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Eicosanoids mediate nodulation reactions to bacterial *Escherichia coli* K 12 infections in larvae of the oriental blowfly, *Chrysomya megacephala*

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Abstract Nodulation is the predominant cellular defense reaction to bacterial challenges in insects. In this study, third instar larvae of *Chrysomya megacephala* were injected with bacteria, *Escherichia coli* K 12 (10^6 CFU/mL, 2 μL), immediately prior to injection of inhibitors of eicosanoid biosynthesis, which sharply reduced nodulation response. Test larvae were treated with specific inhibitors of phospholipase A_2 (dexamethasone), cyclo-oxygenase (indomethacin, ibuprofen and piroxicam), dual cyclo-oxygenase/lipoxygenase (phenidone) and lipoxygenase (esculetin) and these reduced nodulation except esculetin. The influence of bacteria was obvious within 2 h of injection (5 nodules/larva), and increased to a maximum after 8 h (with 15 nodules/larva), and then significantly reduced over 24 h (9 nodules/larva). The inhibitory influence of dexamethasone was apparent within 2 h of injection (4 *vs.* 5 nodules/larva), and nodulation was significantly reduced, compared to control, over 24 h (5 *vs.* 8 nodules/larva). Increased dosages of ibuprofen, indomethacin, piroxicam and phenidone led to decreased numbers of nodules. Nodules continued to exist during the pupal stage. However, the effects of dexamethasone were reversed by treating bacteria-injected insects with an eicosanoid-precursor polyunsaturated fatty acid, arachidonic acid. These findings approved our view that eicosanoid can mediate cellular defense mechanisms in response to bacterial infections in another Dipteran insect *C. megacephala*.

Key words bacteria, *Chrysomya megacephala*, eicosanoids, *Escherichia coli* K 12, nodulation

Introduction

The insect immune system contains humoral and cellular immune components (Stanley, 2005; Hoffmann, 2003). The significant cellular immune response mechanisms of insects include nodulation, phagoytosis and encapsulation (Strand & Pech, 1995; Schmidt *et al.*, 2001). Nodulation is the predominant cellular defense reaction to bacterial infection in insects (Stanley & Miller, 2006; Yonce Durmus *et al.*, 2008). It is a complex process that begins with micro-aggregation of hemocytes, which entrap large numbers of micro-organisms and grow via additionally recruited hemocytes (Franssens *et al.*, 2005; Büyükgüzel *et al.*, 2007). Eventually the nodules appear and melanization occurs, and the dark nodules attach to the body wall or various internal organs of the insect (Horohov & Dunn, 1983).

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Eicosanoid is a collective term for all oxygenated metabolites of three C20 polyunsaturated fatty acids, namely prostaglandins, thromboxanes and epoxyeicosatrienoic acids. Many studies suggest that eicosanoids play a very important role in cellular response of some insects, such as *Pseudaletia unipuncta*, *Agrotis ipsilon* (Lepidoptera) (Jurenka *et al.*, 1997), *Bombyx mori* (Lepidoptera) (Stanley-Samuelson *et al.*, 1997), *Gryllus assimilis* (Orthoptera) (Miller *et al.*, 1999), *Magicicada septendecim*, and *Magicicada cassini* (Orthoptera) (Tunaz *et al.*, 1999), as well as *Lymantria dispar* (Stanley & Shapiro, 2007). These studies showed that inhibitors of eicosanoid biosynthesis inhibit nodulation response to bacterial and other infections. Franssens *et al.* (2005) and Hoffmann (2003) reported that bacteria induced similar nodulation reaction in *Neobellieria bullata* and *Drosophila melanogaster*, respectively, from which it appears that the bacteria inhibits cellular immune reactions in at least two major species of Diptera.

It is known that *Chrysomya megacephala* (D.), can transmit several pathogenic microbes, but cannot be infected, thus providing a good model for an immunity study (Faraldo *et al.*, 2008). Detailed research on its immunity mechanism may provide more recent evidence for immunology. Here we demonstrate that, after treating larvae of *C. megacephala* with *Escherichia coli* K 12 (*E. coli* K 12), hemocytic nodules formed within their bodies in response to the bacterial infection, a cellular defense mediated by different eicosanoids.

Materials and methods

Chemicals, insects and bacteria

The reagents which we used in our experiments are listed in Table 1. All of them were dissolved in 30% ethanol. The concentration of arachidonic acid was 25 mg/mL, while others were 12.5 mg/mL except in dose-response experiments. The concentration of *E. coli* K 12 was 10^6 CFU/mL. Control groups were injected with 30% ethanol each time. All of the standard volumes were 2 μL.

Chrysomya megacephala were reared at $25 \pm 2^\circ$C under a photoperiod of 12 : 12 h (L : D), at the Insect Resources Institute of Huazhong Agriculture University. Third instar larvae (1 day-old), during their wandering stage, were washed and kept in a ventilated glass box for approximate 24 h and were not used in any experiments until their crops were entirely empty.

The bacteria, *E. coli* K 12 were cultured in 20 mL of liquid broth medium in a shaker at 37°C and at 120 r/m until growth reached to a titre of 10^6 colony forming units (CFU)/mL, and then stored at -4°C.

Injections and nodulation assay

Before injection, larvae of *C. megacephala* were washed with distilled water. The test larvae were infected with *E. coli* K 12, and subsequently injected with dexamethasone, ibuprofen, indomethacin, piroxicam, esculetin or phenidone. In the dexamethasone treatment, larvae were also injected with arachidonic acid. Control larvae were injected with 30% ethanol or *E. coli* K 12. All reagents were injected into the opposite side of the abdomen in 2-μL volumes via a 10-μL Hamilton syringe (Hamilton, Reno, NV, USA).

Nodulation was assessed at selected times post-injection (PI). Larvae were anatomized on ice and their haemocoel was exposed under a stereomicroscope at a magnification of 40× to count melanized and darkened nodules (Fig. 1). The number of nodules reflected the extent of the nodulation response to infections (Miller *et al.*, 1994, 1996). After the primary counting, the alimentary canal was removed to count the nodules which were in previously unexposed areas and internal tissues.

Table 1 The reagents used in all experiments.

Sort	Reagent	Origin
Phospholipase A_2 inhibitor	Dexamethasone	Serva Company (Heidelberg, Germany)
Cyclo-oxygenase inhibitor	Ibuprofen	
	Indomethacin	MP Biomedicals (Irvine, CA, USA)
	Piroxicam	
Lipoxygenase inhibitor	Esculetin	MP Biomedicals (Irvine, CA, USA)
Dual cyclo-oxygenase/lipoxygenase inhibitor	Phenidone	MP Biomedicals (Irvine, CA, USA)
Fatty acid	Arachidonic acid	Serva company (Heidelberg, Germany)

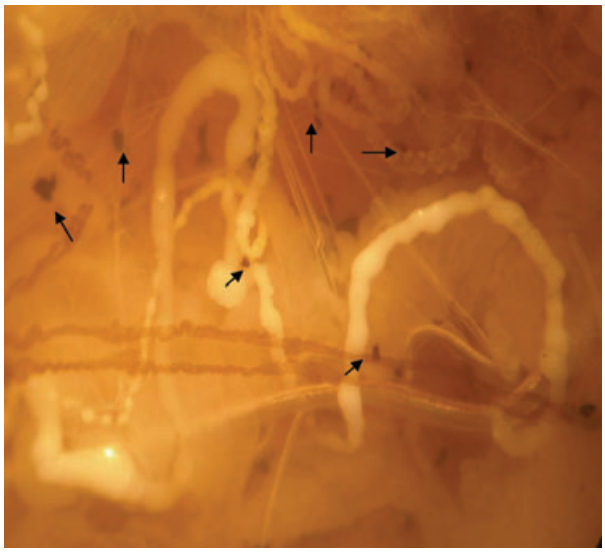

Fig. 1 Photomicrograph of melanized nodules in larvae flies, *Chrysomya megacephala* in response to bacterial infection. One of several visible nodules (black arrow), seen against the background of fat body. This picture was taken during routine assessment of nodulation at 8 h post-infection using an SC 7500 Olympus camera (Olympus, Guangdong, China).

Immune challenge, drug treatment and nodule counting

A series of general control experiments and assessments were conducted to determine the level of background nodulation in larvae. Before injection of control experiments, the larvae were grouped into six groups with 20 larvae in each group. Nodulation in untreated larvae was recorded at various times during this experiment. The influence of injection wound on nodule formation was determined with the needle of the micro-syringe. We considered the possibility that nutrient broth could stimulate nodulation via directly injected broth. The effect of 30% ethanol or *E. coli* K 12 was tested in untreated larvae. All larvae were anesthetized on ice for 3 min and nodulation was assessed at 8 h PI.

Two groups of larvae were first injected with bacteria, then immediately with dexamethasone as described above 2, 4, 8, 12, 16, 20 and 24 h later. Insects were anesthetized on ice and nodulation was assessed.

Individuals in four groups were treated with 2 μL of a standard dosage of bacteria, and then injected with 1.25×10^{-3}, 1.25×10^{-2}, 1.25×10^{-1}, 1.25 or 12.5 μg of indomethacin, ibuprofen, piroxicam or phenidone in 2 μL of ethanol. At 8 h PI, the larvae were anesthetized to count the extent of nodulation.

Table 2 The influence of reagents and infection methods on nodulation responses in larvae of *Chrysomya megacephala*.

Treatment	Nodules/larva	Death
No treatment	0.55 ± 0.11	−
Nutrient broth	4.25 ± 0.25	−
Ethanol	10.35 ± 0.64	+
Bacteria in broth	14.10 ± 1.10	−
Dexamethasone + ethanol	7.40 ± 0.36	+
Injection wound	0.90 ± 0.16	−

Nodulation values are mean numbers of nodules ($\pm$ SE, $n = 20$). The presence of death is indicated by + and − at 24 h post-injection.

Fatty acid rescue experiment

Larvae were injected with bacteria as described, then injected with either 2 μL ethanol or 12.5 μg of dexamethasone in 2 μL ethanol. Immediately after injection, the dexamethasone-treated larvae were divided into two sub-groups. Larvae in one sub-group were treated with 25 mg/mL of arachidonic acid in 2 μL of ethanol. Another sub-group was treated with 2 μL of ethanol to investigate the effects of the extra injection on nodulation. At 8 h PI, the larvae were anesthetized and nodulation was assessed.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). To determine significant differences between means the shortest significant ranges (SSR) test was used. When the F estimate exceeded the probability of 0.05 the differences were considered significant.

Results

Control experiments

Control experiments are designed to eliminate any impact from mechanical damage of physical handling and nutrient broth (Table 2). There was approximately one nodule/larva in injection-wound treatments. Two-microliter ethanol injection caused formation of 10 nodules/larva, while nutrient broth treatments resulted in four nodules/larva. The standard dosage of bacteria resulted in 14 nodules/larva. After 24 h, mortality was assessed. No death was detected in non-treatment, nutrient-broth and injection-wound treatments.

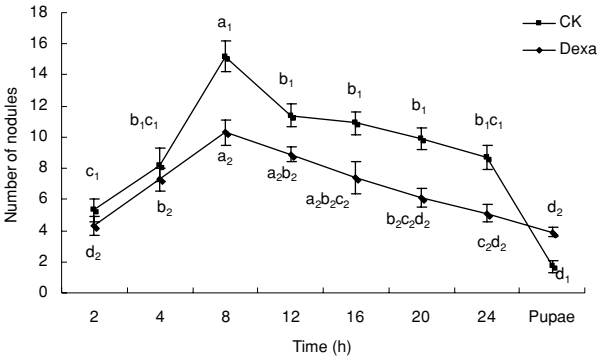

Fig. 2 Time course of nodulation in *Chrysomya megacephala* larvae in response to dexamethasone injection. Control larvae (CK) were treated with 30% ethanol. Dexa represents the PLA_2 inhibitor, dexamethasone. Each point indicates the average value of nodules, and the error bars represent 1 SE. Each experiment was replicated three times with 20 larvae. Values on the same line annotated with the same letter are not significantly different ($P < 0.05$).

Time-course of nodulation

Nodules produced from the ethanol-treated control insects started from five nodules/larva at 2 h PI, increasing to a maximum of 14 nodules/larva by 8 h PI, then decreased with time (Fig. 2). Nodulation was also observed in pupae, but less than in larvae. Dexamethasone treatment resulted in significantly fewer nodules at any time point from 8 h PI than in controls. The number of nodules also reached a maximum value of 10 nodules/larva at 8 h PI. However, the number of nodules in treatments was more than that in controls.

Dose response for indomethacin, ibuprofen, piroxicam and phenidone treatment

Ten-fold increases in inhibitor dosages significantly reduced nodulation ($P < 0.05$). The number of nodulations had a linear correlation with dosage of injections ($P < 0.01$). The number of nodulations started from about 23, 21, 24 or 22 nodules/larva in ethanol-treated control larvae to about 2, 2, 7 or 2 nodules/larva in larvae treated with the highest phenidone, piroxicam, indomethacin or ibuprofen dosage, respectively (12.5 mg/mL) (Fig. 3).

Influence of other eicosanoid biosynthesis inhibitors on nodulation

To assess the influence of several eicosanoid biosynthesis inhibitors on nodulation in response to infections with bacteria, test larvae were treated with the inhibitors. Compared with control larvae, nodules were significantly reduced in all experimental groups except the esculetin-treated group ($P < 0.05$) (Table 3). There were no significant differences in numbers of nodules among different inhibitor-treated groups.

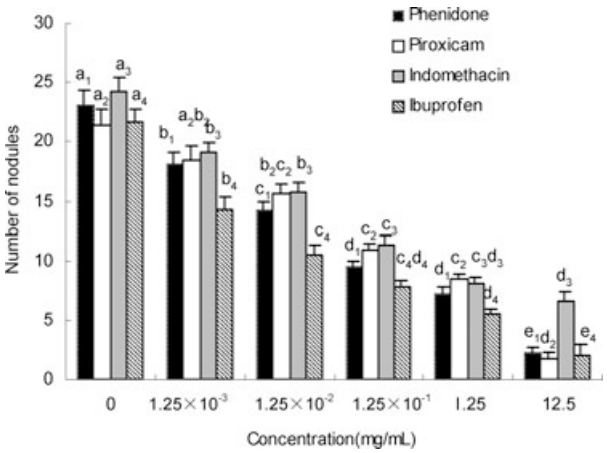

Fig. 3 Dose influence of indomethacin, ibuprofen, piroxicam or phenidone on nodulation formation in larvae of *Chrysomya megacephala*. Test larvae were first injected with bacteria, and control insects were injected with ethanol, and others were treated with different dosages of inhibitors. At 8 h PI, the insects were anesthetized on ice, and nodulation was assessed. Each point represents the average value of nodules (± SE, $n = 20$). Values on columns in the same color annotated with the same letter are not significantly different ($P < 0.05$).

Table 3 Effects of eicosanoid biosynthesis inhibitors on nodule formation in larvae and pupae of *Chrysomya megacephala* in response to *Escherichia coli* K 12 injection.

Treatment	Concentration (mg/mL)	Nodules/ larva	Nodules/ pupa
Ethanol	30% (v)	13.10 ± 0.66 c	1.84 ± 0.21 d
Ibuprofen	12.5	8.15 ± 0.53 b	4.31 ± 0.46 ab
Esculetin	12.5	11.75 ± 0.67 c	2.21 ± 0.25 cd
Dexamethasone	12.5	6.20 ± 0.48 ab	3.42 ± 0.32 abc
Indomethacin	12.5	5.75 ± 0.32 a	2.80 ± 0.41 bcd
Phenidone	12.5	5.30 ± 0.39 a	3.10 ± 0.20 abcd
Piroxicam	12.5	4.80 ± 0.37 ab	4.50 ± 0.52 a

Nodulation values are mean numbers of nodules (± SE, $n = 20$). Means annotated with the same letter are not significantly different ($P < 0.05$).

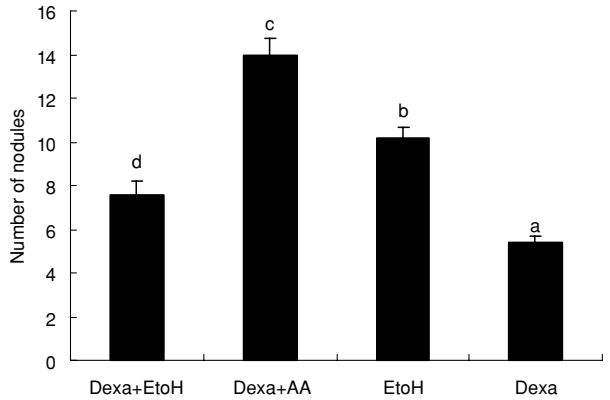

Fig. 4 Arachidonic acid reverses the effect of dexamethasone on nodulation in larvae of *Chrysomya megacephala*. Dexa, EtoH and AA represent dexamethasone, ethanol and arachidonic acid, respectively. The histogram bars represent means (± SE) of three replications in each treatment. Means topped by different letters are significantly different ($P < 0.05$).

Fatty acid rescue experiment

Larvae were injected with bacteria and dexamethasone, then treated with arachidonic acid and ethanol, respectively. The ethanol-injected larvae resulted in about 10 nodules/larva and dexamethasone-treated larvae about five nodules/larva (Fig. 4). However, arachidonic acid caused about five nodules/larva. During the pupal stage, nodules still existed. This indicates that arachidonic acid treatments can reverse the effects of dexamethasone on nodulation in larvae of *C. megacephala* ($P < 0.05$).

Discussion

In this study, our results show that eicosanoids can mediate nodulation reaction in *C. megacephala* after the injection of *E. coli* K 12. Several lines of data presented here support this hypothesis. Dexamethasone significantly reduced nodulation throughout the time course experiment. The higher the dosages of indomethacin, ibuprofen, piroxicam and phenidone, the fewer the number of nodules, which indicated an obviously negative correlation between them. All of the inhibitors significantly reduced nodulation in infected larvae except for lipoxygenase inhibitor. Treatment of arachidonic acid, an eicosanoid precursor and a polyunsaturated fatty acid, on infected larvae could reverse the influence of dexamethasone on nodulation. Thus, we infer from these data that eicosanoids can mediate nodulation in response to bacterial infections in *C. megacephala*.

To consider and eliminate the effect of physical damage or injection that may result in nodulation and mortality, we designed several background control experiments. In pilot experiments we found that the optimum concentration of ethanol was 30%, thus we used this concentration as control treatments. Although larvae of *C. megacephala* in this study were not maintained in a bacteria-free environment, very few nodules were assessed from larvae taken directly from culture. We found injections of dexamethasone dissolved in ethanol and nutrient broth, respectively, resulted in about seven nodules/larva and four nodules/larva (Table 2). Nodulation following ethanol injections was higher than other reports of similar treatments with other insects (Miller *et al.*, 1994, 1996), and this instance was not caused by mechanical injection. We speculate that the larvae may be more sensitive to ethanol. When the time course of dexamethasone treatment extended, its inhibition to nodulation strengthened, indicating that it inhibited immune reactions *in vivo* and lead to larval inability to clean out bacterial infections effectively.

Miller *et al.* (1996) and Howard *et al.* (1998) have reported that the differences in nodulation reaction is probably due to differences in insect type and hemolymph content. Our time course experiments show that larvae produce a maximum of about 15 nodules/larva on each time, which is significantly lower than that seen in larvae of *Manduca sexta* (Horohov & Dunn, 1983) and *Zophobas atratus* (Miller *et al.*, 1996). However, 80 nodules were recorded each time in silkworms (Stanley-Samuelson *et al.*, 1997). So we speculate that the potential for nodule formation in response to similar challenges may be related to insect size (Howard *et al.*, 1998; Miller *et al.*, 1999).

We also noted that in larvae of *C. megacephala*, the number of nodules was significantly reduced, along with the increased dosages of indomethacin, ibuprofen, piroxicam or phenidone. As reported, they could influence the ability of nodule formation in response to bacterial infections in insects (Miller *et al.*, 1999; Stanley-Samuelson *et al.*, 1997). Arachidonic acid, an eicosanoid precursor and a polyunsaturated fatty acid, can reverse dexamethasone effects on nodulation in larvae of *C. megacephala.* This strongly supports the hypothesis that insect cellular immune reactions responsible for clearing bacterial cells from circulation are mediated by eicosanoids (Stanley-Samuelson *et al.*, 1991). Moreover, other eicosanoid biosynthesis inhibitors, specific for cyclo-oxygenase and the dual cyclo-oxygenase/lipoxygenase, can effectively inhibit nodule formation, except lipoxygenase inhibitors. It has been known that nodule formation is a complex process of many separate blood-cell reactions. Inhibitors of cyclo-oxygenase and dual cyclo-oxygenase/lipoxygenase

pathway may inhibit one or more of the nodule formation steps, thus impacting the overall nodulation process.

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References

Büyükgüzel, E., Tunaz, H., Stanley, D. and Büyükgüzel, K. (2007) Eicosanoids mediate *Galleria mellonella* cellular immune response to viral infection. *Journal of Insect Physiology*, 53, 99–105.

Durmus, Y., Büyükgüzel, E., Terzi, B., Tunaz, H., Stanley, D. and Büyükgüzel, K. (2008) Eicosanoids mediate melanotic nodulation reactions to viral infection in larvae of the parasitic wasp, *Pimpla turionellae*. *Journal of Insect Physiology*, 54, 17–24.

Faraldo, A.C., Gregorio, E.A. and Lello, E. (2008) Morphological and quantitative aspects of nodule formation in hemolymph of the blowfly *Chrysomya megacephala* (Fabricius, 1794). *Experimental Parasitology*, 118, 372–377.

Franssens, V., Simonet, G., Bronckaers, A., Claeys, I., De Loof, A. and Broeck, J.V. (2005) Eicosanoids mediate the laminarin-induced nodulation response in larvae of the flesh fly, *Neobellieria bullata*. *Archives of Insect Biochemistry and Physiology*, 59, 32–41.

Hoffmann, J.A. (2003) The immune response of *Drosophila*. *Nature*, 426, 33–38.

Horohov, D.W. and Dunn, P.E. (1983) Phagocytosis and nodule formation by hemocytes of *Manduca sexta* following injection of *Pseudomonas aeruginosa*. *Journal of Invertebrate Pathology*, 41, 203–213.

Howard, R.W., Miller, J.S. and Stanley, D.W. (1998) The influence of bacterial species and intensity of infections on nodule formation in insects. *Journal of Insect Physiology*, 44, 157–164.

Jurenka, R.A., Miller, J.S., Pedibhotla, V.K., Rana, R.L. and Stanley-Samuelson, D.W. (1997) Eicosanoids mediate microaggregation and nodulation responses to bacterial infections in black cutworms, *Agrotis ipsilon*, and true armyworms, *Pseudaletia unipuncta*. *Journal of Insect Physiology*, 43, 125–133.

Miller, J.S., Howard, R.W., Nguyen, T., Nguyen, A., Rosario, R.M.T. and Stanley-Samuelson, D.W. (1996) Eicosanoids mediate nodulation responses to bacterial infections in larvae of the tenebrionid beetle, *Zophobas atratus*. *Journal of Insect Physiology*, 42, 3–12.

Miller, J.S., Howard, R.W., Rana, R.L., Tunaz, H. and Stanley, D.W. (1999) Eicosanoids mediate nodulation reactions to bacterial infections in adults of the cricket, *Gryllus assimilis*. *Journal of Insect Physiology*, 45, 75–83.

Miller, J.S., Nguyen, T. and Stanley-Samuelson, D.W. (1994) Eicosanoids mediate insect nodulation responses to bacterial infections. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 12418–12422.

Schmidt, O., Theopold, U. and Strand, M. (2001) Innate immunity and its evasion and suppression by hymenopteran endoparasitoids. *BioEssays*, 23, 344–351.

Stanley, D. and Shapiro, M. (2007) Eicosanoid biosynthesis inhibitors increase the susceptibility of *Lymantria dispar* to nucleopolyhedrovirus LdMNPV. *Journal of Invertebrate Pathology*, 95, 119–124.

Stanley, D.W. (2005) *Eicosanoids. Comprehensive Molecular Insect Science*. Elsevier Pergamon, Amsterdam. Pp. 307–339.

Stanley, D.W. and Miller, J.S. (2006) Eicosanoid actions in insect cellular immune functions. *Entomologia Experimentalis et Applicata*, 119, 1–13.

Stanley-Samuelson, D.W., Jensen, E., Nickerson, K.W., Tiebel, K., Ogg, C.L. and Howard, R.W. (1991) Insect immune response to bacterial infection is mediated by eicosanoids. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 1064–1068.

Stanley-Samuelson, D.W., Pedibhotla, V.K., Rana, R.L., Nor Aliza, A.R., Hoback, W.W. and Miller, J.S. (1997) Eicosanoids mediate nodulation responses to bacterial infections in larvae of the silkmoth, *Bombyx mori*. *Comparative Biochemistry and Physiology*, 118, 93–100.

Strand, M.R. and Pech, L.L. (1995) Immunological basis for compatibility in parasitoid-host relationships. *Annual Review of Entomology*, 40, 31–56.

Tunaz, H., Bedick, J.C., Miller, J.S., Hoback, W.W., Rana, R.L. and Stanley, D.W. (1999) Eicosanoids mediate nodulation reactions to bacterial infections in adults of two 17-year periodical cicadas, *Magicicada septendecim* and *M. cassini*. *Journal of Insect Physiology*, 45, 923–931.

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