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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	$_{b}88.35\pm10.1$	$_{c}88.58\pm12.9$	$_{b}90.10\pm11.4$
	0‰		$_{b}86.96\pm13.3^{C}$	$_{ab}109.81\pm11.9^{A}$	$_{ab}101.32\pm9.8^{AB}$
	15‰		$_{b}89.38\pm11.3^{B}$	$_{a}121.51\pm16.4^{A}$	$_{a}112.04\pm14.2^{A}$
	35‰		$_{a}110.48\pm13.9^{A}$	$_{bc}99.70\pm15.3^{A}$	$_{ab}100.72\pm12.06^{A}$
Total carbohydrates	Control	4.53±0.55	$_{c}4.59\pm0.53$	$_{c}4.46\pm0.58$	$_{b}4.49\pm0.52$
	0‰		$_{a}6.50\pm0.75^{A}$	$_{b}6.15\pm0.51^{A}$	$_{b}5.0\pm0.66^{B}$
	15‰		$_{c}4.62\pm0.38^{C}$	$_{a}7.13\pm0.59^{A}$	$_{a}6.46\pm0.61^{B}$
	35‰		$_{b}5.56\pm0.67^{A}$	$_{b}5.71\pm0.58^{A}$	$_{b}5.06\pm0.41^{A}$
Total free amino acids	Control	3.03±0.38	$_{b}3.09\pm0.45$	$_{ab}3.08\pm0.42$	$_{b}3.12\pm0.33$
	0‰		$_{a}3.88\pm0.52^{A}$	$_{b}2.69\pm0.33^{B}$	$_{c}2.36\pm0.5^{B}$
	15‰		$_{b}3.09\pm0.36^{B}$	$_{a}3.43\pm0.46^{AB}$	$_{a}3.79\pm0.39^{A}$
	35‰		$_{b}2.52\pm0.46^{AB}$	$_{c}2.16\pm0.3^{B}$	$_{bc}2.81\pm0.42^{A}$
Total lipids	Control	1.94±0.15	$_{a}1.92\pm0.16$	$_{a}1.9\pm0.14$	$_{a}1.89\pm0.16$
	0‰		$_{b}1.54\pm0.18^{A}$	$_{c}1.31\pm0.18^{B}$	$_{c}1.29\pm0.19^{B}$
	15‰		$_{a}1.91\pm0.18^{A}$	$_{ab}1.79\pm0.21^{AB}$	$_{b}1.62\pm0.17^{B}$
	35‰		$_{a}2.05\pm0.13^{A}$	$_{b}1.6\pm0.2^{B}$	$_{ab}1.71\pm0.2^{B}$
Glucose	Control	0.322±0.03	$_{b}0.318\pm0.04$	$_{b}0.315\pm0.04$	$_{b}0.316\pm0.05$
	0‰		$_{c}0.227\pm0.04^{B}$	$_{b}0.295\pm0.05^{A}$	$_{c}0.232\pm0.05^{B}$
	15‰		$_{b}0.322\pm0.06^{B}$	$_{a}0.397\pm0.04^{A}$	$_{a}0.423\pm0.06^{A}$
	35‰		$_{a}0.385\pm0.06^{A}$	$_{c}0.216\pm0.04^{B}$	$_{c}0.248\pm0.05^{B}$
Cholesterol	Control	0.537±0.06	$_{c}0.524\pm0.03$	$_{c}0.53\pm0.07$	$_{ab}0.533\pm0.06$
	0‰		$_{b}0.619\pm0.06^{A}$	$_{b}0.581\pm0.06^{A}$	$_{b}0.479\pm0.06^{B}$
	15‰		$_{c}0.515\pm0.04^{B}$	$_{ab}0.62\pm0.06^{A}$	$_{b}0.499\pm0.07^{B}$
	35‰		$_{a}0.739\pm0.08^{A}$	$_{a}0.668\pm0.06^{A}$	$_{a}0.577\pm0.05^{B}$

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

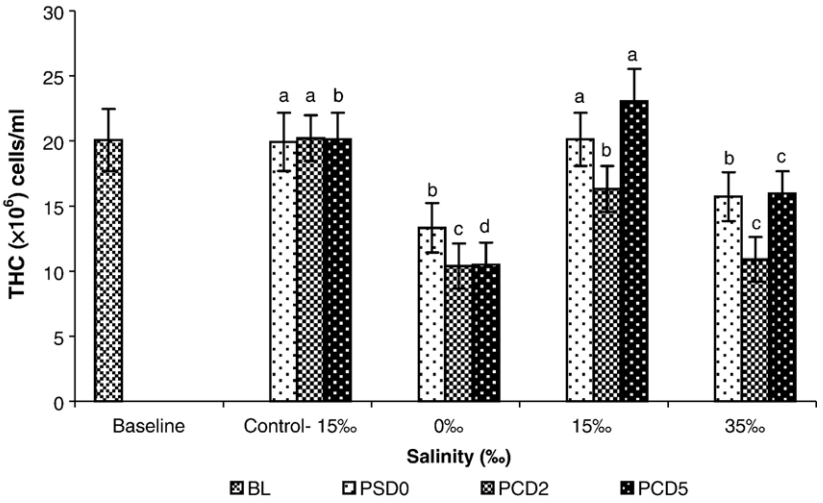

Fig. 1. Total haemocyte count (Mean±S.D.) of *Penaeus monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$). BL = baseline, PSD0 = post salinity change day, PCD = post challenge day.

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.).

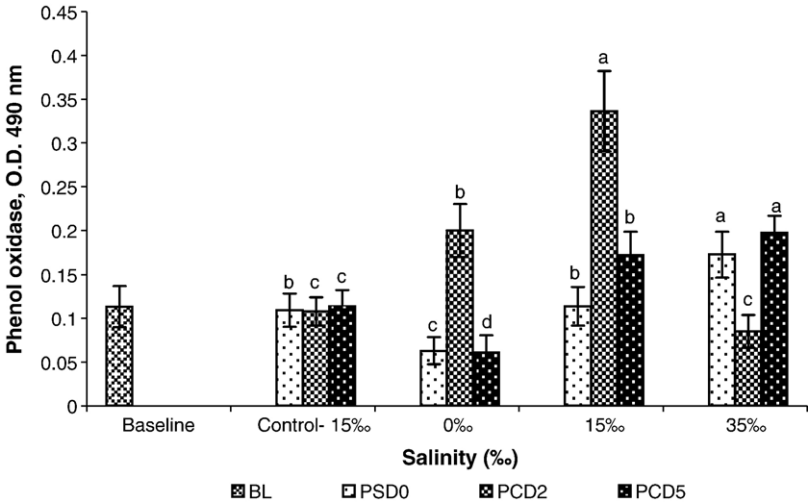

Fig. 2. Phenol oxidase activity (Mean±S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

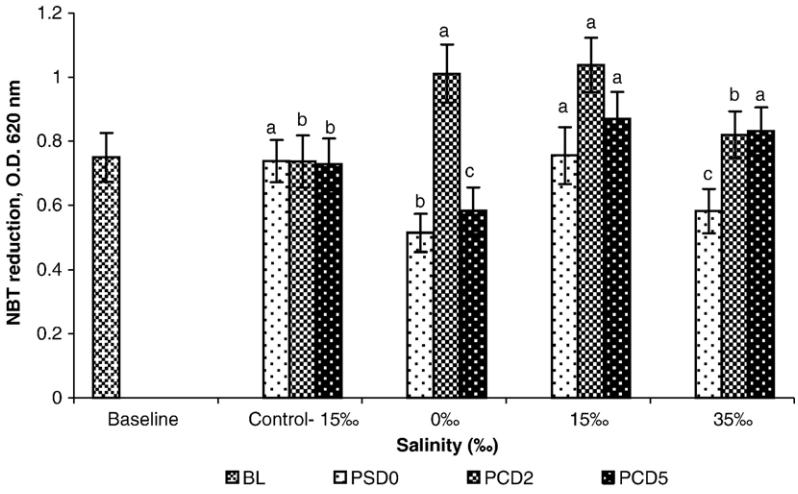

Fig. 3. NBT reduction (Mean ± S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

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

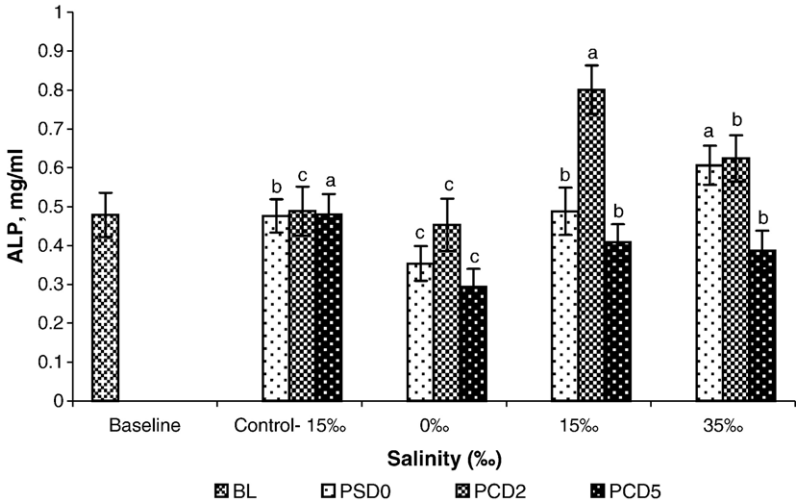

Fig. 4. Alkaline phosphatase activity (Mean ± S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

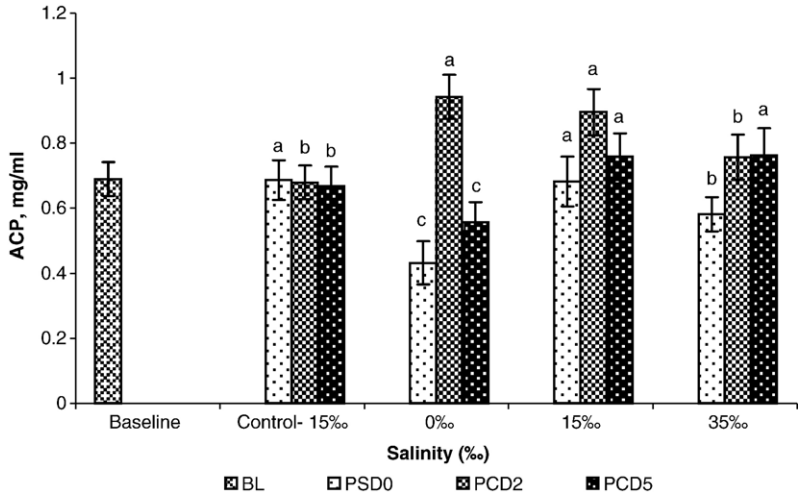

Fig. 5. Acid phosphatase activity (Mean±S.D.) of *P. monodon* subjected to acute salinity stress and then challenged with WSSV. Data at the same exposure time with different letters are statistically different ($P<0.05$).

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

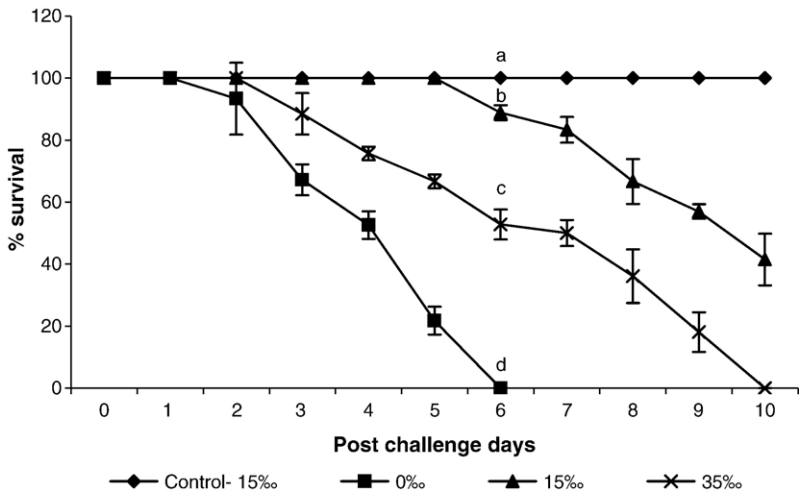

Fig. 6. Post challenge survival (Mean±S.D.) of *P. monodon* subjected to acute salinity stress against experimental infection with WSSV.

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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