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](image)

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 Whitspot 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 molting 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 activities 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, molting 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 mg/l 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 (PSDO) 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 \(-20^\circ\text{C}\) for the analysis of metabolic variables.

3.2.6 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).

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 100μl⁻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 phosphatase 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 haemolymph 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 osmotic 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 patterns 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 *Hemtoodinium* (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. 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.
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 a consequence of changes in the immune system due to salinity stress.
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.
**Fig. 3.1** Total protein in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.
**Fig. 3.2** Total carbohydrates in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.
Fig. 3.3 Total free amino acids (TFAA) in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.

*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.
Fig. 3.5 Glucose levels in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.
Fig. 3.6 Cholesterol levels in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.
Fig. 3.7 Total haemocyte count (THC) in the haemolymph of *P. monodon* subjected to salinity stress and challenged with WSSV.

### Total Haemocyte Count (x10^6 cells ml⁻¹)

<table>
<thead>
<tr>
<th>Salinity</th>
<th>BL</th>
<th>PSD0</th>
<th>PCD2</th>
<th>PCD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.04 ± 2.4</td>
<td>19.90 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.19 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.10 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0%&lt;sub&gt;e&lt;/sub&gt;</td>
<td>13.29 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.38 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.50 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>15%&lt;sub&gt;e&lt;/sub&gt;</td>
<td>20.10 ± 2.01&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>16.28 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.03 ± 2.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>35%&lt;sub&gt;e&lt;/sub&gt;</td>
<td>15.70 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.89 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.95 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

*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.*
**Phenol oxidase activity (increase in OD min⁻¹ 100μl⁻¹)**

<table>
<thead>
<tr>
<th>Salinity (%)</th>
<th>BL</th>
<th>PSD0</th>
<th>PCD2</th>
<th>PCD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.113 ± 0.02</td>
<td>0.109 ± 0.02⁺</td>
<td>0.108 ± 0.02⁺</td>
<td>0.114 ± 0.02⁺</td>
</tr>
<tr>
<td>0%</td>
<td>0.063 ± 0.02₁</td>
<td>0.2 ± 0.03₂</td>
<td>0.061 ± 0.02₂</td>
<td>0.172 ± 0.03²</td>
</tr>
<tr>
<td>15%</td>
<td>0.114 ± 0.02²</td>
<td>0.336 ± 0.05³</td>
<td>0.172 ± 0.03³</td>
<td>0.197 ± 0.02³</td>
</tr>
<tr>
<td>35%</td>
<td>0.172 ± 0.03³</td>
<td>0.085 ± 0.02³</td>
<td>0.197 ± 0.02³</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Salinity (%)</th>
<th>BL</th>
<th>PSD0</th>
<th>PCD2</th>
<th>PCD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.749 ± 0.08</td>
<td>0.738 ± 0.07&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.736 ± 0.08&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.727 ± 0.08&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>0%&lt;sub&gt;e&lt;/sub&gt;</td>
<td>0.513 ± 0.06&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.01 ± 0.09&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.582 ± 0.07&lt;sup&gt;II&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>15%&lt;sub&gt;e&lt;/sub&gt;</td>
<td>0.755 ± 0.09&lt;sup&gt;C&lt;/sup&gt;</td>
<td>1.037 ± 0.08&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.869 ± 0.08&lt;sup&gt;II&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>35%&lt;sub&gt;e&lt;/sub&gt;</td>
<td>0.581 ± 0.07&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.82 ± 0.07&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.831 ± 0.07&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Salinity (%)</th>
<th>BL</th>
<th>PSDO</th>
<th>PCD2</th>
<th>PCD5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.477±0.06</td>
<td>0.476±0.04</td>
<td>0.488±0.06</td>
<td>0.48±0.05</td>
</tr>
<tr>
<td>0%</td>
<td>0.353±0.04</td>
<td>0.453±0.07</td>
<td>0.294±0.05</td>
<td></td>
</tr>
<tr>
<td>15%</td>
<td>0.488±0.06</td>
<td>0.8±0.06</td>
<td>0.409±0.05</td>
<td></td>
</tr>
<tr>
<td>35%</td>
<td>0.606±0.05</td>
<td>0.624±0.06</td>
<td>0.387±0.05</td>
<td></td>
</tr>
</tbody>
</table>

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

*Different letters indicate statistical difference among different treatments*