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# Eicosanoids mediate *Galleria mellonella* cellular immune response to viral infection ☆

Ender Büyükgüzel[a], Hasan Tunaz[b], David Stanley[c,*], Kemal Büyükgüzel[a]

[a]*Department of Biology, Faculty of Arts and Science, Karaelmas University, Zonguldak, Turkey*
[b]*Department of Plant Protection, Faculty of Agriculture, Kahramanmaraş Sütçü İmam University, Kahramanmaraş, Turkey*
[c]*USDA/Agricultural Research Service, Biological Control of Insects Research Laboratory, 1503 S. Providence Road, Columbia, MO 65203, USA*

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**Abstract**

Nodulation is the predominant insect cellular immune response to bacterial and fungal infections and it can also be induced by some viral infections. Treating seventh instar larvae of greater wax moth *Galleria mellonella* with *Bovine herpes simplex virus*-1 (BHSV-1) induced nodulation reactions in a dose-dependent manner. Because eicosanoids mediate nodulation reactions to bacterial and fungal infection, we hypothesized that eicosanoids also mediate nodulation reactions to viral challenge. To test this idea, we injected *G. mellonella* larvae with indomethacin, a nonsteroidal anti-inflammatory drug immediately prior to intrahemocoelic injection of BHSV-1. Relative to vehicle-treated controls, indomethacin-treated larvae produced significantly reduced numbers of nodules following viral infection (down from approximately 190 nodules/larva to $<50$ nodules/larva). In addition to injection treatments, increasing dietary indomethacin dosages (from 0.01% to 1%) were associated with decreasing nodulation (by 10-fold) and phenoloxidase activity (by 3-fold) reactions to BHSV-1 injection. We infer from these findings that cyclooxygenase products, prostaglandins, mediate nodulation response to viral infection in *G. mellonella*.


*Keywords:* *G. mellonella*; Cellular immunity; Nodulation; Indomethacin; Phenoloxidase

## 1. Introductiom

Insects rely on innate immune mechanisms, comprised of cellular and humoral responses, for recognizing and protecting themselves from pathogens (Stanley, 2005; Hoffmann, 2003). Cellular (or hemocytic) immune responses include nodulation (viral, bacterial and fungal spore infections), encapsulation (organisms larger than hemocytes and parasitoid eggs) and phagocytosis (Strand and Pech, 1995). These responses involve direct interaction between circulating hemocytes and invading microorganisms. Humoral immune responses involve the induced biosynthesis of various antibacterial and antifungal proteins, and induction of the prophenoloxidase (PPO) cascade (Gillespie et al., 1997, 2000; Hoffmann, 2003). Nodulation is the predominant cellular defense reaction to bacterial infection in insects (Stanley and Miller, 2006). It is a complex process that begins with microaggregation of hemocytes, which entrap large numbers of microorganisms. These micro-aggregates grow by recruiting additional hemocytes. Eventually a final layer of plasmatocytes surrounds the nodules and melanization occurs. The darkened nodules become attached to the body wall or to various internal organs of insects (Horohov and Dunn, 1983).

While research into the mechanisms of humoral immunity has led to discovery of novel intracellular signal transduction systems (Hoffmann, 2003), far less is known about mediators of insect cellular immunity. Stanley-Samuelson et al. (1991) suggested that eicosanoids mediate one or more cellular reactions responsible for clearing bacterial infections from hemolymph circulation. Eicosanoids are oxygenated metabolites of polyunsaturated fatty acids, especially arachidonic acid. Eicosanoid chemical

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*Corresponding author. Tel.: +1 573 875 5361; fax: +1 573 875 5364.
*E-mail address:* stanleyd@missouri.edu (D. Stanley).

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structures and biosynthetic pathways are detailed elsewhere (Stanley, 2000, 2005). These compounds mediate a broad range of physiological functions in vertebrates and invertebrates (Stanley, 2000). Miller et al. (1994) suggested that eicosanoids mediate nodulation reactions to bacterial infections. This idea has been tested by several research groups in over 20 insect species. Eicosanoids mediate nodulation reactions to challenge by bacteria, fungi (Dean et al., 2002; Lord et al., 2002) and protozoans (Garcia et al., 2004; reviewed in Stanley, 2005, 2006; Stanley and Miller, 2006). Eicosanoids may also mediate encapsulation reactions to parasitoid challenge (Carton et al., 2002).

Eicosanoids influence several aspects of cellular immunity. Mandato et al. (1997) found that cell spreading, a distinct phase of nodulation, and phagocytosis are mediated by eicosanoids in larval waxmoths, *Galleria mellonella*. Miller (2005) reported that eicosanoids mediate cell spreading in hemocytes isolated from tobacco hornworms, *Manduca sexta*. Eicosanoids also mediate microaggregation reactions (another step in nodulation) to bacterial challenge (Miller and Stanley, 2001; Phelps et al., 2003; Miller and Stanley, 2004). Aside from direct cell actions, Bundey et al. (2003) reported that eicosanoids mediate behavioral fever responses to infection in the locusst *Schistocerca gregaria*. The eicosanoid hypothesis is also supported by two lines of work on humoral immunity. Morishima et al. (1997) found that biosynthesis of antibacterial proteins also depends on eicosanoids in the silkworm, *Bombyx mori*. And Yajima et al (2003) reported a functional coupling between the immune deficiency pathway and eicosanoid biosynthesis in *Drosophila*. We surmise eicosanoids are crucial elements in insect immunity.

While cellular protection from bacterial, fungal, protozoan and parasitoid challenges has been recorded, insect cellular immune reactions to viral challenge is an emerging field. We are investigating the hypothesis that eicosanoids also influence insect immune reactions to viral challenge. Using *Bovine Herpes Simplex Virus*-1 (BHSV-1) and larvae of the greater wax moth, *G. mellonella*, we found that wax moth larvae form hemocytic nodules in response to viral infection and this anti-viral nodulation reaction is mediated by eicosanoids.

## 2. Materials and methods

### 2.1. Insects

Waxmoth, *G. mellonella,* larvae and pupae were collected from infected hives in apicultural areas around Zonguldak, Turkey. The newly emerged adults were used to maintain a stock insect culture. The adults were placed in 1000-mL glass jars and provided with synthetic diet (Bronskill, 1961) to lay eggs. The diet was composed of bran (420 g) filtered honey (150 mL), glycerol (150 mL), ground old dark honeycomb (20 g) and distilled water (30 mL). Neonate larvae were reared in similar glass jars at $30 \pm 1\,^{\circ}C$ and constant dark in an incubator (Nüve ES500 Nüve Co. Ankara, Turkey). Seventh-instar larvae (250–350 mg each) were used for experiments and as a source of hemolymph samples.

### 2.2. Virus

Stock BHSV-1 was kindly donated by Dr. Aykut Özkul (Veterinary Faculty, Ankara University). The virus stock was kept at $-70\,^{\circ}C$ until needed. The concentrations of virus solutions ($4 \times 10^7$, $4 \times 10^5$, $4 \times 10^3$ plaque-forming units (PFU)/ml) were prepared by serially diluting the original liquid suspension with distilled water. Dilutions of the stock solution were made immediately before injection.

### 2.3. Reagents

The COX-1 and COX-2 inhibitor, indomethacin (1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid), dopamin, bovine serum albumin (BSA), and Folin-Ciolcalteu Reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.4. Injections and nodulation assay

Insects were challenged by injection to ensure precise specification of challenge dosages. Before injection, larvae were anesthetized by chilling them on ice for 5 min and surface sterilized by swabbing their cuticles with 95% ethanol (EtOH). Injections were carried out with a 25 μL Hamilton micro-syringe (Hamilton, Reno, NV). Injections were performed dorso-laterally in the intersegmental region between last two abdominal segments, with the pharmaceutical treatments and BHSV-1 challenge doses on opposite sides. The abdomen was palpated gently after injection to mix the contents of the haemocoel. Preliminary results showed that EtOH was not lethal for the *Galleria* larvae (İçen et al., 2005) and indomethacin was dissolved in EtOH at 10 mg/mL.

Control insects were injected with 70% EtOH (5 μL) and experimentals with indomethacin (50 μg/5 μL EtOH). All larvae were immediately challenged with BHSV-1 in a standard dosage of $2 \times 10^5$ PFU in 5 μL of culture medium, except in dose–response experiments, following the injection protocols of Miller and Stanley (1998). We assessed nodulation at selected times after injections. The larvae were anesthetized by chilling them on ice, then their hemocoels were exposed to count melanized, brownish black nodules under a stereomicroscope at 45x. After initial counting, the alimentary canal was removed and nodules in the remaining internal tissues were then counted. The nodules were distinct and direct counting reliably reflected the extent of the nodulation response to infections (Miller and Stanley, 1998).

### 2.5. Control experiments

Several control experiments were conducted to determine the level of background nodulation in larvae. Because the larvae were reared in nonsterile conditions, control experiments (no treatment) were performed to register the background number of nodules in the larvae. Nodulation in unchallenged larvae was assessed by randomly taking 10 larvae from culture, chilling them on ice, and counting the nodules, as described above. The effect of wounding on nodulation was determined by wounding 10 larvae with the needle of the micro-syringe. Nodulation was assessed 4 h post-injection (PI). The effect of COX inhibitor, indomethacin, BHSV-1 and their solvent vehicles, ethanol and distilled water, respectively, was tested in unchallenged larvae in the same concentration (50 μg/larva for indomethacin and $2 \times 10^5$ PFU/larva for virus) and volume (5 μL/larva for both solvents) as used for challenge larvae. Nodulation was assessed 4 h PI.

### 2.6. Time course of nodulation: Influence of BHSV-1

Four groups of larvae ($n = 10$) were chilled on ice and injected with 5 μL BHSV-1 ($2 \times 10^5$ PFU/larva) as described. The number of nodules was counted at 2, 4, 6, and 8 h PI.

### 2.7. Dose–response curve for BHSV-1

For the dose–response curve, four groups of larvae ($n = 10$) were chilled on ice and injected with 5 μL BHSV-1 suspension containing 20, $2 \times 10^3$ and $2 \times 10^5$ PFU/larva. Nodulation was assessed 4 h later as described. Control larvae were injected with distilled water.

### 2.8. Influence of indomethacin on nodulation

Four groups of larvae ($n = 10$) were tested. A first group was injected with 5 μL ethanol (70%) as control. The second group was injected with 5 μL BHSV-1 ($2 \times 10^5$ PFU /larva)). Third group was injected with 5 μL indomethacin (50 μg/larva). Fourth group was injected with 5 μL indomethacin (50 μg/larva) and then injected with BHSV-1 ($2 \times 10^5$ PFU/larva). Nodulation was assessed 4 h later by chilling them on ice and counting the nodules, as described above.

### 2.9. Oral treatment with indomethacin and BHSV-1 inoculations

Appropriate amounts of indomethacin were dissolved in glycerol (a diet ingredient) and then mixed with the remaining diet ingredients to achieve the final concentrations of 0.01, 0.1 and 1.0 g of indomethacin/100 g diet.

First instar larvae of *G. mellonella* were reared on these artificial diets up to seventh instar. A group of larvae were reared on diet without indomethacin as control (no treatment).

Groups of seventh-instar larvae from these diets were inoculated with BHSV-1 ($2 \times 10^5$ PFU/larva) by intrahemocoelic injection as described. At 4 h PI, nodulation was assessed. Each feeding experiment was replicated 4 times with 10 larvae. Hemolymph from all groups of larvae was also collected for determination of phenoloxidase (PO) activity. Melanization was assessed visually and the presence of melanization indicated as (+).

### 2.10. Collection of hemolymph

Larvae were chilled on ice for 5 min and surface sterilized in 95% ethanol. Hemolymph was collected by cutting the second proleg with sterile micro-scissors and drawing the hemolymph into an Eppendorf tube. Ten microliters of hemolymph could be collected from single larvae weighing 150–200 mg each. Hemolymph was dispensed into 1.5-mL microcentrifuge tubes, diluted 1:10 (v/v) in 90 μL of 100 mM sodium phosphate buffer, pH 7, and centrifuged at 10,000*g* for 5 min at 4 °C to pellet hemocytes. Clear supernatant was used in PO activity assays. Protein concentrations in the supernatants were determined by the method of Lowry et al (1951) using BSA as quantitative standard.

### 2.11. Analysis of PO activity

PO (EC1.14.18.1) activity was assayed by using a method modified from from Hall et al. (1995) and Hartzer et al. (2005). Diluted hemolymph (50 μL) was added to 1.5 mL glass spectrophotometer cuvettes containing 1 mL phosphate buffer (100 mM, pH 7.0). After 20 min, 100 μL dopamine (10 mM in sodium phosphate buffer) was added and PO activity (mOD/min) was determined by measuring absorbance at 492 nm at 5-min intervals for 30 min at 30 °C using a UV/Visible spectrophotometer (Shimadzu 1700, Kyoto Japan). Enzyme activity is expressed in absorbance unit (au)/min/mg protein at 492 nm.

### 2.12. Statistical analysis

Data were analyzed by one-way ANOVA. To determine significant differences between means the least significant difference (LSD) test (PROC GLM, SAS Institute and Inc, 1989) was used. When the $F$ estimate exceeded the probability of 0.05 the differences were considered significant.

## 3. Results

### 3.1. Control experiments

The results of the control experiments are displayed in Table 1. Generally, experimental, viral-challenged larvae produced far more nodules (ca. 190 nodules/larva) than any of the control treatment groups (< 40 nodules/larva).

Table 1
The results of background control experiments

| Treatment | Concentration | Nodules | Melanization |
|---|---|---|---|
| Nontreatment | | 12.1±1.9 | − |
| Ethanol | | 35.8±6.2 | + |
| Distilled water | | 25.2±4.3 | − |
| Viral culture media | | 7.3±1.5 | − |
| Injection wound | | 8.9±1.6 | − |
| Indomethacin | 50 μg/larva | 15.4±2.3 | − |
| Viral challenge | ($2 \times 10^5$ PFU/larva) | 189.5±16.7 | + |

Seventh-instar *G. mellonella* larvae were treated as indicated and nodulation was assessed 4 h later as described in M&Ms. Nodulation values are mean numbers of nodules (±SE, $n = 10$ larvae in all treatments). The presence of melanization is indicated by + and −, determined by visual inspection.

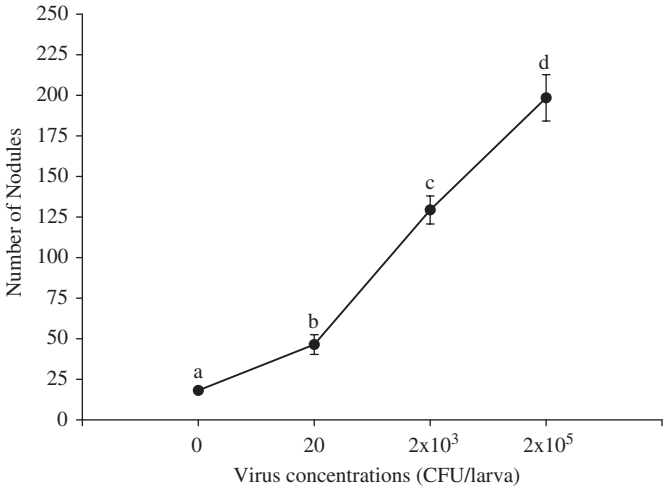


Fig. 1. The influence of increasing BHSV-1 concentrations on nodulation reaction in *G. mellonella* larvae. Larva were injected with the indicated concentrations of BHSV-1 (distilled water for zero). Nodulation was assessed at 4 h PI as described in M&Ms. Each point is the mean (± SE) of 4 replications for each treatment with 10 larvae. Means annotated with the same letter are not significantly different ($P > 0.05$ (LSD test).

### 3.2. Dose–response curve for BHSV-1

The data presented in Fig. 1 indicate that nodulation reactions to BHSV-1 challenge were expressed in a dose-dependent manner. We registered about 50 nodules/larva at 20 PFU/larva which increased to approximately 198 nodules/larva at the highest dose of $2 \times 10^5$ PFU/larva. For larvae treated with high viral doses, we frequently noted nodules in the fat body and tissues surrounding the midgut as well as tightly grouped nodule accumulations in tissues around the posterior midgut.

### 3.3. Time course of nodulation: Influence of BHSV-1

We investigated the influence of incubation time on nodule formation (Fig. 2). Nodulation was clearly visible by as soon as 2 h PI (ca. 130 nodules/larva). We recorded highest nodulation (ca. 190 nodules/larva) at 4 h PI. Longer incubation periods did not yield higher numbers of nodules, which appeared to decline by 8 h PI. In subsequent experiments nodulation was assessed 4 h PI.

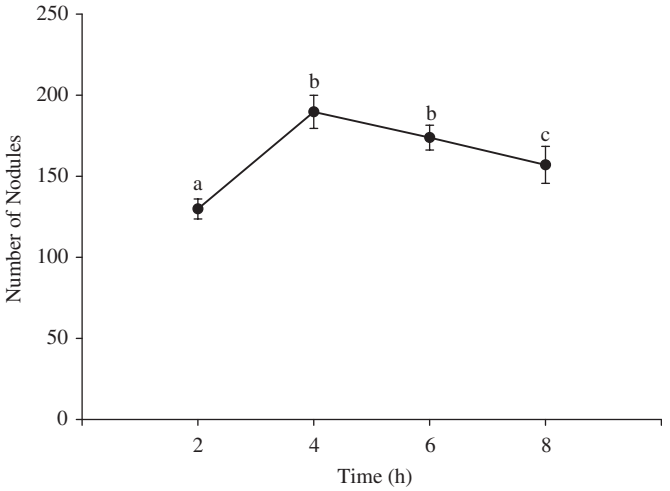


Fig. 2. Time course for nodulation reactions in response to BHVS-1 injection in larvae of *G. mellonella*. Larvae were injected with BHSV-1 ($2 \times 10^5$ PFU/larva). At the indicated times PI nodulation was assessed as described in M&Ms. Each point is the mean (± SE) of 4 replications for each treatment with 10 larvae. Means annotated with the same letter are not significantly different $P > 0.05$ (LSD test).

### 3.4. Influence of indomethacin on nodulation

The data presented in Fig. 3 indicates that larvae challenged with the standard viral dose produced about 198 nodules/larva. Contrarily, nodulation reactions were strongly attenuated in larvae treated by co-injection of indomethacin and BHSV-1 (ca. 44 nodules/larva).

### 3.5. The influence of per os indomethacin treatments on nodulation and PO activity

Larvae reared on media amended with increasing indomethacin concentrations were immunocompromised in terms of nodulation and PO reactions to our standard viral challenge. We recorded decreasing numbers of nodules, reduced PO activity and decreased melanization as a function of increasing dietary indomethacin concentrations (Table 2). The influence of indomethacin was expressed in a dose-dependent manner, with significant declines in immunocompetence at each dose. Nodulation decreased by an order of magnitude from over 200 nodules/larva in larvae reared in the absence of indomethacin to about 20 nodules/larva in larvae reared on medium supplemented with 1% indomethacin. Similarly, hemolymph PO activity declined >3-fold in larvae reared on media amended with 1% indomethacin.

## 4. Discussion

The data reported in this paper support the idea that BHSV-1 challenge stimulates eicosanoid-mediated nodulation reactions and PO activation in *G. mellonella* larvae. Several points are relevant. First, viral challenge provoked nodulation responses that increased in a dose-dependent manner with increasing viral load. Second, the time-course experiment indicates that numbers of nodules increased with incubation time to a maximum of about 200 nodules/larvae at 4 h PI. Third, compared to control treatments, nodulation was severely impaired in larvae treated with the eicosanoid biosynthesis inhibitor (EBI), indomethacin. Finally, nodulation, PO activity and melanization were impaired in a dose-dependent way in larvae treated *per os* with increasing concentrations of indomethacin prior to infecting them with the virus. We infer from these data that eicosanoids mediate *G. mellonella* nodulation response to viral infection. There is now considerable evidence for the involvement of eicosanoids in immune reactions to bacteria, fungal, protozoan and parasitoid challenge in a phylogenetically wide range of insects. The data in this report extends the eicosanoid hypothesis to nodulation reactions to viral challenge.

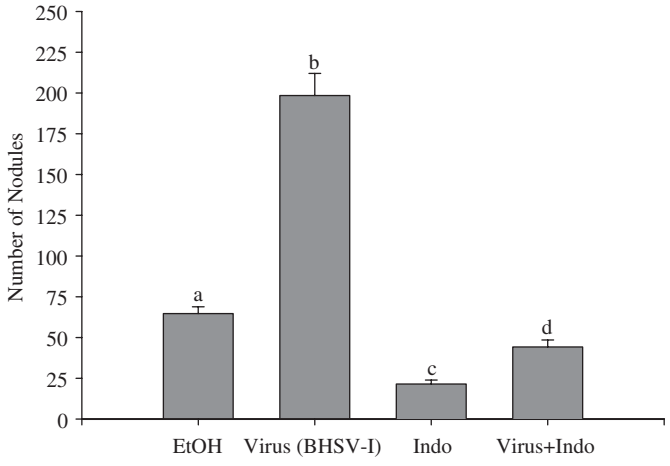


Fig. 3. Influence of indomethacin on BHSV-1 induced nodulation reactions in *G. mellonella* larvae. One group of larvae (EtOH) was injected with 5 μL 70% ethanol as control. A second group (Virus, BHSV-1) was injected with BHSV-1 ($2 \times 10^5$ PFU/larva)). A third group (Indo) was injected with indomethacin (50 μg/larva). A fourth group (Virus + Indo) was treated with indomethacin (50 μg/larva) prior to BHSV-1 ($2 \times 10^5$ PFU/larva) injection. The histogram bars represent means (±SE) of 4 replicates in each treatment. Means topped by the same letter are not significantly different $P > 0.05$ (LSD test).

Most research using EBIs as probes to assess possible roles of eicosanoids in insect immunity involved treating experimental insects by injecting EBIs into the hemocoels. We tested the hypothesis that treating test larvae with indomethacin *per os* would similarly impair nodulation reactions to viral challenge. Our data indicate that orally administered indomethacin strongly impaired nodulation reaction and PO activation in *G. mellonella* larvae. Orally administered EBIs also impaired immune reactions in the termite *Coptotermes formosanus* (Connick et al., 2001) and in the blood-sucking bug *Rhodnius prolixus* (Garcia et al., 2004). On the other hand, in their characterization of prostaglandin (PG) biosynthesis in whole houseflies, *Musca domestica*, Wakayama et al. (1986) found that various EBIs inhibited PG biosynthesis when included in enzyme preparations but not when added to the housefly diets. In their pioneering study of PG biosynthesis in the reproductive tissues of male crickets *Acheta domesticus*, Destephano et al. (1976) reported that indomethacin did not inhibit PG biosynthesis. Contrarily, Murtaugh and Denlinger (1982) reported that indomethacin and other COX inhibitors reduced PG biosynthesis in *A. domesticus* testes preparations. In our hands, indomethacin and naproxen strongly inhibited PG biosynthesis in cricket testes preparations (Tunaz, Büyükgüzel and Stanley, unpublished observations). We infer that most pharmaceutical EBIs known from biomedical research effectively inhibit eicosanoid biosynthesis in insect preparations, including *per os* treatment in whole animals.

The role of eicosanoids in PO activation appears to vary across insect species. Our data indicate that BHSV-1 challenge stimulates PO activation and the activation is attenuated in *G. mellonella* larvae treated with EBIs. This accords with the work of Mandato et al. (1997) who demonstrated that EBIs attenuated the PO activity in *G. mellonella* challenged with bacteria. Our visual observations

Table 2
The influence of orally administered indomethacin on larval *G. mellonella* nodulation reactions, PO activity and melanization reactions to intrahemocoelic BHSV-1 ($2 \times 10^5$ PFU/larva) challenge

| Indomethacin (g/100 g diet) | Number of larvae | Nodules/larva | PO activity | Melanization |
|---|---|---|---|---|
| 0.0 | 10 | 225.9 ± 9.4a | 1.67 ± 0.07a | + |
| 0.01 | 10 | 115.8 ± 5.7b | 1.02 ± 0.05b | + |
| 0.1 | 10 | 88.4 ± 6.4c | 0.61 ± 0.04c | ± |
| 1.0 | 10 | 20.9 ± 3.2d | 0.49 ± 0.05d | − |

Larvae were reared on media amended with the indicated amount of indomethacin. Seventh instars were challenged by injection with BHSV-1 and immune parameters were recorded at 4 h PI. Each experiment was replicated 4 times with 10 larvae/replication. Means (±SE with the same letter are not significantly different ($P < 0.05$).

of reduced melanization support the idea that indomethacin inhibits PO activation in *G. mellonella*. Garcia et al. (2004) similarly found that oral EBI treatments reduced PO activity in *R. prolixus* challenged with the protozoan parasite *Trypanosoma rangeli*. On the other hand, EBIs did not influence PO activation in *M. sexta* larvae challenged with spores of the fungus *Beauveria bassiana* (Lord et al., 2002). This finding aligns with the work of Goldsworthy et al. (2003), who reported that eicosanoids mediate cellular immune reactions, but not PO activation, to challenge with laminarin in *Locusta migratoria*.

Let us turn, now, to the rapidly growing area of insect defenses against viral infection, where we press the point that eicosanoids potentially act in a range of defense mechanisms. Insects express several defense reactions to viral challenge, although these reactions are not expressed to a similar extent in all species. One of the most extensively studied reactions to viral challenge is apoptosis and midgut cell sloughing (Clem, 2005). Popham et al. (2004) suggested that constitutive plasma PO of larval lepidopterans may act as a antiviral defense. In some insect species resistance to baculovirus infection is mediated by dietary phytochemicals. For example, larvae of *Heliothis virescens* reared on cotton were more resistant to baculovirus infection than larvae reared on lettuce (Hoover et al., 2000). The authors inferred that sloughing of infected midgut cells (a major mechanism of increasing resistance to baculoviral attack) took place at higher rates in larvae reared on cotton. Insects also protect themselves from viral infection by hemocytic encapsulation of virus-infected cells (Washburn et al., 1996). The nodules recorded here appear to emerge from this encapsulation process.

Two newly discovered anti-viral mechanisms highlight the novelity and future potentials that will emerge from research in insect/virus interactions. Ponnuvel et al. (2003) isolated a lipase from the digestive fluids of the silkworm *B. mori*. The gene for this lipase, *Bmlipase-1*, is expressed solely in midgut, from which the authors concluded that a digestive lipase may also act as a physiological deterrent to viral infection. More attention has been devoted to study of anti-viral RNA silencing, which may occur in all eurykaryotic cells (Lu et al., 2004; Saumet and Lecellier, 2006), although it may not be so for mammalian cells (Cullen, 2006). While the detailed mechanisms of RNA silencing can be drawn from these citations, the authors note that specific silencing of infecting viral RNAs is induced naturally in insect cells. The silencing pathways are of such evolutionary significance that virally encoded suppressors of RNA silencing have evolved and are necessary for viral infection (Li et al., 2002).

Eicosanoids, particularly PGs act in virus–cell interactions. In terms of inhibitory actions, Amici et al. (1994) reported that $PGA_1$ inhibits viral protein biosynthesis. They used green monkey kidney cells infected with Sendai virus to demonstrate that $PGA_1$ induced HSP-70 synthesis, which in turn inhibited Sendai virus protein biosynthesis. Conti et al. (2001) reported a similar finding with the influenza A virus Ulster 73 in LLC-MK2 cells. Turning to another PG action, Zhu et al. (2002) found that elevated levels of $PGE_2$ are necessary for human cytomegalovirus replication in human fibroblasts. Depending on the particular virus–cell system, PGs are necessary for viral replication or for inhibition of viral replication.

PGs also act in inhibition of viral replication in insect cells. Barbosa and Rebello (1998) indicated that $PGA_1$ treatments inhibited Mayaro virus replication in a cell line established from the mosquito *Aedes albopictus*. Mayaro virus is the agent of Mayaro fever, a nonlethal disease of humans vectored by mosquitoes in the genus *Haemagogus* (Torres et al., 2004). Hence, it seems reasonable to propose that PGs act in insect anti-viral events at the cellular and intracellular levels.

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