INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-7, Issue-2, April-June-2016 Coden IJABFP-CAS-USA Copyrights@2016

Received: 4th Mar 2016 *Revised: 10th April 2016* *Accepted: 12th April 2016*

Research article

STUDIES ON CELLULAR IMMUNITY IN FRESH WATER CRAB *BARYTELPHUSA GUERINI*

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ABSTRACT: Hemocyte classification is based on the presence and size of cytoplasmic granules. In this work we have recognized three types of circulating haemocytes in *Barytelphusa guerini*, like hyalinocytes, Small granulocytes and large granulocytes. It was observed that 17% Hyalinocytes, 51% small granulocytes and 32% large granulocytes were present. THC in male control crab was counted as 5101cells/mm³ and in female crab it was 5810cells/mm³ and on challenge with bacteria the THC count was noticed to be decreased from 2 hrs to 48 hrs gradually and the percentage of decrease was more in first 6 hrs showing involvement of haemocytes in the immunity of the crab. The protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 6hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs and 48 hrs gradually in case of both male and female crabs challenged with Gram positive and Gram Negative bacteria. Based on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 48 hrs and the percentage of decrease is highest, 26% in male crab challenged with *E.Coli* and lowest i.e 21.6% in female crab challenged with *S.aureus. T*he lipid content increased gradually from 2 hrs to 24 hrs and decrease from 24 hrs to 48 hrs in male and female crabs challenged with *E.coli* and *S.aureus.* The percent of increase from 2hrs to 24 hrs is is 18% in male crab challenged with *E.Coli* and 14 % in male crab challenged with *S.aureus* and 19% in female crab challenged with *E.Coli* and 10 % in female crab challenged with *S.aureus.*

Key words: *Barytelphusa guerini*, Cellular immunity, Haemocytes, Haemolymph

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INTRODUCTION

Carus studied crustacean haemocytes as early as 1824, and ever since, haemocytes are being studied along two main lines of investigation: the flrst directed towards the characterization of different morphological types of cells while the other has sought to determine their particular role in blood clotting (Mats et al 2005; Matozzo et al 2010). There has been great interest in haemocytes due to their involvement in wound repair and defense mechanisms as an open circulatory system is always prone to intrusion from the surrounding environment (Mary Leema et al 2010). Several attempts to classify crustacean haemocytes were made over the years. But a lack of logical guidelines in the classiflcation schemes and sufficient caution in sampling procedures has led to a state of confusion in haemocyte classiflcation (Lorenzo et al 1999). This has resulted in many terms and names. In the early studies, haemocyte was known as pale amoeboid cell, amoebocyte hyaline, explosive corpuscle, eosinophilic corpuscle, hyaline thigmocyte, thigmotactic amoebocyte and acidophilic granular cell (Mix et al 1980). More recent research supports this view of a developmental sequence, which successively gives rise to three main forms; hyaline, semi or small granular and granular or large granule haemocytes (Sanjayan et al 1996; Manjula et al 1997; Clare at al 1994; Ravindranath et al 1980). The American lobster, Homarus americanus was found to possess both granulocytes and non-granulocytes.

Based on the shape and staining characteristics, haemocytes were classifled into three types; cells containing acidophilic granules, called eosinophil, spindle shaped haemocytes called spindular basophil, containing basophilic granules and oval shaped ovoid basophil, containing basophilic (Jussila et al 1997; Johansson et al 1989). Another classification is that recognized four types of cells in H americanus. These were based on the size and refractile nature of granules, ratios of cytoplasm to nucleus, and Geimsa staining characteristics (Johansson et al 2000). Two hyaline types were designated as prohyalocytes and hyalocytes, and two granular types termed eosinophilic and chromophobic granulocytes (Subramanyam et al 1983). There was no significant variation in the percentage of cells among different sexes. However, the percentage of eosinophils and hyalinocytes differed significantly between different populations of lobsters (Rakesh Kumar Gupta et al 2013). This work was designed to find different types of Haemocytes in *Barytelphusa guerini* a fresh water crab, and to study the changes after injecting *E.coli* and *S.aureus* bacteria into the haemolymph.

MATERIALS AND METHODS

Experimental animals

The experimental animal selected for the present study was Fresh water crab, *Barytelphusa guerini*. This is a fresh water crab lives among the crevices of rocks.

For the present work, male and female healthy adult animals (6±1 cm carapace width) were used. Healthy adult crabs (5.5 cm mean carapace width, 105 gms wt. females and 6 cm mean carapace width 120 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, *Escherichia coli (Gram -ve), Staphyloccoccus aureus (Gram +ve)* were used for inoculation during the study. The Bacterial strains were obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India. Small amount of bacterial culture, Gram negative (*Escherichia Coli*, MTCC-1687) and Gram Positive (*Staphyloccocus aureaus*, MTCC-3160) were taken from the Glycerol stock and spread on to the Lurea Bertani agar Plates.

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 4ºC and the supernatant was used for assessment of biochemical parameters. 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, 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 unsclerotized arthrodial membrane with Insulin syringe. The smear was air dried and on to this 10 to 15 drops of Geimsa stain (Giemsa stain : MilliQ water in 1: 9 ratio) 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.

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 4ºC 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 *Barytelphusa guerini* 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 *E.coli*, Male crabs challenged with *S.aureus*, Female crabs challenged with *E.coli*, and Female crabs challenged with *S.aureus*. 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.

Hyalinocyte **Small Granulocyte** **Large Granulocyte**

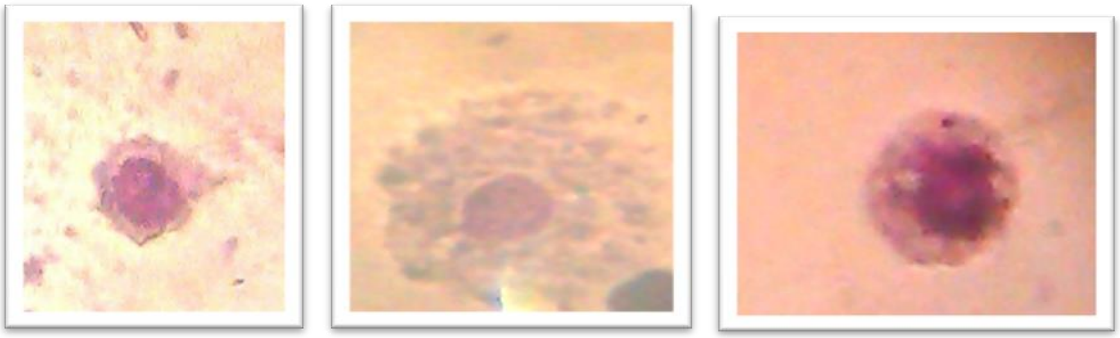

Fig 1: Three types of heamocytes

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- 17%, Small Granulocytes-56% and Large Granulocytes-27%.

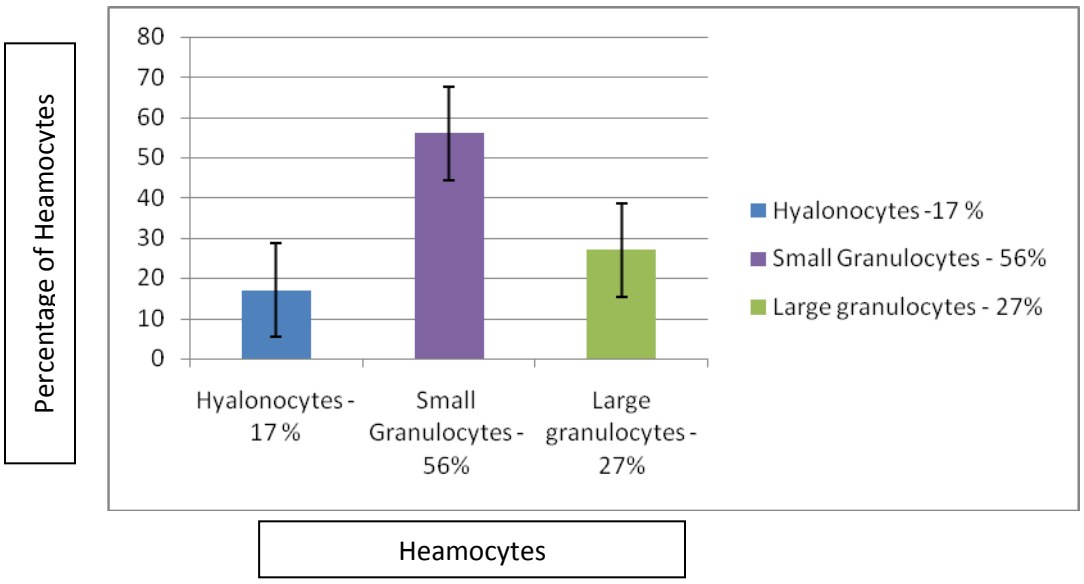

Fig 2: Types of Hemocytes in crab, *Barytelphusa guerini*

Deferential Haemocytes in Control and Challenged crabs:

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., 10% in case of male crab challenged with *E.coli* and highest in case of male crab challenged with *S.aureus*.

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 6% in case of male crab challenged with *E.coli* and 9% in case of male crab challenged with *S.aureus*. Large Granulocytes number is also increased by 5% gradually from 2 hrs to 24 hrs after bacterial challenge in case of male crab challenged with *E.coli* and female crab challenged with *S.aureus* and 8% in case of female crab challenged with *E.coli.*

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

	***E.coli* (Male)**						***S.aureus* (Male)**				
Hemocyte	**2hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**48 hrs**		**2hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**48 hrs**
Hyalinocytes	18	14	12	10	8		21	18	14	8	6
Small Granulocytes	51	55	56	57	57		49	51	53	58	58
Large granulocytes	31	31	32	33	35		30	31	33	34	36
	***E.coli* (Female)**						***S.aureus* (Female)**				
Hemocyte	**2hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**48 hrs**		**2hrs**	**6 hrs**	**12 hrs**	**24 hrs**	**48 hrs**
Hyalinocytes	20	12	9	8	7		19	16	13	8	7
Small Granulocytes	51	55	56	60	59		50	53	55	58	58
Large granulocytes	29	33	35	33	37		31	31	32	34	35

The hemocyte profile of Male crabs challenged with *E.coli* at different time intervals of 2hrs, 6hrs, 12hrs 24 hrs and 48 hrs shows that the Hyalinocytes number is reduced by 10% from 2hrs to 48 hrs, Small Granulocytes number is increased by 6% and Large Granulocytes number increased by 4% gradually from 2 hrs to 48 hrs after bacterial challenge.

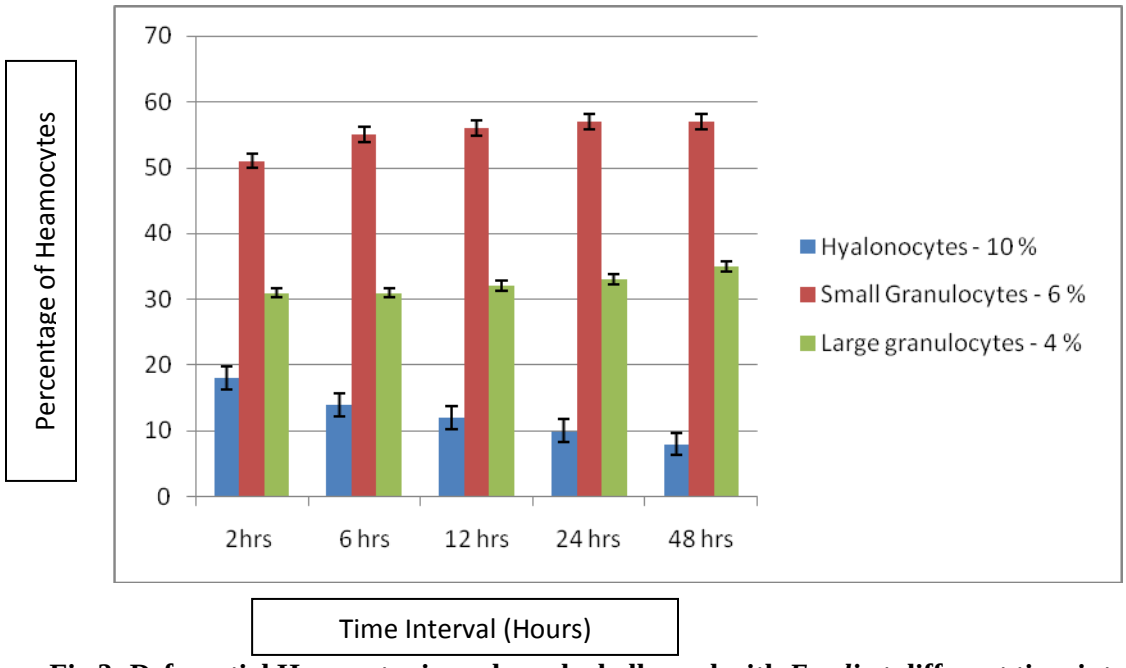

Fig 3: Deferential Hemocytes in male crab challenged with *E.coli* at different time intervals

The heamogram of Male crabs challenged with *S.aureus* at different time intervals of 2hrs, 6hrs, 12hrs 24 hrs and 48 hrs was observed as the Hyalinocytes number is reduced by 15% gradually from 2hrs to 48 hrs, Small Granulocytes number is increased by 9% and Large Granulocytes number is also increased by 6% gradually from 2 hrs to 48 hrs after bacterial challenge.

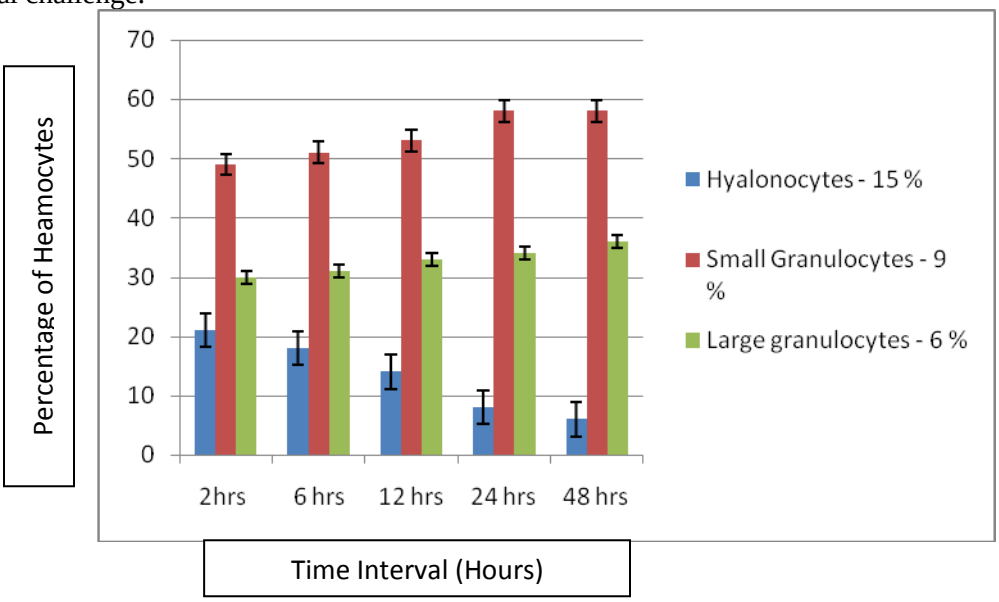

Fig 4: Deferential Hemocytes in male crab challenged with *S.aureus* at different time intervals

In case of female crabs challenged with *E.coli* at different time intervals of 2hrs, 6hrs, 12hrs 24 hrs and 48 hrs shows that the Hyalinocytes number is reduced by 13% from 2hrs to 48 hrs, Small Granulocytes number is increased by 8% and Large Granulocytes number is also increased by 8% gradually from 2 hrs to 48 hrs after bacterial challenge.

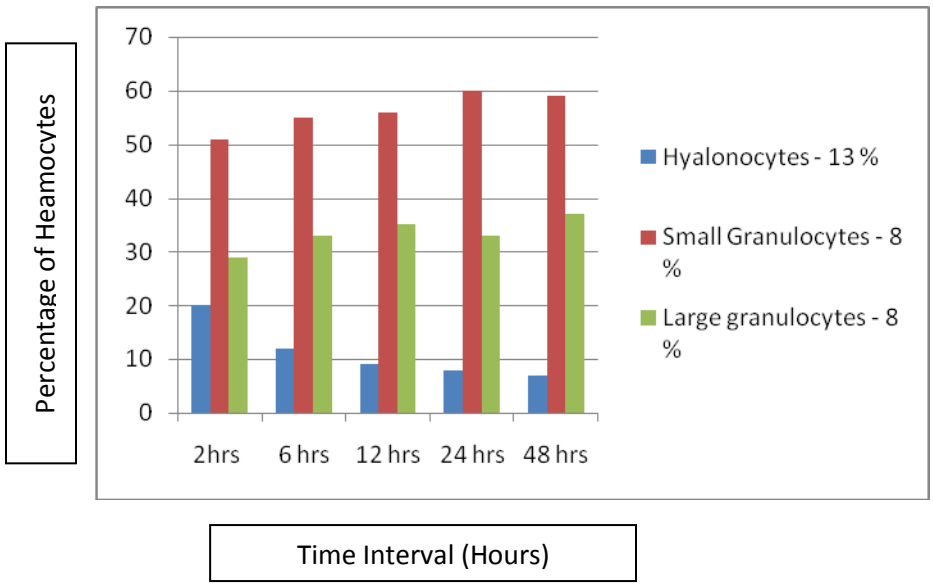

Fig 4: Deferential Hemocytes in female crab challenged with *E.coli* at different time intervals

The differential haemocytes of Male crabs challenged with *S.aureus* 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 15% gradually from 2hrs to 48 hrs, Small Granulocytes number is increased by 9% and Large Granulocytes number is also increased by 6% gradually from 2 hrs to 48 hrs after bacterial challenge.

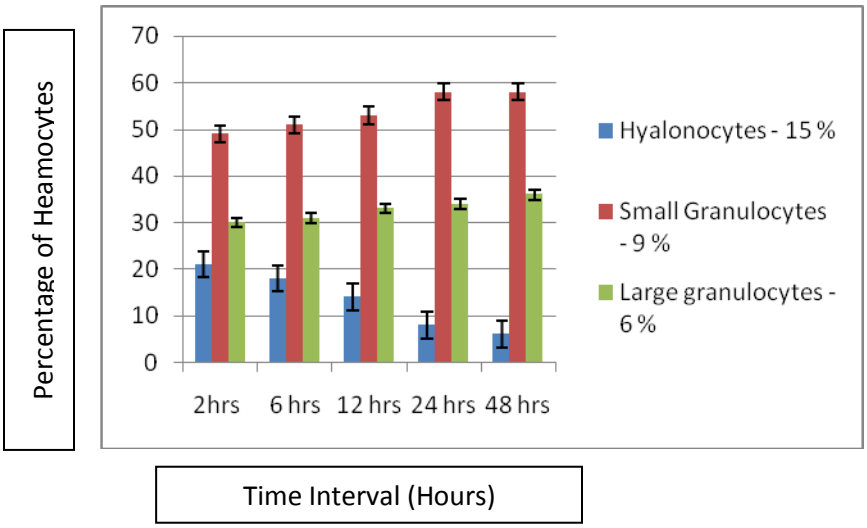

Fig 5: Deferential Hemocytes count in female crab challenged with *S.aureus* bacteria at different time intervals

Total Heamocyte Count (THC):

The Total Haemocyte Count (THC) of haemocytes was counted by using Neubar Heamocytometer.

Table 2: Total Hemocyte count control male and female crabs

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean ± SD
THC in Male (Control)	6078	6600	4008	5802	3018	5101 ±1358
THC in Female (Control)	6874	3278	5802	6642	6456	5810 ±1315

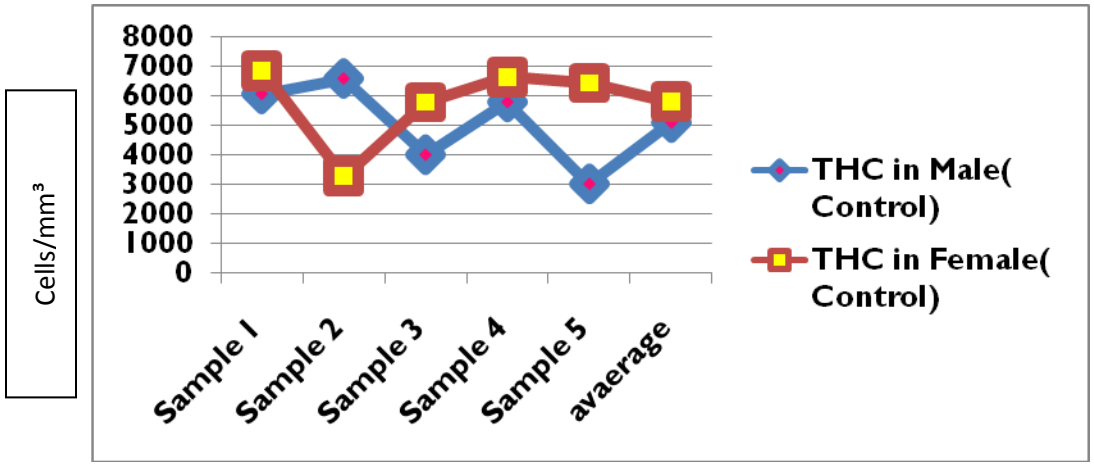

Fig 6: Total Hemocyte count control male and female crabs

The THC in male control crab is counted as 5101cells/mm³ and in female crab it was 5810cells/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.

The THC profile in the male crab challenged with *E.coli* it was observed that 12% of heamocytes were decreased in the first 2 hrs of bacterial challenge and 20% by 6 hrs and gradually 29% of heamocytes reduction was observed by 48 hrs after challenge with bacteria.

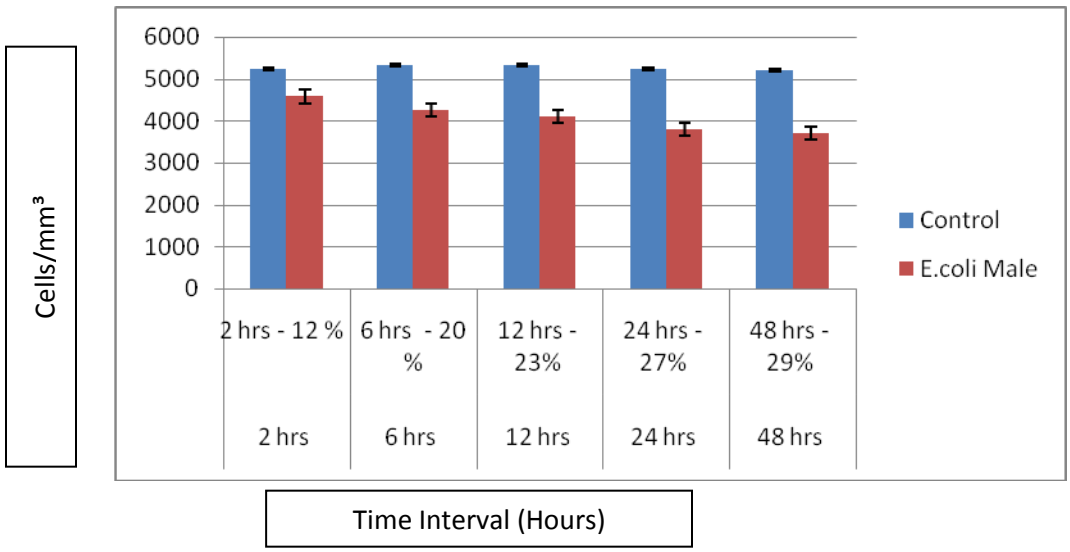

Fig 7: Total Hemocyte Count (THC) in the haemolymph of Male crab challenged with *E.coli* at different time intervals

The THC profile in the male crab challenged with *S.aueus* 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.

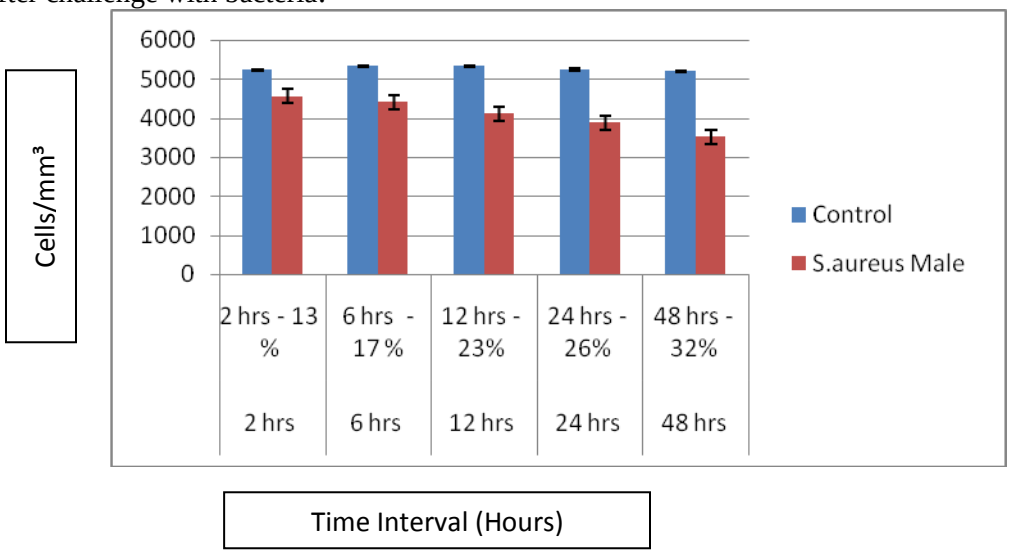

Fig 8: Total Hemocyte Count (THC) in the haemolymph of Male crab challenged with *S.aureus* at different time intervals

It was observed that 14% of heamocytes were decreased gradually from 2hrs to 48 hrs after challenge with *E.coli* 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

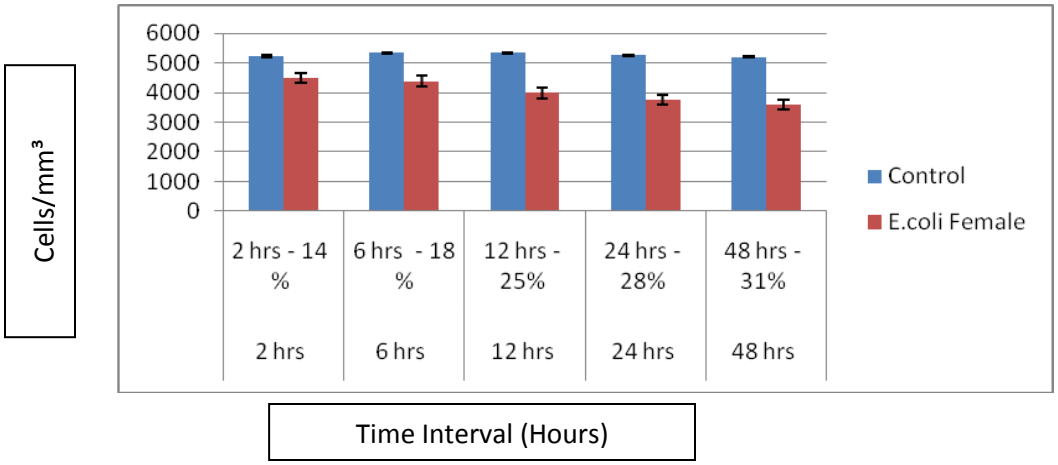

Fig 9: Total Hemocyte Count (THC) in the haemolymph of Female crab challenged with E.coli at different time intervals

The total heamocytes were decreased gradually by 16% from 2hrs to 35% by 48 hrs after challenge with *S.aureus* bacteria in the female crab.

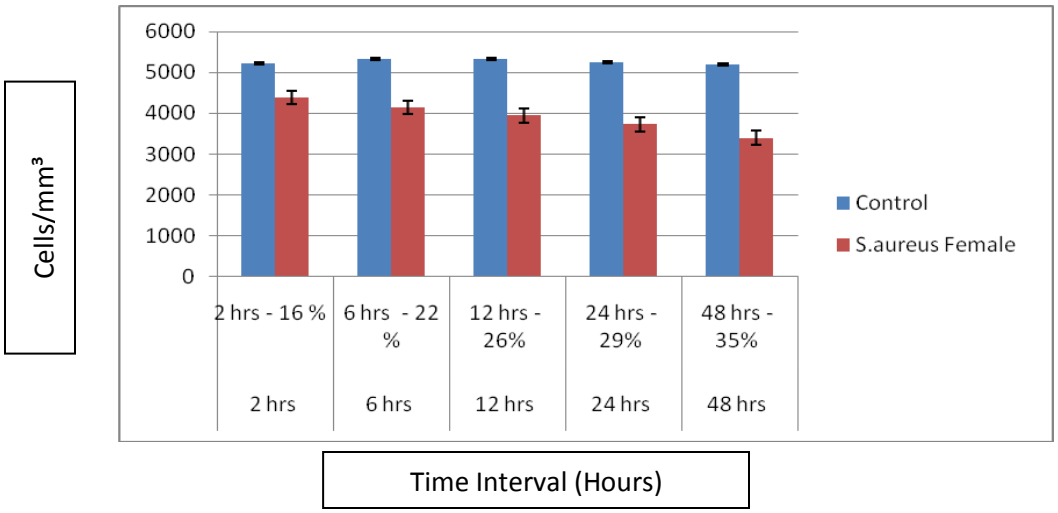

Fig 10: Total Hemocyte Count (THC) in the haemolymph of Female crab challenged with *S.aureus* at different time intervals

It was observed that the THC decrease was observed to be highest in female crab challenged with *S.aureus* and lowest in male crab challenged with *E.coli*

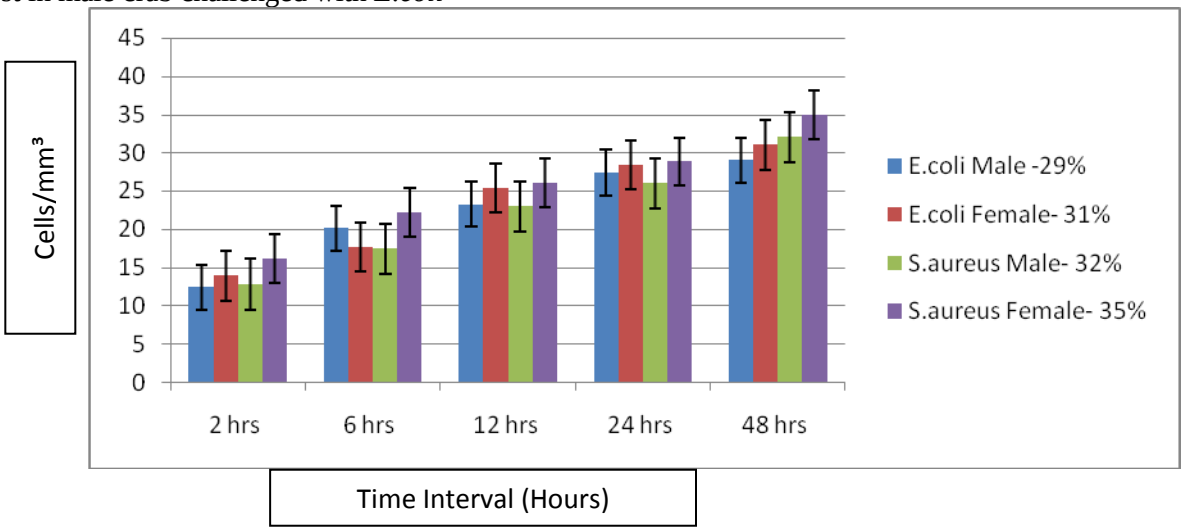

Fig 11: Percentage of Total Hemocyte Count (THC) decreased in the haemolymph of Male and Female crab challenged with *E.coli* and *S.aureus* at different time intervals

CONCLUSION

In this work we have identified three types of circulating haemocytes in the crab, *Barytelphusa guerini,* ie. hyalinocytes, Small granulocytes and large granulocytes. It was observed that 17% Hyalinocytes, 51% small granulocytes and 32% large granulocytes were present. THC in male control crab is counted as 5101cells/mm³ and in female crab it was 5810cells/mm³. The cell count increased from 2 hours after inoculation of S. aureus and E. coli 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. S. aureus and E. coli. This indicates that cellular immune response is exhibited in the test animal that is used in our present experiments.

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ISSN : 0976-4550

INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

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