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Identification of haemocytes in sugar beet weevil, *Lixus incanescens* (Coleoptera: Curculionidae)

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Abstract: The role of blood cells as the main components of the physiological defense of insects has been reported. Blood cells are the first factors of the circulatory system that participate in dealing with all kinds of stress and foreign factors. In the present study, four types of blood cells were identified in the hemolymph of the sugar beet weevil *Lixus incanescens*, which include prohemocytes, granulocytes, plasmatocytes and oenocytoids. Plasmotocytes were the largest hemocytes in terms of size, and the abundance of granulocytes and plasmotocytes in third (70 %) and fourth instar larvae (more 75 %) was higher than other cells. These findings are important as the first step in immunological studies of this insect.

Keywords: Blood cell, Insect immunology, Beetle, Light microscopy, Defence

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Lixus incanescens Boh. (Coleoptera: Curculionidae) is one of the major sugar beet pests, which has spread in recent years to almost all beet-growing areas of Iran (Fathi & Abedi, 2014). Feeding this weevil on the petiole tissue leads to a decrease in root weight and sugar (Kheiri, 1990).

Insects are constantly exposed to biotic and abiotic stresses in their environment, including bacterial and fungal spores, toxic particles, and alterations in chemical and physical parameters. Therefore, their survival is closely related to their physiology and in particular, to an effective immune response (Duarte *et al*, 2020). The role of immunocompetent cells present in the haemolymph (haemocytes) and their morphological and functional changes during immunization has been widely demonstrated.

Haemocytes engage against invading agents by performing phagocytosis, nodulation, and capsule formation activities (Lavine & Strand, 2002). Thus, insects with strong immune systems can prevent the progression of infection in the haemolymph; therefore, it is important to identify insect haemocytes as key indicators of defensive responses.

To identify and investigate the blood cell populations in *L. incanescens*, infested sugar beet plants were collected from Miami fields (80 km from Shahrood, Iran) and transported to the laboratory where they were maintained under controlled conditions (temperature 25±1 ◦C, humidity 50%, and light: dark 14:10 h). The leaves infested with larvae were placed in breeding containers, the dimensions of which were 40×30×15 cm. The method of Gupta (1985) was used to identify blood cells in weevil larvae. First, the abdominal area was pierced with a fine sterile needle and the haemolymph was gently placed on a normal slide. The Giemsa solution was then placed on the haemolymph for about ten minutes to stain blood cells. After washing the solution from the slide surface, the blood cells were identified using valid keys (Gupta, 1985) and light microscopy at 40x magnification (Olympus, BH2). Then morphometric parameters of them were determined. In addition, Differential haemocyte count (DHC) was determined in the larval and pupal stages of the insect. To perform this experiment, haemolymph was first extracted from larval and pupal stages, placed on a normal slide, and stained with Giemsa. The slide was then placed under a light microscope and 100 blood cells were randomly selected and differentially counted. In this way, the abundance percentage of cell types was

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obtained. Thirty numbers from each larval and pupal stage were tested to determine the abundance percentage of blood cells (Yeager *et al*., 1945). All data obtained from a complete randomized design were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test when significant differences were found at $P \leq 0.05$ (Sas, 1997). Differences between samplings were considered statistically significant with a probability of less than 5 %.

Figure 1 shows the larval and pupal stages of *L. incanescens*. The haemocytes were determined in the haemolymph of the fourth instar larvae of this insect. The findings indicated that this weevil's haemolymph contained four major types of blood cells: prohemocyte, plasmatocyte, granulocyte, and oenocytoid. Spherulocytes were not present. Spherulocytes have occasionally been found to be absent in the haemolymph of some insects, despite the fact that reports claim that most insects have five different types of haemocytes in their blood such as *Melipona scutellaris* L (Hymenoptera: Apidae), *Pieris rapae* (Linnaeus) (Lepidoptera: Pieridae), *Sesamia cretica* Lederer (Lepidoptera: Noctuidae) and *Osphrantria coerulescense* Redt (Coleoptera: Cerambycidae) like *L. incanescense* (Amaral *et al*., 2010, Zhang. 2012, Sadeghi *et al*., 2017, Ajamhassani, 2019).

The morphological characteristics of the cells were consistent with observations of haemocytes reported by other researchers (Ebrahimi & Ajamhassani, 2020; Ajamhassani, 2021). Prohemocytes are the smallest cells (5±1.2 micrometers) with a large central nucleus (Fig. 2) (Table 1). Prohemocytes are undifferentiated cells that differentiate into other cell types during immune activity, so their numbers are likely to decrease as a result of the immune challenge (Ling *et al*, 2005). Prohemocyte density in the haemolymph of the first (36.5±4.7 %) and second larval instar (38.6±3.6 %) was higher than the other larval stages ($F= 56.4$, $df=29$, $p\leq0.001$) The granulocytes were larger than the prohemocytes and the cytoplasmic surface of these cells was filled with granules.

Plasmatocytes have various sizes and polymorphous profiles. In some cases, the largest cells were observed with one or two cytoplasmic cells (Fig. 2) (Table 1). Granulocytes and plasmatocytes are the most involved in some immune processes, these cells are activated after recognizing the invaders by lectins-like molecules. Granulocytes and plasmatocytes change their morphology when activated; in some cases, they swell and many become irregular in shape due to ruptures in the plasma membrane through which cell contents leak (Moushumi *et al.* 2008).

In the third and fourth instar larvae, the abundance of granulocytes and plasmatocytes was higher than in the other cell (Table 2). It has been shown that the presence of immunocytes is greater in the larvae of late-stage insects, which have a larger size and higher nutritional level (Strand, 2008). Oenocytoids, observed in larval haemolymph of *L. incanescens,* are round cells with nuclei located in the peripheral area (Fig. 2). Population of oenocytoids was the highest in pupa haemolymph (23.7±1 %.)

Granulocyte and plasmatocyte counts in pupal hemolymph decreased, resulting in respective frequencies of 20.6±4.6 and 19.5±5.5 % for plasmatocytes and granulocytes. Instead, the number of prohemocytes as basal cells increased (35.7±5.6 %). Due to some physiological activities during the pupal stage, such as the cessation of feeding, the decrease in the number of plasmatocytes and granulocytes may be associated with a decrease in immunity during this biological stage.

Identifying blood cells is the first step in immunological studies. This study is performed for the first time on *L. incanescens* and can therefore provide a basis for further research focusing on the cellular immune processes triggered by this insect in the presence of stress and infectious events.

Table 1. Morphometric size of haemocytes of fourth instar larvae of *L. incanescens* (n=20)

Haemocytes	Size (µm)	
	Length (mean±se)	Width (mean±se)
Prohemocytes	5±1.2	4.5±1.5
Plasmotocytes	22.4±8.2	4.4±2
Granulocytes	6.7±2.3	5.5±2.1
Oenocytoids	5.4±1.3	5±1.1

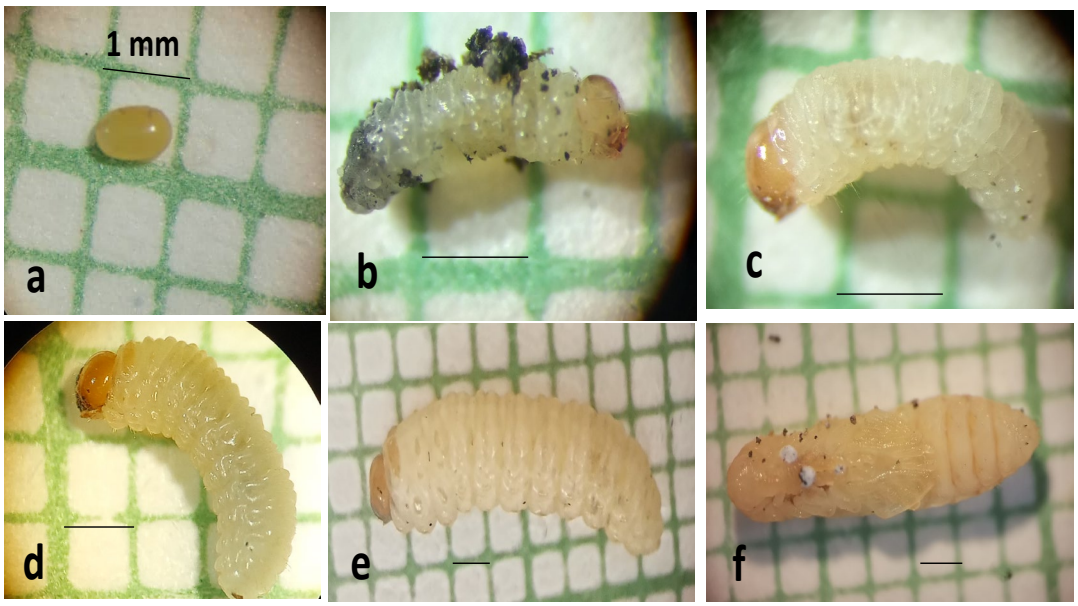

Fig 1. Life stages of *Lixus incanescens.* a, egg; b, first instar larva; c, second instar larva; d, third instar larvae; e, fourth instar larva; f, exarate pupa (Original).

Table 2. Percentage of haemocytes in larval and pupal stages of *L. incanescens* (n=30)

Developmental stages	Frequency of haemocyte (%)			
	Prohemocytes	Plasmotocytes	Granulocytes	Oenocytoids
First instar larva	36.5±4.7^{a}	21.5±3.2^{b}	27±1.5^{b}	17.4±0.5^{b}
Second instar larva	38.6±3.6^{a}	20.4±4.2^{b}	25.4±3.2^{b}	14±4.7^{bc}
Third instar larva	17.8±1.3^{b}	35.7±3.3^{ab}	35±5.3^{a}	11.5±2.7^{bc}
Fourth instar larva	9±0.55^{c}	41.5±3.2^{a}	38.8±4.2^{a}	8.8±2.43^{c}
Pupa	35.7±5.6^{a}	20.6±4.6^{b}	19.5±5.5^{c}	23.7±1^{a}

Different letters in each column show statistical differences among larval instars and pupa (Tukey's test, $p \leq 0.05$).

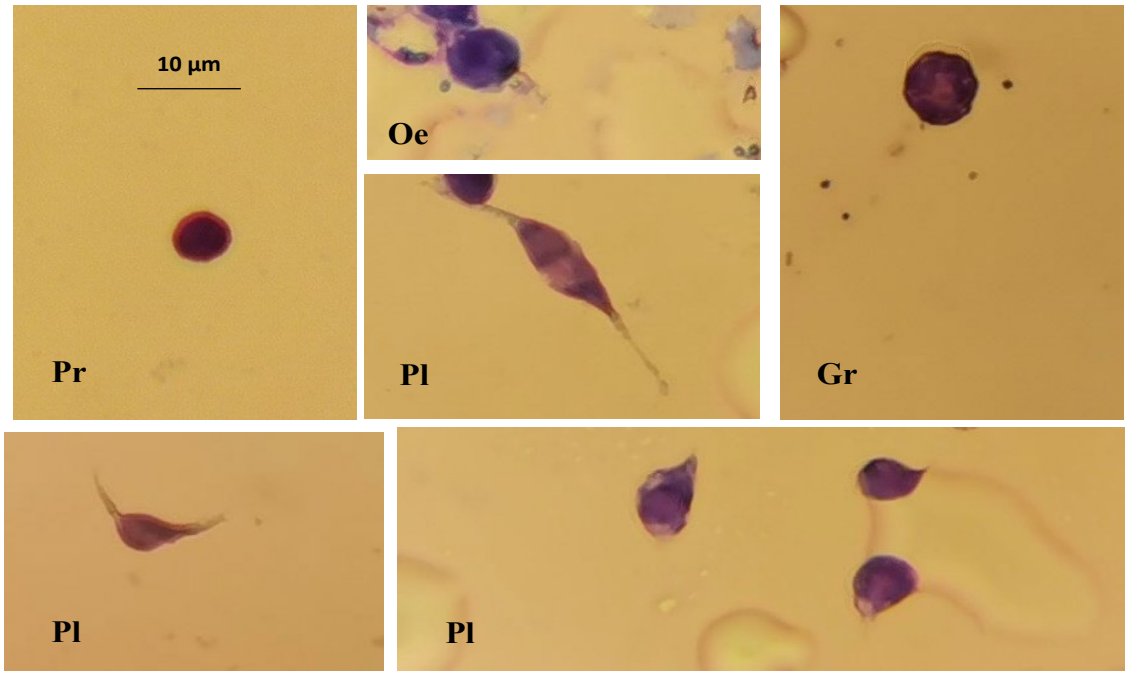

Fig 2. Morphology of haemocyte types of *Lixus incanescens* larvae by Giemsa staining under light microscopy (40x magnification). Pr=Prohemocytes, Pl=Plasmotocytes, Gr=Granulocytes, Oe=Oenocytoids.

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شناسایی سلولهای خونی خرطوم بلند دمبرگ چغندر قند *Lixus incanescens* (Coleoptera: Curculionidae)

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چکیده

نقش سلولهای خونی به عنوان اجزای اصلی دفاع فیزیولوژیک حشرات مهم گزارش شده است. سلولهای خونی، اولین فاکتورهای سیستم گردش خون هستند که در مقابله با انواع تنشها و عوامل بیگانه مشارکت می کنند. در تحقیق حاضر، چهار نوع هموسیت در همولنف لارو سن چهارم خرطوم بلند دمبرگ چغندرقند، *Lixus incanescens* (Coleoptera: Curculionidae) شناسایی شد که شامل پروهموسیتها، گرانولوسیتها، پلاسموتوسیتها و انوسیتوئیدها می‌باشد. پلاسموتوسیتها، بزرگترین سلولهای خونی از نظر اندازه بودند و مجموع فراوانی گرانولوسیتها و پلاسموتوسیتها در لاروهای سنین سوم (حدود ۷۰٪) و چهارم (بیش از ۷۵٪) بالاتر از سایر سلولها بود. این یافته ها، به عنوان اولین گام در مطالعات ایمنی شناسی این حشره، دارای اهمیت می باشد.

کلمات کلیدی: خرطوم بلند دمبرگ چغندرقند،ایمنی شناسی حشرات، میکروسکوپ نوری، هموسیت، دفاع

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