The influence of chronic eicosanoid biosynthesis inhibition on life history of the greater waxmoth, Galleria mellonella and its ectoparasitoid, Bracon hebetor

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Keywords: Galleria mellonella, Bracon hebetor, Survivorship, Longevity, Fecundity, Eicosanoids

ARTICLE INFO

Article history:
Received 21 October 2010
Received in revised form 27 January 2011
Accepted 27 January 2011
Available online 15 February 2011

Abstract

Eicosanoids are oxygenated metabolites of three C20 polyunsaturated fatty acids, mainly arachidonic acid (AA; 20:4n-6), but also 20:3n-6 and 20:5n-3. Aside from their importance in biomedicine, eicosanoids act in invertebrate biology. Prostaglandins (PGs) influence salt and water transport physiology in insect rectal epithelia and in Malpighian tubules. PGs also influence a few insect behaviors, including releasing oviposition behavior and behavioral fever. Eicosanoids act in ovarian development and in insect immunity. Because eicosanoids act in several areas of insect biology, we posed the hypothesis that chronic inhibition of eicosanoid biosynthesis, in the absence of microbial challenge, can influence insect life table parameters, including developmental time, survival, adult longevity and parasitoid fecundity. Here we report that inhibiting eicosanoid biosynthesis throughout the larval life exerted minor influences on some life table parameters of the greater wax moth, Galleria mellonella and its ectoparasitoid, Bracon hebetor, however, the inhibitors strongly reduced the production and hatchability of the parasitoids’ eggs. The significance of the work relates to the potentials of understanding and targeting eicosanoid systems as a platform for developing new technologies of insect pest management. As seen here, the impact of targeting eicosanoid systems is seen in crucial moments of insect life histories, such as reproduction or immune challenge rather than in overall larval development.

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1. Introduction

Eicosanoids are oxygenated metabolites of three C20 polyunsaturated fatty acids, mainly arachidonic acid (AA; 20:4n-6), but also 20:3n-6 and 20:5n-3. Eicosanoid structures and biosynthetic pathways are treated in detail elsewhere (Stanley, 2000, 2005). Most of our understanding of eicosanoids comes from a very large literature on the biomedical significance of these compounds in humans and other mammals. For example, prostaglandins (PGs) are potent pro-inflammatory signal molecules whose actions are ameliorated by aspirin and other over-the-counter analgesics and a mixture of leukotrienes make up the slow-reacting substance of anaphylaxis (Austen et al., 2009). Because of these and many other biological actions, it would be quite difficult to overstate the significance of eicosanoids in mammalian systems.

PGs and other eicosanoids also exert important actions in invertebrates, including insects, although these actions are not as thoroughly documented compared to the biomedical background (Stanley, 2000). Eicosanoids act in salt and water transport physiology in locust rectal epithelia (Radallah et al., 1995) and in Malpighian tubules of the yellow fever mosquito, Aedes aegypti (Petzel and Stanley-Samuelson, 1992) and the forest ant, Formica polyctena (Kerkhove et al., 1995). PGs also influence a few insect behaviors. PGE2 releases oviposition behavior in some cricket species, including Teleogryllus commodus (Loher et al., 1981) and Acheta domesticus (Destephano et al., 1982). Eicosanoids act in regulation of behavioral fever following fungal infections in the desert locust, Schistocerca gregaria (Bundey et al., 2003). Aside from releasing egg-laying behavior, PGs act in signaling ovarian follicle development in the silk moth, Bombyx mori (Machado et al., 2007). It follows that PGs and other eicosanoids act in important areas of insect biology.

Eicosanoids also mediate several aspects of insect immunity (Stanley et al., 2009). Insect immunity is typically seen as an amalgam of physical barriers, humor reactions and cellular defense effectors. Most research on eicosanoid actions in insect immunity has focused on cellular immunity, although there are a few reports that eicosanoids also act in aspects of humoral immunity (Stanley, 2006; Stanley et al., 2009). Eicosanoids mediate phagocytosis, microaggregation and nodulation, the predominant cellular immune reactions to bacterial infection. Aside from mediating a host of cellular actions that make up the overall process of...
hemocytic immunity to bacterial infections, eicosanoids also act in cellular immune reactions to infections of fungal spores, protozoans, parasitoids and viruses (Stanley et al., 2009).

Because eicosanoids act in a very wide spectrum of insect physiological systems it may be thought these compounds are essential to insect life, as they are in mammals. On the other hand, in their experiments on immune reactions to a virus infection, Büyükgüzel et al. (2007) reared wax moth larvae from the first through seventh instars on culture media supplemented with substantial concentrations of indomethacin, a potent cyclooxygenase (COX) inhibitor. The indomethacin treatments impaired immune reactions to viral challenges, however, they did not visibly influence larval development. In a related experiment with the parasitoid wasp Pimpla turionellae, Durmuş et al. (2008) reared wasp larvae on diets charged with up to 0.1% indomethacin, esculetin (a lipoxygenase [LOX] inhibitor) or dexamethasone (a glucocorticoid that inhibits phospholipase A₂). Again, these inhibitors of eicosanoid biosynthesis impaired immune reactions without a visible influence on development of the wasp larvae. Nonetheless, these and other inhibitors of eicosanoid biosynthesis have potential to influence insect lives in ways that cannot be seen on external examination. We posed the hypothesis that chronic inhibition of eicosanoid biosynthesis, in the absence of microbial challenge, can influence insect life table parameters. Here we report that inhibiting eicosanoid biosynthesis throughout the larval life exerted minor influences on some life table parameters of the greater wax moth, Galleria mellonella and its ectoparasitoid, Bracon hebetor, reared on inhibitor-treated larvae, however, the host-derived inhibitors strongly influenced the production and hatchability of the parasitoids’ eggs.

2. Materials and methods

2.1. Insect stock culture

Greater wax moth, G. mellonella (L.) larvae were reared in 1000-ml glass jars with an artificial diet (Bronskill, 1961), at 30 ± 1 °C in constant darkness. The standard diet was composed of 420 g of bran, 150 ml of filtered honey, 150 ml of glycerol, 20 g of ground old dark honey comb, and 30 ml of distilled water. Fifteen newly emerged wax moth females from each of the experimental and control groups were transferred into 30-ml plastic cups covered with a screen lid. To determine average adult longevity, the number of dead adults in each treatment group was recorded every day until all adults died. Experiments were replicated four times with 20 larvae/replicate.

2.4. Survival rate and development

2.4.1. Wax moth larvae

Developmental time from first- to seventh-instar, pupal and adult stages, and survivorship in these stages were recorded. The seventh-instar larvae were transferred into another jar lined with frilled filter paper for pupation and then adult emergence. Developmental time from first- to seventh-instar, pupal and adult stage, and survivorship in these stages, as well as adult longevity, were recorded for each replicate. Each experiment, including four EBI concentrations and one control, was replicated four times with 20 larvae/replicate.

2.4.2. Ectoparasitoids

Late last instar wax moth larvae treated with given concentrations of EBIs were used as host for rearing the parasitoids. Ten treated host larvae were offered to mated females (2–3-day-old) for 4 h in plastic cups with screen lids. After the parasitization period, females were removed. Except one egg, other eggs laid on a host larva were removed and destroyed with a fine-tipped forceps without damaging the host. Control wasps were reared on untreated wax moths. Each parasitized host larva with its one parasitoid egg was placed in a 30-ml plastic cup covered with a screen lid to ensure emergence of wasp adults. Developmental time from first- to third-instar, pupal and adult stage, and survivorship in these stages were recorded for each replication. Larval mandibles of parasitoid were measured using a eyepiece micrometer attached to a stereomicroscope to assess parasitoid growth and larval development stages using methods of Consoli and Vinson (2002). Each experiment, including four concentrations of EBIs and one control, was replicated four times with 10 newly hatched wasp larvae/replicate.

2.5. Adult longevity

2.5.1. Wax moth larvae

Newly emerged wax moth females from each of the experimental groups were transferred into 30-ml plastic cups covered with screen lid. To determine average adult longevity, the number of dead adults in each treatment group was recorded every day until all adults died. Experiments were replicated four times with 10 adults/replicate.

2.5.2. Ectoparasitoids

The effects of EBIs on longevity of the B. hebetor females were investigated by rearing the parasitoid on last instar hosts reared in...
the presence of EBIs (at 0.001, 0.01, 0.1 or 1.0 g/100 g diet). Control wasps were reared on untreated host larvae. Newly emerged adult mated wasp females from each of the treatments groups were paired with males in 30-ml plastic cups covered with screen lids. After mating was observed, males were removed from the cups. Females were provided with 50% honey solution saturated cotton pad for 1 h/day. To determine average adult longevity, the time from emergence to death for each female was recorded every day until all adults died. Experiments were replicated four times with 10 newly emerged adults/replicate.

2.6. Fecundity of *B. hebetor*

Ten newly emerged mated *B. hebetor* females from EBI-exposed hosts were placed individually in 30 ml plastic cups with screen lids. The females from stock culture were used as control group. They were fed with 50% honey solution for 1 h/day. Late last instar host larvae obtained from the stock culture were presented to the wasp females in the cup for oviposition for 4 h/day. Each of 10 female parasitoids was provided a host for oviposition. Parasitized hosts were removed immediately after oviposition and held for 24 h to complete parasitoid embryonic development; parasitoid larvae were allowed to hatch on the host larvae. Eggs were counted daily and thereafter fecundity (total number of eggs produced/female/day) was recorded for all females beginning with the 3rd day after emergence throughout their life spans (26–30 days). Each experiment, including five EBI concentrations and one control, was replicated four times with 10 females/replicate. Egg production was calculated as the total number of eggs divided by the number of females and collecting days (whole life span). The fecundity is recorded as the number of eggs and hatching rate/female/day.

3. Results

3.1. Developmental times

The influence of EBIs on host developmental times is summarized in Fig. 1A. For *Galleria*, control larvae reached their 7th instars.
in about 25 days, pupated by about day 32 and eclosed as adults soon after 40 days. Dietary Dex did not exert significant influence on development time, with exception of larvae reared on the highest Dex dosage, which reached adulthood about 5 days faster. Dietary Esc did not influence developmental time. The dual COX/LOX inhibitor, Phe, increased time to pupation and adult eclosion.

Parasitoid developmental times were not strongly influenced by host dietary Dex or Esc except for hosts reared in the presence of the highest dietary inhibitor concentration (Fig. 1B). Dietary Phe increased time to pupation for wasps reared on Phe-treated hosts.

3.1.1. Survival rates

For the host larvae, dietary Dex reduced survival of late instars, pupae and adults, relative to controls (Fig. 2A). Larval survival decreased from about 80–90% for controls to about 40% or less for larvae reared in the presence of 0.01% or higher concentrations of Dex. Dietary Esc reduced pupal and adult survivorship even at the lowest concentration of 0.001%, while Phe exerted less impact on survival.

Dex decreased parasitoid survival, but only at high host dietary concentrations (0.01, 0.1 or 1%) and only for adult wasps (Fig. 2B). The highest dietary concentrations of the LOX inhibitor, Esc, reduced larval survival, but pupal and adult wasps reared on larvae developed in the presence of all Esc concentrations experienced reduced survival. Dietary Phe did not influence survival of the parasitoids.

3.2. Longevity

Dex increased adult wax moth longevity from about 8 days for controls to about 14 days for insects reared on diets supplemented with 0.1 and 1% Dex (Fig. 3A). The Dex effect on longevity was

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Fig. 2. The influence of three anti-inflammatory pharmaceuticals (Dex, Dexamethasone; Esc, Esculetin; Phe, Phenidone) on survival of the indicated life stage in the greater wax moth, G. mellonella (Panel A) and the parasitoid wasp B. hebetor (Panel B). Histogram bars depict mean number of days (1 SE; n = four biological replicates, 20 insects per replicate) to the indicated life stage. Means followed by the same letter are not significantly different (P > 0.05, LSD test).
expressed in a dose-dependent manner. Dietary Esc and Phe exerted negligible effects on adult lifespans.

The longevity of adult wasps reared on experimental hosts was reduced from about 25 days to about 15 days (Fig. 3B) for all dietary Dex concentrations. Wasp longevity increased slightly following development on experimental hosts reared in the presence of Esc and of Phe.

3.3. Fecundity and hatch rates

Adult wasps reared on experimental larvae expressed reduced fecundity, recorded as about 70 eggs/day for controls and about 12 eggs/day for the highest Dex concentration. About 60% of eggs from control wasps hatched, which declined to about 30% of eggs from wasps reared on diets supplemented with the highest Dex concentration. Approximately similar patterns obtained with wasps reared on Esc- and Phe-treated host larvae (Fig. 4).

4. Discussion

Most research with insects on the influence of inhibiting eicosanoid biosynthesis have been short-term experiments, testing hypotheses about primary urine formation in Malpighian tubules (Petzel and Stanley-Samuelson, 1992; Kerkhove et al., 1995) or about cellular immunity (Stanley, 2006; Stanley et al., 2009). In the work on urine formation we addressed the effects of inhibiting eicosanoid biosynthesis in isolated Malpighian tubules, not whole animals. Similarly, most research on eicosanoids in insect immunity reported on the short-term consequences of treating insects with inhibitors of eicosanoid biosynthesis on various immune parameters (Stanley et al., 2009). The data reported in this paper address the broader issue of how chronic inhibition of eicosanoid biosynthesis influences insect biology. Our hypothesis suggested that chronic inhibition of eicosanoid biosynthesis influences life table parameters of G. mellonella and its ectoparasitoid, B. hebetor. In the most general statement, the
Eicosanoids act in two general areas of the body, where they have enormous clinical significance. PGs are present and exert biological actions in every mammalian tissue. Eicosanoids also act in crucial points in insect biology. In the mating system of the Australian field cricket, *Teleogryllus commodus*, males transfer both the enzyme and substrate necessary to produce PGE$_2$ to the spermathecae of newly mated females. PGE$_2$ migrates into the hemolymph and circulates to the terminal abdominal ganglion, where it releases egg-laying behavior. PGs also release oviposition behavior in other insect species. This PG-mediated egg-laying behavior appears to be a general maturity assurance mechanism (Simmons, 2005). Eicosanoids also mediate several cellular immune reactions to infection and invasion in insects (Stanley et al., 2009). Hence, the impact of chronic inhibition of eicosanoid biosynthesis would not be registered in terms of life history unless the experimental insects passed through a crucial eicosanoid-dependent point, such as a key reproductive process or responding to infection, in their lives.

Relative to the influence of the inhibitors on development times of *Galleria* larvae, development time was slightly shortened at the highest Dex concentration and lengthened by a few days at the highest dosage of Phe. Dietary Esc did not influence development times. Overall, chronic inhibition of eicosanoid biosynthesis did not influence larval development. For the ectoparasites reared on experimental larvae, the highest larval dietary dosage of Dex, Esc and Phe significantly increased development times. The effects of these compounds on wasp development did not obtain in a dose-related manner, even though the dosages were increased 10-fold intervals. We take this finding to indicate the inhibitors did not influence a physiological mechanism in development.

Dietary Dex reduced survival of *Galleria* larvae, pupae and adults, even at low inhibitor concentrations (0.01 g/100 g diet). With approximately similar patterns for the other inhibitors, chronic Dex treatments certainly exerted a negative influence on survival. Dex also decreased survival of the ectoparasites, but only in adults, not juveniles while Esc influenced larvae, pupae and adults. Phe did not influence the ectoparasite survival. Again, the data do not indicate statistically significant dose-related effects and it is questionable that the reduced survival followed from influencing physiological processes.

For the experimental insects that survived, however, dietary Dex increased adult longevity of wax moths from about 8 days to about 14 days, and this was expressed in a dose-dependent manner. No other treatments influenced adult longevity. The same may be said of the parasitoids, although the point is somewhat obscured by the shape of the graphs. *Bracon* adults reared on Dex-treated larvae experienced reduced adult longevity, however, this was apparently not a physiological effect because all four treated larvae experienced reduced adult longevity, however, this was apparently not a physiological effect because all four experimental treatments led to subtle, if any, biologically meaningful changes in life history other than strongly impacting fecundity of the parasitoid wasps that fed on treated hosts.

This is a reasonable outcome in light of the biological significance of PGs and other eicosanoids in mammals. Eicosanoids are present and exert biological actions in every mammalian tissue and body fluid, where they have enormous clinical significance (Haegstrom et al., 2010). Eicosanoids act in two general areas of animal biology. One, they are important modulators of cellular homeostatic functions, such as ion exchange physiology. These actions are often called housekeeping roles. Eicosanoids also serve as mediators of inflammation, fever and several important disease states, including osteoarthritis, thrombosis, glaucoma, atherosclerosis, asthma and many cancers. Many over-the-counter and prescription pharmaceuticals designed to inhibit PG biosynthesis are widely used on a daily basis to ameliorate symptoms of these conditions without influencing human life histories. While these inhibitors do not impact the life histories of humans or of mammalian models, inhibition of PG biosynthesis can aggravate some conditions. For example, in the mammalian stomach, PGs exert a protective effect on gastric mucosa. Using a rat model of gastric ulcer, Schmassmann et al. (1998) found that inhibition of PG biosynthesis in the stomach delayed ulcer healing in a dose-dependent manner.

Eicosanoids also occur and are biologically active in all invertebrates, including insects, that have been investigated (Stanley, 2000). Although the literature on invertebrates is rather thin compared to the biomedical literature, eicosanoids act in invertebrate housekeeping functions. For example, eicosanoids mediate ion transport physiology in bivalve gills, and in the rectal tissue and Malpighian tubules of insects (Stanley, 2000). Eicosanoids also act in crucial points in insect biology. In the mating system of the Australian field cricket, *Teleogryllus commodus*, males transfer both the enzyme and substrate necessary to produce PGE$_2$ to the spermathecae of newly mated females. PGE$_2$ migrates into the hemolymph and circulates to the terminal abdominal ganglion, where it releases egg-laying behavior.
mellonella specifically (Stanley-Samuelson and Dadd, 1984), recording the presence of AA in all tested insect species. Esc and Phe may influence parasite life span, but the effect was registered only at the highest esc and phe doses.

All three of the tested anti-inflammatory drugs potently reduced fecundity of adult wasps that had been reared on drug-treated host larvae. The experimental treatments also reduced hatchability of eggs produced by adult wasps reared on drug-treated hosts because PGs and other eicosanoids act in insect ovarian development. Both of these patterns were expressed in a dose-related manner, from which we infer the drugs acted in a physiological way. This is congruent with recent findings on the significance of PGs and other eicosanoids in insect ovarian development. Medeiros et al. (2002) reported that PGs are involved in vitellogenesis in the blood sucking bug, Rhodnius prolixus. More recently, Machado et al. (2007) reported on ovarian follicle development in the silk moth, B. mori. Using their cultured ovariole system, they showed that treating ovarioles with the COX inhibitors, aspirin and indomethacin, blocked the transition from vitellogenic to chorionic follicles. They followed this with more detailed experiments showing that ovarioles treated with aspirin did not express several stage-specific genes, including the transcription factor BmGATAβ and the orphan nuclear receptor BmE75C. These PCR results were backed up with Western blots showing the expected proteins were not present in experimental ovarioles. These studies show that the biological significance of PGs and other eicosanoids in insect reproduction extends far beyond releasing egg-laying behaviors. A detailed picture of eicosanoid actions in insect reproduction becomes more interesting with data showing that robust immune reactions to fungal challenge in R. prolixus is linked to reduced fecundity. The fungal challenge stimulates increased hemolymph PGE2 titers and the PGF2α down-regulated follicular development (Medeiros et al., 2002) reported that PGs are involved in vitellogenesis in the blood sucking bug, Rhodnius prolixus. More recently, Machado et al. (2007) reported on ovarian follicle development in the silk moth, B. mori. Using their cultured ovariole system, they showed that treating ovarioles with the COX inhibitors, aspirin and indomethacin, blocked the transition from vitellogenic to chorionic follicles. They followed this with more detailed experiments showing that ovarioles treated with aspirin did not express several stage-specific genes, including the transcription factor BmGATAβ and the orphan nuclear receptor BmE75C. These PCR results were backed up with Western blots showing the expected proteins were not present in experimental ovarioles. These studies show that the biological significance of PGs and other eicosanoids in insect reproduction extends far beyond releasing egg-laying behaviors. A detailed picture of eicosanoid actions in insect reproduction becomes more interesting with data showing that robust immune reactions to fungal challenge in R. prolixus is linked to reduced fecundity. The fungal challenge stimulates increased hemolymph PGE2 titers and the PGF2α down-regulated follicular development (Medeiros et al., 2002). This emerging literature demonstrates eicosanoid actions in ovarian development and it strongly supports the concept that chronic inhibition of eicosanoid biosynthesis during key reproductive events can translate into reduced fecundity.

We note that the anti-inflammatory drugs exerted their influence in a tri-trophic relationship, from larval diet to larvae and on to their ectoparasites. If the drugs were rapidly modified within the host diet or within the host body, little influence on the ectoparasite would be expected. There is very little information on the fate of anti-inflammatory drugs within insects. In the sole study in this area, Miller and Stanley-Samuelson (1996) injected radioactive indomethacin (COX inhibitor) into fifth-instar tobacco hornworms. Almost half of the injected radioactive was recovered from the frass between 4 and 12 h after injection, indicating that a substantial amount of indomethacin remained within the body through the first 12 h after treatment. The radioactivity was characterized by radio-high performance liquid chromatography, which showed that most of the injected material was not metabolized within the insect body. Based on these data, at least one COX inhibitor is not substantially metabolized within insects. If this is true, also, for the inhibitors used in this study, the tri-trophic movement of the inhibitors is a reasonable possibility.

Acknowledgements

We are grateful to Deva Holding (Istanbul, Turkey) for providing dexamethasone as a gift. We also are grateful to Dr. Adem Güler and Dr. Eylem Akman Gündüz (Ondokuz Mayıs University, Faculty of Science and Arts, Department of Biology) for providing the B. hebeter culture used in these experiments. This article reports the results of research only and mention of a proprietary product does not constitute an endorsement or recommendation for its use by the USDA.

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