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# Different pistachio cultivars impair hemocyte frequencies in diapausing and nondiapausing larvae of pistachio seed chalcid, *Megastigmus pistaciae* (Hymenoptera: Torymidae)

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**Abstract.** Cellular immunity with the activity of hemocytes is one of the important phases of innate immunity in insects. The role of hemocytes is key in dealing with the stresses and contaminations introduced to the insect hemolymph. These cells react by changing their number, shape, and type during stress. Identifying hemocytes and their diversity is the first step in immunological studies. Humoral defense usually starts with a little delay after cellular immunity, which is associated with the activity of phenol oxidase and antimicrobial peptides. The present study was conducted to investigate the immunology of diapausing and non-diapausing larvae of *Megastigmus pistaciae* fed on different varieties of pistachio. The pistachio seed chalcid, *M. pistaciae*, has been one of the key and destructive pests of pistachio in recent years. After collecting infected fruits and transferring them to the laboratory, hemolymph of larvae was extracted and hemocytes were identified after staining with Giemsa solution. Four types of hemocytes were seen in the hemolymph of larvae: prohemocytes, plasmatocytes, granulocytes and oenocytoids. The morphometric sizes of the cells were determined by light microscopy. Differential hemocyte count in hemolymph of larvae, pupae, and adult was determined. The frequency of granulocytes in the larvae was the highest (more than 35±2.4%) and higher than the frequency of granulocytes in pupa and adult. In the study of changes of blood cells in diapausing and non-diapausing larvae feeding on different cultivars, results showed that the hemocyte density of non-diapause larvae that fed on all cultivars was significantly higher than the number of diapause larval hemocytes. In addition, the total hemocyte count, granulocytes, oenocytoids, and prohemocytes in larvae which fed on Kaleghochi and Shapasand cultivars was higher than similar cases on larvae fed on Khanjari, Akbari and Nokhodi cultivars. Changes in phenol oxidase activity in different treatments were similar to changes in blood cells. On the other hand, the activity of this enzyme was higher in non-diapausing larvae than diapausing larvae and more than on larvae that fed on Kaleghochi and Shapasand cultivars as compared with other cultivars. Identification of hemocytes and their changes in diapausing and nondiapausing of pistachio seed chalcid, *M. pistaciae*, by feeding on different pistachio cultivars, is done for the first time and could be the basis for further studies to identify the immunological characteristics of this pest. It seems that diapause and type of the diet can determine the immune response of the insects against the entomopathogens. Additional research in this field can be effective in the microbial control methods of this pest.

**Keywords:** Insects immunity; cellular defense; humoral defense; feeding; diapause; blood cell

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## Introduction

Hemocytes are the first components of insect immune responses to stresses and pathogens, and they function through changes in shape, number, type, phagocytosis, and nodulation. This cell defense is the first step for the

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participation of antimicrobial peptides, phenol oxidases, and lysozymes, and in fact, humoral immunity is usually activated with a relative delay, leading to melanization and excretion of pathogenic agents (Lavin & Strand, 2002; Wodja *et al*., 2008). Numerous factors, such as temperature, diapause, age, gender, and diet, have been reported to affect immune responses (Siva-jothy & Tompson, 2002; Duarte, 2020). Food is important for growth, reproduction, oviposition rate, and immune system activity (Vengateswari *et al*., 2020).

Insects feed on various plant hosts, and the quality of the food varies from host to host. The protein and carbohydrate content and quality of food have a significant impact on the physiological characteristics of insects (Mason *et al*., 2014; Vogelweith *et al*., 2016). If insects were to benefit from rich food sources, they would exhibit their defense response more successfully, and if their diet is inadequate or they remain hungry, the number of hemocytes involved in immunity and phenoloxidase activity decreases, and the insect becomes more susceptible to the pathogenic agent (Manjula *et al*., 2020).

An immunological study of *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae) larvae fed on chickpeas and raisins, walnuts, pistachios, and artificial foods revealed that total hemocytes, prohemocytes, plasmatocytes, and granulocytes increased in groups fed on artificial foods compared to larvae fed on other treatments. (Ebrahimi & Ajamhassani, 2020). There were also significant changes in hemocyte count and activity of digestive enzymes of *Ephestia kuehniella* zeller (Lepidoptera: Pyralidae) that were fed with a diet containing ascorbic acid (Ajamhassani & Amirijami, 2019). In this regard, Gosh *et al* (2018) compared the effect of natural and artificial diets on the hemocytes of the larvae of *Hyposidra* sp. (Lepidoptera: Geometridae) butterfly. They found that the total hemocyte count was much higher in the above larvae than in the larvae that were fed with natural food (tea) (Gosh *et al*., 2018).

Diet also had a significant impact on the blood components of the greater wax moth larvae, *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae). It has been proven that the hemolymph volume and the total number of hemocytes of the larvae of this insect are higher when they are fed with artificial food containing soy than the larvae that are fed with natural food (beeswax) (Mohamad & Amro, 2022).

In addition to diet, environmental and temperature changes are also factors influencing insect immune responses. Temperature plays a vital role in the growth and homeostasis of insects, and temperature changes lead to a drastic change in hemocyte shape and count. The immune response of insects usually changes with the change in environmental conditions and the onset of cold, which coincides with the beginning of the diapause stage. A drop in the ambient temperature in winter naturally leads to a decrease in larval blood volume (Sinclair *et al*., 2013). As the temperature decreases, sodium (Na+) ions enter the intestinal lumen from the hemolymph with a gradual slope. As sodium ions continue to flow, water moves from the hemolymph into the gut, resulting in a decrease in the concentration of potassium ions (Alvarado *et al*., 2015).

Hemocytes are the main components of insect hemolymph, and their density changes as the volume of the hemolymph changes, affecting the immune responses of diapausing insects (Nappi & Silvers, 1984). The surface area of cells under cold stress was lower than that of control larval cells because the number of circulating hemocytes and phagocytes in *Gromphadorhina coquereliana*, which tolerated 4°C, decreased significantly; in other words, the size of the cells was smaller under cold stress (Lubawy & Stocinska, 2020).

Chalcidoidea is the most diverse superfamily of the Hymenoptera orders. Many species of this superfamily have been identified as insect parasites and thus have a diverse host range. However, some Torymidae and Eurytomidae species, such as pistachio seed chalcid, *Megastigmus pistaciae*, and the pistachio seed wasp, *Eurytoma plotnikovi*, have a vegetarian diet as larvae and live inside the pistachio fruit. *M. pistaciae* has two generations per year and, along with the pistachio seed wasp, causes irreparable damage to this product every year. This insect spends the winter in full larval form in pistachios left on the tree or under trees during diapause (Karadag *et al*., 2011). Infected pistachios have dark and wrinkled skin. The first-generation adult wasps usually emerge in mid-spring. At this time, the physical growth of the pistachio is complete, but the pistachio kernel is not complete. Adult females lay their eggs in the pistachio kernel after mating, and the larvae feed on the fruit kernel after hatching but leave the seed shell intact (Rice & Michailides, 1988). The larval period of the summer generation is shorter and the larvae of this generation are attacked by parasitoids more frequently. In fact, these are the larvae that gradually become inactive with the onset of cold and begin diapause inside the fruit, and remain inside the fruit until mid-May of the following year.

So far, the physiological properties of blood circulation and the immune system of the pistachio seed wasp have not been reported in the literature. On the other hand, an important first step in immunological studies of *M. pistaciae* is to study the status of hemocytes under diapause and environmental conditions as well as diets. Therefore, the aim of the present study was to identify hemocytes and compare their changes in diapausing larvae and in larvae fed with common pistachio cultivars.

## Materials and methods

### Insect collection

Wasps were sampled from infected pistachio orchards (including Khanjari, Kaleghochi, Akbari, Nokhodi and Shapasand varieties) in Semnan province in mid-December 2020 and early September 2021. Mean ambient temperature in winter and summer sampling was 3 ± 1 °C and 27 ± 1 °C, respectively. Infested and wrinkled pistachios were transferred to the Plant pathology Laboratory of Bastam College of Agriculture and were placed in 20 × 20 × 20 cm rearing containers. These fruits were considered for diapausing and nondiapausing larvae removal and immunological tests.

The late instar larvae were pulled out from the pistachios using forceps. Since pistachio seed wasp larvae were also present in infested pistachios and the larvae of both species were creamy white, curved, about 6-7 mm long, and had no legs, first it was necessary to separate and identify them. In order to separate *M. pistaciae* larvae from pistachio seed wasp, the mandibles were examined. The brown mandibles of pistachio seed wasp larvae have two distinct teeth, but *M. pistaciae* larvae have four or more distinct teeth on their mandibles (Basirat & Seyedoleslami, 2000). Therefore, pistachio seed wasps were separated after identification of their larvae. The laboratory storage conditions of infested summer sampled pistachios included temperature: 27 ± 1 ° C, relative humidity: 40%, and light: dark ratio, 10:14 hours. Infested pistachios obtained from winter sampling were transferred to the laboratory and stored in a refrigerator at 3 ± 1° C until testing.

### Hemocyte identification

The method of Gupta (1985) was used to identify blood cells in wasp larvae. First, the abdominal area was pierced with a fine sterile needle and the hemolymph was gently placed on a clean glass slide. The Giemsa solution (1:9) was then placed on the hemolymph for about ten minutes to stain the blood cells. After washing the solution from the slide surface, the blood cells were identified using valid keys (Gupta, 1985; Jones, 1962), and the morphometric sizes of the cells were determined using BH2 light microscopy at 40x magnification.

### Differential hemocyte count (DHC) in the late nondiapausing larvae, pupae, and adults of *M. pistaciae*

For DHC calculations, larvae fed on Khanjari variety (as the most common pistachio variety in Semnan) were used. Infected fruits were collected in summer and grown in the laboratory. Fruits were kept until the larvae completed their development period and turned into pupa and matured. Then, differential hemocyte count of larvae, pupae and adults was calculated.

At first, using a sterile needle, the abdominal region was slightly opened and hemolymph was collected using a micropipette and placed on the slide. Giemsa solution (1:9) was used to stain the cells. Then Giemsa was washed and lithium carbonate was used as a fixative. The slide was then placed under a light microscope (BH2) and 100 blood cells were randomly selected with magnification 40 and differentially counted. In this way, the abundance percentage of cell types (DHC) was obtained. Twenty numbers from each biological stage were tested to determine the abundance percentage of blood cells (Yeager *et al.*, 1945).

### Total hemocyte count, plasmatocytes, granulocytes, oenocytoids, and prohemocytes count in diapausing and non-diapausing larvae fed by different pistachio cultivars

To perform this test, diapausing and non-diapausing larvae were used in Khanjari, Kaleghochi, Akbari, Nokhodi and Shapasand varieties. The late larvae were pulled out from the pistachios using forceps. To count total blood cells, plasmatocytes, granulocytes, oenocytoids, and prohemocytes, the hemolymph of three intact wasp larvae (approximately 1 µl) was collected through a capillary tube and mixed with 10 µl of Tyson anticoagulant solution.

A slide is used to keep the blood and Tyson constant on the Neubauer slide (hemacytometer). The Jones formula and light microscopy at 40× magnification were used to count cells (Jones *et al*., 1967).

$$\frac{\text{Hemocyte in} \times 1\ \text{mm}^2 \times \text{Dilution} \times \text{Depth factor of chamber}}{\text{No. of squares counted}}$$

Dilution= 10 times
Depth factor of the chamber = 10
No. of squares counted = 5
This experiment consisted of ten treatments (diapausing and non-diapausing larvae on five cultivars of Akbari, Kaleghochi, Shapasand, Khanjari, and Nokhodi) and each treatment included five replications.

### Phenoloxidase activity

Hemocyte lysate method was used to determine phenoloxidase activity in diapausing and non- diapausing larvae of five pistachio cultivars (Leonard *et al.*, 1985). In this method, twenty wasp larvae were collected for hemolymph for each treatment and centrifuged at 10000 rpm for 2 minutes. After removing the supernatant, 100 l of phosphate buffer (pH=7) was added to the sediments and homogenized.

The latter solution was centrifuged again at 12,000 rpm for 10 minutes, and the resulting supernatant was used in enzymatic estimates. For this purpose, 20 µl of the samples were added to 20 µl of 10 mM (L-DOPA) L-dihydroxyphenylalanine solution and 20 µl of phosphate buffer. The mixture was incubated for two minutes at 30 °C and the wavelength was read at 490 nm by ELX800 ELISA reader (BioTek, USA).

### Protein determination emory

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, USA) as standard.

### Statistical analysis

All data obtained from a complete randomized design were compared by one-way analysis of variance (ANOVA) followed by Tukey's test when significant differences were found at $P \leq 0.05$ (SAS, 9.4). Differences between samplings (n = 3) were considered statistically significant at a probability less than 5 % and marked in figures and tables.

## Results

### Identification of hemocytes in *M. pistaciae* larvae and determination of their abundance percentage in larval, pupal, and adult stages

Four types of hemocytes including Prohemocytes, granulocytes, plasmatocytes, and oenocytoids have been observed in the hemolymph of *M. pistaciae* larvae. Prohemocytes are small, round cells with a distinct nucleus in the center. In Giemsa staining, the nucleus is more pigmented than the cytoplasm and occupies a large portion of the cell (Fig. 1 and Table 1). Prohemocyte abundance is less than 15±2% in the larval stage (late instar larvae) but increases to about 28±2.5% in the pupal and adult stages' hemolymph (Fig. 2).

Plasmatocytes have polymorphic profiles in most insect hemolymph, but an eye-like shape in *M. pistaciae* larvae. The nucleus of these cells is central and distinct. The cytoplasm is stained pink and contains small granules. These cells vary in size (Fig. 1 and Table 1) and have a density higher than 31±1% in larval hemolymph. However, the number of plasmatocytes in pupal and adult hemolymph decreases (Fig. 2).

Granulocytes are found in medium to large sizes and are the largest cells in some cases. The surface of these cells is full of granules that are involved in phagocytic activities (Fig. 1 and Table 1). These cells have the highest abundance in the wasp larvae (37±2%). Together with plasmatocytes, they make up the highest number of cells in the hemolymph of wasps. The frequency of granulocytes was 27±1% and 25±3.2 % in pupa and adults, respectively, which is significantly lower than in larvae.

Oenocytoids were identified as egg-shaped cells with slightly larger lateral nuclei than prohemocytes (Fig. 1 and Table 1). The abundance percentage of cells in the larval stage is 15±2% but gradually increases as the insect continues to grow (Fig. 2). Prohemocytes were the least abundant in wasp larvae (15±1.5%) compared to other cells. It seems that the mitotic divisions of prohemocytes in the larval stage are high and have resulted in the increase of immunocytes. (Fig. 2).

**Table 1**. Morphometric measurements of hemocytes in nondiapausing larvae of *Megastigmus pistaciae*. (n=20). (Different letters in each column show significance using Tukey's test at $p < 0.05$)

| hemocyte type | Size (µm) | |
|---|---|---|
| | length (mean±se) | Width (mean±se) |
| Prohemocyte | 2.4±0.3c | 2.2±0.2cd |
| Plasmatocyte | 6.5±2.5a | 3.6±0.7b |
| Granulocyte | 6±2.2a | 5±2.1a |
| Oenocytoid | 3±0.3b | 2.7±0.4c |

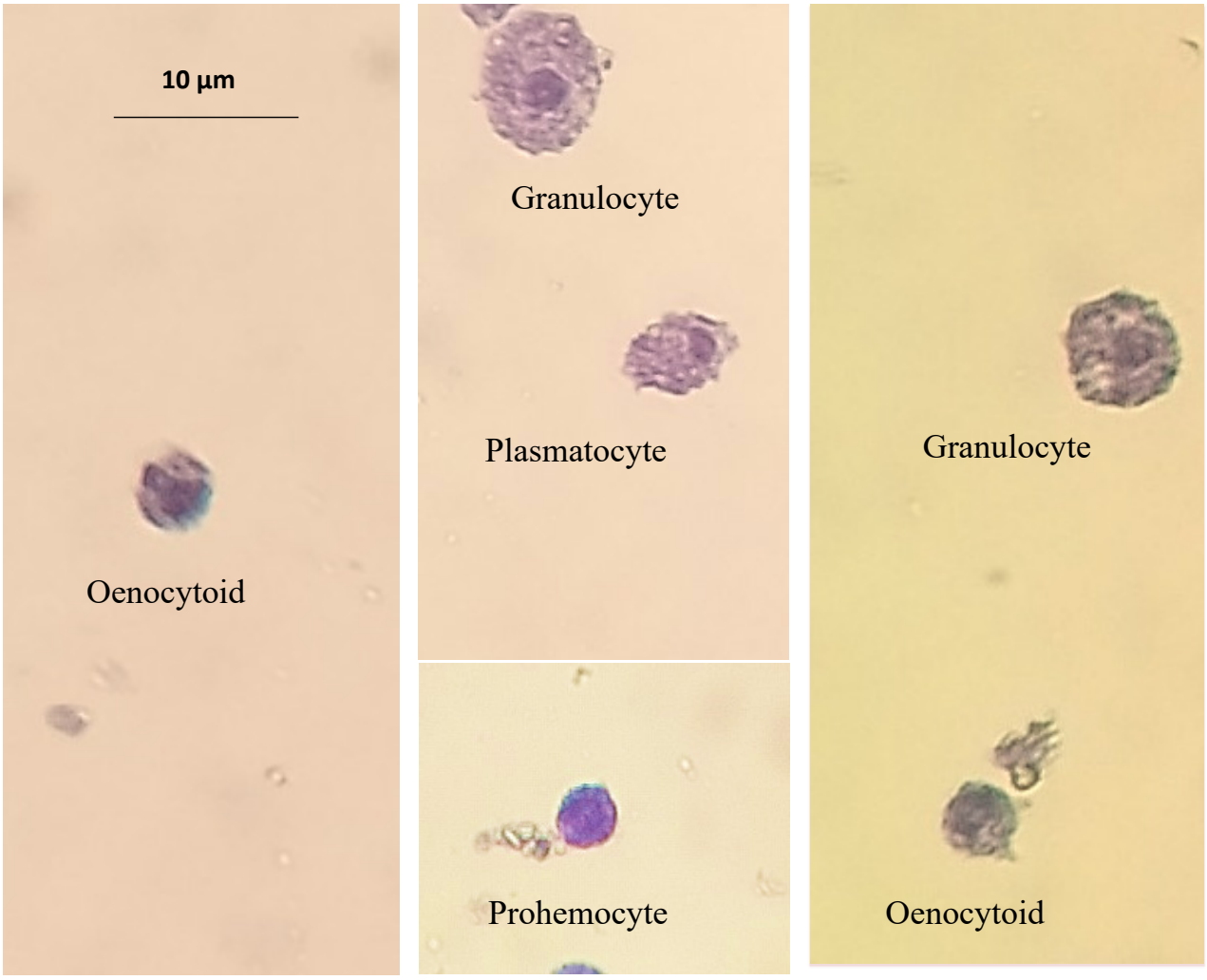


**Fig. 1**. Light microscopy pictures of *Megastigmus pistaciae* hemocytes stained with Giemsa. PR (Prohemocyte), PL (Plasmatocyte), OE (Oneocytoid), GR (Granulocyte). Scale bar = 10 µm

## Total hemocyte count, plasmatocytes, granulocytes, oenocytoids, and prohemocytes count of diapausing and non-diapausing larvae fed by different cultivars

There were significant changes in the number of hemocytes of diapausing and non-diapausing larvae of *M. pistaciae* that were fed with Akbari, Kaleghochi, Khanjari, Shapasand, and Nokhodi cultivars. The results showed that the

total hemocyte count (F = 155.6, $df_{t,e}$ = 9.30, p≤0.0001), granulocytes (F = 104.5, $df_{t,e}$ = 9,30, p≤0.0001), plasmatocytes (F = 107, dft, e = 9,30, p≤0.0001), oenocytoids (F=58.5 $df_{t,e}$ = 9,30, p≤0.0001), and prohemocytes (F = 123, $df_{t,e}$ = 9,30, p≤0.0001) of larvae that were fed with all cultivars of pistachio kernels in summer were significantly higher than diapausing larvae. The total hemocyte count in Kaleghochi and Shapasand-fed larvae (4375 ± 135.5 and 4408 ± 142.6 in $mm^3$ of blood, respectively) was significantly higher than that of larvae that were fed with Nokhodi, Akbari, and Khanjari cultivars (Fig. 3).

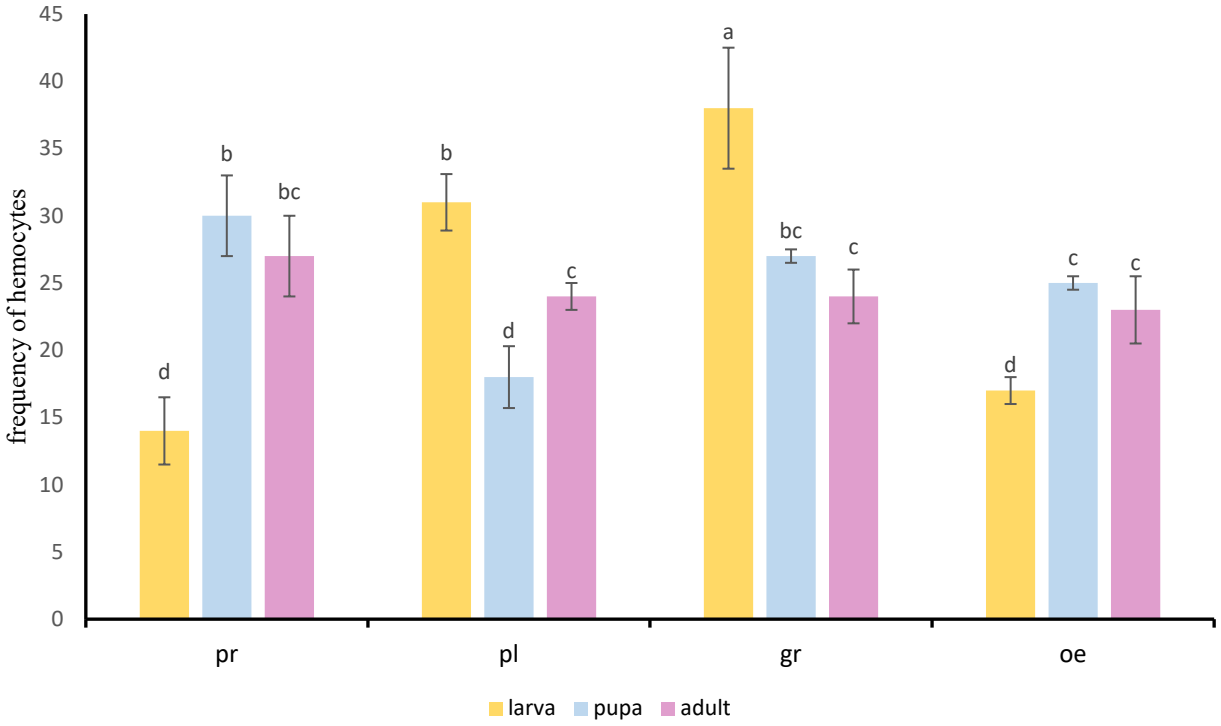


**Fig. 2**. Frequency of hemocytes in nondiapausing larvae, pupa and adults of *Megastigmus pistaciae*.

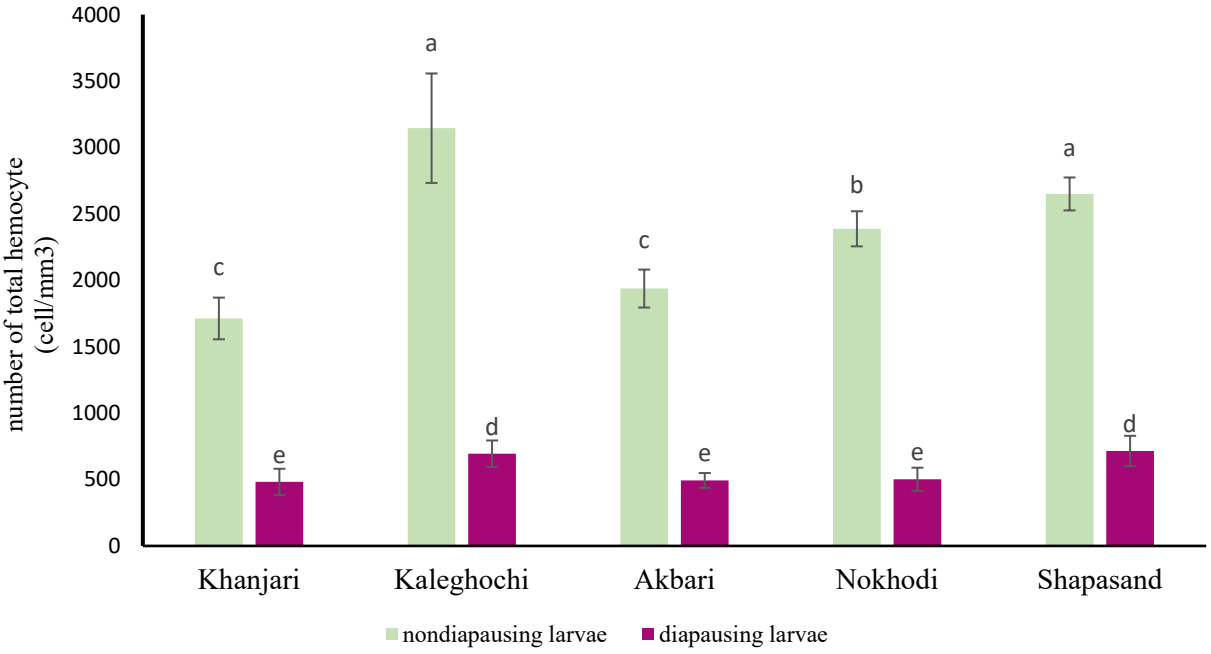


**Fig. 3**. Total hemocyte count of diapausing and nondiapausing larvae of *Megastigmus pistaciae* feeding on different pistachio cultivars. (Different letters show significance using Tukey's test at p < 0.05)

Furthermore, granulocyte count was higher in Kaleghochi and Shapasand-fed larvae (3145 ± 112.4 and 2650.5 ± 155.2 in mm 3 of blood, respectively) than in larvae fed other pistachio cultivars (Fig. 4). The number of plasmatocytes in larvae fed on cultivar Shapasand kernels (760 ± 40 in mm 3 of blood) was greater than in other experimental treatments (Fig. 5). Similar results were observed with respect to the number of oenocytoids and prohemocytes of larvae fed with Kaleghochi and Shapasand cultivars, indicating a significant increase in these cells in larvae fed on these two cultivars (Fig. 6 and 7).

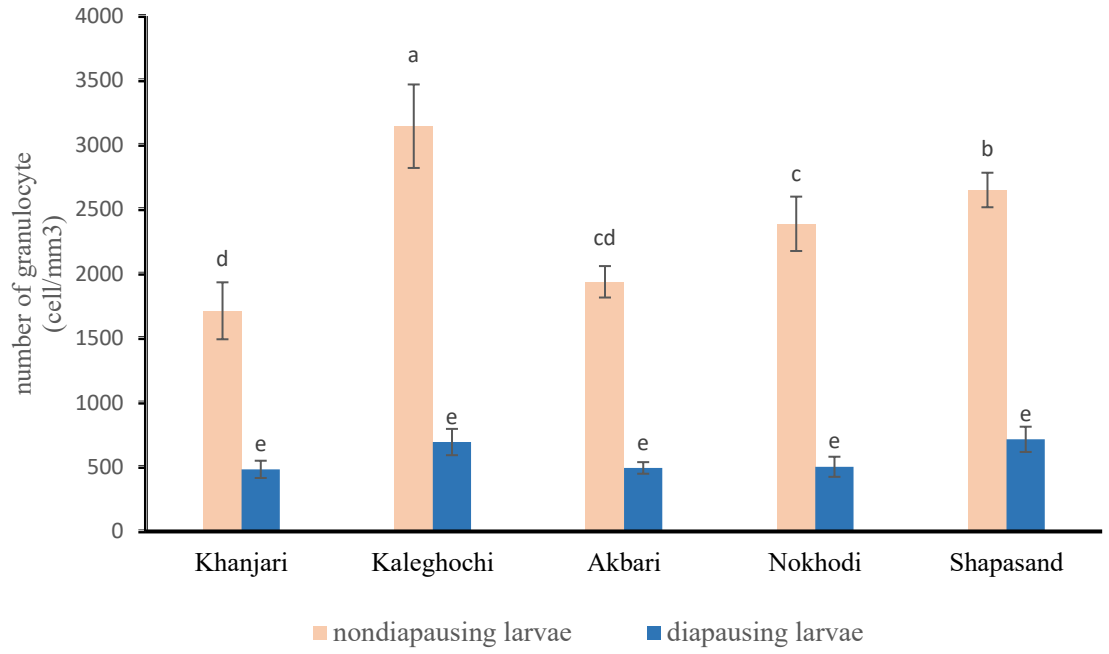


**Fig. 4**. Number of granulocytes of diapausing and nondiapausing larvae of *Megastigmus pistaciae* feeding on different pistachio cultivars (Different letters show significance using Tukey's test at $p < 0.05$).

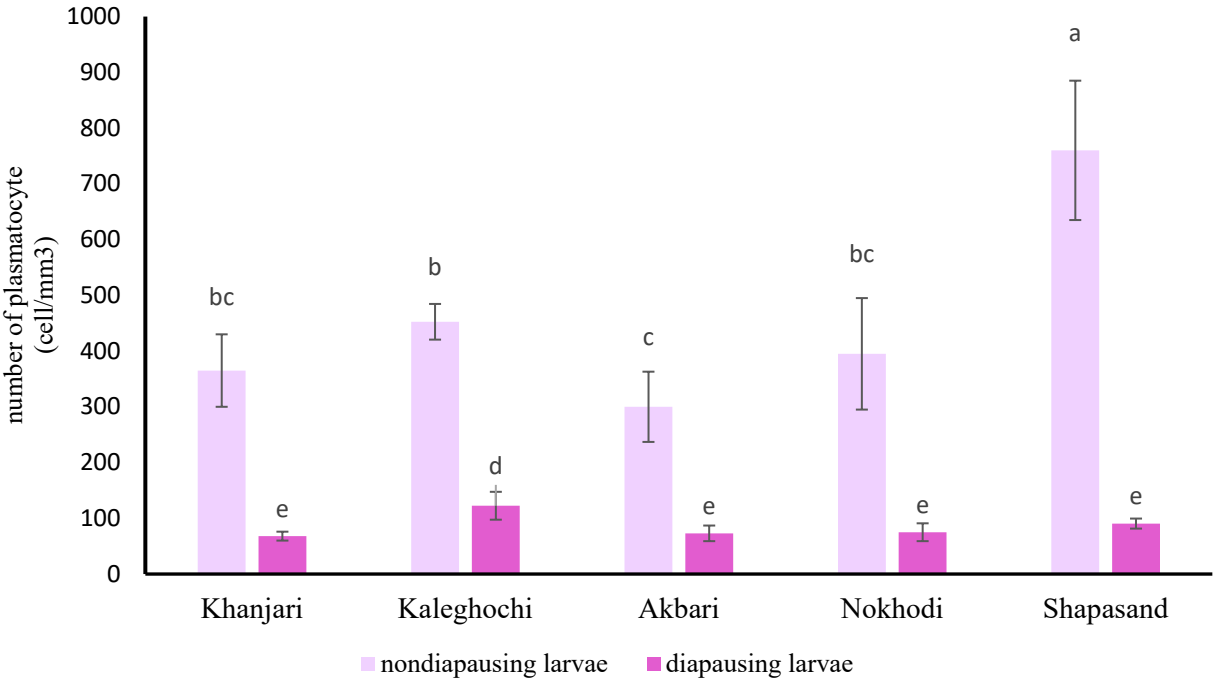


**Fig. 5**. Number of plasmatocytes of diapausing and nondiapausing larvae of *Megastigmus pistaciae* feeding on different pistachio cultivars. (Different letters show significance using Tukey's test at $p < 0.05$).

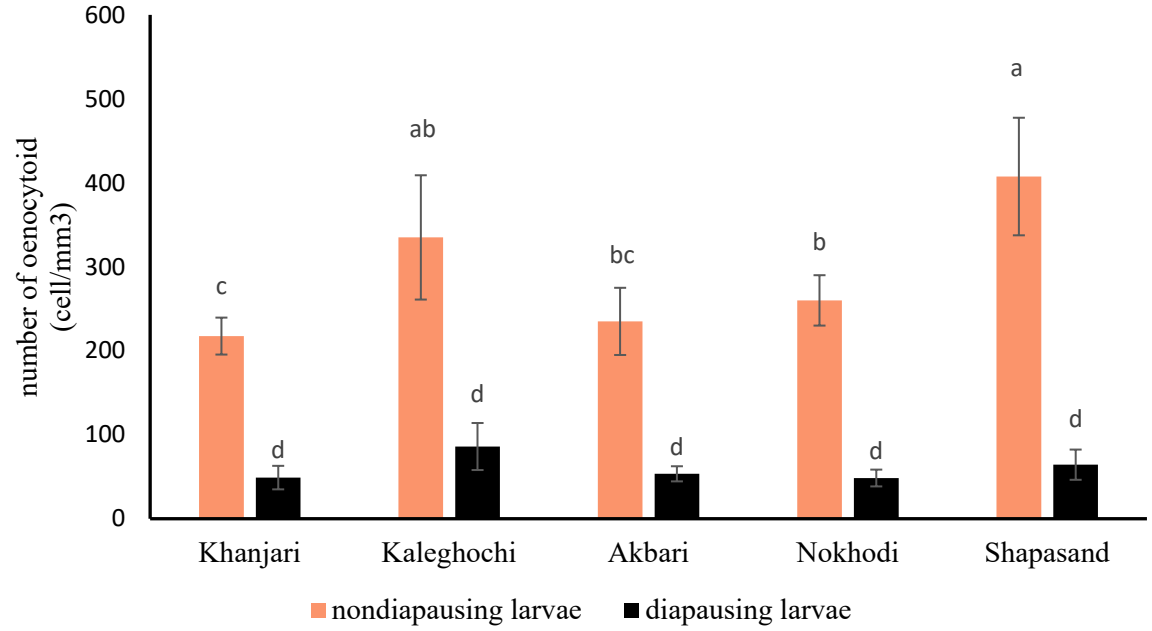


**Fig 6**. Number of oenocytoids of diapausing and nondiapausing larvae of *Megastigmus pistaciae* feeding on different pistachio cultivars. (Different letters show significance using Tukey's test at $p < 0.05$)

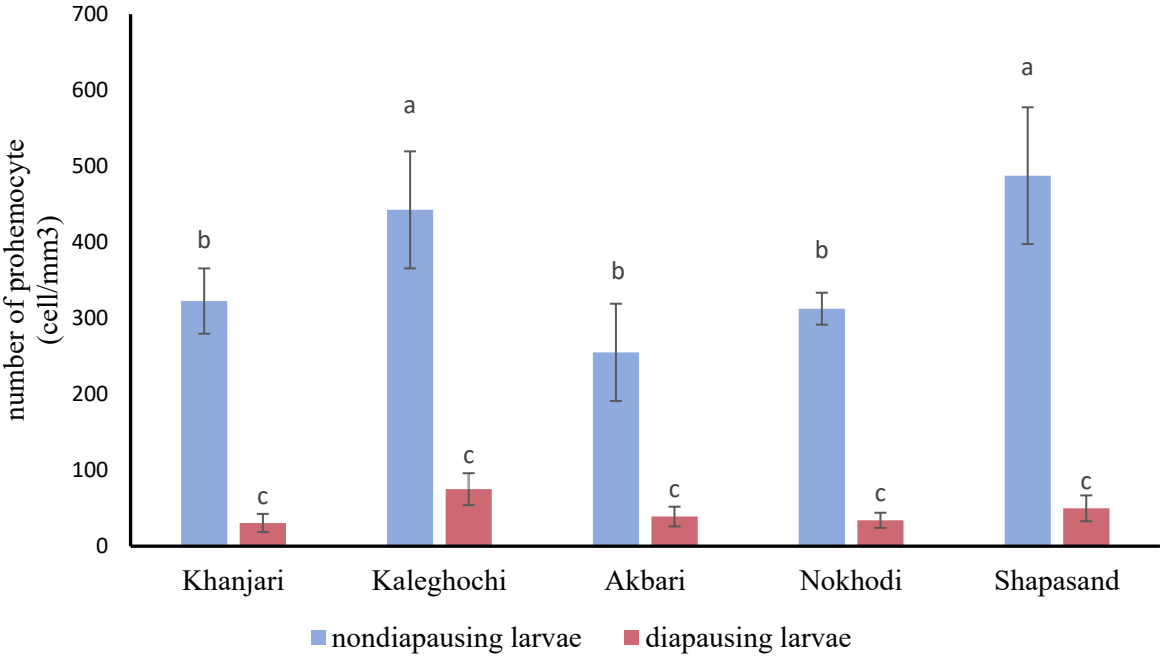


**Fig 7**. Number of prohemocytes of diapausing and nondiapausing larvae of *Megastigmus pistaciae* feeding on different pistachio cultivars. (Different letters show significance using Tukey's test at $p < 0.05$)

## Phenoloxidase activity

This experiment also revealed an increase in phenoloxidase activity in larvae fed on different pistachio cultivars versus diapausing ones ($F = 92$, $df_{t,e} = 9, 30$, $p \leq 0.0001$). In other words, as the larvae consume food, phenoloxidase activity increases, as does blood volume and hemocyte count. Also, the activity of phenoloxidase enzyme in larvae fed on the Kaleghochi kernel cultivar (0.72 ± 0.08 µg/min/mg protein) was higher than all treatments and also in larvae fed on Shapasand was in second order (0.65±0.08 µg/min/mg protein), although there was no significant difference between these two treatments. Other treatments had lower phenoloxidase activity than the first two, but more enzyme activity was obtained when compared to diapausing larvae of all cultivars (Table 2).

**Table 2**. Phenoloxidase activity in diapausing and nondiapausing larvae of *Megastigmus pistaciae* feeding on different pistachio cultivars. (Different letters in each column show significance using Tukey's test at $p < 0.05$)

| | Phenoloxidase enzyme activity (µmol/min/mg protein) | |
|---|---|---|
| pistachio cultivars | nondiapausing larvae | diapausing larvae |
| Khanjari | 0.27±0.05b | 0.18±0.003c |
| Kaleghochi | 0.72±0.08a | 0.17±0.002c |
| Akbari | 0.18±0.03c | 0.10±0.04d |
| Nokhodi | 0.22±0.06b | 0.16±0.01c |
| Shapasand | 0.65±0.08a | 0.2±0.003c |

## Discussion

According to the results, four hemocytes are present in the hemolymph of *M. pistaciae*, which include prohemocytes, granulocytes, plasmatocytes, and oenocytoids, and not spherulocytes. Most insects, particularly lepidopteran larvae, have five types of hemocytes, but these types change and sometimes some hemocytes are absent in some insects (Starnd *et al.*, 2008). For example, spherulocytes were not observed in the hemolymph of *Melipona scutellaris* L (Hymenoptera: Apidae), *Pieris rapae* (Linnaeus) (Lepidoptera: Pieridae), *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae), *Sesamia cretica* Lederer (Lepidoptera: Noctuidae), *Arge ochropus* (Gmelin) (Hymenoptera; Argidae) , *Osphrantria coerulescense* Redt (Coleoptera: Cerambycidae), and *Lixus incanescense* (Coleoptera: Curculionidae) like *M. pistaciae* (Amaral *et al.*, 2010; Zhang, 2012; Zibaee & Malagoli, 2014; Sadeghi *et al.*, 2017; Valizadeh *et al.*, 2017; Ajamhassani, 2019; Ajamhassani & Aghaei, 2022).

Environmental stresses have a continuous impact on the physicochemical properties of insect blood (Duarte *et al.*, 2020). As it has been proven, environmental stresses such as changes in temperature, type of diet, and the entry of pathogenic agents will change the abundance, type, and shape of hemocytes as the most important blood factors of insects and will affect defense activities. Because maintaining blood homeostasis is critical for insect survival, new hemocytes are constantly produced to replace dead, old, and deformed ones (Nakahara *et al.*, 2003). In fact, lowering the hemocyte count weakens the insect's resistance to microbial or chemical control methods and causes it to die (Zhu *et al.*, 2012).

The current experiments focused on two types of insects. The first group consisted of larvae that were found in various pistachio cultivars and survived the cold winter temperatures or lived in the diapause period. The second group consisted of larvae that fed on pistachio cultivars during the summer.

As in summer-fed larvae, regardless of pistachio cultivar, the total number of plasmatocytes, granulocytes, oenocytoids, prohemocytes, and phenoloxidase activity was significantly higher than that of non-fed diapausing larvae, which depends on food energy in fed larvae (Siva jothy, 2005). Besides, food type and quality are effective in the immune response of insects. The results of the present study showed a significant increase in the total number of hemocytes in Kaleghochi and Shapasand-fed larvae as compared to other treatments. Obviously, the number of macromolecules such as carbohydrates, proteins, and fats in food are effective in the growth and immune responses of insects (Manjula *et al.*, 2020). Carbohydrates are essential for activities with high-energy demands such as movement, growth, and cell division (Maklakov *et al.*, 2008). Proteins are involved in the reproduction of adult insects and the levels of hemocytes and hemolymph enzymes (Lee *et al.*, 2008; Garham *et al.*, 2014). Fatty acids are vital energy sources for molting.

To determine the effect of pistachio cultivars on changes in hemocytes and phenoloxidase activity, differences in the number of macromolecules in different pistachio cultivars should be identified, but it should be noted that there is little information on the exact amount of these components in different diets. In fact, the effect of different pistachio cultivars on the abundance of hemocytes is related to their overall energy. As a result, it is possible to conclude that the Kaleghochi and Shapasand kernels are more nutritious than other cultivars for feeding wasp larvae, resulting in a significant increase in cell number and phenoloxidase activity.

Similar findings have been reported regarding the effect of various diets on blood cell changes. The hemocyte count and activity of glutathione s-transferases (GSTs), phenoloxidase, melanization, and catalase differed significantly against *Bacillus thuringiensis* in *Spodoptera litura* (Fabricius) larvae (Lepidoptera: Noctuidae), which fed

on a variety of hosts such as cabbage, tobacco, and cotton (Venjateswari *et al*., 2020). Furthermore, zink supplementation has been shown to improve the immune response of *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae to the pathogenic nematode which is a species of *Mesorhabditis belari* (Manjula *et al*., 2020). The immune response of *Eupoecilia ambiguella* (Hubner) (Lepidoptera: Tortricidae) larvae fed on different diets was studied, and results showed that larvae fed on grape seeds with higher sugar content had higher immunity against *Bacillus cereus* bacterium and *B. bassiana* pathogenic fungus (Vogelweith *et al*., 2016). Diapause and cold stress are among other factors that affect immunity. Insects can undergo diapause during the summer or winter as eggs, larvae, pupae, or adults. Insects' ability to survive in adverse environmental conditions such as low temperatures and limited food resources demonstrates their adaptability (Saunders, 2009). Inactive and immobile diapausing insects, on the other hand, lack the ability to clean themselves or move, making them more susceptible to pathogens and having a higher rate of disease development (Schmid Hempel, 1998). Diapausing insects have minimal metabolic activities such as respiration and cell defense (Nakamura *et al*., 2011). Many insects lose body water and blood volume in cold conditions to increase survival at low temperatures. Measuring hemolymph volume can be a basic method for assessing cold tolerance in insects (Sinclair *et al*., 2015). Trehalose and blood glucose levels increase during cold stress, which is vital in maintaining body water and homeostasis (Teets *et al*., 2013).

The results of the present study showed that the hemocyte density in the hemolymph of diapausing larvae is significantly lower than non-diapausing larvae, which can be related to a possible reduction in blood volume. According to reports, a number of hemocytes undergo severe morphological changes during the cold, and as a result of decreased nutrition, their walls shrink and the nucleus compresses and dies (Pourali & Ajamhassani, 2018) and some are removed from blood circulation and are attached to the body walls (Rowley & Ratcliffe, 1978), so, obviously these cells are not among the circulating cells. The results of other studies also confirmed the effect of cold stress on the reduction of the immune activities of various insects.

The hemocyte count decreased gradually during diapause and winter in *Pectinophora gossypiella* (Sunders) (Lepidoptera: Gelechiidae), *S. cretica*, and *Nicrophorus vespilloides* H. (Coleoptera: Silphidae) (Urbanski *et al*., 2017). In fact, the insect's immune response changes as the ambient temperature decreases at the beginning of the cold season, which often coincides with the onset of diapause. In *Drosophila melanogaster* (Diptera: Drosophilidae), for example, hemocytes successfully encapsulate parasitoid eggs at 29 °C, but as the temperature decreases, the encapsulation mechanism decreases (Nappi & Silvers, 1984).

Cold stress has also been shown to reduce encapsulation activity in *Coccus hesperidum* L. (Hemiptera: Coccidae) (Blumberg *et al*., 1976). Another study found that winter-fed pupae of *Samia cynthia* pryeri (Lepidoptera: Saturnidae) at 4 ° C had significantly lower phagocytosis than pupae at 20 ° C. (Nakamura *et al*., 2011). The results of the present study demonstrated that cold stress, diapause, and subsequent inadequate nutrition, as well as different pistachio cultivars, had a significant effect on changes in the abundance of all hemocytes and phenoloxidase activity in *M. pistaciae*.

## Conclusion

The findings of this research can be considered when deciding to apply microbial agents and anti-pest toxins. It has been proven that reducing the blood volume and the hemocyte count during diapause and the possible shrinkage and compression of cells due to cold stress weaken the immune system of insects and make them more susceptible to contamination and attack by fungi, bacteria, and chemical compounds. Are wasps larvae more susceptible to pathogenic fungi and toxins in winter? Do wasps show different susceptibility to pathogenic agents when feeding on different pistachio cultivars? What is the effect of diapause and temperature on the activity of glutathione transferases, lysozyme, and oxygen and nitrogen mediators? To answer these questions, it is definitely necessary to conduct further research to be able to use the results more confidently and adopt more appropriate control approaches.

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# تاثیر ارقام پسته بر فراوانی هموسیتهای لاروهای دیاپوزی و غیر دیاپوزی زنبور مغزخوار طلایی پسته (Hymenoptera: Torymidae) *Megastigmus pistaciae*

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## چکیده

ایمنی سلولی همراه با فعالیت سلول‌های خونی، یکی از جنبه‌های مهم ایمنی ذاتی در حشرات است. نقش هموسیتها در مقابله با تنش‌ها و آلودگی‌های وارد شده به همولنف، کلیدی است .آنها با تغییر در تعداد، شکل و تیپ در مقابل تنش‌ها واکنش نشان می‌دهند و شناسایی این سلولها و تنوع آنها اولین مرحله از مطالعات ایمنی شناسی محسوب می‌شود. دفاع هیومرال معمولا با اندکی تاخیر نسبت به دفاع سلولی و با فعالیت آنزیم فنل اکسیداز و پپتیدهای ضدمیکروبی همراه است. تحقیق حاضر به بررسی ویژگی‌های ایمنی شناسی لاروهای دیاپوزی و غیردیاپوزی لاروهای زنبور مغزخوار طلایی پسته با تغذیه از رقم‌های مختلف پسته می‌پردازد. زنبور *Megastigmus pistaciae* یکی از آفات مهم و مخرب پسته در سالهای اخیر است. بعد از جمع‌آوری میوه های آلوده و انتقال آنها به آزمایشگاه، همولنف لاروها استخراج شد و هموسیتها بعد از رنگ آمیزی با گیمسا شناسایی شدند. چهار نوع سلول خونی در همولنف شناسایی شد: پروهموسیت‌ها، پلاسماتوسیت‌ها، گرانولوسیت‌ها و اونوسیتوئیدها. اندازه مرفومتریک هموسیت‌ها با استفاده از میکروسکوپ نوری تعیین شد. شمارش تفرقی هموسیت‌ها در همولنف لارو، شفیره و حشرات کامل انجام شد. فراوانی گرانولوسیت‌ها در لاروها (بیشتر از ۲/۴ ±۳۵ ٪) و بالاتر از فراوانی گرانولوسیت‌ها در شفیره و بالغین به دست آمد. در مطالعه تغییرات سلولهای خونی در لاروهای دیاپوزی و غیردیاپوزی با تغذیه از رقم‌های مختلف پسته، نتایج نشان داد که تراکم هموسیتها در لاروهای غیر دیاپوزی تغذیه کرده از تمام رقمها به طور معنی داری بیشتر از لاروهای دیاپوزی بود. به علاوه، تعداد کل سلولهای خونی، گرانولوسیتها، اونوسیتوئیدها و پروهموسیت‌ها در لاروهای تغذیه کرده از رقم‌های پسته کله‌قوچی و شاپسند، بیشتر از موارد مشابه در لاروهای تغذیه کرده از رقم‌های خنجری، اکبری و نخودی بود. نتایج مربوط به تغییرات آنزیم فنل اکسیداز مشابه نتایج مربوط به تغییرات هموسیت‌ها بود. به عبارت دیگر، فعالیت این آنزیم در لاروهای غیر دیاپوزی بیشتر از لاروهای دیاپوزی و در لاروهای تغذیه کرده از ارقام کله قوچی و شاپسند بیشتر از لاروهای تغذیه کرده از سایر ارقام بود. شناسایی هموسیت‌ها و تغییرات آنها در لاروهای دیاپوزی و غیردیاپوزی زنبور مغزخوار طلایی پسته با تغذیه از رقم‌های پسته برای اولین بار انجام شده است و می‌تواند زمینه‌ساز مطالعات بعدی ایمنی‌شناسی این حشره باشد. به نظر می‌رسد دیاپوز و نوع رژیم غذایی می‌تواند به نوعی تعیین کننده واکنش ایمنی حشره در مقابل بیمارگرها باشد. تحقیقات تکمیلی در این زمینه می تواند در روش‌های کنترل میکروبی این آفت موثر باشد.

**کلمات کلیدی:** ایمنی حشرات، دفاع سلولی، دفاع پلاسمایی، تغذیه، دیاپوز، سلول خونی

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