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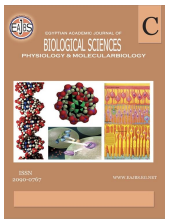

Hemolymph Changes Resulting from Injection of *Escherichia coli* Into the Larvae of the Wax Moth, *Galleria mellonella* (Lepidoptera: Pyralidae)

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ABSTRACT

The present investigation used *Galleria mellonella* larvae as an infection model to describe the virulence of *Escherichia coli*, the most frequent causes of several common bacterial infections in humans and animals. Some hemolymph physical properties such as hemolymph volume - and its relation to body water content, hemolymph density, and pH, along with a quantitative estimation of hemolymph proteins, lipids and carbohydrates were recorded in *G. mellonella* larvae at different time intervals post-injection with a sub-lethal dose (LD_{20}) of *E. coli* into the larval hemocoel. A decrease in fresh body weight and body water content with an increase in the hemolymph volume was observed at all time intervals post-larval treatment. This may be due to the loss of tissue water and gained it into the hemolymph. At the same time, bacterial injection decreased the hemolymph density and pH immediately following injection, while the viscosity and acidity of the hemolymph restored its original level with time. The bacterial injection also recorded an obvious decrease in the hemolymph proteins and lipids of the treated larvae at all time intervals post-treatment. This may be due to their elimination and/or their involvement in immune defence reactions or may be due to the intensive consumption and depletion of nutrition during infection. On the contrary, the levels of hemolymph carbohydrates increased at all-time intervals post bacterial injection into larvae. This increase may be due to the release of stored sugars (treehouse) which is responded strikingly due to bacterial infection causing an increase in the level of glucose and glycogen in the hemolymph. These results may lead to a better understanding of the regulatory events and the physiology of infected insects.

INTRODUCTION

Disease pathogens can dramatically reduce the fitness of their hosts, and natural selection should favor defense mechanisms that can protect hosts against disease (Medzhitov *et al.*, 2012). Much work has focused on understanding physiological immunity against pathogens to avoid infection or alleviate disease symptoms. It is increasingly recognized that the immune system has two strategies designed to avoid the infection. One which is the more complex found only in vertebrates and called-specific adaptive immune system. The other which is an older evolutionary defense strategy and the dominant response found in plants, fungi, insects, and primitive multicellular organisms is the innate immune system (Müller *et al.*, 2008).

Unlike the adaptive immune system, the innate system is not specialized for specific pathogen (i.e., it does not need a long start-up phase) (Litman *et al.,* 2010). Because of this broad effect, it is only capable, to a certain degree, of stopping infection (Retschnig *et al.,* 2014).

In insects, there is no specific or adaptive immunity but only an innate response that includes cellular and humoral factors. The cellular response consists mainly of phagocytosis and encapsulation, while the humoral immune response includes the rapid synthesis of a battery of antimicrobial peptides. Such mechanisms provide strong protection against pathogens (Barakat, 2003). The insect immune system and mammalian innate immune system show vast similarities in the cellular and humoral responses (Alghoribi *et al.,* 2014; Maguire, 2017). Investigation of virulence factors of different pathogens and insect immune system indicated that there are common strategies of host attack and pathogen suppression (Barakat *et al.,* 2002; Vallet-Gely *et al.,* 2008; de Roode and Lefèvre, 2012; Radwan *et al.,* 2019).

The larvae of the wax moth, *Galleria mellonella* have been used as an infection model to describe and evaluate microbial pathogenicity for a number of bacterial pathogens. Previous studies have shown a strong and positive correlation of virulence of different pathogens between mouse infection systems and *G. mellonella* (Alghoribi *et al.,* 2014). Hence, employing *G. mellonella* as model systems for the determination of microbial pathogenesis, toxicity testing, and antimicrobial therapies is extremely suitable and relevant.

Priming of the insect innate immune system involves the exposure of insects to dead or a sub-lethal dose of microbes in order to elicit an initial response. Comparing subsequent infections in primed insects to non-primed individuals indicates that the innate immune response may possess some of the qualities of an adaptive immune system. Although some studies demonstrate that the protective effects of priming are due to a "loitering" innate immune response, others have presented more convincing elements of adaptively. While an immune mechanism capable of producing the same degree of recognition specificity as seen in vertebrates has yet to be discovered in insects, a few interesting cases have been identified and discussed (Cooper and Eleftherianos, 2017).

The hemolymph, 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 hemolymph 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, hemolymph 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 an integrated picture with hemolymph about the effect of treatment. Therefore, this study was conducted to investigate the hemolymph changes of *G. mellonella* larvae during the course of infection by *Escherichia coli,* the major cause of extraintestinal infections.

MATERAILS AND METHODS

Maintenance of Experimental Insect:

The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) was obtained from Plant Protection Research Institute, Agricultural Research Center, Egypt. The colony was kept in constant darkness at 30± 2°C and reared on an artificial diet, reported by Kulkarni *et al*. (2012). Relative humidity was not controlled. Last instar larvae, when they became motionless and just started spinning the cocoon, were used in the present investigation.

Preparation of the Bacterial Pathogen:

Escherichia coli (*E. coli*) ATCC 8739, isolated from feces was supplied as actively growing cultures from the Microbiological Resources Centre (Cairo MIRCEN). Muller-Hinton agar (MHA) from (BBL ™, Becton, Dickinson and Company, USA) was used as media for bacterial growth. The bacterial suspension was adjusted to a concentration of 1×10^8 cells/ml by the pour plate count method according to Campbell and Konowalchuk (1948). A stock suspension of a sub-lethal dose of *E. coli* that produces 20% mortality was prepared. Subsequently, 10 µl of this dose was injected into the hemocoel of the experimental insect for enhancing the immunity of the insect and investigating the influence of infection on the various parameters studied.

Injection Technique:

Groups each with 10 experimental larvae (200–250 mg) normal group, kept without injection, control group, injected with sterile distilled water, and treated group (bacterial injected insects) were selected and injected with 1.2×10^4 cells/larva (LD_{20}). Injection of insects 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 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 and injected insects after 6, 12, 24, and 48 h, along with water –injected insects were used. They were weighed on electronic balance (METTLER, type BB300, Switzerland). Water content was expressed on a wet weight basis. i.e., the relation between water content and fresh body weight was simplified by calculating water content percent wet weight.

Collection of Hemolymph:

Hemolymph samples from normal, control and bacterial injected insects were collected at 6, 12, 24, and 48 h after injection. Larvae bled by puncturing the cuticle on the first hind leg with a fine sterile dissecting needle, taking care not to puncture the gut or other organs. The hemolymph was collected by capillary suction with a fine-tipped calibrated glass capillary tube and immediately transferred to sterile and chilled Eppendorf tubes containing 1 mg of phenylthiourea (Sigma) to prevent melanization, which was kept at –20ºC until further analyses.

Determination of the Hemolymph Volume:

The haemolymph volume was determined according to the method described by Yeager and Munson (1950) and modified by Lee (1961), using 2% amaranth red dye (20 mg/ml of 0.5% NaCl). In this method, a dye solution of known concentration is injected into the hemolymph. After complete circulation of the dye, a sample is drawn off and the absorbency was read at 515 nm and compared with that of a standard solution. The weight of amaranth dye in the unknown sample was first calculated and the blood volume (V) was calculated from the following equation:

$$V = (dg_1/g_2) - a.$$

Where (d) is the volume of sample, (g1) is the weight of dye injected, (g2) is the weight of dye in the sample, and (a) is the volume of distilled water injected with the dye.

Hemolymph Density:

The hemolymph density was determined according to the method described by Carrel *et al.* (1990). Hemolymph was collected immediately into micro-capillary tubes calibrated at 1 µl and pre-weighed using an electronic balance. The filled tube was quickly re-weighed and the hemolymph density was expressed as mg/µl.

Hemolymph pH:

The hemolymph pH was 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 hemolymph. All measurements were accomplished at 28 ± 2ºC.

Processing of Hemolymph Plasma For Biochemical Analysis:

According to Hoffmann (1980), about 3-5 insects were then transferred to a micro-centrifuge tube (1 ml) with a finely perforated bottom, fitted in another (2 ml) micro-centrifuge tube previously cooled on an ice bath. The hemocytes were removed by centrifugation in cold at 3000 rpm for 10 min at 4 ºC. The supernatant (referred to as plasma) was immediately transferred into sterile and chilled Eppendorf tubes and stored at -18 ºC until use.

Estimation of Hemolymph Protein:

According to the method of Bradford (1976), the total protein content of the larval plasma 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.

Estimation of the Total Hemolymph Lipids:

The total lipid content of the larval plasma was estimated using phosphorvanilin reagent according to the method of Frings *et al.* (1972). The standard and unknown samples were read against blank at 540 nm. The total lipid 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.

Estimation of the Total Hemolymph Carbohydrates:

The total carbohydrates of the larval plasma were assessed according to the method described by Van Handel (1985). The absorbance was recorded at 625 nm, and the concentration of the carbohydrates (mg/ml) was valued using the formula resulted from the standard calibration curve using glucose solutions.

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., 2012). The measurements were replicated 5 times at each time interval. 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

Effects of *E.coli* on body weight and water content of *G. mellonella* larvae:

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 at 6 and 12 h post-injection ($P > 0.05$) as compared with water-injected control larvae (Fig. 1).

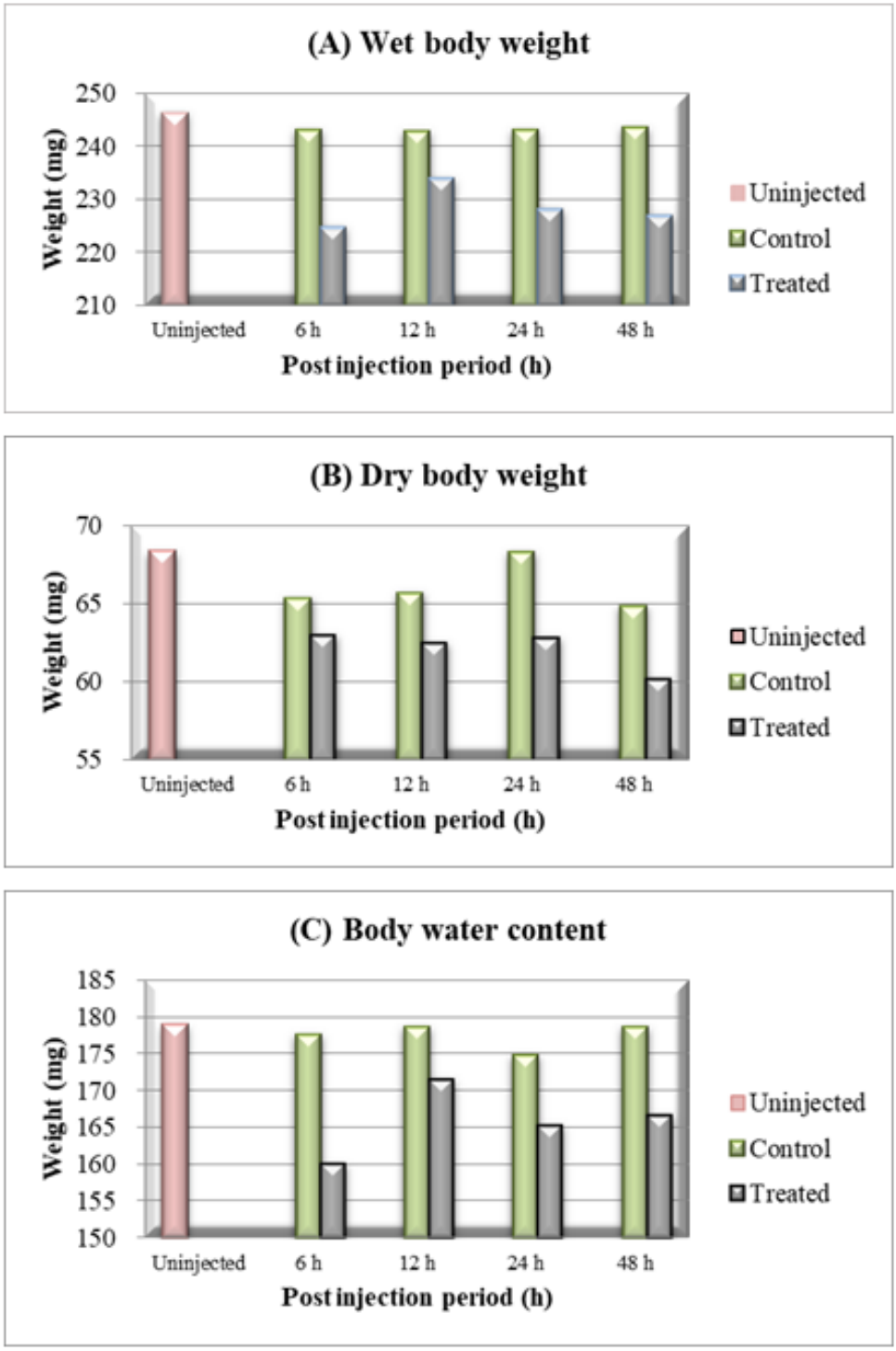

Fig. 1: (A) Total body weight, (B) Dry body weight, and (C) Body water content of *G. mellonella* larvae determined at different time intervals post-injection with *E. coli*.

Effects of *E. coli* on Hemolymph Volume, Density, and pH of *G. mellonella* Larvae:

After bacterial treatment, the hemolymph volume, density, and pH of larvae showed a significant increase ($P < 0.05$) at different time intervals as compared to the water injected controls (Fig. 2).

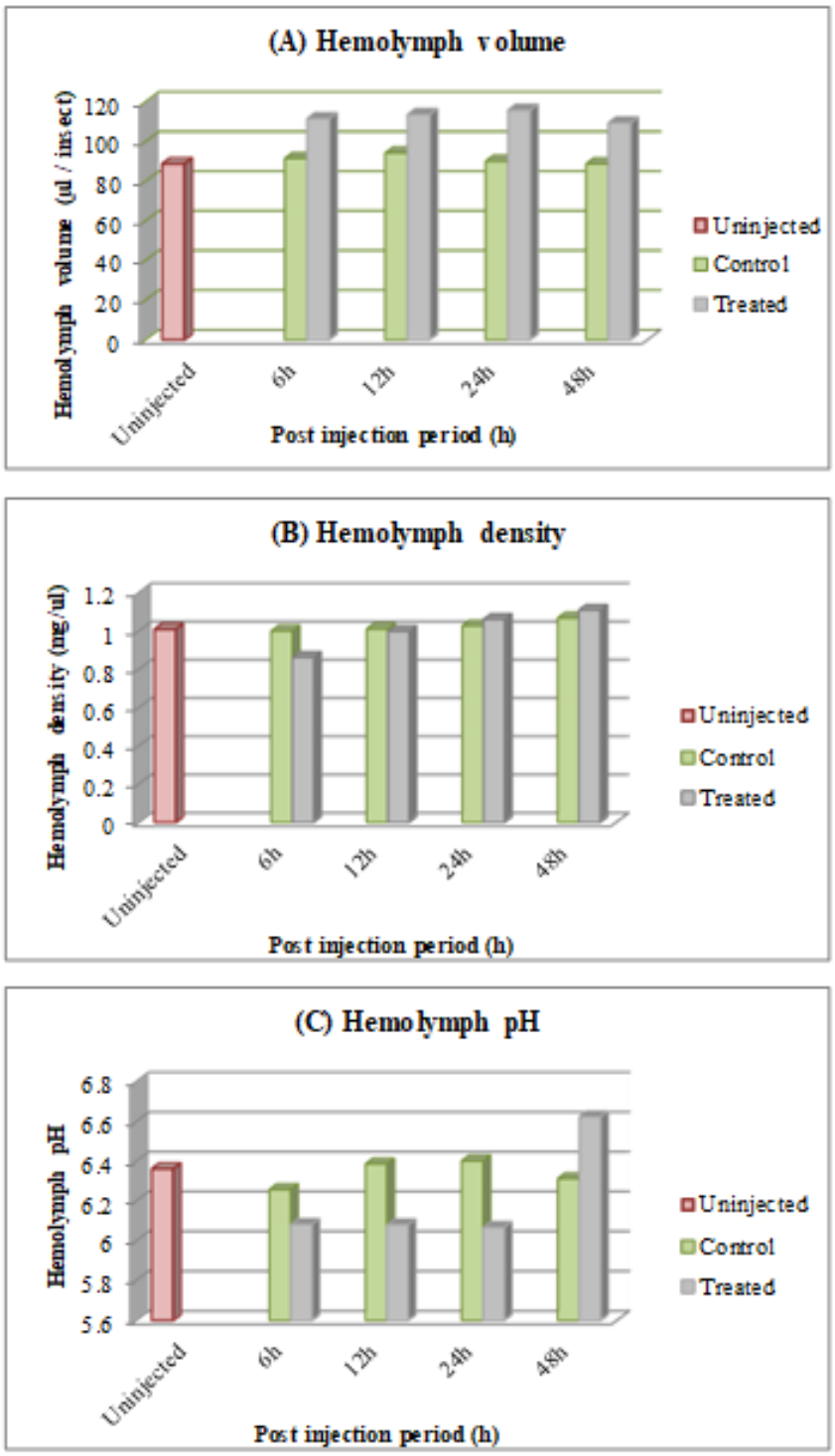

Fig. 2: (A) Hemolymph volume (µl), (B) Hemolymph density (mg/ul), and (C) Hemolymph pH of *G. mellonella* larvae determined at different time intervals post-injection with *E. coli.*

Effects of *E. coli* on Total Contents of Plasma Proteins, Lipids, and Carbohydrates of *G. mellonella* Larvae:

In *E. coli* -injected larvae, there were significant decreases ($P<0.05$) in the contents of plasma proteins and lipids at all time intervals post-injection. On the contrary, the levels of plasma carbohydrates increased ($P<0.05$) at all-time intervals due to the bacterial injection into larvae (Fig. 3).

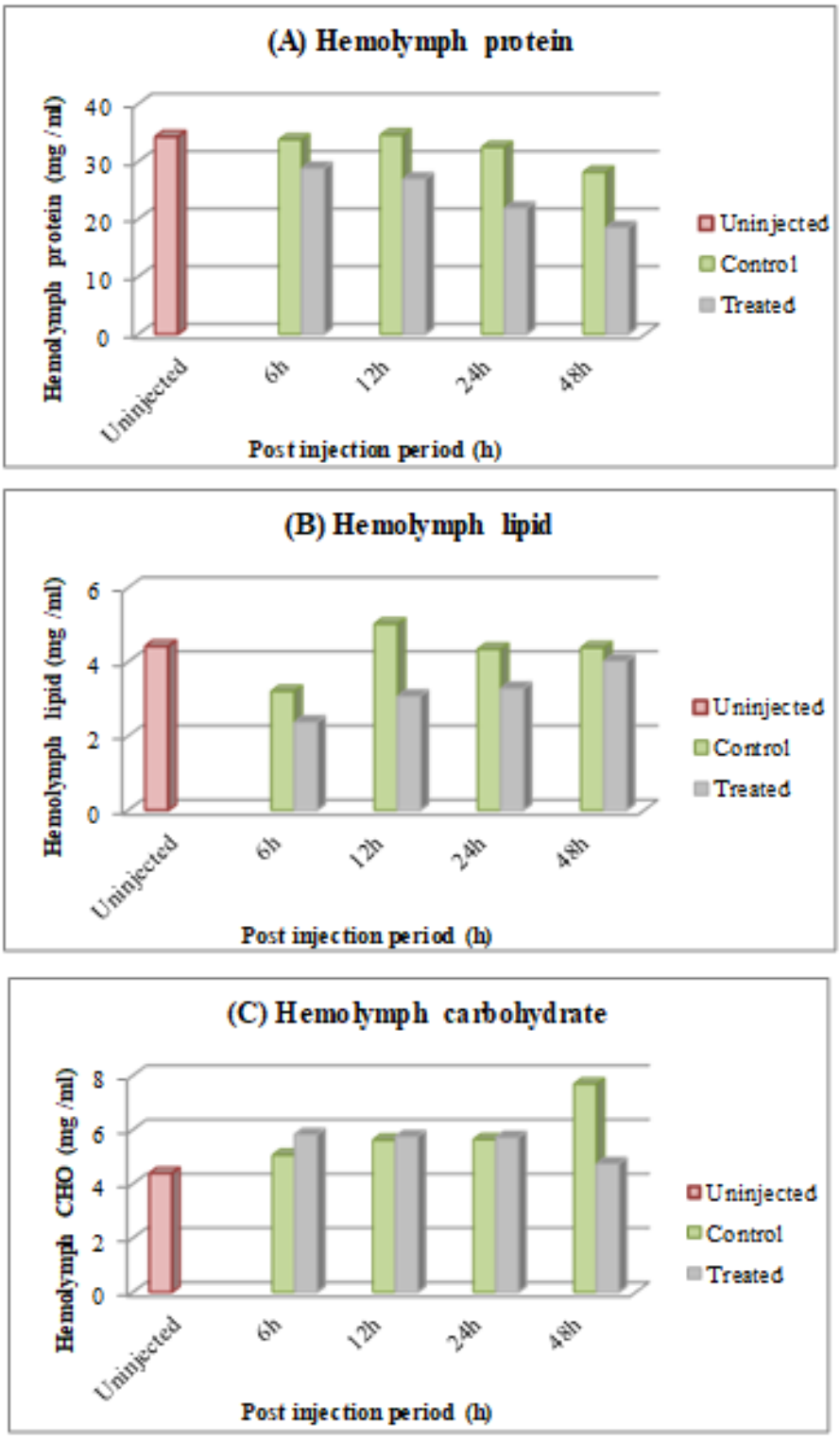

Fig. 3: The total contents (mg/ml) of (A) hemolymph proteins, (B) hemolymph lipids and (C) hemolymph carbohydrates of *G. mellonella* larvae determined at different time intervals post-injection with *E. coli*

DISCUSSION

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; Junqueira and Mylonakis, 2019), and this substitution has several benefits. Within this frame, *G. mellonella* has proved to be an ideal infection model for studying insect physiology due to a number of reasons: (1) it is one from most widely used insects in physiology, immunology and biochemistry, even when is considered as a pest for the apiculture, (2) it has a fast life-cycle, suitable size of all immature stages, (3) easy reared on natural or artificial diets, (4) there are a lot of similarities between the insect and mammalian gastrointestinal tracts, and (5) The insect fat body functions in drug metabolism in a similar way to the mammalian liver (Kavanagh and Reeves, 2004; Andrejko *et al.*, 2009; Champion *et al.*, 2009; Rejasse *et al.*, 2010; Wand *et al.*, 2011). *Galleria* is also being used as a model for ethical reasons that minimize the usage of mammals as models for studying. Given the size of the insect, it is possible to obtain easily hemolymph and other tissues (Wojda, 2017).

Typically, the insect responds 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 (Barakat and Meshrif, 2007). Results are expressed as percentages of the total weight, thus permitting comparisons of relative values demonstrated at different intervals during the course of infection.

Changes in the body weight attracted the immunologists in the past since it gives an integrated picture with hemolymph volume about the effect of treatment. Since the total body water content could be partitioned into two fractions: tissue water and hemolymph water. The decrease in body weight of the *Galleria* larvae 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 Mo`men *et al.* (2019) working on *G. mellonella* and with those of Carrel *et al.* (1990), Bardoloi and Hazarika (1992), Barakat and Meshrif (2007) who obtained similar results with other agents and other insect species.

Many reports demonstrated that hemolymph is the water reservoir and that the hemolymph 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.

It was obvious thus, that *E. coli* decreased body water content, and at the same time, caused a loss of water in the tissues (which was reflected from the decrease in the dry body weight) and gained it in the hemolymph. Similar results were obtained Mo`men *et al.* (2019) 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 hemolymph (i.e. decrease in the hemolymph 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 hemolymph was observed.

The hemolymph density and pH were also increased by the injection of bacteria into *G. mellonella* larvae at almost all post-

injection periods. This may be due to the decrease in blood volume as well as the increase of bacterial metabolites. These results are in agreement with those of Wemer and Jones (1969) Mo`men *et al.* (2019) on *G. mellonella* larvae.

The hemolymph additionally bears various immunity-related products that function in protecting the insect against systemic infection. These products are components of the humoral part of the insect immune system. To a large extent, the humoral effectors are inducible, i.e., they are undetectable (or nearly so) in uninfected insects, and increase to high titers in response to mechanical wounding and infection by bacteria, fungal pathogens, nematodes, etc. Therefore, our study attempts to characterize the hemolymph biochemically to clarify changes that occur due to infection.

This study observed a decrease in the level of hemolymph proteins of *G. mellonella* larvae at all time intervals following intrahemocoelic injection of *E. coli*. We can attribute this decrease to the intensive consumption of hemolymph proteins during the growth and multiplication of bacteria. The hemocytes (Barakat *et al.,* 2002), or some native proteins may be converted into lipoproteins or glycoproteins after injection (Meshrif *et al.,* 2010). These observations agree with the results of Sabbour (2001) on *Earias insulana* larvae treated with *Beuveria bassiana,* Meshrif *et al.* (2007) on *S. littoralis* following infection with hyphomycete fungi and Mo`men *et al.* (2019) on *G. mellonella*.

Abdeen *et al.* (1986) worked on the American bollworm, *Heliothis armigera*, reported that the observed decrease of hemolymph protein contents post-bacterial injection may be attributed to the induction of antibacterial proteins. Bacteria may cause a complete elimination of some hemolymph enzymes and some hemolymph soluble and sticky proteins involved in the attachment of the injected bacteria to the hemocytes.

Similarly, the levels of hemolymph lipids showed a significant decrease at all-time intervals following the injection of *E. coli* into *Galleria* larvae. This decrease may be a consequence of depletion of nutrition during infection in which the body physiology is unable to meet the requirements of the insects. Our results are in agreement with those of Lim and Lee (1981) on starved grasshoppers and Meshrif *et al.* (2007) on fungal infected *S. littoralis* larvae.

On the contrary, the levels of hemolymph carbohydrates in *G. mellonella* larvae increased at all-time intervals following the injection of *E. coli*. This increase may be a natural phenomenon because the hemolymph trehalose levels respond strikingly to the physiological conditions such as infection or starvation (Nowosielski and Patton, 1964). Additionally, the fact that trehalose acts as an immediately available carbohydrates reserve (Clegg and Evans, 1961). Similar results were observed by Lim and Lee (1981) on adult grasshopper deprived of food.

Otherwise, the late decrease of hemolymph carbohydrates in *E. coli*-injected larvae at 48 h post-injection, to maintain the original level, may be considered as additional evidence that these insects recovered from the infection, due to production of antibacterial compounds that function in protecting the insect against infection.

Investigations on the induction of antibacterial activity in the hemolymph of immunized insects provide important information concerning innate immunity, which in many aspects is conserved in animals. This is one of the reasons why insects serve as model organisms to study virulence mechanisms of human pathogens.

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ARABIC SAMMARY

التغيرات الدموية الناتجة عن حقن الإشريكية القولونية في يرقات فراشة الشمع، غاليريا ميلونيلا (حرشفيات الأجنحة: بايراليدى)

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استخدم البحث الحالي يرقات فراشة الشمع، غاليريا ميلونيلا كنموذج لوصف ضراوة الإشريكية القولونية، المسبب الرئيسى للعديد من الالتهابات البكتيرية الأكثر شيوعًا لدى البشر والحيوانات. تم تسجيل بعض الخواص الفيزيائية للهيموليمف مثل حجم الدم - وعلاقته بمحتوى ماء الجسم، وكثافتة والأس الهيدروجيني له، إلى جانب التقدير الكمي لبروتينات الدم والدهون والكربوهيدرات في اليرقات على فترات زمنية مختلفة بعد الحقن بجرعة غير مميتة من الإشريكية القولونية داخل التجويف الدموى لليرقات. لوحظ انخفاضا في وزن الجسم ومحتواه المائى، مع زيادة في حجم الدم في جميع الفترات الزمنية بعد معالجة اليرقات بالبكتيريا. قد يكون هذا بسبب فقدان الماء من الأنسجة واكتسابه في الدم. في نفس الوقت، قلل الحقن البكتيري من كثافة الدم والرقم الهيدروجيني له بعد الحقن مباشرة بسبب انخفاض محتوى ماء الجسم، بينما عادت اللزوجة وحموضة الدم للمستوى الأصلي بمرور الوقت. كما سجل الحقن البكتيري انخفاضاً واضحاً في بروتينات ودهون الدم في اليرقات المعالجة في جميع الفترات الزمنية التالية لعملية الحقن. قد يكون هذا الإنخفاض بسبب التخلص منها أو مشاركتها أثناء التفاعلات المناعية أو بسبب الاستهلاك المكثف لهذه المواد الغذائية واستنزافها أثناء نمو وتكاثر البكتيريا. بينماعلى العكس من ذلك، زادت مستويات كربوهيدرات الدم في جميع الفترات الزمنية بعد الحقن البكتيري في اليرقات. قد تكون هذه الزيادة بسبب تحرر السكريات المخزنة، استجابة للعدوى البكتيرية، وتسببها فى زيادة مستويات الجلوكوز والجليكوجين فى الدم. قد تؤدي هذه النتائج إلى فهم أفضل للأحداث التنظيمية وفسيولوجيا الحشرات المصابة.

الكلمات المفتاحية: يرقات فراشة الشمع، الإشريكية القولونية، محتوى ماء الجسم، حجم الدم، كثافة الدم، الأس الهيدروجيني للدم، بروتينات الدم، الدهون والكربوهيدرات.