

## Response of adult desert locust, *Schistocerca gregaria* towards *Bacillus thuringiensis israelensis* treatment

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### Abstract

The present study was designed to evaluate the effect of *Bacillus thuringiensis israelensis* (*Bti*) on the immune system of the adult desert locust, *Schistocerca gregaria* through quantitative estimation of some physical and biochemical parameters. Locusts were enhanced by injection of a sublethal dose of bacteria into the insect hemocoel at different time intervals. Results showed variable fluctuations in the body water content, hemolymph volume, density and pH after bacterial injection. Also, drastic changes in the concentrations of hemolymph carbohydrates, lipids and proteins were recorded. The protein banding pattern of the hemolymph documented certain variables as the induction of new proteins and the vanishing of others that may be retained to their contribution in the immune response towards the infection. Hemocytic examination by Transmission Electron Microscope (TEM) revealed 4 types of hemocytes; Prohemocytes (PRs), Plasmatocytes (PLs), Granular cells (GRs) and Coagulocytes (COs). Certain pathological effects were observed in the hemocyte populations following injection including vacuolization in the cytoplasm, distortion of the cell membrane and pyknosis in the nuclei. These outcomes confirm that *Bti* has potential role in the suppression of the desert locust immune system and can be applied in incorporation with the integrated pest management (IPM) strategies as a biocontrol agent.

**Keywords:** *Schistocerca gregaria*, *Bacillus thuringiensis israelensis*, insect hemocytes and hemolymph proteins

### Introduction

Insects have a well-developed innate immunity depends on encoded factors for recognition and killing of foreign invaders [16]. Insect hemolymph is very effective weapon in elimination of invaders from hemocoelic circulation by internal defense system that is comprised of cellular components and humoral mechanisms that cooperatively interact to destroy non-self-elements [23]. These defense reactions were observed against pathogens, parasites and other foreign bodies, which entered in the hemocoel [38]. The cellular defense mechanisms are achieved by hemocytes, that have the ability to discriminate non-self-agents, and mediate phagocytosis, encapsulation, wound repair and coagulation [30]. Thus, much interest is needed in describing the role of hemocytes in the defense mechanisms.

Insect hemocytes have been identified and classified using morphological, histochemical, functional characteristics and recently by the use of antigenic and molecular markers [27, 24]. Insect hemocytes, like vertebrate leucocytes are a mixture of cell types with different morphological and biological functions. It is difficult to classify insect hemocytes, according to the classification scheme for vertebrate white blood cells, into well-defined ontogenetic classes due to cell diversity as well as the limited knowledge of their development and differentiation [42]. Therefore, classification of insect hemocytes is a subject of controversy and the terminology used to designate each cell type is often different from one species to another. However, several literatures reviewed the insect hemocytes and suggested that there was some resemblance among most of the cell types and functions, in different insect species [10, 28]. Due to lack of adaptive immunity in invertebrates, lately the use of single-cell technologies have improved descriptions of 39

previously described cell types and have redefined cell complexity [45, 12, 41, 46, 25]. Used single-cell RNA sequencing to characterize 21 mosquito immune cells, signifying an increased complexity to previously Defined prohemocyte, oenocytoid, and granulocyte sub-types.

The desert locust, *Schistocerca gregaria* (Forsk.) represents a relatively important group of plant-feeding insects with strong immune responses against bacteria [36, 33]. To reduce the harmful effects of insecticides, alternative control strategies have been suggested [21, 15] including biopesticides like *Bacillus thuringiensis* (Bt).

Based on the success of *Bacillus thuringiensis* var. *israelensis* (*Bti*) as a bio-control agent in the Egyptian fields against Lepidoptera and Diptera, the present study deals with the interactions among herbivorous insect (*S. gregaria*) and bacterial pathogen (*Bti*). Bt, a spore-forming Gram-positive bacterium, present in soil, leaf litter, and the microflora on the surface of leaves, is widespread in nature [1]. It produces many kinds of insecticidal crystal proteins, including proteins which are toxic to lepidopterans, and are encoded by crystal (cry) and cytolytic (cyt) Genes [14]. Cry toxins, unlike most chemical insecticides, have a distinct mode of action that involves toxin solubilization, proteolytic activation in the midgut of the insect, and binding to larval midgut proteins. In the pore formation model, toxin binding results in the formation of pores in membranes and the lysis of cells in the midgut ultimately, resulting in the death of the insect [39, 51].

The present study forms a part of a wide study aims to gain information about *Schistocerca* immune response through quantitative determination of some hemolymph physical properties and some biochemical parameters. Also, characterization of the hemocyte types morphometrically to

give clearer image to the immune response of the infected Locusts towards the bacterial infection. In order to encourage the use of *Bti* as a biocontrol agent against this dangerous pest in combination with the programs of integrated pest management.

## Materials and Methods

### Insects

The Desert Locusts *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) was obtained from Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt. Rearing of the locusts was carried out in an insectarium in the Faculty of Science, Ain Shams University and had shown no infectious diseases, regulated at 30°C, 65% relative humidity, under a 12h light / 12h dark photoperiod. A wood stick was placed under the bulb to permit the vertical movement of nymphs, to cling, molt and stand close to electric lamps to regularize their body temperature [2]. All experiments outlined below were carried out with adult (both sexes) 2–4 days after ecdysis.

### Bacterial culture

The bacterium, *B. thuringiensis israelensis* (*Bti*) was produced by the Agricultural Genetic Engineering Research Institute (AGRI), Ministry of Agriculture, Egypt, and was grown aerobically at 28 ± 2°C in nutrient broth tubes for 48 h. Inoculates of the grown bacteria were cultured on nutrient agar plates at 28 ± 2°C for another 48 h. After growth, pure isolates were selected, cultured on nutrient agar slants and incubated at 28 ± 2°C for 48 h, and then kept in refrigerator at 4°C until used.

The grown bacteria were harvested by suspending in a physiological saline (0.5% NaCl) and centrifuged at 6000 rpm for 30 min. The sediment bacteria were washed several times with a sterile saline solution and centrifuged again at the same rate till the saline solution becomes completely clear.

### Injection technique

The bacterial suspension was adjusted to a sublethal concentration of 3.1 x 10<sup>4</sup> cells/ml. A dose 10 µl of this concentration was injected into each insect. Control insects were injected only with equivalent volumes of saline solution. Injection of insects was made with a 10 µl Hamilton micro-syringe fitted with a 26-gauge needle according [34].

### Estimation of body weight and water content

The body water content was determined in normal and treated locusts after 6, 12, 24 and 48 h according to the method of [29]. The body weight of locusts was determined gravimetrically for each individual insect. The body water content of locusts was determined as the difference between fresh (total) body weight and body weight after drying for 2–3 days at 80 °C in an oven to constant weight (dry weight). The measurements were replicated 10 times.

### Hemolymph collection for Physical properties

Normal and bacterial injected locusts after 6, 12, 24 and 48 h, along with saline-injected controls were collected and weighed individually. Locusts were submerged in hot water bath at 60 °C for 2–5 min. The heat-killed insects were amputated at the arthropodial membrane of the hind coxa

with fine scissors. Gentle pressure was applied to the thorax until a drop of hemolymph appeared at the point of amputation. All the subsequent measurements were accomplished at 28 ± 2 °C and samples were replicated times at each time interval.

### Determination of hemolymph volume

The hemolymph volume HV (µl/insect) was determined by the amaranth red dye method described by [53] and modified by [29] using amaranth red dye (20 mg/ml of 0.5% NaCl). The optical density was recorded at 515 nm using 1 ml cuvette against the standard solution (prepared by diluting 10 µl of the dye solution in 1 ml of saline solution) to construct a standard calibration curve.

### Assessment of Hemolymph density and pH

The hemolymph densities (mg/µl) were determined following the method described by [11] by using micro-capillary tubes calibrated at 1 µl and pre-weighed using an electronic balance.

The hemolymph pH was determined according to the method described by [15] and using the bulb of the microelectrode (Model 671, pH meter, Exttech., USA).

### Hemolymph collection for the Biochemical parameters

Hemolymph of normal and treated locusts were drowned out after 6, 12, 24 and 48 h from the coxal joint into Eppendorf tube containing traces of phenylthiourea and then diluted 5× with saline solution 0.5%. The hemolymph samples were then centrifuged at 2000 rpm for 5 min, and only the supernatant fractions were used for assays directly or frozen until use.

### Estimation of the total Hemolymph Carbohydrates

Total carbohydrate content in hemolymph was quantitatively estimated by using the Anthrone reagent according to [48] spectrophotometrically (UNICO Spectrophotometer, SP2100 UV, China) at 625 nm. The concentration of the carbohydrates (mg/ml) was calculated using the formula resulted from the standard calibration curve using glucose solution.

### Determination of the total Hemolymph Lipids

The total lipids content of the hemolymph was assessed using phosphorvanilin reagent according to the method of [17]. The standard and unknown samples were read against blank at 540 nm.

The total lipids content was estimated as mg/ml using the formula derived from the equation of the regression line obtained from the standard calibration curve using olive oil as a standard.

### Quantification of the total Hemolymph Protein

The total protein content of the hemolymph was quantified using Coomassie brilliant blue G-250 (CBB) by spectrophotometer where the absorbency was measured at 595 nm. According to the method designated by [8], a standard calibration curve was constructed using Bovine serum albumin (BSA) solutions as the standard proteins.

### Electrophoretic analysis of the total Hemolymph Protein

SDS polyacrylamide gel electrophoresis was carried out for the hemolymph proteins. According to the protocol mentioned by [26].

### Examination of the Hemocytes

For Transmission Electron Microscope (TEM) the hemolymph of normal and infected insects was collected as described above and fixed in 2.5% glutaraldehyde, 5 mM calcium chloride, and 2% sucrose in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. The samples were rinsed in 0.1 M cacodylate buffer (pH 7.2) containing 2% sucrose and post-fixed in 1% osmium tetroxide ( $O_8O_4$ ), 0.8% potassium ferrocyanide and 5 mM calcium chloride in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. They were then dehydrated in graded acetone and embedded in PolyBed 812 epoxy resin. Ultrathin sections obtained using a Leica ultramicrotome (Nussloch, Germany) were stained with uranyl acetate and lead citrate and subsequently observed using a FEI Morgagni F268 transmission electron microscope (operating at 80 kV).

### Statistical analysis

Data of all investigate were expressed as mean  $\pm$  standard error (SE) and analyzed by using the SPSS11.5.0 software. The differences between means were analyzed by independent samples *t*-test and one-way ANOVA. The level of significance differences between the untreated and the controls when  $P \leq 0.01$  and between the controls and the bacterium-injected insects when  $P \leq 0.025$  based on Bonferoni correction.

### Results and Discussion

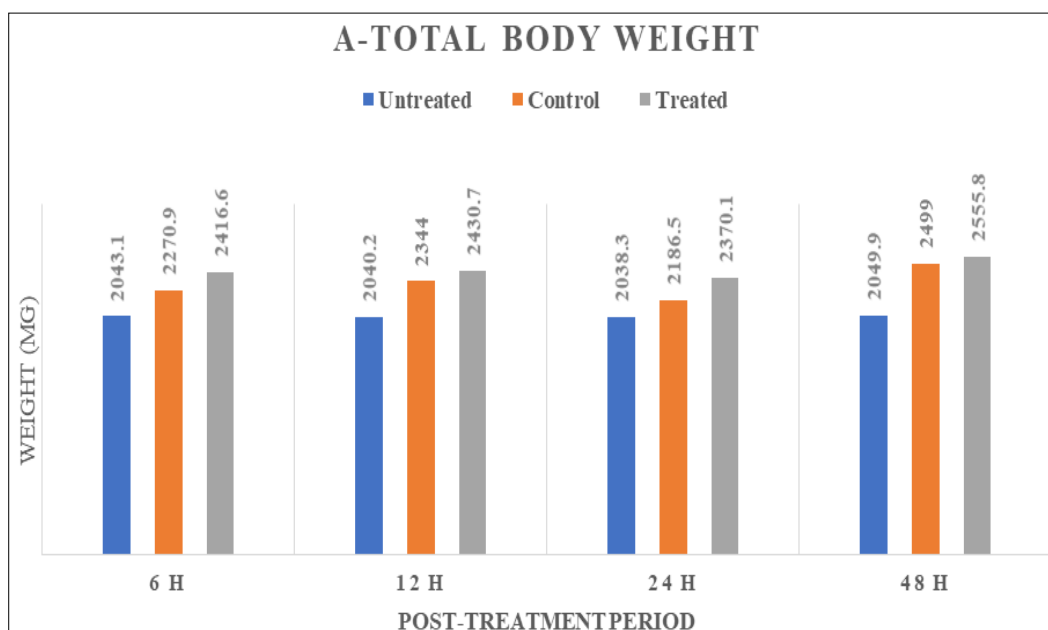
#### Physical properties of the Hemolymph

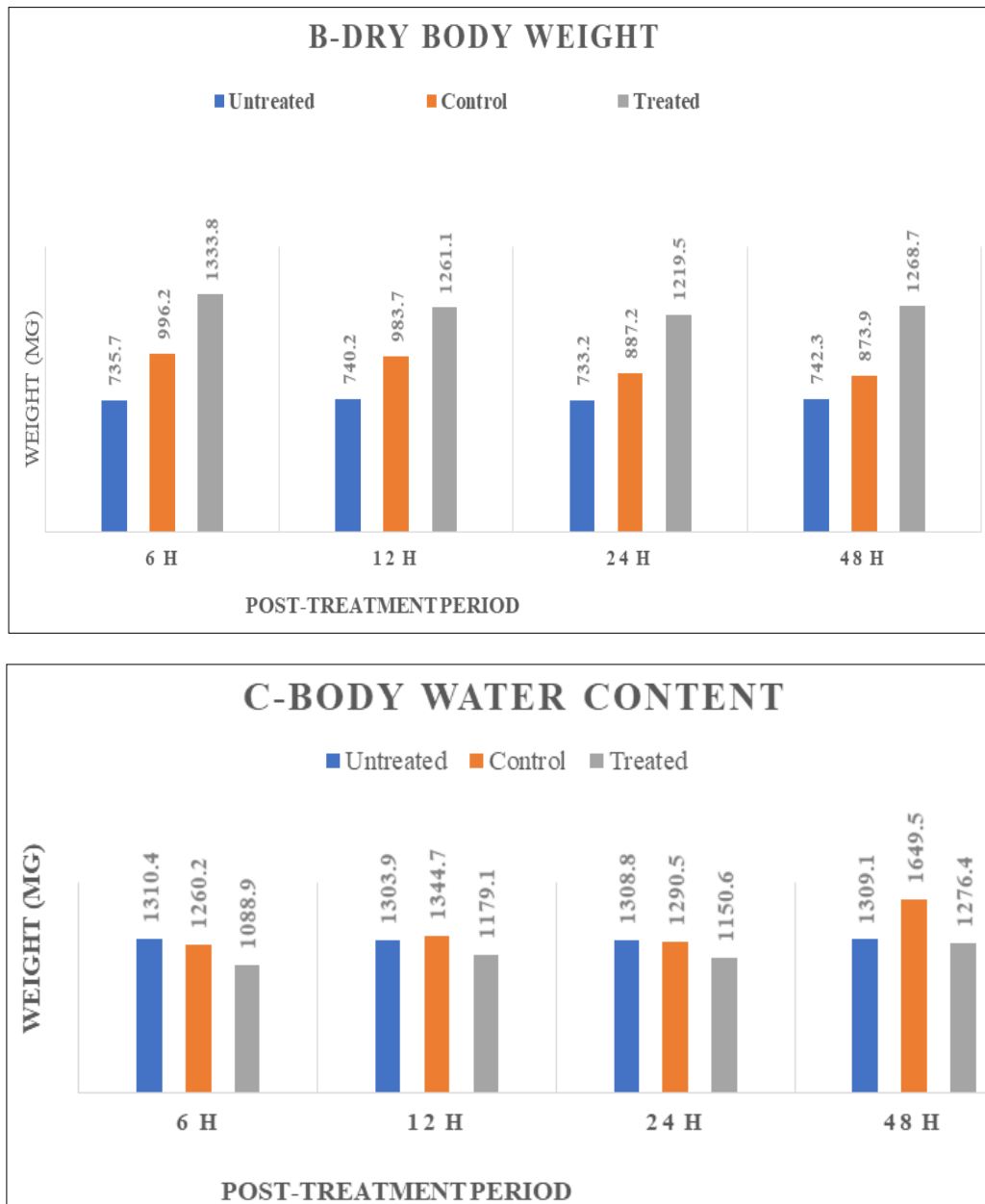
The use of bioinsecticides is considered a promising biological alternative to control insect pests. *Bacillus thuringiensis* produced an assortment of insecticidal proteins, with varied pathogenicity, are active against several pest species [47]. The pathogenesis mechanism of Bt against insects is a complex process that includes numerous factors in addition to the activation of immune responses of insects to combat infection, which is also considered as a factor causative to tolerance against Bt [13]. Changes in body weight and water content did not interest the immunologists in the past, while it gives an integrated image with hemolymph volume and density about the effect of treatment. Insect hemolymph is affected on its physical

properties such as volume, density and pH by several factors like; age, diet, temperature and disease [11]. Results represented in (Fig 1 A, B and C) demonstrate the total (fresh) body weight of the untreated adult, the dry body weight and the water content that representing about 64% of the fresh body weight. The bacteria-treated insects showed a slight difference ( $P > 0.05$ ) in the fresh body weight whereas; the dry body weight increased significantly ( $P < 0.025$ ) at all post-treatment periods. However, a significant decrease ( $P < 0.025$ ) in body water content was observed at all post-treatment periods examined. Typically, the insect responds to a bacterial infection on protein distribution, as well as water distribution. It was considered that the results of blood volume would be most informative with knowledge of dry weight and water content for comparison. Results are expressed as percentages of total weight, allowing comparisons of the relative values of individuals of different weight but of the same age. The total body water content could be subdivided into two fractions: tissue water and hemolymph water. In the present investigation the calculated water content value was 1308.05 mg comprising 64.03 % of the total body weight. These findings were supported by the work of [50] in which, the estimated percentage of the body water content ranged from not less than 50% to not more than 90 % of the total body weight. Also, agree with those of [6] who found that in most insects, the hemolymph represents from 10 to 40% of the total body weight.

From our investigation we revealed that the *S. gregaria* adults have greater body water content and was accompanied by a loss in their dry weight. These variations may be due to the uptake of water during their feeding. The bacterial treatment did not affect the fresh weight, while it increased the dry weight and, in the same time, decreased the body water content.

The increase in dry body weight may be attributed to the increase of the blood density. The detected decrease in body water content may be due to the loss of tissue water and the decrease of the hemolymph volume. These observed changes are in accordance with the findings of [3] and may be attributed to the induction of bacterial metabolites and the intensive loss of tissue water.





**Fig 1:** (A) Total (fresh) body weight (mg), (B) Dry body weight (mg) and (C) Body water content (mg) of adult *S. gregaria* determined in untreated, control, and at different time intervals post-treatment with Bti.

The mean hemolymph volume (HV) of untreated adult locust was 235.77  $\mu$ l. Bacteria-treated insects possessed a significant decrease ( $P \leq 0.025$ ) in HV as compared with saline-treated insects at all periods post-treatment (Fig 2A). The estimated value of the hemolymph density of untreated adults was 1.03 mg/ $\mu$ l. After bacterial-treatment a significant decrease was recorded at all post-treatment periods compared to the saline-treated insects (Fig 2B). However, the value of hemolymph pH of untreated adults was 7.12 (slightly alkaline). In bacteria-treated adults, there was a significant increase only at the 6 h post-injection ( $P < 0.025$ ) as compared to the saline-injected insects. These changes push the hemolymph pH from slight alkaline area

into slight acidic area (Fig 2C). The observed increase of the hemolymph density and pH of adults *S. gregaria* at almost all post-injection periods may be due to the increase of bacterial metabolites [2,3] the increase in total hemocyte count [5], as well as the observed decrease of the blood volume. These findings are also in accordance with those of [4] on the 5<sup>th</sup> nymphal instar and adults of *S. gregaria* [49]. The value of hemolymph volume of the normal (un-injected) adult locusts is close to the findings of [29] on nymphs and adults of *S. gregaria*. The estimated decrease of the blood volume during the course of infection may be attributed to water loss from blood and tissues as a result of bacterial infection.

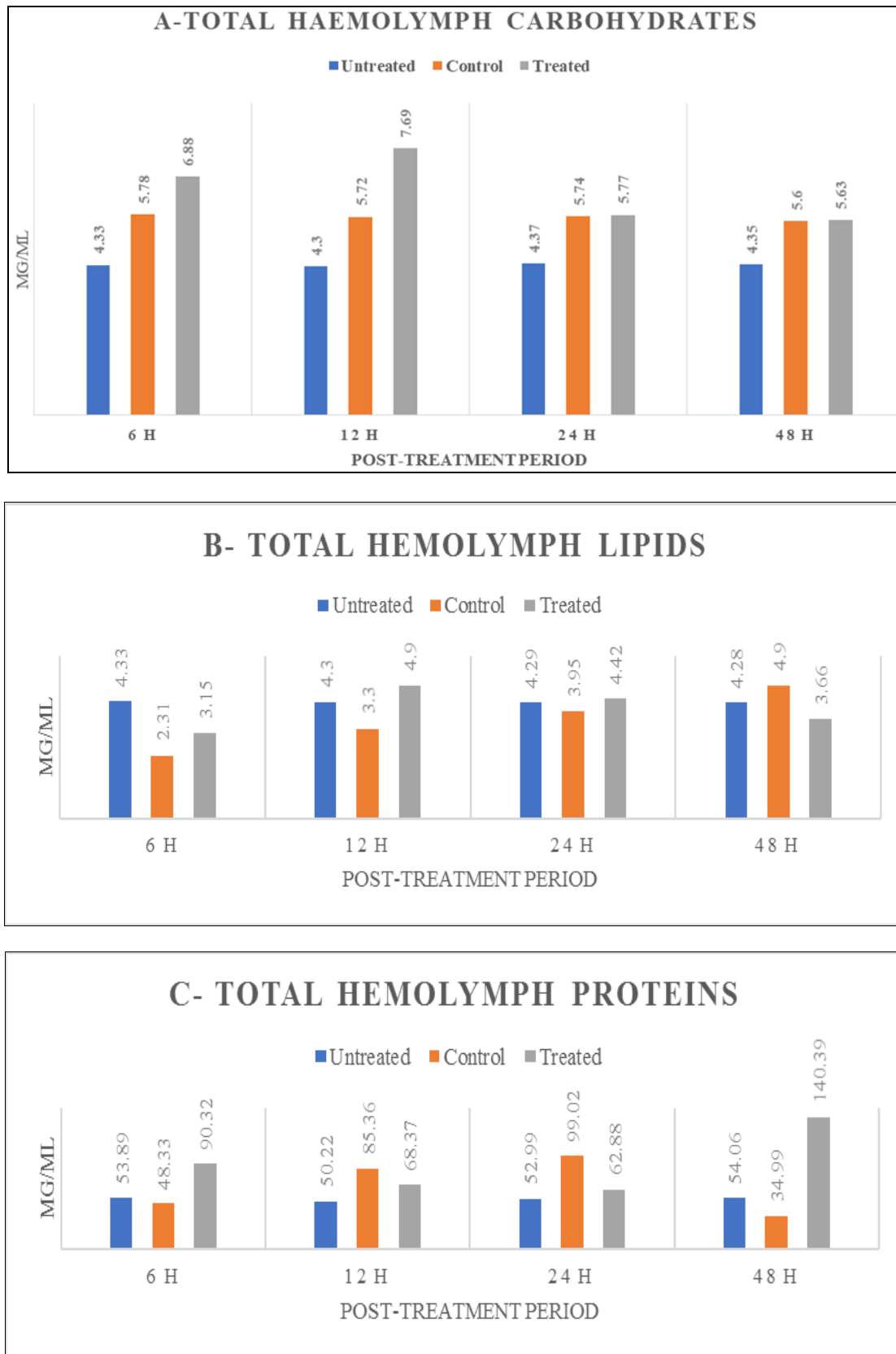


**Fig. 2:** (A) Hemolymph volume ( $\mu\text{l/insect}$ ), (B) Hemolymph density and (C) Hemolymph pH of adult *S. gregaria* determined in untreated, control, and at different time intervals post-treatment with *Bti*.

### Biochemical properties of the Hemolymph

Concerning the hemolymph biochemical composition of *S. gregaria* adults, the present results estimated that the THC, THL and THP of untreated locusts, were 4.34, 4.30 and 52.79 mg/ml, respectively (Fig 3A, B and C). While, *Bti*-treated insects, the THC and the THL showed a significant increase ( $P \leq 0.025$ ) at the 6 and 12 h post-injection compared with the saline-treated insects, but remained unchanged ( $P > 0.05$ ) later at the 24 and 48 h post-treatment. However, there was a significant increase ( $P \leq 0.025$ ) in the THP at 6 h post-injection followed by significant decrease as compared with the saline-treated insects. At the 48 h, a significant increase was observed again. An initial increase in THC after bacterial infection, which is gradually decreased to reach a normal level over time. This increase may be a natural phenomenon because levels of hemolymph trehalose respond extraordinarily to physiological states such as infection or starvation<sup>[37]</sup>. Additionally, the fact that trehalose acts as a carbohydrate reserve is readily available.<sup>[7]</sup> Also suggested that blood glucose be maintained through a dynamic equilibrium between the synthesis and breakdown of trehalose. Similar results were observed by<sup>[32]</sup> on an adult food-deprived grasshopper. Otherwise, the late decline in THC may be due to strong glucose demand during intense growth and bacterial multiplication. The nutritional requirements of entomopathogenic bacteria

include an organic source of carbon<sup>[44]</sup>. This can also be interpreted as a balanced work of the body's physiology. Additionally, the THL and THP of normal (untreated) *S. gregaria* adult stages, estimated in the present study similar to those of<sup>[9]</sup> on *L. migratoria* and<sup>[35]</sup> on *M. sanguinipes*. The decrease in THL following bacterial treatment may be a consequence of depletion of nutrition during infection in which the body physiology is unable to meet the requirements of the insects. Furthermore, the observed decrease of THP contents following bacterial infection may be as a result of the bacterial pathogenicity. Bacteria may cause a complete elimination of some hemolymph enzymes, some hemolymph soluble and sticky proteins, which may be involved in antibacterial immunity or some native proteins may be converted into lipoproteins or glycoproteins after infection could be attributed to intensive consumption of hemolymph proteins during growth and multiplication of the bacteria, together with cessation of feeding observed on the infected insects. The same explanation was reported by<sup>[43]</sup> on different insect species infected with bacteria. While<sup>[20]</sup> found that the hemolymph protein content of the last nymphal instar and young male adults of the grasshopper *L. migratoria* was not affected by injection with *Bt*. The late increase in total proteins observed at the 48 h post-*Bti*-injection may be caused by the metabolites secreted by the growth of bacteria within the hemocoel.

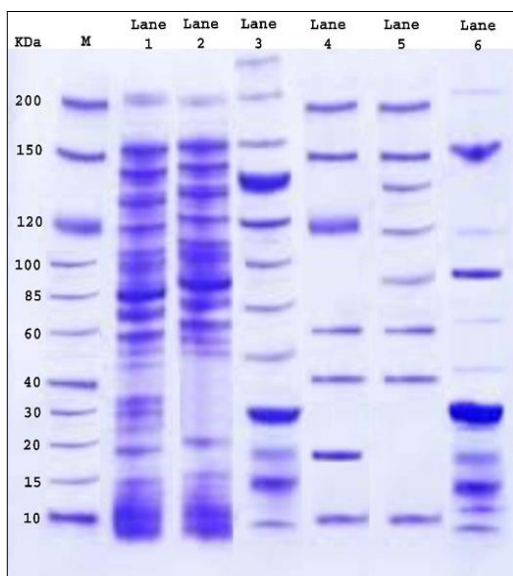


**Fig. 3:** (A) Total hemolymph Carbohydrates, (B) Total hemolymph Lipids and (C) Total hemolymph Proteins of adult *S. gregaria* determined in untreated, control, and at different time intervals post-treatment with *Bti*.

Insects respond to infection by a unique set of proteins that is derived from the gene pool of the insect and distributed into different body tissues. In order to characterize these proteins to determine their relative role in the response towards the infection, Protein banding pattern of hemolymph plasma of adult *S. gregaria* was carried out for

normal, control and treated ones. Data were emphasized in (Plate D), the protein marker was electrophoretically separated into 11 bands with molecular weights (MW) ranged between 200 and 10 kDa. The hemolymph of the normal locust was analyzed into 23 protein bands. Some proteins were disappeared or at least had different band

percentage post injection with water or bacteria. Moreover, new bands were synthesized while other bands were detected as a response to water injection. Plasma proteins of treated locust were separated into bands with MW ranging from 234 to 3 kDa. Results also showed that there were major bands common between normal, control and treated locust, other bands were common between control and treated, appeared to be specific for injection. Certain bands were found to be specific for bacterial injection. Electrophoretic separation revealed that the number and kinds of protein fractions evidenced are highly variable, this observation supported by the fact that these proteins may function in the immune responses, reproduction, storage components, or metabolic processes. The disappearance of some hemolymph proteins after infection may be attributed to their involvement in the immune reactions. While, the synthesis of new immune proteins may be a result simultaneous induction of injected bacteria such explanations similar to that of [36, 33, 40] using different pathogens as inducer to the insect immune response and observed a number of immune proteins were activated leading to the synthesis of antimicrobial peptides. While, few reports of reduced expression of antimicrobial peptides in response to pathogens and parasites in *Manduca sexta* [52]. [31] approved that Bt played a novel role in controlling *P. xylostella*, by suppressing the immune system through the reduced expression of antimicrobial peptides may be due to the release of other toxins and secondary metabolites along with toxin produced by Bt to overcome the immune system and likely to aid fungal and bacterial survival in a variety of insect hosts. Although our knowledge of insect-pathogen interaction (fungi as a pathogen) has increased in recent years [52]. [51] Indicated that the mode of action of a novel toxin from Bt could have a potential application for development of Bt insecticide.



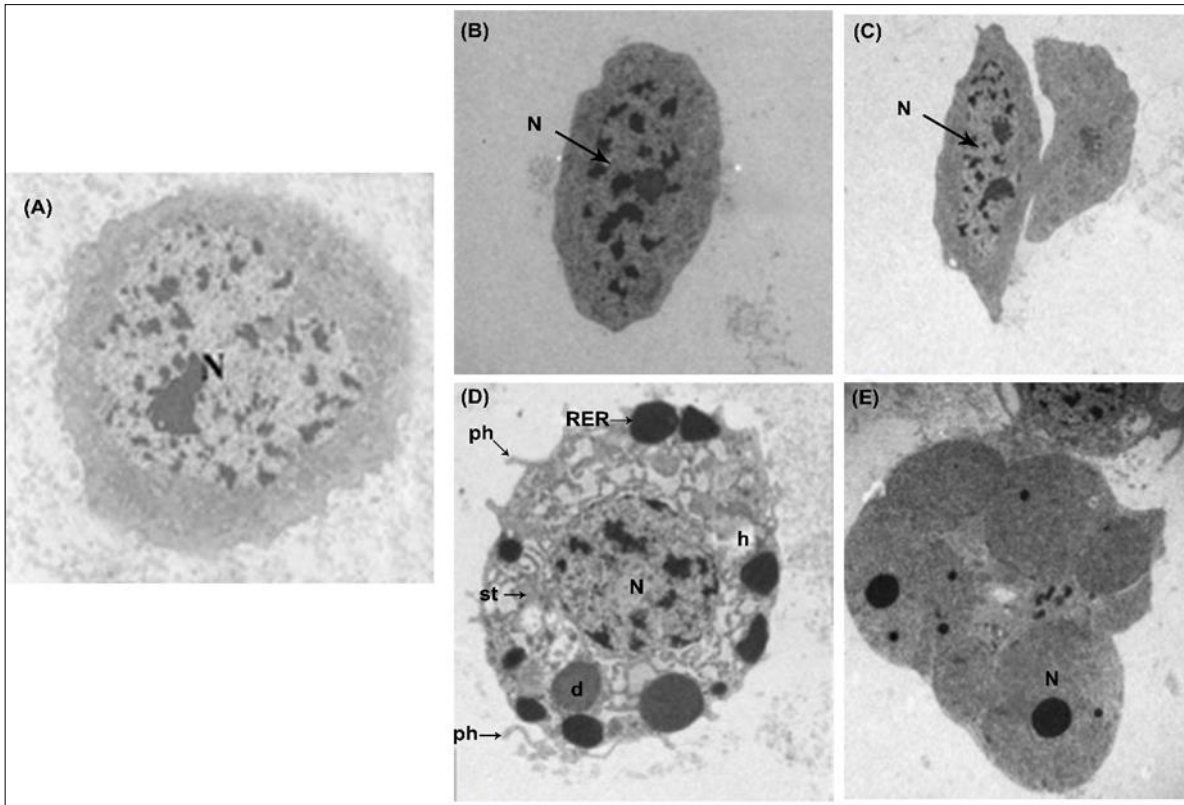
**Plate I:** Changes in the plasma protein banding patterns of *S. gregaria* normal, control and *Bti* injected locusts. M: protein molecular weight marker; Lane 1: plasma of normal Locust; Lane 2: plasma of water injected Locust (Control), Lane 3-6: plasma of *Bti* injected Locust at 6, 12, 24, and 48hr subsequently.

### Description of adult *S. gregaria* Hemocytes

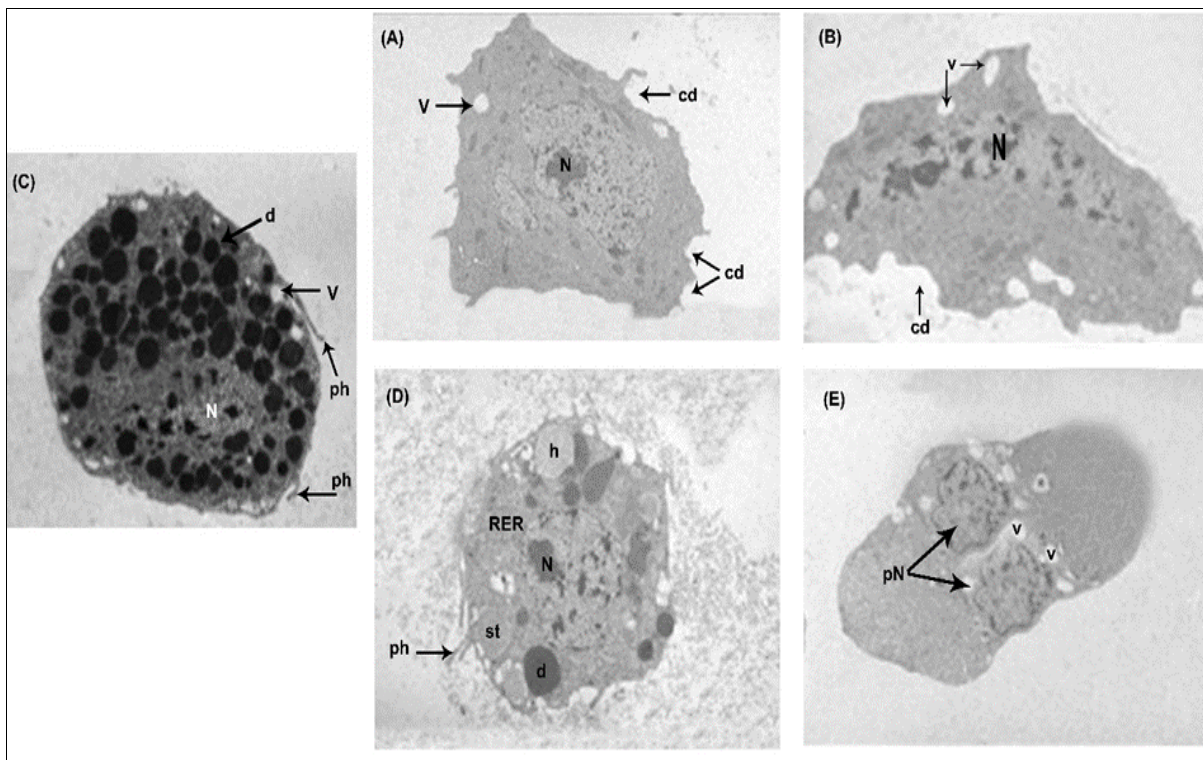
For describing the exact modifications of the blood picture in the course of Bti-injection into *S. gregaria*, it is necessary to describe the types of hemocytes. Depending on morphological classification based on observation of philobodia-like or fan-like structures around the cell membrane [22, 30]. Transmission Electron Microscopy (TEM) revealed, four main hemocyte types in *S. gregaria* adult hemolymph. These were Prohemocytes (PRs), Plasmatocytes (PLs), Granular cells (GRs) and Coagulocytes (COs) (Plate 2).

Prohemocytes (Plate 2A) were the smallest type and ranged 7-18 $\mu$ m. They usually seemed round in shape. The nucleus occupies most of the cell volume, had poorly developed rough endoplasmic reticulum and mitochondria in its cytoplasm. Plasmatocytes (Plate 2B, C) were large in size with diameter of 15-42 $\mu$ m polymorphic but commonly spindle in shape. The nucleus occupies about half of the cell volume. Contained well developed rough endoplasmic reticulum and many mitochondria. Granular cells (Plate 2D) were round or ovoid with diameter ranged 8-21  $\mu$ m. The nucleus was centric spherical or ovoid, they appeared to have a well-developed endoplasmic reticulum (it is a membranous network which is responsible for protein biosynthesis, also acts as calcium storage) and were rich in mitochondria. Their plasma membrane showed filopodial projections. These cells were characterized by the presence of 3 types of granular inclusions: inclusions exhibit an internal structure comprising numerous short microtubules, inclusions contain homogenous electron-dense content, and inclusions with heterogeneous bodies. When cells have richness of endoplasmic reticulum, means that these cells are trying to produce and secrete more protein [54]. Coagulocytes (Plate 2E) were round or ovoid in shape cell diameter ranged between 9 - 16  $\mu$ m with small nuclei and pale hyaline cytoplasm containing scattered granules. Their nuclei were round or ovoid and may be centric or eccentric and occupies about 75.0% of the cell volume. they contain granules and structured globules. Certain pathological observations were recorded in the hemocytes following Bti-injection into the hemocoel of adult locusts (Plate 3). Prohemocytes haven't been detected because these cells may have differentiated into granulocytes and plasmatocytes since the prohemocyte is considered the ancestor of other hemocytes.

The PLs (Plate 3A, B) exhibited great variations in the cell volume, vacuolization in the cytoplasm and distortion of the cell membrane. Some nuclei lost part of their chromatin materials and showed vacuoles in their nucleoplasm. The GRs (Plate 3C, D) have been affected severely in the cytoplasm and nucleus, cells were characterized by highly granulated and deeply stained nucleoproteins and some cells became lysed. In the COs (Plate 3E) the nucleus seemed bilobed and Pycnosis were appeared, the cytoplasm may be eroded, ruptured, and the cytoplasmic contents were extruded.



**Plate 2:** Photomicrographs of normal hemocytes of *S. gregaria* adults, examined by Transmission Electron Micrographs (TEM): (A) Prohemocyte, (B,C) Plasmatocytes, (D) Granular hemocyte, and (E) Coagulocytes; (N: nucleus, RER: rough endoplasmic reticulum, h: heterogeneous granule, d: electron-dense granule, st: structured granules, and ph: filopodia). Bar is 3 μm in PROs, 5 μm in PLs and 4 μm in the rest cells



**Plate 3:** Photomicrographs of treated hemocytes of *S. gregaria* adults examined by Transmission Electron Micrographs (TEM): (A,B) Plasmatocytes, (C,D) Granular hemocyte, and (E) Coagulocyte; (N: nucleus, RER: rough endoplasmic reticulum, h: heterogeneous granule, d: electron-dense granule, st: structured granules, V: vacuoles, cd: cell membrane distortion, pN Pycnosis in nucleus and ph: filopodial). Bar is 4 μm in PLs and 5 μm in the rest cells.

In conclusion, this study has provided an explanation on the response of the immune system of adult *S. gregaria* to Bti exposure at different time intervals. *Bacillus thuringiensis*

*israelensis* infection led to a noticeable reduction in the response of the immune system of locust. In the light of our findings, we speculate that Bti might have released several

other toxins and secondary metabolites, in order to overcome the insect immune system. So, further studies are recommended to evaluate the immunity related genes for this response in this study.

## References

1. Aptosoglou SG, Sivropoulou A, Koliais SI. Distribution and characterization of *Bacillus thuringiensis* in the environment of the olive in Greece. *New Microbiol.* 1997; 20:6976.
2. Azizi N, Ghadraoui El, Petit LD, Fadil F, Mohim A. A simple diet for the rearing success of the Desert Locust, *Schistocerca gregaria* (Forskål, 1775) (Orthoptera, Acrididae). *Bulletin de la Société Entomologique de France.* 2010; 115(4):445-450.
3. Barakat EMS, Meshrif WS. Physiological changes in the desert locust, *Schistocerca gregaria* in response to diet and injection with *Bacillus thuringiensis*. *Journal of Environmental Science,* 2007, 210-239.
4. Barakat EMS, Meshrif WS, Shehata MG. Changes in the hemolymph of the desert locust, *Schistocerca gregaria* after injection with *Bacillus thuringiensis*. *Journal of Egyptian Academic Society for Environmental Development.* 2002; 2(1):95-115.
5. Barakat EMS, El-Monairy MO, El-barky NM, Abd-EL Aziz MF, Abd-El HF, khalek HF, *et al.* Effect of diet on the hemolymph protein profile of the desert locust, *Schistocerca gregaria* Forskal (Orthoptera: Acrididae) and susceptibility of adult to *Bacillus thuringiensis israelensis*. *African Journal of Biological Science.* 2013; 9(1):81-93.
6. Benz G. Physiological and histochemistry. In: *Insect Pathology, an advanced treatise.* (Steinhaus, E.A. Ed). 1963; 1:299-338. Academic Press. New York.
7. Boctor IZ. Carbohydrates in the hemolymph of the cotton leafworm, *Spodoptera littoralis* Boisduval larvae (Lepidoptera: Noctuidae). *Comparative Biochemistry & Physiology.* 1974; 49(B):79-82.
8. Bradford MM. A rapid and sensitive method for the quantitation of the microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* 1976; 72:248-254.
9. Brehélin M, Drif L, Baud L, Boemare N. Insect hemolymph: Cooperation between humoral and cellular factors in *Locusta migratoria*. *Insect Biochemistry,* 1989; 19(3):301-307.
10. Brehélin M, Zachary D. Insect hemocytes: A new classification to rule out the controversy. In: *Immunity in invertebrates.* (Brehélin, M. Ed.), 1986, 36-46. Springer-Verlag.
11. Carrel JE, Wood JM, Yang Z, Mecairel MH, Hindman EE. Diet, body water, and hemolymph content in the Blister beetle, *Lytta polita* (Coleoptera: Meloidae). *Environmental Entomology.* 1990; 19(5):1283-1288.
12. Bumsik Cho, Sang-Ho Yoon, Daewon Lee, Ferdinand Koranteng, Sudhir Gopal Tattikota, Nuri Cha, *et al.* "Single-cell transcriptome maps of myeloid blood cell lineages in *Drosophila*." *bioRxiv,* 2020.
13. Contreras E, Benito-Jardón M, López-Galiano MJ, Real MD, Rausell C. *Tribolium castaneum* immune defense genes are differentially expressed in response to *Bacillus thuringiensis* toxins sharing common receptor molecules and exhibiting disparate toxicity. *Developmental and Comparative Immunology.* 2015; 50:139-145.
14. Crickmore N, Zeigler D, Feitelson J, Schnepf E, Van Rie J, Lereclus D. *et al.* Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews.* 1998; 62:807-813.
15. Diez MC, Gallardo F, Tortella G, Rubilar O, Navia R, Bornhardt C. Chlorophenol degradation in soil columns inoculated with *Anthracophyllum discolor* immobilized on wheat grains. *Journal of Environmental Management.* 2012; 95: S83-S87.
16. Fearon DT, Seeking wisdom in innate immunity. *Nature.* 1997; 388:323-324.
17. Frings CS, Fendley TW, Dunn RT, Queen CA. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. *Clinical chemistry.* 1972; 18(7):673-674.
18. Heimpel AM. The pH in the gut and blood of the larch sawfly, *Pristiphora erichsonii* (HTG.), and other insects with reference to the pathogenicity of *Bacillus cereus*. *Canadian Journal of Zoology.* 1955; 33:99-106.
19. Hill L, Goldsworthy GJ. Growth, feeding activity, and the utilization of reserves in larvae of *Locusta*. *Journal of insect Physiology.* 1968; 14(8):1085-1098.
20. Hoffmann D. Induction of antibacterial activity in the blood of the migratory locust, *Locusta migratoria* L. *J. Insect Physiol.* 1980; 26:539-549.
21. Hussain S, Sorensen SR, Devers-Lamrani M, El-Sebai T, Martin-Laurent F. Characterization of an isotopuron mineralizing bacterial culture enriched from a French agricultural soil. *Chemosphere.* 2009; 77:1052-1059. doi: 10.1016/j.chemosphere.2009.09.020
22. Hwang S, Bang K, Lee J, Cho S. Circulating hemocytes from larvae of the Japanese rhinoceros beetle *Allomyrina dichotoma* (Linnaeus) (Coleoptera: Scarabaeidae) and the cellular immune response to microorganisms. *PloS one.* 2015; 10(6):e0128519.
23. Jiang H, Vilcinskis A, Kanost MR. Immunity in lepidopteran insects. In: *Invertebrate Immunity.* (Soederhaell K, Ed.) New York, Landes Bioscience and Springer Science & Business Media. 2010; 708:181-204.
24. Jung SH, Evans CJ, Uemura C, Banerjee U. The *Drosophila* lymph gland as a developmental model of hematopoiesis. *Development.* 2005; 132(11):2521-2533.
25. Kwon H, Franzén O, Mohammed M, Ankarklev J, Smith RC. Single-cell analysis of mosquito hemocytes identifies signatures of immune cell sub-types and cell differentiation. *bioRxiv,* 2020.
26. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227(5259):680.
27. Lanot R, Zachary D, Holder F, Meister M. Post embryonic hematopoiesis in *Drosophila*. *Dev. Biol.* 2001; 230:243-257.
28. Lavine MD, Strand MR. Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology.* 2002; 32:1295-1309.
29. Lee RM. The variation of blood volume with age in the desert locust, *Schistocerca gregaria* (Fosk.). *Journal of Insect Physiology.* 1961; 6:36-51.
30. Lee J, Hwang S, Cho S. Immune tolerance to an intestine- adapted bacteria, *Chryseobacterium* sp.,

- injected into the hemocoel of *Protaetia brevitarsis seulensis*. Scientific reports. 2016; 6(1):1-14.
31. Li S, Xu X, Shakeel M, Xu J, Zheng Z, Zheng J, *et al.* *Bacillus thuringiensis* suppresses the humoral immune system to overcome defense mechanism of *Plutella xylostella*. *Frontiers in physiology*. 2018; 9:1478.
  32. Lim SJ, Lee SS. The effect of starvation on hemolymph metabolites, fat body and ovarian development in *Oxya japonica* (Acrididae: Orthoptera). *Journal of Insect Physiology*. 1981; 27(2):93-96.
  33. Mahmoud DM, Salem DAM, Mo'men SA, Barakat EMS, Salama MS. Purification and characterization of Phenoloxidase from immunized hemolymph of *Schistocerca gregaria*. *African Journal of Biotechnology*. 2015; 14(44):3027-3036.
  34. Miranpuri GS, Khachatourians GG. Haemocyte surface changes in the migratory grasshopper, *Melanoplus sanguinipes* in response to wounding and infection with *Beuveria bassiana*. *Journal of Entomology Experiment*. 1993; 68:157-164.
  35. Miranpuri GS, Bidochka MJ, Khachatourians GG. Morphology and chemistry of hemocytes and analysis of hemolymph from *Melanoplus sanguinipes* (Orthoptera: Acrididae). *Journal of Economic Entomology*. 1991; 84(2):371-378.
  36. Momen SA, Salem AM, Barakat MS, Salama S. Activation of Prophenoloxidase during Bacterial Injection into the Desert Locust, *Schistocerca gregaria*. *World Academy of Science, Engineering and Technology, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*. 2012; 6(8):585-594.
  37. Nowosielski JW, Patton RL. Variation in the hemolymph proteins, amino acids and lipid levels in adult house crickets, *Acheta domesticus* (L.) of different ages. *Journal of Insect Physiology*. 1964; 11:263-270.
  38. Ottaviani E. Insect immunorecognition. *ISJ*. 2005; 2:142-151. ISSN 1824-307X.
  39. Pigott CR, Ellar DJ. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiol. Mol. Biol. Rev*. 2007; 71:255-281.
  40. Radwan MH, Mo'men SA, Abdou MA, El-Gohary EGE, ElShafe AM, Barakat E, *et al.* Induction of antimicrobial peptides in the hemolymph of *Spodoptera littoralis* larvae following treatment with *Salmonella typhimurium*. *Egyptian Academic Journal of Biological Sciences. A, Entomology*. 2019; 12(4):9-21.
  41. Rady SA, Barakat E, Elmahalawy AR, Elsayed AK, Radwan MH. Hemolymph Changes Resulting from Injection of *Escherichia coli* Into the Larvae of the Wax Moth, *Galleria mellonella* (Lepidoptera: Pyralidae). *Egyptian Academic Journal of Biological Sciences. C, Physiology and Molecular Biology*. 2020; 12(1):93-105.
  42. Ribeiro C, Brehélin M. Insect hemocytes: What type of cell is that? *Journal of Insect Physiology*. 2006; 52:417-429.
  43. Sabbour M. Biochemistry of hemolymph of *Earias insulana* larvae treated with *Bacillus thuringiensis* and *Beauveria bassiana*. *Journal of Egyptian German Society Zoology*. 2001; 36(E):19-27.
  44. Samson AR, Evans HC, Latge J. Atlas of entomopathogenic fungi. Heidelberg, Springer Verlag, 1988.
  45. Severo MS, Landry JJ, Lindquist RL, Goosmann C, Brinkmann V, Collier P, *et al.* Unbiased classification of mosquito blood cells by single-cell genomics and high-content imaging. *Proceedings of the National Academy of Sciences*. 2018; 115(32): E7568-E7577.
  46. Tattikota SG, Cho B, Liu Y, Hu Y, Barrera V, Steinbaugh MJ, *et al.* single-cell survey of *Drosophila* blood. *Elife*, 9, e54818; 2020.
  47. Van Franken huyzen K. Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. *Journal Invertebrate Pathology*. 2013; 114:76-85.
  48. Van Handel E. Rapid determination of total lipids in mosquitoes. *Journal of American Mosquito Control Association*. 1985; 1(3):302-304.
  49. Werner RA, Jones JC. Phagocytic hemocytes in unfixed *Galleria mellonella* larvae. *Journal of Insect Physiology*. 1969; 15(3):425-437.
  50. Wigglesworth VB. The principles of insect physiology. 7th Ed., ELBS and Chapman and Hall Ltd, London, 1976.
  51. Wu Y, Lei CF, Yi D, Liu PM, Gao MY. *Novel Bacillus thuringiensis*  $\delta$ -endotoxin active against *Locusta migratoria manilensis*. *Applied and environmental microbiology*. 2011; 77(10):3227-3233.
  52. Xu, XX, Zhong X, Yi HY, Yu XQ. *Manduca sexta* gloverin binds microbial components and is active against bacteria and fungi. *Developmental & Comparative Immunology*. 2012; 38(2):275-284.
  53. Yeager JF, Munson SC. Blood volume of the roach, *Periplaneta americana* determined by several methods. *Arthropoda*. 1950; 1:255-256.
  54. Zhang, K., and Kaufman, R. J. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008; 454, 455–462. doi: 10.1038/nature07203.