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Physical variances in the hemolymph of *Galleria mellonella L.* (Lepidoptera: Pyralidae) following immune induction

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ABSTRACT

The present study recorded the variations in the body water content and its relation to haemolymph volume, density and pH of the greater wax moth (*Galleria mellonella*) across different developmental stages at different time intervals post injection with *Bacillus thuringiensis kurstaki*. Preliminary results concerning Bt toxicity at a sub lethal concentration indicated that pupae were more resistant while, the adults were highly susceptible. Results concerning physiological investigations indicated that larvae had more body water content than pupae and adults, while haemolymph volume and density increased directly with insect development. Haemolymph pH had the same values in all developmental stages. Bt-injection decreased the body water content and, in the same time, increased the haemolymph volume, density and pH in all developmental stages. Maximum values attained at 48 h post injection. Pupae developed from previously treated larvae, and adults developed from previously challenged larvae and pupae showed significant changes compared with the normal ones and no changes as compared with treated insects.These findings show that different age groups respond to the impact of pathogenic bacteria by the same degree and the physiological changes did not affect the growth and transformation of this insect.

Key words*:* *Galleria mellonella*, *Bacillus thuringiensis*, water content, haemolymph volume, density and pH.

INTRODUCTION

The economic importance of the greater wax moth, *Galleria mellonella* has led to a number of investigations on its life history, biology, behavior, ecology, physiology and control. However, an effective method of controlling this pest has not been developed. Physical and chemical methods are imperfect (Burges, 1978). Therefore, many studies have been conducted to find ways for controlling it. One new trend is applying microbial control agents, especially bacteria.

The association of bacteria with insects has been recorded since Pasteur's time and many species of pathogenic bacteria have been described. Since the description of *Bacillus thuringiensis* Bt by Berhelin (1986), perhaps more is known of its mechanism of pathogenicity than of any other invertebrate bacterial pathogen. Several authors reviewed the toxicity of this organism, in addition to the developmental studies by (Rogoff *et al.* 1969) which led to its use as a biological control agent for insect pests.

The high interest in biological means of controlling insects intensifies the need for investigating the response of insect to disease organisms and foreign proteins. The haemolymph, the tissue made up of fluids and different types of cells, offers a readily accessible criterion of this response. It can undergo quantitative changes to an extent virtually unknown for other tissues. Insect haemolymph is influenced at least on the level of its physical properties such as volume, density and pH, or on its biochemical composition by several factors, among them are: diet, temperature and disease (Carrel *et al.*, 1990), the physiological condition of the insect (Chapman, 2013), and the developmental stage (Jones, 1977). In addition, haemolymph serves as a major

compartment and storage reserve for water (Atmowidjojo *et al*., 1999). Changes in body water content did not attract the physiologists in the past, although it gives integrated picture with haemolymph about effect of treatment.

Due to the succeeded use of Bt against many lepidopteran crop pests, it was necessary to evaluate the use of this pathogen against *G. mellonella*. Furthermore, there are many physiological changes take place during the insect development, therefore, the response of insect to disease organisms are adapted to the specific needs of each developmental stage. In addition, the majority of the previous studies on this framework have been conducted on insect larvae and little or relatively no studies have been published comparing the changes induced in the haemolymph of larvae, pupae and adults of these insects. Therefore, this study was conducted to investigate and compare the effect of Bt challenge on the haemolymph of larvae, pupae and adults of *G. mellonella*, and also to determine the relevance of sex towards this challenge in the adult stage. More particularly, to detect if a previous challenge would enhance the same response of the later developmental stage, indicative of trans stadial transfer of response.

MATERIALS AND METHODS

Maintenance of insect:

Galleria mellonella (L.) was collected and reared on an artificial diet, reported by Kulkarni *et al*. (2012). Last instar larvae, fresh pupae and adult moths (both sexes) on the day of their eclosion were used in the present investigation. They were collected and placed individually in small containers (approx. 13 cm^3) for further development.

Preparation of the bacterial pathogen:

Bacillus thuringiensis kurstaki (Bt) as wettable powder formulation (AGERIN, 3200 IU/mg) 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. Pure isolates were cultured on nutrient agar slants and incubated at 28 ± 2°C for 48 h, and then kept in the refrigerator at 4°C until used. Prior to use, pure isolates were grown on a nutrient agar medium at 28 ± 2°C for 24 h, harvested by suspending in a sterile distilled water and centrifuged at 6000 rpm for 30 min. The resultant sediment bacterial cells were washed three times with sterile distilled water and centrifuged again at the same rate till the solution becomes completely clear. The obtained bacterial pellet was then stored at _18ºC until required.

Susceptibility levels to Pathogenic Agent:

Bacterial suspension was adjusted to a concentration of 2.5×10^9 cells/ml by the pour plate count method according to Campbell and Konowalchuk (1948).Different developmental stages of the *G. mellonella* were divided into three groups; normal insects (negative control without injection), control insects and bacterial injected insects. Larvae, pupae and adults of *G. mellonella* were injected with 2 µl of each bacterial concentration; 2.5×10^3, 2.5×10^4, 2.5×10^5, 2.5×10^6 and 2.5×10^7 cells/ml. Injection was made with a 10 µl Hamilton micro-syringe fitted with a 26-gauge needle according to Miranpuri and Khachatourians (1993).

Determination of the body weight and water content:

Water content was determined gravimetrically for each individual larva, pupa and adult male and female as the difference between fresh (total) body weight to the nearest 0.10 mg (wet weight) and the weight after drying for 2-4 h at 80ºC till constant weight (dry weight). Normal insects and injected insects after3, 6, 12, 24 and 48 h, along with control (water –injected) insects were used. They were weighed on electronic balance (METTLER, type BB300, Switzerland).

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Determination of the haemolymph volume:

The haemolymph volume was determined for normal and injected insects at6, 12, 24 and 48 h post-injection, following the method described by Yeager and Munson **(1950)** and modified by Lee **(1961**). The tested insects were injected with 10 µl of 0.2% amaranth red dye (20 mg/ml of 0.5% NaCl). After allowing 5 min for the dye to mix thoroughly with the blood, 10 µl of blood were extracted and diluted to 1ml with distilled water and mixed thoroughly to lyse the haemocytes and clear the sample. The absorbency was read by UNICO Spectrophotometer (SP2100 UV, China) at 515 nm using 1ml cuvette against the standard solution.

A series of saline solutions containing 0.05–0.3 mg of amaranth dye were made to carry out a standard calibration curve. The absorbency of dyed solutions was measured at 515 nm against the blank prepared previously. The measurements were replicated 5 times. The weight of amaranth dye in unknown sample was calculated from the equation obtained from the standard calibration curve.

Haemolymph density:

The haemolymph densities of normal and injected insects at 6, 12, 24 and 48 h postinjection were determined according to the method described by Carrel *et al.* (1990). Haemolymph of the experimental insect was collected immediately into microcapillary tubes calibrated at 1 µl and pre-weighed using an electronic balance (BRAINWEIGH B100, OHAUS Scale, CORP. USA). The filled tube was quickly reweighed. The haemolymph density was expressed as mg/µl.

Haemolymph pH:

The haemolymph pH was determined for normal and injected insects at 6, 12, 24 and 48 h post-injection were determined according to the method described by Heimpel (1955). The bulb of the microelectrode (Model 671, pH meter, Extech, USA) was brought into contact with a drop of oozed haemolymph. All measurements were accomplished at 28 ± 2ºC and samples were replicated 5 times from each insect at each time intervals.

Statistical analysis:

Results of susceptibility tests were analyzed statistically by using software: Probit Analysis Program, Version 4.0. All the rest data were expressed as mean ± standard error (SE) and analyzed by using the SPSS11.5.0 software (SPSS Inc.). The differences between means were analyzed by independent samples *t*-test and one-way ANOVA. The level of significance for each experiment was set at $P < 0.05$ or $P<0.01$.

RESULTS

Susceptibility of *G. mellonella* to bacterial pathogen:

The estimated LC_{50} values, at 95% probability, were 2.5×10^4, 6.7×10^4, and 3.5×10^3 cells/ml for the larvae, pupae and adult insects, respectively (Table 1).

Table 1: Susceptibility of last instar larvae, pupae and adults of *G. mellonella* to *B.t* cells.

Concentration (cells/ml)	Larval stage		Pupal stage		Adult stage	
	Observed mortality (%)	Expected mortality (%)	Observed mortality (%)	Expected mortality (%)	Observed mortality (%)	Expected mortality (%)
2.5×10^3	10	8.6	20	20.9	20	23.4
2.5×10^4	20	27.2	40	38.2	40	45.6
2.5×10^5	70	48.3	60	86.3	70	68.0
2.5×10^6	80	74.3	90	90.5	90	91.3
Control	0	0	0	0	0	0
Slope	0.7914 ± 0.3365		0.6689 ± 0. 1431		0.7126 ± 0.1983	
LC_{50}	2.5×10^4 cells/ml		6.7×10^4 cells/ml		3.5×10^3cells/ml	

Effects of Bt on body weight and water content of *G. mellonella*:

The body water content of un-injected larvae was 180.68±2.09 mg. Bacterial injection decreased the values for the fresh body weight, dry body weight and water content significantly at 24 h ($P < 0.05$) and at 48 h ($P < 0.01$) post-injection, but no changes were observed at3, 6 and 12 h post-injection ($P > 0.05$) as compared with water injected control larvae (Table 2)

Table 2: Total body weight, dry body weight and water content of *G. mellonella* (L.) larvae (last instar) determined at different time intervals post-injection with *Bt.*

Hours post-injection	Total body weight (mg) Mean ± SE		Dry body weight (mg) Mean ± SE		Body water content (mg) Mean ± SE	
	Control	Treated	Control	Treated	Control	Treated
3	249.33 ± 1.93	244.27 ± 2.07	70.40 ± 1.92	67.38 ±2.38	178.93 ± 2.79	176.90 ± 3.18
6	245.74 ± 1.90	241.81 ± 1.81	69.73 ± 2.43	66.12 ±1.80	176.01 ± 3.08	175.69 ± 1.14
12	244.98 ± 2.36	235.79 ± 2.12	68.59 ± 2.47	65.64 ±2.00	176.39 ± 3.11	170.14 ± 2.52
24	242.77 ± 2.63	227.26 ± 2.32	67.45 ± 2.02	60.60 ±1.61	175.32 ± 3.45	166.66 ± 2.77
48	239.33 ± 2.81	225.44 ± 1.96	65.89 ± 2.05	58.68 ± 1.78	173.44 ± 4.09	166.76 ± 2.96
Un-injected	246.88 ± 1.92		73.48 ± 1.77		180.68 ± 2.09	

n = 10 insects per test. Significat difference ($P < 0.05$).

The body water content of the normal pupae was 128.99 ± 3.73 mg. Following Bt injection, the whole parameters showed a significant decrease ($P < 0.05$) at 48 h postinjection **(**Table 3)

Table 3: Total body weight, dry body weight and water content of *G. mellonella* (L.) pupae determined at different time intervals post-injection with *Bt.*

Hours postinjection	Total body weight (mg) Mean ± SE		Dry body weight (mg) Mean ± SE		Body water content (mg) Mean ± SE	
	Control	Treated	Control	Treated	Control	Treated
3	181.70 ± 2.46	175.27 ± 2.64	51.15 ±1.89	48.36 ± 2.62	130.55 ± 3.06	126.91 ± 1.75
6	177.65 ± 2.64	171.31 ± 1.64	50.01 ±2.48	46.41 ± 2.08	127.63 ± 3.36	124.90 ±2.28
12	175.92 ± 2.76	167.41 ± 1.64	48.88 ±2.47	45.10 ± 1.95	127.03 ± 4.32	122.31 ±2.43
24	175.01 ± 2.74	163.46 ± 1.86	47.74 ±2.20	42.64 ± 1.88	127.27 ± 3.61	120.82 ±2.90
48	171.24 ± 1.50	160.44 ± 1.50	45.11 ±1.93	40.67 ± 1.18	126.13 ± 1.99	119.77 ±1.76
Un-injected	179.66 ± 2.65		72.07 ± 2.46		128.99 ± 3.73	

n = 10 insects per test. Significant difference ($P < 0.05$).

The body water content of the un-injected female adults was 89.23 ± 2.28 mg. *B.t*-injection apparently does not affect the dry body weight at all time intervals, while the mean values of the wet weight and water content showed a significant decrease ($P < 0.05$) at 24 and 48 h post-injection. No change was observed at 3, 6 and 12 h after injection as compared to the water injected control insects **(**Table 4).

Table 4: Total body weight, dry body weight and water content of *G. mellonella* (L.) adult females determined at different time intervals post-injection with *Bt.*

Hours postinjection	Total body weight (mg) Mean ± SE		Dry body weight (mg) Mean ± SE		Body water content (mg) Mean ± SE	
	Control	Treated	Control	Treated	Control	Treated
3	139.02 ± 2.00	132.79 ± 2.25	48.28 ± 1.68	46.29 ± 1.49	90.74 ± 3.12	86.48 ± 3.37
6	135.58 ± 2.61	128.42 ± 2.32	45.83 ± 1.48	43.38 ± 1.62	89.74 ± 2.51	85.05 ± 2.28
12	133.12 ± 1.96	123.91 ± 1.53	43.83 ± 1.68	40.64 ± 1.04	89.30 ± 1.53	83.27 ± 2.11
24	129.79 ± 2.01	118.91 ± 1.72	39.75 ± 1.64	36.55 ± 1.24	90.03 ± 2.49	82.36 ± 2.47
48	126.85 ± 1.87	115.89 ± 2.39	37.84 ± 2.53	34.53 ± 1.42	89.01 ± 3.53	81.35 ± 2.92
Un-injected	135.19 ± 1.98		45.96 ± 2.14		89.23 ± 2.28	

n = 10 insects per test. Significant difference ($P < 0.05$).

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The body water content of the un-injected adult males was 72.84 ± 4.39mg. Injection of adult males with *B.t* induced slight changes (P > 0.05) at 6 h post injection in all studied parameters. A significant decrease (P < 0.05) was observed in the fresh body weight and the water content at 12 and 24 h, while a highly significant change (P < 0.01) was noticed at 48 h after injection as compared with controls **(**Table 5)

Table 5: Total body weight, dry body weight and water content of *G. mellonella* (L.) adult males determined at different time intervals post-injection with *Bt.*

Hours post-injection	Total body weight (mg) Mean ± SE		Dry body weight (mg) Mean ± SE		Body water content (mg) Mean ± SE	
	Control	Treated	Control	Treated	Control	Treated
3	106.30 ±2.83	102.49±1.91	35.31 ± 2.32	33.44 ± 1.73	70.99 ±2.57	69.05 ± 2.58
6	102.62 ±1.95	98.42 ±2.78	32.79 ± 1.69	31.56 ±2.37	69.34 ± 1.51	66.86 ±3.19
12	101.68 ± 1.89	91.52 ±2.69	33.27 ± 1.71	29.37 ±2.41	68.89 ±2.36	62.15 ±3.77
24	99.26 ± 2.93	88.78 ±2.14	30.10 ± 2.38	26.41 ±2.32	69.16 ±3.49	62.38 ±4.09
48	97.55 ± 3.66	84.65 ±2.89	28.01 ± 2.43	23.47 ±2.45	69.53 ±4.28	61.18 ±4.04
Un-injected	105.25 ± 2.50		32.40 ± 2.32		72.84 ± 4.39	

n = 10 insects per test. Significant difference (P < 0.05).

Pupae developed from challenged larvae were found to have a total body weight of 164.38±2.61 mg, dry body weight of 44.37± 1.92mg and body water content of 120.01± 2.38 mg representing 73.01±4.2% of the insect fresh body weight . Adult developed from previously challenged larvae had a body weight that accounts for 102.56±2.65mg, dry body weight of 34.29±1.11 mg and water content 68.27±2.06 mg, representing 66.75% of insect fresh weight. Those developed from challenged pupae had 100..81±0.21mg, 33.89±1.82 mg and 66.92±2.62 mg for total body weight, dry weight and water content, respectively. The water content account for 66.38% of total body weight.The total body weight and dry weight and water content of pupae coming from previously challenged larvae, and adults developed from treated larvae and pupae showed no significant changes (P> 0.05) with treated insects, while it were significantly different (P<0.05) as compared with normal ones (Table 6)

Table 6: Total body weight, dry body weight and water content of *G. mellonella* (L.) pupae and adults developed from previously challenged stagesdetermined at different time intervals postinjection with *Bt.*

Developmental Stage	Treatment	A (mg) Mean ± SE	Dry body weight (mg) Mean ± SE	Body water content (mg) Mean ± SE
Pupae	Normal	178..99 ± 2.65 a	50.00± 2.4 a	128.99 ± 3.73 a
	Treated	160.44 ± 1.50 b	42.74± 1.18 b	117.70 ± 1.76 b
	Developed from treated larva	164. .38 ± 2.61b	44.37± 1.92a	120.01± 2.38 b
Adults	Normal	116.41 ± 2.62a	40.60 ±1.91 a	75.81 ± 2.08 a
	Treated	97.28 ± 3.01b	32.00 ± 2.12 b	65.28± 1.39b
	Developed from treated larva	102.56 ± 2.65 b	34.29 ± 1.11 b	68.27 ± 2.06 b
	Developed from treated pupa	100..81 ± 0.21 b	33.89± 1.82 b	66.92 ± 2.62 b

Different letters for each stage indicate significant change (P<0.05).

Effect of Bt on haemolymph volume of *G. mellonella*:

The normal (un-injected) larvae, pupae, adult females and adult males of *G. mellonella* contained 89.79 ± 2.19, 43.27 ± 2.76, 16.06 ± 2.47 and 11.12 ± 2.11 µl of haemolymph per insect, respectively. These values contributed to about 49.70, 33.55, 18.00 and 16.88 % of the total water content of the larva pupae, adult females and adult males, respectively, and about 36.51, 24.17, 11.88 and 11.11 % of their total body weights, respectively. After bacterial treatment, haemolymph volumes of larvae, pupae, adult females and adult males showed significant increase ($P< 0.05$) at different time intervals as compared to the water injected controls (Table 7).

Table 7: Haemolymph volume (µl/larva) of *G. mellonella* larvae, pupae, adult females and adult males determined at different time intervals post-injection with Bt using amaranth dye method

Hours post injection	Haemolymph volume (µl / larva) (Mean ± SE)							
	Larval stage		Pupal stage		Adult females		Adult males	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
6	94.21 ± 2.40	116.82 ± 1.38	52.81 ± 2.53	68.94 ± 1.53	22.01 ± 2.43	34.99 ± 1.61	16.18 ± 2.68	28.82 ± 2.29
12	95.01 ± 2.98	122.14 ± 1.63	55.01 ± 2.29	69.81 ± 2.16	17.47 ± 2.45	3798 ± 0.95	13.09 ± 1.71	32.14 ± 1.51
24	91.67 ± 2.69	120.08 ± 1.55	51.07 ± 2.86	70.45 ± 2.90	15.12 ± 1.61	37.14 ± 0.95	10.25 ± 2.44	29.84 ± 2.51
48	86.61 ± 2.95	115.41 ± 1.49	47.84 ± 2.21	66.77 ± 1.37	15.91 ± 2.33	31.92 ± 2.84	8.12 ± 2.22	27.31 ± 2.32
Un-injected	89.79 ± 2.19		43.27 ± 2.76		16.06 ± 2.47		11.12 ± 2.11	

Significant difference ($P < 0.05$).

Pupae developed from challenged larvae were found to have about 59 .91 ± 2.09 µl of haemolymph/pupa. This value contributed to about 49. 92 % of the total water content of these insects and about 36.45 % of the fresh body weight. Continuously, adults developed from these challenged larvae had 25.12 ± 0.87 µl of haemolymph/insect. This account for 36.80 % of total body water content and about 24.49 % of the wet body weight, while those developed from previously challenged pupae account had 24.76 ± 1.43 µl of haemolymph/insect, constituting about 37.00% of the water content and about 24.56 % of the total fresh weight. The haemolymph volume of pupae coming from previously challenged larvae, and adults developed from treated larvae and pupae showed no significant changes ($P> 0.05$) with treated insects, while it significantly increase ($P<0.05$) as compared with normal ones (Table 8)

Table 8: Haemolymph volume (µl / insect) of *G. mellonella* pupae and adults developed from previously challenged stages, using amaranth dye method.

Developmental Stage	Treatment	Haemolymph volume (µl / larva) (Mean ± SE)
Pupae	Normal	43.27 ± 2.76 a
	Treated	66.77 ± 1.37 b
	Developed from treated larva	59 .91 ± 2.09b
Adults	Normal	14.51 ± 1.34a
	Treated	30.12 ± 2.71b
	Developed from treated larva	25.12 ± 0.87 b
	Developed from treated pupa	24.76 ± 1.43 b

Different letters for each stage indicate significant change ($P<0.05$).

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Effect of Bt injection on haemolymph density of *G. mellonella*:

The estimated mean values of the haemolymph density of un-injected larvae, pupae, adult females and adult males of *G. mellonella* were 0.98 ± 0.03, 1.04 ± 0.02, 1.03 ± 0.03 and 1.01 ± 0.02 mg/µl, respectively. In bacterial injected insects, the haemolymph density increased significantly ($P < 0.05$) only at 6, 12 and 24 h, and then decreased to the original level at 48 h (Table 9).

The haemolymph density of pupae developed from challenged larvae were found to have 1.44 ± 0.04 mg/µl, and adults developed from these larvae had 1.33 ± 0.04 mg/µl, while those developed from previously challenged pupae account 1.28 ± 0.03 mg/µl. The haemolymph density of pupae coming from previously challenged larvae, and adults developed from treated larvae and pupae showed no significant changes ($P > 0.05$) with treated insects (Table 10).

Table 9: Haemolymph density (mg/µl) of *G. mellonella* larvae, pupae, adult females and adult males determined at different time intervals post-injection with Bt

Hours post injection	Haemolymph density (mg/µl) (Mean ± SE)							
	Larval stage		Pupal stage		Adult females		Adult males	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
6	0.976 ± 0.01	1.27 ± 0.02	1.02 ± 0.03	1.44 ± 0.05	1.04 ± 0.02	1.34 ± 0.03	0.973 ± 0.02	1.23 ± 0.02
12	1.05 ± 0.02	1.19 ± 0.03	0.99 ± 0.01	1.47 ± 0.05	1.14 ± 0.04	1.22 ± 0.04	1.03 ± 0.03	1.18 ± 0.02
24	0.94 ± 0.02	1.06 ± 0.03	1.00 ± 0.03	1.35 ± 0.06	0.98 ± 0.02	1.31 ± 0.03	1.06 ± 0.03	1.53 ± 0.04
48	1.01 ± 0.01	1.08 ± 0.03	1.01 ± 0.03	1.33 ± 0.04	0.99 ± 0.03	1.38 ± 0.03	0.98 ± 0.02	1.32 ± 0.03
Un-injected	0.98 ± 0.03		1.04 ± 0.02		1.03 ± 0.03		1.01 ± 0.02	

Significat difference ($P < 0.05$).

Table 10: Haemolymph density (mg/µl) of *G. mellonella* pupae and adults developed from previously challenged stages

Developmental Stage	Treatment	Haemolymph density (mg/µl) (Mean ± SE)
Pupae	**Normal**	1.04 ± 0.02a
	Treated	1.38 ± 0.03b
	Developed from treated larva	1.44 ± 0.04b
Adults	**Normal**	1.02 ± 0.03a
	Treated	1.35 ± 0.04b
	Developed from treated larva	1.33 ± 0.04b
	Developed from treated pupa	1.28 ± 0.03b

Different letters for each stage indicate significant change ($P<0.05$).

Effect of Bt on haemolymph pH of *G. mellonella*:

Table (11) indicated that the mean haemolymph pH values of un-injected larvae, pupae, adult females and adult males of *G. mellonella* were 7.13 ± 0.03, 7.10 ± 0.04,7.08 ± 0.03 and 7.10 ± 0.04, respectively (i.e., slightly alkaline haemolymph). Although, there were significant increases ($P < 0.05$) at 6, 12 and 24 h in the haemolymph pH of Bt-treated insects, the haemolymph pH was still slightly alkaline.

Table 11: Haemolymph pH of *G. mellonella* larvae, pupae, adult females and adult males determined at different time intervals post-injection with Bt

Hours post injection	Haemolymph pH (Mean ± SE)							
	Larval stage		Pupal stage		Adult females		Adult males	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
6	7.03 ± 0.02	7.30 ± 0.01	6.90 ± 0.06*	6.80 ± 0.02	7.05 ± 0.02	7.10 ± 0.04	7.01 ± 0.04	7.10 ± 0.04
12	7.03 ± 0.02	7.12 ± 0.01	6.81 ± 0.04	6.99 ± 0.02	7.02 ± 0.02	7.10 ± 0.04	7.06 ± 0.02	7.14 ± 0.04
24	7.06 ± 0.02	7.16 ± 0.02	7.09 ± 0.03	7.14 ± 0.02	7.07 ± 0.03	7.14 ± 0.04	7.04 ± 0.02	7.16 ± 0.04
48	7.09 ± 0.03	7.11 ± 0.02	7.03 ± 0.03	7.17 ± 0.02	7.10 ± 0.04	7.14 ± 0.04	7.08 ± 0.03	7.13 ± 0.04
Un-injected	7.13 ± 0.03		7.10 ± 0.04		7.08 ± 0.03		7.10 ± 0.04	

Significant difference ($P < 0.05$).

The haemolymph mean pH value of pupae developed from challenged larvae were found to be 7.15 ± 0.02, and adults developed from these larvae had pH value of 7.14 ± 0.02, while those developed from previously challenged pupae account 7.13 ± 0.02. The haemolymph pH of pupae coming from previously challenged larvae, and adults developed from treated larvae and pupae showed no significant changes ($P > 0.05$) with treated insects (Table 12).

Table 12: Haemolymph pH of *G. mellonella* pupae and adults developed from previously challenged stages, using amaranth dye method

Developmental Stage	Treatment	Haemolymph pH (Mean ± SE)
Pupae	**Normal**	7.10 ± 0.04a
	Treated	7.17 ± 0.02b
	Developed from treated larva	7.15 ± 0.02b
Adults	**Normal**	7.09 ± 0.02a
	Treated	7.16 ± 0.02b
	Developed from treated larva	7.14 ± 0.02b
	Developed from treated pupa	7.13 ± 0.02b

Different letters for each stage indicate significant change ($P<0.05$).

DISCUSSION

Not only *G. mellonella* are important to apiculture industry, but also they are investigated considerably more as a model organism for studying insect physiology. This comes from the fact that they can be easily reared and maintained in large numbers. There are no ethical constraints and their short life cycle makes them ideal for large-scale studies. Given the size of the insect, it is possible to obtain easily hemolymph and other tissues (Wojda, 2017). Many studies have performed using insects as an alternative model host for investigating virulence factors of human pathogenic bacteria (Scully and Bidochka, 2006; Lionakis, 2011), and this substitution has several benefits. Within this frame, *G. mellonella* are used in our study to answer several questions; the most important are; (1) what are the physiological changes, due to infection, that take place during the insect development, (2) what is the developmental stage of an insect that has more susceptibility to microbial invasion,

(3) is the sex has a relevance towards this response, and finally (4) is a previous challenge would enhance the same response of the later developmental stage?

Bacterial formulations, especially Bt are receiving more attention as microbial insecticides and also are being identified as key natural mortality factors in the environment of many important insect pests. It is active against many pest species including the greater wax moth (Bravo *et al*., 2005). However, lack of information on the quantity of test pathogen that reach to the test insect makes comparison of the infectivity of different or the same pathogen, extremely difficult. This in turn would lead to difficulty in the standardization of insect pathogenic micro-organisms, and hence also in the evaluation of their potential in insect control. This factor has attracted considerable attention in recent times, to quantify the actual amount of pathogen introduced into the tested insect. Therefore, the pathogen in the present study was placed in direct contact with the susceptible tissue (hemocoel) by injection, so that variation induced by loss of dose and irregularities of invasion was avoided (Barakat, 2001). Several workers intended to use this technique especially for the study of insect immunity and the observation of the biochemical and physiological changes induced by pathogenic infection. Among the numerous authors who have used the same technique are: Meshrifand Barakat (2002); Krishnedu *et al.* (2010); Mo'men *et al*. (2012); Mahmoud *et al.* (2015); Pereira *et al.* (2015) using different insects and different bacterial pathogens.

Based on qualitative descriptions on how different developmental stages of *G. mellonella* suffered from infection with the chosen bacteria and the resulting mortality patterns, the present study clearly demonstrated that the pupae of are more resistant to Bt (LC_{50} = 5.9 × 10^5 cells/ml). In addition, the presence of proteases and other metabolic compounds, due to the degradation of larval tissues, will add to a bacteria unfriendly environment. Similar explanation was given by Kurata *et al.* (1992); Natori *et al*. (1999) who attributed the high level of resistance found in pupae, against bacteria to the presence of defense proteins that function within development and expressed during the pupal stage. Also the present study stated that the adult resistance was found to be the least effective (LC_{50} = 4.6 × 10^4 cells/ml). This may be resulted from that, the adult immune defense system is traded off against some other fitness components as survival and reproduction. This observation was confirmed by some studies (Sheldon and Verhulst 1996; Schmid-Hempel 2003 a & 2005).

Inter- and intra-specific variation in pathogenicity may be attributed to secondary metabolites and/or enzymes, bacteria secret into their hosts.Such compounds have been suggested to be important virulence factors allowing pathogens to successfully invade hosts (Meshrif *et al*., 2007).

Typically, the insect responses to the infection on protein distribution, as well as on water distribution. It was considered that the results for blood volume would be more informative with knowledge of the dry weight and water values for comparison. Desiccations were carried out concomitant with those on microbial variation (Barakatand Meshrif, 2007). Results are expressed as percentages of the total weight, thus permitting comparisons of relative values from individuals of different weight during development.

The present results indicated that the estimated percentage of body water content of different developmental stages of *Galleria* ranges from more than 50% to less than 90% of the total body weight. These results are supported by the work of

Wigglesworth (1974) on different insect species from different orders.

Changes in the body weight attracted the immunologists in the past, since it gives an integrated picture with haemolymph volume about the effect of treatment. Since the total body water content could be partitioned into two fractions: tissue water and haemolymph water. The decrease in body weight of the *Galleria* larvae, pupae and adults post-bacterial injection, as observed in the present study, may be attributed basically to the decrease of body water content. This decrease may be due to the loss of tissue water. These observations are in agreement with those of(Radwan,2015) working on *G. mellonella* and with those of Carrel *et al.* (1990); Bardoloi and Hazarika (1992); El-Kattan (1995) and Barakat and Meshrif (2007) who obtained similar results with other agents and other insect species.

With respect to sex, the fresh body weight of normal adult females was found to be much greater than males, i.e. the male moths displayed a greater loss of mass during the development in comparison to females. As there is no uptake of food during metamorphosis and the larval gut content has been purged prior to cocooning, so this difference in body mass is attributed to difference in excretion rate (Meylaers *et al.*,2007). When an infection occurs, the mass loss is affected in a sex-specific way, increasing in males over females. Since for female moths, it is important to retain as much mass as possible during development, as it is directly correlated with reproductive success (Honek, 1993; Nylin and Gotthard, 1998), but it is not the case in males. Similar observations on sexual dimorphism in mass loss were detected by Fischer and Fiedler (2000) in the Lepidopteran *Lycaenatityrus* and by Meylaers *et al.* (2007) on the same insect, *G. mellonella* infected by *E. coli.*

Concerning pupae coming from treated larvae and adult insects developed from previously challenged stages, no changes were detected in their body weights (wet and dry) and water content as compared with treated insects, but a significant decrease was found as compared with normal ones, indicating the appearance of a resistance transfer in the subsequent stages.

Many reports demonstrated that haemolymph is the water reservoir and that the haemolymph volume varies considerably with numerous factors such as age, developmental status, diet and hydration state (Edney, 1977; Barakat and Meshrif, 2007) with slight differences due to rearing conditions and diet.

An increase in blood volume was observed, in this study, in all tested developmental stages of *Galleria* following bacterial injection. It was obvious thus, that Bt decreased body water content, and in the same time, caused loss of water in the tissues (which was reflected from the decrease in the dry body weight) and gained it in the haemolymph. Similar results were obtained by (El-Kattan,1995) on *Plodia interpunctella* larvae and (Barakat,1997) on *G. mellonella*, larvae injected with different species of bacteria. Water withdrawal from the cells and tissues following bacterial infection may be due to the increase in the fluidity of the cell membrane (Bardoloi and Hazarika, 1992).

In contrast, some investigators including (Bucher,1957) on his study on *Malacosoma pluviale* infected with a spore-forming bacteria reported an extreme loss of water in the cells and tissues and subsequently in the haemolymph (i.e. decrease in the haemolymph volume). This is the case when bacteria invade the gut, producing diarrhea and/or vomiting. But in the present situation, when bacteria invade the hemocoel, diarrhea or vomiting was not observed and a water gain in the haemolymph was observed.

The current results also indicated that the haemolymph volume of normal

Galleria females is found to be greater than males this may be due to the increase of total haemocyte population of females over males as reported by (Sanjayan *et al.* 1996) worked on the milkweed bug, *Spilostethus hospes*. In addition, both males and females respond to the Bt infection by an increase in the haemolymph volume.

The haemolymph density and pH were also increased by the injection of bacteria into the different developmental stages of *G. mellonella* at almost all post-injection periods. This may be due to the decrease of blood volume as well as the increase of bacterial metabolites. These results are in agreement with those of Werner and Jones (1969), (Barakat,1997) on *G. mellonella* larvae and Angus and Heimpel (1956) on the larvae of *Bombyx mori* infected with *Bacillus sotto*.

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الفروق الجسدية في الدم لغاليريا ميلو نيلا (لبيدوبترا:بيرلدى) بعد الحث المناعى

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المستخلص

سجلت هذه الدراسة الاختلافات في محتوى الماء في الجسم وعلاقته بحجم هيموليمف وكثافة ودرجة الحموضة للعثة الشمع أكبر (غاليريا ميلونيلا) عبر مراحل تطور مختلفة في فترات زمنية مختلفة بعد الحقن باسيلس ثيورنجينسيز كيورستاكيبا.أشارت النتائج الأولية المتعلقة إلى أن الشرانق كانت أكثر مقاومة بينما كان البالغون معرضين بدرجة عالية. كما أشارت النتائج المتعلقة بالتحقيقات الفسيولوجية إلى أن اليرقات تحتوي على كمية أكبر من الماء في الجسم مقارنة بالشرانق والبالغين ، بينما زاد حجم وكثافة اللهيموليمف بشكل مباشر مع تطور الحشرات.كان للهيموليمف نفس قيمة درجة الحموضة في جميع مراحل النمو. بعد الحقن باسيلس ثيورنجينسيز انخفضمحتوى الماء في الجسم ، وفي الوقت نفسه، زاد حجم الدم ، الكثافة ودرجة الحموضة في جميع مراحل النمو. القيم القصوى تحققت في 48 ساعة بعد الحقن .تطورت الشرانق من يرقات سبق علاجها ، و كذلك البالغون من يرقات وشرانق سبق معالجتهما تغيرات مهمة مقارنةً بالمتغيرات الطبيعية ولم تظهر أي تغييرات مقارنةً بالحشرات المعالجة. تبين هذه النتائج أن المجموعات العمرية المختلفة تستجيب لتأثير البكتيريا المسببة للأمراض بنفس الدرجة وأن التغيرات الفسيولوجية لم تؤثر على نمو وتحول هذه الحشرة.