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Effects of Starvation, Dietary Regimes, and Temperature Stress on Hemocyte Profiles and Phenoloxidase Activity in Larvae of *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae)

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Abstract

This study examines changes in hemocyte profiles and phenoloxidase activity in larvae of *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) under conditions of starvation, dietary variation, and thermal stress in laboratory settings. *T. absoluta*-infected tomato fruits were collected from fields and transported to laboratory, where larvae were extracted from fruits after two generations of rearing, Hemolymph was extracted with a capillary tube and placed on a slide. Hemocytes were identified through Giemsa staining and observed under light microscopy at 40× magnification. Starvation stress was induced for 12 and 24 hours while the control group remained unstressed. Hemocyte counts were determined using a hemocytometer under light microscopy at 40× magnification. Starved larvae exhibited significantly reduced total hemocyte counts, plasmatocytes, and granulocytes compared to the control group. Larvae reared on eight tomato varieties (Superchef, Basimo, Hartiva, Berantta, Breivio, Gs15, 1012, and 8320) displayed variable hemocyte densities, with the highest counts observed in those fed on Superchef and Gs15 cultivars. For thermal stress experiments, third- and fourth-instar larvae were exposed to 28°C and 4°C for 12 and 24 hours. Control groups for third and fourth instar larvae maintained at 25 ± 1°C. In total hemocyte and granulocyte densities were significantly reduced across all thermal treatments compared to controls. Plasmatocyte counts in third-instar larvae subjected to 12 hours of heat stress (327.5±18 cell/mm^3 hemolymph) and cold stress (320±34.3 cell/mm^3 hemolymph) were higher than those in control (294.3±23.3 cell/mm^3 hemolymph). Phenoloxidase activity exhibited a direct correlation with hemocyte alterations across all experimental conditions. This study provides a foundation for further investigations into the pest's physiological defense mechanisms.

Keywords: *Tuta absoluta*, hemocyte, food deprivation, nutrition, thermal stress.

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Introduction

The tomato leaf miner, *Tuta absoluta* (Meyrick, 1994) (Lepidoptera: Gelechiidae), is a highly destructive and globally pest of tomato and solanaceous plants (Ferracini *et al*., 2012). In Iran, *T. absoluta* was classified as a quarantine pest until its initial detection in West Azarbaijan Province in 2010, after which it rapidly dispersed throughout the country (Gharekhani and Salek, 2014). The larvae invade stems, leaves, terminal buds, and fruits, mining mesophyll tissues between the epidermal layers. This feeding behavior significantly reduces photosynthetic surface area, impairing plant growth and yield (Desneux *et al*., 2011).

Immunity in insects includes cellular and humoral immunities (Beckage, 2007; Vengateswari *et al*., 2020). Hemocytes constitute the primary components of cellular immunity, exhibiting changes in morphology, type, number, phagocytic activity, and nodulation in response to foreign agents (Strand, 2008). In contrast, humoral immune responses emerge several hours post-infection (Zhong *et al.*, 2017). Phenol oxidase and antimicrobial peptides play crucial roles in humoral immunity. Phenol oxidase, secreted by malpighian tubules and epidermal cells, becomes activated during defense responses, leading to the melanization of foreign agents and their sequestration through quinone secretion. Melanin also binds to pathogens, immobilizing them and enhancing their susceptibility to host defense mechanisms, including phagocytosis and encapsulation (Cerenius & Söderhäll, 2004; Castillo *et al*., 2006). Multiple factors influence insect immune responses, including diet, food deprivation, environmental changes, and hemolymph contamination (Mowlds *et al*., 2008; Strand, 2008). The impact of diet on immunological responses relates to the quality and quantity of macromolecules. Energy derived from macromolecules plays a vital role in insect growth, metabolism, reproduction, survival, and immunity (Triggs and Knell, 2011; Kang *et al*., 2011). Insects subjected to low-quality diets or short- and long-term starvation often exhibit prolonged development, reduced reproductive rates, and altered longevity. Dietary deprivation also diminishes hemocyte density, weakens immune responses, and decreased resistance mechanisms to pathogens invasion (Stączek *et al*., 2020; Siva-Jothy and Thompson, 2002). For instance, low-protein diets adversely affected immunity in bumblebees (Roger *et al*., 2017). In damselfly larvae *Coenagrion puella* (L.), Odonata: Coenagriidae), one week of starvation led to a 10% reduction in weight compared to controls, lower male emergence rates, and significantly reduced hemocyte density and phenoloxidase activity in both sexes (Campero *et al*., 2008).

Temperature fluctuations also significantly impact insect physiology, as insects typically develop and reproduce within narrow temperature ranges (Chown and Nicolson, 2004).

Environmental temperature changes affect body water content, osmolality, hemolymph volume, hemocyte density, and morphology (Lubawy and Slocińska, 2020). For instance, in *Dacus ciliatus* Loew (Diptera: Tephritidae) larvae, heat (30°C) and cold (4°C) stress increased total hemocyte counts (THC), but cold stress reduced granulocyte and plasmatocyte (Ajamhassani *et al*., 2024). Similarly, in *Danaus chrysippus* (L.) (Lepidoptera: Danaidae), cold stress reduced hemocyte counts, while heat stress increased them (Pandey *et al*., 2008). Temperature stress has caused hemocyte morphological changes, nuclear division anomalies, and cell wall ruptures, further highlighting its profound effects on insect immunity (Ghasemi *et al*., 2013).

Understanding hemocyte morphology is a foundational step in insect immunology research (Zibaee and Malagoli, 2014). Investigating the effects of food deprivation and temperature stress on hemocyte dynamics enhances provide a basis for understanding interactions between insect immune systems and biological, microbial, and chemical control agents (Lubawy and Sticinska, 2020). The tomato leaf miner is a significant pest of tomatoes across various climatic regions of Iran, affecting both greenhouse and field-grown crops. Different tomato varieties exhibit varying degrees of resistance or susceptibility to this pest, influenced by trichome morphology, plant volatiles, structural traits, and nitrogen content in leaves and fruits. These factors and temperature fluctuations have been shown to impact the pest's development, fecundity, and survival (Ghaderi *et al*., 2017; Rostami *et al*., 2017; Coqueret *et al*., 2017). Accordingly, this study aims to identify hemocyte types and evaluate the effects of food deprivation and temperature stress on hemocyte density and phenoloxidase activity in *T. absoluta* larvae.

Materials and Methods

Insect rearing

Infected tomato fruits (Gs15 cultivar) were collected from tomato fields in Miami County (36°24′54″North, 55°39′42″East), Semnan Province, Iran. The fruits were transferred to a laboratory growth chamber maintained under controlled conditions: temperature 25 ± 1°C, relative humidity 50%, and a photoperiod of 14:10 (light: dark) hours. Larvae were identified at different instars based on Dyar's rule (Dyar, 1980). Developmental stages of *T. absoluta* was showed in Figure 1. Using forceps, larvae were carefully extracted from the infected fruits and placed on fresh, healthy tomato fruits. After two generations of rearing, the third and fourth instar larvae were selected for experiments. Rearing was conducted in plastic containers (30

cm length × 30 cm width × 25 cm height) covered with white organza mesh to ensure ventilation. As the fruits began to decay, larvae were transferred to fresh, healthy tomatoes to support continuous development (Krechemer and Foerster, 2015).

Hemocyte identification and determination of hemocyte frequency

Hemocytes were identified using a procedure described by Gupta, 1991 and Jones, 1962. The ventral surface of the larval body was punctured with a sterile needle, and hemolymph was collected using a capillary tube and placed onto a microscope slide. Hemocytes were stained with Giemsa solution (Merck KGaA, Germany) for 10 minutes. The stain was then rinsed off, and the slides were observed under a BH2 light microscope at 40× magnification (Yeager, 1945; Gupta, 1991). After staining, the abundance of hemocytes was quantified in second, third, and fourth instar larvae and pupae. One hundred hemocytes were randomly selected at 40× magnification and differentially counted using an Olympus BH2 microscope. For each developmental stage, 25 samples were examined.

Effects of starvation on total hemocytes, plasmatocytes and granulocytes

Fourth instar larvae of *T. absoluta* were subjected to starvation stress for 12 and 24 hours. The experiment consisted of three treatments: a control group (larvae feeding within fruits) and two starved groups (12-hour and 24-hour starvation). Each treatment included six replicates, with hemolymph extracted from four larvae per replicate (4 µL). The extracted hemolymph was mixed with 24 µL of Tyson buffer as an anticoagulant solution containing methyl violet (0.06 mM), glycerol (43 mM), sodium chloride ($NaCl_2$, 72 mM), sodium sulfate (Na_2SO_4, 9 mM), and distilled water (250 mL). (The Chemical compounds were obtained from Merck, Germany) The hemolymph-Tyson buffer mixture was loaded onto a Neubauer hemocytometer (HBG, Germany) for analysis. THC, plasmatocyte count, and granulocyte count were determined under a light microscope at 40× magnification using Jones' formula (Jones, 1967).

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

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

Effect of tomato cultivars on total hemocytes, plasmatocytes and granulocytes

Eight tomato cultivars: Superchef, Basimo, Hartiva, Berantta, Breivio (prepared from Yekan Bazr company, Iran) Gs15, 1012, and 8320 (obtained from Golsam company, Iran) were used in this experiment. Newly emerged adults of *T. absoluta* were allowed to mate and oviposit on

each cultivar. Fourth-instar larvae reared on these cultivars were subsequently used for immunological assessments. As in previous experiments, each treatment included six replicates, with hemolymph collected from four larvae per replicate (4 µL). The hemolymph was mixed with 24 µL of Tyson buffer solution. THC, granulocyte density, and plasmatocyte density was recorded.

Effect of temperature stress on total hemocytes, plasmatocytes and granulocytes

Third and fourth instar larvae of *T. absoluta* were used in this experiment, which included ten treatments: control groups for third and fourth instar larvae maintained at 25 ± 1°C, larvae exposed to heat stress at 28°C for 12 and 24 hours (both instars), and larvae exposed to cold stress at 4°C for 12 and 24 hours (both instars). Each treatment consisted of six replicates, with hemolymph collected from four larvae per replicate (4 µL). The hemolymph was mixed with 24 µL of Tyson buffer solution. Hemocyte counts, including THC, were performed using a Neubauer hemocytometer.

Effect of temperature stress on hemocyte morphology

For this experiment, hemolymph samples from larvae subjected to heat and cold stress were analyzed, with 40 larvae included in each temperature stress treatment. Hemocytes were examined under a microscope to assess plasmatocytes and granulocytes for signs of cellular wall wrinkling, ruptures, or nuclear divisions. After the analysis, the percentage of damaged cells was calculated for each type of temperature stress.

Effect of starvation and temperature stress on phenoloxidase activity

The hemocyte lysate method assessed the effects of starvation periods and temperature stress on phenoloxidase activity in *T. absoluta* larvae (Leonard *et al.*, 1985). Larval rearing conditions in this experiment were consistent with previous studies. Fourth-instar larvae were used to assess the effects of starvation. The experimental treatments included three groups: larvae subjected to starvation for 12 and 24 hours and a control group. Each treatment consisted of 40 replicates (larvae), with the hemolymph from each replicate pooled together. In the experiment examining temperature stress, fourth-instar larvae were similarly used. The control group was maintained at 25 ± 1°C, while treatment groups were exposed to heat stress at 28°C for 12 and 24 hours or cold stress at 4°C for the same duration. Each treatment included 40 replicates. Hemolymph from each treatment group was collected separately and centrifuged at 10,000 rpm for 5 minutes. After removing the supernatant, 100 µL of phosphate buffer (pH 7) was added

to the pellet homogenized. The homogenized solution was centrifuged again at 12,000 rpm for 15 minutes, and the resulting supernatant was used for enzymatic analysis. To estimate enzyme activity, 25 µL of each sample was mixed with 50 µL of a 10 mM solution of L-DOPA (L-dihydroxyphenylalanine) and 50 µL of phosphate buffer. The mixture was incubated at 30°C for 5 minutes and analyzed using an ELISA reader (Model ELX800, BioTek, USA) at a wavelength of 490 nm. unit of phenoloxidase activity is µmol min^{-1} mg $protein^{-1}$.

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

Figure 2 illustrates the types of hemocytes identified in the fourth instar larvae of *T. absoluta*. The hemocyte types and their morphological characteristics are summarized in Table 1.

Prohemocytes were the smallest hemocytes, round in shape, with prominent nuclei. The cytoplasmic area was minimal, extending along the cell wall margin. The highest abundance of prohemocytes was observed in first instar larvae (26.5 ± 2%), with numbers decreasing in subsequent developmental stages (Table 2).

Plasmatocytes were medium-sized cells, often with one or two projections, and occasionally oval in appearance. The nuclei were typically centrally located and stained darker with Giemsa than the cytoplasm. Plasmatocyte frequency was highest in third (23.2±0.7%) and fourth instar larvae (22.1±1.5%).

Granulocytes varied in size, ranging from small to medium, and contained numerous granules in their cytoplasm. These hemocytes were the most abundant cell type across all larval instars, with their population peaking in the fourth instar (50.2 ± 2.5%) (Table 2).

Oenocytoids were circular cells with large peripheral nuclei. They were larger than prohemocytes and the same size as granulocytes and plasmatocytes. The frequency of oenocytoids was lower than that of plasmatocytes and granulocytes across the developmental stages.

Spherulocytes were rarely observed. These cells had central nuclei with visible spherules on their cytoplasmic surface (Figure 2).

Effect of starvation stress on THC, plasmatocytes, granulocytes and phenoloxidase activity

The effect of starvation stress on THC (F = 171.5, $df_{t,e}$ = 2,15, $p \leq 0.0001$), plasmatocyte count (F = 94.5, $df_{t,e}$ = 2,15, $p \leq 0.0001$), and granulocyte count (F = 75.2, $df_{t,e}$ = 2,15, $p \leq 0.0001$) was significant (Table 3). THC decreased progressively with starvation, reducing to nearly half of the control group count (442 ± 32.2 cells/mm³ of hemolymph) after 12 hours of starvation. By 24 hours, the count declined to 118.16 ± 15 cells/mm³ (Table 3).

Plasmatocyte and granulocyte counts followed a similar pattern, with significant reductions observed after 12 hours of starvation, reaching 135.32 ± 12.6 cells/mm³ and 134 ± 15.5 cells/mm³, respectively. These counts continued to decline with prolonged starvation, showing further reductions by 24 hours (Table 3).

In addition to the decrease in hemocyte density, phenoloxidase activity in fourth instar larvae of *T. absoluta* also declined under starvation stress. After 12 hours of starvation, phenoloxidase activity dropped to 0.073 ± 0.004 µmol/min/mg protein, and after 24 hours, it decreased further to 0.055 ± 0.008 µmol/min/mg protein. Both values were significantly lower than the control group (Table 4).

Effect of tomato cultivars on THC, plasmatocytes and granulocytes

Feeding *T. absoluta* larvae on different tomato cultivars significantly influenced THC (F = 614.3, $df_{t,e}$ = 7,40, p ≤ 0.002), plasmatocyte count (F = 225.3, $df_{t,e}$ = 7,40, p ≤ 0.03), and granulocyte count (F = 277.3, $df_{t,e}$ = 7,40, $p \leq 0.000$) (Table 5). The highest THC was recorded in larvae reared on the Superchef cultivar (1188 ± 64.5 cells/mm³ of hemolymph), while the lowest THC was observed in larvae fed on the Breivio cultivar (735 ± 34.7 cells/mm³ of hemolymph). Larvae fed on Superchef and Gs15 cultivars exhibited the highest plasmatocyte and granulocyte count values. In contrast, larvae reared on the Breivio cultivar showed the lowest frequency of these hemocyte types compared to larvae fed on other cultivars (Table 5).

Effect of temperature stress on THC, plasmatocytes, granulocytes, and phenoloxidase activity

Temperature stress, including heat and cold, significantly influenced THC (F = 90.4, $df_{t,e}$ = 9,157, p ≤ 0.002) and plasmatocyte and granulocyte counts in *T. absoluta*. All treatments showed a reduction in THC and granulocyte counts compared to the control groups. The most significant decreases in THC were observed in fourth- and third-instar larvae subjected to 24

hours of heat stress, with counts of 192.6 ± 4.5 cells/mm³ and 243 ± 8.8 cells/mm³, respectively, indicating that heat stress had a more pronounced impact on hemocyte reduction than cold stress (Table 6).

The lowest granulocyte counts were recorded in third- and fourth-instar larvae exposed to 24 hours of heat stress, as well as in third-instar larvae subjected to 24 hours of cold stress ($F = 78.5$, $df_{t.e} = 9,157$, $p \leq 0.0001$). Plasmatocyte counts, however, displayed a slightly different trend. While fourth-instar larvae exposed to 24 hours of temperature stress exhibited the lowest plasmatocyte counts across all treatments, third-instar larvae subjected to 12 hours of heat or cold stress showed higher plasmatocyte counts than their respective control groups. This suggests that plasmatocyte numbers temporarily increase under short-term (12-hour) temperature stress, but decline with prolonged exposure (24 hours), aligning with the trends observed in other treatments (Table 6) ($F = 121.4$, $df_{t,e} = 9,157$, $p \leq 0.0001$).

Temperature stress also significantly reduced phenoloxidase activity in fourth-instar larvae. After 24 hours of heat stress, phenoloxidase activity decreased to 0.058 ± 0.003 µmol/min/mg protein, while cold stress reduced activity to 0.066 ± 0.005 µmol/min/mg protein. These levels represented approximately half the enzymatic activity observed in the control group (Table 7). In third-instar larvae, phenoloxidase activity was similarly reduced under temperature stress, with cold stress causing a more pronounced inhibitory effect on enzyme activity than heat stress (Table 7).

Effect of temperature stress on hemocyte morphology

Temperature stress-induced significant morphological changes in the hemocytes of *T. absoluta*, particularly in granulocytes and plasmatocytes (Figure 3). Under heat stress, approximately 27% of granulocytes and 18% of plasmatocytes exhibited cell wall wrinkling (Figure 4). In contrast, cold stress had a more pronounced effect on granulocyte morphology, with approximately 70% of granulocytes displaying severe wrinkling, the most notable morphological alteration observed under cold conditions (Figure 4). Cold stress also caused approximately 10% wrinkling in plasmatocytes and induced granulocyte nuclear divisions.

Discussion

The insect circulatory system is vital in transporting nutrients, metabolites, hormones, water, and ions. Hemolymph is a medium for carrying waste products and toxins to the Malpighian tubules, acting as a final defense barrier against stresses and infections (Sinclare *et al.*, 2015). Hemocytes are the primary cellular components of the insect's physiological defense system.

These cells are synthesized continuously in hematopoietic organs, replacing aged or damaged cells, a process critical for maintaining hemostasis (Nakahara *et al*., 2003).

In the hemolymph of *T. absoluta* larvae, five types of hemocytes were identified: prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes. Another form of hemocyte, adipohemocytes, has been observed in the hemolymph of *T. absoluta* adult (Maingi *et al*., 2023), but it was absent in the hemolymph of larvae. Similar hemocyte classifications have been reported in various insects, particularly in Lepidoptera (Liu *et al*., 2013; Blanco *et al*., 2017, Ajamhassani, 2021; Gogoi *et al*., 2023). Our findings indicated that the size and frequency of hemocytes in the larval hemolymph of the tomato leaf miner were lower than those in adult hemolymph regarding to Maingi *et al*., (2023), potentially due to genetic factors, nutritional regimes, temperature variations, and climate differences. (Mason *et al*., 2014). On the other hand, the abundance of hemocytes in insects is diverse and even these differences were documented depending on the developmental stage and gender in one species. It seems that nutrition, hormonal changes and antimicrobial peptides during growth can also affect the variation of hemocyte density (Shapiro, 1979). This variability suggests that there is no same hemocyte pattern within this order (Bruno *et al*., 2022). Usually, the abundance of granulocytes and plasmatocytes as the main cells participating in the immune processes in the late instar larvae of Lepidoptera are more than other hemocytes (Kholghahmadi *et al*., 2025). Our finding also confirmed that the granulocytes and plasmatocyte counts were are the highest in hemolymph of third and fourth instar larvae of *T. absoluta*.

Hemocytes morphology and abundance changed in response to food deprivation, dietary modifications, and temperature stress similar to finding of Carper *et al*., 2019 and Ayres, 2024. Starvation and dehydration affect insect growth, survival, longevity, reproduction, movement, and adaptability, depleting the energy required for these processes (Chapman, 2013). Our findings indicate that the circulatory system of *T. absoluta* is susceptible to food deprivation, even over short periods. Starvation for 12 and 24 hours significantly reduced plasmatocytes, granulocytes, and phenoloxidase activity in the hemolymph of *T. absoluta* larvae. This reduction may be explained by hemocytes exiting circulation and adhering to the body wall, decreasing their numbers in the hemolymph. In fact, reduced digestion and nutrient absorption due to malnutrition likely affect circulatory system physiology, causing hemocytes to migrate from the bloodstream to the body wall until refeeding occurs. Similar observations have been reported in *Galleria mellonella* Fabricius (Lepidoptera: Pyralidae) and *Malacosoma pluvial* Dyar (Lepidoptera: Lsiaocampidae) larvae, where food deprivation reduced hemocyte density

and phenoloxidase activity (Banville *et al*., 2012; Myers *et al*., 2011). Siva-Jothy and Thompson (2002) reported that starvation significantly reduces the hemocyte population in the hemolymph of both male and female *Plodia interpunctella* despite the presence and maintenance of relatively large fat reserves. The study found that phenoloxidase activity increased soon after food became available. This finding suggests that maintaining high phenoloxidase activity is metabolically costly, explaining its lower levels during periods of food limitation. Based on reports, that starvation weakens insect immunity, potentially increasing the pest's susceptibility to microbial and chemical control methods (Lord, 2010; Zhu *et al*., 2012).

In examining the effects of dietary regimes on *T. absoluta* immune responses, our results showed that larvae fed on the Superchef and Gs15 cultivars exhibited significantly higher hemocyte counts and phenoloxidase activity compared to larvae fed on other cultivars. This underscores the influence of diet on hemocyte dynamics and immune function. However, the specific quantities of macromolecules (e.g., carbohydrates, proteins, and lipids) in mentioned varieties remain unknown and warrant further investigation (Littlefair and Knell, 2016). Nutritionally richer diets, significantly those rich in carbohydrates and proteins, enhance insect immune responses and physiological functions (Vogelweith *et al*., 2016). Insects feeding on nutrient-dense resources display higher hemocyte densities and phenoloxidase activity, while poor-quality diets reduce immune capacity and increase pathogen susceptibility (Manjula *et al*., 2020). Our findings suggest that Superchef and Gs15 are more palatable cultivars for *T. absoluta* larvae, likely due to their nutritional composition. Additionally, fruit size, physical structure, firmness and plant volatile may influence feeding efficiency, hemolymph volume, and hemocyte density. Based on our observation the fruits of the Superchef variety have thin skin, whereas Gs15 fruits are larger and juicier, making Superchef more susceptible to larval penetration than other varieties. In contrast, the superior nutritional quality of larger fruits may significantly influence larval weight and, consequently, the density of circulating hemocytes (Kholghahmadi *et al*., 2025). Mirhosseini *et al*., (2022) reported that tomato cultivars vary in their suitability for the survival and development of the tomato leaf miner. The role of secondary metabolites, such as alkaloids, in pest feeding should not be overlooked, as many of these compounds contribute to host plant resistance against pests (Veyrat *et al*., 2016). Additionally, the resistance of certain tomato varieties to leaf miners, such as *T. absoluta*, is likely influenced by nutritional availability and larval physiological characteristics, including

immune factors. Supporting this, Venjateswari *et al*., 2020 and Ajamhassani *et al*., 2023 highlighted the critical role of diet in the immunological and physiological responses of insects. Temperature is another critical factor influencing insect growth, fecundity, dispersal, and survival (Klepsatel *et al*., 2019). Polyols and lipids in hemolymph increase during cold exposure, preventing freezing (Goodhead and MacMillan, 2017), while specific genes are expressed under high temperatures to maintain protein structure and prevent denaturation (Nyamukondiwa *et al*., 2010). Temperature stress also affects hemocyte structure, morphology, and abundance, central components of insect immunity. For example, hemocytes of *Gromphadorhina coquereliana* exposed to 4°C were smaller than those in control insects (Lubawy and Stocinska, 2020). Heat stress in *Antherarea mylitta* resulted in compacted cytoplasmic projections in plasmatocytes, vacuolization in plasmatocytes and granulocytes, nuclear fragmentation in prohemocytes, and, in some cases, cell death at 42°C (Pandey *et al*., 2010).

Our findings revealed that temperature stress similarly impacted *T. absoluta* hemocyte profiles. THCs and granulocyte numbers significantly decreased in all stress treatments compared to controls. Interestingly, plasmatocyte counts in third-instar larvae exposed to 12 hours of heat or cold stress were higher than in control groups. However, prolonged exposure (24 hours) led to a decline, aligning with the trends observed in other hemocyte types. The high proportion of disintegrated granulocytes and plasmatocytes under temperature stress suggests that these cells became compacted, leading to cell wall rupture and eventual death. Figures 3 and 4 show that many immunocytes, particularly granulocytes, shrank and disintegrated under cold and heat stress. Consequently, these hemocytes were no longer detectable in circulating hemolymph. Similarly, Maingi *et al*., (2023) revealed that when *T. absoluta* moths were treated with *Metarhizium anisopliae* and exposed to temperatures of 15–25°C, a significant reduction in total hemocyte counts (THCs) occurred. This effect may be attributed to the ability of *M. anisopliae* isolates to produce toxins that impair hemocyte viability or function (Maingi *et al*., 2023).

Temperature-induced variations in hemocyte abundance differ across insect species. Some studies have documented enhanced immune responses with increasing temperature (Laughton *et al*., 2017), whereas others have reported weakening certain immune functions, such as melanization (Ehrlich & Zuk, 2019). Generally, thermal effects on the immune system remain complex and unpredictable (Chaui-Berlinck et al., 2004). For instance, hemocytes of *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) increased significantly under heat

stress at 35°C, while cold stress at 4°C reduced hemocyte counts in *Nicrophorus vespilloides* Herbst (Coleoptera: Silphidae) (Pourali and Ajamhassani, 2018; Urbanski *et al*., 2017). Conversely, hemocyte counts in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) decreased under short-term heat stress (Herren *et al*., 2023).

Phenol oxidase activity and melanization responses also vary with temperature fluctuations. Our findings demonstrated that in *T. absoluta* larvae, phenol oxidase activity declined under both heat and cold stress, whereas in different populations of *Sepsis thoracica* (Robineau-Desvoidy) (Diptera: Sepsidae), phenol oxidase activity positively correlated with developmental temperature (Gourgoulianni *et al*., 2023). Similarly, *T. molitor* larvae maintained at 30°C exhibited increased phenol oxidase activity and antibacterial responses compared to those kept at 10°C or 20°C (Catalán *et al*., 2012). These findings underscore the complexity and diversity of cellular and humeral immune to thermal stress, highlighting the intricate relationship between temperature fluctuations and insect immunity.

Conclusions

This study demonstrated that starvation, dietary composition, and temperature fluctuations significantly affected the hemocyte profile and phenoloxidase activity of *T. absoluta*. The findings highlight the high sensitivity of *T. absoluta* to food deprivation, diet quality, and temperature stress. Stress conditions induced notable changes in the shape and abundance of hemocytes, emphasizing the variability in immune responses of *T. absoluta* larvae. To deepen our understanding of the immunological mechanisms of this pest, future research should focus on field-level studies and investigate the effects of prolonged starvation and extended exposure to temperature stress on hemocyte activity and detoxifying enzymes. Can temperature and climate fluctuations weaken an insect's immune system? Does feeding on resistant plant varieties influence an insect's immune responses to natural enemies or chemical compounds? Addressing these questions through further research will provide valuable insights into effective control measures and management strategies for *T. absoluta*.

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Table 1. Morphometric measurements of hemocytes larvae of *Tuta absoluta* (n=20).

Hemocyte type	Size (µm)	
	Length (mean±se)	Width (mean±se)
Prohemocyte	2.8±0.3	2.4±0.2
Plasmatocyte	6.7±2.5	2.8±0.5
Granulocyte	6±3.2	4.5±2.1
Oenocytoid	6.6±2.4	5.8±1.3
Spherulocyte	3.1±0.3	2±1.2

Table 2. Frequency of hemocytes in developmental stages of *Tuta absoluta*. (n=25)

Developmental stages	Hemocyte frequency (%)				
	Prohemocyte	Plasmatocyte	Granulocyte	Oenocytoid	Spherulocyte
Second instar larvae	26.5±2a	18.7±0.4c	40±2.7c	15.5±0.35b	-
Third instar larvae	24.8±2.4a	23.2±0.7a	45.4±2.2b	6.4±0.2d	1.2±0.2
Fourth instar larvae	17.2±1b	22.1±1.5a	50.2±2.5a	10.2±1.4c	-
Pupa	15.2±1b	20±2.5b	46±1.7b	18.2±1.1a	-

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

Table 3. Effect of starvation period on hemocyte number of fourth instar larvae of *Tuta absoluta*.

Hemocyte number (cell/mm^3)	Starvation period		
	Control	12h	24h
Total hemocyte	961.33±35.5a	442±32.2b	118.16±15c
Granulocyte	407.66±42.6a	134±15.5b	80±12.23c
Plasmatocyte	328.84±24.4a	135.32±12.6b	61.88±10.5c

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

Table 4. Effect of starvation period on phenoloxidase activity in fourth instar larvae of *Tuta absoluta*.

Phenoloxidase activity (µmol min^{-1}mg protein^{-1})	Starvation period		
	Control	12h	24h
	0.11±0.02a	0.073±0.004b	0.055±0.008c

Different letters in the row show significance using Tukey's test at $p< 0.05$

Table 5. Effect of tomato cultivar on hemocyte number of fourth instar larvae of *Tuta absoluta*.

Cultivar	Hemocyte number (cell/mm^3)		
	Total hemocyte	Granulocyte	Plasmatocyte
Gs15	948±24.7b	415±18.6b	338±13.67b
Superchef	1188±64.5a	603±42.2a	482±34.6a
Breivio	735±34.7d	312±14.4d	221±24.4d
Basimo	880±17.7c	388±15.55b	277±12.2c
Berantta	910±32bc	355±20.3bc	273±30c
Hartiva	861±28cd	390±21.2b	290±27.5c
1012	921±21.6bc	344±28.4c	287±24.5c
8320	870±25cd	350±18.8bc	267±10c

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

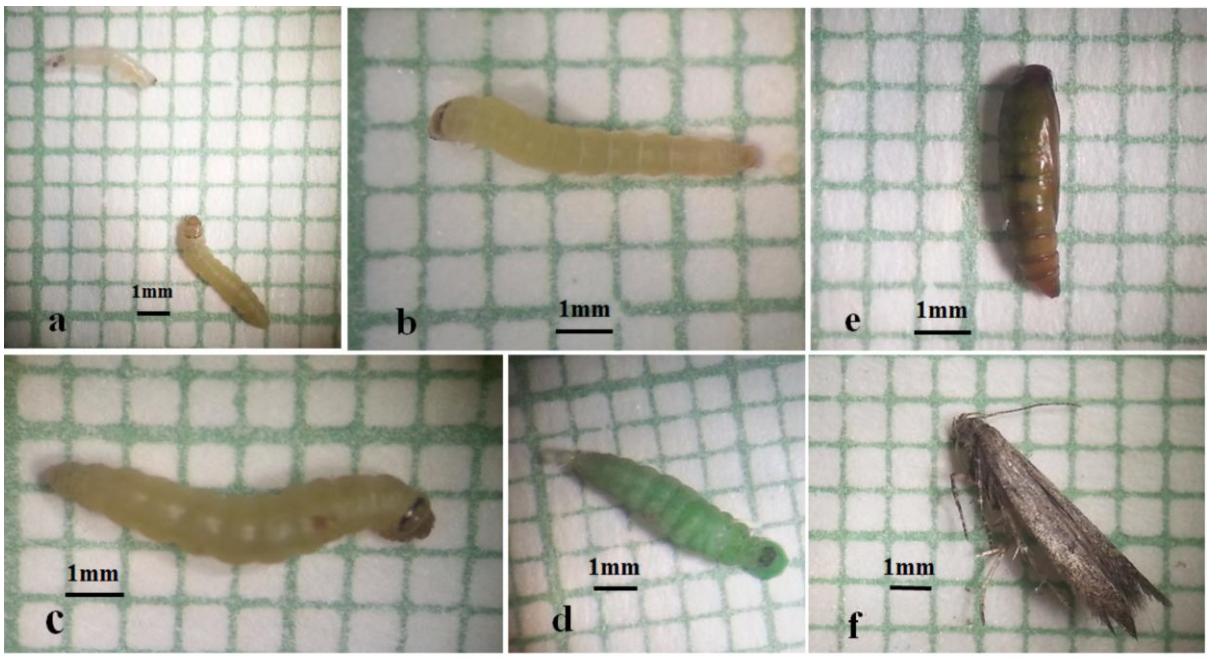
584 **Table 6**. Effect of thermal stress on hemocyte number of third and fourth instar larvae of *Tuta*
585 *absoluta*.

Treatment	Hemocyte number (cell/mm^3)		
	Total hemocyte	Granulocyte	Plasmatocyte
Third instar larvae (Control)	782±31.5b	358.3±42b	294.3±23.3ab
Fourth instar larvae (Control)	959±55.3a	468.8±33.6a	327.3±31.5a
Third instar larvae (heat stress 12 h)	712±21.4c	264.5±25.3c	327.5±18a
Third instar larvae (heat stress 24 h)	243.1±15g	115.8±22.3f	103±17.7e
Fourth instar larvae (heat stress 12 h)	404.5±37.8e	201.8±31.4d	175.5±32.5c
Fourth instar larvae (heat stress 24 h)	192.3±32.2	95±16.7g	73.2±7.4f
Third instar larvae (cold stress 12 h)	702.8±34.4cd	297.4±15.5bc	320±34.3a
Third instar larvae (cold stress 24 h)	331±23.7f	104±12.2g	141.3±14.3d
Fourth instar larvae (cold stress 12 h)	648±43.3d	308.6±25.5bc	281.5±33b
Fourth instar larvae (cold stress 24 h)	360.8±27.3ef	174.5±34.5e	141.6±10.5d

586 Different letters in each column show significance using Tukey's test at $p < 0.05$
587
588 **Table 7**. Effect of thermal stress on phenoloxidase activity in third and fourth instar larvae of
589 *Tuta absoluta*.

Larval stages	Phenoloxidase activity (µmol min^{-1}mg protein^{-1}) in different temperature (°C)		
	Control (25±1)	4	28
Third instar larvae	0.101±0.002a	0.022±0.001c	0.053±0.003b
Fourth instar larvae	0.134±0.003a	0.066±0.005b	0.058±0.003b

590 Different letters in each row show significance using Tukey's test at $p < 0.05$
591



592
593 **Figure 1**. Developmental stages of *Tuta absoluta*, a) first and second instar larva, b) third instar
594 larva, c) fourth instar larva, d) prepupa, e) pupa, f) adult (original photo).
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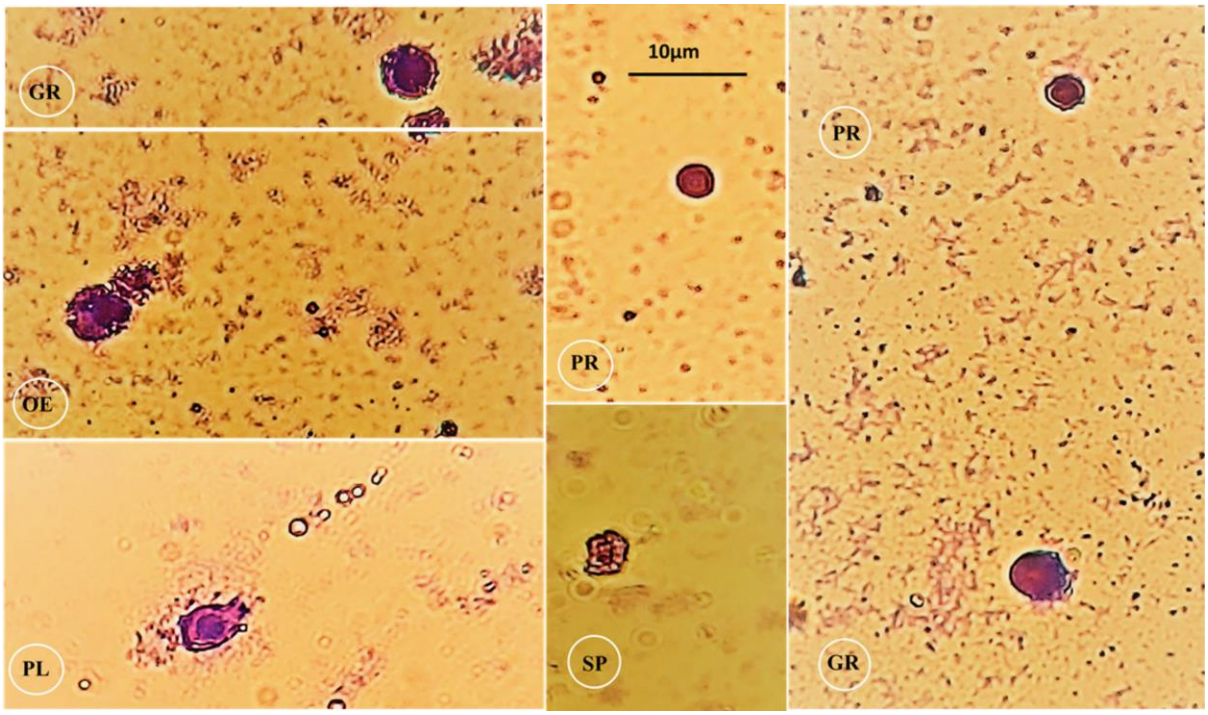

Figure 2. Light microscopy pictures of *Tuta absoluta* hemocytes stained with Giemsa. PR (Prohemocyte), PL (Plasmatocyte), OE (Oneocytoid), GR (Granulocyte), SP (Spherulocyte), Scale bar = 10 µm.

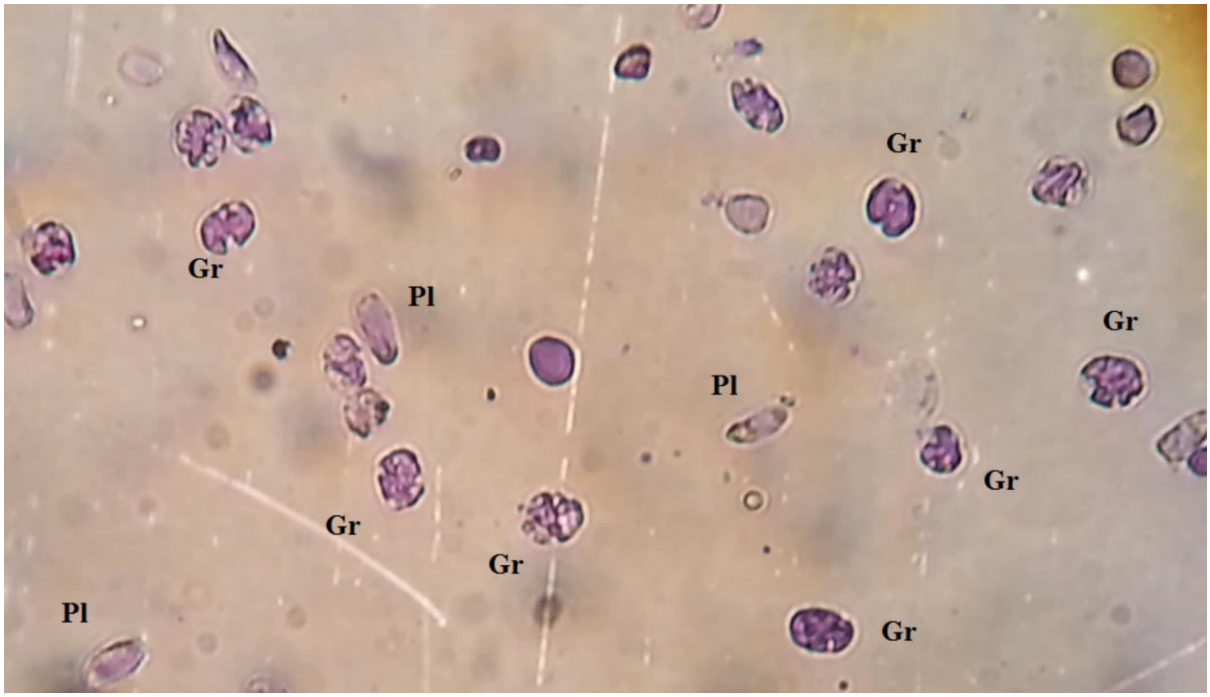

Figure 3. Morphological changes of granulocytes and plasmatocytes of *Tuta absoluta* affected by cold stress.

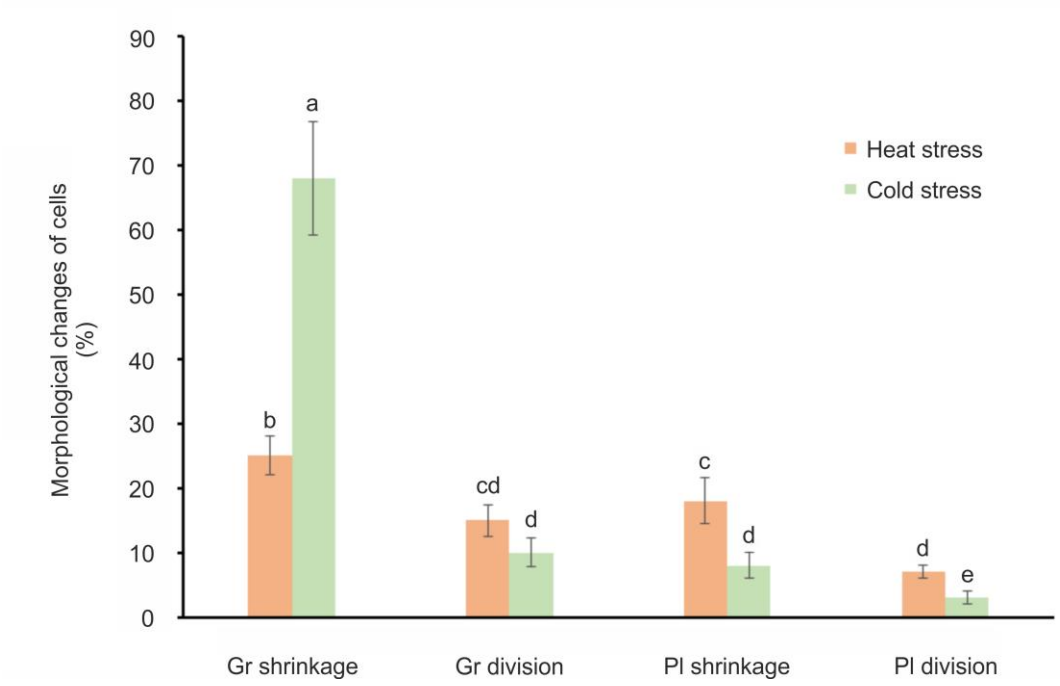

Figure 4. Hemocyte deformation percentage of *Tuta absoluta* affected by heat (28°C) and cold (4°C) stress.