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Shaimaa AA Moamen
Department of Entomology, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt

Emad MS Barakat
Department of Entomology, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt

Mohamed O Abokersh
Department of Zoology, Faculty of Science, Al-Asmarya Islamic University, Zeletin, Lybia

Correspondence
Shaimaa AA Moamen
Department of Entomology, Faculty of Science, Ain Shams University, Abbassia, Cairo, Egypt

The biochemical alternations of *Galleria mellonella* hemolymph following induction of immune response

Shaimaa AA Moamen, Emad MS Barakat and Mohamed O Abokersh

Abstract
The present study investigated the quantitative assessments of hemolymph biochemical composition at different developmental stages of *Galleria mellonella* post-injection with a sub-lethal concentration of *Bacillus thuringiensis* (Bt). The estimated LC_{50} values were 1.3×10^5, 5.9×10^5 and 4.6×10^4 cells/ml for the larvae, pupae, and adults, respectively. Pupae were highly resistant, while the adults showed more susceptibility without any effect of sex on the response. The levels of carbohydrates, lipids, and proteins were of great value during the larval stage, followed by a reduction in the pupal stage finally a sharp decline had been observed in the adult ones. A time-dependent study revealed that the carbohydrate levels augmented significantly ($P<0.05$), while the levels of lipids and proteins reduced significantly at all developmental stages and there were no significant differences between males and females in their responses. Pupae and adults developed from previously treated stages showed no significant changes ($P\geq0.05$) than treated insects. Finally, we deduced that adult was the more susceptible stage to begin the control strategies and the immune activation in the *G. mellonella* was transstadially transmitted from larvae to pupae and adults emerged from primary challenged stage.

Keywords: Biochemical composition, *Galleria mellonella*, *Bacillus thuringiensis*, transstadial transmission

1. Introduction
Many studies had been directed to find ways to control the greater wax moth, *Galleria mellonella* as a result of the damage caused by its larvae, which is severe in tropical and sub-tropical regions and is believed to be one of the contributing factors to the decline in wild honeybee populations [34]. This pest has received more attention as a model organism for immunological and toxicological examinations, with more focus on proven control measures [10-16]. *G. mellonella* larvae can be used in infectivity trials and toxicity testing, and that these essays represent an inexpensive and readily executable alternative to testing in rodents [19].
Entomo-pathogenic bacterial formulations, mainly *B. thuringiensis* (Bt) are being identified as a key natural mortality factor in the environment of many important insect pests, it is active against more than 150 pest species including the greater wax moth [4]. Several scientists planned to place the pathogen in direct contact hemocoel by injection [25-24]. The hemolymph is composed of fluids and dissimilar types of cells offer an available criterion of the response and it can undergo measurable alterations. Insect hemolymph is influenced at least in the level of its physical properties or in its biochemical composition [9]. Through the regulation of ionic and chemical composition, hemolymph conserves the appropriate immune response that includes cellular and humoral factors. The cellular response consists mainly of phagocytosis and encapsulation. The humoral immune response includes the rapid synthesis of a battery of antimicrobial peptides [2]. Insect plasma is the transport system for nutrients, hormones, and metabolic wastes, and contains elements of the immune system [14]. After infection, antimicrobial peptides are synthesized in the fat body and then released to the hemolymph [42]. The knowledge of ecology and biochemistry including proteins, lipids, and carbohydrates of insects necessary to achieve successful control [15]. Invertebrate defense system may be capable of a specific memory, the degree of specificity and memory in invertebrate immunity is unclear. Although a defense reaction can be induced in invertebrates against pathogens, the responses may not be specific as they can distinguish only between different classes of a pathogen. The studies on immune memory in invertebrates vary considerably regarding their experimental design but often use a repeated challenge (or 'priming' followed by 'challenge')

approach [22]. Recently studies dealing with so-called 'trans-generational immune priming', where priming of the parental generation leads to stronger immune reactions or improved resistance of the offspring [5-6].

Therefore, this study designed to (1) Follow up the biochemical drastic changes at different developmental stages of tested insect before and after induction of the hemolymph to detect the more susceptible stage to be controlled. (2) Reconnoiter whether the immune response induced in the hemocoel of *Galleria mellonella* is transstadially transmitted to pupae and adults developed from previously treated stages through the fluctuations of the estimated levels of plasma carbohydrates, lipids and proteins.

2. Materials and Methods

2.1. Insect rearing

A colony of the greater wax moth, *Galleria mellonella* (L.) used in the present study, was gained from Plant Protection Research Institute, Agricultural Research Center, Dokki, Egypt, was reared on an artificial diet described by [20] for several generations and had shown no infectious diseases.

2.2. Pathogenic agent

The bacterium, *Bacillus thuringiensis kurstaki* (Bt) as wettable powder formulation (AGERIN, 3200 IU/mg) made by the Agricultural Genetic Engineering Research Institute, Ministry of Agriculture, Egypt. The bacterial suspension was adjusted to a concentration of 2.5×10^9 cells/ml by the pour plate count method described by [8]

2.3. Susceptibility level of insect to bacterial pathogen

From 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, two µl were injected into each insect. The treated insects were maintained at 30±2ºC and final mortality percentages were scored 48 h post-injection. Control insects were treated with equivalent volumes of sterile distilled water only. A stock suspension of a sub-lethal concentration of Bt that produces 50% mortality was prepared. Consequently, 2 µl of this concentration was injected into the hemocoel of the experimental insect for investigating the influence of infection on the various parameters studied.

2.4. Preparation of hemolymph plasma

According to [18], hemolymph samples from normal, control and immune enhanced insects were collected after injection, the hemocytes were removed by centrifugation (Human Centrifuge, TGL-16XYJ-2, 16000 rpm, Korea) in cold at 3000 rpm for 10 min at 4ºC. The supernatant (referred to as plasma) was removed from the hemocyte pellet, and immediately transferred into sterile and chilled Eppendorf tubes and stored at -18ºC until use.

2.5. Assessment of the total hemolymph carbohydrates

A total carbohydrate content of the plasma was assessed according to the method described by [40-41], the absorbance was recorded by (UNICO Spectrophotometer, SP2100 UV, China) at 625 nm, and the concentration of the carbohydrates (mg/ml) was valued using the formula resulted from the standard calibration curve using glucose solution.

2.6. Estimation of the total hemolymph lipids

The total lipids content of the hemolymph was estimated using phosphorvanilin reagent according to the method of [13]. 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.

2.7. Quantification of hemolymph protein

According to the method designated by [3], the total protein content of the hemolymph was estimated by using Coomassie brilliant blue G-250 (CBB) and the absorbency at 595 nm was measured. A standard calibration curve was constructed using Bovine serum albumin (BSA) solutions as the standard proteins

2.8. Statistical analysis

Data obtained from the susceptibility test were estimated according to [12] using "LdPLine®"software, [http://embakr.tripod.com/ldpline/ldpline.htm]. Data of all investigate were expressed as mean ± standard error (SE) and analyzed by using the SPSS11.5.0 software (SPSS Inc., 2012). 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 $P<0.05$.

3. Results

3.1. Susceptibility of *G. mellonella* to bacterial pathogen

The estimated LC_{50} values (Table 1) were 1.3×10^5, 5.9×10^5 and 4.6×10^4 cells/ml for the larvae, pupae, and adult insects, respectively. While, LC_{20} for the same stages were 1.2×10^4, 3.2×10^4 and 3.0×10^3cells/ml respectively, these concentrations were used to investigate the subsequent tests.

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

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	17.9	20	18.4
2.5×10^4	20	28.2	40	40.2	40	42.6
2.5×10^5	70	58.5	60	66.3	70	70.0
2.5×10^6	80	84.3	90	86.2	90	89.2
Control	0	0	0	0	0	0
X^2 calculated	1.043		0.3286		0.0499	
Slope	0.7914 ± 0.2259		0.6689 ± 0.2101		0.7126 ± 0.2159	
LC_{50}	1.3×10^5 cells/ml		5.9×10^5 cells/ml		4.6×10^4cells/ml	
LC_{20}	1.2×10^4 cells/ml		3.2×10^4 cells/ml		3.0×10^3cells /ml	

Observed mortality (%) is means 3 replicates.

Chi^2 (χ^2) tabulated = 6.0

χ^2 calculated = (Observed mortality- Expected mortality)^2 - Expected mortality.

3.2. The total hemolymph carbohydrates

Plasma Carbohydrate content of un-injected larvae, pupae, adult female and adult males were 6.82±1.01, 5.18±1.13, and 4.37±0.93and 4.79±0.16 mg/ml, respectively. At all determined time intervals, levels of plasma carbohydrates of different developmental stages after induction of the immune system were significantly reduced than those of controls (Table 2, Fig. 1). Moreover, there is no significant difference in the hemolymph carbohydrates between both sexes of an adult.

The plasma carbohydrate content of pupae, adult female and adult male developed from previously induced larvae were 6.78±1.12 4, 14.86±1.43 and 7.97±0.94 mg/ml, respectively. While adult females and males emerged from challenged pupae were 15.20±0.76 and7.37±1.01mg carbohydrate/ml plasma, respectively. These levels showed no significant differences than treated insects (Table 3, Fig. 2).

Table 2: Total hemolymph carbohydrates content (mg/ml) of *G. mellonella* larvae, pupae, adult females and adult males at different time intervals post-injection.

Treatment	Hemolymph carbohydrates (mg/ml) (Mean ± SE)			
	Larval stage	Pupal stage	Adult females	Adult males
Un-injected	6.82±1.01	5.18±1.13	4.37±0.93	4.79±0.16
control	8.79±1.07	6.91±1.10	5.69±0.02	4.23±1.05
6h	34.78±0.00*	17.94±1.01*	15.81±0.10*	8.12±1.12*
12h	29.52±0.01*	16.92±0.92*	9.76±0.02*	4.62±1.06*
24h	25.19±0.00*	14.72±1.11*	5.13±0.04*	3.65±0.88*
48h	8.18±0.01	10.64±1.01	2.73±0.01	1.75±1.06

n = 5 replicates per test.
* Significat difference ($P<0.05$).

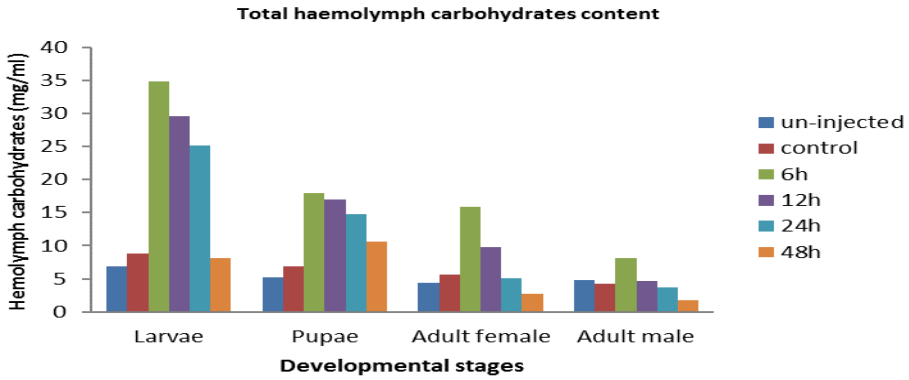

Fig 1: Total hemolymph carbohydrates content (mg/ml) of *G. mellonella* larvae, pupae, adult females and adult males at different time intervals post-injection

Table 3: Total hemolymph carbohydrates content (mg/ml) of *G. mellonella* pupae and adults developed from previously challenged stages

Developmental Stage	Treatment	Haemolymph carbohydrates (mg/ml) (Mean ± SE)
Pupae	Treated	7.44±1.15[b]
	Developed from treated larva	6.78±1.12[b]
Adult female	Treated	15.81±0.18[a]
	Developed from treated larva	14.86±1.43[a]
	Developed from treated pupa	15.20±0.76[a]
Adult male	Treated	8.12±1.12[b]
	Developed from a treated larva	7.97±0.94[b]
	Developed from a treated pupa	7.37±1.01[b]

The same letters for each stage are not significantly different (P≥0.05)

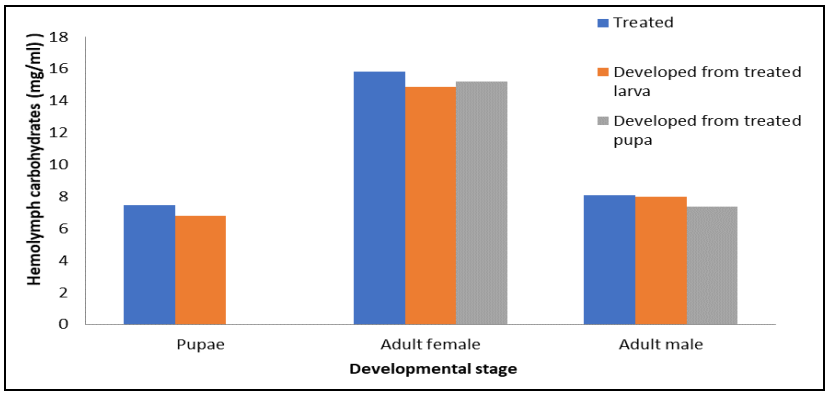

Fig 2: Total hemolymph carbohydrates content (mg/ml) of *G. mellonella* pupae and adults developed from previously challenged stages

3.3. The total hemolymph lipids

The plasma lipid content of un-injected larvae, pupae adult female and adult males were 4.25±0.38, 4.03±0.57, 3.84±0.39 and 3.95±0.46 mg lipids/ml plasma, respectively. A significant decline had been recorded at different developmental stages following injection of pathogenic agent compared with those of controls (Table 4 and Fig.3). There weren't any significant differences between the adult of both sexes.

Pupae, adult female and adult male developed from previously challenged larvae contained4.37±0.93, 3.81±0.97 and 2.12±1.06 mg lipid/ml plasma, respectively. Adult female and male emerged from challenged pupae contained 3.20±1.07 and 2.34±0.98 mg lipids/ml plasma. These levels showed no changes compared with treated one (Table 5 and Fig. 4).

Table 4: Total hemolymph lipids content (mg/ml) of *G. mellonella*, larvae, pupae, adult females and adult males determined at different time intervals post-injection

Treatment	Hemolymph lipids (mg/ml) (Mean ± SE)			
	Larval stage	Pupal stage	Adult females	Adult males
Un-injected	4.25±0.38	4.03±0.57	3.84±0.39	3.95±0.46
control	3.16±0.19	4.02±0.19	3.11±0.27	3.44±0.19
6h	32.29±0.11*	13.86±0.15*	2.18±0.11*	2.49±0.11*
12h	41.91±0.63*	8.33±0.27*	3.81±0.43*	3.79±0.63*
24h	22.27±0.24*	5.61±0.30*	3.55±0.15*	3.52±0.24*
48h	21.98±0.09*	3.72±0.29*	2.10±0.29*	2.65±0.09*

n=5 replicates per test.
* Significat difference (P<0.05).

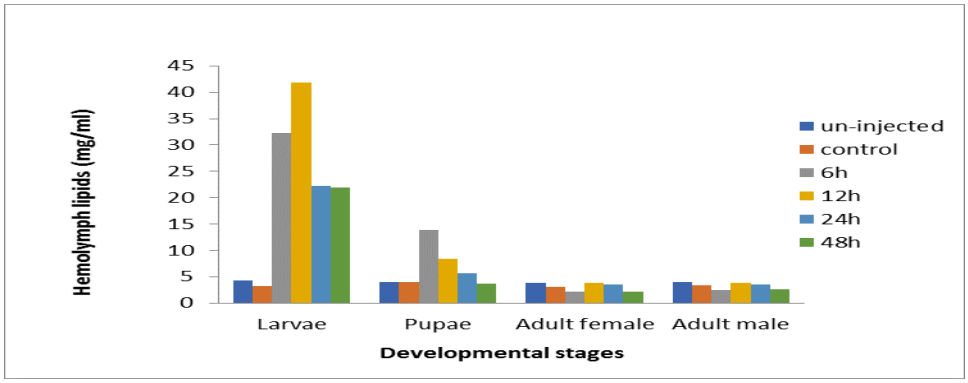

Fig 3: Total hemolymph lipids content (mg/ml) of *G. mellonella*, larvae, pupae, adult females and adult males determined at different time intervals post-injection

Table 5: Total hemolymph lipid content (mg/ml) of *G. mellonella* pupae and adults developed from previously challenged stages

Developmental Stage	Treatment	Hemolymph lipids (mg/ml) (Mean ± SE)
Pupae	Treated	3.66±0.38^{b}
	Developed from a treated larva	4.37±0.93^{b}
Adult female	Treated	3.05±1.83^{a}
	Developed from treated larva	3.81±0.97^{a}
	Developed from a treated pupa	3.20±1.07^{a}
Adult male	Treated	2.29±1.27^{b}
	Developed from a treated larva	2.12±1.06^{b}
	Developed from a treated pupa	2.34±0.98^{b}

The same letters for each stage are not significantly different (P≥0.05)

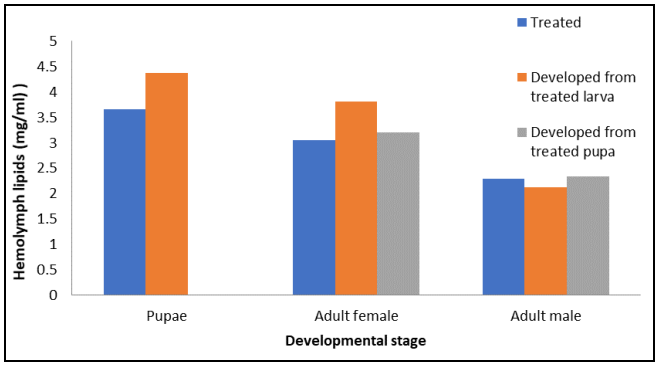

Fig 4: Total hemolymph lipid content (mg/ml) of *G. mellonella* pupae and adults developed from previously challenged stages with Bt

3.4. The total hemolymph proteins

The plasma protein of un-injected larvae, pupae adult female and adult males were 102.01±2.04, 98.46±2.64, 72.24 ± 0.28and 12.00±1.67 mg proteins /ml plasma, respectively. The levels of plasma proteins measured at different developmental stages decreased sharply at all examined time intervals after pathogenic infection than those of controls (Table 6 and Fig. 5). No significant difference in the plasma protein content had been estimated in adult of both sexes. Pupae, adult female and adult male developed from previously challenged larvae contained 121.49±0.03, 109.64±0.98, 9.54±0.87 mg protein/ml plasma, respectively. Adult female and adult male emerged from challenged pupae contained 110.98±1.82 and 8.01±1.43 mg protein/ml plasma respectively. These levels showed no deviations from treated one (Table 7 and Fig. 6).

Table 6: Total hemolymph protein content (mg/ml) of *G. mellonella*, larvae, pupae, adult females and adult males determined at different time intervals post-injection

Treatment	Hemolymph protein (mg/ml) (Mean ± SE)			
	Larval stage	Pupal stage	Adult females	Adult males
Un-injected	102.01±2.04	98.46±2.64	72.24 ± 0.28	12.00±1.67
Control	131.52±1.67	115.08±2.01	89.34±1.04	18.93±0.94
6h	182.92±1.94*	124.23±1.24*	111.28±0.56*	10.76±0.53*
12h	177.55±0.27*	98.36±1.92*	87.81±0.83*	8.79±0.83*
24h	135.12±0.30*	87.64±2.30*	83.55±0.63*	2.52±0.44*
48h	70.45±0.62*	51.56±1.89*	42.18±0.11*	2.49±0.13*

n = 5 replicates per test.
* Significat difference (P<0.05).

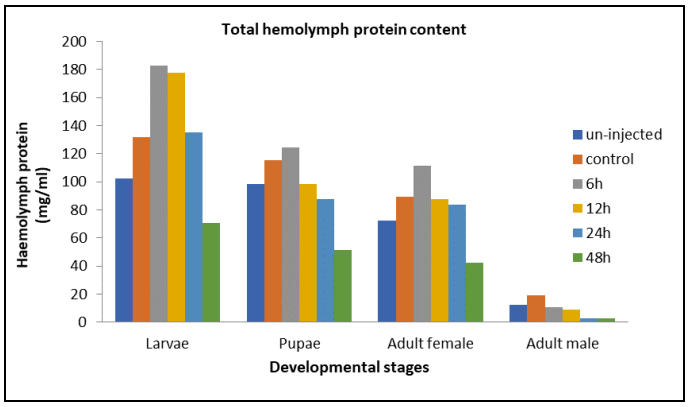

Fig 5: Total hemolymph protein content (mg/ml) of *G. mellonella*, larvae, pupae, adult females and adult males determined at different time intervals post-injection

Table 7: Total hemolymph protein content (mg/ml) of *G. mellonella* pupae and adults developed from previously challenged stages

Developmental Stage	Treatment	Hemolymph protein (mg/ml) (Mean ± SE)
Pupae	Treated	123.93 ± 0.75^{b}
	Developed from treated larva	121.49±0.03^{b}
Adult female	Treated	110.54±1.43^{a}
	Developed from treated larva	109.64±0.98^{a}
	Developed from treated pupa	110.98±1.82^{a}
Adult male	Treated	10.08 ± 0.28^{b}
	Developed from treated larva	10.08 ±0.87^{b}
	Developed from treated pupa	8.01±1.43^{b}

The same letters for each stage are not significantly different (P≥0.05)

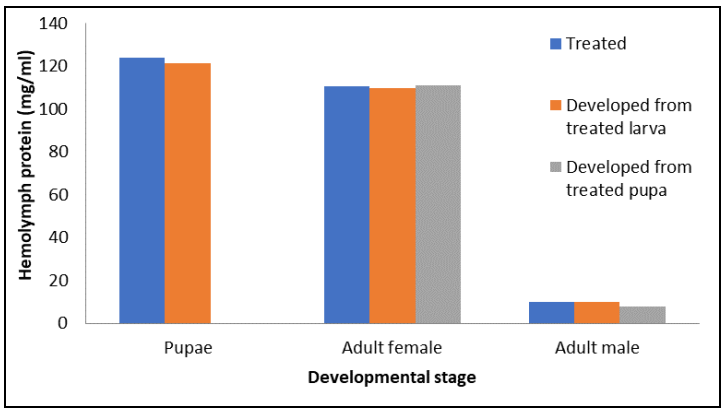

Fig 6: Total hemolymph protein content (mg/ml) of *G. mellonella* pupae and adults developed from previously challenged stages

4. Discussion

Recently many pest control methods are being interested in biological warfare. To produce effectively control methods it is important to know biology, physiology, and biochemistry of the insect [15]. Owing to the economic importance of the wax moth, as an important pest for honey bees and apiculture industry [34], its susceptibility to a wide range of disease agents and the succeeded use of Bt in controlling some insects and failed towards others [4], extensive researches have focused on the greater wax moth [10-16].

To quantify the real amount of pathogen presented into the tested insect. We placed the pathogenic agent in direct contact with the susceptible tissue by injection; to avoid the differences resulted from the loss of dose and irregularities [29]. Several authors [2-26-27-29-33] had applied the same technique for the study of insect immunity and the observation of the biochemical and physiological alternations induced by sublethal doses of pathogenic infection. Depending on the quantitative explanations on how *G. mellonella* suffered from infection with the selected bacteria and the resulted mortality percentages. The value of LC50 was 4.6×10^4 cells/ml for the adult stage this demonstrated that it is the most susceptible stage, without any variances between males and females in their immunological response. The high susceptibility of adults towards Bt may be due to, the adult immune defense system is traded off against some other fitness components as survival and reproduction, this conclusion was in agreement with [38-37]. While pupae are the least susceptible to a value of LC_{50} 5.9×10^5 cells/ml, this high resistance may be related to the presence of some enzymes and metabolic compounds resulted from degradation of larval tissues and defense proteins that function within development and expressed during the pupal stage. Similar results were concluded by [21-31].

Little is known about the effect of pathogenic infection to the hemolymph biochemical composition of different developmental stages of *G. mellonella*. Therefore, this work focused on hemolymph biochemical composition to illuminate alterations that happen as a result of immune induction infection. The level of hemolymph carbohydrates, lipids and proteins were augmented during the larval stage followed by a diminution in the pupal stage and a further sharp decline in the adult one. This may be due to their consumption during adult morphogenesis and the completion of generation, this explanation is in agreement with [23] on *Calliphora vicina.*

Time-dependent study after induction of immune response revealed a significant elevation in the level of hemolymph carbohydrates at different developmental stages. Such elevation may be a natural phenomenon because the hemolymph trehalose levels respond patently to the physiological conditions such as infection or starvation. Similar results were detected by [27] on nymph and adult grasshopper infected with fungi. Otherwise, the hemolymph carbohydrates retained to its original level at 48h post-injection, this may reflect an additional confirmation that these insects improved from infection due to the production of antibacterial compounds that function in protecting the insect against infection, this explanation was supported by investigations of [1] on the American bollworm, *Heliothis armigera*.

Proteins are the most essential organic components of insect tissues and play an important role in energy production. In the development process, protein is required for the synthesis of ATP [39]. We recorded a significant reduction in the levels of plasma proteins at all-time intervals post-injection, such decrease may be attributed to a) the induction of antibacterial proteins, b) bacteria may cause a complete elimination of some hemolymph enzymes and some hemolymph soluble and c) sticky proteins involved in attachment of the injected bacteria to the hemocytes these clarifications are in agreement with [1]. Concerning sex, female *Galleria* was found to have a greater amount of hemolymph proteins, this may be attributed to the incorporation of blood protein in the yolk deposition and the oocyte maturation, this agrees with [35] worked on *Panorpa communis*.

Lipids are used in numerous insects as an energy source, hormone precursors and structural members. It is stored in different regions in the insect body. Also, lipids located in the egg play an important role in meeting the energy needs of developing embryo [7]. This study noted a significant reduction the levels of plasma lipids after induction by the pathogenic agent, such decrease can be attributed to serious depletion of nutrients during growth, or maybe a consequence of lessening of nutrition during infection in which the body physiology is unable to meet the necessities of the insects this conclusion was confirmed by the results of [36-26].

Both sexes showed a significant decrease in the total hemolymph lipid and proteins at all times post-injection, this indicated that there is no strong effect of sex on the mounting of an immune response in *G. mellonella*. The fact of no gender-specific difference that was detected may be due to the growing conditions, optimal temperature. It might be that differential effect would come out only under suboptimal conditions, where the immune challenge would have a harsher effect on the organism's physiology. This explanation can be supported by the studies on the bumblebees, where the effect of immune insult came out only under starving conditions [30]. This observation alerts to the fact that specific species physiology or life history and experimental conditions may have a significant impact on sex immune-competence [43-11-32]. On the other hand study on the hoverflies immune system by [17] reported instar specific variation in immunity; they also suggested that females suffer more from inducing an immune response.

Results mentioned above indicate that a detailed knowledge of biochemistry including carbohydrates, lipids, and proteins of *Galleria mellonella* are necessary to select the more susceptible stage to achieve successful control.

The phenomena of immune memory and underlying mechanisms show significant diversity. Remarkably, many promising candidate mechanisms have been studied in taxa where there is currently no clear proof of the phenomenon of memory. So we should learn a lot more about immune memory in insects [22-28-44]. In our attempt to clarify the immunological memory in subsequent developmental stages of *Galleria mellonella*, pupae and adults of both sexes emerged from earlier treated stages showed no significant changes ($P\geq0.05$) in the levels of plasma carbohydrates, lipids and proteins than the challenged insect. Therefore we deduced the transstadial immune activation in *Galleria mellonella* from larvae to pupae and adults emerged from the primary challenged stage. Our conclusion is confirmed by [5] who investigated that bacterial infection in the hemocoel of the African malaria mosquito is transstadially transmitted from larvae to pupae and from pupae to adults. Also**,** [6] evaluated

the effect of a larval infection on the ability of female and male mosquitoes to fight a successive infection and found evidence for transstadial immune activation in that a larval infection results in adults with a greater ability to kill bacteria in their hemocoel. Therefore, it is recommended for more investigations to understand this mechanism in *Galleria mellonella*.

This study investigated that adult is the more susceptible stage to start the control strategies. Furthermore, the immunological response in the *G. mellonella* is transstadially transmitted from larvae to pupae and adults emerged from previously challenging stage.

5. Acknowledgment

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6. References

1. Abdeen SAO, Gadalla AI, Saleh WS, Hossein NM, Moawad GM. Some toxicological and biochemical effects of the bacterium, *Bacillus thuringiensis* on the American bollworm, *Heliothis armigera* Hbn. Annals of Agricultural Sciences. 1986; 31(2):1445-1462.
2. Barakat EMS, Meshrif WS, Shehata MGL. Changes in the hemolymph of the desert locust *Schistocerca gregaria* after injection with Bacillus thuringiensis. Journal of Egyptian Academic Society for Environmental Development and Molecular Biology. 2002: 2(1):95-115.
3. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Journal of Analytical Biochemistry. 1976; 72:248.
4. Bravo A, Gill SS, Soberon M. *Bacillus thuringiensis* mechanisms and use. Comprehensive Molecular Insect Science. 2005; 6:175-205.
5. Brown LD, Thompson GA, Hillyer JF. Transstadial transmission of larval hemocoelic infection negatively affects development and adult female longevity in the mosquito *Anopheles gambiae*. Journal of Invertebrate Pathology. 2018; 151:21-31.
6. Brown LD, Shapiro LLM, Thompson GA, Estévez Lao TY, Hillyer JF. Transstadial immune activation in a mosquito: Adults that emerge from infected larvae have stronger antibacterial activity in their hemocoel yet increased susceptibility to malaria infection. Journal of Ecology and Evolution. 2019; 9:6082-6095.
7. Boz A, Gülel A. The effects of temperature and time after parasitization on the total amount of protein, lipid and carbohydrate in the hemolymph of host larvae, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). Turkish Journal of Entomology. 2012; 36(2):239-247.
8. Campbell JJR, Konowalchuk J. Comparisons of drop and pour plate counts of bacteria in raw milk. Canadian Journal of Research, Section E. 1948; 26:327-329.
9. 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; 79(5):1283-1288.
10. Ellis JD, Graham JR, Mortensen A. Standard methods for wax moth research. Journal of Apiculture Research. 2013; 52:1-17.
11. Fedorka KM, Zuk M, Mousseau TA. Immune suppression and the cost of reproduction in the ground cricket, *Allonemobius socius*. Evolution. 2004; 58:2478-2485.
12. Finney DJ. Profit Analysis Cambridge university press. Cambridge. 1971, 333.
13. 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):673-4.
14. Gillott C. Entomology; Dordrecht, the Netherlands, Springer-Verlag, 2005.
15. Hakan Bozdoğan, Mahmut Erbey, Hatice Avan Aksoy. Total amount of protein, lipid and carbohydrate of some adult species belong to curculionidae family (Coleoptera: Curculionidae). Journal of Entomology and Zoology Studies. 2016; 4(5):242-248.
16. Harding CR, Schroeder GN, Collins JW, Frankel G. Use of *Galleria mellonella* as a model organism to study Legionella pneumophila infection. Journal of Visualized Experiments. 2013; (81):50964.
17. Hazell SP, Wenlock C, Bachel S, Fellowes MD. The costs and consequences of parasitoid attack for the predatory hoverfly, *Episyrphus balteatus*. Evolution and Ecology Research. 2005; 7(5):669-679.
18. Hoffmann D. Induction of antibacterial activity in the blood of the migratory locust, *Locusta migratoria* L. Journal of Insect Physiology. 1980; 26:539-549.
19. Ignasiak K, Maxwell A. *Galleria mellonella* (greater wax moth) larvae as a model for antibiotic susceptibility testing and acute toxicity trials. BMC Research Notes. 2017; 10(1):428.
20. Kulkarni N, Kushwaha DK, Mishra VK, Paunikar S. Eeffect of economic modification in artificial diet of greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae). Indan Jourl of Entomology. 2012; 74(4):369-374.
21. Kurata S, Saito H, Natori S. The 29 kDa hemocyte proteinase dissociates fat body at metamorphosis of Sarcophaga. Developmental Biology. 1992; 153(1):115-121.
22. Kurtz J, Franz K. Innate defence: evidence for memory in invertebrate immunity. Nature. 2003; 425(6953):37-38.
23. Levenbook L, Bauer AC. The fate of the larval storage protein calliphorin during adult development of *Calliphora vicina*. Insect biochemstry. 1984; 14(1):77-86.
24. Mahmoud DM, Salem DAM, Mo'men SA, Barakat EMS, Salama MS. Purification and characterization of phenoloxidase from immunized haemolymph of *Schistocerca gregaria*. African journal of Biotechnology. 2015; 14(44):3027-3036.
25. Meshrif WS, Barakat EMS. Cell mediated immunity in the Locust, *Shistocerca gregaria* against the bacterium, *Bacillus thuringiensis*. Journal of Egyptian Academic Society for Environmental Development. 2002; 2(1):117-130.
26. Meshrif WS, Barakat EMS, Rohlfs M, Shehata MG, Seif AI, Hegazi MAM. Characterization of extracellular enzymes and their role in studying the pathogencity of the Hyphomycete fungi to the cotton leafworm, *Spodoptera littoralis*. Ain Shams Science Bulletin. 2007; 45:1-16.
27. Meshrif WS, Marko Rohlfs, Mohamed AM, Hegazi Magdi G, Shehata Emad, Barakat MS *et al*. Seif. : Humoral response and plasma changes of *Spodoptera littoralis* (Lepidoptera: Noctuidae) following injection

with the entomopathogenic fungi: Beauveria bassiana and Nomuraea rileyi. Proc. 6th International Conference of Biological Science (Zool), 2010.
28. Milutinovi´ B, Kurtz J. Immune memory in invertebrates, Seminar Immunology, 2016.
29. Mo'men SHA, Salem DAM, Barakat EMS, Salama MS. Activation of Pro-phenoloxidase during Bacterial Injection into the Desert Locust, *Schistocerca gregaria*. International Journal of Medical and Biological Sciences. 2012, 291-300.
30. Moret Y, Schmid-Hempel P. Survival for immunity: the price of immune system activation for bumblebee workers. Science. 2000; 290(5494):1166-1168.
31. Natori S, Shiraishi H, Hori S, Kobayashi A. The roles of *Sarcophaga* defense molecules in immunity and metamorphosis. Developmental and Comparative Immunology. 1999; 23:317-328.
32. Ots I, Freitak D, Vanatoa A. Expression of immunity and general condition in the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), in relation to origin and gender. Entomological Science. 2005; 8:173-178.
33. Pereira MF, Rossi CC, Queiroz MV, Martins GF, Isaac C, Bosse JT *et al*. *Galleria mellonella* is an effective model to study *Actinobacillus pleuropneumoniae* infection. Journal of Microbiology. 2015; 161:387-400.
34. Ramarao N, Nielsen-Leroux C, Lereclus D. The insect *Galleria mellonella* as a powerful infection model to investigate bacterial pathogenesis. Journal of Visualized Experiments. 2012; 70:4392.
35. Ramamurty PS. On the contribution of the follicle epithelium to the deposition of yolk in the oocyte of *Panorpa communis* (Mecoptera). Experimental Cell Research. 1964; 33(3):601-605.
36. Sabbour M. Biochemistry of hemolymph of *Earias insulana* larvae treated with *Bacillus thuringiensis* and *Beauveria bassiana*. Journal of the Egyptian-German Society of Zoology. 2001; 36(E):19-27.
37. Schmid-Hempel P. Evolutionary ecology of insect immune defenses. Annual Review of Entomology. 2005; 50:529-551.
38. Sheldon BC, Verhulst S. Ecological immunology: costly parasite defenses and trade-offs in evolutionary ecology. Trends in Ecology and Evolution. 1996; 11:317-321.
39. Taşkın AD, Aksoylar MY. *Itoplectis melanocephala* (Gravenhorst, 1829) (Hymenoptera: Ichneumonidae)' nınergin öncesi dönemleri ile erginlerinin total lipid ve totalyağ asidi yüzdeleri. Turkish Journal of Entomology. 2011; 35(4):641-649.
40. Van Handel E. Rapid Determination of Glycogen and Sugars in Mosquitoes. Journal of the American Mosquito Control Association. 1985a; 1:199-301 20.
41. Van Handel E. Rapid Determination of Total Lipid's Mosquitoes. Journal of the American Mosquito Control Association. 1985b; 1:302-304.
42. Wojda I, Kowalski P, Jakubowicz T. Humoral immune response of *Galleria mellonella* larvae after infection by Beauveria bassiana under optimal and heat-shock conditions. Journal of Insect Physiology. 2009; 55:525-531.
43. Youth CP, Forbes MR, Baker RL. Sex differences in melanotic encapsulation responses (immunocompetence) in the damselfly, *Lestes forcipatus* Rambur. Canadian Journal of Zoology. 2002; 80(9):1578-1583.
44. Zanchi C, Troussard JP, Martinaud G, Moreau j, Moret Y. Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. Journal of Animal Ecology. 2011; 80:1174-1183.