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Haemocyte Activity and Cellular Defense Reactions in Various Larval Instars of Honey Bee (*Apis mellifera* L.) following Natural and Experimental Bacterial Infections

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ABSTRACT

Haemocyte activity of the third, fourth and fifth larval instars of *Apis mellifera* (L.) was observed following artificial injection with a sublethal doses of the bacterium, *Paenibacillus larvae larvae* (White) the causal agent of American foul brood disease, and compared with naturally infected larvae at different disease intensities (low, medium and high). Thirteen types of haemocytes were described in healthy larvae: prohaemocytes, granulocytes, eosinophil cells, oenocytes, plasmatocytes, spindle-shaped cells, micronucleocytes, macronucleocytes, spherulocytes, pycnonucleocytes, basophil cells, adipohaemocytes and neutrophil cells. Plasmatocytes represent 60-90% of the total haemocyte population and the other cells represent 10-40%. No marked changes were observed in percentages of plasmatocytes at all examination periods post-infection in all larval stages. While, in naturally infected larvae, their percentages increased significantly at all intensities of infection. In total haemocyte counts (THCs), there was a significant increase in the third larval instar, and a significant decrease in the fourth and fifth larval instars was observed at all examination periods. In naturally infected larvae, a significant decrease in THCs was observed at all intensities of the disease. Phagocytic response was observed in all tested instars, the maximum rate was recorded at 24 h post-injection. Percentages of phagocytosis increase as the larval age increases. Also, in naturally infected larvae, phagocytosis was observed at all intensities of infection, the maximum rate was at the high intensity of infection. Injection of *P. I. larvae* caused a significant increase in nodule formation in third and fourth larval instars. In contrast, a significant decrease in nodule formation in fifth larval instar was observed. Nodule formation was not determined in naturally infected larvae due to the extensive destruction of bee colonies.

Keywords: *Apis mellifera*, *Paenibacillus larvae larvae*, haemocytes, cellular defense mechanisms, phagocytosis and nodule formation.

INTRODUCTION

Like all living organisms, honey bees are susceptible to a wide variety of parasites and pathogens. Bee pathogens are varied, ranging from Gram-negative and Gram-positive bacteria to fungi, viruses, microsporidia and amoebae (Morse, 1997). Bees are also parasitized by mites and other arthropods, elevating risks of pathogen infection (Kanbar, 2003; Chen, 2004) and lower capabilities to combat disease (Gregory *et al.*, 2005; Yang, 2005). The common disease agents of bees are restricted to several pathogens, of them the bacterium, *Paenibacillus larvae* and the fungus, *Ascosphaera apis* are predominant. In fact, bacteria are the most abundant type of microorganisms associated with the honey bee (Gilliam, 1997). Both saprophytic and pathogenic bacteria are attached with the bee. The most important bacterial diseases are: American foulbrood, European foulbrood, *Paenibacillus alvei*, Powdery Scale Disease, Septicemia and Spiroplasmas.

American foulbrood (AFB) is an infectious disease of *Apis mellifera* larvae and pupae, caused by the spore forming bacteria, *Paenibacillus larvae larvae* (White, 1906). Larvae become infected by ingesting food contaminated with spores. The number of spores required to infect a larva increases with larval age. As few numbers, less than ten spores, can infect 24 h old larvae, whereas larger numbers are needed to infect older bee larvae, over 2-days-old (Brodsgaard, 1998). *P. I. larvae* spores can remain viable for over 35 years (Haseman, 1961). The only remedy is to completely destroy the hive.

Differences in susceptibility between different developmental stages of honey bees have been suggested, regardless the initial infection (Bailey and Ball, 1991), although there have been no confirmatory studies of physiological and biochemical differences. The ability of insect to escape infection has become already a matter of interest. Therefore, potential immune defense mechanisms of bees need to be studied, and incorporated into breeding programs if possible. They include the alterations of haemocytes and haemolymph to restrict the infection. Several studies on the circulating haemocytes of honey bee have been carried out following infection (Van Steenkiste, 1988; Papadopoulou-Karabela *et al.*, 1993; Gliniski and Grazgorczyx, 1995; Barakat, 2000; Zakaria, 2007). Unfortunately, most of them relied on the role of haemocytes in some physiological processes and did not provide sufficient data necessary for the accurate identification of the different haemocyte types.

The study of insect defense mechanisms is of great practical importance because we need to assess the ways by which insects might avoid infection by disease agents to ensure that colonies and cultures will be strong. In addition, defense mechanisms play a critical but underestimated role in the complex relationships that exist between insect and its pathogens. The present work aims generally to characterize the haemolymph of the honey bee, *A. mellifera* (L.) following infection with entomopathogenic bacterium, *P. larvae* ssp. *larvae*, to investigate the haemocytic changes of this species as well as the different mechanisms of cellular defense. Understanding how the insect defensive system interacts with pathogens may contribute to development of new strategies to block transmission of disease agents.

MATERIALS AND METHODS

Insects

The present study was carried out in the apiary yard of the Apiculture Research Department, Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt. Five colonies of healthy craniolian hybrid honey bees; *A. mellifera carnica*, however, ten diseased colonies by AFB disease were used as a natural infection source. Tested worker larvae were collected from tested colonies with a special grafting tool. The routine work for keeping and developing the colonies was carried out during the experimental period.

Bacteria

The bacterium, *P. larvae larvae* was isolated from ropy remains of died honey bee larvae. Ropy remains were suspended in 10 ml sterile distilled water and kept at room temperature for 10 min, then the suspension is heat-shocked at 80 °C for 10-15 min (effective time to kill non-spore-forming bacteria). Identification of bacteria was done according to methods of Shimanuki and Knox (1988). Bacterial stock suspension is directly inoculated onto J-agar plate, prepared according to methods of Piccini and Zunino (2001). After growth, pure isolates were inoculated onto J-agar slants and incubated at $28 \pm 2^\circ\text{C}$ for 48 h, to get a pure strain of *P. l. larvae*.

Prior to use, the spore suspension was prepared and counted where all viable cells form colonies and each colony counted is formed from one bacterial cell. Calculations of total numbers of viable bacteria from these counts were expressed as colony formed unit per milliliter (CFU/ml). The concentration of the spore stock suspension was determined and adjusted to sublethal doses of 1.67×10^{-6} , 2.91×10^{-2} and 4.49×10^{-3} CFU/larva injected into the haemocoel of the 3rd, 4th and fifth larval stages, respectively.

Injection technique

Injection of larvae was made with a 10 µl Hamilton microsyringe fitted with a 26-gauge needle according to Mullett *et al.* (1993). Direct injections into larval body cavities were taken place dorsally (Randolt *et al.*, 2008). After injection, the larvae were placed in Petri-dishes with drops of royal jelly and incubated at $34 \pm 2^\circ\text{C}$. Control insects were injected only with equivalent volumes of autoclaved distilled water. Another negative control group of insects were not injected.

Also naturally infected larvae with *P. l. larvae* have been tested. The intensity of the natural infection was estimated according to methods of Sorescu and Dragomir (2003). The percentages of infected larval cells may be calculated by the formula:

$$\frac{\text{Number of infected cells / inch}^2}{\text{Total number of cells / inch}^2} \times 100$$

Extraction and collection of haemolymph

The haemolymph samples were obtained by gently puncturing the larval cuticle from the dorsal site, with a fine-tipped calibrated glass capillary, which were kept at -20°C. The haemocytes were separated by centrifugation in cold at 2500g for 5 min.

Haemocyte identification and estimation of differential haemocyte counts (DHCs)

Haemolymph or separated haemocytes were smeared on clean glass slides and then stained with Giemsa's solution according to Arnold and Hinks (1979). Cells and nuclei-shapes, cytoplasmic inclusions, vacuoles and other characteristics were determined and used for the classification and assessment of frequency of haemocytes types. This is achieved by using haemolymph scanning by light microscopy (XSZ-107BN).

Various haemocytes were differentially counted by examining approximately 200 cells per slide and 5 slides (prepared from 5 larvae) were used for each count. The percentages of haemocyte types may be calculated by the formula:

$$\frac{\text{Number of each haemocyte type}}{\text{Total number of haemocytes examined}} \times 100$$

The measurements were replicated 5 times for each larval instar and at different time intervals; 3, 6, 12 and 24 h post-injection, and for naturally infected larvae at different intensities of infection.

Total haemocyte counts (THCs)

Total haemocyte counts (THCs) were estimated according to methods proposed by Tauber and Yeager (1935) by using a spencer bright-line haemocytometer with improved Neubauer ruling. At least 3 drops from each larva were counted and 3 replicates were used for different larval instars at different time intervals and for different intensities of natural infection. The number of haemocytes per cubic millimeter was calculated according to the formula of Jones (1962).

$$\frac{\text{Number of haemocyte counted per chamber} \times \text{dilution} \times \text{depth factor}}{\text{Number of 1 mm squares counted}}$$

The depth factor is usually 10

Estimation of phagocytosis:

Phagocytosis was examined from fixed Giemsa stained slides by light microscopy for different larval instars at different time intervals following injection. In all cases, five larvae were used for each determination and for each examination period. For each sample, at least 200 cells were evaluated at magnification 640X. Percentages of haemocytes engulfed bacterial cells were calculated according to the method described by (Rowley and Ratcliffe, 1980).

Estimation of nodule formation

The injected larvae were dissected (using a hypodermic needle) under a binocular microscope (M6C-9, N841757, USSR) at magnification 87.5X. The number of nodules per insect was counted. Only haemocyte aggregations consisting of more than approximately 10 haemocyte were considered. Numbers of nodules were estimated after different post-injection periods for different larval stages. In all cases, five larvae were used for each determination for each examination period. In general, appearance and the size (small < 40 µm, medium 40–100 µm, large > 100 µm) of the nodules were noted (Gunnarsson and Lackie, 1985).

Data analysis

Data were expressed as mean ± standard error (SE), and compared using Student's "t test" for paired samples. The level of significance for each experiment was set at P < 0.05.

RESULTS

Haemocyte identification

The different types and characteristics of haemocytes were showed in Fig. 1. Thirteen haemocyte types have been found in healthy (un-injected) third, fourth and fifth larval instars of honey bees: Prohaemocytes (PRs): are the smallest haemocytes, usually round to ovoid in shape, with large nuclei. Granulocytes (GRs): are medium to large, round to ovoid cells and regular in shape. Eosinophil cells (EOs): have a large red-staining nucleus, and the hyaline cytoplasm is seldom visible. Oenocytoids (OEs): are medium to large, round or oval cells with an accentric small nucleus. Plasmatocytes (PLs): are larger than PRs, they are round to ovoid and polymorphic, their nucleus is centric or accentric. Spindle shaped cells (SPLs): are small, spindle in shape and have a small nucleus. The nucleus is centric and round in shape. Micronucleocyte cells (MIs): are medium to large round cells. They have a paler staining cytoplasm and granular nuclei which are smaller in their proportion to the total cell volume. Macronucleocyte cells (MAs): are medium to large, round cells, with large nuclei. Spherulocytes (SPs): are often large haemocytes, round to ovoid in shape. Their cytoplasm is filled with large-sized inclusions known as spherules that obscure the small nucleus. Pycnonucleocytes (PYs): are large cells, have more than one side. Basophil cells (BAs): are small round cells with a small nucleus which may be centric or eccentric in position. Adipohaemocytes (ADs): are small to large cells, usually spherical in shape. The cytoplasm contains small fat droplets and vacuoles with small nucleus which is round to ovoid and mostly eccentric in position. Neutrophil cells (NEs): are small cells with a small eccentric nucleus.

Fig. 1: Light microscopy photomicrographs of healthy haemocytes from third, fourth & fifth larval instars of *A. mellifera* (L.). Bar=10 μm

Where EO (Eosinophil cell), PR (Prohaemocyte), PL (Plasmatocyte), SPL (Spindle shaped cell), OE (Oenocyte), NE (Neutrophil cell), AD (Adipohaemocyte cell), BA (Basophil cell), GR (Granulocyte), MA (Macronucleocyte), MI (Micronucleocyte), PY (Pycnonucleocyte), and SP (Spherulocyte).

Table 1: Differential haemocyte counts (DHCs) of third, fourth and fifth larval instars of *A. mellifera* (L.) determined at different time intervals post-injection with *P. I. larvae*

Stage	Hr #	Test	Differential haemocyte counts (percentages of haemocyte types) Mean $\pm$ SE												
			PRs	GRs	EOs	OEs	SPLs	PLs	MI	MA	SP	PY	PA	AD	NE
Third larval instar	3	Control	8.8 $\pm$ 2.7	15 $\pm$ 6.0	0.3 $\pm$ 0.1	1.5 $\pm$ 0.4	0.3 $\pm$ 0.2*	70.0 $\pm$ 7.0	0.0 $\pm$ 0.0	3.0 $\pm$ 1.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2	0.0 $\pm$ 0.0	0.2 $\pm$ 0.1	0.0 $\pm$ 0.0
		Treated	2.6 $\pm$ 0.9	5.6 $\pm$ 2.2	23. $\pm$ 7.3*	1.6 $\pm$ 0.7	0.3 $\pm$ 0.1	65.0 $\pm$ 11.0	2.5 $\pm$ 1.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	6	Control	1.3 $\pm$ 0.7	10.6 $\pm$ 3.8	11. $\pm$ 1.3*	5.0 $\pm$ 1.4	0.9 $\pm$ 0.4*	69.0 $\pm$ 4.3	0.2 $\pm$ 0.1	2.0 $\pm$ 1.7	0.0 $\pm$ 0.0	0.1 $\pm$ 0.07	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
		Treated	1.1 $\pm$ 0.2	32 $\pm$ 6*	22 $\pm$ 4.6	1.9 $\pm$ 0.8	1.9 $\pm$ 0.8	40.0 $\pm$ 7.8	3.1 $\pm$ 1.3*	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	12	Control	3.8 $\pm$ 1.1	16 $\pm$ 6.0	1.0 $\pm$ 0.8	4.0 $\pm$ 1.0	2.0 $\pm$ 1.1	73.8 $\pm$ 6.8	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
		Treated	1.5 $\pm$ 1.1	24.4 $\pm$ 4.6	6.0 $\pm$ 1.9	3.0 $\pm$ 0.4	0.2 $\pm$ 0.1	62.4 $\pm$ 3.7	0.2 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	24	Control	3.2 $\pm$ 1.7	2.8 $\pm$ 1.5	0.8 $\pm$ 0.5	1.5 $\pm$ 0.7	1.5 $\pm$ 0.6	90.0 $\pm$ 2.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.4 $\pm$ 0.3	0.0 $\pm$ 0.0	0.2 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
		Treated	0.8 $\pm$ 0.1	26 $\pm$ 11.8	3.5 $\pm$ 1.9*	2.0 $\pm$ 0.8	1.0 $\pm$ 0.6	66.8 $\pm$ 10.4	0.1 $\pm$ 0.06	0.1 $\pm$ 0.04	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Un-injected		8.4 $\pm$ 2.7	5.0 $\pm$ 2.8	2.0 $\pm$ 0.9	5.4 $\pm$ 2.0	4.0 $\pm$ 1.2	74.0 $\pm$ 6.1	0.3 $\pm$ 0.1	0.3 $\pm$ 0.2	0.4 $\pm$ 0.4	0.3 $\pm$ 0.2	0.0 $\pm$ 0.0	0.2 $\pm$ 0.2	0.3 $\pm$ 0.1
Fourth larval instar	3	Control	7.6 $\pm$ 1.4	10 $\pm$ 3	2.3 $\pm$ 1.7	4 $\pm$ 0.9	1.9 $\pm$ 0.8	74 $\pm$ 5.7	0	0.2 $\pm$ 0.2	0	0	0	0	0
		Treated	2.5 $\pm$ 2.3	13.4 $\pm$ 6.4	12.8 $\pm$ 2	2.7 $\pm$ 0.8	0.3 $\pm$ 0.2	62.4 $\pm$ 10.3	6 $\pm$ 5.3	0.2 $\pm$ 0.1	0	0	0	0	0
	6	Control	4.2 $\pm$ 1.2	11 $\pm$ 6	6.4 $\pm$ 1.5	3.4 $\pm$ 0.7	2.3 $\pm$ 0.7	73 $\pm$ 5.3	0	0	0	0	0	0	0
		Treated	0.5 $\pm$ 0.4	5.2 $\pm$ 1.1	12. $\pm$ 6.2	1 $\pm$ 0.7*	1.1 $\pm$ 0.4	79.8 $\pm$ 7.4	0.1 $\pm$ 0.06	0	0	0	0	0.3 $\pm$ 0.2	0
	12	Control	6 $\pm$ 1.3	4.4 $\pm$ 1.9	3.3 $\pm$ 1.3	3.8 $\pm$ 1	2.6 $\pm$ 1.9	80.8 $\pm$ 3.9	0	0	0	0	0	0	0
		Treated	2 $\pm$ 0.6	10.6 $\pm$ 4.2	10. $\pm$ 3.7	3.2 $\pm$ 1.3	0.4 $\pm$ 0.4	74 $\pm$ 7.2	0	0	0	0	0	0	0
	24	Control	6.2 $\pm$ 1.6	8.5 $\pm$ 5	1.1 $\pm$ 0.6	5 $\pm$ 2	1.1 $\pm$ 0.4*	78.2 $\pm$ 7.4	0	0	0.2 $\pm$ 0.1	0	0.2 $\pm$ 0.1	0	0
		Treated	2.8 $\pm$ 0.7	8.7 $\pm$ 3	0.7 $\pm$ 0.2	0.2 $\pm$ 0.2	0.44 $\pm$ 0.2	87.4 $\pm$ 3.6	0.4 $\pm$ 0.4	0	0	0	0	0.2 $\pm$ 0.14	0
	Un-injected		11.0 $\pm$ 3.3	1.8 $\pm$ 0.7	6.8 $\pm$ 1.9	5.0 $\pm$ 1.5	2.4 $\pm$ 0.5	73.0 $\pm$ 6.1	0.1 $\pm$ 0.07	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.1	0.0 $\pm$ 0.0	0.1 $\pm$ 0.12	0.4 $\pm$ 0.4
Fifth larval instar	3	Control	6 $\pm$ 1.9*	6 $\pm$ 16	4 $\pm$ 1.6	5 $\pm$ 1.2*	0.9 $\pm$ 0.5	79.6 $\pm$ 3.1	0	0	0	0	0.2 $\pm$ 0.2	0.3 $\pm$ 0.2	0
		Treated	2.2 $\pm$ 0.7	2.2 $\pm$ 1	9 $\pm$ 3	12 $\pm$ 0.4	0.32 $\pm$ 0.1	85 $\pm$ 3.8	0	0	0	0	0.2 $\pm$ 0.1	0	0
	6	Control	6 $\pm$ 1.7*	16 $\pm$ 6.8	0.7 $\pm$ 0.2	2 $\pm$ 0.9	0.8 $\pm$ 0.4	74.4 $\pm$ 5.2	0	0	0	0	0	0.2 $\pm$ 0.07	0
		Treated	3 $\pm$ 1	4 $\pm$ 1.7	7 $\pm$ 2.5	2.4 $\pm$ 1.4	0.6 $\pm$ 0.1	82.2 $\pm$ 4.1	0.3 $\pm$ 0.2	0.1 $\pm$ 0.08	0	0	0	0	0
	12	Control	5.2 $\pm$ 2.6*	10 $\pm$ 6.2	4 $\pm$ 1.7	7 $\pm$ 2.4	2.2 $\pm$ 0.2	68.2 $\pm$ 5.6	0	0	0	0	0	0	
		Treated	1.83 $\pm$ 1	3.3 $\pm$ 3	10 $\pm$ 1.1*	4 $\pm$ 1.6	0.34 $\pm$ 0.2*	80 $\pm$ 4.8	0	0	0	0	0	0	0
	24	Control	6.2 $\pm$ 3.4*	0.5 $\pm$ 0.2*	1.7 $\pm$ 1.1	2.6 $\pm$ 1	0.9 $\pm$ 0.46	88 $\pm$ 2.3*	0	0	0.1 $\pm$ 0.08	0	0	0	0.4 $\pm$ 0.3
		Treated	2.9 $\pm$ 1	12 $\pm$ 7.5	10.2 $\pm$ 3.5*	2.7 $\pm$ 1.3	0.12 $\pm$ 0.07	71.6 $\pm$ 12	0.14 $\pm$ 0.14	0	0	0	0	0	0
	Un-injected		19 $\pm$ 1.7	4 $\pm$ 1	4 $\pm$ 2	1.2 $\pm$ 0.6	3.6 $\pm$ 0.1.2	67.4 $\pm$ 4.6	0.2 $\pm$ 0.12	0.5 $\pm$ 0.4	0.1 $\pm$ 0.2	0.4 $\pm$ 0.4	0	0.3 $\pm$ 0.2	0.4 $\pm$ 0.4

#Hr: hours post-injection

N=5

*significant (p < 0.05)

PR (Prohaemocyte), GR (Granulocyte), EO (Eosinophil cell), OE (Oenocyte), SPL (Spindle shaped cell), PL (Plasmatocyte), MI (Micronucleocyte), MA (Macronucleocyte), SP (Spherulocyte), PY (Pycnonucleocyte), BA (Basophil cell), aAD (Adipohaemocyte cell), NE (Neutrophil cell).

Table 2: Differential haemocyte counts (DHCs) in different larval instars (third, fourth & fifth) of *A. mellifera* (L.) naturally infected with *P. I. larvae* at different intensities of infection (low, medium & high)

Haemocyte type	Differential haemocyte counts (percentages of haemocyte types) Mean $\pm$ SE								
	Intensity of disease infection								
	Third larval instar			Fourth larval instar			Fifth larval instar		
	Low	Median	High	Low	Median	High	Low	Median	High
Prohaemocytes	6.5 $\pm$ 2.4	5.4 $\pm$ 1.7	1.7 $\pm$ 0.9	9.0 $\pm$ 2.2	3.0 $\pm$ 0.72	4.2 $\pm$ 2.6	3.2 $\pm$ 0.6*	1.4 $\pm$ 0.4*	2.7 $\pm$ 1.2*
Granulocytes	7.4 $\pm$ 1.7	7.6 $\pm$ 3.3	24.6 $\pm$ 6.7*	3.4 $\pm$ 1.0	4.3 $\pm$ 2.7	16.6 $\pm$ 5.7	2.0 $\pm$ 1.0	1.1 $\pm$ 0.2	5.0 $\pm$ 1.4
Eosinophil cells	9.30 $\pm$ 4	6.0 $\pm$ 2.1	6.6 $\pm$ 1.8	8.4 $\pm$ 1.9	11.2 $\pm$ 2.9	5.6 $\pm$ 1.9	7.0 $\pm$ 2.8	3.0 $\pm$ 0.4	7.0 $\pm$ 2.9
Oenocytes	2.6 $\pm$ 0.5	0.94 $\pm$ 0.	1.5 $\pm$ 0.5	2.0 $\pm$ 0.72	0.82 $\pm$ 0.06	0.8 $\pm$ 0.3	1.8 $\pm$ 0.6	1.2 $\pm$ 0.6	1.6 $\pm$ 0.3
Spindle shaped	2.2 $\pm$ 0.6	0.9 $\pm$ 0.3	1.0 $\pm$ 0.4*	2.3 $\pm$ 1.2	1.2 $\pm$ 0.3	0.64 $\pm$ 0.2	1.0 $\pm$ 0.5	1.0 $\pm$ 0.5	0.2 $\pm$ 0.16
Plasmatocytes	70.6 $\pm$ 3.5	49.4 $\pm$ 3.1	64.6 $\pm$ 8.5	74.6 $\pm$ 2.4	79.4 $\pm$ 2.4	72.0 $\pm$ 3.0	84.4 $\pm$ 4.3*	90.6 $\pm$ 2.2*	83.2 $\pm$ 2.7*
Micronucleocyte	0	0	0	0	0	0.5 $\pm$ 0.2	0	0	0.2 $\pm$ 0.2
Macronucleocyte	0	0	0	0.5 $\pm$ 0.4	0	0	0	0	0
Spherulocyte	0	0	0	0	0	0	0	0	0
Pycnonucleocyte	0	0	0	0	0	0	0	0	0
Basophil cells	0	0	0	0	0	0	0	0	0
Adipohaemocytes	0	0	0	0	0	0	0	0	0
Neutrophil cells	0	0	0	0	0	0	0	0	0

N=5

* Significant (P<0.05).

Differential haemocyte counts (DHCs)

Percentages of different cell types were estimated in un-injected and injected third, fourth and fifth larval instars of *A. mellifera* at 3, 6, 12 and 24 h post-injection with *P. I. larvae*. Results are presented in Table 1. Haemocyte percentages were also estimated in naturally infected larvae at different disease intensities (Table 2). Results indicated that PLs are the most abundant cell type in circulation, representing about 60-90 % of the total cell population.

In the third larval instar, percentages of GRs and MIs at 6 h and of EOs at 3 and 24 h post-injection increased significantly. In naturally infected larvae, percentages of GRs increased significantly while percentages of SPLs decreased significantly at high intensity. The injection of bacteria into the fourth larval instar induced a significant increase in percentages of OEs at 6 h following injection. Percentages of GRs showed a significant decrease at all periods post-injection. In naturally infected larvae, SPLs decreased significantly at high intensity of infection. Injection of fifth larval instar with bacteria induced a significant increase in percentages of EOs at 12 and 24 h following injection. But a significant decrease in percentages of SPLs was observed at 12 h post-injection. In naturally infected larvae, PLs percentages increased significantly, while percentages of PRs decreased significantly at all intensities of infection.

Total haemocyte counts (THCs)

There was a significant increase in THCs of the third larval instar, at 3 h post-injection (Table 3). In contrast, in naturally infected larvae (Table 4) a significant decrease in THCs was observed at all intensities of disease (low, medium and high). The infection caused a significant decrease in THCs of the fourth and fifth larval instars at 24 and 6 h post-injection, respectively. Also, a significant decrease at high intensity of infection was observed for both instars of naturally infected larvae.

Table 3: Total haemocyte counts (THCs) (cell/mm³) of third, fourth and fifth larval instars of *A. mellifera* (L.) determined at different time intervals post-injection with *P. I. larvae*

Hours post injection	Total haemocyte counts (cells/mm ³) Mean $\pm$ SE					
	Third larval instar		Fourth larval instar		Fifth larval instar	
	Control	Treated	Control	Treated	Control	Treated
3	1185 $\pm$ 129.7*	1956.7 $\pm$ 160*	1471.7 $\pm$ 95.5*	1666.7 $\pm$ 199	1353.3 $\pm$ 91.4	1051.7 $\pm$ 111
6	1566.7 $\pm$ 256	1538.3 $\pm$ 158	937.7 $\pm$ 121.8	1118.3 $\pm$ 101.4	1671.7 $\pm$ 134.3	803.3 $\pm$ 36.6*
12	1053.3 $\pm$ 24.9*	1598.3 $\pm$ 225	1271.7 $\pm$ 216.7	1018 $\pm$ 167	1335 $\pm$ 175.6	1326.7 $\pm$ 143
24	1115.3 $\pm$ 367	993.3 $\pm$ 176	1207.5 $\pm$ 114	671.7 $\pm$ 26.2*	1438.3 $\pm$ 170	1333.3 $\pm$ 120.2
Un-injected	1598.3 $\pm$ 92.2		1111.7 $\pm$ 79.3		1341.7 $\pm$ 29.5	

N=5

* Significant (P<0.05).

Table 4: Total haemocyte counts (THCs) (cell/mm³) in different larval instars (third, fourth & fifth) of *A. mellifera* (L.) naturally infected with *P. I. larvae* at different intensities of infection (low, medium & high)

Stage	Total haemocyte counts (cells/mm ³) Mean $\pm$ SE			
	Healthy	Intensity of disease infection		
		Low	Medium	High
Third larval instar	1598.3 $\pm$ 92.2	1000 $\pm$ 29*	131.2 $\pm$ 75.7*	266.7 $\pm$ 50.7*
Fourth larval instar	1111.7 $\pm$ 79.3	1325 $\pm$ 71.8	523.3 $\pm$ 91.8	233.3 $\pm$ 36.3*
Fifth larval instar	1341.7 $\pm$ 29.5	1450 $\pm$ 104.1	900 $\pm$ 80	491.7 $\pm$ 145*

N=5

* Significant (P<0.05)

Estimation of Phagocytosis

The results indicated that PLs were the essential phagocytic cell type followed by the GRs. Phagocytosis reaction was shown to be composed of three phases: attachment and recognition; activation of pseudopodia and engulfing and then digestion (Fig.2). Phagocytosis response was observed in all tested larval instars at all post-injection periods, and the maximum phagocytic rate was at 24 h post-injection. Also, in naturally infected larvae, the phagocytosis response was observed at all intensities of infection, the maximum phagocytic rate was at the high intensity of infection.

Fig 2: Photomicrographs of phagocytic events caused by different haemocytes towards injected bacteria. Bar = 10 μ m

Where, (A1, 2, 3) attachment; (A4, 5, 6) activation of pseudopodia; (A7, 8) engulfing of bacteria; (A9, 10, 11) vacuoles formation and (A12) cell with phagocytosed bacteria.

Nodule formation

No nodule formation was recorded in the un-injected (healthy) third, fourth and fifth larval instars of *A. mellifera*. Nodules were observed as result of bacterial injection in all tested larval instars at different time intervals 3, 6, 12 and 24 h post-injection. Nodule formation was not determined in naturally infected larvae due to the extensive bee colonies destruction. The collected nodules were of variable sizes and centrally melanized (Fig. 3). The number of nodules induced after bacterial infection was related to the larval age. The size and maturation of nodules depends upon time and the immune capacity, where a significant increase in nodule formation in third and fourth larval instars was observed at 12, 24 h and 3, 24 h post-injection, respectively. In contrast, a significant decrease in nodule formation reaction was observed in fifth larval instar at 6 h post-injection.

Fig. 3: Photomicrographs of phagocytic events caused by plasmatocytes (PLs) (from A1 to A6) and granulocytes (GRs) (from B1 to B6) towards injected bacteria. Bar = 15 μ m

Where, (A1, B1) attachment; (A2, 3 & B2, 3) activation of pseudopodia; (A4 & B4) engulfing of bacteria and (A5, 6 & B5, 6) vacuoles formation.

Fig. 4: Photomicrographs of nodule formation of different sizes observed in third, fourth and fifth larval instars of *Apis mellifera* (L.). Bar= 50 μ m

Where (A1) Nodules of different sizes; (A2, 3, 4, 5) small nodules after 3, 6, 12 & 24 h, respectively; (A6, 7, 8, 9) medium nodules after 3, 6, 12 & 24 h, respectively; (A10, 11, 12, 13) large nodules after 3, 6, 12 & 24 h, respectively.

DISCUSSION

In this study, it is interesting to note that there was a great variation in the numbers and in the proportions of the different haemocyte types, although the bees from each larval instar were homogenous as far as age and origin were considered. Furthermore, we have identified staining affinity (by Giemsa) which facilitate discrimination of different haemocyte types according to cytological parameters such as shape, size, nucleus position and cytoplasmic constituents. The size of haemocytes and the shape and location of the nucleus can not be used as single and good criteria in classification of haemocyte population in insects. However, all haemocyte populations are incorporated within the same cellular or nuclear diameter. Indeed, the cytoplasmic constituents and their staining properties can be used as good criteria in the classification of insect haemocytes. In accordance with the scheme of honey bee by Van Steenkiste (1988) thirteen types of haemocytes were observed in our stained smears. Similar results have been obtained by Zakaria (2007) on the *A. mellifera* naturally infected with *P. I. larvae*.

The present study on DHCs showed that there was an increase in PLs and GRs percentages in naturally infected and bacteria injected fifth larval instar, and an increase in GRs and MLs percentages in naturally infected and bacteria injected third larval instar. This increase may be attributed to the release of sessile haemocytes after wounding. This concept is supported by the observations of Van Steenkiste (1988) on honey bees injected with carbon dust particles, Papadopoulou-Karabela (1993) and Barakat (2000) on *A. mellifera* artificially infected with *Pseudomonas aeruginosa*. They recorded a decrease in the number of phagocytic cells, at least temporary, due to phagocytosis. In contrast, Wienands *et al.* (1987) and Zakaria (1997) detected a decrease in the percentage of the PLs at all developmental stages of honey bees following *Varroa* infestation. Also, EOs increased significantly post bacterial injection in third and fifth larval instars. These findings are in agreement with Glinski and Klimont (1987a; b) and Zakaria (1997), on honey bees infected with *varroa* mite. While, percentages of PRs and SPLs showed a decrease in naturally infected and bacteria injected fifth larval instar, and percentages of OEs and SPLs in naturally infected and bacterial injected fourth larval instar. This may be due to the non-phagocytic affinity of these cells, and the increase in number of phagocytic cells.

There were great differences in percentages of different haemocyte types after *P. I. larvae* attack; this may be due to the bacterial effect or larval age. Our results agree, in this respect, with Barakat (2000) on honey bee workers following infection with bacteria, Sorescu and Dragomir (2003) and Zakaria (2007) on the same insect naturally infected with *P. I. larvae*. However, Gilliam (1973), Van Steenkiste (1988) and Zakaria (1997) mentioned that, in developing worker honey bee, the DHCs varied with the age of bees. Contrarily, Wille and Vecchi (1974) did not report any changes in the proportions of the different haemocyte types in bees injected with bacteria or protozoa.

The THC's may normally vary greatly with the amount of available blood, sex, stage of development, and other physiological states (Jones, 1962). Various abnormal conditions may profoundly affect the THC's such wounding, extreme low or high temperature and infection with various diseases (Shapiro, 1979). In the present study, there was a decrease in THC's in the fourth and fifth larval instars observed after bacterial-injection. Also, in naturally infected larvae of third, fourth and fifth larval instars there was a definite decrease in THC's observed at low and medium intensities, and a sharp decrease at high intensity compared with healthy larvae. This may be due to the involvement of haemocytes in phagocytosis and nodule formation which always accompanied with the death of defensive haemocytes (Bedick *et al.*, 2001), or may be due to the action of the released toxins and its lytic action in the haemocoel and degradation of haemocytes (Faye, 1978 and Gregore and Bowen, 1998). The overall results agree, in this respect, with the findings of Abrol (1996) who found a significant increase in THC's of healthy individuals of *A. mellifera* and *A. cerana indica* over those infected with *Tropilaelaps clareae* mite. Barakat (2001) worked on *A. mellifera* injected with *P. aeruginosa* noted the same effect, but Zakaria (2007) observed a sharp decrease in THC's of *A. mellifera* naturally infected with *P. I. larvae* when the immunity process takes a downfall. The observed remarkable increase in THC's in the third larval instar of *A. mellifera* after bacterial-injection may be due to the release of sessile haemocytes and the activation of mitotic activity of the haemocytes. These results are similar to the findings of Glinski and Grzagorczyk (1995) who found a greater increase in the haemocyte counts of infected bee workers by some bacterial spores. Also, Sarag El-Dien (1999) observed a significant increase in THC's of worker bees infected with *varroa* mite.

In the past few years the knowledge of insect defense mechanisms against pathogenic microorganisms and parasites has significantly increased on both the molecular and the organismic levels. The primary response of haemocytes to small particles such as bacteria is phagocytosis. It is well established that insects possess phagocytic cells, which is believed to have a function in insect immunity. The immediate response of insects to microbial invaders is mediated by these cells. In the present investigation, no phagocytosis was recorded in the un-injected (healthy) third, fourth and fifth larval instars of *A. mellifera*. However, the haemocytes of all injected larvae at different time intervals were capable of phagocytosing the injected bacteria. Phagocytosis of *P. I. larvae* by larval haemocytes was recorded as a multiple step process including; (1) attachment; (2) activation of pseudopodia formation; (3) actual engulfment by pseudopodia and digestion. This agrees with the results of Barakat (2001) on honey bees.

The present results indicated that the PLs are the main phagocytic cell type followed by GRs. These results agreed with Mitro (1994) who reported that the predominant cells involved in phagocytosis are PLs, followed by GRs. There is a considerable disagreement about the cell type mainly responsible for phagocytosis in insects. Tojo *et al.* (2000) reported that both GRs and PLs are involved in this reaction. Ratcliffe and Rowley (1979) reported that the PLs are the most important phagocytic cells, whereas, Brehelin and Zachary (1986) believed that the GRs may be the main effective cells. These differences probably result from the large variations occurring in haemocyte types even between closely related species.

Generally in the third, fourth and fifth larval instars, the rate of phagocytosis increased gradually till the end of experiment at 24 h post-injection; also in naturally infected larvae, the phagocytic rate increased gradually from low to high intensity. This may be due to the immunity system breakdown. Our results in this concept are in agreement with those of Zakaria (2007), who reported that the immune system was completely breakdown when the infection reached the high level. Cheung *et al.*, (1978) who found that the direct injection of *Bacillus thuringiensis kurstaki* into the haemocoel of *Heliothis zea* is followed by a rapid phagocytosis and extensive removal of the organisms within 24 h post-injection. Additionally, it is interesting to notice that the phagocytic response against bacteria varies according to the larval age (i.e. the phagocytosis achieved by the haemocytes of the third larval instar is less than that achieved by the haemocytes of the fourth larval instar which is also less than the fifth larval instar). These results agree with those of Abu El-Magd *et al.* (1991) on *Periplaneta americana* and Meshrif and Barakat (2002) on *Schistocerca gregaria*.

Nodule formation reaction is a type of cellular defense mechanisms, which is a multicellular hemocytic aggregate. This may entrap a large number of bacteria in an extracellular material when phagocytosis is inadequate to face those (Franssens *et al.*, 2005). In the present study, no nodules were observed in the healthy (un-injected) insects. Nodules were observed within few hours post-injection with *P. l. larvae* into the third, fourth and fifth larval instars of *A. mellifera*. In the same time, water-infected control larvae also succeeded to induce nodule formation, but the reaction was weaker comparable with bacteria, where the control nodules are mostly small in size, but in bacterial injected larvae the medium and large nodules represent the higher percentage. After contact with haemocytes, bacteria are entrapped in the material released by exocytosis from the GRs and melanization then occurs externally. Then PLs adhere and flatten the necrotic core forming the typical nodule. Our results are supported by the results of Barakat (2001) on honey bees, Ratcliffe and Gagen (1977) on *Galleria mellonella* larvae, Tackle (1988), Abu El-Magd (1992) and Meshrief and Barakat (2002) on *S. gregaria*.

After bacterial-injection into bee larvae, changes in nodule counts were recorded at different time intervals post-injection. The decrease in nodules may be attributed to the conclusion that stated: small nodules eventually disintegrate and disappear, but the larger ones, especially those with a melanized core, persist during the remainder of the insect life (Salt, 1970). Bacteria in nodules come to one of the three ends: (1) in most insect species they are destroyed by digestion (Bucher, 1959), (2) in a few species they can endure the reaction and persist alive throughout metamorphosis when enclosed in nodules as in *G. mellonella* (Cameron, 1934), and (3) they are pathogenic to the blood cells, probably prevent the full development of nodules or multiply in spite of the reaction that took place (Walters and Ratcliffe, 1983).

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REFERENCES

- Abrol DP (1996). Effect of mite parasitosis on blood counts of honey bees *Apis mellifera* L. and *Apis cerana indica* F. (Hymenoptera: Apidae). *Insect Environ.*, 2(1):18-19.
- Abu El-Magd AA (1992). Modifications of the haemogramme and of some cellular defense reactions of the desert locust, *Schistocerca gregaria* 5th nymphs after activation of the prophenoloxidase system. *J. Egypt Ger. Soc. Zool.*, 8(A): 23-36.
- Abu El-Magd AA, Gabry MSI, Shalaby OIM (1991). The ontogeny of phagocytic activity in haemocytes of the American cockroach, *Periplaneta americana* (Linne.). *Sci. bull. Minia Univ.*, 4(1): 359-377.
- Arnold JW, Hinks CF (1979). Insect haemocytes under light microscopy: technique. In: *Insect Haemocytes* (A.P. Gupta, ed.). Cambridge Univ. Press, Cambridge.
- Bailey L, Ball BV (1991). *Honey Bee Pathology*. 2nd edn. Academic Press, London.
- Barakat EMS (2000). Haemocytic changes in honey bee, *Apis mellifera* (L.) following injection of bacteria. *Ain Shams Sci. Bull.* 38: 500-517.
- Barakat EMS (2001). The cellular defence reactions of the honey bee, *Apis mellifera* in response to injected bacterium, *Pseudomonas aeruginosa*. *Ain Shams Sci. Bull.*, 39: 214-227.

- Bedick JC, Tunaz H, Aliza ARN, Putnam SM, Ellis MD, Stanley DW (2001). Eicosanoids act in nodulation reactions to bacterial infections in newly emerged adult honey bees, *Apis mellifera*, but not in older foragers. *Comp. Biochem. Physiol.*, 130:107–117.
- Brehélin M, Zachary D (1986). Insect haemocytes: A new classification to rule out the controversy. In: *Immunity in invertebrates*. (M. Brehélin, ed.). 36-46, Springer-Verlag.
- Brodsgaard CJ (1998). Response of in vitro reared bee larvae to various doses of *Paenibacillus larvae larvae* spores. *Apidol.* 29: 569-578.
- Bucher GE (1959). Bacteria of grasshopper of western Canada. III. Frequency of occurrence, pathogenicity. *J. Insect Pathol.*, 1: 391-405.
- Cameron GR (1934). Inflammation in the caterpillars of Lepidoptera. *J. Pathol. Bacteriol.*, 38: 441-466.
- Chen Y (2004). Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. *Apidol.* 35: 441–448.
- Cheung PYK, Grula EA, Borton RL (1978). Haemolymph responses in *Heliothis zea* to inoculation with *Bacillus thuringiensis* or *Micrococcus lysodeikticus*. *J. Invertebr. Pathol.*, 31: 148-156.
- Faye I (1978). Insect immunity: early fate of bacteria injected in Saturniid pupae. *J. Invertebr. Pathol.*, 31: 19-26.
- Franssens V, Simonet G, Bronckaers A, Claeys I, De Loof A, Broeck JV (2005). Eicosanoids mediate the laminarin-induced nodulation response in larvae of the flesh fly, *Neobellieria bullata*. *Arch. Insect Biochem. Physiol.*, 59:32–41.
- Gilliam M (1973) - Age-dependent variation of differential haemocyte counts of developing worker honeybees. *J. Apic. Res.*, 12 (1): 61-64.
- Gilliam M (1997). Identification and roles of non-pathogenic microflora associated with honey bees. *FEMS Microbiol Lett.*, 155:1–10.
- Gliniski Z, Grazgorczyk K (1995). Cellular defense reactions in the honey bee in environment non-polluted with heavy metals. *Annales-Universitatis Mariae – Curie Skodwska –Sectio dd, Medici. Veterinar.*, 50(13): 131-137.
- Gliniski Z. Klimont (1987 a). Effect of *Varroa jacobsoni* infestation on the blood cells of worker bees. *Medycyna Weterynaryjna*, 43(9): 546-549; (Review of Applied Entomology, 6730/88).
- Gliniski Z, Klimont (1987 b). Activity of haemocytes of worker honeybee during nature infestation with *Varroa jacobsoni*. *Medycyna Weterynaryjna*, 43(11): 664-667; (Apic. Abst., 251/90).
- Gregore A, Bowen ID (1998). Histopathological and histochemical changes in honey bee larvae (*Apis mellifera*) after infection with *Bacillus larvae*, the causative agent American foulbrood disease. *Cell-Biol.-Internatio.*, 22(2): 137-144.
- Gregory PG, Evans JD, Rinderer TDG (2005). Conditional immune-gene suppression honey bees parasitized by *Varroa* mite. *J. insect sci.* 5:7, available online: Insect science. Org 15.7 pp:5.
- Gunarsson SG, Lackie AM (1985). Haemocytic aggregation in *Schistocerca gregaria* and *Periplaneta americana* as a response to injected substances of microbial origin. *J. Invertebr. Pathol.*, 46: 312-319.
- Haseman L (1961). How long can spores of American foulbrood live? *Amer. Bee J.*, 101: 298-299.
- Jones JC (1962). Current concepts concerning insect haemocytes. *Amer. Zool.*, 2:209-246.
- Kanbar G (2003). Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by *Varroa* mites. *Parasitol Res.*, 90:349–354.
- Meshrif WS, Barakat EMS (2002). Cell mediated immunity in the Locust, *Shistocerca gregaria* against the bacterium, *Bacillus thuringiensis*. *J. Egypt. Acad. Soc. Environ. Develop.*, 2(1): 117-130.
- Mitro S (1994). Cellular defense reaction of the haemolymph, and description of specific cells in the seminal plasma of *Apis mellifera* L. *Apidol.*, 25(4): 361-366.
- Morse RA (1997). Honey Bee Pests Predators and Diseases. *Medina, OH: A.I. Root Co.*, editor.
- Mullet H, Ratcliffe NA, Rowley AF (1993). Analysis of immune defense of the wax moth, *Galleria mellonella* with anti-haemocytic monoclonal antibodies. *J. Insect Physiol.*, 93(10): 897-902.
- Papadopoulou-Karabela K, Lliads N, Liakos V (1993). Haemocyte changes in honeybee (*Apis mellifera* L.) artificially infected by *Pseudomonas aeruginosa*. *Apidol.* 24: 81-82.
- Piccini C, Zunino P (2001). American foulbrood in Uruguay: Isolation of *Paenibacillus larvae larvae* from larvae with clinical symptoms and adult honeybees and susceptibility to oxytetracycline. *J. Invertebr. Pathol.* 78: 176-177.
- Randolt K, Gimple O, Geissendorfer J, Reinders J, Prusko C, Martin JM, Albert S, Tautz J, Beier H (2008). Immune-Related Proteins Induced in the Hemolymph After Aseptic and Septic Injury Differ in Honey Bee Worker Larvae and Adults. *Archiv. Insect Biochem. Physiol.*, 69:155–167.
- Ratcliffe NA, Gagen SJ (1977). Studies on the *in vivo* cellular reactions of insects: An ultrastructural analysis of nodule formation in *Galleria mellonella*. *Tissue and Cell*, 9(1): 73-85.
- Ratcliffe NA, Rowley AF (1979). Role of hemocytes in defense against biological agents. In *Insect Hemocytes: Development, Forms, Functions and Techniques* (A.P. Gupta, ed.) pp. 331–414. London: Cambridge Univ. Press.
- Rowley AF, Ratcliffe NA (1980). Insect erythrocyte agglutinines. *In vitro*, opsonization experiments with *Glitummus extradentatus* and *Periplaneta americana* haemocytes. *Immunol.*, 40: 483-492.

- Salt G (1970). *The cellular defense reactions of insects*. Cambridge Monographs in Experimental Biology. No 16, pp. 118, Cambridge Univ. Press. London and New York.
- Sarag El-Dien FS (1999). Studies on the varroaosis "*Varroa jacobsoni* Oud." Ph. D. Thesis, Fac. Agric., Tanta Univ., Egypt.
- Shapiro M (1979). Changes in the haemocyte populations *In: Insect Haemocytes* (Gupta AP, ed) Cambridge Univ. Press, Cambridge.
- Shimanuki H, Knox DA (1988). Improved method for the detection of *Bacillus larvae* spores in honey. *Amer. Bee J.* 12: 353-354.
- Sorescu I, Dragomir C (2003). Differential haemocyte count and the phagocytic index in experimental bacterial infections and in field ascospheerosis of honey bee (*Apis mellifera* L.) at various ontogenetic stages. *Studies-and-Researchers-in-Veterinary-Medicine*, 9: 133-168.
- Tackle GB (1988). Studies on the cellular immune responses of insects toward the pathogen *Trypanosoma rangeli*. *J. Invertebr. Pathol.*, 51: 64-72.
- Tauber OE, Yeager JF (1935). On the total blood counts of insects. I. Orthoptera, Odonata, Hemiptera and Homoptera. *Ann. Entomol. Soc. Amer.*, 28: 229-240.
- Tojo S, Naganuma F, Arakawa K, Yokoo S (2000). Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. *J. Insect Physiol.*, 46: 1129-1135.
- Van-Steenkiste D (1988). The haemocytes of the honeybee: typology, blood spectrum and cellular defence reactions. *Bdo.proefschrift*, Doctor in de wetenschappen, Rijksuniversiteit Gent, Belgium, (Apic. Abst 1173/89).
- Walters JB, Ratcliffe NA (1983). Variable cellular and humoral defense reactivity of *Galleria mellonella* larvae to bacteria of differing pathogenicities. *Dev. Comp. Immunol.*, 7: 661-664.
- White GF (1906). The bacteria of the apiary with speed reference to bee disease. US Department of Agriculture, Bureau of Entomology, Technical Series. 14: 50.
- Wienands A, Strich H, Madel G (1987). Haemocytic and bacteriological aspects of the haemolymph of *Apis mellifera* L. (Hymenoptera, Apidae). *Zentrlblatt-fur-Bakteriologie,-Mikrobiologie-und-Hygiene,-A.* pp. 265, 489; Ba (Apic. Abst. 1264/89).
- Wille H, Veechi MA (1974). Untersuchungen Über die haemolymph der honigbienen (*Apis mellifera* L.) 5. Morphologie der Leukozyten und vier krankheits elementen. *Mitt Schweiz Entomol. Ges.*, 47: 133-149.
- Yang X (2005). Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proc. Natl. Acad. Sci. USA.*, 102:7470-7475.
- Zakaria ME (2007). The cellular immunity responses in the haemolymph of honeybee workers infected by American foulbrood disease. *J. Appl. Sci. Res.* 3(1): 56-63.

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