The Journal of Basic & Applied Zoology (2013) **66**, 1–11

The Egyptian German Society for Zoology

The Journal of Basic & Applied Zoology

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Immune and cellular impacts in the autogenous *Aedes caspius* larvae after experimentally-induced stress: Effects of *Bacillus thuringiensis* infection

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Received 23 September 2012; revised 20 January 2013; accepted 22 January 2013
Available online 11 March 2013

KEYWORDS

Immune response;
Phenoloxidase;
Nitric oxide;
Apoptosis;
Gastric caeca;
Flow cytometry;
Aedes caspius

Abstract Insects possess effective defense mechanisms against pathogens *via* induction of antimicrobial immune and oxidative stress responses. In this study, immune impact and histological damages in the gastric caeca have been investigated in the 3rd instar larvae of the autogenous *Aedes caspius* upon infection with *Bacillus thuringiensis* (*Bt*). Data showed a significant increase in phenoloxidase (PO) activity by 1.23 folds at 4 h post-infection which then reduced to the normal level at 8 h post-infection and until larval death. Besides, the nitric oxide (NO) titer was significantly increased by 1.4 folds at 4 h post-infection, then, reduced down to its normal level at 8 h post-infection, after which, it was significantly decreasing by time until being hardly detected at 44 h post-infection compared to that of control mosquitoes. Moreover, percentages of cellular apoptosis were significantly elevating from 6 to 48 h post-infection. Consequently, cytological damages in the epithelium and the microvilli of the gastric caeca were observed at 48 h post-infection. Finally, larval body sizes were significantly smaller prior to death (at 48 h post-infection). Taken together, these data suggest further modes of action of *Bt* as inhibiting the antibacterial immune responses, inducing cellular apoptosis prior to damaging the epithelium of gastric caeca. This may explain – partially at least – the

Abbreviations: *Bt*, *Bacillus thuringiensis*; GSH, reduced glutathione; L-DOPA, 3,4-dihydroxy-L-phenyl-alanine; NO, nitric oxide; OD, optical density; $ONOO_2$, peroxynitrite; PO, phenoloxidase; pPO, prophenoloxidase; Prxs, peroxiredoxins; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; SEM, scanning electron microscope; TEM, transmission electron microscope; TP, thioredoxin peroxidase; TR, thioredoxin reductase; Trx, thioredoxin

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Peer review under responsibility of The Egyptian German Society for Zoology.

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http://dx.doi.org/10.1016/j.jobaz.2013.01.001

irresistibility and high pathogenicity of *Bt* against mosquito vector, which may help in understanding, and hence, overcoming the developing resistance by some mosquito vectors to some mosquitocidal bacteria. This may help in improving the biocontrol measures against mosquito vectors.

Introduction

Mosquitoes are intermediate hosts of several human pathogens, and thus, serve as vectors of several human diseases that kill several million annually worldwide (Service, 1993; Tolle, 2008). Besides, reducing vector populations using insecticides is very costly, toxic and has led to the development of vector resistance. Therefore, there was an urgent need to create new alternative control strategies. Thus, discovery of insecticidal bacteria, began a century ago, and consequently, have led to the development of commercial bacterial insecticides, the most successful and widely used environmentally safe microbial control agents (Federici, 2005; Lord, 2005). Most of these products were based on several spore-forming bacteria, for example, *Bacillus thuringiensis* (*Bt*), that kills insects *via* a particular Cry proteins (endotoxins) (Boonserm et al., 2005). These bacterial products are effective against larvae of several insect taxa including dipterans, such as mosquito vectors (Boonserm et al., 2005).

Basically, insect resistance encompasses both passive and active mechanisms that prevent successful infection by pathogen. On one hand, passive resistance includes physical or chemical barriers (e.g., cuticle or integument and peritrophic membrane) that deny pathogen entry into the body of the host, or provide a physiologically inadequate environment for pathogen development (e.g., pH) (Su and Mulla, 2004). On the other hand, active resistance entails innate immune responses produced in response to infection (Nirmala and James, 2003; Ahmed et al., 2008, 2010). However, some parasites and pathogens have developed some counter-mechanisms to evade or block the defense systems, and hence, manage to develop successfully (Riley, 1988; Casadevall et al., 2000). Yet, *Bt* faced no resistance from insect host due to both the interactions among its multiple toxins and their respective receptors in mosquito midgut, and it is likely that these multiple intermolecular interactions are the major reasons for the absence of passive resistance to *Bt* in mosquitoes (Federici et al., 2007; Park and Federici, 2009). Thus, both mechanisms of the bacterial endotoxin's activity and the emergence of insects' resistance are being extensively studied (Adamo, 2004; Brar et al., 2007). Yet, the efficiency of the active resistance, together with the innate antibacterial immune response, against *Bt* is still to be investigated in mosquito vector.

Insect, in fact, is capable of fighting back against most pathogens *via* two innate strategies; the first is to put the pathogen under oxidative stress *via* inducing particular reactive oxygen species (ROS) in its tissues as a direct effective weapon against pathogens (Peterson et al., 2007; Matozzo et al., 2011). The second is to induce immune antimicrobial compounds to destroy the pathogen (Hoffmann, 1995; Ratcliffe et al., 2011) and by altering or inactivating its cellular components (Hoffmann, 1995). However, this also may induce ROS causing oxidative stress to the pathogen as well as to the insect's own cells (Nappi and Vass, 2001). Thus, insects have developed a protective strategy to minimize ROS-induced damage against its own cells. This acts *via* induction of complex antioxidants, of both enzymatic and non-enzymatic nature, comprising molecules that act as free radical scavengers (Bauer et al., 2003; Ahmed, 2011). For instance, ascorbic acid, tocopherols and reduced glutathione (GSH), as non-enzymatic molecules, and catalase enzymes related to GSH, like glutathione peroxidase, and glutathione S-transferase, as enzymatic molecules. This self-antioxidant mechanism strongly supports the insect active resistance against pathogen (Bauer et al., 2003).

A prerequisite to understanding the outcome of a host/pathogen encounter is knowledge of the molecular bases of host resistance and pathogen virulence. Unfortunately, there is little evidence to document molecular determinism in such relationships (Ahmed et al., 2001, 2010; Ahmed and Hurd, 2006; Al-Roba et al., 2011; Cancino-Rodezno et al., 2010; Ahmed, 2011). This situation has limited our ability to understand the evolutionary developments of such dynamically interacting populations. Hence, previous studies have contributed, in part, to understanding this relationship in mosquito-*Bt* model by different mechanisms. For example, mitogen-activated protein kinase p38 pathway that activates a complex defense response against *Bt* Cry toxins (Cancino-Rodezno et al., 2010) or alterations of antioxidant defenses as well as decline in nourishment reservation in infected larvae (Ahmed, 2011, 2012a,b). The present study provides additional evidences for interrupting innate immunity, in terms of prophenoloxidase system and nitric oxide production, and cellular damage mechanism, in terms of inducing apoptosis proceeding cellular damage in gastric caeca upon *Bt* infection of the autogenous *Aedes caspius* larvae. This may partially improve our understanding of the mosquito/*Bt* interaction scenario, which may participate in the biotechnological applications for enhancing the activity of some *Bt* Cry toxins against specific mosquito vector.

Materials and methods

Experimental mosquitoes

This study was conducted on the autogenous *Ae. caspius* mosquito. Larvae were originally collected from the Eastern region of the Kingdom of Saudi Arabia (Ahmed et al., 2011) and reared under standard insectarium conditions (26 °C, 8 h/16 h light/dark period) in the insectary of Zoology Department, College of Science, King Saud University, as previously outlined in Ahmed (2012a). After adult emergence, mosquitoes of the same age were used for experimental purposes of the current study. To maintain a stock of mosquito colony, adults were kept accessing 10% glucose *ad libitum* since blood meal is not urgently needed for triggering vitellogenesis.

Bacterial preparations and larval infection

Mosquito-larvicidal bacterium, *Bacillus thuringiensis* (*Bt*) (serotype H-3a and 3b, strain Z-52, Biotech International Ltd, India) was obtained from the Saudi Ministry of Agricul-

ture as a spore-crystal powder [formulation contains 5–8% spores (w/w) and 5–8% delta endotoxins (w/w) based on the company's instructions]. Third instars were treated with the LC_{50} of bacterial formula (0.058 mg/l) for 48 h according to Ahmed (2011) for the experimental purposes of the current study. Control larvae were kept without bacterial treatment.

Immunological assays

Immunological assays, in terms of phenoloxidase (PO) and nitric oxide (NO) titers, were performed in larval homogenates according to Li et al. (2005); Whittena et al. (2007) throughout a complete time course post-*Bt* infection until larval death (4, 8, 12, 16, 20, 24, 28, 32, 36, 40 and 44 h). Briefly, 15 *Bt*-infected or control 3rd instar larvae were completely homogenized in 300 µl of phosphate buffer saline (PBS, pH 7.8, Biorad) in a 1.5 ml eppendorf tube, then topped up to 500 µl of PBS in total. Homogenates were then centrifuged at 3000*g* for 20 min at 4 °C. Supernatants were then removed and stored at −80 °C until used, or immediately used for carrying out the immunological assays, the PO and NO titers.

Phenoloxidase activity assay

Phenoloxidase activity was investigated in *Bt*-infected or control 3rd instar larvae of autogenous *Ae. caspius* using L-DOPA (3,4-dihydroxy-L-phenyl-alanine, Sigma) as substrate according to Matozzo et al. (2011). Briefly, aliquots of 1:10 mixture [20 µl of larval homogenate in 180 µl of L-DOPA (prepared as 1 mg/ml PBS)] at each time point of the experiment, from infected or control larvae, were incubated in a 96-well microtiter plate at room temperature for 30 min. Absorbance at 490 nm was then recorded in a microtiter plate reader spectrophotometer (Anthos2010, Biochrom, England). Preliminary experiments showed the linear phase of the reaction began shortly after the addition of L-DOPA and continued for 30 min. The activity of phenoloxidase per larva was expressed as change in optical density per minute per single larva (OD min^{-1}/larva). Data from five different replicas of each sample ($N = 5$) at each time point of the experiment were performed. This experiment was repeated twice and similar observations were obtained.

Nitric oxide assay

Nitric oxide was determined in control or *Bt*-infected 3rd instar larvae of autogenous *Ae. caspius* by comparative spectrophotometric measurements of the nitrite ion (NO_2^-) concentration in the larval homogenates at each time point of the experiment. Nitrite ion concentration assay was performed using Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4) (Green et al., 1982; Nappi et al., 2000). Briefly, 100 µl from each larval homogenate, of control or *Bt*-infected larvae at each time point, was incubated in 100 µl Griess reagent in wells of a 96-well microtiter plate at room temperature for 30 min prior to spectrophotometric analyses. The absorbance at 532 nm was then recorded in a microtiter plate reader spectrophotometer (Anthos2010, Biochrom, England), and NO (µM/larva) concentration was quantified using $NaNO_2$ to establish a standard reference curve (1–100 µM). Data from five different replicas of each sample ($N = 5$) at each time point of the experiment were performed. This experiment was repeated twice and similar observations were obtained.

Investigation of cellular apoptosis

Apoptosis in control or infected 3rd instar was investigated using flow cytometry (BD FACSCalibur, US) according to the manufacturer's instructions. Briefly, larval cells were manually isolated from control or *Bt*-infected larvae (at 6, 12, 24, 36 and 48 h post-infection) by gentle homogenization of larvae at each time point (20 larvae each) in 10 ml mixture of PBS and 0.04% EDTA. Larval homogenates were then filtered through a suitable fine-mesh refinery to collect single cells. Resulting cellular suspensions were then centrifuged for 30 min at 3000*g* at 4 °C. Supernatants were removed and cellular pellets were washed twice in PBS-EDTA buffer. Cellular pellets were then fixed and permeabilized by incubation in 70% ice-cooled ethanol for overnight. Cells were then washed twice in PBS-EDTA buffer and incubated at 37 °C for 1 h in a mixture of 40 µg/ml propidium iodide and 100 µg/ml DNase free RNase in PBS-EDTA buffer for staining DNA. Samples were then analyzed by assessing FL2 red fluorescence on a linear scale using flow cytometry. The resulting percentages of apoptotic cells were determined as the percentages of hypodiploid cells (sub G0/G1 peaks).

Electron microscopy

Third instar of control or infected (alive, but sluggish) larvae were routinely prepared, at 44 h post-infection, for investigating the body size and ultrastructure alteration in the gastric caeca under scanning and transmission electron microscopes respectively according to the instructions of the Electron Microscopy Unit in Zoology Department, King Saud University. Resulting images from control or treated larval preparations were imported into Adobe Illustrator Cs, 2003 Software and adjusted for contrast and suitable qualities.

Measurements of body size

Measurements of body size, in terms of head and thorax widths, were performed under SEM. Briefly, control or *Bt*-infected larvae (36 h post-infection) were immediately fixed in 2.5% glutaraldehyde in phosphate buffer. Following a minimum of 24 h of fixation, larvae were rinsed with 0.1 M PBS three times at 10 min intervals then fixed in 2% osmium tetroxide in distilled water. Larvae were then rinsed three times in 0.1 M PBS, dehydrated in ethanol series (30%, 50%, 70%, 95%) for 10 min each, then rinsed three times in 100% ethanol. This was followed by treatments with 50% ethanol:50% acetone, 100% acetone, and 50% acetone:50% hexamethyldisilazane (HMDS), each for 15 min. Finally, larvae were rinsed twice in100% HMDS for 15 min and allowed to air dry overnight. Larvae were then gold coated using a sputter machine (SPI Module Sputter Coater, USA) and observed under the SEM (JSM-6380 LA, Japan). Measurements of larval heads and thoraces were performed directly under the microscope using SEM Control User Interface Program (Version 7.06, 2004). Five different head and thorax measurements were performed using five different control or infected larvae ($N = 5$).

Ultrastructure investigation of gastric caeca

Ultrastructure of gastric caeca was investigated in control or *Bt*-infected larvae (at 24 and 40 h post-infection) under the transmission electron microscope (TEM). Larvae were chilled on ice and midguts were excised under a binocular microscope and midgut dissection and removing gastric caeca were carried out as detailed in Silva-Filhaa and Peixoto (2003); Clark et al. (2005). Gastric caeca were then processed for TEM analysis. Briefly, specimens were fixed overnight in cold 0.8% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate, (pH 7.0) and fixed for 4 h at 4 °C in 1% OsO4 in 0.1 M sodium cacodylate (pH 7.0). Specimens were then dehydrated through an ethanol series, treated with propylene oxide and embedded in Epon Araldite resin (1:1). Ultrathin 4-μm sections were mounted on slides, stained with 2% uranyl acetate for 30 min and then incubated in lead citrate for 10 min. Ultrasections were then investigated using TEM (JEM-1010 LA, Japan).

Data analysis

Statistical analyses of data were undertaken using MINITAB software (MINITAB, State College, PA, v: 13.1, 2001). Data were first tested for normality (using Anderson–Darling Normality test) and for variances in homogeneity prior to any further analysis. Data pertaining to body measurements were normally distributed, and thus, a two-sample *t*-test (for individual comparison) was used for comparing differences between *Bt*-infected and control larvae in each case. Data pertaining to the titers of NO and PO, and % apoptosis were normally distributed and had homogeneous variances, and thus, comparisons between treatments were made using One-way analysis of variance (ANOVA), and differences between individual pairs of data were analyzed using the multiple comparison Tukey's test (Morrison, 2002) in each case. Five replicas ($N = 5$) in each experiment were carried out using new groups of mosquito larvae in each case.

Results

Phenoloxidase activity

Prophenoloxidase (PO) titer has been assayed in *Bt*-infected or control 3rd instar larvae of autogenous *Ae. caspius* using L-DOPA. Spectrometric measurements of PO activity in pooled larval homogenate samples (15 larvae) were performed at each time point (4, 8, 12, 16, 20, 24, 28, 32, 36, 40 and 44 h post-*Bt*-infection). No significant differences in larval phenoloxidase activity were detected between the *Bt*-infected and control larvae ($F_{11,48} = 12.15$, $P > 0.05$, One-way ANOVA) except at 4 h post-infection (Fig. 1). Infected larvae showed a significant higher PO activity only at 4 h post-*Bt* infection, averaged approximately 16.7% higher than that of control ones ($F_{11,48} = 12.15$, $P < 0.05$, One-way ANOVA) (Fig. 1). Subsequently, this activity was restored to the normal level, as control level, throughout the rest of experimental time points. This may indicate that larvae were able to exhibit PO activity as early as 4 h post-*Bt* infection, but this activity was quickly ceased afterward until larval death.

Nitric oxide level

Nitric oxide level in *Bt*-infected or control 3rd instar larvae of autogenous *Ae. caspius* was assayed by Griess staining and subsequent spectrometric measurements of pooled larval homogenate samples (15 larvae each) at each time point post-treatment (4, 8, 12, 16, 20, 24, 28, 32, 36, 40 and 44 h post-infection). Means of five separate experiments of infected

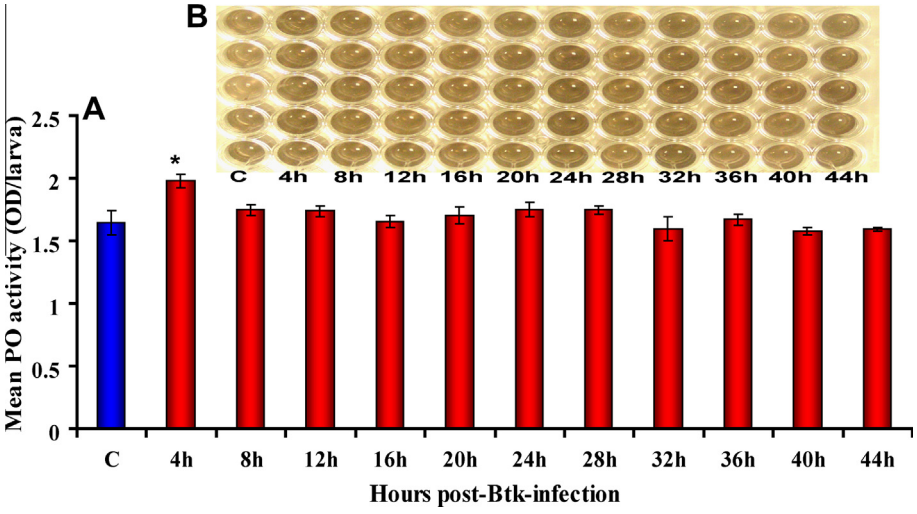

Figure 1 Phenoloxidase activation in control (C) 3rd larval instar of autogenous *Ae. caspius* and *Bt*-infected larvae at different times of post-infection (numbers underneath figures). An amount of 20 μl of larval homogenate (at each time point) was incubated with 180 μl of L-dopamine substrate (1:9), or with PBS alone, in a 96-well microtiter plate for 30 min at room temperature. PO activity was then spectrophotometrically monitored at 490 nm. Values are expressed as means ± standard error (SE). Error bars represent means of five different experimental replicates ($N = 5$), each involving a homogenate pooled from 15 individual larvae. The significance of difference was calculated using One-Way ANOVA (Tukey's Pairwise Test). The kinetic activity of PO per larva was expressed as change in optical density units per minute per single larva (OD min^{-1}/larva). An asterisk (*) indicates significantly higher activity compared to control ($P < 0.05$).

or control larvae are presented in Fig. 2. Based on $NaNO_2$ standard reference curve established for this assay, data showed that larvae exhibited significantly higher concentrations of NO averaged approximately 29.2% higher than those of control ones as early as 4 h post-infection ($F_{11,48} = 159.72$, $P < 0.05$, One-way ANOVA) (Fig. 2). This level was restored to the normal level as that of control one at 8 h post-infection ($F_{11,48} = 159.72$, $P > 0.05$, One-way ANOVA) (Fig. 2). A significant decrease in NO level was detected starting from 12 h post-infection and until hardly detected at 44 h post-infection (just before larval death) ($F_{11,48} = 159.72$, $P < 0.05$, One-way ANOVA) (Fig. 2). These data may indicate that infected larvae were able to induce NO as early as 4 h post-infection, and that *Bt* was able to inhibit this effective antibacterial response at 12 h post-infection and until larval death.

Cellular apoptosis

Flow cytometry is nowadays an extremely useful and reliable technology for biosafety and public health studies related to infectious disease (Marrone, 2009). This technology was utilized in this experiment to monitor the population homogeneity of whole larval cells *via* side and forward scatter parameters (Fig. 3). The percentages of apoptotic cells in control or infected larvae were determined as the percentages of gated cells located in the sub G1 histograms. As shown in Table 1, very low % of apoptosis (9.09 ± 0.24) was detected in cells isolated from control larvae. However, cells from *Bt*-infected larvae showed significantly elevating percentages of apoptosis from 6 up to 44 h post-infection (from 15.5 ± 0.88 up to 47.61 ± 0.93 respectively) compared to that of control ones ($F_{24,29} = 532.64$, $P < 0.05$, $N = 5$, One-way ANOVA, Tukey's Pairwise Test).

Body size alteration

Both control and *Bt*-infected larvae were observed under SEM for investigating the body size (in terms of head and thorax widths) alterations at 40 h post-infection. SEM investigation revealed that the size of thoracic region was considered as an indication of the body size alteration in this study. As shown in Fig. 4, a significant shrinkage in the thoracic region of infected larva (Fig. 4B) is obvious compared to that of control ones (Fig. 4A). Thorax of Bt-infected larvae showed a significant 50% reduction in size compared to that of the control ones (545 ± 15.0 *v* 1003.8 ± 12.4 µm, respectively) ($P < 0.05$, $N = 5$, student *t*-test) (Fig. 4C, D and Fig. 5). However, head capsule was similar in both infected and control larvae (775 ± 12.0 *v* 780.8 ± 13.4 µm, respectively) ($P > 0.05$, $N = 5$, student *t*-test) (Fig. 4C, D and Fig. 5). Moreover, the mean thorax size was ~1.3 folds of that of the head in control larvae, while it was ~0.7 of that of the head in infected larvae (Fig. 4C, D and Fig. 5). These data clearly show a significant shrinkage in the body size of *Bt*-infected larvae.

Structure alteration of gastric caeca

Epithelium of the gastric caeca of both control and *Bt*-infected larvae was investigated under TEM. Cytoplasmic vacuoles were noticed within the epithelial cells of gastric caeca of *Bt*-infected larvae (Fig. 6B) at 24 h post-infection. Moreover, microvilli were fewer in number compared to control larvae, as well as showing evidence of degeneration at 40 h post-infection (Fig. 6C and D). In addition, partial degeneration of nucleus was also observed starting from 24 h post infection (Fig. 6B). These data suggest that cytological destructions in the gastric caeca epithelium were more pronounced just before larval death at 40 h post-infection.

Discussion and conclusion

Bacterial toxins are important virulent factors produced by several pathogenic bacteria against its insect host. Attack by these toxins represents a fundamental threat to the host post-infection, and consequently, host defense responses might be evolved against some of these toxins. Among them, is the

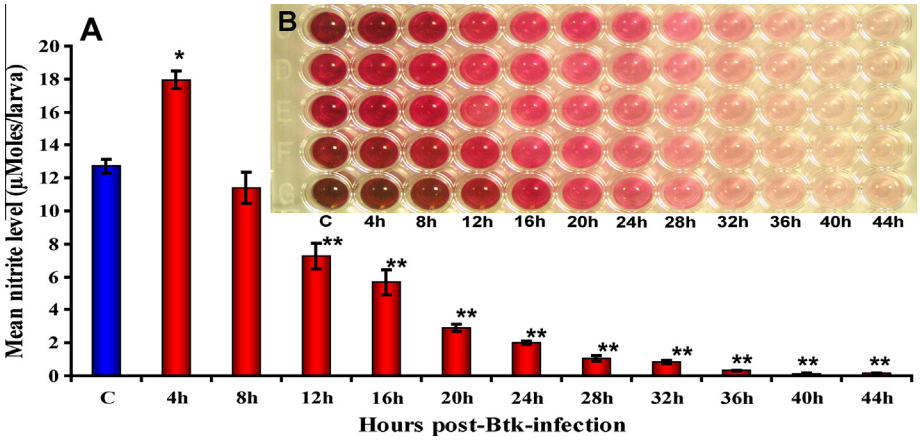

Figure 2 Nitric oxide concentrations in homogenate samples of control (C) and *Bt*-infected 3rd instar larval of autogenous *Ae. caspius* at different time points of post-infection (numbers underneath figures). NO generation in the homogenates was determined by the Griess reaction. Each data point represents the mean of five separate replicates ($N = 5$), each involving homogenate pooled from 15 individual larvae. The significance of difference between means was calculated using One-Way ANOVA (Tukey's Pairwise Test). An asterisk (*) indicates the significantly higher concentration compared to control ($P < 0.05$), while double asterisks (**) indicate the significantly lower concentration compared to control ($P < 0.05$).

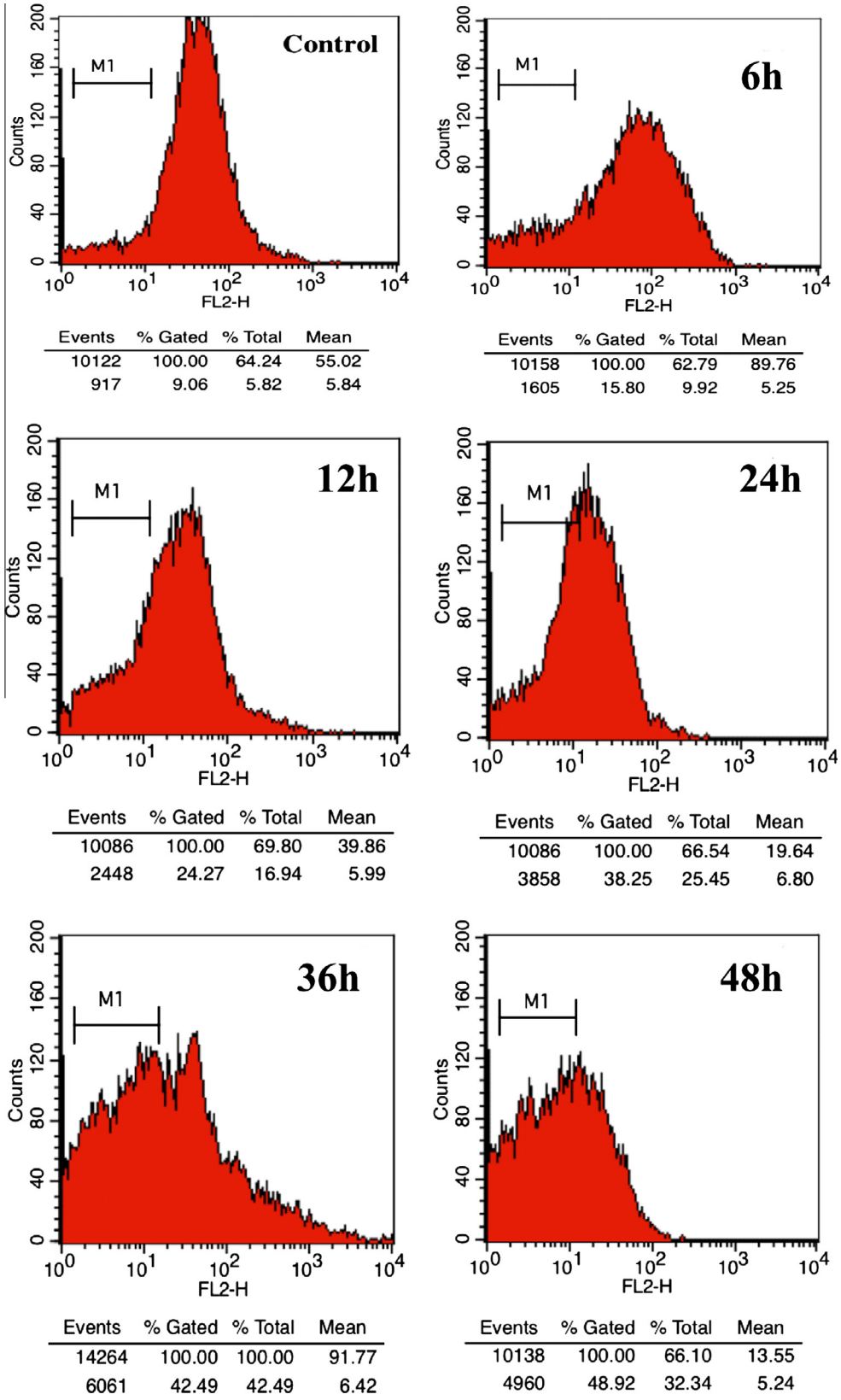

Figure 3 Flow cytometric analysis enrolled to determine the percentage of cells undergoing apoptosis in control and *Bt*-infected 3rd instar larvae of the autogenous *Ae. caspius*. Apoptosis was determined as the percentage of hypodiploid cells in control and Bt-infected larvae. Histograms represent one replicate of the experiment showing the staining with propidium iodide in control and Bt-infected larvae (at 6, 12, 24, 36 and 48 h post-infection). M1: represents mean number of cells in sub G1 stage (apoptotic). Five replicates using five different mosquito groups were carried out.

Table 1 Percentages of cells undergoing apoptosis in control and *Bt*-infected 3rd instar larvae of the autogenous *Ae. caspius* mosquito at 6, 12, 24, 36 and 28 h post-infection as detected by flow cytometry.

	Hours post-infection					
Number (Mean ± SE)	Control	6 h	12 h	24 h	36 h	48 h
Total examined cells	10187 ± 32	10153 ± 14	10441 ± 139	10517 ± 157	14235 ± 87	10246 ± 73
Apoptotic cells	915.2 ± 5.32	1619 ± 9.3	2442 ± 35	4023 ± 58.8	6104.2 ± 65.6	5042 ± 57.1
% of apoptotic cells	9.092 ± 0.141	15.94* ± 0.08	23.45* ± 0.73	38.36* ± 1.23	42.88* ± 0.41	49.23* ± 0.79

Each data point represents the mean of five separate replicates ($N = 5$), each involving pooled cells from 20 individual larvae. The significance of difference between means was calculated using One-Way ANOVA (Tukey's Pairwise Test). Asterisk (*) indicates significantly higher percentages compared to control ($F_{24,29} = 532.64$, $P < 0.05$).

mitogen-activated protein kinase p38 pathway that triggers survival response (resistance) in mosquitoes against some *Bt* Cry toxins (Cancino-Rodezno et al., 2010). Moreover, induction of immune and oxidative stress responses is another main defense mechanism adopted by insects against pathogens involving immune peptides in the hemolymph and reactive oxygen species (ROS) in tissues respectively (Hoffmann, 1995; Michel and Kafatos, 2005; El-Gendy et al., 2009; Cerenius et al., 2008; Maroi, 2011; Ahmed, 2011). The humoral immune responses act by altering or inactivating proteins, lipid membranes, and DNA of the pathogen (Hoffmann, 1995; Zhao et al., 2011). The involvement of ROS and other antibacterial protein pathways as effective resistance mechanisms against bacteria (Hurst and Lymar, 1997; Cancino-Rodezno et al., 2010), parasites (Denicola et al., 1996) and fungi (Hurst and Lymar, 1997) has also been documented. However, some pathogens are capable of blocking these immune-mediated cytotoxicity by oxidizing or reducing these harmful substances (Riley, 1988; Schnitzler et al., 1999; Casadevall et al., 2000; Jacobson, 2000; Ahmed, 2011). In fact, this may explain the sudden significant elevation of PO at 4 h post-*Bt*-infection in the current study as an immediate immune response against the ingested *Bt*, after which it has quickly restored to its normal level (as that of the control), starting from 8 h post-infection and onwards. This could be referred to the capability of *Bt* to inhibit this antibacterial immune response, *via* unknown mechanism(s). And hence, PO level has been kept only at the level essential for the other vital biological process like pigmentation and sclerotization in many tissues (Jiravanichpaisala et al., 2006; Cerenius et al., 2008; Matozzo et al., 2011; Wang et al., 2011). These data may indicate that *Bt* has only inhibited PO activation system from working as antibacterial immune activity (Hauton et al., 1997).

It has also been reported that there is a trade-off between PO and other immune antibacterial peptides (Cotter et al., 2008; Povey et al., 2009; Rao et al., 2010). Moreover, GSH has been found to be an inhibitor for the production and activity of PO (Nayar and Bradley, 1994; Nayar and Knight, 1995).

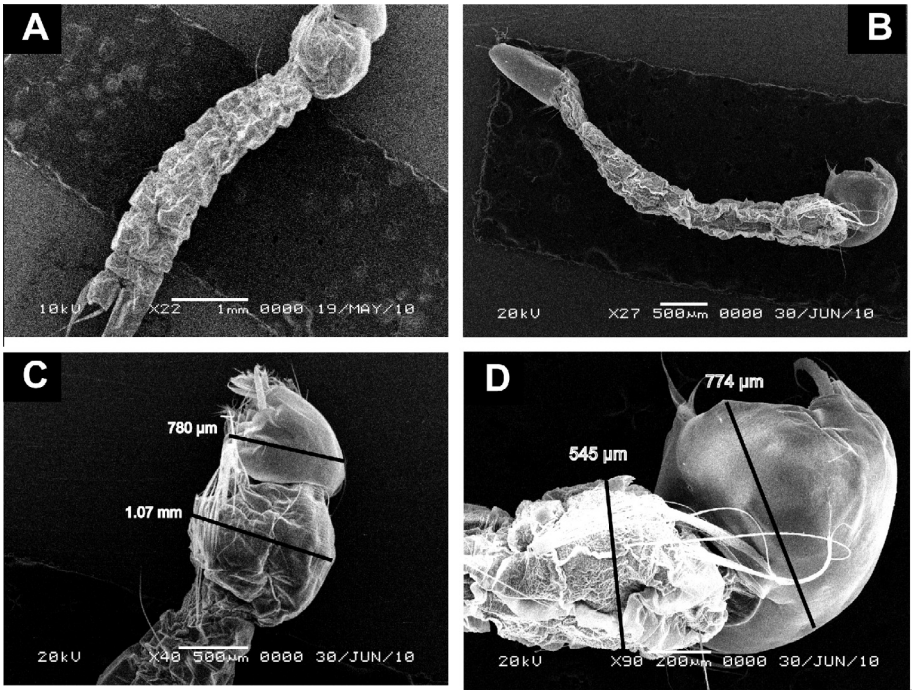

Figure 4 Scanning electron microscopy of body regions of *Bt*-infected, at 40 h post-infection, or control 3rd instar larvae of the autogenous *Ae. caspius*. A and B are whole mounts of control and infected larvae respectively at low magnifications. C and D are control and infected larvae, respectively, at higher magnifications showing heads and thoracic regions. Black lines and numbers on heads and thoracic regions represent diameter width (µm/mm).

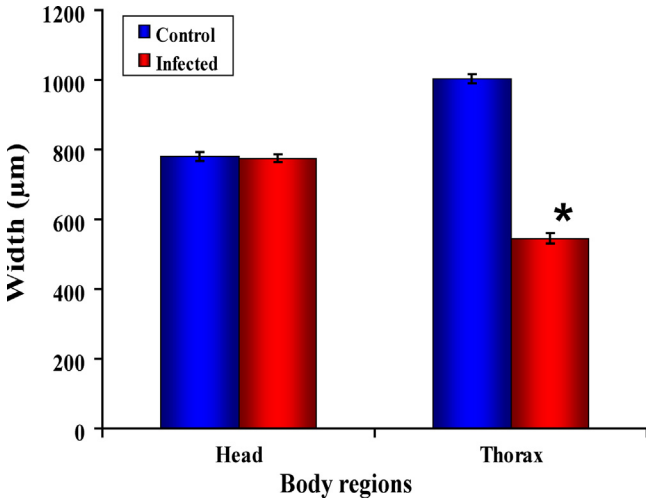

Figure 5 Histogram showing the diameters (widths) of heads and thoracic regions in control and *Bt*-infected (40 h post-infection) 3rd larval instar of the autogenous *Ae. caspius*. An asterisk (*) indicates significantly smaller value compared to that of control ($P < 0.05$, $N = 5$, Student's *t*-test).

This conflict happens probably because hemocytes are the sole source of both PO and GSH production upon infection by microorganism (Clark et al., 2010; Wang et al., 2011). This may interpret the reported elevation of GSH in *Bt*-infected *Ae. caspius* (Ahmed, 2011) and the inhibition of PO activity upon *Bt*-infection in the current study as reported in other Bt-insect models (Giannoulis et al., 2008; Ericsson et al., 2009). Taking all together, I would suggest that *Bt* may have inhibited the PO activation system in infected mosquito larvae of the current study.

Nitric oxide (NO) is another key immune effector and signaling molecule in many organisms. However, the contribution that it makes toward insect immunity has received little attention. NO has been considered as a key phagocyte-derived effector molecule that initiates cytotoxic activity against pathogens *via* both cell-mediated and humoral immune responses in some insects (Nappi et al., 2000; Peterson et al., 2007). It is frequently produced by activated cells and can react with O to form peroxynitrite ($ONOO_2$) (Hogg and Usmar, 1992) and with H_2O_2 to form the very reactive hydroxyl radical (**.**OH) and reactive nitrogen oxide species (RNOS) (Nappi and Vass, 1998). This may explain the immediate elevation of NO in the *Bt*-infected larvae at 4 h post-infection as a quick immune response against *Bt*. It has been proven that anopheline malaria-infected mosquitoes induced nitrosative and oxidative stresses that limit malaria development in its midgut (Brunet, 2001; Herrera-Ortíz et al., 2004; Peterson et al., 2007). On the other hand, mosquito vector produces peroxiredoxins (Prxs), enzymes known to detoxify ROS and RNOS, in order to protect its own cells from oxidative stresses resulting from these radicals (Radyuk et al., 2003; Bauer et al., 2003; Bartholomay et al., 2004). All these studies provide evidences that reactive intermediates of both ROS and RNOS constitute a further part of the cytotoxic arsenal employed by insects in defense

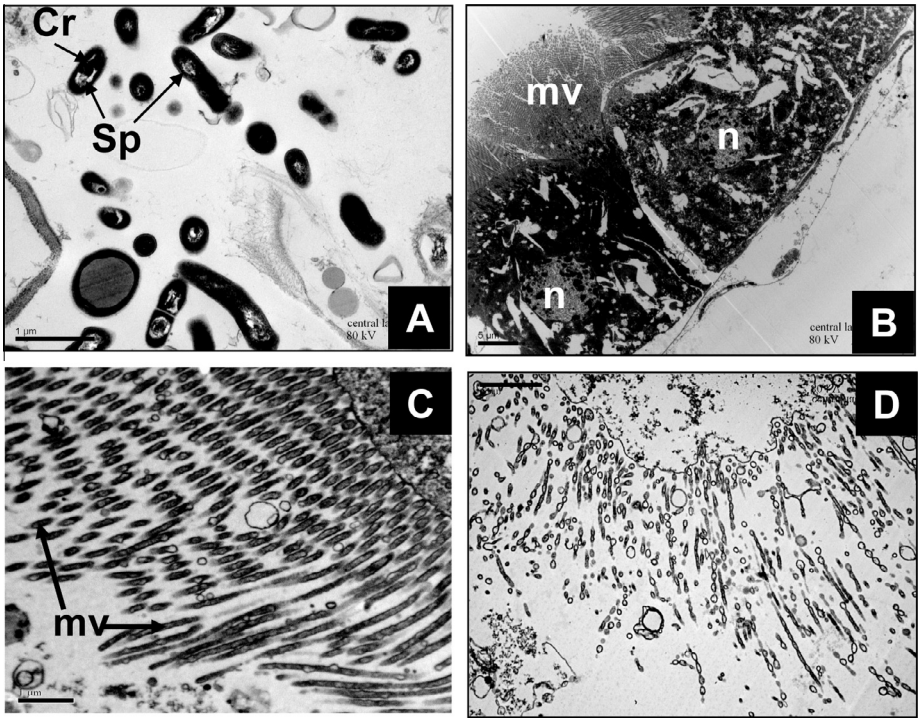

Figure 6 Transmission electron micrographs showing the ultrastructure of the gastric caeca of control and infected 3rd larval instar of autogenous *Ae. caspius*. A: represent sporangia of *Bt* bacteria in the midgut of intoxicated larvae at 12 h post-infection. The parasporal crystals (Cr), which contain the entomopathogenic toxins, are visible close to the spore (Sp) (Scale bar = 1.0 µm). B: Gastric cecum of *Bt*-infected larva at 24 h post-infection showing several cytoplasmic vacuoles, and partial disruption of nuclei (n) and microvilli (Mv) (Scale bar = 5.0 µm). C: Microvilli (Mv) of a control gastric cecum (Scale bar = 1.0 µm) and D: Severe disruption of microvilli of an infected gastric cecum at 40 h post-infection just before death (Scale bar = 2.0 µm).

against pathogens. Thus, the significant time-dependent NO decline detected in *Bt*-infected mosquito larva of the current study may be explained as *Bt* has inhibited this antibacterial key immune effector starting from 4 h post-infection. Evidence for this in the other model has been provided by Herrera-Ortíz et al. (2004) who detected no NO induction against the *in vitro* midgut inoculated *Klebsiella pneumoniae*, *Escherichia coli* or *Serratia marcescens* bacteria. Furthermore, *Bt* might have interrupted the self-protection antioxidant system, and hence, exposed mosquito cells to the cell damaging oxidative stress leading to the reported apoptosis and eventually cellular damage in the gastric caeca.

Basically, apoptosis is essential for the removal of unwanted cells, and is critical to restricting cell numbers and tissue patterning during development (Hengartner, 2000; Honegger et al., 2011). Apoptosis also plays a key role in the development, regulation and function of the immune system, and can be a response of cells to general stress, including the stress incurred as a result of immune induction and infection with intracellular pathogens (Ahmed and Hurd, 2006; Rost-Roszkowskaa et al., 2010; Zhao et al., 2011). Thus, the significant higher percentage of apoptosis recorded in the current study could be attributed to the *Bt*-infection oxidative stress that triggered the apoptotic cascade through the activation of caspases, the family of cysteine proteases (Richardson and Kumar, 2002; Kornbluth and White, 2005; Coopera et al., 2007). Flow cytometry detected apoptosis as early as 12 h post-*Bt*-infection, which may imply that apoptosis proceeds to cellular damage in the larval mosquito gut.

Epithelial damage of the midgut upon Bt-infection has been studied in several insect models (e.g. de Maagd et al., 2001). The current study investigated the late stage of cytological damage in the gastric caeca in particular, as an important site of the final stage of *Bt*-mosquito larva interaction scenario. Basically, in mosquito vector, the midgut is structurally divided into three major regions. The most anterior portion is the cardia, followed by lateral pouches called gastric caeca, then the ventriculus, a long straight tube (Clark et al., 1999; Clements, 2000). All chemical digestion and absorption of nutrients occur in the midgut, and it appears that the gastric caeca, although located at the anterior end of the midgut, are primarily involved in nutrient absorption and ion regulation (Volkmann and Peters, 1989; Clark et al., 1999; Clements, 2000). Moreover, it has been investigated that the posterior region of the midgut is the main target area of the bacterial toxin (de Maagd et al., 2001; Al-Roba et al., 2011). However, data from ultrastructural investigation in the current study showed massive damage in the epithelium of the gastric caeca and the microvilli. Although this has been recorded in several other studies (e.g. Clark et al., 2005), this is the first time to be studied in larvae of the autogenous *Ae. caspius* which may indicate that *Bt* toxins also bind with high affinity to the epithelium of the gastric caeca in this *Bt*-mosquito model. Consequently, *Bt*-infection might have affected the metabolism of infected larvae through manipulating the availability of free amino acids, the host protein synthesis and the gluconeogenesis process (Nakamatsu and Tanaka, 2004; Salvador and Cónsoli, 2008). Taking all together, I would refer the reported decline in the nourishment reservation in *Bt*-infected larvae of the autogenous *Ae. caspius* reported by Ahmed (2012b) to similar mechanisms, as well as, the consumption of some of these nutrient-rich reservations by *Bt* for proliferation. This, in fact, may explain the decrease in body sizes of infected larvae reported in the current study, and hence, may affect the autogeny parameters of this autogenous mosquito vector (Attardo et al., 2005; Ahmed, 2012b).

In conclusion, understanding the mechanisms of mosquito host responses to *Bt* toxins would provide ways to deal with different pathogens and to improve the action of toxins that have biotechnological applications. Different levels of resistance to pathogens have been earlier reported in mosquito populations (Rao, 1995) depending on various factors, including genetic background, selection pressure, the insect generation turnover, migration level and the genetic dominance of resistance (Wirth et al., 2000). Thankfully, field resistance to *Bt* has hardly occurred so far due to its toxin complex of Cry proteins which act in synergy and apparently with different target molecules (Wirth, 1998). However, some of these Cry toxins may face resistance by mosquito vector (e.g. Cancino-Rodezno et al., 2010). Thus, based on the findings of the current study, I would refer this advantageous aspect of irresistible *Bt* to some further mechanisms; (a) inhibiting the antimicrobial NO and PO induction, (b) interrupting the antioxidant self-protection system of mosquito host, and hence, exposing mosquito cells and tissues to oxidative stress and hence (c) triggering cellular apoptosis resulting in cellular damage of midgut, and finally death. So, these data may contribute – in part – to improving our understanding of the different levels of pathogenicity of mosquitocidal bacteria. Nevertheless, investigating NO, PO and apoptosis in particular sites (midgut, fat body and hemolymph) upon infection with *Bt* and some other mosquitocidal bacteria is still to be investigated in both refractory and susceptible mosquito models, which may help in improving the biocontrol measures against mosquito vectors.

Acknowledgements

Author would like to thank Dr Gamaal Badr, Zoology Dept., College of Science, Assiut University, Egypt, for his assistance with the Flow Cytometry, and the anonymous referees for their helpful comments and suggestions.

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