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Characterization of hemocyte types in *Bombyx mori* throughout pupa, larva, and adult stages by light microscopy

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Abstract. Hemocytes are specialized hemolymph cells found in insects, responsible for a variety of important physiological functions such as immunity, wound healing, and the regulation of the insect's circulatory system. The study of insect hemocytes has become increasingly important in recent years due to the emergence of insect-borne diseases and the need to develop new strategies for insect control. Understanding the biology of insect hemocytes has the potential to lead to the development of novel insecticides and strategies for controlling insect-borne diseases. This study aimed to examine the hemocytes in *Bombyx mori* to determine if there were any variations in the types of hemocytes to other arthropods. By comprehensively characterizing hemocyte types across developmental stages, this research sheds light on the intricate immune responses and adaptations of the silkworm. Such insights could contribute to the understanding of basic immunological mechanisms in insects. Hemolymph of *B. mori* was collected, and hemolymph smears were stained with Wright's stain. In *B. mori*, four distinct types of hemocyte prohemocyte, plasmatocyte, granulocyte, and oenocytoid were examined. Using light microscopy, the hemocyte types were identified based on their size, the presence or absence of granules, and the ratio of their nucleus to cytoplasm.

Keywords: hemolymph, insect, prohemocyte, plasmatocyte, granulocyte, oenocytoid.

Introduction

Insects are one of the most diverse and abundant groups of animals in the 3-billion-year history of life on Earth (Grimaldi et al. 2005). They are also some of the hardiest, capable of surviving in almost every habitat on the planet. One key reason for their success is their immune system, which helps them fend off a variety of diseases and pathogens (Sheehan et al. 2020).

Unlike the vertebrate immune system, which relies on antibodies and T-cells, the insect immune system is based on innate immunity (Müller et al. 2008). This means that insects don't have an adaptive immune system that can specifically recognize and respond to a particular pathogen. Instead, they have a range of physical, chemical, and cellular mechanisms that work together to protect them from infection (Owens 2019).

Silkworms are known for their ability to produce silk and their unique bodily fluids, including hemolymph. Hemolymph is crucial in the insect immune system, nutrient transport, and waste removal. Silkworm hemolymph is of particular interest to researchers because of its high protein content and potential uses in various industries, including medicine and cosmetics. The study of silkworm hemolymph has led to the discovery of antimicrobial peptides, immune-related proteins, and growth factors that have been used in the development of novel drugs and biotechnologies (Inagaki et al. 2012, Soumya et al. 2017, Yang et al. 2018). Insects, along with other animals and plants, have been utilized in traditional medicine in China for a significant period. Silkworms (scientifically known as *Bombyx mori* L.) were reportedly used for medicinal purposes dating back 3,000 years in ancient Chinese references. *B. mori* were incorporated into medical treatments (Zimian et al. 1997).

Understanding the insect immune system is important for understanding the biology of these fascinating creatures and for developing new strategies for controlling insect-borne diseases. The purpose of this study is to comprehensively examine and compare the diversity and morphological characteristics of hemocyte types present in *B. mori* during its pupa larva and adult stages using light microscopy, providing valuable insights into the hemocyte dynamics and potential immune responses during different developmental phases of this economically significant insect species.

Material and methods

Experimental setup and sample collection

This study was conducted at the Invertebrate Culture and Research Laboratory, Ege University, Türkiye, maintaining controlled environmental conditions (temperature: 25 ± 1°C; relative humidity: 70 - 85 %; photoperiod: natural). *B. mori* samples were obtained from the Ege University Silkworm Culture Laboratory. Hemolymph samples were collected from the thorax of each specimen using heparinized hematocrit capillary tubes. The hemolymph of each specimen is immediately used to prepare air-dried smears, subsequently stained with Wright's stain.

Hemocytes measurement

One hundred hemocytes were measured, and measurements were taken directly from the samples using a MOB1-15x micrometric ocular for each species. For spherical cells, the formula πr^2 was applied, while for oval cells, the formula $L.W.\pi/4$ was utilized, as outlined in previous research (Öztürk et al. 2018). Additionally, hemocyte types were counted using a Neubauer hemocytometer. The dilution solution used for counting hemocyte types was the standard Hayem solution.

Results

According to light microscopy analysis of the hemolymph in holometabolous *B. mori*, four distinct types of hemocytes (prohemocyte, plasmatocyte, granulocyte, and oenocytoid) were identified in the hemolymph of all developmental stages, including larva, pupa, and adult (Figure 1). The hemocyte numbers counted at different stages of *B. mori* are presented in Table 1. Although most hemocyte types were similar in number in pupal and adult stages, a decrease in spherical plasmatocyte type was observed (Table 1). Prohemocytes, the fundamental type of hemocyte, were smaller than other hemocytes (Table 2) and had a nucleus that occupied almost the entire cell, along with a thin cytoplasmic ring (Figure 1). In adults, their quantity was reduced when compared to that in larvae and pupae.

Plasmatocytes exhibited a polymorphous shape that could either be spherical or oval. In smear preparations, plasmatocytes that had elongated cytoplasmic extensions could be seen either individually or in groups. Spherical ones had a central nucleus that occupied most of the cell. In oval plasmatocytes, the nucleus could be oval or spherical. The plasma membrane showed irregular extensions of filopodia and pseudopodia. Granulocytes are spherical cells that vary in shape and size, and their basophilic granular cytoplasm helps identify them.

The oenocytoid exhibited a circular shape, and its nucleus was also circular, large, and located near the center (Figure 1).

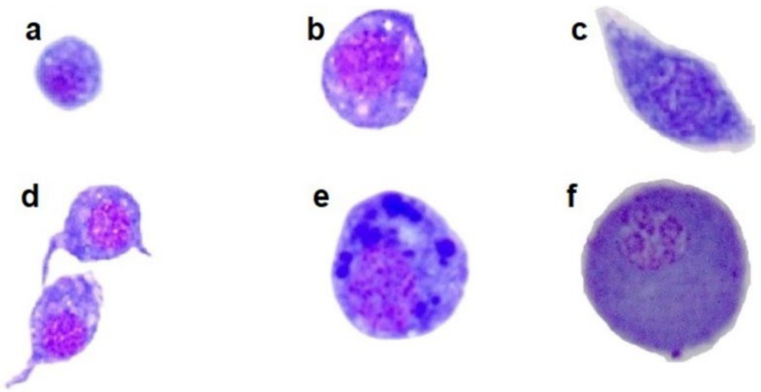

Figure 1. Micrographs of *B. mori* hemocytes a) prohemocyte at the larval stage b) spherical plasmatocyte at the pupal stage c) oval plasmatocyte at the adult stage d) plasmatocyte at the pupal stage, e) granulocyte of the adult animal f) oenocytoid at the pupal stage

In the larval stage of *B. mori*, it was generally observed that hemocytes were smaller in size. Additionally, a notable size difference was observed in spherical plasmatocytes between larvae and adults. A significant difference was detected between pupa and adult in terms of oval plasmatocyte size. When granulocytes were compared in terms of both mean diameter and area at different developmental stages, a remarkable difference was found (Table 2). According to the analysis of variance in granulocytes, the difference between developmental stages in terms of mean diameter was statistically significant. Significant differences were detected in the mean diameter and area of oenocytoids during various developmental stages, indicating a notable variation between larvae and adults (Table 2).

Discussion

Insects possess a variety of hemocyte types that play important roles in their immune system, like recognizing, engulfing, and killing pathogens. The most common types of insect hemocytes are plasmatocytes, granulocytes, oenocytoids, spherulocytes, adipohemocytes and coagulocytes (Kwon et al. 2014).

This study employed light microscopy to identify the composition of hemolymph in three stages (larvae, pupa, and adult) of *B. mori*. In holometabolous *B. mori*, four distinct hemolymph types (prohemocytes, plasmatocytes, granulocytes, and oenocytoids) were identified during different developmental stages such as larvae, pupae, and adults. According to Wu et al. (2016), four distinct types of hemocytes (plasmatocytes, granular cells, spherical cells, and oenocytoids) circulate in the hemolymph of *Galleria mellonella* larvae. Manfredini et al. (2008) discovered three hemocyte types, namely prohemocytes, plasmatocytes, and granulocytes, that circulate in the larvae of the European paper wasp, *Polistes dominulus*.

Plasmatocytes are hemocytes characterized by polymorph-shaped bodies with cytoplasmic extensions like filopodia and pseudopodia. These cells exhibit various shapes, including spherical, oval, and irregular forms. In *B. mori*, the average diameter of spherical plasmatocytes was measured as 11.78 ± 3.77 μm in larvae, 12.5 ± 1.42 μm in pupae, and 12.75 ± 1 μm in adults. For comparison, Kirdök et al. (2020) reported the diameter of spherical plasmatocytes as 13.93 ± 0.41 (L1), 13.07 ± 0.45 (L2), and 13.45 ± 0.45 μm (L3) in different larval stages of *Lucilia sericata*.

Table 1. Average number of hemocytes detected in 1 mm^3 hemolymph at different developmental stages of *Bombyx mori*

Hemocyte Types	Larva	Pupa	Adult
Prohemocyte	26 ± 0.86	27 ± 0.86	16 ± 0.79
Plasmatocyte (spherical)	34 ± 1.48	23 ± 0.84	23 ± 0.88
Plasmatocyte (oval)	16 ± 1.09	17 ± 0.89	17 ± 0.88
Granulocyte	26 ± 1.12	27 ± 0.96	37 ± 1.34
Oencytoid	8 ± 0.99	6 ± 0.70	7 ± 0.75

Table 2. Micrometric measurements of hemocytes in *Bombyx mori*. Values are given as mean ± standard deviation (SD). A indicates a small diameter of elliptical plasmatocytes, while B indicates a large diameter of elliptical plasmatocytes.

Hemocyte types	*Bombyx mori*					
	Larva		Pupa		Adult	
	Diameter (μm)	Area (μm^2)	Diameter (μm)	Area (μm^2)	Diameter (μm)	Area (μm^2)
Prohemocytes	7.25 ± 1.32	42.45 ± 15.38	7.66 ± 1.58	47.65 ± 19.09	9.17 ± 2.06	68.74 ± 31.94
Plasmatocytes (Spherical)	11.78 ± 3.77	118.98 ± 40.32	12.5 ± 1.42	124.02 ± 27.04	12.75 ± 1	128.27 ± 19.52
Plasmatocytes (Elliptical)	^{A}10.06 ± 2.24 ^{B}12.66 ± 2.2	100.97 ± 29.3	^{A} 8.2 ± 1.3 ^{B} 12.4 ± 2.07	81.48 ± 26.47	^{A}10.13 ± 0.6 ^{B}13.31 ± 1.43	106.27 ± 17.68
Granulocytes	11.06 ± 1.28	97.16 ± 21.98	12.97 ± 1.06	132.8 ± 21.72	14.61 ± 0.9	168.04 ± 20.54
Oenocytoid	18.29 ± 2.86	280.92 ± 88.7	17 ± 2.72	254.79 ± 76.12	17.13 ± 1.6	231.21 ± 42.78

Silva et al. (2002) found that spherical plasmatocytes in *A. obliqua* larvae had a diameter of 13 - 26 μm, while oval plasmatocytes were 26 - 34 μm in length and 15 - 30 μm in width. Plasmatocytes, similar to vertebrate macrophages, play a role in phagocytosis, eliminating apoptotic cells during development and encapsulating or digesting pathogens (Evans et al. 2003, Hartenstein 2006).

Plasmatocytes were the most abundant type of hemocytes in the hemolymph of *B. mori* across all developmental stages, following granulocytes. Similarly, some research reported that plasmatocytes were the most abundant type of hemocyte in many species and they were involved in phagocytosis, the process of engulfing and destroying pathogens (Giglio et al. 2008, Browne et al. 2013, Cinege et al. 2020). In addition, they play a role in various processes, such as removing cells that have undergone apoptosis during development, encapsulation, nodule formation, wound repair, and clot formation (Evans et al. 2003, Hartenstein 2006, Strand et al. 2006).

Spherical in shape granulocytes could be identified by their basophilic granular cytoplasm in *B. mori*. The average diameter of granulocytes was found to be 11.06 ± 1.28 µm in larvae, 12.97 ± 1.06 µm in pupae, and 14.61 ± 0.9 µm in adults. In a study conducted by Silva et al. (2002), it was observed that the diameter of granulocytes in larvae of *A. obliqua* was in the range of 15 - 22.25 µm. Kirdök et al. (2020) reported that granulocytes of *L. sericata* were measured at 17.47 ± 0.47 (L1), 22.28 ± 1.93 (L2), and 16.37 ± 1.75 (L3) µm in diameter. Granulocytes play a role in both growth and metabolic functions, as well as in immune-related activities such as wound healing, blood clotting, engulfing and destroying foreign particles, isolating pathogens, and shaping tissues during metamorphosis (Rheuben 1992, Murray et al. 1995, Kiger et al. 2001, Nardi et al. 2001, Hartenstein 2006). Phenoloxidase (PO) is a key enzyme that causes melanotic encapsulation of parasites in insects (Beerntsen et al. 2000). It was discovered that granulocytes, plasmatocytes, and oenocytoids exhibit PO activity in *G. mellonella* (Wu et al. 2016).

The present study determined that oenocytoids, spherical hemocytes, are the largest type of hemocyte found in all stages of development of *B. mori*. The average diameter of oenocytoids in *B. mori* was found to be 18.29 ± 2.86 µm in larvae, 17 ± 2.72 µm in pupae, and 17.13 ± 1.6 µm in adults. According to Silva et al. (2002), the size range of oenocytoids in *A. obliqua* larvae was observed to be between 22 and 35.5 µm in diameter. Kirdök et al. (2020) detected the presence of oenocytoids only in the L3 developmental stage of *L. sericata* larvae and measured their diameter as 8.92 ± 0.89 µm. Overall, the diameter of hemocytes in insects can be influenced by a range of factors, including developmental changes, immune responses, and physiological changes. Understanding these factors can provide insight into the role of hemocytes in insect immunity and health.

According to the literature data, the percentage of oenocytoids is lower than that of other hemocytes. In contrast to granulocytes, oenocytoids do not display phagocytic characteristics (Amaral et al. 2010, Ravaiano et al. 2018, Yelkovan et al. 2021). According to our microscopic observations, oenocytoids were found to be the least commonly occurring type of hemocyte. Oenocytoids, unlike granulocytes, do not exhibit phagocytic properties. Oenocytoids are involved in producing melanin that encapsulates pathogens (Kurihara et al. 1992, Flores-Villegas et al. 2015).

These hemocyte types undergo significant changes in their proportions during the larval developmental stages (Wu et al. 2016). The dominant type of hemocyte is granulocytes, as seen in *Pseudoplusia includens*, a species of moth (Strand et al. 2006). We also observed the same result in all developmental stages of *B. mori*, where granulocytes are the most frequently occurring type of hemocyte. Based on the literature data, insects are incredibly diverse and have evolved a range of strategies for dealing with immune challenges. Therefore, hemocytes can vary in terms of their size, shape, and function, and different insect species may have different combinations of these cell types. Additionally, the density of hemocytes in the insect's hemolymph can also vary depending on a range of factors, including the insect's life stage, the type of infection or immune challenge it is facing, and the specific hemocyte types involved. Overall, the complex interplay between different types of hemocytes and their densities is a fascinating area of research that continues to yield insights into the remarkable immune defenses of insects.

In conclusion, this study uses light microscopy to provide valuable insights into the diversity and morphological characteristics of hemocyte types in *B. mori* during its pupa larva and adult stages. The results offer a basis for further investigations into the intricate interactions between hemocyte types and their functional roles during the different life stages of this economically important insect species.

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