J. Aqua., **14** (2006): 77-82

EFFECT OF WATER TEMPERATURE ON IMMUNE PARAMETERS OF GIANT FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII*

The giant freshwater prawn *Macrobrachium rosenbergii* is an economically important farmed crustacean species cultured in Caribbean countries and south-east Asia including India. Crustaceans have evolved a complex, efficient and highly developed innate immune system based largely upon circulatory haemocytes, various defence proteins *viz*., prophenoloxidase (proPO), serine proteases/PPO activating enzyme (ppA), lectins, β-glucan binding proteins, lysozyme, antibacterial peptides, α2 macroglobulins etc. (Smith *et al*., 2003). It is well established that, in arthropods, the defence of the host against invasive or opportunistic microorganisms is effected principally through the phagocytic, encapsulating and agglutinating activities of the circulating haemocytes (Ratcliffe *et al*., 1982) as well as by antimicrobial factors in the plasma (Gotz and Boman, 1985). The proPO system seems to participate in host defence by enhancing phagocytosis, melanin formation and initiating nodule or capsule formation (Soderhall *et al*., 1986). Based upon the recent classification of *M. rosenbergii* haemocytes (Sierra *et al*., 2001), large ovoid haemocytes and undifferentiated round haemocytes might be carrying out the functions of the proPO system, like semigranular and granular haemocytes in other crustaceans (Johansson and Soderhall, 1989). The activity of phenoloxidase has already been reported in *M. rosenbergii* (Kumari *et al*., 2004).

In recent years, environmental changes have become an important cause for increased prevalence of shrimp disease, leading to production loss. Therefore, there is an effort to study the effects of environmental factors on immune function of shrimp (Lu-Qing *et al*., 2007). Changes in physico-chemical parameters *viz*., pH, temperature, salinity, dissolved oxygen, presence of pollutants and toxicants such as ammonia and nitrite have been reported to affect the disease resistance of freshwater prawn *M. rosenbergii* and other decapod crustaceans (Cheng and Chen, 2000; Le Moullac and Haffner, 2000; Chand and Sahoo, 2006). Water temperature is probably the most important environmental factor, which directly affects metabolism, growth, oxygen consumption and survival and influences environmental parameters such as salinity and oxygenation of the water (Le Moullac and Haffner, 2000). Seasonal ranges of water temperature in scampi farms of southern and eastern parts (major scampi producing parts) of India mostly vary from 19 to 32˚C. *M. rosenbergii* can tolerate a wide range of temperature (14-35°C) and the optimal temperature for growth is 29-31°C (New, 1995). The effects of pH, temperature and salinity on the oxygen consumption and nitrogen excretion of *M. rosenbergii* have been studied by Nelson *et al.* (1977). In a preliminary study, Cheng and Chen (2000) observed a lower THC at 33-34°C (at 1.5% feeding rate) and a higher THC at 27-28°C (at 0.6% feeding rate), the highest PO activity at 30-31°C (at 1.5% feeding rate) and at 27-28°C (at 0.6% feeding rate) while experimenting with two prawns only in triplicate at four different

temperature ranges (20-21°C, 27-28°C, 30-31°C and 33-34°C). Lu-Qing *et al.* (2007) observed a significant effect of temperature on THC and PO, antibacterial and bacteriolytic activities in haemolymph of *L. vannamei*, and advocated a range of 24-30°C as suitable temperature for culture without its fluctuation beyond 3°C. Cheng *et al.* (2005) found a reduction in immune capability and resistance of *L. vannamei* to *Vibrio alginolyticus* infection when the shrimp were transferred to 32-34°C from 27 or 28°C. Thus, the present study aims at drawing a clear picture on few of the immune parameters that are being influenced by various temperature ranges in *M. rosenbergii* and discusses a suitable temperature range for culture of this species from immune capability point of view.

M. rosenbergii (15-20 g, in the intermoult stage) were collected from monoculture ponds of the Central Institute of Freshwater Aquaculture, Bhubaneswar. The prawns were acclimated in the wet laboratory in FRP tanks at a stocking density of 2 g/l for 1 week before experimentation. A pellet feed was provided twice daily during acclimation and also during the experiment as described earlier (Kumari *et al.,* 2004). The basic physico-chemical parameters of the water of the ponds (from where the samples were collected) and experimental tanks (where similar pond water was used) were as follows: pH, 6.8-7.5, dissolved oxygen 5.2-6.4 mg/l, ammonia < 0.1 mg/l, total hardness 70-80 mg/l, and total alkalinity 30-40 mg/l.

Thirty numbers of intermoult prawns (male:female::1:1) were maintained in triplicate in three FRP tanks for each temperature range study. Three different temperature ranges (*viz.,* 19-21°C; 25-27°C; 30-32°C) were setup using electronically controlled water heater based on field observations on temperature fluctuations in prawn farms of coastal area (P.K. Sahoo, unpublished observation). The prawns were acclimatized in the above temperatures for a period of 7 days. Ten percent of water was renewed daily (with preset desired temperature) during removal of waste feed and faecal materials. A continuous aeration was provided during the experimental period.

After 7 days of acclimatization, haemolymph of 100 µl was collected from the ventral sinus of fifteen prawns of each temperature group in to a 1 ml syringe (26 gauge) containing 900 µl anticoagulant (sodium chloride 0.45 M, glucose 0.1 M, sodium citrate 30 mM, citric acid 26 mM, EDTA 20 mM, pH 4.5). Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxy phenylalanine (L-DOPA, a product of Hi Media, Mumbai) following Hernandez-Lopez *et al.* (1996) and Chand and Sahoo (2006). The phenoloxidase activity optical density was expressed as dopachrome formation per 50 µl haemolymph.

Haemolymph (50 µl) was immediately withdrawn into another syringe containing 0.45 ml of anticoagulant with fixative solution (sodium cacodylate 0.10 M, and 1.5% glutaraldehyde) in 1:1 ratio. A drop of haemolymph was placed on a

haemocytometer to measure THC and DHC using an inverted phase contrast microscope. The various cell types were identified following Sierra *et al.* (2001).

From rest of the fifteen prawns of each group, haemolymph (~ 300 µl) was withdrawn using 2 ml syringe and 26 gauge needle from the ventral sinus and dispensed in to 2 ml eppendorf. Haemolymph was allowed to clot at 4^{0}C. After 1 h, the clot was broken using sterile needle and the tube was further kept at 4^{0}C for 3 h. The tube was then centrifuged at 11,000 x g for 30 min at 4^{0}C and the supernatant collected was stored at 30^{0}C till further analysis. Part of the supernatant was used to measure total protein concentration following Bradford (1976), using bovine serum albumin as a standard protein. Rest part of the supernatant was used to measure bacterial agglutination and haemagglutination titres.

Rabbits maintained in the Institute animal house were bled from the ear veins. The blood samples were collected aseptically into Alsever's solution in 1:1 ratio and the blood was centrifuged at 4000 x g for 15 min to prepare packed red blood cells (RBCs). The supernatant fluid was discarded. The packed RaRBC were then washed thrice by centrifugation with sterile PBS (containing Ca^{++} and Mg^{++}). A 1.5% (v/v) RaRBC suspension was prepared in the same buffer for studying haemagglutination (HA) titre of the collected prawn haemolymph. Haemagglutination assay (HA) was performed in U-bottom microtitre plates using 1.5% rabbit RBC (RaRBC) suspension. Two-fold serial dilutions of serum samples were made in PBS (with Ca^{2+} and Mg^{2+}, pH 7.3). Equal volume of 1.5% (v/v) RaRBC was added to each dilution of serum. The plates were incubated at 25ºC for 1 h. HA titre was read as the reciprocal of the last serum dilution showing agglutination after 1 h of incubation.

A known pathogenic isolate of *Aeromonas hydrophila* was grown on tryptone soy broth (TSB, Difco) for 24 h at 30ºC. The bacteria to be used for bacterial agglutination assay was treated with 1% formalin and kept overnight at 4ºC. The formalin-killed cells were washed twice with sterile PBS and suspended in PBS to 2.7 x 10^8 cells/ml. The formalin-killed cells were stored at - 4ºC until use. The test was performed in a similar fashion to HA test using formalin-killed *A. hydrophila*. The plates were incubated overnight at 25ºC before reading.

The mean values of each parameter were calculated for all the groups. Data were analyzed using one-way ANOVA. Means were compared using Duncan's multiple range tests (Duncan, 1955). Difference was considered significant when $P < 0.05$.

The total haemocyte count (THC) was increased corresponding to the rise in acclimatization temperature. The mean THC varied from 13.53±1.65 x 10^6 to 19.9±0.03 x 10^6 cells/ml. An increase of 47% in THC value was observed between the lowest and highest temperature ranges. Similarly, a higher bacterial agglutination titre in the

haemolymph of prawn maintained at the highest temperature was observed. On the other hand, the numbers of undifferentiated round haemocytes were significantly less at the highest temperature. The three temperature ranges have no effect on total protein content, PO activity, HA titre, fusiform or large ovoid cell populations. However, a wide individual variation in haemagglutin and total protein levels was observed (Table 1).

Table 1. Effect of acclimatization temperature on haemolymph parameters of prawn

Temperature range	THCx10^6 cells/ml	Fusiform cells (%)	Large ovoid cells (%)	Undifferentiated cells (%)	PO activity (OD/ 50 µl haemolymph)	Haemagglutination titre	Bacterial agglutination titre	Total protein (g/dl)
30-32°C	19.90 ± 0.03[b]	74.70 ± 2.03	19.96 ± 1.33	5.34 ± 0.70[a]	0.46 ± 0.01	111.68 ± 16.82	7.50 ± 0.50[b]	41.89 ± 20.45
25-27°C	15.32 ± 1.23[ab]	69.42 ± 1.05	21.44 ± 0.87	9.14 ± 0.18[b]	0.39 ± 0.05	177.87 ± 41.79	4.87 ± 0.37[a]	44.34 ± 17.69
19-21°C	13.53 ± 1.65[a]	69.73 ± 1.90	21.50 ± 1.40	8.77 ± 0.49[b]	0.44 ± 0.02	52.54 ± 22.36	4.71 ± 0.42[a]	44.64 ± 17.90

Data represent mean±SE (n=45). Statistical differences ($P<0.05$) among different temperature ranges are indicated by different letters (a, b).

Prawns are poikilothermic animals, and as such, any fluctuation in environmental temperature changes their body temperature. This fluctuation in environmental factors affects directly the metabolism and physiological adjustment. Lin *et al.* (1999) suggested that oxygen consumption and nitrogenous excretion of *M. rosenbergii* juveniles increased directly as the temperature increased from 24 to 32ºC. Circulating haemocytes are affected by extrinsic factors such as temperature, pH, salinity, dissolved oxygen and ammonia in several species of decapod crustaceans (Le Moullac and Haffner, 2000; Cheng and Chen, 2002). *M. rosenbergii* reared in 20ºC had significantly lower THC and PO activity as compared to the prawn reared at 27 and 30ºC (Cheng and Chen, 2000). Blue shrimp *Litopenaeus stylirosttris* reared in 18ºC had a significantly lower THC, as compared to the shrimp reared at 27ºC (Le Moullac and Haffner, 2000). *L. vannamei* showed a lower THC and PO activity at 20 or 24ºC as compared to that of 28ºC. A similar phenomenon was also observed in the present study in case of THC value that revealed a significantly higher value in prawns reared at the temperature range of 30-32ºC and the lowest value at temperature range of 19-21ºC. However, PO activity did not show any significant fluctuation in the present study. Similar type of observations has also been marked in earlier study in *Litopenaeus setiferus* maintained in laboratory for 7 days at two different temperatures (27 and 31ºC) (Sanchez *et al.*, 2001). They observed a significantly higher THC at 31ºC than that of 27ºC, and no change in PO activity between two different temperature groups. It has been observed that after a certain period of immune regulation

(3-6 days), all immune parameters tended to be stable in shrimps leading to immune adaptation (Cheng *et al.*, 2005; Lu-Qing *et al.*, 2007). Similar phenomenon might have occurred in prawns in the present study where they were maintained for a period of 7 days before bleeding to measure immune parameters, thus leading to no major changes in PO activity, haemagglutinin and total protein levels, and fusiform and large ovoid cell populations. However, a positive correlation between the haemocytes and bacterial agglutinin level with temperature in this study clearly indicated influence of temperature on immune capability of prawn. Thus, it may be concluded that the higher range of temperature above 25ºC is beneficial for raising *M. rosenbergii* as the immune capability of the animal is enhanced at higher ranges of temperatures examined.

This research was supported by AP Cess Project of Indian Council of Agricultural Research, New Delhi (Code No. 0614025). Thanks are due to the Director, CIFA, for providing necessary facilities during the study.

REFERENCES

Bradford, M. M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein. *Analyt. Biochem.*, **72**: 248.

Chand, R. K. and P. K. Sahoo, 2006. Effect of nitrite on the immune response of freshwater prawn *Macrobrachium malcolmsonii* and its susceptibility to *Aeromonas hydrophila*. *Aquaculture*, **258**: 150-156.

Cheng, W. and J. Chen, 2000. Effect of pH, temperature and salinity on immune parameters of the freshwater prawn *Macrobrachium rosenbergii*. *Fish Shellfish Immunol.*, **10**: 387-391.

Cheng, W., J. C. Chen, 2002. The virulence of *Enterococcus* to freshwater prawn *Macrobrachium rosenbergii* and its immune resistance under ammonia stress. *Fish Shellfish Immunol.*, **12**: 97-109.

Cheng, W., L. U. Wang and J.C. Chen, 2005. Effect of water temperature on the immune response of white shrimp *Litopenaeus vannamei* to *Vibrio alginolyticus*. *Aquaculture*, 250: 592-601.

Duncan, D. B., 1955. Multiple range and multiple 'F' tests. *Biometrics,* **11**: 1-42.

Gotz, P. and H. G. Boman, 1985. Insect immunity. In: Kerkat G.A, Gilbert A, editors. Comprehensive insect physiology, biochemistry and pharmacology, Oxford: Pergamon Press; 1985. p. 453-485.

Hernandez-Lopez, J., T. Gollas-Galvan and F. Vargas-Albores, 1996. Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes). *Comp. Biochem. Physiol.*, **113C**: 61-66.

Johansson, M. W., K. Soderhall, 1989. A cell adhesion factor from crayfish haemocytes has degranulating activity towards crayfish granular cells. *Insect Biochem.*, **19**: 183-190.

Kumari, Jaya, P. K. Sahoo, S. S. Giri and Bindu R. Pillai, 2004. Immunomodulation by 'ImmuPlus (Aqualmmu)' in giant freshwater prawn *Macrobrachium rosenbergii* (de Man). *Indian J. Expt. Biol.*, **42**: 1073-1077.

Le Moullac, G. L. and P. Haffner, 2000. Environmental factors affecting immune responses in crustacea. *Aquaculture*, **191**: 121-131.

Lin, X. T., X. G. Liang, J. Wu, Y. X. Qu and M. F. Chen, 1999. Effect of temperature on metabolism of parent female of *Macrobrachium rosenbergii*. *J. Ecol. China*, **18**: 15-18.

Liu, C. H. and J. C. Chen, 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus. Fish Shellfish Immunol.*, **16**: 321-334.

Lu-Qing, P., F. Bo, J. Ling-Xu, and L. Jing, 2007. The effect of temperature on selected immune parameters of the white shrimp, *Litopenaeus vannamei*. *J. World Aqua. Soc.*, **38**: 326-332.

Nelson, S.G., D.H. Armstrong, A.W. Knight, and W.H. Li, 1977. The effect of temperature and salinity on the metabolic rate of juvenile *Macrobrachium rosenbergii* (Crustacea: Palaemonidae). *Comp. Biochem. Physiol.*, **56A**: 533-537.

New, M. B., 1995. Status of freshwater prawn farming. *Aqua. Res.*, **26**: 1-54.

Ratcliffe N.A., K.N. White, A.F. Rowley and J.B. Walters, 1982. Cellular defense system of the arthropods. In: Cohen N, Sigel MM, editors. The reticuloendothelial system 3, New York, London: Plenum Press; pp. 167-255.

Sanchez, A., C. Pascual, A. Sanchez, F. Vargas-Albores, G.L. Moullac and C. Rosas, 2001. Hemolymph metabolic variables and immune response in *Litopenaeus setiferus* adult males: the effect of acclimation. *Aquaculture*, **198**: 13-28.

Sierra, C., J. Guevara, R. Lasscurain, A. Perez, C. Agundis, E. Zenteno and L. Vazquez, 2001. Sialylation is modulated through maturation in hemocytes from *Macrobrachium rosenbergii*. *Comp. Biochem. Physiol.(C)*, **130**: 179-189.

Smith, V. J., J. H. Brown, C. Hauton, 2003. Immunostimulation in crustaceans: does it really protect against infection? *Fish Shellfish Immunol.*, **15**: 71-90.

Soderhall, K., V. J. Smith and M.W. Johansson, 1986. Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: Evidence for cell cooperation in the defence reactions of arthropods. *Cell Tissue Res.*, **245**: 43-49.

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