

# Salinity Induced Haematological Inflection in Indian White Shrimp, *Fenneropenaeus indicus* (H. Milne-Edwards, 1837)

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## Abstract

The haematological profile of Indian white prawn (*Fenneropenaeus indicus* (H. Milne-Edwards, 1837), Penaeidae) at different salinities, i.e. 0, 5, 10, 15, 20, 25, 30 and 35 ppt was studied after acclimating the animals for 7 days in each saline condition. The haematological parameters, i.e. total haemolymph protein (THP), plasma protein (PP), serum protein (SP), haemocyte protein (HP), phenoloxidase, intracellular anion (NBT reduction), alkaline phosphatase (ALP), acid phosphatase (ACP) and total free amino acids (TFAA) were analysed and the data were statistically evaluated. These haematological parameters except ACP were at elevated level at 5 and 10 ppt indicating the animal's hyper immune response to the stressful ambient conditions. ACP was significantly low at 5 and 10 ppt.

## Introduction

Salinity is an important water quality parameter in aquaculture and is one of the main environmental factors that wield selective pressure on aquatic organisms (Charmantier and Charmantier-Daures, 2001). Salinity affects food consumption, food conversion efficiency, growth, survival, haemolymph osmolarity and immune mechanism in penaeids (Venkataramaiah et al. 1972; Raj and Raj, 1982; Staples and Heales, 1991; Silva et al. 2010; Yeh et al. 2010). Studies with juvenile Indian white shrimp, *F. indicus* revealed that this species is capable of osmotic adaptation at salinities between 3 and 40 ppt and *F. indicus* grows well at 25 ppt (780 mOsm $\cdot$ kg<sup>-1</sup>) (Parado-Esteva et al. 1987; Diwan and Laximinarayana, 1989). It has been suggested that the maximum growth of an organism occurs in iso-osmotic medium, since the animal would be expending minimal amount of energy in osmoregulation (Panikkar, 1968).

Mass mortality of the stock may occur quite frequently, due to adverse environmental conditions. Euryhaline shrimps adapt physiologically to the alteration in the surrounding medium. These changes are most obviously manifested in the composition of the haemolymph (Pequeux, 1995). In shrimps the internal medium, the haemolymph, reflects its physiological status as well as environmental fluctuations through the up and down regulation of its components. Thus, haemolymph composition of shrimps will provide an indication about the physiological modifications associated with moulting process, developmental stages, defence mechanism and environmental stress. Haemocyanin and other haemolymph proteins and the acid base balance of crustaceans were found to be altered by changes in ambient salinity (Li et al. 2008), temperature, dissolved oxygen (Ferraris et al. 1986), ammonia-N (Chen and Cheng, 1993) and nitrite-N (Chen and Cheng, 1995). Comprehensive data denoting the immune status of *F.*

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*indicus* with respect to changes in ambient salinity is lacking. Therefore, the present study was aimed to get the haematological profile of *F. indicus* acclimatised to different salinity levels.

## Materials and Methods

### Experimental animals and rearing conditions

Adult *F. indicus* were brought to the laboratory within 1 hr of capture from a commercial shrimp farm located at Panangad, Kochi, India. The average wet weight of the shrimp was  $15.24 \pm 1.68$  g (mean  $\pm$  SD). Shrimps were reared in rectangular concrete tanks containing 25 ppt clean seawater and allowed to acclimate for a period of 7 days. Continuous aeration was provided using air pumps and shrimps were maintained on a commercial shrimp diet (Higashimaru, Pvt. Ltd., Kochi). Water quality parameters, *viz.* temperature, dissolved oxygen, NH<sub>3</sub>, NO<sub>2</sub> and NO<sub>3</sub> were monitored daily following standard procedures (Eaton et al. 1995) and maintained at optimal levels as shown in Table 1. Unused feed and faecal matter were siphoned out daily and 30% water was exchanged every alternate day. A biological filter was set up to maintain the appropriate levels of water quality parameters.

**Table 1.** Rearing conditions and water quality.

Animal used	<i>F. indicus</i>
Size of animal	15 $\pm$ 1.68 gm
Stocking density (per tank)	35
Tank capacity	500 L
Feeding level	5-10% body weight.
Feeding frequency	Twice daily.
Feeding period	7 days.
Water temperature	25-27 °C
pH	7.5- 8.0
NH <sub>3</sub> -N	0.01-0.02 ppm
NO <sub>3</sub> -N	below detectable
NO <sub>2</sub> -N	0.00-0.01 ppm
Dissolved oxygen	6-7 mg L <sup>-1</sup>

### Experimental design

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

Group 2 shrimps maintained at 35 ppt were used for salinity treatment and the Group 1 shrimps held at 25 ppt were treated as the control group. After 1 week acclimation at these salinity levels, six shrimps were randomly sampled from the control (G1) and the salinity treatment (G2) groups. In order to avoid any effects due to food material, the shrimps were starved 12 hr prior to sampling (Hall and van Ham, 1998). Subsequently the salinity of Group 2 shrimps was reduced to 30, 25, 20, 15, 10, 5 and 0 ppt giving 1 week duration for acclimation to

each salinity level and six shrimps were sampled each time. The control group remained at 25 ppt throughout the experimental period and shrimps were sampled from the control group as well.

### ***Extraction of haemolymph***

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

### ***Haematological analysis***

Haemolymph biochemical parameters, viz. total haemolymph protein (THP), plasma protein (PLP), serum protein (SRP), haemocyte protein (HCP) (Bradford, 1976) and total free amino acids (TFAA) (Yemm and Cocking, 1955) were determined spectrophotometrically employing standard techniques and expressed as  $\text{mg mL}^{-1}$  haemolymph. The immune assays, viz. phenol oxidase (Soderhall, 1981), intracellular anion (NBT) reduction (Song and Hsieh, 1994), alkaline phosphatase (ALP) and acid phosphatase (ACP) (Gonzalez et al. 1994) were also done.

### ***Statistical analysis***

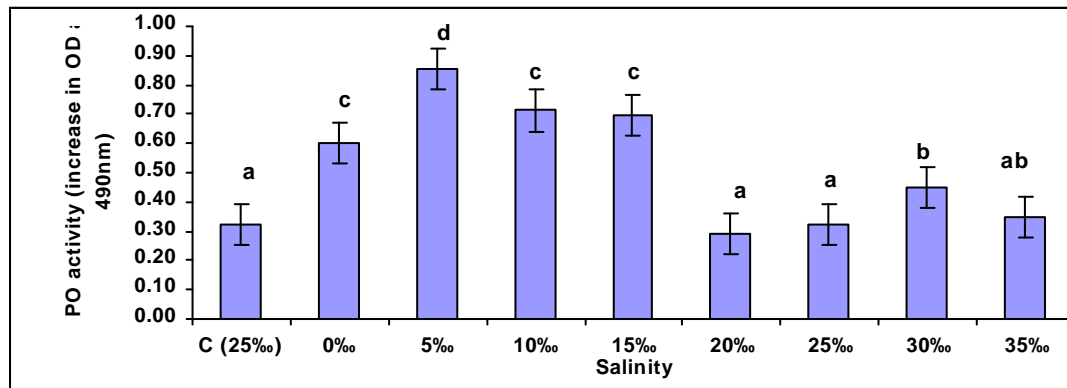
One-way ANOVA was done followed by Duncan's multiple range test in order to determine the significant differences between the means. All the data were presented as mean  $\pm$  SD and differences were regarded as statistically significant when  $p < 0.01$ .

## **Results**

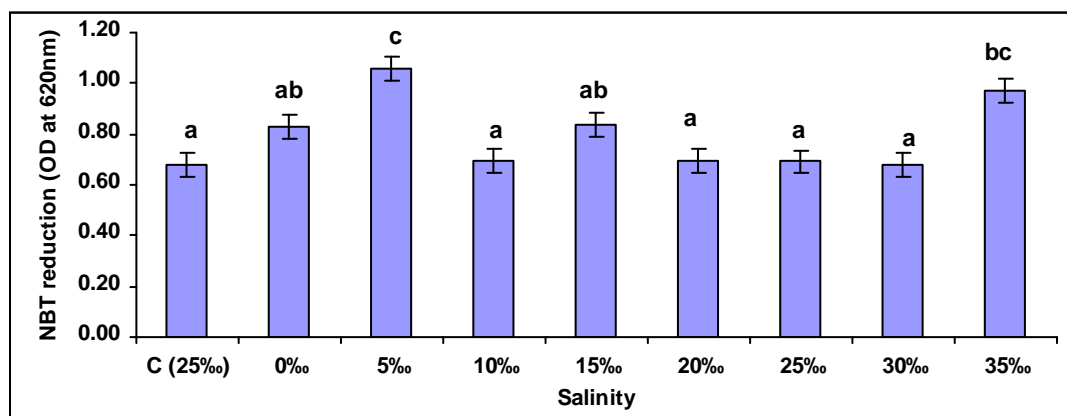
An elevation in haematological parameters i.e. THP, PLP, SRP and HCP could be observed at 5 and 10 ppt compared to other salinity levels (0, 15, 20, 25, 30 and 35 ppt), the lowest being recorded at 20–25 ppt. The haematological profile of prawns maintained at 15–35 ppt did not vary much between groups. Total free amino acid level was found to be exceptionally high at 0 ppt. The immune parameters viz. phenol oxidase, NBT reduction and ALP were found to be significantly higher in prawns maintained at 5 and 10 ppt compared to other salinity levels, the maximum being at 5 ppt for all parameters ( $p < 0.05$ ). Interestingly, ACP were found to be significantly lower at 5 and 10 ppt and comparatively higher at other salinity levels (0, 15, 20, 25 and 30 ppt) contrary to other parameters (Table 2 and Fig. 1-4).

**Table 2.** Total haemolymph protein (THP), plasma protein (PLP), serum protein (SRP) and total free amino acid concentration (TFAA) of *F. indicus* held at different salinity levels. Each data represents mean  $\pm$ SD of six separate determinations. Data with same lowercase letter do not vary significantly.

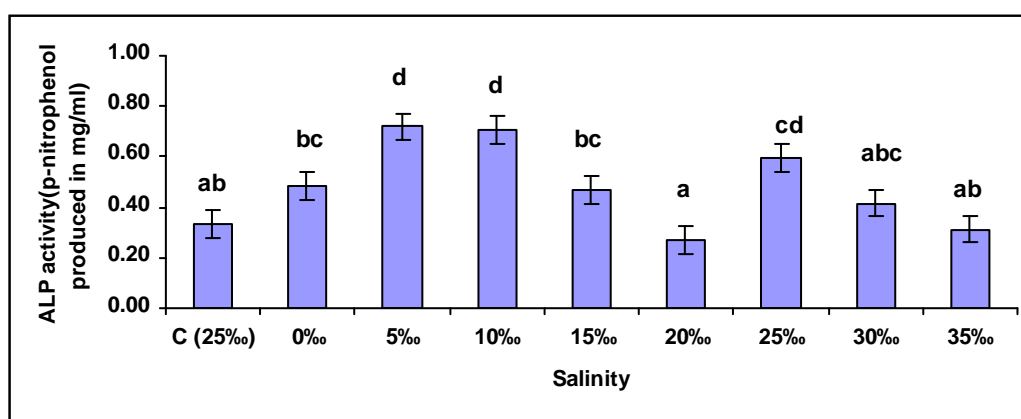
Salinity (%)	Haematological parameters ( $\text{mg mL}^{-1}$ )				
	THP	PLP	SRP	HCP	TFAA
Control (25)	90.16 $\pm$ 11.03	82.23 $\pm$ 10.61	64.64 $\pm$ 9.82	8.94 $\pm$ 0.99	5.64 $\pm$ 0.93
0	100.64 $\pm$ 5.00 <sup>a</sup>	93.28 $\pm$ 5.33 <sup>a</sup>	68.80 $\pm$ 5.65 <sup>a</sup>	6.25 $\pm$ 0.33 <sup>a</sup>	17.36 $\pm$ 1.63 <sup>e</sup>
5	141.19 $\pm$ 6.35 <sup>c</sup>	130.52 $\pm$ 6.24 <sup>c</sup>	91.33 $\pm$ 12.35 <sup>bc</sup>	10.10 $\pm$ 0.75 <sup>d</sup>	9.93 $\pm$ 0.86 <sup>cd</sup>
10	133.49 $\pm$ 6.06 <sup>c</sup>	122.19 $\pm$ 5.40 <sup>c</sup>	96.18 $\pm$ 8.96 <sup>c</sup>	9.77 $\pm$ 0.85 <sup>cd</sup>	10.30 $\pm$ 0.81 <sup>cd</sup>
15	108.68 $\pm$ 7.03 <sup>b</sup>	100.30 $\pm$ 6.44 <sup>b</sup>	67.87 $\pm$ 5.04 <sup>a</sup>	7.65 $\pm$ 0.64 <sup>abc</sup>	11.59 $\pm$ 1.66 <sup>d</sup>
20	94.20 $\pm$ 4.75 <sup>a</sup>	87.14 $\pm$ 4.82 <sup>a</sup>	68.25 $\pm$ 4.45 <sup>a</sup>	8.02 $\pm$ 0.57 <sup>abcd</sup>	9.10 $\pm$ 1.43 <sup>bcd</sup>
25	103.03 $\pm$ 4.80 <sup>ab</sup>	96.01 $\pm$ 4.65 <sup>ab</sup>	78.29 $\pm$ 3.51 <sup>ab</sup>	6.36 $\pm$ 0.50 <sup>ab</sup>	5.12 $\pm$ 1.13 <sup>a</sup>
30	109.82 $\pm$ 6.98 <sup>b</sup>	101.34 $\pm$ 7.33 <sup>b</sup>	75.93 $\pm$ 6.95 <sup>ab</sup>	8.50 $\pm$ 2.00 <sup>bcd</sup>	7.74 $\pm$ 1.18 <sup>abc</sup>
35	104.28 $\pm$ 8.83 <sup>bc</sup>	95.84 $\pm$ 9.35 <sup>ab</sup>	81.52 $\pm$ 9.82 <sup>abc</sup>	9.24 $\pm$ 1.14 <sup>cd</sup>	6.91 $\pm$ 1.06 <sup>ab</sup>



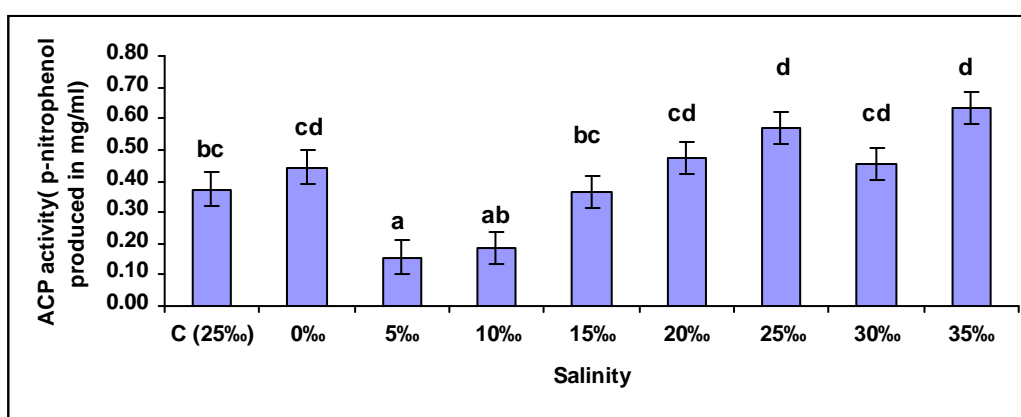
**Fig 1.** Phenoloxidase activity (mean $\pm$ SD) in the haemolymph of *F. indicus* held at different salinity levels. The lowercase letters show significant variations in the PO activity among different salinity levels at  $p < 0.01$ . Data with same lowercase letter do not vary significantly.



**Fig 2.** Intracellular super anion production (mean  $\pm$  SD) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.1 for statistical details).



**Fig 3.** Alkaline phosphatase activity (mean  $\pm$  SD) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.1 for statistical details).



**Fig 4.** Acid phosphatase activity (mean  $\pm$  S.D) in the haemolymph of *F. indicus* held at different salinity levels (See Fig.1 for statistical details).

## Discussion

It has been well demonstrated that *F. indicus* is able to withstand a wide range of salinity (3-40 ppt) although its optimum salinity level is 25 ppt. However, a detailed metabolic response in terms of haematological parameters has not been attempted so far. The present investigation has demonstrated that *F. indicus* acclimatised at different salinity levels exhibited measurable amount of variations in the haemolymph protein components *viz.* THP, PLP and SRP concentrations compared to the control group of shrimps. A considerable increase in the haematological parameters was found at the lower (5 and 10 ppt) and higher salinity level (35 ppt). Post hoc tests showed that the elevation in the haemolymph variables was significant at  $p < 0.01$ . The increase in the haemolymph protein components may be attributed to the increased production of haemolymph proteins such as haemocyanin and other stress proteins in response to the hypo- and hyper-osmotic environment. Hypo-osmotic condition has been reported to trigger increase in the production of haemocyanin in tiger shrimp, *Penaeus monodon* Fabricius, 1798 (Chen et al. 1994). Plasma protein concentration of penaeid shrimp correlates with the pathogenic infection (Vogan and Rowley, 2002; Song et al. 2003) as well as with environmental stress (Chen et al. 1994). Alterations in salinity have been reported to modulate the immune

vigour of crustaceans (Hauton et al. 2000; Wang and Chen, 2006; Joseph and Philip, 2007). Further, Li et al. (2008) showed that low salinity (3 ppt) induced increase in haemolymph protein and haemocyanin concentration in white shrimp *Litopenaeus vannamei* (Boone, 1931).

In the present study, prominent variation in total haemocytes could be observed in terms of haemocyte protein concentration in shrimps at different salinity levels. Shrimps held at 5 and 10 ppt displayed higher HCP concentration compared to other salinities. Many authors have demonstrated that hyper osmotic conditions can increase the number of circulating haemocytes (Le Moullac and Haffner, 2000; Cheng et al. 2004). We observed an increase in HCP at lower salinity indicating an increase in haemocyte population at hypo osmotic conditions in *F. indicus*. The circulating haemocyte number can be a stress indicator but this parameter varies non-specifically according to the natural rhythm of the environment, and chemical/ physico-chemical stress. The observation in the present study highlights the importance of further studies to reveal the impact of salinity stress on haemocytes of shrimps.

Free amino acids (FAA) are known to play a major role in osmoregulation of marine invertebrates (Dalla Via, 1986). In cray fish and crab, more than 40- 60% of the intracellular osmolarity has been contributed by FAA (Shaw, 1958; Robertson, 1961). In the present study, increased concentration of FAA was noted at lower salinity, the highest being at 0 ppt, which may be a physiological adaptation for osmoregulation. However, this observation does not agree with the findings of Huong et al. (2001) who demonstrated that hyper osmotic condition was directly correlated with high amino acid concentration in crustaceans.

We found that phenoloxidase activity was high for shrimps held at lower salinity levels, the maximum being at 5 ppt salinity. Previous reports indicated that perturbations in the ambient salinity had profound influence on the phenoloxidase system of crustaceans. Lu Qing et al. (2005) have shown that salinities between 5-30 ppt were found to peak the phenol oxidase activity in *L. vannamei* at the 12<sup>th</sup> hr of exposure. Similarly, in yellow leg shrimp, proPO was found to increase directly proportional to salinity (Vargas-Albores et al. 1998). The significant increase in phenol oxidase activity observed in our study may be attributed to the hyper immune response of the organism under hypo osmotic stress. Cheng and Chen (2000) observed that phenoloxidase activity of *Macrobrachium rosenbergii* (De Man, 1879) was significantly higher in animals reared at 5 and 10 ppt than those in fresh water and 15 ppt. Lamela et al. (2005) reported that phenoloxidase activity decreased in *Litopenaeus schmitti* (Burkenroad, 1936) exposed to low salinity for 48 hr.

Intracellular superoxide anions production increases because of elevated activity of NADPH oxidase and by the decreased activity of superoxide dismutase. In the present study, it was found that superoxide anion production was high at lower salinity (5 and 10 ppt) and higher salinity (35 ppt) levels. It has been reported that production of intracellular superoxide anions have been influenced by the fluctuations in the ambient salinity. According to Cheng et al. (2004), the super oxide anion production has been found to decrease in *Halotis diversicolor* Reeve, 1845 when transferred to 20, 25 and 35 ppt from 30 ppt.

In the present study, ALP was found to be significantly higher at lower salinity levels (5 and 10 ppt.) but the ACP activity was lower at these salinity levels. The results suggest the possible implication of the phosphatases in acute osmotic stress in shrimps. Previous studies showed that the ACP activity in serum can reflect the immune state of the scallop (Zhang et al. 2005). Lovett et al. (1994) reported the presence of an ALP at pH 9.1 in the posterior gills of *Callinectes sapidus* Rathbun, 1896 and the activity was sensitive to environmental salinity. The specific activity of this ALP was greater in *C. sapidus* acclimated to 35 ppt than crabs acclimated to 10 ppt. Lovett et al. (1994) postulated the role of alkaline phosphatase in modulating the osmoregulatory response of *C. sapidus* by varying the expression in response to acclimation salinity; our results also support the possible role of phosphatases in osmoregulation in crustaceans.

It has been well demonstrated that 25 ppt salinity is the best salinity for culture of Indian *F. indicus* strains (Kumlu and Jones, 1993 and 1995). The alterations in salinity beyond the optimum level have evoked stress responses in the haematological variables. According to Charmantier (1987), *F. indicus* adults are not as capable of withstanding lower salinities as post larvae and juveniles do. The unsuitability of *F. indicus* as a candidate species for culture at salinities lower than 10 ppt has been discussed by Parado-Estapa et al. (1987) and Kumlu and Jones (1995). Our results indicated that the adult *F. indicus* acclimated to extreme salinities were under stress. These stress responses have been reflected in the haematological profile emphasising the unsuitability of lower salinity (below 10 ppt) for the culture of *F. indicus*.

## Conclusion

In conclusion, the haematological alterations were prominent at lower salinity levels (5 and 10 ppt) showing maximum stress on the animals. Significant increase of haemolymph protein and immune factors in shrimps maintained at lower salinities is a reflection of the immuno-physiological responses to cope with the ambient stressful condition. The presence of ACP at significantly low level at lower salinities would be worth investigating. Salinity of 15-35 ppt was found to be the desirable range for culture of *F. indicus* to avoid stress and related physiological malfunctions. These haematological profiles can be used as an index for monitoring the health conditions of shrimps, thereby preventing reduced growth and diseases often associated with stressors.

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