

**Orijinal araştırma (Original article)**

**Effect of eicosanoid biosynthesis inhibitors  
on the haemolymph protein profile of *Galleria  
mellonella* (Linnaeus, 1758) larvae  
(Lepidoptera: Pyralidae)**

Pavel HYRŠL<sup>1\*</sup> Ender BÜYÜKGÜZEL<sup>2</sup> Kemal BÜYÜKGÜZEL<sup>2</sup>

**Summary**

Eicosanoids mediate insects cellular and humoral immune reactions and stress responses. Function of these mediators can be specifically blocked using different eicosanoid biosynthesis inhibitors (EBIs). Effects of EBIs on total haemolymph protein composition have not been extensively studied in insects. We posed the hypothesis that eicosanoids also mediate physiological homeostasis by regulating protein profiles involved in stress response and other defensive reactions. To test this idea, we reared greater wax moth *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) larvae on artificial diets containing 0.001, 0.01, 0.1 or 1.0% of specific EBIs with different mode of action: Esculetin, dexamethasone and phenidone. Feeding larvae with esculetin caused significantly dose-dependent changes in 45 kDa protein fraction (one of 16 proteins detected) using sodium dodecyl-sulphate polyacrylamide gradient gel electrophoresis followed by silver staining. Other main haemolymph proteins, lipophorins (ApoLP-I) and storage proteins, were not affected by EBIs treatments. Dexamethasone and phenidone caused no significant differences in detected protein fractions. We infer from these findings that eicosanoids, at least lipoxigenase products, have been implicated in the protein composition of insect tissues as structural and functional concept. Although it has not yet been possible to use directly EBIs for insect pest control, our results bring new data to understand physiological signaling systems in insects.

**Key words:** Eicosanoids, esculetin, *Galleria mellonella*, haemolymph, protein profile

**Anahtar sözcükler:** Eikosanoidler, eskuletin, *Galleria mellonella*, hemolenf, protein profili

<sup>1</sup> Department of Animal Physiology and Immunology, Institute of Experimental Biology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37, Brno, Czech Republic

<sup>2</sup> Department of Biology, Faculty of Arts and Science, Karaelmas University, 67100, Zonguldak, Turkey

\* Sorumlu yazar (Corresponding author) e-mail: hyrsl@mail.muni.cz

Alınış (Received): 25.08.2010

Kabul edilmiş (Accepted): 25.01.2011

## Introduction

Eicosanoids are produced by enzymatic oxygenation of arachidonic acid and two other C20 polyunsaturated fatty acids derived from phospholipids by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (Stanley, 2006). The major groups of eicosanoids are cyclooxygenase (COX) products, prostaglandins (PGs) and many lipoxygenase (LOX) products. These pathways can be specifically blocked by different eicosanoid biosynthesis inhibitors (EBIs).

Eicosanoids are important in insects cellular immune reactions such as haemocyte-spreading and migration, microaggregation, nodulation, encapsulation and phagocytosis to bacterial, fungal, viral and metazoan and protozoan infectious agents (Miller & Stanley, 2001; Dean et al., 2002; Tunaz, 2006; Büyükgüzel et al., 2007; Stanley & Shapiro, 2007; Durmuş et al., 2008; Figueiredo et al., 2008; Merchant et al., 2008). In humoral immunity, eicosanoids mediate biosynthesis of the antimicrobial peptides in some insect groups (Morishima et al., 1997; García Gil de Muñoz et al., 2008), and are also included in the activation of prophenoloxidase in *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) larvae (Mandato et al., 1997; Downer et al., 1997). Although intensive research of eicosanoids function and EBIs during last years, information on their influence on total haemolymph protein composition have not been extensively studied in insects. In our study we focused on phenidone (dual inhibitor of COX and LOX), dexamethasone (phospholipase A<sub>2</sub> inhibitor) and esculetin (LOX inhibitor). Here we report that, relative to controls, EBIs treatments changed protein composition of larval haemolymph.

## Materials and Methods

### Insects and collection of the haemolymph

Last-instar larvae (upon reaching VII<sup>th</sup> instars) of *G. mellonella* obtained from laboratory cultures maintained on a semiartificial diet at 29 ± 1°C (Bronskill, 1961) in constant darkness were used in all experiments. The methods used to prepare and dispense diets into container; to obtain eggs and placements of larvae onto diets were described in our previous study (Hyršl et al., 2008). Last instar larvae were used in the biochemical analyses because our preliminary experiments demonstrated that EBIs exerted its main effects on mostly larval stages of the insect (Büyükgüzel et al., 2007). For collecting of the haemolymph, the larvae were chilled on ice for 5 min and surface sterilized in 95% ethanol. Haemolymph was collected into cold Eppendorf tubes by amputating the second pair of prolegs. A few crystals of phenylthiourea (PTU) were added to each sample to prevent melanization. The samples were frozen until use.

### **Chemicals and experimental design**

Phenylthiourea (PTU), esculetin (6,7-dihydroxycoumarin) and phenidone (4-methyl-1-phenyl-3-pyrazolidinon) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone [(11 $\beta$ ,16 $\alpha$ )-9-fluoro-11-17,21-trihydroxy-16-methylpregna-1,4-diene-3, 20-dione] was kindly gift from Deva Holding (Levent, Istanbul, Turkey). Chemicals for electrophoresis were obtained from Fluka Chemie GmbH (Steinheim, Germany) except acrylamide 30% solution (AppliChem GmbH, Darmstadt, Germany), ammonium persulphate (Bio-Rad, Ramsey, MN, USA) and glycine (Lachema, Brno, Czech Republic). All reagents were analytical grade.

Control larvae were reared on the normal diet without EBIs. Desired amounts of EBIs, esculetin (98% pure by manufacturer's analysis), dexamethasone (98%) and phenidone (97%) were first diluted in 1 ml of ethanol (70%) and completed with distilled water to prepare solutions of the required concentrations at 0.001, 0.01, 0.1 or 1.0 g in 100 g of diets. Phenidone was tested only at concentrations 0.001, 0.01 or 0.1% because this inhibitor at 1.0% did not produce seventh-instar larvae. Solutions of EBIs were then added into the diets as water source of diets during preparation. First instar larvae were reared through VII<sup>th</sup> instars on an artificial diet amended with given concentrations of EBIs. Preliminary experiments showed that ethanol at this volume did not affect biochemical responses. Each feeding experiment was run in duplicate with eight control or EBI-exposed larvae in one replication.

### **Protein electrophoresis**

Haemolymph samples were denatured by heating at 90°C for 2–5 min in a diluted sample buffer containing 7%  $\beta$ -mercaptoethanol and 0.37% of the anionic detergent sodium dodecyl sulphate (SDS). After dilution, the samples applied into the gels contained finally 1.25  $\mu$ g of proteins. Individual as well as mixed samples (haemolymph mixed from all the larvae in one experimental group) were used for the analysis.

Sodium dodecyl-sulphate polyacrylamide gradient gel electrophoresis (SDS-PAGE) was performed in a discontinuous buffer system according to Laemmli (1970) modified as described by Hyršl et al. (2008). Briefly, samples were applied to a 5% stacking gel (1 mm thickness) and 7.5–20.0% gradient separating gel in the electrophoresis unit (Hoefer Scientific Instruments SE 600 device, San Francisco, CA, USA) with mini chiller (model 1000, Bio-Rad, Ramsey, MN, USA). The gels were fixed and stained with silver nitrate at room temperature following the technique of Kirkeby et al. (1993). Broad range protein standards (161-0317, Bio-Rad, Ramsey, MN, USA) were used for calibration. The molecular weight and peak area of separated protein fractions were determined from absorption curves by densitometry using an imaging densitometer (GS-670, Bio-Rad, Ramsey, MN, USA) and Molecular Analyst software (Bio-Rad). Statistical analysis includes ANOVA followed by Tukey's test for significance.

## Results

Firstly, larval weight was checked to avoid possible negative effect of EBIs during the treatments. There was no significant difference between control larvae ( $0.158 \pm 0.025$  g) and EBIs treated larvae except lower weight in the group of dexamethasone at 0,01% concentration ( $0.088 \pm 0.012$  g, Figure 1).

Approximately 16 protein fractions were detected after separation of *G. mellonella* larval haemolymph proteins on gradient acrylamide gel with following silver staining (Figure 2). Larval haemolymph includes a dominant group of protein bands ranging in molecular mass approximately 72-84 kDa. These are generally recognized as storage proteins. Other well-determined fractions in larval haemolymph are lipophorins including e.g. ApoLP I (230-250 kDa). Some unidentified protein fractions varying from 6.5 to 260 kDa were also detected in larval haemolymph.

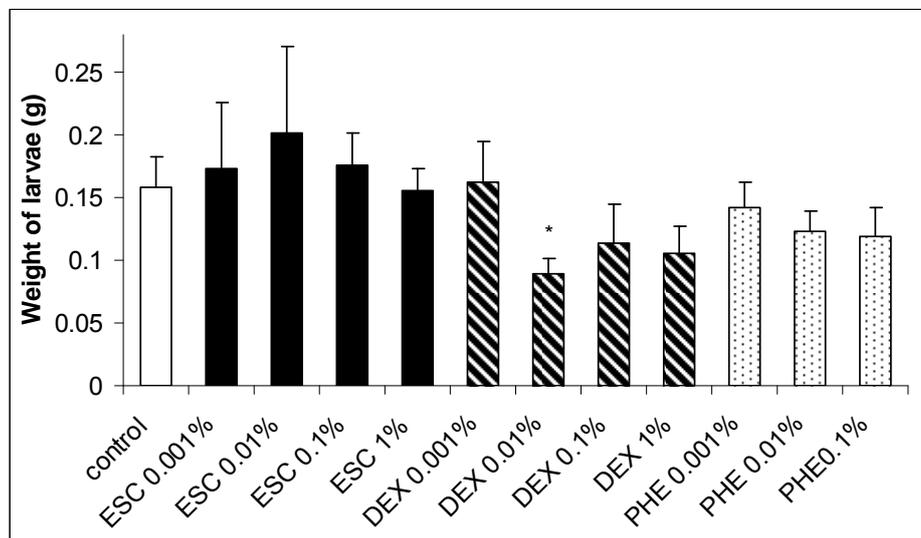


Figure 1. Weights of *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) larvae during the EBIs treatment of indicated concentrations. Average values  $\pm$  S.D. are indicated, significance level is  $<0.05$  (\*).

Therefore, we focused on the effects of EBIs on protein composition of larval haemolymph. The quantity of one 45 kDa protein fraction (Figure 2, 3) was increased dose dependently with dietary esculetin while it was significantly unaffected with dexamethasone- and phenidone-treated groups as determined in individual samples as well as mixed samples from each experimental group. Higher dietary concentrations of esculetin led to increase the quantity of the 45 kDa fraction up 60% with significance  $<0.01$  comparing to control and two lower esculetin concentrations (0.001 and 0.01%). The influence of EBIs on expression of the 45 kDa protein was accompanied by changes also by the

small nearest fraction (48 kDa), but less visible and thus we focused on the 45 kDa fraction only. The other proteins including main protein groups such as ApoLP-I and storage proteins were not altered in insects reared on diets amended with EBIs.

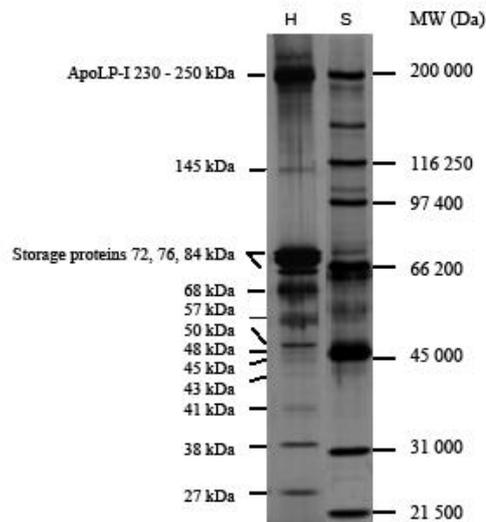


Figure 2. Acrylamide gradient gel of haemolymph proteins extracted from last instar larvae of *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) (H). Main protein groups are described on the left margin according to molecular weight (MW) of protein standards (S).

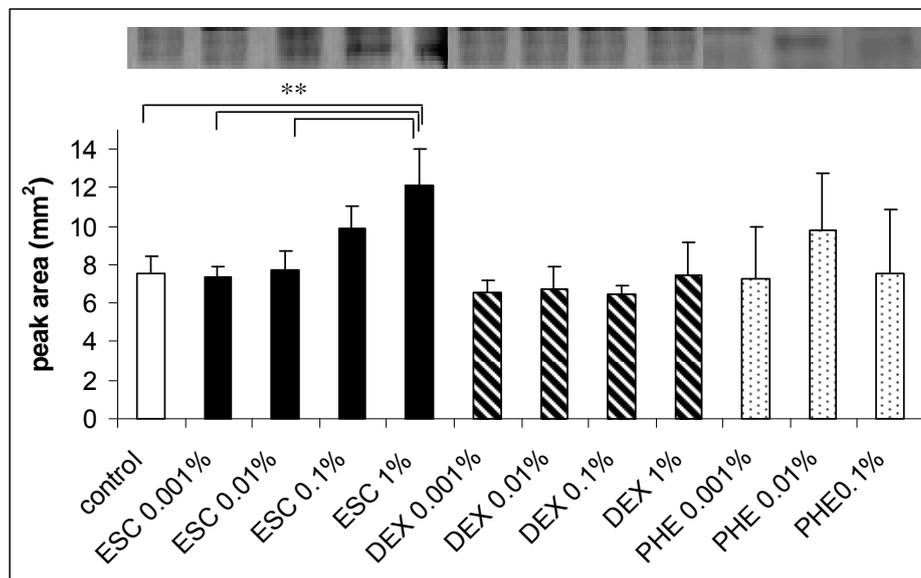


Figure 3. Profile analysis of 45 kDa protein fraction (shown on gel in upper part of the figure) quantified by densitometry. Data are presented as average peak area in mm<sup>2</sup> ± S.D., significance level is <0.01 (\*\*).

## Discussion

We recorded 16 protein fractions in larval haemolymph including a dominant group of protein bands ranging in molecular mass from 72 to 84 kDa (storage proteins according to Miller & Silhacek, 1982; Godlewski et al., 2001). Other well-determined fractions in larval haemolymph are lipophorins, ApoLPI (230-250 kDa) was identified in our study similar to Wiesner et al. (1997) and Halwani et al. (2001). The changes of total protein, free amino acids and some enzymes in midgut of PGF<sub>2 $\alpha$</sub> -treated silkworm, *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) indicate that eicosanoids modulate protein synthesis which might influence on the growth and development of the larvae (Miao & Jiang, 2003). Our experiments show no effect on growth of larvae except one concentration of dexamethasone, thus we suggest that block of LOX pathways by esculetin modulates protein synthesis with no correlation to larval growth. We report significant increase in the 45 kDa protein fraction in the larvae reared on esculetin- and nonsignificantly on phenidone-supplemented diets. Altered protein profiles may be attributed to synthesis of aberrant or stress-related proteins in response to inhibition of eicosanoid biosynthesis, mainly LOX pathway specifically blocked by esculetin. This is supported also with higher dietary concentration of a dual inhibitor of COX and LOX, phenidone (0.01%) led to nonsignificant variation of 45kDa fraction; while all dietary concentrations of a phospholipase A<sub>2</sub> inhibitor, dexamethasone doesn't mark any changes. This could be caused by redundancy of eicosanoid metabolism, where effect of blocking of one pathway can be partly compensated by products of other unaffected enzymes. When the system is impaired by more severe damages, e.g. after using dexamethasone, which inhibits the inception of eicosanoid metabolism – phospholipase A<sub>2</sub> mediated release of arachidonic acid; it can lose also the ability to recover by using alternative products of redundant pathways. Our suggestion is consistent with the results of Stanley et al. (2008) who reported that PGA<sub>1</sub> and PGE<sub>1</sub> modulate gene expression of heat shock proteins (HSPs) in a cell line established from *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) as they play major signalling roles in stress responses in vertebrates (Negishi & Katoh, 2002). They also observed that these PGs mediate gene expression of some cell protection proteins including GST-like protein, GST subunit 2, catalase and superoxide dismutase involved in detoxification function and protection from reactive oxygen metabolites.

COX and LOX products mediate cellular and humoral immune responses in *G. mellonella*, in which indomethacin (COX inhibitor) and esculetin (LOX inhibitor) impaired haemocytic phagocytosis and phenoloxidase cascade (Mandato et al., 1997). On the other hand, LOX products mediate cellular protein synthesis implicated in cell cycle regulation and provide oxidative balance (Sandstrom et al., 1995; Aragno et al., 2001). We infer that haemolymph proteins composition may be influenced mainly by block of LOX

pathways in *G. mellonella*. Although it has not yet been possible to use directly EBIs for insect pest control, our results bring new data to understand physiological signaling systems in insects.

## Acknowledgement

Our research was supported by research grant from Grant Agency of Czech Republic (GA206/09/P470). All authors are contributed equally to this research.

## Özet

### Eikosanoid biyosentezi inhibitörlerinin *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) larvalarının hemolenf protein profiline etkisi

Eikosanoidlerin böceklerin hücresel, humoral immün savunma reaksiyonlarına ve strese karşı tepkilerine aracılık ettiği bilinmektedir. Bu aracı moleküllerin işlevi farklı eikosanoid biyosentezi inhibitörleri tarafından spesifik olarak önlenebilir. Bu inhibitörlerin böceklerde total hemolenf protein bileşimi üzerine etkileri detaylı olarak çalışılmamıştır. Eikosanoidlerin böceklerde stress faktörlerine karşı sentezlenen ve diğer savunma reaksiyonlarından sorumlu proteinler üzerinde etkili olarak fizyolojik dengenin sağlanmasına aracılık ettiği düşünülmüştür. Bu sebeple Büyük bal mumu güvesi *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) larvaları farklı etki mekanizmalarına sahip eikosanoid biyosentezi inhibitörleri eskuletin, deksametazon ve fenidonu % 0.001, 0.01, 0.1 ve 1.0 oranında içeren yapay besinler ile beslenmiştir. Larvaların eskuletin ile beslenmesi 45 kDa protein bileşiminde (belirlenen 16 protein bandından biri) uygulanan inhibitörün dozuna bağlı olarak önemli değişime sebep olmuştur. Diğer başlıca hemolenf proteinlerinden lipoforinler (ApoLP-I) ve depo proteinleri üzerinde denenen eikosanoid biyosentezi inhibitörlerinin önemli bir etkisi olmamıştır. Deksametazon ve fenidon hemolenf proteinlerinde önemli bir değişikliğe sebep olmamıştır. Bu çalışmanın sonuçları eikosanoidlerin, en azından lipoksijenaz ürünlerinin, böcek dokularında yapısal ve işlevsel olarak protein bileşimi üzerinde etkili olduğunu göstermiştir. Eikosanoid biyosentezi inhibitörlerinin doğrudan zararlı böceklerin kontrolünde kullanılması yaygın olmasa da bu çalışmadan elde edilen sonuçlar böceklerde eikosanoidlerin fizyolojik sinyal sistemlerindeki rollerinin anlaşılmasına katkıda bulunmaktadır.

## References

- Aragno, M., S. Parola, E. Rignardello, R. Manti, S. Betteto, E. Tamagno, O. Danni & G. Boccuzzi, 2001. Oxidative stress and eicosanoids in the kidneys of hyperglycemic rats treated with dehydroepiandrosterone. **Free Radical Biology and Medicine**, **31**: 935–942.
- Bronskill, J., 1961. A cage to simplify the rearing of the greater wax moth, *Galleria mellonella* (Pyralidae). **Journal Lepidopterist's Society**, **15**: 102-104.

- Büyükgüzel, E., H. Tunaz, D. Stanley & K. Büyükgüzel, 2007. Eicosanoids mediate *Galleria mellonella* cellular immune response to viral infection. **Journal of Insect Physiology**, **53**: 99-105.
- Dean, P., J. C. Gadsden, E. H. Richards, J. P. Edwards, A. K. Charnley & S. E. Reynolds, 2002. Modulation by eicosanoid biosynthesis inhibitors of immune responses by the insect *Manduca sexta* to the pathogenic fungus *Metarhizium anisopliae*. **Journal of Invertebrate Pathology**, **79**: 3-101.
- Downer, R. G., S. J. Moore, W. Diehl-Jones & C. A. Mandato, 1997. The effects of eicosanoid biosynthesis inhibitors on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella*. **Journal of Insect Physiology**, **43**: 1-8.
- Durmuş, Y., E. Büyükgüzel, B. Terzi, H. Tunaz, D. Stanley & K. Büyükgüzel, 2008. Eicosanoids mediate melanotic nodulation reactions to viral infection in larvae of the parasitic wasp, *Pimpla turionellae*. **Journal of Insect Physiology**, **54**: 17-24.
- Figueiredo, M. B., E. S. Garcia & P. Azambuja, 2008. Blockades of phospholipase A<sub>2</sub> and platelet-activating factor receptors reduce the hemocyte phagocytosis in *Rhodnius prolixus*: In vitro experiments. **Journal of Insect Physiology**, **54**: 344-350.
- García Gil de Muñoz, F. L., J. Martínez-Barnette, H. Lanz-Mendoza, M. H. Rodríguez & F. C. Hernández-Hernández, 2008. Prostaglandin E<sub>2</sub> modulates the expression of antimicrobial peptides in the fat body and midgut of *Anopheles albimanus*. **Archives of Insect Biochemistry and Physiology**, **68**: 14-25.
- Godlewski, J., B. Kłodkiewicz, K. Grzelak & B. Cymborowski, 2001. Expression of larval hemolymph proteins (Lhp) genes and protein synthesis in the fat body of greater wax moth (*Galleria mellonella*) larvae during diapause. **Journal of Insect Physiology**, **47**: 759-766.
- Halwani, A. E., D. F. Niven & G. B. Dunphy, 2001. Apolipoprotein III in the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae). **Archives of Insect Biochemistry and Physiology**, **48**: 135-143.
- Hyršl, P., E. Büyükgüzel & K. Büyükgüzel, 2008. Boric acid-induced effects on protein profiles of *Galleria mellonella* hemolymph and fat body. **Acta Biologica Hungarica**, **59**: 281-288.
- Kirkeby, S., D. Moe & T. C. Bog-Hansen, 1993. The silver staining procedure of sodium dodecyl sulphate-gels may be accelerated by shortening fixation time. **Electrophoresis**, **14**: 51-55.
- Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, **227**: 680-685.
- Mandato, C. A., W. L. Diehl-Jones, S. J. Moore & R. G. H Downer, 1997. The Effects of eicosanoid biosynthesis inhibitors on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella*. **Journal of Insect Physiology**, **43**: 1-8.
- Merchant, D., R. L. Ertl, S. I. Rennard, D. W. Stanley & J. S. Miller, 2008. Eicosanoids mediate insect hemocyte migration. **Journal of Insect Physiology**, **54**: 215-221.
- Miao, Y. & L. Jiang, 2003. Changes in growth and lipid profiles of silk gland, midgut biochemical composition of silkworm, *Bombyx mori* L. on exposure to prostaglandin F<sub>2α</sub>. **Prostaglandins & Other Lipid Mediators**, **70**: 331-338.

- Miller, S. G. & D. L. Silhacek, 1982. The synthesis and uptake of hemolymph storage proteins by the fat body of the greater wax moth *Galleria mellonella* (L.). **Insect Biochemistry**, **12**: 293-300.
- Miller, J. S. & D. W. Stanley, 2001. Eicosanoids mediate microaggregation reactions to bacterial challenge in isolated insect hemocyte preparations. **Journal of Insect Physiology**, **47**: 1409-1417.
- Morishima, I., Y. Yamano, K. Inoue & N. Matsuo, 1997. Eicosanoids mediate induction of immune genes in the fat body of the silkworm, *Bombyx mori*. **FEBS Letters**, **419**: 83-86.
- Negishi, M. & H. Katoh, 2002. Cyclopentenone prostaglandin receptors. **Prostaglandins & Other Lipid Mediators**, **69**: 611-617.
- Sandstrom, P. A., D. Pardi, D. M. Terrian, T. M. Folks & T. M. Buttke, 1995. Lipid hydroperoxide-induced apoptosis: lack of inhibition by bcl-2 over-expression. **FEBS Letters**, **365**: 66-70.
- Stanley, D. W., 2006. Prostaglandins and other eicosanoids in insects: biological significance. **Annual Review of Entomology**, **51**: 25-44.
- Stanley, D. W., C. Goodman, S. An, A. McIntosh & Q. Song, 2008. Prostaglandins A<sub>1</sub> and E<sub>1</sub> influence gene expression in an established insect cell line (BCIRL-HzAM1 cells). **Insect Biochemistry and Molecular Biology**, **38**: 275-284.
- Stanley, D. & M. Shapiro, 2007. Eicosanoid biosynthesis inhibitors increase the susceptibility of *Lymantria dispar* to nucleopolyhedrovirus LdMNPV. **Journal of Invertebrate Pathology**, **95** (2): 119-124.
- Tunaz, H., 2006. Eicosanoid biosynthesis inhibitors influence mortality of *Pieris brassicae* larvae co-injected with fungal conidia. **Archives of Insect Biochemistry and Physiology**, **63**: 93-100.
- Wiesner, A., S. Losen, P. Kopacek, C. Weise & P. Götz, 1997. Isolated apolipoprotein III from *Galleria mellonella* stimulates the immune reactions of this insect. **Journal of Insect Physiology**, **43**: 383-391.

