*J. Crop Prot. 2026, 15 (2): 167-180*

Research Article

# Hemocytes of the *Cydia pomonella* (Lep.: Tortricidae), *Sesamia cretica* (Lep.: Noctuidae), and *Antigastra catalaunalis* (Lep.: Crambidae): Morphology and hemogram

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**Abstract:** Insect circulatory physiology studies begin with analyzing hemocyte morphology and the hemogram. Hemocytes are critical blood components in insects and are vital to physiological defense responses. This research examined the hemocyte profile and hemogram across larval and pupal stages in three major pests: *Cydia pomonella* L. (Lep.: Tortricidae), *Sesamia cretica* Lederer (Lep.: Noctuidae), and *Antigastra catalaunalis* Duponchel (Lep.: Crambidae). Apple fruits, corn, and sesame plants infested with larvae were collected from the field and brought to the laboratory, where fourth- and fifth-instar larvae were extracted from plant tissue. Hemolymph was collected and stained using Giemsa solution to identify hemocytes under a light microscope. Five hemocyte types were identified in all examined species: prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes. Hemogram analysis included measurements of Differential Hemocyte Count (DHC), Total Hemocyte Count (THC), and hemolymph volume across larval and pupal stages. In fourth- and fifth-instar larvae of the three species, plasmatocytes and granulocytes were the most abundant hemocytes, constituting approximately 80% of the hemocyte population. Conversely, prohemocytes were most prevalent in first-instar larvae, comprising 28–32% of the hemocyte population. Blood volume and THC were highest in fourth- and fifth-instar larvae of three pests, showing a strong correlation between larval weight, hemolymph volume, and THC. This study is the first report on hemocyte identification and hemogram analysis in these three pests, providing a foundation for future immunological research.

**Keywords:** Codling moth, corn stem borer, sesame webworm, hemocyte profile, hemocyte frequency

## Introduction

*Cydia pomonella* L. (Lepidoptera: Tortricidae), *Sesamia cretica* Lederer (Lepidoptera: Noctuidae), and *Antigastra catalaunalis* Duponchel (Lepidoptera: Crambidae) are significant pests in the world, causing extensive damage to crops annually. The codling moth, *C. pomonella*, is a serious global pest of various fruits, including apples, quince, pears, walnuts, and some stone fruits (Furmanczyk *et al*., 2022). The larvae feed on fruit flesh, burrow into the seeds, and reduce fruit quality, leading to premature fruit drop. At high population densities, damage can reach up to

Handling Editor: Saeid Moharramipour

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Received: 31 January 2025, Accepted: 07 January 2026
Published online: 08 November 2026

80% of apple production (Pajač *et al*., 2012; Kuyulu and Genç, 2019).

The corn stem borer, *S. cretica*, attacks all types of corn, including cob and fodder varieties. Larvae feed on stalks, flowers, cobs, and seeds. Severe infestations hollow out corn stalks, making them brittle, and infect corn cobs with larval excrement, leading to rotting and unusable crops. Alongside larval feeding, fungal pathogens invade the damaged stalks, further increasing crop loss (Johnson *et al*., 1995; Nikpay and Volpe, 2016).

The sesame leafroller and capsule borer, *A. catalaunalis*, is a multivoltine pest of sesame, one of the oldest cultivated oilseed crops globally (Mishra *et al*., 2023). The larvae feed on various parts of the sesame plant except for the root. During the vegetative stage, larvae feed on tender leaves by binding the top leaves together. In the flowering stage, they enter buds and flowers and consume floral tissues. Eventually, they penetrate the capsules, feed on developing seeds, and wholly or partially destroy the pod structure (Gupta *et al*., 2002).

Insect defense mechanisms are categorized as morphological, physical, and physiological. Morphological defenses involve changes in body color and size, aiding camouflage or mimicry to evade predators. Features such as integument structure, spines, and sensory hairs play a defensive role against natural enemies (Eisner *et al*., 2007). Physical defenses rely on the integument's cuticular layers, including wax, cement, protein, and lipid, which prevent contaminants from entering the hemolymph. Still, weak, easily penetrated pores on the body surface, including the mouth, anus, spiracles, and reproductive pore, allow pathogens to penetrate the insect and enter the hemolymph (Narayanan, 2004). At this point, physiological defenses are activated. These include cellular and humoral responses involving hemocytes, phenol oxidase enzymes, antimicrobial peptides, and antibacterial glycoproteins (Lavine and Strand, 2002; Strand, 2008). Cellular defense reactions are usually fast, but humoral defense occurs a few hours after infection (Stanley and Miller, 2006).

Insect immunity reflects the circulatory system's response to stressors such as temperature changes, diet, starvation, diapause, and pathogen entry (fungi, bacteria, toxins, or pollutions) (Siva-Jothy and Thompson, 2002; Ajamhassani, 2021; Suwannakhon *et al*., 2024). The first stage of immunity is recognition of the foreign agent by granulocytes; then, depending on the density and size of the foreign agent, hemocytes destroy it through phagocytosis and nodulation (Borges *et al*., 2008). The insect immune response is an important indicator of host susceptibility to contamination. Resistant hosts can stop the infection from progressing (Washburn *et al*., 2000).

The basic method for identifying hemocytes is based on morphological characteristics, tissue chemistry, and physiological roles (Liu *et al*., 2013). After Giemsa staining, hemocytes can be identified using light and electron microscopy. Among these cells, granulocytes and plasmatocytes are the primary mediators of cellular immunity and are found at the highest densities in older larvae (Lavine and Strand, 2002; Strand, 2008). As larvae grow, feeding increases, leading to higher hemolymph volume (HV) and hemocyte density (Ajamhassani *et al*., 2025). The increase in cell number during larval development shows that blood cells multiply in Lepidoptera, as in other insects (Jalali and Salehi, 2008). Hemocytes of lepidopteran larvae increase both by mitotic division and production in hematopoietic organs (Yamashita and Iwabuchi, 2001; Holz *et al*., 2003). Consequently, older larvae exhibit stronger immune responses and greater resistance to pathogens and environmental stress than younger larvae (Valades-Lira *et al.*, 2011).

Understanding insect immune system characteristics begins with identifying hemocytes and studying their morphology and abundance. The hemogram defines changes in hemocyte abundance during specific developmental stages or time points. Given the agricultural importance of *C. pomonella*, *S. cretica*, and *A. catalaunalis*, this research focuses on identifying hemocytes and the factors influencing their hemogram. Investigating these

pests' circulatory systems and hemocytes provides a foundation for further studies on their immune responses to stress and infections.

## Materials and Methods

### Insect Rearing

Infested apple fruits, corn, and sesame were collected from apple orchards, corn, and sesame fields in the Bakharz region, Khorasan Razavi Province (60°10′15″ E, 34°58′10″ N), Kheirabad region, Semnan Province (53°25′14″ E, 35°32′37″ N), and the central region of Neyshabur County, Khorasan Razavi Province (58°10′20″ E, 35°54′31″ N), Iran, respectively. The plants were transferred to a laboratory growth chamber with controlled conditions (25 ± 1 °C, 50% RH, and a 14:10 h light: dark). Larvae were identified at different instars using Dyar's rule (Dyar, 1980).

Using forceps, larvae were carefully extracted from infected plants and placed on fresh, healthy plants. After one generation of rearing, third- and fourth-instar larvae were selected for hemocyte identification, and experiments were performed to determine the hemogram using larval and pupal stages. Rearing was conducted in plastic containers (40 × 40 × 30 cm) covered with white organza mesh for proper ventilation, with each pest reared separately on its primary hosts-apple, corn, and sesame, respectively.

### Hemocyte Identification

Hemocytes were identified from the third and fourth instar larvae hemolymph using Gupta's keys and Giemsa staining (Gupta, 1991). The ventral surface of the larval body was punctured with a sterile needle, and hemolymph was collected with a capillary tube and placed onto a microscope slide. Hemocytes were stained with Giemsa solution (Merck KGaA, Germany) for 10 minutes, rinsed, and observed under a BH2 light microscope at 40 × magnification (Yeager, 1945; Jones, 1962). Permanent slide preparations were made using Canada balsam. After staining, hemocyte size was measured, and abundance was quantified across all developmental stages, including first-, second-, third-, fourth-, and fifth-instar larvae and pupae.

### Hemogram

### Differential Hemocyte Count (DHC)

After Giemsa staining, we randomly selected 100 hemocytes and differentially counted them at 40 × magnification using an Olympus BH2 microscope. For each developmental stage, we examined 30 samples.

### Total Hemocyte Count (THC)

For THC, approximately 3 µL of hemolymph was collected from each larva or pupa using a capillary tube and mixed with 10 µL of Tyson buffer as an anticoagulant solution ($NaCl_2$ 72 mM, $Na_2SO_4$ 9 mM, glycerol 43 mM, methyl violet 0.06 mM, distilled water) (Mahmood and Yusaf, 1985). The hemolymph-Tyson solution mixture was placed on Neubauer slides (HBG, Germany), and hemocytes were counted using the Jones formula under light microscopy at 40 × magnification (Jones *et al*., 1962).

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

Dilution = 10 times, Depth factor of the chamber = 10, No. of squares counted = 5

### Hemolymph Volume (HV)

Hemolymph volume was measured directly across developmental stages using micropipettes (Terra *et al*., 1975). We first recorded the weight of a dry filter paper sheet. A proleg was then cut from the larval abdomen, and the hemolymph was extracted using a Hamilton syringe (10 µL, Switzerland), placed onto the filter paper, and weighed. We also sampled pupae and adults. The difference between the wet and dry filter paper weights was calculated and recorded as the hemolymph volume (Terra *et al*., 1975). Fifteen insects from each developmental stage were included in the analysis.

### Statistical Analysis

All data were analyzed using a completely randomized design (CRD) and compared with a one-way analysis of variance (ANOVA),

followed by Tukey's test for significant differences at $p \leq 0.05$ (SAS, 9.4). Differences between samples (n = 3) were considered statistically significant at $p < 0.05$ and were marked in the corresponding figures and tables.

## Results

### Identification of Hemocytes

Five types of hemocytes were observed in the hemolymph of *C. pomonella*, *S. cretica*, and *A. catalaunalis* larvae: prohemocytes, granulocytes, plasmatocytes, oenocytoids, and spherulocytes. Prohemocytes were round, the smallest hemocytes in size, and had a central nucleus that occupied most of the cell volume (Figs. 1, 2, and 3). In *C. pomonella*, the prohemocyte nucleus was smaller than the nuclei of other cells. However, in *S. cretica* and *A. catalaunalis*, oenocytoids and granulocytes had the smallest nucleus sizes, respectively (Table 1). Plasmatocytes in *C. pomonella* displayed various shapes (spindle, eye, and cylindrical) ranging from small to large (Fig. 1). Some plasmatocytes in *C. pomonella* had one short or elongated germ, while others had two, with a nucleus that appeared brighter than the cytoplasm under Giemsa staining. In contrast, plasmatocytes in *S. cretica* were eye-shaped (Fig. 2), and those in *A. catalaunalis* were spindle-shaped without germs (Fig. 3). In *C. pomonella*, some plasmatocytes were the largest hemocytes, while in *S. cretica*, plasmatocytes were comparable in size to oenocytoids and granulocytes. In *A. catalaunalis*, plasmatocyte sizes were close to those of spherulocytes (Table 1). The nucleus of *C. pomonella* plasmatocytes was the largest among all hemocytes, whereas in the other pests, plasmatocyte nuclei were similar in size to those of other hemocytes (Table 1). Granulocytes were round or oval, with cytoplasm containing numerous granular grains. In *S. cretica*, granulocytes were sometimes the largest hemocytes (Table 1). The most significant variation in granulocyte size was observed in *A. catalaunalis* hemolymph (Fig. 3).

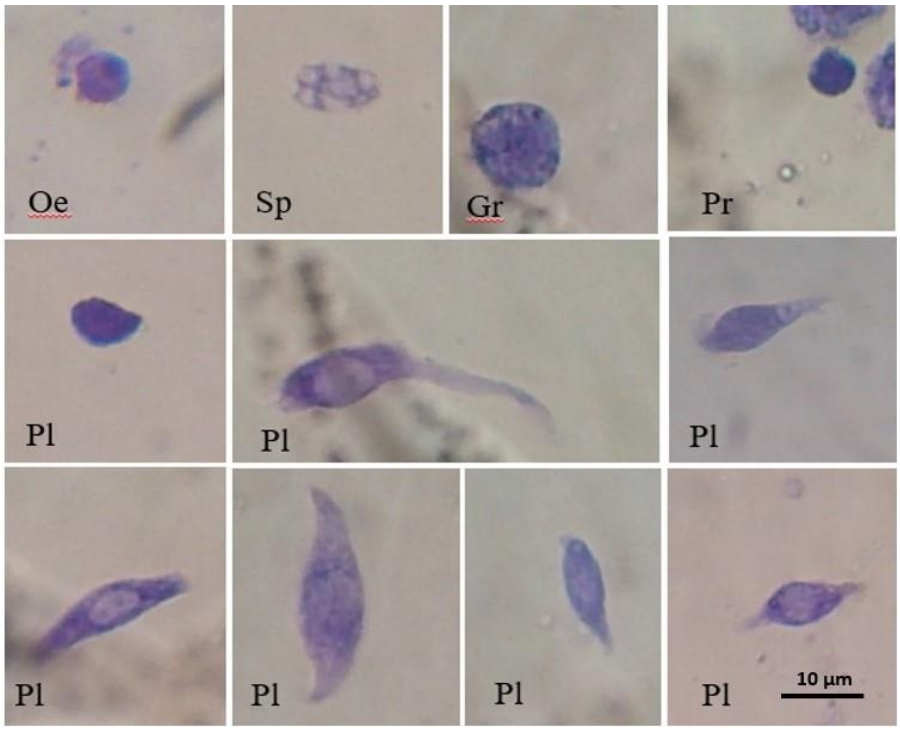



**Figure 1** Light microscopy pictures of *Cydia pomonella* hemocytes stained with Giemsa. PR (Prohemocyte), PL (Plasmatocyte), OE (Oneocytoid), GR (Granulocyte), SP (Spherulocyte), Scale bar = 10 µm.

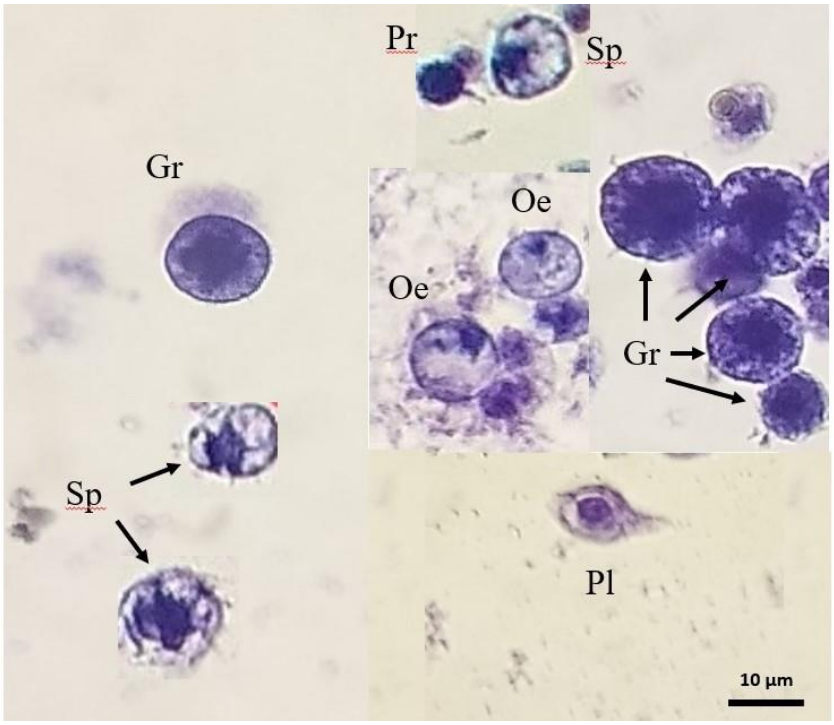



**Figure 2** Light microscopy pictures of *Sesamia cretica* hemocytes stained with Giemsa. PR (Prohemocyte), PL (Plasmatocyte), OE (Oneocytoid), GR (Granulocyte), SP (Spherulocyte), Scale bar = 10 µm.

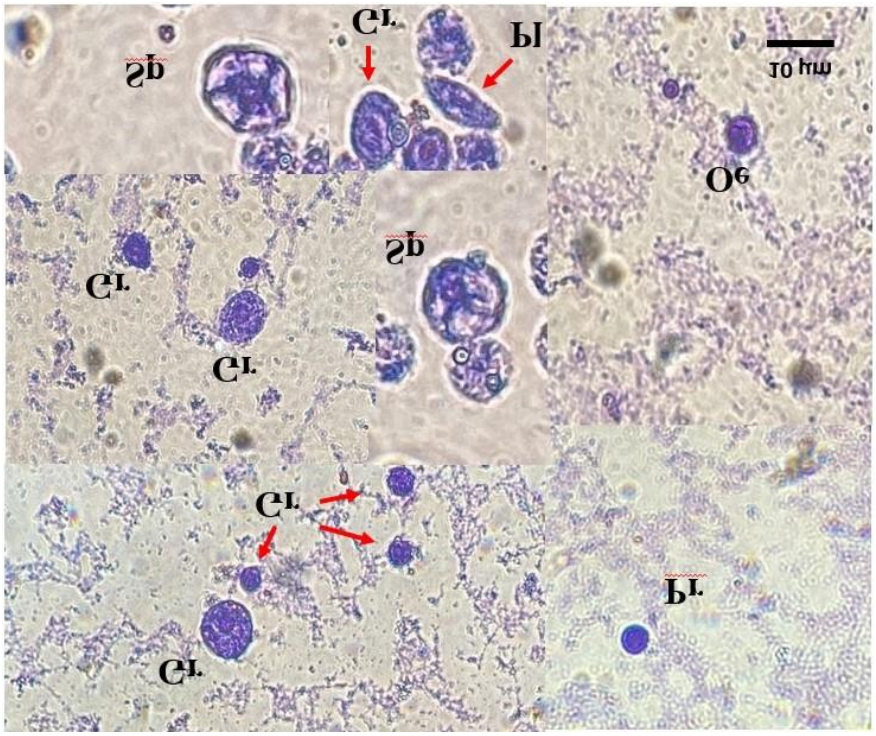


**Figure 3** Light microscopy pictures of *Antigastra catalaunalis* hemocytes stained with Giemsa. PR (Prohemocyte), PL (Plasmatocyte), OE (Oenocytoid), GR (Granulocyte), SP (Spherulocyte), Scale bar = 10 µm.

**Table 1** Morphometric measurements of hemocytes in larvae of *Cydia pomonella*, *Sesamia cretica* and *Antigastra catalaunalis* (n = 20).

| | | Cell size (µm) | | Nucleus size (µm) | |
|---|---|---|---|---|---|
| Insect | Hemocyte type | Length (mean ± SE) | Width (mean ± SE) | Length (mean ± SE) | Width (mean ± SE) |
| *Cydia pomonella* | Prohemocyte | 3.3 ± 1.8f | 3.0 ± 1.5d | 2.5 ± 0.6c | 2.6 ± 0.5c |
| | Plasmatocyte | 21.0 ± 11.7a | 6.2 ± 2.3c | 5.5 ± 2.8a | 5.0 ± 1.7ab |
| | Granulocyte | 7.5 ± 3.2de | 8.0 ± 2.6b | 3.7 ± 1.8ab | 3.6 ± 1.7c |
| | Oenocytoid | 5.0 ± 1.1.0e | 4.6 ± 1.5cd | 1.5 ± 1.2d | 1.4 ± 1.0e |
| | Spherulocyte | 8.5 ± 1.7d | 5.2 ± 0.7c | 2.8 ± 0.5c | 3.0 ± 0.6bc |
| *Sesamia cretica* | Prohemocyte | 3.8 ± 1.0f | 3.7 ± 1.2d | 3.2 ± 1.1c | 3.0 ± 1.2c |
| | Plasmatocyte | 12.5 ± 2.4b | 7.3 ± 1.0b | 4.0 ± 1.3b | 3.8 ± 0.9bc |
| | Granulocyte | 12.8 ± 4.5bc | 11.0 ± 3.2a | 6.5 ± 3.7a | 6.2 ± 3.0a |
| | Oenocytoid | 11 ± 1.8b | 10.0 ± 2.1a | 1.5 ± 0.6d | 1.3 ± 0.4d |
| | Spherulocyte | 12.1 ± 3.3b | 8.0 ± 1.8ab | 6.3 ± 2.6a | 6.0 ± 2.4a |
| *Antigastra catalaunalis* | Prohemocyte | 2.5 ± 1.7g | 2.7 ± 1.4d | 2.3 ± 1.5cd | 2.3 ± 1.4cd |
| | Plasmatocyte | 10.8 ± 2.2bc | 5.0 ± 1.3c | 4.6 ± 0.7a | 4.0 ± 1.1.0b |
| | Granulocyte | 5.1 ± 3.2f | 4.9 ± 3.0d | 1.5 ± 0.6d | 1.4 ± 0.5d |
| | Oenocytoid | 4.6 ± 0.7e | 4.8 ± 0.8c | 2.8 ± 0.4c | 2.7 ± 0.5c |
| | Spherulocyte | 11.7 ± 2.5b | 12.0 ± 1.7a | 5.3 ± 1.8a | 5.2 ± 1.5ab |

Different letters in each column show significance using Tukey's test at $p < 0.05$.

In some cases, *A. catalaunalis* granulocytes were small and similar to prohemocytes. However, *C. pomonella* and *S. cretica* granulocytes were generally more prominent than prohemocytes (Figs. 1 and 2). The granulocyte nuclei in *S. cretica* were the largest among the pests studied. Oenocytoids were round or ovoid and contained a lateral nucleus in all three pests. The size of oenocytoids was largest in *S. cretica* hemolymph, although their nuclei were relatively small (Table 1). Oenocytoid cytoplasm was smooth and uniform across all three pests (Figs. 1, 2, and 3). Spherulocytes, relatively large hemocytes, were observed in all three pests (Table 1). They had a central nucleus and pitted cytoplasm, which visually distinguished them from other hemocytes (Figs. 1, 2, and 3).

**Differential Hemocyte Count (DHC)**

Table 2 shows that prohemocytes were the most abundant hemocytes in young larvae of all three insects, with their abundance decreasing significantly as larval age increased. The highest prohemocyte density was observed in the first instar larvae of *S. cretica* (32.2 ± 3.5%) and *A. catalaunalis* (30.5 ± 1.3%). In contrast, the fourth and fifth instar larvae of *C. pomonella* had the lowest prohemocyte densities, with frequencies of 6 ± 0.55% and 4.45 ± 1.8%, respectively ($F = 112.45$, $df_{t,e} = 14,60$, $p \leq 0.0001$).

Plasmatocytes and granulocytes, the primary hemocytes involved in immunity, were the most abundant in the fourth and fifth instar larvae of pests. At advanced larval stages, the combined frequency of plasmatocytes and granulocytes was higher than that of other hemocytes. Among these, granulocytes were more abundant than plasmatocytes in older larvae of all insects. The highest abundance of plasmatocytes was recorded in the fourth (42 ± 1.8%) and fifth instar larvae (40 ± 1.4%) of *C. pomonella* (Table 2) ($F = 125.33$, $df_{t,e} = 14,60$, $p \leq 0.0001$).

Similarly, the fifth instar larvae of *A. catalaunalis* and *C. pomonella* exhibited the highest granulocyte densities at 47 ± 3.1% and 46.5 ± 2.21%, respectively. ($F=142.24$, $df_{t,e} = 14,60$, $p \leq 0.0001$).

Oenocytoids were observed at low to moderate levels (4–21%) across all larval stages. Their densities were typically higher in younger larvae compared to older ones ($F = 140.7$, $df_{t,e} = 14,60$, $p \leq 0.0001$). In contrast, spherulocytes were the least abundant hemocytes in all insects. The highest density of spherulocytes was observed in the third and fourth instar larvae of *S. cretica* (9–11%). Notably, spherulocytes were absent in the first and second instar larvae of *C. pomonella* and the first instar larvae of *S. cretica* (Table 2). ($F = 80.15$, $df_{t,e} = 14,60$, $p \leq 0.0001$).

**Hemolymph Volume (HV) and Total Hemocyte Count (THC)**

Tables 3, 4, and 5 show that the body weight of the tested pests' fourth- and fifth-instar larvae was significantly higher than that of other developmental stages. Among the insects, fourth instar larvae of *S. cretica* (210 ± 12.3 mg) were approximately four times heavier than those of *C. pomonella* (49.2 ± 0.45 mg) and seven times heavier than those of *A. catalaunalis* (30 ± 1.5 mg). Similarly, the fifth instar larvae of *S. cretica* (220 ± 28.4 mg) were three times heavier than *C. pomonella* (60.45 ± 1.24 mg) and five times heavier than *A. catalaunalis* (43.6 ± 2.54 mg).

HV increased with larval age, and the older larvae of *C. pomonella* ($F = 76.22$, $df_{t,e} = 4,14$, $p \leq 0.0001$), *S. cretica* ($F = 70.77$, $df_{t,e} = 4,14$, $p \leq 0.0001$), and *A. catalaunalis* ($F= 55.53$, $df_{t,e} = 4,14$, $p \leq 0.0001$) had a significantly higher HV than younger larvae and pupae. Consequently, the THC was also higher in older larvae of *C. pomonella* ($F = 27.14$, $df_{t,e} = 4,14$, $p \leq 0.001$), *S. cretica* ($F = 21.24$, $df_{t,e}=4,14$, $p \leq 0.001$), and *A. catalaunalis* ($F=35.6$, $df_{t,e} = 4,14$, $p \leq 0.0001$). The highest HV and THC were observed in the fifth instar larvae of *S. cretica*, with values of 25.5 ± 2.2 µL and 1155 ± 72.5 cells in $mm^3$, respectively. These values were significantly higher than the HV and THC of other developmental stages and species (Tables 3, 4, and 5).

**Table 2** Differential hemocyte count in developmental stages of *Cydia pomonella*, *Sesamia cretica* and *Antigastra catalaunalis* (n = 20 for each insect).

| Insect | Developmental larvae | Frequency of hemocytes (± SE) (%) | | | | |
|---|---|---|---|---|---|---|
| | | Prohemocyte | Plasmatocyte | Granulocyte | Oenocytoid | Spherulocyte |
| *C. pomonella* | 1th instar | 28.0 ± 2.5ab | 25.5 ± 3.1d | 26.8 ± 4.3g | 21.2 ± 1.5a | - |
| | 2nd instar | 26.5 ± 2.7b | 31.0 ± 0.7c | 29.1 ± 2.7f | 14.7 ± 1.6c | - |
| | 3rd instar | 9.6 ± 1.1g | 37.7 ± 1.3b | 38.5 ± 3.3d | 12.4 ± 0.6c | 0.5 ± 0.2e |
| | 4th instar | 6.0 ± 0.5h | 42.0 ± 1.8a | 44.4 ± 2.1b | 8.6 ± 1.5d | 1.0 ± 0.3d |
| | 5th instar | 4.45 ± 1.8hi | 40.0 ± 1.4a | 46.5 ± 2.2b | 7.3 ± 1.7e | 1.0 ± 0.2d |
| *S. cretica* | 1th instar | 32.2 ± 3.5a | 18.0 ± 4.3g | 30.4 ± 2.2f | 17.5 ± 3.1b | - |
| | 2nd instar | 18.8 ± 2.1d | 18.7 ± 3.3g | 35.5 ± 3.2e | 20.3 ± 2.7a | 2.3 ± 0.2c |
| | 3rd instar | 10.5 ± 0.5fg | 21.2 ± 2.5f | 41.6 ± 3.7c | 15.5 ± 1.7b | 11.4 ± 1.7a |
| | 4th instar | 14.1 ± 2.2e | 26.7 ± 2.6d | 43.3 ± 2.2b | 10.4 ± 2.5d | 9.0 ± 1.5a |
| | 5th instar | 15.35 ± 2.2e | 25.5 ± 2.1d | 42.3 ± 2.5bc | 12.2 ± 2.5cd | 3.5 ± 1.4b |
| *A. catalaunalis* | 1th instar | 30.5 ± 1.3a | 17.7 ± 2.6 | 34.8 ± 2.4 | 15.7 ± 4.3c | 1.0 ± 0.2d |
| | 2nd instar | 21.2 ± 2.1c | 23.3 ± 2.4e | 43.3 ± 3.2b | 9.0 ± 2.5d | 2.0 ± 0.2c |
| | 3rd instar | 19.5 ± 1.57d | 27.0 ± 0.3cd | 40.0 ± 2.5cd | 1.01 ± 0.7c | 1.0 ± 0.2d |
| | 4th instar | 10.5 ± 1.4fg | 31.2 ± 3.2c | 42.4 ± 1.6bc | 4.0 ± 0.3f | 1.0 ± 0.1d |
| | 5th instar | 12.3 ± 2.4f | 30.3 ± 2.2c | 47.0 ± 3.1a | 6.5 ± 0.5e | 1.2 ± 0.3d |

Different letters in each column show statistical differences among biological stages (Tukey's test, $p \leq 0.05$).

**Table 3** Body weight, hemolymph volume (HV), total hemocyte count (THC) in developmental larvae and pupae of *Cydia pomonella*.

| Developmental stages | Body length (mm) | Head width (mm) | Weight (mg) | HV (µl) | THC (cell in $mm^3$) |
|---|---|---|---|---|---|
| 1th instar | 2.2 ± 0.3 | 0.2 ± 0.1 | 7.2 ± 0.1f | 1.7 ± 0.0e | 65.5 ± 10.3e |
| 2nd instar | 4 ± 0.4 | 0.2 ± 0.1 | 15.5 ± 0.1e | 2.5 ± 0.0d | 115.7 ± 14.4d |
| 3rd instar | 6.5 ± 0.3 | 0.5 ± 0.1 | 31.3 ± 1.3d | 6 ± 0.3c | 256.8 ± 23.6b |
| 4th instar | 10.2 ± 0.6 | 0.7 ± 0.1 | 49.2 ± 0.4b | 10.8 ± 0.4b | 405.5 ± 22.5a |
| 5th instar | 13.2 ± 1.5a | 0.8± 0.1 | 60.4 ± 1.2a | 12.8 ± 0.2a | 454.7 ± 40.5a |
| Pupa | 9.1 ± 0.4 | - | 41.5 ± 1.4c | 5 ± 0.5c | 126.7 ± 18.5c |

Different letters in each column show statistical differences among biological stages (Tukey's test, $p \leq 0.05$).

**Table 4** Body weight, hemolymph volume (HV), total hemocyte count (THC), in developmental larvae and pupae of *Sesamia cretica.*

| Developmental stages | Body length (mm) | Head width (mm) | Weight (mg) | HV (µl) | THC (cell in $mm^3$) |
|---|---|---|---|---|---|
| 1th instar | 2.1 ± 0.1 | 0.63 ± 0.1 | 21 ± 3.7e | 4.2 ± 0.2e | 147.3 ± 14.6f |
| 2nd instar | 5.2 ± 0.3 | 0.93 ± 0.2 | 60 ± 8.4d | 6.3 ± 0.5d | 218 ± 21.5e |
| 3rd instar | 12.5 ± 1.4 | 1 ± 0.2 | 140 ± 25c | 13.5 ± 1.3c | 556.4 ± 47.5c |
| 4th instar | 19.5 ± 0.7 | 1.82 ± 0.1 | 210 ± 12.3a | 20 ± 2.5b | 924.4 ± 45.4b |
| 5th instar | 25.2 ± 0.8 | 2.23 ± 0.1 | 220 ± 28.4a | 25.5 ± 2.2a | 1155 ± 72.5a |
| Pupa | 9.1 ± 0.2 | - | 180 ± 15.5b | 8.3 ± 0.4d | 312 ± 32.7d |

Different letters in each column show statistical differences among biological stages (Tukey's test, $p \leq 0.05$).

**Table 5** Body weight, hemolymph volume (HV), total hemocyte count (THC) in developmental larvae and pupae of *Antigastra catalaunalis.*

| Developmental stages | Body length (mm) | Head width (mm) | Weight (mg) | HV (µl) | THC (cell in $mm^3$) |
|---|---|---|---|---|---|
| 1th instar | 1.5 ± 0.2 | 0.25 ± 0.0 | 0.6 ± 0.2de | 1 ± 0.0e | 47.6 ± 7.3d |
| 2nd instar | 3.3 ± 0.2 | 0.82 ± 0.0 | 1.4 ± 0.5d | 1.85 ± 0.0d | 103.6 ± 12.4c |
| 3rd instar | 7.1 ± 0.1 | 1.1 ± 0.0 | 8.4 ± 1.2c | 5 ± 0.1c | 217.1 ± 15.5b |
| 4th instar | 12.7 ± 0.3 | 1.1 ± 0.0 | 30 ± 1.5b | 9.8 ± 0.5ab | 416.3 ± 61.5a |
| 5th instar | 16.2 ± 1.8 | 1.2 ± 0.01 | 43.6 ± 2.5a | 10.3 ± 0.2a | 440.3 ± 52.5a |
| Pupa | 9.8 ± 0.3 | - | 10.8 ± 1.7c | 3.7± 0.5c | 90.2 ± 10.5c |

Different letters in each column show statistical differences among biological stages (Tukey's test, $p \leq 0.05$).

## Discussion

Hematopoiesis is the process of synthesizing hemocytes in the hematopoietic organs, replacing dead and released hemocytes in the circulatory system in both embryo and larval development (Nardi *et al*., 2003; Grigorian and Hartenstein, 2013; Bredow *et al*., 2021). Hemocytes are the primary components of insect cellular immunity and respond to stress or foreign agents in the hemocoel by altering their shape, number, and activity, particularly in processes such as phagocytosis and nodulation (Sato, 2023; Campbell *et al*., 2024). Hemocyte patterns vary widely in type, shape, size, and number, even within insects of the same genus

(Gábor *et al*., 2020). Reports indicate five common hemocyte types, prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes in Lepidoptera such as: *Bombyx mori* L. (Lep: Bombicidae) (Tan *et al*., 2013), *Galleria mellonella* Fabricius (Lep: Pyralidae) (Blanco *et al*., 2017), *Samia cynthia* Drury (Lepidoptera: Saturnidae) (Gogoi *et al*., 2023), *Phycodes radiata* (Ochsenheimer, 1808) (Lepidoptera: Brachodidae) (Abdos *et al*., 2025), *Stiphrometasia monialis* Erschoff (Lepidoptera: Crambidae) (Ajamhassani *et al*., 2026) and *Yponomeuta padella* (L) (Lepidoptera: Yponomeutidae) (Ajamhassani, 2026). Our findings confirmed the presence of these hemocyte types in the hemolymph of *C. pomonella, S. cretica*, and *A. catalaunalis*. In a previous report, four hemocyte types were described for *S. cretica* (Sadeghi *et al*., 2017). This research also observed the spherulocyte form in the hemolymph of S. cretica.

Prohemocytes were round, and the smallest hemocytes were observed. These cells were most abundant in younger larvae of *C. pomonella, S. cretica*, and *A. catalaunalis*, with their numbers decreasing as larval age increased. As larvae grew, prohemocytes appeared to contribute to granulocyte and plasmatocyte populations through mitosis (Yamashita and Iwabuki, 2001; Liu et al., 2013), explaining the higher abundance of granulocytes in fourth- and fifth-instar larvae. Granulocytes, present in small to large sizes, were easily identified by their granular cytoplasm, which facilitated their phagocytic activity (Ling *et al*., 2003). Granulocyte performance improves with an increased number of granules, and their surface lectin receptors play a critical role in identifying foreign agents.

Plasmatocytes were polymorphic, appearing in cylindrical, spindle, eye, star, and worm-like shapes. In *C. pomonella*, plasmatocytes exhibited diverse shapes and sizes, whereas in *S. cretica* and *A. catalaunalis*, they were primarily eye- and spindle-shaped. Their long, irregular shapes make them easily identifiable even without staining (Ling *et al*., 2005). This type of hemocyte can adhere to the surfaces of foreign agents using pseudopods (Ling *et al*., 2003). Like granulocytes, plasmatocytes were most abundant in the older larvae of all three insects. Together, plasmatocytes and granulocytes accounted for over 80% of hemocytes in fourth- and fifth-instar larvae, consistent with previous studies on the dominance of these hemocytes in Lepidoptera (Beetz *et al*., 2008). In *Conogethes punctiferalis* (Lepidoptera: Crambidae), the abundance of granulocytes was 64.8% and plasmatocytes 21.4% (Li *et al*., 2022). The combined percentage of these hemocytes in Zeuzera pyrina L. (Lep: Cossidae) was observed to exceed 70%. (Ajamhassani, 2019). Oenocytoids were round or egg-shaped and occurred at low to moderate abundance (typically 5–10%) in our observations of three pests, aligning with reports on other Lepidoptera (Andrade et al., 2010; Ling *et al*., 2003; Ajamhassani et al., 2023). Their abundance is typically lower than that of immunocytes. Spherulocytes, characterized by a large size, central nucleus, and pitted cytoplasm (Akai and Sato, 1973; Ling *et al*., 2003), were the least abundant hemocytes, with the lowest frequency observed in *C. pomonella*, *S. cretica*, and *A. catalaunalis*. DHC showed significant variation in hemocyte populations across the life stages of the tested insects.

In addition to DHC, HV, and THC are critical components of the hemogram. Fourth- and fifth-instar larvae, being larger and consuming more nutrients, had significantly higher hemolymph volumes than younger larvae and pupae (Gardiner and Strand, 2000). Since hemocytes constitute a large proportion of hemolymph, the increased hemolymph volume in older larvae corresponds to a higher THC. (Gupta, 1985). Female larvae of *Trichosia pubescens*, which are larger, have more hemolymph and hemocytes than males (Barracco and Cestari, 1984). Conversely, first-instar larvae had lower hemolymph volumes and THC due to limited feeding, while pupae, which do not feed, also exhibited lower values. Previous studies have demonstrated that the quality and quantity of nutrition significantly influence hemolymph volume and hemocyte density (Manjula *et al*.,

2020). Larger larvae, benefiting from higher concentrations of immunocytes and antimicrobial peptides, exhibit greater resistance to microbial agents.

In conclusion, this study identified five hemocyte types in the hemolymph of *C. pomonella*, *S. cretica*, and *A. catalaunalis*. Hemocyte density increased with larval age and size, with granulocytes and plasmatocytes being the most abundant hemocytes in larger larvae. This explains why younger larvae are more sensitive to environmental pollutants and pathogens. Introducing hemocytes and hemogram factors in these insects provides a foundation for further immunological studies. Understanding the physiology of these pests' circulatory and immune systems could facilitate research on how immune components interact with foreign agents in the hemolymph, contributing to pest management strategies and the development of microbial control methods.

## Conflicts of interest

The authors declare no conflicts of interest.

## Author contributions

Maryam Ajamhassani: conceptualization, data analysis, draft writing and editing, validation; Fatemeh Abdos and Mostafa Aghaei: investigation and references. All authors approved the final manuscript.

## Acknowledgments

The authors greatly appreciate Shahrood University of Technology for their scientific support

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# هموسیت‌های کرم سیب *Cydia pomonella* (Lep.: Tortricidae)، کرم ساقه‌خوار ذرت *Sesamia cretica* (Lep.: Noctuidae) و پروانه بذرخوار کنجد *Antigastra catalaunalis* (Lep.: Crambidae): مرفولوژی و هموگرام

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**چکیده:** مطالعه فیزیولوژی گردش خون در حشرات با بررسی مرفولوژی هموسیتها و هموگرام آغاز می‌شود. هموسیتها اجزای مهم و حیاتی در پاسخ‌های دفاع فیزیولوژیک محسوب می‌شوند. این تحقیق، پروفایل هموسیتها و هموگرام مراحل زیستی سه آفت مهم کرم سیب *Cydia pomonella* L. (Lep: Tortricidae)، کرم ساقه‌خوار ذرت *Sesamia cretica* Lederer (Lep: Noctuidae) و پروانه بذرخوار کنجد *Antigastra catalaunalis* Duponchel (Lep: Crambidae) را مورد بررسی قرار می‌دهد. میوه‌های سیب و گیاهان ذرت و کنجد آلوده به لاروهای آفات جمع‌آوری و به آزمایشگاه منتقل شدند. لاروهای سنین چهار و پنج از بافت‌های گیاهی خارج شد. همولنف لاروها استخراج و پس از رنگ‌آمیزی آن‌ها با گیمسا، شناسایی با میکروسکوپ نوری انجام شد. پنج تیپ هموسیت شامل پروهموسیتها، پلاسماتوسیتها، گرانولوسیتها، اونوسیتوئیدها و اسفرولوسیتها در همه حشرات مورد آزمایش شناسایی شدند. هموگرام شامل محاسبه شمارش تفرقی، شمارش کل هموسیتها و حجم همولنف در همه مراحل زیستی حشرات بود. در لاروهای سنین چهار و پنج در سه آفت، پلاسماتوسیتها و گرانولوسیتها بیشترین فراوانی و حدود ۸۰% تراکم هموسیتها را شامل شدند. در مقابل، پروهموسیتها بالاترین فراوانی را در لاروهای سن اول به میزان ۳۲-۲۸% داشتند. حجم همولنف و تعداد کل هموسیتها در لاروهای سنین چهار و پنج حشرات بالاترین بود و نشان‌دهنده ارتباط مستقیم بین وزن لاروی، حجم همولنف و تعداد هموسیتها بود. مطالعه حاضر، اولین گزارش از شناسایی هموسیتها و هموگرام از کرم سیب، ساقه‌خوار ذرت و بذرخوار کنجد است که می‌تواند مقدمه‌ای برای تحقیقات بعدی در زمینه مطالعات ایمنی‌شناسی باشد.

**واژگان کلیدی:** کرم سیب، کرم ساقه‌خوار ذرت، کرم بذرخوار کنجد، پروفایل هموسیتها، فراوانی هموسیتها