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Full Length Research Paper

Immune and antioxidant defenses in an autogenous *Aedes caspius* mosquito upon infection with *Bacillus thuringiensis kurstaki*

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This study investigates the glutathione-immunity interaction upon infection of both larval and adult stages of *Aedes caspius* with *Bacillus thuringiensis kurstaki* (*Btk*). The anti-oxidative stress, in terms of glutathione (GSH) titer, and melanization immune response, in terms of phenoloxidase (PO) titer, was investigated. Data from *Btk*-infected larvae showed no or significant lower GSH titer at 12 or 24 h post-treatment respectively compared to control larvae. On the other hand, no PO activity was detected at both time points post-*Btk* infection. This may indicate that oxidative stress in larvae was increased while antibacterial response was blocked upon *Btk* infection. Bacteria-inoculated adult mosquitoes showed higher GSH activity at 12 and 24 h post-inoculation with *Btk* and *E. coli* compared to control ones, and was more pronounced in *E. coli*-inoculated mosquitoes. On the other hand, PO titer showed significant higher PO activity at 12 and 24 h post-inoculation with each kind of bacteria, but was more pronounced against *E. coli.* These results may indicate that antibacterial and antioxidtive responses are more pronounced against *Btk* in adult stage compared to larval stage, which may be attributed to the difference in the mode of infection and/or mosquito stage. Moreover, inhibition of both antibacterial and antioxidtive responses upon *Btk* infection in larval stage may explain its high larvicidal activity. More markers of oxidative stress in *Btk*-infected mosquitoes still to be investigated.

Key words: Immune response, antioxidant response, oxidative stress, glutathione, *Aedes caspius*, *Bacillus thuringiensis*.

INTRODUCTION

Although insects are normally living in places that are full of pathogenic microbes, they have no adaptive immunity like humans do. However, they can still develop readily due to the protection of their innate immune systems, which consists of both cellular and humoral mechanisms that are tightly interconnected (Kavanagh and Reeves, 2004). Humoral defense refers to antibacterial proteins and other immune-related molecules generated by the fat body and/or haemocytes that are released into haemolymph to immobilize and kill invading microorganisms or parasites (Vilmos and Kurucz, 1998; Morton et al., 1987). This involves the synthesis of a range of anti-microbial peptides (Boman, 1998; Imler and Bulet, 2005), lysozyme (Fiolka et al., 2005), lectins and the prophenoloxidase cascade (Dunphy et al., 1986), serine proteases and carbohydrases (da Silva et al., 2000) and heat shock proteins (Salzet, 2001). In contrast, cellular defenses refer to responses such as phagocytosis and encapsulation which are directly carried out by haemocytes (Gillespie et al., 1997; Irving et al., 2005; Wang et al., 2011).

One important antibacterial humoral immune response in insects is the prophenoloxidase-activating system (Söderhäll and Cerenius, 1998). This system plays an important role in initiating the humoral and cellular defense responses (Ashida and Brey, 1997). After activation, prophenoloxidase (pPO) becomes the active form, phenoloxidase (PO) which oxidizes phenolic

compounds to quinone intermediates. The last one cross-links with proteins to form melanin (Söderhäll and Cerenius, 1998). Therefore, pPO is a very important humoral protein that is involved in the insect innate immunity. In adult mosquitoes, pPOs are key immunity proteins that are involved in the immune response that kills malaria parasites. Very little is known about which types of haemocytes can produce pPO. However, a very recent study on *Culex pipiens quinquefasciatus* by Wang et al. (2011) detected prophenoloxidase in the plasmatocytes at larval stage alone and in the smallest prohemocytes during almost all developmental stages and in granulocytes from blood-fed female mosquitoes and in oenocytoids in pupae and in adult females after blood-feeding.

The glutathione mediated reaction catalyzed by glutathione S-transferase (GST) (Meister and Anderson, 1983) is one of the important mechanisms that allow insects to survive in a contaminated environment (Poupardina et al., 2008) and insecticide-resistance (Prapanthadara et al., 1995). Many studies of insect glutathione revealed multiple forms exist in different types of insects. Those include housefly (Clark et al., 1984; Fournier et al., 1992), grass grub (Clark et al., 1985) and *Drosophila* (Toung et al., 1990). In mosquitoes, there are at least three GST isoenzymes present in *A. aegypti* (Grant et al., 1991) and *Anopheles dirus* (Prapanthadara et al., 2000) seven in *Anopheles gambiae* (Prapanthadara et al., 1993).

These different forms of glutathione exhibited varying specificities for different insecticides. Prapanthadara et al. (1995) demonstrated that in a DDT-resistant strain of the African mosquito *A. gambiae*, there was an eight-fold increased in DDT-dehydrochlorinase activity due to an increase synthesis of different isoenzymes of glutathione that possessed a greater level of this activity. Observed differences in the glutathione from the two strains demonstrated that expression of the enzymes is influenced by environ-mental factors. Thus, this may indicate that long-term exposure to a toxicant will eventually select for mutations conferring a level of resistance to that toxicant and indeed, insecticide-resistant populations of mosquitoes are now threatening the success of control measures.

Beside playing a central role in the metabolism of insecticides and other xenobiotics (Feyereisen, 2005; Hemingway et al., 2004), glutathione constitutes a second line in insect immunity as it plays a role in the detoxification of toxins in insect body, including toxic immune compounds that involve melanin, and protecting insects from the concomitant oxidative stress (Nappi and Vass, 2001; Kumar et al., 2003). In the current study, the antioxidative response, in terms of glutathione titer (GSH) titer, a tripeptide thiol found in virtually all metazoans, and melanization immune response in terms of phenoloxidase (PO) titer, an essential enzyme to induce melanization in insects, in the autogenous mosquito vector, *Aedes caspius*, will be investigated in both larval and adult mosquito stages upon challenging with the entomopathogenic *B. thuringiensis kurstaki* or non-pathogenic *E. coli* bacteria. Moreover, theses two activities will also be investigated in larval stage upon infection with *B. thuringiensis kurstaki*. This study forms a ground base for further studies on the other anti-oxidative responses and its interaction with innate immune responses aiming at, on one hand, reducing the vectorial capacity of mosquito vector in the battle against mosquito-borne pathogens. And on the other hand, this may help in enhancing the pathogenicity of *B. thuringiensis* against mosquito larvae.

MATERIALS AND METHODS

Experimental mosquitoes

This study has been performed on an autogenous species of *A. caspius*. This species was chosen for this study because of its ability to lay eggs without taking a blood meal, and its availability in the laboratory, which enabled me to obtain numerous adults and third-instar larvae for repeated toxicological experiments. Larvae were originally collected from Eastern region of the Kingdom of Saudi Arabia as detailed in (Ahmed et al., 2011). Collected larvae were identified according to the classification keys of Mattingly and Knight (1956) and confirmed by the Natural History Museum (London, UK). Mosquitoes were reared under standard insectarium conditions (26°C, 8 h/12 h light/dark period) in tap water (or distilled water for toxicity experiments) in the insectary of Zoology Department, College of Science, King Saud University, as previously outlined in Ahmed et al. (1999). Adults emerging within a 24 h period were maintained in rearing cages (30 × 30 × 30 cm each) with continuous access to a 10% glucose solution (w/v). At least 15 generations were produced prior to use for the experimental purposes. After adult emergence, mosquitoes of the same age were used for the relevant experiments in this study. To maintain a stock of mosquito colony, they were kept accessing 10% glucose since blood meal is not urgently needed for triggering vitellogenesis.

Bacterial preparations

Mosquito-larvicidal bacterium, *B. thuringiensis* var *kurstaki* (*Btk*) (serotype H-3a and 3b, strain Z-52, Biotech International Ltd, India) was obtained from the Saudi Ministry of Agriculture 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]. It was used directly for larvicidal activity bioassay as detailed below. For adult mosquito bacterial inoculation, *Btk* bacteria (spores from the spore-crystal powders) or *Escherichia coli* (kindly provided by the Department of Food Sciences and Nutrition, Faculty of Food Sciences and Agriculture, King Saud University) were routinely incubated in nutrient broth (13 g/l) at 37°C for 48 h at 200 rpm in a rotary shaker until an OD_{598} of 0.5-0.7 is reached, then inoculated into mosquitoes as detailed in Nimmo et al. (1997). Bacteria-inoculated mosquitoes were allowed access to sugar solution until used for the relevant experiments. Only active mosquitoes (able to fly) were used for experimental purposes.

Mosquito-larvicidal activity bioassay

Bioassay for mosquito-larvicidal activity was performed using spore-

crystal powders of *B. thuringiensis kurstaki* (*Btk*). Bioassays was performed in five replicates (N = 5), in rearing plastic trays (30 × 15 × 10 cm). Each tray was containing 100 third-instar larvae in 1000 ml dH_2O infused with various concentrations of *Btk* bacteria according to (Rey et al., 1999) in the presence of food (ground Tetramin flakes). In a preliminary study, the bacterial powder was used at 0.0 concentration (control) or 8 ascending concentrations of spore-crystal powders starting from 0.004–0.52 mg/l of 5 independent replicates for each concentration. The medium was not subsequently replaced, and no further food was added, so that larvae were nominally food-deprived after 20 h. Larvae were monitored each 6 h until death or survival for 48 h had occurred. Larval mortality was quantified by counting live larvae remaining after 48 h post-treatment in 5 replicates (N = 5). Based on the resulting mortalities, the suitable concentrations used in the main toxicity experiment of the current study were assessed in 5 concentrations from 0.008 – 0.13 mg/l of 5 replicates each. The relationship between concentrations and mortality was plotted *via* a regression plot using MINITAB software (MINITAB, Stat College, PA, version 13.1, 2001), and the resulting linear equation was used for calculating the fifty and ninety percent lethal concentrations (LC_{50} and LC_{90}, respectively). LC_{50} (0.058 mg/l) was use in the subsequent experiments of the current study.

Bacterial inoculation

Bacteria suspensions of *Btk* or *E. coli* were prepared as detailed above. Fifty adult mosquitoes were immobilized by chilling on ice for 5 min. prior to either sham-injection with 0.25 µl of *Aedes* physiological saline (APS) (13 mM NaCl, 0.5 mM KCl, 0.1 mM $CaCl_2$) as trauma control, or challenged with bacteria by pricking (injection) with a fine capillary needle (pre-dipped in the bacterial suspension of *Btk* or *E. coli*) according to Dimopoulos et al. (1997). Any mosquito that was severely bled after injection was discarded from the study. Mosquitoes were then allowed to recover and maintained in appropriate cages (16 × 16 × 16 cm each) under the usual standard rearing insectarium conditions. Mosquitoes were then used for biochemical analysis assays 24 h post-inoculation. Five independent replicates in each case (from five different individual mosquitoes) (N = 5) were carried out to perform statistical analysis.

Mosquito larval glutathione activity upon bacterial infection

Glutathione (GSH) assay was carried out on body extracts of *Btk*-infected larvae at 12 and 24 h post-treatment according to Clark et al. (2010) with some modifications. Briefly, at the specified time points of experiment, fifty control or bacterial-infected larvae (with LC_{50} = 0.058 mg/l) were homogenized in 500 ml of phosphate buffer (0.05 M, pH 7.2) containing 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma), 0.5 mM dithiothreitol (DTT; Fluka), 0.8 mM phenylmethylsulphonyl fluoride (PMSF; Sigma) and 1.5% polyvinylpyrolidone (PVP; Sigma). Homogenates were then centrifuged at 16,000 g at 4°C for 30 min. The resulting supernatant was boiled to precipitate and deactivate other proteins, but did not alter GSH levels (Clark et al., 2010). GSH concentrations were then measured by adding 100 µl of boiled supernatant to 400 µl PBS [containing 200 mM MCB and 2 U/ml glutathione S-transferase (per 100 µl)]. GSH concentrations were then determined by measuring the absorbance (OD) of the reaction after 1 min at 340 nm using an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech). GSH standards were measured concurrently to obtain a standard curve that was used to calculate GSH concentrations in samples. Results were expressed as µg GSH/g larval tissue. Five replicates at each time point (five measurements from five different independent treated or control mosquito groups; N = 5) were carried out to perform statistical analysis, and each measure was repeated five times. Statistical comparisons of GSH activities between controls and treatments in each case were performed using Minitab statistical program as detailed below.

Adult mosquito glutathione activity upon bacterial inoculation

Mosquitoes were chilled on ice for 5 min prior to bacterial inoculation. Fifty 6-days old mosquitoes were sham-injected (with APS) or bacterial inoculated with *Btk* or *E. coli* as detailed above. Mosquitoes were left until recovered, and 20 active mosquitoes (have the ability to fly) were used for the experiments. At 12 and 24 h post inoculation, mosquitoes were homogenized and processed for GSH assay in the same manner as larvae (as detailed above). Statistical comparisons of GSH activities between controls and treatments were performed by using Minitab statistical program as detailed below.

Melanization assay in larval and adult stages upon bacterial treatments

The early key steps in the melanin formation in insect haemolymph include the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA or dopamine to dopaquinone and dopaminequinine respectively. Dopaquinone and dopaminequinone thereafter proceed through a series of unstable intermediates such as dopachrome and doaminechrome, which lead to the formation of melanin (Kanost and Gorman, 2008). Monitoring of melanization was carried out in terms of phenoloxidase (PO) activity (Clark et al., 2010), in both larval and adult mosquito at 12 and 24 h post-bacterial treatment in each case according to Clark et al. (2010). Prior to carrying out the melanization assays, larvae were infected with the LC_{50} (0.058 mg/l), and adult mosquitoes were inoculated with *Btk* or *E. coli* as detailed above. Homogenate samples from bacterial-treated or control larvae, or bacterial-inoculated or sham injected adults were prepared in the same method used to measure GSH concentrations as detailed above. An amount of 100 µl of supernatant was diluted in 400 µl PBS, and OD was measured at 470 nm according to An et al. (2009) using an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech). Statistical comparisons of PO activities between controls and treatments in larval or adult experiments were performed by using Minitab statistical program as detailed below.

Statistical analysis

All statistical analyses 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 homogeneity prior to any further analysis. Data pertaining to the titers of GSH and PO in larvae at 12 and 24 h were normally distributed (Anderson Darling test) and thus, a two-sample t-test (for individual comparison) was used for comparing differences between *Btk*-infected and control larvae. Data pertaining to the titers of GSH at 12 h and PO at 12 and 24 h post-treatment in adult mosquitoes were normally distributed (Anderson Darling test) 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 comparisons Tukey's test (Morrison, 2002). The non parametric data (measurements) pertaining to the titer of GSH in adult mosquitoes at 24 h post treatment were log transformed, again tested for normality and variance homogeneity and, as they were then normally distributed, they were analyzed as

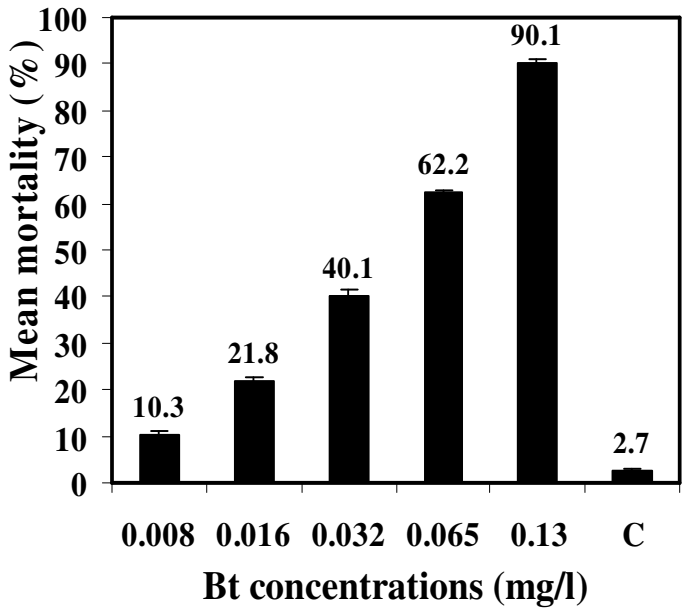

Figure 1. Mean mortalities (%) in the third larval instars of autogenous *Ae. caspius* 24 h post treatment with *B. t. kurstaki* (*Btk*). Larvae were treated with different concentrations of bacterial spore-crystal powder, or left without treatment as control (C). Error bars represent standard error of means of 5 replicates (N = 5) for each concentration. Numbers on bars represent the mean mortality % for each concentration.

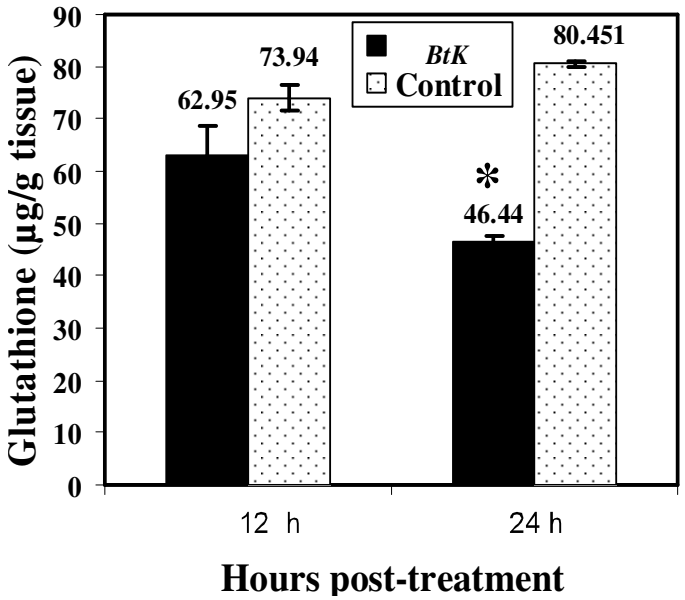

Figure 2. Glutathione (GSH) titer (µg/g larval tissue) in the third larval instars of the autogenous *Ae. caspius* mosquito. Larvae were treated with LC_{50} of *Btk* spore-crystal powder or left without treatment (control). GSH titer was measured at 12 and 24h post-treatment. Error bars represent standard error of means of 5 replicates (N = 5). Asterisk (*) represents significant lower GSH titer comparing to control larvae ($P < 0.05$, two-sample t-test). Numbers on bars represent the amounts of GSH (µg/g larval tissue).

above using ANOVA. Repeated experiments (five replicates in each experiment) were carried out using new groups of mosquitoes.

RESULTS

Mosquito-larvicidal bioassay

This part of study was conducted to determine the LC_{50} of *B. t. kurstaki* (*Btk*) against the 3rd larval instar of the autogenous *A. caspius* mosquito eat 24 h post-treatment. Larvae were treated with wide range of ascending concentrations ranging from 0.004–0.52 mg/l from the spore-crystal powder in a preliminary study. The concentration 0.004 mg/l showed no mortality and concentrations above 0.13 mg/l showed 100% mortality (data not shown). Thus, ascending concentrations range from 0.008-0.13 mg/l was assessed for determining LC_{50} and LC_{90} at 24 h post *Btk* treatment. As shown in Figure 1, the mean larval mortality percentage was increased by increasing concentrations. A linear relationship between concentrations and mean mortality percentages was made *via* a regression plot (not shown) using Minitab statistical program. The resulting linear equation was used to calculate LC_{50} and LC_{90} which were 0.058 and 0.123 mg/l respectively. The LC_{50} (0.058 mg/l) was used for the subsequent experimental purposes of the current study.

Glutathione activity assay in larval mosquito

Third larval instars of the autogenous *A. caspius* mosquito were exposed to LC_{50} (0.058 mg/l) of *Btk* for 24 h. Data from GSH activity assay showed no significant difference in GSH titer between treated and control larvae (62.95 *v* 73.94 µg/g tissue respectively) at 12 h post-treatment ($P > 0.05$, N = 5, student t-test) (Figure 2). While at 24 h, GSH activity was significantly lower in treated larvae (42.3% less) compared to control ones ($P < 0.05$, N = 5, student t-test) (Figure 2). This may indicate that *Btk* infection has significantly decreased GSH activity in the larval stage of the autogenous *A. caspius* at late state of infection.

Glutathione activity assay in adult mosquito

Five-days-old adult autogenous *Ae. caspius* mosquitoes were inoculated with *Btk* or *E. coli* (*Ec*). GSH activity assay showed significant 1.9 folds higher activity in *Btk*-inoculated mosquitoes comparing to control ones at 12h post-inoculation (65.31 *v* 33.03 µg/g tissue) and 2.2 folds higher at 24 h (92.56 *v* 42.463 µg/g tissue) ($P < 0.05$, N = 5, ANOVA) (Figure 3). *Ec*-inoculated mosquitoes showed 1.3 folds higher GSH activity comparing to control ones at 12h (43.64 *v* 33.03 µg/g tissue respectively) and 1.8 folds higher at 24 h post-inoculation (80.021 *v* 42.463 µg/g

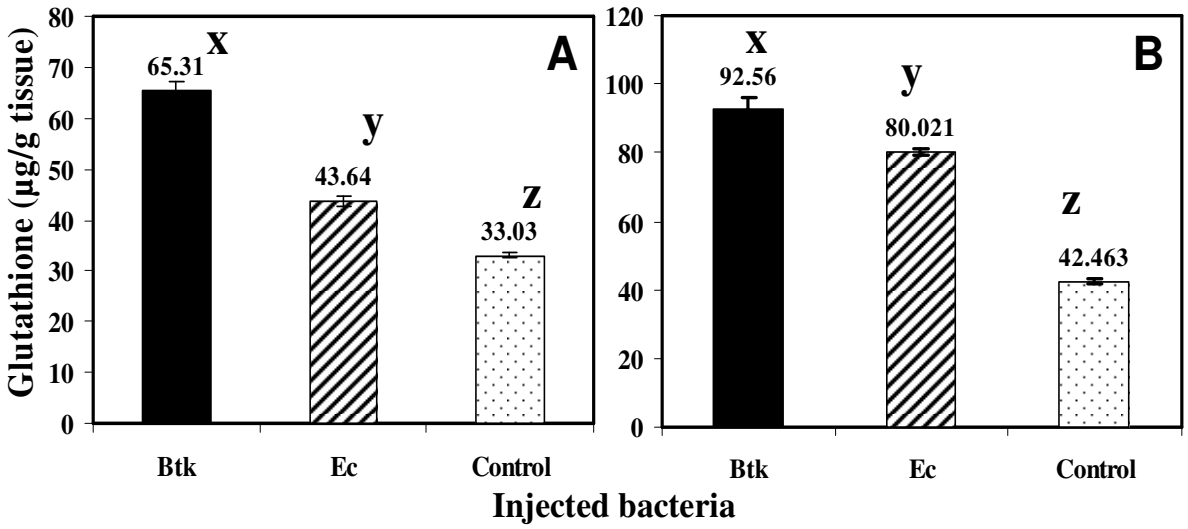

Figure 3. Titer of glutathione (GSH) (µg/g mosquito tissue) in 5-days-old adult *Ae. caspius* mosquito at 12 h (A) and 24 h (B). Mosquitoes were challenged with *B. thuringiensis kurstaki* (Btk) or *E. coli* (Ec), or sham injected with APS (control). GSH titer was measured at 12 and 24 h post-treatment. Error bars represent standard error of means of 5 replicates (N = 5). Within each experiment, different letters above bars (x, y and z) represent significant difference in the amounts of GSH (µg/g mosquito tissue) comparing to control mosquitoes in each case ($P < 0.05$, ANOVA). Numbers on bars represent the amounts of GSH (µg/g larval tissue).

tissue, respectively) ($F_{,2,12} = 167.31$, $P < 0.05$, ANOVA) (Figure 3). Furthermore, ANOVA showed significant 1.5 fold and 1.1 fold higher GSH activity in *Btk*-inoculated mosquitoes compared to *Ec*-inoculated ones at 12 and 24h post-inoculation respectively (65.31 *v* 43.64 and 92.56 *v* 80.02 µg/g tissue respectively) ($F_{,2,12} = 315.71$, $P < 0.05$, ANOVA) (Figure 3). This may indicate that both *Btk*- and *Ec*-inoculation have induced higher GSH activity compared to control ones and that this activity was more pronounced in *Btk*-inoculated mosquitoes.

Melanization assay in larval mosquitoes

Third larval instars of autogenous *A. caspius* were exposed to LC_{50} (0.058 mg/l) of *Btk* for 24h. Data from PO activity assays showed no significant difference in PO titer between treated and control larvae (0.128 *v* 0.131 OD at A_{470}, respectively) at 12 h post-*Btk* infection ($P > 0.05$, N = 5, student t-test) (Figure 4). Data from PO activity assay at 24 h post-*Btk* infection also showed no significant difference in PO titer between treated and control larvae (0.127 *v* 0.125 OD at A_{470}, respectively) ($P > 0.05$, N = 5, student t-test) (Figure 4). This may indicate that *Btk* infection has suppressed the melanization response, or in other words, mosquito larvae were unable to induce melanization against gut-invaded *Btk*.

Melanization assay in adult mosquitoes

Five-days-old adult autogenous *A. caspius* mosquitoes were inoculated with *B. t. kurstaki* (*Btk*) or *E. coli* (*Ec*). PO activity assay showed significant 1.3 folds higher activity in *Ec*-inoculated mosquitoes comparing to control ones at 12 h post-inoculation (0.126 *v* 0.096 OD at A_{470}, respectively) ($F_{,2,12} = 7.34$, $P < 0.05$, ANOVA) (Figure 5). However, ANOVA showed no significant difference between *Btk*-inoculated mosquitoes and control ones (0.116 *v* 0.096 OD at A_{470}, respectively) ($P > 0.05$) (Figure 5). Furthermore, ANOVA showed significant 1.3 folds and 1.26 folds higher in PO activity in *Btk*- and *Ec*-inoculated mosquitoes compared to control ones at 24 h post-inoculation (0.123 and 0.12 *v* 0.095 OD at A_{470}) ($F_{,2,12} = 5.33$, $P < 0.05$, ANOVA) (Figure 5) which may indicate that melanization response was more pronounced against both inoculated bacteria at 24 h post-inoculation. On the other hand, comparing to *B. thuringiensis*. While ANOVA showed no significant difference in PO titer between *Btk*- and *Ec*-inoculated mosquitoes (Figure 5). This in general may indicate that PO activity was highly active against *Ec* compared to *Btk*.

DISCUSSION

As an important vector, mosquitoes transmit a number of human threatening diseases such as malaria, dengue fever and yellow fever. In order to block disease transmission, many studies have been conducted for investigating mosquito immune responses aiming at enhancing their immune capacity that could reduce its vectorial capability *via* killing the pathogen inside its vector before transmission occurs. For this reason, it is

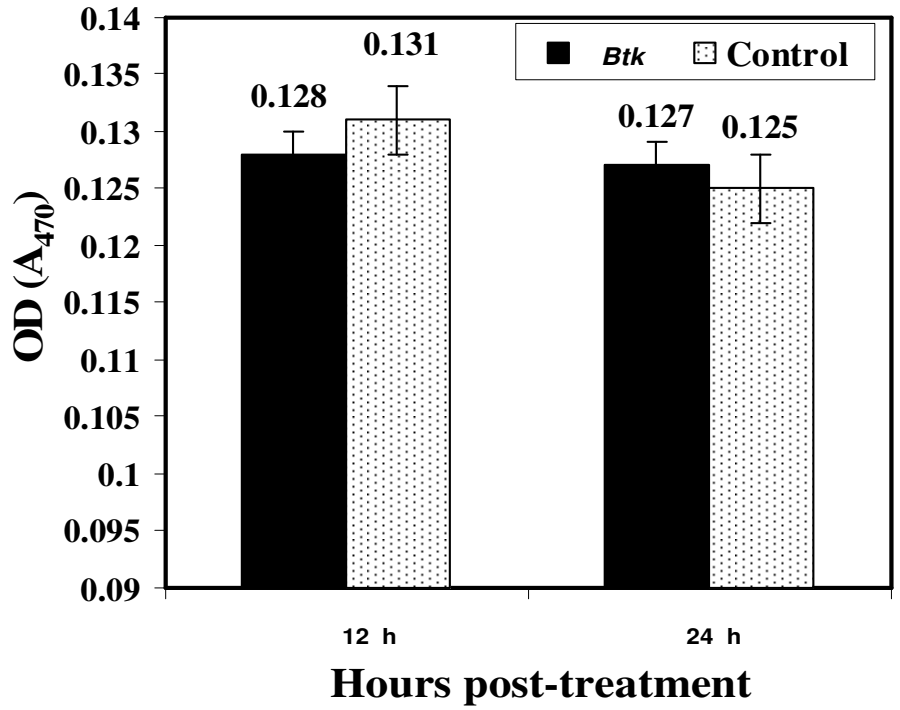

Figure 4. Titer of PO (OD at A_{470}) in the third larval instars of the autogenous *Ae. caspius* at 12 and 24 h post-treatment. Larvae were treated with LC_{50} of *B. t. kurstaki* (*Btk*) or left without treatment (control). PO titer was measured at 12 and 24 h post-treatment. Error bars represent standard errors of means of OD measurements at A_{470} from five different independent assays (N = 5). Numbers on bars represent means of OD measurements at A_{470}.

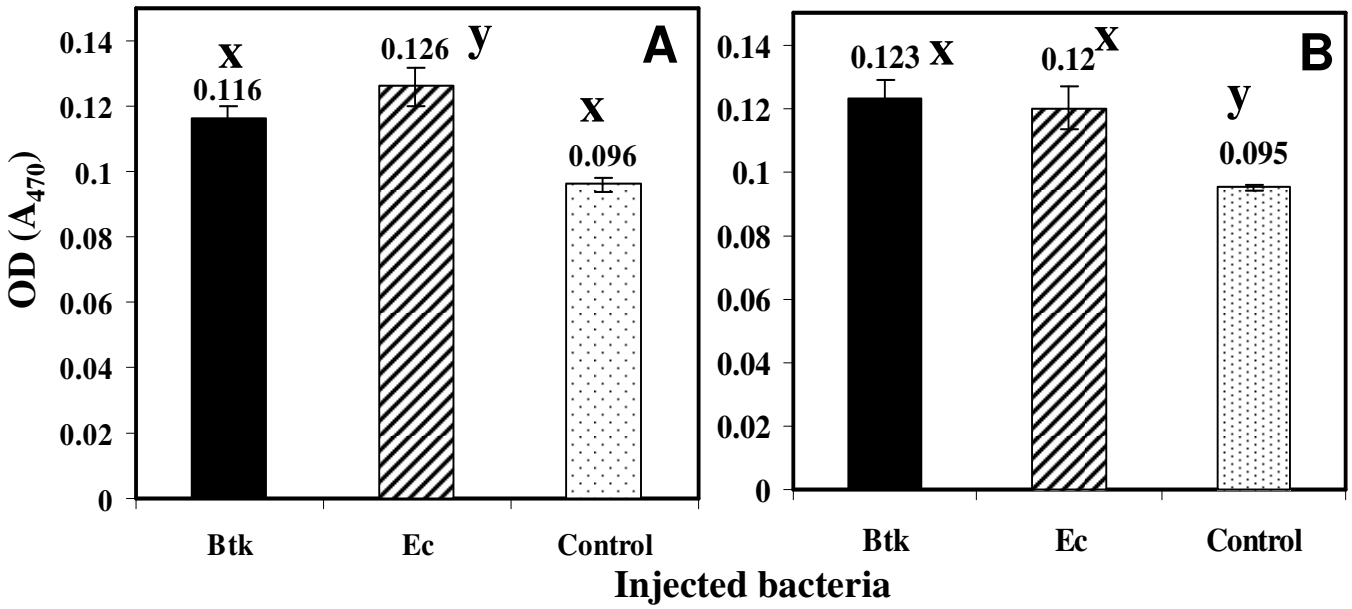

Figure 5. Titer of PO (absorbance (OD) at A_{470}) in adult autogenous *Ae. caspius* mosquitoes at 12h (A) and 24h (B) post-treatment. Five-days old mosquitoes were inoculated with *B. thuringiensis kurstaki* (Btk) or *E. coli* (Ec), or sham injected with APS (control). PO titer was measured at 12h and 24h post-treatment (OD measurements at A_{470}). Error bars represent standard errors of means of OD measurements at A_{470} from five different independent assays (N = 5) in each case. Within each experiment, different letters above bars (x, y & z) represent significant difference comparing to control mosquitoes ($P < 0.05$, ANOVA) in OD measurements at A_{470} in each case. Numbers on bars represent the means of OD measurements at A_{470}.

very important to understand the whole picture of mosquito innate immunity. Thus, it is important to clarify 5 points; 1) a field collected Rift Valley Fever autogenous mosquito vector *Ae. caspius* (Al-Hazmi et al., 2005) from the Eastern region of KSA was targeted by this study, 2) both larval and adult stages have been targeted in this study for comparison, 3) tow complementary harmonic responses, the melanization and anti-oxidative stress responses, in terms of phenoloxidase and glutathione titers respectively, have been carried out in both of mosquito stages 4) a well-known mosquitocidal commercially produced mosquitocidal Gram positive *B. thuringiensis kurstaki* and the Gram-negative non-pathogenic *E. coli* were used for carrying out the immune induction experiments in both mosquito stages for comparison and 5) the time points 12 and 24 h post-treatment were used as they are the times of midgut invasion and osmotic lysis of gut epithelium, respectively by *B. thuringiensis* (Brar et al., 2007).

B. thuringiensis (*Bt*) is a Gram-positive bacterium able to synthesize endotoxin proteins. After ingestion, the crystals produced during sporulation acted at the mid-gut level by rupturing epithelium cells and leading to death (Brar et al., 2007). The strain in this study is highly toxic both to Lepidopteran and Dipteran insect larvae (Tounsi et al., 1999; Tounsi and Jaoua, 2003). Schnepf et al. (1998) and Cinar et al. (2008) mentioned that when susceptible insect larvae ingest *Bt* spore–crystals, the crystal δ-endotoxins are solubilized in the alkaline environment of the midgut and then these protoxins are proteolytically cleaved by midgut proteases into active toxic peptides. The active toxin binds to specific receptors on the surface of midgut cells and is inserted into the membrane to form pores that destroy transmembrane potential, resulting in osmotic lysis of the cells lining the midgut and fatal consequences to the larval mosquito (de Maagd et al., 2001; Brar et al., 2007). The current study showed LC_{50} of 0.058 mg/l against *Ae. caspius* at 24 h post-treatment which was used for infecting mosquito groups of the subsequent experiments.

Melanization is an essential component of the cell-mediated immune response in insects against pathogens that invade the body cavity of the host. In some insects, melanization response represents a type cellular melanization that involves the collaborative interaction of numerous haemocytes to form multicellular structures in which eukaryotic parasites become sequestered (Christensen et al., 2005) or humoral response that occurs against ookinetes on the refractory mosquito midgut, and with melanized filarial worms in Malpighian tubule cells and the thoracic musculature (Reviewed in Beerntsen et al., 2000). Melanization response in mosquetoes against specific bacteria involves melanization in a manner that is different from nodule formation (Hillyer et al., 2003a, b). It is clear that the melanization response is highly specific and elicited only against specific species of bacteria (Hillyer et al., 2003a, b), and parasites (Collins et al., 1986; Beerntsen et al., 2000). Although, Hillyer et al. (2004) investigated that the factors governing phagocytic vs. melanization responses are complex and independent of bacterial Gram type and pathogenicity, I used in the current study *Btk* as a Gram +ve pathogenic bacteria and *Ec* as non-pathogenic Gram –ve one for comparison which showed higher PO activity against *Ec* compared to *Btk* in bacterial-inoculated adult mosquitoes. Moreover, *Btk*-infected larval mosquitoes showed no PO activity against *Btk*. This, in fact, may indicate that *Btk* may have the ability to suppress larval anti-bacterial immune responses as an effective pathogenicity mechanism against susceptible mosquitoes larvae.

Melanization is primarily regulated by phenoloxidase (PO) and involves the cytotoxic melanin in killing pathogens (Chen and Chen, 1995) and parasites (Nappi and Vass, 2001). Infection and other immune challenges activate upstream proteinases in the cascade, which activate prophenoloxidase (pPO) (Kanost and Gorman, 2008) which catalyzes the hydroxylation of monophenols such a tyrosine and the oxidation of o-diphenols like dopamine to form quinones using molecular oxygen (O_2) (Sugumaran, 2002). Further, metabolic processing of quinones results in the formation of cytotoxic melanin (Cerenius and Soderhall, 2004). The melanogenesis-related cytotoxic functions of melanin intermediates are attributed, in part, to their ability to bind covalently to cell-membrane components and other cellular nucleophiles, which promotes free-radical cascades and elevates the levels of superoxide anion ($.O_2^-$), hydrogen peroxide (H_2O_2), some derivatives of nitric oxide (.NO) and others. Melanin cytotoxicity may also involve sulfydryl oxidations, inactivation of DNA polymerase, depolymerization of lipids and lipid peroxidation. Melanogenesis-related cytotoxic reactions in insects have been reviewed (Christensen et al., 2005). Since promotion of free-radical cascades in insect is melanogenesis-related cytotoxic reactions (Nappi and Vass, 2001), mosquitoes adapted a mechanism that protects them from oxidative stressors that are produced during melanization reactions which, in fact, relies heavily on the reduced glutathione (GSH) (reviewed by Christensen et al., 2005). Evidence for this is Nayar and Bradley (1994), Nayar and Knight (1995) who used reduced glutathione as PO inhibitor in mosquitoes. Haemocytes are the main source of PO production upon infection by microorganism (Wang et al., 2011) and likely the source of the GSH detected in plasma too, and capable of recycling oxidized glutathione (GSSG) to GSH (Clark et al., 2010). Further, in the absence of immune challenge, preventing activated proteases, reactive intermediates, and cytotoxic quinones from damaging insect tises (Lu and Jiang, 2007) that takes place *via* glutathione, a tripeptide thiol found in virtually all metazoans, which is well known in mammals to protect cells from the destructive effects of reactive oxygen intermediates and free radicals (Meister and Anderson, 1983).

Based on what mentioned above, glutathione could be an immune inhibitor (Nayar and Knight, 1995). Melanization in insect does not begin until endogenous GSH levels fall below 20 mM (Clark, et al., 2010). Thus high titer of glutathione in *Btk*-inoculated adult mosquitoes in this study may indicate inhibition of the immune response which may explain the high pathogenecity of *Btk* to mosquitoes. However, lower titer of GSH in *Btk*-infected larvae may indicate, in one hand, the induction of immune response of larvae against *Btk* bacteria but, on the other hand, increasing the oxidative stress of free radicals (in the lower titer of GSH) that may have resulted in cytotoxicity beside the cytopathological effect caused to the midgut epithelium (Ahmed et al., 2010), and finally, septicemia caused by the endotoxins of *Btk.* Evidence of this is Clark et al. (2010) who investigated a depletion of endogenous GSH correlated with increased melanization. This could be the case here in *Btk* challenging-induced immune response in the autogenous *Ae. caspius.*

Based on these studies mentioned above, the higher GSH activities investigated in both *Btk*- and *Ec*-inoculated mosquitoes compared to control ones of the current study may indicate higher oxidative stress as a result of bacterial inoculation. This bacterial-induced oxidative stress which may be attributed to induction of a cytotoxic humoral immune response(s) based on previous findings (Ahmed et al., 2008, 2010). Moreover, higher titer of glutathione in untreated larvae could be attributed to the environmental stress due to pollutants, xenobiotics or to the oxidative stress (Lipke and Chalkley, 1962; Reisen et al., 1986; Jovanovic-Galovic et al., 2004) that may exist in its natural aquatic breeding environment. Evidence for this is Poupardina, et al. (2008) who showed expression of detoxification genes as a result of environmental xenobiotics and insecticides in the mosquito *Aedes aegypti* that increases larval tolerance to chemical insecticides. However, lower titer of GSH in *Btk*-treated larvae may be attributed to two factors; 1) that *Btk* inhibited induction of the anti-stress factor, the GSH, as an effective larvicidal toxic mechanism, and hence, larvae died within 24 h post-treatment or 2) *Btk* inhibited the larval immune response as another pathogenicity mechanism. Evidence for this is Shrestha et al. (2010) who identified two novel immune inhibition compounds from the entomopathogenic bacterium, *Xenorhabdus nematophila*. Therefore, these authors demonstrated that these compounds inhibit the mediator of insect immune responses, the eicosanoids, in the diamondback moth, *Plutella xylostella*. They also demonstrated that these compounds have increased the pathogenicity of *B. thuringiensis* against larvae of the moth.

In conclusion; given the increasing evidence for roles of storage proteins in adult insects (Attardo et al., 2005), in the autogenous *Ae. caspius* targeted by the current study, storage proteins in larval stage (the main source of autogeny in adult stage) could serve as an amino acid reserve for oogenesis (Su and Mulla, 1997; Wheeler and Buck, 1996; Telang and Wells, 2004), as well as for anti-stress defence in terms of antioxidant reactions involving GSH. This could explain the high GSH titer in un-treated (control) larval stage comparing to control adult stage. Moreover, the aquatic environment (the breading site of larvae) is a main source of contamination with different pollutants stresses, and hence, GSH production is one of the main mechanisms that allow larval stage to survive in this contaminated environment (Poupardina et al., 2008). Evidence for this is in case of dengue viral infection as the expression of GST was reported to be upregulated in response to viral infection in mosquito cells (Lin et al., 2007) as an antioxidant defense mechanism (Chen et al., 2011). Findings of the current study form a base for further studies on the other anti-free radicals responses in mosquitoes aiming at enhancing the vector immune responses for fighting against mosquito-borne pathogens and/or enhancing the pathogenicity of the biological agents used in biocontrol measures.

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REFERENCES

Ahmed AM, Taylor P, Maingon R, Hurd H. (1999). The effect of Plasmodium yoelii nigeriensis on the reproductive fitness of *Anopheles gambiae*. Invertebr. Reprod. Dev., 36(1-3): 217-222.

Ahmed AM, Al-Olayan EM, Amoudy MA (2008). Enhancing the humoral and melanization responses of *Aedes aegypti* mosquito: a step towards the utilization of immune system against dengue fever. J. Entomol., 5(5): 305-321.

Ahmed AM, Al-Olayan EM, Aboul-Soud MAM, Al- Khedhairy AA (2010). The immune enhancer, thymoquinone, and the hope of utilizing the immune system of *Aedes caspius* against disease agents. Afr. J. Biotechnol., 9(21): 3183-3195.

Ahmed AM, Shaalan EA, Aboul-Soud MAM, Al-Khedhairy AA (2011). Mosquito vectors survey in AL-Ahsaa district, eastern region, Kingdom of Saudi Arabia. J. Insect Sci., (In Press).

Al-Hazmi A, Al-Rajhi AA, Abboud EB (2005). Ocular Complications of Rift Valley Fever Outbreak in Saudi Arabia, Ophthalmology, 112: 313-318.

An CJ, Ishibashi J, Ragan EJ, Jiang HB, Kanost MR (2009). Functions of *Manduca sexta* hemolymph proteinases HP6 and HP8 in two innate immune pathways. J. Biol. Chem., 284: 19716-19726.

Ashida M, Brey PT (1997). Recent advances in research on the insect prophenoloxidase cascade. In: Brey PT, Hultmark D. (Eds.), Molecular Mechanisms of Immune Responses in Insects. Chapman and Hall, London, pp. 135-172.

Attardo GM, Hansen IA, Raikhe AS (2005). Nutritional regulation of vitellogenesis in mosquitoes: Implications for anautogeny. Insect Biochem. Mol. Biol., 35: 661-675.

Beerntsen BT (2000). Genetics of mosquito vector competence. Microbiol. Mol. Biol. Rev., 64: 115-137.

Boman HG (1998). Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. Scandinavian J. Immunol., 48: 15-25.
Brar SK, Verma MRD, Surampalli RY (2007). *Bacillus thurengiensis* proteases: Production and role in growth, sporulation and synergism. Process Biochem., 42: 773-790.
Cerenius L, Soderhall K (2004). The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198: 116-126.
Chen CC, Chen CS (1995). *Brugia pahangi*: Effects of melanization on the uptake of nutrients by microfilariae *in vitro*. Exp. Parasitol., 81(1): 72-78.
Christensen BM, Li J, Chen CC, Nappi AJ (2005). Melanization immune responses in mosquito vectors. Trends Parasitol., 21(4): 193-199.
Cinar C, Apaydin O, Yenidunya A, Harsa S, Gunes H (2008). Isolation and characterization of *Bacillus thurengiensis* strains from olive-related habitats in Turkey. J. Appl. Microbiol., 104: 515-525.
Chen TH, Tang P, Yang CF, Kao LH, Lo YP (2011). Antioxidant defense is one of the mechanisms by which mosquito cells survive dengue 2 viral infection. Virology, 410: 410-417.
Clark AG, Shamaan NA, Dauterman WC, Hayaoka T (1984). Characterization of multiple glutathione transferases from the house fly *Musca domestica* (L). Pestic. Biochem. Physiol., 22: 51-59.
Clark AG, Dick GL, Martindale SM, Smith JN (1985). Glutathione S-transferases from the New Zealand grass grub, *Costelytra zealandica*. Their isolation and characterization and the effect on their activity of endogenous factors. Insect Biochem., 15: 35-44.
Clark KD, Lu Z, Strand MR (2010). Regulation of melanization by glutathione in the moth *Pseudoplusia includens*. Insect Biochem. Mol. Biol., 40: 460-467.
Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, Collins WE, Campbell CC, Gwadz RW (1986). Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. Science, 234: 607-610.
Da Silva CC, Dunphy GB, Rau ME (2000). Interaction of *Xenorhabdus nematophilus* (Enterobacteriaceae) with anti-microbial defenses of the house cricket, *Acheta domesticus*. J. Invert. Pathol., 76: 285-292.
De Maagd RA, Bravo A, Crickmore N (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet., 17: 193-199.
Dimopoulos G, Richman A, Muller HM, Kafatos FC (1997). Molecular immune responses of the mosquito Anopheles gambiae to bacteria and malaria parasites. Proc. Natl. Acad Sci. USA., 94: 11508-11513.
Dunphy G, Morton D, Kropinski A, Chadwick J (1986). Pathogenicity of lipopolysaccharide mutants of *Pseudomonas aeruginosa* for larvae of *Galleria mellonella*: bacterial properties associated with virulence. J. Invert. Pathol., 47: 48-55.
Feyereisen R (2005). Insect cytochrome P450. In: Gilbert LI, Iatrou K, Gill S (Eds.), Comprehensive Mol. Insect Sci., pp. 1-77.
Fiolka MJ, Ptaszynska AA, Czarniawski A (2005). Antibacterial and antifungal lysozyme-type activity in *Cameraria ohridella* pupae. J. Invertebrate Pathol., 90: 1-9.
Fournier D, Bride JM, Poirie M, Berge JB, Plapp FW (1992). Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. J. Biol. Chem., 267: 1840-1845.
Gillespie JP, Kanost MR, Trenczek T (1997). Biological mediators of insect immunity. Annu. Rev. Entomol., 42: 611-643.
Grant DF, Dietze EC, Hammock BD (1991). Glutathione S-transferase isozymes in Aedes aegypti: Purification, characterization, and isozyme-specific regulation. Insect Biochem., 21: 421-433.
Hemingway J, Hawkes NJ, McCarroll L, Ranson H (2004). The molecular basis of insecticide resistance in mosquitoes. Insect Biochem., Mol. Biol., 34: 653-665.
Hillyer JF (2003a). Rapid phagocytosis and melanization of bacteria and *Plasmodium* sporozoites by hemocytes of the mosquito *Aedes aegypti*. J. Parasitol., 89: 62-69.
Hillyer JF (2003b). Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbautus* following immune challenge by bacteria. Cell Tissue Res., 313: 117-127.
Hillyer JF, Schmidt SL, Christ BM (2004). The antibacterial innate immune response by the mosquito *Aedes aegypti* is mediated by hemocytes and independent of Gram type and pathogenicity. Microbes Infect., 6: 448-459.
Imler JL, Bulet P (2005). Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chem. Immunol. Allergy, 86: 1-21.
Irving P, Ubeda J, Doucet D, Troxler L, Lagueux M, Zachary D, Hoffmann J, Hetru C, Meister M (2005). New insights into *Drosophila* larval haemocyte functions through genome wide analysis. Cell. Microbiol., 7: 335-350.
Jovanovic-Galovic A, Blagojevic DP, Grubor-Lajsic G, Worland R, Spasic MB (2004). Role of antioxidant defense during different stages of pre-adult lifecycle in European cornborer (*Ostrinia nubilalis*, Hubn.): diapause and metamorphosis. Arch. Insect Biochem. Physiol., 55: 79-89.
Kanost MR, Gorman M (2008). Phenoloxidases in insect immunity. In: Beckage NE (Ed.), Insect immunity. Academic Press, San Diego, pp. 69-96.
Kavanagh K, Reeves EP (2004). Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev., 28: 101-112.
Kumar S (2003). The role of reactive oxygen species on *Plasmodium melanotic* encapsulation in *Anopheles gambiae*. Proc. Natl. Acad. Sci. USA., 100: 14139-14144.
Lin CC, Yang CF, Tu CH, Huang CG, Shih YT, Chuang CK, Chen WJ (2007). A novel tetraspanin C189 upregulated in C6/36 mosquito cells following dengue 2 virus infection. Virus Res., 124: 176-183.
Lipke H, Chalkley J (1962). Glutathione, oxidized and reduced, in some dipterans treated with 1,1,1-trichloro-2,2-di-(p-chlorophenyl)ethane. Biochem. J., 85: 104-109.
Lu Z, Jiang H (2007). Regulation of phenoloxidase activity by high-and low-molecular-weight inhibitors from the larval hemolymph of *Manduca sexta*. Insect Biochem. Mol. Biol., 37: 478e485.
Mattingly PF, Knight KL (1956). The mosquito of Arabia. I. Bull. Brit. Mus. Nat. Hist. (Ent.)., 4(3): 89-141.
Meister A, Anderson ME (1983). Glutathione. Annu. Rev. Biochem., 52: 711-760.
Morrison DA (2002). How to improve statistical analysis in parasitology research publications, Int. J. Parasitol., 32: 1065-1070.
Morton D, Dunphy G, Chadwick J (1987). Reactions of haemocytes of immune and non-immune *Galleria mellonella* larvae to *Proteus mirabilis*. Develop. Comparat. Immunol., 11: 47-55.
Nappi AJ, Vass E (2001). Cytotoxic reactions associated with insect immunity. In Phylogenetic Perspectives on the Vertebrate Immune System (Cooper EL, ed.), Kluwer Academic-Plemum, pp. 329-348.
Nayar JK, Bradley TJ (1994). Comparative study of hemolymph phenoloxidase activity in *Aedes aegypti* and *Anopheles quadrimaculatus* and its role in encapsulation of *Brugia malayi* microfilariae. Come. Biochm. Physiol., 109A(4): 929-938.
Nayar JK, Knight JW (1995). Wounding increases intracellular encapsulation (melanization) of developing *Brugia malayi* (Nematoda: Filarioidea) larvae in thoracic muscles of *Anopheles quadrimaculatus*. Comp. Biochem. Physiol., 112A(314): 553-557.
Nimmo DD, Ham PJ, Ward RD, Maingon R (1997). The sand fly *Lutzomyia longipalpis* shows specific humoral responses to bacterial challenge. Med. Vet. Entomol., 11: 324-328.
Poupardina R, Reynauda S, Strodeb C (2008). Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Aedes aegypti*: Impact on larval tolerance to chemical insecticides. Insect Biochem. Mol. Biol., 38: 540-551.
Prapanthadara L, Hemingway J, Ketterman AJ (1993). Partial purification and characterization of glutathione S-transferases involved in DDT resistance from the mosquito *Anopheles gambiae*. Pestic. Biochem. Physiol., 47: 119-133.
Prapanthadara L, Promtet, N, Koottathep S, Somboon P, Ketterman AJ (1995). Isoenzymes of glutathione S-transferase from the mosquito *Anopheles dirus* species B: the purification, partial characterization and interaction with various insecticides. Insect Biochem. Mol. Biol., 30: 395-403.
Reisen WK, Milby MM, Bock ME (1986). The effects of immature stress on selected events in the life history of *Culex tarsalis*. Mosq. News. 44: 385-395.
Rey D, Pautou MP, Meyran JC (1999). Histopathological effects of tannic acid on the midgut epithelium of some aquatic Diptera larvae.

J. Invertebrate Pathol., 73: 173-181.
Salzet M (2001). Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. TRENDS Immunol., 22: 285-288.
Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998). *Bacillus thurengiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev., 62: 775-806.
Shrestha S, Hong YP, Kim Y (2010). Two chemical derivatives of bacterial metabolites suppress cellular immune responses and enhance pathogenicity of *Bacillus thuringiensis* against the diamondback moth, *Plutella xylostella*. J. Asia-Pacific Entomol., 13: 55-60.
Söderhäll K, Cerenius L (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol., 10: 23-28.
Su T, Mulla MS (1997). Physiological aspects of autogeny in *Culex tarsalis* (Diptera: Culicidae): influences of sugar-feeding, mating, body weight, and wing length. J. Vector Ecol., 22: 115-121.
Sugumaran M, Nellaiappan K, Amaratunga C, Cardinale ST (2000). Insect melanogenesis. III. Metabolon formation in the melanogenic pathway regulation of phenoloxidase activity by endogenous dopachrome isomerase (decarboxylating) from *Manduca sexta*. Arch. Biochem. Biophys. 378: 393-403.
Telang A Wells MA (2004). The effect of larval and adult nutrition on successful autogenous egg production by a mosquito. J. Insect Physiol., 50: 677-685.
Toung YPS, Hsieh TS, Tu CPD (1990). *Drosophila* glutathione S-transferase 1-1 shares a region of sequence homology with the maize glutathione S-transferase III. Proc. Natl. Acad. Sci. USA., 87: 31-35.
Tounsi S, Jaoua S (2003). Characterisation of a novel cry2Aa-type gene from *Bacillus thuringiensis* subsp. *kurstaki*. Biotechnol. Lett., 25: 1219-1223.
Tounsi S, J'Mal A, Zouari N, Jaoua S (1999). Cloning and nucleotide sequence of a novel cry1Aa-type gene from *Bacillus thuringiensis* subsp. *kurstaki*. Biotechnol. Lett., 21: 771-775.
Vilmos P, Kurucz E (1998). Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol. Lett., 62: 59-66.
Wang Z, Lu A, Li X (2011). A systematic study on hemocyte identification and plasma prophenoloxidase from *Culex pipiens quinquefasciatus* at different developmental stages. Exp. Parasitol., 127: 135-141.
Wheeler DE, Buck NA (1996). A role for storage proteins in autogenous reproduction in *Aedes atropalpus*. J. Insect Physiol., 42(10): 961-966.