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Cytomorphological characterization, classification and counting of haemocytes in freshwater crab, *Varuna litterata* (Crustacea: Decapoda)

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ABSTRACT: Present study aims to characterize and classify the haemocyte structural types and to determine the total and differential haemocyte counts in freshwater crab, *Varuna litterata*. Haemolymph was collected from the crabs and subjected to the light microscopy, transmission electron microscopy, and flow cytometric analysis for the cytomorphological characterization and classification. Total and differential haemocyte counts were also carried out. On the basis of various cytomorphological features, haemocytes of *V. litterata* were classified into hyalinocytes, small granule haemocytes and large granule haemocytes. Hyalinocytes are round, oval, irregular, spindle or ellipsoid shaped cells with low to high nucleo-cytoplasmic ratio and contained a number of cytoplasmic organelles and a few minute-sized granules in the cytoplasm. Small granule haemocytes are large sized ovoid or ellipsoid shaped cells with a relatively high nucleo-cytoplasmic ratio and possessed numerous small sized granules. Large granule haemocytes are comparatively large-size, circular or ovoid shaped haemocytes with a high nucleo-cytoplasmic ratio and contained numerous large round refractile granules and a few small granules. Total haemocyte count of *V. litterata* is noted as 1.021×10^6 to 3.108×10^6 with a mean value of $2.145 \pm 0.84 \times 10^6$ cells ml^{-1}. The relative percentages of hyalinocytes, small granule haemocytes and large granule haemocytes are accounted as 13.93, 55.24 and 30.83% respectively in the haemolymph. Outcome of this study might provide valuable information regarding the haemocyte profile of *V. litterata* that would be helpful to carry out further studies on haemocyte structural types to know about their specific functions and immune mechanisms.
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KEY WORDS: *Varuna litterata*, haemocyte structural types, ultrastructure, flow cytometry, total haemocyte count, differential haemocyte count.

Цитоморфологическая характеристика, классификация и подсчет гемацитов у пресноводного краба *Varuna litterata* (Crustacea: Decapoda)

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РЕЗЮМЕ: Настоящее исследование посвящено характеристике и классификации гемоцитов пресноводного краба *Varuna litterata*. Гемолимфу краба отбирали и фиксировали для изучения методами световой микроскопии, трансмиссионной электронной микроскопии и проточной цитометрии. В ходе работы было определено общее и относительное число гемоцитов разных типов. На основании различных цитоморфологических параметров гемоциты *V. litterata* была подразделены на три группы: гиалиноциты, малые гранулоциты и большие гранулоциты. Гиалиноциты имеют округлую, овальную, неправильную или веретеновидную форму с невысоким или довольно большим ядерно-цитоплазматическим соотношением. Гиалиноциты содержат большое число различных клеточных органелл и немногочисленные крошечные гранулы включений. Малые гранулоциты имеют большие размеры клетки, их форма может быть овальной или элепсоидной. Малые гранулоциты характеризуются большим ядерно-цитоплазматическим соотношением и наличием многочисленных гранул небольшого диаметра. Большие гранулоциты — довольно крупные клетки, округлой или овальной формы с высоким ядерно-цитоплазматическим соотношением. Цитоплазма крупных гранулоцитов содержит многочисленные крупные округлые гранулы, которые способны отражать свет, а так же немногочисленные мелкие гранулы. Тотальное число гемоцитов в гемолимфе *V. litterata* было определено от 1,021 x10^6 до 3,108 x10^6, а среднее как 2,145 ± 0,84 x 10^6 клеток/мл. Процентное соотношение гиалиноцитов, малых гранулоцитов и больших гранулоцитов было определено как 13,93, 55,24 и 30,83%, соотвественно. Результаты исследования вносят новую информацию о структуре гемоцитов *V. litterata*, что может быть использовано в дальнейших исследованиях специфических функций гемоцитов и их роли в иммунном ответе.

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КЛЮЧЕВЫЕ СЛОВА: *Varuna litterata*, типы гемоцитов, ультраструктура, поточная цитометрия, тотальный подсчет гемоцитов, относительное обилие гемоцитов.

Introduction

Crustaceans are frequently exposed to different environmental toxicants, pollutants as well as pathogenic organisms in their aquatic habitats. Like other invertebrates, they lack adaptive immunity and extremely rely on the innate immune system which effectively serves the protective defense against invading foreign components and pathogens in the body (Le Moullac, Haffner, 2000; Galloway, Depledge, 2001). Circulating haemocytes play a key role in innate immune system and are directly involved in the recognition and elimination of the antigens (Smith, Söderhäll, 1986; Johansson, Söderhäll, 1989; Söderhäll, Cerenius, 1992; Johansson *et al.*, 2000). Generally, three structural types of circulating haemocytes have been recognized in crustacean haemolymph based on the presence or absence of the cytoplasmic granules. They are hyalinocytes (or agranular haemocytes), semigranulocytes (or small granule haemocytes), and granulocytes (or large granule haemocytes), executing different vital functions in host immunity (Giulianini *et al.*, 2007; Matozzo, Marin, 2010a). Hyalinocytes are involved in clotting and phagocytosis; semigranulocytes participate in encapsulation, phagocytosis, cytotoxicity, storage and release of prophenoloxidase (pro-PO) system and granulocytes have primary role in cytotoxicity, storage and release of prophenoloxidase (proPO) system (Matozzo, Marin, 2010b). The haematological parameters are considered as a diagnostic tool to evaluate the cellular immunity and the pathophysiological status of animal body (Van de Braak *et al.*, 2002). Total haemocyte counts (THC) and differential haemocyte counts (DHC) are used for monitoring the health condition of various crustacean species (Jussila *et al.*, 1997; Le Moullac *et al.*, 1998; Battison *et al.*, 2003; Lorenzon *et al.*, 2008). Several investigators have also reported these haematological parameters as indicators of different environmental contaminants in various crustacean species (Gessa *et al.*, 2006; Saha *et al.*, 2010; Oanh *et al.*, 2014; Maharajan *et al.*, 2017).

To understand the appropriate functional mechanisms of crustacean immunity, it is essential to classify and characterize, and determine the relative percentages of circulating haemocytes in the haemolymph. However, there is no uniform classification scheme for crustacean haemocytes (Johansson *et al.*, 2000). Proper classification and functional morphology of circulating haemocytes in different crustacean species remain controversial due to the methods and criteria used by the investigators (Parrinello *et al.*, 2015). To the best of our knowledge no study regarding the haemocyte profile of the crab *Varuna litterata* (Fabricius, 1798) has previously been performed. This crab is an ecologically and economically important decapod crustacean species in India and is facing various environmental contaminants in its aquatic habitat (Deyashi *et al.*, 2016, 2019). Thus, the present study has been undertaken to evaluate the classification, morphological characterization, total and differential counting of circulating haemocytes in the freshwater grapsid crab, *V. litterata* based on light microscopy, transmission electron microscopy and flow cytometric assay.

Materials and methods

Test animal and acclimatization

Seventy five apparently healthy male grapsid crabs, *Varuna litterata* (Fabricius, 1798) of intermolt stages (mean carapace length: 2.02 ± 0.38 cm; mean weight: 5.36 ± 3.45 g) were collected from freshwater bodies of Birlapur, South 24 Parganas District, West Bengal, India (22.4217° N, 88.1560° E), brought into the laboratory and stocked at the same time in five large plastic troughs (42 cm bottom diameter; 16 L capacity, 15 crabs per trough) filled with 2 L tap water at natural photoperiod cycle for acclimatization. The physicochemical parameters of water used in this study were as follows: temperature 22.8 ± 0.76 °C, dissolved oxygen 5.88 ± 0.14 mg l^{-1}, pH 7.1 ± 0.17 and total hardness 91.98 ± 4.18 mg l^{-1} as $CaCO_3$. In general, the water physicochemical parameters were

fitted within the range found in the natural freshwater habitats of the crab and could be helpful for *V. litterata* to acclimatize in the laboratory condition. The crabs were fed with the balanced meal and the water of the trough was replaced every 24 h. This acclimatization process has been carried out for 7 days before the examination.

Haemolymph sampling

Crabs were anaesthetized by putting them on ice for 15 min, and the haemolymph (at least 200 ml per crab) was drawn aseptically from the unsclerotized membrane of the base of the third walking leg using a 3 ml sterile plastic syringe equipped with 26-gauge needle. Each syringe was pre-filled with ice-cold citrate EDTA buffer (0.45 M NaCl; 0.1 M glucose; 30 mM trisodium citrate; 20 mM citric acid; 100 mM EDTA, pH 4.6) as anticoagulant (Kumaran *et al.*, 2013). The pooled haemolymph (from 3 crabs) was properly mixed and diluted (1:2) with the anticoagulant solution (Matozzo *et al.*, 2011). This haemolymph suspension was kept in Eppendorf tubes and stored at 4 °C. In each test, five such pools of haemolymph were used.

Light microscopy

Thin film was prepared from the anticoagulant mixed haemolymph suspension. This thin film was air dried and fixed in methanol for 5 min and stained with Giemsa and phosphate buffer (pH 7.2) solution (1:2) for 10 min and finally rinsed with distilled water. After completion of the staining, slides were examined under the brightfield microscope (Dewinter, Italy). Photo documentation was carried out using CCD camera (Debro Microview software, DGI 510C, Dewinter) attached with the microscope.

Cytomorphological parameters measurement

The cytomorphological parameters such as cell length, cell width, nuclear diameter, nucleus/cytoplasm ratio and granule size were measured by using the stage micrometre and ocular micrometer (Erma, Japan). Before starting the measurement, the stage and ocular micrometres were calibrated. Stained haemocytes were examined under the brightfield microscope (Dewinter, Italy) for the cytomorphological measurement.

Transmission electron microscopy

Freshly isolated pooled haemolymph mixed with the ice-cold anticoagulant was centrifuged in 2000 rpm for 10 min. The supernatant was discarded and haemocyte pellet was fixed in Karnovsky's fixative for 6 hours at 4 °C. The pellets were double-washed in 0.1 M PBS (pH 7.2) for 10 min and then post-fixed in 2% osmium tetroxide solution for 2 h. Osmicated pellets were dehydrated with ethanol and acetone and finally embedded in Araldite CY 212. Ultrathin sections (60 nm) were cut using an ultramicrotome (Leica UC6) and stained with 0.5% uranyl acetate and subsequently in 1% ice-cold lead citrate solution. The sections were observed in Tecnai G2 transmission electron microscope operated at 120 kV and photographed.

Flow cytometric analysis

The anticoagulant mixed haemolymph (600 µl) was diluted in phosphate-buffered saline (PBS). The diluted cells were acquired on a flow cytometer (BD Accuri C6, BD Biosciences, USA) fitted with a 488 nm laser beam. The low angle scatter signal was collected and designated as FSC which represented relative size of the cell and large angle scatter was designated as SSC which represented the relative internal granularity of the cells. The distribution of different haemocyte structural types was attributed based on the scatter profile of cells on an FSC vs. SSC dot plot and a total of 10,000 events were acquired for each haemolymph sample. Cell aggregates were excluded from analysis on the FSC vs SSC dot plot.

Total haemocyte count (THC)

THC was determined by using a Neubauer type hemocytometer (Marienfeld-Superior, Germany). A portion (20 µl) of properly diluted haemocyte solution was placed under the cover slip on the haemocytometer chamber and counted under the brightfield light microscope (Dew-

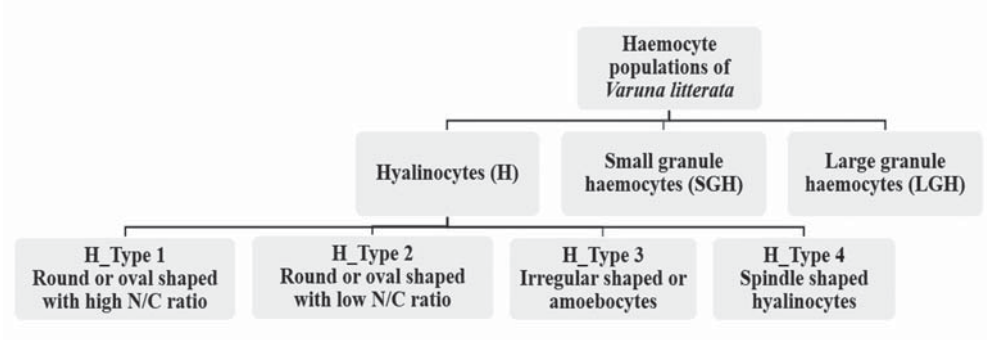

Fig. 1. Classification of the haemocyte populations of *Varuna litterata*.
Abbreviations: H — hyalinocytes; LGH — large granule haemocytes; SGH — small granule haemocytes.
Рис. 1. Классификация популяций гемоцитов у *Varuna litterata*.
Обозначения: H — гиалиноциты; LGH — большие гранулоциты; SGH — малые гранулоциты.

inter, Italy). THC was expressed as the number of haemocytes ($x10^6$) ml^{-1} of haemolymph.

Differential haemocyte count (DHC)

Thin cell smears were prepared from a drop of anticoagulant mixed pooled haemolymph. The monolayer of haemocytes was fixed in methanol and stained by using the Giemsa's staining protocol described in previous section and observed under the brightfield light microscope (Dewinter, Italy). Differential haemocyte count (DHC) was performed by counting the different structural types of haemocytes and calculating their relative percentages as present in the smears. The stained monolayer from each specimen was examined in areas where not less than 200 cells were accounted per slide to ascertain the appropriate percentage of each haemocyte structural type. DHC was expressed as the percentage abundance of the respective haemocyte structural types.

Statistical analysis

The statistical analysis was performed by using the statistical software SPSS Statistics (version 23.0, 2015). All data are expressed in terms of mean ± standard deviation (SD). Values of cytomorphological parameters from different haemocyte structural types were checked for normality and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison tests at the significance level $P < 0.05$.

Results

Cytomorphological characterization and classification of haemocytes

On the basis of different morphological characteristics features such as size, shape, presence or absence of granules and their diameter in the cytoplasm, position of nucleus and nucleo-cytoplasmic (N/C) ratio, mainly three major structural types of haemocytes have been identified in *Varuna litterata* (Figs 1 and 2). These are hyalinocytes (H), small granule haemocytes (SGH) and large granule haemocytes (LGH). The morphological features and relative percentages of different haemocyte types are summarized in Table 1.

Hyalinocytes have different morphological forms like round, oval, irregular, spindle or ellipsoidal and varied in sizes (Fig. 2A–F; Table 1). Cytoplasm of these cells showed purple-pink color and contained centrally or eccentrically placed nucleus which appeared dark blue color after staining with Giemsa stain. Granules might be absent in the cytoplasm or if present, they were very small in size and scanty in numbers. Four structural types of hyalinocytes, designated as H-Type 1, H-Type 2, H-Type 3 and H-Type 4 were identified according to the morphological shape and N/C ratio (Table 1). Type 1 hyalinocytes (Fig. 2A) were small in size (8.45±2.70 x 7.65±2.25 μm) and round in shape with high N/C ratio (0.681±0.10). Type 2 hyalinocytes (Fig. 2B) were round or oval shaped

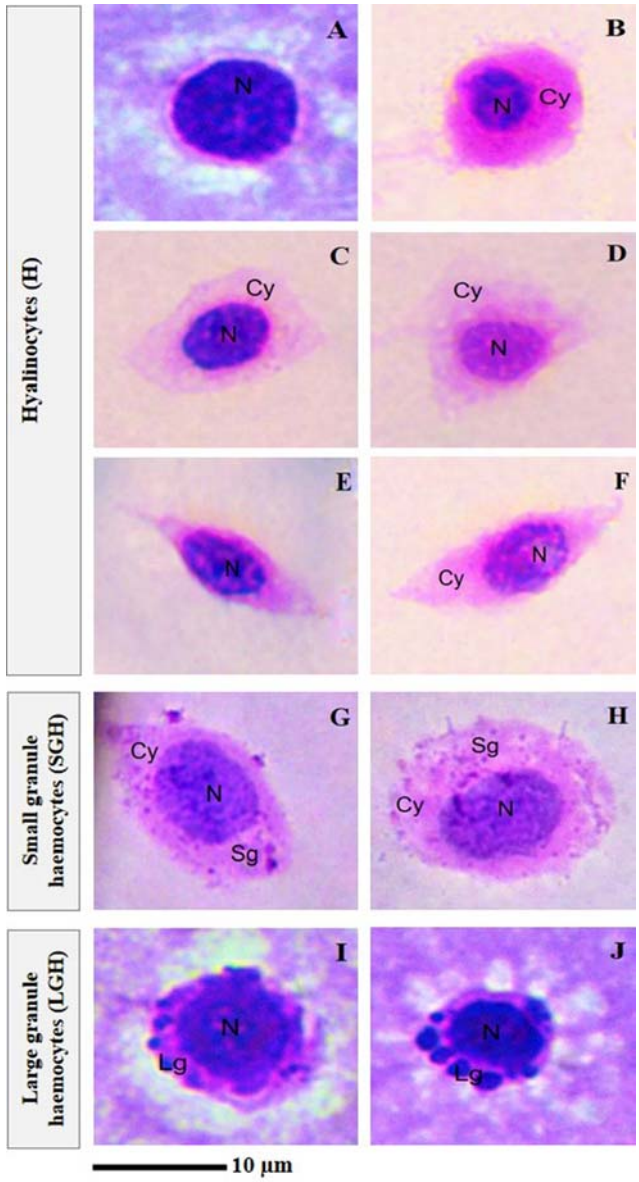

Fig. 2. Light micrographs of the haemocyte populations of *Varuna litterata* (Giemsa stained; magnification: x 1000). A — H type 1: round or oval shaped hyalinocyte with high N/C ratio; B — H type 2: round or oval shaped hyalinocyte with low N/C ratio; C–D — H type 3: irregular shaped hyalinocytes or amoebocytes; E–F — H type 4: spindle shaped hyalinocytes; G–H — small granule haemocytes; I–J — large granule haemocytes.

Abbreviations: Cy — cytoplasm; Lg — large-size granules; LGH — large granule haemocytes; N — nucleus; Sg — small-size granules; SGH — small granule haemocytes. Scale bar 10 µm.

Рис. 2. Световые фотографии гемоцитов разных типов *Varuna litterata* (окраска по Гимза; увеличение 1000 раз). A — тип 1: округлые или овальные гиалиноциты с высоким ядерно-цитоплазматическим соотношением; B — тип 2: округлые или овальные гиалиноциты с низким ядерно-цитоплазматическим соотношением; C–D — тип 3: гиалиноциты неправильной формы (амебоциты); E–F — тип 4: веретеновидные гиалиноциты; G–H — малые гранулоциты; I–J — большие гранулоциты.

Обозначения: Cy — цитоплазма; Lg — гранулы большого диаметра; LGH — большие гранулоциты; N — ядро; Sg — гранулы маленького диаметра; SGH — малые гранулоциты. Масштаб 10 µm.

Table 1. Cytomorphological features and relative percentages of the haemocyte structural types of *Varuna litterata*. Measurements are mean ± SD. Different superscripts indicate significant difference ($P < 0.05$) in mean values within columns.
Таблица 1. Цитоморфологические характеристики и относительный процентный состав различных типов гемоцитов у *Varuna litterata*.

Haemocyte types	Cell length (μm)	Cell width (μm)	Nuclear diameter (μm)	N/C ratio	Relative percentage (%)
H-Type 1	8.45± 2.70 [a]	7.65± 2.25 [a]	5.74± 2.07 [ab]	0.681± 0.10 [b]	4.82
H-Type 2	9.14± 2.72 [a]	8.11± 3.00 [a]	3.88± 1.83 [a]	0.443± 0.16 [a]	2.47
H-Type 3	14.28± 1.47 [ab]	12.14± 2.33 [a]	6.82± 1.22 [bc]	0.527± 0.12 [ab]	2.73
H-Type 4	22.34± 2.99 [c]	7.57± 1.44 [a]	7.44± 2.53 [bc]	0.637± 0.23 [b]	3.91
SGH	16.56± 6.67 [bc]	11.43± 4.51 [a]	8.62± 2.64 [c]	0.616± 0.14 [b]	55.24
LGH	12.10± 3.76 [ab]	10.83± 3.53 [a]	7.13± 2.05 [bc]	0.646± 0.13 [b]	30.83

Note: H — hyalinocyte; SGH — small granule haemocyte; LGH — large granule haemocyte.

cells (9.14±2.72 x 8.11±3.00 μm) with low N/C ratio (0.443±0.16). Type 3 hyalinocytes (Fig. 2C–D) were irregular or amoeboid shaped (14.28±1.47 x 12.14±2.33 μm) with moderate N/C ratio (0.527±0.12) while Type 4 hyalinocytes (Fig. 2E–F) appeared spindle or ellipsoid shaped (22.34±2.99 x 7.57±1.44 μm) with high N/C ratio (0.637±0.23). Cell length of Type 4 hyalinocyte was significantly higher ($P < 0.05$) than other hyalinocytes whereas the cell length variations of Type 1, Type 2 and Type 3 hyalinocytes were non-significant ($P > 0.05$) (Table 1). Nuclear diameter of Type 2 hyalinocytes was significantly lower ($P < 0.05$) than Type 3 and Type 4 hyalinocytes (Table 1). Nucleo-cytoplasmic ratio found in Type 1 hyalinocytes was significantly different ($P < 0.05$) from that found in Type 2 hyalinocytes (Table 1). SGHs were large sized (16.56±6.67 x 11.43±4.51 μm) ovoid or ellipsoid shaped cells with a relatively high N/C ratio (0.616±0.14). The nucleus commonly located in the center and showed dark blue color. Cytoplasm was stained purple color and contained numerous small sized blue-colored granules (<1 mm) (Fig. 2G–H). LGHs were comparatively large-size (12.10± 3.76 x 10.83±3.53 μm), circular or ovoid shaped haemocytes with a high N/C ratio (0.646±0.13). The variations in cell length, nuclear diameter and N/C ratio of SGHs and LGHs were found to be non-significant ($P > 0.05$) (Table 1). Nucleus located eccentrically in the cytoplasm and appeared dark blue color. Their purple-colored cytoplasm was filled with numerous large round refractile granules and a few small granules. The large-size granules stained purple or dark blue and more or less obscured the nucleus (Fig. 2I–J).

Ultrastructure of haemocytes

Based on the ultrastructural studies, three major structural types of haemocyte — hyalinocytes, SGHs and LGHs were identified in *Varuna litterata* (Fig. 3). Hyalinocytes (Fig. 3A–B) contained centrally located large or small-size nucleus with condensed chromatin body. Cytoplasm contained endoplasmic reticulum, Golgi body, mitochondria, ribosomes and vacuoles. Granules may be absent in the cytoplasm or if present, it was very small in size (0.17±0.06 μm) and scarcely distributed. Pseudopodial extension occurred in some hyalinocytes. SGHs (Fig. 3C–D) showed numerous small sized gran-

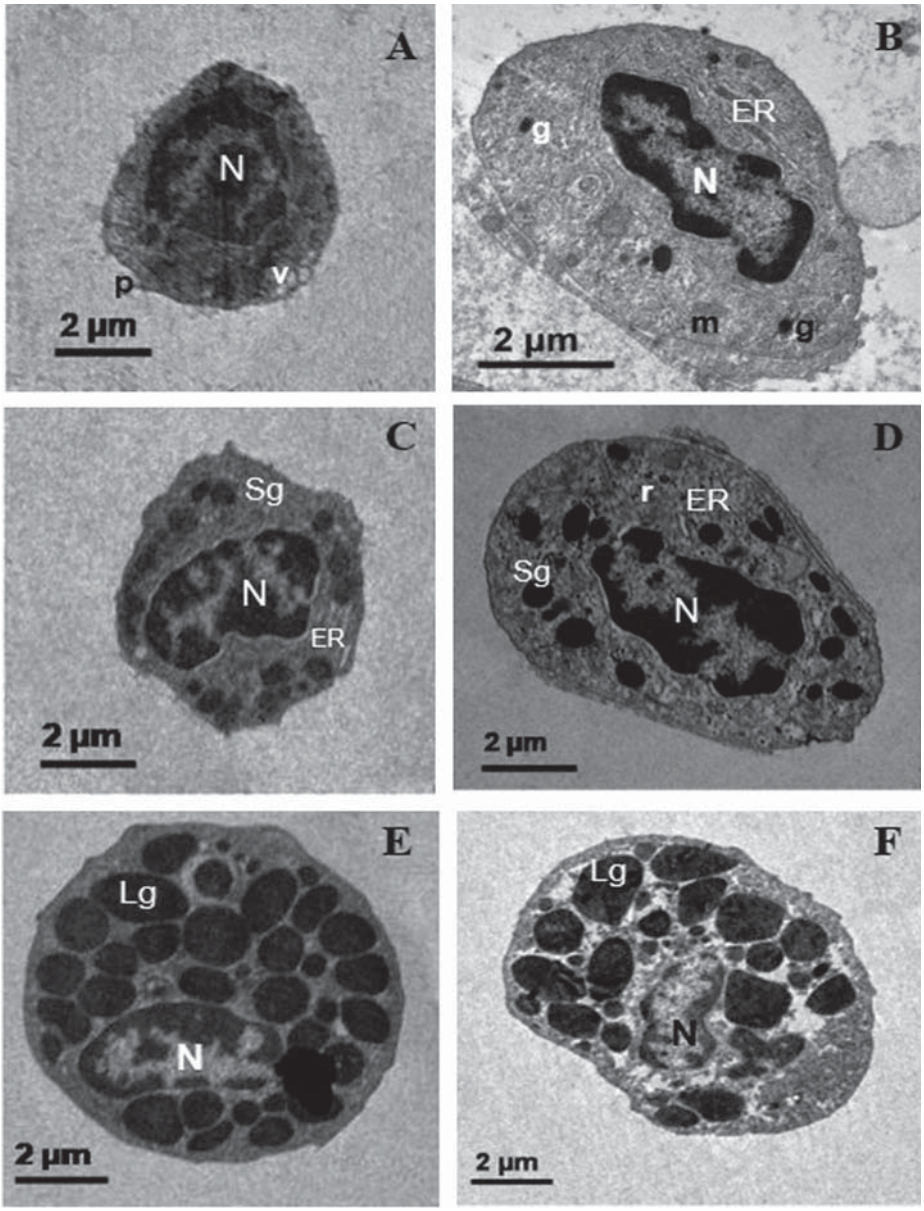

Fig. 3. Transmission electron micrographs of the haemocyte populations of *Varuna litterata*. A — hyalinocyte with high N/C ratio; B — hyalinocyte with low N/C ratio; C–D — small granule haemocytes; E–F — large granule haemocytes.

Abbreviations: ER — endoplasmic reticulum; g — minute granules; Lg — large-size granules; m — mitochondrion; N — nucleus; p — pseudopodia; r — ribosome; Sg — small-size granules; v — vesicles. Scale bar 2 µm.

Рис. 3. Ультраструктурные особенности гемоцитов разных типов у *Varuna litterata* (трансмиссионная электронная микроскопия). A — гиалиноцит с высоким ядерно-цитоплазматическим соотношением; B — гиалиноцит с низким ядерно-цитоплазматическим соотношением; C–D — малые гранулоциты; E–F — большие гранулоциты.

Обозначения: ER — эндоплазматический ритикулюм; g — крошечные гранулы; Lg — гранулы большого диаметра; m — митохондрия; N — ядро; p — псевдоподия; r — рибосомы; Sg — гранулы малого диаметра; v — везикулы. Масштаб 2 µm.

ules (0.57±0.19 μm) present in the cytoplasm around the large-size nucleus. Nucleus may be spherical or bilobate with condensed chromatin and located centrally or eccentrically in the cytoplasm. Endoplasmic reticulum, free ribosomes, mitochondria were present in the cytoplasm. LGHs (Fig. 3E–F) showed a small-size, eccentric, bilobate, spherical or irregular nucleus. Cytoplasm contained numerous large electron-dense granules (1.23±0.36 μm) and few small-size granules (0.56±0.17 μm). These granules were densely packed in the cytoplasm and almost obscured the nucleus. A Golgi body, endoplasmic reticulum, mitochondria and free ribosomes were also present in the periphery of the cytoplasm.

Flow cytometric analysis of haemocytes

Heterogenous structural types of haemocytes in *Varuna litterata* were subjected to flow cytometry for the further classification on the basis of cell size (FSC) and cell complexity/granularity (SSC). The dot-plot of FSC versus SSC was recorded and gated into three distinct regions, named as P1, P2 and P3 (Fig. 4). The P1 haemocyte structural type, having low FSC and low SSC value, were mainly composed of small sized cells with low granularity and designated as agranular cells or hyalinocytes. Similarly, P2 structural type, having high FSC and low SSC value, were designated as SGHs and P3 structural type, having high FSC and high SSC value, were designated as LGHs.

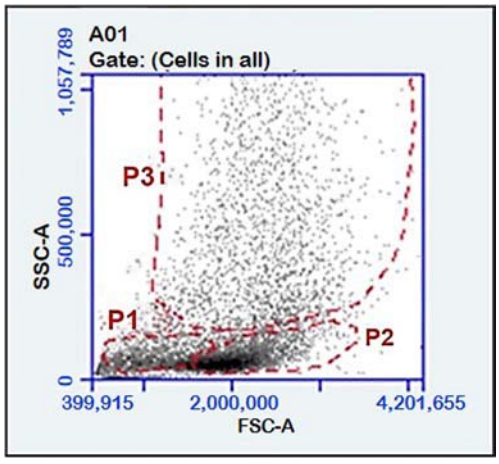

Fig. 4. Flow cytometry of haemocyte populations of *Varuna litterata*. P1, P2 and P3 gates represent hyalinocytes, small granule haemocytes and large granule haemocytes, respectively along FSC vs. SSC axes. The dot-plot presentation of haemocytes by flow cytometry is representative of one test over five determinations.
Abbreviations: FSC — forward scatter; SSC — side scatter.
Fig. 4. Проточная цитометрия гемоцитов разных типов у *Varuna litterata*. P1, P2 и P3 — популяции гиалиноцитов; малые гранулоциты и большие гранулоциты распределены вдоль осей FSC и SSC, соответственно.
Обозначения: FSC — передний разброс; SSC — сторонний разброс.

Total haemocyte count (THC) and differential haemocyte count (DHC)

The total haemocyte count ranged from 1.021 to 3.108 (x10^6) cells ml^{-1} haemolymph with mean value 2.145 ± 0.84 x 10^6 cells ml^{-1} in *Varuna litterata*. A summary of the THC reported in other crustacean species along with the present result is represented in Table 2. The differential haemocyte count revealed that the most abundant haemocyte type was SGHs comprising 55.24% of the total circulating haemocytes in *V. litterata* (Fig. 5). The percent values of hyalinocytes and LGHs were 13.93 and 30.83% respectively. In addition, the relative percentages of Type 1, Type 2, Type 3 and Type 4 H cells were 4.82, 2.47, 2.73 and 3.91% respectively (Table 1).

Discussion

Circulating haemocytes are very important cellular component of crustacean immunity. Their identification and classification are significantly important to understand their specific role in immune mechanisms (Ding *et al.*, 2012). The morphological classification of crustacean haemocytes is mainly based on the presence/absence, abundance and size of the cytoplasmic granules and nucleo-cytoplasmic ratio (Hose *et al.*, 1990; Johansson *et al.*, 2000; Matozzo, Marin, 2010a; Lv *et al.*, 2014). In the present work, after examined by light microscopy and transmission electron microscopy, three major structural types of haemocytes are character-

Table 2. Total haemocyte counts along with the habitats and life stages in various crustacean species.
Таблица 2. Общее число гемоцитов у различных видов ракообразных из разных биотопов и на разных стадиях жизненного цикла.

Crustacean species	**Habitats**	**Life stages**	**Total haemocyte counts**	**References**
Potamon fluviatilis	Freshwater	Intermolt	10.53 ±11.10 x 10^5 cells ml^{-1}	Yildiz, Atar, 2002
Macrobrachium rosenbergii	Freshwater	Intermolt	89.2 (± 4.7) x10^5 cells ml^{-1}	Chang *et al.*, 2006
Macrobrachium rosenbergii	Freshwater	Juvenile	7250 cells mm^{-3}	Gessa *et al.*, 2006
Callinectes sapidus	Mediterranean lagoon ecosystem	Mature	242.300±6.113 x10^4 ml^{-1} in female crab 216.434±4.778 x10^4 ml^{-1} in male crab	Gelibolu *et al.*, 2009
Fenneropenaeus indicus	Marine	Adult	5.3 ± 2×10^6 cc	Kakoolaki *et al.*, 2010
Carcinus aestuarii	Marine	Adult	6.4 x10^6 cells ml^{-1}	Matozzo, Marin, 2010b
Scylla serrata	Marine	Juvenile	2.86 x 10^7 cells ml^{-1}	Kumar *et al.*, 2013
Paratelphusa hydrodromous	Freshwater	Adult	140 cells mm^{-3}	Rulprakash *et al.*, 2013
Eriocheir sinensis	Freshwater	Intermolt	3.88 x10^8 cells ml^{-1} in female crab 3.23 x10^8 cells ml^{-1} in male crab	Lv *et al.*, 2014
Cancer borealis	Intertidal and subtidal	Intermolt	4.7 ± 0.4 x10^6 cell ml^{-1}	Parrinello *et al.*, 2015
Cancer pagurus	— " —	— " —	4.4 ± 0.6 x 10^7 cell ml^{-1}	— " —
Scylla tranquebarica	Brackish water	Intermolt	26501 ± 5743.121 cells μl^{-1}	Ramlingam *et al.*, 2015
Callinectes amnicola	Tropical lagoon ecosystem	Adult	3.08 to 3.19 x 10^7 cells ml^{-1}	Adeogun *et al.*, 2015
Penaeus monodon	Marine	Post-larval stage	9.5×10^4 cell mm^{-3}	Paulose, Sherly Williams, 2016
Paratelphusa jacquemontii	Freshwater	Adult	1162 ± 12.6 cells mm^{-3}	Maharajan *et al.*, 2017
Caridina weberi	Freshwater	Intermolt	1011 to 1225 cells mm^{-3}	Babila Jasmine *et al.*, 2018
Scylla paramamosain	Marine	Intermolt	10.92 ± 2.54 × 10^6 cells ml^{-1}	Zhou *et al.*, 2018
Varuna litterata	Freshwater	Intermolt	2.145 ± 0.84 x 10^6 cells ml^{-1}	Present study

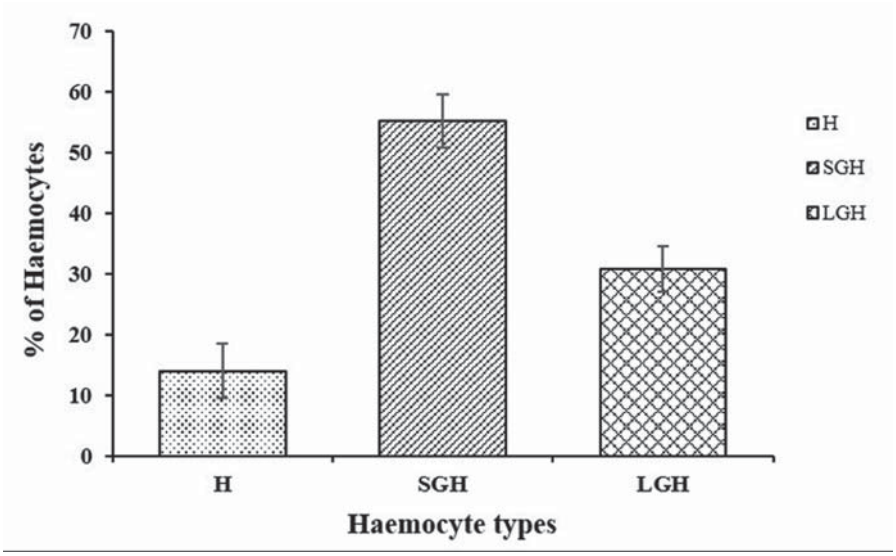

Fig. 5. Differential count of hyalinocytes, small granule haemocytes and large granule haemocytes in *Varuna litterata*. Each bar represents the mean value ± SD.
Abbreviations: H — hyalinocytes; LGH — large granule haemocytes; SGH — small granule haemocytes.
Fig. 5. Лейкоцитарная формула гиалиноцитов, малых и больших гранулоцитов у *Varuna litterata*.
Обозначения: H — гиалиноциты; LGH — большие гранулоциты; SGH — малые гранулоциты.

ized in *Varuna litterata*, named as hyalinocytes (H), small granule haemocytes (SGHs), and large granule haemocytes (LGHs). The present observations are in agreement with Clare and Lumb (1994), who identified three types of haemocytes namely as hyaline cells, small granulocytes and large granulocytes in blue crab, *Callinectes sapidus*. In contrast, Manjula *et al.* (1997) described four types of haemocytes-prohyalocytes, hyalocytes, eosinophilic granulocytes and chromophilic granulocytes in the Indian spiny lobster, *Panulirus homarus*. Morphologically distinct eleven cell types have been reported by Battison *et al.* (2003) in the haemolymph of American lobster, *Homarus americanus*. Other investigators have also reported three types of haemocytes in various crustacean species such as *Fenneropenaeus indicus* (Kakoolaki *et al.*, 2010), *Paratelphusa hydrodromous* (Rulprakash *et al.*, 2013), *Paratelphusa masoniana* (Gupta *et al.*, 2013), *Scylla serrata* (Kumar *et al.*, 2013), *Eriocheir sinensis* (Lv *et al.*, 2014), *Cancer borealis* and *Cancer pagurus* (Parrinello *et al.*, 2015), *Travancoriana schirnerae* (Padmanabhan *et al.*, 2017) and *Scylla paramamosain* (Zhou *et al.*, 2018). As various types of haemocytes have been described by different researchers, the similar scheme of haemocyte classification is not applicable in all crustacean species.

In *Varuna litterata*, hyalinocytes possess different morphological sizes and shapes like round, oval, irregular, spindle or ellipsoid with low to high nucleo-cytoplasmic (N/C) ratio. Ultrastructure of hyalinocytes revealed a number of cytoplasmic organelles and a few minute-sized granules in the cytoplasm. Similar homogeneous granules have been reported in the hyalinocytes of other crustacean species such as *Macrobrachium rosenbergii*, *M. acanthurus*, *Scylla serrata*, *Eriocheir sinensis* etc. (Gargioni, Barracco, 1998; Kumar *et al.*, 2013; Lv *et al.*, 2014). Various cytoplasmic organelles were copiously present in the hyalinocyte having small sized nucleus. Other hyalinocytes with larger nucleus showed least cytoplasmic organelles, pseudopodial extension and vacuoles. Presence of pseudopodia indicates that hyali-

nocytes possess phagocytic activity (Johansson *et al.*, 2000). The ultrastructure of hyalinocytes in *V. litterata* are also similar to those described from *Fenneropenaeus indicus* (Laxmilatha et Laxminarayana, 2004) and *Scylla serrata* (Kumar *et al.*, 2013). In *V. litterata,* various morphological forms and low to high N/C ratio of hyalinocytes indicated that H might be an undifferentiated haemocyte (Lv *et al.*, 2014). In contrast to the hyalinocytes, SGHs contained abundant small-sized granules and LGHs showed very few small-sized and numerous large-sized refractile granules in *V. litterata,* signifying their functional difference. In crustacea, semigranulocytes are generally involved in encapsulation, phagocytosis and cytotoxic responses whereas granulocytes are responsible for storage and release of proPO system and cytotoxicity (Hose *et al.*, 1990; Johansson *et al.*, 2000; Saha, 2011). Both SGHs and LGHs of *V. litterata* were basically large sized round, ovoid or ellipsoid shaped cells with a relatively high N/C ratio and contained comparatively moderate and a smaller number of cytoplasmic organelles, suggesting that these cells might be in different phases of the cell differentiation and maturation. However, semigranulocytes are assumed to be the intermediate cell type between the hyalinocytes and granulocytes (Battistella *et al.*, 1996; Matozzo, Marin, 2010a).

In flow cytometry, similar types of cells are sorted out from the heterogeneous cell populations on the basis of cell size and cytoplasmic complexity/granularity. Beside the conventional methods, flow cytometry technique has been widely used by the investigators for the purpose of suitable counting and isolation of the haemocytes from crustacean haemolymph (Allen, 2011). In the present study, three distinct haemocyte structural types, named as hyalinocytes, SGHs and LGHs were isolated from the haemolymph of *V. litterata* using this technique. The present findings are in agreement with Cheng *et al.* (2012) and Zhou *et al.* (2018) who categorized three structural types of haemocyte (hyalinocyte, granulocyte and semigranulocyte) in the mud crab, *Scylla serrata* and *S. paramamosain* respectively by the flow cytometric assay. Thus, in the present study flow cytometric analysis could help to establish the classification scheme of haemocyte cells in *V. litterata* along with the microscopic observation.

In crustacea, total and differential haemocyte count might be used as a biomarker for monitoring the immune response and health status of that organisms (Battison *et al.*, 2003; Li and Chen, 2008; Du *et al.*, 2012). A wide range of THC and DHC values have already been reported in crustacean species and it is difficult to draw any comparison due to different haemocyte classification scheme (Kumar *et al.*, 2013). In the present study, a wide range of THC value was observed in *V. litterata*, which can be fitted in the range found in other crustacean species (Table 2). Variation in THC might be correlated with the habitat, gender and life stages of the crustacean species (Table 2). DHC data revealed that SGHs comprised the highest percentage (55.24%) of circulating haemocytes in *V. litterata* and it is followed by LGHs (30.83%) and hyalinocytes (13.93%) respectively. The present findings are in agreement with Yildiz and Atar (2002) who found 54.25% semigranulocytes, 30.75% granulocytes and 15% hyalinocytes in the freshwater crab, *Potamon fluviatilis*. Moreover, similar DHC values were observed in the haemolymph of adult freshwater crab, *Paratelphusa masoniana* (Gupta *et al.*, 2013). In contrast, high percentages of hyalinocytes were reported in the lobster, *Panulirus interruptus* (Hose *et al.*, 1990), in the crayfish, *Procambarus clarki* (Lanz *et al.*, 1993), in freshwater prawn, *Macrobrachium rosenbergii* (Vázquez *et al.*, 1997) and in the crab, *Carcinus aestuarii* (Matozzo, Marin, 2010a). The relative proportion of each haemocyte structural type among decapod crustaceans depends on the mitotic activity in hematopoietic tissue and the dynamics of haemocytes production and release in the peripheral circulation (Hose *et al.*, 1992). In crabs, mitotic activity was high during the inter-molt period and low immediately after molting in the hematopoietic tissue and major release of haemocytes occurred during the ecdysial interval (Marrec, 1944; Charmantier, 1972; Johnson, 1980). It has been reported that SGHs

were produced at the highest rate and released during the intermolt and early premolt stages whereas hyalinocytes were produced at fairly constant levels from early intermolt stages until immediately before the molting (Hose *et al.*, 1992). Thus, in the present study, high percent of SGHs and low percent of hyalinocytes in *V. litterata* might be directly associated with the molting stage of the test individuals. In mature blue crab, *Callinectes sapidus*, Gelibolu *et al.*, (2009) found higher amount of total haemocyte, hyaline, semigranule and granule cells in female individuals than that of males during the reproduction period. In addition, Lv *et al.* (2014) recorded the relative percentages of hyalinocytes, semigranulocytes and granulocytes as 59.8, 21.1 and 19.2% respectively in male and 55, 24.9 and 20.1% respectively in female Chinese mitten crab, *Eriocheir sinensis*; both acclimatized in aerated freshwater at 16–18 °C. In another study, Parrinello *et al.* (2015) counted the relative percentages of hyalinocytes, semigranulocytes and granulocytes as 47.6, 45.2 and 7.2% respectively in *Cancer borealis* and 30, 60 and 9.4% respectively in *Cancer pagurus* where both the species were acclimatized in running seawater tank and temperature was 10 °C for *Cancer borealis* and 15 °C for *Cancer pagurus*. Hence, the relative percentages of each type of circulating haemocytes could not be countable unanimously in the crustacean species. It might be presumed that both THC and DHC in decapod crustacea depend on age, sex, species, molting stage, reproduction cycle, habitat, nutritional status of the test animals and also rely on the rearing conditions and experimental procedure followed in the laboratory (Owens, O'Neill, 1997; Yildiz, Atar, 2002; Adeogun, 2015). Outcome of the present study might provide the valuable information regarding the haemocyte profiles in *V. litterata* that would serve as better platform for the further cytochemical and molecular studies on detail functions and immune mechanisms of different haemocyte structural types.

Compliance with ethical standards

Conflicts of interest: The authors declare that they have no conflicts of interest.

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