Characterization of the haemolymph from *Schistocerca gregaria* adults after infection with entomopathogenic fungus *Beauveria bassiana*

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Abstract: The present investigation aims to study the impact of the hyphomycete fungus, *Beauveria bassiana* on the body water content and haemolymph of the desert locust, *Schistocerca gregaria* adults through quantitatively determination of some physical properties such as haemolymph volume, density and pH and through some biochemical parameters such as haemolymph carbohydrate, protein, and lipid contents. Based on the results derived from susceptibility tests, our data demonstrated that *B. bassiana* caused fatal infection to the adult stages of *S. gregaria*. Haemolymph was taken from control and treated locusts, after different time intervals; 1, 2, 3 and 4 days post topical treatment, to characterize its reaction against the fungal infection. The physical and biochemical results demonstrated variable changes in the body water content, haemolymph volume, density and pH as well as drastic changes in haemolymph carbohydrates, lipids and proteins concentrations after fungal injection. This study shows that *B. bassiana* has potential use for control of locusts. Conidia could be used in baits or droplets in a similar way as chemical insecticides for controlling *S. gregaria*. The present study also provides the basis for further studies of biochemical and molecular mechanisms underlying fungal strategies that have evolved for the suppression or evasion of the antifungal immunity in infected locusts.
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Key words: desert locust, entomopathogenic fungi, haemolymph.

1. Introduction

Synthetic chemical insecticides have been considered the major element of most insect control programs due to their low cost, high efficacy, persistence and broad spectrum of activity. However, due to the development of resistance in many target insect populations (Federici, 1999) and apparent hazards to the environment, human health and natural enemies (Tingle *et al*., 2003), there is a strong need for development biological control agents. Biocontrol agents are now considered as best alternatives to hazardous and non-degradable synthetic chemical insecticides (Perior and Greathead, 1989). A large group of insect pathogens such as viruses, bacteria and fungi have been considered in this context. Moreover, it is reported that hyphomycete fungi are target specific and non-toxic to other non-target organisms. As far as hyphomycete fungi are concerned, *Beauveria bassiana* is the most extensively studied (Hajek and St. Leger, 1994).

Fungi have been tested as biocontrol agents against a wide range of agricultural pests, especially the most destructive locusts and grasshoppers (Greathead, 1992). These fungi were reported to reduce vigour, reproduction and ultimately survival of individuals, and thus may contribute to the regulation of pest populations. However, the exact mechanism of these potent organisms on the susceptible tissue of target insect, i.e., the haemolymph, is not fully understood. Therefore, investigation on the changes of haemograme of the target insects due to fungal infection is very essential.

The haemolymph of insects resembles the blood of vertebrates in that it contains, in addition to cellular components, carbohydrates, proteins, lipids, salts, water, hormones, etc. The physical properties and chemical composition of the haemolymph is nearly as variable. This variation is due to different developmental stages of the same species and depending upon the nutrition, environment, and physiological state of the individual. The changes in physical and biochemical parameters of haemolymph are valuable in evaluating and predicting the pathogenic effect on the insects. In addition, the understanding of the potential effects of pathogenic fungi on the physical and biochemical environment of insect's haemolymph are of great practical importance in biological control. Also, the haemolymph alterations induced by these agents may be helpful in establishing the mode of action against the target insect. This response must be measured in an organism or its products and indicate a change compared to the normal state.

There has been a considerable amount of studies on the process of cuticle penetration by entomopathogenic fungi (Clarkson and Chamley, 1996; Chamley, *et al*., 1997). Most of them reported that these agents can affect biochemistry and physiology of insects. The physiological information of fungal pathogenisity on orthopteran locusts is lacked. Also, little is known about the development of the fungus in the haemolymph, in particular assimilation of nutrients and the interactions with haemocytes, therefore, this work focused on those missing gaps in knowledge and this investigation aims to study the impact of *B. bassiana* on the body water content and haemolymph of *S. gregaria* adults through quantitatively determination of some physical properties such as haemolymph volume, density and pH and some biochemical parameters such as haemolymph carbohydrate, protein, and lipid contents.

2. Materials and Methods

1. Insects:

The desert locust, *S. gregaria* (Forskal) used in the present study was originated from the Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Egypt. A colony from this locust was maintained in Faculty of Science, Ain Shams University and had shown no infectious diseases. The culture was raised and handled under crowded breeding conditions described by Hassanein (1965). The insects were reared in groups and maintained at 30±2 °C, 60 - 80% RH under a photoperiod of 16:8 (Light:Dark). Adult locusts were fed on an artificial diet as a dry mixture of: bran, 2; dried whole milk, 2; wheat, 2; dried brewer's yeast, 1 (parts by volume); plus a small quantity of fresh clover leaves. All experiments outlined below were carried out with adult (both sexes), all being within 2–4 days after ecdysis.

2. Tested fungi:

Commercial strain of *B. bassiana* (isolate GHA) obtained from Mycotech (Butte, MT) as a dry technical grade conidial powder was cultured on sabouraud dextrose yeast broth (SDY) at 26±2 ºC in a rotator incubator for 2 days. Blastospores were then collected from filtrate by centrifugation and washed twice with sterile 0.85% saline as reported by Gillespie *et al*. (2000). A concentrated stock suspension in sterile 0.85% saline containing blastospores was prepared, and the blastospores were counted using pour plate method (Campbell and Konowalchuk, 1948).

3. Susceptibility of locusts to the fungal pathogen:

Fungal treatments were topically applied to adult locusts at the posterior dorsum of the pronotum including the following fungal doses suspended in saline solution: $4.5x10^4$, $1.8x10^5$, $4.5x10^5$, $4.5x10^6$ and $9.0x10^7$ Bs/ul. An accurate delivery rate of inoculums was obtained using microapplicator equipped with a syringe according to Miranpuri and Khachatourians (1993). To avoid saprophytic growth of *B. bassiana* from the integument, cadavers were surface sterilized with 1% sodium hypochlorite for 3 min and then rinsed twice in sterile water. Surface sterilization ensured that fungal sporulation resulted from hyphae emerging from the body cavity, providing evidence of infection. Four groups of insects, each containing 15 individuals per dose were used in this assay. Control insects were treated only with equivalent amount of saline solution. Dead locusts were recorded according to a daily scheme in order to assess fungus-borne mortality rates.

One µl of fungal suspension (=LD_{50}) were prepared and used for investigating the influence of pathogenic infection on the body water and haemolymph.

4. Determination of locust`s body weight and water content:

The body water content was determined in normal and treated locusts after 1, 2, 3 and 4 days according to the method of Lee (1961). The body weight of locusts was determined gravimetrically for each individual insect. The body water content of locusts was determined as the difference between fresh (total) body weight and body weight after drying for 2-3 days at 80 ºC in an oven to constant weight (dry weight). The measurements were replicated 10 times.

5. Collection of haemolymph for studying some physical properties:

Normal (un-treated) and fungal-treated locusts after 1, 2, 3 and 4 days along with saline-treated controls were removed from the rearing cages and weighed individually. Locusts were submerged in hot water bath at 60 ºC for 2-5 min; they were allowed to dry on paper towel. The heat-killed insects were amputated at the arthropodial membrane of the hind coxa with fine scissors. Gentle pressure was applied to the thorax until a drop of haemolymph appeared at the point of amputation. All measurements were accomplished at 28±2 ºC and samples were replicated 10 times at each time interval for each determination.

5.1. Estimation of haemolymph volume (HV):

The HV (µl/insect) was determined by the amaranth red dye method described by Yeager and Munson (1950) and modified by Lee (1961). The optical density was measured spectrophotometrically by Nova spec, (Pharmacy Biotech.) at 515 nm.

5.2. Estimation of haemolymph density (HD):

The haemolymph densities (mg/µl) were determined following the method described by Carrel *et al.* (1990) by using micro-capillary tubes calibrated at 1 µl and pre-weighed using an electronic balance.

5.3. Estimation of haemolymph pH (HpH):

The haemolymph pH was determined according to the method described by Heimpel (1955) and using the bulb of the microelectrode (Model 671, pH meter, Extech., USA). Determination of the haemolymph pH was completed within a maximum of 15 sec, to reduce the possibility of altering the pH value by absorption of carbon dioxide.

***6.* Collection of haemolymph for studying some biochemical properties:**

Haemolymph from normal and treated locusts after 1, 2, 3 and 4 days was drawn out from the coxal joint into Eppendorff Pipetman containing traces of phenylthiourea to prevent melanization and then diluted 5× with saline solution 0.7%. The haemolymph samples were then centrifuged at 2000 r.p.m. for 5 min, and only the supernatant fractions were used for assays directly or frozen until use. Ten replicates were used for each determination and the haemolymph of two individuals were never mixed.

6.1. Estimation of the total haemolymph carbohydrate (THC):

Total carbohydrate content in haemolymph was quantitatively determined by using the anthrone reagent according to Singh and Sinha (1977) and utilizing the spectrophotometer at 620 nm.

6.2. Estimation of the total haemolymph lipid (THL):

Quantitative determination of the total lipid content in haemolymph was conducted according to the technique of Folch *et al.* (1957) and lipid estimation was taken place by phosphovanilin reagent depending on Knight *et al.* (1972) and using the spectrophotometer at 520 nm.

6.3. Estimation of the total haemolymph protein (THP):

Quantitative determination of the total protein content in haemolymph was conducted according to Bradford (1976) with bovine serum albumin (BSA) as the standard protein solution. The method depends on the protein forms a violet complex with cupric ions in alkaline medium, and then measured the absorbance at 595 nm using a spectrophotometer.

***7.* Statistical analysis:**

Results of susceptibility tests were subjected to probit analysis using LDP Line (LdP Line, 2000 by Ehab Mostofa Bakr, Cairo, Egypt). Statistical analysis of data was made using the chi^2 test. The lethal dose and the confidence limits of LD_{50} values were done according to the method of Litchfield and Wilcoxon (1949). Data of the rest tests were expressed as the mean ± standard error (SE). Levels of significance for differences of the means were determined using the Student's "*t*- test" for paired samples with equivalent variance. The level of significance for each experiment was set at $P< 0.05$. Comparisons were carried out between untreated and saline-treated (controls) and between control and fungus-treated insects. For multiple comparisons, the α-level was Bonferoni-corrected.

3. Results

***1.* Susceptibility of locusts to the fungal pathogen:**

In the control, mortality did not exceed 5%, and no sign of infection was observed on the dead locusts. After fungal treatment, mortality occurred within two days (cadavers exhibited fungal growth) and significantly increased four days after inoculation and continued to increase over 90% by nine days post inoculation. The lethal dose to achieve 50% mortality (LD_{50}) was estimated to be 6.83×10^5 blastospores per insect (Table 1).

***2.* Estimation of locust`s body water content:**

The mean total (fresh) body weight of the untreated adult was 2040.8 ± 127.6 mg, while the dry body weight was 732.9 ± 58.7 mg and the water content was 1307.9 ± 738 mg representing about 64% of the fresh body weight. The control insects showed insignificant difference ($P> 0.05$) at the first three days and eventually showed a significant increase ($P< 0.01$) in both fresh body weight and body water content only at the 4th day post-treatment. The dry body weight showed a significant increase ($P< 0.01$) at all time intervals after saline-treatment compared to the untreated insects. The fungal-treated insects showed a slight difference ($P> 0.05$) in the fresh body weight whereas; the dry body weight increased significantly ($P< 0.025$) at all post-treatment periods. However, a significant decrease ($P< 0.025$) in body water content was observed at all post-treatment periods examined (Table 2).

***2.* Estimation of some physical properties of haemolymph:**

2.1. Haemolymph volume (HV):

The HV of untreated adult was 236.6 ± 9.7 µl. In saline-treated insects, the HV showed no changes ($P> 0.05$) when compared to the untreated insects. Fungal-treated insects possessed a significant decrease ($P\leq 0.025$) in HV compared to saline- treated insects at all periods post-treatment (Figure 1).

2.2. Haemolymph density (HD):

The estimated mean value of the HD of untreated adults was 1.04 ± 0.02 mg/µl. The HD of control insects showed non-significant difference ($P> 0.05$) at all post-treatment periods compared to the untreated insects. However, after fungal-treatment, the HD increased significantly ($P< 0.025$) at all post-treatment periods compared to the saline-treated insects (Figure 2).

2.3. Haemolymph pH (HpH):

The mean value of HpH of untreated adults was 7.15 ± 0.03 (slightly alkaline). The HpH values were

significantly decreased at the 1st and 2nd day's post-saline-treatment ($P< 0.01$) in controls and then increased to the normal level. In fungal-treated adults, there was a significant increase only at the 2nd day ($P< 0.025$) as compared to the saline-treated insects. These changes push the HpH from slight alkaline area into slight acidic area (Figure 3).

Table 1: Pathogenicity of *Beauveria bassiana* for adult *Schistocerca gregaria* based on mortalities 9 days after topical application.

LD_{50} (conidia/insect)	95% Confidence Limits	Slope (SE)	Chi-Square (P) *	g**
6.5×10^5	$4.0 \times 10^5 - 1.5 \times 10^6$	0.885 (0.171)	6.745 (0.08)	0.144

*Chi-square of heterogeneity: measures goodness of fit to the weighted regression line with ($P> 0.05$) indicating a good fit of the data to the line. D.F. = 5; **g is the index of regression significance.

Table 2: Total body weight, dry body weight and body water content of adult *Schistocerca gregaria* determined at different time intervals post-treatment with *Beauveria bassiana*.

Days post-treatment	Total body weight (mg) Mean ± SE		Dry body weight (mg) Mean ± SE		Body water content (mg) Mean ± SE	
	Control	Treated	Control	Treated	Control	Treated
1	2256.6 ± 135.6	2412.6 ± 076.1	997.4 ± 83.4*	1328.7 ± 044.2*	1259.2 ± 58.8	1083.9 ± 37.5*
2	2313.0 ± 066.6	2427.7 ± 087.6	971.5 ± 43.0*	1253.1 ± 39.2*	1341.5 ± 31.2	1174.6 ± 59.3*
3	2165.5 ± 080.1	2366.3 ± 154.0	883.0 ± 56.9	1211.1 ± 106.4*	1282.5 ± 41.0	1155.2 ± 50.8*
4	2516.0 ± 116.8*	2532.2 ± 122.5	870.8 ± 26.6*	1261.4 ± 071.6*	1645.2 ± 97.8*	1270.8 ± 64.3*
Untreated	2040.8 ± 127.6		732.9 ± 58.7		1307.9 ± 738	

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

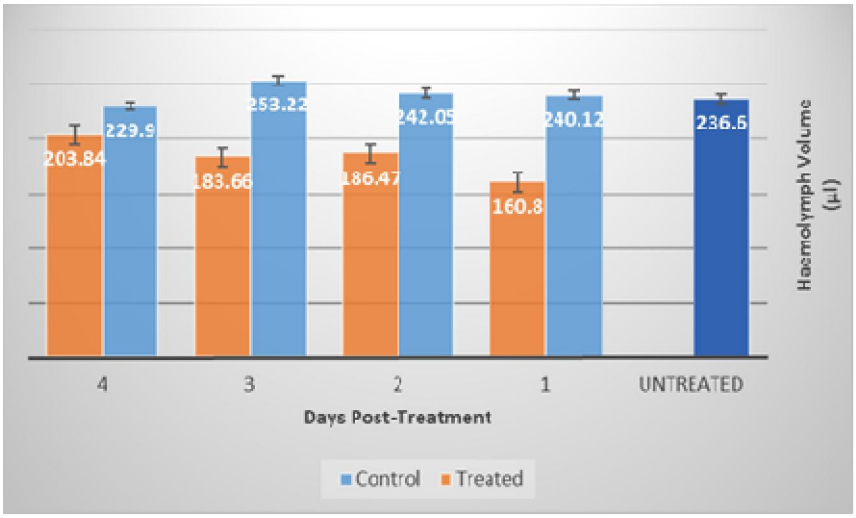

Figure 1: Haemolymph volume (µl/insect) of adult *Schistocerca gregaria* determined at different time intervals post-treatment with *Beauveria bassiana* using Amaranth dye method.
n = 10 insects per test.

***3.* Estimation of some biochemical parameters of haemolymph:**

3.1. Total haemolymph carbohydrates (THC):

In untreated locusts, the THC was 4.52 ± 0.1 mg/ml. In saline-treated insects, the THC were significantly higher ($P\leq 0.01$) at all time intervals post-treatment compared with those of untreated insects. In *B. bassiana*-treated insects, the THC showed a significant increase ($P\leq 0.025$) at the 1st and 2nd days compared with the saline-treated insects, but remained unchanged ($P> 0.05$) later at the 3rd and 4th days post-treatment (Figure 4).

3.2. Total haemolymph lipids (THL):

The THL in untreated adult locusts was 4.25 ± 0.38 mg/ ml. In saline-treated insects, the THL were significantly decreasing ($P\leq 0.01$) within the 1st day, followed by significant increase thereafter, compared with untreated insects. In *B. bassiana*-treated insects, the THL showed a significant increase ($P\leq 0.025$) at the 1st and 2nd day's post-treatment, and then followed

by significant decrease as compared with saline-treated insects (Figure 5).

3.3. Total haemolymph proteins (THP):

In untreated adult locust, the THP was 62.33 ± 2.03 mg/ml. In saline-treated insects, the THP were significantly higher ($P \leq 0.01$) at the first three days post-treatment, but lower at the 4th day post-treatment compared with untreated insects. The same change was observed in *B. bassiana*-treated insects. There was a significant increase ($P \leq 0.025$) at the 1st day followed by significant decrease thereafter, as compared with the saline-treated insects. At the 4th day, a significant increase was observed again (Figure 6).

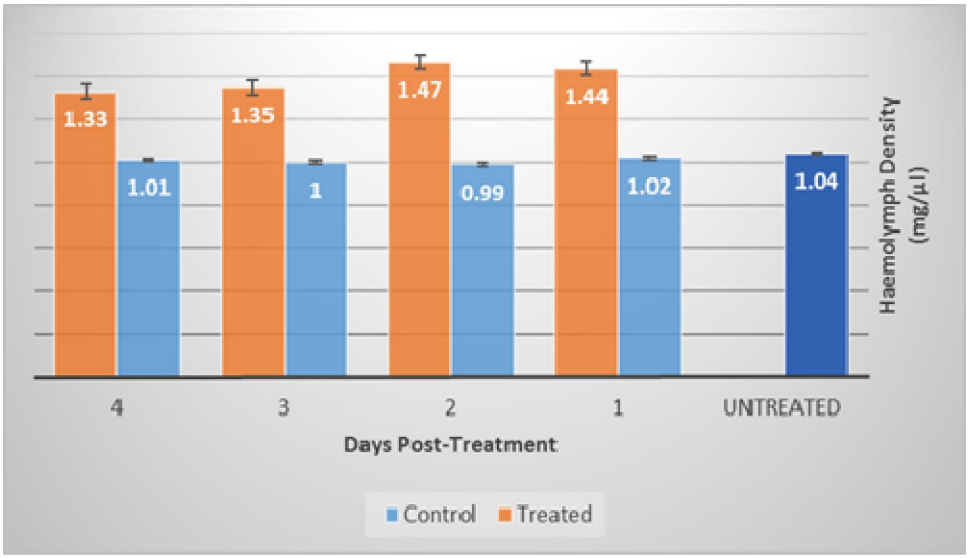

Figure 2: Haemolymph density (mg/µl) of adult *Schistocerca gregaria* determined at different time intervals post-treatment with *Beauveria bassiana*.
$n = 10$ insects per test.
* Significant differences between the untreated and the controls when $P \leq 0.01$ and between the control and the fungus-injected insects when $P \leq 0.025$ based on Bonferoni correction.

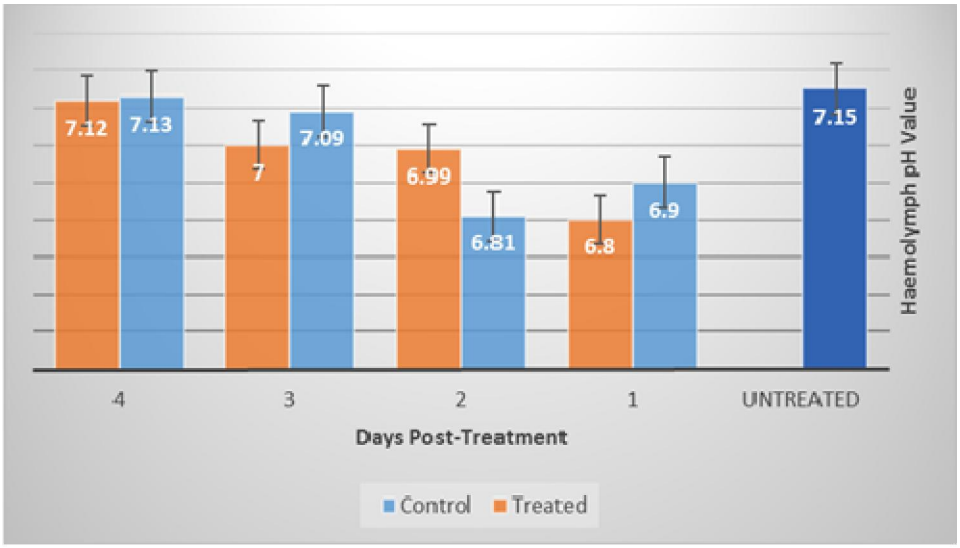

Figure 3: Haemolymph pH of adult *Schistocerca gregaria* determined at different time intervals post-treatment with *Beauveria bassiana*.
$n = 10$ insects per test.
* Significant differences between the untreated and the controls when $P \leq 0.01$ and between the control and the fungus-injected insects when $P \leq 0.025$ based on Bonferoni correction.

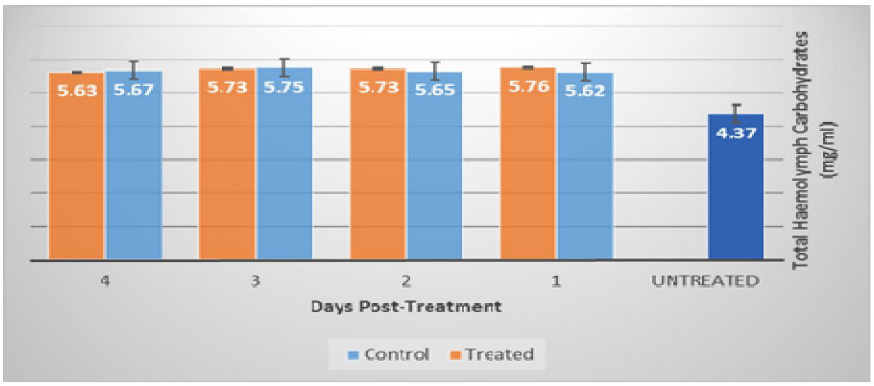

Figure 4: Total haemolymph carbohydrates of adult *Schistocerca gregaria* determined at different time intervals post-treatment with *Beauveria bassiana*.
n = 10 insects per test.
* Significant differences between the untreated and the controls when $P \leq 0.01$ and between the control and the fungus-injected insects when $P \leq 0.025$ based on Bonferoni correction.

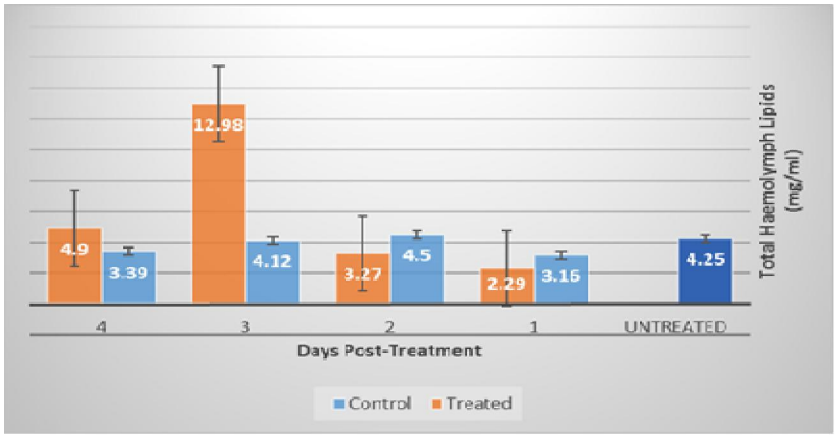

Figure 5: Total haemolymph lipids of adult *Schistocerca gregaria* determined at different time intervals post-treatment with *Beauveria bassiana*.
n = 10 insects per test.
* Significant differences between the untreated and the controls when $P \leq 0.01$ and between the control and the fungus-injected insects when $P \leq 0.025$ based on Bonferoni correction.

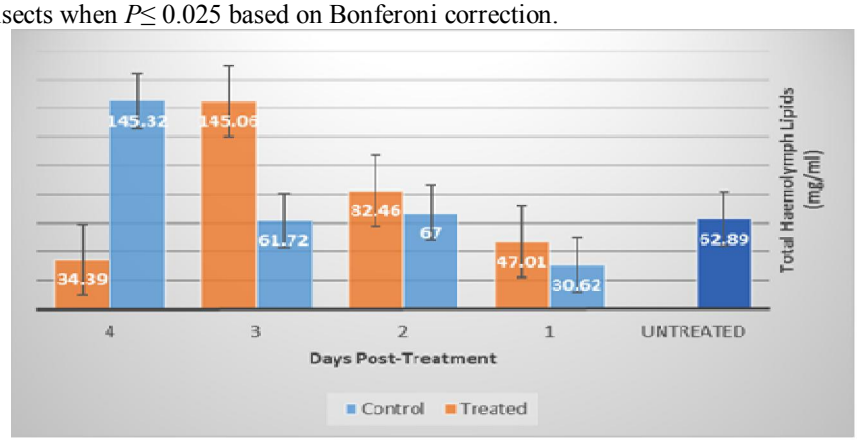

Figure 6: Total haemolymph proteins (mg/ml) of adult *Schistocerca gregaria* determined at different time intervals post-treatment with *Beauveria bassiana*.
n = 10 insects per test.
* Significant differences between the untreated and the controls when $P \leq 0.01$ and between the control and the fungus-injected insects when $P \leq 0.025$ based on Bonferoni correction.

4. Discussion

Pathogenicity is the ability of a pathogen to cause disease. Virulence is the quantitative measurement of this ability. The factors that govern pathogenicity of entomopathogenic fungi are not fully understood. There are suggestions that enzymatic secretions on the insect cuticle, toxins or conidial growth within the host might play a role (Bidochka and Khachatourians, 1987). Pathogenicity bioassays are the simplest way of determining the activity of fungi (Hajek and St. Leger, 1994). The selected criteria in bioassays include the number of insects that die from fungal infection and the length of time before death. Upon these criteria, *B. bassiana* was found to be a good fungal pathogen, because it kills the target insects, locusts – in relatively short time under stimulated laboratory conditions of temperature, light and relative humidity similar, to large extent, to field conditions. There have been many reports on *B. bassiana* pathogenicity against locusts and grasshoppers (Clarkson and Chamley, 1996; Chamley, *et al*., 1997; Seyoum *et al.*, 2002). Based on these studies, *B. bassiana* is pathogenic at doses ranging from 10^2 to 10^6 conidia per insect. This variation in pathogenicity might be due to the method of inoculation used, fungus formulation and age of the insect.

It is not known how locust physiology is affected by *B. bassiana*. Also the mechanism of infection is not known exactly. Most of the previous studies agreed that *B. bassiana* is capable of producing pathogenesis by the digestive system (Broome *et al.,* 1976) however; Ferron (1981) reported that fungal spores do not germinate in digestive system. However, Dillon and Charnley (1988) showed that bacteria in the gut of *S. gregaria* produced toxins capable of inhibiting the infectivity of the fungus, *Mitarhizium anisoplia.* In the present study, the lake of visible external fungal growth on some locusts that died within the first days following fungal infection may indicate that the mortality caused by septicemia due to fungal proliferation or by a secondary infection with bacteria through wounds caused by fungal penetration. Similar observation was noticed by Dillon and Charnley (1988). Alternatively, the development of intestinal bacteria after host death may have masked fungal growth (Ferron, 1977).

The body water content of normal (untreated) adult stage represents 64% of the total body weight. These results were supported by the work of Wigglesworth (1976) in which, the estimated percentage of the body water content ranged from not less than 50% to not more than 90 % of the total body weight. The total body water content could be partitioned into two fractions: tissue water and haemolymph water. Indeed, the HV was 236.6 ± 9.66 µl with a density of 1.04 ± 0.02 mg/µl. So the calculated haemolymph content value was 246.20 mg comprising about 12% of the total body weight. These findings agree with those of Benz (1963) who found that in most insects, the haemolymph accounts from 10 to 40% of the total body weight and the results of Jones (1977) who found that the ratio of haemolymph content: fresh body weight is from 15 to 25% in most orthopteran insects.

Following fungal treatment, no effect on the fresh body weight was observed. The dry body weight increased at all periods, while the body water content decreased. The increase in dry body weight may be attributed to the increase of the blood density. The observed decrease in body water content may be due to the loss of tissue water and the decrease of the HV. These results are close to the findings of Lee (1961) on *S. gregaria* and Hill and Goldsworthy (1968) on *Locusta migratoria* and Milat-Bissaad *et al.* (2011) with slight differences due to species difference, rearing conditions and diet. The observed decrease in the HV of adult locusts during the course of infection may be attributed to water loss from blood and tissues.

On the other hand, a significant increase in HD was observed due to fungal infection onto the locusts at almost all post-treatment periods. This may be due to the the decrease of HV as well as the increase of fungal metabolites. These results are in agreement with those of Barakat *et al.* (2002) on the same insect. In addition, the significant increase in the HpH in adults after fungal injection may be due to the induction of fungal metabolites.

Concerning the biochemical parameters of the locust haemolymph, the present results showed an initial increase in the THC after fungal infection, which is gradually decreased to attain the normal level with time. This increase may be a natural phenomenon because the haemolymph 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 (Boctor, 1974). Similar results were observed by Lim and Lee (1981) on adult grasshopper deprived of food. Otherwise, the late decrease in the THC may be due to the strong demand of glucose during the extensive growth and multiplication of fungi. The nutritional requirements of entomopathogenic fungi include an organic source of carbon (Samson *et al*., 1988). This could be also explained that as a balance action of the body physiology. Boctor (1974) suggested that blood glucose is maintained by a dynamic equilibrium between synthesis and breakdown of trehalose.

In these insects infected with *B. bassiana*, the THL showed significant decrease at all post-treatment periods. 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.

The haemolymph protein contents in normal (untreated) adult stages, estimated in the present study are in agreement with those of Brehélin *et al*. (1989) on *L. migratoria* and Miranpuri *et al*. (1991) on *M. sanguinipes*. The observed decrease of THP contents following fungal infection may be attributed to the fungal pathogenicity. Pathogens may cause a complete elimination of some haemolymph enzymes, some haemolymph soluble proteins and sticky proteins, which may be involved in anti-fungal immunity or some native proteins may be converted into lipoproteins or glycoproteins after infection could be attributed to intensive consumption of haemolymph proteins during growth and multiplication of the fungi, together with cessation of feeding observed on the infected insects.. The same explanation was reported by Bread (1945), Miranpuri *et al*. (1991), Sabbour (2001) and Seyoum *et al*. (2002) on different insect species infected with the same fungi. In contrary, the late increase of total proteins observed at the 4th day following infection may be caused by the metabolites secreted by the growth of fungi within the haemocoel.

In conclusion, the results obtained in the present study show an upsetting interfering of *B. bassiana* with the metabolism of proteins and the essential energy sources, carbohydrates and lipids, in adults of the desert locust *S. gregaria* which can afford an evidence to a promising use of these biocontrol agents against this destructive pest as environmentally-friendly alternatives of the synthetic chemical insecticides. However, more research is needed to investigate the *in vivo* and *in vitro* determination of virulence factors of entomopathogenic fungi.

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