Pakistan J. Zool., vol. 44 (2), pp. 525-536, 2012.

Lipid Peroxidation and Oxidative Protein Products as Biomarkers of Oxidative Stress in the Autogenous Mosquito, *Aedes caspius*, Upon Infection with the Mosquitocidal Bacterium, *Bacillus thuringiensis kurstaki*

Ashraf M. Ahmed[1,2]
[1]*Zoology Department, College of Science, King Saud University, PO. Box 2455, Riyadh 11451, Saudi Arabia*
[2]*Zoology Department, Faculty of Science, Minia University, El-Minia, Egypt*

Abstract.- It has recently been shown that *Bacillus thuringiensis kurstaki* (*Btk*) suppressed both immune and antioxidant responses in the autogenous mosquito, *Aedes caspius*. The purpose of the presented study herein was to investigate the concomitant oxidative stresses upon infection with *Btk*. Data showed elevation in the level of both lipid peroxidation and protein oxidation, as important oxidative stress biomarkers, in *Btk*-infected *Ae. caspius* mosquitoes. In larval mosquitoes, *Btk*-infection induced significant higher levels of both lipid peroxidation and protein oxidation at 12 and 24h post-infection compared to those of control ones. In adult mosquitoes, *Btk*-inoculation has induced significant higher lipid peroxidation at 12 and 24h post-inoculation compared to control ones, while *E. coli*-inoculation induced significant higher lipid peroxidation at 12h post-inoculation only compared to control ones. However, significant high protein oxidation was shown only at 12h post-*Btk* inoculation but not in *E. coli*-inoculated mosquitoes compared to control ones in each case. These data may indicate that oxidative stress was more pronounced in *Btk*-inoculated adult mosquitoes than that in *E. coli*-inoculated ones. Moreover, *Btk*-induced oxidative stress was more pronounced in larvae than that in adult mosquitoes. Consequently, these findings suggest that the oxidative stress-induced cellular damage in mosquito larvae may be considered as an important pathogenicity mechanism of *Btk* against host mosquito larvae.

Keywords: Lipid peroxidation, oxidative protein products, *Bacillus thuringiensis*, *Aedes caspius*, oxidative stress.

INTRODUCTION

Insects live in all environments everywhere on the globe. Thus they are exposed to different challenging factors such as pathogens and contaminants. Humoral defense against pathogens is one of the crucial immune responses that refer to antibacterial proteins and other immune-related molecules generated by the fat body and/or haemocytes (Kavanagh and Reeves, 2004). These molecules are released into haemolymph to immobilize and kill invading microorganisms or parasites (Vilmos and Kurucz, 1998; Morton *et al.*, 1987) which involves the synthesis of a range of anti-microbial peptides (Boman, 1998; Imler and Bulet, 2005). Cellular immune responses refer to responses such as phagocytosis nodule formation and encapsulation which are directly carried out by haemocytes (Gillespie *et al.*, 1997, Irving *et al.*, 2005; Wang *et al.*, 2011).

Although reactive oxygen species (ROS) are used by insect as cytotoxic materials against invading pathogens and parasites (Fang, 1999; Peterson and Luckhart, 2006), they also cause oxidative stresses in insect itself, leading to cellular damage (Fang, 1999). This is because ROS are radicals that can cause cellular toxicity like lipid peroxidation which disrupts membrane fluidity and the degradation products can initiate cellular apoptosis (Halliwell and Gutteridge, 1999; Kannan and Jain, 2000). Oxidative damage to proteins is another oxidative stress biomarker that can range

* Corresponding author: amahmedkeele@yahoo.co.uk - aalii@ksu.edu.sa - a.ahmed@biol.keele.ac.uk
0030-9923/2012/0002-0525 $ 8.00/0

Abbreviations: AOPP, advanced oxidative protein products; APS, *Aedes* physiological saline; *Btk, Bacillus thuringiensis kurstaki*; DENV-2, dengue virus; *Ec, Escherichia coli*; GPx, glutathione peroxidase; GSH, glutathione reduced form; GST, glutathione S transferase; LP, lipid peroxidation; LPS, lipopolysaccharide; MDA, malondialdehyde, NO, nitric oxide; PHGPx: phospholipid hydroperoxide glutathione peroxidases; ROS, reactive oxygen species; SOD, superoxide dismutase; Prxs: peroxiredoxins.

from specific amino acid modifications and fragmentation of the peptide chain to total enzyme inactivation by superoxide anions (Stadtman, 1986). ROS can also lead to DNA damage *via* deletions, mutations, base degradation, single strand breakage and cross-linkage of proteins (Imlay and Linn, 1988; Imlay, 2003). Superoxide radicals generated by oxidative stress act as oxidants or reductants that lead to the production of hydroxyl radicals (Fridovich, 1995). Therefore, host protection against oxidative stress is vital for homeostasis and hence survival.

Antioxidant defense refers to producing particular antioxidents that keep the balance between oxidants and antioxidants levels. Insects express a suite of antioxidant enzymes that may form a concatenated response to an onslaught of dietary and endogenously produced oxidants leading to oxidative stress (Felton and Summer, 1995; Hyršl *et al.*, 2007). These antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GSTs) and ascorbate peroxidase (APOX) (Ahmad, 1995; Halliwell and Gutteridge, 1999; Radyuk *et al.*, 2003). Transferrine and glutathione (GSH) are important antioxidants that help keeping this oxidative/antioxidative balance. The former, is an antioxidant that controls the level of free iron (Yoshiga *et al.*, 1997) and has been shown to be upregulated upon microbial infection (Valles and Pereira, 2005; Kim *et al.*, 2008). Hence, it has been suggested that a possible important role of insect transferrin could be in forming a general part of the insect immune response and functioning as an antibiotic against pathogens. The later is a tripeptide thiol found in virtually all metazoans, mediates reaction catalyzed by GST (Meister and Anderson, 1983) as one of the important antioxidative mechanisms that allow insects to survive in a contaminated environment (Poupardina, *et al.*, 2008) and insecticide-resistance (Prapanthadara *et al.*, 1993). Beside playing a central role in the metabolism of insecticides and other xenobiotics (Feyereisen, 2005; Hemingway *et al.*, 2004), GSH 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 a previous study, the antioxidative response, in terms of high GSH titer and its interaction with melanization immune response, in terms of phenoloxidase (PO) titer, have been investigated in mosquitoes upon infection with *B. thurengiensis kurstaki* (Ahmed, 2011). In this study, infection of mosquitoes with *B. thuringiensis kurstaki* (*Btk*) has been shown to induce suppression in antibacterial immune response in terms of low PO titer and antioxidant defense in terms of low GSH titer. Thus, it would be interesting to support these finding by investigating oxidative stress biomarkers in *Btk*-infected mosquitoes. Thus, in the study proposed herein, lipid peroxidation and protein oxidation will be investigated as important biomarkers of oxidative stress-induced pathogenicity of *Btk*-infection in the autogenous mosquito, *Ae. caspius*.

MATERIALS AND METHODS

Mosquito rearing

An autogenous *Ae. caspius* mosquitoes were reared under standard insectarium conditions (26ºC, 12h/12h light/dark period and 80–82% humidity). Larvae were reared in tap water (or distilled water for experimental purposes) in the insectary of Zoology Department, College of Science, King Saud University, as previously detailed in Ahmed *et al.* (1999). Adults emerging within a 24h period were maintained in rearing cages (30 × 30 × 30 cm each) with continuous access to a 10% glucose solution (w/v). 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 (Clements, 1992).

Experimental bacteria

Mosquito-larvicidal bacterium, *Bacillus 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)

and used based on the company's instructions]. For adult mosquito 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 37ºC for 48 h in a rotary shaker at 200 rpm until a spectrophotometrical optical density OD_{598} of 0.5-0.7 is reached (Nimmo *et al.,* 1997) using UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech), then used for inoculating mosquitoes according to Nimmo *et al.* (1997) as briefed below.

Infecting larvae with Btk

A rearing tray of one liter distilled water containing 100 3rd instar larvae of *Ae. caspius* was left without treatment (control) or treated with LC_{50} (0.058mg/l) (Ahmed, 2011) of spore-crystal powders of *Bacillus thuringiensis kurstaki* (*Btk*) (serotype H-3a and 3b, strain Z-52, Biotech International Ltd, India) based on the company's instructions. Larvae were allowed access to food (ground Goldfish Flake Food, Wardley®, USA: www.wordley.com) throughout the experiments. Alive larvae were used for estimating lipid peroxidation or advanced oxidative protein products at 12 and 24h post-treatment as detailed below.

Mosquito inoculation with bacteria

Bacterial suspensions of *Btk* or *E. coli* (OD_{598} of 0.5-0.7) were prepared as detailed above. Fifty 6-days old adult mosquitoes were immobilized, by chilling on ice for 5 min, prior to inoculation (piercing) with a hand made glass capillary needle. Ice-chilled mosquitoes were pierced through a lateral side of the thoracic cavity, deeply enough to penetrate the layer of fat body beneath the epidermis, but at an angle oblique enough to avoid piercing the gut. Piercing needle was pre-dipped in *Aedes* physiological saline (APS) (13 mM NaCl, 0.5 mM KCl, 0.1 mM $CaCl_2$) as trauma control, *Btk* or *E. coli* bacteria according to Dimopoulos *et al.* (1997) and Ahmed (2011). Any mosquito that was severely bled after inoculation 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 assays at 12 and 24h post-inoculation. Only active mosquitoes (able to fly) were used for experimental purposes. Five independent replicates (from five different individual mosquitoes) (N = 5) were carried out in each experiment to perform statistical analysis.

Determination of lipid peroxidation (LP)

Endogenous lipid peroxidation in mosquito homogenate was estimated spectrophotometrically following the method described by Okhawa *et al.* (1979), with some modifications, expressed in a nano-moles of malondialdehyde (MDA) per milliliter mosquito homogenate (nmole/ml). Fifty control or bacterial-infected 3rd instar larvae (with LC_{50} = 0.058mg/l), or fifty bacterial-inoculated 6-days old mosquitoes (with *Btk*, *E. coli* or APS) were homogenized in 500 ml of phosphate buffer (0.05 M, pH 7.2) containing 2mM ethylenediaminetetra-acetic acid (EDTA; Sigma), 0.5mM dithiothreitol (DTT; Fluka), 0.8mM phenylmethylsulphonyl fluoride (PMSF; Sigma) and 1.5% polyvinyl-pyrolidone (PVP; Sigma). Homogenates (from larvae or adult mosquitoes) were then centrifuged at 16,000g at 4ºC for 30 min. An amount of 0.5 ml of the resulting supernatant was shaken with 2.5 ml of 20% trichloroacetic acid (TCA). To the resulting the mixture, 1 ml of 0.67% thiobarbituric acid (TBA) was added, shaken, and wormed for 30 min in a boiling water bath and followed by immediate rapid cooling in ice for 5 min. After cooling, 4 ml of n-butyl-alcohol was added and shaken well. The resulting mixture was then centrifuged at 16,000g for 5 min. The resultant n-butyl-alcohol layer was moved into a separate tube and MDA content was determined spectrophotometrically at 535 nm using an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech).

Determination of advanced oxidative protein products (AOPP)

Protein oxidation was measured in homogenates (of larvae or adult mosquitoes) that prepared at 12 or 24h post-treatment by determining the AOPP levels spectrophotometrically (Rugale *et al.,* 2007; Aiassa *et al.,* 2007). One milliliter of homogenate (diluted 1/5 in PBS) was analyzed with

0.1 ml of glacial acetic acid and 50 µl of 1.16 M potassium iodide. One milliliter of 0–100 µM chloramine-T was used as standard (Correa Salde and Albesa, 2009). The absorbance of the reaction mixture was read at 340 nm using an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech) to calculate the chloramine-T equivalents protein products in µM/ml.

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 test) and for variances homogeneity prior to any further analysis. Data pertaining to the LP and AOPP were normally distributed (Anderson Darling test) and thus, a two-sample t-test (for individual comparison) was used for comparing differences between treated and control insect groups in each case (Morrison, 2002). Five replicates (five different mosquito groups in each case: N = 5) were carried out for better statistical analysis.

RESULTS

Measuring oxidative stress is not straightforward, and standardized methods are still lacking. The current study investigates the possibility that oxidants could induce stress that may result in damage to cellular lipid and protein molecules in *Btk*-infected mosquitoes. Thus, the current study focused mainly on lipid peroxidation and protein oxidation as important biomarkers of cellular collapse upon *Btk* infection in mosquito larvae and adults. This is based on modifying the characteristics of proteins and lipids which can be fractionated by size exclusion spectrophotometrically at 340 and 535nm, respectively.

LP in larval mosquitoes

Third instar larvae of the autogenous *Ae. caspius* mosquito were exposed to LC_{50} (0.058 mg/l) of *Btk* for 24h. Data from LP assay showed a significant higher MDA concentration in the homogenate of *Btk*-infected larvae compared to that of control ones at 12h post-treatment (3.667±0.435 *v* 1.66±0.153 nmole/ml respectively) ($P < 0.05$, N = 5, student t-test) (Fig. 1). Moreover, MDA concentration was still significantly higher in *Btk*-infected larvae at 24h post-infection compared to that of control ones (2.92 ± 0.374 *v* 1.533 ± 0.17 nmole/ml respectively) ($P < 0.05$, N = 5, student t-test) (Fig. 1).

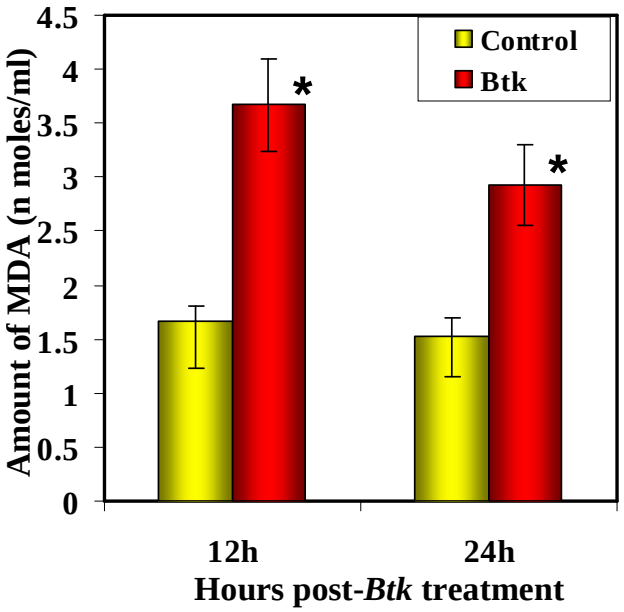

Fig. 1. Malondialdehyde (MDA) titer (nmole/ml) in third larval instars of the autogenous *Ae. caspius* mosquito. Larvae were treated with LC_{50} (0.058 mg/l) of *B. thuringiensis* (*Btk*) spore-crystal powder or left without treatment (control). MDA titer was measured spectrophotometrically (at 535 nm) at 12 and 24h post-*Btk* infection. Error bars represent standard errors of means of 5 replicates (N = 5). Asterisk (*) represents significantly higher MDA level compared with control larvae ($P < 0.05$, two-sample t-test).

LP in adult mosquitoes

Six-days old autogenous *Ae. caspius* mosquito were inoculated with *B. thuringiensis* (*Btk*), *E. coli* (Ec) or APS (control) prior to LP bioassay in terms of Malondialdehyde (MDA) titer. Data from LP assay showed significant higher MDA concentration in the homogenate of mosquitoes at 12h post-inoculation with *Btk* bacteria compared to that of control ones (18.33 ± 2.41 *v* 4.067 ± 0.287 nmole/ml respectively) ($P < 0.05$, N = 5, student t-test) (Fig. 2). Moreover, MDA concentration was

significantly higher at 12h post-inoculation with *E. coli* compared to that of control ones (21.67 ±2.56 *v* 4.067 ± 0.287 nmole/ml, respectively) ($P < 0.05$, N = 5, student t-test) (Fig. 2). This may indicate that *Btk* and *E. coli* inoculation has significantly increased lipid peroxidation in adult autogenous *Ae. caspius* mosquitoes.

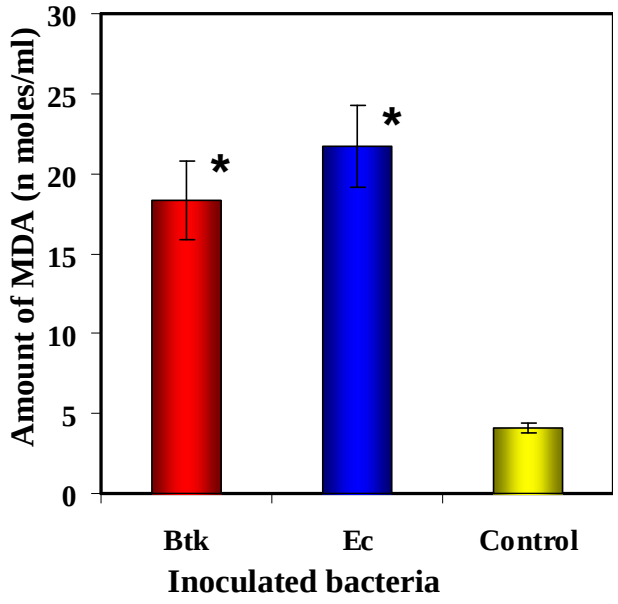

Fig. 2. Malondialdehyde (MDA) titer (nmole/ml) in 6-days old autogenous *Ae. caspius* mosquitoes at 12h post-inoculation with *B thuringiensis* (Btk), *E. coli* (Ec) or APS (control). In each case, MDA concentration was measured spectrophotometrically (at 535 nm) at 12h post-treatment. Error bars represent standard errors of means of 5 replicates (N = 5). Asterisk (*) represents significant higher MDA level comparing to control mosquitoes ($P < 0.05$, two-sample t-test).

For investigating long-term oxidative stress upon inoculation with *Btk* or *E. coli*, MDA levels were measured at 24h post-inoculation with either of bacterial types in comparison with control mosquitoes. Data from LP assay showed significant higher MDA concentration in the homogenate of mosquitoes at 24h post-inoculation with *Btk* bacteria compared to that of control ones (7.267 ± 0.878 *v* 3.6 ± 0.71 nmole/ml respectively) ($P < 0.05$, N = 5, student t-test) (Fig. 3). However, MDA concentration in *E. coli*-inoculated mosquitoes was similar to that of control ones at 24h post-inoculation (4.2 ± 0.94 *v* 3.6 ± 0.71 nmole/ml respectively) ($P > 0.05$, N = 5, student t-test) (Fig. 3). This may indicate that *Btk* inoculation has a long-term adverse oxidative effect on adult mosquitoes comparing to that of *E. coli* inoculation in terms of lipid peroxidation.

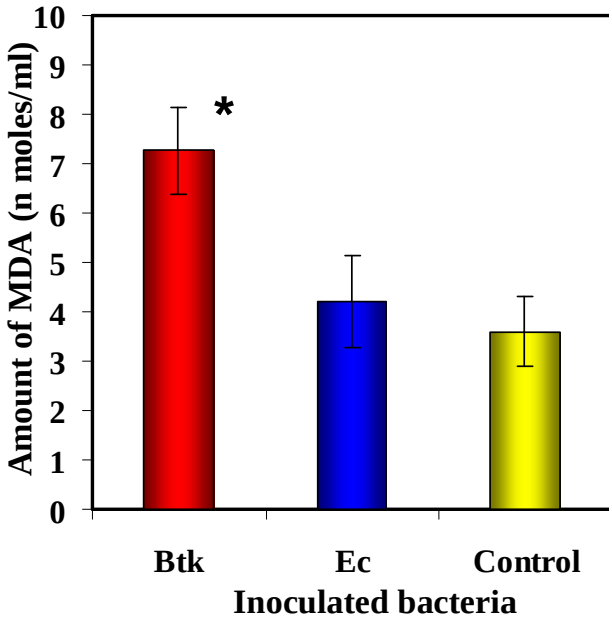

Fig. 3. Malondialdehyde (MDA) titer (nmole/ml) in 6-days old autogenous *Ae. caspius* mosquitoes at 24h post-inoculation with *B thuringiensis* (Btk), *E. coli* (Ec) or APS (control). In each case, MDA concentration was measured spectrophotometrically (at 535 nm) at 24h post-treatment. Error bars represent standard errors of means of 5 replicates (N = 5). Asterisk (*) represents significant higher MDA titer comparing to control mosquitoes ($P < 0.05$, two-sample t-test).

AOPP in larval mosquitoes

The oxidative effect of *Btk* infection on proteins in the 3rd larval instar was spectrophotometrically investigated in larval homogenate. This experiment showed that infecting larvae with *Btk* induced a significant increase of AOPP compared to control ones (77.21±4.48 *v* 37.378±0.47 µmole/ml respectively) ($P < 0.05$, N = 5, student t-test) (Fig. 4) at 12 h post-infection (Fig. 4). This significant increase in AOPP was also

monitored at 24h post-infection compared to that of control larvae (78.05±0.28 *v* 48.052±4.48 µmole/ml respectively) (P<0.05, N = 5, student t-test) (Fig.4).

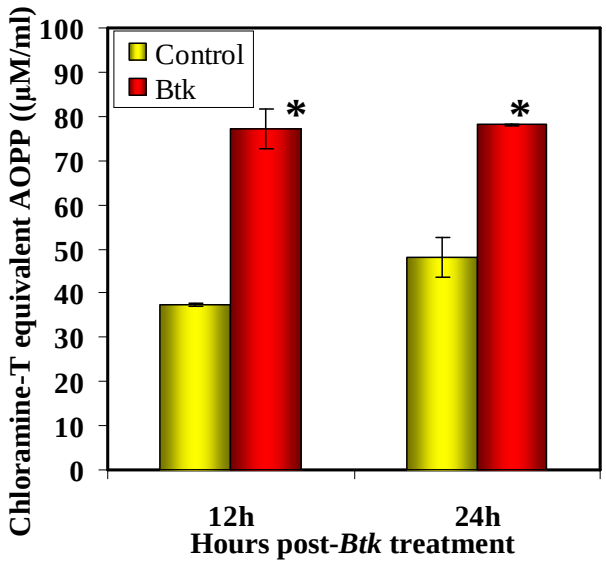

Fig. 4. Oxidation of proteins to AOPP in the 3rd larval instar of autogenous *Ae. caspius* mosquitoes at 12 and 24h post-infection with *B thuringiensis* (Btk). AOPP concentration was measured spectrophotometrically (at 340 nm). Error bars represent standard errors of means of 5 replicates (N = 5). Asterisk (*) represents significant higher AOPP comparing to control mosquitoes (P < 0.05, two-sample t-test).

AOPP in adult mosquitoes

Protein damage, in terms of advanced oxidative protein products (AOPP), was investigated spectrophotometrically in 6-days old autogenous *Ae. caspius* mosquito upon inoculation with *B. thuringiensis* (*Btk*), *E. coli* (Ec) or APS (control). Data from bacteria-inoculated mosquito homogenates showed significant higher AOPP concentration in *Btk*-inoculated mosquitoes at 12h post-inoculation compared to that of control ones (42.81 ± 1.77 *v* 35.14 ± 0.27 µmole/ml respectively) (P < 0.05, N = 5, student t-test) (Fig. 5). However, AOPP concentration in *E. coli*-inoculated mosquitoes was similar to that in control ones at 12h post-inoculation (35.752 ± 0.35 *v* 35.14 ± 0.27 µmole/ml respectively) (P > 0.05, N = 5, student t-test) (Fig. 5). This may indicate that *Btk*, but not *E. coli*, has induced protein damage in adult autogenous *Ae. caspius* mosquitoes as early as 12h post-inoculation.

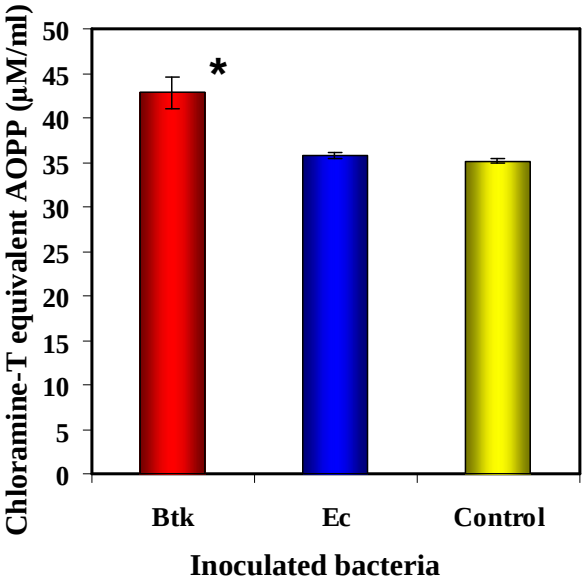

Fig. 5. Oxidation of proteins to AOPP in 6-days old autogenous *Ae. caspius* mosquitoes at 12h post-inoculation with *B thuringiensis* (Btk), *E. coli* (Ec) or APS (control). In each case, AOPP concentration was measured spectrophotometrically (at 340 nm) at 12h post-inoculation. Error bars represent standard errors of means of 5 replicates (N = 5). Asterisk (*) represents significant higher AOPP level comparing to control mosquitoes (P < 0.05, two-sample t-test).

For investigating long-term protein damage in adult mosquitoes upon inoculation with *Btk* or *E. coli*, AOPP level was measured at 24h post-inoculation with either of bacterial types in comparison with control mosquitoes. Data from each experiment showed no significant differences in AOPP concentration in the homogenate of mosquitoes at 24h post-inoculation with *Btk* or *E. coli* compared to APS-inoculated ones (31.462 ± 0.125 or 31.605 ± 0.186 *v* 31.437 ± 0.123 µmole/ml respectively) (P > 0.05, N = 5, student t-test) (Fig. 6). This may indicate that *Btk* has short-term protein damage effect in adult mosquitoes (Fig. 5) and *E. coli* has no protein damage effect on adult mosquitoes (Figs. 5, 6).

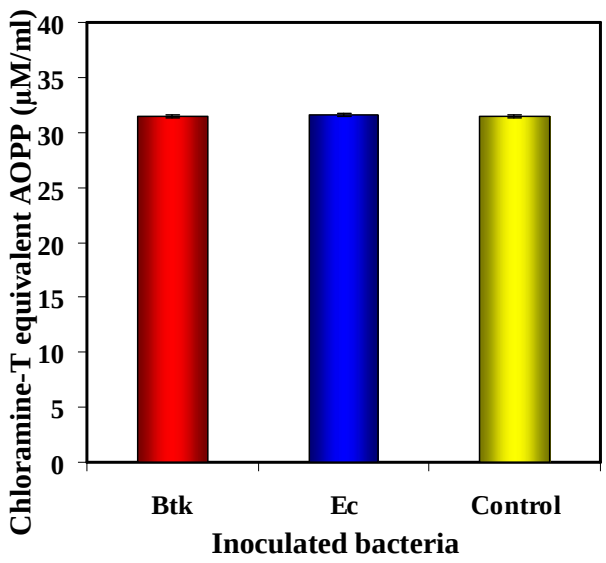

Fig. 6. Oxidation of proteins to AOPP in 6-days old autogenous *Ae. caspius* mosquitoes at 24h post-inoculation with *B thuringiensis* (Btk), *E. coli* (Ec) or APS (control). In each case, AOPP concentration was measured spectrophotometrically (at 340 nm) at 24h post-inoculation. Error bars represent standard errors of means of 5 replicates (N = 5). No significant differences in AOPP levels in bacterial inoculated mosquitoes comparing to control ones were detected ($P > 0.05$, two-sample t-test).

DISCUSSION

Phospholipids make up the bulk of all internal and external cellular membranes in eukaryotic cell. Most cellular membrane proteins are embedded in or attached to membrane phospholipids. The structure and function of membrane proteins depend on the precise composition of its immediate phospholipid environment. Oxidative stress is known to be associated with damaged cell membranes and cell death (Klein and Ackerman, 2003). Under normal conditions**,** a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell (Stohs and Bagchi, 1995). ROS includes superoxide, hydroxyl, peroxyl and nitric oxide (NO) free radicals (Stohs and Bagchi, 1995). Superoxide (O_2^-) is an important source of free radicals (Fridovich, 1986). Hydrogen peroxide

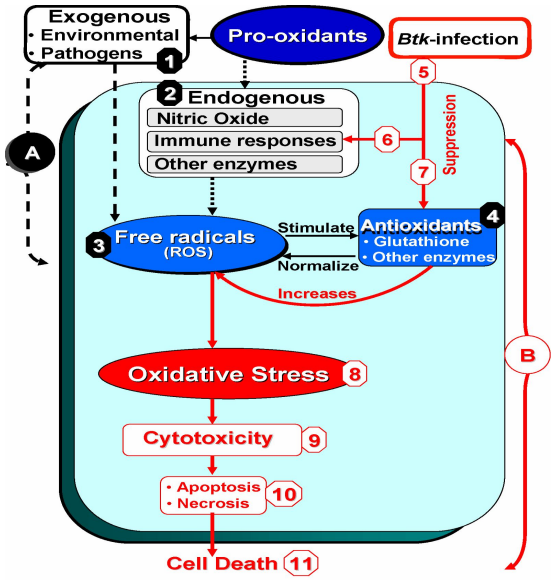

Fig. 7. Speculative schematic diagram suggesting a potential mechanism of oxidative stress in *Btk*-infected *Ae caspius* larvae (modified from Chauhan and Chauhan, 2006). **Part A** in the diagram (shown in **black** dotted arrows and numbering) illustrates the normal balance between generation of Reactive Oxygen Species (ROS), that called free radicals (3) caused by endogenous/exogenous pro-oxidants and the defense mechanism against ROS by antioxidants (4). Increasing in ROS levels may be due: I) exogenous environmental pro-oxidant factors (1) such as heavy metals, pollutants, toxins and microbial infections, II) increased production of endogenous pro-oxidants (2) such as NO, xanthine oxidase, homocysteine, or both which result in high level of ORS. On the other hand, antioxidants within the cell (4) such as ceruloplasmin, transferrin, superoxide dismutase, catalase and reduced glutathione are neutralizing these extra ORS. This oxidant/antioxidant balance is essential for normal functioning of these cells. **Part B** in the diagram (shown in **red** solid arrows and numbering) illustrates suggested mode of actions of *Btk* infection (5) in mosquito larva. *Btk*-infection may suppress both the immune responses of infected larvae and antioxidant defense (6 and 7, respectively) (Ahmed, 2011). Subsequently, this increases the level of ROS which imposes cellular oxidative stress. Increased oxidative stress (8) may lead to cytotoxicity (9) in terms of lipid peroxidation, protein oxidation, DNA oxidation, mitochondrial dysfunction and impaired energy production. These cellular abnormalities trigger apoptosis and/or necrosis (10) which ultimately results in cellular collapse (11).

(H_2O_2) reacts with iron to produce the highly reactive hydroxyl radical (McCord and Day, 1978) which causes the most cytotoxic effects as well as initiation of lipid peroxidation (McCord and Day, 1978).

In the current study, lipid peroxidation and advanced oxidation protein products bioassays, as important biomarkers for oxidative stress, were investigated in both larval and adult stages of the autogenous mosquito, *Ae. caspius*. Bioassays were carried out in adult mosquitoes (6 days-old) or 3rd larval instars at 12 and 24h post-*Btk* infection or inoculation respectively. Basically, lipid peroxidation is a chain reaction between polyunsaturated fatty acids and ROS, and it produces lipid peroxides and hydrocarbon polymers that are both highly cytotoxic (Horton and Fairhurst, 1987), and hence, increase oxidative stress. It has been shown that ROS cause lipid peroxidation, protein carbonylation, DNA oxidation and glutathione depletion, leading to oxidative damage and alterations in radical scavenging enzymes in insect tissues (Ahmad, 1995). The current study reported significant 2.2 and 1.9 folds increase in TBA-reactive Malonyldialdehyde at 12 and 24h respectively post-infection with *Btk* in mosquito larvae as compared to un-infected controls. This may indicate *Btk*-induced high lipid peroxidation that may have lead to cytotoxicity in infected larvae (Jain, 1984). Functional studies in mammals showed that phospholipid hydroperoxide glutathione peroxidases (PHGPx) prevent lipid peroxidation and protect biomembranes against oxidative stress (Ursini *et al.,* 1982). This is because PHGPx are antioxidant enzymes that can directly reduce peroxidized phospholipids and cholesterols within membranes (Ursini *et al.,* 1985; Thomas *et al.,* 1990). Similar function for PHGPx in insect has also been demonstrated (Li *et al.,* 2003). Based on these studies, the observed significant increase of lipid peroxidation upon *Btk* infection in the current study may be attributed to *Btk* suppression/deactivation of the production of PHGPx in infected mosquito larvae. Furthermore, *Btk* has been shown to have suppressive effect on both glutathione production (an important antioxidant) and antibacterial immune activity in *Btk*-infected larvae (Ahmed, 2011) which may constitute a further mechanism of this elevated oxidative stress. Taken all together, I would suggest that these effects might be considered as pathogenecity mechanisms of *Btk* against mosquitoes larvae (Fig. 7).

Exposure of proteins to ROS gives both side-chain oxidation and backbone fragmentation (Davies and Dean, 1997). Ultimately, these processes can result in the loss of structural or enzymatic activity of the protein and, hence, biological perturbations (Davies and Dean, 1997). Cytological dysfunction is believed to be correlated with oxidative stress *via* modification of biological structures and formation of AOPP. These protein products might be regarded as a family of compounds containing abundantly dityrosines, which allow cross linking, disulfide bridges, and carbonyl groups (Capeille're-Blandin *et al.,* 2004). This may indicate that AOPP act as markers of oxidative stress, may contribute to cytological dysfunction (Descamps-Latscha *et al.,* 2005). Oxidation of amino acid residues such as tyrosine, leading to the formation of dityrosine, protein aggregation, cross-linking and fragmentation is an example of ROS-mediated protein damage *in vitro* (Dean *et al.,* 1997). This may have happened in *Btk*-infected larvae of the current study as they showed significant 2 and 1.6 folds increase in AOPP at 12 and 24h post-infection respectively compared to control ones. This elevation in AOPP was more pronounced in larvae compared to that in adult mosquitoes which showed significant 1.2 folds increase and no significant difference at 12 and 24h post-*Btk* inoculation respectively compared to control ones. In insects, bacterial challenge normally activates upstream proteinases in the cascade, which activate prophenoloxidase (pPO) (Kanost and Gorman, 2008) leading to hydroxylation of tyrosine and oxidation of o-diphenols like dopamine to form quinones using molecular oxygen (O_2) (Sugumaran *et al.,* 2000). Further metabolic processing of quinones results in the formation of the cytotoxic melanin (Cerenius and Soderhall, 2004). The melanogenesis-related cytotoxicity of melanin is attributed, in part, to its ability to bind covalently to cell-membrane components and other cellular nucleophiles. This subsequently promotes free-radical cascades and involves sulfydryl oxidations,

inactivation of DNA polymerase, depolymerization of lipids and lipid peroxidation (Nappi and Vass, 2001; Christensen *et al.,* 2005). Thus, in the evolutionary point of view, I may suggest that mosquitoes adapted a mechanism that protects them from this immune-related oxidative stressor *via* the antioxidant GSH (Erden-Inal *et al.,* 2002; and reviewed by Christensen *et al.,* 2005). Thus, the low GSH titer in *Btk*-infected mosquitoes that observed by Ahmed (2011) explains the elevated level of AOPP recorded in *Btk*-infected mosquitoes in the current study.

From the point of view of immune defense of insects, ROS inducers are involved in immune responses against foreign invaders. Herrera-Ortiz *et al.* (2011) investigated the involvement of H_2O_2 and Nitric oxide NO in the activation of the anti-malarial immune response in malaria-refractory strain of *An. gambiae* (compared to a susceptible strain) post-infected blood meal (Kumar *et al.,* 2003; Herrera-Ortíz *et al.,* 2004). NO is considered as a main immune factor against malaria development (Peterson *et al.,* 2007; Herrera-Ortiz *et al.,* 2011) as it induces the production of antimicrobial peptide in mosquito gut. NO also induced the production of antimicrobial peptide in uninfected *Drosophila larvae* (Foley and O'Farell, 2003) and in *Bombyx mori* (Imamura *et al.,* 2002). To my knowledge, the concomitant oxidative stress in these insects as a result of NO induction is yet to be investigated. Mosquitoes, on the other hand, protect themselves against this immune-induced oxidative stresses through antioxidant defensive mechanism for their survival (Townsend, 2007). Evidence for this is study of Chen *et al.* (2011) who recorded endoplasmic reticular stress in mosquito cell line upon infection with dengue virus (DENV-2). This virus-induced cytotoxicity was characterized as changed mitochondrial membrane potential and generated superoxide production. On the other hand, they recorded significant elevation of GST minimized the resulting cellular damage as antioxidative response. Further evidence from malaria parasite infection in anopheline mosquitoes is the study of Peterson and Luckhart (2006) who observed the induction of oxidative stresses upon malaria infection that damaged mosquito tissues. They reported cellular defenses in mosquito that minimized the resulting cellular damage *via* peroxiredoxins (Prxs), enzymes known to detoxify ROS, as antioxidative response. Data of the current study shows clearly that *Btk* has induced oxidative stress in infected mosquitoes. This could be explained by the observed low level of glutathione in *Btk*-infected mosquito larvae (Ahmed, 2011), and subsequently, interrupted the antioxidant-oxidant balance in infected mosquitoes. Hence, this *Btk*-induced oxidative stress may be considered as a mechanism behind high pathogenicity of *Btk* against mosquitoes (Fig. 7). Collectively, data of the current study demonstrated that *Btk* infection induced oxidative stress in *Ae. caspius* producing higher levels of ROS and this oxidative imbalance playing an important role in the larvicidal effect of this bacteria as shown for other toxins (Wei *et al.,* 2010; Beckon *et al.,* 2008). The short-term protein damage effect of *Btk* infection in adult mosquitoes (Fig. 5) compared to no protein damage in *E. coli* infected adult mosquitoes (Figs. 5, 6) may indicate that this oxidative stress is pathogenicity-dependent.

In conclusion, increased oxidative stress in *Btk*-infected mosquitoes (in terms of high levels of lipid peroxidation and protein oxidation) may be due to a) increased production of endogenous or exogenous pro-oxidants such as NO, xanthine oxidase, homocysteine (Stohs and Bagchi, 1995a), b) deficiencies of antioxidants (ceruloplasmin, transferrin, SOD, GPx, catalase, reduced glutathione) or (c) both. This increased oxidative stress may have lead to cytotoxicity which ends up with cellular death (Kannan and Jain, 2000) (Fig. 7). Thus, the suggested scenario that may have happened in *Btk*-infected larvae is suppression of antioxidants leading to elevation of ROS resulting in oxidative stress in terms of membrane lipid abnormalities, mitochondrial dysfunction, excitotoxicity and apoptosis (cellular damage), and innate immunity dysregulation in parallel (Ahmed, 2011). This scenario may explain the mechanism behind the high mosquitocidal pathogenecity of *Btk* within 24h post-infection. Therefore, I would suggest that the cytopathological effects of *Btk* in larval gut epithelial cells (Al-Robal *et al.,* 2011) may have happened *via* this suggested scenario. Finally, the question now is "would the inhibition of ROS (through oral feeding of antioxidants) reduce

cytotoxicity (pathogenecity) of entomopathogenic microorganisms against beneficial insects and/or activation of endogenous ROS *via* adding exogenous antioxidant-inhibitors to the formula of biocontrol agents be in the favor of biocontrol measures?" Although trials of activating the antioxidant defense has been recently toke place (Alquicer *et al.*, 2009), this exciting question awaits to be tackled.

ACKNOWLEDGEMENT

The author extends his appreciation to Deanship of Scientific Research at King Saud University for funding this work through the research group project No: RGP-VPP-075. Author would like also to thank Prof. Ali M. Metwally, Department of Food Sciences and Nutrition, Faculty of Food Sciences and Agriculture, King Saud University, for provision of *E. coli* and help with culturing and maintaining the bacterial strains used in this study.

REFERENCES

AHMED, A.M., 2011. Immune and antioxidant defenses in an autogenous *Aedes caspius* mosquito upon infection with *Bacillus thuringiensis kurstaki*. *Afr. J. Microbiol. Res.* **5**: 3848-3857

AHMAD, S., 1995. Oxidative stress from environmental pollutants. *Arch. Insect Biochem. Physiol.*, **29**: 135–157.

AHMED, A.M., TAYLOR, P., MAINGON, R. AND HURD, H., 1999. The effect of *Plasmodium yoelii nigeriensis* on the reproductive fitness of *Anopheles gambiae*. *Inverteb. Reprod. Develop*., **36**: 217-222.

AIASSA, V., BARONETTI, J.L., PAEZ, P. L., BARNES, A.I., ALBRECHT, C., PELLARIN, G., ERASO, A. J. AND ALBESA, I., 2011. Increased advanced oxidation of protein products and enhanced total antioxidant capacity in plasma by action of toxins of *Escherichia coli* STEC. *Toxicology in Vitro*, **25**: 426–431.

AL-ROBA, A.A., ABOUL-SOUD, M.A.M., AHMED, A.M. AND AL-KHEDHAIRY, A.A., 2011. The gene expression of caspasses is up-regulated during the signaling response of *Aedes caspius* against larvicidal bacteria. *Afr. J. Biotechnol*., **10**: 225-233.

ALQUICER, G., KODRÍK, D., KRISHNAN, N., VEČEŘA, J. AND SOCHA, R., 2009. Activation of insect anti-oxidative mechanisms by mammalian glucagon. *Comp. Biochem. Physiol*., Part B **152**: 226–233.

BECKON, W.N., PARKINS, C., MAXIMOVICH, A. AND BECKON, A.V., 2008. A general approach to modeling biphasic relationships. *Environ. Sci. Technol*., **42**: 1308–1314.

BOMAN, H.G., 1998. Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. *Scand. J. Immunol*., **48**: 15–25.

CAPEILLE'RE-BLANDIN, C., VALE'RIE, G., DESCAMPS-LATSCH, B. AND WITKO-SARSAT, V., 2004. Biochemical and spectrophotometric significance of advanced oxidized protein products. *Biochim. biophys. Acta*, **1689**: 91–102.

CERENIUS, L. AND SODERHALL, K., 2004. The prophenoloxidase-activating system in invertebrates. *Immunol. Rev*., **198**: 116–126.

CHAUHAN, A. AND CHAUHAN, V., 2006. Oxidative stress in autism. *Pathophysiology*, **13**: 171–181.

CHEN, T.-H., TANG, P., YANG, C.-F., KAO, L.-H., LO, Y.-P., CHUANG, C.-K.., SHIH, Y.-T. AND CHEN, W.-J., 2011. Antioxidant defense is one of the mechanisms by which mosquito cells survive dengue 2 viral infection. *Virology*, **410**: 410–417.

CHRISTENSEN, B.M., LI. J., CHENG-C. AND NAPPI, A.J., 2005. Melanization immune responses in mosquito vectors. *Trends Parasitol*., **21**: 193-199.

CLEMENTS, A.N., 1992. Vitellogenesis, In: *The biology of mosquitoes*, V.1. Cambridge University Press, pp. 360-379.

CORREA SALDE, V. AND ALBESA, I., 2009. Reactive oxidant species and oxidation of protein and haemoglobin as biomarkers of susceptibility to stress caused by chloramphenicol. *Biomed. Pharmacother*., **63**: 100–104.

DAVIES, M.J. AND DEAN, R.T., 1997. *Radical-mediated protein oxidation*: *from chemistry to medicine*. Oxford University Press, Oxford.

DEAN, R.T.S., FU, R. AND DAVIES, M.J., 1997. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem. J*., **324**: 1.

DESCAMPS-LATSCHA, B., WITKO-SARSAT, V., NGUYEN-KHOA, T., NGUYEN, A.T., GAUSSON, V., MOTHU, N., LONDON, G.M. AND JUNGERS, P., 2005. Advanced oxidation protein products as risk factors for atherosclerotic cardiovascular events in nondiabetic predialysis patients. *Am. J. Kidney Dis*., **45**: 39–47

DIMOPOULOS, G., RICHMAN, A., MULLER, H-M. AND KAFATOS, F.C., 1997. Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc. natl. Acad. Sci. U.S.A.*, **94**: 11508-11513.

ERDEN-INAL, M,, SUNAL, E. AND KANBAK, E.G., 2002. Age-related changes in the glutathione redox system. *Cell. Biochem. Funct*., **20**: 61–66.

FANG, F.C. (Ed.), 1999. *Nitric oxide and infection.* Kluwer Academic/Plenum, New York.

FELTON, G.W. AND SUMMERS, C.B., 1995. Antioxidant systems in insects. *Arch. Insect Biochem. Physiol.*, **9**: 187–197.

FEYEREISEN, R., 2005. Insect Cytochrome P450. In: *Comprehensive molecular insect science* (eds. L.I. Gilbert, K. Iatrou and S. Gill), vol. 4. Elsevier, Oxford, pp. 1–77.

FOLEY, E. AND O'FARELL, P.H., 2003. Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. *Genes Dev*., **17**: 115–125.

FRIDOVICH, I., 1986. Biological effects of the superoxide radical, *Arch. Biochem. Biophys.*, 247: 1–11.

FRIDOVICH, I., 1995. Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem*., **64**: 97–112.

GILLESPIE, J.P. AND KANOST, M.R., TRENCZEK, T., 1997. Biological mediators of insect immunity. *Annu. Rev. Ent.*, **42**: 611–643.

HALLIWELL, B. AND GUTTERIDGE, J. M. C., 1999. *Free radicals in biology and medicine.* Oxford University Press, New York.

HEMINGWAY, J., HAWKES, N.J., MCCARROLL, L. AND RANSON, H., 2004. The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem. mol. Biol*. **34**: 653–665.

HERRERA-ORTÍZ, A., LANZ-MENDOZA, H., MARTÍNEZ-BARNETCHE, J., HERNÁNDEZ-MARTÍNEZ, S., VILLARREAL-TREVIÑO, C., AGUILAR-MARCELINO, L. AND RODRÍGUEZ, M.H., 2004. *Plasmodium berghei* ookinetes induce nitric oxide production in *Anopheles pseudopunctipennis* midguts cultured *in vitro*. *Insect Biochem. mol. Biol*., **34**: 893–901.

HERRERA-ORTIZ, A., MARTÍNEZ-BARNETCHE, J., SMIT, N., RODRIGUEZ, M.H. AND LANZ-MENDOZA, H., 2011. The effect of nitric oxide and hydrogen peroxide in the activation of the systemic immune response of *Anopheles albimanus* infected with *Plasmodium berghei*. *Dev. Comp. Immunol*., **35**: 44–50.

HORTON A.A. AND FAIRHURST, S., 1987. Lipid peroxidation and mechanisms of toxicity. *CRC Crit. Rev. Toxicol*., **18**: 27–79.

HYRŠL, P., BÜYÜKGÜZEL, E. AND BÜYÜKGÜZEL, K., 2007. The effects of boric acid-induced oxidative stress on antioxidant enzymes and survivorship in *Galleria mellonella*. *Arch. Insect Biochem. Physiol*., **66**: 23–31.

IMAMURA, M., YANG, J. AND YAMAKAWA, M., 2002. cDNA cloning, characterization and gene expression of nitric oxide synthase from the silkworm, *Bombyx mori*. *Insect mol. Biol*., **11**: 257–265.

IMLAY, J.A., 2003. Pathways of oxidative damage. *Annu. Rev. Microbiol*., **57:** 395–418.

IMLAY, J. A. AND LINN, S., 1988. DNA damage and oxygen radical toxicity. *Science*, **240**: 1302–1309.

IMLER, J.L. AND 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. AND MEISTER, M., 2005. New insights into *Drosophila* larval haemocyte functions through genome wide analysis. *Cell. Microbiol*., **7**: 335–350.

JAIN, S.K., 1984. The accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes, *J. biol. Chem*., **25**: 3391–3394.

KANNAN, K. AND JAIN, S.K., 2000. Oxidative stress and apoptosis, *Pathophysiology*, **7**: 153–163.

KANOST, M.R. AND GORMAN, M., 2008. Phenoloxidases in insect immunity. In: *Insect immunity* (ed. N.E. Beckage). Academic Press, San Diego, pp. 69-96.

KAVANAGH, K. AND REEVES, E.P., 2004. Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. *FEMS Microbiol. Rev*., **28**: 101–112.

KIM, B,Y., LEE, K.S., CHOO, Y.M., KIM, I. JE, Y. H., WOO, S.D**.,** LEE, S.M., PARK, H.C., SOHN, H. AND JIN, B.R., 2008. Insect transferrin functions as an antioxidant protein in a beetle larva. *Comp. Biochem. Physiol.*, Part B, **150**: 161–169.

KLEIN, J.A. AND ACKERMAN, S.L., 2003. Oxidative stress, cell cycle, and neurodegeneration. *J. clin. Invest*., **111**: 785–793.

KUMAR, S., CHRISTOPHIDES, G.K., CANTERA, R., CHARLES, B., HAN, Y.S., MEISTER, S., DIMOPOULOS, G., KAFATOS, F.C. AND BARILLAS-MURY, C., 2003. The role of reactive oxygen species on *Plasmodium melanotic* encapsulation in *Anopheles gambiae*. *Proc. natl. Acad. Sci. U.S.A*., **100**: 14139–14144.

LI, D., BLASEVICH, F., THEOPOLD, U. AND SCHMIDT, O., 2003. Possible function of two insect phospholipid-hydroperoxide glutathione peroxidases. *J. Insect Physiol*., **49**: 1–9.

MCCORD, J.M. AND DAY, E.D., 1978. Superoxide dependent production of hydroxyl radical catalyzed by iron-EDTA complex, *FEBS Lett.*, **86**: 139–142.

MEISTER, A. AND ANDERSON, M.E. 1983. Glutathione. *Annu. Rev. Biochem*., **52**: 711-760.

MORRISON, D.A., 2002. How to improve statistical analysis in parasitology research publications. *Int. J. Parasitol*., **32**: 1065–1070.

MORTON, D., DUNPHY, G. AND CHADWICK, J., 1987. Reactions of haemocytes of immune and non-immune *Galleria mellonella* larvae to *Proteus mirabilis*. *Develop. Comp. Immunol*., **11**: 47–55.

NAPPI, A.J. AND VASS, E., 2001. Cytotoxic reactions associated with insect immunity. In: *Phylogenetic*

perspectives on the vertebrate immune system (ed. E.L. Cooper), Kluwer Academic-Plemum., pp. 329–348.

NIMMO, D.D., HAM, P.J.; WARD, R.D. AND MAINGON, R., 1997. The sand fly *Lutzomyia longipalpis* shows specific humoral responses to bacterial challenge. *Med. Vet. Ent.*, **11**: 324–328.

OKHAWA, H., OHISHI, N. AND YAGI, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem*., **95**: 351-358.

PETERSON, T.M.L. AND LUCKHART, S., 2006. A mosquito 2-Cys peroxiredoxin protects against nitrosative and oxidative stresses associated with malaria parasite infection. *Free Rad. Biol. Med.,* **40**: 1067 – 1082.

PETERSON, T.M.L., GROW, A.J. AND LUCKHART, S., 2007. Nitric oxide metabolites induced in *Anopheles stephensi* control malaria parasite infection. *Free Radic. Biol. Med*., **42**: 132–142.

POUPARDINA, R., REYNAUDA, S. AND 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. AND KETTERMAN, A.J., 1993. Partial purification and characterization of glutathione S-transferases involved in DDT resistance from the mosquito *Anopheles gambiae*. *Pestic. Biochem. Physiol*., **47**: 119–133.

RADYUK, S.N., SOHAL, R.S. AND ORR, W.C. 2003. Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and underexpression of thioredoxin peroxidase in *Drosophila* cells. *Biochem. J*., **371**: 743–752.

RUGALE, C., DELBOSC, S., MIMRAN, A. AND JOVER, B., 2007. Simvastatin reverses target organ damage and oxidative stress in Angiotensin II hypertension: comparison with apocynin, tempol, and hydralazine. *J. Cardiovasc. Pharmacol*., **50**: 293–298.

STADTMAN, E.R., 1986. Oxidation of proteins by mixed-function oxidation systems: implication in protein turnover, aging and neutrophil function. *Trends Biochem. Sci*., **11**: 11–12.

STOHS, S.J. AND BAGCHI, D., 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med*., **18**: 321–336.

SUGUMARAN, M., NELLAIAPPAN, K., AMARATUNGA, C. AND CARDINALE, S.T., 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.

THOMAS, J., MAIORINO, M., URSINI, F. AND GIROTTI, A., 1990. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. *J. biol. Chem*., **265**: 454–461.

TOWNSEND, D.M., 2007. S-glutathionylation: indicator of cell stress and regulator of the unfolded protein response. *Mol. Interv*., **7**: 313–324.

URSINI, F., MAIORINO, M., VALENTE, M., FERRI, L. AND GREGOLIN, C., 1982. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochim. biophys. Acta*, **710**: 197–211.

URSINI, F., MAIORINO, M. AND GREGOLIN, C., 1985. The selenoenzyme phospholipids hydroperoxide glutathione peroxidase. *Biochim. biophys. Acta*, **839**: 62–70.

VALLES, S.M. AND PEREIRA, R.M., 2005. *Solenopsis invicta* transferrin: cDNA cloning, gene architecture, and up-regulation in response to *Beauveria bassiana* infection. *Gene*, **358**: 60–66.

VILMOS, P. AND KURUCZ, E., 1998. Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunol. Lett*., 62: 59–66.

WANG, Z., LU, A. LI, X., SHAO, Q., BEERNTSEN, B.T. LIU, C., MAD, Y., HUANG, Y., ZHU, H. AND LING, E., 2011. A systematic study on hemocyte identification and plasma prophenoloxidase from *Culex pipiens quinquefasciatus* at different developmental stages. *Exp. Parasitol*., **127**: 135–141.

WEI, W., MA, B., LI, H. Y., JIA, Y., LV, K., WANG, G., ZHANG, J., ZHU, S., TANG, H., SHENG, Z. AND XIA, Z., 2010. Biphasic effects of selective inhibition of transforming growth factor beta1 activin receptor-like kinase on LPS-induced lung injury. *Shock*, **33**: 218–224.

YOSHIGA, T., HERNANDEZ, V.P., FALLON, A.M. AND LAW, J.H., 1997. Mosquito transferrin, an acutephase protein that is up-regulated upon infection. *Proc. natl. Acad. Sci. USA.*, **94**: 12337–12342.

(Received 14 October 2011, revised 30 November 2011)