CELLULAR IMMUNITY STUDIES OF FRESH MUD CRAB SCYLLA SERRATA FORSSKAL, 1775

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ABSTRACT

Mud crabs that dwell constantly exposed to biotic stress throughout the year. Therefore, it is interesting to know how crabs regulate their biotic stress physiology to counteract the bacterial induction. To understand the relationship between bacterial induction and biotic stress physiology two experiments were designed. The present study is a comprehensive attempt to examine the relationship between bacterial induction of biotic stress physiology of mud crabs (S. serrata). In the first experiment, various biochemical components physiology of mud crabs collected in the fields were studied at different seasons. In the second experiment, parameters were investigated in animals that were first acclimatized in the laboratory and then exposed to different bacteria for the biochemical changes. In both the experiments, the objective was achieved by quantifying selected biochemical parameters related to biotic stress and antioxidant defences in different tissues of mud crabs such as muscle, gills and hepatopancreas. Hemocyte classification is based on the presence and size of cytoplasmic granules into 3 types: hyaline, semi-granular and granular hemocytes. Following this criterion, three types of circulating haemocytes were recognised in the crab, Scylla serrata, hyalinocytes, Small granulocytes and large granulocytes.

KEYWORDS: Biotic Stress, Hemocytes, Granulocytes, Scylla serrata.

INTRODUCTION

Biotic stress is considered as an important biomarker of the health status of organisms (Halliwell and Gutteridge, 2001). Mud crab (S. serrata) is an important commercial arthropod belonging to estuarine ecosystem. It generally inhabits intertidal zones of estuaries throughout Indo-Pacific regions including Chilika lagoon of India. It is a well known fact that fluctuations in both biotic and abiotic components of the marine environment with relation to...
seasons have considerable impact on the metabolic activities of its inhabitants particularly of the invertebrates (Abele and Puntarulo, 2004; Lesser, 2006; Almeida et al., 2007). Biotic stress of an organism is a reflection of its metabolic state. Although several studies have clearly demonstrated a strong correlation between seasonal changes and biotic stress status of marine invertebrates such as molluscs (Viarengo et al., 1991; Sheehan and Power, 1996; Filho et al., 2001; Manduzio et al., 2004; Verlecar et al., 2008b) and cephalopods (Zielinski, and Portner, 2000), not much information is available on arthropods in general and mud crabs in particular. Out of all abiotic factors of marine or estuarine environments, salinity deserves special attention. Salinity of marine or estuarine habitat significantly changes with seasons (Verlecar, 1987). Although several aspects of S. serrata are reported to be modulated by environmental salinity (Hill, 1974; Hai et al., 1998), effect of salinity on oxidative stress parameters and antioxidant defence system of mud crabs is lacking. In aerobes, oxygen is reduced tetra-electronically to water in mitochondria during oxidative phosphorylation. In the process, role of the respiratory enzymes are vital in transferring electrons successfully from one complex to another. Leaking of electrons from mitochondrial complexes leads to production of reactive oxygen species which if not efficiently neutralized lead to biotic stress.

MATERIALS AND METHODS

Male and female healthy adult animals (7±1 cm carapace width) were used. Healthy adult crabs (6.3 cm mean carapace width, 110 gms wt. females and 7 cm mean carapace width 140 gms wt. males) were purchased from regular animal supplier kept in the laboratory in disinfected plastic tubs, the water in the tubs was changed every day and fed with minced chicken. The crabs were acclimatized in the laboratory for 7 days.

Bacterial strains and culture

Two different strains of bacteria, Klebsiella pneumonia (Gram -ve), Bacillus cereus (Gram +ve) were used for inoculation during the study.

Klebsiella pneumonia, Gram Negative bacteria do not retain crystal violet dye. The cells are typically rod shaped, facultative anaerobic bacteria can live on wide variety of substrates. The optimal growth is at 37°C. Bacillus cereus, Gram Positive coccal bacterium retains crystal violet dye during Gram staining. The bacterium frequently found in human respiratory tract and on the skin and causes common skin and respiratory diseases.

The Bacterial strains were obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India. Small amount of bacterial culture, Gram negative (Klebsiella pneumonia
MTCC-4030) and Bacillus cereus (MTCC 430) were taken from the Glycerol stock and spread on to the Lurea Bertani agar Plates. The Agar plates were incubated for 24 hours at 37°C. Pure colonies were picked from the Overnight culture and inoculated into an autoclaved broth for 12 hours and incubated at 37°C on a rotor shaker at 150 rpm. 1 ml of this culture was introduced for 3 hrs culture and incubate for 3 hrs at 37°C in shaker and 1 ml of 3 hrs culture was taken, centrifuged at 2000xg for 10 min at 4°C and the pellet was taken. 100 ml Milli Q water was added to the pellet and subjected to serial dilutions (9 times) and 1 ml of 10³ cultures was used for inoculation.

**Experimental Groups**

1. **Group I**: Control crabs without any bacterial inoculation
2. **Group II**: Control injured crabs, these crabs were injected with saline/ 0.9% NaCl
3. **Group III**: Male Crabs challenged with Bacillus cereus, Gram +ve bacteria
4. **Group IV**: Female Crabs challenged with Bacillus cereus, Gram +ve bacteria
5. **Group V**: Male Crabs challenged with Klebsiella pneumonia, Gram -ve bacteria
6. **Group VI**: Female Crabs challenged with Klebsiella pneumonia, Gram -ve bacteria

**Collection of Hemolymph**

Hemolymph was collected from unsclerotized membrane from the ventral side with Insulin syringe and each crab was subjected to a single bleed amounting to 1–2 ml of Hemolymph at different time intervals 2h, 6h, 12h, 24h and 48 hrs. The collected hemolymph was immediately diluted with 1:1 ice cold anticoagulant solution for further biochemical studies.

The hemolymph was collected and diluted with anticoagulant and other chemicals (1ml Hemolymph + 1ml Anticoagulant + 10 µl Phenylthiourea + 10 µl of Aprotin) and centrifuged at 2000 rpm for 15 min at 4°C and the supernatant was used for assessment of biochemical parameters (G. Rameshkumar et al 2009). In the citrate–EDTA buffer used, citric acid serves to delay cell break down while EDTA inhibits prophenoloxidase (proPO) activation and prevents the clotting reaction, (Hall et al., 1999), and this buffer at low pH, in combination with citrate, glucose and NaCl, provides a medium optimal for maintenance of cell integrity without significant loss of cell viability.

All the experiments were conducted three times and the results were put to statistical analysis

**Morphology of Hemocytes**

**Differential Hemocyte Count**
A smear was prepared using a fresh drop of hemolymph collected from the ventral side of the un sclerotized arthrodial membrane with Insulin syringe. The smear was air dried and on to this 10 to 15 drops of Geimsa stain (Giemsas stain: MilliQ water in 1: 9 ratio) (Matozzo and Marin, 2010) was poured and kept for 20 minutes. The smear was washed with Milli Q water air dried and studied under Olympus compound microscope at different magnifications and micro photography was done at 100x.

The hemocytes were counted in shape on the slide in three tiers. The counting starting from upper right corner to left side and then in the middle part from left side to right side followed by lower row from right lower corner to right left corner to avoid duplication of counting of cells. 100 to 120 cells were counted on each slide and the percentage of the cells was estimated by using statistical methods.

**Total Hemocyte Count**

The hemolymph was collected and diluted with 1:1 anticoagulant and stained with Trypan blue (dilute with 1:1 Trapan blue: MilliQ water). 10 µl of sample was taken on to a Neubar Hemocytometer for total hemocytes count (Jussila et al 1997).

**Cytochemical Studies**

**Biochemical Parameters**

The hemolymph was collected and diluted with anticoagulant and other chemicals (1ml Hemolymph + 1ml Anticoagulant + 10 µl Phenylthiourea + 10 µl of Aprotin) and centrifuged at 2000 rpm for 15 min at 4°C and the supernatant was used for assessment of biochemical parameters.

**RESULTS AND DISCUSSION**

Studies on cellular and humoral immune response of crab hemolymph were conducted in *Sylla serreta* with Gram positive and Gram negative bacteria at different time intervals of 2h, 6h, 12h, 24h and 48 hrs. The experiments were conducted in all groups of crabs i.e., control, control injured, Male crabs challenged with *Klebsiella pneumonia*, Male crabs challenged with *Bacillus cereus*, Female crabs challenged with *Klebsiella pneumonia*, and Female crabs challenged with *Bacillus cereus*. Each experiment was conducted thrice and the results were analysed with statistical methods.

**Differential hemocytes**
Investigations into hemocyte under the electron microscopy studies reveals that, three types of hemocytes were identified based on cell size, shape, Nucleocytoplasmic ratio and presence of granules. 100 to 150 cells were observed in each smear and the percentage of the cells was calculated. The three types of cells are Hyalinocytes, Small Granulocytes and Large Granulocytes.

A) **Hyalinocytes** are smaller than other cells, round or oval shape cells with high Nucleocytoplasmic ratio. Nucleus is in the middle, round in shape and large compared with other cells

B) **Small Granulocytes** are round small in size and contain few to many dark granules with varying size, lower Nucleocytoplasmic ratio than hyalinocytes and contain small centrally placed nucleus.

C) **Large Granulocytes** are comparatively larger in size, circular or oval in shape as small granulocytes and packed with larger refractile granules. The nucleus is eccentric in position (Fig 1).

Fig 1: Three types of heamocytes

The occurrence of the granulocytes is more when compared with hyalanocytes. Among granulocytes also the small granulocyte number is more when compare with the large granulocytes. The number of different types of cells in normal control crab is Hyalinocytes-18%, Small Granulocytes-55% and Large Granulocytes-35% (Fig 2).
Fig 2: Types of Hemocytes in crab, *Sycilla serreta*

**Deferential Haemocytes in Control and Challenged crabs**

In case of granulocytes gradual increase is observed from 2 hrs to 48 hrs after bacterial challenge. The percent of increase of Small Granulocytes is 8% in case of male crab challenged with *Klebsiella pneumonia* and 10% in case of male crab challenged with *Bacillus cereus*. Large Granulocytes number is also increased by 6% gradually from 2 hrs to 24 hrs after bacterial challenge in case of male crab challenged with *Klebsiella pneumonia* and female crab challenged with *Bacillus cereus* and 10% in case of female crab challenged with *Klebsiella pneumonia* (Table 1). The hemocyte profile of Male and female crabs challenged with Gram positive and Gram negative bacteria at different time intervals of 2hrs, 6hrs, 12hrs, 24 hrs and 48 hrs shows that the Hyalinocytes number was reduced gradually from 2hrs to 48 hrs and the percent of decrease is lowest, i.e., 12% in case of male crab challenged with *Klebsiella pneumonia* and highest in case of male crab challenged with *Bacillus cereus*.

**Table 1: Deferential Hemocytes in crab challenged with bacteria at different time intervals.**

<table>
<thead>
<tr>
<th>Hemocyte</th>
<th><em>Klebsiella pneumonia</em> (Male)</th>
<th><em>Bacillus cereus</em> (Male)</th>
<th><em>Bacillus cereus</em> (Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2hrs 6 hrs 12 hrs 24 hrs 48 hrs</td>
<td>2hrs 6 hrs 12 hrs 24 hrs 48 hrs</td>
<td>2hrs 6 hrs 12 hrs 24 hrs 48 hrs</td>
</tr>
<tr>
<td>Hyalinocytes</td>
<td>20 16 10 8 6</td>
<td>22 17 15 10 8</td>
<td>24 22 18 13 9</td>
</tr>
<tr>
<td>Small Granulocytes</td>
<td>55 58 59 60 60</td>
<td>52 55 58 60 62</td>
<td>53 56 59 57 57</td>
</tr>
<tr>
<td>Large granulocytes</td>
<td>35 36 38 40 42</td>
<td>36 39 42 45 48</td>
<td>33 33 34 36 37</td>
</tr>
<tr>
<td></td>
<td>2hrs 6 hrs 12 hrs 24 hrs 48 hrs</td>
<td>2hrs 6 hrs 12 hrs 24 hrs 48 hrs</td>
<td>2hrs 6 hrs 12 hrs 24 hrs 48 hrs</td>
</tr>
<tr>
<td>Hyalinocytes</td>
<td>25 16 12 9 7</td>
<td>24 22 18 13 9</td>
<td>24 22 18 13 9</td>
</tr>
<tr>
<td>Small Granulocytes</td>
<td>55 59 62 64 61</td>
<td>53 56 59 57 57</td>
<td>53 56 59 57 57</td>
</tr>
<tr>
<td>Large granulocytes</td>
<td>32 35 37 38 39</td>
<td>33 33 34 36 37</td>
<td>33 33 34 36 37</td>
</tr>
</tbody>
</table>
The hemocyte profile of Male crabs challenged with *Klebsiella pneumonia* at different time intervals of 2hrs, 6hrs, 12hrs 24 hrs and 48 hrs shows that the Hyalinocytes number is reduced by 12% from 2hrs to 48 hrs, Small Granulocytes number is increased by 8% and Large Granulocytes number increased by 5% gradually from 2 hrs to 48 hrs after bacterial challenge (Fig 3).

![Fig 3: Different Hemocytes count in Male crab challenged with Klebsiella pneumonia bacteria at different time intervals.](image)

The heamogram of Male crabs challenged with *Bacillus cereus* at different time intervals of 2hrs, 6hrs, 12hrs 24 hrs and 48 hrs was observed as the Hyalinocytes number is reduced by 16% gradually from 2hrs to 48 hrs, Small Granulocytes number is increased by 10% and Large Granulocytes number is also increased by 7% gradually from 2 hrs to 48 hrs after bacterial challenge (Fig 4).

![Fig 4: Different Hemocytes count in Male crab challenged with Bacillus cereus bacteria at different time intervals.](image)
In case of female crabs challenged with *Klebsiella pneumonia* at different time intervals of 2hrs, 6hrs, 12hrs 24 hrs and 48 hrs shows that the Hyalinocytes number is reduced by 15% from 2hrs to 48 hrs, Small Granulocytes number is increased by 5% and Large Granulocytes number is also increased by 9% gradually from 2 hrs to 48 hrs after bacterial challenge (Fig 5).

Fig 5: Differential Hemocytes count in Female crab challenged with *Klebsiella pneumonia* bacteria at different time interval.

The differential haemocytes of Male crabs challenged with *Bacillus cereus* at different time intervals of 2hrs, 6hrs, 12hrs 24 hrs and 48 hrs was observed. It was noticed that the Hyalinocytes number is reduced by 16% gradually from 2hrs to 48 hrs, Small Granulocytes number is increased by 10% and Large Granulocytes number is also increased by 8% gradually from 2 hrs to 48 hrs after bacterial challenge (Fig 6).

Fig 6: Differential Hemocytes count in Female crab challenged with *Bacillus cereus* bacteria at different time intervals.
Total Haemocyte Count (THC)

The Total Haemocyte Count (THC) of haemocytes was counted by using Neubar Heamocytometer. The mean value of 5 samples was done for both male and female control crabs and the THC was counted (Table 2).

Table 2: Total Hemocyte count control male and female crabs

<table>
<thead>
<tr>
<th>Sample</th>
<th>THC in Male(Control)</th>
<th>THC in Female(Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>6254</td>
<td>6645</td>
</tr>
<tr>
<td>Sample 2</td>
<td>6500</td>
<td>4365</td>
</tr>
<tr>
<td>Sample 3</td>
<td>5487</td>
<td>5238</td>
</tr>
<tr>
<td>Sample 4</td>
<td>5672</td>
<td>6459</td>
</tr>
<tr>
<td>Sample 5</td>
<td>3542</td>
<td>4386</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5491 ± 1567</td>
<td>5418 ± 1345</td>
</tr>
</tbody>
</table>

The THC in male control crab is counted as 5491 cells/mm³ and in female crab it was 5418 cells/mm³ and on challenge with bacteria the THC count was observed to be decreased from 2 hrs to 48 hrs gradually and the percent of decrease was more in first 6 hrs showing involvement of haemocytes in the immunity of the crab (Fig 7).

The THC profile in the male crab challenged with *Klebsiella pneumonia* it was observed that 14% of haemocytes were decreased in the first 2 hrs of bacterial challenge and 20% by 6 hrs and gradually 29% of haemocytes reduction was observed by 48 hrs after challenge with bacteria (Fig 8).
Fig 8: Total Hemocyte Count (THC) in the haemolymph of Male crab challenged with *Klebsiella pneumonia* at different time intervals.

The THC profile in the male crab challenged with *Bacillus cereus* it was observed that 13% of heamocytes were decreased in the first 2 hrs of bacterial challenge and 17% by 6 hrs and gradually 32% of heamocytes reduction was observed by 48 hrs after challenge with bacteria (Fig 9).

Fig 9: Total Hemocyte Count (THC) in the haemolymph of Male crab challenged with *Bacillus cereus* at different time intervals.

It was observed that 14% of heamocytes were decreased gradually from 2hrs to 48 hrs after challenge with *Klebsiella pneumonia* bacteria in the female crab. The THC decreased by 14% in the first 2 hrs of bacterial challenge, 18% by 6 hrs, 25% by 12 hrs, 28% by 24 hrs and 32% of heamocytes reduction was observed by 48 hrs (Fig 10).
Fig 10: Total Hemocyte Count (THC) in the haemolymph of Female crab challenged with *Klebsiella pneumonia* at different time intervals.

The total hemocytes were decreased gradually by 16% from 2hrs to 35% by 48 hrs after challenge with *Bacillus cereus* bacteria in the female crab (Fig 11).

Fig 11: Total Hemocyte Count (THC) in the haemolymph of Female crab challenged with *Bacillus cereus* at different time intervals.

It was observed that the THC decrease was observed to be highest in female crab challenged with *Bacillus cereus* and lowest in male crab challenged with *Klebsiella pneumonia* (Fig 12).
CONCLUSION

In this work we have identified three types of circulating haemocytes in the crab, *Scylla serrata*, ie. hyalinocytes, Small granulocytes and large granulocytes. It was observed that 18% Hyalinocytes, 55% small granulocytes and 35% large granulocytes were present. THC in male control crab is counted as 5101cells/mm³ and in female crab it was 5418cells/mm³. The cell count increased from 2 hours after inoculation of *Klebsiella* and *Bacillus* until 48 hours. One more interesting thing recorded is the THC is always higher than the control insects. This suggests that there is a kind of cellular defence mechanism is evolved and it worked well and protected the crabs from foreign antigens that are used in our study i.e. *Klebsiella* and *Bacillus*. This indicates that cellular immune response is exhibited in the test animal that is used in our present experiments. This study indicates that the haemolymph of crab would be a good source of antimicrobial agents and may be useful for provision of cost effective antibiotics.

REFERENCES


