV, particularly at low salinity stress conditions.

Keywords: *Penaeus monodon*; White spot syndrome virus; Salinity; Haemolymph; Immune response

1. Introduction

White spot syndrome, first reported in Taiwan in 1992 (Chou et al., 1995), has emerged as the most serious threat to commercial shrimp farming. White spot syndrome virus (WSSV), a member of the genus Whispovirus within a new virus family Nimaviridae is a circular, double-stranded DNA virus (Vlak et al., 2005). Tiger shrimp, *Penaeus monodon*, the widely cultured shrimp species is highly susceptible to WSSV infection (Chen, 1995; Hameed et al., 2006). Susceptibility is often intensified by the highly stressful environment in culture systems.

Stress responses to environmental fluctuations are well reflected in the composition of haemolymph, the prime component involved in the defense mechanism of

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crustaceans. Haemocytes, along with proPhenol oxidase activity, respiratory burst activity and phagocytic activity have been used as indices of immune capability in penaeid shrimps (Le Moullac et al., 1998; Cheng et al., 2007). Extrinsic factors like temperature (Pascual et al., 2003; Wang and Chen, 2006b), salinity (Vargas-Albores et al., 1998), pH (Cheng and Chen, 2000) dissolved oxygen (Jiang et al., 2005) etc. are reported to affect immune responses in several species of decapod crustaceans. Biochemical variables in haemolymph have also been identified as indicators of stress as stress leads to the onset of a cascade of molecular and biochemical responses. Haemolymph metabolic variables viz., proteins, glucose, cholesterol, triacylglycerol etc. were found to vary in response to captivity stress (Sanchez et al., 2001), temperature alterations (Pascual et al., 2003), depleted dissolved oxygen (Hall and van Ham, 1998), high ambient ammonia (Racotta and Hernandez-Herrera, 2000) etc. Stress therefore disrupts the immune ability and metabolic performance of shrimps, increasing the susceptibility to microbial infections. However, there are very few scientific data supporting the link between environmental stress and increased susceptibility to diseases in shrimps.

P. monodon with an iso-osmotic point of 750 mOsm kg^{-1} (equivalent to 25‰) is very often cultured at a salinity range of 10‰–20‰, as they are believed to exhibit better growth in brackish water than in pure seawater under culture conditions (Fang et al., 1992). Being a euryhaline form having wide salinity tolerance ranging from 1‰ to 57‰ (Chen, 1990), the fluctuations are usually neglected in culture ponds. The salinity of culture ponds may decrease suddenly to as low as 0‰ after a heavy rainfall. There are reports of WSSV outbreaks with the onset of monsoon in Malaysia when intense rainfall decreased the salinity of aquaculture areas (Oseko, 2006). It is possible that acute salinity changes over a particular range weaken the immune system of shrimp and make them highly vulnerable to pathogens. Drastic salinity changes may also affect the feed intake, metabolism, and higher energy utilization for osmoregulation resulting in poor growth. However, there are very few works on the effects of environmental stress on infection, particularly WSSV.

Present study on *P. monodon* was therefore aimed at determining the: (i) effect of acute salinity change on the metabolic and immune variables of haemolymph (ii) effect of acute salinity stress on the susceptibility to WSSV infection. (iii) effect of WSSV infection on the haemolymph metabolic variables and immune response of shrimps maintained at optimal salinity and those subjected to acute salinity stress.

2. Materials and methods

2.1. Experimental animals and rearing conditions

Adult *P. monodon* obtained from a commercial farm in Panangad, Kochi were used as experimental shrimps in the present study. They were transported to the laboratory within 1 h of capture. Average wet weight of the shrimp was 19.85± 2.01 g (Mean±S.D.). Shrimps were reared in rectangular concrete tanks containing 15‰ sea water and allowed to acclimate for a week. Continuous aeration was provided and shrimps were fed on a commercial shrimp diet (Higashimaru, Kochi). Water quality parameters viz., salinity, 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 1. Unused feed and faecal matter were siphoned out daily and 25% water exchanged every second day. A biological filter was set up to maintain the appropriate levels of water quality parameters. After acclimation for a period of 7 days, the biochemical and immunological profile was obtained from a group of shrimps (n=6) as the baseline (BL) data.

2.2. Experimental design

Shrimps were distributed in the experimental tanks containing 500 L of seawater (n=30/tank). Shrimps in the intermoult stage (Robertson et al., 1987) only were used. There were 4 treatments (Group-I, Group-II, Group-III and Group-IV) and the experiment was conducted in triplicate i.e., 3 tanks per treatment. Salinity of all the tanks was adjusted to 15‰ prior to the experiment.

2.3. Salinity stress

After 2 days, the shrimps of Group-II and Group-IV were subjected to sudden salinity changes. Shrimps were starved for 12 h prior to salinity change. Salinity of Group-II was lowered from 15‰ to 0‰ by diluting with fresh water. Whereas, the salinity of Group-IV was raised from 15‰ to 35‰ by adding sea water. The desired salinity was adjusted

Table 1
Rearing conditions and water quality

Stocking density	30 shrimps/tank
Tank capacity	500 L
Feeding level	10–15% body weight
Feeding frequency	twice daily
Water temperature	24–27 °C
pH	7.5–8.0
Salinity	15‰
NH_3-N	0.01–0.02 mgl^{-1}
NO_3-N	below detectable level
NO_2-N	0.00–0.01 mgl^{-1}
Dissolved oxygen	6–7 mgl^{-1}

over a period of 7 h. Shrimps of Group-I and Group-III was maintained at 15‰ itself with no salinity change. Ten minutes after the desired salinity level was reached, 6 shrimps from each group ($n=6$) were sampled (post salinity change day 0, PSD0).

2.4. WSSV challenge

The shrimps of Group-II, Group-III and Group-IV were then challenged with WSSV. Challenge was performed through oral administration i.e., by feeding white spot virus infected frozen tissue at the rate of 1 g/shrimp. Group-I was maintained as the unchallenged control. Shrimps were sampled ($n=6$) after 48 h (post challenge day 2, PCD2) and 120 h of challenge (post challenge day 5, PCD5). Before each sampling the shrimps were starved for 12 h 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.

2.5. Extraction of haemolymph

Anticoagulant for haemolymph extraction was prepared by adding 10 mM EDTA-Na_2 salt to the Shrimp Salt Solution (45 mM NaCl, 10 mM KCl, 10 mM HEPES, pH 7.3, 850 mOsM kg^{-1}, Vargas-Albores et al., 1993). Haemolymph was withdrawn aseptically from rostral sinus using specially designed sterile capillary tubes of diameter 0.5 mm, rinsed thoroughly with pre-cooled anticoagulant. The samples were transferred to sterile eppendorf vials containing pre-cooled anticoagulant. Haemolymph collected from six shrimps ($n=6$) of each treatment group was analysed separately. The immune parameters were analysed immediately and the samples stored at −20 °C for the analysis of metabolic variables.

2.6. Biochemical assays

The metabolic variables of haemolymph viz., total protein, total carbohydrates, total free amino acids, total lipids, glucose and cholesterol were determined spectrophotometrically employing standard techniques and expressed in mg ml^{-1} haemolymph. Protein determination was done employing the Bradford method (1976) using Coomassie Brilliant Blue G-250 (OD at 595 nm). Total carbohydrates were determined (OD at 620 nm) by the anthrone method (Hedge and Hofreiter, 1962). Total free amino acids (TFAA) were determined using the ninhydrin method (OD 570 nm) by Yemm and Cocking (1955). Total lipids were determined by the sulphophosphovanillin method (OD 520 nm) by Barnes and Blackstock (1973). Glucose concentration (OD 625 nm) was estimated according to the method of Marks (1959) and cholesterol concentration (OD 540 nm) was determined according to the method of Zak (1957).

2.7. Immune assays

2.7.1. Total haemocyte count(THC)

A drop of anticoagulant-haemolymph mixture was placed on a Neubaeur's haemocytometer immediately after extraction. The haemocytes were counted using a bright field microscope and the values expressed as THC ml^{-1} haemolymph.

2.7.2. Phenoloxidase (PO) activity

A sample of 100 μl of haemolymph was incubated for 10 min at 20 °C with 0.1 ml of SDS and 2.0 ml of substrate (L-DOPA in Tris-HCl buffer) was added. The dopachrome formed was measured in a U-V Visible spectrophotometer at 490 nm, every 30 s for 3 min and the activity expressed as increase in absorbance $minute^{-1}$ 100 μl^{-1} haemolymph (Soderhall, 1981).

2.7.3. NBT reduction

Respiratory burst activity of haemocytes was quantified based on the reduction of nitroblue tetrazolium (NBT) to blue formazan as a measure of superoxide anion production (Song and Hsieh, 1994). 100 μl of haemolymph was incubated with 100 μl of NBT for 1 h at 20 °C. The cells were centrifuged and fixed in 100% methanol. Supernatant was removed after centrifugation and the cells were dried, rinsed in 50% methanol and solubilised in 140 μl of DMSO and 120 μl of 2 M KOH. The absorbance at 620 nm was recorded and the activity expressed as O.D. 100 μl^{-1} haemolymph.

2.7.4. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activity

A sample of 100 μl of haemolymph was incubated for 30 min at 37 °C with 2.0 ml of substrate (*p*-nitrophenyl phosphate in citrate buffer- pH 5 for ACP and *p*-nitrophenyl phosphate in glycine-NaOH buffer- pH 9 for ALP). Then 2.9 ml of 0.1 N NaOH was added and the absorbance measured spectrophotometrically at 405 nm and the activity expressed as mg ml^{-1} *p*-nitrophenol released. (Gonzalez et al., 1994).

2.8. Statistical analysis

Statistical analyses were carried out using the software SPSS 10.0. One-way ANOVA and Duncan's multiple comparison of the means were done to compare the data obtained. To find out the relationships between survival rate and haematological parameters, correlation and regression analyses of the post challenge data was done.

3. Results

Significant variations could be observed in the metabolic and immune variables when shrimps were subjected to salinity stress. In the case of metabolic variables, an increase could be observed at 35‰ except for TFAA and total carbohydrates, which were maximum in shrimps at 0‰. Whereas, in the case of immunological parameters a decrease could be observed both at 35‰ and 0‰ except for PO and ALP activity and the

Table 2
Metabolic variables in the haemolymph of *Penaeus monodon* subjected to acute salinity stress and then challenged with WSSV

Haemolymph metabolic Variables (mg/ml)	Salinity	Baseline	PSD0	PCD2	PCD5
Total protein	Control	89.28±9.2	$_{b}$88.35±10.1	$_{c}$88.58±12.9	$_{b}$90.10±11.4
	0‰		$_{b}$86.96±13.3^{C}	$_{ab}$109.81±11.9^{A}	$_{ab}$101.32±9.8^{AB}
	15‰		$_{b}$89.38±11.3^{B}	$_{a}$121.51±16.4^{A}	$_{a}$112.04±14.2^{A}
	35‰		$_{a}$110.48±13.9^{A}	$_{bc}$99.70±15.3^{A}	$_{ab}$100.72±12.06^{A}
Total carbohydrates	Control	4.53±0.55	$_{c}$4.59±0.53	$_{c}$4.46±0.58	$_{b}$4.49±0.52
	0‰		$_{a}$6.50±0.75^{A}	$_{b}$6.15±0.51^{A}	$_{b}$5.0±0.66^{B}
	15‰		$_{c}$4.62±0.38^{C}	$_{a}$7.13±0.59^{A}	$_{a}$6.46±0.61^{B}
	35‰		$_{b}$5.56±0.67^{A}	$_{b}$5.71±0.58^{A}	$_{b}$5.06±0.41^{A}
Total free amino acids	Control	3.03±0.38	$_{b}$3.09±0.45	$_{ab}$3.08±0.42	$_{b}$3.12±0.33
	0‰		$_{a}$3.88±0.52^{A}	$_{b}$2.69±0.33^{B}	$_{c}$2.36±0.5^{B}
	15‰		$_{b}$3.09±0.36^{B}	$_{a}$3.43±0.46^{AB}	$_{a}$3.79±0.39^{A}
	35‰		$_{b}$2.52±0.46^{AB}	$_{c}$2.16±0.3^{B}	$_{bc}$2.81±0.42^{A}
Total lipids	Control	1.94±0.15	$_{a}$1.92±0.16	$_{a}$1.9±0.14	$_{a}$1.89±0.16
	0‰		$_{b}$1.54±0.18^{A}	$_{c}$1.31±0.18^{B}	$_{c}$1.29±0.19^{B}
	15‰		$_{a}$1.91±0.18^{A}	$_{ab}$1.79±0.21^{AB}	$_{b}$1.62±0.17^{B}
	35‰		$_{a}$2.05±0.13^{A}	$_{b}$1.6±0.2^{B}	$_{ab}$1.71±0.2^{B}
Glucose	Control	0.322±0.03	$_{b}$0.318±0.04	$_{b}$0.315±0.04	$_{b}$0.316±0.05
	0‰		$_{c}$0.227±0.04^{B}	$_{b}$0.295±0.05^{A}	$_{c}$0.232±0.05^{B}
	15‰		$_{b}$0.322±0.06^{B}	$_{a}$0.397±0.04^{A}	$_{a}$0.423±0.06^{A}
	35‰		$_{a}$0.385±0.06^{A}	$_{c}$0.216±0.04^{B}	$_{c}$0.248±0.05^{B}
Cholesterol	Control	0.537±0.06	$_{c}$0.524±0.03	$_{c}$0.53±0.07	$_{ab}$0.533±0.06
	0‰		$_{b}$0.619±0.06^{A}	$_{b}$0.581±0.06^{A}	$_{b}$0.479±0.06^{B}
	15‰		$_{c}$0.515±0.04^{B}	$_{ab}$0.62±0.06^{A}	$_{b}$0.499±0.07^{B}
	35‰		$_{a}$0.739±0.08^{A}	$_{a}$0.668±0.06^{A}	$_{a}$0.577±0.05^{B}

Data (Mean±S.D.) in the same column (for each parameter) with different subscripts are statistically different ($P<0.05$) among treatments at the same exposure time and data (Mean±S.D.) in the same row (for each parameter) with different superscripts are statistically different ($P<0.05$) among different time periods.
PSD=post salinity change day. PCD=post challenge day.

reduction was maximum at 0‰. Following WSSV infection, the metabolic and immune parameters were maximum at 15‰ compared to those at 35‰ and 0‰. This could be correlated with the survival also. Immune responses of shrimps under salinity stress were better at 35‰ compared to those held at 0‰, which shows that salinity change to a lower level is more stressful and shrimps are highly susceptible to infection (Table 2 and Figs. 1–5).

3.1. Haemolymph metabolic variables

A significant increase in the total protein concentration was noted in shrimps at 35‰ after salinity change ($P<0.05$). Post challenge total protein levels were significantly higher in shrimps at all salinities compared to the control ($P<0.05$) (Table 2).

Total carbohydrate concentration in haemolymph significantly increased after acute salinity change to 0‰ and 35‰, the variation being higher at 0‰ ($P<0.05$). Compared to the 0‰ and 35‰ Group, 15‰ Group registered significantly higher total carbohydrate levels following infection ($P<0.05$).

TFAA was found to increase significantly in response to acute salinity change to 0‰ ($P<0.05$). The concentration decreased after challenge in shrimps held at 0‰. Shrimps held at 35‰ showed an increase in TFAA on PCD5. A progressive elevation in the haemolymph TFAA concentration was observed in shrimps maintained at 15‰ following WSSV challenge ($P<0.05$) (Table 2).

Significantly lower lipid levels were observed in shrimps at 0‰ on PSD0 and on post challenge days compared to the 15‰ and 35‰ Group ($P<0.05$). Haemolymph total lipid concentration decreased in the challenged shrimp compared to the control (Table 2).

There was a slight elevation in the glucose level of shrimps at 35‰ after salinity change and a slight decrease at 0‰ ($P<0.05$). An elevation in haemolymph glucose concentration was noted in shrimps maintained at 15‰ following challenge. Significantly lower glucose levels were recorded on post challenge days in shrimps subjected to salinity stress ($P<0.05$) (Table 2).

The cholesterol concentration significantly increased after salinity change in shrimps at 0‰ and 35‰ respectively ($P<0.05$). Following challenge, the cholesterol concentration showed a declining trend on PCD5 (Table 2).

3.2. Immune response

Significantly lower THC was recorded in shrimps held at 0‰ and 35‰ stress immediately after salinity change and on post challenge days compared to the control shrimps and those held at 15‰ ($P<0.05$). A general decline in THC was observed

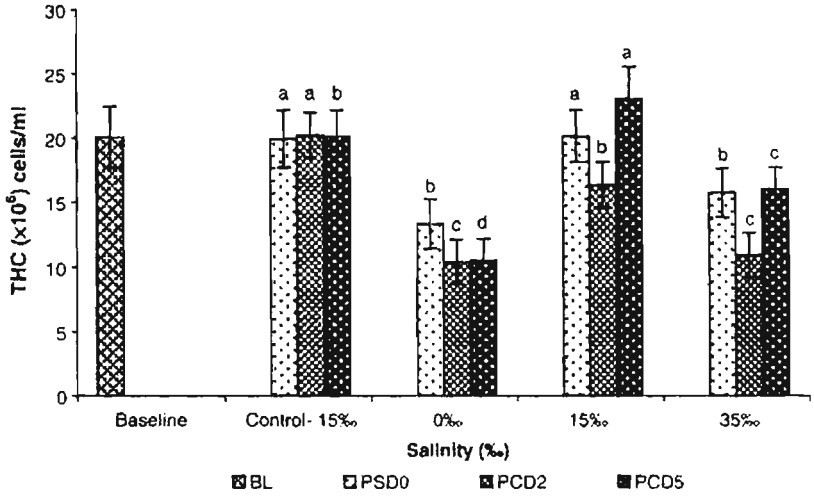

Fig. 1. Total haemocyte count (Mean±S.D.) of *Penaeus monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$). BL = baseline, PSD0 = post salinity change day, PCD = post challenge day.

on PCD2 at all salinities. THC slightly improved on PCD5 for shrimps held at 15‰ and 35‰, being significantly higher at 15‰ ($P<0.05$) (Fig. 1.).

PO activity registered a slight increase in shrimps subjected to a salinity change to 35‰ and a slight decrease in those subjected to a change to 0‰ ($P<0.05$). Following WSSV challenge, the activity increased significantly in shrimps held at 15‰ and 0‰, being higher at 15‰ ($P<0.05$). The activity showed a declining trend on PCD5. In the case of shrimps held at 35‰ an increased PO activity was seen on PCD5 compared to the lower activity on PCD2 (Fig. 2.).

NBT reduction was significantly low in *P. monodon* subjected to salinity stress ($P<0.05$). Following challenge significantly higher activities were recorded at all salinities on PCD2 ($P<0.05$). The activity significantly declined on PCD5 in shrimps held at 0‰. (Fig. 3.).

ALP activity increased after the sudden increase in salinity to 35‰ and decreased with the sudden decrease to 0‰ ($P<0.05$). A significant elevation in ALP activity was noticed in shrimps at 15‰ on PCD2 ($P<0.05$). The activity declined on PCD5 at all salinities. Post challenge ALP activity was significantly lower for the shrimps held at 0‰ compared to those at 15‰ and 35‰ ($P<0.05$) (Fig. 4.). ACP activity significantly reduced after salinity stress, being lower at 0‰ than at 35‰. PCD2 showed a significant elevation in the activity at all salinities ($P<0.05$). ACP activity was considerably low on PCD5 at 0‰ compared to the 15‰ and 35‰ Group (Fig. 5.).

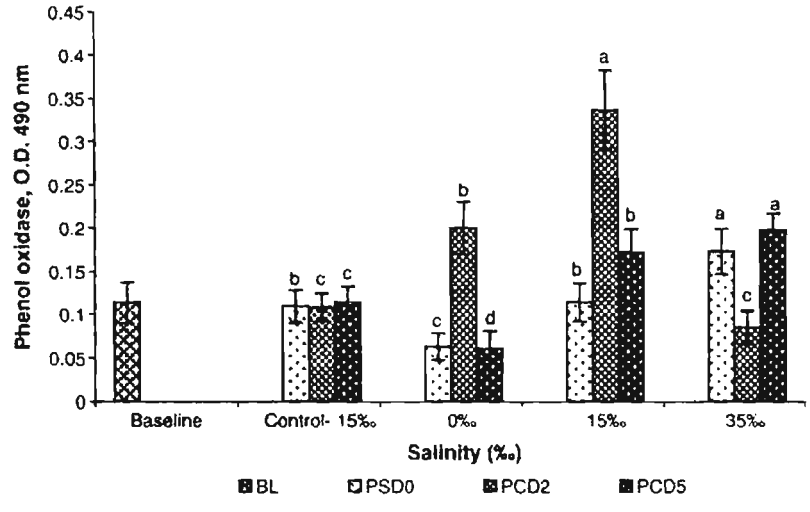

Fig. 2. Phenol oxidase activity (Mean±S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

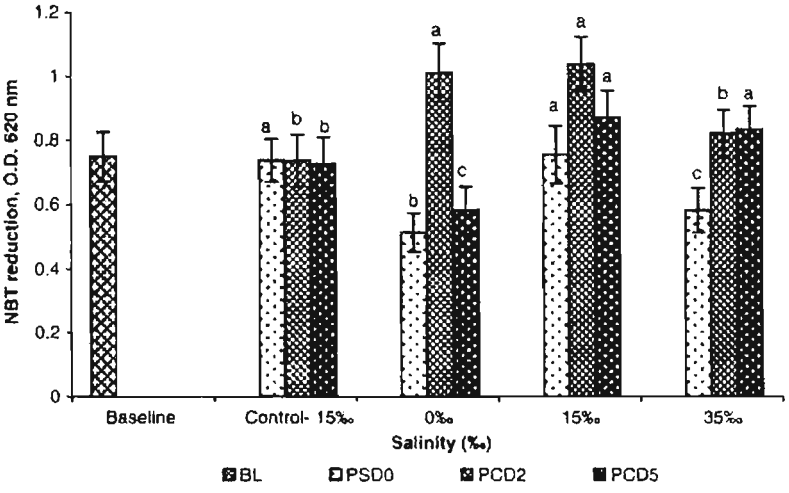

Fig. 3. NBT reduction (Mean±S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

3.3. Post challenge survival

The unchallenged control shrimps showed 100% survival. Percentage survival rates of *P. monodon* maintained at 15‰ were significantly higher than that for the shrimps held at 35‰ and 0‰. Least survival rate was recorded for shrimps subjected to 0‰ stress, which succumbed to death (100%) within 6 days of challenge. Percentage survival of 35‰ group reached 0 by PCD10 when the 15‰ Group showed a relatively higher survival (41.2%) ($P<0.05$) (Fig. 6).

3.4. Correlation and regression

Pearson correlation co-efficients showed that all variables except cholesterol exhibited positive correlation with the survival rate. Metabolic and immune variables exhibited a greater degree of correlation with each other (Table 3). When multiple regression of survival rate on all parameters were considered, the amount of variability explained was 89% (R Square=0.890). When significant regression co-efficients were taken into account, it was found that THC ($P<0.001$), ALP ($P<0.01$) and PO ($P<0.05$) together are explaining the 80% (R Square=0.804) of variability, indicating that these three are mainly responsible for the survival rate (Table 4).

4. Discussion

Metabolic adjustments could be observed in *P. monodon* subjected to acute salinity changes in the

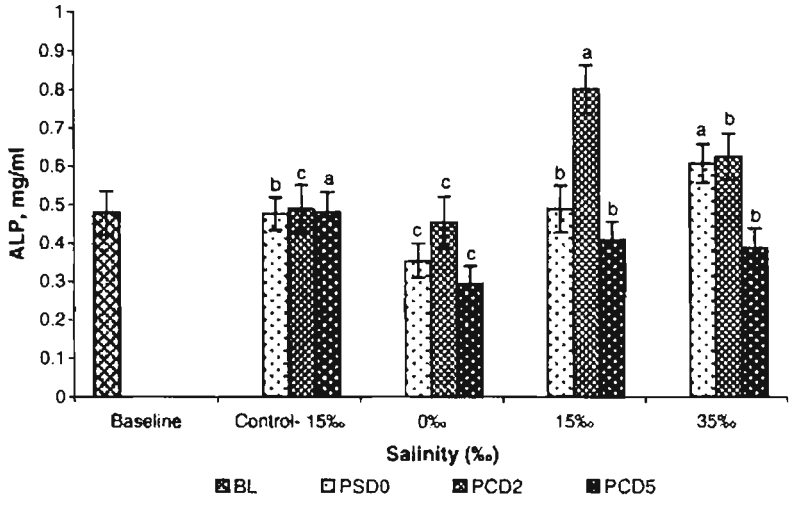

Fig. 4. Alkaline phosphatase activity (Mean±S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

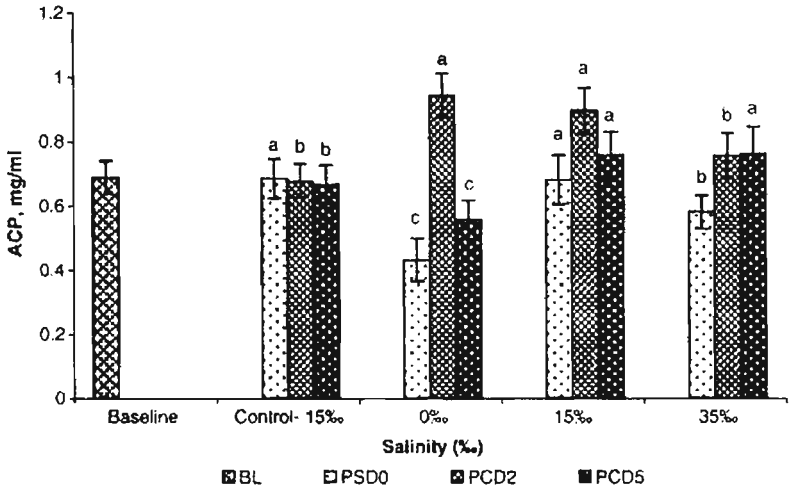

Fig. 5. Acid phosphatase activity (Mean±S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

present study. Initial response to cope with the hypo-saline environment (0‰) was probably an increase in the level of TFAA as they are proposed to function as osmotic effectors in haemolymph (Smith and Dall, 1991). Significantly high TFAA were found in *Fenneropenaeus indicus* from less saline mud bank area compared to the non-mud bank sample (Jayasree and Selvam, 2000). Haemolymph proteins probably assisted in adjusting to the hyper-saline environment (35‰) that showed a significant increase. A similar elevation in HSP (heat shock protein or stress protein) mRNA expression after 0.5 h of osmotic stress has been observed in American lobster (Chang, 2005). An immediate variation in proteins and free amino acids could be a consequence of cellular release (Jury et al., 1994) that has been suggested as a passive mechanism to maintain internal osmolality in crustaceans. Lipid mobilization and the involvement of these compounds in osmotic acclimation process after an osmotic shock has been verified in the euryhaline crab *Chasmognathus granulata* (Luvizotto-santos et al., 2003). A preferential usage of lipids as an energy source was evident at a lower salinity stress. However, cholesterol seemed to be spared and presumably retained as an osmolyte at 0‰. Higher cholesterol level noticed at both salinities was a clear indication of lipid transport that occurred since shrimps cannot synthesise cholesterol de novo (Teshima and Kanazawa, 1971). Generally, glucose and total carbohydrates in haemolymph increase in stressed shrimps to meet the energy demands to ward off stress. Though an increase in total carbohydrates was

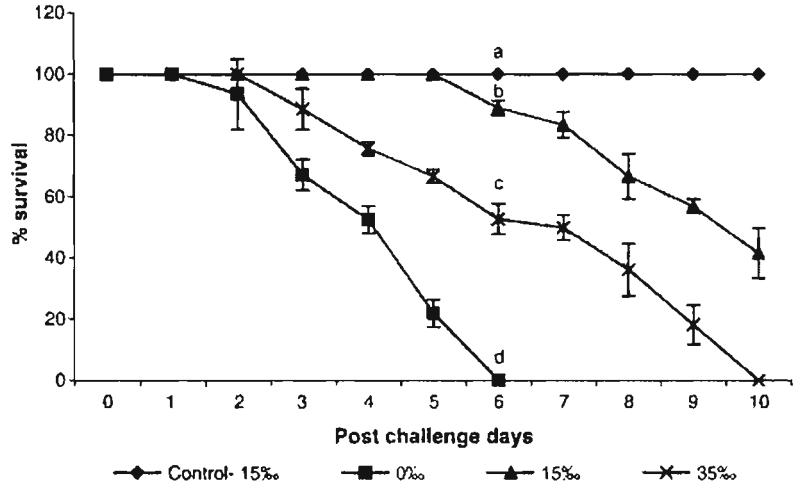

Fig. 6. Post challenge survival (Mean±S.D.) of *P. monodon* subjected to acute salinity stress against experimental infection with WSSV.

Table 3
Correlation matrix between survival rate and haematological parameters of WSSV-infected *P. monodon* maintained at optimal salinity or subjected to salinity stress

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	Gl	Ch	Surv
THC	1.000											
PO	0.350*	1.000										
NBT	0.211	0.722**	1.000									
ALP	0.056	0.628**	0.567**	1.000								
ACP	0.097	0.635**	0.814**	0.493**	1.000							
TP	0.270	0.407*	0.404*	0.337*	0.274	1.000						
TC	0.334*	0.533**	0.566**	0.512**	0.513**	0.390*	1.000					
TFAA	0.387*	0.519**	0.36*	0.113**	0.310	0.256	0.552*	1.000				
TL	0.708**	0.455**	0.296	0.486	0.124	0.028	0.296	0.230	1.000			
Gl	0.387*	0.525**	0.451**	0.288	0.367*	0.410*	0.641**	0.707**	0.354*	1.000		
Ch	−0.227	0.202	0.306	0.572**	0.263	0.039	0.246	−0.312	0.461**	−0.158	1.000	
Surv	0.680**	0.589**	0.591**	0.621**	0.470**	0.331*	0.627**	0.608**	0.609**	0.631**	0.295	1.000

**$P<0.01$, *$P<0.05$.
THC—total haemocyte count, PO—phenol oxidase activity, NBT—NBT reduction, ALP—alkaline phosphatase activity, ACP—acid phosphatase activity, TP—total protein, TC—total carbohydrates, TFAA—total free amino acids, TL—total lipids, Gl—glucose, Ch—cholesterol, Surv—survival.

observed both at lower and higher salinity stress, increase in glucose was observed only at 35‰, which is indicative of a rapid and selective consumption of the simple sugar at 0‰. Lamela et al. (2005) has observed a reduction in haemolymph glucose of *Litopenaeus schmitti* exposed to lower salinities.

Following infection, a prominent increase could be observed in haemolymph metabolic variables except lipids in shrimps maintained at 15‰. Yoganandhan et al. (2003) reported similar increase in haemolymph metabolites in WSSV-infected *F. indicus*. Increase in haemolymph metabolites during initial stages of infection may be attributed to the mobilization of energy reserves from the reserve tissues, hepatopancreas and muscle, to meet the energy requirements to ward off infection. Significant reduction of haemolymph metabolites in infected shrimps under salinity stress could be explained as a deviation in the energy flow to support osmotic work as they are under dual stress (salinity stress and pathogenic stress). Since metabolic variables had correlation with some or all of the immune variables, it is clear that a poor metabolic response may lead to a lower level of immunocompetence.

According to Yoganandhan et al. (2003), sharp increase in the haemolymph total protein of WSSV-infected shrimp might owe to an increase in the amount of virus. Taking into account the significantly higher total protein in shrimps held at 15‰ we suggest that enzymes involved in immune function that display elevated transcription during pathogenic stress are also contributing to the increase. Rameshthangam and Ramasamy (2005) could detect new and intensely expressed protein patterns in WSSV-infected *P. monodon*. Further research on protein

Table 4
Multiple regression of survival rate on haematological parameters of WSSV-infected *P. monodon* maintained at optimal salinity or subjected to salinity stress

R Square—0.890
Predictors—THC, PO, NBT, ALP, ACP, TP, TC, TFAA, TL, Gl, Ch
Dependent variable—Survival

	THC	PO	NBT	ALP	ACP	TP	TC	TFAA	TL	Gl	Ch
Significance	0.000**	0.042*	0.212	0.001**	0.456	0.841	0.780	0.058	0.256	0.777	0.234

R Square—0.804
Predictors–ALP, THC, PO
Dependent variable–Survival

**$P<0.01$, *$P<0.05$.
THC—total haemocyte count, PO—phenol oxidase activity, NBT—NBT reduction, ALP—alkaline phosphatase activity, ACP—acid phosphatase activity, TP—total protein, TC—total carbohydrates, TFAA—total free amino acids, TL—total lipids, Gl—glucose, Ch—cholesterol, Surv–survival.

profile in shrimps under salinity stress may provide better clarification. Decrease of fatty acid level in haemolymph is a usual phenomenon in the infected shrimp (Hameed, 1989), the reason of which is yet to be defined. Hyperglycemia on WSSV infection was evident only in shrimps maintained at 15‰. Increased secretion of CHH (Crustacean hyperglycemic hormone) may cause hyperglycemia. An increase in plasma CHH concentration was reported in Norway lobsters infected with *Hemtodinium* (Stentiford et al., 2001). Hyperglycemia may also be an indication of the stimulation of other compensatory mechanisms. Glucose was found to exhibit positive correlation with majority of other variables. Even though an increase was noted at 0‰, the glucose levels were seen to decrease on PCD5. Further research is needed to clarify whether the decrease in glucose was due to less release of CHH as the total carbohydrate level increased.

Immunological analysis has shown that an immediate effect of acute salinity change in *P. monodon* is the depression of immune response, which was maximum at a lower salinity stress than at a higher level. A similar immunosuppression has been reported in *P. monodon* (Wang and Chen, 2006a) transferred to 5‰, 15‰ and 35‰ from 25‰ after 12 h. Immune response following WSSV infection was surprisingly high in case of shrimps maintained at optimal salinity (15‰), particularly on PCD2, compared to those under salinity stress. Generally the activities showed a declining trend on PCD5. A similar upward trend in immune response on PCD3 and a declining trend on PCD5 were also observed in *F. indicus* challenged with WSSV (Sajeevan et al., 2006). In spite of the enhanced PO activity, respiratory burst activity and ACP activity on PCD2, shrimps at 0‰ succumbed to death on PCD6. Presumably, the shrimps suffered an immune fatigue after the enhanced response on PCD2, as the immune system was weak at the time of WSSV challenge due to acute salinity stress. Significant reduction in the immune response was noted on PCD5 in shrimps at 0‰ stress. Shrimps with 35‰ showed responses similar to that of the challenged shrimps at 15‰ on PCD5, exhibiting a comparatively better resistance to WSSV than those at 0‰.

Decrease in THC exhibited by WSSV- infected shrimps at all salinities is most likely caused by haemocytic accumulation at the site of injection for wound healing and phagocytosis (Ratcliffe and Rowley, 1979). In Taura Syndrome Virus infected *L. vannamei* THC was reported to decrease significantly (Song et al., 2003). A low circulating haemocyte count is strongly correlated with a greater sensitivity to pathogens (Persson et al., 1987). Reduction in THC that occurred after salinity stress due to cell lysis, diapedesis or osmosis of the water between haemolymph and medium (Pipe and Coles, 1995) may therefore be interpreted as a major factor that decreased the immunocompetence. Positive correlation could be established between THC and PO activity. Previous workers have found both negative (Hauton et al., 1995; Le Moullac et al., 1998) and positive (Cheng et al., 2004) correlation between THC and PO activity. Variations in PO activity may also be related to alterations in the regulatory mechanisms. Variations in respiratory burst activity could be attributed to the disparity in NADPH oxidase activity, phagocytic rate and/or the number of hyaline cells (Holmblad and Soderhall, 1999; Sajeevan et al., 2006). The activity of ALP and ACP that play a key role in destroying the extracellular invaders (Cheng and Rodirick, 1975) could be related to the phagocytic ability of haemocytes.

Sudden salinity changes were found to reduce the survival rate of *P. monodon*. It was previously reported in *P. monodon* that salinity variations lowered the disease resistance to *Photobacterium damselae* (Wang and Chen, 2006a). Shrimps were highly susceptible to WSSV infection at 0‰ stress compared to 35‰. Chang et al. (1998) could prove that salinity has little effect on the infectivity of WSBV. Hence the higher susceptibility of *P. monodon* at 0‰ cannot be related to the virulence of WSSV. It may particularly be noted that all the analysed parameters in haemolymph except cholesterol could be correlated with the survival rate. However, THC, ALP and PO that were mainly responsible for the survival rate, as shown by regression analysis, could be proposed as the most potential biomarkers of health in haemolymph that may be used in periodic assessment of the health status of shrimps.

In accordance with the above results it can be concluded that acute salinity stress induces alterations in haemolymph metabolic variables and affects the immunocompetence of *P. monodon* resulting in increased susceptibility to WSSV infection, being significantly more at a lower salinity stress. Shrimps maintained at optimal salinity (15‰) though could not completely eliminate virus particles from circulation and thwart an infection, their powerful immune defense and metabolic response could overwhelm the pathogen during early stages of infection that delayed the onset and pace of mortality. Study hence points to the significance of appropriate management measures to be adopted to minimize acute salinity stress in *P. monodon* culture ponds to minimize loss from WSSV infection.

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