

# Leishman staining for the identification of haemocytes in beetles

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## Abstract

Haemocytes in insects play a vital role in immune responses, and their general appearance varies in different groups. Leishman stain is widely used for the staining and identification of blood cells. The present study was conducted to identify the haemocytes of two adult beetles, *Pheropsophus sbrinus* and *Oryctes rhinoceros*. Haemocytes were stained with Leishman stain, examined, counted under a microscope and photographed. The cells were identified based on their general appearance, size, shape and position of the nucleus. Prohaemocytes, plasmatocytes, granulocytes, oenocytes, spherulocytes and adipohaemocytes are the major cells identified in the haemolymph. Differential haemocytes count showed a higher percentage of prohaemocytes in both the beetles. Plasmatocytes and adipohaemocytes had the second highest percentages in *O. rhinoceros* and oenocytes in *P. sbrinus*. The study enabled us to understand how the haemocytes of different insect species differ in terms of their appearance and number in concomitant with their mode of living.

**Keywords:** haemocytes, *Pheropsophus sbrinus*, *Oryctes rhinoceros*, plasmatocytes, adipohaemocytes, oenocytes

## 1. Introduction

Arthropods, the members of the largest phylum Arthropoda, include insects, arachnids, myriapods and crustaceans. Insects are the largest group, with more than one million species, and are adapted to live in almost all

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habitats on earth. They have a characteristic segmented body structure with the head, thorax and abdomen; an exoskeleton formed of chitin; six legs; two antennae; and frequently one or two sets of wings. Many insects are capable of flight and are relatively small, enabling them to escape danger and disperse to new locations. The insect groups include bedbugs, houseflies, clothes moths, Japanese beetles, aphids, mosquitoes, fleas, horseflies, hornets, butterflies, moths, and beetles. Coleoptera, comprising beetles and weevils, is the largest and most diverse order in the class Insecta, with more than 3,50,000 identified species of beetles, contributing to about 40% of all insects and 30% of all animals [1].

Haemolymph is the fluid in the circulatory system of insects and is analogous to the blood and interstitial fluid in vertebrates [2]. It consists of plasma and blood cells called haemocytes, various organic and inorganic constituents. Haemocytes are involved in both cellular and humoral immune responses in insects. These responses include phagocytosis, encapsulation, secretion of antimicrobial peptides, production of cell adhesion molecules, lysozyme and lectins, detoxification and activation of the prophenoloxidase system [3,4]. Several classes of haemocytes have been morphologically and functionally characterized in different group of insects [5,6]. The most common types of haemocytes reported in the literature are prohaemocytes, granulocytes, adipohaemocytes, plasmatocytes, spherulocytes and oenocytes. Gupta [7] has described seven basic types of haemocytes common among the different orders of insects. Amaral et al. [8] have identified prohaemocytes, plasmatocytes, granulocytes and oenocytoids in the haemolymph of *Melipona scutellaris*. Six types of haemocytes have been identified in the haemolymph of third instar larvae of *O. rhinoceros* [9]. Also, many insects have several sub-populations of haemocytes which differ in morphology and function but are known by common names [10].

Different haemocytes have their own functions, contributing to the overall immune response of the animal. Plasmatocytes are involved in capsule formation and phagocytosis, granulocytes are involved in phagocytosis, oenocytes produce enzymes involved in the melanization cascade [5]. The spherulocytes are involved in regeneration of tissues, transport of chemical messengers and the synthesis of proteins. Adipohaemocytes, which are similar to the adipose tissues and liver in vertebrates, are the primary storage organs for glycogen, lipids, and proteins in insects and are important in energy metabolism [11].

The identification of haemocytes in insects is of vital importance regarding their immunological adaptations and survival. Adult *Oryctes rhinoceros* (rhinoceros beetle) is a serious pest of coconut palm and belongs

to the family Scarabaeidae, subfamily Dynastinae. *Pheropsophus sbrinus* (bombardier beetle) is a ground beetle that belongs to the family Carabidae and the subfamily Brachininae. The Leishman stain is a polychromic stain widely used for the staining and identification of blood cells under microscopy. It is a type of Romanowsky differential stain that contains the basic dye methylene blue and the acidic dye eosin, dissolved in an alcohol medium (methanol). It is named after the Scottish pathologist Sir William Boog Leishman, who, along with Karl Reuter, independently developed the stain in 1901 as a practical modification of Malachowski's stain. It imparts a violet colour to the nuclei and provides a clear contrast between the nucleus and the cytoplasm. This contrast results primarily from the combined action of two staining components, Azure B and Eosin Y. Azure B is an oxidation product of methylene blue that stains nucleic acids [12].

Insects are adapted to diverse ecological conditions, environmental stresses, and pathogen pressures. Correspondingly, haemocytes exhibit considerable variation in their morphology, abundance, and functional specialization across insect species. Therefore, detailed studies on haemocyte diversity and function provide valuable insights into the evolutionary and physiological mechanisms that enable insects to survive and thrive in diverse habitats. Furthermore, knowledge of haemocyte biology can aid in the development of effective insect control strategies by identifying potential targets for disrupting immune functions, enhancing the efficacy of biological control agents, and improving pest management programs. Such investigations are also valuable for understanding host-pathogen interactions and the overall health and resilience of insect populations. The current study aimed at Leishman staining, observation and identification of haemocytes present in the beetles, *P. sbrinus* and *O. rhinoceros*.

## 2. Materials and methods

### 2.1 Collection of haemolymph

Adult *Pheropsophus sbrinus* were collected from damp places in Thiruvananthapuram district of Kerala state (8.5017° N latitude, 76.9596° E longitude; altitude 58m.) and identification was done by comparing the morphological features [13]. Adult *Oryctes rhinoceros* were collected from coconut gardens. Species identification was done by observing their morphological features [14]. The bodies of the beetles were cleaned and kept at 4°C for five minutes. The prolegs of the inactive beetle were cut and the haemolymph was collected by gently squeezing the body.

### 2.2 Differential haemocyte count (DHC)

On one end of a clean slide, the haemolymph was collected, as previously

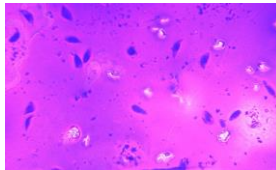
mentioned. After that, a clean slide was moved onto the slide positioned at a 45° angle to form a smear and air dried. The smear is then stained with filtered Leishman stain, which is then left on for five to ten minutes. With distilled water, excess stain is removed, and then it is allowed to dry. The cells on the slides were subsequently observed under a compound microscope and identified based on their distinguishing morphological characteristics [15,16]. The cells were photographed using Labomed digital microscope (Lx 400, magnification 400X). For each slide, one hundred cells were counted, and the relative proportions of the different haemocyte types were calculated [9,17].

### 3. Results

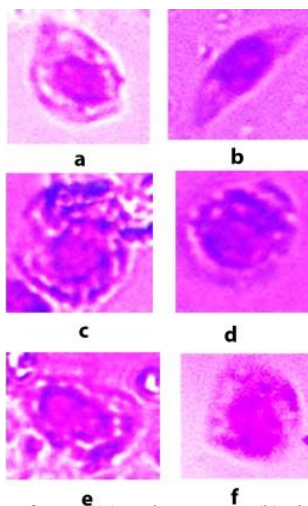
Leishman stain provided better contrast between the nucleus and cytoplasm of haemocytes, facilitating the identification and study of different cell types.

#### 3.1 Haemocytes of *P. sobrinus*

Six types of haemocytes could be identified in the haemolymph of *P. sobrinus* (Fig. 1, 2, Table 1).



**Figure 1:** Microscopic field of view of haemocytes identified in *P. sobrinus*



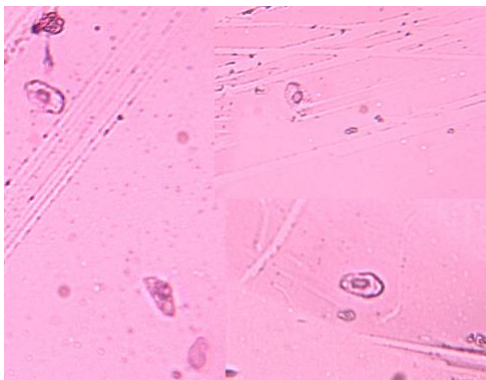
**Figure.2:** Haemocytes of *P. sobrinus*, (a) prohaemocyte, (b) plasmatocyte, (c) granulocyte, (d) adipohaemocyte, (e) spherulocyte, (f) oenocyte

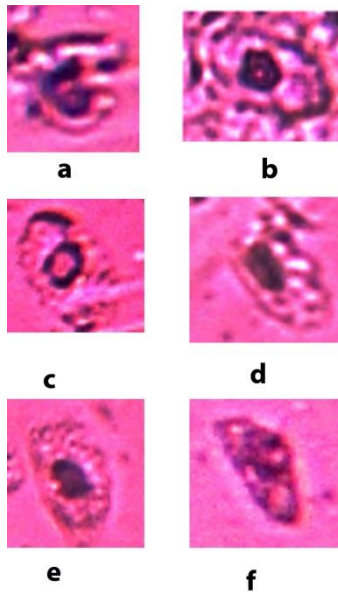
**Table 1:** Haemocytes identified in *P. sobrinus*

Cell types	Description
<b>Prohaemocytes</b> (Figure 2a)	The smallest haemocytes, irregular in shape with relatively large nucleus and a few cytoplasmic granules. The nucleus occupies the cell's center and almost completely fills it.
<b>Plasmatocytes</b> (Figure 2b)	Elongated, spindle shaped cells with a large nucleus, and a large mass of granular cytoplasm.
<b>Granulocytes</b> (Figure 2c)	Large cells with a relatively large nucleus and granular cytoplasm.
<b>Adipohaemocyte</b> (Figure 2d)	Irregular in shape with a large nucleus and cytoplasm with lipid droplets.
<b>Spherulocytes</b> (Figure 2e)	Large, irregularly shaped cells, with a large nucleus and many spherules around it.
<b>Oenocytes</b> (Figure 2f)	Oenocytes showed varied shape and nucleus is eccentric in position.

### 3.2 Haemocytes of *O. rhinoceros*

Six types of haemocytes could be identified in the haemolymph of *O. rhinoceros* (Fig.3, 4, Table 2).

**Figure 3:** Microscopic field of view of haemocytes identified in *O. rhinoceros*



**Figure 4:** Haemocytes of *O. rhinoceros*, (a) prohaemocyte, (b) plasmatocyte, (c) granulocyte, (d) adipohaemocyte, (e) spherulocyte, (f) oenocyte

**Table 2:** Haemocytes Identified in *O. rhinoceros*

Cell types	Description
Prohaemocytes (Figure 4a)	The smallest haemocytes, oval in shape with relatively large nucleus and a few cytoplasmic granules.
Plasmatocytes (Figure 4b)	Large, oval shaped cells with a large nucleus and a large mass of granular cytoplasm.
Granulocytes (Figure 4c)	Large cells with a large conspicuous nucleus and granular cytoplasm.
Adipohaemocyte (Figure 4d)	Elongated cells and contain numerous large lipid droplets and granules in the cytoplasm.
Spherulocytes (Figure 4e)	Large oval shaped cells, with a large nucleus and spherules almost filling the cytoplasm.
Oenocytes (Figure 4f)	Oenocytes with granular cytoplasm have a nucleus that is clearly projecting towards the periphery.

DHC showed the highest proportion of prohaemocytes in both *O. rhinoceros* and *P. sobrinus* compared to other types of cells; *O. rhinoceros* has a higher proportion than *P. sobrinus* (Table 3). Plasmatocytes and adipohaemocytes were in the second highest percentage in the rhinoceros beetle, whereas oenocytes were in the bombardier beetle.

**Table 3:** DHC in *P. sobrinus* and *O. rhinoceros*

Differential haemocyte count (%)*						
Cells	PR	PL	GR	AD	OE	SP
<i>P. sobrinus</i>	32.04 ±	12.57 ±	15.11 ±	12.49 ±	16.58 ±	11.45 ±
	0.93	0.37	0.92	0.69	0.30	0.40
<i>O. rhinoceros</i>	44.33 ±	13.00 ±	12.00 ±	13.00 ±	8.00 ±	12.67 ±
	1.75	1.32	1.00	1.00	1.41	1.08

\*Values represent mean of three replicates ± SE

PR- prohaemocyte, PL- plasmatocyte, GR - granulocyte, AD - adipohaemocyte, OE- oenocyte, SP- spherulocyte

## Discussion

The present study reveals the presence of different types of haemocytes in the haemolymph of the beetles, *P. sobrinus* and *O. rhinoceros* employing Leishman staining. Leishman stain gives better contrast between the nucleus and cytoplasm. Cytoplasm and granules are basophilic and have a strong affinity for acidic stains, whereas the nucleus is acidophilic and has a strong affinity for basic dyes, giving blue to purple shades. Compared with other stains used in haemocyte studies, Leishman staining is a rapid, simple, and cost-effective technique in which methanol serves as a fixative. It preserves cellular morphology by preventing further cellular alterations and also facilitating cell adhesion to the glass slide.

Haemocytes are important components of the insect immune system, enabling insects to thrive in adverse environments where many other organisms cannot survive. Haemocytes provide cellular immune responses through various mechanisms, thereby enhancing insect survival [18]. This enables insects to control a wide range of harmful microorganisms that have emerged throughout different periods of evolution. Such capabilities contribute to the evolutionary success of insects across diverse ecological niches and help maintain species diversity [19]. The antibacterial activity of haemocytes in *Bombyx mori* larvae that have been infected with *E. coli* already reported [20]. According to Li et al., (2019), melanisation process induced by haemocytes has been shown to be an effective method of

preventing pathogen invasion. All these defense response of insects depends on the cellular types involved in the process [22]. Mosquitoes have phagocytic granulocytes, phenoloxidase-producing oenocytoids and prohaemocytes [23]. Likewise, *Drosophila melanogaster* has plasmatocytes, crystal cells and lamellocytes [24]. These observations show that types of haemocytes vary from insect to insect and depend on their mode of life. In the present study, six types of haemocytes were identified in the haemolymph of the beetles. The cells show variation among themselves and between the beetles. The presence of all the basic types of haemocytes in the beetles is one of the contributing factors to their survival.

The present study reports variations in the morphology of different types of haemocytes, each of which performs distinct roles in the immune response. DHC shows a higher proportion of prohaemocytes in the beetles. Prohemocytes are stem cells that have a high mitotic index and are capable of dividing into specific cell lines. Pathogenic and non-pathogenic agents are known inducers of haemocytic responses and variation in differential haemocyte counts [25]. The higher proportion of prohaemocytes acts as a reservoir for the formation of other types of cells in need. Plasmatocytes and adipohaemocytes are the second largest proportion of cells in the haemolymph of *O. rhinoceros* whereas in *P. sobrinus* it is oenocytes. This might be correlated with the conditions in which they live. Beetles live in damp environments and have to protect themselves from various infections and pathogens. The number of each type of haemocyte in the haemolymph also varies with the stages of development and the life cycle. Wheeler (1963) reported an increase in cystocytes in *Periplanata americana* before ecdysis and a decrease in number after ecdysis. Also the total haemocyte count of *Oryctes* larvae infected with *B. thuringiensis* decreased, but the number of granulocytes increased dramatically [9]. These observations substantiate the role of the identified haemocytes present in *P. sobrinus* and *O. rhinoceros* in immune responses and adaptations in insects in general. *P. sobrinus* is a bombardier beetle that prefers moist habitats and is commonly found under stones and logs. The conservation of bombardier beetles is of vital importance because they serve as natural biological control agents against many agricultural pests [26]. The present study revealed that six types of haemocytes contribute to the normal immunological responses of *P. sobrinus*, which are essential for its survival. But the haemocyte count are influenced by toxic substances such as heavy metals, micro- and nano-plastics, pesticides, hydrocarbons, and oil spills and are used as a tool for assessing the toxicity of the environment [27]. Therefore, the identification of haemocytes in *P. sobrinus* is significant for studying the adverse effects of pollutants on these cells and for developing effective conservation strategies for this beetle. Further studies investigating the effects of specific

pollutants on the haemocytes of *P. sobrinus* are needed, as such research could contribute to the conservation and maintenance of this beneficial insect within ecosystems. The identification of haemocyte types is the first and essential step in this process, and this objective has been accomplished in the present study. *O. rhinoceros* is a pest of coconut palms. Haemocytes are one of the targets of insecticides, impeding the immune response of pests. Organochlorines are reported to cause a decrease in the granulocytes and an increase in the prohemocytes and plasmatocytes [28]. The identification of haemocytes in *O. rhinoceros* is important for the development of novel insecticidal agents against this pest. Future studies should focus on the evolutionary processes of these cells, their diversity and the genetic basis underlying such variations. Targeting specific agents against these cells holds potential for advancing the study of immune responses, as well as improving insect rearing and control strategies; however, this approach requires more detailed investigation. Also, within our limited knowledge, this is the first report of the different types of haemocytes in the beetles, adult *P. sobrinus* and adult *O. rhinoceros*.

#### 4. Conclusion

Leishman stain could be used for the identification of haemocytes, which are the major components of the immune response in insects. They are also involved in intoxication processes induced by different toxic substances, enabling the survival of the insect. Six types of haemocytes were identified in the beetles, *P. sobrinus* and *O. rhinoceros*, which impart immune responses and other physiological mechanisms essential for their survival. A higher proportion of prohaemocytes might have significant roles in maintaining haemocyte count and differential count of cells as a mechanism of physiological adaptation. *P. sobrinus* is a biocontrol agent and *O. rhinoceros* is a pest. Further studies, including the isolation of haemocytes and their cytological studies, and responses to toxic chemicals in the environment or pesticides, are required to fully understand the immune mechanisms and their role in the developmental processes of the beetles and to develop conservation strategies or control measures.

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## Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

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## Author contributions

Bhavya M P and Pradeep Kumar R conceived and designed the experiments: Bhavya and Farhana J and Revathy A S performed the experiments: Jaya Chithra S K and Farhana analyzed the data: Bhavya and Pradeep wrote the paper: Jaya and Revathy edited the manuscript.

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