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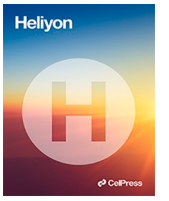

Research article

Immune modulation by dexketoprofen trometamol, a selective eicosanoid biosynthesis inhibitor of cellular immune response and phenoloxidase reaction in response to viral infection in *Pimpla turionellae* adults

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A B S T R A C T

Nodulation is the first immune defence mechanism related to melanisation in response to microbial infections in insects. Adult parasitoid insects have been hypothesised to produce nodules with melanisation in response to viral infections and, eicosanoids, to mediate nodulation reactions and phenoloxidase (PO) activation in this type of infections. To test this hypothesis, endoparasitoid *Pimpla turionellae* adults were first inoculated with a novel generation nonsteroidal anti-inflammatory drug (NSAID) dexketoprofen trometamol (DT) (5 μg/adult), which is a selective cyclooxygenase-1 (COX-1) inhibitor. These adults were then immediately injected with intrahaemocoelic injection of Bovine *herpes simplex virus-1* (BHSV-1) as a model insect-virus interaction. Additionally, adults were fed on artificial diet with increasing concentrations of DT (0.001, 0.01, or 0.1 g/100 ml diet) *per os* prior to intrahaemocoelic injection of BHSV-1 (2×10^3 PFU/adult) and nodulation and PO activity were recorded at 2 h post inoculation (PI). BHSV-1-treated newly emerged adults fed with inhibitors showed low levels of nodulation and increased PO enzyme activity. DT-treated *Pimpla* adults produced significantly fewer nodules (approximately nine nodules/adult), whereas viral infection provoked nodules (approximately 33 nodules/adult) in comparison with needle (vehicle)-treated controls (approximately five nodules/adult). Increasing dietary dexketoprofen trometamol concentrations decreased nodulation (by 12-fold at the highest concentration) and increased PO reactions (by approximately 3-fold at the highest concentration) to BHSV-1 injection. Compared with control adults, adults orally fed on the lowest DT concentration (0.001 %) significantly increased PO activity (1.22 ± 0.23–2.74 ± 0.31 unit/min/mg protein) while nodules significantly decreased (43.19 ± 4.26–17.84 ± 2.19) in response to virus infections. These findings suggest that eicosanoid biosynthesis, at least in the context of prostaglandins (PGs) formed by COX-1, mediates nodulation reactions and PO activation in response to viral infection in adults of this endoparasitoid. This is the first demonstration that the immune response of *P. turionellae* adults to viral pathogens is modulated by DT, which initiates haemolymph PO activation.

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1. Introduction

Insects rely on cellular responses as part of their innate immune system, along with humoral responses, as a defence against pathogenic infections and other abiotic invaders [1]. Cellular immune responses such as haemocyte-mediated immune functions include nodulation, encapsulation, and phagocytosis [2]. These reactions comprise a crucial interaction between haemocytes in the circulation and attack by microbial infection agents such as bacteria, viruses, and other biotic factors. Humoral immune responses include the production of antibacterial and antifungal proteins in response to bacteria and fungi and the activation of prophenoloxidase (PPO) [3,4]. Nodule formation is the predominant haemocytic defense response, producing microaggregates of haemolymph cells that entrap many bacteria, viruses, and fungi in insects [5,6]. These microaggregations are enlarged by the movement of many other circulating haemocytes. Eventually, plasmatocytes surround the nodules as the last cellular layer, and melanisation occurs around the nodules. These darkened nodules are fixed on the walls of various internal organs in insects [7,8].

Although studies to unravel the mechanisms underlying the humoral immune system have led to the characterisation of novel biomolecules involved in immune signalling mediators [9,10], the mechanisms of mediators in insect haemocyte-mediated immune defense remain unclear. Pioneers of insect immune mediators (such as eicosanoids), Stanley-Samuelson et al. [11] were the first to suggest that eicosanoids are potent mediators of cellular responses involved in clearing microbial invaders, mainly bacterial infections, from the haemolymph. Eicosanoids play crucial roles as mediators of physiological systems in all animals [12]. Eicosanoid biosynthesis is required for nodule formation to defend against bacterial [13,14] and fungal infections [15] and protozoal attacks on insects [5,16]. Carton et al. [17] showed that eicosanoids, especially prostaglandins (PGs), may also be crucial mediators of the encapsulation of parasitoid attacks by host insects. Approaches which entail exploiting the immune system of insects with the aim of developing biological control programs have been developed [18,19]. For example, the endoparasitoid, *Pimpla turionellae* L., attacks many lepidopteran pest insect pupae and is used as an important biological control agent alone or in combination with chemical control agents as an integrated pest management program.

Haemolymph phenoloxidase (PO) activity in lepidopteran insect larvae ensures resistance to baculoviral infections and other single nucleopolyhedrosis viruses (HzSNPV) [20-22]. Several *in vitro* studies have indicated that the haemolymph PO action of the tobacco budworm *Heliothis virescens* (F.) shows a virucidal action on some vertebrate viruses, including *herpes simplex virus*, vesicular stomatitis virus, parainfluenza-3, coxsackie B3, Sindbis virus, and HIV-1 [23,24]. Eicosanoids mediated PO enzyme activation and nodulation reactions in greater wax moths *Galleria mellonella* larvae and its endoparasitoid *P. turionellae* challenged with *Bovine herpes simplex virus*-1 (BHSV-1) [8,18]. Therefore, eicosanoids may also mediate PO-dependent humoral and haemocytic immune reactions to viral infection in parasitoid insects.

Pharmacological inhibitors have different effects. Unlike conventional nonspecific and nonsteroidal anti-inflammatory drugs (NSAIDs) including indomethacin, naproxen, and ibuprofen [14,25,26] which exhibit antiviral activity to highly virulent human viruses, such as herpes viruses [27-30] and drive epithelial damage andnecrosis in insects [31], the effect of DT has been investigated in this study, which is a selective and most active COX-1 inhibitor [32] that has no antiviral activity and does not cause gastric damage. Eicosanoid biosynthesis can be modulated by different eicosanoid biosynthesis inhibitors (EBIs) which have a selective mode of action on cyclooxygenase (COX) enzymes, to effectively manage pest control. Conventional nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g. indomethacin, aspirin, and ibuprofen) are nonselective COX-1 and COX-2 inhibitors that induce the formation of gastric and duodenal lesions [32]. Dexketoprofen trometamol (DT) is a nonsteroidal anti-inflammatory drug which is a selective COX-1 inhibitor used to treat pain in mammals [33]. This feature prioritises DT as an important candidate with the least environmental impact in the fight against pests by selectively disrupting eicosanoid biosynthesis.

Haemocyte-mediated cellular immune responses related to PO activity in microbial, protozoan, and possibly parasitoid infections have been observed in most lepidopteran species. However, insect immune responses to viral infections have not been comprehensively elucidated. Recently, eicosanoids were suggested to be mediators for melanotic nodulations to BHSV-1 infection in larvae [18] and adults [14] of the highly diverse endoparasitoid, *P. turionellae*. These parasitoids acquire viral infections from host plants while feeding on their nectar [34]. This study aimed to test the hypothesis that eicosanoids also mediate the cellular reactions and PO activation of adult hymenopteran parasitoids in response to a to viral infection. Therefore, a system consisting of a model virus (BHSV-1) and a selective COX-1 pharmaceutical DT was used. This study reported that viral challenge crippled haemocytic nodulation and induced PO action in *P. turionellae* adults; these processes were mediated by eicosanoids, at least in the context of PG biosynthesis modulated by a selective COX-1 inhibitor, DT.

2. Materials and methods

2.1. Insects

To maintain stock culture of *P. turionellae* L., adults were reared on the pupae of the greater wax moth, *G. mellonella* L., at 23 ± 1 °C, 75 ± 5 % RH.; these adults were reared in laboratory conditions using a photoperiod of 16:8 (LD) h. After the females laid eggs inside the host pupae, the hatched wasp larvae developed inside the pupae of *G. mellonella*. Adults were fed on filtered honey solution (50 %) and host pupal haemolymph every other day to maintain *Pimpla* stock culture. Newly emerged virgin female adults which had not been previously fed were used for injection and *per os* experiments. Female adults were used in the nodulation response and PO action experiments because of their importance in the biological control of pest insects.

2.2. Virus and reagents

Stock BHSV-1 was stored in liquid nitrogen at −86 °C until subsequent use. The viral concentration (2×10^3 plaque-forming units (PFU)/ml) that severely impaired nodule formation and PO action in *P. turionellae* adults in our previous study [14] was used for viral infection and prepared by serially diluting the original liquid suspension (4×10^3 PFU) with distilled water, as described previously [14]. The original solution was diluted immediately prior to injection.

Selective COX-1 inhibitor DT (≥98 %, white crystalline powder, IC50 of 1.9 nm for COX-1, DXTD 220026, and PHR2626, Saurav Chemicals Limited, India) was obtained from Berko Drug and Chemical Ind. Inc. İstanbul, Turkey. Finally, Folin–Ciocalteu reagent, dopamine, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Injections and nodulation assay

Adults were chilled on ice for 5 min and their surfaces were sterilised by swabbing their cuticles with ethanol (95 %) before injection. Injections were administered using a 10 μl micro-syringe (Hamilton, Reno, NV); specifically, they were performed dorso-laterally in the intersegmental region between last two abdominal segments. Doses of DT and BHSV-1 were injected on opposite sides. The abdomen was palpated gently after the injection to mix the contents of the haemocoel. DT was dissolved in distilled water as a stock solution as it is freely soluble in water at 1.0 mg/ml as stock solution.

Control adult insects were injected with distilled water and experimentals with DT (5 μg/5 μl distilled water/adult). Adults were immediately challenged with BHSV-1 in a standard dose of 2×10^3 PFU in 5 μl of viral culture medium by the injection per previously published protocol [7,14]. Nodulation at 2 h post-injection (PI) was assessed which caused the maximum nodulation reaction, as previously determined by Büyükgüzel [14] for the same virus (BHSV-1) and concentration (2×10^3 PFU). The adults were chilled on ice and their internal tissues were dissected to count the melanised, brownish-dark nodules under a stereomicroscope at 45x. After the initial counting, the alimentary canal was removed and the remaining nodules in the tissues were reported. The nodules were prominent and direct counting reliably reported the extent of the nodulation response to infection, as outlined by Miller and Stanley [7].

2.4. Control experiments

The background level of adult nodulation was determined using seven control experiments. No treatment experiments were conducted to determine the background number of nodules in the adults as they were grown under non-sterile conditions. Randomly

Table 1
Composition of the diet used to rear *P. turionellae* adults.

Constituent	mg/100 ml diet	Constituent	mg/100 ml diet
L-Amino acid mixture	**3000.00**	**Lipids**	**540.96**
Alanine	210.00	Cholesterol	138.84
Arginine-HCl	150.00	Linoleic acid	8.03
Aspartic acid	195.00	Linolenic acid	25.55
Cystein-HCl	39.00	Oleic acid	10.59
Glutamic acid	315.00	Palmitic acid	0.67
Glycine	192.00	Stearic acid	0.23
Histidine-HCl	120.00	Tween 80	357.02
Hydroxyproline	57.00	**Water-Soluble Vitamins**	**284.38**
Isoleucine	156.00	Ascorbic acid	10.61
Leucine	231.00	Biotin	0.03
Lysine	159.00	Calcium panthothenate	2.80
Methionine	90.00	Cholin chloride	246.31
Phenilalanine	165.00	Folic acid	0.11
Proline	246.00	Inositol	17.05
Serine	195.00	Nicotinic acid	5.68
Threonine	165.00	Pyridoxine-HCl	0.28
Tryptophane	60.00	Riboflavin	1.32
Tyrosine	120.00	Thiamine-HCl	0.15
Valine	135.00	**Miscellaneous**	
Inorganic salt mixture	**75.00**	Sucrose	14,000.00
$CaCl_2$	3.66	Ribonucleic acid	75.00
$CoCl_2.6H_20$	0.57	KOH (2N)	280.00
$CuSO_4.5H_2O$	0.67	K_2HPO_4 (2N)[a]	14.03
$FeCl_3.6H_2O$	2.15	Distilled water to 100 ml	
K_2HPO_4	45.01		
$MgSO_4.7\ H_2O$	15.78		
$MnSO_4.H_2O$	0.04		
$Na_2HPO_4.12\ H_2O$	6.22		
$ZnCl_2$	0.85		

[a] Added into water-soluble vitamins mixture solution.

selected 10 newly emerged adults from the stock *Pimpla* culture were used for nodulation assessment of the unchallenged groups, as aforementioned. The effect of wounding on nodulation was determined by wounding the adults with a microsyringe needle. Nodulation was assessed 2 h post-injection (PI). The effect of an adult artificial diet on nodulation was ascertained by injecting the same volume (5 μl/adult) of this diet into adults. The effects of the selective COX inhibitor, DT (5 μg/adult), BHSV-1 (2×10^3 PFU/adult), and their solvent distilled water (5 μl) were tested in unchallenged adults at the same concentration and volume as those used for challenged adults. The treatments were repeated four times and 10 adults were used for each treatment.

2.5. Influence of DT on nodulation

The influence of DT on nodulation was determined using four groups of adults, with 10 adults per group. The experiments were replicated four times. A first group adults was injected with distilled water (5 μl) as solvent control as DT is easily soluble in water. The second was inoculated with BHSV-1 (5 μl, 2×10^3 PFU/adult), the third with 5 μl of DT (5 μg/adult), the fourth with 5 μl DT (5 μg/adult) and then individuals in fourth group adults were injected with BHSV-1 (5 μl, 2×10^3 PFU/adult). Each adult group were chilled on ice before counting the nodules. Nodules were observed and counted 2 h later as aforementioned.

2.6. Oral treatment with DT and virus injections

Newly emerged virgin females which had not been previously fed were used for oral treatment. The effects of *per os* DT on nodule production and haemoplymph PO activities were determined by rearing newly emerged adults on a chemically defined synthetic diet [35] containing DT at concentrations of 0.001, 0.01, and 0.1 % (g of 100 ml diet). This synthetic diet contained amino acids, carbohydrates, lipids, vitamins, inorganic salts, and other nutrients (Table 1). Concentrations of DT at 0.001, 0.01, and 0.1 % (g of 100 ml diet) were first diluted in 1 ml of distilled water and completed with distilled water to prepare the selected concentrations of the solution. DT solutions were then added to the diets during diet preparation. The total volume of the diet was completed using distilled water to prepare the final DT concentrations. Adults were fed on equal amounts of the test diets and placed on aluminum foil for 1 h. A group of adults was fed a diet without DT in each experiment as a non-treatment control. The treatments were performed under the same laboratory conditions as those used for the stock culture of *P. turionellae*.

For oral treatment, the adults were first fed a control diet and diets containing DT. These adults were then inoculated with BHSV-1 (2×10^3 PFU/adult) as previously described. Nodulation was assessed 2 h PI. Haemolymphs from all adult groups were collected to determine PO activity. Each feeding experiment was replicated four times and 10 adults were used in each experiment.

2.7. Haemolymph collection

Haemolymph (2 μl of haemolymph per adult weighing 25–30 mg each) were extracted by piercing the lateral side of abdomen of individual adults with a 10 μl Hamilton micro-syringe (Hamilton, Reno, NV) after chilling the adults on ice for 5 min and surface sterilising in 95 % ethanol. Haemolymph was prepared to obtain clear supernatants (pellet haemocytes) for PO activity and protein concentration assays as described by Büyükgüzel [14]. Protein levels in the haemolymph were determined and bovine serum albumin was used as a quantitative standard [36].

2.8. PO activity

Using a modified method [37,38] as recently described [8,14], phenoloxidase enzyme (PO: L-DOPA: oxygen oxidoreductase; EC 1.14.18.1) activity was determined. Diluted haemolymph (10 μl) was poured into 1.5 ml glass spectrophotometre cuvettes and then added 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 spectrophotometre (Shimadzu 1700, Kyoto Japan). Enzyme activity was defined as absorbance units (au)/min/mg protein at 492 nm.

Table 2
The data of control experiments. Nodulation is mean numbers of nodules (±SE, n = 10 adults in each treatment). Asterisk shows significant differences ($P < 0.05$) compared to any of the control treatment. The presence of melanisation is indicated by + and −; determined by visual inspection.

Treatments	Concentration	Nodules/Adult Mean ± SE	Melanisation
Non-treatment		5.51 ± 0.94	–
Distilled water		8.37 ± 0.42	–
Vıral culture diet		4.58 ± 0.61	–
Insect artificial diet		5.41 ± 0.62	–
Injection wound		4.60 ± 0.55	–
Dexketoprofen trometamol	5 μg/adult	8.93 ± 1.12	+
Viral Challenge	(2×10^3 PFU/adult)	32.67 ± 4.18*	+

2.9. Statistical analysis

One-way 'analysis of variance' (ANOVA) (IBM SPSS Statistics, [39]) was used to analyse data. Significant differences between means were determined by using least significant difference (LSD) test (IBM SPSS Statistics, [39]). Significance was considered at a *P* value 0.05 level.

3. Results

3.1. Control treatments

Table 2 shows the results of the background control experiments in which the inocultated adults produced more nodules than the control groups (i.e., as an immune response to the viral treatment). A few nodules (5–6 nodules/adult) in untreated adults (5.51 ± 0.94 nodules/adult) and in adults challenged by injection wound (4.60 ± 0.55), by injecting viral culture medium as viral media control (4.58 ± 0.61) were recorded. Adults injected with artificial diet as insect diet control had fewer nodules (about 5.42 ± 0.62 nodules/adult). In addition, adults injected with DT or distilled water (dH2O) also produced fewer nodules (8–9 nodules/adult). Endoparasitoid adults injected with BHSV-1 (2×10^3 PFU/adult) yielded significantly more nodules (ca. 32.67 ± 4.18 nodules/adult) than their background controls (<9 nodules/adult) ($F = 103.270$, $df = 6$, $P = 0.001$).

3.2. Influence of DT on nodulation reactions to viral challenge

Fig. 1 presents adults inoculated with the standard BHSV-1 concentration (2×10^3 PFU/adult) produced about 38.65 ± 2.63 nodules per adult. Contrarily, nodulation reactions were strongly reduced (approximately 3.91 ± 0.25 nodules/adult) in adults treated with co-injection of DT and BHSV-1 ($F = 334.976$, $df = 3$, $P = 0.001$). Based on these results, 5 μg DT doses was used in the following *per os* experiments.

3.3. The influence of per os DT treatments on nodulation and PO activity

A decreased number of nodules and increased PO activity were recorded as a function of high dietary concentrations of DT (Table 3). The lowest dietary DT concentration provoked significantly increased PO activity (1.22 ± 0.23–2.74 ± 0.31 unit/min/mg protein) ($F = 26.255$, $df = 3$, $P = 0.001$) and significantly decreased nodulation (43.19 ± 4.26–17.84 ± 2.19 nodule/adult) ($F = 131.946$, $df = 3$, $P = 0.001$). The effect of DT was dose-dependent, with a decrease in immunocompetence in nodule production and an increase in the PO reaction at high inhibitor doses. Nodulation was decreased by approximately 12-fold, from over 43 nodules (per adult) in adults reared on an artificial diet without DT, while challenged with virus to approximately 4 nodules (per adult) in adults reared with a diet containing 0.1 % DT and viral co-injection ($F = 131.946$, $df = 3$, $P = 0.001$). However, haemolymph PO activity raised to 3.47 ± 0.40 unit/min/mg protein in adults (2.84-fold) reared with diet containing 0.1 % DT ($F = 26.255$, $df = 3$, $P = 0.001$).

4. Discussion

Adults fed on diets with DT of varying concentrations exhibited compromised cellular responses, as evidenced by decreased nodulation. However, these adults exhibited an enhanced humoral response to viral inoculation, as driven by PO reactions. The results also revealed that adults treated with intrahaemocoelic injections of DT were immunocompromised in response to viral infection. These data support the hypothesis that BHSV-1 infection provokes eicosanoid-mediated or prostaglandin-mediated nodulation reactions and PO activation in *P. turionellae* adults. Overall, by inhibiting cyclooxygenase-1 (COX-1), DT prevents the biosynthesis of PGs (COX-1 products), which are immune mediator molecules in insects. Therefore, DT may modulate the immune response by reducing

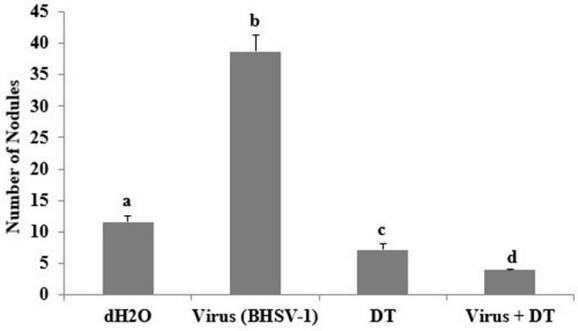

Fig. 1. Immunomodulation of DT on BHSV-1 induced nodulation reactions in *P.turionellae* adults. The histogram bars represent means (±S.E.) of four replicates in each treatment with 10 adults. Means indicated with different letters are significantly different; $P < 0.05$ (LSD test).

Table 3
The effects of DT on adult *P. turionellae* nodulation reactions and PO activity and melanisation reactions *per os* to intrahaemocoelic BHSV-1 (2×10^3 PFU/adult) infections. Each experiment was replicated four times with 10 adults per replication. Means ± SE with the different letters are significantly different ($P < 0.05$).

Dexketoprofen trometamol (g/100 ml Diet)	Number of Adults	Nodules/Adult Mean ± SE	PO Activity Mean ± SE	Melanisation
0.0	10	43.19 ± 4,26a	1.22 ± 0.23a	+
0.001	10	17.84 ± 2,19b	2.74 ± 0.31b	+
0.01	10	12.61 ± 1.56c	2.95 ± 0.33bc	+
0.1	10	3.58 ± 0.86d	3.47 ± 0.40c	++

cellular immune reactions.

COX-1 is primarily responsible for PG synthesis which is important for maintaining normal physiological functions [40]. Water-soluble DT salt is rapidly absorbed with the maximum plasma concentration. It is eliminated after extensive biotransformation into inactive glucuroconjugated metabolites with hydroxyl derivatives by the hepatic cytochrome P450 enzymes [32,41]. These properties enable DT a good candidate for immunomodulation by direct inhibition of eicosanoid- or protaglandin-mediated immune responses to viral challenge, with fewer side effects in the wasp. If its pharmacokinetics are the same in insects, this selective EBI, DT, can be used as a potent immunosuppressant for the control of pest insects in an environmentally safe manner compared to nonselective conventional EBIs.

Contrary to current PO results, a previous *per os* study with nonselective COX inhibitor indomethacin resulted in decreased PO activity and nodulation reactions in *P. turionellae* adults challenged with the same concentrations of BHSV-1 (2×10^3 PFU viral load) [14]. In comparison with the selective COX-1 inhibitor DT used in this study, the modulation of nodulation and PO action by eicosanoids appears to vary with the inhibitors in terms of stimulatory or inhibitory activity. Thus, considerable evidence for the involvement of eicosanoids, at least PGs, in immune defense against bacterial, fungal, protozoan, and parasitoid challenges in a phylogenetically wide range of insects, as recently stated by Kim and Stanley [1]. Recent findings support the role of eicosanoids in immune response to BHSV-1 as viral challenge in *P. turionellae* larvae [18] and its host *G. mellonella* [8] challenged with conventional NSAIDs which are nonspecific inhibitors of both COX-1 and COX-2, lipoxynegase and phospholipase A2 inhibitors. The data reported in this study extend and strengthen the hypothesis that eicosanoids, at least COX-1 products, modulate nodule formation and PO activation in response to viral challenge in *Pimpla* adults exposed to this selective COX-1 inhibitor and this modulation of EBIs varies with their selectivity, pharmacological actions, and life stages of the insects.

Eicosanoids mediate cellular immunity of the social hymenopteran, *Apis mellifera* challenged with microbial infection [42]. Nodulation decreases in honeybee workers with increasing age, suggesting that constitutive eicosanoid biosynthesis is impaired in older workers, possibly because of deteriorative effects [43]. The PO level increases with age, reaching maximum activity within the first week of adult emergence in worker bees [44]. Eicosanoids also act in haemocytic immunity of the parasitoid wasp, *P. turionellae* larvae and adults [8,14,18]. In this study, eicosanoid actions, especially those of COX-1 products, were confirmed to mediate the cellular and humoral immunity of an adult parasitoid with reduced nodules compared to larvae, supporting the assumption of reduced cellular immunocompetence through the adult stage. As many studies on parasitoids have focussed mainly on host immune defence to attack of parasitoid, this study draws attention to the hypothesis that parasitoids might also be exposed to bacterial, fungal, and protozoal infections and protect themselves as preadult stages within the host or in their natural environment during their adulthood [8,14,18].

Most studies using EBIs to evaluate the possible function of eicosanoids in insect immune responses have involved experimental insects treated with intrahaemocoelic injection of EBIs. The hypothesis that treated adults with DT *per os* would similarly decrease nodulation responses and PO level to viral infection as in recent studies [8,14,18] was tested. However, the results of the present study showed that oral administration of DT severely deteriorated nodulation and increased PO activity in *P. turionellae* adults to BHSV-1 infection. As this selective COX-1 inhibitor crippled the nodulation response, COX-1 products, prostaglandins (PGs), were inferred to mediate the nodulation response to viral challenge, as observed in most insects with bacterial infection [5]. Consistent with this assumption, a more recent study by Camara et al. [45] reported that decreased nodulation by phenidone confirmed that the dual COX/LOX products are responsible for nodulation in *Locusta migratoria manilensis* leading to significantly increased larval mortality of fungus-phenidone-treated locusts.

EBIs also deteriorated immune responses in the orally fed termite *Coptotermes formosanus* [46] and the blood-sucking insect *Rhodnius prolixus* [47]. Wakayama et al. [48] reported that several EBIs inhibited PG biosynthesis in whole houseflies, *Musca domestica* in characterisation of PG biosynthesis experiments with *in vitro* enzyme preparations, though not oral feeding on housefly diets. A pioneering study of PG biosynthesis revealed that indomethacin was not effective in inhibiting PG biosynthesis in the reproductive tissues of male cricket *Acheta domesticus* [49]. In contrast, Murtaugh and Denlinger [50] indicated that indomethacin and other COX inhibitors decreased PG biosynthesis in the testes of *A. domesticus*. An unpublished observation (Tunaz, Büyükgüzel, and Stanley) also reported that naproxen and indomethacin strongly inhibited PG biosynthesis in testis preparations of crickets. Pletora of pharmaceutically important EBIs from clinical treatments are inferred to have effectively inhibited eicosanoid biosynthesis in different insect tissues or organ preparations, including *per os* treatments in whole animals. The data of the present study also suggest that they modulate immune responses related to different modes of action, pharmacokinetics of pharmaceuticals, and different tissues and developmental stages of insects.

Important functions of eicosanoids in PO action in adult wasp were recently addressed [8,14,18] with a suggestion that eicosanoids roles in PO activity appears to vary across insect species and stages. The results of the current study show that BHSV-1 infection provokes the PO reaction and the activity is increased in *P. turionellae* adults fed on high DT doses. This result may be attributed to decreased PG biosynthesis, leading to curtailed nodulation and subsequently increased PO activation which may act as an antiviral humoral defense, enabling melanisation to compensate for crippled cellular immune defense. This increased PO activity is inconsistent with the most recent works of our research team, showing that several conventional EBIs attenuated the PO activity in larvae of *G. mellonella* [8] and its parasitoid *P. turionellae* [18] and adults [14] challenged with BHSV-1. Contrary to our results, Mandato et al. [51] found that EBIs decreased PO activation in *G. mellonella* challenged with bacterial infection. Garcia et al. [47] similarly reported that feeding *R. prolixus* EBI after injecting the protozoan parasite *Trypanosoma rangeli* reduced PO activity. Conversely, EBIs had no effects on PO enzyme activity in larvae of *Manduca sexta* treated with spores of the fungus *Beauveria bassiana* [52]. This finding confirms that eicosanoids act in haemocytic immune responses, though not PO action, in *Locusta migratoria* challenged with laminarin [53]. Except for the increased PO activity, the findings of these studies also align with the results of this study; specifically, that eicosanoids mediate the immune response in terms of decreased cellular immune reaction, though not PO activity, suggesting that the influence of EBIs somehow varies with infection agents, insect species, developmental stages, and pharmaceutical action. The PO response is important in the insect immune defense involved in the melanisation of encapsulated infectious agents and nodulation after infection invaders. This enzyme is mostly produced in haemocytes as an inactive PPO called zymogen and is released into the haemolymph to perform humoral immune defense [12]. Our results indicate the need for a more detailed evaluation to confirm the mechanisms underlying the effects of EBIs on increased PO activity.

Serpins have been shown in many animals to be key regulators of innate immune reactions. Serine proteases inhibitor serpins in relation to Spaetzle or Toll pathway is crucial role in regulating prophenoloxidase activating enzyme (inactive PPAE) to activate proPO to PO in humoral response of insects producing melanisation and cytotoxic reactive intermediate. The prophenoloxidase (PPO) activation pathway and Toll pathway are two critical insect immune responses against microbial infection. Activation of these pathways is mediated by an extracellular serine protease cascade, which is negatively regulated by serpins [54]. In study, specific COX-1 inhibitor dexketoprofen trometamol may have possibly impaired serpin regulation of PPO pathway by inhibiting COX-1 products at least prostaglandins mediating cellular and humoral immune response of this parasitoids confirmed in the previous studies [14,18]. However further experiments are needed to determine the exact role of dexketoprofen trometamol in molecular mechanisms of complicated pathway of serpin in increased PO activity in this parasitoid *P. turionellae*.

The increasingly important subject and the range of insect immune responses to viral infections potentially mediated by eicosanoids were focussed on in this study. Insects exert several immune defense reactions against viral infections, although not all insect species react to viral challenges to a similar extent. Consistent with this knowledge, increased PO activity was observed in the haemolymph of *Pimpla* adults fed on DT in response to BHSV-1. The results of Popham et al. [20] that suggested that constitutive haemolymph PO may act as a antiviral defence for a larval lepidopteran, is consistent with the findings of this study. Midgut cell sloughing (a major mechanism for increasing resistance to baculovirus infection) and apoptosis which are crucial responses to viral challenges, have been extensively studied [55]. Insects also respond to viral attacks by cellular (haemocytic) encapsulation of virus-infected cells, accompanied by melanisation [56]. The nodules recorded 2 h after the PI of BHSV-1 in this study appear to emerge from this encapsulation process mediated by COX-1 products and are accompanied by increased PO activity leading to melanisation. Nodules are generally attached to body walls or internal organs, where they remain throughout the lifespan of an insect. These nodules were cleared from wasp larvae and other stages after the initial virus-provoked nodulation reaction. This may be a general feature of cellular immunity in parasitoid wasps, as shown by Camara et al. [45] for other insect species 3 h after the PI of the fungus following longer incubation periods.

PGs inhibit viral replication in insect cells. In cell culture of the mosquito *Aedes albopictus,* Barbosa and Rebello [57] reported that PGA_1 blocks Mayaro virus replication. The Mayaro virus causes Mayaro fever, a non-lethal disease in humans transmitted by mosquitoes belonging to the genus *Haemagogus* [58]. Decreased nodule production in response to BHSV-1 infection in adults treated with increasing DT concentrations appeared to increase PO activities to compensate for the crippled cellular immune response. Therefore, suggesting that cyclooxygenase products, at least COX-1 products, mediate insect antiviral responses, and that the PO system contributes to this process at the cellular and intracellular levels seems to be reasonable.

Ethics declarations

Review and/or approval by an ethics committee was not needed for this study.

Data availability

All data generated or analyzed during this study are included in this published article.

CRediT authorship contribution statement

Cihat Çelik: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The author declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study.

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