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Study of Haemocytes of the Rice Brown Planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae)

Amani Mannakkara*

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ABSTRACT

Purpose: *The brown planthopper (BPH), Nilaparvata lugens, is one of the most serious insect pests of rice (Oryza sativa L.) in South and South East Asia and also parts of the Pacific and Australia. The BPH, like other insects, is able to build up powerful innate immune responses against invading pathogens and cellular immunity mediated by haemocytes. Hence, an attempt was made to investigate the detailed microscopic analysis of the haemocytes of BPH.*

Research Method: *The study was primarily concerned with the haemocyte profile of BPH with emphasis on the changes in the total and differential haemocyte population with three different life stages; 5th nympal stadium, newly emerged adults (post-moulting adult of 1-3 days old) and matured adults (15 days old) for an insight of the physiological events during the morphogenesis.*

Findings: *According to light microscopic examinations, the haemogram of the BPH comprised six types of haemocytes viz. prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), adipohaemocyte (ADs) oenocytoids (OEs) and spherulocytes (SPs). It was estimated that the mean numbers of total haemocyte count are varied from 6096.4 to 7393.6 cells per mm3 of haemolymph with the different physiological stages of the insects and it was statistically increased in the mature adults. PLs, GRs and PRs are the most abundant cells in the haemolymph in all development stages of BPH while SPs is the scarcest type.*

Originality/value: *To our knowledge, this is the first report that has detected the haemogram of BPH and these data are valuable for future avenues of immunological research of BPH.*

Keywords: *Differential haemocyte count, Haemocyte profile, Total haemocyte count*

INTRODUCTION

Insects have to depend on innate immunity which is consisted of humoral and cellular components, to counteract themselves from infections and invasions (Ratcliffe *et al.*, 1985; Lavine and Strand, 2002; Gupta *et al.*, 2005; de Andrade *et al.,* 2010; Mangalika *et al*., 2010). Humoral immunity involves induced antimicrobial peptides and other immune-related molecules generated by fat body and haemocyte that regulate extracellular and intracellular signal transduction which leads to induce melanization and clotting to immobilize and/or kill invading foreign bodies (Lavine and Strand, 2002; Kanost *et al.*, 2004; Shrestha *et al.,* 2010; Wang *et al.*, 2011). Cellular defense involves with haemocyte-mediated immune responses such as phagocytosis, nodulation and encapsulation that are mainly mediated by the interaction of haemocytes (Lavine and Strand, 2002; Jiravanichpaisal *et al.,* 2006; Wang *et al.,* 2011). Other than the immune reactions, haemocytes have multiple functions such as wound repair and coagulation to prevent loss of blood (Lai *et al.,* 2001; Rowley and Powell 2007; Cerenius and Soderhall, 2011),

* *Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna, Kamburupitiya 81100, Sri Lanka.*
amani@agbio.ruh.ac.lk
https://orcid.org/0000-0002-3457-9781

direct nutrients storage them (Meloni *et al.*, 1999; Pandey *et al.,* 2010; Pandey and Tiwari, 2012), transporting hormones (Meloni *et al.,* 1999; Pandey *et al.,* 2010; Pandey and Tiwari, 2012), detoxification of metabolites and biological active materials (Patton, 1961; Qamar and Jamal 2009), etc. Therefore, haemocytes endowed with a wide variety of physiological, biochemical and immunological functions of insects.

Haemocytes have been studied mostly in Lepidoptera, Hymenoptera, Coleoptera, and Diptera (Gupta, 1985). The haemocytes, circulating in the haemocoel of insects are categorized into several types by their morphology, functions, and staining or histochemical reactions, but, clear guidelines for classification of these cells are still lacking. The most common types of haemocytes in various insect orders are prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SPs), adipohaemocytes (ADs), and oenocytoids (OEs) (Lavine and Strand, 2002) and their characteristics slightly differ across various insect species (Ribeiro and Brehelin, 2006).

The brown planthopper (BPH) *Nilaparvata lugens,* is one of the most important sap sucking insect pests of rice, causing huge economic damage to the rice production, directly by feeding and also indirectly by transmitting viruses (Dyck and Thomas, 1979; Win *et al.,* 2011). Opposite to the economic importance of this insect pest, little or no work has been carried out on the BPH haemocytes. The present study was primarily concerned with the haemocyte profile of the BPH with emphasis on the changes in the total and differential haemocyte population across three different life stages: 5th nympal stadium, newly emerged adults (post-moulting adult of 1-3 days old) and aged adults (15 days old) for an insight of the physiological events during development.

MATERIALS AND METHODS

Experimental Insects

Initially, the BPH population collected from farmer paddy fields were reared in cages (2′x2′x3′) having aluminium frames and fine-mesh nylon sidewalls under room temperature at day light conditions at least for one generation. Two-month-old potted rice plants of variety Bg 380 were used to feed the insects and these potted plants were replaced by new ones as necessary. Enough numbers of newly emerged adults were transferred to another cage and maintained 15 days to obtain mature insects for experiments. Fifth nymphal stadium and newly emerged adults were collected directly form the original cages. While the population of BPH consists of both brachypterous and macropterous, only macropterous morph was used for the experiment. We studied both sexes separately in adult stages except the nymphal stage. All individuals used for the experiment were randomly selected.

Haemocyte Morphotypes: Observation of Haemocytes on Stained Haemolymph Smears

Newly emerged adult female BPHs were used to study the morphotypes of haemocytes. To collect haemolymph from each insect, one front leg was cut in midway and allows a small bead of hemolymph placed on a glass slide. Pooled samples of haemolymph from 5 insects were used to prepare one slide. Thereafter, the slide was air-dried for 2-3 minutes and fixed with 100% methanol at 4 C for 5 minutes. Before sample collection, the insects were anaesthetized by slow cooling on ice to facilitate an easy handling of insects. Haemocytes were then stained with few drops of Giemsa dye for 2-5 minutes for morphological examination. Finally, Smears were washed with running distilled water until dye residues disappeared and cover-slip was mounted on a glass slide with a commercial aqueous mounting medium. These smears were observed under a light microscope and types of hemocytes in BPH hemolymph were described with shape, size, nuclear/cytoplasmic ratio, staining reactions and position of the nucleus. Haemocyte morphotypes were classified using previously established morphological criteria and terminology with special emphasis on Jones' classification (Jones, 1962; Price & Ratcliffe, 1974; Brehelin *et al.,* 1978; Gupta, 1979a; Gupta, 1994; Lavin & Strand, 2002; Ribeiro & Brehelin,

2006; Stand, 2008). Photographs were taken with NIKON camera which was connected to the microscope and images were captured. Size of each type of haemocytes (n=10) was measured using the same software.

Haemocyte Morphotypes: Observation of Haemocytes Immediately after Bleeding

As well, haemolymph samples were observed immediately after bleeding to view live. For that, haemolymph were dropped directly on the glass side and cover-slip was mounted immediately (Barracco & Cestari, 1984; Hillyer & Christensen, 2002). These slides were observed under light microscope and digital images were captured connected to a colour digital NIKON camera. Structural changes of hemocytes during incubation were observed for 60 minutes.

Differential Haemocyte Count

Insects at 5th nymphal stadium, newly emerged adult stage (1-3 day old adults) and aged adult stage(15 days old) were used to determine the differential haemocyte count (DHC) using the hanging-drop technique (Gupta, 1979b; Gupta, 1979c). Hemolymph was drawn directly into the drop of 0.1 M PBS (pH 7.2) on glass cover-slip after cutting off one front leg in the midway with gentle abdominal pressure from anaesthetized insects at different age groups separately. Then, the cover-slip was set in a humid chamber and haemocytes were allowed to spread and adhere to glass surfaces for 5 minutes at room temperature. Thereafter, the cover-slip was removed from the chamber and carefully turned upside down and placed it over the cavity slide. Finally, the edges of the cover-slip to the slide were sealed with colorless, transparent nail polish. Due to the small size of insects, the hemolymph from 5-10 individuals was pooled to prepare one slide. For each age category of the insects, 5 slides were prepared. Two hundred cells were counted per slide using the random sampling method using light microscope.

Total Haemocyte Count

Total haemocyte count (THC) was determined in three different age categories; 5th stadium nymphs, newly emerged adult stage (1-3 day old adults) and aged adult stages (15 days old). Haemolymph was collected after cutting off one front leg in the midway of cooled anaesthetized insects with gentle abdominal pressure to a glass micro-capillary tube as it pearled from the wound. A total of 2-10µl of hemolymph was collected from 50 to 200 individuals and immediately transferred into the 1.5 ml Eppendorf tube containing 0.1 M, pH 7.2 phosphate buffer solution in a proportion of 1µl of hemolymph to the 9 µl of buffer solution. After mixing by gentle pipetting, one drop of haemolymph was mounted near the edge of the cover slip of the Neubauer haemocytometer to facilitate to automatically fill the entire chamber by capillary action. After allowing several minutes to settle down the haemocytes, the haemocytes were counted in four corners and central ruled squares in each of the two chambers of haemocytometer under a microscope (Jones, 1962). Each was replicated five times. The number of circulating hemocytes per cubic millimeter (mm^3) was computed by using the formula suggested by Jones (1962).

Statistical Analysis

The differential haemocyte count and total haemocyte count were analyzed by one-way analysis of variance using SPSS 18 for Windows (SPSS Inc.).

RESULTS AND DISCUSSION

Morphological Observations

The examination of the haemocytes from BPH indicates the presence of six (6) types of haemocytes: PRs, PLs, GRs, ADs, OEs and SPs were morphologically distinct using light microscope (Figure 01) with different percentages at different physiological stage of BPH (Figure 02).

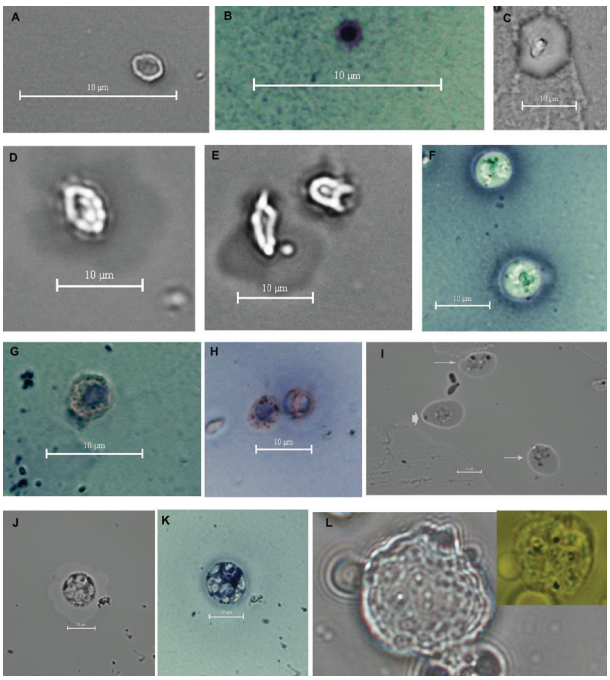

Figure 01. **Light micrographs of haemolymph smear of BPH showing different types of haemopcytes (A) PRs. The nucleus occupies a large part of the cytoplasm, (B) PRs stained with Giemsa; the cytoplasm is with acidophilic characteristics and basophilic nucleus (under oil immersion x 100 using light microscope). (C) Round plasmatocyte (D) and (E) Fusiform plasmatocytes. (F) Round plasmatocyte (stained with Giemsa; note nucleus with basophilic characteristics (40×)). (G and H) GRs. stained with Giemsa (40×). (I) OEs with exocentric nucleus (thin arrow) and central nucleus (broad arrow)(40 ×). (J) ADs without Giemsa staining (40×). (K)ADs stained with Giemsa (40×), (L) SPS observed in wet smears with the light microscope (40×).**

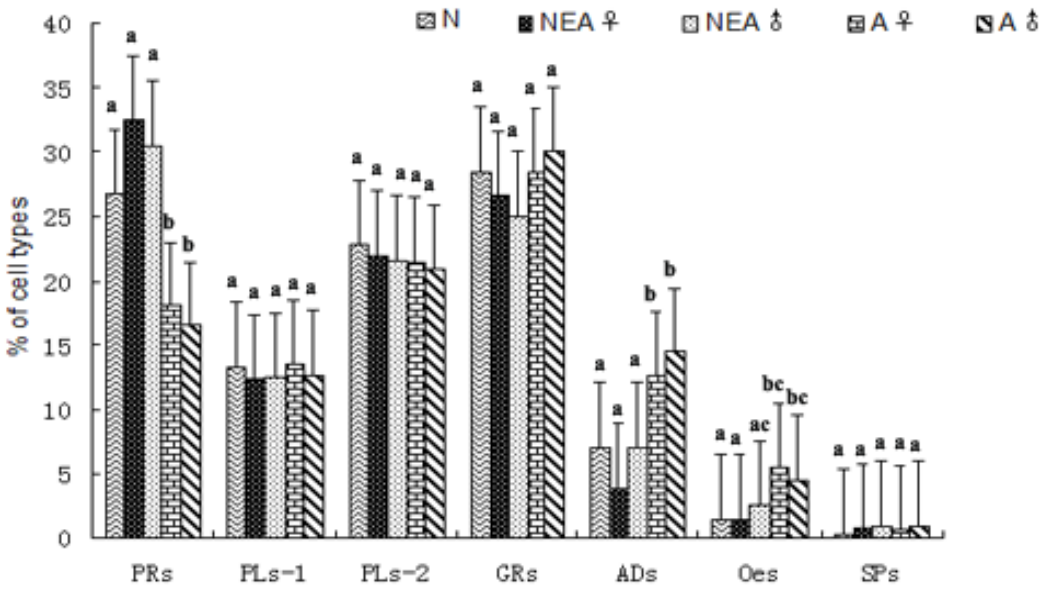

Figure 02: **Percentage of different type of hemocytes of Rice brown plant hopper; Nilaparvata lugens Stål (Hemiptera: Delphacidae) at the different physiological stages. Randomly selected 200 cells/slide were assessed for calculate the percentage of different types of cells. Pooled samples of haemolymph from 5-10 insects were used to prepare one slide. PLs-1 – Round shape PLs; PLs-2 - Fusiform shape PLs; N - Nymph; NEA♀ - Newly emerged Adult ♀; NEA♂ - Newly emerged Adult ♂; A♀ - Adult ♀; A♂ - Adult ♂.**

Prohaemocytes

PRs are small, round cells with variable sizes (0.5-3 µm, average of 1.5µm). The centrally located nucleus is larger and almost fills the cell containing high nuclear/cytoplasmic ratio (Figure 01(A)). With Giemsa staining, cytoplasm stained with an acidophilic characters and the nucleus exhibited basophilic affinity (Figure 01(B)). In monolayer, most prohaemocytes remained unchanged within the 60 minutes incubation experimental time period at room temperature (25 ℃).

Plasmatocytes

Plasmatocytes are the most abundant cell type present. These are polymorphic cells of variable sizes measuring approximately 5 - 15 µm in diameter. The round or elongate nucleus is commonly located in the central of the cell (Figure 01 (C), exocentric nucleus was observed rarely. The cytoplasm is abundant containing low nuclear/cytoplasmic ratio. Cytoplasm softly stained in a light grey and the nucleus appears paler blue with Giemsa staining (Figure 01F). These cells started to form surface projections (pseudopodia) after 5 -10 minutes of bleeding and completed these changes after 30 minutes of bleeding at 25^0C. Being polymorphic, two distinct forms of plasmatocytes were observed for the round shape plasmatocyte (Figure 01 C,F) and fusiform shape plasmatocytes (Figure 01 D,E). Fusiform shape plasmatocytes were more common in all the tested age categories than round shaped ones ranging 61% to 64% among different physiological life stages from whole plasmatocytes population.

Granulocytes

Granulocytes are the second most common cell type with round or ovoid cells of variable sizes (3-9 µm). The relatively small, rounded or elongate nucleus is generally located in the cell center, exocentric nucleus was observed rarely. The cytoplasm characteristically consists of numerous small granules. The nuclear chromatin appears as blue and the cytoplasm stains up pale pink after Giemsa staining (Figure-1(G-H)). In monolayer, after few minutes of incubation, granulocytes were readily attached to glass and begun to spread out on the glass surface. These cells spread on the glass surface forming large and thin cells reaching diameters that may exceed 10 µm and completed the spreading fully within 35 minutes of incubation at 25℃. After about 15 minutes of incubation the initial refractiveness of the cells became declined due to gradual lose of cytoplasmic granules. Most of the cytoplasmic granules were disappeared and cells gradually lose their initial refractiveness within the incubation period. At the 60 minuets incubation time period, these cells did not form any surface projections (pseudopodia).

Oenocytoids

Oenocytoids are large cells (15- 35 µm) with round or ovoid in shape that measure approximately 15 - 35 µm in diameter. They contain large, often eccentrically positioned nucleus. The cytoplasm is abundant containing a low nuclear/cytoplasmic ratio (Figure-(I)). These cells are characteristic by rapid lysis. Immediately, after bleeding, these cells were shown to have very smooth surface and without any cytoplasmic extensions. These cells started to lysis within 5-10 minutes after bleeding and completely lysed within 10 -15 minutes at 25℃. These hemocytes were observed very rarely with Giemsa stain technique.

Adipohaemocyte

These are oval cells with 10 - 15 µm length. Compared with that of the plasmatocytes, the nucleus is relatively small, elongate and is mostly eccentrically located. The cytoplasm is abundant containing a low nuclear/cytoplasmic ratio. Several large lipid droplets are seen in the cytoplasm (Figure-1(J-K)). Light microscopic observations did not show any morphological changes on the cells during the incubation time of 60 minutes, in monolayer of haemolymph.

Spherulocytes

Spherulocytes are large cells (15- 35 µm) with irregular in shape (Figure 1-(L)). The cytoplasm was characteristically filled with a small number of prominent spherules (large inclusions). These cells were scarce in BPH with remaining very low in immature stages. Cell margins may appear rough due to the presence of marginal lumps.

Differential Haemocyte Count

Result of one-way ANOVA showed the statistically significance on PRs among the different stages of the life cycle of BPH (Table 01). Last nympal stadium of BPH possessed a significantly high number of PRs than both sexes of aged adults. While, there are no significant difference, the number of PRs was observed between last nympal and newly emerged adult stages and the mean number of PRs are significantly lower in aged adults than newly emerged ones, but there were no significant differences between the male and female insect.

Mean numbers of two different morphotypes of PLs, GRs and SPs did not show any significant differences with comparison of age groups or sexes as determined by Tukey post-hoc test at 0.05 probability level. The SPs were numerically less abundant in all age categories. Mean number of ADs were significantly high in aged adults compared to other life stages and the percentage increased from 3.9±0.4 to 12.5±3.0 in females and 7.0±2.4 to 14.5±3.0 in males with the maturity of adult stage. There was a statistically high number of OEs in aged adults (5.5% and 4.5% for both female and male respectively) compared to nympal and newly emerged adults female at P≤0.05 level. The mean number of OEs in adult males was not significantly different at newly emerged adult male at P≤0.05 level. But, the mean number of OEs in adult females was significantly higher than both sexes of newly emerged adults at P≤0.05 level (Table 01).

'Total Haemocyte Population'

One-way ANOVA reported in Table 02 shows a statistical difference of total circulating haemocytes among the different age categories of BPHs. A Tukey post-hoc test revealed that the total haemocyte count of nymphs was significantly higher (7101 ± 192.399, *P* =.002) than newly emerged males (6096.40 ±135.228). Mature adult females possessed a significantly higher number of circulating haemocytes (7393.60±575.366) than both sexes of newly emerged stage; female (6600.60 ±272.762, *P* =.013) and male (6096.40±302.379, *P* =.000) while aged adult male possessed (7119.40±566.857) a significantly higher amount compared to newly emerged adult male (*P* =.001) only.

Table 01: Differential haemocyle count of Rice brown plant hopper; *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) in relation to mean number of cells out of 200 cells

Life Stages	% of Cell types (mean±StDev)						
	PRs Mean± sd	PLs-1 Mean± sd	PLs-2 Mean± sd	GRs Mean± sd	ADs Mean± sd	OEs Mean± sd	SPs Mean± sd
N	26.7±2.1 ^{a}	13.2±1.0 ^{a}	22.8±2.3 ^{a}	28.5±3.6 ^{a}	7.0±1.4 ^{a}	1.5±0.7 ^{a}	0.3±0.4 ^{a}
NEA♀	32.5±4.3 ^{a}	12.3±2.5 ^{a}	22.0±3.9 ^{a}	26.6±2.2 ^{a}	3.9±0.4 ^{a}	1.5±0.7 ^{a}	0.7±0.5 ^{a}
NEA♂	30.5±2.2 ^{a}	12.4±1.5 ^{a}	21.6±2.4 ^{a}	25.0±5.9^{a}	7.0±2.4 ^{a}	2.5±1.4 ^{ab}	1.0±0.7 ^{a}
A♀	18.0 ±2.3 ^{b}	13.5±1.6 ^{a}	21.5±1.4^{a}	28.4±5.6 ^{a}	12.5±3.0 ^{b}	5.5±1.6 ^{c}	0.6±0.6 ^{a}
A♂	16.5 ±3.8 ^{b}	12.6±2.3 ^{a}	20.9±2.3 ^{a}	30.0±3.3 ^{a}	14.5±3.0 ^{b}	4.5±0.9 ^{bc}	1.0±0.5 ^{a}

Values within a given column not followed by the same superscript letter are significantly different at 0.05 probability level as determined by tukey's. HSD. PLs-1 - Round shape PLs; PLs-2 - Fusiform shape PLs. N - Nymph; NEA♀ - Newly emerged Adult ♀; NEA♂ - Newly emerged Adult ♂; A♀ - Adult ♀; A♂ - Adult ♂. PRs- prohemocytes; PLs - 1 plasmatocytes (round shaped); PLs - 2 plasmatocytes (fusiform shaped); GRs- granulocytes; ADs – adipohemocytes; OEs – oenocytoids; Sps – spherulocytes.

Table 02: Total Circulating Haemocytes of Rice brown plant hopper; *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) per cubic millimeter(mm^3) of haemolymph.

Life stages	Mean ± SD	Std. Error	95% Confidence Interval for Mean	
			Lower Bound	Upper Bound
Nymph	7101.0±192.3 [bc]	86.044	6862.10	7339.90
Newly emerged Adult ♀	6600.6±272.7 [ab]	121.983	6261.92	6939.28
Newly emerged Adult♂	6096.4±302.3 [a]	135.228	5720.95	6471.85
Adult ♀	7393.6±575.3 [c]	257.311	6679.19	8108.01
Adult ♂	7119.4±261.7 [bc]	117.053	6794.41	7444.39

Values followed by the same superscript letter in the column are statistically not different at the .05 level as determined by Tukey HSD ($P<0.05$).

Numerous works on the classification of insect haemocytes by using light microscopic observations in different group of insects have been published with remarkable differences of opinions concerning haemocyte classifications and terminologies. Types of haemocytes identified in insects are not always present in all species (Wigglesworth, 1959; Jones, 1962; Price and Ratcliffe, 1974; Sahayarij *et al*., 2007; Josef and Klara, 2008) and there is an inherent variability of haemocytes within a species as well as among closely related species (Price and Ratcliffe, 1974). Therefore, classification of insect haemocytes is the subject of controversy, and the terminology used to assign each cellular type is often different from one species to another.

In this experiment, we did not observe surface projections (pseudopodia) of GRs of BPH when these cells spread out on the glass surface. These observations are in accordance as well as differ with some previous findings. With the accordance with many previous studies of different kinds of insects, PLs of BPH showed many surface projections (pseudopodia). For an example, in 1974, Price and Ratcliffe observed blood films of fifteen insect orders and according to their viewed GRs might be existed with or without surface projections and PLs begun to spread out after bleeding with forming surface projections in many insect orders. Prominent lipid-like inclusions in the cytoplasm of ADs are characteristic to many other insects (Sanjayan, *et. al.*, 1996; Silva, *et. al.*, 2002; Lea and Gilbert, 1966; Fevzi and Olga, 2010). Similar to the OEs of some other insects like *Spodoptera litura* (Kurihara *et al.,* 1992), and *Pseudoplusia includens* (Strand and Noda, 1991), OEs of BPH also underwent rapid lysis after bleeding. SPs in BPH are haemocytes exhibiting large inclusions in cytoplasm. Their characteristics are similar to those observed in other insect species (Miyuki and Kikuo, 2001; Brayner *et al.,* 2005). PRs are believed to be the basic stem-type cells that divide frequently and give rise to other types of haemocytes (Suhail *et al.,* 2007). In the present study, the percentage of pohaemocytes decreased while increasing the THC with their maturity also shows the greater transformation of pohaemocytes into other type of cells.

There are no available data to compare the different types of haemocytes of BPH with other delphacid of order homoptera. There are few published data of different types of haemocytes of the same order, but, comparing different types of haemocytes within the order is not ideal. Similar to the *Agallia constricta,* the BPH has PRs, PLs, GRs, ADs, OEs and SPs (Granados & Meehan 1973). Haemocyte profile of *Spilostethus hospes* showed PRs, PLs, Grs, ADs and SPs (Sanjayan *et al.*, 1996). According to the Patro *et al.* (2005), five types of haemocytes were found in the haemolymph of *Aphis gossypii*, namely PRs, PLs, spindle shaped haemocytes, GRs and ADs. Furthermore, his study clearly pointed out

that the spindle shaped haemocytes is a kind of plasmatocytes. However, PRs, OEs, and GRs were observed in *Phenacoccus manihot* and only GRs were observed in *Planococcus citri* by Russo *et al.* (1994). Four (4) distinct types of haemocytes : PRs, GRs, PLs, and SPs were identified in the *Pyrrhocoris apterus* by Josef and Klara (2008).

The changes in both, the number of circulating hemocytes and in the relative proportions of different hemocyte types in the hemolymph of insects are a normal response to immune defense. Cellular defense mechanism of insects is primarily depending upon the availability of circulatory hemolymph and the changes on the THC are a good indicator for the activation of the mechanisms of innate immune system of insects. According to Lavine & Strand (2002), the increases in THC also show the activation of the mechanisms responsible for phagocytosis, nodule formation, encapsulation, recognition of foreign bodies and wound healing. On the other hand, decreases in THC show a decline in the proportion of metabolically active cells which suggests to a reduction of the ability of insects cellular defense system. Therefore, these findings are essential towards the advancement of insect immunological studies.

prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), adipohemocytes (ADs), oenocytoids (OEs) and spherulocytes (SPs). It was estimated that the mean numbers of total haemocyte count are varied from 6096.4 to 7393.6 cells per mm^3 of haemolymph with the different physiological stages of the insects and it statistically increased in the aged adults. PLs, GRs and PRs are the most abundant cells in the haemolymph in all development stages of BPH while SPs are the scarcest type.

Small size of the BPH with a limited amount of hemolymph was the key challenge to collect hemocyte in this experiment. However, up to date, using different names for the hemocytes often leads to confusing identification and of hemocytes. Therefore, it is important to develop a more uniform terminology for naming insect hemocytes in different insect species. The gap is rapidly closing as more studies on insect hemocytes enter the literature.

To the best of our knowledge, this is the first study to detect the hemocytes of BPH and these data will be important for future immunological research using BPH. However, further studies are needed in order to improve the knowledge on the functional aspects of each hemocyte types in a cellular manner.

CONCLUTIONS

Examination of hemocytes from BPH indicated the presence of six types of hemocyte:

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