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# The Chemistry of Lipid Signal Molecules in Insects

by

Sean M. Putnam

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Entomology

Under the Supervision of Professor David W. Stanley

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# The Chemistry of Lipid Signal Molecules in Insects

Sean M. Putnam, Ph.D.

University of Nebraska, 2009

Adviser: David W. Stanley

I report on the chemical identification of lipid signaling molecules in three species of insects and describe the chemical isolation of a bacterial product that inhibits eicosanoid biosynthesis.

Chapter 2 reports that eicosanoids mediate nodulation reactions to bacterial infection in newly-emerged, but not forager honeybees. Cyclooxygenase and lipoxygenase inhibitors attenuate nodulation, which is reversed in the presence of arachidonic acid. Older adult honeybees do not produce bacterial-induced nodules, and they have fewer circulating hemocytes, from which I infer that foraging honeybees express a physiological trade-off between maintaining a biologically expensive hemocytic immune system and flight activity associated with foraging.

Chapters 3 and 4 report on eicosanoid production in two insect tissues, *Manduca sexta* midgut and *Zophobus atrata* fat body. Optimal reaction conditions for prostaglandin biosynthesis were developed. Microsomal-enriched fractions of the tissues produced four PGs, PGA/B<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2α</sub>. Chemical structures of each PG was confirmed by gas chromatography-mass spectrometry. PG biosynthesis was reduced by two cyclooxygenase inhibitors, indomethacin and naproxen. In contrast to the mammalian model, PG biosynthesis was more prevalent in cytosolic fractions compared to microsomal fractions. The chemical confirmation of PG structures provides strong

evidence that PGs are definitely produced by insect tissue and that these lipid mediators facilitate important roles in biological actions.

Chapter 5 describes the chemical properties of a factor produced from an insect pathogenic bacterium, *Xenorhabdus nematophilus*, which attenuates immune responses. The bacterium suppresses nodulation responses to bacterial infections by inhibiting eicosanoid biosynthesis. The immunity-suppressing factor from living *X. nematophilus* was present in the organic, and not aqueous, fraction of the bacterial culture medium. This chemical work was the first step in identification of a new class of eicosanoid biosynthesis inhibitor.

This work advances the eicosanoid hypothesis by chemically confirming that PGs are synthesized in insect tissues and that they represent significant mediators of biological actions in insects. Beyond that, the work illustrates the power of multidisciplinary research to advance our understanding of insect biology generally.

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## TABLE OF CONTENTS

	Page
<b>Chapter 1:</b> Endocrine and Eicosanoid Signaling in Insect Biology .....	1
<b>Chapter 2:</b> Eicosanoids act in nodulation reactions to bacterial infections in newly emerged adult honey bees, <i>Apis mellifera</i> , but not in older foragers	
Abstract .....	30
Introduction .....	31
Materials and Methods .....	33
Results .....	39
Discussion .....	42
References .....	48
Figure legends .....	51
<b>Chapter 3:</b> Prostaglandin biosynthesis by midgut tissue isolated from the tobacco hornworm, <i>Manduca sexta</i>	
Abstract .....	59
Introduction .....	60
Materials and Methods .....	62
Results .....	65
Discussion .....	69
References .....	74
Figure legends .....	78

<b>Chapter 4:</b>	Prostaglandin Biosynthesis by Fat Body from Larvae of the Beetle <i>Zophobas atratus</i>	
	Abstract .....	90
	Introduction .....	91
	Materials and Methods .....	93
	Results .....	97
	Discussion .....	101
	References .....	107
	Figure legends .....	112
 <b>Chapter 5:</b>	 The Bacterium <i>Xenorhabdus nematophilus</i> Depresses Nodulation Reactions to Infection by Inhibiting Eicosanoid Biosynthesis in Tobacco Hornworms, <i>Manduca sexta</i>	
	Abstract .....	123
	Introduction .....	124
	Materials and Methods .....	126
	Results .....	131
	Discussion .....	134
	References .....	138
	Figure legends .....	142
 <b>Chapter 6:</b>	 Summary and Conclusions.....	 150
 <b>Appendix:</b>	 Extra-Dissertation Experiences, teaching and grant writing Experiences .....	  157



# Chapter 1

## Endocrine and Eicosanoid Signaling in Insect Biology

## Endocrine mediators of Insect Biology

Beginning in the 1930's with the work of Sir Vincent Wigglesworth, who demonstrated the functional role of developmental hormones in the blood-sucking bug, *Rhodnius prolixus* (Wigglesworth, 1934), the following decades have witnessed a tremendous growth in knowledge of endocrine signaling moieties in insect biology. Hormones, the chemical messengers of the endocrine system, are produced within organisms to coordinate physiological and biological activities. Biological functions affected by hormones include embryogenesis, postembryonic development, behavior, water balance, metabolism, caste determination, polymorphism, mating, reproduction and diapause (Klowden, 2007). Compared to the short-term and rapid responses of the nervous system, hormones can impart long-lasting effects due to their influence on gene expression. Although distinct in function, the nervous and endocrine systems coordinate most, if not all, biological functions in animals, including insects. Cells and tissues respond to hormones via hormone-specific receptors, which can be localized in the plasma- and nuclear membranes as well as within the cytosol. Hormones are generally categorized according to chemical properties, such as lipophilic (steroids and sesquiterpenoids), peptidal and biogenic amines.

Early researchers in insect endocrinology focused on the molting hormones, ecdysone (E) and juvenile hormone (JH; Wigglesworth, 1936; Karlson, 1996). E, first identified by Karlson (Butenandt & Karlson, 1954), is a steroid hormone derived in most insects from exogenous cholesterol in the prothoracic glands and in some insects, the epidermal and gonadal cells (Gilbert et al., 2002). E is subsequently metabolized into its active form, 20-hydroxyecdysone (20E), in most insect tissues by an intracellular 20E-

monooxygenase (Sakuri et al., 1989; Greenwood & Rees, 1984). 20E is principally responsible for the first step in molting, apolysis, or the separation of an old cuticle from the epidermal cells of the integument. Long after the discovery of 20E, a second discovery revealed that the actual shedding of the old cuticle, ecdysis, was signaled by another hormone, eclosion hormone (EH; Truman, 1992). EH is a peptide hormone synthesized by the ventral medial cells of the brain in conjunction with declining ecdysteroid levels (Zitnan & Adams, 2000). In molting insects, ecdysis-triggering hormone precedes the release of EH that in turn releases a third ecdysal hormone, crustacean cardioactive peptide (Zitnan et al., 1996; Gammie & Truman, 1999). This latter hormone acts directly on ventral nerve cord ganglia, releasing the pattern of behavior necessary to complete ecdysis from the old cuticle.

The other central developmental hormone, JH, acts on the epidermal cells to determine the subsequent body form following a molting period. Wigglesworth (1940), who first coined the term juvenile hormone, reported when homogenates of corpora allata (CA), the JH synthesizing tissue, were implanted into the last larval instar of *Rhodnius prolixus*, the recipients molted into supernumerary larvae rather than the expected adult form. In the simplest sense, 20E functions to promote metamorphic events, whereas JH regulates developmental stages (Nijhout, 1994). Berger & Dubrovsky (2005) concluded that JH acts primarily at the level of gene expression as a direct transcriptional regulator of JH target genes and through indirect suppression of 20E-dependent transcriptional regulation. The six major JH forms also exert regulatory roles in embryonic development, adult behaviors, vitellogenin synthesis, and pheromone production (Bellés et al., 2005; Klowden, 2007). JH expression is tightly regulated by two sets of neuropeptide

regulators: allatotropins, which stimulate JH biosynthesis, and allatostatins, which inhibit JH biosynthesis. JH levels are also regulated through degradation by two sets of hydrolytic enzymes: JH epoxidase hydrolases and JH esterases.

During the 1980's and beyond, the biological significance of biogenic amines in insect physiology and behavior became apparent. Biogenic amines are biologically active neurotransmitters, or neuromodulators. They are synthesized in specific neurons from amino acid precursors. Dopamine, a catecholamine, is a derivative of tyrosine, while histamine, serotonin and  $\gamma$ -aminobutyric acid (GABA) are synthesized from the amino acids histidine, tryptophan and glutamate, respectively (Voet & Voet, 2004). Octopamine, a homolog of the mammalian neurotransmitter norepinephrine (Roeder, 1999), functions in insects to modulate the effects of other neurotransmitters, such as the neuromuscular neurotransmitter L-glutamate (Nishikawa & Kidokoro, 1999). Contemporary research into biogenic amine biochemistry and modulation-effects will provide greater insights into our understanding of insect physiology.

### **The emergence of eicosanoids**

The 1980's also saw the discovery of prostaglandins (PGs) and other eicosanoids in insects. The term 'eicosanoid' was coined by Corey et al. (1979) as an umbrella term for oxygenated metabolites of 20-carbon polyunsaturated fatty acids (PUFA), including arachidonic acid ( $\Delta^{5,8,11,14}20:4n-6$ ; AA). The eicosanoids are lipid mediators of many physiological and pathophysiological processes (Funk, 2001; Murphy et al., 2004). Figure 1 presents an overview of eicosanoid-mediated actions in vertebrates. For most animals, AA, is derived from essential fatty acids, molecules that are required in the diet

because the organism lacks the ability to produce them *de novo*. Generally speaking, insects are capable of synthesizing most polyunsaturated fatty acids given the appropriate dietary substrate(s), usually linoleic acid ( $\Delta^{9,12}$ 18:2n-6; LA; Stanley, 2000). Figures 2 and 3 outline the biosynthetic pathways for n-6 & n-3 long-chained PUFAs in insects (from Stanley, 2000). The general consensus for insects is that virtually all insects contain C20 PUFAs, but typically in very low concentrations (Stanley, 2000).

Development of the mammalian model of PGs, and ultimately eicosanoids, began with Kurzrok & Lieb's (1930) discovery that semen facilitates marked responsiveness of uterine smooth muscle. The semen-mediated smooth muscle contraction and relaxation demonstrate that the same tissue can express opposite responses. Even though significant biological actions were identified from vesicular and prostate gland secretions, this work did not generate serious consideration because the nature and specificity of the active constituents were then unknown. von Euler (1935, 1936) first coined the term "prostaglandin", along with the term "vesiglandin", as pharmacological descriptors for the prostate and vesicular gland extracts. The characterization of their chemical structures provided directions for identifying, isolating and, much later, synthesizing these compounds *in vitro*.

Bergström and his colleagues forged our current understanding of PG biochemistry by determining the chemical structures of PGs (Bergström et al., 1962a & b; Bergström, 1982). Bergström and Sjövall (1957) reported the first isolation of PGs from sheep prostate gland, designated as PGs E and F (PGE & PGF, respectively). They determined the empirical formulae and structures of PGE and PGF by rigorous chemical procedures (Bergström & Sjövall; 1957, 1960a, 1960b, Bergström & Samuelsson, 1965).

They also showed that AA is converted into PGE<sub>2</sub>, whereas PGE<sub>1</sub> and PGE<sub>3</sub> are synthesized from homo- $\gamma$ -linolenic acid (all-*cis*-eicosa-8,11,14-trienoic acid:  $\Delta^{8,11,14}20:3n-6$ ) and eicosapentaenoic acid (all-*cis*-eicosa-5,8,11,14,17-pentaenoic acid;  $\Delta^{5,8,11,14,17}20:5n-3$ ), respectively (van Dorp et al., 1964; Bergström et al., 1964). These structures are represented in Figure 4. The significance of PG biochemistry and pharmacology was recognized early in Bergstrom's writings, as cited in his short communication, *"It does not appear unlikely that the prostaglandins are representatives of a group of hormonal compounds of general importance. Their high biological activity makes pharmacological exploration of the activity of similar compounds of interest"* (Bergström, 1962a). The broad biological significance of PGs emerged as scientists pursued PGs in tissues other than the male reproductive systems, such as pig lung, guinea pig uterus, human myometrium, rabbit jejunum, and sheep iris. (Hall & Pickles, 1963; Änggård & Samuelsson, 1964).

In the mammalian model, the first step in eicosanoid biosynthesis is the catalytic hydrolysis of AA from the *sn*-2 position of cellular phospholipids (PLs) by phospholipases A<sub>2</sub> (PLA<sub>2</sub>s; Dennis, 1994). PUFAs are generally associated with the *sn*-2 position within cellular PLs, whereas saturated or monounsaturated fatty acids are typically linked to the *sn*-1 position. This asymmetric composition within PLs allows enzyme specificity for C20 PUFAs. Another consideration of PUFA accessibility is their bilayer-specific distribution within cell membranes. The external and cytoplasmic leaflet compositions of the phospholipid bilayer was compared by Sandra and Pagano (1978) where the cytoplasmic leaflets were found enriched with unsaturated fatty acids. This biased distribution provides cytoplasmic enzyme systems a greater abundance of PUFAs.

PLA<sub>2</sub> is a superfamily of enzymes that hydrolyzes AA, and other PUFAs, from the *sn*-2 position of PLs resulting in the release of free AA and a lysophospholipid. Enzymes of the PLA<sub>2</sub> superfamily vary in cellular function, localization, regulation, amino acid sequencing, protein structure and metal ion cofactor requirements. PLA<sub>2</sub> activity was first characterized in pancreatic juices, snake venoms and bee venoms (Dennis, 1994). These enzymes are secretory PLA<sub>2</sub>s (sPLA<sub>2</sub>) because they are secreted and act outside of the cells that produce them. A second general group, the cytosolic PLA<sub>2</sub>s (cPLA<sub>2</sub>s) are active within intracellular environments. Currently 15 PLA<sub>2</sub> groups are recognized (Schaloske & Dennis, 2006). Regarding eicosanoid biosynthesis, both sPLA<sub>2</sub>s and cPLA<sub>2</sub>s act in AA hydrolysis (Dennis, 1994; Balestrieri & Arm, 2006; Schaloske & Dennis, 2006). Group IVA PLA<sub>2</sub>s are the central enzymes responsible for launching eicosanoid biosynthesis (Ghosh et al., 2006; Lambeau & Gelb, 2008). These PLA<sub>2</sub>s exhibit strong preference for AA-specific *sn*-2 phospholipids (20-fold increase over other PUFAs in the *sn*-2 position) resulting in agonist-induced release of AA for eicosanoid production (Leslie, 2004). The gene for this enzyme has 95% identity between human and mouse and over 80% identity between humans and other vertebrates. This is a highly conserved enzyme structure in many species, including a filamentous fungus, *Aspergillus nidulans* (Ghosh et al., 2006; Hong et al., 2005). The dynamics of PLA<sub>2</sub>-activated AA release points to complex regulatory mechanisms, responsible for finely-tuned responses associated with eicosanoid production.

Following hydrolysis from PL fractions, free AA is commonly oxygenated into eicosanoids via three metabolic pathways. The cyclooxygenase (COX) pathway produces the prostanoids, which include PGs and thromboxans, the lipoxygenase (LOX) pathway

leads to leukotriene production, and the cytochrome P-450 epoxygenase pathway produces epoxyeicosatrienoic acids. The structures and biosynthetic pathways for these molecules are depicted in Figure 5.

Eicosanoid biosynthesis, commonly called AA-metabolism because AA is the prevalent C20 PUFA in mammalian cells, proceeds through the formation of endoperoxide intermediates (Hamberg and Samuelsson, 1973). Hamberg et al. (1974) identified two PG intermediates, PGG<sub>2</sub> and PGH<sub>2</sub>. COX catalyzes the conversion of AA into PGG<sub>2</sub> by oxygenation at C-11 and C-15 positions within the AA chain. PGG<sub>2</sub> is converted into PGH<sub>2</sub> by an associated peroxidase. PGH<sub>2</sub> is converted into most of the primary PGs by cell specific enzymes. Hamberg et al. (1974) bioassayed PGG<sub>2</sub> and PGH<sub>2</sub> for biological activity using rabbit aorta smooth muscle contraction, finding that PGG<sub>2</sub> and PGH<sub>2</sub> were 50-100 times and 100-450 times more active than PGE<sub>2</sub>, respectively. These intermediates also stimulate platelet aggregations whereas PGE<sub>1</sub> and PGE<sub>2</sub> inhibit platelet aggregation. These findings make the point that the intermediates, as well as the end products, of PG biosynthesis are biologically active.

Understanding of PG biosynthesis advanced considerably with the characterization of COX, which contains two enzyme activities in the same protein, COX and peroxidase (Miyamoto et al., 1976; Kiefer et al., 2000). Until the early 1990s it was thought only one form of COX is expressed in mammalian tissues, although that view changed with discovery of COX-2 (Masferrer, et al. 1990; Kraemer et al. 1992). COX-1 is a constitutive form, responsible for “housekeeping”, homeostatic functions. COX-2, an inducible form of the enzyme, is up-regulated in the presence of a wide range of inducers in a variety of different cell types (Herschman, 2004). These two isoenzymes share about



80% sequence identity, differing principally in the N- and C-termini. Both are located in the endoplasmic reticulum and nuclear membranes. Both COX and peroxidase active sites are conserved in the two forms, providing a common catalytic mechanism. The pathway of PGH<sub>2</sub> synthesis is outlined in Figure 6.

Discovery that aspirin inhibits PG biosynthesis (Vane, 1971) opened research into pharmacological significance of PG biosynthesis. A second set of studies showed that indomethacin also inhibits PGs production in dog spleens. Both compounds exert anti-inflammatory, antipyretic and analgesic actions. These pharmaceutical compounds are now called non-steroidal anti-inflammatory drugs (NSAIDs) and they act via inhibiting COX (Ferreira et al., 1971 & 1973). This work launched an industry that led to discovery of many pharmaceutical inhibitors of eicosanoid biosynthesis, some of which are now common over-the-counter analgesics. Samuelsson, Berström and Vane shared the 1982 Nobel Prize in Physiology or Medicine for their work on PGs and other eicosanoids.

The biological significance of PGs in insects was first recognized with the work of Destephano and Brady, (1977), who demonstrated that PGE<sub>2</sub> released egg-laying behavior in the cricket, *Acheta domesticus*. Soon after, Loher et al. (1981) reported on the detailed mechanism of PG actions in releasing egg-laying behavior in another cricket species, *Teleogryllus commodus*. Specifically, a COX is transferred from male to female in seminal fluids within spermatophores. Once in the spermathecae of newly-mated females, COX converts AA into PGE<sub>2</sub>. The PGE<sub>2</sub> moves into hemolymph circulation, where it interacts with the terminal abdominal ganglion to release oviposition behavior. Later Stanley-Samuelson and Loher (1986) showed that spermatophores also deliver

substantial quantities of AA to spermathecae. This early work showed that an entirely new class of signal molecules, PGs, also act in insect biology.

### **Eicosanoids in Insect Immunity**

It is now understood that PGs and other eicosanoids exert crucial actions in several areas of insect biology. PGs modulate water and ion transport in Malpighian tubules of the mosquito, *Aedes aegypti* (Petzel and Stanley-Samuelson, 1992), and the ant, *Formica polyctena*, (Van Kerkhove et al., 1995), in rectal tissues of the locust, *Locusta migratoria* (Radallah et al., 1995) and salivary glands of female ticks, *Amblyomma americanum* (Bowman et al., 1995). Midgut cells from the tobacco hornworm, *Manduca sexta*, biosynthesize substantial amounts of PGs, from which I infer that PGs exert so far unidentified actions in insect midgut tissue.

Possibly the most important actions of PGs and other eicosanoids are in insect immunity (Stanley et al., 2009). Immunity is a complex area of insect biology that requires some introductory remarks. Insects protect themselves from microbial and parasite invasions by three broad systems. The physical barriers of the integument and peritrophic membrane comprise the first line of immunological defense. Once invaders breach these physical barriers they are met with the latter two components, humoral and cellular immune processes, collectively called the innate immune system.

Humoral immunity is characterized by induced biosynthesis of antimicrobial peptides, complement-like proteins, and enzymes that regulate melanin production and clotting. These factors usually appear in the hemolymph of infected insects 6 – 12 hours after infection. Current research into pathogen-specific recognition of the innate system

functionally involves a series of proteins called pattern recognition receptors (PRRs), which selectively bind to pathogen-associated molecular patterns (PAMPS; Siva-Jothy et al., 2005). These hemoceolic proteins initiate the cascades of immunological reactions, such as the production of clotting fibers, activation of the prophenoloxidase pathway, and antimicrobial peptide generation (De Gregoria et al., 2002; Kanost et al., 2004; Siva-Jothy et al., 2005; Strand, 2008a).

Cellular (also called hemocytic) immunity depends on direct interactions between circulating hemocytes and the invaders, which begin immediately an infection is detected (Stanley et al., 2009). Hemocyte-mediated processes include phagocytosis, encapsulation and nodulation, a form of encapsulation (Strand, 2008b). Cell adhesion to both invaders and other hemocytes is tied directly to specific immune functions, including microaggregation, nodulation, encapsulation, phagocytosis, and wound repair. (Irving et al., 2005; Strand, 2008a). Eicosanoids are involved in many, if not all of these defense actions (Stanley, 2006).

While the biological significance of PGs and other eicosanoids in insect biology is by now well established, there remains a critical lacuna in overall understanding. PUFAs and their oxygenated metabolites make up a very wide range of chemical species. These molecules are fairly similar in their structures and in many systems they can not be adequately resolved on chromatography. At the time I began my graduate research, most of these molecules from insect sources had not been rigorously identified. In the absence of rigorous chemical identification, hypotheses that PGs and other eicosanoids are crucial elements of insect biology lack a crucial pillar of support. I developed an independent hypothesis that, although they are present in insect tissues in very small amounts, the

major eicosanoids in insect tissues could be extracted from the tissues without serious chemical degradation, could be derivatized for gas chromatography and the chemical structures could be confirmed by analyses of mass spectra. Similar chemical approaches could lead to identification and chemical synthesis of an insect pheromone. In the chapters following this introduction, I substantively support my hypothesis by reporting on the biosynthesis and chemical structures of PGs involved in three insect systems. Chapter 2 (Bedick et al., 2001) reports that newly-emerged honey bees were competent to form nodules in response to bacterial challenges but foraging bees were not. This is apparently due to a physiological trade-off in which adult honeybees lose almost all circulating hemocytes. This new finding was later confirmed by Schmid et al. (2008) who reported that honeybee adult queens, drones and workers abandon hemocytic, but not phenoloxidase-based immunity.

Rigorous chemical verification of the structures of PGs and other eicosanoids is a crucial step forward in grasping the biological significance of lipid mediators in insect physiology. Mammals produce a wide range of eicosanoids, all the known ones of which have been identified by chemical methods. As the mammalian model can serve as an imprecise guide for research into insects, it becomes clear the chemical identification of these compounds from insect sources is an important component of future work.

Chapters 3 & 4 (Büyükgüzel et al., 2002; Tunaz et al., 2002) provide substantial chemical evidence supporting the eicosanoid hypothesis in insects. The midgut of *Manduca sexta* and the fat body of *Zophobus atratus* both produce PGs, and their chemical structures were verified by gas chromatography-mass spectroscopy.

I also report on chemical separation of bacterial factors responsible for impairing insect immune functions (Park et al., 2003). Chapter 5 furthers the eicosanoid hypothesis by establishing a process to chemically identify a factor that the insect pathogen *X. nematophila* produces and secretes to inhibit the activity of sPLA<sub>2</sub>s. Blocking this first step in eicosanoid biosynthesis effectively compromises insect immune reactions to the presence of the bacterium's mutualist nematodes. Identifying the chemical nature of enzyme inhibitors provides a foundation for developing compounds that have potential for inhibiting insect immunity in pest insects. Understanding the chemical interactions of regulators, inhibitors and enzymes in insect eicosanoid systems potentially enhances our abilities to develop greater specificity in pest management protocols.

Looking back to my Master's thesis, in the Appendix I review the discovery of two novel polyunsaturated fatty acid metabolites in insects and present other extra-dissertation activities. I complete this dissertation with brief consideration of the scientific significance of interdisciplinary research in the chemistry and biology of insects. It will become clear that my independent leadership is based on my background as a chemist. I contributed rigorous chemical analyses of prostaglandins, pheromones and a new bacterial factor responsible for inhibiting insect immune reactions to infection.

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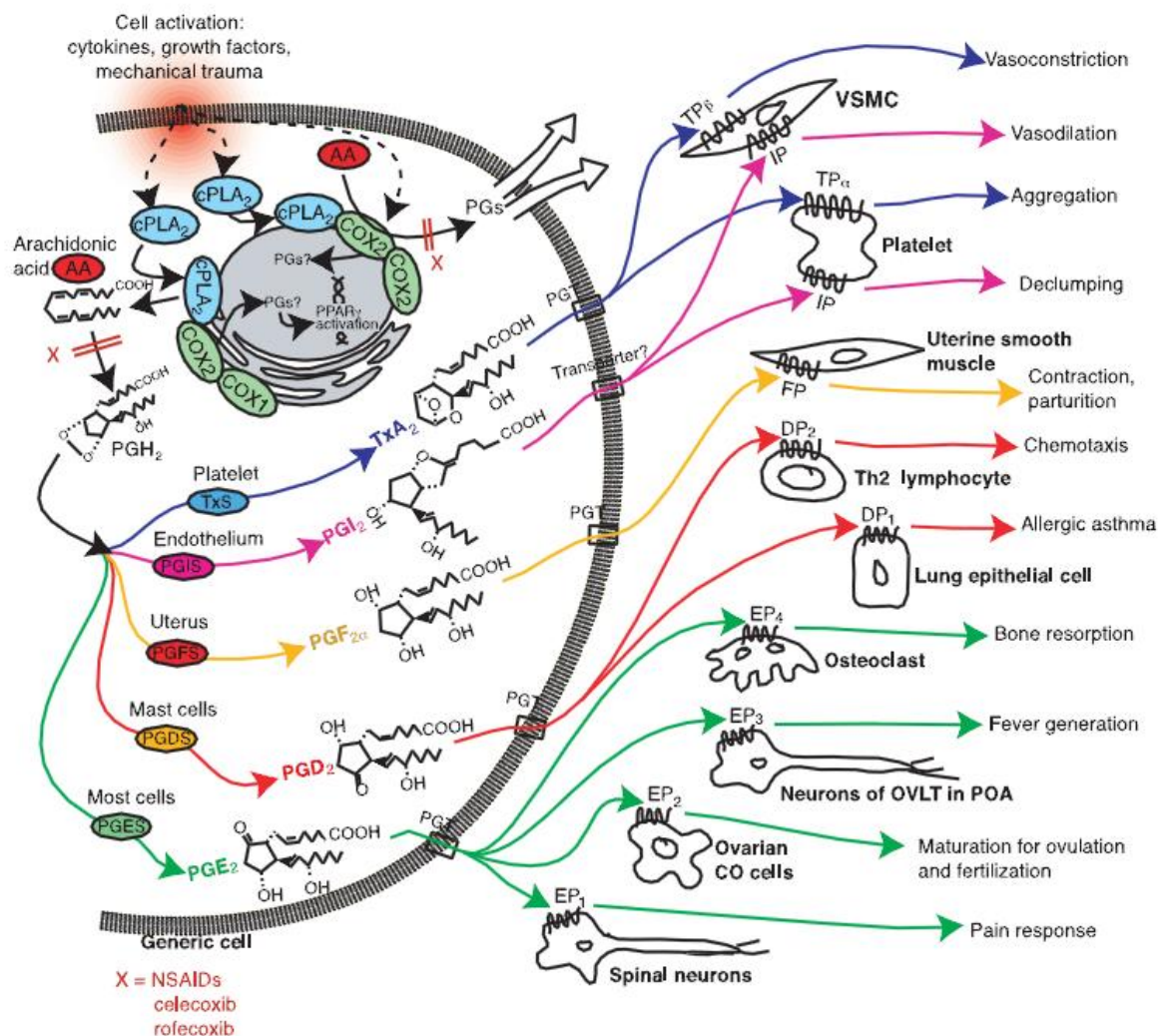


Figure 1. Prostaglandin synthesis and physiological actions. Prostanoid biosynthesis is stimulated in a “generic” mammalian cell by cytokines, growth factors, inflammatory stimuli and mechanical trauma. The generalized pathway follows: stimulation, PLA<sub>2</sub> activation, AA conversion to PGH<sub>2</sub> via prostaglandin H synthase (COX), followed by prostanoid-specific enzyme systems with specific cells. Funk (2001)

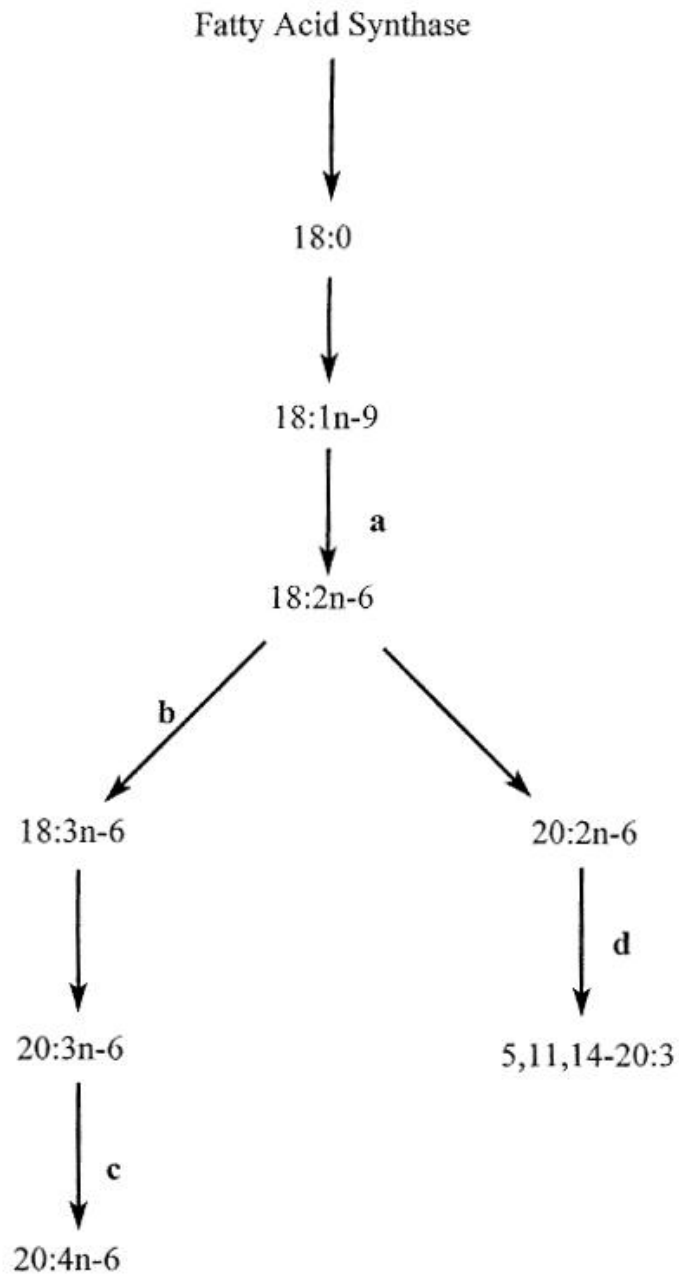


Figure 2: Elongation desaturation pathways responsible for biosynthesis of 18:2n-6, and subsequent elongation desaturation to C20 polyunsaturates. a =  $\Delta^{12}$  desaturase. b =  $\Delta^6$  desaturase. c =  $\Delta^5$  desaturase. d =  $\Delta^5$  desaturase. Stanley (2000)



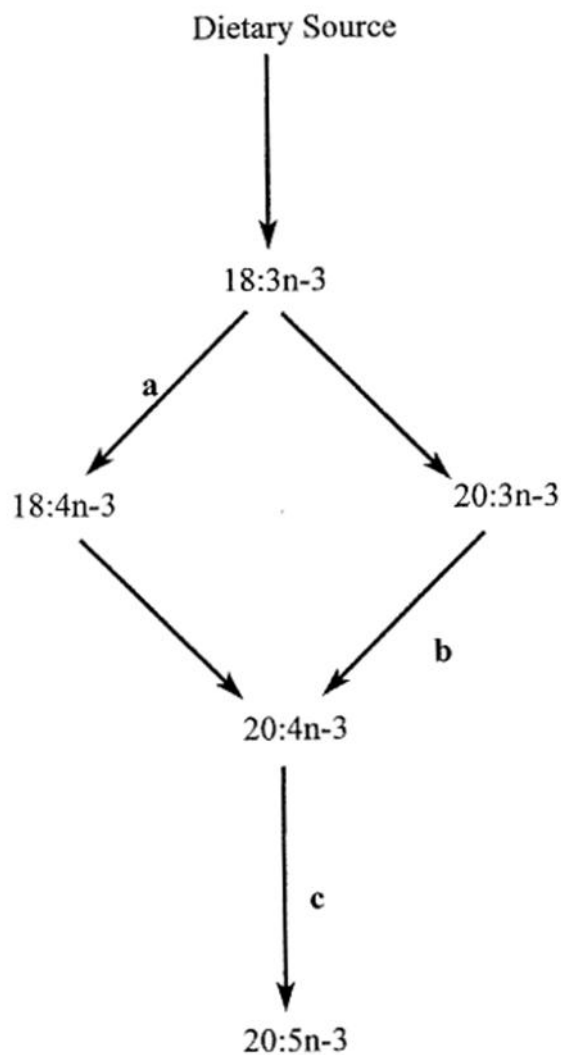


Figure 3. Proposed pathways for elongation desaturation for 18:3n-3 into C20 members of the n-3 family. a =  $\Delta^6$  desaturase. b =  $\Delta^8$  desaturase. c =  $\Delta^5$  desaturase. Stanley (2000)

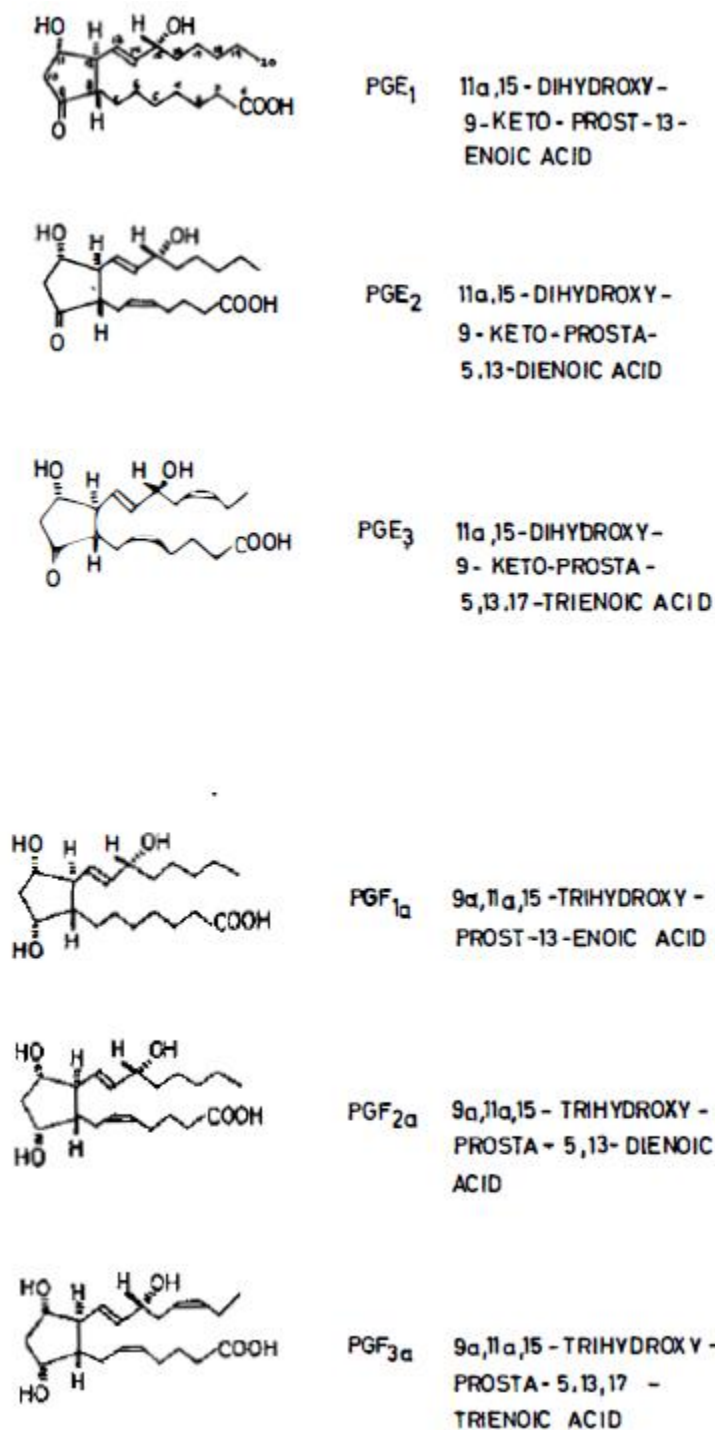


Figure 4. Structures of Prostaglandins E & F. The numeric classification of prostaglandins refers to the degrees of unsaturation within the hydrocarbon chain, dependent upon various PUFA precursors. Bergström, S. & Samuelsson, B. (1965).

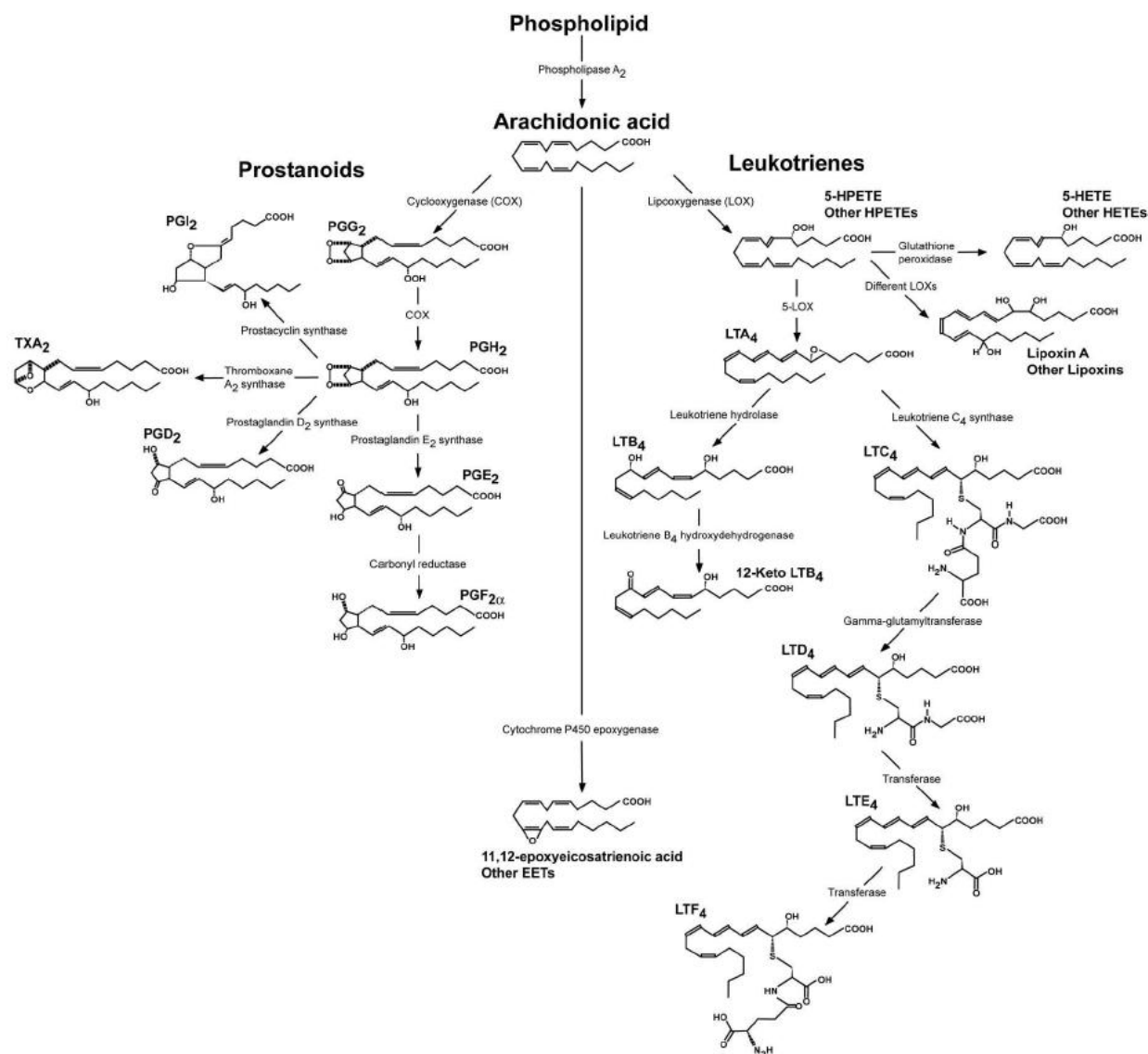


Figure 5: Overview of eicosanoid biosynthesis based on current knowledge from mammalian models. The three major pathways, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 epoxidase, are shown displaying major metabolites. Prostanoids cover prostaglandins (PG) and thromboxane (TX), while leukotrienes (LT) include LTs and lipoxins. PGD<sub>2</sub> and PGE<sub>2</sub> may be transformed into PGJ<sub>2</sub> and PGA<sub>2</sub> through either non-enzymatic rearrangement or dehydration, respectively. Abbreviations: EET, epoxyicosatrienoic acids; HETE, hydroxyeicosatetraenoic acids; HPETE, hydroperoxyeicosatetraenoic acids. Heckmann et al. (2008).

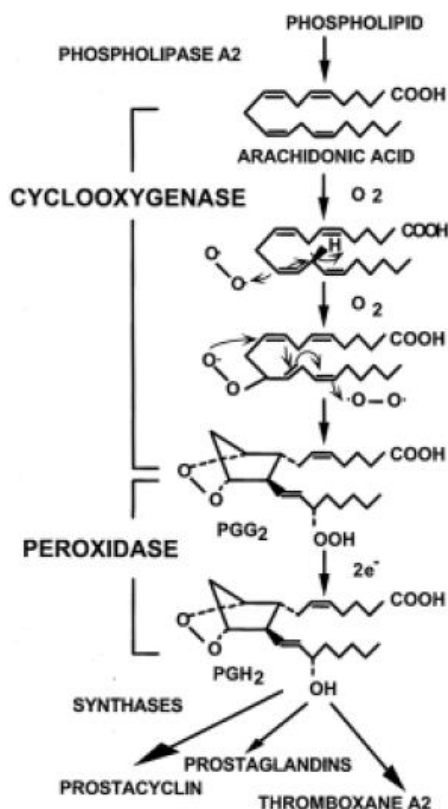


Figure 6. Biosynthetic pathway of prostanoid biosynthesis derived from arachidonic acid. The bis-oxygenase (cyclooxygenase) addition to arachidonic acid yields PGG<sub>2</sub> followed by the electron-reduction producing PGH<sub>2</sub>. The pathway for cell-specific prostanoids diverges from the PGH<sub>2</sub> precursor into prostacyclin, prostaglandins and thromboxanes. Smith et al. (2000)

## Chapter 2

Eicosanoids act in nodulation reactions to bacterial infections in newly emerged adult honey bees, *Apis mellifera*, but not in older foragers

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Tunaz, A.R. Nor Aliza, S.M. Putnam, M.D. Ellis, D.W. Stanley (2001)

**Abstract**

Nodulation is the first, and qualitatively predominant, cellular defense reaction to bacterial infections in insects. We tested the hypothesis that eicosanoids also mediate nodulation reactions to bacterial challenge in adults of a social insect, the honey bee, *Apis mellifera*. Treating newly-emerged experimental bees with the eicosanoid biosynthesis inhibitor, dexamethasone, impaired nodulation reactions to bacterial infections, and the influence of dexamethasone was reversed by treating infected insects with arachidonic acid, an eicosanoid precursor. Several other eicosanoid biosynthesis inhibitors, including the cyclooxygenase inhibitor, indomethacin, and the dual cyclooxygenase/lipoxygenase inhibitor, phenidone, also impaired the ability of experimental honeybees to form nodules in reaction to bacterial challenge. The influence of phenidone on nodulation was expressed in a dose-dependent manner. However, in experiments with older honey bee foragers, similar bacterial challenge did not evoke nodulation reactions. We infer from our results that while eicosanoids mediate cellular immune responses to bacterial infections in newly emerged honey bees, and more broadly, in most insect species, nodulation reactions to bacterial challenge probably do not occur in all phases of insect life cycles.

## Introduction

Hemocytic immune reactions to bacterial infections involve direct cellular interactions between circulating hemocytes and bacteria. Specific cellular defense mechanisms include phagocytosis and nodulation (Gupta, 1991). While humoral and hemocytic immune reactions to bacterial infections are well documented, until recently, there was virtually no information on the biochemical events responsible for mediating insect immune reactions. Drawing on the background of signal transduction systems in mammalian immunity, Stanley-Samuelson et al. (1991) suggested insect cellular immune reactions are mediated by eicosanoids. Eicosanoids are oxygenated metabolites of arachidonic acid and two other polyunsaturated fatty acids (Figure 1), the structures and biosynthesis of which are outlined elsewhere (Stanley, 2000). Eicosanoids are very well understood in the contexts of human and animal medicine, where they influence many pathophysiological events, including inflammation. Beyond their actions in mammals, recognition of the biological significance of eicosanoids in invertebrates is steadily growing, as detailed in recent reviews (Stanley and Howard, 1998; Howard and Stanley, 1999; Stanley, 2000). In our initial investigations into the possible roles of eicosanoids in invertebrate immunity, we determined that treating tobacco hornworms, *Manduca sexta*, with pharmaceutical inhibitors of eicosanoid biosynthesis rendered experimental hornworms unable to clear bacterial infections from hemolymph circulation. We inferred from these observations that some or all of the cellular defense reactions responsible for clearing bacterial infections from hemolymph are mediated by eicosanoids (Stanley-Samuelson et al., 1991). This was the first suggestion of a signal transduction system in invertebrate cellular immunity. Nodulation is an insect cellular defense reaction

responsible for clearing large numbers of bacterial cells from circulation during the first 2 h of an infection (Horohov and Dunn, 1983). Because nodulation is the predominant cellular reaction to bacterial infections, we hypothesized that eicosanoids mediate nodulation reactions to bacterial infections. We tested this idea by injecting hornworms with an eicosanoid biosynthesis inhibitor, then infecting them with bacteria. Compared to ethanol-treated controls, the experimental larvae produced significantly fewer nodules in response to similar bacterial challenges. Moreover, the influence of the eicosanoid biosynthesis inhibitor, dexamethasone, could be reversed by treating the experimental larvae with arachidonic acid, the immediate precursor of eicosanoids. These findings supported the idea that nodulation is one of the cellular immune responses to bacterial infections that is mediated by eicosanoids (Miller et al., 1994). On the basis of these findings in a single lepidopteran species, we developed the hypothesis that eicosanoids mediate nodulation reactions to bacterial infections in most, if not all, insect species, which for convenience we refer to as the eicosanoid hypothesis (Stanley and Howard, 1998; Stanley, 2000). Using similar experimental protocols, we have obtained comparable results with several insect species, including the tenebrionid beetle, *Zophobas atratus* (Miller et al., 1996), the silkworm, *Bombyx mori* (Stanley-Samuelson et al., 1997) the larvae of two other moths, black cutworms, *Agrotis ipsilon* and true armyworms, *Pseudaletia unipuncta* (Jurenka et al., 1997), adults of the cricket, *Gryllus assimilis* (Miller et al., 1999), the cockroach, *Periplaneta americana* (Tunaz and Stanley, 1999) and the 17-year periodical cicadas, *Magicicada septendecim* and *M. cassini* (Tunaz et al., 1999). In related work, Mandato et al. (1997) found that cell spreading and prophenoloxidase activation, two distinct phases of nodulation, as well as another cellular



defense reaction, phagocytosis, also are mediated by eicosanoids in waxmoths, *Galleria mellonella*. These findings uniformly support the eicosanoid hypothesis. Such positive support notwithstanding, innate immunity can be regarded as an adaptive trait whose expression is linked to life-history (Dunn, 1990). Under this view, there remains the question of whether all insects are competent to mount cellular defense reactions. In this paper we report on experiments designed to test the eicosanoid hypothesis in a social insect, the honey bee, *Apis mellifera*. We observed nodulation reactions to bacterial infections in newly-emerged adults, and found that these reactions depend on eicosanoid biosynthesis. However, we were unable to record nodulation reactions to bacterial challenge in older adults, those which had reached the age of foragers. We infer that the eicosanoid hypothesis is a useful organizing concept in insect immunity, however, it must be taken within the broader idea that the various expressions of insect immunity are adaptive traits which may or may not be present in any given life-history stage within a species.

## **Materials and methods**

### *Organisms*

Honey bees, *A. mellifera*, were taken from hives maintained on the University of Nebraska, Lincoln, campus during late summer. Pupae were removed from combs and used immediately. Newly-emerged adult bees were taken from combs within 24 h of emergence. Foraging bees were captured near the entrances of their hives. Cultures of a pigmented strain of the bacterium *Serratia marcescens* were taken from the microbe collection at UNL, and nutrient broth (Difco) was purchased from Carolina Biological

Supply (Burlington, NC). Bacteria were grown in 50 ml of nutrient broth in an environmental shaker at 37°C and 100 rev./min. The bacteria were freeze-dried, and this material was taken up into pyrogen-free water for injection into the bees.

### *Injections and assays for nodulation*

We followed the protocols formalized by Miller and Stanley (1998). Before injections, the bees were chilled, and then surface sterilized by swabbing their cuticle with 95% ethanol. We injected adults with either the phospholipase A<sub>2</sub> (PLA<sub>2</sub>) inhibitor dexamethasone {(11β, 16α)-9-fluoro-11, 17, 21-trihydroxy-16-methylpregna-1, 4-dione}, the cyclooxygenase inhibitor indomethacin {1-P- (chlorobenzyl)-5-methoxy-2-methyl-3-indolyl-acetic acid} or the dual cyclooxygenase and lipoxxygenase inhibitor phenidone {1-phenyl-3-pyrazolidinone} (all inhibitors purchased from BioMol, Plymouth Meeting, PA.) In rescue experiments, adults were injected also with arachidonic acid {5,8,11,14-eicosatetraenoic acid}, purchased from Sigma Chemical Co. (St. Louis, MO). Control insects were injected with 95% ethanol. Drugs and control substances were injected into the opposite side of the abdomen using a 10-μl Hamilton 701 syringe. All injections of pharmaceuticals were in a standard dosage of 52 μg in 4 μl of ethanol, except in dose-response experiments. The fatty acids were injected at dosages of 20 μg in 2 μl ethanol per adult honey bee.

Immediately after the drug injections, bees were challenged by injecting 50 μg of freeze-dried bacterial preparation, made up in pyrogen-free water, into each bee, following injection protocols of Miller and Stanley (1998). Bacteria were injected in 10-

μl aliquots, using a 26 gauge 0.5-inch needle attached to a 50-μl syringe (Hamilton, Reno, NV).

We assessed nodulation at selected times post-infection (PI). We anesthetized the bees by chilling them on ice, and then exposed their hemocoels. Melanized, black nodules were counted under a stereo microscope at 60×. The nodules were distinct, and direct counting reliably reflected the extent of the nodulation response to infections (Miller and Stanley, 1998). After the first counting, the alimentary canal was removed. Nodules in the previously unexposed areas and remaining internal tissues were then counted and the two counts were summed.

#### *Control experiments*

We conducted control experiments to register the background numbers of nodules in honey bees. To record the nodulation in unchallenged adults, 19 bees were taken from culture at various times in this project. We anesthetized bees on ice for 10 min, then assessed nodulation. To determine the influence of the drug vehicle, ethanol, on nodule formation, four adults were injected with 4 μl of ethanol. Nodulation was assessed 4 h later, following the standard protocol. To assess the effect of the pharmaceutical products on nodulation in unchallenged bees, a standard dosage of phenidone, in 4 μl of ethanol was injected into four bees. Nodulation was assessed by standard methods 4 h later. Finally, we tested the possibility that water or simple wounding could stimulate nodulation by injecting 4 μl of water into five honey bees or piercing the integument of four honey bees. Nodulation was assessed by standard methods 4 h later.

### *Dose-response curve for freeze-dried bacteria*

The freeze-dried bacterial preparation was made up in pyrogen-free water in four concentrations, 5, 10, 25 and 50 µg per injection. Newly emerged honey bees were anesthetized, sterilized and injected as described. Nodulation was assessed 4 h later.

### *Time course of nodulation: influence of Phenidone*

Individuals in two groups of bees were injected with 4 µl of ethanol or with 52 µg of phenidone in 4 µl of ethanol. The bees were immediately injected with bacteria as described. At 1, 2 and 4-h PI, sub-groups of control and experimental insects were anesthetized, and nodulation was assessed.

### *Dose-response curve for phenidone*

Individuals in four groups of bees were injected with 4 µl of ethanol, or 0.52, 5.2 or 52 µg of phenidone in 4 µl of ethanol, then challenged with a standard solution of freeze-dried bacteria. At 4-h PI, the bees were anesthetized, and nodulation was assessed.

### *Influence of other eicosanoid biosynthesis inhibitors on nodulation*

We divided bees into two groups and injected individuals in each group with either the cyclooxygenase inhibitor indomethacin, or the dual cyclooxygenase /lipoxygenase inhibitor phenidone, in standard dosages of 52 µg in 4 µl of ethanol. Control insects were injected with 4 µl of ethanol. Following injections, the bees were infected with a standard dosage of bacteria as described. At 4-h PI, the bees were anesthetized and nodulation was assessed.

### *Fatty acid rescue experiment*

Individuals in two groups of adult bees were injected with either 4  $\mu$ l of ethanol or 52  $\mu$ g of dexamethasone in 4  $\mu$ l of ethanol and then infected with bacteria as described. Immediately after challenge, the dexamethasone-treated bees were divided into two sub-groups. Individuals in one sub-group were treated with 20  $\mu$ g of arachidonic acid in 2  $\mu$ l of ethanol. Another sub-group was treated with 2  $\mu$ l of ethanol to control for the effects of the extra injection on nodulation. At 4-h PI, bees were anesthetized and nodulation assessed.

### *Determining biosynthesis of prostaglandins by abdominal preparations from newly-emerged honey bees*

We investigated eicosanoid biosynthesis by microsomal- enriched preparations of gut-free honey bee bodies. These experiments followed protocols developed for fat body from *M. sexta* (Stanley- Samuelson and Ogg, 1994). Briefly, tissues prepared from 15 honey bees were mechanically ground using a 1-ml glass homogenizer. The homogenates were sonicated for 40 s at 30 W using a VibraCell sonicator (VibraCell, Danbury, CT). This preparation was centrifuged for 10 min at 735 $\times$ g, and the supernatant was centrifuged for another 20 min at 16 000 $\times$ g, both steps at 4°C. The 16 000 $\times$ g supernatants were microsomal-enriched preparations used in all experiments. Protein concentrations in these preparations were determined as described by Stanley-Samuelson and Ogg (1994).

Radioactive arachidonic acid (5,6,8,9,11, 12,14,15-[ $^3$ H]-20:4, 60-100 Ci/mmol) was purchased from DuPont. The incubation buffer was 0.05 M  $\text{KH}_2\text{PO}_4$  , pH 8.0,

amended with a standard co-factor cocktail (2.4 mM reduced glutathione, 0.25 mM hydroquinone and 25  $\mu$ g hemoglobin per reaction). For each PG biosynthesis reaction, 0.4  $\mu$ Ci of labeled arachidonic acid was dispensed into reaction tubes and the solvent was evaporated. The reactions were carried out in 1.0-ml total volume. The experiments were preceded by a 10-min pre-incubation at 32°C with all reaction components, except the protein source. After a 2-min reaction period at 32°C, the reactions were stopped by addition of 500  $\mu$ l 0.1 N HCl. Reaction products were extracted from the acidified reaction mixture three times in ethyl acetate. The combined extracts, containing PGs and possibly lipoxygenase products, were evaporated under N<sub>2</sub>. A mixture of appropriate eicosanoid standards was added to each sample, then samples were applied to thin-layer chromatography plates (described above). The plates were developed and fractions observed as described (Stanley-Samuelson and Ogg, 1994). Bands corresponding in  $R_f$  to selected authentic eicosanoid standards and to free fatty acids were transferred to liquid scintillation vials. Radioactivity in each fraction was determined by adding Ecolite scintillation cocktail (ICN Biomedicals, Irvine, CA) and counting on a LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Pharmacia, Turku, Finland) at 50% counting efficiency for [<sup>3</sup>H]. Eicosanoid biosynthesis was calculated from the liquid scintillation data. In control experiments, microsomal-enriched preparations were heated in boiling water for 15 min before the experiments, and processed as just described. The results of these control experiments were used to correct values from biosynthesis experiments.

### *Statistical analysis*

Nodulation data were analyzed by analysis of variance in the General Linear Models procedure, and mean comparisons were made using the Least Significant Difference (LSD) test (SAS Institute Inc., 1989).

## **Results**

### *Control experiments*

Table 1 displays the results of control experiments. We recorded less than one nodule/bee ( $n=19$  honey bees) in untreated insects taken directly from the culture. Injections with water resulted in approximately four nodules/insect, and simple wounding induced virtually no nodulation. Similarly, we observed approximately eight nodules/insect in bees injected with phenidone. Pure ethanol seemed to induce a moderate level of nodulation because we recorded 46 nodules/insect in bees injected with ethanol. By comparison, challenges with standard dosages of the freeze-dried *S. marcescens*, prepared in pyrogen-free water, resulted in approximately 134 nodules per adult.

### *Dose-response curve for S. marcescens*

We recorded increased nodulation with increasing doses of the freeze-dried bacterial preparation, from approximately 10 nodules/insect at the lowest dose to approximately 50 nodules/insect at the highest dose (Figure 2). As a practical matter, these data informed our use of 50  $\mu\text{g}$ /insect in subsequent experiments.

### *Time course of nodulation*

The time course of visible nodule formation in two groups of bees, experimentals and controls, is shown in Figure 3. Phenidone-treated adults formed approximately three nodules/insect at 1-h PI, which increased to seven at 2-h PI, and to approximately 11 at 4 h. The ethanol-treated control adults produced 20 nodules at 1-h PI and 32 at 2-h PI. By 4-h PI, the control bees yielded significantly more nodules, approximately 130/insect.

### *Dose-response curve for phenidone*

The influence of phenidone on nodulation in response to bacterial infections was expressed in a dose-dependent manner (Figure 4). Nodulation declined from approximately 130 nodules/bee in ethanol-treated control bees, to approximately 11 nodules/insect in bees treated with the highest phenidone dosage (Figure 4). Intermediate dosages produced intermediate nodulation reactions.

### *Influence of other eicosanoid biosynthesis inhibitors on nodulation*

We considered the influence of three pharmaceutical inhibitors of eicosanoid biosynthesis on nodulation in response to bacterial infections (Figure 5). Compared to control (EtOH) bees, all experimental bees exhibited significantly reduced nodulation in response to bacterial infections (LSD,  $P < 0.05$ ). We obtained significant differences between the influence of phenidone, which severely inhibited nodule formation, and the influences of indomethacin and dexamethasone.



*Arachidonic acid reversed the influence of dexamethasone on nodulation*

According to the evidence taken from research in mammalian physiology, dexamethasone, as one of its actions, inhibits eicosanoid biosynthesis through its effect on PLA<sub>2</sub>. On this idea, injecting the eicosanoid-precursor polyunsaturated fatty acid, arachidonic acid, into dexamethasone-treated infected adults should reverse the effects of dexamethasone on nodulation. We used the following protocol to test this. After injection with dexamethasone, adults were infected with bacteria and then immediately treated with arachidonic acid. To control for the influence of the third injection on nodulation, an additional control group of bees was injected with ethanol. As can be seen in Figure 6, arachidonic acid treatments reversed the effects of dexamethasone on nodulation (LSD,  $P < 0.05$ ). The ethanol-injected control bees yielded approximately 160 nodules/honey bee and dexamethasone-treated bees approximately 37 nodules/honey bee, both in line with expectation. The arachidonic acid-treated bees produced approximately 190 nodules/bee, also in line with expectation for control animals. The second control group, injected with a second dose of ethanol, yielded approximately 50 nodules/bee.

*The influence of age on nodulation reactions to bacterial challenge*

Newly-emerged honey bees produced high numbers of nodules, usually well over 130 nodules/bee, in reaction to our standard challenge. The situation was entirely different for older, foraging honey bees. At 4 h after injection with our standard bacterial challenge, we recorded no nodules in foragers. This seemed a little odd, compared to our results with younger bees, and we investigated the point a little further. While we were

able to withdraw hemolymph and register the presence of hemocytes with younger bees, we were not able to withdraw hemolymph from foragers.

#### *Eicosanoid biosynthesis by abdominal tissues*

The gut-free bodies of newly-emerged adult honey bees converted arachidonic acid into two prostaglandins,  $\text{PGA}_2$  (1.05 pmol/mg protein/h) and  $\text{PGF}_{2\alpha}$  (0.07 pmol/mg protein/h). As seen in many insect preparations,  $\text{PGA}_2$  appears to be the predominant product.

### **Discussion**

In this paper we report on the outcomes of experiments designed to test the eicosanoid hypothesis in adults of the honey bee, *Apis mellifera*. The results of all experiments support the hypothesis for newly-emerged, but not for foraging bees. First, treating experimental bees with phenidone prior to challenging them with freeze-dried bacterial preparations significantly reduced nodulation at all points in the time course experiments. Second, the influence of phenidone on nodulation was expressed in a dose-dependent manner. Third, three different eicosanoid biosynthesis inhibitors significantly reduced nodulation relative to control treatments. Fourth, the influence of dexamethasone on nodulation was reversed by treating infected bees with arachidonic acid. Finally, we found that gut-free tissue homogenates were able to convert arachidonic acid into two prostaglandins. This indicates that honey bee tissues express the enzymes required for eicosanoid biosynthesis. Taken together, these separate lines of evidence strongly support the overall hypothesis.

The results of the time course experiment indicate that bees treated with phenidone produced significantly fewer nodules than the control bees at all time points in the experiment. We infer from this finding that inhibition of eicosanoid biosynthesis influences the cellular events involved in nodulation early in the infection process, and continues to exert a negative influence for many hours PI.

The time course experiment also indicates the upper limits of nodulation reactions to bacterial infections in bees. The experimental honey bees produced a maximum of approximately 130 nodules/individual by 4-h PI. This value is in line with the outcomes of similar experiments with other insect species. Larvae of the tenebrionid beetle, *Z. atratus* produced approximately 50 nodules/individual at 6-h after infection with the same number of bacterial cells (Miller et al., 1996). The larvae of several Lepidoptera produced more nodules in response to similar challenges. Tobacco hornworms, *M. sexta* yielded approximately 120 nodules/individual by 6-h PI (Miller et al., 1994), and silkworms, *B. mori*, formed approximately 80 nodules/individual at 15-h PI (Stanley-Samuelson et al., 1997). In our work with another hemimetabolous species, Miller et al. (1999) recorded approximately 45 nodules at 4-h PI in identical experiments with adult crickets, *G. assimilis*.

Howard et al. (1998) suggested that differences in nodulation intensity might be due to differences in circulating hemocyte populations. In work with tobacco hornworms and larvae of *Z. atratus*, Howard et al. (1998) recorded numbers of nodules formed in reaction to similar bacterial challenges as a function of insect size, weight and age. These experiments showed that nodulation was not influenced by these three parameters. Noting that insects tend to maintain fairly similar concentrations of circulating hemocytes

(approximately  $4\text{-}6 \times 10^6$  cell/ml hemolymph), those insect species with copious amounts of hemolymph, such as tobacco hornworms, would have far larger absolute numbers of circulating hemocytes. The authors speculated that if circulating hemocyte population sizes account for differences in nodulation capacity, then, it should not be surprising to record considerable differences among insect species in nodulation responses to similar infection challenges.

These comments are germane to our finding that older, foraging honey bees did not form visible nodules in reaction to bacterial challenge. We were unable to prepare hemolymph samples from foragers, even at the  $\mu\text{l}$  scale, from which we inferred that older honey bees have very few circulating hemocytes. The very low, for practical purposes, absent volumes of collectable hemolymph, with attendant low populations of circulating hemocytes, may be a form of immune senescence. Honey bees go through a lengthy excursion of age-related tasks, beginning with in-hive tasks such as tending brood and comb building and ending with outside tasks, including ventilating and finally foraging. While newly-emerged and cell-cleaning bees do not overlap in age with foragers, there is considerable overlap in age-related task performance (Winston, 1987). The mean age at first foraging is approximately 23 days, ranging from 18 to 28 days, which overlaps the age of several advanced in-hive tasks. Immune senescence may occur in honey bees, however, until this is investigated in more detail, it cannot be said whether the phenomenon is linked to physiological age or the onset of a certain category of tasks, such as outside tasks.

We considered influence of phenidone dosage on nodulation reactions to similar bacterial challenges. Dose-response relationships are basic to physiological research, and

the approximately linear negative relationship we obtained for phenidone strongly supports the idea that eicosanoids mediate nodulation reactions to bacterial infections in the honey bee.

Due to their importance in human medicine, many different inhibitors of eicosanoid biosynthesis are available. Some, such as aspirin and ibuprofen, are available as analgesic drugs for relief of minor pains, while many others are not yet approved for use in humans. These compounds, which we refer to with the general term ‘eicosanoid biosynthesis inhibitors’, exert different actions in cellular eicosanoid biosynthesis. For example, dexamethasone inhibits PLA<sub>2</sub>. Dexamethasone exerts other actions, as well, including influence on gene expression. Several compounds specifically inhibit cyclooxygenase, the first step in prostaglandin biosynthesis (Figure 1). Our experiments with different inhibitors showed that all three of the compounds we tested resulted in similar reductions in nodulation. The observation that separate experiments with different inhibitors of eicosanoid biosynthetic pathways similarly retarded nodulation in adult honey bees indicate that eicosanoids act in nodule formation.

The results of the rescue experiments strongly support our hypothesis that eicosanoids mediate nodulation in honey bees. Dexamethasone is thought to act by inhibiting PLA<sub>2</sub>, the enzyme responsible for releasing arachidonic acid from cellular phospholipids. This is the first and rate-limiting step in eicosanoid biosynthesis. Dexamethasone inhibits eicosanoid biosynthesis by inhibiting the release of substrate from cellular phospholipids, which in effect withholds substrate from cyclooxygenase and other eicosanoid biosynthesizing enzymes. If this is so, then providing arachidonic acid to the immunity-conferring cells within the honey bees would be expected to reverse

the influence of dexamethasone on nodulation. Indeed, the arachidonic acid treatments restored the honey bees' ability to produce nodules in response to bacterial infections.

Stanley-Samuelson (1994) indicated that experiments with eicosanoid biosynthesis inhibitors were based, in part, on the assumption that the experimental insects were competent to biosynthesize eicosanoids. The presence of eicosanoid precursor polyunsaturated fatty acids and the enzymes responsible for eicosanoid biosynthesis have been documented for honey bees (Stanley-Samuelson and Dadd, 1983). In this paper, we document the presence of eicosanoid biosynthesizing enzymes in fat body of adult honey bees. The honey bee preparations yielded  $\text{PGA}_2$  (at approx. 1.05 pmol/mg protein/h) as the major product. Rates of prostaglandin biosynthesis recorded from in vitro insect preparations are typically low, and the values recorded with honey bee preparations are similar to values recorded from larvae of the butterfly, *Colias eurytheme* (Stanley-Samuelson et al., 1997) and adults of cicadas, *Magicicada septendecim* (Tunaz et al., 1999). Hence, a basic assumption of this line of experimentation has been met.

Our background control experiments indicate that the nodules we recorded were due to the experimental treatments, and not to adventitious infections. Honey bees taken directly from the colony had a low background of nodulation. The injection treatments, similarly, did not influence the low background of nodulation. We note that control experiments with ethanol yielded higher numbers of nodules than recorded with other insect species (Miller et al., 1994, 1996), however, the key point is that the drug vehicle did not, in itself, diminish the bees' ability to form nodules. We note that control treatments with phenidone in the vehicle yielded very few nodules, indicating that the

ethanol-induced nodulation also is mediated by eicosanoids. We infer that the experimental protocols allow a physiological interpretation of the data, inhibition of eicosanoid biosynthesis impairs immunity in newly-emerged honey bees.

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Table 1: Outcomes of background control experiments <sup>a</sup>		
<b>Treatment</b>	<b>Number of Individuals</b>	<b>Nodules per bee (mean <math>\pm</math> S.E.M.)</b>
No treatment	19	0.05 $\pm$ 0.05
Inject EtOH	4	46.2 $\pm$ 12.4
Inject water	5	3.6 $\pm$ 1.0
Bacterial challenge	15	133.9 $\pm$ 11.7
Wound with needle	4	0.2 $\pm$ 0.2
<i>Inject phenidone</i>	4	7.7 $\pm$ 3.9

<sup>a</sup>Newly-emerged honey bees were treated as specified in the left column, and nodulation was assessed at 4-h post-treatment, except for bees taken directly from combs to assess nodulation in untreated individuals. The phenidone was dissolved in 95% EtOH, and the vehicle control was 95% EtOH. The bacterial challenge was our standard dosage of freeze-dried bacteria prepared in pyrogen-free water.

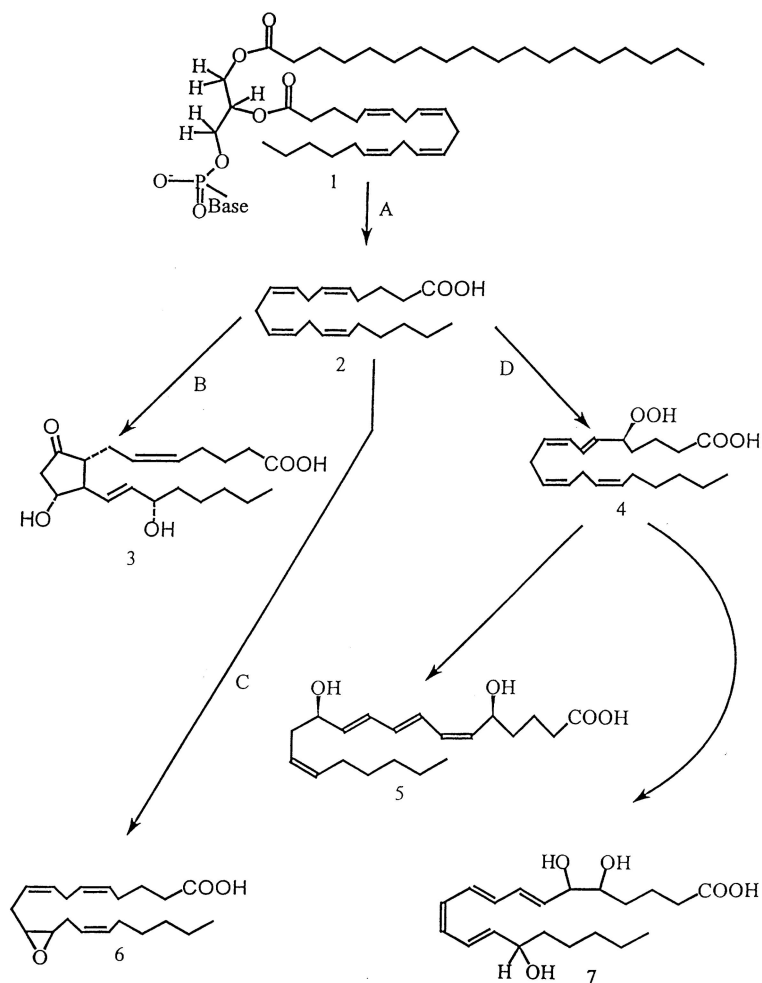


Figure 1. An overview of 20:4 $n$ -6 metabolism as understood from mammalian physiology. Three polyunsaturated fatty acids, 20:3 $n$ -6, 20:4 $n$ -6 and 20:5 $n$ -3 are potential substrates for eicosanoid biosynthesis. Of these, metabolism of 20:4 $n$ -6 is most well studied. Chemical structures are denoted by numerals. **1**=a cellular phospholipid; **2**=hydrolyzed 20:4 $n$ -6; **3**=prostaglandin  $E_2$ ; **4**=5-hydroperoxyeicosatetraenoic acid; **5**=leukotriene B<sub>4</sub>; **6**=11,12-epoxyeicosatrienoic acid; and **7**=lipoxin A. Capital letters indicate major enzyme systems responsible for eicosanoid biosynthesis. A=phospholipase A<sub>2</sub>; B=cyclooxygenase and associated enzyme steps; C=cytochrome P<sub>450</sub> epoxygenase; and D=lipoxygenase.

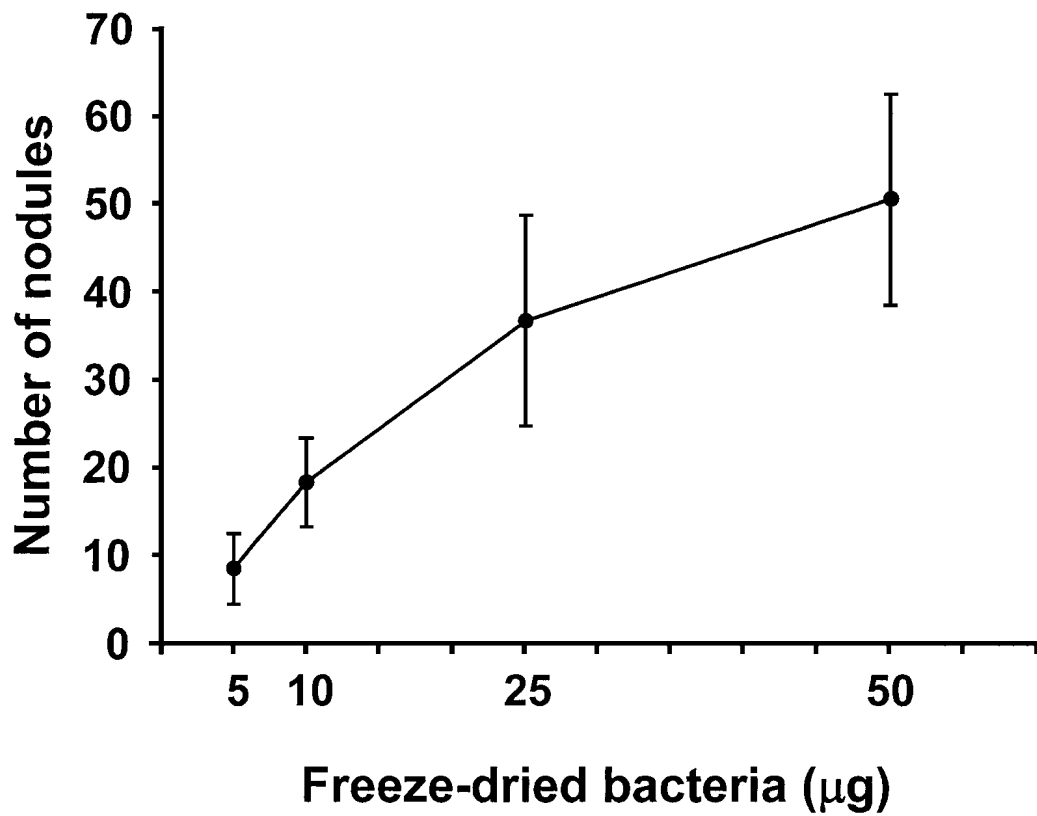


Figure 2. The influence of freeze-dried bacteria, *S. marcescens*, on nodulation reactions in adult honey bees, *A. mellifera*, was expressed in a dose-dependent manner. Bees were challenged with injection of the indicated dosages of freeze-dried bacterial, prepared in water, then nodulation was recorded at 4-h PI. Each point indicates the mean number of nodules found in each insect ( $n=6$  individuals), and the error bars represent 1 S.E.M.

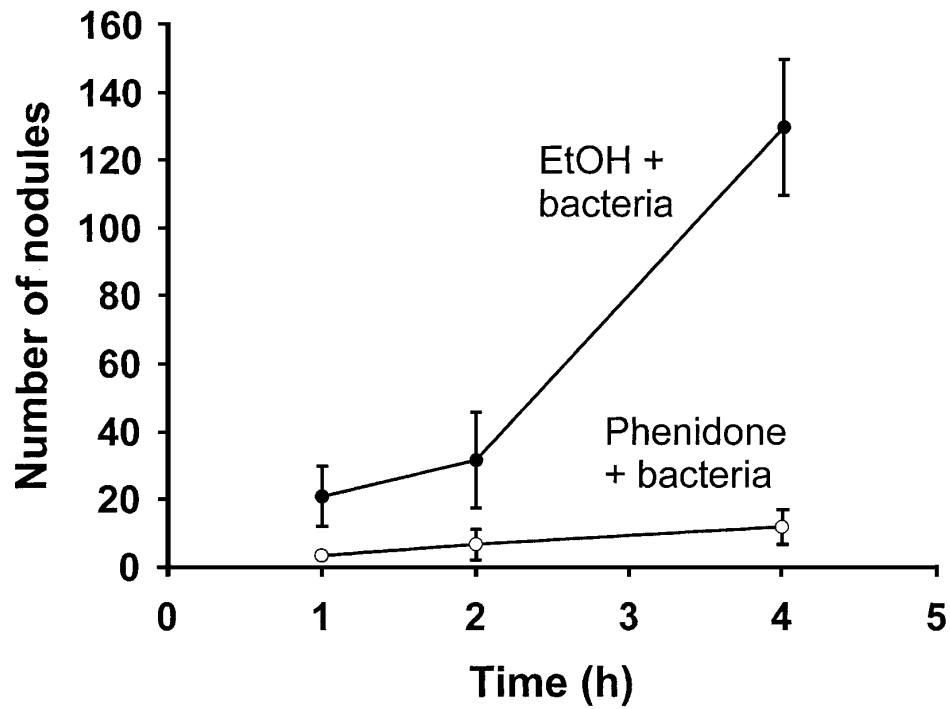


Figure 3. Time course of nodulation in adult honey bees, *A. mellifera*, in response to intrahemocoelic challenge with freeze-dried pathogenic bacterium, *S. marcescens*. Control insects were first treated with ethanol, then injected with bacteria and experimental insects were first treated with phenidone, then injected with bacteria. Each point indicates the mean number of nodules found at the indicated times PI in each insect ( $n=5$  or 6 individuals), and the error bars represent 1 S.E.M.

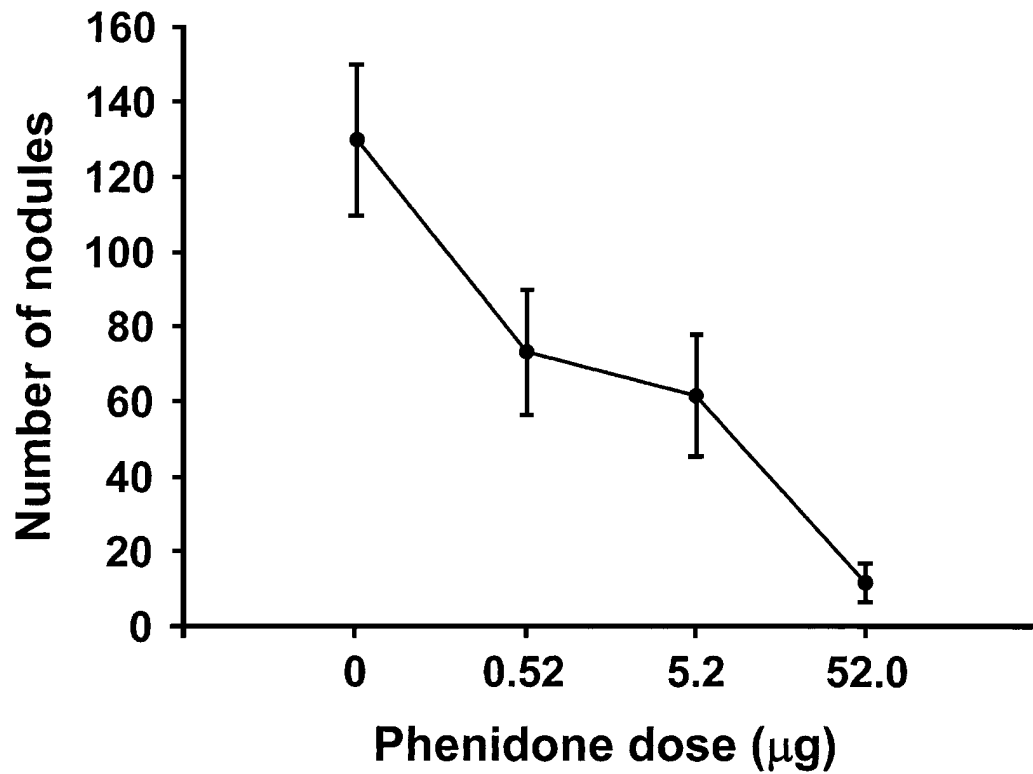


Figure 4. Dose-response curve for the influence of phenidone on nodule formation in adult honey bees. Individuals in four groups of bees were first treated with indicated dosages of phenidone, then challenged with intrahemocoelic injections of freeze-dried bacteria, *S. marcescens*. Nodulation was assessed at 4-h PI. Each point indicates the mean number of nodules found in each insect ( $n=6$ ) and the error bars represent 1 S.E.M.

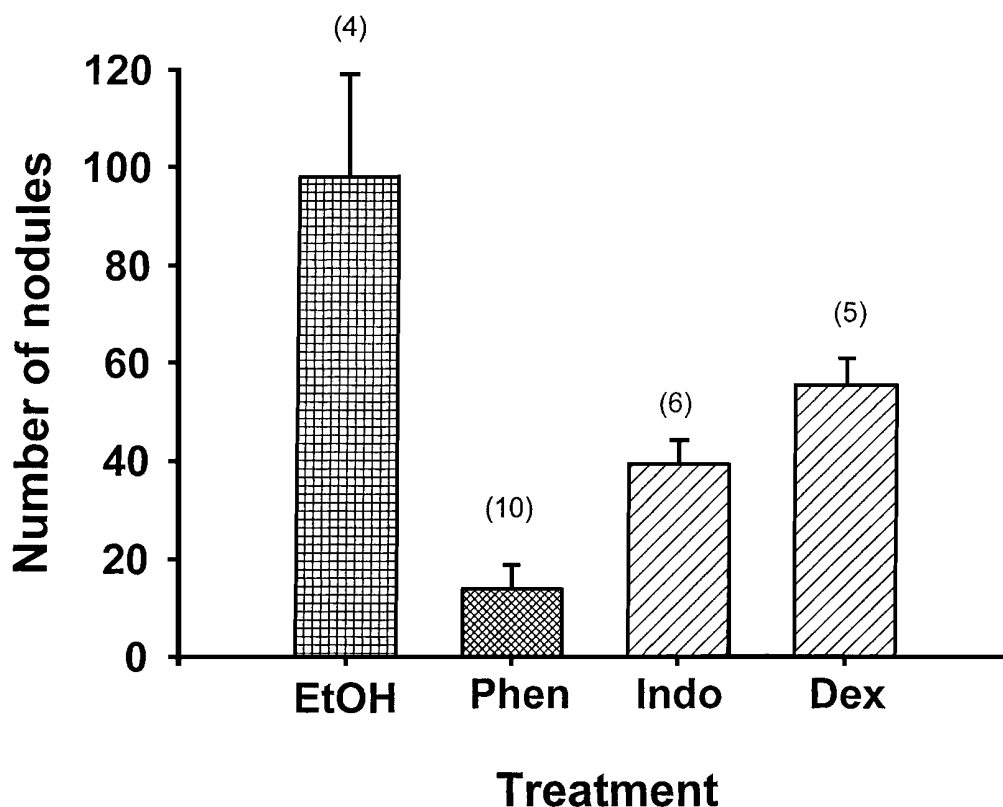


Figure 5. Effect of treating honey bees with individual eicosanoid biosynthesis inhibitors on nodule formation in response to intrahemocoelic infections with the insect pathogen, *S. marcescens*. Test insects were first injected with 52  $\mu$ g of either dexamethasone (Dex, PLA<sub>2</sub> inhibitor), indomethacin (Indo, cyclooxygenase inhibitor) or phenidone (phen, dual cyclooxygenase/lipoxygenase inhibitor), then challenged with intrahemocoelic injections of freeze-dried bacteria, *S. marcescens*. Control insects were first injected with ethanol (EtOH), then similarly challenged. Nodulation was assessed at 4-h PI. The height of the histogram bars represents the mean number of nodules found in each insect and the error bars represent 1 S.E.M. The number in parentheses represents the number of individuals in each treatment. Histogram bars with the same fill pattern are not significantly different from each other (LSD,  $P < 0.01$ ).



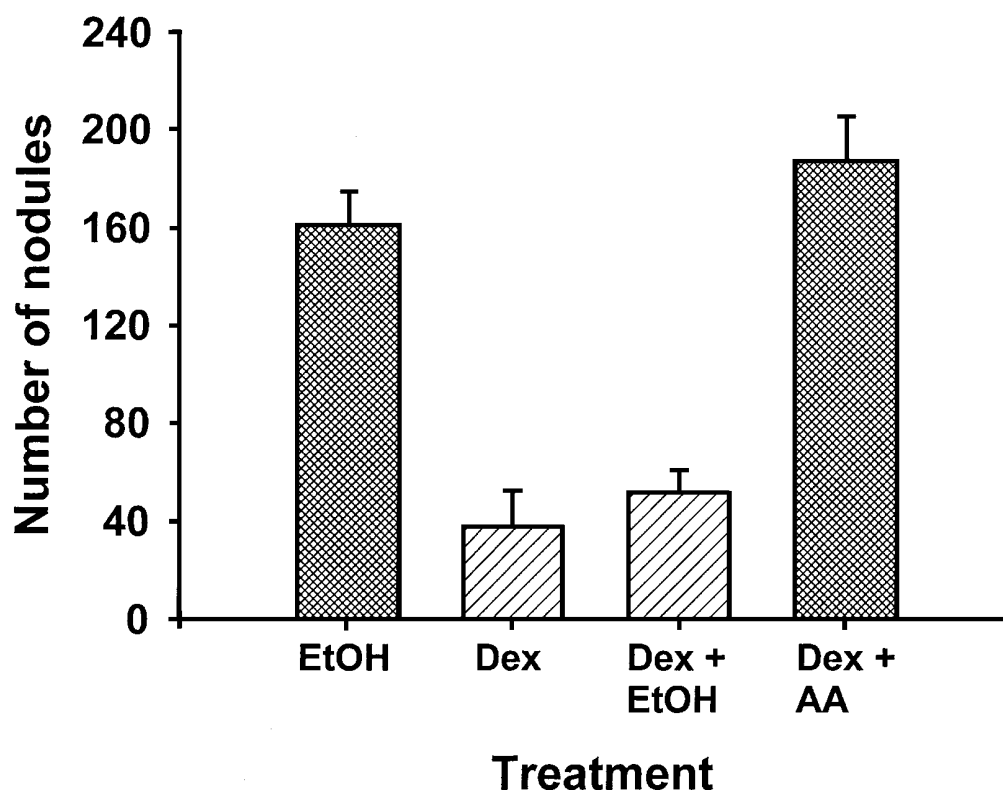


Figure 6. The eicosanoid-precursor fatty acid, arachidonate, reversed the effect of dexamethasone on nodulation in adults of *A. mellifera*. Honey bees were treated with ethanol (EtOH) or dexamethasone (Dex) and then challenged with intrahemocoelic injections of freeze-dried bacteria, *S. marcescens*. Immediately after challenge, test insects were treated with 20  $\mu$ g of arachidonic acid (Dex + AA). Control insects were treated with dexamethasone and ethanol (Dex + EtOH). The height of histogram bars represents the mean number of nodules found at 4-h PI in each insect ( $n=6$  individuals) and the error bars represent 1 S.E.M. Histogram bars with the same fill pattern are not significantly different from each other (LSD,  $P<0.01$ ).

## Chapter 3

Prostaglandin biosynthesis by midgut tissue isolated from  
the tobacco hornworm, *Manduca sexta*

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## Abstract

I describe prostaglandin (PG) biosynthesis by isolated midgut preparations from tobacco hornworms, *Manduca sexta*. Microsomal-enriched midgut preparations yielded four PGs, PGA/B<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2α</sub>, all of which were confirmed by analysis on gas chromatography–mass spectrometry (GC–MS). PGA and PGB are double bond isomers which do not resolve on TLC but do resolve by GC; for convenience, we use the single term PGA<sub>2</sub> for this product. PGA<sub>2</sub> was the major product under most conditions. The midgut preparations were sensitive to reaction conditions, including radioactive substrate, protein concentration (optimal at 1 mg/reaction), reaction time (optimal at 0.5 min), temperature (optimal at 22°C), buffer pH (highest at pH 6), and the presence of a co-factor cocktail composed of reduced glutathione, hydroquinone and hemoglobin. In vitro PG biosynthesis was inhibited by two cyclooxygenase inhibitors, indomethacin and naproxen. Subcellular localization of PG biosynthetic activity in midgut preparations, determined by ultracentrifugation, revealed the presence of PG biosynthetic activity in the cytosolic and microsomal fractions, although most activity was found in the cytosolic fractions. This is similar to other invertebrates, and different from mammalian preparations, in which the activity is exclusively associated with the microsomal fractions. Midgut preparations from *M. sexta* pupae, adult cockroach, *Periplaneta americana*, and corn ear worms, *Helicoverpa zea*, also produced the same four major PG products. I infer that insect midguts are competent to biosynthesize PGs, and speculate they exert important, albeit unrevealed, actions in midgut physiology.

## Introduction

Eicosanoids are oxygenated metabolites of arachidonic acid (20:4n-6) and two other C20 polyunsaturated fatty acids (Figure 1). Major groups of eicosanoids include prostaglandins (PGs), products of the cyclooxygenase pathway, epoxyeicosatrienoic acids (EETs) products of the cytochrome P-450 epoxygenase pathways, and various lipoxygenase products. The structures and biosynthetic pathways of eicosanoids are described in detail elsewhere (Stanley, 2000). Although they are most well understood in the contexts of mammalian physiology and pathophysiology, eicosanoids exert important biological actions in insects and other invertebrates (Rowley et al., 1998; Stanley, 2000).

The significance of eicosanoids bears on fundamental areas of insect biology. In reproduction, PGE<sub>2</sub> releases egg-laying behavior in crickets and a smattering of other species (Stanley, 2000). PGE<sub>2</sub> also acts in modulating basal fluid secretion rates in Malpighian tubule physiology (Petzel and Stanley-Samuelson, 1992; Van Kerkhove et al., 1995). Still other eicosanoids mediate innate immune reactions to bacterial infection including nodule formation (Miller et al., 1994), phagocytosis and prophenoloxidase activation (Mandato et al., 1997) and expression of silk worm fat body genes for cecropin and lysozyme (Morishima et al., 1997). Other eicosanoid-mediated biological actions will undoubtedly come to the surface in the future.

Recognition of the biological significance of eicosanoids has stimulated inquiry into the biosynthesis of PGs and other eicosanoids in various insect systems. The presence and biosynthesis of PGs have been recorded at the organismal level in only a few species, such as houseflies, *Musca domestica* (Wakayama et al., 1986). PG biosynthesis has been studied in reproductive tissues of various species, including the

crickets, *Acheta domesticus* (Destephano et al., 1974). PG biosynthesis has been characterized in detail for fat body from a few species, including tobacco hornworms, *Manduca sexta* (Stanley-Samuelson and Ogg, 1994) and true armyworms, *Pseudaletia unipuncta* (Tunaz et al., 2001). Several studies report the presence of PG biosynthetic activity in fat body from other species, including cockroaches, *Periplaneta americana* (Howard et al., 1986), the silkworm, *Bombyx mori* (Stanley-Samuelson et al., 1997), adults of the 17-year periodical cicada, *Magicacada septendecim* (Tunaz et al., 1999), and adults of the cricket, *Gryllus assimilis* (Miller et al., 1999). Eicosanoid biosynthesis has been recorded in a few other specialized arthropod tissues, including salivary glands of the tick, *Amblyomma americanus* (Pedibhotla et al., 1997), Malpighian tubules of adult female mosquitoes, *Aedes aegypti* (Petzel et al., 1993), and hemocytes from the tobacco hornworm (Gadelhak et al., 1995). Together, these reports indicate that arthropod tissues are competent to biosynthesize PGs and other eicosanoids. It appears that PG biosynthesis is a common feature of the biochemistry of specific tissues from many, or possibly all, arthropods, although the point has not been investigated extensively.

In this paper, we report on PG biosynthesis by isolated midguts prepared from tobacco hornworms, *M. sexta*. These preparations are active producers of PGs and possibly other eicosanoids, from which we suggest these molecules are likely to be important mediators in the physiology of the insect midgut.

## Materials and methods

### *Insects*

Eggs of the tobacco hornworm, *M. sexta*, were purchased from Carolina Biological supply (Wilmington, NC). The hornworms were reared on standard culture medium under the semi-sterile conditions described elsewhere (Gadelhak et al., 1995).

### *Isolation of midgut and preparation of microsomal-enriched fractions*

These experiments followed protocols developed for the tobacco hornworm fat body (Stanley-Samuelson and Ogg, 1994). The larvae were anesthetized by chilling on ice, then midgut tissue was dissected in ice-cold phosphate buffer (0.05 M potassium phosphate, pH 8.0). The midgut sections, in 1 ml Eppendorf tubes, were sonicated for 10 s at 30 W using a VibraCell sonicator (VibraCell, Danbury, CT). This preparation was centrifuged for 10 min at 735g, and the supernatant was centrifuged for another 20 min at 16,000g, both steps at 4°C. The 16,000g supernatants were microsomal-enriched preparations used in all experiments. Protein concentrations in these preparations were determined in microtiter format using the bicinchoninic acid reagent (Pierce, Rock-ford, IL), against bovine serum albumin as a quantitative standard. The microtiter plates were read on a BioTek microtiter plate reader at 562 nm.

Radioactive arachidonic acid (5,6,8,9,11,12,14,15-<sup>3</sup>H-20:4, 60–100 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The incubation buffer was 0.05 M K<sub>2</sub>HPO<sub>4</sub>, pH 8.0, amended with a standard co-factor cocktail (2.4 mM reduced glutathione, 0.25 mM hydroquinone and 25 µg hemoglobin; Stanley-Samuelson and Ogg,

1994), except in the experiments designed to assess co-factor requirements. For each PG biosynthesis reaction (unless indicated otherwise), 0.8  $\mu$ Ci of labeled 20:4n-6 was dispensed into reaction tubes and the solvent was evaporated. The reactions were carried out in 0.5 ml total volume. The experiments were preceded by a 10 min pre-incubation at 32°C with all reaction components, except the protein source. The reactions were stopped by acidification to pH 3.5–4.0 by addition of 0.22 ml 0.1 N HCl. Reaction products were extracted from the acidified reaction mixture three times with 0.5 ml ethyl acetate. The combined extracts, containing PGs and possible lipoxygenase products, were evaporated under N<sub>2</sub>. A mixture of appropriate unlabeled eicosanoid standards was added to each sample, then samples were applied to TLC plates (20×20 cm Silica Gel G, 0.25 mm thick, Sigma Chemical Co., St Louis, MO). The plates were developed in the A9 solvent system (Hurst et al., 1987) and fractions observed by exposure to iodine vapors. Bands corresponding to selected authentic eicosanoid standards and to free fatty acids were transferred to liquid scintillation vials. Radioactivity in each fraction was determined by adding 5 ml scintillation cocktail (ICN Biomedicals, Irvine, CA) and counting on a LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Pharmacia, Turku, Finland) at 50% counting efficiency for <sup>3</sup>H. Eicosanoid biosynthesis was calculated from the liquid scintillation data. In control experiments, microsomal-enriched preparations were heated in boiling water for 20 min before the experiments, and processed as just described. The results of these control experiments were used to correct values from biosynthesis experiments as previously described (Stanley-Samuelson and Ogg, 1994).

### *Ultracentrifugation*

The 11,750g supernatants were centrifuged at 100,000g for 90 min in a Beckman Optima TL series ultracentrifuge equipped with a TLA 100.4 rotor (Beckman, Inc., Fullerton, CA). The 100,000g pellets were taken as microsomal fractions, and the corresponding supernatants were taken as cytosolic fractions. Protein concentrations in both fractions were determined as just described. PG biosynthesis was assessed following our standard protocol.

### *Determining PG structures by gas chromatography–mass spectrometry (GC–MS)*

The PG biosynthesis reactions were scaled up to generate enough material for chemical analysis. Microsomal-enriched homogenates from six hornworm midguts yielded sufficient protein for five reactions. Each reaction tube contained 20 mg microsomal-enriched protein, 50 µg arachidonic acid, and 25 µl of the co-factor cocktail in 10 ml total volume. After 1 min at 22°C the reactions were stopped with the addition of 1.6 ml 0.1 N HCl. Products were extracted three times with 3 ml ethyl acetate. The ethyl acetate extracts were combined and dried under a stream of N<sub>2</sub>. The combined extract was then purified and analyzed as described previously (Jurenka et al., 1999). Briefly, the extract was cleaned up on silicic acid chromatography and the acetonitrile/methanol fraction was treated with 100 µl diazomethane in diethyl ether. The resulting methyl esters were then treated with N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and heated at 60°C for 20 min. The reaction was dried under N<sub>2</sub> and reconstituted in isooctane for analysis on GC–MS.



Analyses were conducted by capillary GC–MS using a Hewlett–Packard 6890 GC equipped with a DB-5 column (0.25 mm×30 m). The GC was interfaced with a Hewlett–Packard 5973 Mass Selective Detector operated at 70 eV. Separations were conducted in split mode (50:1) with temperature programming at 80°C for 1 min, then 10°/min to 320°C. Mass spectra were scanned from  $m/z$  50 to 500 and data were collected and analyzed with a Hewlett–Packard Vectra Xm series 4 computer with Hewlett–Packard Chemstation software. The structures of PGs were determined by comparing the obtained spectra with the spectra of chemical standards derivatized as above, and by comparisons with published spectra (Pace-Asciak, 1989).

#### *Statistical analysis*

Data were analyzed using the General Linear Models procedure, and mean comparisons were made using Least Significant Different (LSD) tests (SAS Institute Inc., 1989).

### **Results**

Microsomal-enriched fractions of midguts prepared from fifth-instar tobacco hornworms are competent to biosynthesize PGs. The midgut preparations produced four major products (PGA<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2α</sub>). PGA and PGB are double bond isomers, and they cannot be resolved on TLC, however, for convenience we refer to this product as PGA. PGA<sub>2</sub> was the major product under most experimental conditions, although the overall profiles indicate the other PGs were also produced in substantial abundance. The identities of all four PGs were confirmed by analysis on GC–MS. The mass spectra of

PGA<sub>2</sub> and PGF<sub>2α</sub> from insect sources have been published (Wakayama et al., 1986; Jurenka et al., 1999; Tunaz et al., 2001), and the spectra of PGD<sub>2</sub> and PGE<sub>2</sub> are displayed in Figure 2. Our data indicate that changes in reaction conditions influenced the overall profile of PG biosynthesis. The influence of reaction conditions on PG biosynthesis is reported in the following paragraphs.

#### *Influence of radioactive substrate on PG biosynthesis*

The influence of radioactive substrate on PG biosynthesis is shown in Figure 3. Total PG biosynthesis increased from ca. 0.04 pmol/mg protein/h in the presence of 0.2 μCi of substrate to >0.32 pmol/mg protein/h with 1.6 μCi of substrate. PG biosynthesis increased in an approximately linear way with increasing radioactive substrate. Highest PG biosynthesis obtained in the presence of 1.6 μCi of substrate (significantly higher than other amounts), however, we used 0.8 μCi of substrate per reaction in subsequent experiments to balance optimal use of radioactive material with a reasonable level of PG biosynthesis.

#### *Influence of protein concentration on PG biosynthesis*

Figure 4 shows the relationship between midgut microsomal-enriched protein concentration and PG biosynthesis. The optimal protein concentration was in the range of 0.5–2 mg/reaction, which yielded significantly higher product formation. These findings informed the use of 1 mg protein/reaction in subsequent experiments. We also observed the influence of protein concentration on the overall profiles of PG biosynthesis. PGA<sub>2</sub>

was the major product at 1 mg protein/reaction while  $\text{PGF}_{2\alpha}$  was not recorded in experiments in the presence of 4.0 mg protein/reaction.

#### *Influence of reaction time on PG biosynthesis*

The influence of reaction time on PG biosynthesis is shown in Figure 5. Total PG biosynthesis was higher in short incubations, from 0.125 to 0.5 min, than in longer incubations, 1–5 min. We used 0.5 min incubations in subsequent experiments.

#### *Influence of reaction temperature on PG biosynthesis*

Total PG biosynthesis increased significantly from 2 pmol/mg protein/h at 2°C to a high of nearly 9 pmol/mg protein/h at 22°C (Figure 6). The results from incubations at 32°C were statistically similar to the results at 22°C.  $\text{PGA}_2$  was the major product at all temperatures, particularly 22°C.

#### *Influence of reaction pH on PG biosynthesis*

Figure 7 shows the influence of reaction pH on PG biosynthesis. Significantly higher PG biosynthesis, approximately 11 pmol/mg protein/h, was obtained at pH 6, which was not statistically different from the results of incubations at pH 8. We recorded only very low levels of PG biosynthesis at pH 4 and 10.

#### *The influence of exogenous co-factors on PG biosynthesis*

We investigated the possibility that the tobacco hornworm midgut preparation does not depend on exogenous co-factors that are routinely used in mammalian and

invertebrate PG biosynthesis reactions (Stanley-Samuelson and Ogg, 1994; Stanley, 2000). Microsomal-enriched midgut preparations were incubated with radioactive 20:4n-6 under the usual conditions, with the exception of the co-factor cocktail which was present in three concentrations, 0, 5 and 10  $\mu$ l per reaction (2.4 mM reduced glutathione, 0.25 mM hydroquinone and 25  $\mu$ g bovine hemoglobin per 10  $\mu$ l aliquot). At the end of 2 min incubations, the products were extracted and separated as usual. Figure 8 shows that optimal PG biosynthesis obtained in the presence of 5  $\mu$ l of the co-factor cocktail. While some PG biosynthesis obtained in the absence of the cocktail, PG biosynthesis was nearly abolished in reactions conducted in the presence of 10  $\mu$ l of cocktail.

*The midgut preparation is sensitive to non-steroidal anti-inflammatory drugs*

Total midgut PG biosynthesis was inhibited in reactions conducted in the presence of indomethacin and naproxen (Figure 9a and b). PG biosynthesis declined significantly from about 8 pmol/mg protein/h in incubations conducted in the absence of indomethacin to approximately 1 pmol/mg protein/h in the presence of 1 mM indomethacin. PG biosynthesis was significantly reduced from about 9 pmol/mg protein/h to 2 pmol/mg protein/h in the presence of 1 mM naproxen.

*Subcellular localization of PG biosynthetic activity*

PG biosynthetic activity is virtually always associated with the microsomal fractions of mammalian cell preparations. To determine the subcellular localization of PG biosynthetic activity in the midgut preparations, the 11,750g supernatants prepared from midguts were fractionated into cytosolic and microsomal fractions by ultracentrifugation.

Unlike mammalian preparations, PG biosynthetic activity was associated with both fractions of the midgut preparations (Figure 10). Most activity (70%) obtained in the cytosolic fractions, and the remaining 30% in the microsomal fractions. We also note that  $\text{PGA}_2$  was the major product in the cytosolic fraction, and only a minor product in the microsomal fraction.

#### *PG biosynthesis in midgut preparations from other insect species*

We assessed the capacity for PG biosynthesis in other midgut preparations, specifically American cockroaches, *P. americana* and corn ear worms, *Helicoverpa zea*, taken from routine cultures in our laboratory. Figure 11 is a composite, showing substantial PG biosynthetic activity in midguts from newly pupated tobacco hornworms, in midguts from the American cockroach, *P. americana*, and from the corn ear worm, *H. zea*.

### **Discussion**

In this paper, we document PG biosynthesis by isolated midgut preparations from tobacco hornworms, *M. sexta*, and two other insect species. Our data indicate that PG biosynthesis in these preparations is sensitive to reaction conditions, including radioactive substrate, microsomal-enriched protein concentration, reaction time, incubation temperature, buffer pH, the presence of co-factors, and the influence of pharmaceutical inhibitors of PG biosynthesis. These findings support the idea that PG biosynthesis in the insect midgut is an enzyme-mediated process.

The accumulation of information on PG biosynthesis by various insect systems has a long, albeit slow-moving history. The emerging picture, however, suggests that PG biosynthesis proceeds through a fairly common theme which is punctuated by interesting comparative differences. Over 20 years ago, Destephano et al. (1974) reported the biosynthesis of mg quantities of PGE using 1 g tissue samples of reproductive tissues from house crickets, *Acheta domesticus*. This first effort did not provide a characterization of the biosynthetic system, but the products were confirmed by analysis on GC–MS.

A few years later, Wakayama et al. (1986) presented a characterization of PG biosynthesis in the house fly, *Musca domestica*. This work showed that PG biosynthesis in whole-animal preparations increased with protein concentration, substrate concentration, and reaction time. This work showed that PG biosynthetic rates in an insect preparation are generally lower than the rates recorded for mammalian preparations.

More recent characterizations of PG biosynthesis in insects similarly report relatively low rates of synthesis, and they indicate subtle variations in the biosynthesizing systems. Fat body preparations from tobacco hornworms (Stanley-Samuelson and Ogg, 1994) and true armyworms (Tunaz et al., 2001), as well as the midgut work presented here, yielded increasing PG biosynthesis with increasing radioactive substrate concentrations, as reported for the house fly preparations. Unlike the house fly data, the fat body and midgut preparations expressed an optimal protein concentration of about 1 mg/reaction for highest PG biosynthesis, and higher protein concentrations yielded lower rates of PG biosynthesis. Data on hornworm hemocytes (Gadelhak et al., 1995) were

slightly different because the hemocyte preparations expressed an optimal protein concentration of 1.5 mg for PG biosynthesis, and no apparent optimum for production of lipoxygenase products. We also recorded temperature optima of about 32°C for tobacco fat body preparations, 30°C for hemocytes and about 22°C for the midgut reactions. Data on the influence of reaction time on PG biosynthesis have been interpreted in terms of the reaction mechanisms. The tobacco hornworm fat body preparations generated highest PG biosynthesis in 1 min or shorter incubations, beyond which we recovered less product (Stanley-Samuelson and Ogg, 1994). This is consistent with the mechanism of PG biosynthesis, which is thought to take place in rapid bursts in mammals, after which the central enzyme, cyclooxygenase, undergoes a suicidal inactivation. We recorded a similar pattern with hemocytes, at 2 min, and the true armyworm fat body (Tunaz et al., 2001), although the optimal reaction time was 7.5 min. For still another variation, the house fly work indicated continued product formation over 60 min incubations (Wakayama et al., 1986). The true armyworm fat body and hornworm midgut express differing pH optima, about pH 8 for the fat body and pH 6 for the midgut. Overall, the insect PG biosynthesizing systems respond to variations in the usual biophysical parameters in different ways, from which we infer the underlying enzymes differ among insects.

Most studies indicate that PG biosynthesis in insect preparations is inhibited in reactions conducted in the presence of pharmaceutical non-steroidal anti-inflammatory drugs, which in mammals act through inhibition of cyclooxygenase. This is so for house flies (Wakayama et al., 1986), fat body from tobacco hornworms and true armyworms (Stanley-Samuelson and Ogg, 1994; Tunaz et al., 2001), and for the midgut preparations.

All of these drugs act as competitive inhibitors at the active site of cyclooxygenase, and it appears that at least the catalytic portions of cyclooxygenases are fairly similar.

In all mammalian preparations so studied, PG biosynthetic activity is localized within the endoplasmic and nuclear membranes (Otto and Smith, 1995). In practice, the activity is uniformly associated with the microsomal fractions of tissue preparations. The situation is otherwise for insects. Wakayama et al. (1986) recorded varying distributions of PG biosynthesizing activity according to the protocol under which the tissues were processed. For hornworm hemocytes, Gadelhak et al. (1995) found about 58% of the activity in the microsomal fraction and the remainder in the cytosolic and mitochondrial fractions. Again, for midguts, we found most activity (70%) in the cytosolic fraction. We also note that the cytosolic fraction yielded relatively high levels of  $\text{PGA}_2$ , while very little  $\text{PGA}_2$  was recovered from reactions with the microsomal fraction. The distribution of PG biosynthetic activity in insect cells has long been enigmatic (Stanley-Samuelson and Loher, 1986), from which it appears that the organization of the insect systems within cells differs in a fundamental way from their mammalian counterparts.

Although the tobacco hornworm is a well-studied model of insect physiology, we briefly considered the issue of whether midguts from other insect species also produced PGs. Figure 11 shows that midguts from newly pupated tobacco hornworms, from cockroaches, *P. americana*, and from corn ear worms, *H. zea*, all provided evidence of PG biosynthesis. We infer that PG biosynthesis is a common feature of insect midgut biochemistry. Such observation clearly begs the issue of the biological significance of PGs in midgut physiology. Certainly, midgut cells are very active in secretion and uptake of many substances, and in regulation of lumenal conditions, including pH and redox



potential, all potential activities which PGs may influence (Lehane and Billingsley, 1996). Perhaps, too, as recently suggested for tick salivary glands (Qian et al., 1998), PGs modulate midgut protein secretory physiology.

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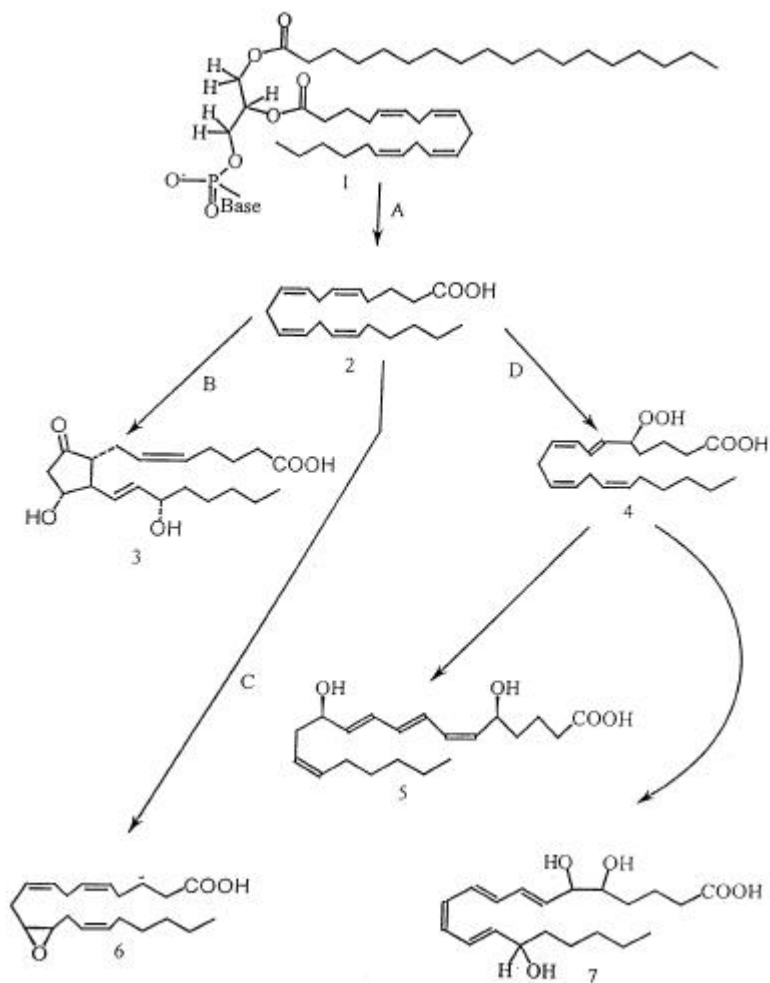


Figure 1. An overview of 20:4n-6 metabolism as understood from the mammalian background. Three polyunsaturated fatty acids, 20:3n-6, 20:4n-6 and 20:5n-3 are potential substrates for eicosanoid biosynthesis. Of these, metabolism of 20:4n-6 is most well studied. Chemical structures are denoted by numerals. 1=cellular phospholipid. 2=hydrolyzed 20:4n-6. 3=prostaglandin E<sub>2</sub>. 4=5-hydroperoxyeicosatetraenoic acid. 5=leukotriene B<sub>4</sub>. 6=11,12-epoxyeicosatrienoic acid. 7=lipoxin A. Capital letters indicate major enzyme systems responsible for eicosanoid biosynthesis. A=phospholipase A<sub>2</sub>; B=cyclooxygenase and associated enzyme steps; C=cytochrome P<sub>450</sub> epoxygenase; D=lipoxygenase.

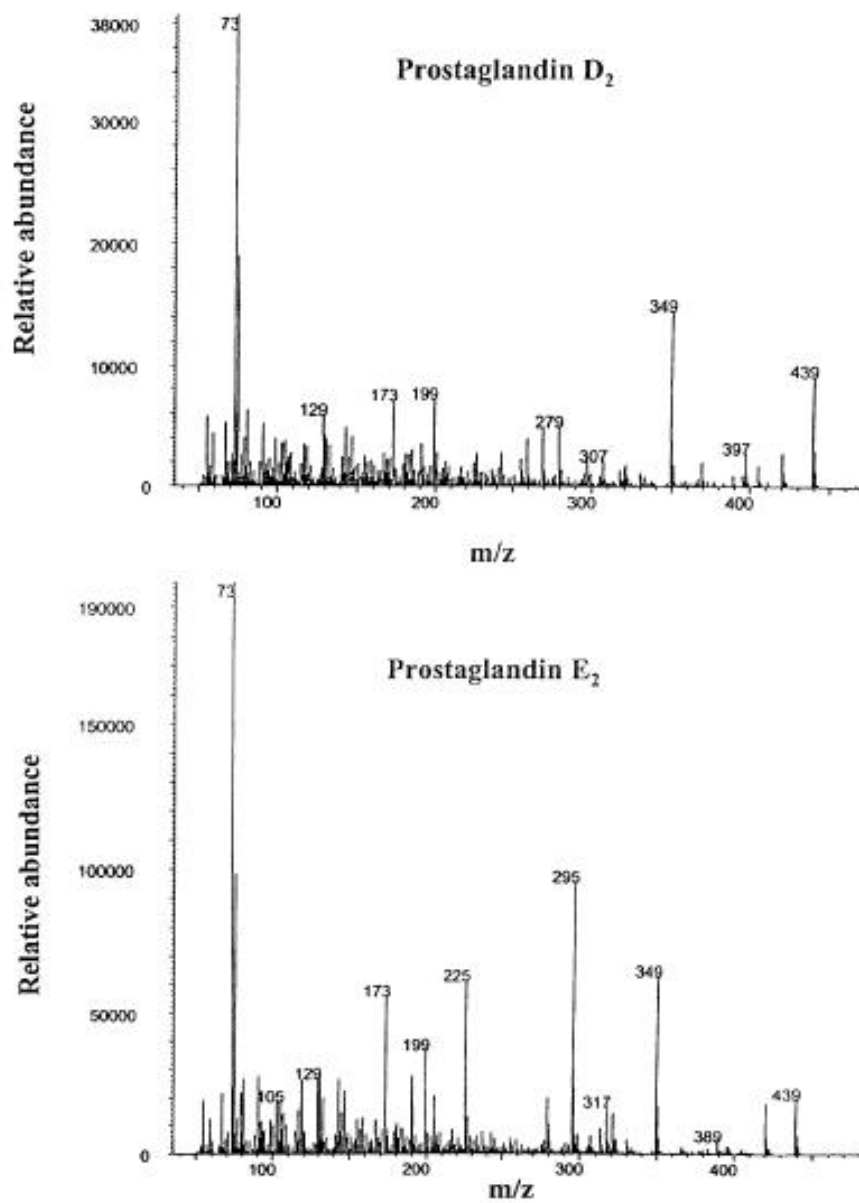


Figure 2. A total ion scan of the methyl ester trimethylsilyl derivatives of PGE<sub>2</sub> and PGD<sub>2</sub>, obtained from the incubation of tobacco hornworm midgut preparations with arachidonic acid.

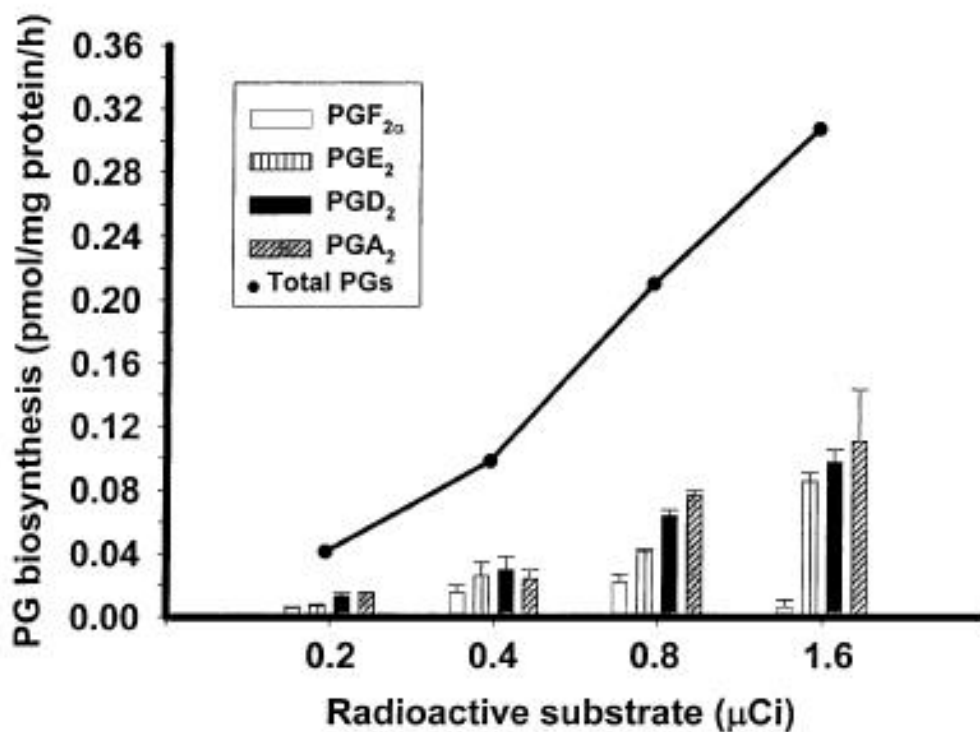


Figure 3. The influence of radioactive substrate (20:4n-6) on prostaglandin biosynthesis by microsomal-enriched preparations of tobacco hornworm midguts. The 0.5 ml reaction mixtures containing 0.2, 0.4, 0.8 and 1.6  $\mu\text{Ci}$   $^3\text{H}$ -20:4n-6, 1 mg of microsomal-enriched protein, and the co-factor cocktail in 50 mM potassium phosphate buffer, pH 8.0, were incubated at 32°C. After 2 min incubations, the reactions were stopped, and the reaction products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and the line represents total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each substrate concentration. The error bars, where visible, indicate 1 SEM.



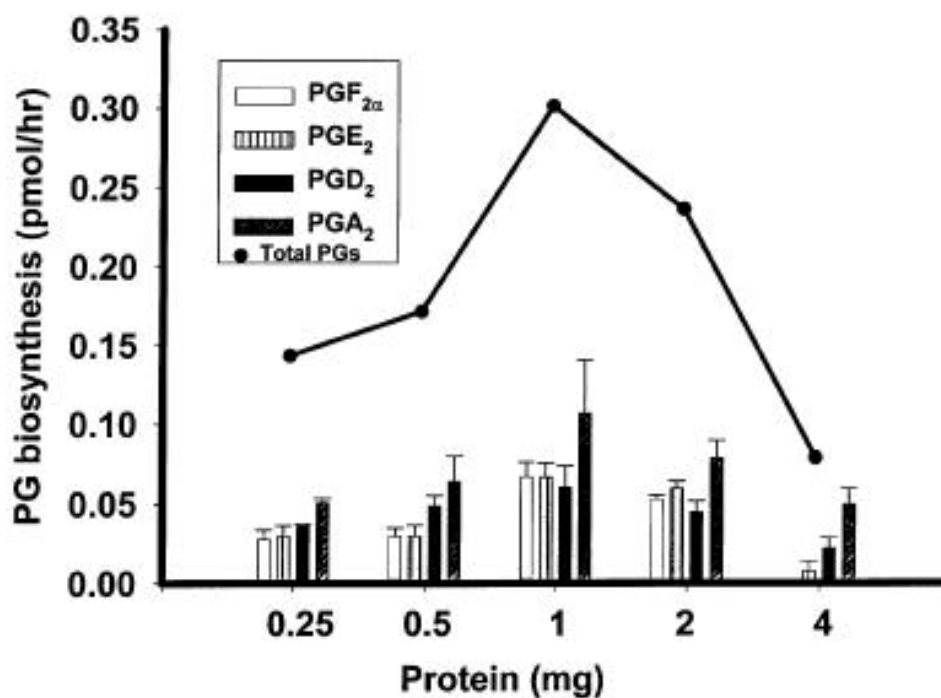


Figure 4. The influence of midgut microsomal-enriched protein concentration on prostaglandin biosynthesis. The 0.5 ml reaction mixtures containing the indicated amounts of microsomal-enriched protein, 0.8  $\mu\text{Ci}$   $^3\text{H}$ -20:4n-6 and the co-factor cocktail in 50 mM potassium phosphate buffer, pH 8.0, were incubated at 32°C. After 2 min incubations, the reactions were stopped, and the reaction products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and the line represents total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each protein concentration. The error bars, where visible, indicate 1 SEM.

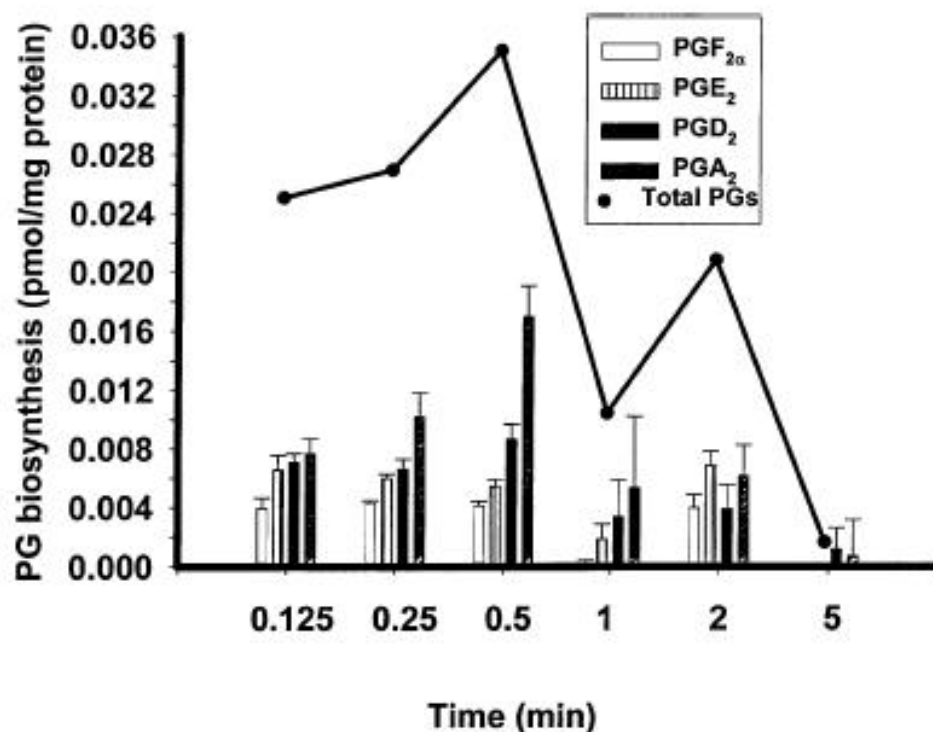


Figure 5. The influence of incubation time on prostaglandin biosynthesis by microsomal-enriched preparations of tobacco hornworm midguts. The 0.5 ml reaction mixtures containing 1 mg of microsomal-enriched protein, 0.8  $\mu\text{Ci}$   $^3\text{H}$ -20:4n-6 and the co-factor cocktail in 50 mM potassium phosphate buffer, pH 8.0, were incubated at 32°C. At the indicated times, reactions were stopped and the products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and the line represents total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different incubation time. The error bars, where visible, indicate 1 SEM.

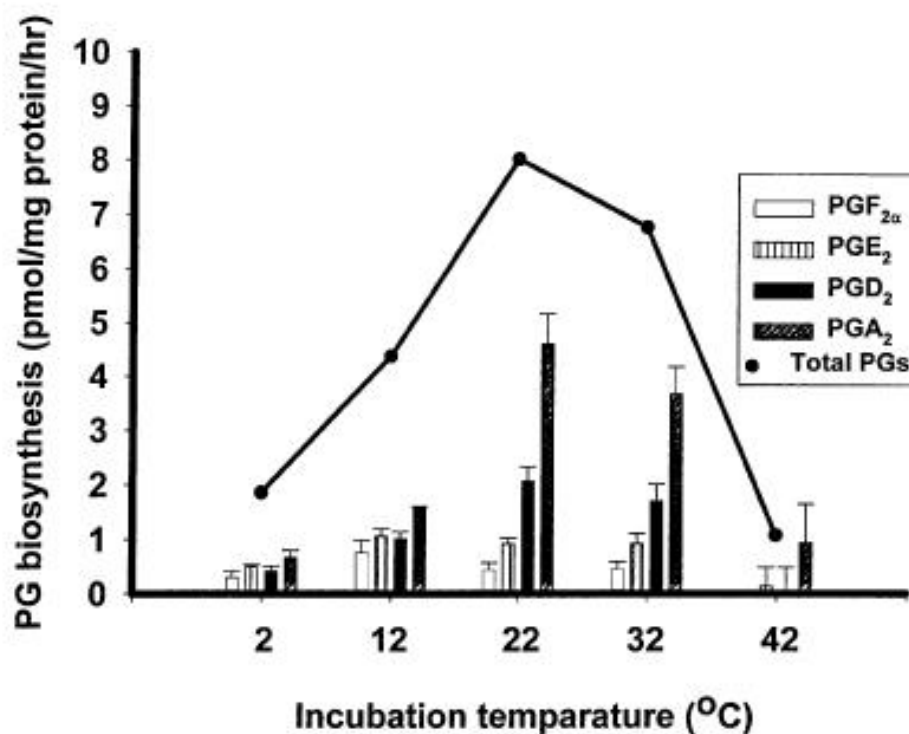


Figure 6. The influence of incubation temperature on prostaglandin biosynthesis by microsomal-enriched midgut preparations. The 0.5 ml reaction mixtures containing 1 mg of microsomal-enriched protein, 0.8  $\mu\text{Ci}$   $^3\text{H}$ -20:4n-6 and the co-factor cocktail in 50 mM potassium phosphate buffer, pH 8.0, were incubated for 0.5 min at 32°C. The products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and the line represents total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different incubation time. The error bars, where visible, indicate 1 SEM.

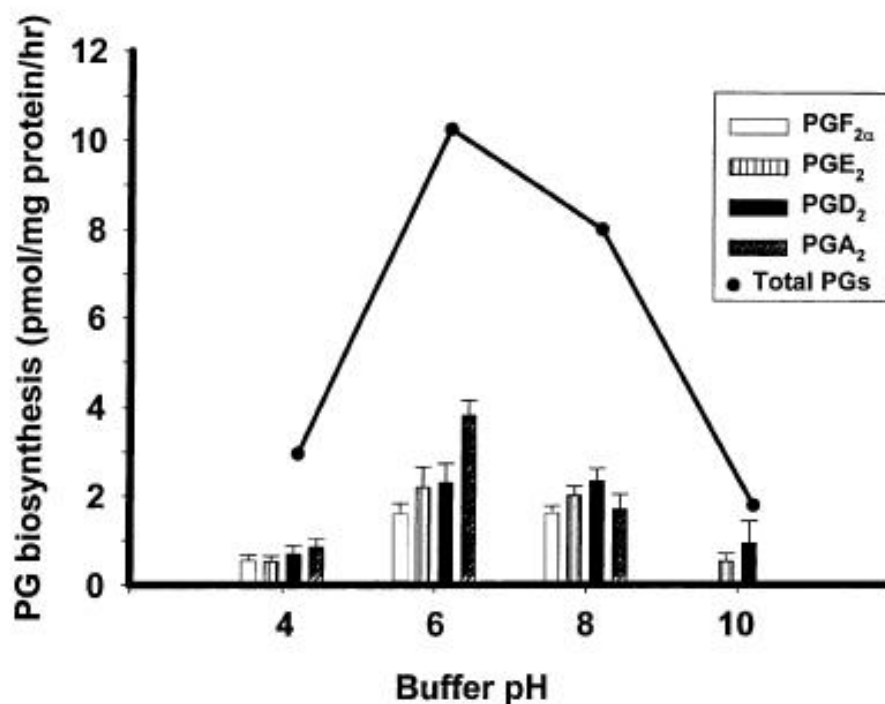


Figure 7. The influence of buffer pH on prostaglandin biosynthesis by microsomal-enriched midgut preparations. The 0.5 ml reaction mixtures containing 1 mg of microsomal-enriched protein, 0.8  $\mu\text{Ci}$   $^3\text{H}$ 20:4n-6 and the co-factor cocktail in 50 mM potassium phosphate buffer, pH 4.0, 6.0, 8.0 or 10.0, were incubated 0.5 min at 32°C. The products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and the line represents total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different buffer pH. The error bars, where visible, indicate 1 SEM.

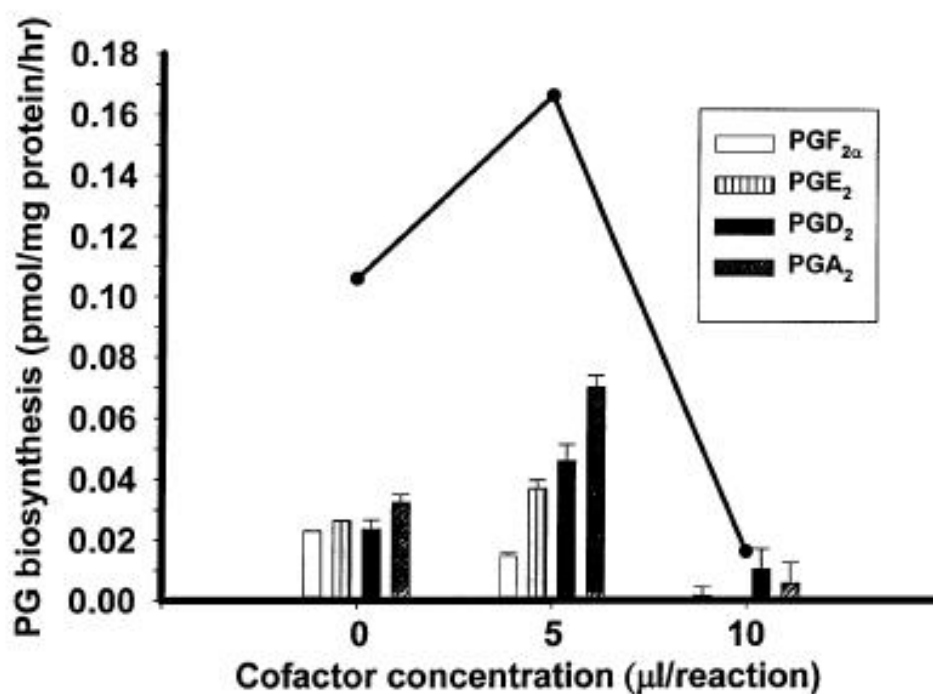


Figure 8. The influence of co-factor concentration on prostaglandin biosynthesis by microsomal-enriched midgut preparations. The 0.5 ml reaction mixtures containing 1 mg of microsomal-enriched protein, 0.8  $\mu\text{Ci}$   $^3\text{H}$ -20:4n-6 and either 0, 5.0 or 10  $\mu\text{l}$  of co-factor cocktail (2.4 mM reduced glutathione, 0.25 mM hydroquinone and 25  $\mu\text{g}$  bovine hemoglobin per 10  $\mu\text{l}$  aliquot) in 50 mM potassium phosphate buffer, pH 4.0, 6.0, 8.0 or 10.0, were incubated 0.5 min at 32°C. The products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and the line represents total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each co-factor concentration. Error bars, where visible, indicate 1 SEM.

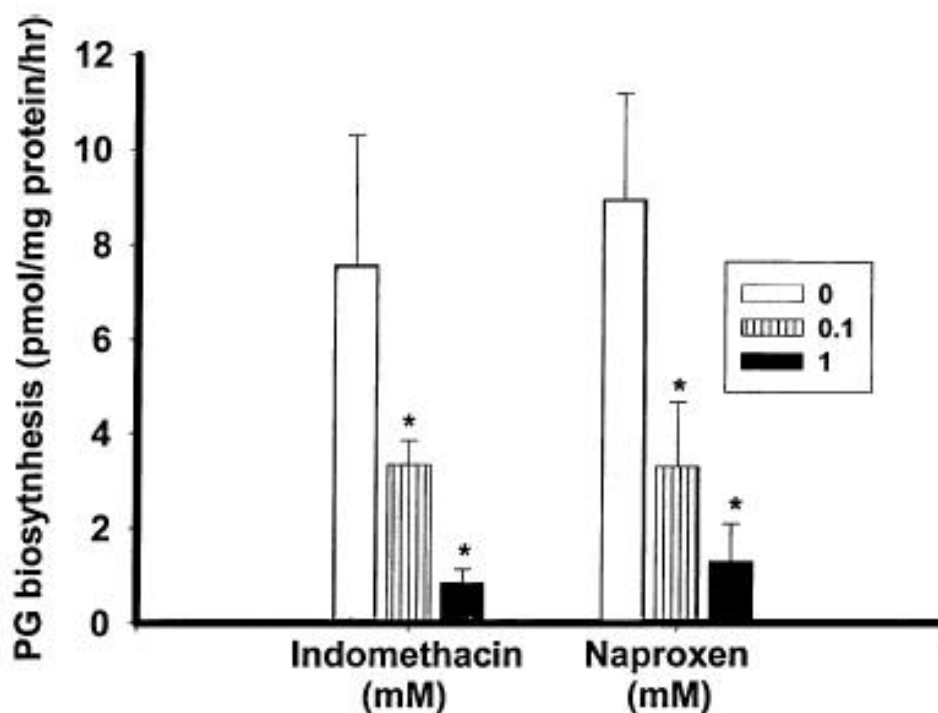


Figure 9. The influence of cyclooxygenase inhibitors, indomethacin and naproxen on prostaglandin biosynthesis by microsomal-enriched midgut preparations. The 0.5 ml reaction mixtures containing the indicated amounts of inhibitor, 1 mg of microsomal-enriched protein, 0.8  $\mu\text{Ci}$   $^3\text{H}$ -20:4n-6 and the co-factor cocktail in 50 mM potassium phosphate buffer, pH 8.0, were incubated 0.5 min at 32°C. The products were extracted and separated as described in Section 2. Each bar represents the mean of three separate experiments at each concentration. The error bars indicate 1 SEM. \* indicates significant difference from 0 inhibitor treatment.

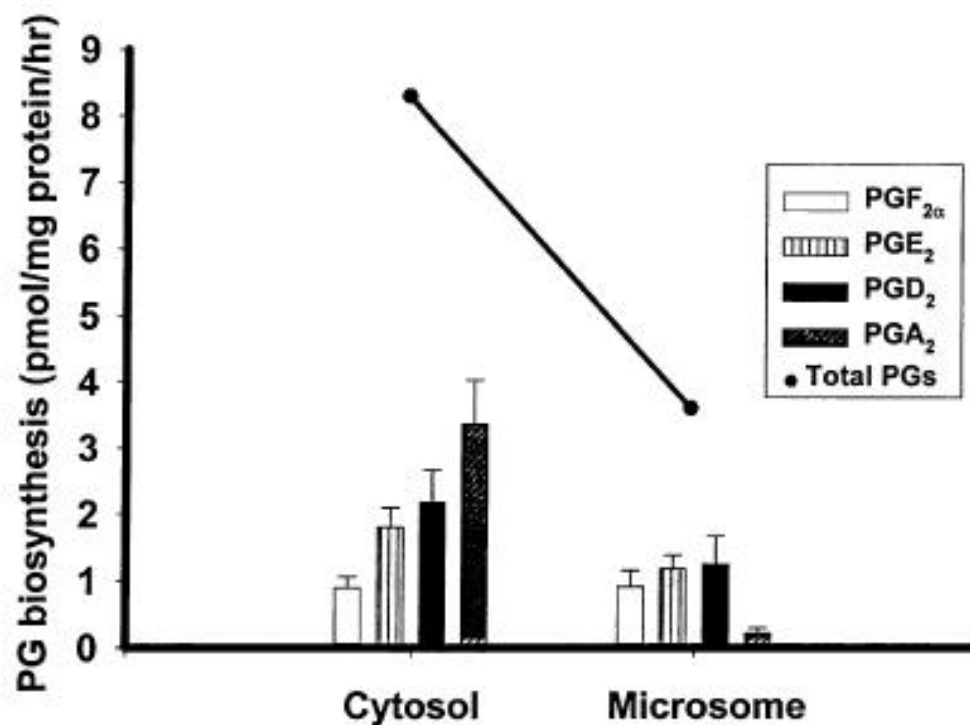


Figure 10. Localization of prostaglandin biosynthetic activity in the cytosolic and microsomal fractions of midguts prepared from tobacco hornworms. The reactions were conducted and the products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and the line represents total prostaglandin biosynthesis. Each point represents the mean of three separate experiments. Error bars indicate 1 SEM.

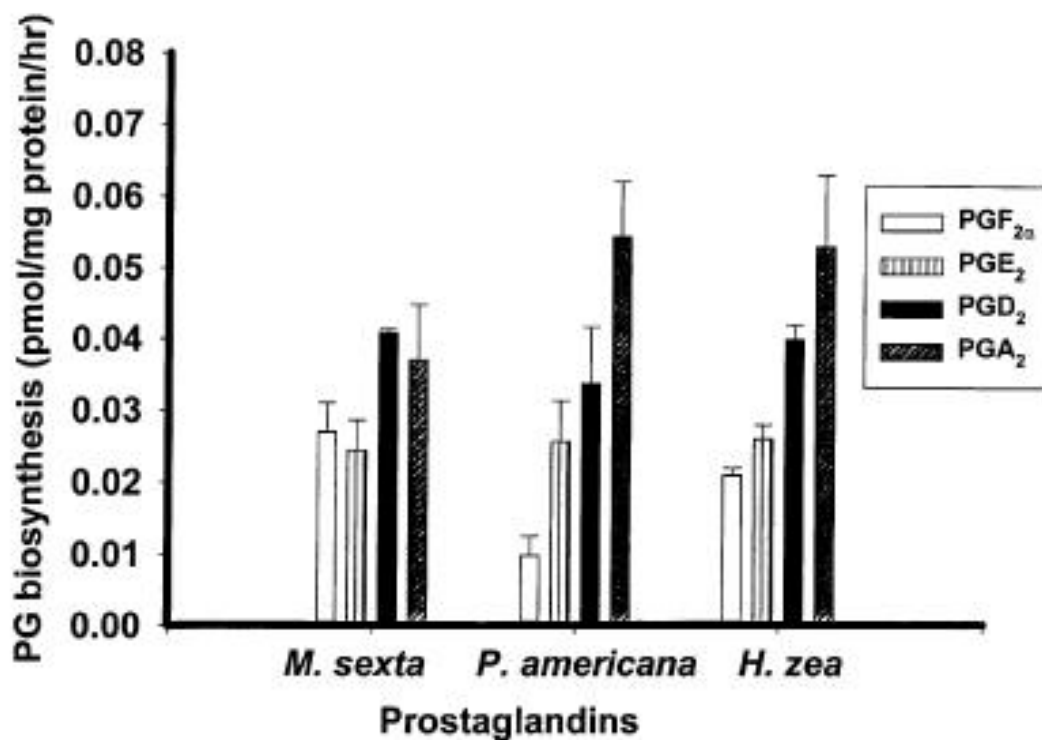


Figure 11. Prostaglandin biosynthesis by microsomal-enriched midgut preparations from *M. sexta* pupae (*M. sexta*), cockroach adults (*P. americana*), and corn ear worms (*H. zea*). The reactions were conducted and the products were extracted and separated as described in Section 2. The histogram displays the biosynthesis of individual prostaglandins, and each bar represents the mean of three separate experiments. Error bars indicate 1 SEM.



## Chapter 4

Prostaglandin biosynthesis by fat body from larvae of the  
beetle *Zophobas atratus*

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Putnam, and D.W. Stanley (2002)

## Abstract

I describe prostaglandin (PG) biosynthesis by microsomal-enriched fractions of fat body prepared from larvae of the tenebrionid beetle, *Zophobas atratus*. PG biosynthesis was sensitive to incubation time, temperature, pH, substrate and protein concentration. Optimal PG biosynthesis conditions of those we examined included 2 mg of microsomal-enriched protein, incubated at 22°C for 2 min at pH 6. These preparations yielded four major PGs:  $\text{PGA}_2$ ,  $\text{PGE}_2$ ,  $\text{PGD}_2$  and  $\text{PGF}_{2\alpha}$ .  $\text{PGA}_2$  and  $\text{PGF}_{2\alpha}$  were the predominant eicosanoids produced under these conditions. Two non-steroidal anti-inflammatory drugs, indomethacin and naproxen, effectively inhibited PG biosynthesis in low concentrations. In vitro PG biosynthetic reaction conditions, using vertebrate or invertebrate enzyme sources, usually include a cocktail of reaction co-factors. The *Z. atratus* preparation similarly performs better in the presence of co-factors.

## Introduction

We have set forth the hypothesis that eicosanoids mediate insect immune reactions to bacterial infections. Eicosanoid is a collective term originally coined by Corey et al. (1981) for all biologically active, oxygenated metabolites of 20:4n-6 and two other C20 polyunsaturated fatty acids. The structures and biosynthetic pathways of these compounds are detailed elsewhere (Stanley, 2000). Most experiments designed to test this hypothesis are based on selective inhibition of putative eicosanoid biosynthetic pathways, done by treating experimental animals or tissues with a variety of anti-inflammatory drugs. Nodule formation is thought to be the predominant mechanism of clearing bacterial cells from hemolymph (Horohov and Dunn, 1983) and, using pharmaceutical inhibitors of eicosanoid biosynthesis, We found that microaggregation (an early phase of nodule formation) and nodule formation were severely impaired in inhibitor-treated insects. On the basis of these results, we suggested that eicosanoids are responsible for mediating nodulation, a specific cellular reaction to bacterial infections.

So far, results with several insect species support the hypothesis. These include tobacco horn-worms, *Manduca sexta* (Stanley-Samuelson et al., 1991; Miller et al., 1994), larvae of a beetle, *Zophobas atratus* (Miller et al., 1996), and several lepidopterans, including the silkworm, *Bombyx mori* (Stanley-Samuelson et al., 1997), cutworms, *Agrotis ipsilon*, true armyworms, *P. unipuncta* (Jurenka et al., 1997) and larvae of the butterfly, *Colias eurytheme* (Stanley et al., 1999). We also tested our idea with adults of three hemimetabolous insect species, the cricket *Gryllus assimilis* (Miller et al., 1999) and two 17-year periodical cicadas, *Magicicada septendecim* and *M. cassini*

(Tunaz et al., 1999). Downer's group (Mandato et al., 1997) identified two additional distinct phases of nodulation, cell spreading and prophenyloxidase activation, which were inhibited in waxmoths, *Galleria mellonella*, that had been treated with eicosanoid biosynthesis inhibitors. They also suggested that eicosanoids mediate phagocytosis, another independent cellular reaction to bacterial infections. Beyond this, appreciation of the significance of eicosanoids in signal transduction in insect immunity was considerably broadened by Sun and Faye (1995), who reported that arachidonic acid strongly induced expression of a gene for the antibacterial protein attacin in fat body of the giant silk moth, *Hyalophora cecropia*. Morishima et al. (1997) extended this work. Based on their work with anti-inflammatory drugs, this group showed that eicosanoids also mediate expression of two silkworm fat body genes for antibacterial proteins, cecropin and lysozyme. We infer from the work with all these systems that eicosanoids are important elements of insect immunity (Stanley, 2000).

The hypothesis that eicosanoids act in insect immunity has stimulated research on the biochemical pathways involved in eicosanoid production. We characterized eicosanoid biosynthesis in tobacco hornworm fat body and hemocytes (Stanley-Samuelson and Ogg, 1994; Gadelhak et al., 1995), and similarly characterized eicosanoid biosynthesis in true armyworm fat body (Tunaz et al., 2001). These studies documented the ability of insect immune tissues to biosynthesize eicosanoids, and they revealed substantial insights into the biochemical requirements for eicosanoid biosynthesis. Such biochemical findings are important because they lie at the foundation of understanding the biological significance of eicosanoids in immunity or any other physiological arena.

In an earlier paper, we suggested that eicosanoids mediate nodulation reactions to bacterial infections in the tenebrionid beetle, *Z. atratus* (Miller et al., 1996). Although beetles comprise the largest group of animals, we have very little information on eicosanoid biosynthesis in representatives of this group. Here, we report on PG biosynthesis by fat body from *Z. atratus*.

## **Materials and Methods**

### *Insect*

Larvae of the tenebrionid beetle, *Z. atratus*, were purchased from a local pet shop, where they are maintained and sold as food for various herpetological pets. Larvae were maintained on a laboratory bench at room temperature and natural photoperiod in terraria. The terraria were charged with bran in which the larvae burrowed themselves and fed. Water was provided by keeping damp paper towels on top of the bran. These beetles express indeterminate numbers of molts, and late instar larvae we previously classified as “large” (Howard et al., 1998) were used for this study.

### *Isolation of Fat Body and Preparation of Microsomal-Enriched Fractions*

These experiments followed protocols developed for the tobacco hornworm fat body (Stanley-Samuelson and Ogg, 1994). The larvae were anesthetized by chilling on ice, then fat body tissue was dissected in ice-cold phosphate buffer (0.05 M potassium phosphate, pH 8.0). The fat body tissues, in 1.5 ml Eppendorf tubes, were sonicated for 10 s at 30 W using a VibraCell sonicator (VibraCell, Danbury, CT). This preparation was centrifuged for 10 min at 735g, and the supernatant was centrifuged for another 20 min at

16,000g, both steps at 4°C. The 16,000g supernatants were microsomal-enriched preparations used in all experiments. Protein concentrations in these preparations were determined in microtiter format using the bicinchoninic acid reagent (Pierce, Rockford, IL), against bovine serum albumin as quantitative standard. The microtiter plates were read on a BioTek microtiter plate reader at 562 nm. Radioactive arachidonic acid (5,6,8,9,11,12, 14,15-<sup>3</sup>H-20:4, 60–100 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and used without further purification. The incubation buffer was 0.05 M KH<sub>2</sub>PO<sub>4</sub>, pH 8.0, amended with a standard co-factor cocktail (2.4 mM reduced glutathione, 0.25 mM hydroquinone, and 25 µg hemoglobin) (Stanley-Samuelson and Ogg, 1994). For each PG biosynthesis reaction (unless indicated otherwise), 0.4 µCi of labeled 20:4n-6 in ethanol was dispensed into reaction tubes and the solvent was evaporated under N<sub>2</sub>. The reactions were carried out in 0.5 ml total volume. The experiments were preceded by a 10-min pre-incubation at 32°C with all reaction components, except the protein source. The reactions were stopped by acidification to pH 3.5–4.0 by addition of 0.2 ml 0.1 N HCl. Reaction products were extracted from the acidified reaction mixture three times with 0.5 ml ethyl acetate. The combined extracts, containing PGs and possible lipoxygenase products, were evaporated under N<sub>2</sub>. A mixture of appropriate unlabeled eicosanoid standards was added to each sample, then samples were applied to TLC plates (20 × 20 cm Silica Gel G, 0.25 mm thick, Sigma Chemical Co., St Louis, MO). The plates were developed in the A9 solvent system (Hurst et al., 1987) and fractions observed by exposure to iodine vapors. Bands corresponding to selected authentic eicosanoid standards and to free fatty acids were transferred to liquid scintillation vials. Radioactivity in each fraction was determined by adding 5 ml

scintillation cocktail (ICN Biomedicals, Irvine, CA) and counting on a LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Pharmacia, Turku, Finland) at 50% counting efficiency for  $^3\text{H}$ . Eicosanoid biosynthesis was calculated from the liquid scintillation data. In control experiments, microsomal-enriched preparations were heated in boiling water for 15 min before the experiments, and processed as just described. The results of these control experiments were used to correct values from biosynthesis experiments as previously described (Stanley-Samuelson and Ogg, 1994). The influence of reaction parameters on PG biosynthesis was assessed by varying reaction conditions in a systematic way, as indicated in Results.

#### *Ultracentrifugation*

The 11,750g supernatants were centrifuged at 100,000g for 90 min in a Beckman Optima TL series ultracentrifuge equipped with a TLA 100.4 rotor (Beckman, Inc., Fullerton, CA). The 100,000g pellets were taken as microsomal fractions, and the corresponding supernatants were taken as cytosolic fractions. Protein concentrations in both fractions were determined as just described. PG biosynthesis was assessed following our standard protocol.

#### *Influence of Sample Storage on PG Biosynthesis*

Samples were prepared as described, then stored at  $-20^{\circ}\text{C}$  for 1, 7, and 14 days. The frozen samples were thawed on ice, then PG biosynthesis was assessed according to our usual protocol.

In another series of experiments, fresh and 7day stored samples were mixed prior to assessing PG biosynthesis. Five mixtures, 100% frozen, 75% frozen/25% fresh, 50% each, 25% frozen/75% fresh and 100% fresh samples, were used to assess PG biosynthesis.

*Determining PGF<sub>2α</sub> by Gas-Chromatography/Mass Spectrometry (GC-MS)*

Five beetles were injected with 50 µg of the eicosanoid biosynthesis precursor, 20:4n-6. After 5 min incubations, the larvae were anesthetized by chilling on ice and the abdominal sections were placed in silanized tissue grinder tubes containing water and petroleum ether. The tissues were homogenized, and neutral lipids, which confound extraction of eicosanoids, were removed with the petroleum ether layer. Acidified ethyl acetate was added to the tubes, after homogenization the ethyl acetate layer was focused by centrifuging the tubes at 12,000g for 10 min.

The extract was then purified and analyzed as described previously (Jurenka et al., 1999). Briefly, the extract was cleaned up on silicic acid chromatography (using 1 ml pipette tips for the columns prepared with 750 mg of Silicar CC-4 [Mallinckrodt, Paris, KY]). The columns were eluted in a step-wise mode, 375 µl per wash. First step, ethyl acetate, second step, ethyl acetate: acetonitrile (1/1, v/v), third step, acetonitrile, fourth step, acetonitrile:methanol (1/1, v/v), and finally the column was washed with methanol. The acetonitrile:methanol fraction was dried under nitrogen, then treated with 100 µl diazomethane in diethyl ether. The resulting methyl esters were then treated with N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and heated at



60°C for 20 min. The reaction was dried under N<sub>2</sub> and reconstituted with isooctane for analysis on GC-MS.

Analyses were conducted by capillary GC-MS using a Hewlett-Packard 6890 GC equipped with a DB-5 column (0.25 mm × 30 m). The GC was interfaced with a Hewlett-Packard 5973 Mass Selective Detector operated in scan mode. Separations were conducted in split mode with temperature programming at 60°C for 1 min, then 10°C/min to 300°C. Mass spectra were scanned from 50 to 500 m/z and data were collected and analyzed on a Hewlett-Packard Vectra Xm Series 4 computer using HP Chemstation software. The chemical structure of PGF<sub>2α</sub> was determined by comparison to published spectra (Pace-Asciak, 1989) and by comparing obtained spectra with those of authentic standards analyzed in our laboratory.

#### *Statistical Analysis*

Data were analyzed using the General Linear Models procedure, and mean comparisons were made using Least Significant Different (LSD) test (SAS Institute Inc., 1989).

### **Results**

Microsomal-enriched fractions of fat bodies prepared from larvae of the beetle, *Z. atratus*, were competent to biosynthesize PGs. The fat body preparations produced four major products (PGA<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2α</sub>). PGA<sub>2</sub> was the major product under most experimental conditions, although our data indicate that changes in reaction conditions influenced the overall profile of PG biosynthesis. Because PGF<sub>2α</sub> has been

implicated in insect immunity (Jurenka et al., 1999), we confirmed the identification of PGF<sub>2α</sub> by GC-MS. Figure 1 shows a total ion scan the methyl ester trimethylsilyl derivative of PGF<sub>2α</sub>; we did not obtain mass spectra of the remaining PGs. The influence of reaction conditions on PG biosynthesis are reported in the following paragraphs.

#### *Z. atratus System Required Exogenous Co-Factors for Prostaglandin Biosynthesis*

PG biosynthesis in all mammalian preparations studied so far, and in most invertebrate systems depends on the presence of exogenous co-factors, although some insect systems do not. In the early phase of our work on the *Z. atratus* system we assessed the need for co-factors in the fat body enzyme source preparations. We found reactions conducted in the presence of the cocktail (0.054 pmol/mg protein/hr) yielded over 3-fold greater PG biosynthesis than reactions run in the absence of the cocktail (0.017 pmol/mg protein/hr; significantly different at  $P < 0.05$ ), and all subsequent reactions included the co-factor mix.

#### *Influence of Radioactive Substrate Concentration on Prostaglandin Biosynthesis*

The influence of four concentrations of radioactive substrate on PG biosynthesis is shown in Figure 2. Total PG biosynthesis increased from approximately 0.02 pmol/mg protein/h in the presence of 0.2 μCi of substrate to > 0.3 pmol/mg protein/h with 1.6 μCi of substrate. Except for 0.8 μCi of substrate, PG biosynthesis increased in an approximately linear way with increasing substrate. The highest PG biosynthesis was obtained in the presence of 1.6 μCi of substrate (significantly higher than other

concentrations). However, we used 0.4  $\mu\text{Ci}$  of substrate per reaction to balance optimal use of radioactive material with a reasonable level of PG biosynthesis.

We note that substrate concentrations influenced the profiles of radioactive PG products. At 0.4 and 1.6  $\mu\text{Ci}$  of substrate  $\text{PGF}_{2\alpha}$  was the predominant product, while  $\text{PGD}_2$  was not detected at 0.2 and 0.8  $\mu\text{Ci}$  of substrate.

#### *Influence of Protein Concentration on Prostaglandin Biosynthesis*

Figure 3 shows the relationship between fat body microsomal-enriched protein concentration and PG biosynthesis. The optimal protein concentration was 2 mg/reaction, which yielded significantly higher product formation. These findings informed the use of 2 mg protein/reaction in subsequent experiments. We also observed the influence of protein concentration on the overall profiles of PG biosynthesis.  $\text{PGF}_{2\alpha}$  and  $\text{PGA}_2$  were the major products at 2 mg protein/reaction while  $\text{PGA}_2$  was substantially reduced compared to  $\text{PGF}_{2\alpha}$  at 1 mg protein/reaction.

#### *Influence of Reaction Time on Prostaglandin Biosynthesis*

The influence of reaction time on PG biosynthesis is shown in Figure 4. Total PG biosynthesis increased from about 0.003 pmol/mg protein at 0.5 min to a high of nearly 0.1 pmol/mg protein at 1 and 2 min. Total PG production was diminished in longer incubations. We used 2.0-min incubations in subsequent experiments.

#### *Influence of Reaction Temperature on Prostaglandin Biosynthesis*

Total PG biosynthesis increased significantly from 0.02 pmol/mg protein/h at 12°C to a high of 0.05 pmol/mg protein/h at 22°C (Figure 5). The results from incubations at 22°C were statistically higher than the other temperatures examined. Higher temperatures yielded similar or lower amounts of product. PGA<sub>2</sub>, PGD<sub>2</sub>, and PGE<sub>2</sub> were the major products at 22°C, which was used in all subsequent experiments.

#### *Influence of Reaction pH on Prostaglandin Biosynthesis*

Figure 6 shows the influence of reaction pH on PG biosynthesis. Under each of the pH conditions below pH 8, obtained by adjusting the buffer to the indicated pH, the fat body preparations yielded all four PGs. Substantial PG biosynthesis, in the range of 0.2 pmol/mg protein/h, was obtained under neutral and acidic conditions. We recorded negligible biosynthesis at pH 10. We used pH 6 in all subsequent reactions.

#### *Fat Body Preparation Is Sensitive to Two Non-Steroidal Anti-Inflammatory Drugs*

Total fat body PG biosynthesis was inhibited in reactions conducted in the presence of indomethacin and naproxen (Figures. 7a,b). In reactions with indomethacin (1 mM), PG biosynthesis declined significantly from about 0.16 pmol/mg protein/h to approximately 0.02 pmol/mg protein/h. Naproxen reduced PG biosynthesis from about 0.18 pmol/mg protein/h to 0.08 pmol/mg protein/h at 1 mM.

#### *Subcellular Localization of PG Biosynthetic Activity*

PG biosynthetic activity is virtually always associated with the microsomal fractions of mammalian cell preparations. To determine the subcellular localization of PG

biosynthetic activity in the midgut preparations, the 11,750g supernatants prepared from midguts were fractionated into cytosolic and microsomal fractions by ultracentrifugation. Unlike mammalian preparations, PG biosynthetic activity was associated almost entirely with the cytosolic fraction (Figure 8). Virtually all of the activity obtained in the cytosolic fractions.  $\text{PGA}_2$  was the major product in the cytosolic fraction.

#### *Influence of Sample Storage on PG Biosynthesis*

We considered the influence of storing fat body enzyme preparations on PG biosynthetic activity. Figure 9 shows that, relative to results with fresh samples, we recorded higher PG biosynthesis in samples that were stored for 1 day (approximately 5-fold higher) and for 7 days (about 6-fold higher). PG biosynthesis declined in samples stored for 14 days.

We investigated the increased PG biosynthesis a little bit further by assessing PG biosynthesis in mixtures of fresh and frozen samples. Figure 10 shows that experiments composed of 100% frozen material yielded the highest PG biosynthetic activity, which declined with increasing proportions of fresh material.

## **Discussion**

In this paper, we document PG biosynthesis by microsomal-enriched preparations of fat bodies taken from larvae of the beetle, *Z. atratus*. Under our experimental conditions,  $\text{PGE}_2$ ,  $\text{PGD}_2$ ,  $\text{PGF}_{2\alpha}$ , and  $\text{PGA}_2$  were formed. While  $\text{PGA}_2$  was the quantitatively predominant product under most conditions, we recorded substantial biosynthesis of all four PGs. Identification of  $\text{PGF}_{2\alpha}$  by GC-MS (Figure 2) adds

considerable verisimilitude to studies of PG biosynthesis in insects. As seen in other studies of insect tissues (Stanley-Samuelson and Ogg, 1994; Stanley, 2000), PG biosynthesis in the *Z. atratus* fat body preparations was sensitive to reaction conditions, including the presence of the cofactor cocktail, temperature, pH, incubation time, and protein concentration. In our protocols, optimal reaction conditions for PG biosynthesis include reacting 2.0 mg microsomal-enriched protein with 0.4  $\mu\text{Ci}$  of  $^3\text{H}$ -20:4n-6 at 22°C and pH 6.0 for 2 min. Also, PG biosynthesis by the *Z. atratus* preparations was inhibited in reactions conducted in the presence of two non-steroidal anti-inflammatory drugs. We infer from these findings that the *Z. atratus* fat body is competent to biosynthesize PGs.

We have discussed most aspects of PG biosynthesis, including the influence of reaction conditions and non-steroidal anti-inflammatory drugs on PG biosynthesis, in our work on other species (Stanley-Samuelson and Ogg, 1994; Gadelhak et al., 1995; Pedibhotla et al., 1995; Tunaz et al., 2001). The *Z. atratus* fat body provides additional comparative information. For one point, we note that the temperature optimum is more sharply defined than our findings with the true armyworm fat body (Tunaz et al., 2001). For another, the optimal pH for PG biosynthesis was in the acidic range (pH 4 to 6) for *Z. atratus*, and in the alkaline range (pH 8) for the true armyworm. we also note a novel intracellular distribution of the PG biosynthetic activity in the *Z. atratus* system.

Drawing on the mammalian background (Stanley, 2000), the central enzymes in the PG biosynthetic pathway are cyclooxygenase and peroxidase, both associated with a single protein that was called PGH synthase. In the current idiom, it is most often called COX, for cyclooxygenase. COX is localized within the membranes of the endoplasmic reticulum and the nuclear envelope of mammalian cells, and in practice is always

associated with the microsomal fraction of cellular preparations. The situation is otherwise for insect preparations previously examined, in which the PG biosynthetic activity is more or less evenly distributed in the cytosolic and microsomal fractions (Stanley-Samuelson and Loher, 1986). In this study, we recovered virtually 100% of the PG biosynthetic activity in the cytosolic fraction, and none in the microsomal fraction. We infer that the PG biosynthetic activity of insects differs substantially from the mammalian COXs on the point of how the enzymes are organized within cell structures.

In this work with *Z. atratus*, and with several other insect tissues referenced just above, we have noted the influence of reaction conditions on the overall profile of PG biosynthesis. In Figure 3, for example, we recorded a higher production of PGD<sub>2</sub> than PGA<sub>2</sub> at 1 mg/ml of enzyme source protein concentration, and the opposite at 2 mg/ml. Stanley-Samuelson and Ogg (1994) considered this observation in detail, suggesting that the differing profiles of PG biosynthesis could be understood in terms of the PG biosynthetic pathways. PG biosynthesis from free arachidonic acid entails three enzymatic steps. The first two are associated with COX, which yields PGH<sub>2</sub> as just described. The third step is responsible for converting PGH<sub>2</sub> into one of the major PGs, and this step is catalyzed by a specific enzyme. PGE<sub>2</sub> isomerase convert PGH<sub>2</sub> into PGE<sub>2</sub>, while PGF<sub>2α</sub> reductase is responsible for production of PGF<sub>2α</sub>. We speculate that each of these enzymes are differentially influenced by reaction conditions. If so, changes in reaction conditions can favor biosynthesis of some PGs over others, which would be registered as changes in the overall profile of PG biosynthesis.

Based on studies with mammalian systems, PG biosynthesis reactions with invertebrate preparations are conducted in the presence of a more or less standard

cocktail of co-factors that provide a heme group, reduced glutathione and hydroquinone. We found that tobacco hornworm fat body preparations required the cocktail for PG biosynthesis (Stanley-Samuelson and Ogg, 1994). The results indicated that PG biosynthesis was completely abolished in the absence of added cofactors, from which we inferred that insect PG biosynthesizing systems require a mixture of cofactors similar to the co-factors required by mammalian PG biosynthesizing enzymes. Analogous experiments with internal-tissue preparations from the tick, *Amblyomma americanum*, showed the tick systems also require the added co-factor cocktail to support PG biosynthesis (Pedibhotla et al., 1995). We similarly assessed the need for co-factors in true armyworm fat body preparations (Tunaz et al., 2001), and in whole-animal preparations of primary screwworms, *Cochliomyia hominivorax* (unpublished observations). Contrary to expectation, we recorded no difference in PG biosynthesis in the presence or absence of our standard co-factor cocktail in the latter two insect systems. The outcomes of similar experiments indicated that the *Z. atratus* fat body preparations require the exogenous co-factor cocktail for optimal PG biosynthesis (although we recorded some product in the absence of co-factors), a point of similarity with mammalian and most invertebrate systems.

We found that *Z. atratus* fat body preparations that had been stored at  $-20^{\circ}\text{C}$  for 1 or 7 days yielded enhanced PG biosynthesis. The point of this experiment, in the first instance, was to assess the stability of the preparations for storage as a practical matter of accumulating material for research. If the preparations were stable for storage, we would expect to record similar PG biosynthetic activity in freshly-prepared and stored samples. We speculate, on this observation, that there is a storage-labile factor that inhibits PG



biosynthesis in the fresh samples. The factor could be a completely adventitious artifact, to be seen only when normal tissue and cellular compartmentalization is disrupted, or it could be a natural component of the eicosanoid system, acting to set an upper limit on PG biosynthesis. In either case, the inhibitory influence of the factor is attenuated after storage. We investigated this point by assessing PG biosynthesis in mixtures of fresh and frozen samples. Samples composed of 100% frozen material yielded the highest PG biosynthesis. Samples composed of 25% fresh material yielded significantly reduced PG biosynthesis. Samples with 50, 75, and 100% fresh material yielded still lower PG biosynthesis. We infer the presence of a storage-labile factor, which either naturally or by artifact inhibits PG biosynthesis in the *Z. atratus* fat body preparations.

The presence of such a factor in insect systems is so far known only for *Z. atratus*. However, such a factor may have considerable significance in understanding insect eicosanoid systems. The insect preparations examined so far all seem to produce PGs at substantially lower rates than mammalian preparations. Howard et al. (1986) used bovine seminal vesicles and cockroach, *Periplaneta americana*, fat body to assess the influence of defensive secretions on PG biosynthetic activity, and their results provide a fairly direct comparison of the two systems. In their assays, reactions using 2 mg of the bovine preparation yielded about 3 nmol/ h of PGE<sub>2</sub> while reactions with 10 mg of the cockroach fat body yielded about 126 pmol/h. Most studies of PG biosynthesis in invertebrate preparations report biosynthetic rates in terms of pmol. This is so for whole houseflies, *Musca domestica* (Wakayama et al., 1986), tobacco hornworm, *M. sexta*, hemocytes (Gadelhak et al., 1995), and tick, *Amblyomma americanum*, internal tissues (Pedibhotla et al., 1995). The biochemical basis for the lower rates of PG biosynthesis recorded in work

with these preparations is not known. However, our observation with the *Z. atratus* fat body raises the possibility that tissue homogenates of the invertebrate systems may contain inhibitory factors, which, by nature or by artifact, act to restrict PG biosynthesis.

The influence of various pharmaceutical inhibitors of PG biosynthesis on invertebrate tissue preparations is typically expressed in a dose-related manner. This is so with naproxen and indomethacin in tobacco hornworm fat body preparations (Stanley-Samuelson and Ogg, 1994), tick salivary gland preparations (Pedibhotla et al., 1997), and with naproxen in true armyworm fat body preparations (Tunaz et al., 2001). In the work reported here with *Z. atratus* fat body preparations, we recorded substantial inhibition of PG biosynthesis in reactions conducted in the presence of indomethacin and naproxen. However, the inhibition did not obtain in the usual dose-related pattern. While judging that the point does not support additional investigation, I suspect this reflects the sensitivity of the enzyme preparations to these pharmaceuticals.

PG biosynthesis has been recorded in fat body preparations from several insect species, including cockroaches (Howard et al., 1986), tobacco hornworms (Stanley-Samuelson and Ogg, 1994), and true armyworms (Tunaz et al., 2001). Fat body is a central tissue in insect biology, and the PGs may act in many roles, including immune signaling (Morishima et al., 1997) and hormone signal transduction (Keeley et al., 1996; Ali and Steele, 1997a,b). Other important PG actions will emerge in the future.

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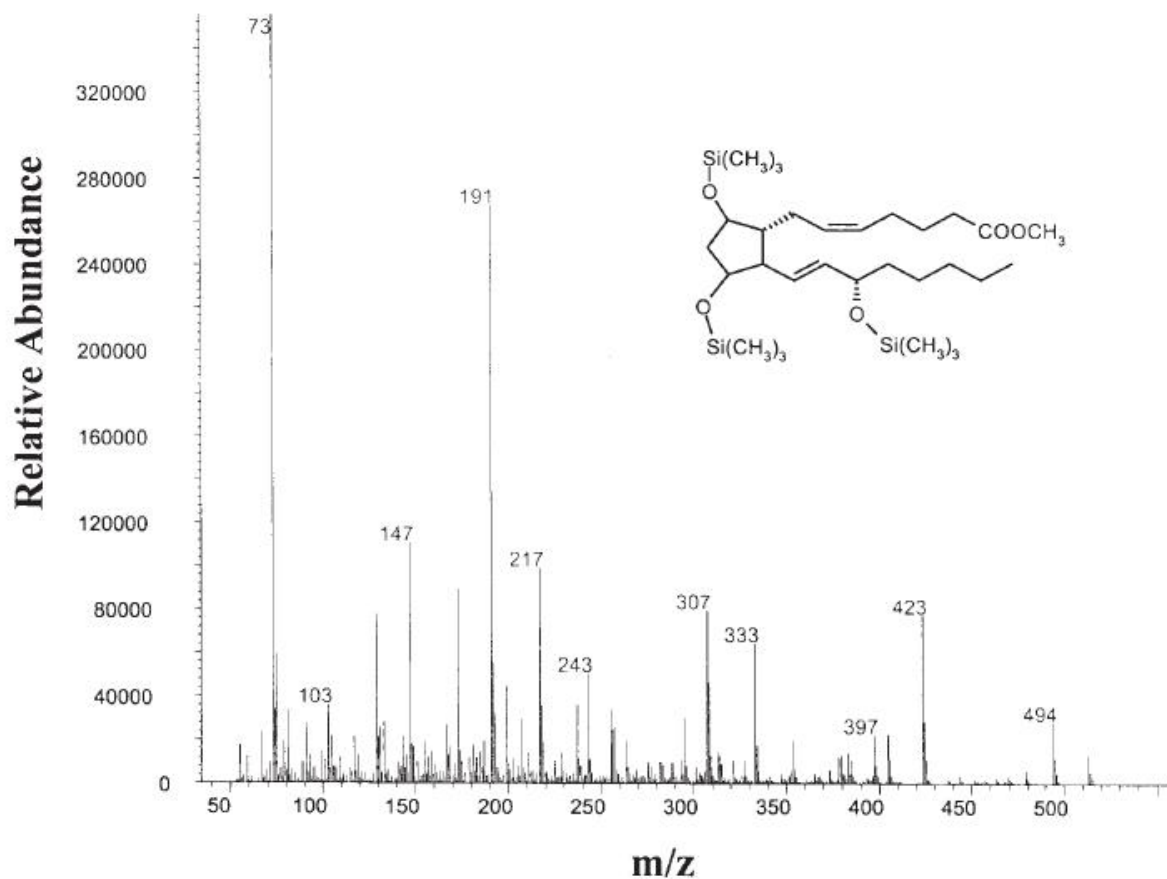


Figure 1. A total ion scan of the methyl ester trimethylsilyl derivative of PGF<sub>2α</sub> obtained from the incubation of *Z. atratus* fat body with arachidonic acid.



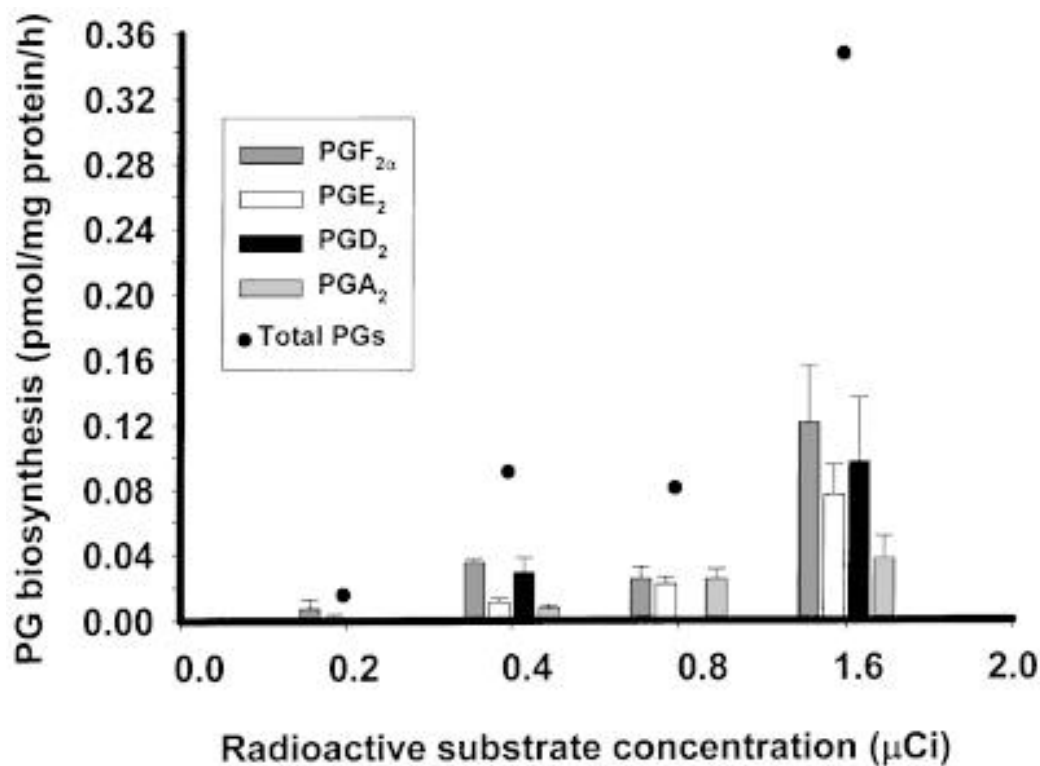


Figure 2. The influence of four concentrations of radioactive 20:4n-6 on prostaglandin biosynthesis by microsomal-enriched preparations of fat body from larvae of the beetle *Z. atratus*. The 0.5-ml reaction mixtures contained 0.2, 0.4, 0.8, and 1.6  $\mu\text{Ci}$   $^3\text{H}$ -20:4n-6, 2 mg of microsomal-enriched protein, and the co-factor in 0.05 M potassium phosphate buffer, pH 8.0. The reactions were incubated 32°C. After 2-min incubations, the reactions were stopped, and the reaction products were extracted and separated as described in Materials and Methods. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each substrate concentration. The error bars, where visible, indicate 1 SEM.

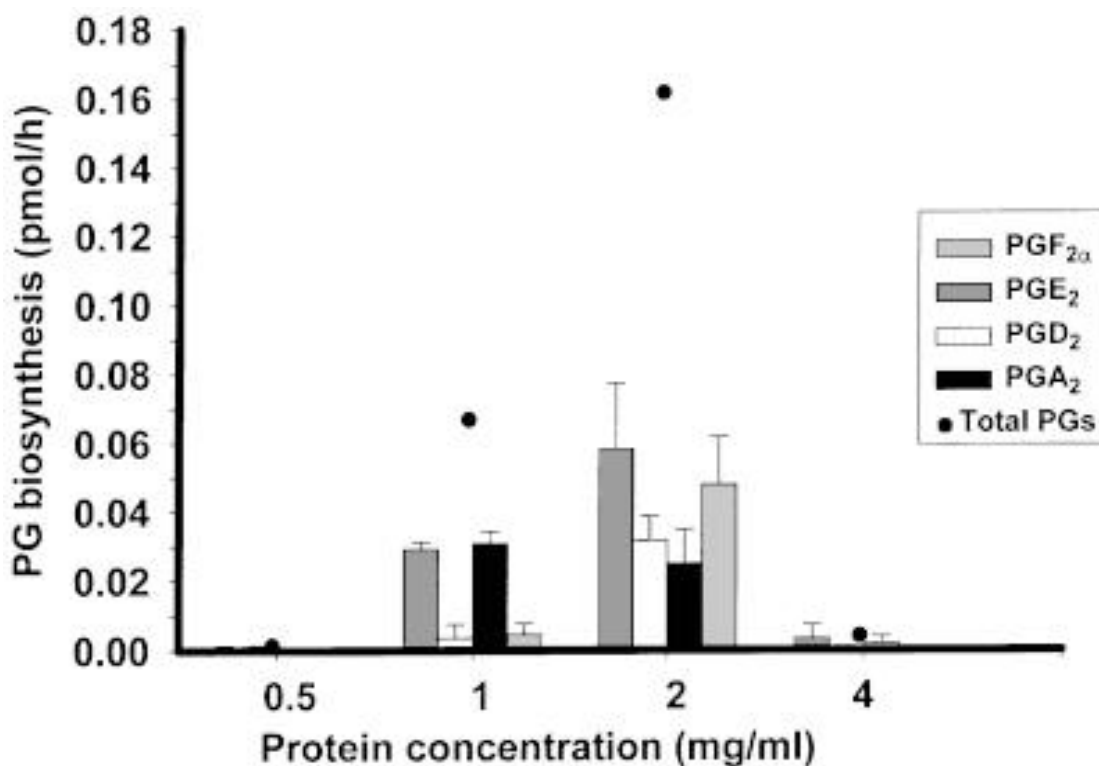


Figure 3. The influence of *Z. atratus* fat body microsomal-enriched protein concentration on prostaglandin biosynthesis. The reactions were conducted and the products were extracted and separated as described in Figure 2 and Materials and Methods. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each protein concentration. The error bars, where visible, indicate 1 SEM.

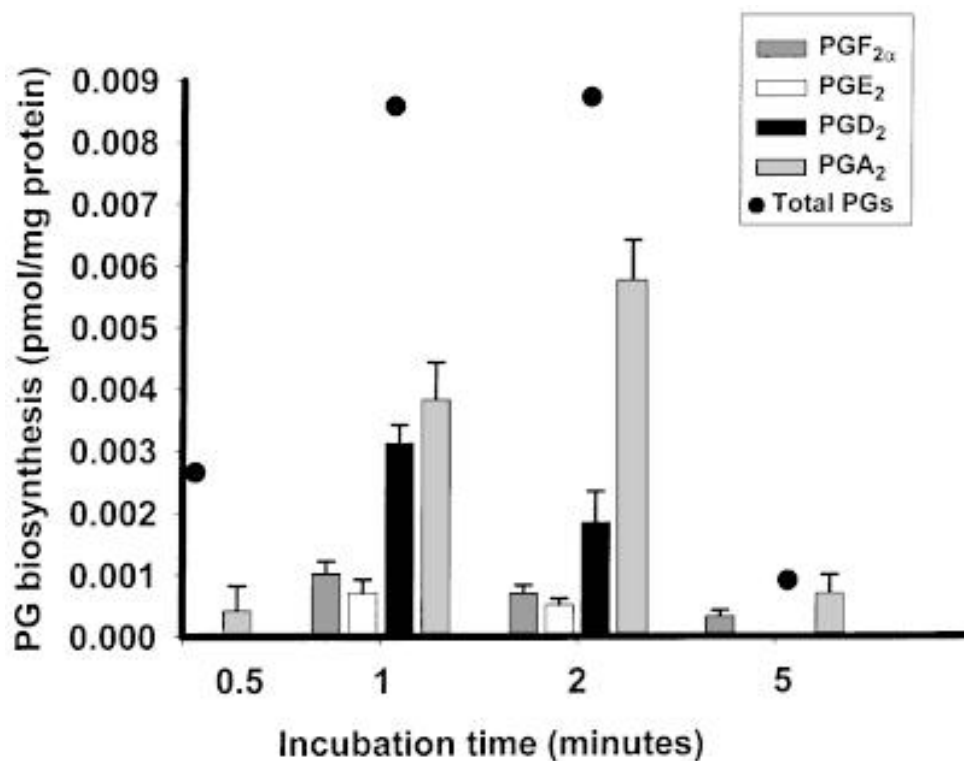


Figure 4. The influence of incubation time on prostaglandin biosynthesis by microsomal-enriched preparations of fat body from larvae of the beetle, *Z. atratus*. The reactions were conducted and the products were extracted and separated as described in Figure 2 and Materials and Methods. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different incubation time. The error bars, where visible, indicate 1 SEM.

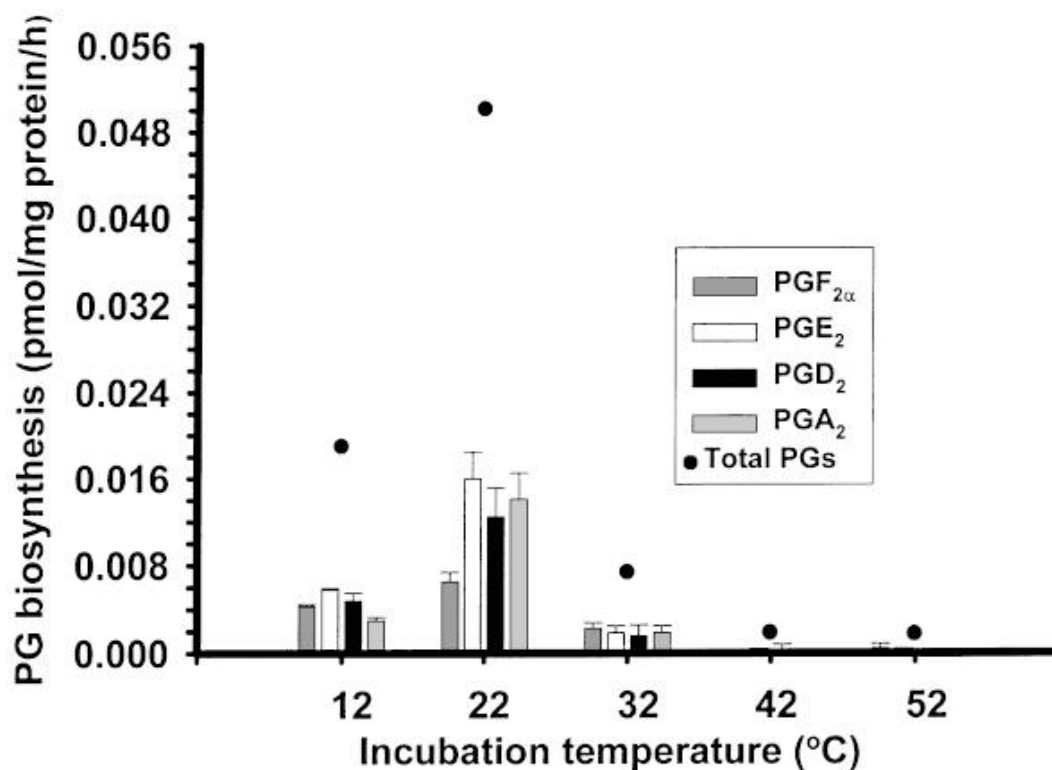


Figure 5. The influence of incubation temperature on prostaglandin biosynthesis by microsomal-enriched preparations of fat body from larvae of the beetle, *Z. atratus*. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different temperature. The error bars, where visible, indicate 1 SEM.

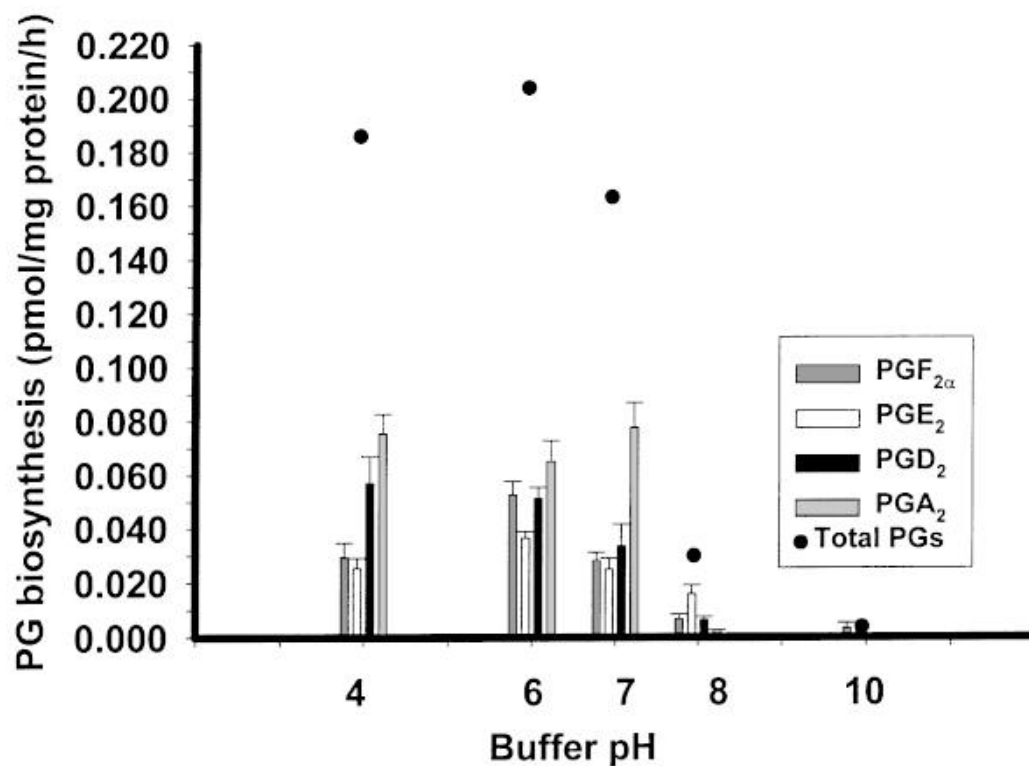


Figure 6. The influence of buffer pH on prostaglandin biosynthesis by microsomal-enriched preparations of fat body from larvae of the beetle, *Z. atratus*. The reactions were conducted and the products were extracted and separated as described in Figure 2 and Materials and Methods. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different buffer pH. The error bars, where visible, indicate 1 SEM.

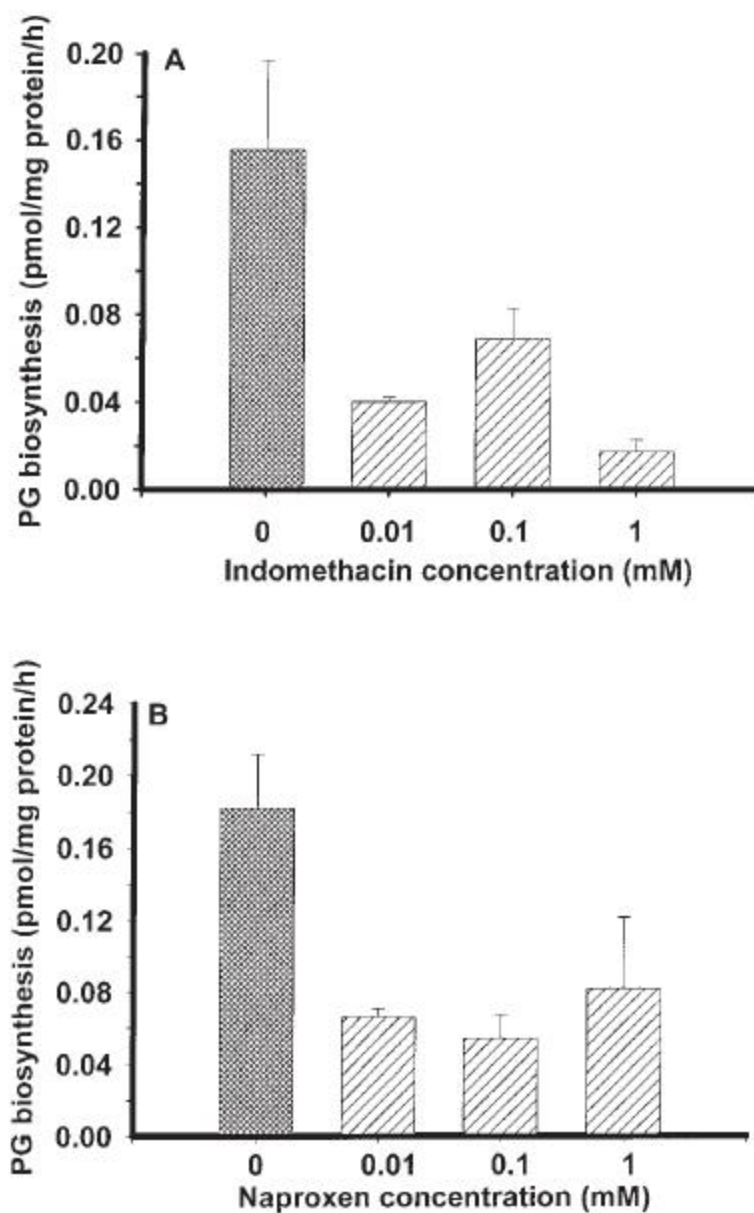


Figure 7. The influence of indomethacin (A) and naproxen (B) on prostaglandin biosynthesis by microsomal-enriched preparations of fat body from larvae of the beetle, *Z. atratus*. The reactions were conducted and the products were extracted and separated as described in Figure 2 and Materials and Methods. Each bar represents the mean of three separate experiments at each concentration of indomethacin. The error bars, where visible, indicate 1 SEM. Bars with different fill patterns represent statistical differences.

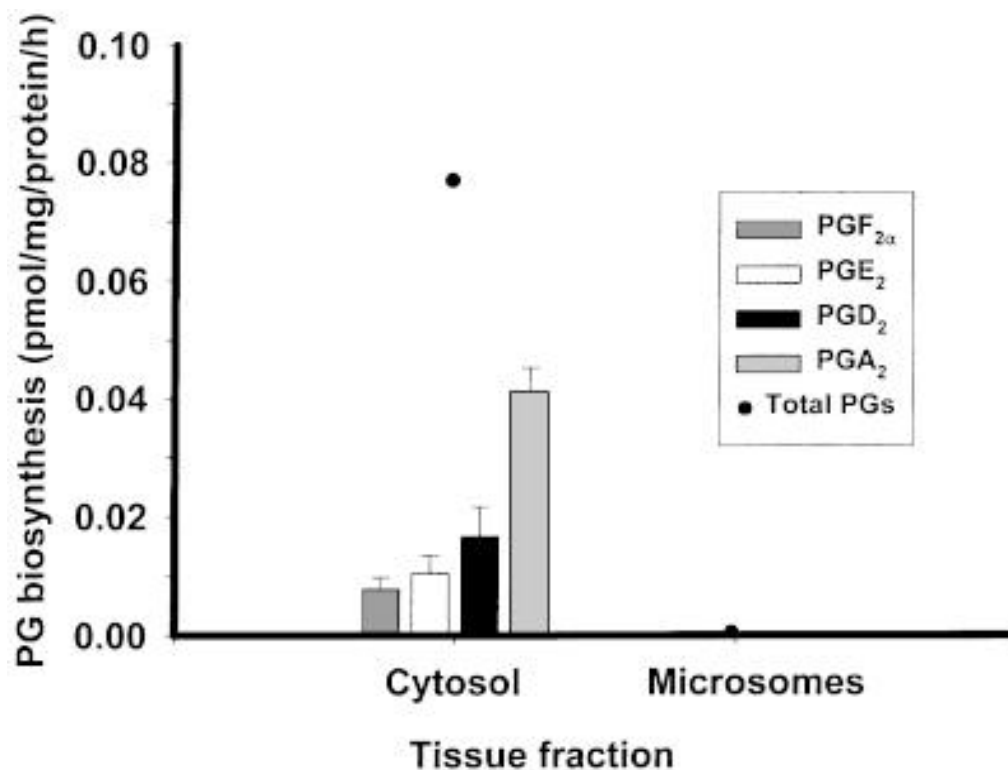


Figure 8. Localization of prostaglandin biosynthetic activity in the cytosolic and microsomal fractions of fat body prepared from larvae of the beetle, *Z. atratus*. The reactions were conducted and the products were extracted and separated as described in Figure 2 and Materials and Methods. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments. Error bars indicate 1 SEM.

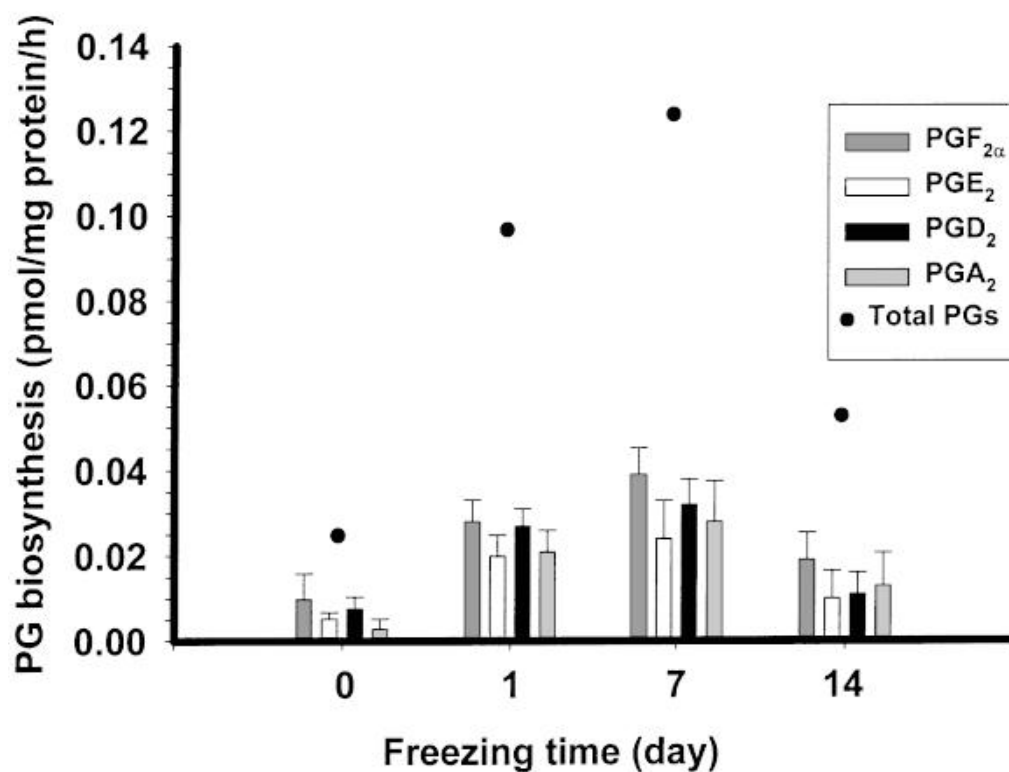


Figure 9. The influence of storage at  $-20^{\circ}\text{C}$  on prostaglandin biosynthesis by microsomal-enriched preparations of fat body from larvae of the beetle, *Z. atratus*. The reactions were conducted and the products were extracted and separated as described in Figure 2 and Materials and Methods. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different buffer pH. The error bars, where visible, indicate 1 SEM.



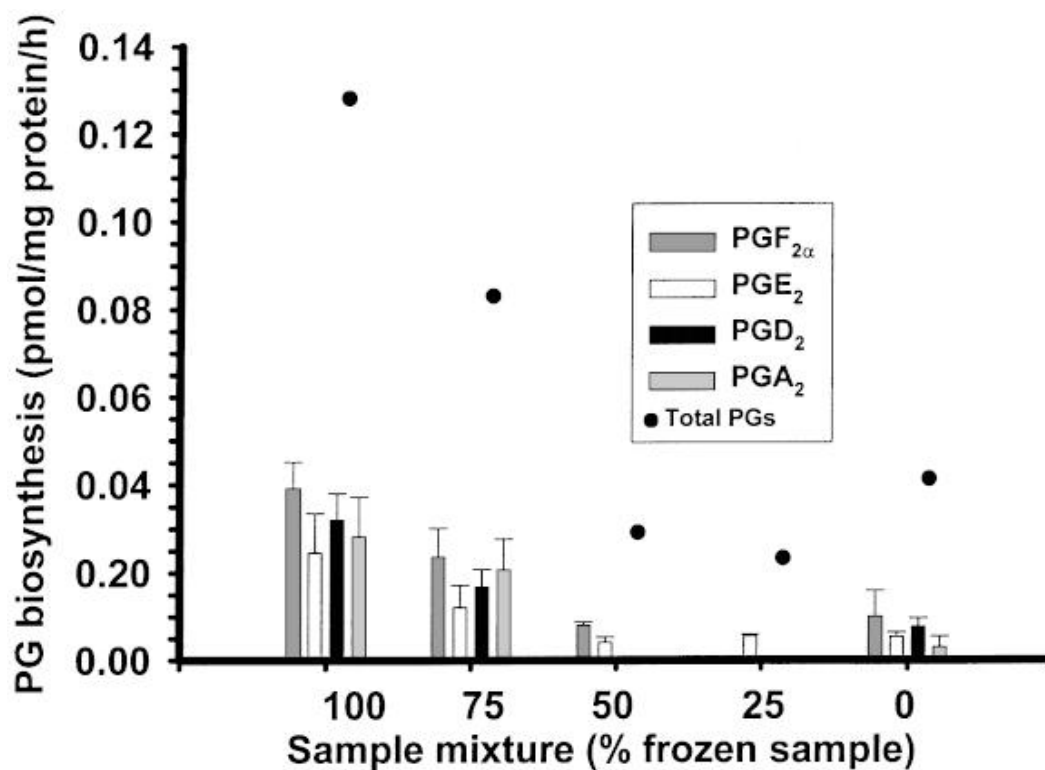


Figure 10. Prostaglandin biosynthesis recorded in mixtures of fresh and frozen samples of microsomal-enriched fat body preparations from larvae of the beetle, *Z. atratus*. The reactions were conducted with the indicated proportions of fresh and frozen sample, and the products were extracted and separated as described in Figure 2 and Materials and Methods. The histogram displays the biosynthesis of individual prostaglandins, and the solid circles represent total prostaglandin biosynthesis. Each point represents the mean of three separate experiments at each different buffer pH. The error bars, where visible, indicate 1 SEM.

## Chapter 5

The bacterium *Xenorhabdus nematophilus* depresses  
nodulation reactions to infection by inhibiting eicosanoid  
biosynthesis in tobacco hornworms, *Manduca sexta*

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**Abstract**

The bacterium, *Xenorhabdus nematophilus*, is a virulent insect pathogen. We tested the hypothesis that this bacterium impairs insect cellular immune defense reactions by inhibiting biosynthesis of eicosanoids involved in mediating cellular defense reactions. Fifth instar tobacco hornworms, *Manduca sexta*, produced melanized nodules in reaction to challenge with living and heat-killed *X. nematophilus*. However, the nodulation reactions were much attenuated in insects challenged with living bacteria (approximately 20 nodules/larva for living bacteria vs. approximately 80 nodules/larva in insects challenged with heat-killed bacteria). The nodule-inhibiting action of living *X. nematophilus* was due to a factor that was present in the organic, but not aqueous, fraction of the bacterial cultural medium. The nodule-inhibiting factor in the organic fraction was labile to heat treatments. The immunodepressive influence of the factor in the organic fraction was reversed by treating challenged hornworms with arachidonic acid. The factor also depressed nodulation reactions to challenge with the plant pathogenic bacteria, *Pseudomonas putida* and *Ralstonia solanacearum*. These findings indicate that one or more factors from *X. nematophilus* depress nodulation reactions in tobacco hornworms by inhibiting eicosanoid biosynthesis.

## Introduction

Insect immune reactions are broadly categorized into two expressions: humoral and cellular (Strand and Pech, 1995; Gillespie et al., 1997). Humoral immunity involves induced synthesis of various antibacterial proteins and enzymes, which usually appear in hemolymph 6 to 12 h post-infection (PI). Cellular, or hemocytic, immunity involves direct contact between circulating hemocytes and the invaders. Small invaders, such as bacterial cells, are cleared from circulation by phagocytosis and nodulation, a process of entrapping bacteria in aggregates of hemocytes. The internalized or entrapped bacteria are secondarily killed by various killing mechanisms involving reactive intermediates of oxygen (Nappi and Vass, 1998; Carton and Nappi, 1997). Insects protect themselves from larger invaders that cannot be taken into individual cells via phagocytosis or entrapped in hemocyte-micro-aggregation forms (nodules) by encapsulating the invaders in layers of hemocytes (Strand and Pech, 1995; Carton and Nappi, 1997).

While insect innate immunity provides effective protection from many invaders, some are able to overcome or evade the defense reactions. *Xenorhabdus nematophilus* is an intestinal symbiotic bacterium of the entomopathic nematode *Steinernema carpocapsae* (Akhurst, 1980) and a potent insect pathogen. This bacterium enters insects with its host nematode, and is released from the nematode into the insect hemolymph, where it multiplies and eventually kills the insect (Poinar and Thomas, 1966). The freshly-killed insect is the appropriate microenvironment for nematode reproduction and development. The mutualism between the bacterium and the nematode depends on the

ability of the bacterium to evade or somehow impair the normal robust insect immune reactions to the bacterial infection.

Park and Kim (2000) proposed the hypothesis that *X. nematophilus* impairs insect innate cellular immune reactions by inhibiting the biosynthesis of eicosanoids.

Eicosanoids are oxygenated metabolites of arachidonic acid and two other C20 polyunsaturated fatty acids. Major groups of eicosanoids include prostaglandins and various lipoxygenase products, and these compounds are thought to mediate cellular and some humoral immune reactions to bacterial infection (Jurenka et al., 1997, 1999; Miller et al., 1994, 1996, 1999; Morishima et al., 1997; Tunaz et al., 1999; Stanley, 2000). Park and Kim (2000) showed that treating infected larvae of the moth *Spodoptera exigua* with arachidonic acid reduced the mortality caused by *X. nematophilus* by 40% and that the arachidonic acid effect was expressed in a dose-dependent manner. They also showed that treating *S. exigua* larvae with pharmaceutical inhibitors of eicosanoid biosynthesis exacerbated the lethality of *X. nematophilus* infection. They demonstrated that live, but not heat-killed, bacteria exert pathogenicity by impairing the ability of infected insect larvae to form nodules in reaction to infection. In this study, we extend these findings along two axes. First, we report that challenge with heat-killed *X. nematophilus* stimulated intense nodulation reactions in another insect species, the tobacco horn-worm, *Manduca sexta*, and that the nodulation reactions were severely attenuated by similar challenge with live bacteria. Second, the nodulation-impairing action of the bacteria is due to a factor that can be extracted into organic solvents from the culture media of living, but not heat-killed, bacteria.

## Materials and methods

### *Organisms*

Eggs of the tobacco hornworm, *M. sexta*, were purchased from Carolina Biological Supply (Wilmington, NC). The hornworms were reared on standard culture medium under the semi-sterile conditions described elsewhere (Gadelhak et al., 1995). Fifth instar larvae were used in all experiments. Larvae of *Spodoptera exigua* were cultured as described (Park and Kim, 1999), and fifth in-star larvae were used in one experiment.

*Steinernema carpocapsae* were collected in Pochon, Korea, and donated by Prof. H. Y. Choo (Kyungsang National University, Korea). Entomopathogenic bacteria, *Xenorhabdus nematophilus*, were isolated from the hemolymph of fifth instars of *Spodoptera exigua* infected with *Steinernema carpocapsae* (Park et al., 1999). The bacteria were cultured in tryptic soy agar (Difco, Detroit, MI) at 28°C for 48 h. Bacteria were lyophilized, then stored at –70°C.

Plant pathogenic bacteria, *Pseudomonas putida*, *P. syringae*, and *Ralstonia solanacearum* were provided by Prof. Y. Yi (Andong National University, Korea) and cultured on nutrient agar (Difco) at 28°C for 48 h.

### *Chemicals*

Materials for *Manduca* saline buffer (MSB: 1.7 mM PIPES {piperazine-N-N'-bis [2-ethanesulfonic acid]}, 4 mM NaCl, 40 mM KCl, 18 mM MgCl<sub>2</sub>, 3 mM CaCl<sub>2</sub>, 243 mM sucrose, 15 mg/l polyvinylpyrrolidone, pH 6.5), and ethylene glycol bis (Baminoethyl

ether)-N,N,N',N'tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (St. Louis, MO).

#### *Injections and Nodulation Assay*

The experimental treatments followed the protocols described by Miller and Stanley (1998). Tobacco hornworms were chilled on ice and surface-sterilized with 95% ethanol. A live (in some experiments a heat-killed) bacterial suspension (10  $\mu$ l), was injected to each test larva through the first abdominal proleg using a 50  $\mu$ l Hamilton micro-syringe (Hamilton, Nevada). Control larvae were injected with the same volume of MSB. The treated larvae were incubated at room temperature. After 24 h (or other incubation period indicated in Results), the hemocoels were exposed and numbers of nodules were counted under a stereomicroscope.

#### *Dose-Response Curve for Bacterial Challenge*

The bacterial samples were grown on nutrient agar and counted to estimate the mean colony forming units (cfu), as described by Park and Kim (2000). Bacterial dosages were prepared by diluting the estimated stock suspension with MSB. The treated larvae were incubated at room temperature. After 24 h, the numbers of nodules were counted. Each dosage treatment (0,  $10^1$ ,  $10^2$ ,  $10^4$ ,  $10^6$ , and  $10^8$  cfu/treatment) consisted of six test larvae.

### *Time Course of Nodulation*

Test hornworms were injected with  $10^6$  cells of live bacteria in 10 ml MSB as just described. Control larvae were injected with the same volume of MSB. After six incubation periods, 0, 1, 2, 4, 8, and 16 hours, nodulation was determined by direct counting. Each treatment consisted of six test larvae.

### *Influence of Heat-Killed Bacteria on Nodulation*

Live bacteria ( $10^6$  cells/10ml) were heat killed at 98°C for 30 min in a shaking water bath. Experimental hornworms were injected with the heat-killed bacterial suspension. Control larvae were injected with the same volume of live bacterial suspension. After 4-h incubations at room temperature, nodulation was determined by direct counting. Each treatment consisted of six test larvae.

### *Preparing Fractions of Bacterial Culture Media*

*X. nematophilus* were cultured in 200 ml of tryptic soy broth (Difco) at 28°C for 48 h. Bacterial suspension medium (200 ml) was extracted in ethyl acetate (200 ml) in a 1-liter separation funnel. After centrifugation at 4,000 rpm for 30 min, the organic and aqueous fractions were separated. The organic fraction was evaporated on a rotary evaporator at 30°C and the aqueous fraction was evaporated on a speed vacuum. The organic fraction was resuspended in 100 ml of 50% ethanol, and the aqueous fraction was resuspended in 100 ml of 0.7% NaCl. Tobacco horn-worms were injected with 20  $\mu$ l of heat-killed bacterial suspension using a 50  $\mu$ l Hamilton micro-syringe. Within 5 min, the hornworms were treated with a second injection of either the organic or aqueous fraction



(at 0, 5, 10, and 20  $\mu$ l/ hornworm). After 4 h incubations, nodulation was assessed by direct counting. Each treatment consisted of six test larvae.

#### *Influence of Heat-Treating the Organic Fraction*

The organic fraction was heat-treated at 98°C for 30 min in a shaking water bath. Tobacco horn-worms were challenged with 20  $\mu$ l of heat-killed bacteria. Within 5 min, the hornworms were injected with the heat-treated organic fraction (20  $\mu$ l). Control larvae were injected with the same volume of non heat-treated organic fraction. After 4 h incubations, nodulation was assessed by direct counting. Each treatment consisted of six test larvae.

#### *Preparing Sub-Fractions of the Organic Fraction*

We prepared micro-columns using 1.0-ml pipette tips plugged with silanized glass wool. The columns were charged with 750 mg acidified silica gel, then washed with 3 ml ethyl acetate. The organic fractions were dried, then re-suspended in 100 ml ethyl acetate and loaded onto the columns. The columns were eluted in step-wise fashion with five solvents, using two-375 ml washes for each step. Solvent A was 100% ethyl acetate, solvent B was ethyl acetate/acetonitrile, 50%/50%, solvent C was 100% acetonitrile, solvent D was acetonitrile/ methanol, 50%/50%, and solvent E was 100% methanol. Each eluent was dried and taken up in 100 ml of 50% ethanol.

To assess the influence of each fraction on nodulation, tobacco hornworms were challenged with *X. nematophilus*, then treated with one of the five sub-fractions. Control

larvae were injected with the same volume of live bacterial suspension. After 4 h incubations at room temperature, nodulation was determined by direct counting. Each treatment consisted of six test larvae.

### *Rescue Experiments*

Tobacco hornworms were injected with heat-killed bacteria as described. Individuals in two groups of test larvae were injected with either 20 ml 95% ethanol or the organic fraction. Then, the organic fraction-treated hornworms were divided into two sub-groups. Individuals in one sub-group were treated with 20 mg arachidonic acid in 20 ml 95% ethanol. Insects in the other sub-group were treated with 20 ml of the vehicle, 95% ethanol. After 4 h incubations, nodulation was assessed by direct counting. Each treatment consisted of six test larvae.

### *Influence of *X. nematophilus* on Immune Reactions to Other Bacterial Species*

Three species of plant pathogenic bacteria were cultured on nutrient agar (Difco) at 28°C for 48 h, then counted to estimate the mean number of cfu. The bacteria were diluted to  $10^7$  cfu/ml in 0.7% NaCl. *S. exigua* larvae were injected with 2 ml of bacterial suspension, then 2 ml of either the organic or aqueous fractions prepared from *X. nematophilus* culture medium. At 24 h post challenge, nodulation was assessed by direct counting.

### *Data Analysis*

Treatment means and variances of the transformed data were analyzed by PROC GLM of SAS program (SAS Institute, 1989).

## **Results**

### *Dose-Response Curve for Bacterial Challenge*

The intensity of nodulation, determined by numbers of visible nodules formed by 24 h PI, was dependent on the dosage of infecting bacteria. It can be seen in Figure 1 that very few nodules, <2 nodules/larva, were recorded in control insects. The highest nodulation, approximately 16 nodules/ larva, was registered in response to bacterial dosages of  $10^6$  and  $10^8$  cfu/larva, which declined in a statistically significant way in reaction to lower bacterial dosages. As a practical matter, we used  $10^6$  cfu/larva as a standard challenge dose in subsequent experiments.

### *Time Course Of Nodulation*

The time course of visible nodule formation in tobacco hornworms challenged with  $10^6$  cfu/larva is shown in Figure 2. Nodulation increased from approximately 4 nodules/larva at 1 h PI to a maximum of approximately 16 nodules/larva at 4 h PI. Longer incubation periods did not yield further increases in nodulation intensity.

### *Influence of Heat-Killed Bacteria on Nodulation*

Whereas hornworms challenged with standard dosages of live bacteria produced low numbers of nodules, typically <20 nodules/larva, we recorded a much higher

nodulation intensity from horn-worms challenged with the same dosages of heat-killed bacteria, approximately 75 nodules/larva (Fig. 3).

*A Nodulation-Inhibiting Factor in the Organic Fraction of X. nematophilus Culture Media*

It appeared from the foregoing results that challenge with living *X. nematophilus* somehow impaired the ability of tobacco hornworms to elaborate innate cellular immune reactions. To investigate this in more detail, we prepared organic and aqueous extracts of living bacterial culture media. We then assessed the ability of these extracts to influence nodulation reactions to challenge with standard dosages of heat-killed bacterial. Organic and aqueous extracts were dried and taken up in 100 ml of solvent, 50% ethanol for the organic fraction and 0.7% NaCl for the aqueous. Horn-worms were first treated with four volumes of the organic, or separately with the aqueous, extract, 0, 5, 10, and 20  $\mu$ l. The insects were then challenged with heat-killed bacteria. It can be seen in Figure 4 that treatment with the aqueous extracts did not significantly influence the nodulation reaction to the heat-killed bacterial challenge. We recorded approximately 75 nodules/larva in all insects treated with the aqueous extracts. The situation is otherwise for hornworms treated with the organic extracts. Insects treated with the organic extracts produced significantly reduced nodulation intensity (Fig. 4), which was expressed in a dose-dependent manner.

### *Influence of Heat-Treating the Organic Fraction*

We also considered the possibility that heating the organic fraction would similarly attenuate nodulation intensity in response to challenge with heat-killed bacteria. It can be seen in Figure 5 that the organic extracts from heat-killed bacteria did not negatively influence nodulation.

### *Immune-Impairing Factor Appeared in a Single Sub-Fraction of the Organic Fraction*

The influence of five sub-fractions of the organic fraction on nodulation reactions to bacterial challenge are shown in Figure 6. Control hornworms yielded about 80 nodules/larva, which was unchanged in larvae treated with fractions A, B, and C. Nodulation was significantly impaired in larvae treated with fraction D (acetonitrile/methanol).

### *Arachidonic Acid Reversed the Influence of the Organic Extract*

The nodulation-impairing influence of the factor in the organic extract could be reversed by treating experimental hornworms with arachidonic acid, a substrate for eicosanoid biosynthesis (Fig. 7). In this experiment, insects treated with heat-killed bacteria, then injected with ethanol, produced approximately 75 nodules/larva. Nodulation was attenuated to approximately 15 nodules/larva in hornworms treated with heat-killed bacteria, then injected with the organic extract of living bacteria. The influence of the factor in the organic extract was reversed, however, in insects that had been challenged with heat-killed bacteria, then injected with the organic extract, and then treated with arachidonic acid in a third injection. To control for the possibility that the

third injection with ethanol stimulated nodulation in a non-physiological way, individuals in a fourth group of larvae were challenged with heat-killed bacteria, then injected with the organic extract, and then injected with ethanol, the vehicle for arachidonic acid injection. We recorded a very low nodulation, <15/larvae, in these insects.

#### *Influence of X. nematophilus on Immune Reactions to Other Bacterial Species*

We considered the possibility that the immune-depressing factor from *X. nematophilus* may impair insect immune reactions to other bacterial challenges. In these experiments, separate groups of *S. exigua* larvae were challenged with either of three plant pathogenic bacteria, *P. putida*, *P. syringae*, or *R. solanacearum*, then treated with either the organic or the aqueous fraction prepared from *X. nematophilus* culture medium. The data represented in Figure 8 indicate that larvae treated with the organic fraction produced significantly fewer nodules than larvae treated with the aqueous fraction in reaction to two species of bacteria, *P. putida* and *R. solanacearum*. The organic fraction did not influence nodulation reactions to *P. syringae*.

### **Discussion**

The results of the experiments reported in this study support the hypothesis put forth by Park and Kim (2000) that the insect pathogen *X. nematophilus* impairs the innate immune system of host insects by exerting an inhibitory influence on eicosanoid biosynthesis. Several lines of evidence support this hypothesis. First, challenge with a wide range of dosages of living *X. nematophilus*, from  $10^1$  to  $10^8$  cfu/larva, elicited very little nodulation reactions. Second, nodulation did not increase above the minimal

intensity level of approximately 15 nodules/larva throughout the time course of these experiments. Third, challenge with comparable dosages of heat-killed bacteria stimulated a 4-fold increase in nodulation, compared to the reaction to living bacteria. Fourth, the nodulation-attenuating factor was present in the organic, but not the aqueous, fraction of the bacterial culture medium. Fifth, challenge with the heat-treated organic fraction did not impair nodulation, while a similar challenge with organic extracts from living bacteria severely impaired nodulation. Finally, the influence of the factor in the organic fraction of the bacteria could be reversed by treating experimental hornworms with arachidonic acid, substrate for eicosanoid biosynthesis. We infer that a heat-labile factor in the organic fraction of the bacterium *X. nematophilus* somehow inhibits biosynthesis of immunity-mediating eicosanoids and thereby impairs the normal insect innate immune reactions to bacterial infection.

Steinernema and Heterorhabditis nematodes live in mutualistic relationships with a variety of *Xenorhabdus* species. Nematodes in these families exploit an unusually wide range of potential hosts that may include representatives of virtually all insect orders (Kaya and Gaugler, 1993). The nematodes rely on their bacterial partners to kill the insect host and to suppress potential competing secondary microbes. These actions serve to establish an appropriate microenvironment for nematode development. The bacteria cannot kill their insect hosts immediately on infection, however, and it is possible that innate immune reactions to the presence of nematodes could prevent the parasitization cycle. The bacterially induced down-regulation of insect immunity also may serve to protect the invading nematodes from insect hemocytic defense reactions. Hence, the idea that a bacterial pathogen can influence the innate immunity of infected insects, which

helps in the protection of its symbiotic partner, reveals a novel dimension of the chemical ecology of host-parasite biology.

The bacterial partners of the nematodes are very virulent insect pathogens. In terms of pathological microbiology, a wide range of virulence factors are associated with bacterial invasion (Salyers and Whitt, 1994). These include structural features, pili, for example, that promote colonization, adhesins that promote binding to host cells, and siderophores that serve the bacteria in iron acquisition. Bacteria also secrete toxic compounds into their host tissues and cells. Various hydrolytic enzymes (proteases, for example) break down extracellular matrix and thereby disrupt host tissue structure. Bacterial cell wall components, such as lipopolysaccharide (LPS; also known as endotoxin) also are toxic to hosts. Aside from these factors, which are under genetic control and thereby help in understanding the variance in virulence among bacterial strains, bacteria can also evade and disrupt mammalian host defenses. Variations in bacterial cell surface antigens, for example, serve to evade antibody reactions.

The virulence of *Xenorhabdus* as an insect pathogen also can be understood in terms of factors that enhance colonization, suppress secondary microbial invasions, and attenuate insect innate immune reactions. The idea that an insect pathogen can attenuate insect immunity through its influence on eicosanoid biosynthesis is an attractive and operationally useful model.

In interpreting our findings, there is concern that the organic fractions of living bacteria may inhibit nodulation due to the potential cytotoxicity of the solvents used to prepare the fractions. Hence, the reduced nodulation seen in Figure 4 could be an artifact.



The work represented in Figure 5, however, shows that the organic fraction of heat-killed bacteria did not inhibit nodulation, which allays the concern.

The idea that various bacterial species can induce differing levels of nodulation intensity has been explored (Ratcliffe and Walters, 1983; Rahmet-Alla and Rowley, 1989). For example, Howard et al. (1998) found that tobacco hornworms challenged with *Serratia marcescens* and *Escherichia coli* produced many nodules, >80 nodules/larva, while relatively few nodules were recorded in hornworms infected with *Bacillus subtilis* (>20 nodules/larva) and *Sarcina flava* (about 40 nodules/larva). Such differences were ascribed to features of the bacterial species, such as LPS structure, and to features of the interaction between bacteria and their insect hosts (Howard et al., 1998). With the eicosanoid inhibition hypothesis, Park and Kim (2000) open the intriguing possibility that many microbial invaders manipulate to their own advantage the innate immunity of their hosts.

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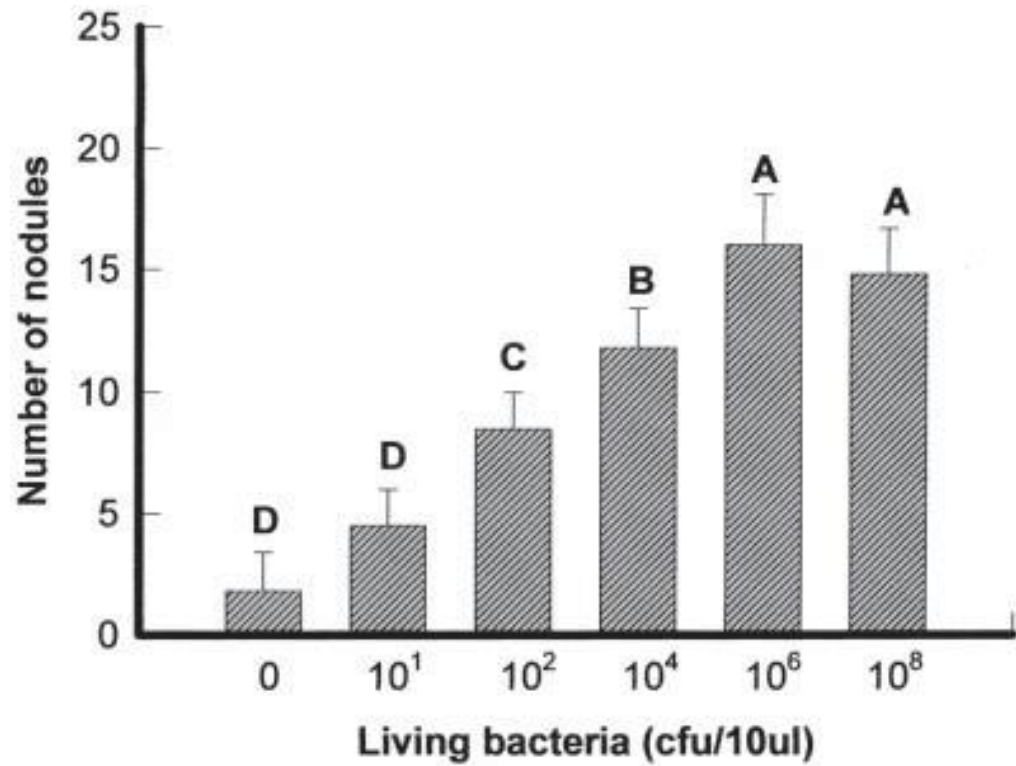


Figure 1. The influence of dosages of living bacteria, *Xenorhabdus nematophilus*, on nodulation intensity in fifth instar tobacco hornworms, *Manduca sexta*. Hornworms were intrahemocoelically injected with the indicated dosage of living bacteria, then nodulation was assessed at 24 h post-challenge. Histogram bars with the same letter are not statistically different,  $P < 0.05$ .

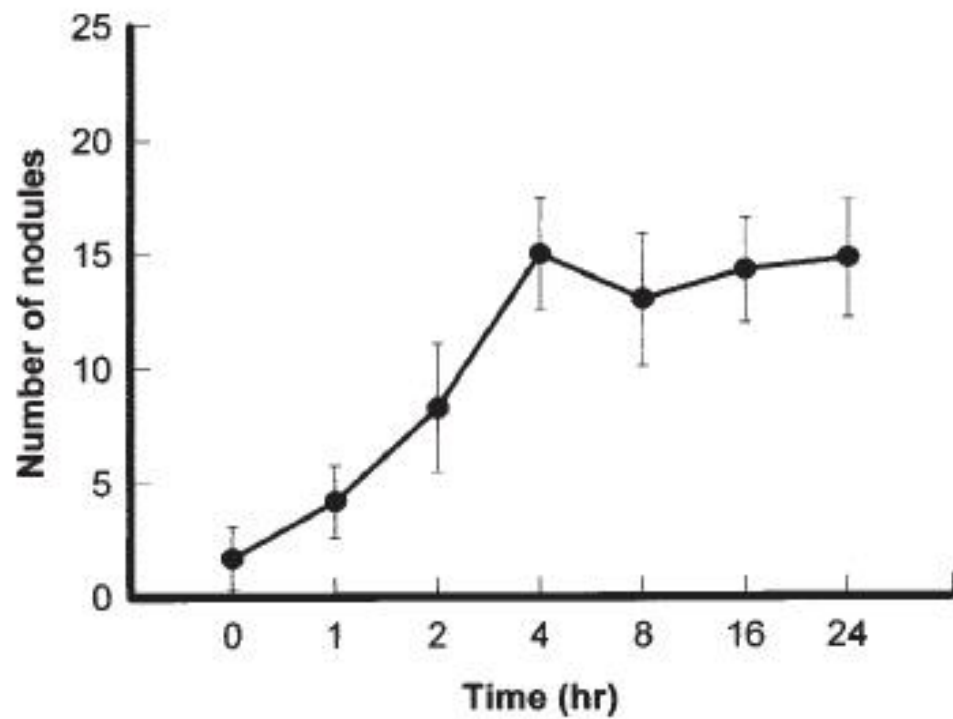


Figure 2. A time course of nodule formation in fifth instar tobacco hornworms, *M. sexta* challenged with living bacteria, *X. nematophilus*. Hornworms were intrahemocoelically injected with living bacteria ( $10^6$  cfu/hornworm), then nodulation was assessed at 24 h post-challenge. Each point represents the mean number of nodules/larva ( $n = 6$ ) and the error bars represent 1 SEM.

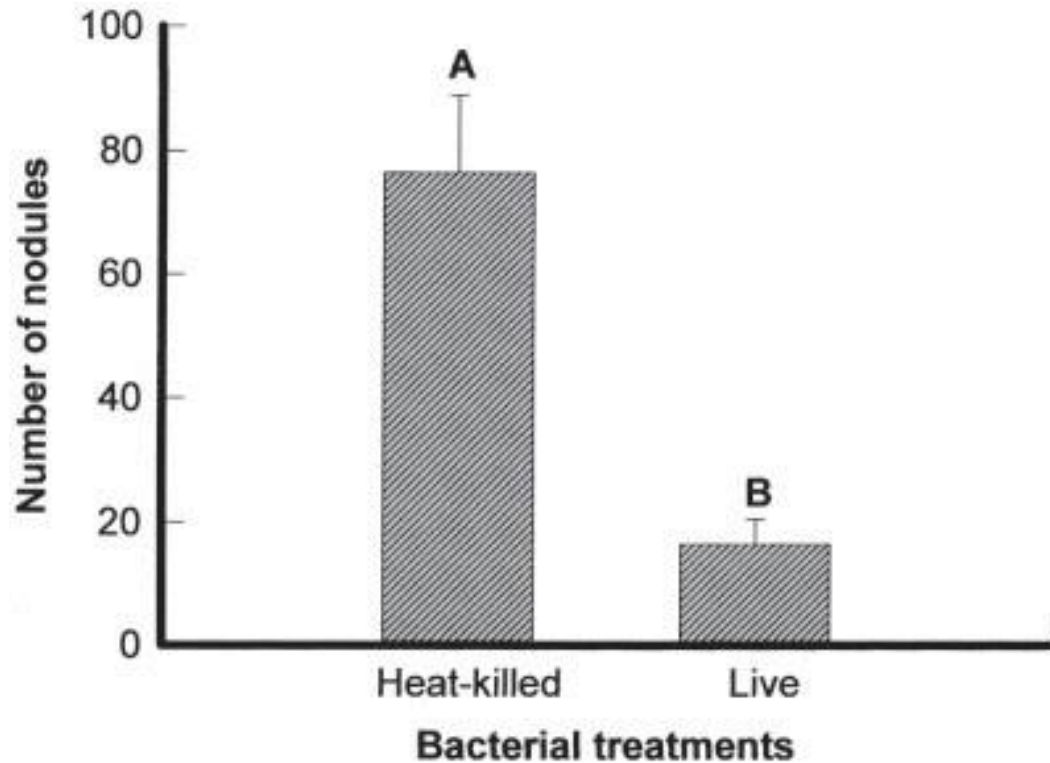


Figure 3. A comparison of the influence of living and heat-killed bacterial challenge on nodulation intensity. Horn-worms, *M. sexta*, were intrahemocoelically injected with  $10^6$  cfu/hornworm of living or heat-killed bacteria, *X. nematophilus*, then nodulation was assessed at 24 h post-challenge. Each histogram bar represents the mean number of nodules/larva ( $n = 6$ ) and the error bars represent 1 SEM. Histogram bars with different letters are statistically different,  $P < 0.05$ .



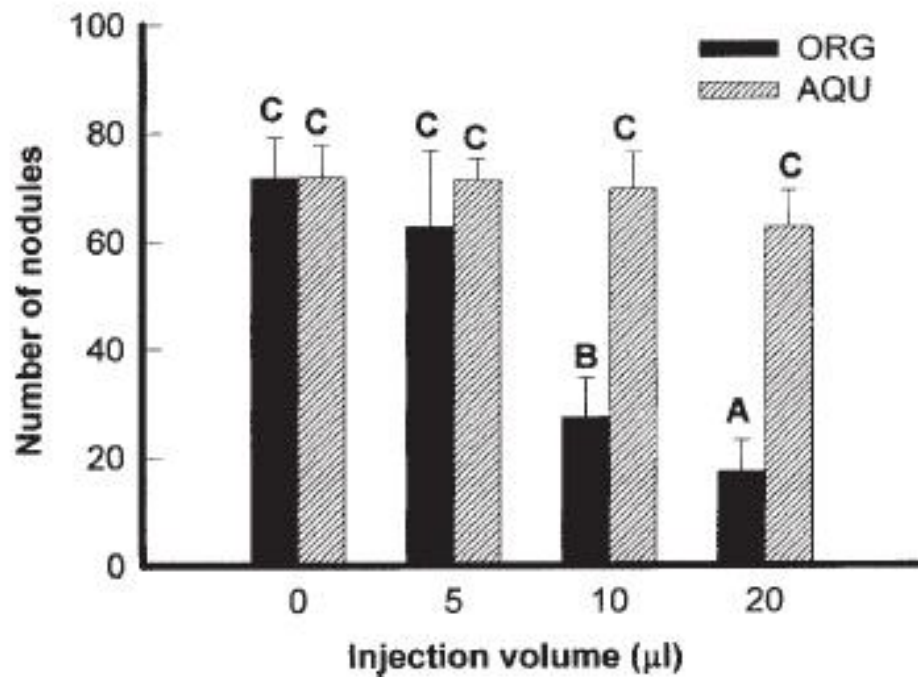


Figure 4. The influence of organic and aqueous extracts of living bacteria, *X. nematophilus* culture medium, on nodulation reactions to intrahemocoelic challenge with heat-killed bacteria, *X. nematophilus*. Hornworms, *M. sexta*, were injected with the organic or aqueous extract, then intrahemocoelically injected with  $10^6$  cfu/hornworm of heat-killed bacteria. Nodulation was assessed at 24 h post-challenge. Each histogram bar represents the mean number of nodules ( $n = 6$ ) and the error bars represent 1 SEM. Histogram bars with the same letter are not statistically different,  $P < 0.05$ .

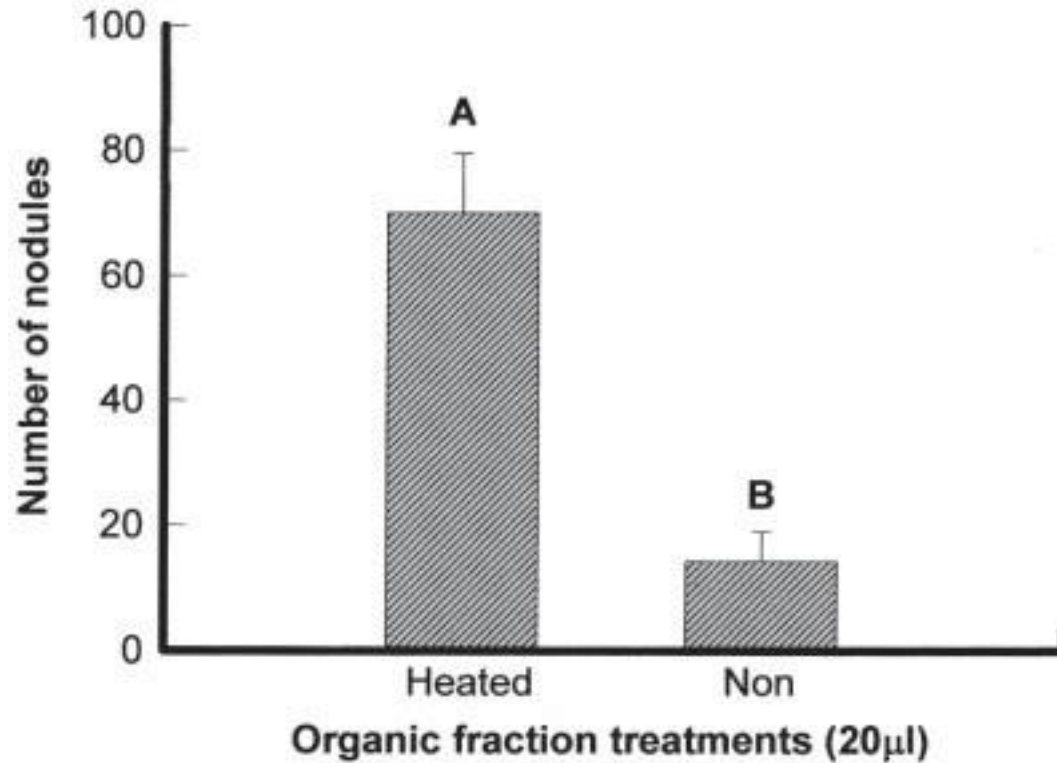


Figure 5. The influence of organic extracts of *X. nematophilus* culture medium, on nodulation reactions to intrahemocoelic challenge with heat-killed bacteria, *X. nematophilus*. Hornworms, *M. sexta*, were injected with the indicated organic extract, then intrahemocoelically injected with  $10^6$  cfu/hornworm of heat-killed bacteria. Nodulation was assessed at 24 h post-challenge. Each histogram bar represents the mean number of nodules ( $n = 6$ ) and the error bars represent 1 SEM. Histogram bars with different letters are statistically different,  $P < 0.05$ .

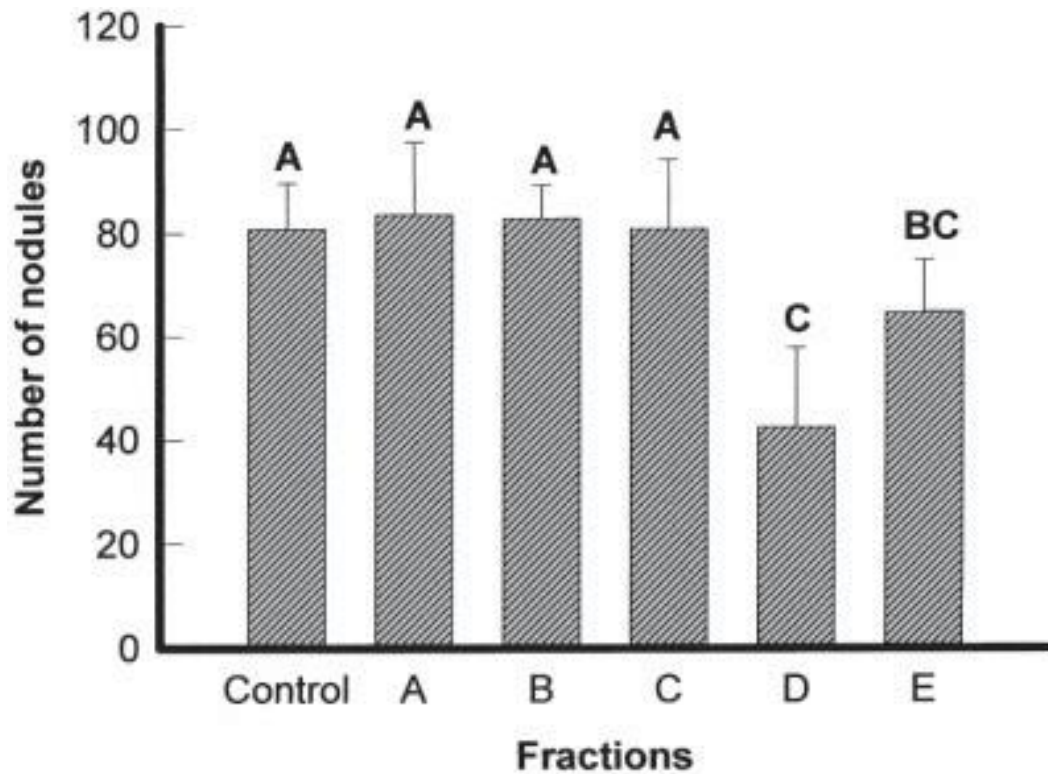


Figure 6. The immunosuppressive factor appears in a single sub-fraction of the organic extract of *X. nematophilus* culture medium. The organic extract was fractionated on a silica gel column, then separate groups' tobacco horn-worms, *M. sexta*, were treated with one of the fractions and challenged with heat-killed bacteria ( $10^6$  cfu/hornworm). Nodulation was assessed as 24 h post-challenge. Each histogram bar represents the mean number of nodules ( $n = 6$ ) and the error bars represent 1 SEM. Histogram bars with the same letter are not statistically different,  $P < 0.05$ .

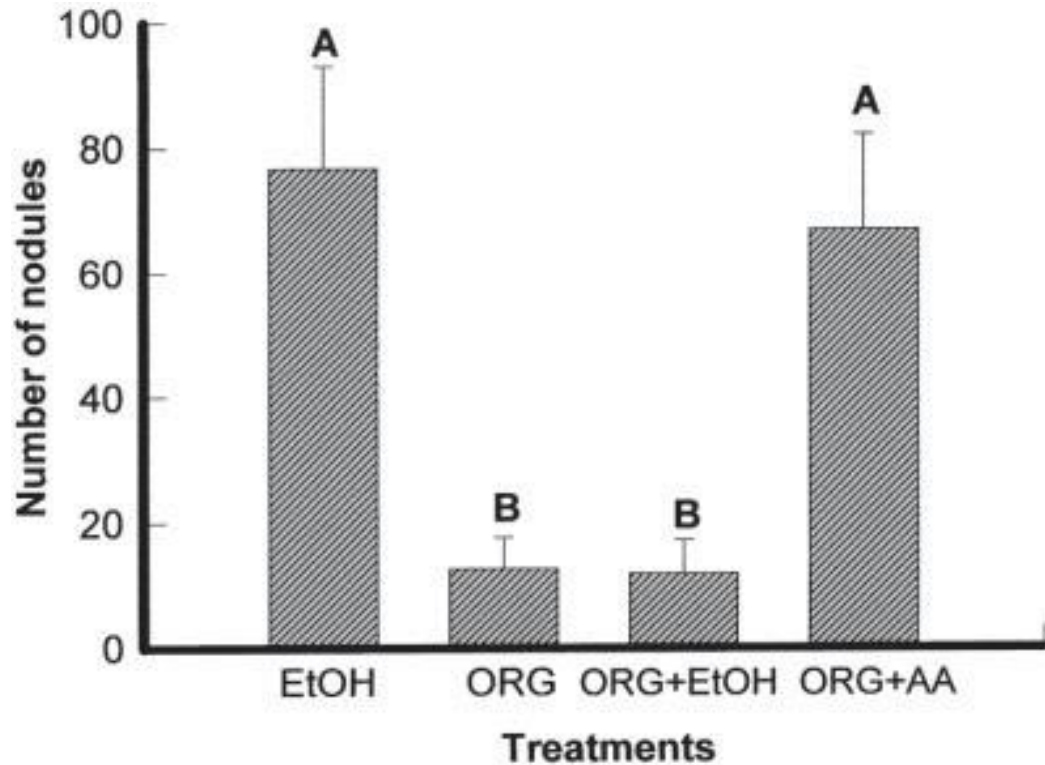


Figure 7. Arachidonic acid reversed the effect of the organic extract on nodulation. Tobacco hornworms, *M. sexta*, were treated with ethanol (EtOH) or the organic extract (ORG) and then challenged with heat-killed bacteria ( $10^6$  cfu/ hornworm). Immediately after challenge, test insects were treated with 50 mg of arachidonic acid (ORG+AA). Control insects were treated with ethanol (ORG+EtOH). Nodulation was assessed at 24 h post-challenge. The histogram bars represent the mean number of nodules ( $n = 6$ ) and the error bars represent 1 SEM. Histogram bars with the same letter are not significantly different from each other,  $P < 0.05$ .

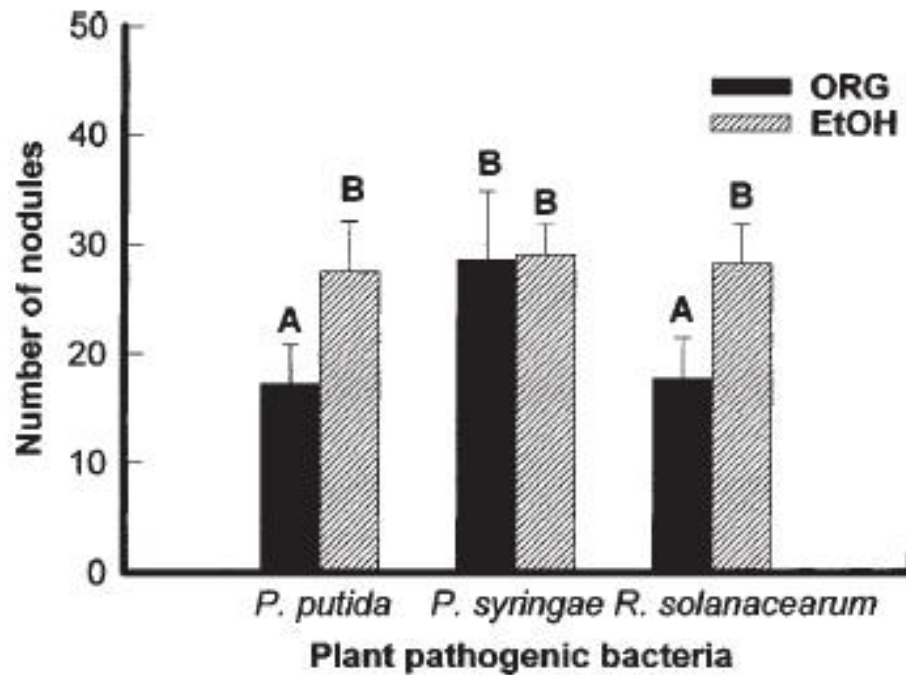


Figure 8. The influence of organic extracts of *X. nematophilus* culture medium on nodulation reactions to intrahemocoelic challenge with three species of plant pathogenic bacteria. Tobacco hornworms, *M. sexta*, were injected with either the organic or aqueous extract, then intrahemocoelically injected with  $10^6$  cfu/hornworm of heat-killed bacteria. Nodulation was assessed at 24 h post-challenge. Each histogram bar represents the mean number of nodules ( $n = 6$ ) and the error bars represent 1 SEM. Histogram bars with the same letter are not statistically different,  $P < 0.05$ .

## Chapter 6

### Summary and Conclusion

This dissertation bolsters the eicosanoid hypothesis (Stanley and Howard; 1998; Stanley; 2000; Stanley et al., 2009) by using rigorous chemical methods to determine the structures of prostaglandins (PGs) synthesized from two insect species. Also, I describe a bioassay for a natural product that inhibits PG biosynthesis. I used the bioassay to isolate and partially identify an inhibitor from the insect pathogenic bacterium, *Xenorhabdus nematophila*. Taken together, the chemical identification of PGs and the biological significance of inhibiting their synthesis, provide strong evidence that PGs are important lipid mediators in insects.

PGs are a subgroup of the eicosanoids, a group of biologically active, oxygenated metabolites of arachidonic acid (AA) and two other C20 polyunsaturated fatty acids (PUFAs). The presence and biological activity of eicosanoids have been identified in nearly all major animal phyla, including single-celled organisms (Stanley, 2000). In mammals, eicosanoids mediate fever induction, contraction (and relaxation) of smooth muscle, down-regulation of mast cell function, ion-transport physiology, cellular immunity and gene expression. Many pathophysiological actions in humans are directly related to eicosanoids. Gauging from the prevalence of these compounds in most metazoan organisms and their parallel modes of action, I surmise that eicosanoids represent an ancient signaling system, widely adapted in diverse phyla for many biological actions.

In insects, eicosanoids exert regulatory roles in immunity, reproduction, behavior, host-parasite relationships, stress induction, thermoregulation and ion transport (Stanley, 2000; Stanley 2006). Studies of several insect immune reactions to infection and invasion, including nodulation, microaggregation, cell spreading, cell migration and

encapsulation strongly support the idea that eicosanoids mediate insect immune responses (Miller et al., 1994; Stanley et al., 2009). The eicosanoid hypothesis is supported by the outcomes of experiments with over 20 species representing holo- and hemimetabolous orders as well as juvenile and adult insects. Further support for the hypothesis will depend on determining the chemical structures of known and unknown eicosanoids, as described just below.

The catalog of eicosanoid mediators represents a large group of compounds from many enzymatic, and some non-enzymatic, processes. Although studies in mammalian systems have outlined the synthesis these compounds, the evidence for these pathways is scant in insects systems. As eicosanoid research in invertebrates continues, entirely new compounds are coming to light. The “classical” eicosanoids are lipoxygenase (LOX) and cyclooxygenase (COX) products, commonly identified as leukotrienes (LTs) and prostanoids, respectively. The prostanoids include the prostaglandins, prostacyclins (PIs) and thromboxanes (TXs). A large body of literature on vertebrates describes the chemical and biological characteristics of these lipid mediators. The recent discoveries of other C20 eicosanoids, now known as “nonclassic eicosanoids”, are providing new directions for investigating biological actions in insects.

Recalling Chapter 1, the epoxygenase pathway produces epoxide metabolites of C20 PUFAs. In mammals, these compounds function as autocrine and paracrine effectors that modulate ion transport, anti-inflammatory responses and anti-fibrinolytic effects in the heart and kidneys (Spector et al., 2004). Epoxygenases are cytochrome P450 monooxygenases and in insects they are crucial enzymes in insecticide resistance. The eicosanoid products of these enzymes remain unknown in insects and they provide one of



many new topics for future research in insect biochemistry. Other nonclassic eicosanoids are derivatives of C20 PUFAs or even other eicosanoids. These include hepoxilins, lipoxins, resolvins, epi-lipoxins, isofurans, isoprostanes, endocannabinoids, oxo-eicosanoids and levuglandins (Salomon et al., 1984; Stanley, 2000; Fessel et al., 2003; Serhan et al., 2004). These molecules are strongly associated with lipid-mediated, oxidative injury in humans (Salomon, 2005). LOX products also act in insect immunity (Stanley et al., 2009). Discovery of these and other novel eicosanoids in insects depends entirely on rigorous chemical procedures needed to identify them. This fledgling field of insect lipid chemistry and biochemistry provides fertile ground for advancing our overall understanding of insect physiology and biology.

An uncharted course in lipid biochemistry of insects is the oxygenated products of the C18 PUFA linoleic acid (LA). As presented in my extra-dissertation activities, I outline work showing that insect tissue is competent to synthesize two products, 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE). These two metabolites may provide an entirely new direction for identifying lipid mediators in insect systems. HODEs mediate a wide range of actions in mammals including regulation of platelet function, maintenance of vascular thromboresistance, transduction of cellular responses to growth factors, stimulation of cell proliferation, extracellular matrix synthesis, inhibition of triacylglycerol-rich lipoprotein secretions and selective inhibition of protein kinase C (Spindler et al., 1996; Murthy et al., 1998; Pongracz and Lord, 1999; Negishi et al., 2004). With the exception of the work presented in the Appendix, these compounds remain completely unknown in insects and therefore represent still another potentially significant line of research in lipid signaling molecules.

Research presented in this dissertation and elsewhere on the biological significance of eicosanoids and other lipid mediators in insects highlights the growing value of multidisciplinary research in biology. Multidisciplinary research is not new. Looking back at the Loher (1984) work on the roles of PGs in releasing oviposition behavior in Australian field crickets, I note that research was led by a classically-trained behaviorist with no formal training in chemistry or biochemistry. Loher assembled a multidisciplinary team composed of an endocrinologist, a biochemist and two chemists in addition to a behaviorist to generate a break-through study of cricket behavior. He was pointing the way toward multidisciplinary research nearly 3 decades ago. Going a little further back, the discovery that insect cuticular hydrocarbons carried biological information is another breakthrough in insect science coming from interdisciplinary research between a chemist and a biochemist (Howard et al., 1980). Here, I suggest that continued advances in insect science will come from the work of well-coordinated, multidisciplinary teams of scientists. My goal is to play an active role in some of these teams.

## **Resources**

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## Appendix

Extra-dissertation research, teaching  
and grant writing experiences

I entered the graduate program in the Department of Entomology as an experienced professional teacher of high school chemistry and biology. My goal was to participate in a graduate experience in which I would contribute to the program while growing professionally through coursework, seminars, interactions with faculty members, working in a research group and conducting research. My experiences went far beyond my expectations of coursework and research.

The following sections outline extra-dissertation experiences in international research, teaching, developing curricula, developing web-sites, engaging field research, conducting independent chemical and biochemical research for my M.S. program and writing successful grant proposals to link high school and university instruction for advanced high school chemistry students. Taken together, these activities helped me gain the knowledge base, experience and confidence that are integral features of graduate programs leading to the Ph.D. degree.

### **Research at the International Level**

I gained valuable experience as a research scientist through collaborations at the international level. I worked on pheromone chemistry with an international scientist, Dr. Juan Cibrian-Tovar, during his sabbatical in Dr. Stanley's lab. I worked out a plan and chemically synthesized two sets of pheromones, then confirmed structures by mass spectrometry and nuclear magnetic resonance spectroscopy. Dr. Cibrian field-tested the pheromones, finding they did not attract the target pest species. While we did not publish the results, I gained synthetic chemistry experience and improved my knowledge of pheromone chemistry by spending a great deal of time with Dr. Cibrian. I also worked

with Youngjin Park, then a graduate student visiting for 5 months from South Korea, as described just below. I also collaborated with Park's advisor, Professor Yonggyun Kim, who spent time in Dr. Stanley's laboratory furthering their research on eicosanoid inhibitors. I collaborated with Dr. Kemal Büyükgüzel during his sabbatical research in Dr. Stanley's lab, contributing chemical identifications. All these experiences broadened my appreciation and understanding of scientific research at the international level.

### **Teaching Insect Physiology**

During the fall semesters of 2007-08 and 2009-10, I have been solely responsible for the administration of the Department of Entomology's Insect Physiology 401/801 course. As part of my responsibility of this course, I developed the curriculum, presented lectures, created assessments, developed the laboratory program and evaluated student progress. The central context of the course was designed around the concept of integrating the endocrine system as a central theme in the study of insect physiological systems. The students in this course were exposed to the principal components of several systems along with the underlying mechanism that coordinates biological activities. The challenge of teaching both graduate and undergraduate students in the same course relies on the ability to create a curriculum that engages students in the course content at a level commensurate with their academic backgrounds. This necessitates providing instruction and conversations that allows the undergraduates to gain an understanding of the complexities of a system without the biochemical background. Many of my lessons incorporate analogies and examples that make even the most complex interactions relevant to all students, in some accord. Utilizing concrete examples that serve to

correlate new learning can help undergraduates experience lessons from a relevant point of view.

The laboratory portion of the 401/801 course incorporates an exploratory set of activities that allows students an opportunity to interact with insects through a physiologist's point of view. The 496/896 course is part of the distance program where students work much more independently compared to direct contact with an instructor. Mastery learning strategies are incorporated into this course where students become able to demonstrate understanding by using online quizzes through Maple TA, a software program offered through UNL's Blackboard program. The opportunity to engage a curriculum through repetition and critical analysis provides a mechanism where conversations through the discussion board become consistent with mastery-level students. Student feedback was very positive and reaffirms my professional goals of developing a curriculum that engages students at a critical thinking level but providing instruction that focuses on student learning.

I was also responsible for facilitating the evaluative component of Entomology 496/896 distance course during three semesters. This course was designed to use Dr. David Stanley's recorded lectures while he taught the course. The students were provided online reading assignments that were put together by both Dr. Stanley and myself, and then were evaluated by questions presented through the EDU software system. This was a mastery-level course where students read, listened to lectures, presented questions via emails and completed the evaluation components. By evaluating students based on Dr. Stanley's lectures and curriculum, I could engage his thinking processes as he created his course. His approach to course content was centered about contemporary research and



how it applies to insect physiology. His immense knowledge of scientists and their work is amazing. I hope that as my course and experiences continue, a greater scope of research findings are integrated into the class.

### **Websites**

Insect Physiology 496/896. Sean M. Putnam and Dr. David W. Stanley.

<http://lhs2.lps.org/staff/sputnam/Ent801/Ento801.htm>

Insect Physiology 496/896. Sean M. Putnam.

[http://lhs2.lps.org/staff/sputnam/Ento801\\_07/ENTO401801.htm](http://lhs2.lps.org/staff/sputnam/Ento801_07/ENTO401801.htm)

Insect Physiology 496/896 EDU evaluation page. Sean M. Putnam

<http://calculus.unl.edu/edu/classes/IPSp09a2/>

### **Curriculum development for an insect molecular biology course**

In collaboration with Dr. John Foster, I have been developing a graduate-level course in insect molecular biology. Dr. Foster presented his plans of creating a three-pronged curriculum aimed at introducing graduate students to physiology, biochemistry and molecular biology. These three scientific scopes are incorporated into many biological research programs. In correlation with my insect physiology curriculum, I set out to create a curriculum which takes a molecular approach to entomological research. Physiology represents the biological action of organisms, whereas molecular biology represents the genomic expressions that underlie these actions. The third component, biochemistry, aims at describing the interactions of the chemicals that facilitate biological actions in organisms. All biological actions within in an organism fundamentally can be approached by any one of these scientific approaches. It is the understanding that all three

areas function cohesively to mitigate any biologic action that forms a strong academic program.

As the course content developed, my intent was to make a curriculum that represented our current knowledge in molecular biology come alive for the students. By developing a curriculum that can be presented using current instruction technologies, this course can be integrated into the direct instruction practices of on-campus courses or via the internet, as a developing practice for distance courses. Dr. Stanley and I began the development of interactive instructions with the insect physiology course. The lab component presented introductory information which included interactive components. The molecular biology course also contains interactive components where students are able to engage the content through various forms of on-line instruction. I am excited to see this course come to fruition and plan to continue developing coursework within the biochemical scope.

### **Mark-recapture analysis of Western corn rootworm, *Diabrotica virgifera virgifera*, using rubidium labeling**

Mark-recapture studies that assess intra- and inter-field dispersion of agricultural pests have become a useful tool for ecological entomologists. Pest migration from pesticide treated fields to untreated fields diminishes the effectiveness of pest-management programs and therefore identifying the migration patterns may help develop new practices that improve agricultural pest control.

Using trace elements as a marking strategy for characterizing pest migration emerged in the 1970s (Hagler and Jackson, 2001). This strategy utilizes elements that exist at very low levels within insect tissues and the environment. These trace elements

are taken up by insects, typically from feeding them diets supplemented with the trace element. The concentrations of trace elements then rise above native concentrations, thus providing a means for analyzing insect mobility by using trace element levels. Rubidium (Rb), an alkali metal, exists at trace levels within the lithosphere. Coupled with its ability to substitute for sodium and potassium within tissues, Rb makes an excellent candidate for mark-recapture studies. Sodium and potassium exist at much higher levels than Rb in insect tissues and can easily be replaced within biological systems with virtually no changes to a population. Once taken into tissues, the Rb can be extracted and quantified by atomic absorption spectroscopy. This type of spectroscopy measures the amount of energy absorbed by a solution upon exposure to a specific wavelength of light, 780.0 nm for Rb.

Nowatzki et al. (2003) studied the intra-field movement of western corn rootworm, *Diabrotica virgifera virgifera*, by labeling the larvae with rubidium chloride. Optimal labeling for western corn root worm larvae was identified where  $\text{RbCl}_{(\text{aq})}$  was applied to the soil, at the base of the corn plant, and within the whorl at concentrations of 1.0 g Rb/plant. Assessing rubidium levels within both insect and plant tissues, along with the soil, required extracting Rb from these samples. As a member of this project, I helped develop the protocols for extracting Rb. Initially, all samples were dried at 80 °C for 24 hours followed by dissolution in an extraction solution. Soil samples were exposed to 1N ammonium acetate (pH 7.0) and subsequently centrifuged at 4000 rpm for 3 min. After centrifugation, 10 ml of supernatant was removed for analysis. For plant and insects tissues, 70% nitric acid digestion for 12 hours followed by immersion in an 80 °C dry bath for 1 hour was sufficient for Rb extraction. Rb levels in these samples were

measured in a graphite furnace by atomic absorption spectroscopy. From this work, it was found that larvae were sufficiently labeled, above background, for up to 6 days, post-emergence. This study provides the evidence to show that migration of western corn rootworm by Rb mark-recapture techniques is feasible.

### *Publication*

Nowatzki, T.M., Niimi, B.; Warren, K.J. **Putnam, S.**, Meinke, L.J., Gosselin, D.C., Harvey, F.E., Hunt, T. E., and Siegfried, B.D. 2003. In-field labeling of western corn rootworm adults (Coleoptera: Chrysomelidae) with rubidium. *Journal of Economic Entomology*. **96**(6):1750-9

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## **In vitro synthesis of insect pheromones**

First evidence that insects utilize chemical messengers to attract the opposite sex dates to the 1870s when two scientists, Joseph A. Lintner and Jean-Henri Fabre, independently showed that newly emerged female moths are able to attract male moths. German biochemist Peter Karlson and Swiss entomologist Martin Lüscher were the first to describe pheromones as compounds which are given off by an animal which in turn triggers a specific behavioral or developmental response in another animal of the same species. Another German biochemist, Adolf Butenandt, painstakingly isolated and

characterized the first pheromone from silkworm moths. These scientists laid the foundation for utilizing pheromones as a component of modern pest-managements systems (Patlak et al., 2003).

Showing that insect pheromones are capable of attracting members of the same species has been known for over 130 years but using pheromones to control agricultural pests has emerged only in the last 30 years. Identifying pheromones was facilitated with development of sophisticated analytical instrumentation. Isolating, characterizing and synthesizing them for use in field trials is a highly sophisticate area of chemistry. Although sometimes an arduous task, utilizing these compounds for pest management offers significant economic and ecological benefits. Although pheromone trapping has not been as successful in pest management as once hoped, insect pheromones are in wide use in pest monitoring programs. Of particular importance, some pheromones are used to monitor for possible pest at international borders.

Working with Dr. Juan Cibrian-Tovar, I synthesized two sets of insect pheromones beginning with unsaturated fatty acids as initial substrates. The first set of pheromones were isolated and characterized by GC/MS from *Copitarsia consueta* (Lepidoptera: Noctuidae), a moth which plagues cabbage crops in Mexico. The second insect characterized was *Copitarsia incommada* Walker (Lepidoptera: Noctuidae). The two sets of pheromones for these two insects were synthesized from the fatty acids,  $\alpha$ -linolenic acid ( $Z,Z,Z$ -18:3 $\Delta^{9,12,15}$ ) and myristoleic acid ( $E$ -14:1 $\Delta^9$ ), respectively.

The synthesis of *C. consueta* pheromones involved the production of two different compounds from  $\alpha$  -linolenic acid. The first step involved the lithium aluminum hydride reduction of the  $\alpha$  -linolenic acid carboxyl group to a hydroxyl group, producing the

alcohol,  $\alpha$  -linolenol (18:3-OH). The second step, oxidizing the alcohol to an aldehyde,  $\alpha$  -linolenal, required using NMO/TPAP (N-methylmorpholine-N-oxide/tetrapropylammonium perruthenate) as the oxidizing agent. Both products were purified, concentrated and analyzed by NMR. The products were subsequently taken to Mexico for pheromone capturing field trials.

The second synthesis, pheromones of *C. incommada*, required the reduction of myristoleic acid to the alcohol followed by acetylation producing an ester, myristoyl acetate. As in previous reduction of  $\alpha$  -linolenic acid, the reduction of myristoleic acid using lithium aluminum hydride produced the alcohol of myristoleic acid, myristol (cis 9-tetradecen-1-ol). Acetylation of myristol was accomplished by reacting the alcohol with acetic anhydride in the presence of the catalyst, N,N-dimethylaminopyridine (DMAP). Purification, concentration and analysis by NMR verified the products. These products were also taken to Mexico for pheromone capture field trials.

### *Presentation*

Cibrian-Tovar, J., Rojas, J., **Putnam, S.**, Calyecac, G., Cruz-Lopes, L., Malo, E., Diaz-Gomez, O. Identification of sexual pheromone of *Copitarsia incommoda* (Lepidoptera. Noctuidae). Annual meeting Entomological Society of America. Oct. 26, 2003.

### *Reference*

Patlak, M., Baker, T., Berenbaum, M., Carde', R., Eisner, T., Meinwald, J., Roelofs, and Wood, D. (2003, Jan.) Insect pheromones: Chemicals of Communication. For

Beyond Discovery: The path from research to human benefit. Nat. Acad. Sci.

<http://www.beyonddiscovery.org/content/view.page.asp?I=2709>

### **Laboratory instructor for Insect Physiology 401/801**

During the fall semester of 2003-2004, I taught the lab component of Insect Physiology 401/801. I was responsible for developing the online lab manual, lab preparation, introducing lab objectives and procedures, and monitoring students during lab activities. The evaluation component for the lab was coordinated with lecture assessments, which employed the UNL-hosted EDU system.

As a high school science teacher, working with undergraduate and graduate students allowed me to further enhance my teaching experience. Teaching students who have a larger knowledge of science and better understanding of the scientific method allowed me to utilize more inquiry-based strategies where students could critically evaluate protocols and data while investigating scientific principles. By comparing high school and college students, it is easy to see that academic success at any level is predicated on the prepared mind. As expected, students in the lab course who prepared for each activity by reading the lab introduction and procedures, and developing a model for the activity were more successful. This experience has reinforced my belief that learning is a skill that requires continual reinforcement and reflection. By understanding what makes us successful as learners helps establish a foundation for successful teaching.

Developing the online lab manuals for the physiology lab provided the students with a resource which incorporated more dynamics models and ancillary resources which is concurrent with our knowledge of insect physiology research. Developing dynamic

resources, such as an online lab manual, helps foster a student's understanding of scientific concepts. I have utilized these kinds of resources in my high school teaching and believe that students who actively engage new learning in multiple ways are able to master learning objectives and develop a deeper understanding of science.

### **Cooperative LHS/UNL research practices for high school chemistry students**

As a non-traditional graduate student who also teaches high school science classes, I have the rare opportunity to connect my research experiences to learning objectives and teaching in my classroom. The analytical equipment and research practices we use in the insect physiology lab are common to many organic and biochemical labs. Through my experiences and with the assistance of Dr. Stanley, I have been able to develop research activities where my advanced chemistry students use this equipment and protocols to answer significant scientific questions.

These activities have been supported by grants from the Lincoln Public Schools Foundation and Sun Microsystems. Through these grants we have introduced high school students to study prostaglandin biosynthesis and fatty acid composition in insect tissues, identify uptake of deuterated polyunsaturated fatty acids into the midgut of *Manduca sexta*, and analyze hydrocarbon profiles of termite caste members. Through these experiences, my students have developed a better sense of how research is conducted and with the use of analytical equipment, such as GC, TLC, & GC/MS, have developed skills which they can use later in their education. Students who have graduated from this program have gone on to represent many facets of science education, such as medical school, chemical engineering, pharmacy, science research (one graduate is



published in Virology detailing research with HIV matrix domain replacements), science education, international public health, materials science and engineering. The experiences that high school educators can integrate into higher level science classes provide a richer set of experiences for students.

*Grant support for teaching*

**Putnam, S.M.**, and Stanley, D.W. (2001) LHS/UNL collaborative research experience for advanced chemistry students grant. Lincoln Public Schools Foundation.  
\$1000.00.

**Putnam, S.M.**, and Stanley, D.W. (2002) LHS/UNL collaborative research experience for advanced chemistry students grant. Lincoln Public Schools Foundation.  
\$2350.00

Stanley, D.W., and **Putnam, S.M.** (2003) Connecting university scientific equipment to high school laboratory teaching grant. Sun Microsystems. \$30,000.00

**M.S. research: Linoleate metabolism by fat body of the tobacco hornworm, *Manduca sexta***

This researched reported on the presence and biosynthesis of 9- and 13-hydroxyoctadecanoic acid (9- and 13-HODE) in fat body of tobacco hornworms, *Manduca sexta*. The HODEs are oxygenated metabolites of linoleic acid, 18:2n-6, making them 2 carbons shorter than eicosanoids. Fat body preparations were treated with 18:2n-6. After 20 min incubations at 30°C, two HODES, 9- and 13-HODE, were extracted from the fat body preparations, derivatized and analyzed on gas chromatography-mass spectrometry. The identity of the two HODEs was confirmed by

analysis of the mass spectra. After confirming the structure of the HODEs, HODE biosynthesis was characterized. In the hornworm fat body preparations, HODE biosynthesis was sensitive to incubation time, pH, radioactive substrate, and protein concentration. In mammals, both cyclooxygenases and lipoxygenases are responsible for HODE biosynthesis. The hornworm fat body preparation was sensitive to the dual cyclooxygenase/lipoxygenase inhibitor, phenidone, from which I infer insects, as seen in mammals, biosynthesize HODEs via enzymatic oxygenation. HODEs represent another group of the growing list of lipid mediators in insect biology.

The HODEs make up a group of lipid mediators new to our understanding of insect physiology. Because recognition of these mediators is new, their biological significance is not yet plain. It appears that the main actions of HODEs lie in their influence on intracellular signal transduction systems. In their study of HODE actions in melanoma cells, Liu et al. (1995) reported that 12-hydroxyeicosatetraenoic acid (12-HETE, a LOX product of arachidonic acid) activated protein kinase C- $\alpha$ , leading to increased adhesion of the cells to a matrix protein. 13-HODE effectively inhibited this and the authors speculated that the actions of 12-HETE and 13-HODE take place via interactions with a specific receptor site. Hui et al. (1997) showed that 13-HODE acts as a specific enhancer of epidermal growth factor-dependent DNA synthesis. Similarly, Shureiqi et al. (2003) suggested that 13-HODE induces apoptosis in colorectal cancer cells by down-regulating peroxisome proliferators-activated receptors, which act as nuclear receptors for 18:2n-6, 20:4n-6 and their oxygenated metabolites. I suggest that HODEs also influence signal transduction systems in insect systems.

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