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STUDIES ON THE PROFILE OF HEMOCYTES WITH AN ATTEMPT TO SEPARATE DIFFERENT HEMOCYTE SUB – POPULATIONS FROM THE HEMOLYMPH OF THE SAND LOBSTER, *THENUS ORIENTALIS* (LUND, 1793)

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Abstract: **In this work, a general profile of circulating cells (or hemocytes) in the hemolymph of the sand lobster, *Thenus orientalis* has been studied, which includes total hemocyte count (THC), identification of various hemocyte morphotypes and their relative proportion in circulation (differential hemocyte count or DHC). Mean THC was found to be 3×10^6 cells/ml. Three distinct hemocyte morphotypes were identified in the systemic circulation of the lobster namely, hyaline, semigranular and granular cell types with a relative proportion of 43 %, 30 % and 27 % respectively In addition, this paper also highlight an attempt to isolate different hemocyte morphotypes using percoll by discontinuous density gradient centrifugation. Separation of lobster hemocyte morphotypes through this method yielded partially successful result, in which two bands were obtained. The first band was found in between 20 and 40 % percoll gradient and was populated with large number of hyaline hemocytes and semigranular type 1 cells. The second band appeared in between 40 and 60 % percoll gradient and majorly consisted of granular and semigranular type 3 cells. A minor population of semigranular type 2 cells were seen in both the bands.**

Keywords: **Hemocytes, THC, DHC, Morphotype separation, Percoll density gradient centrifugation.**

I. INTRODUCTION

Crustaceans such as crabs, shrimps, lobsters and several other zooplankton have an important role in aquaculture industry due to its potential as food and feed. Studies on the defense system of crustaceans play an important role for their successful survival in diverse habitats. Studies on crustacean defense mechanism have become a separate area of study, owing to the importance of crustaceans in food web.

Hemocytes of crustaceans are highly reactive cells which undergo tremendous degree of structural alterations in extravasated hemolymph, and this is one of the major limiting factors in studying different hemocyte types *in vitro*. Anticoagulants have to be used to prevent considerably such alterations in hemocytes, thereby enabling identification of various hemocyte morphotypes. The classification of crustacean hemocytes is either based on morphological identity, functional characteristics, or combination of both these features. Total hemocyte count (THC) shows a great degree of variation among different crustacean species. In most crustaceans, three major hemocyte types have been identified: hyaline cells are the smallest and most fragile cells, with a large central nucleus, few endoplasmic reticulum, ribosomes

and mitochondria (Amirante, 1986). In these cells, granules are generally absent, but a few very small granules can be seen (Smith & Söderhäll, 1983). Previous studies have shown that hyaline cells are actively phagocytic and initiate the process of hemolymph coagulation (Omori *et al*., 1989). Semigranular cells have lobed, central or eccentric nucleus with presence of ribosomes, endoplasmatic reticulum, Golgi bodies, and granules in the cytoplasm (Amirante, 1986). In most crustaceans, semigranular cells are further subdivided into two or three subtypes depending on the relative abundance and size of the granules, and they are considered as transitional cell type between hyaline and granular cells. In a few crustaceans, these cells have been found to be phagocytic and their granules stain positive for prophenoloxidase, the enzyme responsible for melanin synthesis (Smith & Söderhäll, 1983). The semigranular cells have been reported to react to microbial cell wall components such as lipopolysaccharides and β -1,3 glucans as evident from degranulation response and release of prophenoloxidase, thereby triggering the melanization process (Johansson & Söderhäll, 1989). Granular cells are the largest of all the three hemocyte morphotypes, and they have a small, eccentric nucleus. All the cytoplasmic organelles are present but are masked by the presence of large, membrane bound, electron dense, refractile granules which cover the entire cytoplasm. The granules are usually acidophilic, containing various biochemical components such as mucopolysaccharides and hydrolytic enzymes (Estrada *et al*., 2016). However, hyaline cells have been found to be most abundant in many species of crustaceans. It is also important to note that the proportions of different hemocyte morphotypes vary with moult cycle and environmental conditions (Hose *et al*., 1990; Maggioni *et al*., 2004).

Separation of different hemocyte types are performed to study the functions of each distinct morphotypes. In invertebrates, separation of blood cells has been attempted by density gradient centrifugation. Hemocyte separation was well studied in molluscan system using this technique (Bachère *et* al., 1988; Friebel & Renwrantz, 1995; Xue *et al*., 2000). Peake (1979) was the first to develop a Ficoll–based density gradient centrifugation method for insect system. Most of the subsequent studies have utilized continuous or discontinuous gradient of ficoll to separate various hemocyte morphotypes.

In crustaceans, attempts have been made to separate three main types of hemocytes from hemolymph of different species by a single – step density gradient centrifugation on Percoll. Percoll is considered as an ideal density gradient for separation of cells when compared to other density gradients. This is because Percoll has very low osmolality, non toxic to living cells and does not penetrate or damage the cells. The cell separation profiles of studies performed using percoll indicate a successful separation of all the three hemocyte morphotypes from the hemolymph of crustaceans. However, these studies also reveal certain problems that include low recovery or enrichment, fragility, lysis of hyaline cells, and poor attachment of semi-granular and granular cells on the glass surface. Other problems include incomplete separation of one or more cell types with low recovery of semi-granular cells (Söderhäll *et al.*, 1986) and poor viability of separated cell types (Peyre & Chu, 1990).

The present study, focuses on identification and classification of hemocytes found in the sand lobster, *Thenus orientalis* and an attempt has been made to separate three different hemocyte population.

II. MATERIALS AND METHODS

Collection of lobster hemolymph samples and preparation of hemocyte monolayers;

100 µl of hemolymph sample was collected from *T.orientalis* in 1 ml of cysteine-anticoagulant saline or CACS (379 mM NaCl, 13 mM KCl, 11 mM $MgSO_4.7H_2O$, 10 mM $MgCl_2.6H_2O$, 0.3 mM NaH_2PO_4 and 1.6 mM glucose, 20 mM L-cysteine, pH 7.4) by cutting the dactylus region of the walking leg. The hemocyte monolayers were prepared following the procedure described by Renwrantz *et al*. (1985). The hemocytes were observed under the phase contrast microscope (20x, 40x objective lens) to monitor the process of formation of hemocyte monolayer (Carl Zeiss Axiolab microscope).

Trypan blue dye exclusion test for viability of hemocytes:

The hemocyte monolayers were prepared as mentioned above. The viability of hemocytes held in the monolayer *in vitro* was assessed up to three hours by Trypan blue dye exclusion test following the method of Garvey *et al*., 1979.

Determination of total hemocyte count:

100 µl of hemolymph sample was collected in lobster saline (379 mM NaCl, 13 mM KCl, 11 mM $MgSO_4.7H_2O$, 10 mM $MgCl_2.6H_2O$, 0.3 mM NaH_2PO_4 and 1.6 mM glucose) containing 30 mM N-ethyl maleimide and the total hemocyte count was determined using improved Neubauer hemocytometer (Garvey *et al*., 1979) and was observed under the 40 x objective of Carl Zeiss Axiolab microscope.

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The total hemocyte count in the hemolymph was calculated using the following formula:

$$\text{Total number of hemocytes / ml} = \frac{\text{Total cell count in 4 large squares} \times \text{dilution factor (10)} \times 10^4}{\text{Number of large squares counted (4)}}$$

Identification of various hemocyte morphotypes:

Hemolymph samples from lobster was collected in CACS and the hemocyte monolayers were prepared as described above. The hemocytes were allowed to settle, attach and spread on the surface of the slides. The morphology of fully spread live hemocytes in monolayer were resolved with phase and DIC optics of Carl Zeiss Axioskop 2 plus microscope using plan-apochromat objective lens (100x with NA 1.4), and the images were acquired with Axiocam MRc digital camera and Axiovision software (Carl Zeiss, Germany).

Enumeration of various hemocyte morphotypes (Differential hemocyte count):

After extensive spreading of hemocytes, the monolayers were observed under 40x objective of Carl Zeiss Axioskop 2 plus phase contrast microscope. The different hemocyte morphotypes were identified based on their cytoplasmic granules after inspecting at least 10 random microscopic fields on each monolayer. The proportion of each hemocyte morphotype present in the systemic circulation was enumerated and expressed in percentage.

Separation of hemocytes using density gradient centrifugation:

Attempts have been made to separate various hemocytic populations from the hemolymph of *T.orientalis* by discontinuous density gradient centrifugation in Percoll.

Preparation of discontinuous density gradient:

Various concentrations of Percoll (20, 40, 60 and 80 %) were prepared by mixing it with appropriate hyperosmotic buffers (TBS) that did or did not contain cysteine. The pH of each gradient was determined using pH indicator paper.

TBS I: 61 mM Tris, 383 mM NaCl, 121 mM D-glucose, 6 mM KCl, 3 mM $MgCl_2.6H_2O$, 24 mM cysteine, pH 6.8, 1054 Osm

TBS II: (77 mM Tris, 502 mM NaCl, 155 mM D-glucose, 8 mM KCl, 4 mM $MgCl_2.6H_2O$, pH 6.8, 1295 mOsm

TBS III: 107 mM Tris, 693 mM NaCl, 213 mM D-glucose, 11 mM KCl, 5 mM $MgCl_2.6H_2O$, pH 6.8, 1771 mOsm

TBS IV: (171 mM Tris, 1.14 M NaCl, 342 mM D-glucose, 17 mM KCl, 8 mM $MgCl_2.6H_2O$, pH 6.8, 2971 mOsm

Concentration of Percoll (%)	Volume of undiluted Percoll (ml)	Type of TBS	Volume of TBS (ml)	pH of final density medium
20	0.353	TBS I	1.646	7.2 – 7.4
40	0.706	TBS II	1.294	7.2 – 7.4
60	1.061	TBS III	0.938	7.4 - 7.6
80	1.414	TBS IV	0.586	7.6 – 7.8

The final osmolality of these hyperosmotic buffers after mixing with Percoll was calculated to be 850 mOsm.

The discontinuous gradients of Percoll were formed in U bottom polycarbonate tubes (15 × 90 mm; 16 ml) by first adding 2 ml of 80 % Percoll. The medium for other gradients were then layered (1 ml each). Finally, 2 ml of CACS containing hemolymph sample was layered on the top of lowest Percoll concentration.

Separation of hemocyte morphotypes:

An aliquot of 150 µl hemolymph from the lobster was collected in 1 ml of ice-cold CACS. Two aliquotes of these hemolymph samples collected from each lobster were pooled and was layered on the top of the preformed density gradient and subjected to centrifugation in a swinging bucket rotor (Model: Heraeus, Biofuge primo R) at various speed and time. A balancing tube with equal weight was used during this step. After centrifugation, the tubes were taken out and were examined for the presence of opaque bands containing the hemocytes. These bands were carefully removed and the hemocytes were examined under Carl Zeiss Axiolab phase contrast microscope and their images were acquired using Carl Zeiss Axioskop 2 plus under phase or DIC optics.

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III. RESULTS

Viability of hemocytes:

98 %, 97 % and 31 % of hemocytes were viable at 1, 2 and 3 hours respectively on hemocyte monolayers prepared in CACS as shown by trypan blue dye exclusion test (Table 1).

Table 1: *In vitro* viability of hemocytes of *Thenus orientalis* determined by trypan blue dye exclusion test

Time of incubation	Percentage of viable hemocytes*
1 hour	98 ± 1
2 hours	97 ± 2
3 hours	31 ± 2

*Values are expressed as mean ± standard error (n = 5)

Total hemocyte count and enumeration of various hemocyte morphotypes:

The mean total hemocyte count in the hemolymph of sand lobsters was found to be 3×10^6 cells/ml. Three distinct hemocyte morphotypes, namely agranular or hyaline, semigranular and granular hemocytes were identified under phase and DIC optics. The semigranular cells showed great degree of variation with respect to the size and number of cytoplasmic granules and could be further subdivided into semigranular type 1 or SG1, semigranular type 2 or SG2 and semigranular type 3 or SG3 types (Figures 1A-J). However, the general proportion of hyaline, semigranular (SG1+ SG2 + SG3) and granular hemocytes was found to be 43 %, 30 % and 27 % respectively.

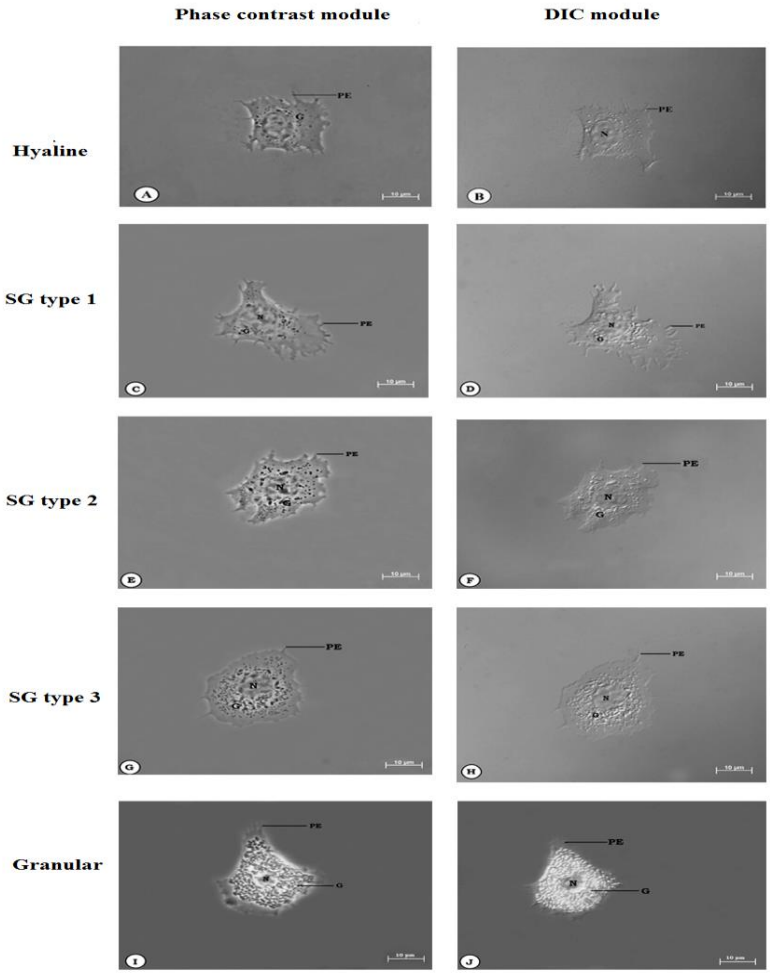

Fig 1: Different hemocyte morphotypes found in the hemolymph of sand lobster

N: Nucleus; PE: Protoplasmic extension; G: Granules; Scale bar = 10 µm

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Separation of hemocytes from the hemolymph of sand lobster:

After various attempts, separation of the hemocyte population from the lobster hemolymph was partly successful with a discontinuous gradient of 20, 40, 60 and 80 % Percoll prepared in TBS. Two opaque bands were obtained each at the interface between 20-40 % and 40-60 %, respectively (Fig. 2A). The first band comprised predominantly hyaline and SG 1 cells (Fig. 2B), and the second opaque band primarily made of SG 3 cells and granular hemocytes (Fig.2C). Besides, a very small proportion of SG 2 cells was present in both opaque bands. The cells recovered in both bands were moderate with 3 – 4 cells per field under 40x objective lens. The cells in band 1 showed good spreading response. However, the granular cells found in band 2 showed only 30-40 % spreading response.

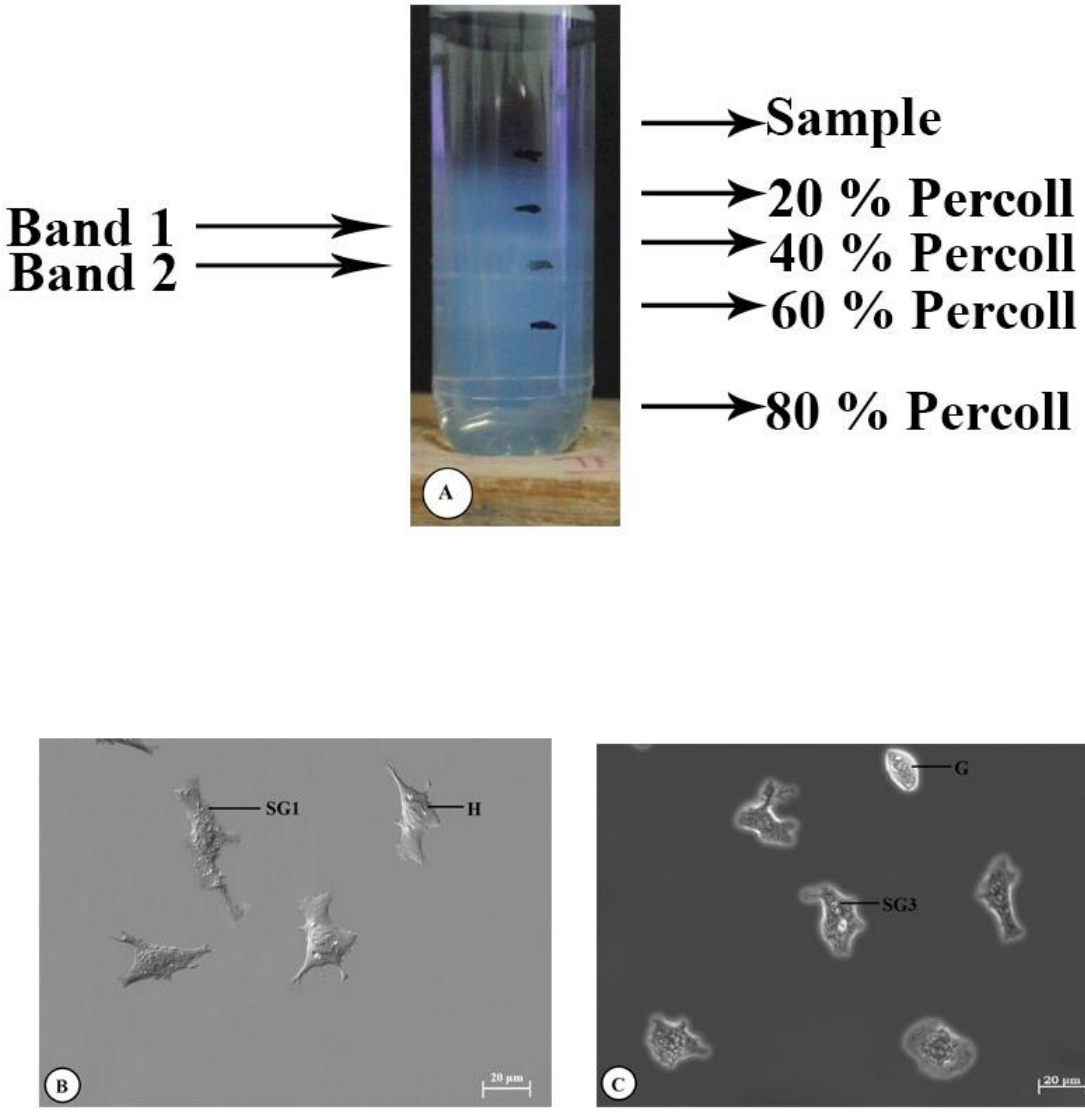

Fig 2: Separation of hemocyte morphotypes using Percoll

A: Two opaque bands seen between 20-40 % and 40-60 % gradient of Percoll.

B: Hyaline (H) and SG1 cells obtained from band 1

C: Granular (G) and SG3 cells obtained from band

Scale bar = 20 µm

IV. DISCUSSION

Hemocytes or circulating blood cells are the major effector components of cellular immune system of crustaceans (Jiravanichpaisal *et al*., 2006; Vázquez *et al*., 2009). The importance of hemocytes in cellular defense process is apparent from the fact that these are the most abundant cells in the systemic circulation (Van *de* Braak *et al*., 1996), and freely suspended in the hemolymph of crustaceans. The previous studies have established that hemocytes play an indispensable role in hemolymph coagulation process (Wood *et al*., 1971; Omori *et al*., 1989; Martin *et al*., 1991). All the hemocytes undergo extensive morphological alterations during this process to accomplish effective hemolymph clotting. *In vitro* studies on hemocytes therefore, require efficient anticoagulant that not only curbs the coagulation reaction but also preserve native hemocyte morphology and behavior.

The most ideal hemocyte preparation in our study was obtained with L-cysteine as anticoagulant, and this aminoacid (20 mM) in CACS was found to arrest hemolymph coagulation and aided in obtaining hemocytes in native form. Most of the hemocytes from this preparation were viable up to 2 hours and declined thereafter. Besides, these hemocytes were free, intact and refractile, and able to attach within 10 minutes with differential spreading response. Most of the hemocytes were able to extensively spread within 45 minutes which enabled differentiation of the morphotypes based on cytoplasmic granulation.

Total hemocyte count or THC is an important indicator to assess the health status of any crustacean (Jussila *et al.*, 1997; Yildiz & Atar, 2002). The count in systemic circulation varies among different crustacean species and ranges from 4.5×10^4 cells/ml to 5.1×10^7 cells/ml. Environmental toxicants including ammonia or mercury toxicity, microbial infections or any other physiological conditions such as moulting or starvation can directly inflict modulations in total hemocyte counts (Manjula *et al.*, 1997; Johansson *et al.*, 2000; Le Moullac & Haffner, 2000). Besides, the studies on crustacean system reveal that animals with very low THC are more susceptible to microbial infections (Le Moullac & Haffner, 2000). In our study, a mean THC value of 3×10^6 cells/ml was recorded in the hemolymph of the sand lobster, and this count is well within the range of previous reports.

In crustaceans, three different hemocyte morphotypes based on their cytoplasmic granulation have been identified, which include (1) hyaline cells with little or no granules, (2) semigranular cells with numerous small sized granules and (3) granular cells with densely packed, membrane bound granules. These cell types not only differ in their morphological characters but also in biochemical and functional characteristics. The proportion of these cell types varies among different groups of crustaceans (Smith, 1991). In the present study, the proportions of hyaline, semigranular and granular cells in hemolymph of the sand lobster, *T. orientalis* were found to be 43 %, 30 % and 27 % respectively. This shows that the proportion of hyaline cells is marginally higher than the two other hemocyte morphotypes identified in the lobster hemolymph. It is important to note that in these lobsters, semigranular cells could be classified into three subtypes based on the abundance of small cytoplasmic granules: SG type 1 (a few small granules), SG type 2 (moderate number of small granules) and SG type 3 (moderate to numerous small granules) cells.

Separation of hemocytes has been attempted in various crustaceans with differential success rates. Separated hemocyte population can give a better insight into nature and behavior of each hemocyte morphotype. The commonest method employed for separation of hemocytes in crustaceans is density gradient centrifugation. Even though, various density media such as sucrose, caesium chloride and Ficoll are available, they are not widely used for separation of live hemocytes as they could inflict damage to the cells due to incompatible osmolality. Percoll density gradient centrifugation is one of widely used method for separation of hemocytes. Percoll with negligible osmolality is an ideal density medium and it helps to recover hemocytes with native morphology and behavior. Both continuous and discontinuous density gradient centrifugation have been attempted with Percoll to separate the hemocyte population in crustaceans (Söderhall & Smith, 1983; Loret, 1993; Johansson & Söderhall, 1995).Even though many studies claim successful separation of the hemocyte populations, they have also nevertheless disclosed various problems encountered in hemocyte separation. In crayfish, often low recovery of hyaline hemocytes has been reported (Smith & Söderhall, 1983; Kobayashi *et al*., 1990; Wang *et al*., 2001). In case of most crabs, there was incomplete separation of semigranular cells and they were co-sedimented with hyaline cells (Söderhall & Smith, 1983; Peyre & Chu, 1990; Loret, 1993; Bell & Smith, 1993). In the present study, a series of our attempts resulted only in a partial success in separation of hemocytes from the hemolymph of the sand lobster, *T.orientalis*. Two opaque bands at 20-40 % and 40-60 % Percoll interface were obtained. The first band primarily was composed of hyaline and SG type 1 hemocytes with a few SG type 2 cells. The second band consisted

of SG type 3 and granular cells with a few SG type 2 cells. The cells recovered from both the bands were moderately high with atleast 3 cells per field under 40x. The hyaline and semigranular cells (all types) were able to attach and spread on the glass surface within 50 minutes, whereas the granular cells showed attachment with poor spreading response tested up to an hour. Co- sedimentation of semigranular cells (SG type 1 with hyaline cells and SG type 3 cells with granular cells) is due to their differential degree of cytoplasmic granulation.

Thus, in this study a general profile with respect to hemocytes of sand lobster has been discussed which includes total hemocyte count and different hemocyte types with their relative proportion in circulating hemolymph. Additionally, an attempt was made to isolate different hemocyte morphotypes which was partially successful.

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