# A DIGESTIVE PHOSPHOLIPASE $A_2$ IN MIDGUTS OF WORKER HONEY BEES, *APIS MELLIFERA*: SIGNIFICANCE IN LIPID NUTRITION AND HEALTH

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*APIS MELLIFERA*:

SIGNIFICANCE IN LIPID NUTRITION AND HEALTH

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic Acid |
| AdPLA | Adipose-specific Phospholipases $A_2$ |
| AMP | Antimicrobial Peptides |
| BPB | p-Bromophenacyl Bromide or 2,4′-Dibromoacetophenone |
| CCD | Colony Collapse Disorder |
| $cPLA_2$ | Cytosolic Phospholipases $A_2$ |
| DMSO | Dimethyl Sulfoxide |
| EGTA | Ethylene Glycol Tetraacetic Acid |
| HTH | Hypertrehalosemic Hormones |
| $iPLA_2$ | $Ca^{2+}$-independent Phospholipases $A_2$ |
| $LPLA_2$ | Lysosomal Phospholipases $A_2$ |
| MAFP | Methyl Arachidonyl Fluorophosphonate |
| PAF-AH | Platelet-activating Factor Acetylhydrolase |
| PBS | Phosphate Buffer Saline |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanolamine |

| | |
|---|---|
| PG | Prostaglandin |
| PI | Phosphatidylinositol |
| PL | Phospholipid |
| PLA | Phospholipases A |
| PLB | Phospholipases B |
| PLC | Phospholipases C |
| PLD | Phospholipases D |
| PMSF | Phenylmethane Sulfonyl Fluoride |
| PS | Phosphatidylserine |
| PUFA | Polyunsaturated Fatty Acids |
| $sPLA_2$ | Secreted Phospholipases $A_2$ |
| TAG | Triacylglycerol |

# ABSTRACT

Phospholipase $A_2$ ($PLA_2$) catalyzes hydrolysis of the fatty acid moiety from the *sn-2* position of phospholipids. At least 16 groups of $PLA_2$s are known in mammals. In insects, $PLA_2$s occur in several tissues, where they act in a variety of biological functions, such as digestion, immune signaling, development and reproduction. However, other than the venom $PLA_2$s, characterization of their physiological functions has not been reported in Hymenoptera, particularly in honey bees (*Apis mellifera*). Among many studies on honey bee nutrition and physiology, the enzymes acting in digesting dietary lipids remain understudied. This is an important lacuna because it bears directly on meeting essential dietary fatty acid requirements, which I addressed by characterizing a digestive $PLA_2$ in midgut preparations of honey bee workers. The honey bee midgut $PLA_2$ is sensitive to reaction conditions, including incubation time, substrate and enzyme concentrations, reaction temperature and pH. It is $Ca^{2+}$-independent and susceptible to specific $PLA_2$ inhibitors. Through a cryopreservation study of this $PLA_2$ in honey bee midgut preparations, I confirmed that the honey bee digestive $PLA_2$ preparations are physically stable, with high activity after storage at -80°C for at least three months. These findings open a new corridor of research into honey bee dietary lipid digestion and nutrition.

I used classical molecular tools to identify genes encoding honey bee midgut $PLA_2$s, to clone the genes from midgut tissue preparations and express one of the identified genes in competent *E. coli* cells. Although the enriched $PLA_2$ from *E. coli* cultures was not biologically active, this work is a meaningful aspect of my training and education, on which I offer my thoughts in Chapter 4.

# CHAPTER 1

# LITERATURE REVIEW

## 1.1 A brief introduction to the phospholipases

Phospholipases are a class of enzymes that catalyze the hydrolysis of various phosphate and acyl esters of cellular phospholipids (PLs; all abbreviations are defined in the List of Abbreviations). They occur in water-soluble and membrane-bound forms, most of which require association with membrane PLs for enzyme activity. Here, I present a general introduction to the phospholipases, then shift focus to my research on a specific phospholipase. This introduction draws heavily on the biomedical background, the source of almost all information on the subject.

PLs are present in virtually all organisms, including viruses, bacteria, yeast, plants and animals, where they have tremendous biological significance. These enzymes function in many aspects of physiology and pathophysiology. Some phospholipases act within cells, hydrolyzing fatty acids associated with biomembranes. In some cases, they remove polyunsaturated fatty acids (PUFAs) that had been damaged, often by reactive oxygen species. Other phospholipases hydrolyze specific PLs to release precursors of biochemical signals, such as prostaglandins (PGs) and other eicosanoids, powerful mediators of inflammation.

Phospholipases also act in extracellular spaces. They often act in various pathologies in serum and synovial fluid (Dennis, 2015). For example, some of them are released into

synovial fluids, where they act in some forms of arthritis (Pruzanski *et al*., 1985). Others are related to human diseases, such as cancers, brain disorders, cardiovascular diseases, infertility and hematologic diseases (Cocco *et al*., 2015; Nelson and Frohman, 2015; Federico *et al*., 2016).

Phospholipases are defined by their site of hydrolysis. For example, phospholipases A (PLAs) hydrolyze a fatty acid from the hydrophobic triacylglycerol backbone. PLAs are divided into $PLA_1$s and $PLA_2$s based on their preference for the *sn-1* or *sn-2* position of the phospholipids. Phospholipases B (PLBs) cleave acyl chains from both the *sn*-1 and *sn*-2 positions, thus it is an enzyme with both $PLA_1$ and $PLA_2$ activities. Phospholipases C (PLCs) and phospholipases D (PLDs) cleave on either side of the polar head group of the amphipathic PLs, releasing either the phospho-head group [i.e., choline phosphate from phosphatidylcholine (PC)] or just the head group (i.e., choline from PC). Common phospholipase substrates include PC, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), classified by their head group. Fig. 1 shows the specific hydrolysis site of each phospholipase on PC.

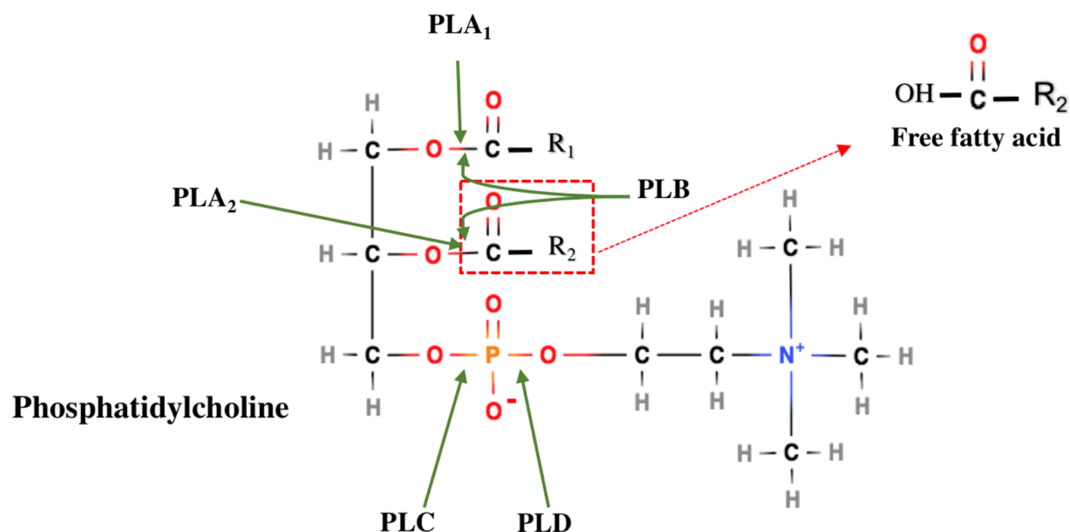


**Figure 1. PLA$_1$, PLA$_2$, PLC and PLD cleavage positions for PC.**

As seen in Figure 1, there are several groups of phospholipases, including PLA$_1$, PLA$_2$, PLB, PLC and PLD.. In this thesis, I focus on reviewing one group of them, the PLA$_2$s.

## 1.2 Discovery and significance of the expanding PLA$_2$ superfamily

### 1.2.1 Classification of PLA$_2$s

The most well-studied phospholipase group is the superfamily made of the many PLA$_2$s. Dennis *et al*. (2011) assorted PLA$_2$s into 16 groups. Most groups have several subgroups, representing more than 30 proteins with known amino acid sequences. In the early years, PLA$_2$s were named in chronological order, as they were discovered. This arrangement was less useful than the newer classification based on the enzyme sequences, structural commonalities and/or functioning properties (Dennis *et al*., 2011; Guijas *et al*., 2014). The 16 PLA$_2$ groups fall into six main types: secreted PLA$_2$ (sPLA$_2$), cytosolic PLA$_2$

($cPLA_2$), $Ca^{2+}$-independent $PLA_2$ ($iPLA_2$), platelet-activating factor acetylhydrolase (PAF-AH), lysosomal $PLA_2$ ($LPLA_2$), and adipose-specific $PLA_2$ (AdPLA). Here, I briefly review some of the most well-studied $PLA_2$s with details of their discovery, structural properties, regulatory mechanisms and the biological functions.

### 1.2.2 Secreted $PLA_2$s

$sPLA_2$s are the earliest identified and most studied $PLA_2$s. There are 17 forms of $sPLA_2$ identified in mammals, insects, plants, reptiles and bacteria. A typical $sPLA_2$ (group I, II, V and X; Murakami *et al*., 2015) usually has a conserved $Ca^{2+}$ binding loop and a His/Asp catalytic dyad. The lytic actions of snake venom were recognized in the 19th century, yet the $sPLA_2$s causing this toxicity were not identified until the 1970s when protein sequencing methodology such as Edman degradation (Edman, 1950) was developed (Heinrikson *et al*., 1977; Dennis *et al*., 2011). Venom $sPLA_2$s were identified in cobras and rattlesnakes, recognized as Group I and Group II $sPLA_2$, respectively (Heinrikson *et al*., 1977). They share six disulfide bonds. More catalytically active venom $sPLA_2$s were identified, with more than 200 amino acid sequences known from snakes (Koh *et al*.; 2006), scorpions (Valdez-Cruz *et al*., 2007), jellyfish (Nevalainen *et al*., 2004), bees and wasps (Habermann,1972; King and Spangfort, 2000). They are somewhat identical with their $Ca^{2+}$ binding loops and active sites. A unique venom $PLA_2$ was identified in a marine snail, displaying no structural homology with other venom $PLA_2$s (McIntosh *et al*., 1995).

The first reported mammalian $sPLA_2$ was the porcine pancreatic enzyme belonging to group IB (Seilhamer *et al*., 1986). It was initially recognized as a digestive enzyme because of its very high expression level in pancreas and role in dietary lipid digestion, yet later,

additional functions were recognized as its low but significant expression was detected in other organs such as lung, kidney and ovary (Valentin and Lambeau, 2000). Inhibition of group IB $sPLA_2$ through genetic disruption (Hollie and Hui, 2011) or dietary $sPLA_2$ inhibitors (Hui *et al*., 2009) confers protection against metabolic diseases, such as diet-induced obesity.

In the late 1980s, the second mammalian $sPLA_2$ was detected as group IIA $sPLA_2$, also known as the inflammatory $sPLA_2$ (Seilhamer *et al*., 1989). As its name indicates, this $sPLA_2$ is often highly expressed under pathological conditions, such as, synovial fluids and tears of patients with severe inflammatory diseases (Valentin and Lambeau, 2000). The most important physiological role of group IIA $sPLA_2$ is the degradation of gram-negative and -positive bacterial membranes. The group IIA $sPLA_2$ has a higher affinity for PE and PG susbstrates, the two major PLs in bacterial membranes, compared to PC, which is rich in mammalian cell membranes. The cationic nature of group IIA $sPLA_2$ is required for bacterial membrane hydrolysis. These specificities make this type of $sPLA_2$ a potent antimicrobial agent implicated in host defense and, when over-expressed in host immunity, leads to inflammation (Harwig *et al*., 1995; Weinrauch *et al*., 1998; Koduri *et al*.,2002; Murakami *et al*., 2015). Other $sPLA_2$s involved in inflammation include groups IID, V and X $sPLA_2$s, each playing tissue-specific roles. These are sometimes controversial because they target different PLs and produce molecules for various signaling pathways (Murakami *et al*., 2015).

Most $sPLA_2$s are low molecular weight enzymes, within 14-18 kDa except the mammalian group III $sPLA_2$, at around 55 kDa. Although it has a central group III $sPLA_2$ domain and is similar to the bee venom $sPLA_2$, its unique N- and C-terminal domains are

larger than other known $sPLA_2$s (Valentin and Lambeau, 2000). It promotes the maturation of human mast cells, which act in anaphylaxis, and of sperm cells, a crucial step for fertilization (Murakami *et al*., 2015).

Ten of the 17 known $sPLA_2$ forms were identified in mammals. All ten forms were found in the mouse genome and nine found in humans (Dennis *et al*., 2011). Studies on the biological functions of $sPLA_2$s are mostly done on transgenic mice. Some, but not all, of them could be translated to human physiology. Nonetheless, understanding the regulation, mechanisms and functions of human $sPLA_2$s can aid in the development of therapeutics for many diseases.

### 1.2.3 Other $PLA_2$s

$cPLA_2$s (also known as group IV $PLA_2$s) are larger enzymes (60-114 kDa) widely expressed in mammalian cells (Dennis *et al*., 2011). To date, there are six $cPLA_2$ isoforms sharing 30% structural homology. Although the differences of regulation and function among groups are manifest, little is known about their activation mechanisms and biological functions except the well-studied $cPLA_2\alpha$. In response to cellular stimulation, it is activated by intracellular calcium, which facilitates translocation to cell membranes, where they function. Besides the dependency on calcium, $cPLA_2\alpha$s have a strong preference for PL substrate with arachidonic acid (AA) in the *sn-2* position. The hydrolyzed AA is available for conversion into lipid mediators such as leukotrienes and PGs (Leslie, 2015).

$cPLA_2\alpha$ enzyme activity was first reported in human platelets (Kramer *et al*., 1986) and neutrophils (Alonso *et al*., 1986). Over the ensuing 30 years, it was found in cells of

all human and mouse tissues. Homologues in other vertebrates, including chicken, zebrafish and *Xenopus* were also reported (Ghosh *et al*., 2006). With its wide expression, $cPLA_2\alpha$ plays very complex and sometimes controversial roles in disease processes. Gene-inactivated mice (knockout mice, generated by Bonventre *et al*., 1997) and indole $PLA_2$ inhibitors (McKew *et al*., 2008) were used in evaluating the biological significance of the enzyme in various disease states, such as high-fat diets (Ishihara *et al*., 2012), thromboembolism (Wong *et al*., 2002), neurodegenerative diseases (Sanchez-Mejia *et al*., 2008; Desbène, 2012), auto-immune diseases (Hegen *et al*., 2003) and non-obese diabetes (Oikawa *et al*., 2005).

Unlike the $sPLA_2$s and $cPLA_2$s, members in the $iPLA_2$ family are independent of calcium with respect to translocation to membranes or catalysis. The earliest records on $iPLA_2$ come from Wolf *et al*. (1985), however, the identification and characterization of this new $PLA_2$ family was not reported until the mid 1990s, from mammalian macrophages (Ackermann *et al*., 1994), insulinoma cells (Ramanadham *et al*., 1994), renal proximal tubules (Portilla *et al*., 1994) and ovary cells (Tang *et al*., 1997). The seven recognized $iPLA_2$s are $iPLA_2\beta$ which is divided into two subgroups (a and b), $iPLA_2\gamma$, $iPLA_2$ $\delta$, $iPLA_2$ $\varepsilon$, $iPLA_2$ $\zeta$ and $iPLA_2$ $\eta$ (Ramanadham *et al*., 2015). Structurally, they are partially homologous to $cPLA_2$, but do not have the substrate preference for AA in the *sn-2* position. The $iPLA_2$ isoforms share a patatin-like lipase domain, yet they show diverse catalytic activities in addition to $PLA_2$ activity (i.e., $PLA_1$, lysophospholipase, transacylase and thioesterase) and thus produce multiple metabolites exhibiting different biological functions (Ramanadham *et al*., 2015). For instance, the most well-described isoform, $iPLA_2\beta$, is ubiquitous in human cells and essential for membrane homeostasis and

remodeling (Balsinde *et al*., 1997), cell proliferation (Balboa *et al*., 2003), bone formation (Ramanadham *et al*., 2008) and sperm development (Bao *et al*., 2004). The expression level differs among organs and its overexpression or underexpression can induce multiple diseases, such as cancer and various neurological disorders, such as Parkinson's and Alzheimer's diseases (Ramanadham *et al*., 2015).

PAF-AH, also known as lipoprotein-associated $PLA_2$ (Lp-$PLA_2$), was first isolated and cloned from human plasma (Prescott *et al*., 1990; Tjoelker *et al*., 1995). PAF-AHs have a substrate preference for a short acyl chain in the *sn-2* position. They have anti-inflammatory properties because they hydrolyze the pro-inflammatory PAF into an inactive form (Tjoelker *et al*., 1995; Stafforini *et al*., 2014). In this role PAF-AH acts in resolution of inflammation. However, PAF-AH may also play a controversial role because it generates pro-inflammatory and pro-apoptotic lipid mediators such as lyso-PC (Dennis *et al*., 2011). Dysregulations of PAF-AH activities can cause various diseased conditions. For example, catalytically inert PAF-AH is a key factor of inflammatory disorders in Japanese populations (Stafforini *et al*., 1996). Mice models with genetically deficient plasma PAF-AH were protected from early mortality in reaction to bacteria, but developed much more susceptibility to necrotizing enterocolitis after 24 h (Lu *et al*., 2010). Recently, Huang *et al*. (2018) found that PAF-AH concentrations from sepsis-infected patients correlated with the severity of disease, and thus suggested its role as a potential prognostic biomarker.

The $LPLA_2$, initially purified from bovine brain (Abe and Shayman, 1998), has been cloned in cow, mouse, rat and human (Dennis *et al*., 2011). It exhibits 1-*O*-acylcer-amide synthase (ACS), $PLA_2$ and $PLA_1$ activities (Shayman *et al*., 2011). One important biological

role of $LPLA_2$ is the degradation of lysosomal phospholipids. Inhibition of $LPLA_2$ with specific inhibitors showed it acts in cellular phospholipidosis (Halliwell, 1997). $LPLA_2$ deficiency can impair PC and PE degradation, which is linked to the phospholipidosis induced by cationic amphiphilic drugs (Abe *et al*., 2007). Also, $LPLA_2$ has high expression in alveolar macrophages (Abe *et al*., 2004). PC and PE accumulation in alveolar macrophages of $LPLA_2$ knockout mice could cause a surfactant catabolic disorder, which is similar to conditions of human pulmonary alveolar proteinosis (Shayman *et al*., 2011).

Another $PLA_2$ group, AdPLA, was identified in the 1990s as a tumor suppressor (Hajnal *et al*., 1994; Sers *et al*., 1997). It is expressed in adipose tissue and is involved in obesity as shown by AdPLA knockout mice studies (Jaworski *et al*., 2009).

A subset of these phospholipases have been reported in insects, including $cPLA_2$, $sPLA_2$ and $iPLA_2$. We turn, now, to these insect enzymes.

## 1.3 Insect $PLA_2$s

### 1.3.1 Discovery, functions and applications of the insect venom $PLA_2$s

Compared to the numerous studies on mammalian $PLA_2$s, studies on insect $PLA_2$s have only scratched the surface of their mostly unknown biological roles and mechanisms. The only exceptions are the well-known venom $PLA_2$s.

Insect venoms are secreted from their venom glands. Their biological significance includes predation [i.e., the Heteroptera species *Kirkaldyia deyrolli* (Walker *et al*., 2016)], parasitism [e.g., the endoparasitoid wasp *Pteromalus puparum* (Yan *et al*., 2016)] and/or defense [e.g., the *Apis sp*. and *Pogonomyrmex sp*. (Shorter and Rueppell, 2012)]. In some

hymenopteran species such as the genus *Apis*, the spread of venoms onto the cuticle and the nest surface indicated that beyond the classical stereotype of preying or deter predators, venoms can provide external, or social immunity, against pathogens and parasites (Baracchi *et al*., 2011; Baracchi and Tragust, 2017).

Although insect venoms have been known as toxins that sting or even kill humans since prehistoric times, the amino acid sequences of venom proteins were not determined until relatively recently. Insect venom is a complex mixture mostly of water, but also biogenic amines, polypeptides, enzymes, phospholipids, amino acids, sugars, pheromones and minerals, mixed in various cocktails among species (Habermann, 1972; Oršolić, 2012). In *A. mellifera* venom, the small peptide melittin, constituting 40-50% of the venom dry weight, is the main toxin. Depending on its dose, it can directly damage mammalian cells and organs, such as killing erythrocytes and leukocytes. It often creates more extensive damage, shortening skeletal and heart musculature (Habermann, 1972). Also, it activates $PLA_2$, another major component of honey bee venom, at 10-12% of dry weight.

$PLA_2$ is a major allergen of bee venom. After injection into mammals and activation in the presence of melittin, $PLA_2$ induces immune responses, for example, activating T helper type 2 cells and immunoglobulin E responses. The venom $PLA_2$ also leads to pain and neurotoxicity (Lee and Bae, 2016). Despite its expected adverse effects, the venom $PLA_2$ may also have considerable significance in biomedicine, including cytotoxicity of various tumor cells (Oršolić, 2012), anti-inflammatory effects against some autoimmune diseases (rheumatoid arthritis, multiple sclerosis, etc. (Spence *et al*., 2015)) and slowing neurodamage, such as in a mice model of Parkinson's disease (Chung *et al*., 2015). Most studies related to this topic used mice or mammalian cell lines in their experimental

systems, except Zieler *et al*. (2001), who found that the rattlesnake and honeybee venom $PLA_2$s inhibited ookinete association and thus prevented malaria parasite development and transmission in *Aedes aegypti* (Zieler *et al*., 2001; Lee and Bae, 2016).

Insect venom $PLA_2$s have substantial potential in medical research and related applications. Nonetheless, new knowledge on the physiological, rather than toxicological, significance of insect $PLA_2$s is generating new insights into insect biology, physiology and molecular biology. I report on this new information in the next few sections.

### 1.3.2 $PLA_2$ and insect innate immunity

Having survived in the world for over 400 million years (Grimaldi and Michael, 2005), insects have evolved potent innate immune systems that include physical barriers (i.e. the cuticles, the peritrophic membranes and the midgut epithelia) to protect themselves from invaders. For convenience, innate immunity has been divided into cellular and humoral responses. The distinction between them is becoming rather foggy as new knowledge emerges. For example, hemocytes are the drivers of cellular immunity, but they also produce and secrete the antimicrobial peptides that make up humoral immunity. Similarly, many insects generate behavioral fevers by assuming warming basking postures or moving to warmer sites within their locations. These behaviors are neither cellular nor humoral responses to infection. Insects and other invertebrates do not have the antibody-based adaptive immunity of vertebrates, which conveys a very specific memory of previous infections, which leads to heightened reactions to a subsequent infection. Yet insects also have an immune memory, known as 'priming'(Sadd *et al*., 2005; Tidbury *et al*., 2011).

Here, I stress the point that insect immunity is a sophisticated system that partly accounts for the 400 million-year success of insects.

Humoral immunity relates to the biosynthesis of massive amounts of antimicrobial peptides (AMPs) which are mainly produced by the fat body and hemocytes. Some tissues, such a the cuticle epithelium and Malpighian tubules also produce and secrete AMPs. Signal mechanism studies on *Drosophila melanogaster* revealed that two pathways, the Toll and Immune Deficiency pathways, are responsible for producing AMPs (Hultmark, 2003).

Cellular immunity clears invading microorganisms by several mechanisms. Phagocytosis refers to the internalization and killing of bacteria by hemocytes. Hemocytes adhere to infecting bacterial cells, then clump into microaggregates of about 10-15 hemocytes. The microaggregates clump together, which leads to large nodules that can involve millions of individual hemocytes (Stanley and Miller, 2006; Lemaitre *et al*., 2007). Encapsulation is the cellular response to invaders larger than microbes, such as parasitoid eggs. Hemocytes form continuous layers around the invader, which ends in melanization. The cellular immune responses are mediated by a group of lipid mediators, PGs and some other eicosanoids. They are produced from three C20 PUFAs, 20:3n-6, 20:n-6 and 20:n-3 PUFAs that are associated with cellular membrane PLs. Most of these mediators are produced from 20:4n-6, known as AA. Infections lead to activation of $cPLA_2$, which translocates to the cell membrane and releases AA, which is subsequently converted into PGs and other mediators. Hence, $PLA_2$s also directly act in insect immunity and health.

I reviewed the action of $PLA_2$s in insect immunity to form a distinction between the $sPLA_2$s acting in digestion, including the honey bee digestive $PLA_2$, and the intracellular

$PLA_2$s responsible for hydrolyzing AA from cellular PLs. Park *et al*. (2015) described a novel $iPLA_2$ that acts in *Spodoptera exigua* immunity and development. Phylogenetic analysis placed this enzyme with vertebrate $iPLA_2$s. The primary structure of this $iPLA_2$ lacks a calcium binding loop and bacterial challenge led to increased accumulation of mRNA encoding the enzyme. Sadekuzzaman *et al*. (2017) found a second $iPLA_2$ with a considerably different primary structure. This new intracellular enzyme acts in immunity and development and I foresee discovery of similar intracellular $PLA_2$s in insects.

$PLA_2$ activities were characterized by biochemical analyses in fat body cells and hemocytes, the two main tissues related to insect immunity (Uscian and Stanley-Samuelson, 1993; Schleusener and Stanley- Samuelson, 1996). Inhibiting $PLA_2$ negatively affected the fat body metabolism (Orville *et al*., 2016). Similarly, inhibiting $PLA_2$ or silencing genes encoding $PLA_2$ resulted in suppression of immune responses in a range of insect species, including *Manduca sexta* (Park. *et al*., 2004), *Tribolium castaneum* (Shrestha *et al*., 2010), *S. exigua* (Park *et al*., 2015; Sadekuzzaman *et al*., 2017), and *Bactrocera dorsalis* (Hendel) (Li. *et al*., 2017).

In my view, as seen in mammals, insects express a range of secretory and intracellular $PLA_2$s that await discovery.

### 1.3.3 $PLA_2$ and insect lipid digestion

Insects, require dietary lipids, including sterols and PUFAs, to meet their nutritional needs. Humans consume substantial amounts of lipids, of which 90-95% are triacylglycerols (TAGs), with smaller amounts of sterols and PLs (Sizer *et al.*, 2012).

Similarly, insects consume high amounts of TAGs (Klowden, 2013), along with some essential components, sterols and some PUFAs.

Insects digest dietary lipids in the midgut with digestive enzymes, lipases for neutral lipids, such as TAGs and $PLA_2$s for PLs. Stanley (2006) reviewed the two $PLA_2$ roles in lipid digestion. The first action is the direct hydrolysis of dietary PLs into free fatty acids for absorption into midgut cells to meet dietary PUFA requirements. The $PLA_2$ action also generates 2-acyl lysophospholipids. Insects lack the bile salts of vertebrates and their dietary TAGs are emulsified by the lysophospholipids, a necessary step for digestion by lipases.

An enyme in midgut contents of the tiger beetle, *Cicindella circumpicta* was the first reported insect digestive $PLA_2$ (Uscian *et al.*, 1995). The presence of at least one $Ca^{2+}$-dependent $sPLA_2$ was recorded using radioactive PC. Later, similar work was done with the burying beetle *Nicrophorus marginatus* (Rana *et al.*, 1997), the tobacco hornworm, *Manduca sexta* (Rana *et al.*, 1998; Tunaz and Stanley, 2004), the mosquito, *Aedes aegypti* (Nor Aliza and Stanley, 1998) and the screwworm, *Cochliomyia hominivorax* (Nor Aliza *et al.*, 1999). Among carrion feeders, such as *N. marginatus*, the $PLA_2$ activity in salivary secretions was much higher than the midgut contents, which the authors took to indicate the other roles of $PLA_2$ such as microcidal effects to protect their carrion food (Rana *et al.*, 1997; Stanley, 2006). No digestive $PLA_2$ was reported for the Hymenoptera.

### 1.3.4 Other $PLA_2$ actions in insect biology

Aside from defense (venom), innate immunity and digestion, $PLA_2$s act in other biological roles. In insect reproduction, a $PLA_2$ was characterized (Ragab *et al.*, 1992) and

shown to induce egg-laying behavior in *Thermobia domestica* (Bitsch *et al.*, 1995). PGs induce egg-laying behavior in newly-mated females of most cricket species (Stanley, 2006; Stanley and Kim, 2014). $PLA_2$ release of AA from cellular PLs is the first step in PG biosynthesis, from which we infer $PLA_2$ is a key player in PG-mediated reproductive physiology. PGs also mediate follicle development in silk worms (Machado *et al.,* 2007) and in *D. melanogaster* (Tootle and Spradling, 2008;), as well as coordinate expression of genes encoding egg-shell proteins in *D. melanogaster* (Tootle *et al.,* 2011), from which we propose that $PLA_2$ is an essential enzyme in several specific steps in insect reproduction.

Uscian and Stanley-Samuelson (1993) reported on a fat body $iPLA_2$ in the tobacco hornworm *Manduca sexta* (Uscian and Stanley-Samuelson, 1993). This enzyme may act in fat body physiology, but the point has not been investigated. Steele and his colleagues found that injections of the synthetic hypertrehalosemic hormones (HTH- Ⅰ and Ⅱ) led to increased $PLA_2$ activity in the fat body of cockroach, *Periplaneta americana*This $PLA_2$ requires $Ca^{2+}$ to function (Sun and Steele, 2002). The HTH treatments also led to decreased fat body PLs and increased free fatty acids (Oguri and Steele, 2003). Our understanding of the biological significance of PGs and other eicosanoids is also expanding, particularly in insect immunity (Kim *et al.,* 2017). Given that $PLA_2$ is responsible for hydrolyzing these PUFAs from cellular membrane phospholipids, it is reasonable to believe that $PLA_2$s act in various biological, physiological and ecological roles in insects.

### 1.3.5 Genes encoding insect $PLA_2$s

Group III venom $sPLA_2$ has been cloned and sequenced from venom glands of some bees, such as different subspecies of *A. mellifera* (Shen. *et al*., 2002; Hou *et al*., 2014; Lin,

2014) and *Bombus ignitus* (Xin. *et al*., 2009). They are usually low-molecular-weight, secreted proteins (14-18 kDa) and are partially identical to a 55 kDa mammalian group III $sPLA_2$ (Valentin *et al*., 2000). However, studies on generating recombinant proteins from other Hymenopteran venom allergens have very little $PLA_2$ activity (Müller, 2002; Baek and Lee, 2010).

Some non-venom $PLA_2$s have also been cloned in a variety of insect species, including *D. melanogaster* (Ryu *et al*., 2003), *T. castaneum* (Shrestha *et al*., 2010), *S. exigua* (Park *et al*., 2015; Sadekuzzaman *et al*., 2017), *Bombyx mori* (Orville *et al*., 2016) and *B. dorsalis*, (Li *et al*., 2017). All three types of $PLA_2$, $sPLA_2$, $cPLA_2$ and $iPLA_2$ are present in *S. exigua* and at least one of them can be suppressed by *Xenorhabdus hominickii*, as a strategy to suppress host immunity by this entomopathogenic bacterium (Sadekuzzaman and Kim, 2017).

Comparing to the very large background on human pancreatic $PLA_2$, an enzyme produced in the pancreas that acts in the stomach (Seilhamer *et al*., 1986), very little information has appeared on its insect counterparts. Research into the non-venom insect $PLA_2$s is significant for a couple of reasons. It helps us generate a better understanding of insect physiology, and provide us information on potential gene targets in pest control technologies. For insect species that render valuable ecological services such as honey bees, studying digestive $PLA_2$s can help understand the digestive physiology necessary to meet nutritional needs, which I discuss in more detail in the following sections.

## 1.4. Honey bee lipid nutrition and health

### 1.4.1 Honey bees: economic impacts and colony threats

Honey bees (*A. mellifera*) are economically crucial because they perform valuable economic services as agricultural pollinators and as sources of valuable products, including honey, beeswax, royal jelly, pollen and propolis, which are widely used by the food, medicine and cosmetics industries (Jacobs *et al.*, 2006). As pollinators, they pollinate many wild bushes, small trees and herbaceous plants, as well as crop plants. For approximately 352,000 species of flowering plants around the world (Paton *et al.*, 2008), more than 87% of them rely on animals (bees, pollinating wasps, ants, flies, etc.) as their pollen vectors (Ollerton *et al.*, 2011). In the United States, honey bees increase crop productivity worth an estimated $15 billion each year by pollinating more than 2 million acres of crops (Morse and Calderone, 2000). In Missouri, more than 400 bee species act in pollinating a wide range of crops (Foré, 2003). Honey production has been a major economic venture in the U.S., with an annual honey production of more than 150 million pounds per year over the past 20 years (USDA-ERS, 2016). The average price of honey has increased four-fold over the last 22 years (USDA-ERS, 2017).

The well-known honey bee colony collapse disorder (CCD) has led to a rapid decline of colony numbers in recent years. The number of managed honey bee colonies in the U.S. decreased from about 5 million in the 1940s to an estimation of 2.7 million today (Seitz *et al.*, 2016). Consequently, honey productivity has declined by about a third over the last 20 years ago, which led to considerable economic losses (USDA-ERS, 2016). Annual surveys have been conducted since this problem was taken into consideration. Although the total number of lost colonies (including summer and winter losses) has fluctuated over years,

even the best years saw 30 percent colony losses (Vanengelsdorp *et al.*, 2007, 2015a, 2015b; Spleen *et al.*, 2013; Steinhauer *et al.*, 2014; Lee *et al.*, 2015; Seitz *et al.*, 2016). CCD is probably due to a syndrome of serious problems rather than a single clear cause. Most common elements are exposure to chemical insecticides, extreme winter, poor nutrition, queen failure and pests (e.g., *Varroa destructor* and *Deformed wing virus*) (Seitz *et al.*, 2016). The mechanisms of how these elements influence colony health and stability are still unclear. My work on a honey bee $PLA_2$ relates to nutrition and digestion, to which we now turn attention.

### 1.4.2 Honey bee nutrition and digestion

Dadd (1985) laid out the broad nutritional requirement for insects, which are fairly similar to mammalian requirements, including macro- and micronutrients. Insects, including honey bees, require large quantities of carbohydrates, proteins and lipids that support energy metabolism and aspects of body structure. Most insects and mammals require the same essential nutrients (compounds that are needed but cannot be made within the animal), such as the rat-ten essential amino acids, the C18 PUFAs, the water-soluble and most lipoidal vitamins. Micronutrients include various minerals such as zinc, iron and copper, which are necessary in some enzymes, and vitamins, including all the B-vitamins known in vertebrate and invertebrate nutrtion and some fat soluble vitamins. Insects do not require vitamin D, which serves in bone metabolism (Dadd, 1985). Detailed reports indicate that insect diets lacking macronutrients and essential micronutrients suffer reduced performance, slowed development and, often, lethal deformations. Due to their colonial life styles, details of honey bee nutrition have not been investigated in the same detail compared to solitary insects, such as locusts (Dadd, 1961), aphids (Dadd and Mittler,

1965), wax moths (Dadd, 1964) and some mosquito species (Dadd and Kleinjan, 1979). From above, we can draw general conclusions about nutritional requirements of honey bees. Overall, honey bees have similar nutritional needs recorded in insects, and animals, generally.

Here I turn to the literature on honey bee nutrition. The availability and qualities of nutrients can impact honey bee juvenile and adult weight, longevity, internal tissue development and in-hive performance, such as building activity, quality and quantity of reared brood (Haydak, 1970). High quality nutrition enhances cellular and humoral immune capacity, including expression of immune-related genes (mRNA synthesis) and syntheses of AMPs (Yi *et al*., 2014; DeGrandi-Hoffman and Chen, 2015). Improved nutritional inputs led to up-regulated gut detoxification pathways (Mao *et al.,* 2013). After ingesting a commercially available honey bee diet supplemented with proteins and vitamins, honey bees infected by the microsporidium *Nosema ceranae* had significantly higher expression of some immune-related genes, compared to the controls (Glavinic *et al*., 2017). Another line of research showed that bees infected with *Varroa* mites face harsher nutritional challenges, demonstrated by reduced body proteins and elevated free amino acids which indicated depressed protein utilization (Aronstein *et al.,* 2012). Generally, while honey bees have nutritional requirements similar to other animals, nutritional deficiencies lead to visible declines in performance at the individual level.

Honey bees meet their nutritional needs with nectar and pollen. They acquire carbohydrates from nectar, of which the three main sugars are glucose, fructose (a monosaccharide), and sucrose (a disaccharide) (Vaudo *et al.,* 2015). Larvae food is present as one of two jellies. Worker jelly has about 4% sugar. All larvae consume this jelly for

the first 3 or 4 days of larval life. At that point, queen-determined larvae are given royal jelly, with about 12% sugar (Wang and Li-Byarlay, 2015). Adult workers use carbohydrates as their main energy source, averaging about 4 mg utilizable sugars per day (Barker and Lehner, 1974; Brodschneider and Crailsheim, 2010).

Proteins, lipids and micronutrients (i.e. vitamins and minerals) are taken from pollen (Vaudo *et al.,* 2015). Dry pollen pellets contain 2.5% to 61% protein, depending on plant species (Roulston *et al*., 2000). Proteins make up a large amount of adult worker dry weight at about 66-74%, (Hrassnigg *et al*., 2005; Brodschneider and Crailsheim, 2010). At the individual level, protein intake influences fecundity, longevity, metabolic activity, body size and development of wings and internal organs such as hypopharyngeal glands. At the colony level, although bees are able to tolerate some protein deficiency, the brood production can be strongly reduced or even terminated with further protein depletion (Schmickl and Crailsheim, 2002; Brodschneider and Crailsheim, 2010).

Pollen can contain some toxic sugars, such as mannose, xylose, and arabinose (Zheng *et al.,* 2016; Ricigliano *et al.,* 2017). These sugars are metabolized into harmless forms by glucoside hydrolases that are secreted by head glands in the bees and also by the gut microbial community, from which I infer the gut microbiota contributes to honey bee nutritional inputs.

For honey bee foragers, digestion starts before they return to the hive. A specialized proventriculus called the ‘honey stopper’ helps capture pollen grains in the four ‘fingers’ of intermeshing spines. Most nectar remains in the crop for processing into honey and honey production after returning to the hive (Nation, 2002). Most digestion happens in the

midgut using digestive enzymes including invertase and glucose oxidase which break down sugars, and enzymes such as trypsin, chymotrypsin, and elastase, which hydrolyze proteins. (Giebel *et al*., 1971; Wang and Li-Byarlay, 2015). The resulting simple sugars, small peptides and amino acids are absorbed from the midgut lumen into cells for transport into hemolymph for circulation throughout the body.

Lipids, including sterols, fatty acids, TAGs and PLs are present in pollen components, the exine, coat, intracellular oil bodies and membranes (Piffanelli *et al*., 1998; Zhang *et al*., 2016). They constitute 0.8-18.9% of the pollen pellet dry weight depending on plant species (Roulston *et al*., 2000). In general, insect lipids are composed of two major fractions, neutral lipids, mostly TAGs, and PLs. TAGs are stored in the fat body, where they can be mobilized to produce energy. Hymenoptrans power their flights with sugar and use their lipid stores, mostly TAGs for other energy-requiring activities such as ovarian development. PLs are generally structural components of biomembranes. Lipid intake and metabolism take place in the brood, or larval, stages (Brodschneider and Crailsheim, 2010). Adults also have lipids, albeit, in relatively low quantities. Peng *et al*. (1985) indicated that adult bees can digest lipids by removing the lipid-rich pollenkitt layer from the pollen exine. Dietary neutral lipids are digested by lipases, which release free fatty acids for absorption into midgut cells. PLs are digested by $PLA_2$s, which release fatty acids from the *sn*-2 position of PLs, leaving a free fatty acid and a lysophospholipid. A decrease of free fatty acid in honey bee midgut was observed between the transition between nurses to foragers (Toth and Robinson, 2005; Wang and Li-Byarlay, 2015). Workers can convert dietary phytosterols to utilizable forms of sterol, e.g., 24-methylene cholesterol, to support rearing broods (Herbert *et al*., 1980; Svoboda *et al*., 1982; Brodschneider and Crailsheim,

2010). General lipase, but not phospholipase, activity was recorded in worker bee midguts (Ma *et al*., 2015).

Some beekeepers use commercially available supplementary honey bee diets to maintain healthy honey bee colonies. Adding lipids to pollen-containing diets is an effective way to attract bees and increase diet consumption (Schmidt and Hanna, 2006). At the colony level, fatty acids such as the 18:3n-3 can impact the colony size, brood amount and weight of newly emerged adults (Ma *et al*., 2015). Supplementing essential fatty acids like 18:3n-3 and 18:2n-6 to lipid-deprived pollens enhanced forager dance rate, which reflected its subjective value of food resources (Zarchin *et al*., 2017). However, the optimal mixture of dietary lipids has not been completely determined. Adverse effects can occur before signs of lipid malnutrition are visible. For example, Manning *et al*. (2007) found that worker longevity significantly decreased after feeding on pollens containing inappropriate lipid types. On the idea that honey bees require dietary C18 PUFAs, the optimal amounts remain unknown. Dadd (1985) also reported that diets with an overabundance of PUFAs were harmful to larval mosquitoes.

PLs are components of pollens. Piffanelli *et al*. (1997) first used co-chromatography to determine that they are the main components of rapeseed (*Brassica napus*) pollen intracellular membranes. Liang *et al*. (2013) determined that PLs (mainly PS and PC) are the major components of pollen coats among various plant species. These dietary PLs are likely to be digested in the honey bee midgut, with phospholipases, which provides PUFAs to the honey bees.

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# CHAPTER 2

# ENZYMATIC CHARACTERIZATION OF A HONEY BEE DIGESTIVE $PLA_2$

## 2.1. Introduction

Phospholipases catalyze the hydrolysis of various phosphate and acyl esters of cellular PLs. Among them, $PLA_2$s are the most well-studied superfamily, composed of 16 groups (Dennis *et al*., 2011). They are present in almost all organisms except for viruses. In insects, besides the most well-known and medically significant venom $PLA_2$s (Habermann, 1972; Zieler *et al*., 2001; Lee and Bae, 2016), they act in a wide range of other biological actions, including but not limited to: insect immunity (Park *et al.,* 2004, 2015; Li. *et al*., 2017; Sadekuzzaman *et al*., 2017), reproduction (Ragab *et al*., 1992; Bitsch *et al*., 1995; Stanley, 2006; Stanley and Kim, 2014), and digestion (Uscian *et al*., 1995; Rana *et al*., 1997, 1998; Nor Aliza and Stanley, 1998; Tunaz and Stanley, 2004).

Dadd (1985) laid out the broad nutritional requirement for insects, which are fairly similar to mammalian requirements, including macro- and micronutrients. On the aspect of lipid nutrition, insects consume high amounts of TAGs (Arrese *et al*., 2001), along with some essential components, sterols and some PUFAs. Dietary C18 and C20 PUFAs required by many insect species are more abundant in PLs than neutral lipids (Stanley-Samuelson *et al*., 1988). Digestive enzymes in the midgut are associated with digesting the dietary lipids. Neutral lipids are digested through lipases while $PLA_2$s are responsible for digesting the dietary PLs. Stanley (2006) reviewed $PLA_2$ roles in lipid digestion. The first

action, as mentioned above, is the direct hydrolysis of dietary PLs into free fatty acids for absorption into midgut cells to meet dietary PUFA requirements. A second $PLA_2$ action generates 2-acyl lysophospholipids. Insects lack the bile salts of vertebrates and their dietary TAGs are emulsified by the lysophospholipids, a necessary step for digestion by lipases.

The first recorded insect digestive $PLA_2$ was in the tiger beetle, *C. circumpicta*, midgut contents (Uscian *et al*., 1995). The presence of at least one $Ca^{2+}$-dependent $sPLA_2$ was recorded using radioactive PC. Later, similar work was done with the burying beetle *N. marginatus* (Rana *et al*., 1997), the tobacco hornworm, *M. sexta* (Rana *et al*., 1998; Tunaz and Stanley, 2004), the mosquito, *A. aegypti* (Nor Aliza and Stanley, 1998) and the screwworm, *C. hominivorax* (Nor Aliza *et al*., 1999). Among carrion feeders, such as *N. marginatus*, the $PLA_2$ activity in salivary secretions was much higher than the midgut contents, which the authors took to indicate the other roles of $PLA_2$, such as microcidal effects to protect their carrion food (Rana *et al*., 1997; Stanley, 2006).

In a broad view, $PLA_2$ activity is very likely acting in insect digestive physiology, generally. Here, I report on a $PLA_2$ activity in the midgut contents from the honey bee, *Apis mellifera ligustica*. This is the first digestive $PLA_2$ recorded in Hymenoptera.

## 2.2. Materioals and Methods

### 2.2.1 Insects

A colony of *A. mellifera ligustica* were maintained at the USDA-ARS Biological Control of Insects Research Laboratory in Columbia, MO. A Langstroth hive (Langstroth, 1852) was established using the installation methods of Delaplane (2007). The hive was composed of a bottom board, ten-frame deep hive bodies, 2 medium supers and an outer hive cover. Bees were fed a 1:1 sugar syrup (1:2 w/v sugar to water) in early summer (June, 2017) via plastic feeders (Delaplane, 2007). The hive was monitored once a week for presence of a queen, quantity of nectar and pollen, and density of brood pattern to maintain a healthy reproductive colony. They were given no miticides, antibiotics, or supplemental feeding for at least 4 weeks before the experiments. Adult workers were collected in the same time period (noon to 1 pm) under similar environmental conditions (temperature at 19°C±5°C on sunny days). Numbers of insects used vary among studies. Each study was repeated three times on three different experimental days to capture random biological variation.

### 2.2.2 $PLA_2$ source preparation

The adult workers were anesthetized by chilling on ice for 5 min. Dissection was conducted on a pre-chilled sterile wax plate. During dissection, the insect body was soaked in ice-chilled phosphate buffer saline (PBS: pH 6.4, containing 7.7mM $Na_2HPO_4$, 2.65 mM$NaH_2PO_4$ and 150mM NaCl) to keep the tissue cells intact. The alimentary canal was exposed by cutting the integument from the first to the sixth visible segment of the abdomen. The Malpighian tubules were removed carefully from the alimentary canal using

forceps. The midgut was excised from the rest of the alimentary canal, rinsed in 1 mL sterile PBS 3X (pH 6.4, containing 7.7mM $Na_2HPO_4$, 2.65 mM$NaH_2PO_4$ and 150mM NaCl) and transferred into chilled buffers as just described at 66.7µl/midgut. For the $PLA_2$-source determination study, the peritrophic membrane containing the midgut contents and the content-free midgut epithelium were isolated and cleaned seperately.

Midguts in each sample tube were ground on ice using pre-sterilized homogenizers (Axygen Scientific Inc., Union City, CA) and sonicated on ice (Heat-systems Ultrasonics Inc.), three times, at 35 watts for 10 s in each time, with a 10 s break in between. The 10 s interval helps prevent the sample from getting warm. The homogenates were centrifuged at 16,000 x g at 4°C, for 10 min.

The resulting supernatants were transferred to a centrifugal concentrator (500 µL, MWCO 5000 Da Vivaspin, Sigma-Aldrich Inc., St. Louis, MO) and concentrated at 16, 000 x g at 4°C for 30 min, reducing the sample volume to about 25%, which varied slightly due to the textures of the buffers. Standard $PLA_2$ assay buffer (Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 100 mM KCl, and 0.3 mM Triton X-100; 200 µl) was added to each concentrator and centrifuged at 4°C for 30 min at 16,000 x g. The flow-through was discarded and the samples washed 2X as just described. The samples were centrifuged for 30 min, concentrating the samples to approximately 50 µl. The resulting concentrated $PLA_2$ solution was used as the $PLA_2$ source.

### 2.2.3 Determination of protein concentration

Protein concentrations in the prepared $PLA_2$ solutions were determined against bovine serum albumin using the BCA Protein Assay Reagent Kit from Pierce™ (Rockford, IL).

Unknown samples and standard curves were read at 562 nm using a microtiter plate reader (2300 Multilabel Reader, PerkinElmer Inc., Waltham, MA).

### 2.2.4 $PLA_2$ activity assay

$PLA_2$ activities were measured using a commercial assay kit which has diheptanoyl thio-PC as the enzyme substrate ($sPLA_2$ Assay Kit, Cayman Chemical, Ann Arbor, MI). The assay used the Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid]; DTNB) to create a colored product, 5-thio-2-nitrobenzoic acid (λmax: 412 nm; ε: 13,600). The assay was set up according to the manufacturer's instructions and was tested using the bee venom $PLA_2$ (Cayman Chemical, Ann Arbor, MI) as a positive control. Change in absorbance at 414 nm of the reaction product was measured and plotted to obtain the slope of the linear portion of the curve. Absorbance in non-enzymatic blank control wells was calculated and subtracted from the sample wells. The actual extinction coefficient for DTNB at 414 nm was 13.6 $mM^{-1}cm^{-1}$ but this value was adjusted to 10.66 $mM^{-1}$ due to the path-length of the solution in the well (0.784 cm). The following formula was used to calculate the $PLA_2$ activity as the manufacturer's instruction indicated:

$$\text{Enzyme activity (μmol/min/ml)} = \frac{\Delta \text{ A414 per minute}}{10.66\, mM^{-1}} \times \frac{0.225\, ml}{0.01\, ml}$$

### 2.2.5 Statistical analysis

Data were expressed as means ± SD. Statistical significance was evaluated by a *t*-test, and differences were considered to be significant when $P < 0.05$. Statistical differences between treatments were determined using the Least Significant Difference (LSD) test with the Minitab 17 Statistical Software (Minitab Inc., 2016).

## 2.3 Results

Insect digestive enzymes, except for those in the saliva, are synthesized and secreted by the midgut epithelial cells. Experience with digestive $PLA_2$s in tobacco hornworm, *M. sexta* L., (Rana *et al*., 1998) and larval mosquitoes, *A. aegypti* (Nor Aliza and Stanley, 1998), indicated that most $PLA_2$ activity occurred in midgut contents, with very low activity in the midgut epithelium. I hypothesized that the honey bee $PLA_2$ is secreted into and functions in the midgut contents. To test this, I isolated whole midguts, the peritrophic membrane, containing the midgut contents and the content-free midgut epithelium. I performed the $PLA_2$ assay with three midgut protein concentrations: 25, 50 and 100 μg in a 225 μl reaction volume. As shown in Figure 2-1, midgut contents had significantly higher $PLA_2$ activity than the epithelium preparations for all three protein concentrations. The whole midgut had significantly higher $PLA_2$ activity than the separated midgut contents as well as the epithelium and I inferred that most of the midgut $PLA_2$ activity is localized in the lumen. I used the whole midgut preparations to characterize the honey bee digestive $PLA_2$, which I take to be a $sPLA_2$ on the idea that the enzyme was secreted from the midgut epithelium into the lumen.

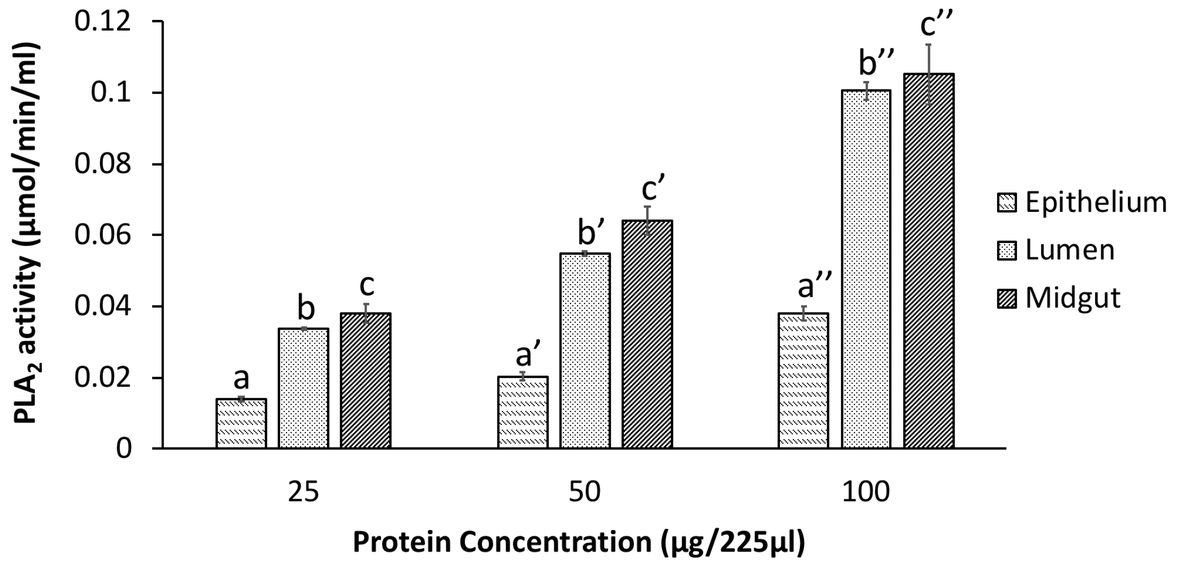


**Figure 2-1. Determination of the honey bee midgut $PLA_2$ source.** $PLA_2$ activities (average ± SE) from different sources, epithelium, lumen and midgut(epithelium + lumen) were compared at each protein concentration, indicated as a, a′ and a′′. Histogram bars labeled with different letters are significantly different from each other ($P<0.05$, LSD).

The human pancreatic $sPLA_2$, a typical mammalian $PLA_2$ associated with digestion, requires binding of $Ca^{2+}$ for catalysis (Burke and Dennis, 2009). Insect digestive $PLA_2$s, however, do not necessarily require $Ca^{2+}$. Ethylene Glycol Tetraacetic Acid (EGTA) is a selective $Ca^{2+}$ chelating agent used to reduce $Ca^{2+}$ concentrations in reaction mixtures (Harafuji *et al*., 1980). To determine whether the honey bee midgut $PLA_2$ requires $Ca^{2+}$, I prepared three reaction buffers containing the indicated concentrations of EGTA (Figure 2-2). When loading 100µg of the honey bee midgut $PLA_2$ and venom $PLA_2$ (Bee venom $PLA_2$ control, Cayman Chemical, Ann Arbor, MI), reactions performed in the presence of 10 mM and 20 mM EGTA did not significantly influence the *Am* digestive $PLA_2$ activity but significantly reduced the *Am* venom $PLA_2$ activity (Figure 2-2). I concluded that the honey bee midgut $PLA_2$ is a calcium-independent enzyme, while the honey bee venom $PLA_2$ is calcium-dependent.

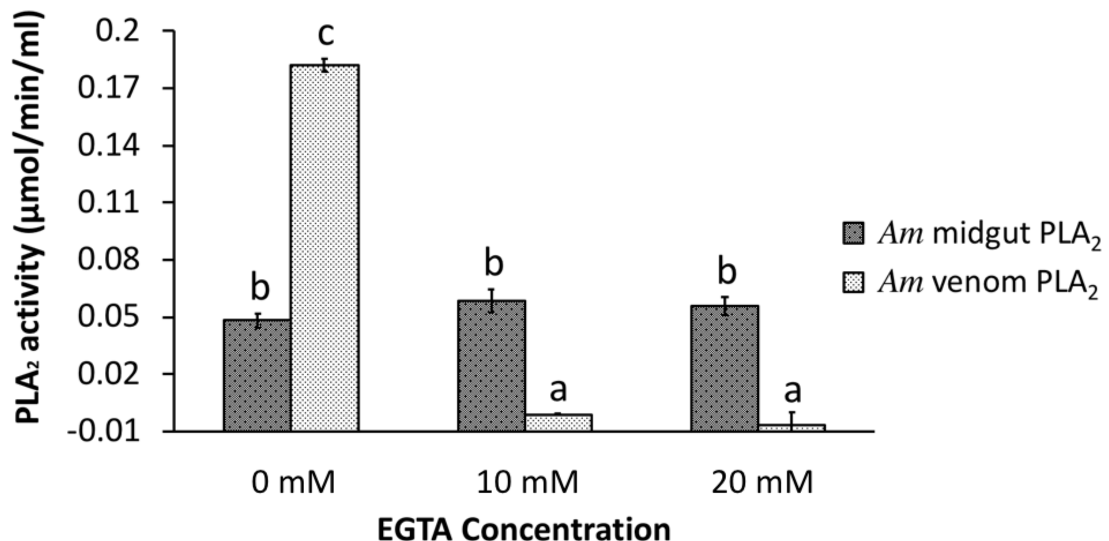


**Figure 2-2. Influence of EGTA on honey bee, *Apis mellifera*, midgut and venom $PLA_2$ activity (average ± SE).** Histogram bars labeled with the same letter are not significantly different from each other (P>0.05, LSD).

Figure 2-3 shows the absorbance of the assay product, 5-thio-2-nitrobenzoic acid, as a function of reaction time. The accumulation of product increased in a linear way for the first hour of reaction and gradually decreased afterwards, until there was almost no product increase at 102 min. The slope represents the change of $PLA_2$ hydrolysis rate determined with 100 µg/225 µl reaction volume. I used 10 min incubations to stay well within the linear reaction phase in my standard reaction condition.

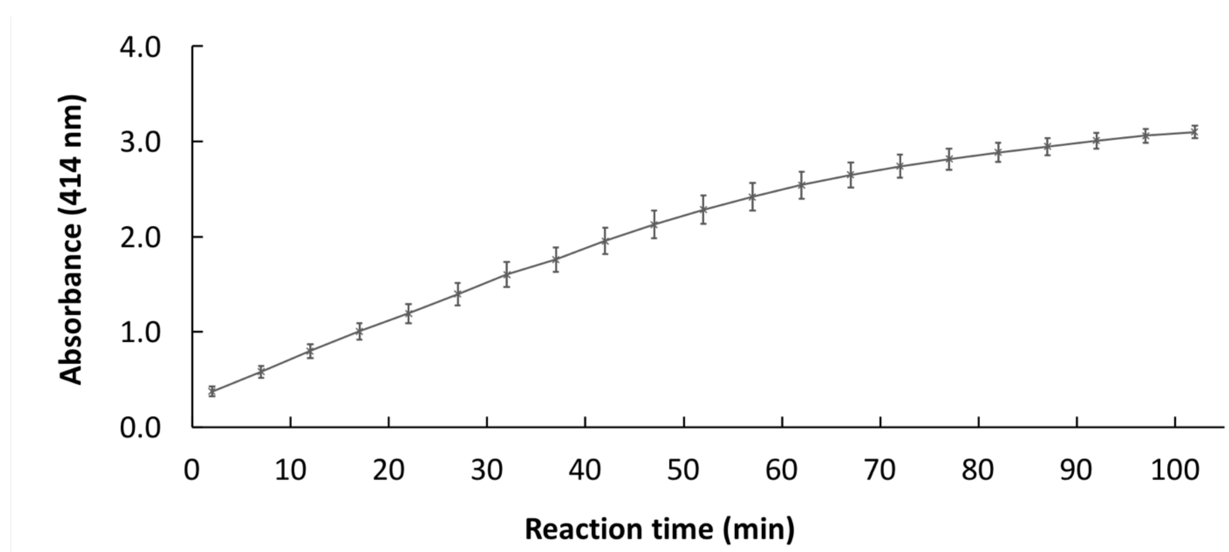


**Figure 2-3. Mean number (± SE) of A414 of the reaction product (5-thio-2-nitrobenzoic acid) as a function of reaction time.** The slope represents the change of $PLA_2$ hydrolysis rate as a function of time, determined with 100 µg/225 µl reaction volume.

The influence of protein concentrations on the $PLA_2$ activity in worker midgut preparations is shown in Figure 2-4. $PLA_2$ activity increased linearly over the protein concentrations ranging from 25-325 µg/225 µl reaction volume. Activity was significantly lower in the presence of 400 µg than 325 µg protein per reaction. I set 100 µg protein/reaction as my routine $PLA_2$ assay condition.

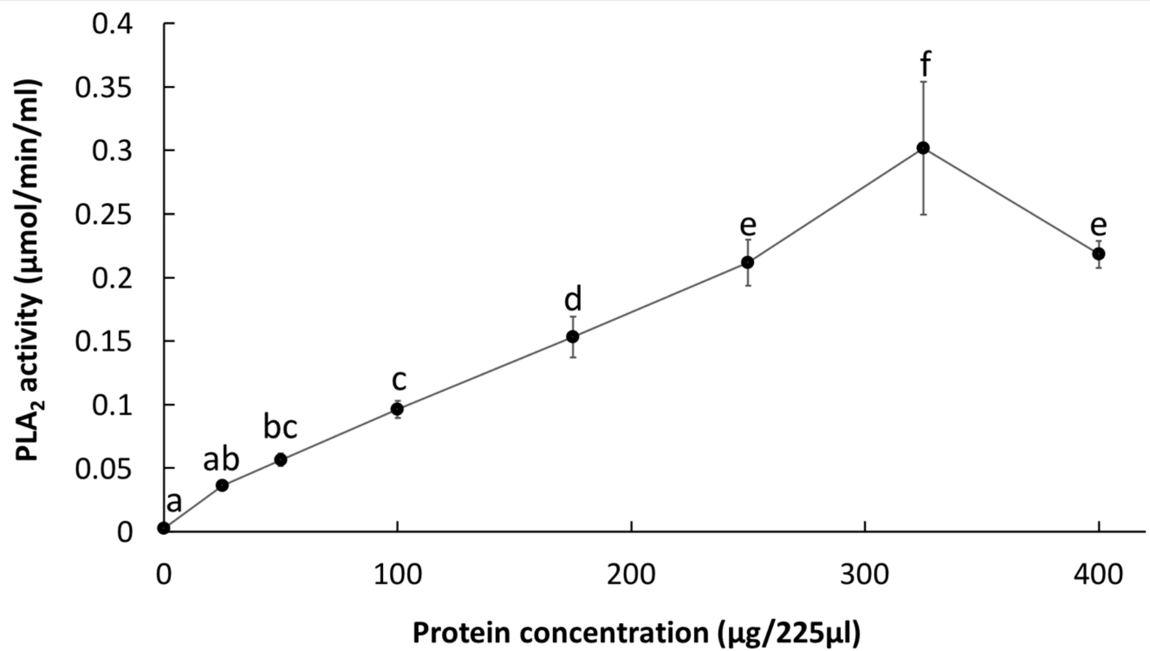


**Figure 2-4. Influence of protein concentrations on *Apis mellifera* midgut $PLA_2$ activity (average ± SE).** Data points labeled with the same letter are not significantly different from each other (P>0.05, LSD).

Two buffers with an overlap in their useful pH ranges were used to determine the optimum pH conditions for the midgut $PLA_2$ to discount the effects of their chemical constituents or ionic strength on $PLA_2$ activity (Figure 2-5). The two buffers are: Citrate-Phosphate Buffer (useful pH range ranges from 2.6 to 7.6 and I used pH 4.0, 5.0, 6.0 and 7.0, buffer strength is 100mM-200mM made with mixture of 100mM citric acid and 200mM $Na_2HPO4$ stock solutions) and Bis Tris Propane (useful pH range ranges from 6.3 to 9.5 and I used pH 7.0, 8.0 and 9.0; working concentration ranges from 10mM to 50mM and I used 25mM buffer strength). Honey bee adult workers have weakly acidic to neutral midgut contents (Zheng *et al*., 2017). To span the bee gut pH, I tested buffers with pH ranges from 4.0 - 9.0. The enzyme preparations were sensitive to the pH of the reactions. $PLA_2$ activity was significantly lower in acidic, than neutral and weakly alkaline conditions. Also, my results were consistent when using my standard assay condition using Tris-HCl buffer in pH 7.5 (also containing 10 mM $CaCl_2$, 100 mM KCl, and 0.3 mM Triton X-100).

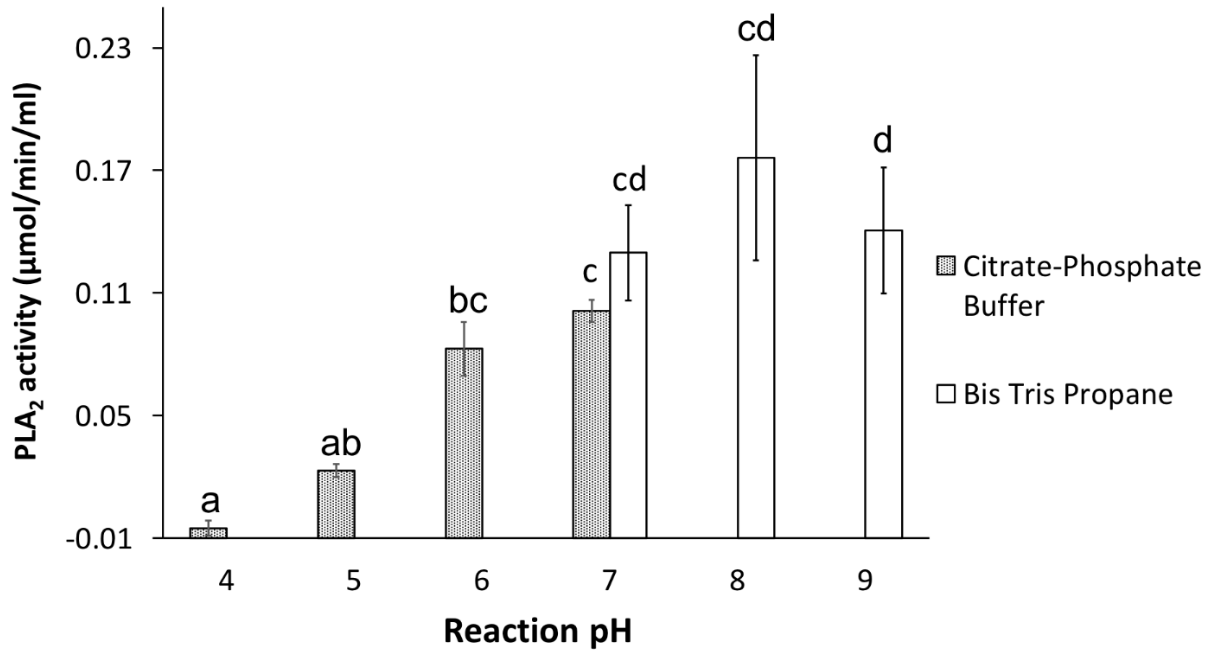


**Figure 2-5. Influence of pH on *Apis mellifera* midgut $PLA_2$ activity (average ± SE).** Citrate-Phosphate Buffer: buffer strength 100mM-200mM, pH 4.0, 5.0, 6.0 and 7.0; Bis Tris Propane: buffer strength 25mM, pH 7.0, 8.0 and 9.0. Histogram bars labeled with the same letter are not significantly different from each other (P>0.05, LSD).

The honey bee midgut $PLA_2$ was sensitive to reaction temperatures (Figure 2-6). I created inactive controls by boiling enzyme preparations for 5 mins, which eliminated all activity, then assayed $PLA_2$ activity over the fairly wide range of 27-62°C. I recorded an optimal temperature at 42°C, after which enzyme activity significantly declined. Figure 2-6 shows fairly high activity at 57°C, which is significantly higher than the activities in 52°C and 62°C. This may be a second temperature optimum, which may be due to the presence of an additional $PLA_2$ in the midgut preparations. The International Union of Biochemistry and Molecular Biology suggested to use a temperature close to or slightly higher than room temperature, such as 30°C, in enzyme assays, unless it is unstable at this temperature or altered for specific purposes (Terra and Ferreira, 2012). This is reasonable, because we want to know how the animal enzymes perform under natural conditions although they might be optimized for higher temperatures. Also, for practical reasons, this temperature is easy to acquire and maintain during the assay. I chose to use 27 °C as my standard reaction temperature in routine assays.

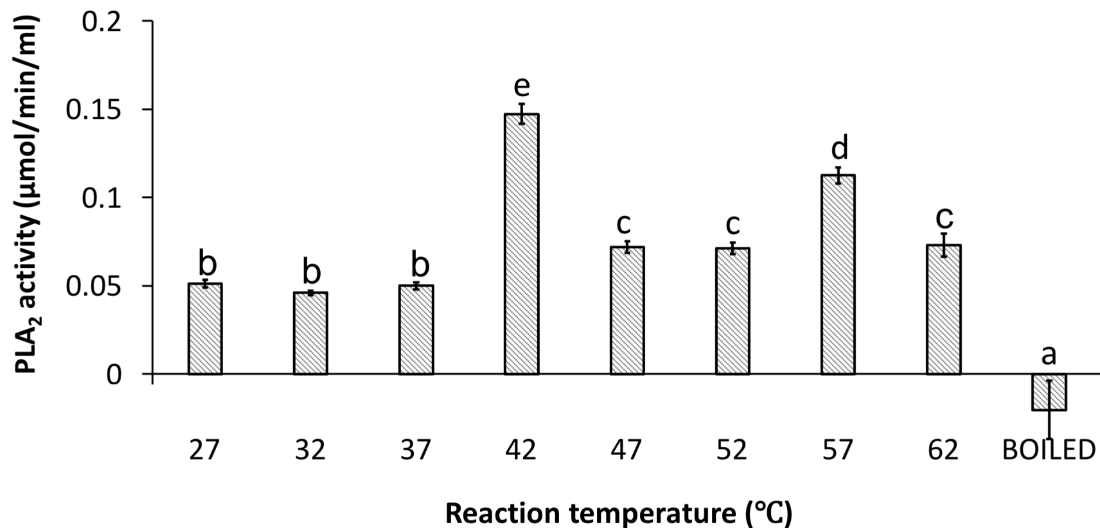


**Figure 2-6. Influence of temperature on *Apis mellifera* midgut $PL_{A2}$ activity (average ± SE).** Histogram bars labeled with the same letter are not significantly different from each other (P>0.05, LSD).

The standard substrate (diheptanoylthio-PC) concentration, indicated by the manufacturer's manual was 1.66 mM. Figure 2-7 showed that a 1:2 dilution did not alter the enzyme activity at a standard reaction time of 10 min, indicating an excessive amount of substrate. Thus, I used 0.83 mM substrate as my standard concentration.

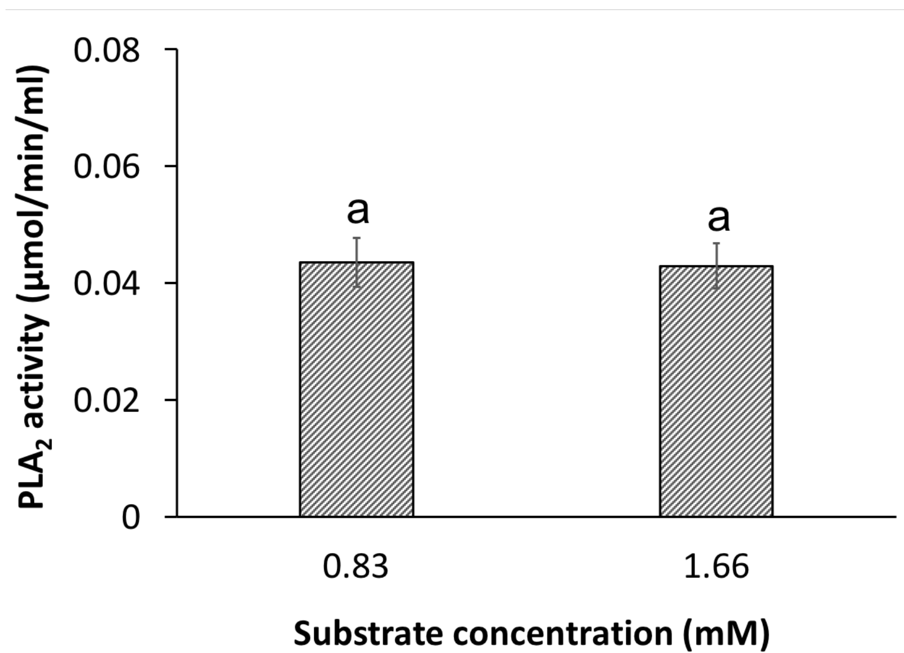


**Figure 2-7. Influence of two substrate concentrations on *Apis mellifera* midgut $PLA_2$ activity (average ± SE).** Histogram bars labeled with the same letter are not significantly different from each other (P>0.05, LSD).

Many $PLA_2$s can be inhibited by inhibitors specific to some $PLA_2$ types. I used p-Bromophenacyl Bromide or 2,4'-Dibromoacetophenone (BPB), an $sPLA_2$ inhibitor (Magolda *et al.*, 1985); and Methyl Arachidonyl Fluorophosphonate (MAFP), a $cPLA_2$ inhibitor (Huang *et al.,* 1994) to determine the class of honey bee midgut $PLA_2$. Figure 2-8 shows that reactions in the presence of MAFP completely inhibited $PLA_2$ activity whereas reactions in the presence of BPB did not influence the $PLA_2$ activity. I will return to this point in the Discussion.

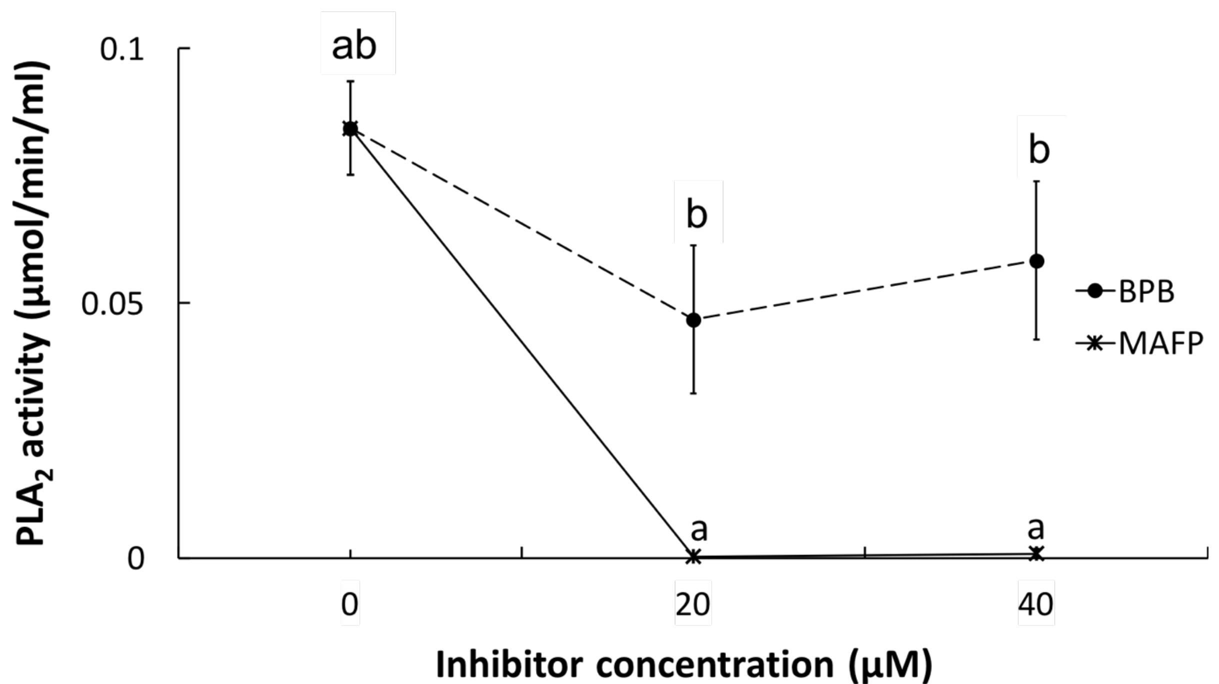


**Figure 2-8. Influence of specific $PLA_2$ inhibitors on *Apis mellifera* midgut $PLA_2$ activity (average ± SE).** Data points labeled with the same letter are not significantly different from each other (P>0.05, LSD).

## 2.4 Discussion

My data strongly support my hypothesis that $PLA_2$ is present in honey bee midguts, and very likely, responsible for digesting the dietary lipids to meet their requirements of PUFAs. The usual biophysical parameters, including reaction time, protein concentration, temperature, pH and the presence of specific inhibitors significantly influenced the $PLA_2$ activity indicating the sensitivity of this enzymatic reaction.

Digestive $PLA_2$s are secreted into the midgut contents by the epithelial cells, as indicated by earlier work in tobacco hornworm, *M. sexta* L., (Rana *et al.*, 1998) and larval mosquitoes, *A. aegypti* (Nor Aliza and Stanley, 1998). *In vitro* secretion of midgut $PLA_2$ by isolated midgut regions documented highest activity in the middle, and lowest in the anterior section of the *M. sexta* midguts (Rana and Stanley, 1999). My data are consistent with these findings. I recorded no $PLA_2$ activity in isolated hindgut preparations (data not shown). Other than the midgut $PLA_2$s mentioned above, digestive $PLA_2$s were recorded in salivary secretions of carrion feeders, such as the burying beetle, *N. marginatus* (Rana *et al.*, 1997) and salivary glands isolated from tobacco hornworms, *M. sexta* (Tunaz and Stanley, 2004).

Most $sPLA_2$s, including those identified in vertebrates and invertebrates are low-molecular-weight secreted proteins (14-18kDa), except for the 55 kDa mammalian group III $sPLA_2$ (Dennis, *et al.*, 2011). Identified insect $sPLA_2$s in the flour beetle, *T. castaneum* (Shrestha *et al.*, 2010) and *D. melanogaster* (Ryu *et al.*, 2003) are less than 30 kDa. Park *et al.* (2015) described a 90.5 kDa novel $iPLA_2$ that acts in *S. exigua* immunity and development. Phylogenetic analysis placed this enzyme with vertebrate $iPLA_2$s. The

primary structure lacks a calcium binding loop and bacterial challenges led to increased accumulation of mRNA encoding the enzyme. Sadekuzzaman *et al*. (2017) found a second $iPLA_2$ in *S. exigua* with a considerably different primary structure and a size of 36.6 kDa. These larger intracellular $PLA_2$s are associated with insect immunity.

The known insect digestive $PLA_2$s have highly variable properties. For example, from mosquito larvae, tobacco hornworms and my data with honey bees, the digestive $PLA_2$ preparations did not require $Ca^{2+}$ for full activity function (Nor Aliza and Stanley, 1998; Rana *et al*., 1998) although those from tiger beetles and burying beetles did (Uscian *et al*., 1995; Rana *et al*., 1997). Reactions in the presence of BPB, an inhibitor of $sPLA_2$, did not influence the honey bee digestive $PLA_2$ activity while the $cPLA_2$ inhibitor, MAFP completely abolished enzyme activity. I suggest to possibilities that may help understand why a $sPLA_2$ inhibitor did not influence the secreted digestive $PLA_2$ activity in honey bee midgut preparations. One, it may be inferred that there is more than one type of $PLA_2$ in the midgut preparations, which await discovery. Alternatively, another digestive $PLA_2$, possibly a $cPLA_2$, is sufficiently different from other digestive $PLA_2$s to be insensitive to BPB.

The pH of the midgut contents is one factor that influences digestive enzymes, including the digestive $PLA_2$s. Midgut pH is not necessarily related with diet and mostly occurs within the range of 6-7.5. Exceptions include Lepidoptera, with highly alkaline midgut contents (pH 9-12) which allows them to feed on tannin-riched plant tissues (Berenbaum, 1980). Flies have very acidic (pH 3.1-3.4) midgut middle sections and true bugs have acidic midgut posterior sections (Terra and Ferreira, 2012). For honey bees, the microelectrode profiles of adult worker midgut pH revealed weakly acidic (6.0-6.5)

anterior to middle regions and neutral posterior midgut contents (Zheng *et al*., 2017). Despite this, my results indicates that honey bee digestive $PLA_2$s prefer neutral to alkaline environments. I suggest that peritrophic matrices and muscles lining the alimentary canals create abundant micro-compartments within the midguts that may be quite variable in local pH values (Boudko *et al*., 2001; Clark *et al*., 2007).

Honey bees meet their nutritional needs with nectar and pollen. They acquire carbohydrates from nectar, and proteins, lipids as well as micronutrients (i.e. vitamins and minerals) from pollen (Vaudo *et al.,* 2015). Honey bees power their flights with sugar and use their lipid stores for other energy-requiring activities such as in-hive duties for workers and ovarian development in queens. Lipid intake and metabolism mostly take place in the larval stages (Brodschneider and Crailsheim, 2010). Adults also have body lipids, albeit, in relatively low quantities. Liang *et al*. (2013) determined that PLs (mainly PS and PC) are the major components of pollen coats among various plant species. Honey bee digestion of these dietary PLs occurs in the honey bee midguts, very likely with $PLA_2$s. The well-known honey bee colony collapse disorder (CCD) has led to a rapid decline of colony numbers and drawn attention to honey bee health. CCD is probably due to a syndrome of serious problems rather than a single clear cause. Most common elements are exposure to chemical insecticides, extreme winter, poor nutrition, queen failure and pests (e.g., *Varroa destructor* and *Deformed wing virus*) (Vanengelsdorp *et al*., 2007, 2015a, 2015b; Hayes *et al*., 2008; Spleen *et al*., 2013; Steinhauer *et al*., 2014; Lee *et al*., 2015; Seitz *et al*., 2016). Understanding honey bee nutrition and digestive physiology may be a breakthrough point in overcoming this situation.

Taken together, my data suggest the existence of more than one type of $Ca^{2+}$-independent honey bee digestive $PLA_2$s in the midgut preparations. It is reasonable to expect new knowledge of honey bee digestive mechanisms to emerge in the near future as we work to understand honey bee nutrition and health.

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# CHAPTER 3

# CRYOPRESERVATION OF THE HONEY BEE MIDGUT PREPARATIONS TO INVESTIGATE A DIGESTIVE $PLA_2$

## 3.1 Introduction

I was introduced to Dr. Xu Han, assistant research professor of Mechanical and Aerospace Engineering in the University of Missouri and founder of CyroCrate, LLC., when our lab started collaborating with him on a project of cell culture cryopreservation. Cryopreservation of cell cultures, embryos and tissues are of great importance in medical and biological research (Rall *et al*., 1985; Engelmann, 2004; Donnez *et al*., 2006).

As mentioned in Chapter 2, I used fresh honey bee midgut homogenates as sources of $PLA_2$. However, honey bees are seasonal animals, with high colony growth and foraging activity in summers and reduced growth and foraging in winters. Generally, summer bees are biologically different from winter bees. I began collaborating with Dr. Han at Dr. Stanley's suggestion because of the possibility that we could prepare and store summer bee midguts for out of season research. The question was whether Dr. Han's propriety cryopreservation product, EZ80, would help stabilize the honey bee midgut preparations during long-term storage. The EZ80 minimizes ice formation during freezing, which also minimizes ice-related damage to cell organelles and membranes. We have been testing this on established insect cell lines and the idea was to expand the possibilities of EZ80 to small tissue preparations. In this brief chapter, I report on the outcomes of the few experiments I did using EZ80.

## 3.2 Methods and materials

### 3.2.1 Cryopreservation buffers

Four buffers were used in the cryopreservation process: 1/ sterilized PBS (pH 6.4, 7.7 mM $Na_2HPO_4$, 2.65 mM $NaH_2PO_4$, 150 mM NaCl); 2/ 1:1 (v/v) mixture of sterilized PBS and EZ80 containing 10% (m/v) (PBS+EZ80); 3/ 1:1 (v/v) mixture of sterilized PBS and 10% (v/v) cell culture grade glycerol (PBS+G); and 4/ 1:1 (v/v) of 10% (v/v) cell culture grade glycerol and 10% (m/v) EZ80 (EZ80+G). Glycerol, rather than the more widely applied cryoprotectant, dimethyl sulfoxide (DMSO), was used because it maintains the natural state of internal proteins whereas DMSO denatures the proteins (Arakawa *et al.*, 2007). Each buffer was placed in cryopreservation tubes at 66.7 µl/midgut and the tubes were chilled by maintaining them on ice during dissections.

### 3.2.2 Midgut tissue preparations

A colony of *Apis mellifera ligustica* was maintained as indicated in 2.2.1. For each preparation, six adult workers were anesthetized by chilling them on ice for 5 min. Dissection was conducted on a pre-chilled sterile wax plate. During dissection, the insect body was soaked in ice-chilled PBS to keep the tissue cells intact. The alimentary canal was exposed by cutting the integument from the first to the sixth visible segment of the abdomen. The Malpighian tubules were removed from the alimentary canal using forceps. The midgut was excised from the rest of the alimentary canal, rinsed 3X in 1 mL sterile PBS and transferred into each of the chilled buffers just described.

Mild agitation improved the efficiency of mixing the buffers with the midgut tissues. The sample tubes were gently shaken on ice in an orbital shaker (LabDoctor™ ) at 100 rpm, for 10 min, to allow the buffers to enter the midgut cells.

### 3.2.3 $PLA_2$ sources

To prepare the homogenate, midguts in each sample tube were ground on ice using pre-sterilized homogenizers (Axygen Scientific Inc., Union City, CA) and sonicated (Heat-systems Ultrasonics Inc.) on ice, three times, at 35 watts for 10s each time, with a 10 s break in between. The 10 s interval helps prevent the sample from getting warm. The homogenates were then centrifuged at 16,000 x g, 4°C, for 10 min.

The resulting supernatants were transferred to a centrifugal concentrator (500 μL, MWCO 5000 Da Vivaspin, Sigma-Aldrich Inc., St. Louis, MO) and concentrated at 16, 000 x g, 4°C, for 30 min, reducing the sample volume to about 25%, which varied slightly due to the textures of the buffers. Standard $PLA_2$ assay buffer (Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 100 mM KCl, and 0.3 mM Triton X-100; 200 ml) was added to each concentrator and centrifuged for 30 min at 16,000 x g, 4°C. The flow-through was discarded and the samples were washed 2X as just described. The samples were centrifuged for 30 min, concentrating the samples to approximately 50 μl. The resulting concentrated $PLA_2$ solution was used as the enzyme source.

### 3.2.4 Cryopreservation and thawing of the homogenate and whole midgut tissues

For the control group, the midgut preparations were processed for the $PLA_2$ assay on the sampling day, as indicated in 3.2.3. For the cryopreserved groups, including the midgut

homogenate and whole midgut tissues, each sample was treated separately with a treatment buffer and stored in -80°C for 90 days (homogenate), or 30 days and 90 days (whole midgut tissues). Slow freezing (at a freezing rate about 1 °C /min) and fast thawing (at a thawing rate >10 °C /min) produces higher activity recovery for enzymes (Cao *et al.*, 2003). Thus, a specialized cryogenic storage container, the Corning® CoolCell® (Corning Inc., New York City) was used during freezing to allow slow freezing and minimize the sample crystallization. Each group was composed of three biologically independent preparations for each of the four treatment buffers. After indicated storage time (30 days or 90 days), samples were thawed at 4°C to prevent the $PLA_2$s from undergoing proteolysis.

### 3.2.5 Determination of Protein Concentration

Protein concentrations in the prepared $PLA_2$ solutions were determined against bovine serum albumin using the BCA Protein Assay Reagent Kits from Pierce ™ (Rockford, IL). Unknown samples and standard curves were read at 562 nm using a microtiter plate reader (2300 Multilabel Reader, PerkinElmer Inc., Waltham, MA).

### 3.2.6 $PLA_2$ activity assay and statistical analysis

$PLA_2$ activities were determined and analyzed statistically as described in Chapter 2.

## 3.3 Results

Relative to freshly prepared controls, cryopreservation of the homogenates for 90 days did not significantly influence $PLA_2$ activity in buffers PBS, PBS+G, and EZ80+G. $PLA_2$ activity after storage in PBS+EZ80 was statistically significant from the fresh sample at the 0.05 level and decreased by 18.7% (Table 3-1 and Figure 3-1). This indicated that although the EZ80 was not an ideal cyroprotectant, the honey bee digestive $PLA_2$ was fairly stable for at least three months at -80°C.

**Table 3-1. Average $PLA_2$ activity (µmol/min/ml) of the same enzyme sources before and after stroring in cyropreservation buffers for 90 days.**

| Average $PLA_2$ activity (µmol/min/ml) | PBS | PBS + EZ80 | PBS + G | EZ80+G |
|---|---|---|---|---|
| 0 day | 0.0675 ± 0.0041[b] | 0.0466 ± 0.0033[b] | 0.1164 ± 0.0126[c] | 0.0627 ± 0.0062[b] |
| 90 days | 0.0613 ± 0.0050[b] | 0.0379 ± 0.0030[a] | 0.1037 ± 0.0112[c] | 0.0598 ± 0.0064[b] |

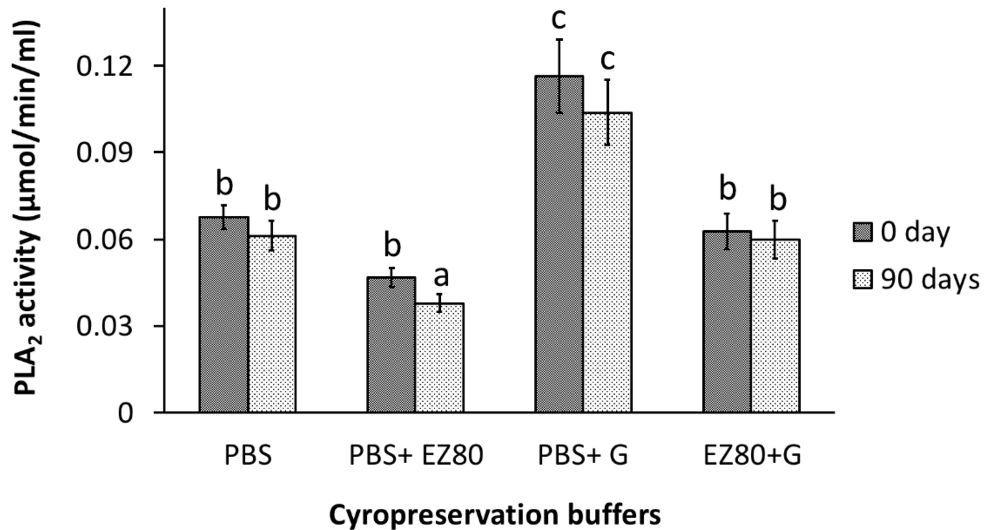


**Figure 3-1. Influence of cyropreservation buffers on honey bee midgut homogenate $PLA_2$ activities after 90 days of cryopreservation in -80°C.** Histogram bars labeled with the same letter are not significantly different from each other (P>0.05, LSD).

Storing whole midguts instead of the homogenate preparations, led to more variation due to the differences among individual bees (Figure 3-2). $PLA_2$ assays conducted with fresh tissues determined that compared to PBS, buffer PBS+EZ80 and EZ80+G did not alter the $PLA_2$ activity. Treatment with buffer PBS+G led to significantly higher enzyme activity. This eliminated the possibility that the cyroprotectants in buffers PBS+EZ80, PBS+G, and EZ80+G would impair the $PLA_2$ activities. After storing the whole midguts for 30 or 90 days, $PLA_2$ activity can be meaningfully assayed in each treatment. I note no clear preference was determined among the four tested cryopreservation buffers.

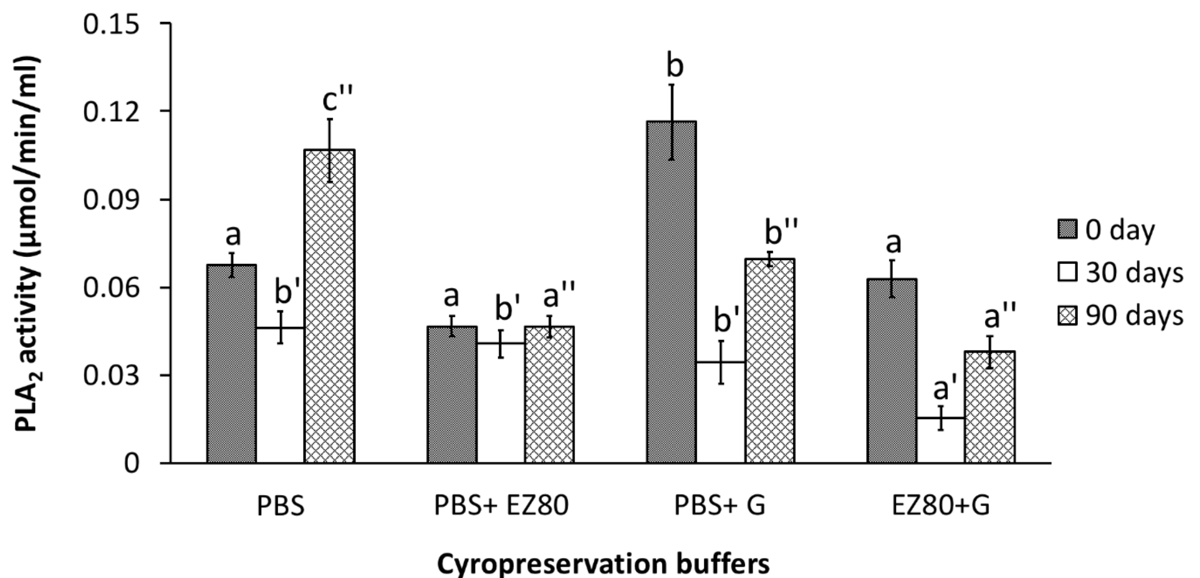


**Figure 3-2. Influence of cyropreservation buffers on honey bee midgut tissue $PLA_2$ activities after 30 and 90 days of cryopreservation in -80°C. Treatment effects were compared at each time point not among time groups.** Histogram bars labeled with the same letter are not significantly different from each other (P>0.05, LSD).

## 3.4 Discussion

My data reveal that the honey bee digestive $PLA_2$s are fairly stable, for at least three months at -80°C in the buffers I used. The overall finding is that the EZ80 cryopreservation medium did not enhance the cryopreservation of the midgut preparations. My experiments were designed to assess the influence of the EZ80 on relatively short-term storage durations, in the range of a few months. While the EZ80 did not enhance the cryopreservation during 90-day durations, it is possible longer-term storage may be facilitated by the EZ80. These experiments lie outside the scope of my program.

Cyropreservation, as the storage of biological material at ultralow temperature, allows a safe and cost-efficient method for long-term conservation of biology genetic resources, such as medicinal plants, agricultural crops and animal breeds. However, most biological experimental materials (cells, embryos, etc.) contain high amounts of intercellular water and are very sensitive to the ice-formation injuries (Mazur, 1970). Techniques such as freeze-induced dehydration and vitrification enhance cryopreservation by minimizing ice-related intracellular injuries (Engelmann, 2004). EZ80 uses nanoparticles to improve the nucleation and decrease the devitrification temperatures of the cryoprotectant solutions during the super-cooling processes (Han *et al*., 2008a, b).

In addition to exploring the application possibilities of EZ80 in tissue cyropreservation, another objective of this project was to test the stability of the honey bee digestive $PLA_2$s in midgut preparations. In general, freezing and thawing effects on proteins can be various depending on their internal structures and external factors. To better preserve the proteins, higher protein concentrations, smaller sample sizes, lower salt contents are factors to consider in future research. It is well understood that buffers with

altered pH during freezing should be avoided (Fishbein and Winkert, 1979). Generally, slow freezing (at a freezing rate about 1 °C /min) and fast thawing (at a thawing rate >10 °C /min) produce higher activity recovery for enzymes (Cao *et al*., 2003). In one of my experimental groups, PBS containing high salt content did not influence the enzyme activity, suggesting that the honey bee digestive $PLA_2$ is, in general, stable, during the freezing and thawing process.

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# CHAPTER 4

# CLONING AND EXPRESSION OF THE HONEY BEE DIGESTIVE $PLA_2$

## 4.1 Introduction

Chapters 1 and 2 review some of the literature on $PLA_2$s, drawing on the biomedical literature to appreciate the broad biological significance of $PLA_2$s generally and on the insect literature to cover the more limited background on insect digestive $PLA_2$s. That information does not need to be repeated in this Chapter.

The original goal of my project was to generate a recombinant honey bee digestive $PLA_2$ that could be used to characterize the enzyme. Here, I describe the molecular approaches I used to identify genes encoding honey bee midgut $PLA_2$s, clone the genes from midgut tissue preparations, verify the sequences of the cloned genes, insert a selected gene into an expression vector, express the gene in competent *E. coli* cells and enrich the recombinant protein from *E. coli* cultures. The recombinant $PLA_2$ did not possess catalytic activity, however, the experiences gained in this work are meaningful aspects of my training and learning throughout my program.

## 4.2 Methods and materials

### 4.2.1 Bioinformatics to search for honey bee digestive $PLA_2$s

The honey bee genome was interrogated (Honeybee Genome Sequencing Consortium, 2006) and eleven sequences encoding honey bee $PLA_2$ were obtained from

GenBank (www.ncbi.nlm.nih.gov). The sequences were aligned and a phylogenetic tree was constructed using MEGA7.0 software (Kumar *et al.*, 2016).

### 4.2.2 RNA extraction and cDNA synthesis

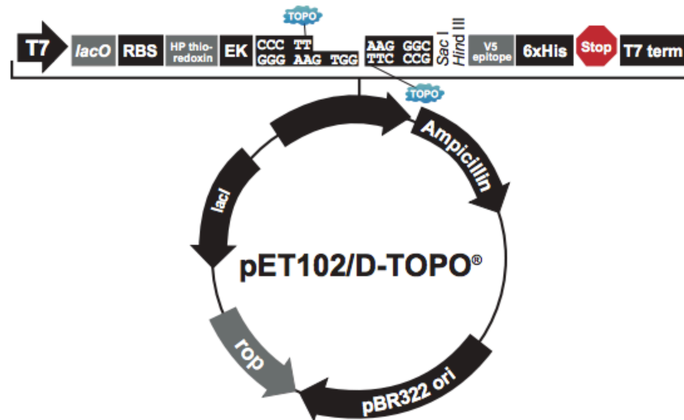
A colony of *A. mellifera ligustica* was maintained as indicated in 2.2.1. Total RNA and mRNA was extracted from whole animals, specific tissues (midgut, fat body) and heads of adult workers pulverized under liquid nitrogen using RNeasy Mini Kits® (Qiagen Co., Germantown, MD) following the manufacturer's instructions. RNA concentrations were determined with a Nanodrop 2000™ spectrophotometer (Thermo Scientific, Waltham, MA) at 260 nm. Three μg total RNA was used to synthesize the first strand cDNA following the instructions of the Omniscript RT Kit® (Qiagen Co., Germantown, MD).

### 4.2.3 Gene cloning, expression and purification

Primers were designed to generate DNA fragments encoding the honey bee $PLA_2$s. The PCR conditions were as the followings: initial denaturation at 95°C for 5 min, 35 cycles of denaturation (95°C, 60s), annealing (58°C, 60s) and extension (72°C, 120s) and a final extension at 72°C for 5 min. The products were stored at 4°C. Agarose gel electrophoresis (2%) was used to isolate and quantitate the DNA amplicons. Single, discrete PCR products, were cut, purified and sequenced (MU Core Facility) to verify their identity to the known sequences from the database and literature (Honeybee Genome Sequencing Consortium, 2006). After eliminating the venom $PLA_2$ (Gene ID: 406141), the entire ORF of one gene (Gene ID: 724436; *Am* $PLA_2$) was amplified using the following

primers: forward: 5’-ATG CGC GTT CTC CAC TCG TCG TTT CTT-3’and reverse: 5’-AAA GCT GGG AAG ATC GAA CCA TTG ATA TTT CTT CG-3’.

The Champion pET102 Directional TOPO Expression Kit® (Invitrogen, Carlsbad, CA) was used to directionally clone the blunt-end PCR products into the pET102/D-TOPO® vector using topoisomerase I. Specific primers were designed by adding 4 bases, CACC, to the forward primer to match with the overhang in the cloning vector (GTGG). Thus, *AmPLA*$_2$ DNA fragments were inserted into the vectors in the correct direction with at least 90% efficiency. The DNA sequences of the vectors I used (pET102 plasmids) contain the ampicillin resistance gene and a T7 promoter (Figure 4-1), which helps with expressing the T7-regulated target gene.




| | pET102/D-TOPO® |
|---|---|
| T7 promoter | 209-225 |
| T7 promoter/priming site | 209-228 |
| *lac* operator (lacO) | 228-252 |
| Ribosome binding site (RBS) | 282-288 |
| His-patch (HP) thioredoxin ORF | 298-627 |
| TrxFus forward priming site | 607-624 |
| EK recognition site | 643-657 |
| TOPO® Cloning site (directional) | 670-683 |
| V5 epitope | 700-741 |
| Polyhistidine (6xHis) region | 751-768 |
| T7 reverse priming site | 822-841 |
| T7 transcription termination region | 783-911 |
| *bla* promoter | 1407-1505 |
| Ampicillin (*bla*) resistance gene | 1506-2366 |
| pBR322 origin | 2511-3184 |
| *ROP* ORF (complementary strand) | 3552-3743 |
| *lacI* ORF (complementary strand) | 5055-6146 |

**Figure 4-1. The pET 102 cloning vector map.**

To express the target gene, chemically competent One Shot™ TOP 10 *E. coli* cells (Invitrogen, CA) were used for the transformation. Lysogeny broth (LB) agar plates were prepared, with each plate containing 25 ml 0.04g/ml LB agar medium. Six experimental plates as well as the negative control plate contained 100 μg/ml ampicillin. For the negative control plate, the *E. coli* cells which did not have vectors expressing ampicillin resistance gene were used. If no colonies grow, the used ampicillin concentration killed the susceptible bacteria cells. For the positive control plate, ampicillin was not added. If the competent *E. coli* cells grow to confluent colonies after overnight incubation, they were functional.

After amplifying the coding region of the three target genes using PCR, cloning reactions were set up with two molar ratios of PCR product: vector (0.5:1 and 2:1) for *Am* $PLA_2$. The cloning reaction and transformation were conducted following the manufacturer's manual. Each transformation was spread on one ampicillin selective plate, sealed with parafilm and incubated at 37°C for 19 h.

Successful colonies were selected to analyze the transformants using a QIAprep Spin Miniprep Kit® (Qiagen Co., Germantown, MD) to purify the plasmid DNA. PCR reactions were set up with the plasmid DNA template, using a forward primer with Trx fusion, 5'-TTC CTC GAC GCT AAC CTG-3' and T7 reverse primer, 5'-TAG TTA TTG CTG AGC GGT GG-3', to confirm the correct insert by secuquencing at the University of Missouri DNA core facility.

During the inoculation, 100 μl of overnight *E.coli* cultures containing the *$AmPLA_2$* expression plasmid were added to 10 ml Super Broth medium (35 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl and 5 ml/L NaOH) with ampicillin selection in a 250 ml flask.

The culture was incubated at 37°C with agitation at 250 rpm until $OD_{600}$ reached 0.500. The cultures were induced with 0 mM, 0.5 mM or 1 mM IPTG (ready-to-use IPTG Solution, Thermo Scientific, Waltham, MA). An additional 5 hours of shaking were allowed to induce protein expression. Aliquots of the cultures were removed, pelleted at 21,000 xg for 1 min and frozen at -20°C.The His-tagged *Am*$PLA_2$ was partially purified using the His SpinTrap columns and His Buffer Kit (GE Healthcare, Marlborough, MA) following the manufacturer's instruction.

The enriched $rPLA_2$ , as well as a negative control from a *E. coli* colony which did not contain $PLA_2$ inserts, were used to assess $PLA_2$ activity. Several assay formats, including the absorbance assays ($sPLA_2$ and $cPLA_2$ Assay Kits, Cayman Chemical, Ann Arbor, MI), florescent assay (EnzChek™ $PLA_2$ Assay Kit, Invitrogen, Carlsbad, CA) and assays using radioactive substrates, L-α-1-Palmitoyl-2-Arachidonyl-Phosphatidylcholine, (Arachidonyl-1-$^{14}$C) and L-α-DiPalmitoyl-Phosphatidylcholine (DiPalmitoyl-1-$^{14}$C), following the protocols described by Rana *et al*. (1998) were conducted.

### 4.2.4 Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining and Western Blot analysis were used to evaluate protein expression. *E. coli* lysates from the samples which contain and did not contain *Am*$PLA_2$ inserts were separated using Mini-PROTEAN® 3 Cell System (BIO-RAD, Hercules, CA), with 4% stacking and 12% resolving gel formula adopted. Precision Plus Protein™ All Blue Prestained Protein Standards (BIO-RAD, Hercules, CA) were used as strandards. Detailed experimental conditions and buffer fomulas are described in the *SDS-PAGE*

*(Laemmli) Buffer System* of the instruction manual (Mini-PROTEAN® 3 Cell System). After running, the gel was stained by gently shaking in Bio-Safe™ Coomassie G-250 Stain (BIO-RAD, Hercules, CA) for 1 hr. Stained gel was rinsed for three times using $ddH_2O$. Resolved proteins were transferred onto a PVDF membrane with Mini Trans-Blot® Electrophoretic Transfer Cell. The membrane was probed with 6x-His Tag Monoclonal MA1-21315 Antibody (Invitrogen, Carlsbad, CA).

## 4.3 Results

Figure 4-2 shows a phylogenetic tree of *A. mellifera* $PLA_2$ genes. The cloned *Am*$PLA_2$ is partially identical to the *Am* venom $PLA_2$.

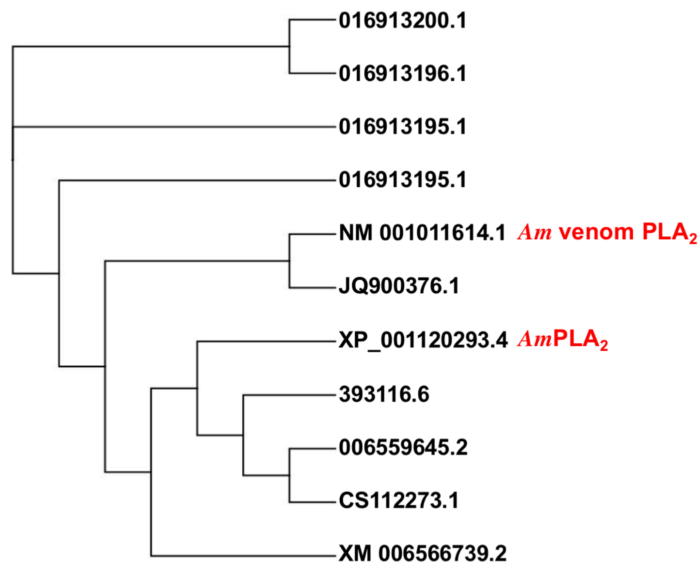



**Figure 4-2. A phylogenetic tree generated with MEGA7.0 software based on the alignment of selected honey bee $PLA_2$ sequences.**

*Am*$PLA_2$ was inserted into the vector and expressed by *E. coli* (Figure 4-3). The nucleotide sequence has 525 base pairs, calculated to encode a protein with 174 amino

acids (Figure 4-4). The computed molecular weight is 20,533.90 Da and the theroretical pI is 8.81. Lys(K), at 11.5%, is the most abundant amino acid. SignalP 4.1 indicated the presence of a signal peptide ((http://www.cbs.dtu.dk/services/SignalP), of which the sequence is MRVLHSSFLLLVLCLFLHVSA (Figure 4-5).

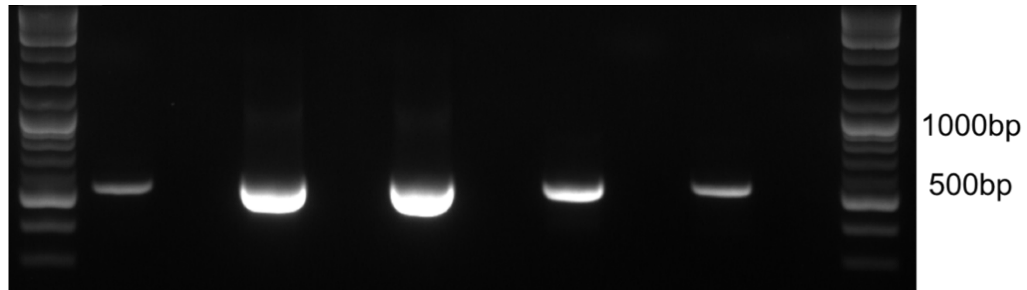



**Figure 4-3. Selected colonies which have inserts of *A.m.* $PLA_2$.**

***Am* $PLA_2$ DNA with highlighted ORF (525 bp)**

```
  1 aagtctcttt tctctcgttt ctcctaaaaa aaaaaaaaaa aaaaaagaaa agaaaagaca
 61 ctctcttctt atcgctgttc cacgatgcgc gttctccact cgtcgtttct tcttctcgtt
121 ctctgcctgt tcctccacgt ttctgcgcgg gaatgggaga ttcaacacaa ggaggccgac
181 gatgagattc aggaacgaat caacacaatt gtaccaagta cgaagtggtg cgggccaggt
241 aacaaagcga aaaactacaa cgatttggga ttcaatcaca taaccgacgc ctgctgcagg
301 gagcacgatt actgtcccga tagcatcaag gctttgagaa gaaaacacaa tctgtggaac
361 gcaagcttat tcctcaggtc aaaatgctcg tgcgatcata agttctacaa gtgtctgaag
421 aattcgacag agctaattgc tgtcggtata ggtaaagttt acttcaacga cataataatt
481 ccaaaatgct tcgaacttga acatcctatc atcggttgca aaaaatacga agattaccgt
541 tgcatcaagt atattgtgga caagaaaaag ccgaagaaat atcaatggtt cgatcttccc
601 agcttttgat tatacaaatt aacctccttc gattcttatt tttcattcga ttgaatatcg
661 taatatttta ctttggaaac aaatttttca atctgtcaaa tttacctaaa tgcaccatgt
721 gaaacgaatt tttttgtttg tgtatcttgt atcaatcctt tgagaattaa gatcaaatta
781 aaaattgtac gctatcgaat ttcgaagaaa
```

***Am* $PLA_2$ protein (174 AA)**

```
  1 mrvlhssfll lvlclflhvs areweiqhke addeiqerin tivpstkwcg pgnkaknynd
 61 lgfnhitdac crehdycpds ikalrrkhnl wnaslflrsk cscdhkfykc lknsteliav
121 gigkvyfndi iipkcfeleh piigckkyed yrcikyivdk kkpkkyqwfd lpsf
```

**Amino acid composition:**

| | | |
|---|---|---|
| Ala (A) | 7 | 4.0% |
| Arg (R) | 8 | 4.6% |
| Asn (N) | 9 | 5.2% |
| Asp (D) | 11 | 6.3% |
| Cys (C) | 11 | 6.3% |
| Gln (Q) | 3 | 1.7% |
| Glu (E) | 10 | 5.7% |
| Gly (G) | 6 | 3.4% |
| His (H) | 8 | 4.6% |
| Ile (I) | 15 | 8.6% |
| Leu (L) | 16 | 9.2% |
| Lys (K) | 20 | 11.5% |
| Met (M) | 1 | 0.6% |
| Phe (F) | 9 | 5.2% |
| Pro (P) | 7 | 4.0% |
| Ser (S) | 10 | 5.7% |
| Thr (T) | 4 | 2.3% |
| Trp (W) | 4 | 2.3% |
| Tyr (Y) | 8 | 4.6% |
| Val (V) | 7 | 4.0% |
| Pyl (O) | 0 | 0.0% |
| Sec (U) | 0 | 0.0% |
| (B) | 0 | 0.0% |
| (Z) | 0 | 0.0% |
| (X) | 0 | 0.0% |

**Figure 4-4. *Am*$PLA_2$ nucleotide and calculated amino acid sequences.**

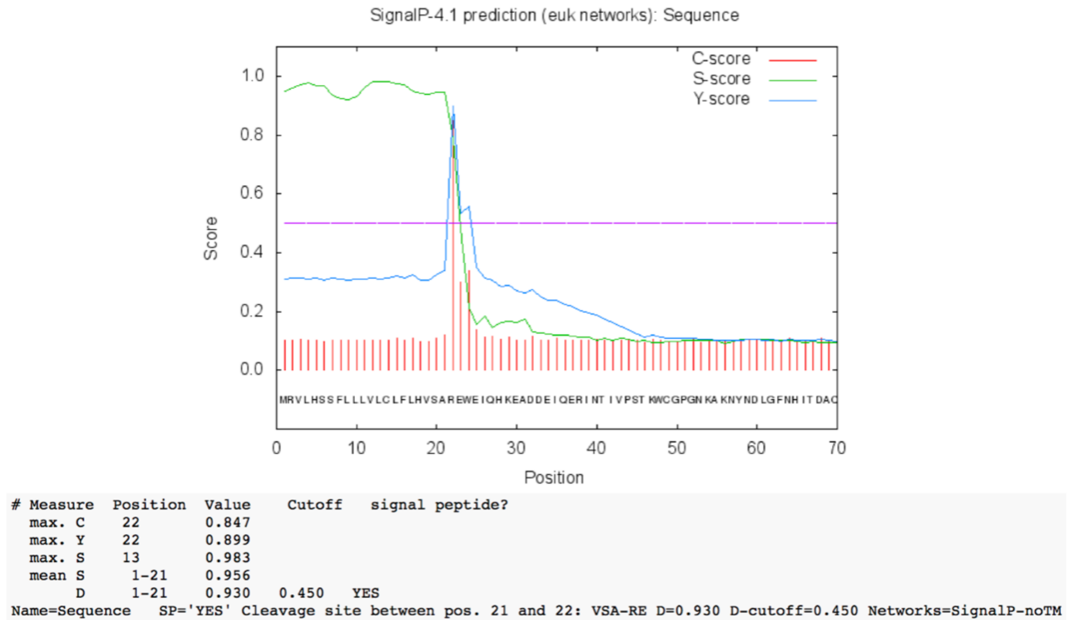



**Figure 4-5. SignalP prediction results of *Am*PLA$_2$.**

The SDS-PAGE followed by Coomassie Blue staining or Western Blot analysis did not indicate an expression of *Am*PLA$_2$ comparing to the negative control using *E. coli* lysates which do not contain *Am*PLA$_2$ inserts (Figure 4-6). From the results, no target band, which is around 20.54 kDa, was identified. Initial efforts on testing the recombinant *Am*PLA$_2$ activities using both radioactive and non-radioactive assay methods did not yield positive PLA$_2$ activities.

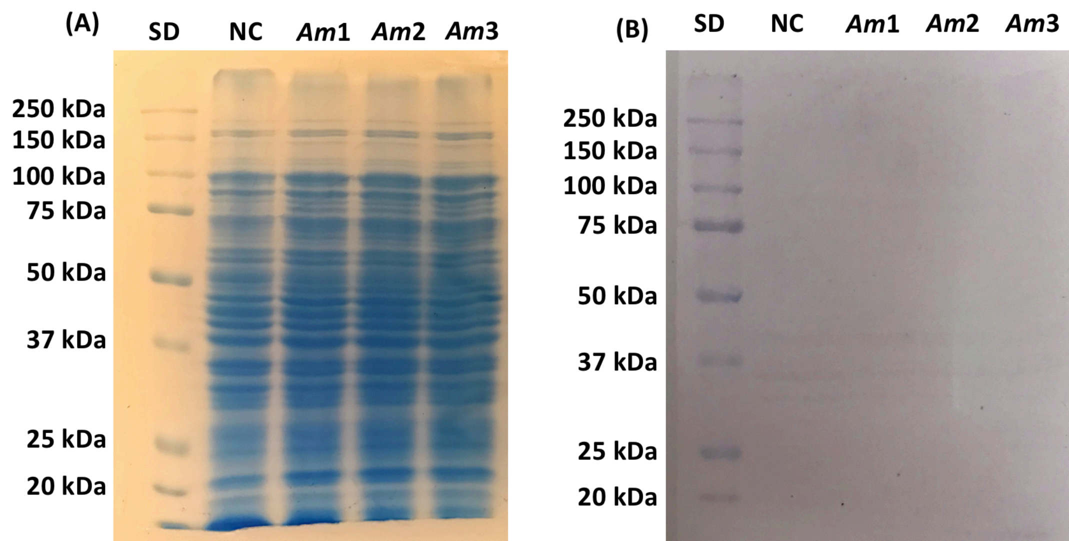



**Figure 4-6. Stained SDS-PAGE gel (A) and Western Blot analysis of *Am*PLA$_2$ expression (B).** SD: Precision Plus Protein™ All Blue Prestained Protein Standards; NC: the negative control using *E. coli* lysates which do not contain *Am*PLA$_2$ inserts; *Am*1: *E. coli* lysates of which the expression was not induced with IPTG; *Am*2: *E. coli* lysates of which the expression was induced with 0.5 mM IPTG; *Am*3: *E. coli* lysates of which the expression was induced with 1 mM IPTG.

## 4.4 Discussion

A few non-venom insect $PLA_2$s have been investigated at the molecular level, as summarized in Table 4-1.

**Table 4-1. A comparison of characterized recombinant insect non-venom $PLA_2$s. These $PLA_2$s are also presented in the text of Chapter 1.**

| Insect | Common name | $PLA_2$ properties | | | | Reference |
|---|---|---|---|---|---|---|
| | | Type | Size (kDa) | $Ca^{2+}$ dependent/ binding loop present | Function | |
| *Drosophila melanogaster* | Common fruit fly | $sPLA_2$ | 22.6 | Yes | Unknown. Expressed throughout development | Ryu *et al*., 2003 |
| *Tribolium castaneum* | Red flour beetle | $sPLA_2$ | 20.5 - 29.4 | Yes | Mediate nodule formation after bacterial infection | Shrestha *et al*., 2010 |
| *Spodoptera exigua* | Beet armyworm | $iPLA_2$ | 90.5 | No | Facilitate larval growth; form nodules during bacterial challenge | Park *et al*., 2015 |
| *Spodoptera exigua* | Beet armyworm | $iPLA_2$ | 36.6 | No | Facilitate larval growth; form nodules during bacterial challenge | Sadekuzzaman *et al*., 2017 |
| *Bombyx mori* | Silkworm | $sPLA_2$ | 31.9 | N/A | Energy storage and lipid metabolism | Orville *et al*., 2016 |
| *Bactrocera dorsalis* | Oriental fruit fly | $sPLA_2$ | N/A | N/A | Activate Toll/Imd signaling pathways in humoral response | Li *et al*., 2017 |

These examples support the idea that the insect $PLA_2$s can be investigated at the molecular level. These successful operations raise questions about why my work did not lead to a catalytic recombinant enzyme.

The first trouble-shooting step was to make sure the gene was inserted into the plasmid DNA in the correct orientation with no in-frame stop codons or other errors. Because I already verified this by sequencing, this was not the issue.

One possibility was that inclusion bodies, instead of correctly folded proteins, were produced. Inclusion bodies are overexpressed recombinant proteins which accumulate as insoluble aggregates, misfolded and devoid of biological functions (Singh and Upadhyay, 2014). They have much higher densities than many other cellular components at about 1.3 mg/ml (Tayler *et al*., 1986).

Methods have been developed to solve this issue. One critical step is to use highly concentrated chaotropic reagents, such as urea, guanidine hydrochloride and detergents such as SDS to solve them (Fischer *et al*., 1993; Singh and Upadhyay, 2014). Next, it is needed to refold, or renature the solubilized protein into a bioactive form, through dilution, dialysis or solid-phase separation (Clark, 1998).

It also occurs in generating recombinant proteins that the protein is degraded by proteases before the protein expression measurements. Protease inhibitors like PMSF and leupeptin might prevent this from occurring (Duong-Ly and Gabelli, 2014).

Theoretically, it is very straight-forward to use a host, commonly *E. coli*, to generate recombinant proteins. But just like other experiments, many possibilities account for unsuccessful trials. My work is a meaningful step in expressing a recombinant digestive $PLA_2$, which has not been accomplished in insect preparations. If given more time, step-by-step troubleshooting can be conducted.

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# CONCLUSION AND PERSPECTIVES

My study confirms the presence of at least one $PLA_2$ in honey bee worker midgut preparations. It is sensitive to the usual biophysical parameters, reaction time, protein concentration, temperature, pH and the action of specific inhibitors. It is stable at -80°C for at least 90 days. The biological significance of digestive $PLA_2$s relates to dietary essential fatty acid requirements and to neutral lipid digestion.

Like vertebrates, many insect species utilize C18 PUFAs, mostly C18:2n-6 and C18:3n-3, as part of their nutritional requirements obtained directly from food (Dadd, 1983). In most cases, plants contain C18, but not C20 PUFAs, thus herbivores generate C20 PUFAs via elongation/desaturation pathways (Stanley-Samuelson *et al*., 1988). Carnivores intake C20 PUFAs through preying, parasitizing or blood-feeding, which were recorded in some insects, such as adult stable flies, *Stomoxys calcitrans* (L.) (Gadelhak *et al*., 1995), and *Anopheles stephensi* mosquitoes (Stanley-Samuelson and Dadd, 1983).

PUFA deficiencies in insects result in retarded development, flight-disabled adults, deformations and death. C20 PUFAs are components of cellular and subcellular biomembranes and are substrates for conversion to oxygenated metabolites, or eicosanoids (Stanley and Kim, 2014). Eicosanoids are divided into three groups: PGs, epoxyeicosatrienoic acids and lipoxygenase products, involved in various aspects of insect development and physiology (Vrablik and Watts, 2013; Stanley and Kim, 2014; Kim *et al*., 2017). For example, impaired PUFA-derived eicosanoids cause sperm migration defects in *Caenorhabditis elegans* and limited egg follicle maturation of *D. melanogaster*, both

can be rescued through PG supplementation (Kubagawa *et al*., 2006; Tootle and Spradling, 2008; Groen *et al*., 2012).

The dietary PUFA requirements are not clear with honey bees. It is likely that they obtain PUFAs through pollen-associated lipids. Lipid contents vary among pollen species, ranging up to 19% of the pollen pellet dry weight (T'ai and Cane, 2000; Arien *et al*., 2015). A recent study recorded a high level of n-3 PUFAs in willow pollens and n-6 PUFAs in chestnut pollens, both preferred food for local bees (Conte *et al*., 2016). Although bees prefer to consume fresh pollens, it is very common that the pollens brought back from foragers are further processed by the hive bees to generate tighter-packed, antimicrobial bee bread, which is better for storage (Carroll *et al*., 2017). In the bee bread, lipid contents decrease to 3-8%, in which digestion of dietary lipids may occur (Human *et al*., 2006; Wright *et al*., 2017). The functional relevance of lipid digestion is to provide PUFA nutritional requirements in both individual development and colony regulation, although this aspect of honey bee nutrition is unclear.

Honey bees have been suffering colony loss over the last twenty years, of which the reason seems to be a combination of multiple environmental stressors. Understanding honey bee nutrition is critical in overcoming the decline, in the idea of preserving these critical pollinators as well as bee product makers. Nutritional deficits, including the qualitative changes in n-3 PUFAs, can impair their learning abilities (Arien *et al*., 2015). Poor nutrition also influences their fecundity, longevity, metabolic activity, body size, development of wings and internal organs such as hypopharyngeal glands. Consequences of poor nutrition may interact with pesticide exposure, as more pesticide residues were

found in honey bees consuming diets with lower floral diversity and quality (Colwell *et al*., 2017).

Insects like bumble bees and *Drosophila* can evaluate and discriminate nutritional differences of diets (Ruedenauer *et al*., 2015, 2016; Lihoreau *et al*., 2016). While honey bees are considered advanced eusocial insects, this feeding behavior is unclear with them. Pernal and Currie (2001) found that the foragers do not increase their access to pollens which rescue their experimentally-imposed protein deficiencies, but instead the forager population increases. Similarly, the foragers do not adjust the duration of the return phase or the number of dance circuits if given more protein (Beekman *et al*., 2016). Controversially, some recent studies suggest that foragers can assess pollen protein deficiencies and adjust related behaviors such as dancing (Hendriksma and Shafir, 2016; Zarchin *et al*., 2017). Nurses, who consume most of the pollen within a bee hive, do not assess pollen nutritional differences in proteins or lipids (Corby-Harris *et al*., 2018). Although it is very possible that the digestive enzymes are present in different castes and ages of bees, their nutritional assessment may vary, leading to an interesting topic of honey bee digestive physiology. My study was conducted with adult workers, without selecting a certain age of bees, which was one component of my experimental variability. I foresee the study of honey bee digestive physiology on different ages, or workers performing different hive duties.

Gut microbiota are exclusive microbial communities found in many animals, including honey bees and they provide many beneficial services to their hosts, such as contribution to digestion and nutrition, protection from parasites and pathogens and modulation of immune responses. Social insects like bees, termites and ants, have some

distinctive species which can interact by social communication (Engel and Moran, 2013). Gut microbiota influence digestion in many insect species. For example, Visôtto *et al*., (2008) found that oral administration of antibiotics can influence many digestive enzyme activities in the midgut, including general proteases, serine-proteinases and lipases. Similarly, inhibiting midgut bacteria retarded the digestion of blood proteins and reduced egg production in *A. aegypti* (Gaio *et al*., 2011). Honey bee gut symbionts showed great genetic diversity (Engel, 2013), but it is unclear how these microbial communities influence digestion, and further, regulate nutrition in the colony level.

Like other insects, honey bee digestive physiology is a large, complex and very interesting area. It involves multiple enzymes, including the $PLA_2$s, regulated by inner and outer environmental factors and is associated with nutrition intake, metabolism and immunity. What makes it even more complicated and fascinating is the nutritional regulation at the colony-level. I look forward to the outcomes of long-term field work in this area.

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