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Jayasree S

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Keerthana CK

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Anjali Menon K

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Bhasura K

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Rahna S

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Reeha Mashirin K

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Nidhila M

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Akshaya V

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Greeshma M

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

K Akhila

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Correspondence

Jayasree S

Associate Professor, Department of Zoology, Mercy College, Palakkad, Kerala, India

Study on the effect of environmental stress on metabolic and immunological parameters in the haemolymph of *Fenneropenaeus indicus* H. Milne Edwards, 1837

Jayasree S, Keerthana CK, Anjali Menon K, Bhasura K, Rahna S, Reeha Mashirin K, Nidhila M, Akshaya V, Greeshma M and K Akhila

Abstract

The present study deals with the comparison of immunological and biochemical parameters in the haemolymph of Indian white shrimp *Fenneropenaeus indicus* collected from mud bank forming area of (chavakkad) and natural habitat (Kozhikode). Higher Total hemocyte count (THC) of 63.7×10^6 cells /mm³ was obtained with Chavakkad sample in contrast to Kozhikode 61.9×10^5 cells millions/mm³. Higher phenoloxidase (PO) activity was found in Chavakkad samples (0.029 units/min) but less in kozhikkode sample (0.011 units/min). The PO activity of *F. indicus* showed highest titre value (0.180) in plasma, with increased concentration of laminarin. Haemagglutination activity against bacterial cells was found to be strong in the plasma of Chavakkad samples (0.0625) in comparison with Chavakkad samples (0.0123). *F. indicus* plasma of Chavakkad samples showed the strongest agglutination titre against human erythrocytes A, B, O and AB (48, 32, 25, 28) than supernatant and pellet. Antimicrobial production assay of Chavakkad sample showed maximum zone of clearance of 10mm with *Bacillus* and 12 mm with *Pseudomonas* whereas Kozhikode samples showed only 5 mm clearance with *Bacillus* and 1mm clearance with *Pseudomonas*. The protein concentration of Chavakkad and Kozhikode samples were 1129.75 μ g/ml and 471.5 μ g/ml respectively. Samples collected from Chavakkad showed maximum glucose concentration of 2757.5 μ g/ml and less glucose in Kozhikode sample (680 μ g/ml). Results evidently reveal that the samples of shrimp haemolymph from non-stressful habitats viz. mud bank formation areas show enhancement in immunological response and biochemical parameters both quantitatively and qualitatively. Studies on the haemolymph provide clear indications about the physiological state and general health of the shrimp which is the most important factor in any aquaculture practice.

Keywords: Prophenoloxidase, haemagglutination, antimicrobial, *Penaeus indicus*

Introduction

Shrimps are an extremely good source of protein, yet are very low in fat and calories, making them a very healthy choice of food. *Fenneropenaeus indicus* is one of the commercially important species in aquaculture along the Indian Ocean especially in east coast of India^[1]. Farming of the species faces lots of problems due to the occurrence of opportunistic microbial pathogens including bacteria and viruses^[2-6]. Nowadays understanding the immune ability of shrimp and their defense mechanisms have become a primary concern in shrimp culture for better production. Invertebrate immune mechanism has the ability to recognize the invading organisms as foreign substance^[7-9], these substances were recognized by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, peptidoglycan and glucan, present on the surface of microorganisms, which trigger the cellular and humoral responses in crustaceans^[10]. Cellular mediated responses include encapsulation, phagocytosis and nodule formation^[11], whereas the humoral responses include the clotting cascade pathway, antimicrobial peptides synthesis, proPO cascade^[11-13]. Studying the biological interaction with the invertebrate immune system is attractive for the advancement of a basic knowledge on invertebrate and vertebrate general immunity, because it offers various possible alternatives for disease management. In recent years blood metabolites have been investigated as a tool for monitoring physiological condition in wild or cultured crustaceans exposed to different environmental

conditions [14-16]. Blood protein levels fluctuate with changes in environmental and physiological conditions and play fundamental roles in the physiology of crustaceans from O₂ transport to reproduction up to stress responses [17-20].

Both the cellular and humoral immune systems of the shrimp function synergistically to protect the shrimp and eliminate foreign particles and pathogens. Low immunity may be caused by factors such as poor water quality, disease and the presence of toxins. However, several factors, including trace nutrients (astaxanthin, vitamins and minerals), probiotics and immune stimulants (β -glucan, peptidoglycan and lipopolysaccharide) have been reported to promote shrimp immunity and thereby increase resistance to disease. In fact, moulting, reproduction, nutritional state, infection, hypoxia, and salinity variations are the major factors affecting the relative proportions and total quantities of the hemolymph proteins [21, 22]. The shrimp immune system response is largely based on proteins and involved in recognizing foreign particles [23] and prevent blood loss upon wounding [24, 25]. Shrimps are well adapted to use protein as a source of energy and molecules [15]. Blood protein concentration has been found since long to be related to nutritional condition in a number of crustaceans [26].

Penaeus indicus is found at depths of 2 to 90 m, inhabiting bottom mud or sand. It is most abundant in shallow waters of less than 30 m depth, on sand or mud [27]. The adults are marine and breed offshore; while post larvae and juveniles are

estuarine [27]. The Chavakkad samples were collected during the time of mudbank formation. The present work deals with a comparative study on the effect of environmental stress on immunological and biochemical parameters in the haemolymph of *Fenneropenaeus indicus* H. Milne Edwards collected from Chavakkad of Thrissur District and Puthiyappa of Kozhikode district.

Materials and Methods

Experimental Animals (*Fenneropenaeus indicus*)

Indian white shrimp *Fenneropenaeus indicus* used in the study were collected from Mudbank forming area of Chavakkad (Lat 10.57'N; 76.007' E) and Puthiyappa(non mudbank area), Kozhikode (11° 19' 7.89" N 75° 44' 46.96" E). Puthiyappa is an important fishing village of Kozhikode district. It is situated to the North of Kozhikode city, 7 km away at latitude 11° 19" N and longitude 75° 44" E respectively. The body weight of the intermoult shrimp ranged from 11.1 to 14.0 g with an average of 12.5 ± 1.5 g (mean ± S.D.). Samples were washed with de ionized water to remove any adhering contamination, drained under folds of filter paper. Samples were then put in crushed ice in insulated containers and brought to the laboratory for preservation prior to analysis.

Scientific Classification

KINGDOM : Animalia
PHYLUM : Arthropoda
SUBPHYLUM : Crustacea
CLASS : Malacostraca
ORDER : Decapoda
SUBCLASS : Dendrobranchiata
FAMILY : Penaeidae
GENUS : Fenneropenaeus
SPECIES : indicus
<i>Fenneropenaeus indicus</i> H. Milne Edwards 1837.



Collection of haemolymph

Hemolymph was withdrawn from the pericardial sinus with a sterile 1mL syringe fitted with a 25-G needle using anticoagulant in theratio 1:1 (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) [28]. The total haemolymph was used for total haemocyte study, whereas for the other determinations haemolymph was allowed to clot at 4 °C for 3hours. Hemolymph was centrifuged for 1min at 10,000 rpm at 4 °C, and the plasma fraction was quickly frozen at -20 °C and stored until required for study.

Separation of plasma and haemocytes

Pooled haemolymph samples were immediately centrifuged (4000 g, 6 min, 4 °C) and the resulting supernatants were used as plasma. The pellet (haemocytes) was gently resuspended and washed once in iso osmotic TBS-I (50 mM Tris; 400 mM NaCl; pH 7.5; 840 mOsm) by centrifugation (4000 g, 5 min, 4 °C). The haemocytes were finally suspended in 2 mL Tris - HCl buffer (250 mM Tris, pH 6.5).

Preparation of haemocyte lysate

Immediately after withdrawal, the haemolymph was centrifuged at 300 g for 3–5 min at 4 °C. The supernatant (plasma) was separated and the cell pellet suspended in 10

mM sodium cacodylate buffer, containing 0.40 M NaCl, (10 mM CaCl₂ and 20 mM MgCl₂) [29]. The cell pellet was washed twice in the same buffer by centrifugation and then suspended in equal volume of the buffer. The mixture was homogenized with a glass piston and centrifuged at 35,000 ×g for 20 min at 4 °C) [28]. The precipitate was discarded and the resulting supernatant represented the haemocyte lysate supernatant (HLS).

Determination of the effectors of the immune response

Total haemocyte count (THC)

Haemolymph was mixed with equal volume of marine anticoagulant (MAC, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M sodium chloride, pH 7.5) to reduce the haemocyte aggregation. This haemocyte suspension was then fixed with 100 μ L paraformaldehyde (4% w/v). The number of haemocytes per ml was estimated individually (60 animals) using an Improved Neubauer haemocytometer.

Phenoloxidase activity assay

Phenoloxidase activity was measured spectrophotometrically using L-3, 4-dihydroxyphenylalanine (L-DOPA) as the substrate) [30]. Optical density (O.D) was measured at 492nm using UV-spectrophotometer. In PO enhancing activity,

haemocyte lysate was serially diluted twofold with TBS/Ca²⁺ (pH 7.4) buffer in flat-bottomed microtitre plates and incubated with 25 µl of laminarin (1 mg/ml in TBS-I, pH 7.4) for 1 h at room temperature^[31]. 25 µl of L-DOPA, 3 mg/ml in distilled water was added and again incubated for 1 h at room temperature. Controls were made with Tris buffer saline buffer instead of haemocyte lysate, replacing them with TBS/Ca²⁺ pH 7.4 buffers. The absorbance at 492 nm was measured at 30 min intervals using UV-spectrophotometer and expressed as units' min⁻¹ mg protein⁻¹.

Haemagglutination assay

The determination of hemagglutinating activity (HA) was done by Vargas-Albores *et al*^[32] method. HA was recorded as the reciprocal of the last dilution in which a positive agglutination was observed in relation to controls, where the shrimp plasma was substituted by PBS.

Haemo agglutination assay (HA) with erythrocytes (RBC)

Blood samples of Human (A, B and O group) originate from healthy male volunteers (age 25-45 years were obtained by venous puncture and collected in sterile Alsever's solution. Human RBC were washed three times with 0.9% saline, once with TBS-II (25 mM Tris HCl, 100 mM NaCl, pH 7.5), and finally resuspended in the same buffer as 1.5% (v/v) suspensions. HA assays were performed in V-bottom microtiter plates by serial dilution of a 25 µl suspension with an equal volume of TBS II. RBC suspension (25 µl) was added to each well, mixed, and incubated for 45 min at 30 °C. The HA titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC. Control for all assays consisted of the substitution of the sample by TBS-II. Each experiment was performed in duplicate.

Zone of Inhibition Test for Antimicrobial activity

A Zone of Inhibition Test (Kirby-Bauer Test) was used to measure the antibiotic resistance of the plasma sample. Overnight cultures of *Pseudomonas*, *Bacillus* and *E. coli* were prepared in Luria-Bertani broth (LB). A hole was made at the centre of the agar plate and 100 µl of plasma samples from shrimp collected from Chavakkad and Kozhikode were applied to the center of the agar plate. The agar plates were incubated for 18-24 hrs (2-3 days for *Bacillus*), at 37 °C. The size of the zone of inhibition is usually related to the level of antimicrobial activity present in the sample.

Biochemical variables

Total Protein

The total protein was estimated as per the Folin-Ciocalteu method^[33] with bovine serum albumin (BSA) as the standard.

Glucose concentration

Nelson^[34] method was adopted for estimation of glucose in the haemolymph with slight modifications. A standard stock solution was prepared using D-glucose in saturated Benzoic acid (20 mg/100 ml). The color intensity was measured at 520 nm against the blank in a spectrophotometer.

Statistical analysis

Mean and standard deviation for each of the biochemical parameter was calculated. T-test for comparison of means was performed for each parameter to test significant difference.

Results

Immunological indicators

The various changes in haemolymph parameters of *F. indicus* collected from Chavakkad (mudbank area) and Kozhikode (non mudbank area) were tabulated. Total haemocyte counts were varied remarkably among individuals, ranging from 63.7 X10⁶ to 61.9 X10⁶ cells/mm³ of haemolymph (n=10) *F. indicus*. High THC count of 63.7 X10⁶ cells /mm³ was found in the samples collected from Chavakkad region and the other in Kozhikode sample(61.9 X10⁵ cells millions/mm³) (Fig.1).

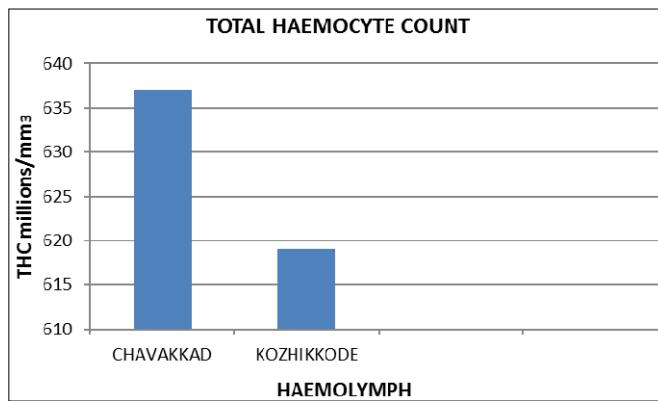
The PO activity of plasma, haemocyte lysate and haemolymph of *F. indicus* were determined. Significant variation was observed in the PO activity between locations and between plasma, hemocyte lysate and hemolymph. The results indicate that the plasma showed highest PO activity of (0.029units/mi) than hemocyte lysate (0.010and units/mi) and hemolymph (0.013and units/mi) in Chavakkad sample. Same trend follows in the Kozhikode sample also. The plasma PO activity was significantly high with Chavakkad sample (0.029units/mi) than Kozhikode sample (0.011units/mi) (Table.1). Dose-dependent enhancement of PO activity in a plasma fraction of *F. indicus* collected from Chavakkad samples were shown in Fig.2. Increase in the concentration of laminarin like PAMPs led to a parallel increase in PO activity in *F. indicus* haemocytes. 5% increase of laminarin resulted with enhancement of PO activity from 0.029units/mi to 0.031units/mi. Dose response increase in PO activity was noticed in plasma from the samples collected from Chavakkad region. 30% enhancement of Laminarin enhanced the PO activity to the maximum of 0.180 units/mi (Fig.2).

Humoral immune response of plasma was measured using haemagglutination activity. In the haemagglutination activity studies using formalin fixed bacterial cells, Chavakkad plasma samples showed positive agglutination against bacterial cells with 0.0625 titre value and 0.0123 in Kozhikode sample (Fig.3). Plasma, supernatant and pellet of Chavakkad samples of *F. indicus* were found to agglutinate with all types of the human erythrocytes. Results indicate that strongest agglutination titre was found in human erythrocytes with plasma. A group erythrocytes showed significantly greater HA titre against supernatant (45), plasma (48) and pellet (15). Minimum HA titre values of 15 (supernatant), 25(plasma) and 10 (pellet) were obtained against O group erythrocytes. HA efficiency was found to be minimum with pellets (Fig.4& Table 2).

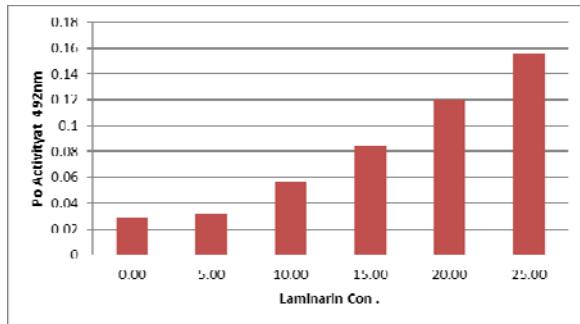
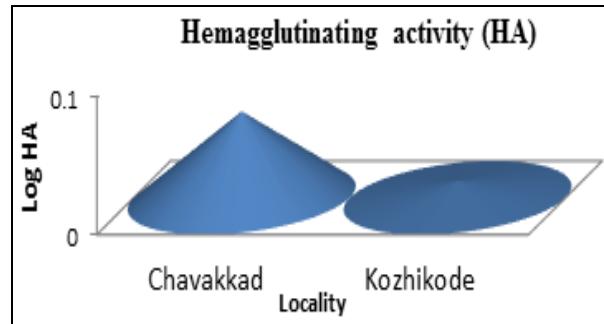
Chavakkad samples showed maximum zone of clearance of 10mm with *Bacillus* and 12mm with *Pseudomonas* and Kozhikode samples showed only 5mm clearance with *Bacillus* and 1mm clearance with *Pseudomonas* (Fig.5).

Biochemical Variables

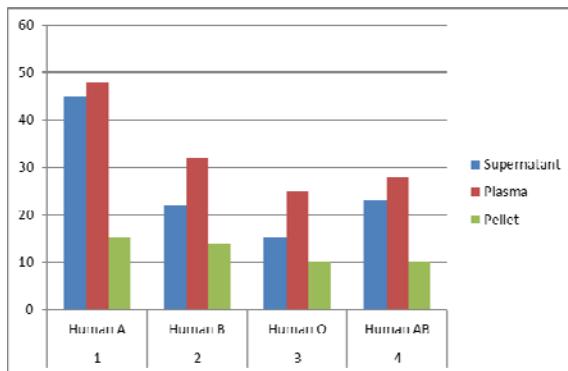
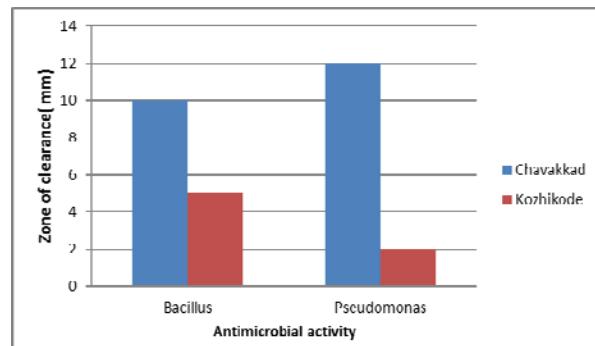
Total protein concentration 1129.75µg/ml, was found to be significantly higher in the plasma sample from the shrimp collected from Chavakkad region compared to Kozhikode sample (471.45µg/ml) Glucose concentration was tested by anthrone method and 2757.5µg/ml of glucose level was recorded in the plasma of shrimp collected from Chavakkad and 680µg/ml of glucose in the shrimp plasma of Kozhikode sample. Only a slight increase in glucose concentration was met with shrimp collected from the Chavakkad region. Chavakkad region showed 2757.5 µg/ml and Kozhikode 680µg/ml (Fig.6. & 7).

**Fig 1:** Total Haemocyte Count in the haemolymph of *Fenneropenaeus indicus***Table 1:** Phenoloxidase activity of *Fenneropenaeus indicus*

Locality	Plasma (units/min)	haemocyte lysate (units/min)	Haemolymph (units/min)
Chavakkad	0.029	0.010	0.013
Kozhikode	0.011	0.009	0.0086

**Fig 2:** Dose-dependent enhancement of PO activity in a plasma fraction of *F. indicus* (Chavakkad sample). Activation of phenoloxidase was observed with the respective dosage of laminarin.**Fig 3:** Hemagglutinating activity in the Plasma of *Fenneropenaeus indicus***Table 2:** HA activity profile of various fractions of *F. indicus* against mammalian RBC

S. No	RBC types	Supernatant	Plasma	Pellet
1	Human A	45	48	15
2	Human B	22	32	14
3	Human O	15	25	10
4	Human AB	23	28	10

**Fig 4:** *F. indicus* haemagglutination profile (Chavakkad) with different types of Human RBCs against supernatant, plasma and pellet.**Fig 5:** Zone of clearance test for antimicrobial activity in the Plasma of *Fenneropenaeus indicus*

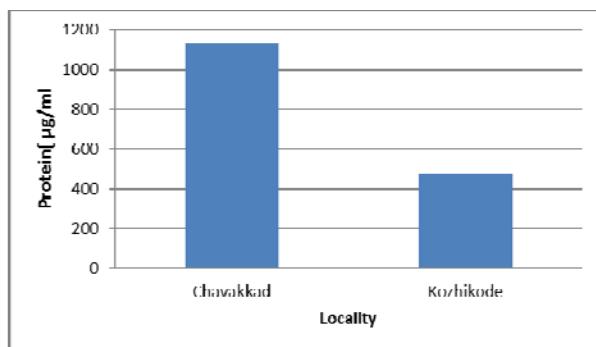


Fig 6: Protein concentration in the Plasma of *Fenneropenaeus indicus*

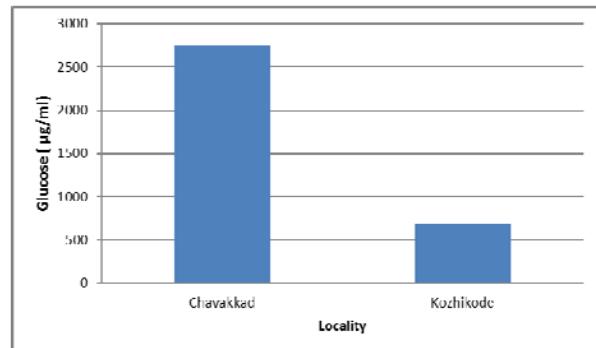


Fig 7: Glucose concentration in the Plasma of *Fenneropenaeus indicus*

Discussion

The chemical composition of the haemolymph in *Penaeus indicus* depends more or less directly on the nature of the environment and varies during development. Despite the ontogenetic and phenotypic variations, taxonomic inferences can often be based on the haemolymph composition. Since the haemolymph is the carrier of every kind of biochemical constituent from one point of the body to the other, haemolymph chemistry is concerned with the number of specific role of the substances in transit^[35].

Among the examined hemato-immunological parameters in this study, the total haemocyte count (THC) was the one that exhibited the greatest variation in shrimps between Kozhikode and Chavakkad sample. Change in environmental stress conditions prevailed at the sampling time of Puthiyappa may be the reason for reduced THC count (619 million/mm³) reported than normal sea coast of Chavakkad with frequent mud bank formation. Mud bank formation creates a low stress environment. At low shear stresses, the fluid mud behaves like a visco-elastic medium hence the growth parameters will be enhanced. A decrease in THC is frequently reported in marine crustaceans exposed to certain stress conditions. THC of male *L. setiferus* maintained in captivity for 7 days at 27 °C decreased by 43% in comparison to freshly caught shrimps^[36]. Also in *P. stylirostris*, a significant decrease in THC was observed in shrimps exposed to hypoxia^[37]. The THC of *P. japonicus* declined after experimental viral infection^[37] or after being fed with a commercial diet containing peptidoglycans. It was recently shown that shrimp hemocytes, besides their role in cellular immune reactions, are the principal site of expression of genes encoding immune effectors^[38]. Therefore, a prolonged decrease in THC in shrimps exposed to some physiological or environmental stress, such as those investigated in this study, could lead to

an animal immune depletion, increasing the risk of infections by opportunistic and/ or pathogenic microorganisms.

Higher phenoloxidase (PO) activity was found in Chavakkad samples (0.029 units/min) but less in kozhikkode sample (0.011 units/min). The crustacean pro-PO system is specifically activated by sugars of microorganism cell walls, and therefore, it apparently participates in nonself recognition and, consequently, in crustacean immune system^[39]. Serum is a practical alternative source for rapid analyses of proPO/PO since serum samples are much more easily obtained than haemocyte lysates. Moreover, the pronounced decrease of the THC in haemolymph may also have contributed to the reduction of the total PO activity, since the haemocytes are the major reservoirs of this enzyme^[40]. A depletion of the proPO system in all animals possibly caused by the stress of captivity conditions^[41]. 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.

Agglutinins and/or lectins are proteins or glycoproteins, naturally occurring in the haemolymph of crustaceans and other invertebrates, that bind specifically to a variety of carbohydrates expressed on different cell surfaces, resulting in cell agglutination. Lectins are believed to participate in the immune system of crustaceans by recognizing specific sugars on microorganism cell walls^[42]. A lectin from *F. paulensis* has already been characterized and partially purified from its haemolymph^[42]. An agglutinin protein from the haemolymph of *F. indicus* was isolated and purified^[43]. In this study, the agglutinating titer of *F. indicus* was found maximum with Chavakkad sample less with samples collected from Kozhikode, Puthiyappa. Among the assessed haemato-immunological parameters, the total haemocyte counts (THC) and the total protein concentration of serum (PC) were the most promising parameters to indicate shrimp stress status.

The immune parameters decreased in shrimp following environmental stressing. For instance, the haemocyte count of blue shrimp *Litopenaeus stylirostris* exposed to 1.5 and 3.0 mg/L ammonia-N decreased by 15% and 50%, respectively after 12 h^[44]. White shrimp *Litopenaeus vannamei* immersed in seawater (35‰) containing *Gracilaria tenuistipitata* extract (GTE) showed decreased levels of immunological parameters^[45]. Changes in haemato-immunological parameters have been reported in some penaeids, as *Penaeus stylirostris*, exposed to hypoxia^[46] or during its molt cycle. Also in *P. japonicas*^[37] and *P. monodon*^[47], the modulation of immuno parameters have been examined in association to viral infection or after the use of immune stimulants. More recently, the influence of captivity stress on various blood and immune parameters was described in males of *L. setiferus*^[48].

Changes in the levels of haemolymph components have been described in shrimps under several physiological conditions. As examples, we can mention the changes in protein composition in some penaeids related to the molt cycle^[49], sex and animal size^[49], water salinity and environmental ammonia-N^[50]. In this study significance increase in protein concentration was met with shrimps collected from Chavakkad sample. PC level can potentially function as a stress indicator to monitor shrimp health status. In the other penaeid, *P. monodon*, PC also decreased after exposure to environmental stress conditions, such as low salinity^[51] and elevated nitrite-N levels^[52]. It shall be emphasized that the respiratory pigment hemocyanin accounts for 90–95% of the serum protein content in crustaceans^[53]. Therefore, it is very

likely that the pronounced reduction of PC in the serum of stressed shrimps is mostly due to a decrease of hemocyanin, but possibly also to specific immune proteins. An important decrease in hemocyanin can have serious reflexes on the oxygen transport to the shrimp tissues and results in a general debilitation of the animals.

The habitat of marine shrimp varies considerably ranging from high saline water in the open seas to regions of freshwater. Chavakkad region showed 2757.5 µg/ml of glucose in the plasma compared to 680µg/ml in Kozhikode sample. Glucose is generally considered the exclusive reducing sugar in the blood of shrimp. Wide range of values reported for reducing sugar in the haemolymph of *P. indicus* suggests that physiological condition of the animal influences the blood sugar levels^[54]. Great individual variation is seen in the blood glucose level of *P. indicus* wide range of 0.1 mg/ml to 1.1 mg/ ml glucose is observed which is related to the size and moult stage. The glucose content in the blood increased with the size of the species (0.267 mg/ml in 60-80 mm to 0.592 mg/ml in 120-140 mm size) but no significant difference between either sex was noticed.

In the present study blood constituents namely total haemocyte count, PO activity, HA, antimicrobial activity, total protein and glucose levels were found to be high in the haemolymph of Chavakkad sample whereas less in the sample from Kozhikode indicates some environmental stress prevailing in that area. Haemolymph from unstressful habitats viz. mud bank formation areas show enhancement in immunological and biochemical parameters both quantitatively and qualitatively. The modulation of some immunological biochemical parameters, as a response to stressful conditions, may function as an important indicator of health status especially in cultivated animals such as shrimps.

Conclusion

In conclusion, the study revealed that haemolymph constituents namely THC, PO activity, HA, antimicrobial activity, total protein and glucose levels were found to be high in the haemolymph of Chavakkad sample (mudbank area) whereas less in Kozhikode samples indicates some environmental stress prevailing in that area. These changes in the immune system of *F. indicus* can provide favorable condition for pathogens present in the aquatic environment to invade the animals and can cause mass mortality. To undertake health monitoring on shrimp farms, and other aquatic environment, it is necessary to monitor the relationships between environmental conditions and normal or abnormal values of immune responses of shrimp. This will help to develop strategies to avoid diseases outbreak in aquatic environment.

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