Asian Fisheries Science **19**(2006):131-139
ISSN: 0116-6514
https://doi.org/10.33997/j.afs.2006.19.2.005

Asian Fisheries Society, Manila, Philippines

Physiological Changes in Indian River Prawn *Macrobrachium malcolmsonii* Experimentally Infected with *Vibrio alginolyticus* and *Vibrio anguillarum*

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Abstract

Outbreak of diseases and health related problems due to vibriosis are common in brackishwater aquaculture and mariculture. However, studies on vibriosis in the fresh water prawn are not well documented. In the present study, biochemical changes under induced stress condition in Indian river prawn *Macrobrachium malcolmsonii* was studied. Prawn juveniles of (90 ± 5 mm) procured from a nearby river were injected with pathogenic bacteria (*Vibrio alginolyticus* and *V. anguillarum*) through the gill $0.3x10^6$cfu of each prawn for a period of 15 days in controlled laboratory environment. Total protein of the haemolymph decreased. Haemolymph cholesterol was elevated significantly ($p \leq 0.05$) in *V. anguillarum* infection, whereas triglyceride and glucose level decreased in comparison to the control. Variation of aspartate amino transferase and alanine amino transferase activities increased under bacterial stress condition. The acid phosphatase level was reduced and alkaline phosphatase activity increased. There was a decrease in bactericidal activity in infected prawns.

Introduction

Exposure of aquatic organisms to even very low levels of pollutants in their environment may result in various physiological alterations in vital tissues. The immune response of prawns is affected by environmental factors, particularly chemical contaminants in water, and exposure to bacterial, viral and parasitic infections (Adams 1991). Survival rates from cultured shellfish are not always predictable, and mortalities associated with bacterial infection are common. Vibriosis is considered as one of the most serious disease problems in the prawn industry in the world and therefore, *Vibrio alginolyticus* and *Vibrio anguillarum* were chosen as bacterial challenge. Several studies have been conducted on vibriosis of kuruma shrimp,

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including isolation and chemotherapy (Takahashi et al. 1985a,b), ecology (De la Pena et al. 1992) and detailed characterization of the organisms (De la Pena et al. 1993).

As the diversification continues in freshwater aquaculture systems in India, Indian river prawn *M. malcolmsonii* is a candidate species in polyculture along with Indian major carps and monoculture practices. Diseases were encountered in their early and grow-out stages (Sethi 2000). There is also paucity of microbiological studies in freshwater prawn and in particular Indian river prawn. This study was initiated as a preliminary attempt to establish the changes in haemolymph biochemistry, haemolymph enzymes and bactericidal activity after challenge with *V. alginolyticus* and *V. anguillarum*. This information will help in the future while diagnosing the infection caused by these species and the changes in the haemolymph profile will act as indicators of stress caused by these pathogens.

Materials and Methods

Prawns and husbandry

Juvenile Indian river prawn, *M. malcolmsonii* (90 ± 5 mm) were collected from the riverine system of Daya (a tributary of river Mahanadi), Bhubaneswar, India. The animals were brought to the laboratory of the Aquatic Animal Health Division and acclimatized in 500 l fiberglass tanks for two weeks. Chlorine free tap water was used throughout the experiment. The physico-chemical characteristics of the test water are as follows: temperature 27 ± 1.0 ^{0}C; pH 7.4; hardness 80 mg•L^{-1} (as $CaCO_3$); alkalinity 88 mg•L^{-1} (as $CaCO_3$); dissolved oxygen concentration 5.6 mg•L^{-1}.

Preparation of bacteria

V. anguillarum (VA1) and *V. alginolyticus* (VAL1) maintained in the Aquatic Animal Health Animal Division of the institute were grown in tryptic soya broth (TSB) with 3% NaCl at 37 ^{o}C for 24 h. The cells were harvested by centrifugation at 3000 g for 15 min at 4 ^{o}C. The bacterial pellet was washed twice with sterile phosphate buffered saline (PBS, pH, 7.2) by spinning at the same speed. The optical density of the bacterial suspension was measured at 540 nm and adjusted to optical density (O.D.) that corresponded to 10^6 cfu•ml^{-1}. The bacterial inocula were pour- plated using standard dilution techniques in TSA to confirm the number of cfu•ml^{-1}.

Experimental design

One hundred and thirty five juvenile prawns were divided into three groups (Group T1, T2 and C), each group in triplicate containing 15 individuals. Juveniles of Group T1 and T2 were challenged with 0.3 ml of bacteria (*V. alginolyticus* or *V. anguillarum*) in PBS corresponding to 1 x 10^6 cfu•ml^{-1} injected in gills using of 2 ml hypodermal syringe and 26 gauge needle. The group C juveniles were control groups injected with 0.3 ml PBS solution. The prawns were then released into the water and kept for another two weeks under observation. The haemolymph was collected using a sterile plastic syringe with 26-gauge needle from the pericardial sinus. The haemolymph was allowed to clot in 2 ml microcentrifuge tubes held in an icebox. In the laboratory, the clot was broken using a sterile needle and kept at 4 ^{0}C for 1 h. The tubes were then centrifuged at 5000 x g at 4 ^{0}C for 3 min and the serum collected and stored at –30 ^{0}C. The biochemical analyses were conducted within 12 h of sample collection.

The following biochemical parameters e.g. haemolymph total protein, cholesterol, triglyceride, glucose, asparate amino transferase, alanine amino transferase, acid phosphatase, alkaline phosphatase were measured.

Assay methods

Total haemolymph protein concentration was determined following the procedure of Weichselbaum (1946). Haemolymph triglyceride was determined using the method of Wahlefeld (1974). Haemolymph cholesterol was determined as per the procedure of Trinider (1969). Haemolymph glucose was determined following the procedure of Schmidt (1974). Haemolymph ASAT and ALAT were determined following the procedure of Wallnofer et al. (1974). Haemolymph ACP was determined following the procedure of Hillmann (1971). Haemolymph ALP was determined following the procedure of Rosalki et al. (1993).

Bactericidal activity of haemolymph was determined following the procedure of Rainger and Rowley (1993) with slight modification. The bacterial cultures were pelleted (3000 g, 15 min) and washed three times with sterile PBS. The bacterial suspension was adjusted to an optical density 0.5 at 540 nm. Then 100 µl of bacterial suspension and 900 µl fresh haemolymph or control group were mixed in sterile Eppendorff tubes. The control groups consisted of bacterial cell suspension and PBS alone. They were incubated at 30°C for 1h and subsequently, A incubation mixtures were used to determine the cfu•ml^{-1} by the spread plate method on Tryptic Soya Agar (TSA). Activities of the sera were expressed in cfu•ml^{-1}.

Statistical analysis

The Analysis of Variance and Duncan's multiple range test (DMRT) were used to investigate the significant difference at 5% ($P \leq 0.05$) level between control and experimental means using Statistical Analysis System (SAS) Computer Software (SAS Institute Inc. 1985).

Results

The total haemolymph protein declined in the treatment group as indicated in Fig. 1. Significantly ($p \leq 0.05$) lower protein value was observed in group T1 (4.06 ± 0.20 g•dl^{-1}) as compared to T2 and control whereas, albumin content increased in treatment group in comparison to control value. It was noticed that there was a rise in the haemolymph cholesterol of prawns under bacterial stress, which was the highest in the case of group T2 followed by group T1 in comparison to the control. Cholesterol activity was highly significant ($p \leq 0.05$) in both the treatments as compared to the control and insignificant between the treatments (Fig. 2). Triglycerides level decreased in group T1 (48.88 ± 11.79 g•dl^{-1}) and increased in group T2 (112.37 ± 32.12 g•dl^{-1}) as compared to the control. The increase in the level of triglyceride was significant ($p \leq 0.05$) in group T1 but insignificant ($p > 0.05$) in group T2 (Fig. 3). There was a reduction in the haemolymph glucose values in both cases. The reduction level was recorded highest in-group T1 (318.58 ± 70.49 g•dl^{-1}) and was significant ($p \leq 0.05$) in comparison to the control group (Fig. 4).

There were significant ($p \leq 0.05$) elevation of ASAT activities in group T1 and group T2 as compared to the control (Fig. 5). ALAT activities increased significant ($p \leq 0.05$) in group T1 (60.0 ± 14.719 U•L^{-1}) in comparison to group T2 and control (Fig. 6). There was a

significant ($p \leq 0.05$) decrease in ACP activity in groups T1 and T2 as compared to the control. The ACP activity in the haemolymph of the control animal was 5.74 $U \cdot L^{-1}$ (Fig. 7). It could be noticed that group T1 showed the highest elevation of ALP (52.13 ± 4.92 $U \cdot L^{-1}$), which was significant ($p \leq 0.05$) against the control value (Fig. 8). Haemolymph bactericidal activity increased significantly ($p \leq 0.05$) in both the treatment groups as compared to the control (Fig. 9). Group T1 showed higher bactericidal activity (1.44 x 10^4 ± 0.32 $cfu \cdot ml^{-1}$) as compared to group T2 (1.32 x 10^4 ± 0.14 $cfu \cdot ml^{-1}$).

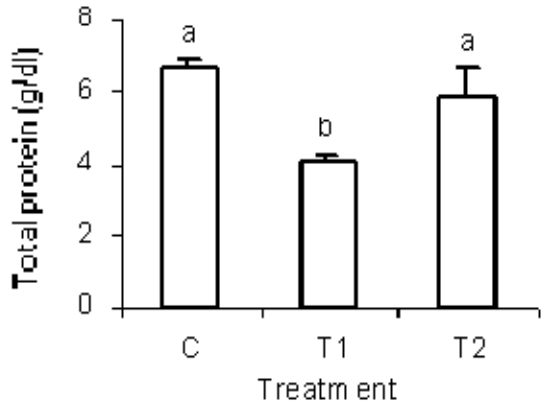

Fig. 1. Changes in total protein of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=3o) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

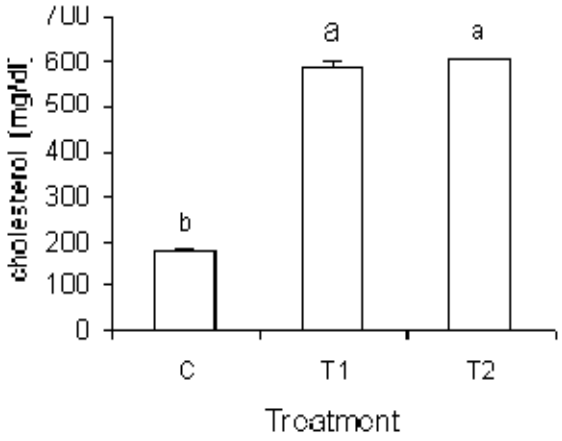

Fig. 2. Changes in cholestrol of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

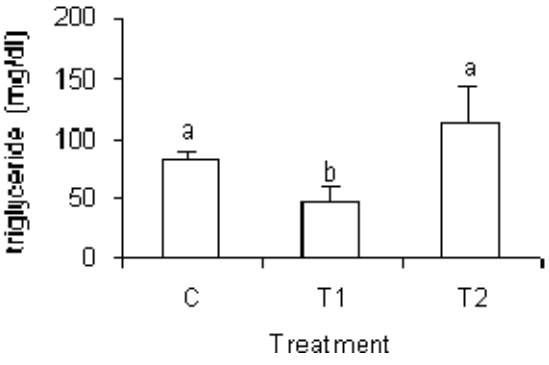

Fig. 3. Changes in triglyceride of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

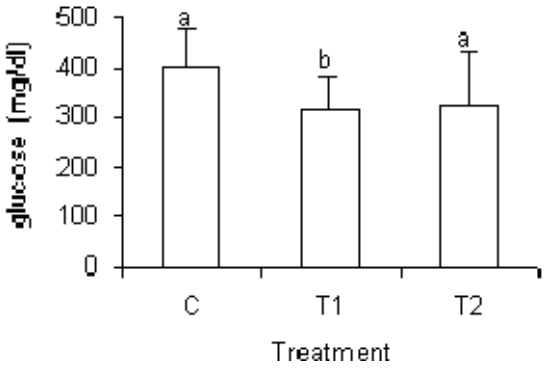

Fig. 4. Changes in glucose value of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

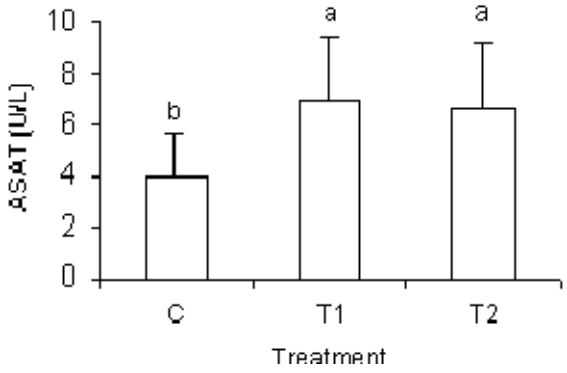

Fig. 5. Changes in ASAT activity of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

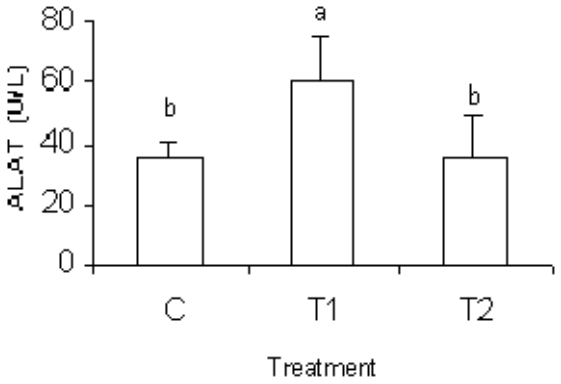

Fig. 6. Changes in ALAT activity of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

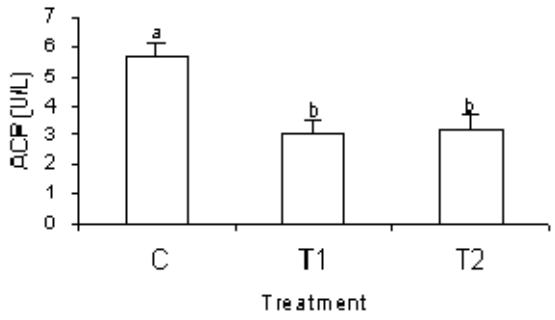

Fig. 7. Changes in ACP activity of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

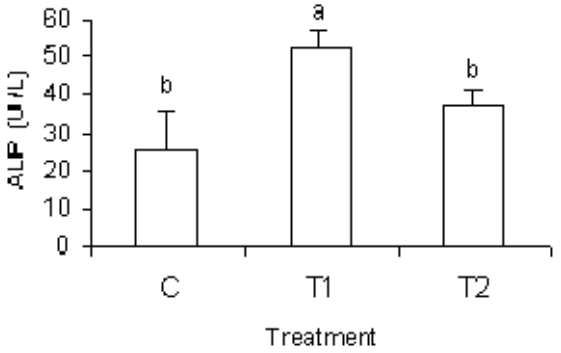

Fig. 8. Changes in ALP activity of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

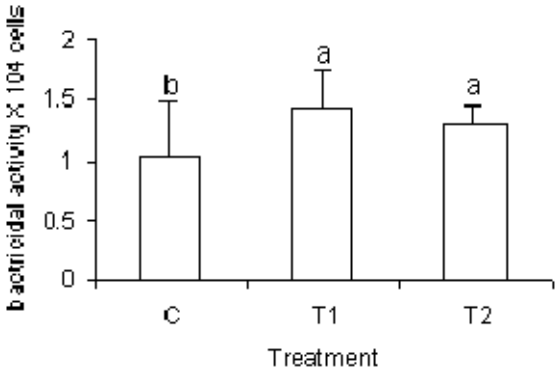

Fig. 9. Changes in bactericidal activity of *M. malcolmsonii* exposed to *Vibrio* sp. [mean (n=30) ± SE bearing common superscript are not significantly ($p > 0.05$) different from each other]. C= control, T1= *V. anguillarum* T2= *V. alginolyticus*

Discussion

Various stress factors bring about changes in the physiological systems and cause behavioral changes. This behavioral and biochemical changes lead to outbreaks of various diseases due to biotic factors and increasing morbidity and mortality (Dunier and Siwicki, 1993; Pipe and Coles 1995; Le Moullac and Haffner 2000; Sethi 2000). In the present context the experiments were directed to observe the role of pathogenic organisms encountered in prawn farming i.e. *V. anguillarum* and *V. alginolyticus* to the different biochemical and histopathological changes of the Indian river prawn, *M. malcolmsonii*.

The biochemical changes induced by stress may lead to disturbance in metabolism. Changes such as reduction in protein and globulin content of the haemolymph and inhibition of activity of certain important enzymes at cellular level lead to retardation of growth, reduction in the fecundity and longevity of organism (Tilak et al. 2000; Acharya et al. 2004). The reduction of total protein content in the treatment group might be attributed to bacterial infection. Prolonged exposure of prawns to stressors interferes with protein metabolism and these observations that the general effect of most stressed environment on protein synthesis is similar. There will be cellular depletion of amino acid resulting in inhibition of protein synthesis (Roger 1980). Decrease in total protein synthesis and hepatopancreatic function is profound which accounts for the progressive reduction in the concentration of total haemolymph protein and globulin content. Stressors induce some sort of changes that alter the homeostasis of the animals (Schreck 1981). Carbohydrates play an important role in cellular communication in the animals (Sharon 1984; Sharon and Lis 1993), and have an immense potential for diversity compared with linear nucleic acids and proteins. There was a decrease in haemolymph glucose level in both treatment groups. The reduction of haemolymph glucose may be due to breakdown of cellular communication as reported by Sharon and Lis (1993). The haemolymph glucose during stress is utilized in cellular growth leading to a hypoglycemic condition. Prawns may show marked hypoglycemic response to stressed environments as a result of incomplete or impaired osmoregulation. Decrease in the haemolymph glucose concentration results from peripheral uptake of sugar or disruption of enzymes associated with carbohydrate metabolism (Heilmeyer et al. 1970).

The ALAT and ASAT are indices for the diagnosis of liver function (Ozaki 1978) and damage (Oda 1990). The elevated level of these enzymes in our experiment corroborate the finding of Song et al. (1993) who demonstrated that *V. damsela* infection could induce *P. monodon* hepatopancreatitis and death.

Phosphatase activity is of significance in pathological conditions (Reddy and Rao 1990). The ACP activity in haemolymph of *M. malcolmsonii* was lower than that of control in the present cases. This was probably due to the rupture of cellular and lysosomal membranes and the release of their contents, resulting in the rapid autolysis of the cells (Mahendru and Agrawal 1983). An increase in the lysosomal activity occurs as a part of pancreatic necrotic changes (Novikoff 1961). Acid phosphatase is a lysosomal enzyme which hydrolyses the phosphorous esters in an acidic medium and also helps in the autolysis of the cell after death. The ALP activity was found to be more in the haemolymph of test prawns when compared to the control prawns. The ALP is a brush border enzyme, which splits various phosphorous esterases at an alkaline pH and mediates membrane transport (Foldfisher et al. 1964). The ALP is also involved in the active transport of glycogen (Denielli 1972), glycogen metabolism (Gupta and Rao 1974), protein synthesis (Pilo et al. 1972), synthesis of certain enzymes

(Summer 1965) and secretary activity (Ibrahim et al. 1974). Thus, any alteration in the activity of ALP may affect an animal in a variety of ways. The ALP, like acetylcholine esterase, contains a serine residue at its active sites (Mahendru and Agarwal 1983). There may be hyperfunction of the hepatopancreas due to stress, which stimulates the release of the enzyme thereby increasing the content in the haemolymph. Antibacterial activity was significantly decreased, suggesting the role of humoral defence mechanism responsible for decreasing resistance to bacteria. The complement system plays a crucial role in humoral defence against microbial pathogens (Taylor 1998). Lysozyme activity contributes to the destruction of bacterial pathogens. The decrease in bactericidal activity in groups T1 and T2 could be due to either a decrease in the number of phagocytes secreting lysozyme, or a decrease in the amount of lysozyme synthesized per cell (Engstad et al. 1992).

Conclusion

Changes in the biochemical alteration of the *Vibrio* challenged prawns lead to structural manifestations of disruptions in the absorptive, storage and secretary functions of haepatopancreas and in the osmoregulatory, respiratory, and physiological mechanisms of gills. Since exposure of prawns to even low levels of bacterial infection can result in such deleterious changes, it is imperative that bacterial infection be prevented.

Acknowledgments

The authors are thankful to the Indian Council of Agricultural Research (ICAR) for providing financial assistance to the senior author as Junior Research Fellow. Acknowledgment is also due to Dr. S. Ayyappan, DDG (Fisheries), ICAR for providing all the necessary facilities for conducting the experimental trials.

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