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# Effect of salinity and pH on selected immune functions of the Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837)

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## ABSTRACT

The Indian white shrimp, *Fenneropenaeus indicus*, was subjected to environmental stresses like high (9) and low (5.5) water pH and decreasing water salinity (34 ‰ to 18 ‰) for a period of one week and certain vital immunological functions like total hemocyte count, total hemolymph protein and phenoloxidase activity were analysed to understand the effect of the environmental stress factors on these functions. The results indicated that while stress induced by change in the salinity had no damaging effects on the immune functions, stress caused by lower water pH induced more immunological damage when compared to higher water pH. Significantly decreased total hemocyte count and phenoloxidase activity were observed in shrimps exposed to lower pH when compared to shrimps exposed to higher pH and control shrimps. Also, lower pH significantly reduced the hemolymph protein values. It is therefore concluded that *Fenneropenaeus indicus* that are exposed to extreme pH show lowered immunological activity which would render the shrimp susceptible to infectious agents.

Keywords: *Fenneropenaeus indicus*, Immune response, pH, Salinity, Shrimp

## Introduction

Shrimp health is influenced by a range of factors, one of the most important being environmental stress. Wide range of environmental factors such as water quality parameters (pH, salinity, temperature, dissolved oxygen, *etc*.) can act as stress factors invoking stress responses (Evans, 2000). Mismanagement of the ecosystem leading to stress in shrimp is the root cause of most shrimp diseases. In culture systems, health problems are usually detected at an advanced stage as reduced growth, abnormal behaviour and widespread mortalities (Perazzolo *et al.*,2002).

Crustacean defence system mainly consists of cellular and humoral components (Ratcliff *et al.*, 1985). The cellular reactions include phagocytosis and nodule formation (Soderhall and Cerenius, 1992; Roch, 1999). Humoral reactions comprise of phenoloxidase system (Soderhall *et al.*, 1996), antimicrobial peptides (Destoumieux *et al.*, 1997) and coagulation mechanisms (Montano-Perez *et al.*, 1999). Under aquaculture conditions, wide range of stresses caused by various adverse environmental factors damage the host defence system resulting in an increased susceptibility to infections (Perazzolo *et al.*, 2002).

Ecological factors like salinity and pH of the water are two of the many major factors to be considered vital in shrimp farming since extreme values are harmful to many species affecting their growth and productivity. Limited information is available on the physiological and immunological changes induced on shrimps by captivity, management and environmental stress conditions. Evaluation of hemato-immunological parameters like hemocyte count, protein profile, phenoloxidase activity and agglutinin activity were done in marine shrimp *Farfantepenaeus paulensis* subjected to varying levels of salinity (Perazzolo *et al.*, 2002). Effects of pH, temperature, salinity and hypoxia on the immune parameters of freshwater prawn *Macrobrachium rosenbergii* has been reported (Cheng and Chen, 2000; Cheng *et al.*, 2002). No published information is available on the defence functions of the Indian white shrimp, *Fenneropenaeus indicus* as affected by altered environmental factors. Hence, it was felt worthwhile to study the effect of environmental stress factors on some of the immunological functions of *F. indicus*.

## Materials and methods

### *Collection of shrimp and experimental design*

Shrimps caught off Calicut ($74^0$ 40' N., $11^0$ 30' E.) were brought to the laboratory and maintained in 5000 l fibre reinforced plastic (FRP) coated cement tanks containing

3000 l of filtered seawater (salinity: 33%; temperature: 29 $^{O}$C; pH: 7.6 and dissolved oxygen: >90% saturation) for 6 days for acclimatisation. Subsequently, the shrimps were divided into different groups consisting of 40 shrimps (15±4 g) in each group and maintained in 1000 l circular FRP tanks containing 500 l of filtered seawater (temperature: 29±0.2 $^{O}$C and dissolved oxygen: >90% saturation). The first group was introduced into seawater with a pH of 9.0 and salinity of 33±1 ‰. Second group was subjected to a pH of 5.5 and salinity of 33±1 ‰. Acidic and alkaline pH values were achieved by adding hydrochloric acid and calcium hyhroxide respectively. Third group was subjected to decreasing salinity from 34 ‰ to 18 ‰ by reducing at the rate of 4 ‰ every 48 h by adding freshwater and pH was maintained at 7.6±0.2. The fourth group served as control where the pH and salinity were 7.6±0.2 and 33±1 ‰ respectively. Duplicate tanks were maintained for each treatment. One third of the water was renewed daily. Shrimps were fed at the rate of 10% of the body weight twice daily with standard shrimp feed procured commercially. Uneaten feed was removed three hours after feeding by siphoning.

*Hemolymph collection*

Hemolymph was collected directly from the heart of shrimp in intermoult stage by inserting a 26 gauze hypodermic needle fitted to a 2 ml syringe. In case of pH experiments, hemolymph was collected at 0, 24, 48, 72 and 96 h after commencement of experiment and subjected to total hemocyte count, serum phenoloxidase activity assay and determination of total hemolymph protein. In the case of salinity experiments, the hemolymph withdrawn at the end of 0, 2$^{nd}$, 4$^{th}$ and 6$^{th}$ day was subjected to total hemocyte count, determination of total hemolymph protein and serum phenoloxidase activity assay. In both the cases, hemolymph was withdrawn from six individual shrimp at a time. For serum separation, approximately 0.5 ml of hemolymph collected was kept at room temperature for 30 min and at 4 $^{O}$C overnight. Serum was then separated by agitating the clot followed by repeated centrifugation.

*Total hemocyte count (THC)*

For evaluating the THC, 0.1 ml of hemolymph was withdrawn into a syringe containing 0.9 ml of 4% neutral buffered formalin. After thorough mixing, a drop of hemolymph-formalin mixture was placed on a naeubauer slide and cells were counted using a phase contrast microscope.

*Estimation of total hemolymph protein*

Total hemolymph protein was estimated as per Lowry *et al.* (1951) using bovine serum albumin as standard after precipitating the hemolymph with 80% ethanol.

*Phenoloxidase (PO) activity assay*

The method adopted by Perazzolo and Barracco (1997) was employed with slight modifications which involved use of microtitre plates and a microplate reader for reading the optical density. Briefly, 15 µl of the serum was dispensed into triplicate wells of a microtitre plate and 15 µl of bovine trypsin (1 mg ml$^{-1}$) was added as an elicitor. The plate was incubated for 20 min at 18 $^{O}$C. Fifteen microlitre of L-DOPA (3 mg ml$^{-1}$) was then added and left for 5 min. The reaction was stopped by adding 255 µl of distilled water and an increase in the absorbance due to the formation of dopachrome was immediately read at 490 nm for 60 min at five min interval in a Biotek ELx 800 microplate reader. Blank wells were prepared by incubating L-DOPA with 0.45 M NaCl. A unit of enzyme activity was expressed as change in the absorbance of 0.001 min$^{-1}$ mg protein$^{-1}$. Part of the serum (50 µl) was subjected to protein estimation by the method of Bradford (1976) using bovine serum albumin as standard.

*Statistical analysis*

The experimental results were statistically analysed by one-way-ANOVA with tukey post-tests using GraphpadPrism (Version: 4) for Windows

## Results

The values of hemocyte count, PO activity and total hemolymph protein of the shrimp subjected to decreasing salinity are shown in Table 1. The values for all the three immunological parameters of the shrimp exposed to decreasing water salinity were not significantly ($p<0.1$) different from those in the control group.

Effect of change in water pH on total hemocyte count is shown in Fig.1. In the case of shrimps exposed to pH 5.5, the THC values at 24 h were significantly ($p<0.01$) lower than those exposed to pH of 9 and control shrimp.

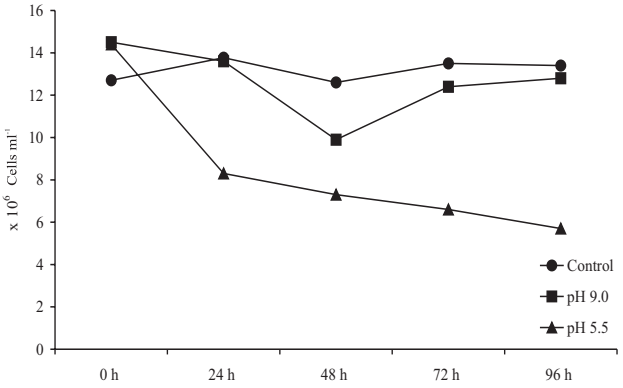


Fig. 1. Total hemocyte count (mean±SE) of the shrimp exposed to pH stress

Table 1. Total hemocyte count (THC), phenoloxidese (PO) activity and total hemolymph protein values of shrimps exposed to salinity stress

| Immune parameters | Treatment groups | Days after commencement of experiment (mean±SE) | | | |
|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 |
| Hemocyte count ($x10^6$ cells $ml^{-1}$) | Experimental group | 9.3±5.7 | 12.9±9.1 | 5.5±4.9 | 14.1±8.8 |
| | Control | 11.2±35.7 | 9.1±3.5 | 8.1±3.1 | 12.1±2.55 |
| PO activity (OD $min^{-1}$ mg $protein^{-1}$) | Experimental group | 4.0±1.6 | 8.8±2.6 | 9.7±15.5 | 6.4±4.3 |
| | Control | 4.9±3.3 | 16.1±29.4 | 5.0±4.2 | 5.8±3.1 |
| Total hemolymph protein (mg $ml^{-1}$) | Experimental group | 53.4±6.2 | 25.8±13.9 | 58.0±28.7 | 67.8±16.0 |
| | Control | 44.9±9.3 | 25.2±12.0 | 55.7±19.7 | 59.7±6.9 |

The hemocyte count values of shrimp subjected to both low and high pH were significantly lower than the control shrimp at 48 h ($p<0.01$). At 72 and 96 h, the THC values of the shrimp exposed to pH 5.5 were significantly ($p<0.01$) lower than those exposed to a pH of 9 and also control shrimp.

Effect of change in water pH on total hemolymph protein is shown in Fig. 2. The total hemolymph protein values of the shrimp exposed to pH 9 were not significantly ($p<0.1$) different from that of the control shrimp throughout

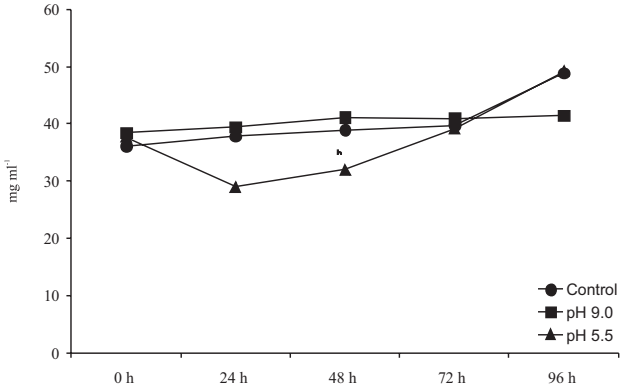


Fig. 2. Hemolymph protein (mean±SE) of the shrimp exposed to pH stress

the period of experiment. Those exposed to pH 5.5 had significantly ($p<0.01$) lower protein values when compared to control animals and also shrimp exposed to higher pH, from 24 to 48 h after the commencement of the experiment.

Effect of pH on PO activity is shown in Fig. 3. The PO activity values in the case of shrimps subjected to pH 9 were significantly ($p<0.01$) lower than control shrimp at 96 h after the commencement of the experiment. In case of shrimps exposed to pH 5.5, the PO activity values were significantly ($p<0.01$) lower than the control animals throughout the experimental period from 24 h after the commencement of the experiment.

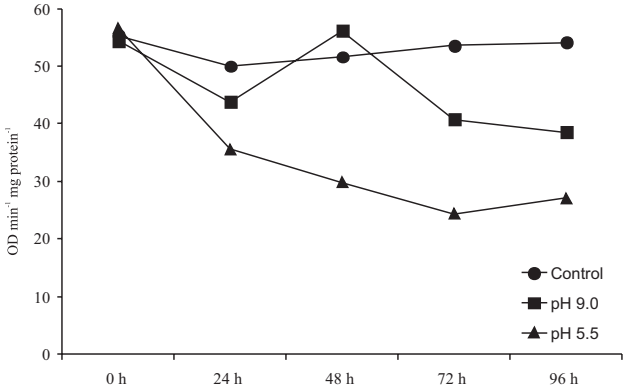


Fig. 3. Phenoloxidase activity (mean±SE) of the shrimp exposed to pH stress

## Discussion

The habitat of marine shrimp varies considerably ranging from high saline water in the open seas to regions of freshwater. It is reported that the concentration of $K^+$, $Na^+$ and $Cl^-$ in the blood of *Macrbrachium mastersii* shows more or less the same changes according to the difference in the concentration of ions in the seawater (Dall, 1957). Absence of any significant variation in the immune functions of *F. indicus* exposed to decreasing salinity when compared to control shrimp which were exposed to a constant salinity in the present study supports the above explanation which upholds the wide tolerance for salinity the penaeid shrimp enjoys.

In the present study, the salinity was gradually reduced at the rate of 4 ‰ every 4 days in order to simulate the culture conditions in field situation where there will be gradual and continuous fall in salinity as monsoon sets in. However, in experiments conducted by keeping the shrimps in extreme but constant salinity, it was observed that the THC, PO activity and total protein were lower at low salinity (13 ‰) than the control shrimp maintained at 34 ‰ (Perazzolo *et al.*, 2002). Also, it is reported that extreme salinities can cause mortality particularly if they are exposed without acclimation (Imai, 1977).

The results of the present study have revealed that among the three immune parameters studied, both THC and PO activity were significantly affected after exposure of the shrimp to altered pH when compared to the total hemolymph protein. Also, increase in the $H^+$ concentration in seawater seems to have more deleterious effects on these immune functions when compared to an increase in the $OH^-$ concentration. In case of shrimp, the ionic concentration of hemolymph is more or less same as in the case of seawater. But the stress induced on the animals by exposing them to change in the water pH resulted in lowered immunological values. Lowered THC and PO activity were observed in *Macrobrachium rosenbergii* and some penaeids, after exposing the animals to various environmental stresses including hypoxia (Le Moullac *et al.*, 1998; Cheng and Chen, 2000; Cheng *et al.*, 2002; Perazzolo *et al.*, 2002). Since precursors of PO system are stored within the hemocytes, decline in the hemocyte count may lead to a lowered serum PO activity. Alternatively, stress caused by altered pH may directly inhibit the conversion of prophenoloxidase to phenoloxidase thus lowering the PO activity as a pathological response to stress.

Acidic and alkaline waters produce stress in fish due to an impairment of oxygen transfer and ammonia accumulation respectively (Schaperclaus, 1986). This may be responsible for more prominent immune damage observed in the present study in the case of shrimps exposed to lower pH, since accumulation of ammonia was not a limiting factor as one third of the water was replaced daily. Hence, more prominent changes in the THC and PO activity observed in the shrimp exposed to lower pH may predominantly be attributed to general tissue hypoxia.

Interestingly, total hemolymph protein was not affected in case of shrimps exposed to higher pH. As majority of the hemolymph protein is constituted by oxygen carrying hemocyanin pigment (Djangman, 1970), the present study revealed that the oxygen carrying function of the hemolymph is not affected at this intensity of stress caused by increased pH of the water. However, significantly low hemolymph protein could deprive the tissue of oxygen due to hypoxia in case of shrimp exposed to low pH.

Besides their role in cellular immune reactions, hemocyte is the principle site of expression of genes encoding immune effectors (Gross *et al.*, 2001). Hence, prolonged decrease in THC together with low PO activity and total protein in cultured shrimp exposed to very high or low water pH may lead to extensive immune damage, thereby increasing the risk of infections by opportunistic and/ or pathogenic microorganisms.

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