Identification of potential biomarker genes for selecting varroa tolerant honey bees (*Apis mellifera*) and biochemical characterization of a differentially expressed carboxylesterase gene in response to mite infestation

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By
Jin Wang
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ABSTRACT

Previously a large number of differentially expressed genes were identified by a DNA microarray analysis of two contrasting honey bee colonies for tolerance and susceptibility to varroa mite infestation. This study initially analyzed the expression patterns of ten of these genes in detail for a wide range of colonies with a range of phenotypes for susceptibility and tolerance to varroa mite infestation using real time qRT-PCR. Dark eyed stage 4 pupae with and without varroa infestation were sampled for the molecular analysis. The results showed that three out of the ten genes, *AmCbE E4*, *AmApoD* and *AmCYP6A1* displayed relatively consistent differential expression patterns among the colonies and could be used as potential biomarkers for identifying varroa tolerant colony phenotypes. In general, these biomarker genes exhibited higher expression in tolerant colonies and lower expression in susceptible colonies with varroa mite infestation, compared to non-infested colonies. Tissue expression analysis showed *AmCbE E4* was more differentially expressed in the head and *AmApoD* was differentially expressed in the abdomen, and *AmCYP6A1* showed more differential expression in the thorax and abdomen among the honey bees differing in varroa tolerance and susceptibility. Expression of the three genes also responded to miticide treatments in the colonies. The miticide treatments (Apistan®, Apivar®, Thymovar®) could stimulate their expression in tolerant colonies, but not in susceptible colonies. In addition, the infection of deformed wing virus (DWV), another biotic stressor for honey bees primarily vectored by the mite, was also quantitatively evaluated by real time qRT-PCR in the varroa tolerant and susceptible honey bee colonies. The results showed that DWV infections were considerably increased in the susceptible colonies infested by varroa mites or treated with miticides (Apistan®, Apivar®, Thymovar®). *AmCbE E4* encoding a putative esterase E4 was identified for its highly differential expression between the susceptible and tolerant bees in response to the mite infestation. Its biochemical function was analyzed by cloning the *AmCbE E4* from the head of the dark eyed stage 4 pupae and heterologously expressing it in *E. coli*. The enzymatic assays revealed that AmCbE E4 could hydrolyze synthetic esterase substrates, α-naphthyl acetate, β-naphthyl acetate and para-nitrophenyl acetate, as well as carbaryl, a carbamate pesticide. This result suggests a defensive function of *AmCbE E4* in protecting the varroa tolerant bees from the toxic stresses of carboxylester miticides and ester compounds possibly produced by the *Varroa destructor* parasitism.

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LIST OF ABBREVIATIONS

ABPV: acute bee paralysis virus

AmApoD: honey bee apolipoprotein D

AmCbE E4: honey bee esterase E4

AmCYP6A1: honey bee cytochrome P450 6A1

AmCYP6A14: honey bee cytochrome P450 6A14

AmCYP6BD1: honey bee cytochrome P450 6BD1

AmCYP6BE1: honey bee cytochrome P450 6BE1

AmCYP9E2: honey bee cytochrome P450 9E2

AmHIP14: honey bee huntingtin-interacting protein 14

AmHsp90: honey bee heat shock protein 90

AmSPH51: honey bee serine protease homolog 51

ANOVA: analysis of variance

ApoD: apolipoprotein D

BLASTn: nucleotide-nucleotide basic local alignment search tool

BLASTx: nucleotide-protein basic local alignment search tool

BSA: bovine serum albumin

CbEs: carboxylesterases

CBPV: chronic bee paralysis virus

CBS: Center for Biological Sequence Analysis

CCD: colony collapse disorder

CCEs: carboxyl/cholinesterases

cDNA: complementary DNA

Contigs: contiguous sequences

CYPs: cytochrome P450s

DGE: digital gene expression

dNTP: deoxynucleotide 5'-triphosphate

dsRNA: double-stranded RNA

DWV: deformed wing virus

E. coli: *Escherichia coli*

EST: expressed sequence tag

G4-: the susceptible honey bee phenotype (G4) without varroa mite infestation (-)

G4+: the susceptible honey bee phenotype (G4) with varroa mite infestation (+)

GABA: gamma-aminobutyric acid

Glu/E: glutamic acid

GMOs: genetically modified organisms

GSTs: glutathione-S-transferases

His/H: histidine

HTT: huntingtin

IAPV: israeli acute paralysis virus

IPTG: Isopropyl β-D-1-thiogalactopyranoside

KBV: kashmir bee virus

LSD: Fisher's Protected Least Significant Difference

LSV: lake sinai virus

miRNA: microRNA

mRNA: messenger RNA

NCBI: National Center for Biotechnology Information

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

OIE: World Organization for Animal Health

ORF: open reading frame

PCR: polymerase chain reaction

PMRA: Pest Management Regulatory Agency

PMS: parasitic mite syndrome

PPhs: primer pheromones

QMP: queen mandibular pheromone

Real time qPCR: real time quantitative polymerase chain reaction

Real time qRT-PCR: real time quantitative reverse transcription-polymerase chain reaction

RISC: RNA-induced silencing complex

RNAi: RNA interference

RpS5: ribosomal protein S5

rRNA: ribosomal RNA

RT-PCR: reverse transcription-polymerase chain reaction

S88-: the tolerant honey bee phenotype (S88) without varroa mite infestation (-)

S88+: the tolerant honey bee phenotype (S88) with varroa mite infestation (+)

SAS: statistical analysis system

SBV: sacbrood virus

CSBV: chinese sacbrood virus

SDS–PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

Ser/S: serine

siRNA: small interfering RNA

SSH: suppression subtractive hybridization

UDG: uracil DNA glycosylase

UTR: untranslated region

VSH: varroa sensitive hygiene

1. INTRODUCTION

The honey bee (*Apis mellifera* L.) is an insect in the animal kingdom, recognized for its social behavior and economic benefits to agriculture and global food production. Honey bees are the most commonly managed bees in the world (vanEngelsdorp and Meixner 2010), contributing multi-billion dollars to the global economy annually. For instance, global production of honey is estimated at approximately 1.7 million metric ton in 2013, increasing 2.5-fold since 1961 (Moritz and Erler 2016). Furthermore, almost 50% of leading global commodity foods depend on pollination by honey bees for either fruit formation or seed set (Klein et al. 2007), as the bees are the most efficient pollinators for most crops, vegetables and fruit trees (Delaplane et al. 2000; McGregor 1976).

In recent years, large scale of enigmatic losses of honey bee colonies have been occurring worldwide, most commonly in North America and some European countries (vanEngelsdorp and Meixner 2010). Apiculture has identified a set of symptoms termed as "Colony Collapse Disorder" (CCD) to denote this unexpected phenomenon (Evans et al. 2009). To understand the mechanism underlying the CCD, a number of traditional and modern techniques, such as sequencing technologies (Cox-Foster et al. 2007), real time qRT-PCR (Bourgeois et al. 2010; Dainat et al. 2012), DNA microarray (Glover et al. 2011; Jiang et al. 2016), proteomics (Han et al. 2013) and RNAi (Campbell et al. 2016; Desai et al. 2012; Garbian et al. 2012) have been recently used for identifying the primary causes and the possible curative strategies. Through these studies, myriad factors have been suggested to give a rise to honey bee colony losses. Among them, *Varroa destructor*, an ecto-parasite of domesticated honey bee (*Apis mellifera*), has been regarded as a flagship stressor (Villalobos 2016). There are two major adverse impacts imposed by the varroa mite: firstly, the mite sucks a substantial amount of hemolymph from the host (larvae, pupae and adult bees), resulting in the honey bee lacking essential nutrients and becoming underdeveloped and weakened (Kanbar and Engels 2003); secondly, it vectors at least eighteen different viruses and spreads the diseases within and among bee colonies, resulting in

"parasitic mite syndrome (PMS)" (Chen and Siede 2007; Mariani et al. 2012; Shimanuki et al. 1994; Villalobos 2016). By 2002, the varroa mite had spread to most beekeeping regions across Canada (Currie et al. 2010), and it is believed that *Varroa destructor* infestation is the main culprit for the population reduction of overwintered honey bee colonies in Ontario (Guzmán-Novoa et al. 2010).

Miticide treatments were introduced as a management strategy for controlling varroa mite (Johnson et al. 2010). However, application of miticides can cause serious contamination of honey bee products, impose adverse effects on honey bee health and impart the pesticide tolerance of varroa mites (Chiesa et al. 2016; Johnson et al. 2013a; Johnson et al. 2010; Lodesani and Costa 2005; Martel et al. 2007). Therefore, breeding varroa tolerant honey bees is considered a valuable strategy to control varroa mite parasitism (Dietemann et al. 2012). Natural selection is thus used to select colony phenotype with varroa tolerant in France (Le Conte et al. 2007), USA (Seeley 2007), Sweden (Locke et al. 2014) and Canada (Jiang et al. 2016; Robertson et al. 2014). The Varroa Sensitive Hygiene (VSH) behavior in honey bees is recognized as one of the more desirable traits able to suppress varroa population growth (Harbo and Harris 2009; Tsuruda et al. 2012). However, selecting varroa tolerance by natural selection (colony survival in the absence of miticide treatments) is difficult and labor intensive, and requires several years of analyses to determine the varroa tolerant colony phenotypes, due to lack of understanding of the mechanism of honey bee tolerance to the mite infestation. Therefore, identification of genes and molecular markers that underlie the tolerant mechanism or are tightly associated with the tolerant traits would be beneficial in rapidly selecting the tolerant phenotypes over the course of months instead of years. It may also predict possible biological mechanisms involved in the complex varroa tolerant traits.

To identify genes that can distinguish varroa tolerant honey bee phenotypes, a high throughput DNA microarray was constructed to investigate genome-wide differential gene expression in two contrasting honey bee colony phenotypes for tolerance (S88) and susceptibility (G4) (Jiang et al. 2016). More than 200 genes were identified with differential expression patterns in these two extreme colony phenotypes. These genes were classified into functional groups that are related to

olfaction, signal transduction, detoxification processes, protein and lipid metabolisms as well as exoskeleton formation, suggesting possible defensive mechanisms of varroa tolerant honey bees in combating with the mites. To confirm the differential expression patterns of the genes and assess their utility as potential biomarkers for selecting varroa tolerant colony phenotypes, the present study analyzed the differential expression patterns of ten genes selected from previous DNA microarray (Jiang et al. 2016) by using real time quantitative PCR in a wide range of honey bee colonies with varying degrees of varroa tolerance to varroa parasitism. The effects of three commonly used miticides (Apistan®, Apivar®, Thymovar®) on the expression of three potential biomarker genes were also investigated. In addition, one of the potential biomarker genes, *AmCbE E4* encoding a putative esterase E4, was further heterologously expressed and functionally characterized in *E. coli*.

2. LITERATURE SURVEY

2.1 Honey bee

2.1.1 Growth and development of the domesticated honey bee (*Apis mellifera* L.)

The honey bee (*Apis mellifera* L.) is an insect well known for its social organization and economic benefits. Honey bees are originally from the southern part of Africa, but with wide environmental adaptation, they have been successfully expanded around the world (Winston 1991). Human transportation also plays an important role in their expansion, stretching from northern Europe to central Asia and Americas (Whitfield et al. 2006). Currently, honey bees have become the most commonly managed bees in the world (vanEngelsdorp and Meixner 2010).

Based on the division of labor tasks, honey bees have three types of colony members or castes: queen, drone and worker bee (Winston 1991). Normally there is only one queen in a colony, who can lay up to thousands of eggs per day once mated (Snodgrass 1984) and produce a variety of primer pheromones (PPhs) affecting worker bee's behavior (Hoover et al. 2003; Pankiw et al. 1998). PPhs released from the queen have been used as model bioactive compounds to elucidate the complexity of pheromonal communication in social insects. For instance, queen mandibular pheromones (QMP) secreted from the mandibular gland of queen bees can inhibit the reproductive ability of worker bees by suppressing the growth and development of workers' ovaries resulting in only 0.01% of the workers containing full-sized eggs in their ovaries (Conte and Hefetz 2008). Depending on the seasons, drones living with the queen and worker bees in a hive range from zero to several thousand (Fathian et al. 2007). Drones are produced by the queen with only one responsibility, mating with a virgin queen during the mating flight. After the copulation, the drones complete their task and die (Fathian et al. 2007). Worker bees are also produced by the queen, and usually perform a wider range of tasks than either queens or drones (Winston 1991). Interestingly, the task specialization of workers corresponds to their ages

(Kolmes 1985). For instance, younger workers are found inside the hive, and their main duty is to clean the cells, take care of brood, build comb and store food, whereas older workers guard the entrance and forage outside to gather food for the hive (Moore et al. 1987).

Development of all three castes of bees goes through four major stages: egg, larva, pupa and adult (Figure 2.1) (Winston 1991). At the very beginning of the larval stage, all fertilized eggs are fed with royal jelly. However, when a larva is fed with royal jelly for the whole larval stage, it will develop into a queen bee; when a larva is fed with honey and pollen at the late larval stage, it will develop into a worker bee. The three major body parts of honey bees, head, thorax and abdomen, appear at the pupal stage. During this stage, the color of both bee eyes and body changes from white to dark. Mature adults get out by breaking the cap of the cell (Winston 1991). Conditions, such as temperature, nutrition and the honey bee genotype can influence the duration of the developmental stages and quantity and quality of the bees in a colony (Page and Peng 2001).

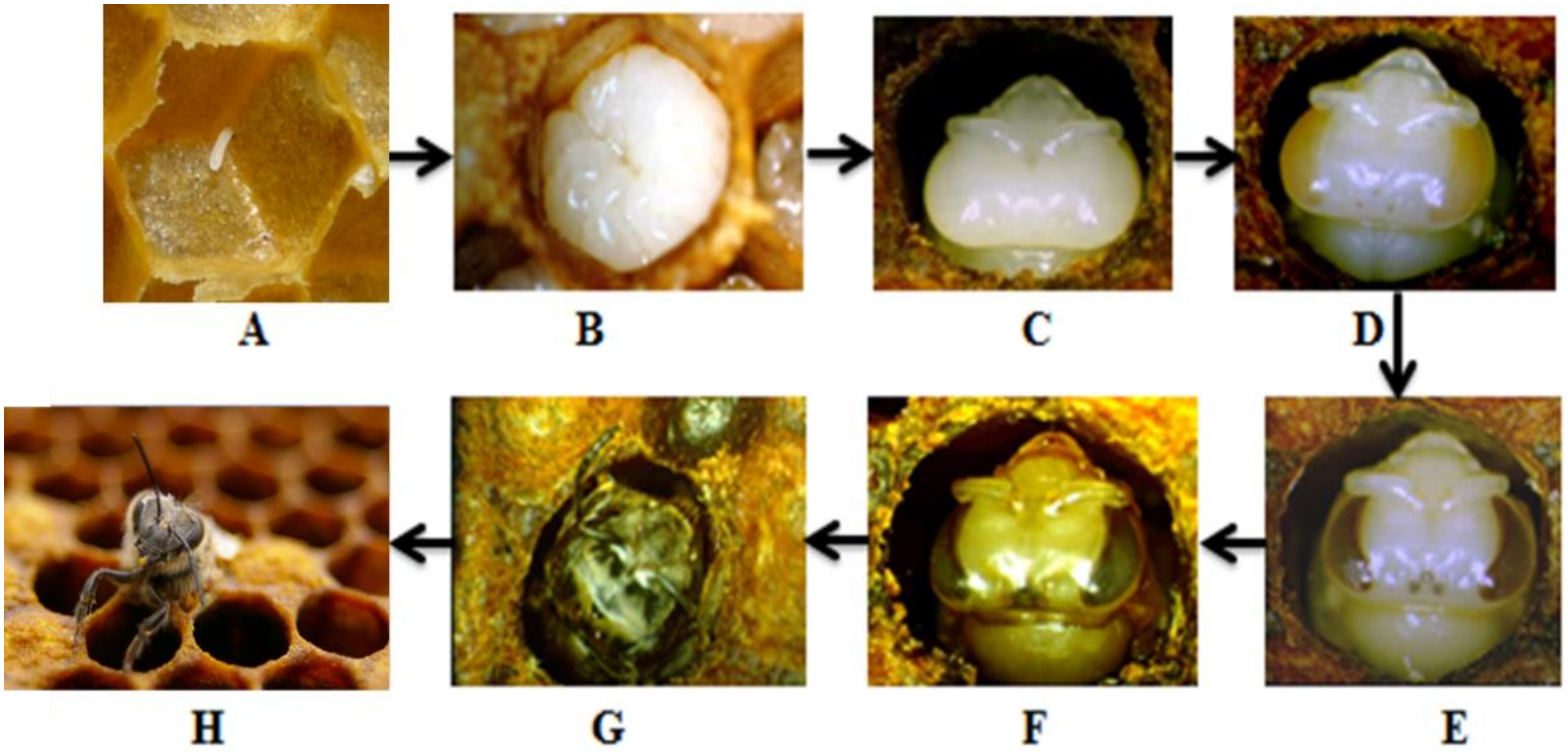

Figure 2.1 Developmental stages of honey bees (*Apis mellifera*).

A. Egg laid in the bottom of a brood cell; B. Larvae; C-F. Pupa: C. White eyed pupae, D. Pink eyed pupae, E. Dark eyed pupae, F. Dark body pupae; G. Pre-emerge adult; H. Adult. (Modified from https://resistantbees.com/blog/?page_id=1757)

2.1.2 Economic benefits from honey bees

The honey bee is one of the most important social insect providing a wealth of economic benefits for human beings. It is well known that a variety of bee products, such as honey, royal jelly, propolis and beeswax, are produced by honey bees. Honey has been used as a traditional medicine since ancient times (Gómez-Caravaca et al. 2006), and is currently considered as a healthy product for human consumption (White Jr 1978). The predominant components of honey are sugars, such as fructose (38%) and glucose (31%), which are commonly used as a sweetener (White Jr 1978). In addition, some minor compounds have been identified in honey, such as vitamins, minerals and antioxidants (phenolic compounds) (Gheldof et al. 2002). The functional properties of honey have been suggested, including anti-bacterial, anti-oxidant, anti-tumor, anti-inflammatory, anti-enzymatic browning and anti-viral capacities, mainly due to the existence of the phenolic compounds (Ahmed and Othman 2013; De la Rosa et al. 2011; Estevinho et al. 2008; Kassim et al. 2010; Watanabe et al. 2014). In 2013, the global production of honey was estimated at approximately 1.7 million metric tons, increasing 2.5-fold since 1961 (Moritz and Erler 2016). In the US alone, the wholesale value of honey was estimated at more than $320 million dollars in 2014 (Dennis and Kemp 2016).

Honey bees play an important role in the pollination of plants in agriculture. Up to 22.6% of all agricultural production benefits from animal pollination in developing countries and 14.7% in the developed countries (Aizen et al. 2008). The commercial value of insect pollination for agricultural products is estimated at approximately US$19.8 billion in Europe and US$20.1 billion in North America (Gallai et al. 2009). Honey bees are considered as the most efficient pollinators in agriculture (Delaplane et al. 2000; McGregor 1976). Almost 50% of leading global commodity foods depend on pollination for fruit formation and seed set (Klein et al. 2007). Without honey bee pollination, honey bee-dependent crops and fruits would have a reduced yield, fruit or seed size, and nutrition (Klein et al. 2007). Because of their importance, honey bees are managed worldwide by beekeepers to improve the crop yield. The honey bee is an important link in the ecological chain, and without this pollinator, the whole ecosystem would suffer or collapse (Evans and Schwarz 2011). The honey bee is sensitive to the environmental changes from the surrounding living species, air quality, temperature, precipitation, light, floral

colors and plants odors, thus it is used as a monitor of the ecological environment (Porrini et al. 2003; Williams et al. 2010).

2.1.3 Honey bee health

Since honey bees are critical to nature and human society, governments and scientific communities around the world pay special attention to the bee population's health. Over the past 50 years the bee population has experienced a significant global increase of colonies by more than 60%, however not all regions have seen equal growth of the honey bee population during this period of time (Moritz and Erler 2016). Particularly in recent years, a large scale of enigmatic loss of bee colonies has been observed in the United States and some European countries (vanEngelsdorp and Meixner 2010). Honey bee colonies were reduced by 26.5% in Europe and 49.5% in the United States in 2009 (vanEngelsdorp and Meixner 2010). The Bee Informed Partnership reported that in the past eight years, 66% of the beekeepers in the U.S. have experienced large losses of honey bee colonies, sometimes referred to as "Colony Collapse Disorder (CCD)" (Lee et al. 2015; vanEngelsdorp et al. 2014).

2.2 Colony collapse disorder

2.2.1 Symptoms of colony collapse disorder

The specific symptoms of honey bee colonies undergoing colony collapse disorder are as follows: (1) a sudden drop of adult worker bees in the affected colonies with only some immature bees (broods) and food left in the hives (Figure 2.2); (2) no dead bodies of worker bees are found within and surrounding the affected colonies; (3) no obvious invasion of hive pests, such as small hive beetles and wax moths, and kleptoparasitism from neighboring honey bee colonies (Evans et al. 2009). The mechanism underlying CCD is still mysterious, even though many possible causes for it have been proposed, reported and discussed in the scientific literature or popular media.

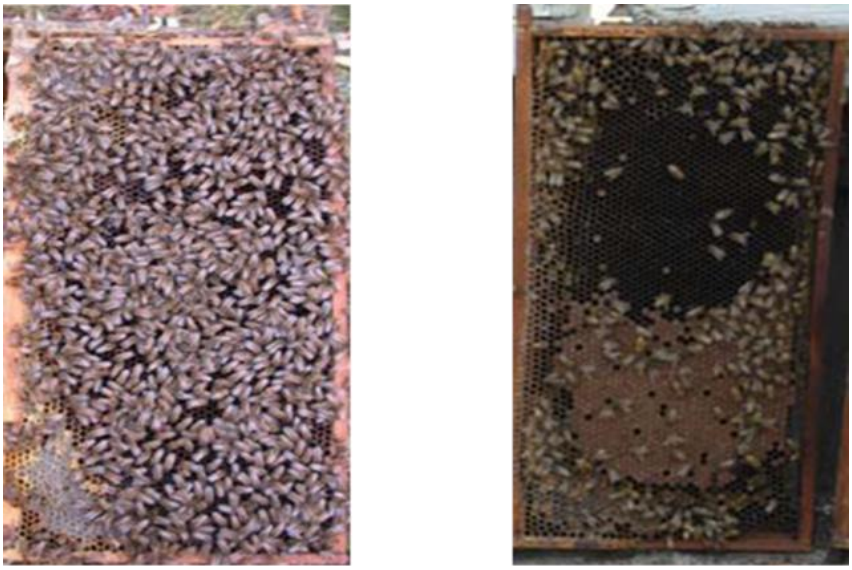

Figure 2.2 Honey bee colony in collapsing disorder.

A. A frame of a healthy honey bee colony, B. A frame of a collapsing honey bee colony. (Modified from https://beespotter.org/topics/ccd/)

2.2.2 Primary causes of colony collapse disorder

Due to large-scaled honey bee colony losses, a number of studies have been conducted to elucidate the primary causes of CCD using functional genomic techniques such as new sequencing technologies (Cox-Foster et al. 2007), real time qPCR (Bourgeois et al. 2010; Dainat et al. 2012), DNA microarray (Glover et al. 2011; Jiang et al. 2016) and proteomics (Han et al. 2013). The results showed that the major responsible factors are likely diseases and parasites that contribute to the health aggravation of honey bee colonies, resulting in a large and swift decline of the population. As a social group, honey bees are readily infested and infected by fungi, bacteria, virus and parasitic mites or harmed by predators (beetles and humans) (vanEngelsdorp and Meixner 2010). In addition, other stressors including pesticide application, and climate have been proposed to influence the population size and health of honey bees (vanEngelsdorp and Meixner 2010). For example, pesticide neonicotinoids, coumaphos and fluvalinate, have been suggested to contribute to honey bee colony losses recently (Johnson 2015; Schmehl et al. 2014).

High-throughput sequencing analysis of the microflora associated with CCD of honey bees (Cox-Foster et al. 2007) reveals five major groups of bacteria, Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Firmicutes and Actinobacteria and four major lineages of fungi, Saccharomycotina, Microsporidia, Mucoromycotina and Entomphthoromycotina are involved. Microsporidia comprises the important bee pathogens, *Nosema apis* and *Nosema ceranae*, which can cause digestive disorders, and shorten the life span of honey bees. Mucoromycotina includes *Mucor hiemalis*, a species known to kill honey bees under certain conditions (Cox-Foster et al. 2007).

Pathogenic viruses have been identified to affect honey bee health (Glover et al. 2011). For instance, deformed wing virus (DWV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), Israeli acute paralysis virus (IAPV), kashmir bee virus (KBV), sacbrood virus (SBV) and lake sinai virus (LSV) have recently been found in honey bees by deep RNA sequencing (Cornman et al. 2012). Most viruses, such as IAPV, CBPV, SBV, and DWV are probably associated with colony collapse disorder (Cox-Foster et al. 2007). The Guts serve as an important interface between honey bees and pathogens or toxins (Johnson et al. 2009a). The whole-genome microarray analysis of transcripts in guts from CCD bees and healthy bees revealed that the increased expression of honey bee ribosomal RNA fragments and DWV simultaneously occurred in the CCD bees, regardless of their collection from different geographical locations (Johnson et al. 2009a). In addition, high loads of a diverse set of the viruses can deplete the honey bee worker population rapidly and turn the whole colony to be vulnerable (Cornman et al. 2012). These results suggested a positive correlation between virus loads and survival numbers of bee colonies, and the importance of the viral pathogens to CCD. Furthermore, the pathological mechanism of honey bee (*Apis cerana*) sacbrood disease caused by Chinese sacbrood virus (CSBV) was recently investigated by an integrated proteomics investigation (Han et al. 2013). Expression of 142 proteins and 12 phosphoproteins were down-regulated in the infected larvae, and 38 proteins and 7 phosphoproteins are up-regulated. Expression-altered proteins were related to carbohydrate and energy metabolism, protein synthesis and folding as well as cytoskeleton formation and development. The infected larvae with the abnormal processes stop development and die eventually (Han et al. 2013).

In order to identify predictive colony collapse markers, Dainat and colleagues screened eleven disease agents and genes involved in immunity of honey bees in the monitored colonies (Dainat et al. 2012). The varroa mite (*Varroa destructor*) was identified as a dominant cause among the factors for colony loss. The number of *V. destructor* mites collected in the dying colonies consistently exceeded that in the surviving colonies during the whole year, even though some organic acids (oxalic acid and formic acid) were applied to control the mite infestation during the fall (Dainat et al. 2012). The parasitic varroa mite was found to possess the most dominant lethality to honey bee colonies by "parasitic mite syndrome (PMS)" (Shimanuki et al. 1994), comprehensive symptoms of honey bees caused by synergetic actions of viruses and the varroa mite.

2.3 Varroa mite

2.3.1 General introduction

The varroa mite, *Varroa destructor* (Anderson and Trueman 2000), is widely recognized as a severe ecto-parasitic pest of honey bees. It spreads rapidly and can infest almost all types of honey bees in the world (Le Conte et al. 2010). *V. destructor* causes a clinical symptom of honey bee colonies called "varroosis" describing a high rate of infestation and the secondary infestation by varroa mites (Boecking and Genersch 2008). Originally *V. destructor* executes parasitism on *Apis ceranae* in Asian countries and later it expands parasitism on *Apis mellifera* in western countries. Most likely due to shipments and importations, this new parasite was introduced into the western countries (Boecking and Genersch 2008). As there is no natural balance between the new host and parasite, *V. destructor* rapidly spreads and brings deadly impacts on *A. mellifera* bees around western countries in a few years (Rademacher and Harz 2006). In Canada, varroa mites were first found in New Brunswick in 1989 (The Canadian Honey Council, http://www.honeycouncil.ca/index.php). By 2002, the mites had spread to most beekeeping regions across Canada (Currie et al. 2010), and now *V. destructor* is the main cause of population reduction of overwintered honey bee colonies in Canada (Guzmán-Novoa et al. 2010).

2.3.2 Impact of varroa mite in apiculture

V. destructor has spread worldwide within a short period of time and it is now hard to find a "Varroa free" honey bee colony anywhere in the world, except in Australia (Rosenkranz et al. 2010). Varroa mites directly affect the population size of managed honey bees and inflict much greater damage and higher economic costs than any other known apicultural diseases (Dainat et al. 2012). Beekeepers have a limited number of effective control measures for varroa mites with only a few synthetic miticides showing good efficacy (Johnson et al. 2010).

V. destructor parasitizes honey bees over the host entire life cycle and it cannot survive without nutrition from the host (Rosenkranz et al. 2010). Varroa females have two distinct phases in their life cycle: a phoretic phase on adult bees and a reproductive phase in brood cells. To reproduce, varroa females enter the brood cells before the cells are sealed (Boot et al. 1992). After the cell capping, a mother mite (foundress) lays her eggs inside which can produce as many as 10 progenies (Sammataro et al. 2000). All the mites in a cell suck the hemolymph from the single feeding object (Kanbar and Engels 2003; Nazzi and Le Conte 2015). The mites are attached to the surviving adult bee when it emerges (Rosenkranz et al. 2010). In addition, the varroa mite can also release bioactive secretions that are detrimental to the host (Kanbar and Engels 2003; Kanbar and Engels 2005). The secretions contain anti-immune/anti-healing factors that can keep the puncture wound open in the body cuticle of the bee, thus the mite re-visitors and pathogens are benefited from a communal feeding and infection site (Kanbar and Engels 2003; Kanbar and Engels 2005). During its life cycle, varroa mite and its offspring suck a substantial amount of hemolymph from larvae, pupae and adult bees (Figure 2.3). A lack of essential nutrients leads to weight loss, underdevelopment and vulnerability of the parasitized bees (Rosenkranz et al. 2010). In comparison, *V. destructor* are rarely found in queen cells, while the drone brood and worker brood of *A. mellifera* are heavily infested with varroa mite, and drone brood even more (Duay et al. 2003; Fuchs 1990).

In addition, the varroa mite can also serve as a vector to spread pathogenic viruses among colonies, causing so called "parasitic mite syndrome (PMS)" (Martin et al. 2012; Shimanuki et al. 1994). So far, eighteen viruses have been isolated from honey bees (Chen and Siede 2007)

and many of them are vectored by varroa mites, such as DWV, ABPV, CBPV, IAPV, KBV and SBV (Boecking and Genersch 2008). DWV is the most well-known virus which can cause the typical symptoms of crippled wings and shorten abdomen in heavily infected honey bee (Boecking and Genersch 2008; De Miranda and Genersch 2010; Martin et al. 2012). A mutualistic symbiosis between DWV and parasitic mites has been suggested to contribute to CCD (Di Prisco et al. 2016). DWV negatively affects the humoral and cellular immune responses of honey bees, resulting in increasing reproduction of parasitic mites in the impaired host (Di Prisco et al. 2016). Along with the increasing number of honey bees infested by varroa mites, the overall reproductive capacity of honey bee colonies declines.

2.3.3 Varroa control

Varroa mite infestation of domesticated honey bees has spread worldwide, and limited control measures are available. More and better varroa control measures are needed to prevent increased colony deaths from varroa parasitism (Rademacher and Harz 2006). Therefore, governments around the world have been increasing the financial support in scientific research to study the mite parasitism of honey bees and pest control methods to solve the problem. Unfortunately, the progress is still limited (Rademacher and Harz 2006).

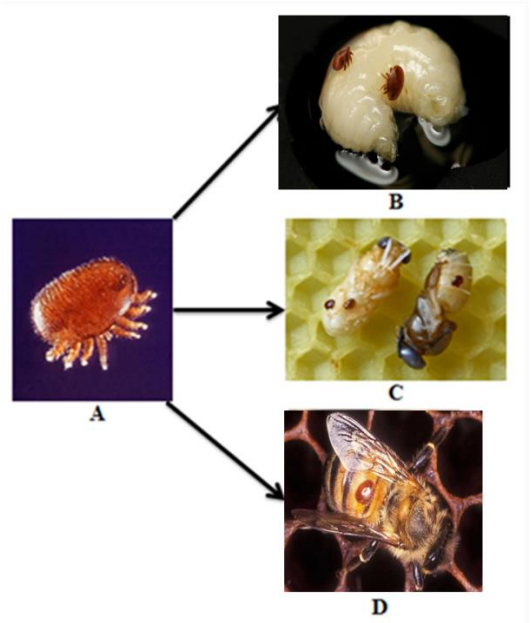

Figure 2.3 Varroa mite-infested honey bees.

A. A *Varroa destructor* female; B. Varroa attached to a larval bee; C. Varroa attached to a pupal bee; D. Varroa attached to an adult bee. (Modified from http://entnemdept.ufl.edu/creatures/misc/bees/varroa_mite.htm and https://en.wikipedia.org/wiki/Varroa_destructor)

2.3.3.1 Synthetic pesticide

Applying synthetic pesticides/miticides directly to the varroa mite-infested colonies is a quick and effective way to control mite reproduction. In recent years, various pesticides have been developed and used by bee industries (Milani and Iob 1998). Miticide treatments have positive effects on reducing the number of parasitic mites in infested honey bee colonies (Locke et al. 2012). Major synthetic acaricides commonly applied to honey bee colonies are organophosphates such as coumaphos (Checkmite®, Asuntol®, Perizin®), pyrethroids such as tau-fluvalinate (Apistan®, Klartan®, Mavrik®) and flumethrin (Bayvarol®), and the formamidine amitraz (Apivar®). These pesticides interference with nerve signaling and energy metabolism in varroa mites and finally kill the mites (Johnson et al. 2010). However, broad and long term usage of these pesticides probably brings more disadvantages than advantages, such as miticide contamination left in bee products, adverse effects on overall honey bee health and generation of varroa mites tolerant to pesticide (Chiesa et al. 2016; Johnson et al. 2013a; Johnson et al. 2010;

Maggi et al. 2012; Martel et al. 2007). Ironically, the pesticide application has also been suggested as an acute stressor likely contributing to colony losses, as evidenced by increased viral loads of honey bees when exposed to pesticides/miticides (Doublet et al. 2015; Locke et al. 2012; Smart et al. 2016). For instance, exposure to neonicotinoid insecticides, clothianidin and imidacloprid, resulted in honey bees becoming more susceptible to DWV infection, due to hosts' reduced immune competence by adversely modulating NF-κB signaling pathways (Di Prisco et al. 2013). Moreover, honey bees lack the capacity to metabolize pesticides, and cannot avoid harm when encountering them (Johnson et al. 2010).

2.3.3.2 Natural chemical compounds

Natural chemical compounds with negligible toxicity to honey bees have been attempted in the control of varroa mite. For instance, plant-derived thymol products, such as Thymovar®, Apilife Var® and Apiguard®, increased mite mortality by approximately 17.8% in cool temperate climates (Coffey and Breen 2013). Recently, the efficacy of Thymovar® against *V. destructor* as a fall treatment was increased to 26.7% (Al Naggar et al. 2015). The methanolic extracts from plant *Lepidium latifolium* and *Zataria multiflora* also elevated varroa mite motility in the field practice (Razavi et al. 2015). Organic acids, such as formic acid and oxalic acid, are also able to control mite populations (Goswami and Khan 2013; Rashid Mahmood et al. 2013), and the mechanism of these natural compounds for varroa mite control is probably employed by the neuro-excitatory effect on the neurons and the interference with the energy metabolism in the mitochondria (Johnson et al. 2010).

The advantages of using the naturally occurring products to control mite populations are environmental friendliness, a lower risk of residue accumulation in honey bees and bee products, and a lower probability of the appearance of mite pesticide resistance, compared to that of synthetic pesticide (Rosenkranz et al. 2010; Serra Bonvehí et al. 2016). However, the disadvantages of this method are treatment conditions and variable efficacies in the control (Adjlane et al. 2015). Therefore, finding the appropriate condition for highly effective application of naturally occurring compounds to control varroa mites in honey bee hives is still a challenging task (Emsen and Dodologlu 2009).

2.3.3.3 Biological agents

Based on the biology of a parasite and its host, a biological method is also considered as a promising alternative for the control of the varroa mite. Pathogenic organisms have been used to control varroa mites, as they could reduce toxic residue contamination in bee products and the adverse effects of chemical compounds on honey bee health. Entomopathogenic fungi *Metarhizium anisopliae*, *Beauveria bassiana* and *Clonostachys rosea* can ease varroa mite damage to honey bee brood by increasing the parasite's mortality and preventing varroa-associated suppression of honey bee immunity (Hamiduzzaman et al. 2012; Meikle et al. 2012; Pirali-Kheirabadi et al. 2013; Sewify et al. 2015). However, fungal pathogens take time, as many as several days, to be effective (Chandler et al. 2001). In addition, the effect is very much dependent on their adaptation to the local climate and honey bee brood conditions (Chandler et al. 2001). The bacterial pathogen *Serratia marcescens* is also considered as a biocontrol agent for varroa mite control (Tu et al. 2010). These antagonists can spread independently among colonies and provide long-term effects on mite control, although the efficacy is still low and variable (Van der Geest et al. 2000).

2.3.3.4 Molecular interference

RNA interference (RNAi) is a powerful technique to biologically silence specific genes for pathogenic intervention. Two types of small RNA molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are central to RNA interference, relying on the formation of double-stranded RNA (dsRNA). The small RNA molecules combining with RNA-induced silencing complex (RISC) aim at specific mRNA sequences to prevent the expression at the post-transcriptional stage. It has been used successfully to silence genes in a range of prokaryotes, nematodes and other invertebrate animals to date (Geley and Müller 2004), and conjectured as a possible way for controlling viral diseases (Desai et al. 2012; Hunter et al. 2010; Maori et al. 2009; NOH et al. 2012) and the prevalence of varroa mites in honey bees (Garbian et al. 2012).

The dsRNA molecules, specific to varroa genes, could be transferred between the honey bee host and varroa mite for silencing the varroa genes and increasing the varroa mortality (killing over 60% of mites) in a 60-day experiment (Garbian et al. 2012). In addition, dsRNAs are stable for

the silencing effect and are friendly to the host and environment. Furthermore, as target genes are specifically defined, it is difficult for the mite to develop resistance (Garbian et al. 2012). Therefore, transferring of gene-silencing-triggering dsRNA molecules between the host and its ecto-parasite could lead to a conceptually novel approach for the control of varroa in apiculture. However, this new method is still at its early stages of development and requires further validation.

2.4 Molecular analysis of mechanisms underlying tolerance of the honey bee to varroa mite parasitism

2.4.1 General introduction

Honey bee health is a decisive factor in apiculture. Expansion of global trade and exchange of bees between countries has contributed to the spread of parasites and pathogens in bee populations (Boecking and Genersch 2008). Because the current methods used to control varroa mites are inadequate, breeding honey bees with tolerance to pathogenic infections and pest infestation would be an ideal solution (Dietemann et al. 2012). This approach is cost effective, environmentally friendly, and with no chemical residue issues. However, it is time-consuming and labor intensive, and requires several years of observation to define colony phenotypes. Honey bee tolerant mechanisms to varroa mite infestation are not well understood at the molecular level, and there are few suitable biomarkers to identify the trait and select varroa tolerant colonies, which makes breeding bees tolerant to the varroa mite a challenging task.

New genomic technologies have been successfully applied to obtain the genetic information of honey bees in response to the mite infestation. The whole genome of honey bees has recently been sequenced and large amounts of sequence data provide an integrated and comprehensive genetic resource for molecular studies on the interaction between honey bees and mites (The Honey bee Genome Sequencing Consortium 2006). Compared to other sequenced insect genomes, such as the fruit fly *Drosophila melanogaster* genome (13,500 genes) and the mosquito *Anopheles gambiae* genome (14,000 genes), honey bee genome contains less annotated genes (only about 11,000 genes) (Holt et al. 2002). This is suggested that honey bee has more

highly specialized genetic information than other insects attributed to pre-adults that have limited exposure to the external environment during its life span and this feature may eliminate certain functional genes involved in environmental interactions in honey bee genome. Technologies for gene cloning and expression analysis also provided numerous opportunities for studying defensive mechanisms of honey bees infested by varroa mites (Garrido et al. 2013). Suppression subtractive hybridization (SSH) had been developed to investigate differentially expressed genes of honey bees induced by *V. destructor*, revealing possible molecular mechanisms of varroa tolerance (Zhang et al. 2010). Digital gene expression (DGE) analysis of bee abdomens indicated that varroa parasitism increased viral population (DWV) and decreased protein metabolism in honey bees (Alaux et al. 2011; Navajas et al. 2008). DNA microarray and RNA sequencing, two powerful genomic tools for transcript profiling, identified a large number of insect genes that were differentially expressed under different parasitic and pathogenic conditions (Jiang et al. 2016; Le Conte et al. 2011; Mondet et al. 2015). Characterized by high sensitivity and specificity, polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) methods can offer fast detection of pathogens and accurate identification of pathogenic species (Dainat et al. 2012). These above methods are being used to explain the molecular mechanisms underlying the pathogenesis and parasitism of honey bees.

2.4.2. High throughput molecular analysis

RNA sequencing was recently performed to investigate the mechanism of VSH behavior, as the VSH bees can remove the varroa-infested broods from the frames to suppress the growth and spread of the varroa mite (Mondet et al. 2015). Comparison of antennal transcriptomes of bees that do and do not perform VSH behavior indicated that antennae likely plays a key role in the expression of VSH behavior. A total of 258 coding transcripts were found to be differentially expressed (173 up- and 85 down-regulated) in the antennae of VSH as compared to non-VSH bees. Biological functions of these genes could be attributed to metabolism (general metabolism and oxidative phosphorylation), motor activity and neuronal process. It was suggested that VSH bees could not only enhance the detection of certain odorants but also increase metabolism and antennal motor activity to response the varroa parasitism (Mondet et al. 2015).

DNA microarrays with hundreds of molecular probes are used to detect the differential expression of a vast number of genes in a genome under different conditions. The analysis could be used to observe the interaction between pathogen and host (Cummings and Relman 2000). Availability of this genomic resource for both honey bees and their primary parasites and pathogens (e.g. *V. destructor* and P. *larvae*) allows us to identify the possible mechanisms underlying host genetic susceptibility and pathogenesis (Evans et al. 2006). High throughput DNA microarray analysis was previously used to investigate genome-wide gene expression of the varroa tolerant (S88) and varroa susceptible (G4) honey bee phenotypes with and without varroa infestation (Jiang et al. 2016). The result showed that 106 genes at the pupal stage and 50 genes at the adult stage were significantly differentially expressed in the mite comparison and 126 genes at the pupal stage and 13 genes at the adult stage were significantly expressed in the phenotype comparison. Identification of the overlapping genes between different comparisons implied that these genes play important roles in host response to varroa mite parasitism. Classification of these genes into functional groups showed they are related to olfaction, signal transduction, detoxification processes, protein and lipid metabolisms as well as exoskeleton formation. This suggested these processes are involved in possible defensive reactions of honey bees in combating varroa mite parasitism. Particularly, high differential expression of cytochrome P450 and esterase genes at both pupal and adult stages indicated their likely involvement in detoxification processes to respond to xenobiotic compounds from the varroa mite parasitism (Jiang et al. 2016).

A bee-specific peptide array for characterizing global cellular kinase activity was also attempted to identify potential tolerant biomarkers in signal transduction pathways of honey bees (Robertson et al. 2014). The array contained 299 unique phosphopeptides, some of which are associated with the innate immune process. Bees with different phenotypes showed distinct, developmentally-specific signaling profiles represented by phosphorylated peptides, and as few as five peptides could distinguish the tolerant and susceptible phenotypes with high confidence, suggesting potential commercialization of this array for selecting varroa tolerant phenotypes. Immune genes were not differentially regulated in either non-infested susceptible or tolerant phenotypes, however, upon infestation the susceptible phenotype (G4) showed down regulation of immune genes while the tolerant phenotype (S88) did not follow the same trend (Jiang et al.

2016). Therefore, more diverse viral infections were detected in susceptible adult bees than tolerant bees (Robertson et al. 2014). This technique appears to be an effective tool for understanding the complex resistance mechanisms of honey bees, and for the discovery and utilization of phosphorylation biomarkers in varroa mite tolerant honey bee breeding programs.

2.4.3 Real time quantitative RT-PCR analysis

Real time qRT-PCR is a sensitive and reliable technique that enables detection of rare mRNAs from small amounts of tissue samples (Dorak 2007). In the work described here, it was used to analyze in detail some of the differentially expressed genes associated with honey bee phenotypes showing varying degrees of tolerance and susceptibility to varroa mites. Genes were examined in different honey bee tissues as well as the responses to miticide treatments used to control varroa mite infestations.

Real time qRT-PCR follows a regular PCR procedure with fluorophore added to detect levels of gene expression in a real time manner (Dorak 2007). The accumulation of fluorescent signals in each cycle indicates the amplification of target genes in a real time manner, which can be used to calculate the gene expression level. SYBR Green fluorescence detection system is commonly used in real time qRT-PCR. SYBR Green I dye can specifically bind to double-stranded DNA and maintain the optical intensity at the binding state. Along with the amplification going on, the number of double-stranded amplicons is increased; the intensity of fluorescent signals from the binding SYBR Green I dye is enhanced. The fluorescent signals could be recorded by the computer and used for calculation of expression levels of the specific genes (Dorak 2007).

The PCR-based diagnostic methods are recommended by the World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (The Terrestrial Manual) (Belák 2007). Real time qRT-PCR has recently been used to detect honey bee viral pathogens and the expression of genes encoding anti-microbial peptides (defensin, abaecin, and hymenoptaecin) between varroa-infested and non-infested honey bees (Aronstein et al. 2012). The mite infestation resulted in significant changes in gene expression in honey bee genome, and

certain transcripts of anti-microbial peptide genes were highly expressed in infested bees (Aronstein et al. 2012). In order to investigate the bee larva resistance to the fungus *Ascosphaera apis* that causes the severe chalkbrood, real time qRT-PCR was performed and two genes (single IgIL-related receptor-like and juvenile hormone-binding protein) were identified as responsible for the resistance and can potentially function as modulators of the innate immunity pathway in insects (Holloway et al. 2013). The increased expression of the honey bee immune genes detected by real time qRT-PCR suggested their effect on varroa mite parasitism, which can be explored to enhance mite control (Kuster et al. 2014).

2.5 Insect metabolic resistance associated with carboxylesterase

2.5.1 General introduction

Insect resistance to chemicals commonly occurs during long term use of a chemical such as a pesticide or an insecticide. The mechanism is primarily associated with a detoxification process gradually developed by insects to degrade the pesticide, thus obtaining metabolic tolerance (Onstad 2013). Three major enzymes responsible for detoxification of toxic xenobiotic compounds in the environment are glutathione-S-transferases (GSTs), cytochrome P450s (CYPs) and carboxyl/cholinesterase (CCEs). Several genes encoding these enzymes have been suggested with detoxification functions in generating insecticide resistance in honey bee (Johnson et al. 2012; Johnson et al. 2009b; Johnson et al. 2006; Mao et al. 2009; Mao et al. 2011; Papadopoulos et al. 2004). For example, the *GSTD1* gene detected by qRT-PCR is widely expressed in honey bee tissues, such as the brain, thorax, abdomen and reproductive tissue, indicating its potential protective role at where it requires (Collins et al. 2004; Corona et al. 2005). However, the exact function of this gene is yet to be confirmed. Members of CYP-4, -6, -9 and -12 groups are implicated in environmental response or functional detoxification in other insects, where CYP4 and CYP6 are involved in insecticide metabolism and resistance (Claudianos et al. 2006). Recently, CYP9Q1, CYP9Q2 and CYP9Q3 identified from honey bee midgut have been reported to contribute to insecticide tolerance by detoxifying pyrethroid tau-fluvalilnate as well as the organophosphate coumaphos, both of which are used for varroa mite control (Mao et al. 2011). Like the CYPs, esterase involved in the detoxification process is reported to contribute to metabolic organophosphate resistance in many insect species, such as the oriental fruit fly

(*Bactrocera dorsalis*), the peach-potato aphid (*Myzus persicae*) and the Australian sheep blowfly (*Lucilia cuprina*) (Bass et al. 2014; Jackson et al. 2013; Wang et al. 2015). However, these genes have not been well characterized and their specific functions associated with the detoxification process have yet to be ambiguously determined in honey bees. The overall defensive mechanisms of these genes for tolerant honey bees remain largely unknown (Oakeshott et al. 1999; Oakeshott et al. 2010). Therefore, determining the activity of these enzymes involved in the detoxification processes is a critical step in understanding the defensive mechanisms of honey bees against the pesticides and possibly varroa mite parasitism.

2.5.2 Carboxylesterases involved in detoxification process

A phylogenic analysis of insect carboxyl/cholinesterase sequences (CCEs) was constructed by Oakeshott, and fourteen major clades were classified (denoted from A to N) in three functional groups: dietary detoxification (A-C), hormone and pheromone degradation (D-G) and neurodevelopment (H-N) (Oakeshott et al. 1999). Carboxylesterase (CbE, EC3.1.1.1) in the dietary detoxification clade has been shown with hydrolysis ability to degrade some pesticides and develop resistance to those esters-like compounds such as carbamates and pyrethroids (Sogorb and Vilanova 2002). The chemical structures of pesticides containing at least one ester bond are shown in Figure 2.4. The hydrolysis reaction of carboxylesterase (Figure 2.5) begins with the nucleophilic attack by the catalytic serine hydroxyl group on the carbonyl carbon of the ester bond, producing the alcohol and the intermediate product (Montella et al. 2012). By incorporating a water molecule, the acid is produced and the enzyme goes back to its resting form (Montella et al. 2012).

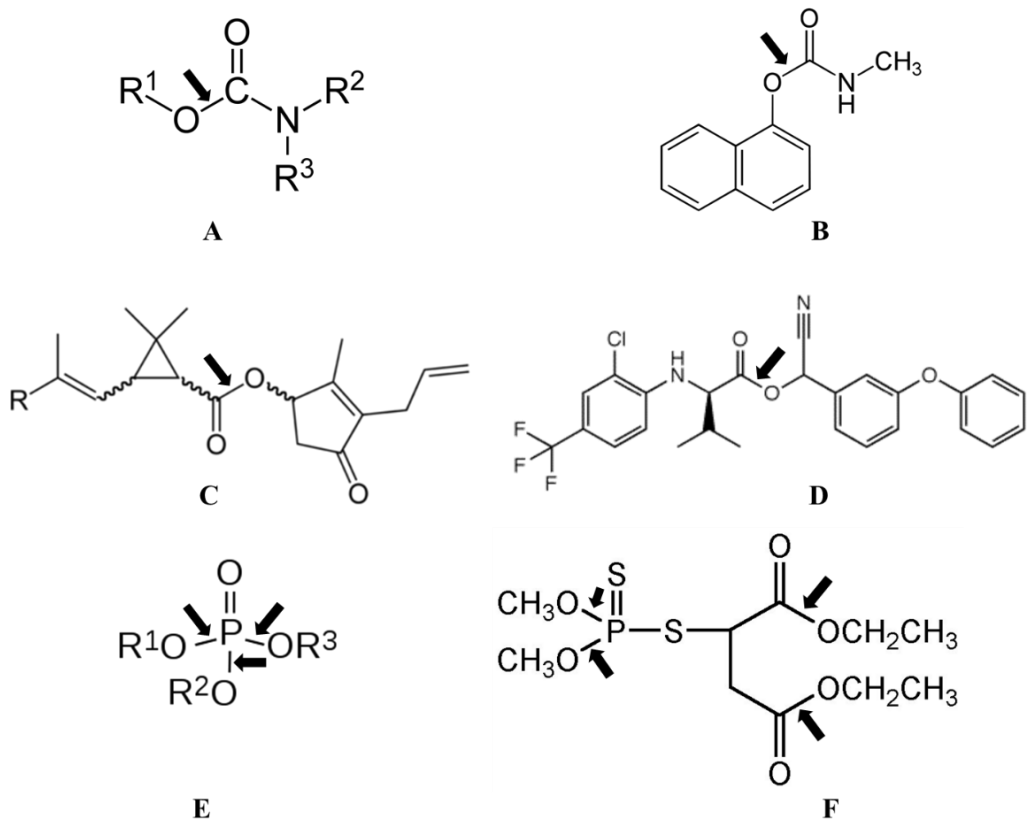

Figure 2.4 Chemical structures of pesticides.

A. Carbamate pesticide family; B. Carbaryl (carbamate); C. Pyrethroid pesticide family; D. Tau-fluvalinate (pyrethroid); E. Organophosphate pesticide family; F. Malathion (organophosphate). Arrows indicate the ester bond.

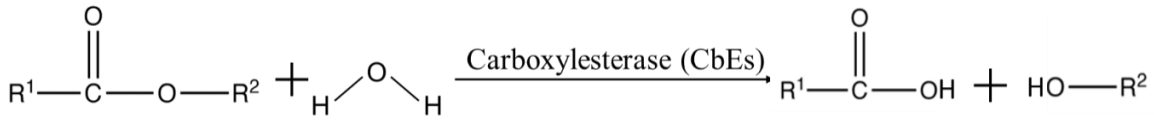

Figure 2.5 Simplified general hydrolysis reaction of carboxylesterases.

Some resistant insects can increase the synthesis of carboxylesterase to protect themselves from the damage of a wide range of pesticides (Devonshire and Moores 1982; Kao et al. 1984). For instance, carboxylesterase *LmCesA20* and *LmCesE1* have been reported playing an important role in detoxification of malathion in the Migratory Locust (*Loocusta migratoria*) (Zhang et al. 2014). Overexpression of two α-esterase genes isolated from fruit fly, *BdCarE4* and *BdCarE6*, is consistent with increased esterase activities in the malathion resistant fly, and knockdown of each of two genes by RNA interference (RNAi) significantly decreases malathion tolerance in the resistant strain (Wang et al. 2015). In the aphid, CbE E4 activity is a major resistant factor responsible for degradation of organophosphates, carbamates and pyrethroid pesticides (Devonshire and Moores 1982). This aphid CbE E4 had been proved with the hydrolysis ability to degrade synthetic esterase substrate β-naphthyl acetate as well as pesticide carbaryl and malathion (Lan et al. 2005). Moreover, the *α-carboxylesterase7* (*LcαE7*), also known as *E3*, isolated from the Australian sheep blowfly (*Lucilia cuprina*), has been confirmed in its important physiological role in lipid metabolism as well as organophosphate resistance (Jackson et al. 2013).

By now, there is no report on the functional characterization of honey bee esterase genes with regards to the detoxification processes. Previous DNA microarray analysis indicated high differential expression of *AmCbE E4* at both pupal and adult bees in response to varroa mite parasitism of two extreme colony phenotypes (tolerant and susceptible) (Jiang et al. 2016). When honey bees are parasitized by varroa mites, increased esterase activity may protect them from the harm of toxic esters possibly brought by varroa mite parasitism (Claudianos et al. 2006). In reality, honey bees have to encounter not only in-hive pesticides to devastate varroa mites, but also the pesticides sprayed in the agricultural field for broad-spectrum pest control. Therefore, understanding of this enzyme (AmCbE E4) in the detoxification process in honey bees is critical for elucidation of the defensive mechanisms underlying tolerant honey bee phenotypes and for developing methods against pesticide-resistant mites as well as for the effective maintenance of a viable apiculture industry.

3. HYPOTHESES AND OBJECTIVES

Phenotypic differences (degrees of susceptibility and tolerance) of honey bee colonies to varroa parasitism result from behavioral mechanisms (grooming behavior, hygienic behavior) and unknown factors. These phenotypic differences result in part from differential expression of the genes involved in the defense mechanisms. A large number of these differentially expressed genes identified in a previous DNA microarray analysis of two extremely contrasting colonies are indicative of possible defensive mechanisms of honey bees in response to varroa mite (Jiang et al. 2016). The usefulness of these genes as potential biomarker genes for screening varroa tolerant phenotype need to be analyzed by another reliable quantitative transcript-profiling tool (real time qRT-PCR) in a wider range of honey bee colonies identified by the Saskatraz breeding program (www.saskatraz.com). Therefore, two major specific hypotheses are as follows:

1. Potential biomarker genes should possess consistent and differential expression patterns in a wide range of varroa tolerant and susceptible honey bee colonies and play defensive roles against mite parasitism and/or miticide applications.

2. A highly differentially expressed gene *AmCbE E4* selected by both DNA microarray and quantitative RT-PCR analyses encodes a functional carboxylesterase in honey bees in response to the varroa mite.

The technical objectives of this project are as follows: (1) To analyze differential expression of ten genes selected by previous DNA microarray analysis in a wide range of honey bee colonies using real time qRT-PCR; (2) To examine expression patterns of three potential biomarker genes in different tissues and in response to miticide treatment and quantify the amount of DWV in honey bee colonies; (3) To biochemically characterize *AmCbE E4* heterologously expressed in *E. coli*.

4. STUDY 1: ANALYSES OF TEN DIFFERENTIALLY EXPRESSED GENES BY REAL TIME QRT-PCR

4.1 Abstract

Real time qRT-PCR was used to analyze the expression of ten genes identified by previous DNA microarray in a wide range of honey bee colonies. The results showed that most of these genes exhibited differential expression in six honey bee colonies with different phenotypes of varroa tolerance and susceptibility. Particularly, gene *GB53798* encoding putative esterase E4 (*AmCbE E4*), gene *GB49888* encoding putative cytochrome P450 6A1 (*AmCYP6A1*) and gene *GB50876* encoding apolipoprotein D (*AmApoD*) displayed relatively higher transcription levels in three tolerant colonies, but lower expression levels in three susceptible colonies in the presence of varroa mites. Afterwards, the expression of these three genes was further examined in another ten more colonies showing varying degrees of tolerance and susceptibility to varroa parasitism. The results suggest that these three genes could be used as potential biomarkers for screening varroa tolerant honey bee colonies in the breeding program.

4.2 Introduction

The honey bee (*Apis mellifera* L.), well known as an indispensable pollinator, has brought a wealth of economic benefits to human beings. Over the past 50 years, the bee population has experienced a significant global increase in the number of colonies (Moritz and Erler 2016). However, in very recent years, a large scale of enigmatic losses of honey bee colonies so called as “Colony Collapse Disorder (CCD)” has been observed in the United States and some European countries (vanEngelsdorp and Meixner 2010). As a social insect, honey bees are readily infected by fungi, bacteria and viruses, and infested by parasitic mites or harmed by predators (beetles, bears, and humans) (vanEngelsdorp and Meixner 2010). Among these agents, *Varroa destructor* has been regarded as a flagship ecto-parasite contributing to colony losses, and it has spread worldwide in a short period of time (Rosenkranz et al. 2010).

Pesticides/miticides are used to control varroa mites in apiculture management practices (Milani and Iob 1998), however, the wide use of these miticides has been problematic. Firstly, miticide contamination has been detected in bee products (Chiesa et al. 2016; Martel et al. 2007). Secondly, it can impose adverse impact on the health of host bees (Johnson et al. 2013a; Johnson et al. 2013b; Johnson et al. 2010). Thirdly, long term use can result in generation of miticide-tolerant pests (Maggi et al. 2012). Thus, breeding honey bees tolerant to the varroa mite would be an attractive alternative for controlling varroa mites in honey bee colonies. However, due to the lack of understanding honey bee tolerant mechanisms to mite parasitism and molecular markers for phenotype selection, breeding varroa tolerant honey bees is still a challenging task.

To investigate possible honey bee defensive mechanisms toward varroa mites, a high throughput DNA microarray analysis was performed to investigate the genome-wide gene expression of two extreme colonies, varroa tolerant phenotype (S88) and varroa susceptible phenotype (G4) with and without varroa infestation (Jiang et al. 2016). The analysis revealed a large number of the genes differentially expressed between colony phenotype comparisons and mite infestation comparisons. Classification of these genes into several functional groups related to olfaction, signal transduction, detoxification processes, protein and lipid metabolism as well as exoskeleton formation suggests possible defensive reactions of honey bees in response to the mite parasitism (Jiang et al. 2016).

In this study, we hypothesized that the effective biomarker genes for selecting varroa tolerant phenotypes should possess consistent and differential expression patterns among a wide range of varroa tolerant and susceptible honey bee colonies. Therefore, expression of ten highly differentially expressed genes identified by previous DNA microarray analysis (Jiang et al. 2016) were assessed using real time qRT-PCR in a wide range of honey bee colonies differing in the tolerance and susceptibility to mite infestation. Colony phenotypes were selected over several years by natural selection (no miticide treatments used) in the presence of varroa mites (Jiang et al. 2016; Robertson et al. 2014, www.saskatraz.com) and were monitored for honey production, phoretic mite populations, brood infestation and survival times in the absence of miticide treatments. The most tolerant colonies had the longest survival times and lowest brood and

phoretic varroa mite infestation levels. Susceptible colonies showed high varroa mite population growth and short survival time.

4.3 Materials and methods

4.3.1 Honey bee breeding project and colony phenotyping

Honey bee colonies differing in tolerance and susceptibility to varroa mite infestation were selected from the Saskatraz Breeding Project (The Saskatchewan Honey bee Breeding and Selection Program). The Saskatraz natural selection apiaries were operated by Meadow Ridge Enterprises LTD Saskatchewan, Canada (52º11' N, 106º63' W). The details of the breeding program such as crossbreeding methods, natural selection results and relevant references are available at www.saskatraz.com. Several parameters such as honey production, winter survival time and varroa mite infestation rate were included to define the phenotype of bee colonies with tolerance or susceptibility to the mite. For instance, the number of varroa mites found in each colony was recorded each year and then used to calculate the means of mites per hundred bees over the survival years.

4.3.2 Sample collection

For the analysis of differentially expressed genes identified by previous DNA microarrays, honey bee samples were collected at dark eyed stage 4 pupae with and without varroa mites. The brood frames were removed from the selected colonies and incubated in the dark at 32°C and 80% humidity in the field laboratory at Meadow Ridge Enterprises LTD. The cap was carefully opened on each brood cell and the dark color of the eye cuticle was used to determine the developmental stage of the pupal bees. Pupae from cells infested with mites were identified and separated from non-infested pupae. A bee was considered to be parasitized if there was at least one mite attached to the pupae, and bee pupae with mites were separated from non-parasitized bees before freezing. All the varroa-free and varroa-infested pupae samples were rapidly frozen in liquid nitrogen, and then stored in a -80ºC freezer. The following sixteen colonies were used in the study: S88, G4, S86E, S86C-1, S85-09, S65 SAT-1 and S88-4 collected in September 2011; S84C-4, S23A, S23A-3 and G8 collected in September 2012; S96-4-12 collected in

September 2013; G50-3 collected in September 2014; Sy26, S14 and JH-8-10 collected in August 2015.

4.3.3 RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis are two key steps to obtain a reliable qRT-PCR result. The total RNAs were extracted from two honey bee heads with biological triplicates by using RNeasy Plant Mini kits (Qiagen, Valencia, USA) and treated with DNase I (RNase-free DNase I Set, Qiagen, Valencia, USA) as described by the manufacturer. RNA purity and integrity were checked by NanoDrop spectrophotometer (Thermo Fisher Scientific, Burlington, CA) and agarose gel electrophoresis (1% agarose gels). Then 2.5 μg RNA samples were reverse synthesized to cDNA by using the SuperScript® III First-Strand Synthesis System (Invitrogen, Burlington, CA) according to the protocol provided by the manufacturer.

4.3.4 Target gene selection, primer design and evaluation of primer amplification efficiency

Genes displaying highly differential expression levels identified in colony phenotype and mite infestation comparisons by previous DNA microarray analysis (Jiang et al. 2016) were chosen as candidate genes for real time qRT-PCR analysis in sixteen different honey bee colonies. Two housekeeping gene, *actin* and *ribosomal protein S5* (*RpS5*) were used as internal references (Jiang et al. 2016). Primers were designed by primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/), their amplification specificities were evaluated by NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), and then synthesized by Sigma-Aldrich, Oakville, CA. The amplification efficiency of each primer set was calculated according to the following formula on the basis of the slope of the linear regression on a series of cDNA template dilutions (Equation 1) (Higuchi et al. 1993; Ramakers et al. 2003).

$$E = 10^{[-1/slope]} \qquad \text{(Equation 1)}$$

E: primer set efficiency.

4.3.5 Real time qRT-PCR

The freshly reverse-transcribed cDNA samples were diluted ten times for real time qRT-PCR assays. A 20 µl reaction mixture contains: 4 µl of diluted cDNA sample (12.5ng/ul), 0.4 µl of each two primers (10 µM), and 10 µl Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Burlington, CA) and 5.2 µl sterilized ddH_2O. UDG (uracil DNA glycosylase) in the SuperMix prevented re-amplification of carryover PCR products between reactions, which was then inactivated by high temperatures during normal PCR cycling, thereby allowing amplification of genuine target sequences (Longo et al. 1990). Amplification reactions were proceeded in 96-well plates by applying CFX96 System (BIORAD, Mississauga, CA) with the following 2-step qPCR program: UDG incubation at 50ºC for 2 min, an initial denaturation at 95ºC for 2 min, followed by 45 cycles of 10 s of denaturation at 95ºC, 30 s of annealing and elongation at 60ºC. Technical duplicate reactions were carried out for each cDNA template.

4.3.6 Data analysis

Real time qRT-PCR data collected by the BIORAD CFX Manager Software was analyzed with custom-designed excel spreadsheets. The relative expression levels of target genes were calculated using the ΔCt method (Equation 2 and 3).

$$\text{Relative expression level (Fold change)} = 2^{\Delta Ct} \quad \text{(Equation 2)}$$

$$\Delta Ct = Ct\ (\text{reference gene}) - Ct\ (\text{target gene}) \quad \text{(Equation 3)}$$

Statistical analysis was performed using the software SAS 9.0 (Statistical Analysis System, Cary, USA). The paired comparison (PROC TTEST) and difference normality test (PROC UNIVARIATE with the NORMAL PLOT) were both conducted for statistical significance analysis for each colony in the mite comparison. When the $p<0.05$, the difference was regarded as statistically significant.

4.4 Results

4.4.1 Honey bee colony phenotyping

Productive colonies, with longer survival time and fewer mites per hundred bees (less variations), were identified as varroa tolerant colonies, such as S88, S23A, S14, Sy26, S85-09, S84C-4 and S23A-3 with varying degrees of tolerance to varroa (Figure 4.1). Conversely, colonies showing shorter survival time and more mites per hundred bees (more variations) were defined as susceptible colonies, such as JH-8-10, S96-4-12, S65 SAT-1, S88-4, G50-3 and G4 (Figure 4.1). For example, S88 showed an extreme varroa tolerant phenotype since it survived 58 months with a low phoretic varroa infestation rate, while G4 was designated a highly susceptible colony phenotype as it survived 17 months reaching a very high phoretic varroa infestation rate in a short time. Detailed varroa analysis on these two extreme colonies was described in Robertson et al. 2014.

4.4.2 Isolation of the RNAs

The total RNAs were extracted from the head tissues of two honey bees. The quality and quantity of the RNA samples were checked by electrophoresis and spectrophotometry (Figure 4.2 and Table 4.1). As shown in Figure 4.2, two ribosomal RNA bands, 28S rRNA and 18S rRNA, were clearly observed on an agarose gel, indicating that quality RNAs were obtained. As seen from Table 4.1, the initial concentrations of RNAs isolated from sixteen colonies with and without mite infestation were around 500 ng/µl, indicating a sufficient quantity of RNAs were extracted.

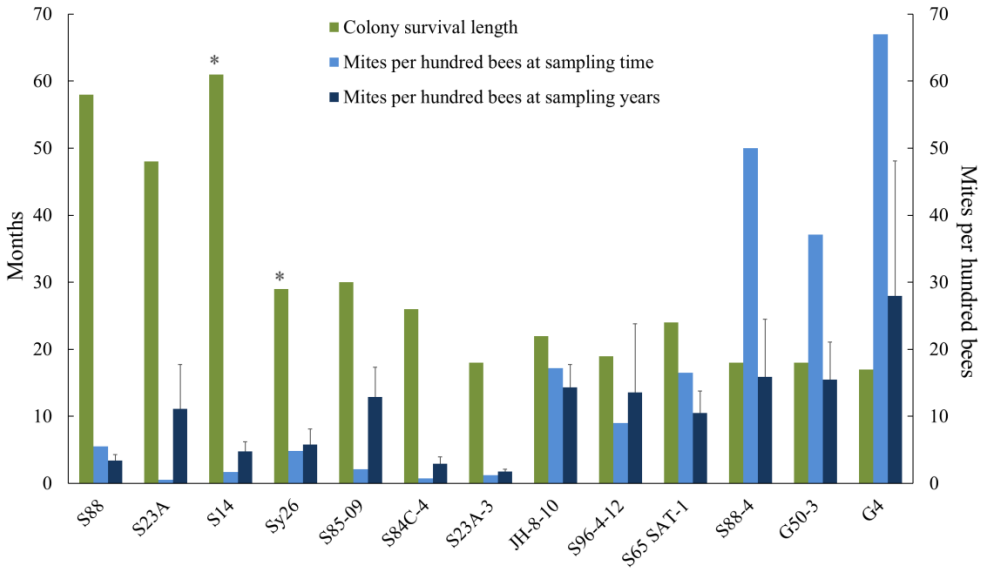

Figure 4.1 The colony survival time and varroa mite infestation of selected honey bee colonies.

The survival time (green bar) is presented in months, and the Varroa mite infestation (blue bars) is presented in Mites per Hundred Bees (MHB). The light blue bar shows the varroa infestation rate at the sampling time. The dark blue bar shows the varroa infestation rate in the sampling years (mean±SEM). A colony with a single star is still alive.

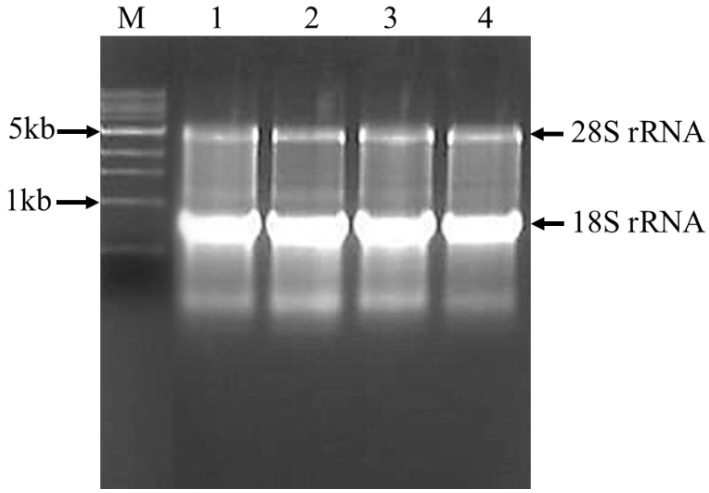

Figure 4.2 Representative agarose gel electrophoresis of RNAs isolated from two heads of dark eyed stage 4 pupae.

Marker: 1 kb DNA ladder. Lanes 1-4: RNA samples extracted from S88 with mite, S88 without mite, G4 with mite and G4 without mite, separately.

Table 4.1 The concentration of the total RNAs isolated from two heads of dark eyed stage 4 pupae.

Honey bee colony	RNA Concentration (ng/µl)			Honey bee colony	RNA Concentration (ng/µl)		
	H1	H2	H3		H1	H2	H3
S88 wo	952.1	1042.5	747.3	S88 w	1130.8	735.6	1126.4
G4 wo	785.6	1016.2	794.9	G4 w	1180.4	1353.7	1369.5
S86E wo	708.2	700.3	563.8	S86E w	831.6	255.0	260.1
S84C-4 wo	240.8	279.6	480.4	S84C-4 w	540.3	1026.5	451.5
S86C-1 wo	792.9	1044.2	699.3	S86C-1 w	573.1	409.0	450.9
S85-09 wo	419.6	472.0	376.1	S85-09 w	273.8	369.9	597.6
S23A wo	570.1	464.4	386.0	S23A w	259.0	398.5	483.9
S23A-3 wo	581.5	370.6	553.3	S23A-3 w	219.5	276.0	465.6
S65 SAT-1 wo	411.7	420.2	839.5	S65 SAT-1 w	378.3	884.8	466.5
S88-4 wo	397.6	226.7	565.3	S88-4 w	765.3	489.5	332.6
G8 wo	525.5	412.2	343.6	G8 w	234.5	665.2	501.7
Sy26 wo	774.8	607.0	850.3	Sy26 w	974.1	1463.7	496.5
S14 wo	964.6	676.9	1024.2	S14 w	609.6	949.4	1069.5
G50-3 wo	1025.2	793.2	1153.3	G50-3 w	1185.6	654.7	913.3
S96-4-12 wo	704.4	637.1	661.2	S96-4-12 w	844.2	778.6	917.6
JH-8-10 wo	915.8	595.2	534.0	JH-8-10 w	1024.9	1160.5	874.1

Note: “wo” represents honey bees without mite infestation; “w” represents honey bees with mite infestation; “H1, H2 and H3” represent the RNA samples of three biological replicates isolated from two heads of dark eyed stage 4 pupae.

4.4.3 Target gene selection and primer design for qRT-PCR analysis

Ten genes, *GB49878* (*AmCYP6A14*), *GB53798* (*AmCbE E4*), *GB49888* (*AmCYP6A1*), *GB50876* (*AmApoD*), *GB41410* (*AmSPH51*), *GB43411* (*AmHIP14*), *GB19967* (*AmCYP9E2*), *GB47279* (*AmCYP6BD1*), *GB46814* (*AmCYP6BE1*), and *GB40976* (*AmHsp90*) (Table 4.2) were selected for real time qRT-PCR analysis based on their highly differential expression obtained in a previous DNA microarray analysis of two extreme colonies for varroa tolerance and susceptibility (Jiang et al. 2016). Predicted biological functions of these genes were involved in detoxification processes, lipid and protein metabolism and the immune response. They were all highly differentially expressed either in the phenotype or varroa mite infestation comparisons.

Table 4.2 Ten genes showing differential expression in phenotype and varroa mite infestation comparisons.

		Differential expression in comparisons			
		The phenotype comparison	**The infestation comparison**		
New Gene ID	**Old Gene ID**	**S88+ /G4+**	**G4+ /G4-**	**S88+ /S88-**	**Putative encoded protein**
GB49878	*GB11754*	0.31		0.34	Cytochrome P450 6a14 isoform 1
GB53798	*GB16889*	3.41		3.92	Esterase E4-like
GB49888	*GB12136*	4.13		6.67	Cytochrome P450 6A1
GB50876	*GB11723*	6.88	0.44	2.58	Apolipoprotein D-like isoform 2
GB41410	*GB13397*	10.23		6.74	Serine protease homolog 51
GB43411	*GB17410*			8.02	Huntingtin-interacting protein 14
GB19967	*GB19967*			0.46	Cytochrome P450 9e2 isoform 4
GB47279	*GB19306*			0.45	Cytochrome P450 6BD1
GB46814	*GB14612*			0.48	Cytochrome P450 6BE1
GB40976	*GB14758*			0.26	Heat shock protein 90

Primers for qPCR analysis were designed (Table 4.3) on several standardized parameters such as similar location, length, GC ratio and product length to warrant comparable amplification efficiencies of different genes in a biological sample. Due to the exponential amplification with two PCR amplicons generated from a single DNA template in each PCR cycle, the theoretical amplification efficiency should be at two. In this study, three representative primer pairs for *GB47279*, *GB49878* and *GB40976* were tested with amplification efficiencies. All three primer sets were located in the middle of the coding region with 22 base pairs in length with 45% to 50% of GC content and amplifying similar length fragments (147, 127 and 138 base pairs, respectively). Their amplification efficiencies were found at around two, indicating that these primer sets could be used for qPCR analysis to compare relative expression of corresponding genes (Figure 4.3).

Table 4.3 Primers used for qRT-PCR.

Gene ID	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'	Source
Actin	GTACCACCATGTATCCTGGAATC	GAGATCCACATCTGTTGGAAGG	(Jiang et al. 2016)
RpS5	CCGCAATGTCCTATAGTCGAAC	GATGATAGCAGTCACAAGAACCTG	
GB49888	GCCCACTTGGAACTCTATAATACG	CCTGAACACGTTTCTCTCTTTCC	
GB50876	GATGGGAAATTCCGTGTCAG	TTTATCTCGCCCTCCAACAC	
GB53798	ACCATATTCCCCGTGTATCG	TGTATGCCGTATCGTTGCTC	
GB46814	CGAAAGGAACTTGCATAGCC	TCTTCGGAAAATCGTTCTGG	
GB47279	TCCTCCGACTCCAATTATCG	AAACGGAGAGGATCTGGATG	
GB19967	TGTTCGGCTTGAGATTCCTC	ATCTGTTGGTGCCCAACTTC	
GB49878	CGGCGTAGAAATGAACTCGTTG	CAATCGCGGCATAAACTCTCTG	This study
GB41410	CATTGTGTCAAAGGCCCCATTT	CACCTGTCATGCTCACCGAA	
GB43411	CAAACTGCCTGTCAAAGCGAA	TCACACGATCCAAAGCTCCAT	
GB40976	TCTGGAGATGAAATGTGCTCGT	ACCACGCTTCTTAACCCTTTCT	

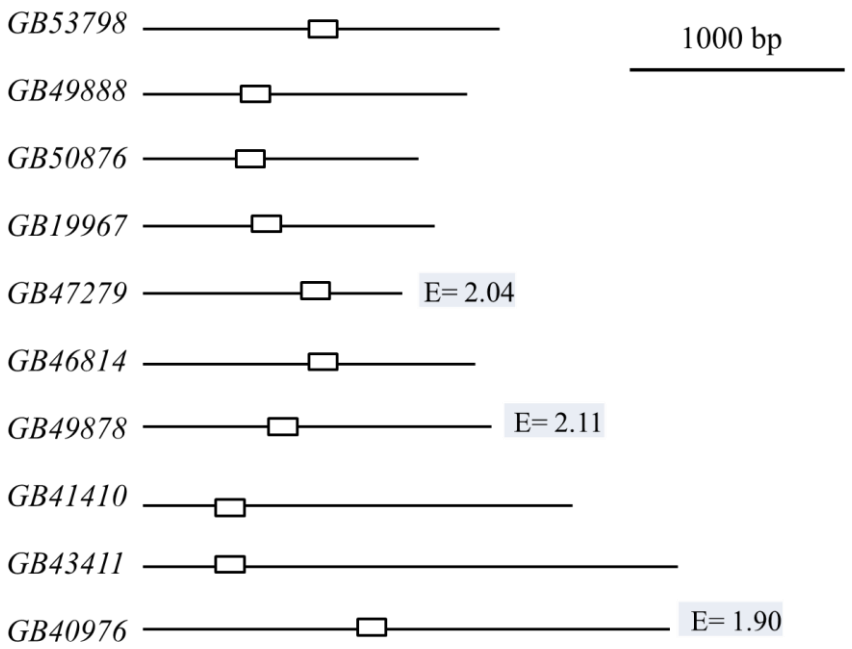

Figure 4.3 Locations and amplification efficiencies of designed primers used for qRT-PCR analysis.

E: Efficiency of primer set.

4.4.4 Differential expression analyses of ten selected genes in varroa tolerant and varroa susceptible colony phenotypes

Relative expression levels of ten selected genes were first examined by real time qRT-PCR in six colonies including two highly contrasting colonies previously used in the DNA microarray analysis (tolerant S88 and susceptible G4). As seen in Figure 4.4, *AmCYP6A1*, *AmCbE E4* and *AmApoD* showed increased expression in S88 in the presence of varroa infestation. However, in G4, varroa infestation decreased the expression of these genes. *AmCYP6BE1* responded similarly in both phenotypes in the presence of varroa. The rest of ten genes showed no significant differences in expression either in the presence or absence of varroa in S88. In G4, varroa infestation resulted in increased expression of *AmCYP6BD1*, *AmCYP9E2* and *AmCYP6A14*. No differences were observed for *AmSP51, AmHIP14* and *AmHsp90*. These results suggested that *AmCYP6A1*, *AmCbE E4* and *AmApoD* are good candidates for identifying varroa tolerant colony phenotypes.

Figure 4.5A showed that S23A, another strong tolerant colony, significantly increased expression of *AmCYP6BE1* and *AmCbE E4* in response to varroa infestation, similar to S88 (Figure 4.4). The expression of *AmCYP6A1* was increased in S23A in response to varroa mites, but not significantly. The expression levels of cytochrome P450 transcripts (*AmCYP*s) were all increased in S23A, but they were relatively low as in S88. The expression of *AmHSP90* was significantly increased in S23A, but not in S88 in response to varroa infestation. S84C-4 (Figure 4.5B), a colony phenotype with less varroa tolerance than S88 and S23A, showed a significantly increased expression of *AmApoD*, an increased, but not significant expression of *AmCbE E4* and *AmHsp90*, and a decreased transcription of *AmCYP6BE1*.

The differential expression of ten genes in dark eyed stage 4 pupae with and without varroa infestation in another two susceptible colonies with varying degrees of susceptibility (S88-4 and S65 SAT-1) were shown in Figure 4.6. In S88-4 (Figure 4.6A), the second most susceptible colony phenotype analyzed, the expression of *AmCYP6A1* was decreased in the presence of varroa, but not significantly as in G4. *AmApoD* was significantly decreased in the presence of varroa as in G4, but no significant differences were observed in the other 8 genes analyzed. In S65 SAT-1 (Figure 4.6B), a phenotype showing less susceptibility than G4 and S88-4, the varroa infestation significantly suppressed the expression of *AmCYP6A14* and *AmApoD*, except for *AmHsp90* showing a significantly increase. None of the other 7 genes analyzed were significantly affected.

These observations suggest that the transcriptional levels of *AmCYP6A1*, *AmCbE E4* and *AmApoD* are generally increased in varroa-infested dark eyed stage 4 pupae from varroa tolerant honey bee colonies, although there is some variability between colonies. Conversely, these genes were suppressed in varroa-infested dark eyed stage 4 pupae from varroa susceptible colonies. These three genes were thus chosen to screen ten more colonies showing a wide range of varroa tolerant and susceptible colony phenotypes (Figure 4.7).

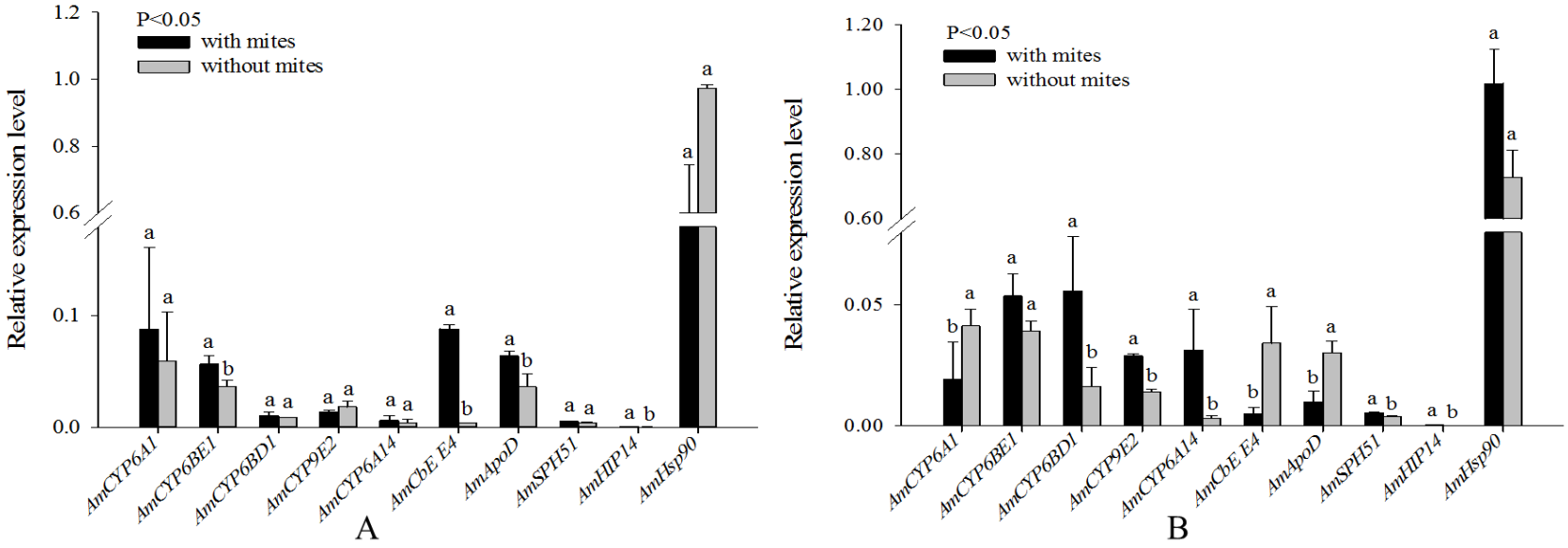

Figure 4.4 Relative expression levels of ten genes in dark eyed stage 4 pupae from varroa tolerant (S88) and susceptible (G4) honey bee colony phenotypes with and without varroa mite infestation.

y axis: relative gene expression levels (mean±SEM, N=3). x axis: ten selected genes. A. S88; B. G4. Relative gene expression levels were normalized by the expression of internal reference genes (*actin* and *RpS5*), and error bars indicated the expression variability of each gene. Value bars signed by different letters are significantly different ($p<0.05$) in varroa mite comparison.

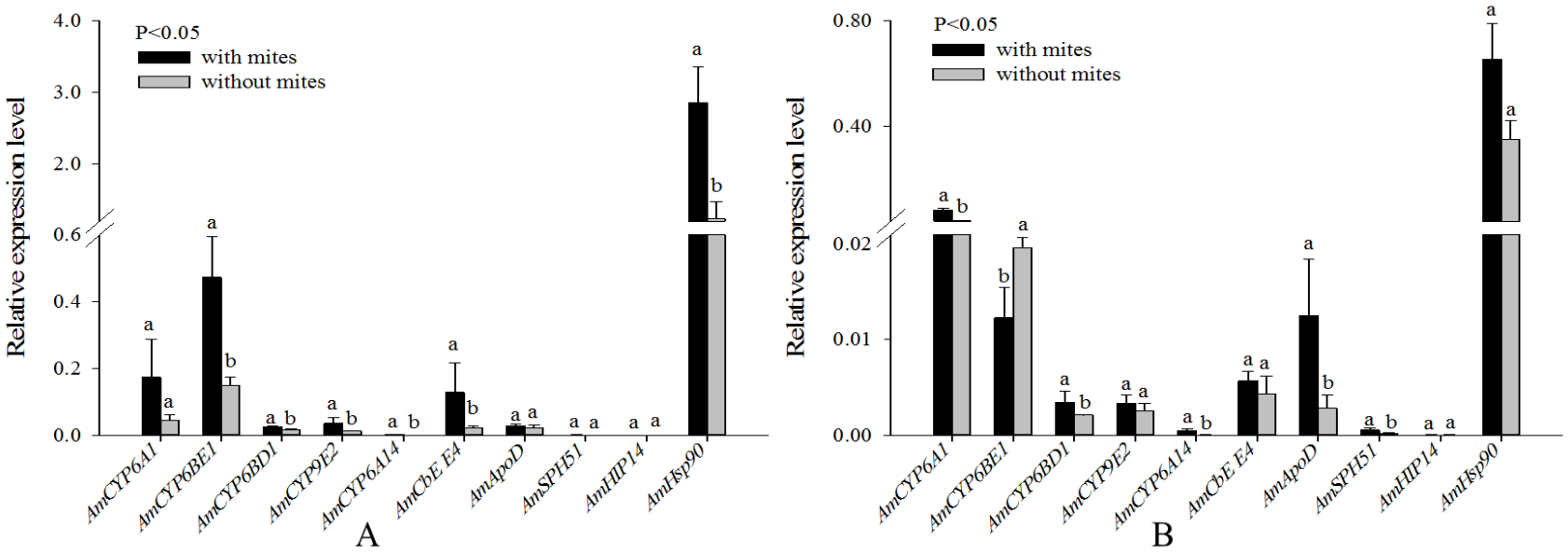

Figure 4.5 Relative expression levels of ten genes in dark eyed stage 4 pupae from two different varroa tolerant honey bee colonies with and without varroa mite infestation.

y axis: relative gene expression levels (mean±SEM, N=3). x axis: ten selected genes. A. S23A; B. S84C-4. Relative gene expression levels were normalized by the expression of internal reference genes (*actin* and *RpS5*), and error bars indicated the expression variability of each gene. Value bars signed by different letters are significantly different ($p<0.05$) in varroa mite comparison.

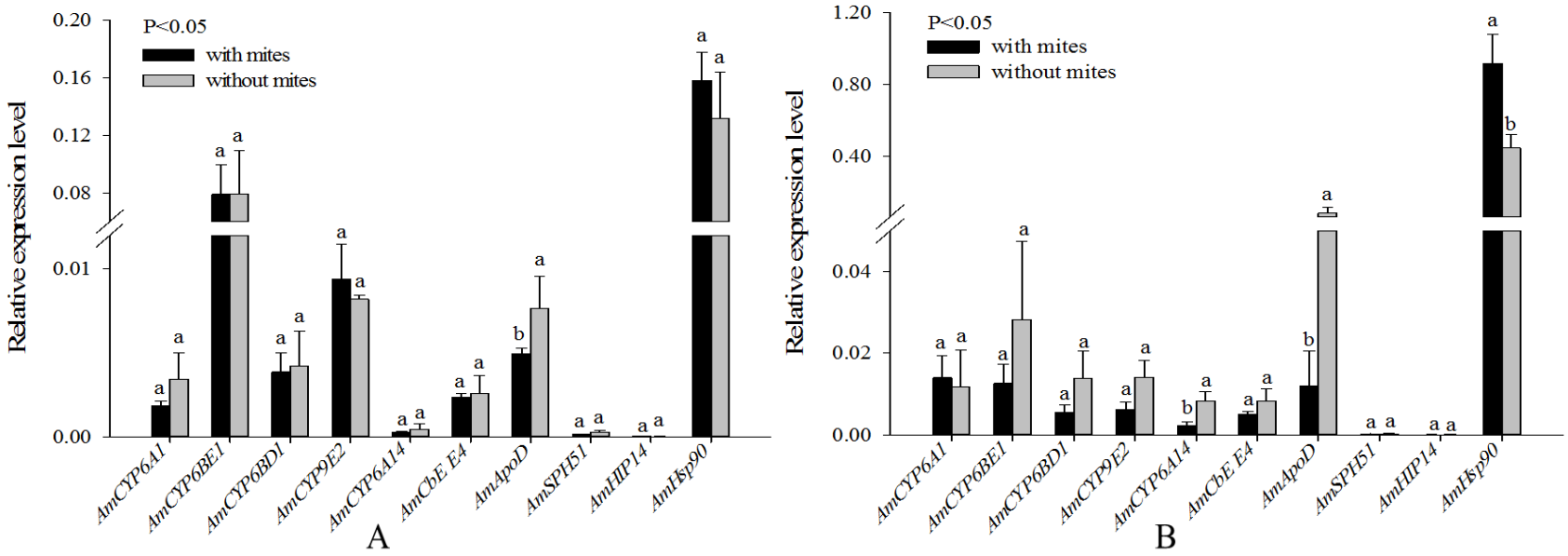

Figure 4.6 Relative expression levels of ten genes in dark eyed stage 4 pupae from two different varroa susceptible honey bee colonies with and without varroa mite infestation.

y axis: relative gene expression levels (mean±SEM, N=3). x axis: ten selected genes. A. S88-4; B. S65 SAT-1. Relative gene expression levels were normalized by the expression of internal reference genes (*actin* and *RpS5*), and error bars indicated the expression variability of each gene. Value bars signed by different letters are significantly different ($p<0.05$) in varroa mite comparison.

4.4.5 Differential expression analyses of *AmCbE E4*, *AmApoD* and *AmCYP6A1* in a wide range of colonies

The expression levels of three selected genes, *AmCbE E4*, *AmApoD* and *AmCYP6A1*, were further evaluated in another ten colonies and compared with the previous qPCR analysis. Three varroa susceptible colonies, S96-4-12, G50-3 and JH-8-10 showed less varroa susceptibility than the first three colonies, G4, S88-4, and S65 SAT-1. Seven colonies showed varying degrees of varroa tolerance, S14, S85-09, S23A-3, Sy26, G8, S86C-1, and S86E (Figure 4.7).

Varroa susceptible colonies (G4, S88-4, S65 SAT-1, and S96-4-12) showed decreased expression of *AmCbE E4*, two of which were significant. G50-3 showed no difference and JH-8-10 showed a significant increase in *AmCbE E4* transcripts (Figure 4.7A). G50-3 was reverted to a susceptible phenotype after prolonged exposure to varroa mites, and JH-8-10 was the most varroa tolerant of sixteen Australian breeder queens evaluated for susceptibility to varroa over a six-month period (data not shown). Nine of the varroa tolerant colonies (S88, S23A, S84C, S14, S23A-3, Sy26, G8, S86C-1, and S86E) showed increased expression of *AmCbE E4* in varroa-infected dark eyed stage 4 pupae, five of which were significant. However, S85-09 showed a decreased expression of *AmCbE E4*.

Four of the six varroa susceptible colonies (G4, S88-4, S65 SAT-1, G50-3) showed significant decreases in *AmApoD* transcripts in the presence of varroa, two of which showed no changes, S96-4-12 and JH-8-10 (Figure4.7B). Seven of the varroa tolerant colonies (S88, S23A, S84C-4, S14, S23A-3, Sy26, and G8) showed increased transcriptional levels of *AmApoD*, five of which were significant. S85-09 showed no significant change, but S86C and S86E both showed significantly decreased expression levels of *AmApoD* in the presence of varroa mite.

Two of the varroa susceptible colonies (G4 and G50-3) showed significantly decreased transcription of *AmCYP6A1*, three (S88-4, S65 SAT-1, and JH-8-10) showed no change and S96-4-12 showed an increase expression in the presence of varroa mite (Figure 4.7C). Nine out of the ten varroa tolerant colonies (S88, S23A, S84C-4, S14, S85-09, Sy26, G8, S86C-1, S86E) showed

increased transcription of *AmCYP6A1* in varroa-infested pupae, and five showed significantly expression ($P<0.05$), and one S23A-3 showed a significant decrease in the expression.

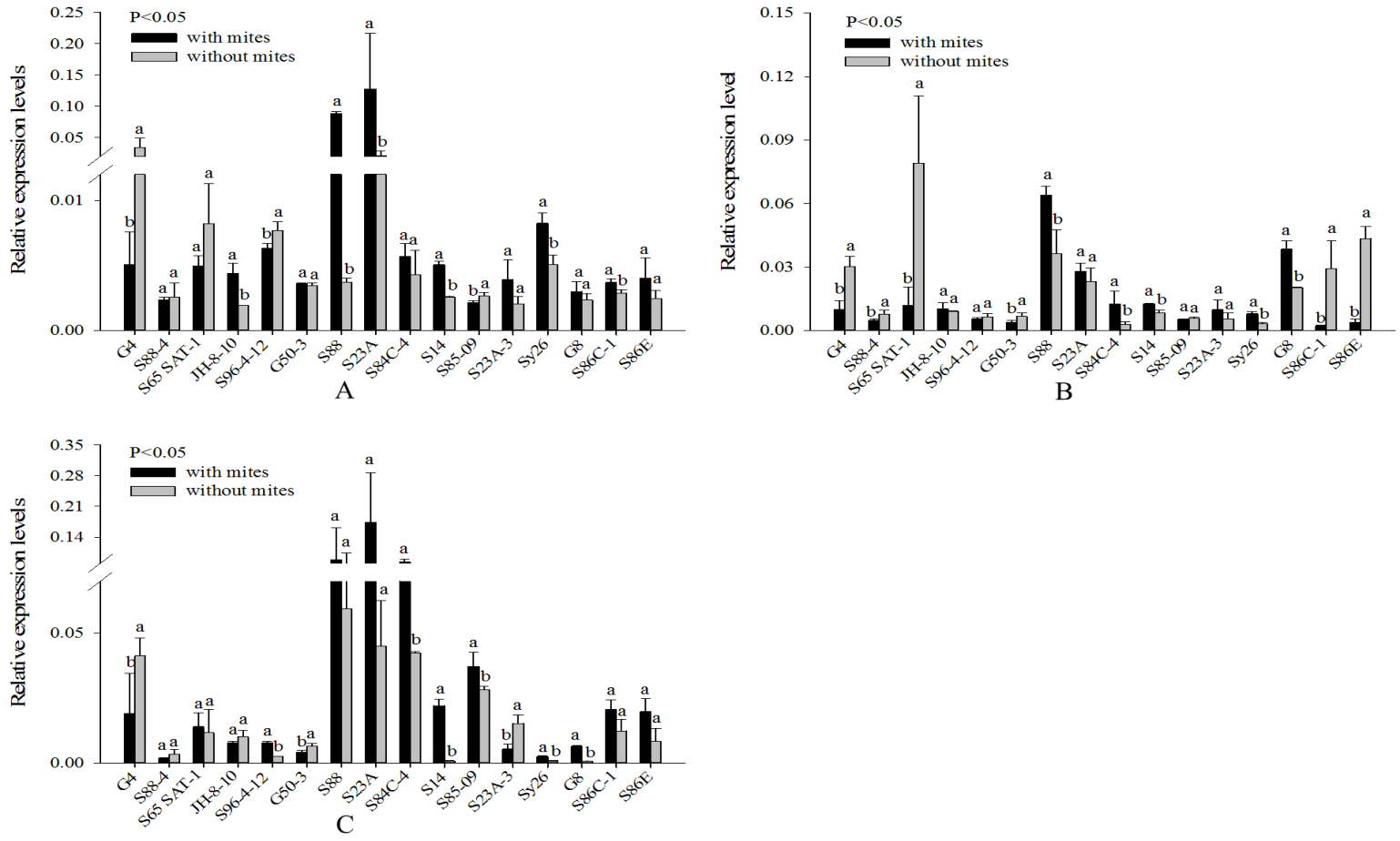

Figure 4.7 Relative expression levels of three genes, *AmCbE E4* (A), *AmApoD* (B), and *AmCYP 6A1* (C) in a wide range of honey bee colonies with and without varroa mite infestation.

y axis: relative gene expression levels (mean±SEM, N=3). x axis: total sixteen honey bee colonies. Relative gene expression levels were normalized by the expression of internal reference genes (*actin* and *RpS5*), and error bars indicated the expression variability of each gene. Value bars signed by different letters are significantly different ($p<0.05$) in varroa mite comparison.

4.5 Discussion

In this study, the differential expression of ten selected genes was evaluated by real time qRT-PCR in a wide range of tolerant and susceptible colony phenotypes. Preliminary analyses of the ten genes in three varroa susceptible and three varroa tolerant colonies showed that the expressions of three genes were most correlated with varroa tolerance and susceptibility. They were *AmCbE E4*, *AmApoD* and *AmCYP6A1* (Figure 4.4 and 4.7). Further analyses of the three genes in a wider range of colony phenotypes showed increased expression of *AmCbE E4* was best correlated with varroa tolerance (Figure 4.7). In the most varroa susceptible colonies, the varroa infestation suppressed the expression of *AmCbE E4* in dark eyed stage 4 pupae. Similar, but more variable correlations were found with *AmApoD* (encoding Apolipoprotein D) and *AmCYP6A1* (encoding cytochrome P450 6A1). In colonies showing the most varroa tolerant phenotypes (long survival time with low phoretic varroa infestations) (Figure 4.1), the increased expression of *AmCbE E4*, *AmApoD* and *AmCYP6A1* were most significant. Phenotypes with intermediate levels of susceptibility to varroa mite showed more variable expression of these genes. The colony phenotypes were identified over several years and assayed for mite infestation and honey production at different times of the year. This might have contributed to the variation in gene expression. Nevertheless, the identification of the genes for consistent differential expression in varroa tolerant and varroa susceptible colony phenotypes makes these genes valuable as biomarkers for selecting colonies with varroa tolerance.

Like most insects, honey bees mainly rely on detoxification processes to protect themselves from harmful, xenobiotic compounds (pesticides, pollutants, etc.) in the environment. Three families of enzymes, glutathione-S-transferases (GSTs), cytochrome P450s (P450s) and carboxyl/cholinesterase (CCEs) are believed to be involved in the process (Claudianos et al. 2006). Two of the three potential biomarker genes *AmCbE E4* and *AmCYP6A1* showing relatively constant differential expression patterns among the colony phenotypes are predicted to be involved in the detoxification processes. Insect *CYP6A1* was first shown to function in the detoxification process since it showed constitutive expression in an organophosphate-resistant housefly strain (Carino et al. 1994). Later on, a similar expression pattern of *CYP6A1* was observed in other house fly strains showing neonicotinoid resistance (Markussen and Kristensen

2010) and spinosad resistance (Højland et al. 2014). Increased expression of *AmCYP6A1* observed in most varroa tolerant colony phenotypes could allow these colonies to better detoxify compounds in the environment and possibly those introduced or generated by varroa mite parasitism

Like P450s, esterases have been reported to contribute to pesticide resistance in many insects, such as the oriental fruit fly (*Bactrocera dorsalis*), the peach-potato aphid (*Myzus persicae*), and the Australian sheep blowfly (*Lucilia cuprina*) (Jackson et al. 2013; Lan et al. 2005; Wang et al. 2015). For instance, overexpression of two α-esterase genes, *BdCarE4* and *BdCarE6*, in the fruit fly resulted in increased resistance to the malathion insecticide, while down-regulating expression of the two genes by RNA interference (RNAi) decreased malathion tolerance in the resistant strain (Wang et al. 2015). The fact that *AmCbE E4* displayed an increase of close to 20-fold expression in the varroa tolerant colony S88 and a significantly reduced expression in the varroa susceptible colony G4 in response to mite parasitism (Figure 4.4). This result may imply *AmCbE E4* play a significant role in varroa tolerant mechanisms, which could help tolerant honey bees detoxify toxic compounds possibly released by the mite.

Apolipoprotein D (ApoD) is a member of lipocalin protein, which function in lipid transports (Rassart et al. 2000). A loss function of ApoD in *Drosophila* mutants resulted in reduced resistance to oxidative stresses and a shortened lifespan as well as a smaller body mass due to lower amount of lipids stored in the body (Sanchez et al. 2006). A high degree (above 50% identity) of sequence similarity of ApoD between human and insects is likely due to an evolutionarily conserved functionality (Drayna et al. 1987). The overexpression of Human ApoD in *Drosophila* can extend its lifespan and increase its stress resistance (Dassati et al. 2014). In honey bees, *AmApoD* shows significantly higher expression in varroa tolerant colonies S88, S14, S84C-4, Sy26 and G8 (Figure 4.7B).This is consistent with its positive role in conferring tolerant bees with an increased rate of lipid transport and metabolism to help cope with varroa mite infestation (Perdomo et al. 2010).

In summary, the increased gene expression of *AmCbE E4*, *AmApoD* and *AmCYP6A1* in honey bee colonies showing varroa tolerant phenotypes may give these colonies increased fitness allowing longer survival times in the presence of varroa mites. In addition, it may also allow these tolerant colonies to better cope with environmental pesticides, and other stressors, including miticides which are commonly used to protect them from varroa infestation.

5. STUDY 2: TISSUE EXPRESSION OF THREE BIOMARKER GENES AND THEIR EXPRESSION IN RESPONSE TO MITICIDE TREATMENTS AND THE INFECTION OF DWV AMONG DIFFERENT HONEY BEE COLONY PHENOTYPES

5.1 Abstract

To further examine the spatial differential expression, three different types of the tissues (head, thorax and abdomen) of two susceptible and two tolerant honey bee colonies with and without mite infestation were dissected and used for the analysis. The results showed that the expression patterns of these genes were displayed in a tissue-differential manner. The differential expression of *AmCbE E*4 was more significant in the head tissue, *AmApoD* showed stronger differential expression in the abdomen tissue while *AmCYP6A1* showed higher differential expression in both thorax and abdomen tissues. In addition, expression of the biomarker genes in three different honey bee colonies treated by three commonly used miticides (Apistan®, Apivar® and Thymovar®) were also investigated. The result showed that *AmCYP6A1* displayed higher expression in the two tolerant colonies (Sy26 and S14) with all three miticide treatments, but not in the susceptible colony (JH-8-10). *AmCbE E4* exhibited higher expression only in the tolerant colony Sy26 with Apivar® treatment. *AmApoD* showed higher expression in the tolerant colony S14 with all three miticide treatments, but not in the susceptible colony JH-8-10. In addition, the amount of deformed wing virus (DWV), a biotic stressor for honey bees primarily vectored by varroa mite, was compared between tolerant and susceptible colony phenotypes. The results showed that the virus load was considerably higher in the susceptible colony phenotype than the tolerant colony phenotype. Miticide treatments increased DWV levels significantly more in susceptible colony phenotype than in the varroa tolerant colony phenotype. These results suggest the mechanisms of varroa tolerant colonies in defense against stresses imposed by varroa, viruses and miticides.

5.2 Introduction

To uncover the molecular mechanism of honey bees for the tolerance to varroa mite parasitism, high throughput DNA microarray was previously performed to analyze genome-wide gene expression in two contrasting colony phenotypes, resulting in identification of a large number of the genes that are differentially expressed. To find potential marker genes that are associated with the tolerant phenotype, ten highly differentially expressed genes were selected for the further expression analysis in a wide range of bee colonies in Study 1, resulting in identification of three genes with more consistent differential expression patterns in varroa tolerant and susceptible colonies in response to the mite infestation. *GB53798* encodes putative esterase E4 (*AmCbE E4*), *GB49888* encodes for putative cytochrome P450 6A1 (*AmCYP6A1*) and *GB50876* encodes for apolipoprotein D (*AmApoD*). *AmCbE E4* and *AmCYP 6A1* are predicted to be involved in the detoxification process, degrading harmful xenobiotic compounds in the environment. AmApoD is a member of lipocalin proteins that may function in lipid transport and lipid metabolism (Rassart et al. 2000). In this study, expression of the three biomarker genes (*AmCbE E4, AmCYP6A1* and *AmApoD*) were further analyzed in three different honey bee tissues (head, thorax, and abdomen) and in response to three different miticide treatments (Apistan®, Apivar® and Thymovar®). In addition, the infection of deformed wing virus, a viral pathogen of honey bees primarily vectored by varroa mite, was assayed by real time qRT-PCR between varroa tolerant and susceptible bees in response to varroa mite infestation and miticide treatments.

5.3 Materials and methods

5.3.1 Sample preparation for detecting differential expression in different tissues

Analysis of differential tissue expression of three potential biomarker genes was carried out in four honey bee colonies, S88 and S23A (tolerant to varroa mite), G4 (susceptible to varroa mite), and S96-4-12 (intermediately susceptible to varroa mite) (Figure 4.1) using three primer pairs for *AmCbE E4, AmApoD* and *AmCYP6A1,* respectively. RNA samples were extracted from two of head, thorax and abdomen tissues of each honey bee colony with and without varroa mite infestation. RNA extraction, cDNA synthesis, real time qRT-PCR assay and data analyses were the same as described in Materials and Methods 4.3.

5.3.2 Sample preparation for detecting differential expression in response to miticide treatments

Analysis of marker gene expression influenced by miticide treatments was carried out in three honey bee colonies, Sy26 and S14 (tolerant to varroa mites), and JH-8-10 (intermediately susceptible to varroa mites) (Figure 4.1). At least 500 bees from sealed brood cells in a brood frame were collected from each of the following colonies: Sy26 (July 28th, 2015), S14 (July 28th, 2015) and JH-8-10 (August 18th, 2015). Three different miticide treatments were used Apistan® anti-varroa mite strips (Wellmark International, USA), Apivar® plastic strips (Veto-pharma S.A., France), and Thymovar® Acaricide wafers (Pronatex Inc., Canada).

One strip or wafer with each miticide was cut into 1 mm x 3 mm (30 pieces) for the treatment of dark eyed stage 4 pupae. Each piece of Apistan® contains approximately 0.23 mg of tau-fluvalinate; each piece of Apivar® contains 0.17 mg of Amitraz and each piece of Thymovar® contains 4.77 mg of thymol. A total of 180 brood cells containing dark eyed stage 4 pupae were randomly selected from three testing frames (60 brood cells per frame) for the treatments. On each frame, a total of 45 pupae were treated with the three miticides (15 pupae per miticide), and an additional 15 pupae were used as the control. The brood cell cap was partially opened with a pair of fine tweezers, and then one 1 mm x 3 mm piece of each miticide was placed into the brood cell by laying it horizontally on top (head) of the worker bee pupa. After treatment the half-opened cell cap was re-sealed and marked with a dark black dot on top of the cell cap. After that, the frame was placed in an incubator at 32ºC for 24 hours and then both the control and treated pupae were carefully collected, and placed in 2 ml microcentrifuge tubes, frozen in liquid nitrogen and stored at -80ºC. RNA samples from different tissues were extracted from each group of honey bees and analyzed as described in Materials and Methods 4.3.

5.3.3 Deformed wing virus detection

The cDNA samples, reverse transcribed from total RNAs obtained from two head tissues of varroa mite-infested and non-infested dark eyed stage 4 pupae, and miticide-treated dark eyed stage 4 pupae, were used in DWV detection. Biological triplicates were applied for each total

RNA sample and qPCR assays were conducted with technical duplicates. The primer set for qRT-PCR analysis of the DWV titer in honey bees was: DWV_F, 5'-GAGATTGAAGCGCATGAACA-3' and DWV_R, 5'-TGAATTCAGTGTCGCCCATA-3' (Boncristiani et al. 2012). The reaction mixture and program for qRT-PCR assays were as described in Materials and Methods 4.3.

5.3.4 Statistical analysis for miticide treatments and DWV infection load

Statistical analysis was performed using the software SAS 9.0 (Statistical Analysis System, Cary, USA). The multi-treatment comparison (PROC MIXED) for each selected gene was fit in a one-way analysis of variance (ANOVA) within three miticide treatments followed by a LSD test (Fisher's Protected Least Significant Difference) at p-value less than 0.05. The residual normality was confirmed by the Shapiro-Wilk test (PROC UNIVARIATE with the NORMAL PLOT) and the common variance was confirmed by the Satterthwaite's Approximation test. Infection load of the DWV (Ct values) in each honey bee colony treated with miticides was statistically analyzed with the same method. The infection load of the DWV (Ct values) in each honey bee colony infested by varroa mites was statistically analyzed with the paired comparison (PROC TTEST) and difference normality test (PROC UNIVARIATE with the NORMAL PLOT). When the p-value was less than 0.05, the difference was regarded as statistically significant.

5.4 Results

5.4.1 Differential expression of *AmCbE E4*, *AmApoD* and *AmCYP6A1* in different honey bee tissues

Total RNAs were extracted from three types of tissues (dark eyed stage 4 pupae), head, thorax and abdomen, of two varroa tolerant colonies S88 and S23A, a susceptible colony G4, and S96-4-12 a colony showing intermediate susceptibility and tolerance to varroa (Figure 4.1), with and without varroa mite infestation. Figure 5.1A showed expression patterns of *AmCbE E4* in the head, thorax, and abdomen of G4 pupae in the presence and absence of varroa infestation. G4 is a varroa susceptible colony and the varroa infestation resulted in a highly significant suppression

of *AmCbE E4* transcripts in all tissues. Non-infested pupae showed good levels of *AmCbE E4* transcripts in all tissues. In S96-4-12, varroa infestation resulted in a significant, but small, decrease in *AmCbE E4* expression in head tissue, an increase in thorax, and no difference in abdominal tissue (Figure5.1B). Good levels of *AmCbE E4* transcripts were present both in the presence and absence of varroa infestation, as S96-4-12 showed an intermediated phenotype in susceptibility and tolerance to varroa mites (Figure 4.1) and excellent honey production (data not shown). In the two varroa tolerant colonies S88 and S23A (Figure 5.1C and D), steady state levels of *AmCbE E4* transcripts were lower in all tissues than in the susceptible colony G4 (Figure 5.1A) and the intermediate colony S96-4-12 (Figure 5.1B) without varroa mites. However, varroa infested pupae showed a large and significant increase (around 8-fold) of *AmCbE E4* transcripts in head tissue from both varroa tolerant colonies (Figure 5.1C and D). Significant, but smaller increases of transcripts in thorax and abdominal tissues were detected. These observations confirm that *AmCbE E4* is a potential biomarker for identifying varroa susceptible and tolerant colony phenotypes.

In general, the highest relative expression of *AmApoD* (encoding apolipoprotein D) was in the thorax and abdominal tissue of honey bee pupae (Figure 5.2). Varroa infestation decreased expression in the most susceptible phenotype G4, in all tissues, with the greatest decreases in thorax and abdominal tissues (Figure 5.2A). In the intermediate phenotype S96-4-12, no differences were observed in head and thorax tissues, but a significant decrease occurred in the abdominal tissue (Figure 5.2B). Relative expression levels of *AmApoD* in S96-4-12 were much lower (more than 10-fold) than in G4 (Figure 5.2A), which made the comparisons more difficult. Figure 5.2A and C showed comparable differential expression levels of *AmApoD*, with thorax and abdominal tissues showing the highest transcriptional levels. The most varroa tolerant colony S88 (Figure 5.2C) showed increased expression of *AmApoD* in all tissues, but most significant in abdominal tissue. S23A (Figure 5.2D), a varroa tolerant colony, also showed increased expression of *AmApoD* in thorax and abdominal tissue, comparable to S88. Expression was increased, but not significantly in S23A head tissue. *AmCYP6A1*, a cytochrome P450 monooxygenase, showed expression in all tissues, but with higher values in abdominal tissues (Figure 5.3). Varroa infestation in the most susceptible colony G4 significantly suppressed

AmCYP6A1 expression in head, thorax and abdominal tissues (Figure 5.3A). In the intermediate phenotype S96-4-12, the expression of *AmCYP6A1* were increased in head tissue, but decreased in both thorax and abdominal tissues (Figure5.3B). In the varroa tolerant colonies S88 and S23A (Figure 5.3C and D), varroa mite infested pupae showed a significant increase in *AmCYP6A1* expression in thorax and abdominal tissues.

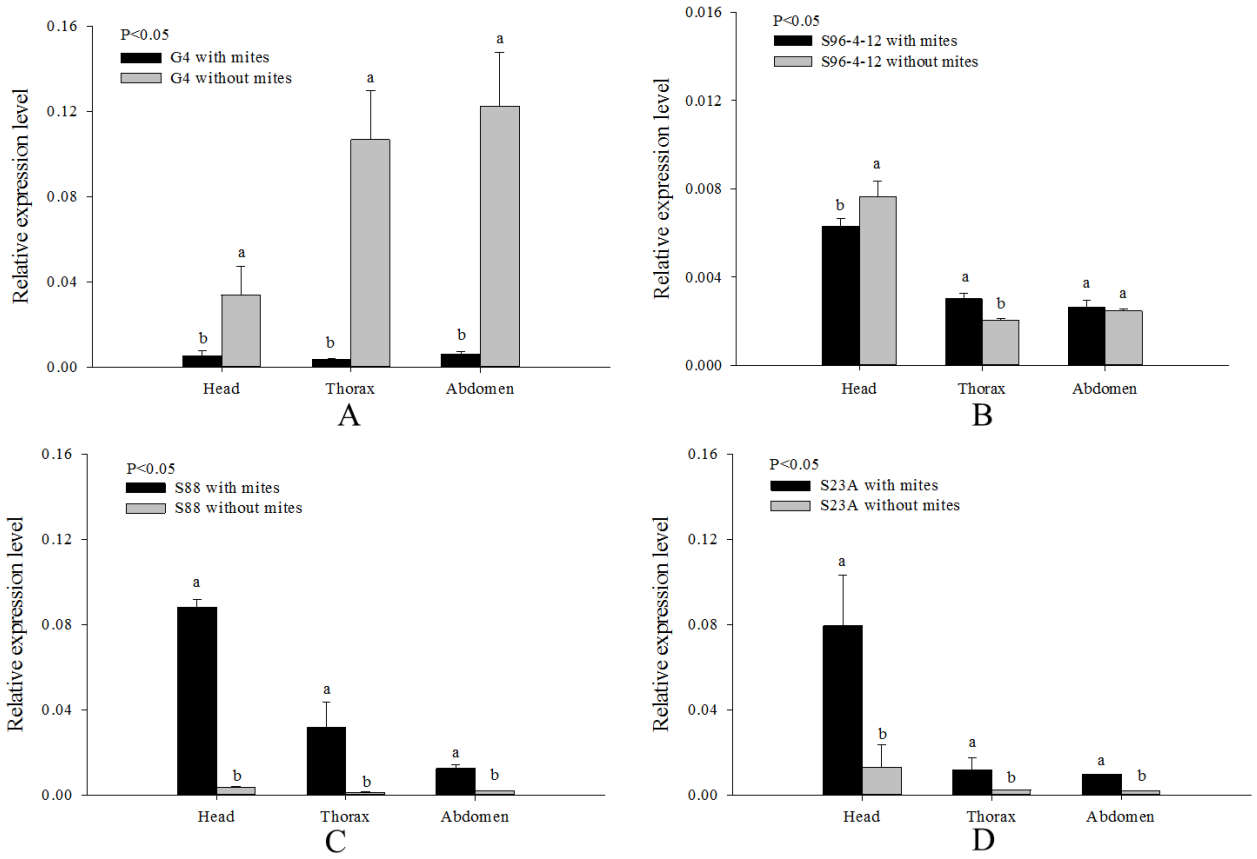

Figure 5.1 Tissue-differential expression of *AmCbE E4* in four honey bee colonies with and without the mite infestation.

y axis: relative gene expression levels (mean±SEM, N=3). x axis: three honey bee tissue. A. G4; B. S96-4-12; C. S88; D. S23A. Relative gene expression levels were normalized by the expression of internal reference genes (*actin* and *RpS5*), and error bars indicated the expression variability of *AmCbE E4*. Value bars signed by different letters are significantly different ($p<0.05$) in varroa mite comparison.

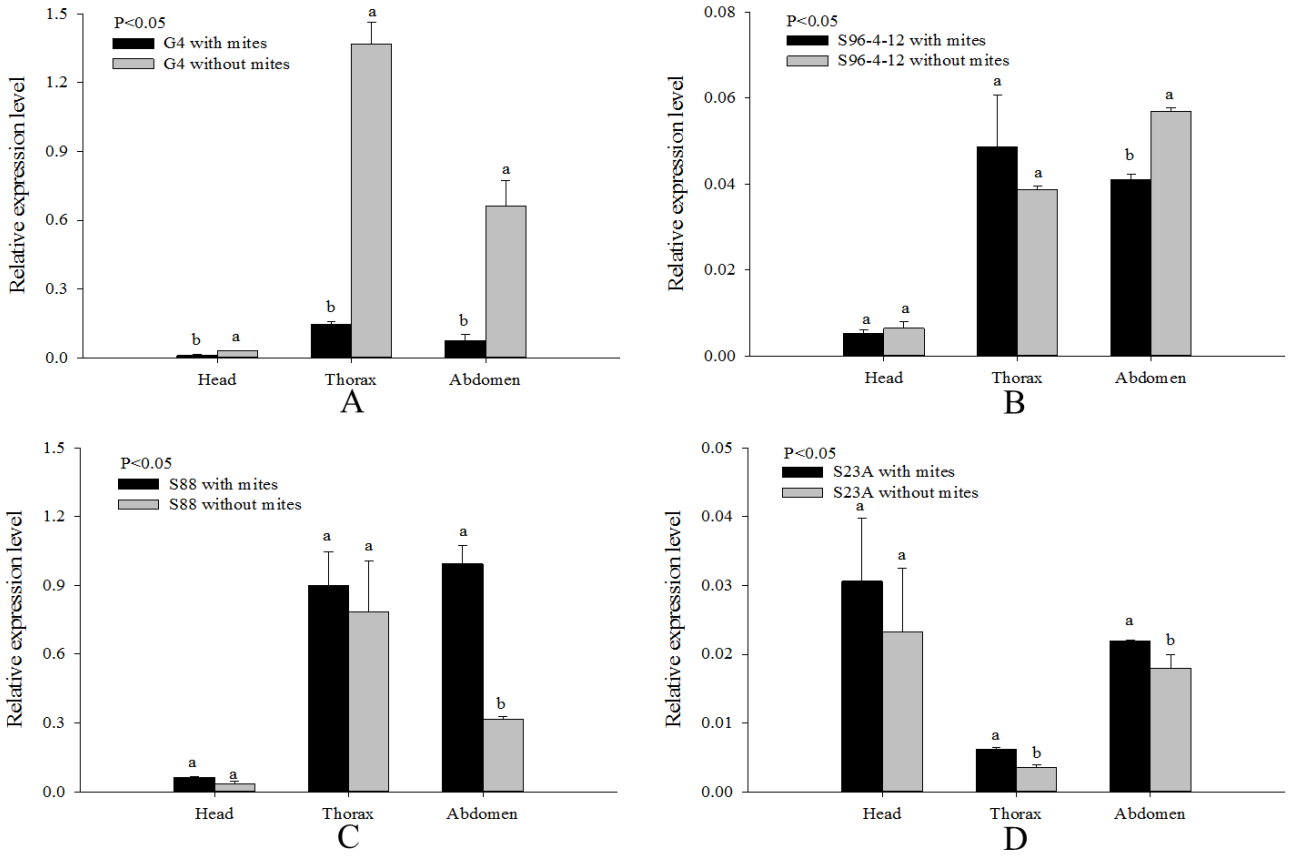

Figure 5.2 Tissue-differential expression of *AmApoD* in four honey bee colonies with and without the mite infestation.

y axis: relative gene expression levels (mean±SEM, N=3). x axis: three honey bee tissue. A. G4; B. S96-4-12; C. S88; D. S23A. Relative gene expression levels were normalized by the expression of internal reference genes (*actin* and *RpS5*), and error bars indicated the expression variability of *AmApoD*. Value bars signed by different letters are significantly different ($p<0.05$) in varroa mite comparison.

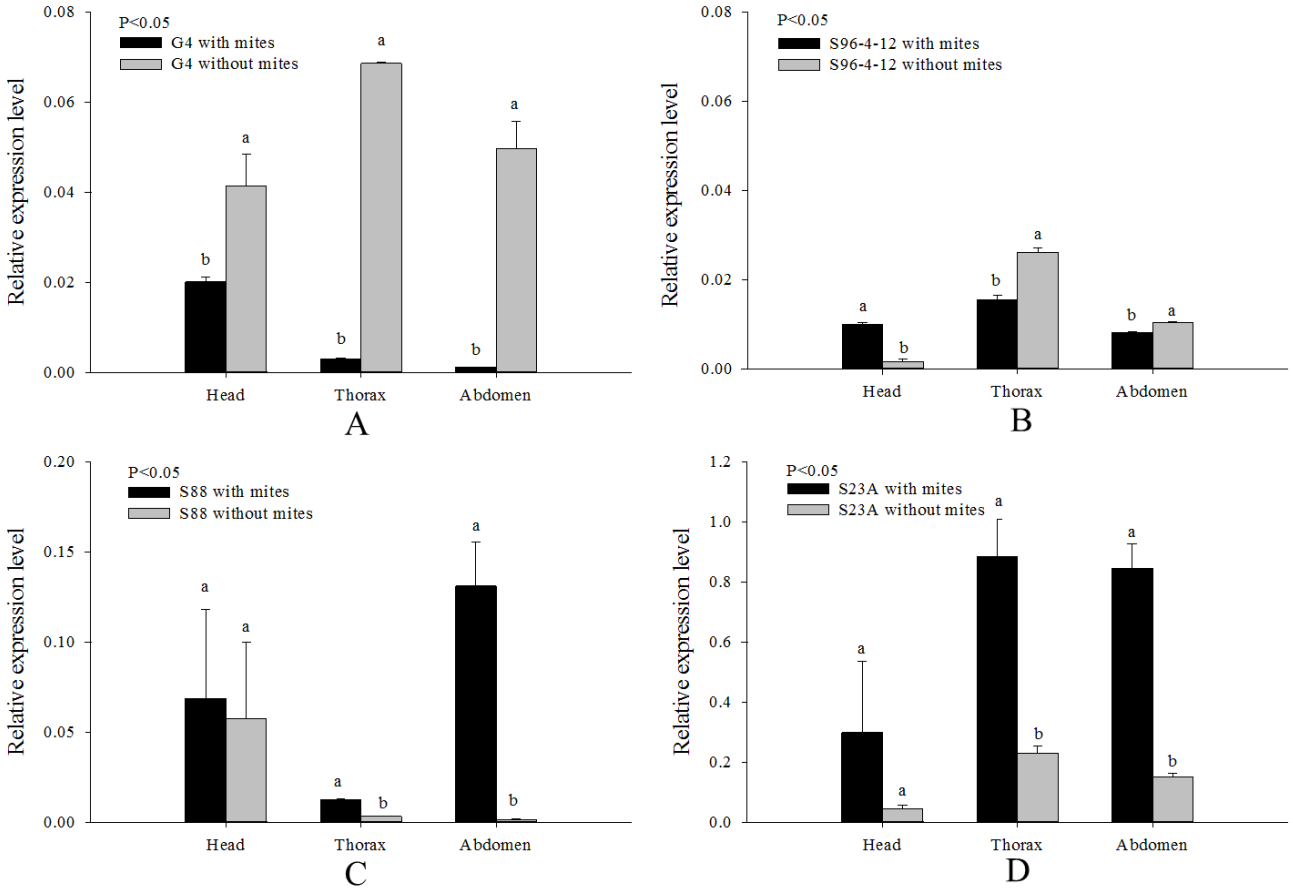

Figure 5.3 Tissue-differential expression of *AmCYP6A1* in four honey bee colonies with and without the mite infestation.

y axis: relative gene expression levels (mean±SEM, N=3). x axis: three honey bee tissue. A. G4; B. S96-4-12; C. S88; D. S23A. Relative gene expression levels were normalized by the expression of internal reference genes (*actin* and *RpS5*), and error bars indicated the expression variability of *AmCYP6A1*. Value bars signed by different letters are significantly different ($p<0.05$) in varroa mite comparison.

5.4.2 Expression of the three selected genes in response to miticide treatments

To investigate the expression of three genes in response to miticides, two different tolerant colonies (Sy26 and S14) and one moderately susceptible colony (JH-8-10) were selected for the analysis. Three different types of miticides (Apistan®, Apivar®, Thymovar®) were applied to dark eyed stage 4 pupae as described in Materials and Methods 5.3.2. After 24 h treatment, the RNAs were extracted from different tissues of treated bees and used for the gene expression analyses. The effects of miticide treatments were compared in the preferential expression tissue of respective genes. The expression of *AmCbE E4*, was compared in head tissues, and the expression of *AmApoD* and *AmCYP6A1* were compared in abdominal tissues.

In Figure 5.4A, *AmCbE E4* transcripts showed a low, but significant increase relative to controls when dark eyed stage 4 pupae were exposed to Apivar®. Apistan® and Thymovar® did not have any effects on *AmCbE E4* expression in Sy26 pupae. However, the expression of *AmApoD* transcripts was decreased or no change among three miticides, and significantly decreased in Apivar® treatment. Transcripts of *AmCYP6A1* showed dramatic increases in Apivar®, Apistan® and Thymovar® treatments over the control values in Sy26. In S14, Apivar® and Apistan® suppressed the expression of *AmCbE E4* transcripts, but not Thymovar®, relative to the control levels (Figure 5.4B). The expression of *AmApoD* transcripts were all significantly increased in S14, relative to the control levels, and Thymovar® induced expression showed a less increase than that of Apivar® and Apistan® (Figure 5.4B). Apivar®, Apistan® and Thymovar® treatments all increased *AmCYP6A1* transcription over the control levels, with Apivar® and Apistan® showing the highest increases in S14 (Figure 5.4B). Figure 5.4C showed the effects of miticide treatments in a moderately varroa susceptible colony, JH-8-10. No significant differences were detected in the expression of *AmCbE E4* transcripts. Apivar® showed no effect on *AmApoD* transcription, but Apistan® and Thymovar® both significantly suppressed *AmApoD* transcription (Figure 5.4C). *AmCYP6A1* increased the expression in response to Apivar® and Thymovar®, but not Apistan®. In the two varroa tolerant colonies (Sy26 and S14), there was a consistent increased level of in *AmCYP6A1* transcripts in response to Apivar®, Apistan® and Thymovar®, but in the more varroa susceptible colony JH-8-10, *AmCYP6A1* only responded to Thymovar® treatment.

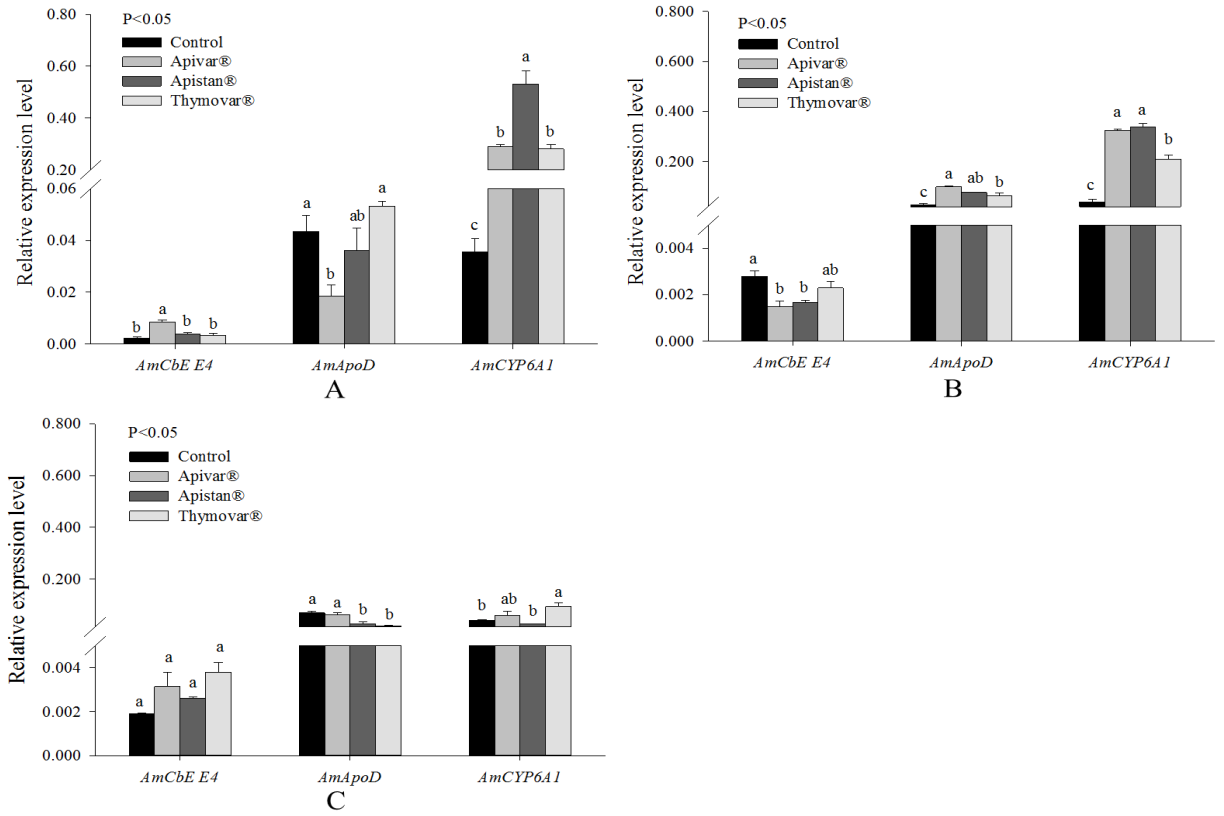

Figure 5.4 Relative expression levels of *AmCbE E4*, *AmApoD* and *AmCYP6A1* in dark eyed stage 4 pupae of three honey bee colonies in response to Apistan®, Apivar®, and Thymovar® miticide treatments.

y axis: relative gene expression (mean±SEM, N=3); x axis: three potential biomarker genes. A. Sy26; B. S14; C. JH-8-10. Notes: Expression of *AmCbE E4* was detected in head tissues, and expression of *AmApoD* and *AmCYP6A1* in abdominal tissues. Values followed by different letters are significantly different ($p<0.05$). The multi-treatment comparisons used LSD (least significant difference) method for difference analysis of each gene.

5.4.3 Effects of varroa mites on infection of deformed wing virus

Deformed wing virus (DWV) is believed to play a role in the honey bee collapse disorder, a large scale of mysterious colony loss (De Miranda and Genersch 2010). To examine the relationship between varroa mite parasitism and virus infection in honey bees, the infection of DWV in three colonies with different phenotypes with and without varroa mite infestation were compared. Table 5.1 compares the levels of DWV infection in head tissues of dark eyed stage 4 pupae of two tolerant colonies (Sy26 and S14) and one colony showing more susceptibility to varroa mites (JH-8-10). Lower Ct values indicate higher virus copy numbers (Schmittgen and Livak 2008). Sy26 showed the highest Ct values in non-infested bees, reflecting lower levels of DWV infections. Infected pupae (heads) showed an 11-fold increase in DWV copies. S14, a second varroa tolerant colony exposed for a longer time period (44 months) to varroa mites (Figure 4.1) showed higher levels of DWV in non-infected pupae (Ct at 26) than Sy26 (exposed for14 months and Ct at 35). S14 also showed a much higher level of DWV copies in mite-infested pupae (2545-fold increase) than Sy26 (11-fold increase). The more varroa susceptible colony JH-8-10 (exposed to varroa for 20 months) showed a lower virus level in non-infected pupae (Ct at 28) than S14, but when infected with varroa mites, the virus copy number increased more than 3.4 million-fold. These results showed varroa mite parasitism could influence the DWV infection. The varroa tolerant colonies showed lower DWV infections in infested pupae than varroa susceptible colony. After mite infestation, the susceptible colony was extremely susceptible to DWV infection, whereas the tolerant bees were better able to suppress varroa mite infestation, and also DWV virus infection.

Table 5.1 Quantitative measurements of DWV infection in three colonies varying in tolerance and susceptibility to varroa mite infestation by qRT-PCR.

Honey bee Colony ID	Ct Value of DWV in non-infested bees	Ct Value of DWV in mite-infested bees	Fold change of DWV (infested/non-infested)
Sy26	35.55±0.54[a]	32.06±0.22[b]	11.24
S14	26.28±0.39[a]	14.97±0.35[b]	2,544.79
JH-8-10	28.13±0.36[a]	6.42±0.70[b]	3,430,529.88

Note: Ct values for DWV detection from pupae heads equal to mean±SEM, N=3; Sy26, S14 are tolerant colonies and JH-8-10 is a susceptible colony; The significant differences were compared by Ct values between infested and non-infested samples in each colony. Values followed by different letters are significantly different ($p<0.05$).

5.4.4 Effects of miticide treatments on infection of deformed wing virus

Miticide treatments were applied to dark eyed stage 4 pupae in brood frames from two varroa tolerant colonies (Sy26 and S14) and one susceptible colony JH-8-10 as described in the section 5.3.3. Table 5.2 showed that in varroa tolerant colony Sy26, three treatments, Apivar®, Apistan® and Thymovar®, had little effects on DWV infection levels by comparing the Ct values among non-treated and treated pupae heads. In S14, a varroa tolerant colony with higher DWV levels (Ct at 24) in non-infested pupae, DWV levels were increased 20-fold by Apivar®, 40-fold by Apistan® and 3.4-fold by Thymovar® treatment, compared to non-treated pupae. In the susceptible colony JH-8-10 (Table 5.2), Apivar® and Apistan® treatments showed dramatic increases in DWV levels in non-mite infested pupae, 1.1 million-fold and 166 thousand-fold increases over non-treated pupae. And Thymovar® treatment showed 32-fold increase in DWV levels in JH-8-10. These observations imply common miticide treatments may also cause increases in DWV levels in the absence of varroa mite infestations.

Table 5.2 Quantitative measurements of the infection of DWV in three different colonies treated with Apivar®, Apistan® and Thymovar® miticide in the absence of varroa mite.

Honey bee Colony ID	Non-treated bees	Apivar®-treated bees	Apistan®-treated bees	Thymovar®-treated bees	Fold change (Apivar®-treated/non-treated)	Fold change (Apistan®-treated/non-treated)	Fold change (Thymovar®-treated/non-treated)
Sy26	30.19±1.57[a]	30.07±1.99[a]	30.66±1.60[a]	31.46±1.78[a]	1.09	0.72	0.41
S14	24.43±0.12[a]	20.10±0.22[c]	19.11±0.25[c]	22.66±0.14[b]	20.07	39.95	3.40
JH-8-10	26.53±0.33[a]	6.36±0.49[c]	9.19±2.60[c]	21.50±0.29[b]	1,176,986.76	165,905.20	32.60

Note: Ct values for DWV detection from pupae heads equal to mean±SEM, N=3; Sy26 and S14 are tolerant colonies and JH-8-10 is a susceptible colony; The multi-treatment comparisons used LSD method (least significant difference) for difference analysis among Ct values of each miticide treatment in each colony. Values followed by different letters are significantly different ($P<0.05$).

5.5 Discussion

Investigations of the expression of *AmCbE E4*, *AmApoD* and *AmCYP6A1* in head, thorax and abdominal tissues showed *AmCbE E4* was expressed to some extent in all tissues, but differential in head tissue. *AmApoD* showed most differential expression in the abdominal tissue, whereas *AmCYP6A1* showed most differential expression in both thorax and abdominal tissue. Differential expression patterns in the varroa tolerant and susceptible colony phenotypes were well defined in different tissues, confirming that *AmCbE E4*, *AmApoD*, and *AmCYP6A1* make good biomarkers for varroa tolerance and susceptibility. Bee heads from varroa-infested dark eyed stage 4 pupae could be used to screen for the expression of *AmCbE E4* and used to predict the colony phenotype of varroa tolerance and susceptibility. Similarly, the data indicated that *AmApoD* and *AmCYP6A1* could also be used to screen for colony phenotypes using thorax and abdominal tissues. These observations are consistent with those of other studies. EST (expressed sequence tag) sequencing revealed that *AmCbE E4* is highly expressed in the integument of the honey bee brain (Claudianos et al. 2006), while most cytochrome P450 genes are expressed in the digestive and immune systems, such as the midgut and fat body, rather than the head (Huang et al. 2013). The expression patterns observed suggest that esterase activity is required in head tissue and cytochrome P450s activity is required in abdominal and thorax tissues for detoxification processes. The differential expression of *AmApoD* in the abdominal tissue supports its positive role in the lipid metabolism that occurs mainly in this tissue, such as in the biosynthesis of storage lipids, signal molecules, and phospholipids of cellular membranes (Rassart et al. 2000). In humans, apolipoprotein D (ApoD) is widely distributed in a number of tissues, including kidney, liver, pancreas, spleen, and intestine (Drayna et al. 1987). The high degree (above 50% identities) of sequence similarity of ApoD between humans and insects likely indicates its evolutionarily conserved function in the tolerant bees for increasing the lipid metabolisms to help cope with varroa mites.

Differential expression of the three potential biomarker genes was also observed in response to three commonly used miticides (Apistan®, Apivar® and Thymovar®) in both tolerant and susceptible honey bee colonies. Dark eyed stage 4 pupae were directly exposed to strips containing the miticides. *AmCYP6A1* consistently showed higher expression in miticide-treated

bees than in control bees. Increased expression was most evident in varroa tolerant colonies (Figure 5.4). *AmCbE E4* showed an increased, but not significant expression in the tolerant colony Sy26 in response to Apistan®, a miticide which has an ester linkage, suggesting a possible role of esterase E4 in hydrolyzing miticide residues (Figure 5.4). In the tolerant colony S14, both *AmApoD* and *AmCYP6A1* showed differential expression in the treatment comparisons. However, in the susceptible colony JH-8-10, *AmCbE E4* and *AmApoD* displayed lower or similar expression levels as the controls. These expression patterns imply that varroa susceptible colonies are not as responsive as tolerant colonies to the stress imposed by miticides, with less expression of those defensive genes (*AmCbE E4*, *AmApoD*, and *AmCYP6A1*) able to detoxify active and toxic ingredients.

Apivar® is the trade name for amitraz, a formamidine pesticide which was registered for varroa control by the Canadian PMRA (Pest Management Regulatory Agency) in 2009. It is an octopaminergic agonist in arthropods and has the potential to influence honey bee behavior (Johnson et al. 2010). The higher differential expression levels of the defensive genes (*AmCbE E4*, *AmApoD* and *AmCYP6A1*) in honey bee coincide with their function in the tissues. Apistan® is a synthetic pyrethroid tau-fluvalinate, and harms the mite by blocking voltage-gated sodium and calcium channels (Johnson et al. 2010). However, tau-fluvalinate is highly tolerated by honey bees, due to the rapid oxidation by cytochrome P450 monooxygenases (Johnson et al. 2010). Significantly higher expression of *AmCYP6A1* in Apistan®-treated varroa tolerant honey bees suggested these phenotypes are able to more rapidly detoxify this miticide. The main ingredient of Thymovar® is thymol, a natural pesticide derived from plant essential oils, belonging to the monoterpenoids. Thymol harms the mite by binding to cellular octopamine or neurotransmitter GABA (gamma-aminobutyric acid) receptors (Johnson et al. 2010). No significant difference in expression of the protective genes in the treatment comparison implies it might not have a serious effect on honey bees when used at the recommended concentrations.

Real time qRT-PCR has been successfully applied for detection of viral loads in honey bees, providing rapid and accurate information for virus epidemiology, pathogenesis and diagnosis (Chen et al. 2005). Deformed wing virus (DWV) is known as one of the factors causing

increased colony losses (Ryabov et al. 2014), with symptoms such as crippled wings, shortened abdomens and increased mortality of the honey bees (Boecking and Genersch 2008; Chen and Siede 2007; De Miranda and Genersch 2010). The virus relies primarily on the ecto-parasitic mite, *Varroa destructor*, as the vector for spreading among colonies (Di Prisco et al. 2016; Schöning et al. 2012). Except for studying the individual primary stressor causing large-scaled colony losses, many researchers also focus on the possible synergistic interaction between those stressors (Nazzi et al. 2012; Nazzi and Pennacchio 2014). For instance, the interoperable parasite-insecticide interactions (*Nosema ceranae* and fipronil) are found to adversely affect honey bee survival (Aufauvre et al. 2012). The mutualistic symbiosis between DWV and the varroa mite can interfere NF-κB signaling (nuclear factor kappa-light-chain-enhancer of activated B cells) and affect humoral and cellular immune responses in honey bees (Di Prisco et al. 2016). However, the causal link between insecticide application and pathogen infection remains to be determined, although the impact of pesticide and/or miticide application on the viral load is known in honey bees (Doublet et al. 2015; Locke et al. 2012; Smart et al. 2016). Exposing honey bees to neonicotinoid insecticides, clothianidin and imidacloprid, results in a reduced immune competence, increasing the viral load (Di Prisco et al. 2013). However, another study showed that the treatment with miticides, such as thymol and organophosphate coumaphos, reduces levels of the pathogen as a result of inducing expression of the genes in the detoxification and immune response in varroa mite-free honey bees (Boncristiani et al. 2012).

This study demonstrated an astonishing contrast in the DWV load between tolerant and susceptible honey bee colony phenotypes in response to varroa mite infestation and miticide treatments. Generally, both stressors, varroa mites and miticides, can enhance DWV loads in both varroa tolerant and susceptible bee colony phenotypes. However, the degree of increase in the load varied with the phenotype. The varroa tolerant bees had much lower DWV infection loads than susceptible bees when infested with mites. For instance, the varroa tolerant colony Sy26 is less susceptible to DWV regardless of the mite infestation or miticide treatments (Table 5.1 and Table 5.2). In contrast, the mite susceptible colony JH-8-10 which is extremely vulnerable to DWV virus infection particularly after miticide treatment, indicating its defense system is less able to cope with the DWV infection (Table 5.1 and Table 5.2). Meanwhile, the role of the varroa mite as the vector for increasing DWV infection is confirmed by the significant

increase in DWV loads in pupae with varroa infestation (Martin et al. 2012; Ryabov et al. 2014). Moreover, the extent of the increase in virus loads in the colony was also highly varied with each individual miticide treatment. Semi-synthetic thymol is less toxic to honey bees (Dahlgren et al. 2012); thus the increased amount of virus was less drastic after its treatment. Apivar® and Apistan® are two synthetic miticides and possess strong influences on honey bee behaviors. The treatments drastically increased the virus loads in the colony (>160,000 times). This implies that the effect of miticide treatment on the virus load may be dependent on the toxicity of a miticide to the bees and the colony's phenotype in response to the mites.

Besides serving as a vector to spread the virus, the varroa mite seriously harms the bees by sucking nutrients from the host and damaging the hosts' defensive system (Rosenkranz et al. 2010). Thus, the varroa mite is still considered the major factor responsible for increased colony losses. Currently, the main effort in coping with varroa mites is to apply the miticide treatments in the hives (Al Naggar et al. 2015). However, despite various miticides having been trialed, the effectiveness of chemical control is still limited as to reduce mite infestation. Because of this, breeding varroa tolerant honey bee colonies is an attractive way to fight against varroa infestation and then reduce colony losses. Therefore, comprehensive understanding of the synergistic relationship among the insecticide, parasitic mite and infected pathogens is very valuable to breed honey bee populations with enhanced fitness, longevity and productivity.

6. STUDY 3: BIOCHEMICAL CHARACTERIZATION OF A DIFFERENTIALLY EXPRESSED CARBOXYLESTERASE IN RESPONSE TO VARROA MITE PARASITISM

6.1 Abstract

AmCbE E4 encoding a putative esterase from honey bee (*Apis mellifera*) was selected for further biochemical characterization because of its increased differential expression between tolerant and susceptible colonies in response to varroa mite infestation. Sequence analysis indicated that the putative protein sequence contained a signal peptide at the N-terminus and three residues presumably as the active triad for esterase activity were conserved. The protein sequence shared 36.2% amino acid identity with a biochemically characterized peach–potato aphid (*Myzus persicae*) esterase E4. To express this gene in *E. coli*, two sets of specific primers were used to amplify intact (full length ORF) and short (the signal peptide removed) *AmCbE E4*, respectively, using cDNA prepared from dark eyed stage 4 pupae as a template for RT-PCR (reverse transcription polymerase chain reaction). The two amplicons were cloned into a bacterial expression vector pET28a and expressed in an *E. coli* strain Rosetta2 (DE3) pLysS for functional analysis. The activity assays were conducted using cell lysates of the transformants as enzyme sources, and α-naphthyl acetate, β-naphthyl acetate and para-nitrophenyl acetate as substrates. It was the short AmCbE E4protein that had catalytic activity on the synthetic substrates. The optimal pH was pH 9 and the optimal temperature was 37◦C when para-nitrophenyl acetate was used as a substrate. The highest specific enzymatic activity was found on para-nitrophenyl acetate, followed by α-naphthyl acetate and β-naphthyl acetate. In addition, AmCbE E4 could hydrolyze carbaryl, a carboxylester pesticide. These results confirm the biochemical function of *AmCbE E4* as an esterase and indicate the possibility that the varroa tolerant honey bees may use it to protect against varroa mites by detoxifying ester-like compounds generated by varroa mite parasitism or to protect the bees from the harm of certain pesticides having ester linkages.

6.2 Introduction

In previous study, *AmCbE E4* encoding a putative esterase E4, showed significant differential expression in the head tissues of honey bees in different honey bee colonies showing varying degrees of tolerance and susceptibility to varroa mite. It showed the best potential as an informative biomarker gene for identifying varroa tolerant and susceptible phenotypes by transcriptional analyses. In addition, miticide treatment was found to induce the expression of *AmCbE E4* in tolerant honey bee colonies, such as Sy26 and S14.

Insect pesticide resistance is generally induced by long term use of a pesticide or an insecticide. Metabolic tolerance is predominantly dependent on the detoxification process gradually developed by insects to degrade the chemical (Onstad 2013). Carboxylesterase (CbE, EC3.1.1.1) has been shown to be involved in the detoxification process of estesr-like compounds such as carbamates and pyrethroids (Bass et al. 2014; Jackson et al. 2013; Sogorb and Vilanova 2002; Wang et al. 2015). To our knowledge, no carboxylesterase has been characterized for detoxification processes in honey bees. In the present study, *AmCbE E4* was cloned from honey bees and expressed in *E. coli* for *in vitro* enzyme assays with both synthetic substrates and pesticides (carbamate and organophosphate). The results provide functional evidence that this gene may play a role in the detoxification process and help defense against varroa mite parasitism and environmental pesticide exposure in honey bee colonies.

6.3 Materials and methods

6.3.1 Bioinformatic sequence analysis

On-line software from the websites, http://www.ncbi.nlm.nih.gov/, http://www.cbs.dtu.dk/, http://www.expasy.org/, and DNASTAR developed by Lasergene (Madison, USA) were used for sequence analyses of *AmCbE E4*. The nucleotide and protein sequences were analysed by ProtParam tool (ExPASy) and open reading frame (ORF) was identified by ORF Finder (National Center for Biotechnology Information, NCBI). Conserved domains were predicted using the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd). Homology alignments of *AmCbE E4* sequences and phylogenetic trees were constructed by ClustalW

method by DNASTAR 7.1. The signal peptide was predicted according to SignalP 4.1 (Center for Biological Sequence Analysis, CBS) and ProtScale (ExPASy). The topology structure was predicted by Pro-origami (Protein Structure Cartoons), and three-dimensional protein structure was simulated by Swiss-Model (ExPASy).

6.3.2 Cloning

6.3.2.1 Primer design and amplification of *AmCbE E4*

The total RNA was isolated from dark eyed stage 4 pupae followed by cDNA synthesis as described in 4.3.3. According to SignalP analysis, *AmCbE E4* contains a 69 base pairs signal peptide sequence for membrane targeting. In order to obtain the soluble active protein, the signal peptide was removed by designing the forward primer targeted just after the 69th bp of the original open reading frame. Two primer sets, WJ15F/R and WJ16F/R, for amplifying short and intact *AmCbE E4s*, separately, were designed with restriction digestion sites attached, according to the full length sequence from GenBank database (GenBank accession number *GB53798*), and then synthesized by Sigma-Aldrich (Oakville, CA) (Table 6.1). The polymerase chain reaction (PCR) mixtures (total volume of 25 µl) consisted of 50 ng of cDNA template, 0.5 µM of each primer, 200 µM of dNTP (deoxynucleotide 5'-triphosphate), 0.5 U of Q5® High-Fidelity DNA polymerase (New England BioLabs Inc, Whitby, CA), 5 µl of 5× Q5 reaction buffer and sterilized ddH_2O up to 25 µl. The PCR program was set: denaturing at 98ºC for 30 s, followed by 35 cycles of 98ºC for 10 s, annealing at 60ºC for 30 s and extending at 72ºC for 1 min, 72ºC for 2 min and 4ºC for holding. After PCR reaction, the products were checked on 1% agarose gel.

Table 6.1 Primers designed for *AmCbE E4* cloning.

Primer	Sequence 5'-3'	Restriction digestion site	Product description
WJ15F	GCGC**GGATCC**ATCGAGCAAC CATTGGTCGAA	BamHI	Short *Am CbE E4* (without predicted signal peptide)
WJ15R	GCGC**AAGCTT**TCAATGTTTCA CTCCTCTCGAAGGT	HindIII	
WJ16F	GCGC**GGATCC**ATGAAGTATA ACACTTTGATATCG	BamHI	Intact *Am CbE E4* (with predicted signal peptide)
WJ16R	GCGC**AAGCTT**TCAATGTTTCA CTCCTCTC	HindIII	

Two amplified bands were cut from DNA gels, followed by gel extraction using the Plasmid DNA, Gel Extraction and PCR Product Purification Kits (Bio Basic Inc., Markham, CA). The purified DNA molecules were double enzyme digested first with BamHI (Invitrogen, Burlington, CA) and then with HindIII (Invitrogen, Burlington, CA). The BamHI digestion reaction mixture contained: 1 µg purified DNA molecules, 1 µl BamHI, 2 µl 10× Rec1 buffer, and sterilized ddH_2O up to 20 µl, then the mixture was incubated at 30^oC for 1 h. After incubation, the digested DNA molecules were purified by PCR Product Purification Kits (Bio Basic Inc., Markham, CA). The HindIII digestion reaction mixture contained: purified DNA molecules, 1 µl HindIII, 2 µl 10× Rec2 buffer, and sterilized ddH_2O up to 20 µl, then the mixture was incubated at 37^oC for 1 h. After incubation, the double digested DNA molecules were purified by PCR Product Purification Kits (Bio Basic Inc., Markham, CA), and then ligated with same double enzyme digested protein expression plasmid pET28a (Novagen, EMD Millipore Ltd, Etobicoke, CA) (Figure 6.1). The ligation mixture contained: 50 ng of digested pET28a, 50 ng of double digested DNA molecules (short and intact *AmCbE E4*), 1 U of T4 ligase (Invitrogen, Burlington, CA), 2 µl of 5× ligase buffer, and sterilized ddH_2O up to 10 µl, then the mixture was incubated at room temperature for 30 min, and then stored at -20^oC in a freezer.

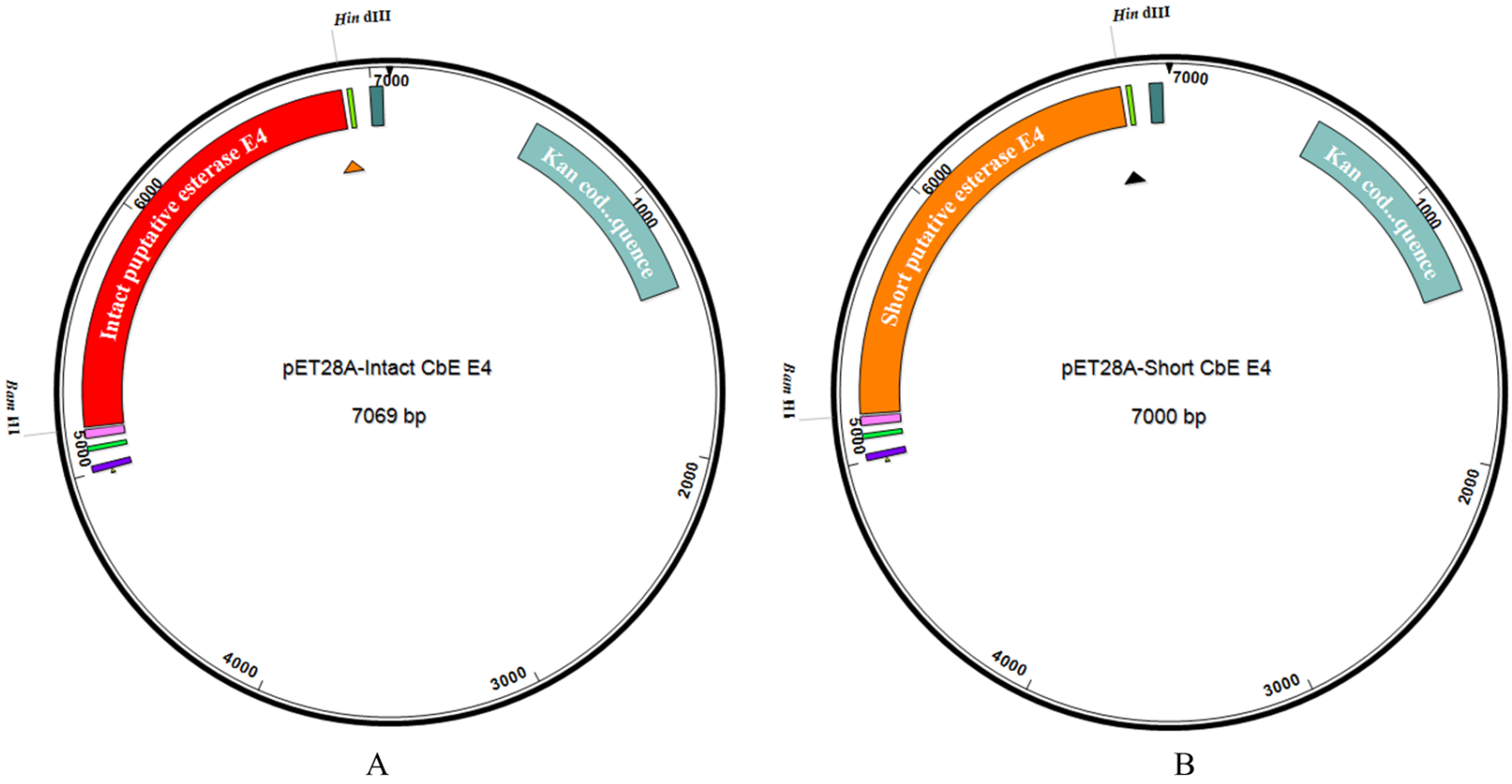

Figure 6.1 Recombinant plasmid maps of pET28a_IntactE4 (A) and pET28a_ShortE4 (B).

Small tri-angle before purple box indicates T7 promotor region; the purple box indicates lac operator; the green box indicates 6×His tag; the pink box indicates T7 tag; the orange box indicates short *AmCbE E4*; the red box indicates intact *AmCbE E4*; the black/orange tri-angles indicates stop codon; the dark blue indicates T7 terminator; the light blue box indicates kanamycin resistance gene expression cassette.

6.3.2.2 Preparation of *E. coli* competent cells

Two host stains were used in this project: *E. coli* Top10 and Rosetta2 (DE3) pLysS (Novagen, EMD Millipore Ltd, Etobicoke, CA). As Rosetta2 (DE3) pLysS strain carries resistance to chloramphenicol, the LB plates and LB liquid medium used for competent cell preparation of Rosetta contained 34 µg/ml chloramphenicol (Sigma-Aldrich, Oakville, CA). The protocol for competent cell preparation was as follows: scrape off a portion from the top of the frozen glycerol *E. coli* Top10 or Rosetta2 (DE3) pLysS stocks, and streak each of them on the LB plates; incubate the plates at 37ºC overnight; inoculate single colony of *E. coli* Top10 and Rosetta2 (DE3) pLysS into 2 ml LB liquid medium and shake at 37ºC overnight; inoculate 1 ml overnight cell culture into 100 ml LB medium (in a 500 ml flask) and shake at 230 rpm in 37ºC incubator until OD600= 0.3-0.5 (usually it takes about 2.5-3 hours); chill the culture on ice for 20 min, and at the same time chill 0.1 M $CaCl_2$ solution and 0.1 M $CaCl_2$ containing 15% glycerol; transfer the culture into two 50 ml tubes, then centrifuge the cells at 3, 000 g for 10 min at 4ºC (Eppendorf centrifuge 5804 R, Fisher Scientific, Burlington, CA); discard the medium and re-suspend the cell pellet in 30-40 ml cold 0.1 M $CaCl_2$; incubate the cells on ice for 30 min; centrifuge the cells as described above; remove the supernatant, and resuspend the cell pellet in 6 ml 0.1 M $CaCl_2$ solution containing 15% glycerol; transfer each 150 µl of the cell suspension into sterile 1.5 ml microcentrifuge tubes; quickly freeze these tubes in the liquid nitrogen and then store them in -80ºC freezer.

6.3.2.3 Recombinant plasmid transformation and sequencing

Recombinant plasmids carrying short *AmCbE E4* (pET28a-ShortE4) and intact *AmCbE E4* (pET28a-IntactE4) were transformed into *E. coli* Top10 competent cells for sequencing. The protocol for the transformation was as follows: thaw competent cells on ice; add 10 µl ligation mixture to the cell and incubate on ice for 30 min; heat shock at 42ºC for 45 s; put back on ice for 2 min; add 1 ml pre-warm LB medium and shake at 220 rpm for 1 h in 37ºC incubator; evenly spread 150-300 µl on the LB plate added with 50 µg/ml kanamycin (Sigma-Aldrich, Oakville, CA) to screen the positive colonies. Positive colonies were picked up for colony PCR tests and re-inoculated to new LB medium containing kanamycin and culture overnight at 37ºC for plasmid extraction and double enzyme digestion verification. The reaction mixture (total

volume of 20 µl) of colony PCR test consisted of the colony cells, 0.5 µM of each primer, 200 µM of dNTP (deoxynucleotide 5'-triphosphate), 0.5 U of Q5® High-Fidelity DNA polymerase (New England BioLabs Inc, Whitby, CA), 4 µl of 5× Q5 reaction buffer and sterilized ddH_2O up to 20 µl. The PCR program was set: denaturing at 98^{o}C for 30 s, followed by 35 cycles of 98^{o}C for 10 s, annealing at 60^{o}C for 30 s and extending at 72^{o}C for 1 min, 72^{o}C for 2 min and 4^{o}C for holding. After PCR reaction, the products were checked on 1% agarose gel. The plasmids were extracted from each colony culture followed by double enzyme restriction digestion as described above. Cultures of the positive in both colony PCR test and enzyme digestion test were stored in -80^{o}C by adding 15% glycerol. Then all positive plasmids were sent out for sequencing (Plant Biotechnology Institute, Saskatoon, Canada). For sequencing *AmCbE E4*, two extra sequencing primers were designed, WJ17F 5'GTTGTGCGGCTACAGCG'3 and WJ18F 5'GATCAGCGAAAGGACTC'3, and synthesized by Sigma-Aldrich (Oakville, CA).

6.3.3 Protein expression in *E.coli*

Correct plasmids containing pET28a-ShortE4 and pET28a-IntactE4 were then transformed into Rosetta2 (DE3) pLysS competent cells for expressing the recombinant proteins. LB medium and LB plates used for transformation and expression all contained kanamycin and chloramphenicol. The protocol for transformation was described in 6.3.2.3. Colonies growing on the plates were screened by colony PCR tests to identify positive transformants.

A single colony with positive transformants from the host Rosetta2 (DE3) pLysS harboring the expression plasmid (pET28a-ShortE4 and pET28a-IntactE4, separately) was inoculated with 3 ml LB medium containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol and shaking at 220 rpm overnight at 37^{o}C. A 150 µl aliquot of pre-culture was inoculated into 15 ml of fresh LB medium containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol and incubated aerobically at 220 rpm in 37^{o}C incubator until OD600 reached to 0.4–0.6. Then IPTG (Isopropyl β-D-1-thiogalactopyranoside, Sigma-Aldrich, Oakville, CA) was added at a final concentration of 0.5 mM to induce protein expression at 37^{o}C for 3 h. After induction, a 15 ml culture was centrifuged at 8000 rpm in 4^{o}C for 5 min, then washed with 5 ml of 50 mM Tris-HCl buffer, pH 8.0 for 3 times, and centrifuged at 10,000 rpm for 5 min in 4^{o}C. The cell pellets were re-

suspended in 1.5 ml of 50 mM Tris-HCl buffer and the mixture was transferred to a 2 ml tube with 500 µl of pre-chilled 0.1 mm diameter glass beads. The cells were disrupted by Mini-Beadbeater-16 (Biospec products, Bartlesville, USA) 3 times by vibrating each time for 45 s, and then placed on ice for 1 min. The cell lysate was centrifuged at 12,000 rpm for 15 min at 4^{o}C (Eppendorf centrifuge 5804 R, Fisher Scientific, Burlington, CA) and the supernatants were transferred to a new 1.5 ml tube as the crude enzyme. SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was applied to evaluate the yield of soluble expressed proteins. The loading sample contained 20 µl of crude enzyme and 20 µl SDS–PAGE loading buffer (19 µl of 2× Laemmli Sample Buffer and 1 µl of β-mercaptoethanol (BIORAD, Mississauga, CA)). Then the mixture was heated at 99^{o}C for 10 min, and 10 µl of the denatured protein sample was loaded for electrophoresis. The gel was stained with PageBlue Protein Staining Solution (Thermo Scientific, Burlington, CA). Empty vector transformant was used as the negative control. In order to obtain a high yield of soluble recombinant proteins, different inducing conditions were tested for both intact and short AmCbE E4, such as IPTG concentrations: 0.1 mM, 0.5 mM and 1 mM; temperatures:16^{o}C, 24^{o}C, 30^{o}C and 37^{o}C; expression durations: 3-4 h, 6-8 h, 16-18 h. Once the optimal expression condition was determined, a large scale cultures were used to express higher yields of crude enzyme.

6.3.4 Enzymatic assays

The protein concentrations of expressed enzymes were determined by the Bradford method with a standard curve generated from a series of protein concentrations of bovine serum albumin (BSA). Chemically synthetic substrates, α-naphthyl acetate, β-naphthyl acetate and para-nitrophenyl acetate (Sigma-Aldrich, Oakville, CA) were used in the characterization of putative AmCbE E4, and specific enzymatic activities were calculated according to standard curves generated from a series of concentrations of hydrolyzed products α-naphthol/β-naphthol/para-nitrophenol at the optimal condition. The protocol of enzymatic assays was modified from the published method (Asperen 1962; Devonshire 1977; Sharma et al. 2001). The total volume of 1.25 ml reaction mixture contained expressed enzyme sample and substrate (0.3 mM α-naphthyl acetate/β-naphthyl acetate/para-nitrophenyl acetate in 0.5% acetone in 50 mM Tris-HCl, pH 8.0) (Sigma-Aldrich, Oakville, CA). After incubation at 37^{o}C for 30 min, 160 µl of freshly prepared

Diazoblue SDS reagent (0.3% Fast Blue B salt (Sigma-Aldrich, Oakville, CA) dissolved in 3.5% aqueous SDS (sodium dodecyl sulphate)) (for reactions of α-naphthyl acetate, β-naphthyl acetate) or 160 µl of 3.5% aqueous SDS (for reactions of para-nitrophenyl acetate) was added to terminate the reaction. The mixture stayed at room temperature for about 5 min until a stable blue color for α-naphthol, a red color for β-naphthol or a yellow color for para-nitrophenol product formed. Then the samples were centrifuged at 1,000 g and the absorbency of α-naphthol/β-naphthol/para-nitrophenol products were measured at 600 nm, 555 nm and 405 nm by a spectrophotometer. Enzymatic reactions with the same amount of cell lysate protein from empty vector expression host were used as negative controls.

Due to its lower molecular weight and no need for a color-reaction reagent, para-nitrophenyl acetate was used to determine the optimal reaction conditions, such as pH and temperature. Different pH buffers used were: 50 mM citrate buffers (pH 3-6), 50 mM Tris buffers (pH 7.2-9) and 50 mM carbonate-bicarbonate buffers (pH 10-10.7); different temperatures applied were: 0^{o}C, 4^{o}C, 10^{o}C, 20^{o}C, 28^{o}C, 37^{o}C, 45^{o}C and 55^{o}C. The amounts of hydrolyzed products were determined by referencing para-nitrophenol standard curves. The specific enzymatic activity was determined as the amount of α-naphthol/β-naphthol/para-nitrophenol produced by the hydrolysis of one gram of AmCbE E4 enzyme on α-naphthyl acetate, β-naphthyl acetate or para-nitrophenyl acetate per minute at 37^{o}C and pH 9.0 (the optimal condition).

6.3.5 Hydrolytic activity of AmCbE E4 on pesticide containing ester linkages

To investigate if AmCbE E4 possessed hydrolytic activity towards pesticides, three pesticides carbaryl (carbamate), parathion and paraoxon (organophosphate) (Sigma-Aldrich, Oakville, CA) were used for these assays, as they all contains ester linkage in the chemical structures. The assay conditions were the same as described in 6.3.4 except the enzymatic reaction time was 3 hours. After the hydrolysis reaction, products α-naphthol for carbaryl and para-nitrophenyl for organophosphate were detected by color changes. The specific hydrolytic activities of crude AmCbE E4 on pesticides were calculated from the amount of α-naphthol/para-nitrophenyl products generated from one gram of crude AmCbE E4 per minute at 37^{o}C and pH 9.0.

6.4 Results

6.4.1 Sequence analysis of AmCbE E4

Genomic sequence analysis showed that *AmCbE E4* contains 8 exons and 7 introns and an open reading frame (ORF) of 1719 bp in length (Figure 6.2). The start codon ATG is located at 147 bp downstream from the transcriptional start site. The ORF of *AmCbE E4* encods a protein of 572 amino acids with a molecular weight of approximately 65kDa that shares sequence similarity to other carboxylesterases in the GenBank. The predicted catalytic triad of the AmCbE E4 protein is located at Ser209, Glu344 and His467 (Figure 6.3). By searching honey bee genome resource databases (NCBI), four mRNA sequences were found to share sequence similarity to *AmCbE E4*. One mRNA sequence was in full length, and the other three mRNAs were short transcripts. All these transcript sequences were transcribed from different *AmCbE E4* genes.

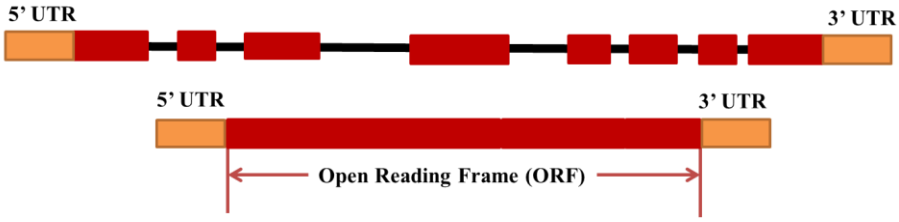

Figure 6.2 Structure of *AmCbE E4*.

Orange boxes represents the 5' or 3' untranslated region (UTR); red boxes represents the exon and the black line represented intron regions.

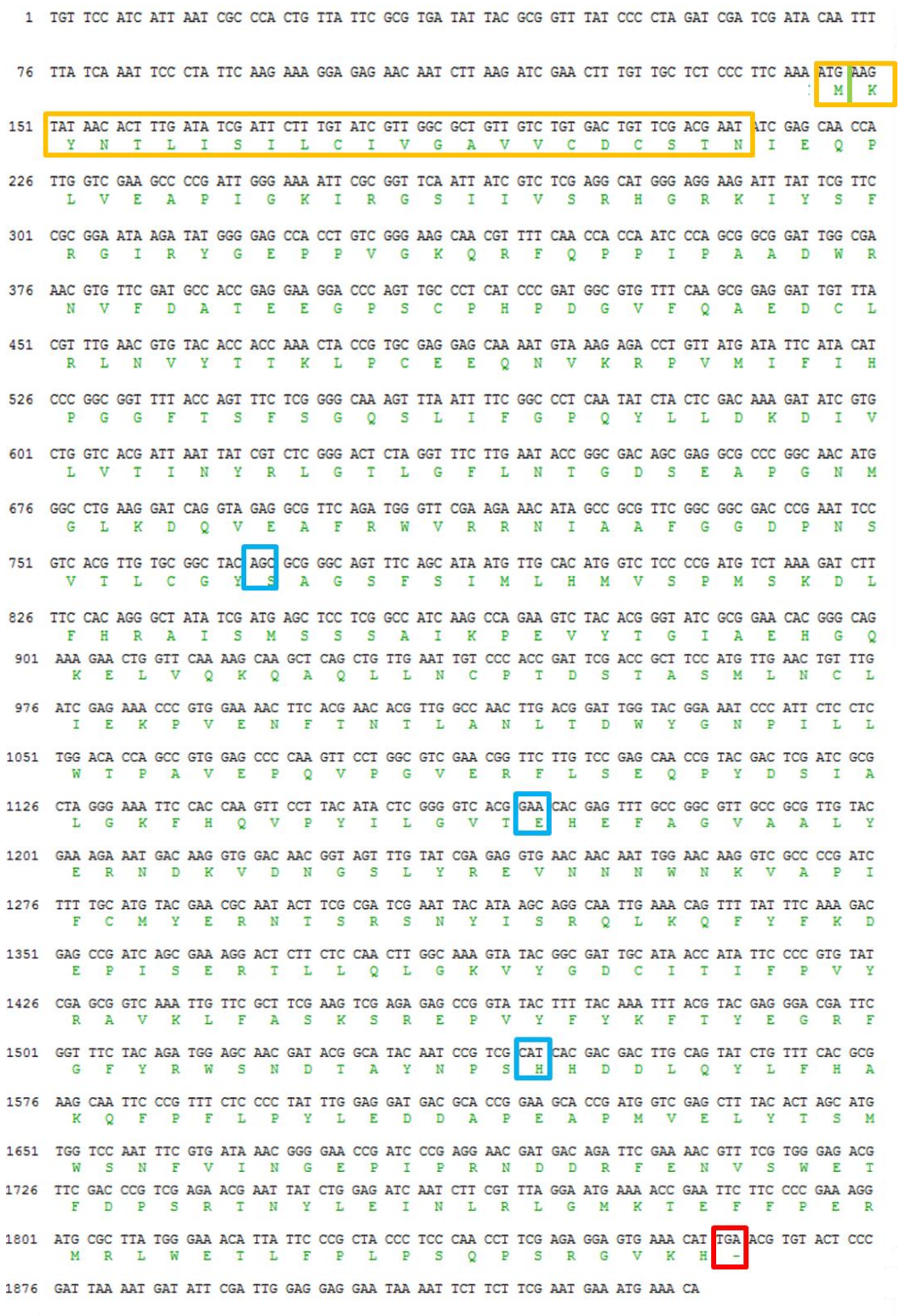

Figure 6.3 Nucleotide and amino acid sequences of *AmCbE E4*.

Green box: start codon; Red box: stop codon; Blue box: catalytic triad; Yellow box: signal peptide.

Hydropathic analysis of AmCbE E4 revealed that it has a highly hydrophobic region at the N-terminus (Figure 6.4) where a twenty-three amino acid sequence was predicted as a signal peptide that might guide the protein either towards the secretory pathway or to reside in the endoplasmic reticulum (Figure 6.3 and Figure 6.5). This result implied that the coding region of this gene encodes a pre-protein and activation of the enzyme might be achieved by removal of the signal peptide from the pre-protein.

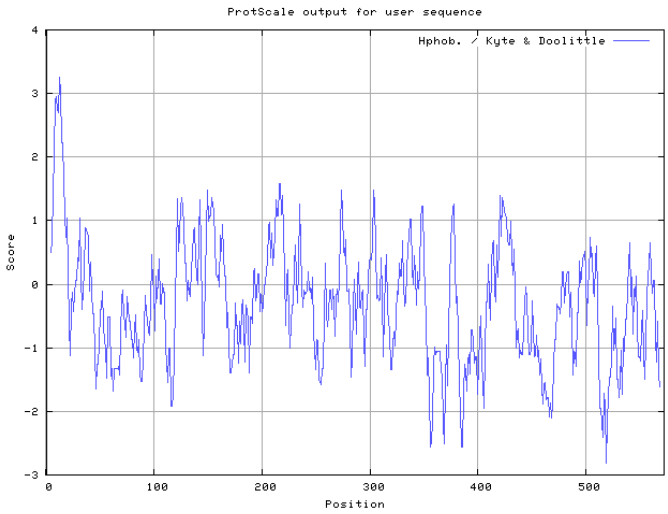

Figure 6.4 Hydropathy profile of the putative AmCbE E4 by ProtScale.

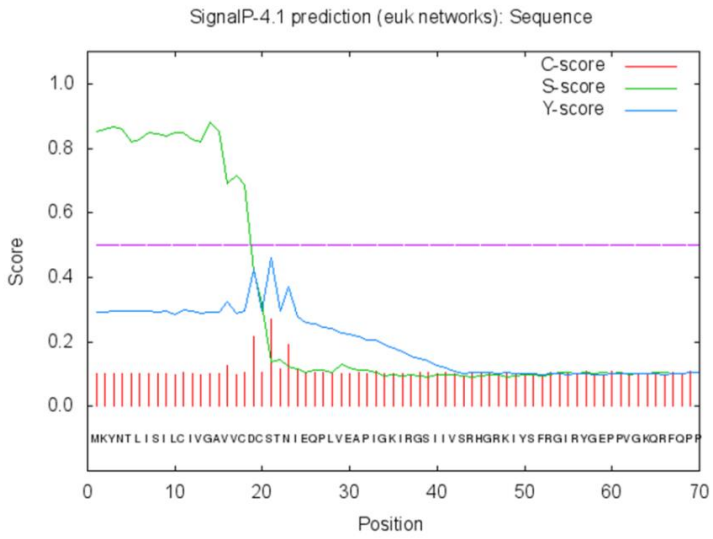

Figure 6.5 Signal peptide prediction of the putative AmCbE E4 by SignalP-4.1.

C-score (raw cleavage site score); S-score (signal peptide score); Y-score (combined cleavage site score).

Amino acid sequence alignment of AmCbE E4 with biochemically characterized insect carboxylesterases showed that the catalytic triad of carboxylesterases is highly conserved in these sequences (Figure 6.6). AmCbE E4 shared 28% and 43% identity, respectively, with BdCarE4 and BdCarE6, two α-esterase identified from the oriental fruit fly (*Bactrocera dorsalis*) for their hydrolysis ability in response to organophosphate insecticide, malathion (Wang et al. 2015). AmCbE E4 also shared high sequence identity (36%) to aphid CbE E4 presumably involved in degradation of organophosphate, carbamate and pyrethroid pesticides (Devonshire and Moores 1982). MpCbE E4, another homologue of AmCbE E4, was identified which can hydrolyze the synthetic esterase substrate β-naphthyl acetate as well as the pesticides carbaryl and malathion (Lan et al. 2005). Lc-αE7 from the Australian sheep blowfly (*Lucilia cuprina*), sharing 33% identity to AmCbE E4, plays a major role in the detoxification of organophosphate insecticides, protecting insects from the harm of pesticides (Jackson et al. 2013). Md-αE7 sharing high sequence identity to Lc-αE7 (75%), but low sequence identity to AmCbE E4 (28%), confers the organophosphate hydrolase activity to the house fly (*Musca domestica*) (Claudianos et al. 1999).

The topology structure of AmCbE E4 was constructed using the Australian sheep blowfly Lc-αE7 as a template (Figure 6.7), as the three dimensional structures of AmCbE E4 and Lc-αE7 showed highest similarities by Swiss-Model (ExPASy). The core structure of a carboxylesterase enzyme was a canonical conformation of the alpha/beta hydrolase fold composed of six alpha helices and eight beta-sheets in a parallel orientation (Montella et al. 2012). The high similarity of the AmCbE E4 in the core structure predicted its biochemical function as a carboxylesterase.

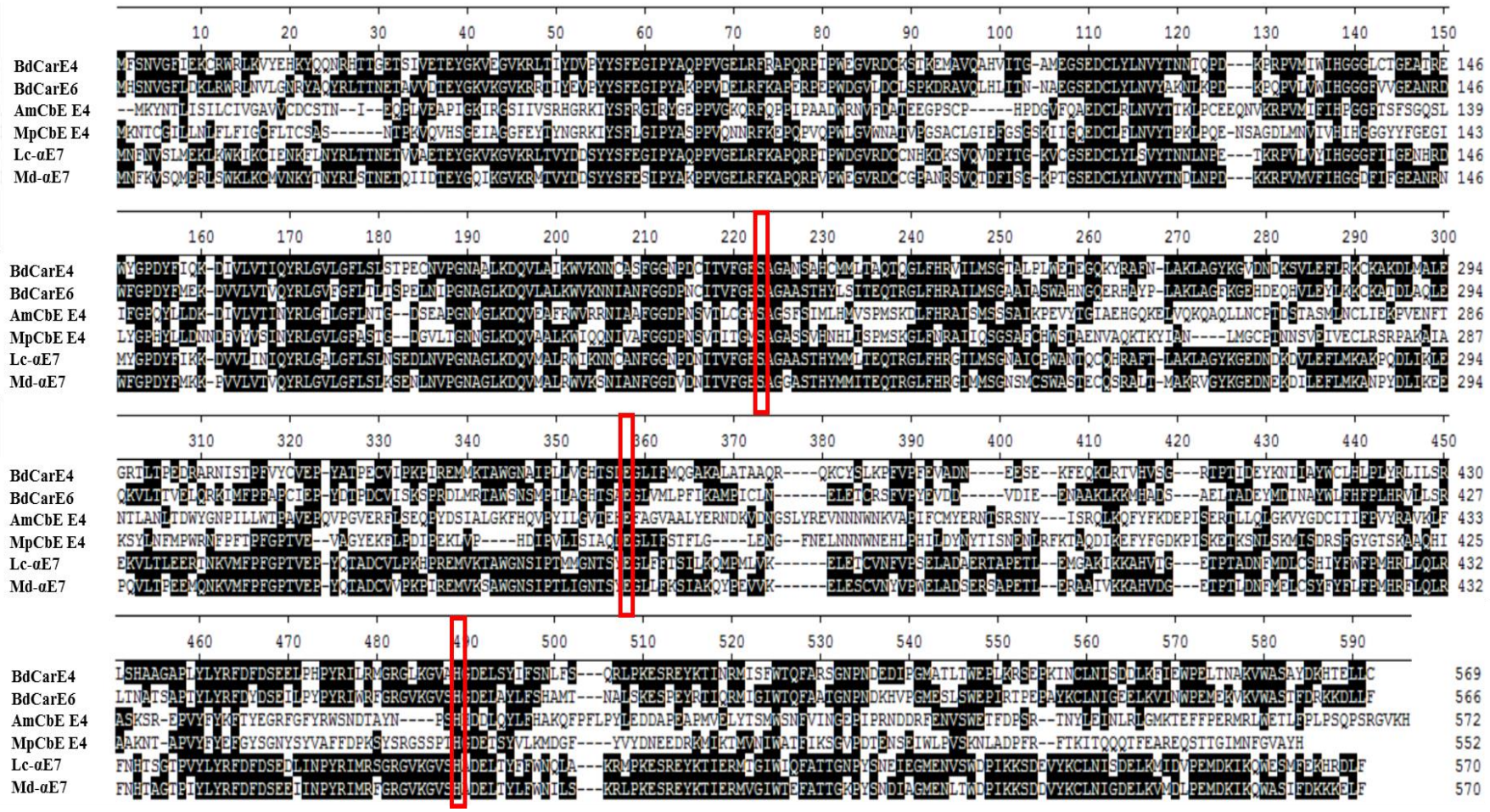

Figure 6.6 Amino acid sequence alignment of AmCbE E4 and functionally characterized CbEs from other insects.

The multiple sequence alignment was performed using DNASTAR 7.1. The conserved catalytic triad is in the red box. All insect esterase sequences were retrieved from the National Center for Biotechnology Information. Abbreviations and GenBank accession numbers are: BdCarE4, *Bactrocera dorsalis*, AKN90082; BdCarE6, *Bactrocera dorsalis*, AKN90083; MpCbE E4, *Myzus persicae*, CAA52648; Lc-αE7, *Lucilia cuprina*, AAB67728; Md-αE7, *Musca domestica*, AF133341.

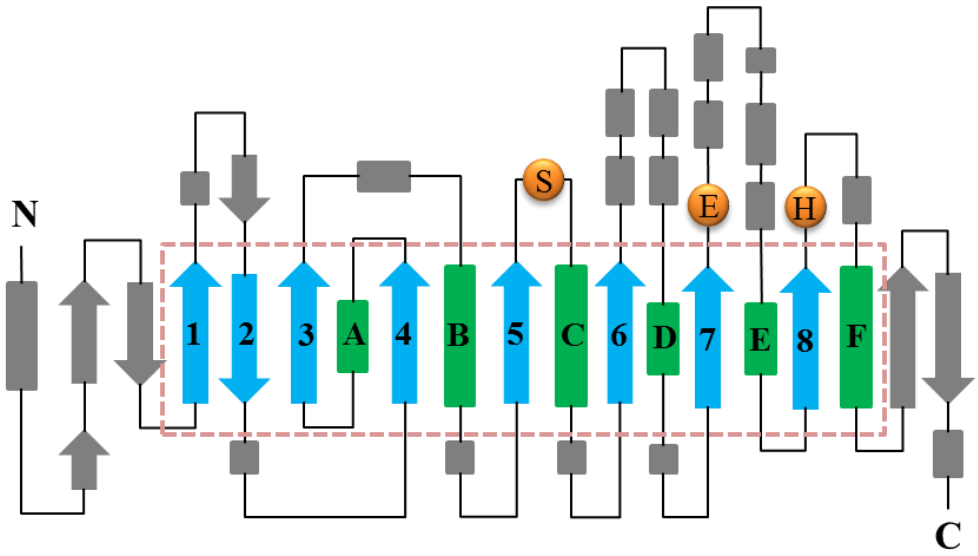

Figure 6.7 Predicted topology structure of AmCbE E4.

In the topological diagram, alpha helices represent by rectangles and beta strands by arrows; the location of the catalytic triad is indicated. Alpha helices of the "canonical" alpha/beta hydrolase fold are in green and beta strands in blue, other secondary structures in gray. Catalytic residues in orange: serine (S), histidine (H) and glutamic acid (E).

6.4.2 Cloning and expression of *AmCbE E4*

The coding region of *AmCbE E4* with and without the signal peptide sequence (intact *AmCbE E4* and short *AmCbE E4*) was amplified from cDNA of head tissues of dark eyed stage 4 pupae by RT-PCR, and cloned into the *E. coli* expression vector pET28a (Figure 6.1). The recombinant plasmids were transformed into an intermediate *E. coli* strain. Positive transformants were identified by colony PCR, showing amplification of the fragment with the right size. The plasmids from the transformants were then isolated and restriction digested for further confirmation on the release of the right fragment (Figure 6.8). After confirmation, the plasmids were then sent for sequencing. Sequencing results showed that the ORF of intact *AmCbE E4* was 1719 bp long encoding 572 amino acids. The ORF of the short *AmCbE E4* was 1650 bp nucleotides encoding 549 amino acids. The ORFs were right behind the T7 inducing promoter in the recombinant plasmids.

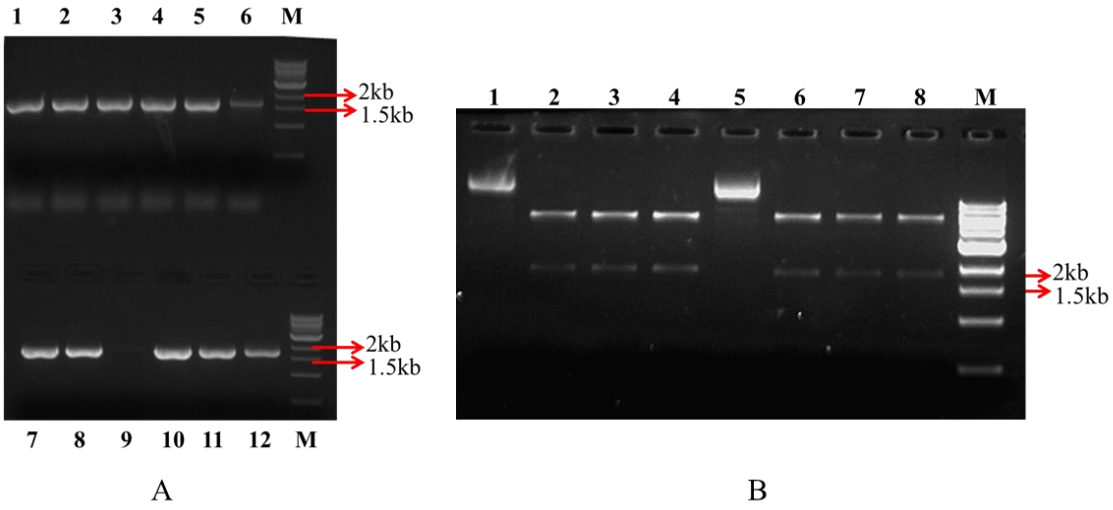

Figure 6.8 Positive transformants identified by colony PCR (A) and double enzyme restriction digestion (B).

A. Colony PCR of transformants: Lane 1-5: short *AmCbE E4* (1650 bp); Lane 6: positive control (short *AmCbE E4* cDNA); Lane 7-11: intact *AmCbE E4* (1719bp); Lane 12: positive control (intact *AmCbE E4* cDNA). B. BamHI and HindIII double digestion of recombinant plasmids from positive transformants. Lane 1: un-digested pET28a-IntactE4; Lane 2-4: digested pET28a-IntactE4; Lane 5: un-digested pET28a-ShortE4; Lane 6-8: digested pET28a-ShortE4. M: 1kb DNA marker (New England Biolabs Inc., NEB).

For functional expression, the two recombinant plasmids, pET28a-IntactE4 and pET28a-ShortE4, were transformed into an expression host strain *E. coli* Rosetta2 (DE3) pLysS, respectively. After induction, the proteins were isolated from the cultures and electrophoresed on a SDS-PAGE gel. The result showed that pET28a-ShortE4 could, but pET28a-IntactE4 could not, express a soluble protein in *E.coli* visualized in an electrophoresis gel (Figure 6.9). It indicated the removal of the signal peptide was essential for increasing the solubility of eukaryotic proteins expressed in a prokaryotic host. This coincided with the activity assays that expression of pET28a-ShortE4, but not that of pET28a-IntactE4 showed the carboxylesterase activity. The optimal expression condition was induction by 0.5 mM IPTG at 24^{o}C for 16-18 hours. On the SDS-PAGE gel, the molecular weight of the expressed short AmCbE E4 was nearly 63 kDa, close to the size of theoretical calculation from the sequence (Figure 6.9).

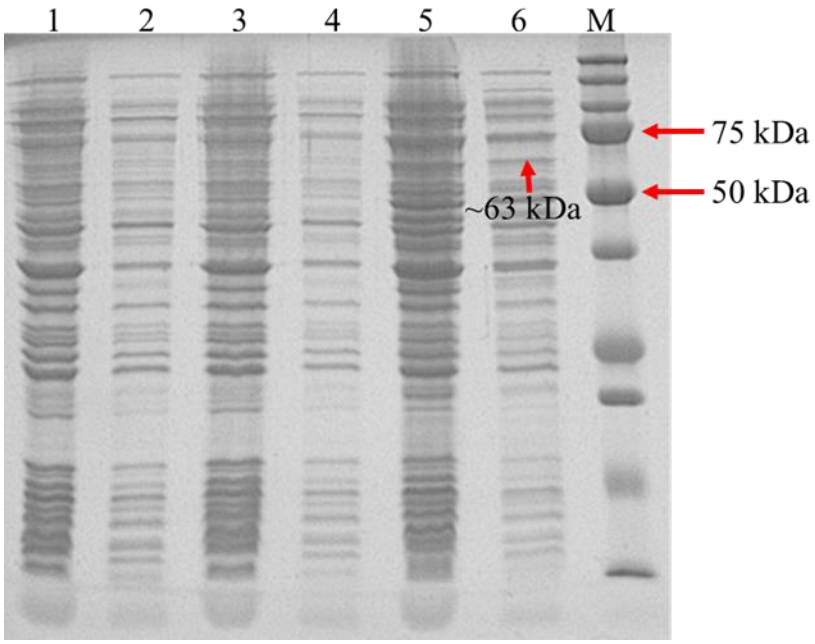

Figure 6.9 SDS-PAGE analysis of expressed AmCbE E4 proteins.

Lane 1 and 2: total and supernatant fractions of protein extracts from the culture with the empty vector pET28a; Lane 3 and 4: total and supernatant fractions of protein extracts from the culture with pET28a-IntactE4; Lane 5 and 6: total and supernatant fractions of protein extracts from the culture with pET28a-ShortE4; M: protein marker (Bio-rad, Mississauga, CA).

6.4.3 Enzymatic assays of AmCbE E4

6.4.3.1 Quantitation of the proteins and products used in the assay

Concentrations of crude protein were determined by the Bradford method with a standard curve generated from a series of bovine serum albumin (BSA) concentrations (Figure 6.10A). The concentrations of the hydrolyzed products were determined using standard curves generated from a series of concentrations of α-naphthol, β-naphthol and para-nitrophenol on their characteristic absorbance. The linear standard curves were used for calculating specific enzymatic activity (Figure 6.10B, C, D).

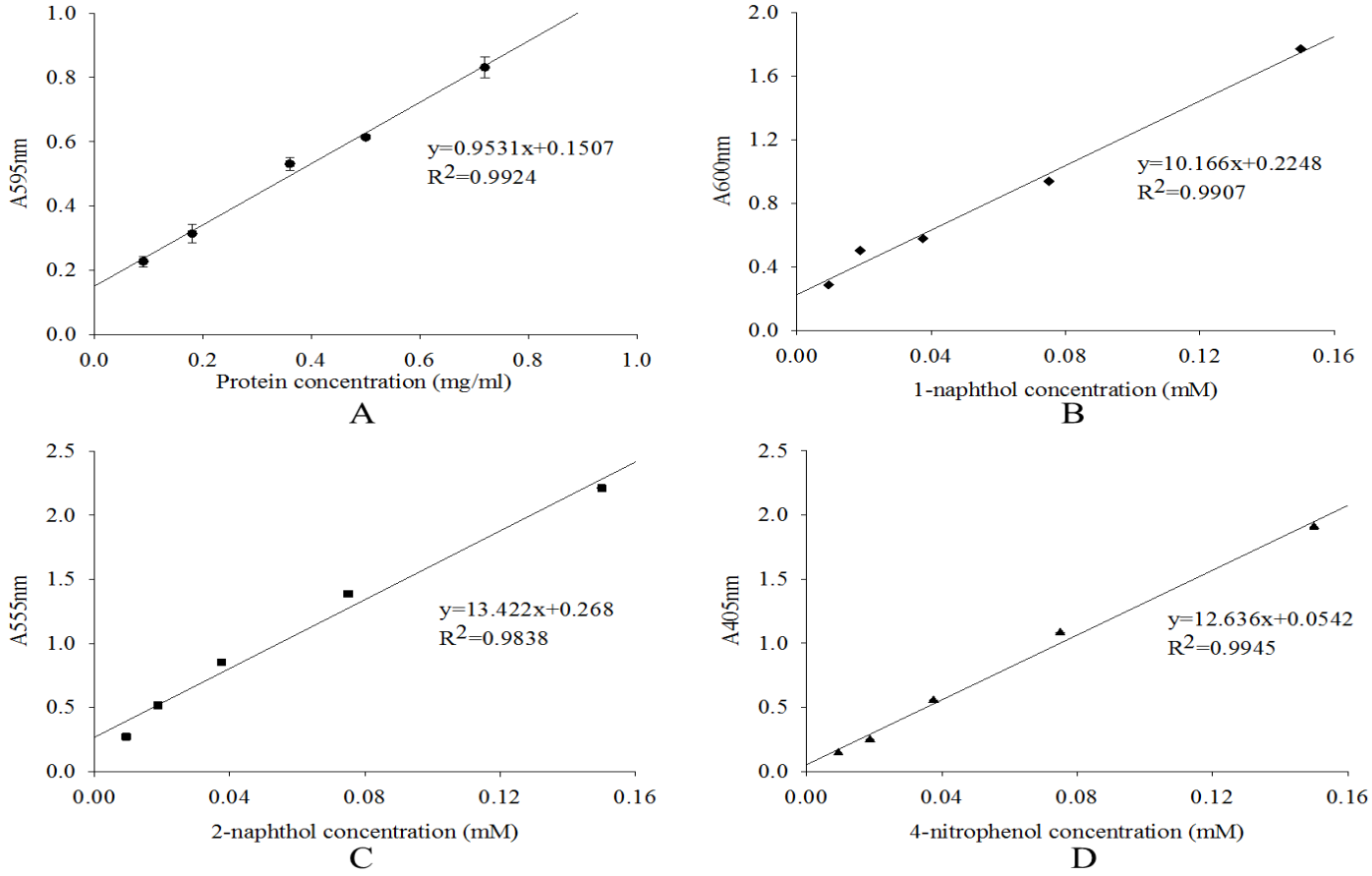

Figure 6.10 Standard curves used to calculate the activity.

A. A standard curve of absorbance at A595 nm for a protein concentration standard using BSA by the Bradford method; B. A standard curve of absorbance at A600 nm for α-naphthol concentration; C. A standard curve of absorbance at A555 nm for β-naphthol concentration; D. Standard curve of absorbance at A405 nm for para-nitrophenol concentration.

6.4.3.2 Functional characterization of AmCbE E4

Enzymatic activity of the expressed proteins was first tested on para-nitrophenyl acetate, a small synthetic substrate that has been widely used for esterase activity assays. The results showed that short AmCbE E4 was active in hydrolyzing the substrate. The optimal pH for the activity was found at pH 9.0 and optimal temperature was at 37^{o}C (Figure 6.11). Afterwards, the enzymatic assay of short AmCbE E4 was extended to two other synthetic substrates. At the optimal condition, specific activities of the expressed AmCbE E4 on the three substrates α-naphthyl acetate, β-naphthyl acetate and para-nitrophenyl acetate were in a range of 2 to 50 µmol/g/min, respectively (Table 6.2) with the highest activity found on para-nitrophenyl acetate, followed by α-naphthyl acetate and then β-naphthyl acetate.

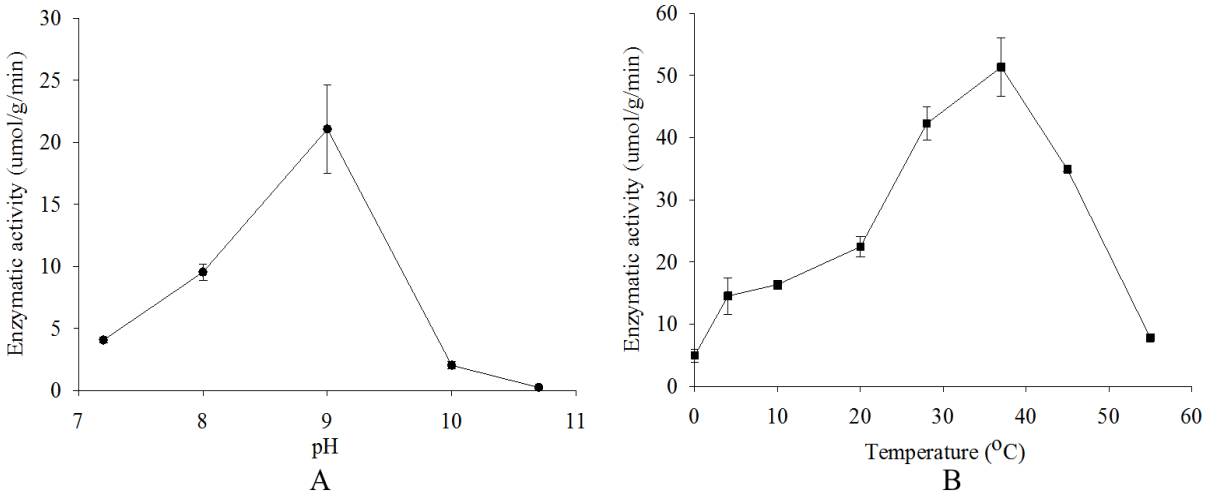

Figure 6.11 Optimal conditions of the enzymatic reaction using the crude enzyme AmCbE E4 on para-nitrophenyl acetate.

A. Enzymatic activity of crude AmCbE E4 at different pHs; B. Enzymatic activity of crude AmCbE E4 at different temperatures.

Table 6.2 Specific enzymatic activities of crude AmCbE E4.

Synthetic Substrates	Specific enzymatic activity of crude enzyme (μmol/g/min)
α-naphthyl acetate	5.18±0.24
β-naphthyl acetate	1.91±0.30
p-nitrophenyl acetate	51.37±4.71

6.4.4 Hydrolytic function of AmCbE E4 on pesticides

Carbamate and organophosphate pesticides have been widely used for the pest control in crop fields (Johnson et al. 2010). Therefore, one carbamate pesticide (Carbaryl) and two organophosphate pesticides (parathion and paraoxon) were used to check the hydrolytic activity of AmCbE E4. As shown in Table 6.3, AmCbE E4 possessed hydrolytic activity on carbaryl, although the activity was not comparable to those on the synthetic substrates. After an extended reaction time, about 11% of carbaryl was converted to α-naphthol by the crude enzyme. However, no hydrolytic activities of AmCbE E4 were detected for both organophosphate pesticides.

Table 6.3 The degradation of carbaryl by crude AmCbE E4.

Duration time (min)	Specific enzymatic activity of crude enzyme (μmol/g/min)	Substrate conversion (%)
180	1.89±0.13	11.34±0.80

6.5 Discussion

Like most insects, honey bees rely on three families of detoxification enzymes, glutathione-S-transferases (GSTs), cytochrome P450s (CYPs) and carboxyl/cholinesterase (CCEs) to protect themselves from harm of toxic, xenobiotic compounds in the environment. Some of the genes in the three families have been examined for their functions in insects (Claudianos et al. 2006). Esterase E4 belongs to carboxylesterase (CbEs, EC3.1.1.1) that can break ester linkages in carboxylesters including some pesticides (Sogorb and Vilanova 2002). Some resistant insects increase the synthesis of these enzymes to protect themselves from the pesticide (Devonshire

1977; Kao et al. 1984). For example, carboxylesterase *LmCesA20* and *LmCesE1* are reported to play an important role in detoxification of malathion in the Migratory Locust (*Loocusta migratoria*) (Zhang et al. 2014). However, there is no report on the functional characterization of honey bee esterase genes in this regard.

In this study, *AmCbE E4* was shown to be differentially expressed in honey bee colonies showing varying degrees of susceptibility and tolerance to varroa mite parasitism. Its relatively consistent expression pattern in a wide range of honey bee colonies suggested that *AmCbE E4* could be used as a potential biomarker gene for screening varroa tolerant honey bee colony phenotype in breeding programs. In addition, *AmCbE E4* also showed the increased expression in varroa mite-tolerant honey bee pupae exposed to miticide treatments. These results suggested its function in protecting the pollinator from the toxicity of not only compounds generated by varroa mite infestation, but also miticide residues and other pesticides used in agriculture.

The honey bee genome has been sequenced and a large amount of sequence data provides an integrated and comprehensive genetic resource for molecular studies on the bee in response to biotic and abiotic stresses (The Honey bee Genome Sequencing Consortium 2006). Surprisingly, compared to other sequenced genomes of insects, such as fruit fly *Drosophila melanogaster* (13,500 genes) and malarial mosquito *Anopheles gambiae* (14,000 genes), honey bee contains fewer genes in the annotated genome (only about 11,000 genes) (Claudianos et al. 2006). This results suggested that honey bee, a highly socialized insect, might have eliminated certain unrequired genes in interactions with the environment in the genome. Four putative *AmCbE E4* genes encoding esterase E4-like proteins were found in the honey bee genome and potentially involved in detoxification mechanisms. The amino acid sequence of AmCbE E4 comprises a signal peptide at the N-terminus where present as a hydrophobic region, suggesting the enzyme might be extracellular (Blobel and Dobberstein 1975). After synthesis, the pre-protein might use the signal peptide to guide the enzyme on the secretory pathway and its activity would be activated by removal of the peptide. This assumption was confirmed by heterologous expression in *E. coli* where only the short AmCbE E4 with the signal peptide removed has an active function. Alignment of AmCbE E4 and homologous sequences from other insects showed that

AmCbE E4 shares high sequence similarity with esterases. The predicted topology structure of AmCbE E4 also showed the canonical conformation of the alpha/beta hydrolase. These results strongly supported AmCbE E4 as a carboxylesterase. Biochemical characterization of AmCbE E4 in *E. coli* confirmed that it indeed can hydrolyze chemically synthetic esterase substrates, α-naphthyl acetate, β-naphthyl acetate and para-nitrophenyl acetate, as well as carbamate pesticide carbaryl. Therefore, AmCbE E4 could functionally equip honey bees with some resistance to pesticides or toxic ester-like compounds associated with voarra mites. A wealth of bacterial carbaryl hydrolases have been investigated (Hayatsu et al. 2001; Kim et al. 2014), however, fewer insect enzymes that can detoxify pesticide are reported. An aphid CbE E4 was the first carbaryl hydrolase identified from insects (Lan et al. 2005). AmCbE E4 identified from honey bee in this study is a new addition to this category. It is noteworthy that, unlike fruit fly BdCar4 and BdCar6 from fruit fly with activity on organophosphates, AmCbE E4 is unable to hydrolyze two organophosphate pesticides, paraoxon and parathion. In agriculture, carbamate and organophosphate are two types of commonly used pesticides (Barata et al. 2004). Honey bees are generally not so sensitive to organophosphate pesticide (coumaphos) and also lack the capacity to metabolize this compound when exposed to over therapeutic doses for mite control (Johnson et al. 2010). Therefore, understanding of honey bees' ability to detoxify different pesticides is critical for breeding new colonies in response to the varroa mite infestation as well as miticide management in the apiculture.

7. SUMMARY AND CONCLUSIONS

The honey bee (*Apis mellifera* L.) is a social insect that brings tremendous ecological benefits to the environment and economic benefits to human society. In recent years, however, honey bees have experienced a serious threat imposed by the synergistic action of pathogens and parasites, leading to large scale colony losses, sometimes called colony collapse disorder (CCD) (Cox-Foster et al. 2007). The varroa mite (*Varroa destructor*), an ecto-parasite for honey bees, is regarded as a flagship agent contributing to CCD and threatening global apiculture (Martin et al. 2012; Villalobos 2016). It parasitizes honey bees at all stages of the life cycle and harms the host by sucking nutritional fluids from the body (Rosenkranz et al. 2010). In addition, the mite can also function as a vector to spread pathogenic viruses among bee colonies resulting in increasing mortality of bees (Chen and Siede 2007; Mariani et al. 2012; Shimanuki et al. 1994; Villalobos 2016). Even though a wealth of chemical control methods has been utilized to combat mites, the efficacy is limited. The wide use of pesticides and miticides has resulted in the residual contamination of honey bee products, detrimental consequences to the environment, adverse effects on honey bee health, and pesticide-tolerant varroa mites (Chiesa et al. 2016; Johnson et al. 2013a; Johnson et al. 2010; Lodesani and Costa 2005; Martel et al. 2007). Therefore, apiculture practices are focusing on integrated pest management methods to cope with varroa mites without the use of toxic chemicals. Breeding varroa tolerant honey bees is considered an alternative and environmentally friendly way to control varroa mite infestation in honey bee colonies (Dietemann et al. 2012). Consequently, identification of biomarker genes for determining varroa tolerant colony phenotypes will be very valuable for bee breeding programs (Jiang et al. 2016; Robertson et al. 2014).

High throughput functional genomics have provided numerous opportunities for studying defensive mechanisms of varroa tolerant honey bees in the presence of varroa mites. Digital gene expression (DGE) analysis of bee abdomens indicated that varroa parasitism increased viral population (DWV) and decreased protein metabolism in honey bees (Alaux et al. 2011; Navajas

et al. 2008). DNA microarray and RNA sequencing, two powerful genomic tools for transcript profiling, identified a large number of honey bee genes that were differentially expressed under different parasitic and pathogenic conditions (Jiang et al. 2016; Le Conte et al. 2011; Mondet et al. 2015). A large number of differentially expressed genes had been identified by DNA microarray analysis of two extreme colonies for varroa tolerance and susceptibility (Jiang et al. 2016). To further identify potential biomarker genes for selecting the varroa tolerant phenotypes, this research started by validating expression of a few of these genes in a wide range of honey bee colonies for varroa tolerance and susceptibility by using real time qPCR. Out of ten selected genes, *AmCbE E4*, *AmApoD* and *AmCYP6A1* showed relatively constant, higher expression levels in dark eyed stage 4 pupae from varroa tolerant colonies in the presence of varroa than those in the absence of the mite. In contrast, significantly lower expression was found in varroa susceptible colonies in the presence of varroa than in the absence of the varroa. This suggests these genes may play important roles in protecting honey bees from varroa parasitism and providing honey bees with increased fitness and survival ability during the mite infestation. Therefore, these three genes could be used as potential biomarkers for selecting varroa tolerant honey bees.

The expression of these genes was further investigated in three different honey bee tissues, head, thorax and abdomen. The results showed that *AmCbE E*4 displayed higher differential expression in the head tissue; *AmApoD* exhibited more differential expression in the abdominal tissue, whereas *AmCYP6A1* showed stronger differential expression in both thorax and abdominal tissues in the colonies differing in the phenotype. Previous EST (expressed sequence tag) sequencing indicated that *AmCbE E4* is expressed in the integument of the honey bee brain for its conceivable guardian role, especially for maintaining normal function of the nerve system (Claudianos et al. 2006). Most insect cytochrome P450 genes are found to be expressed in the digestive and immune systems, such as the midgut and fat body, rather than the head (Huang et al. 2013). The expression patterns of these genes implied their potential functions as detoxification enzymes in the corresponding tissues. On the other hand, the highly differential expression of *AmApoD* in the abdomen tissue supports its positive role in lipid metabolism, such as in the biosynthesis of storage lipids, hydrophobic signaling molecules and membrane phospholipids (Rassart et al. 2000).

Miticides are used to control varroa mite infestation in many parts of the world including North America (Johnson et al. 2013b). Their application has imposed upon the honey bee a hostile environment of toxic chemicals. *AmCYP6A1 and AmCbE E4* displayed relatively higher expression in varroa mite-tolerant bees treated with miticides than non-treated tolerant bees, but this expression pattern did not occur in varroa susceptible colonies. This result suggested that the varroa tolerant colonies could protect themselves from miticide damage by increasing expression of defensive genes during miticide treatments, while the susceptible bees do not activate these defensive genes to cope with the stress brought about miticide application.

DWV has been suggested to play a role in CCD (Ryabov et al. 2014). To examine the relationships among varroa mites, miticides and virus infections in honey bees, the infection of DWV in three colonies with different phenotypes with or without varroa mite infestation were investigated. The results provided direct correlations between the mite and DWV. The virus load in the susceptible colony infested with varroa mites was significantly higher than that without mite infestation and the degree of the virus infection among the colonies infested with the mite dramatically increased with susceptibility of the bees to varroa. The infection of DWV in three different colonies with or without miticide treatments was also investigated. The virus load in mite susceptible colonies after miticide treatment was dramatically increased while there was little change in the titer in the varroa mite-tolerant colonies treated or non-treated with miticides. Collectively, this data suggested that the tolerant bees are less susceptible not only to varroa parasitism and DWV infection, but also to miticide applications, probably because they could stimulate higher expression of defensive genes involved in detoxification processes and lipid metabolism to minimize the damage caused by mites, viruses, and miticides. As such, the susceptible colony is more vulnerable to all the stressors due to a compromised defense system.

AmCbE E4 encoding a putative E4 esterase was identified for its differential expression in varroa susceptible and varroa tolerant bees in response to varroa infestation. In order to elucidate its biochemical function, *AmCbE E4* was cloned from the cDNA of head tissues of dark eyed stage 4 pupae and heterologously expressed in *E. coli*. The result showed that it can hydrolyze the chemically synthetic substrates, α-naphthyl acetate, β-naphthyl acetate and para-nitrophenyl

acetate, as well as a carboxylester pesticide, carbaryl. This result suggested the biological function of AmCbE E4 in protecting the tolerant bees from the harm of carboxylester miticides or possible toxic esters produced by varroa parasitism.

8. PROSPECTIVE RESEARCH

Breeding varroa tolerant honey bees by natural selection is challenging as it requires many years of labor intensive analyses of thousands of colonies to identify varroa tolerant phenotypes. Phenotypes are not always stable and extensive breeding methods involving recurrent selection are required over a number of years (www.saskatraz.com). This study identified and evaluated three suitable biomarker genes to distinguish the tolerance and susceptibility of honey bee colony phenotypes and these results should have important applications in varroa tolerant honey bee breeding. The biological functions of these potential biomarker genes are involved in detoxification processes and lipid transport, indicating the varroa tolerant bees are probably equipped with better capacity in detoxification and lipid metabolism processes against varroa infestation. However, the overall mechanism underlying health and tolerance of honey bees to the mite still remain elusive. Further studies are required to understand varroa tolerance in honey bees.

Firstly, future studies should focus on identification and characterization of more genes in the detoxification processes, particularly those involved in pesticide degradation, as it may confer more tolerance of honey bees to both varroa mites and miticides. Three major superfamilies of enzymes responsible for metabolism or detoxification of toxic xenobiotic compounds in the environment are glutathione-S-transferases (GSTs), cytochrome P450s (CYPs) and carboxyl/cholinesterase (CCEs). Several genes belonging to these families are thought to function in generating insecticide resistance in honey bees (Johnson et al. 2012; Johnson et al. 2009b; Johnson et al. 2006; Mao et al. 2009; Mao et al. 2011; Papadopoulos et al. 2004). In this study, *AmCbE E4* encoding a carboxylesterase, belonging to CCEs family, was functionally characterized. It may play a potential defensive role in detoxifying ester compounds introduced by varroa parasitism or carbaryl pesticide applied by beekeepers in the varroa management. However, genes involved in detoxification of other toxic compounds and pesticides should be

identified and characterized in the future for elucidating the comprehensive detoxification processes in honey bees.

Secondly, more genes with highly differential expression in the tolerant and susceptible bees should be evaluated in a wide range of honey bee colonies to uncover more varroa tolerant mechanism. This study showed that *AmApoD* involved in lipid transport was significantly expressed in the abdomen of tolerant bees infested by varroa mites, which is consistent with its positive role (Perdomo et al. 2010). Serine protease has been reported with physiological functions in immune response (Hedstrom 2002). Therefore, the increased expression of *AmSPH51* encoding the protease in the head tissue of tolerant bees with the mite may involve enhanced protein turnover/metabolism to combat varroa mites. Furthermore, two oncogenic genes, *AmHIP14* and *AmHsp90*, displayed similar or increased expression in both tolerant and susceptible bees with the mite infestation. Huntingtin interacting protein 14 (HIP14) is a palmitoyl acyltransferases (PATs) mediating post-translational addition of long chain fatty acids to proteins in a process called palmitoylation. The unique interaction of HIP14 and HTT (huntingtin protein) determines the palmitoylation of HTT, and reduced interaction leads to HTT toxicity, and finally to huntingtin disease in human (Sanders et al. 2014). Also in humans, Hsp90 has emerged as a viable target for antitumor drug development, as it is important to help cancer cells overcome multiple stresses, including genomic instability/aneuploidy, proteotoxic stress, increased nutrient demands, reduced oxygen levels, and destruction by the immune system (Hong et al. 2013). Evaluation of expression of these homologous genes in honey bees may help uncover new mechanisms for the tolerant bees to combat against varroa mite infestation.

Thirdly, the synergistic relationship between miticides, parasitic varroa mites and associated pathogens should be further investigated. In reality, honey bees have to encounter not only in hive miticides to destroy varroa mites, but also pesticides sprayed in the agricultural fields for broad-spectrum pest control. The concurrent occurrence between pathogenic virus and varroa mites has been demonstrated among the bee colonies in this study, suggesting the mite can spread viruses among bee colonies and the virus may interfere with the host's NF-κB signaling (nuclear factor kappa-light-chain-enhancer of activated B cells) and humoral and cellular

immune responses, which in turn can facilitate reproduction of the parasitic mite (Di Prisco et al. 2016). In addition, the causal link between insecticide application and virus infection has also been demonstrated, indicating that the increased viral loads in the susceptible bees are possible results of the honey bees being exposed to miticides (Doublet et al. 2015; Locke et al. 2012; Smart et al. 2016). Exposure of honey bees to the neonicotinoid insecticide can compromise the host's immune-competence and promotion of the viral infection in healthy honey bees (Di Prisco et al. 2013). Thus, further evaluation of the immune-competences among varroa mite-tolerant and mite susceptible honey bees may give new insight into the defensive mechanism of honey bees against the mite.

9. REFERENCES

Adjlane, N, Tarek, E-O and Haddad, N (2015) Evaluation of oxalic acid treatments against the mite *Varroa destructor* and secondary effects on honey bees *Apis mellifera*. *Journal of Arthropod-Borne Diseases* **10**: 501-509.

Ahmed, S and Othman, NH (2013) Honey as a potential natural anticancer agent: a review of its mechanisms. *Evidence-Based Complementary and Alternative Medicine* **2013**: 1-7.

Aizen, MA, Garibaldi, LA, Cunningham, SA and Klein, AM (2008) Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. *Current Biology* **18**: 1572-1575.

Al Naggar, Y, Tan, Y, Rutherford, C, Connor, W, Griebel, P, Giesy, JP and Robertson, AJ (2015) Effects of treatments with Apivar® and Thymovar® on *V. destructor* populations, virus infections and indoor winter survival of Canadian honey bee (*Apis mellifera* L.) colonies. *Journal of Apicultural Research* **54**: 548-554.

Alaux, C, Dantec, C, Parrinello, H and Le Conte, Y (2011) Nutrigenomics in honey bees: digital gene expression analysis of pollen's nutritive effects on healthy and varroa-parasitized bees. *BMC genomics* **12**: 496.

Anderson, D and Trueman, J (2000) *Varroa jacobsoni* (Acari: Varroidae) is more than one species. *Experimental & applied acarology* **24**: 165-189.

Aronstein, KA, Saldivar, E, Vega, R, Westmiller, S and Douglas, AE (2012) How Varroa parasitism affects the immunological and nutritional status of the honey bee, *Apis mellifera*. *Insects* **3**: 601-615.

Asperen, KV (1962) A study of housefly esterases by means of a sensitive colorimetric method. *Journal of Insect Physiology* **8**: 401-416.

Aufauvre, J, Biron, DG, Vidau, C, Fontbonne, R, Roudel, M, Diogon, M, Viguès, B, Belzunces, LP, Delbac, F and Blot, N (2012) Parasite-insecticide interactions: a case study of *Nosema ceranae* and fipronil synergy on honeybee. *Scientific reports* **2**: 326.

Barata, C, Solayan, A and Porte, C (2004) Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. *Aquatic toxicology* **66**: 125-139.

Bass, C, Puinean, AM, Zimmer, CT, Denholm, I, Field, LM, Foster, SP, Gutbrod, O, Nauen, R, Slater, R and Williamson, MS (2014) The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*. *Insect biochemistry and molecular biology* **51**: 41-51.

Belák, S (2007) Molecular diagnosis of viral diseases, present trends and future aspects: A view from the OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine. *Vaccine* **25**: 5444-5452.

Blobel, G and Dobberstein, B (1975) Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *The Journal of Cell Biology* **67**: 835-851.

Boecking, O and Genersch, E (2008) Varroosis–the ongoing crisis in bee keeping. *Journal für Verbraucherschutz und Lebensmittelsicherheit* **3**: 221-228.

Boncristiani, H, Underwood, R, Schwarz, R, Evans, JD and Pettis, J (2012) Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. *Journal of Insect Physiology* **58**: 613-620.

Boot, WJ, Calis, JN and Beetsma, J (1992) Differential periods of Varroa mite invasion into worker and drone cells of honey bees. *Experimental & applied acarology* **16**: 295-301.

Bourgeois, AL, Rinderer, TE, Beaman, LD and Danka, RG (2010) Genetic detection and quantification of *Nosema apis* and *N. ceranae* in the honey bee. *Journal of Invertebrate Pathology* **103**: 53-58.

Campbell, EM, Budge, GE, Watkins, M and Bowman, AS (2016) Transcriptome analysis of the synganglion from the honey bee mite, *Varroa destructor* and RNAi knockdown of neural peptide targets. *Insect biochemistry and molecular biology* **70**: 116-126.

Carino, F, Koener, J, Plapp, F and Feyereisen, R (1994) Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a house fly strain with metabolic resistance to insecticides. *Insect biochemistry and molecular biology* **24**: 411-418.

Chandler, D, Sunderland, K, Ball, B and Davidson, G (2001) Prospective biological control agents of *Varroa destructor n. sp.*, an important pest of the European honeybee, *Apis mellifera*. *Biocontrol Science and Technology* **11**: 429-448.

Chen, Y, Higgins, J and Feldlaufer, M (2005) Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). *Applied and Environmental Microbiology* **71**: 436-441.

Chen, YP and Siede, R (2007) Honey bee viruses. *Advances in virus research* **70**: 33-80.

Chiesa, LM, Labella, GF, Giorgi, A, Panseri, S, Pavlovic, R, Bonacci, S and Arioli, F (2016) The occurrence of pesticides and persistent organic pollutants in Italian organic honeys from different productive areas in relation to potential environmental pollution. *Chemosphere* **154**: 482-490.

Claudianos, C, Ranson, H, Johnson, RM and Biswas, S (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Molecular Biology* **15**: 615-636.

Claudianos, C, Russell, RJ and Oakeshott, JG (1999) The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. *Insect biochemistry and molecular biology* **29**: 675-686.

Coffey, MF and Breen, J (2013) Efficacy of Apilife Var® and Thymovar® against *Varroa destructor* as an autumn treatment in a cool climate. *Journal of Apicultural Research* **52**: 210-218.

Collins, A, Williams, V and Evans, J (2004) Sperm storage and antioxidative enzyme expression in the honey bee, *Apis mellifera*. *Insect molecular biology* **13**: 141-146.

Conte, YL and Hefetz, A (2008) Primer pheromones in social hymenoptera. *Annual Review of Entomology* **53**: 523-542.

Cornman, RS, Tarpy, DR, Chen, Y and Jeffreys, L (2012) Pathogen webs in collapsing honey bee colonies. *PLoS One* **7**: e43562.

Corona, M, Hughes, KA, Weaver, DB and Robinson, GE (2005) Gene expression patterns associated with queen honey bee longevity. *Mechanisms of ageing and development* **126**: 1230-1238.

Cox-Foster, DL, Conlan, S, Holmes, EC, Palacios, G, Evans, JD, Moran, NA, Quan, P-L, Briese, T, Hornig, M and Geiser, DM (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* **318**: 283-287.

Cummings, CA and Relman, DA (2000) Using DNA microarrays to study host-microbe interactions. *Emerging infectious diseases* **6**: 513-523.

Currie, RW, Pernal, SF and Guzmán-Novoa, E (2010) Honey bee colony losses in Canada. *Journal of Apicultural Research* **49**: 104-106.

Dahlgren, L, Johnson, RM, Siegfried, BD and Ellis, MD (2012) Comparative toxicity of acaricides to honey bee (Hymenoptera: Apidae) workers and queens. *Journal of economic entomology* **105**: 1895-1902.

Dainat, B, Evans, JD, Chen, YP and Gauthier, L (2012) Predictive markers of honey bee colony collapse. *PLoS One* **7**: e32151.

Dassati, S, Waldner, A and Schweigreiter, R (2014) Apolipoprotein D takes center stage in the stress response of the aging and degenerative brain. *Neurobiology of aging* **35**: 1632-1642.

De la Rosa, L, Alvarez-Parrilla, E, Moyers-Montoya, E, Villegas-Ochoa, M, Ayala-Zavala, J, Hernández, J, Ruiz-Cruz, S and González-Aguilar, G (2011) Mechanism for the inhibition of apple juice enzymatic browning by Palo Fierro (desert ironweed) honey extract and other natural compounds. *LWT-Food Science and Technology* **44**: 269-276.

De Miranda, JR and Genersch, E (2010) Deformed wing virus. *Journal of Invertebrate Pathology* **103**: 48-61.

Delaplane, KS, Mayer, DR and Mayer, DF (2000) *Crop pollination by bees*. New York: CAB International.

Dennis, B and Kemp, WP (2016) How hives collapse: Allee effects, ecological resilience, and the honey bee. *PloS one* **11**: e0150055.

Desai, S, Eu, YJ, Whyard, S and Currie, R (2012) Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. *Insect molecular biology* **21**: 446-455.

Devonshire, AL (1977) The properties of a carboxylesterase from the peach-potato aphid, *Myzus persicae* (Sulz.), and its role in conferring insecticide resistance. *Biochemical Journal* **167**: 675-683.

Devonshire, AL and Moores, GD (1982) A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). *Pesticide Biochemistry and Physiology* **18**: 235-246.

Di Prisco, G, Annoscia, D, Margiotta, M, Ferrara, R, Varricchio, P, Zanni, V, Caprio, E, Nazzi, F and Pennacchio, F (2016) A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. *Proceedings of the National Academy of Sciences* **113**: 3203–3208.

Di Prisco, G, Cavaliere, V, Annoscia, D, Varricchio, P, Caprio, E, Nazzi, F, Gargiulo, G and Pennacchio, F (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences* **110**: 18466-18471.

Dietemann, V, Pflugfelder, J, Anderson, D, Charrière, J-D, Chejanovsky, N, Dainat, B, de Miranda, J, Delaplane, K, Dillier, F-X and Fuch, S (2012) *Varroa destructor*: research avenues towards sustainable control. *Journal of Apicultural Research* **51**: 125-132.

Dorak, MT (2007) *Real-time PCR*. Taylor & Francis.

Doublet, V, Labarussias, M, Miranda, JR, Moritz, RF and Paxton, RJ (2015) Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environmental Microbiology* **17**: 969-983.

Drayna, DT, Mclean, JW, Wion, KL, Trent, JM, Drabkin, HA and Lawn, RM (1987) Human apolipoprotein D gene: gene sequence, chromosome localization, and homology to the α2u-globulin superfamily. *DNA* **6**: 199-204.

Duay, P, De Jong, D and Engels, W (2003) Weight loss in drone pupae (*Apis mellifera*) multiply infested by *Varroa destructor* mites. *Apidologie* **34**: 61-65.

Emsen, B and Dodologlu, A (2009) The effects of using different organic compounds against honey bee mite (*Varroa destructor* Anderson and Trueman) on colony developments of honey bee (*Apis mellifera* L.) and residue levels in honey. *Journal of Animal and Veterinary Advances* **8**: 1004-1009.

Estevinho, L, Pereira, AP, Moreira, L, Dias, LG and Pereira, E (2008) Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. *Food and Chemical Toxicology* **46**: 3774-3779.

Evans, J, Aronstein, K, Chen, Y, Hetru, C, Imler, JL, Jiang, H, Kanost, M, Thompson, G, Zou, Z and Hultmark, D (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect molecular biology* **15**: 645-656.

Evans, JD, Saegerman, C, Mullin, C, Haubruge, E, Nguyen, BK, Frazier, M, Frazier, J, Cox-Foster, D, Chen, Y and Underwood, R (2009) Colony collapse disorder: a descriptive study. *PloS one* **4**: e6481.

Evans, JD and Schwarz, RS (2011) Bees brought to their knees: microbes affecting honey bee health. *Trends in microbiology* **19**: 614-620.

Fathian, M, Amiri, B and Maroosi, A (2007) Application of honey-bee mating optimization algorithm on clustering. *Applied Mathematics and Computation* **190**: 1502-1513.

Fuchs, S (1990) Preference for drone brood cells by *Varroa jacobsoni* Oud. in colonies of *Apis mellifera* carnica. *Apidologie* **21**: 193-199.

Gallai, N, Salles, J-M, Settele, J and Vaissière, BE (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological economics* **68**: 810-821.

Garbian, Y, Maori, E, Kalev, H, Shafir, S and Sela, I (2012) Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: Varroa gene silencing reduces Varroa population. *PLoS Pathogens* **8**: e1003035.

Garrido, PM, Antunez, K, Martin, M, Porrini, MP, Zunino, P and Eguaras, MJ (2013) Immune-related gene expression in nurse honey bees (*Apis mellifera*) exposed to synthetic acaricides. *Journal of Insect Physiology* **59**: 113-119.

Geley, S and Müller, C (2004) RNAi: ancient mechanism with a promising future. *Experimental gerontology* **39**: 985-998.

Gheldof, N, Wang, X-H and Engeseth, NJ (2002) Identification and quantification of antioxidant components of honeys from various floral sources. *Journal of agricultural and food chemistry* **50**: 5870-5877.

Glover, RH, Adams, IP, Budge, G, Wilkins, S and Boonham, N (2011) Detection of honey bee (*Apis mellifera*) viruses with an oligonucleotide microarray. *Journal of Invertebrate Pathology* **107**: 216-219.

Gómez-Caravaca, A, Gómez-Romero, M, Arráez-Román, D, Segura-Carretero, A and Fernández-Gutiérrez, A (2006) Advances in the analysis of phenolic compounds in products derived from bees. *Journal of Pharmaceutical and Biomedical Analysis* **41**: 1220-1234.

Goswami, V and Khan, M (2013) Management of varroa mite, *Varroa destructor* by essential oil and formic acid in *Apis mellifera* Linn. Colonies. *Journal of Natural Products* **6**: 206-210.

Guzmán-Novoa, E, Eccles, L, Calvete, Y, McGowan, J, Kelly, PG and Correa-Benítez, A (2010) *Varroa destructor* is the main culprit for the death and reduced populations of overwintered honey bee (*Apis mellifera*) colonies in Ontario, Canada. *Apidologie* **41**: 443-450.

Hamiduzzaman, MM, Sinia, A, Guzman-Novoa, E and Goodwin, PH (2012) Entomopathogenic fungi as potential biocontrol agents of the ecto-parasitic mite, *Varroa destructor*, and their effect on the immune response of honey bees (*Apis mellifera* L.). *Journal of invertebrate pathology* **111**: 237-243.

Han, B, Zhang, L, Feng, M, Fang, Y and Li, J (2013) An integrated proteomics reveals pathological mechanism of honeybee (*Apis cerena*) sacbrood disease. *Journal of Proteome Research* **12**: 1881-1897.

Harbo, JR and Harris, JW (2009) Responses to Varroa by honey bees with different levels of Varroa Sensitive Hygiene. *Journal of apicultural research* **48**: 156-161.

Hayatsu, M, Mizutani, A, Hashimoto, M, Sato, K and Hayano, K (2001) Purification and characterization of carbaryl hydrolase from *Arthrobacter* sp. RC100. *FEMS microbiology letters* **201**: 99-103.

Hedstrom, L (2002) Serine protease mechanism and specificity. *Chemical reviews* **102**: 4501-4524.

Higuchi, R, Fockler, C, Dollinger, G and Watson, R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology* **11**: 1026-1030.

Højland, DH, Jensen, K-MV and Kristensen, M (2014) Expression of xenobiotic metabolizing cytochrome P450 genes in a spinosad-resistant *Musca domestica* L. strain. *PloS one* **9**: e103689.

Holloway, B, Tarver, MR and Rinderer, TE (2013) Fine mapping identifies significantly associating markers for resistance to the honey bee brood fungal disease, Chalkbrood. *Journal of Apicultural Research* **52**: 134-140.

Holt, RA, Subramanian, GM, Halpern, A, Sutton, GG, Charlab, R, Nusskern, DR, Wincker, P, Clark, AG, Ribeiro, JC and Wides, R (2002) The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* **298**: 129-149.

Hong, DS, Banerji, U, Tavana, B, George, GC, Aaron, J and Kurzrock, R (2013) Targeting the molecular chaperone heat shock protein 90 (HSP90): lessons learned and future directions. *Cancer treatment reviews* **39**: 375-387.

Hoover, SE, Keeling, CI, Winston, ML and Slessor, KN (2003) The effect of queen pheromones on worker honey bee ovary development. *Naturwissenschaften* **90**: 477-480.

Huang, Y, Shen, G-M, Jiang, H-B, Jiang, X-Z, Dou, W and Wang, J-J (2013) Multiple P450 genes: Identification, tissue-specific expression and their responses to insecticide treatments in the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidea). *Pesticide Biochemistry and Physiology* **106**: 1-7.

Hunter, W, Ellis, J, Hayes, J, Westervelt, D, Glick, E, Williams, M, Sela, I, Maori, E, Pettis, J and Cox-Foster, D (2010) Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). *PLoS pathogens* **6**: e1001160.

Jackson, CJ, Liu, J-W, Carr, PD, Younus, F, Coppin, C, Meirelles, T, Lethier, M, Pandey, G, Ollis, DL and Russell, RJ (2013) Structure and function of an insect α-carboxylesterase (αEsterase7) associated with insecticide resistance. *Proceedings of the National Academy of Sciences* **110**: 10177-10182.

Jiang, S, Robertson, T, Mostajeran, M, Robertson, A and Qiu, X (2016) Differential gene expression of two extreme honey bee (*Apis mellifera*) colonies showing varroa tolerance and susceptibility. *Insect molecular biology* **25**: 272-282.

Johnson, RM (2015) Honey bee toxicology. *Annual review of entomology* **60**: 415-434.

Johnson, RM, Dahlgren, L, Siegfried, BD and Ellis, MD (2013a) Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). *PLoS One* **8**: e54092.

Johnson, RM, Dahlgren, L, Siegfried, BD and Ellis, MD (2013b) Effect of in-hive miticides on drone honey bee survival and sperm viability. *Journal of Apicultural Research* **52**: 88-95.

Johnson, RM, Ellis, MD, Mullin, CA and Frazier, M (2010) Pesticides and honey bee toxicity – USA. *Apidologie* **41**: 312-331.

Johnson, RM, Evans, JD, Robinson, GE and Berenbaum, MR (2009a) Changes in transcript abundance relating to colony collapse disorder in honey bees (*Apis mellifera*). *Proceedings of the National Academy of Sciences* **106**: 14790-14795.

Johnson, RM, Mao, W, Pollock, HS, Niu, G, Schuler, MA and Berenbaum, MR (2012) Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. *PloS one* **7**: e31051.

Johnson, RM, Pollock, HS and Berenbaum, MR (2009b) Synergistic interactions between in-hive miticides in *Apis mellifera*. *Journal of economic entomology* **102**: 474-479.

Johnson, RM, Wen, Z, Schuler, MA and Berenbaum, MR (2006) Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. *Journal of Economic Entomology* **99**: 1046-1050.

Kanbar, G and Engels, W (2003) Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by Varroa mites. *Parasitology research* **90**: 349-354.

Kanbar, G and Engels, W (2005) Communal use of integumental wounds in honey bee (*Apis mellifera*) pupae multiply infested by the ectoparasitic mite *Varroa destructor*. *Genetics and Molecular Research* **4**: 465-472.

Kao, L, Motoyama, N and Dauterman, W (1984) Studies on hydrolases in various house fly strains and their role in malathion resistance. *Pesticide Biochemistry and Physiology* **22**: 86-92.

Kassim, M, Achoui, M, Mustafa, MR, Mohd, MA and Yusoff, KM (2010) Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutrition research* **30**: 650-659.

Kim, I, Kim, D-U, Kim, N-H and Ka, J-O (2014) Isolation and characterization of fenobucarb-degrading bacteria from rice paddy soils. *Biodegradation* **25**: 383-394.

Klein, A-M, Vaissiere, BE, Cane, JH, Steffan-Dewenter, I, Cunningham, SA, Kremen, C and Tscharntke, T (2007) Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences* **274**: 303-313.

Kolmes, SA (1985) An information-theory analysis of task specialization among worker honey bees performing hive duties. *Animal behaviour* **33**: 181-187.

Kuster, RD, Boncristiani, HF and Rueppell, O (2014) Immunogene and viral transcript dynamics during parasitic *Varroa destructor* mite infection of developing honey bee (*Apis mellifera*) pupae. *Journal of Experimental Biology* **217**: 1710-1718.

Lan, WS, Cong, J, Jiang, H, Jiang, SR and Qiao, CL (2005) Expression and characterization of carboxylesterase E4 gene from peach-potato aphid (*Myzus persicae*) for degradation of carbaryl and malathion. *Biotechnology Letters* **27**: 1141-1146.

Le Conte, Y, Alaux, C, Martin, JF, Harbo, J, Harris, J, Dantec, C, Severac, D, Cros-Arteil, S and Navajas, M (2011) Social immunity in honeybees (*Apis mellifera*): transcriptome analysis of varroa-hygienic behaviour. *Insect molecular biology* **20**: 399-408.

Le Conte, Y, De Vaublanc, G, Crauser, D, Jeanne, F, Rousselle, J-C and Bécard, J-M (2007) Honey bee colonies that have survived *Varroa destructor*. *Apidologie* **38**: 566-572.

Le Conte, Y, Ellis, M and Ritter, W (2010) Varroamites and honey bee health: can Varroa explain part of the colony losses? *Apidologie* **41**: 353-363.

Lee, KV, Steinhauer, N, Rennich, K, Wilson, ME, Tarpy, DR, Caron, DM, Rose, R, Delaplane, KS, Baylis, K and Lengerich, EJ (2015) A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie* **46**: 292-305.

Locke, B, Forsgren, E and de Miranda, JR (2014) Increased tolerance and resistance to virus infections: a possible factor in the survival of *Varroa destructor*-resistant honey bees (*Apis mellifera*). *PLoS One* **9**: e99998.

Locke, B, Forsgren, E, Fries, I and de Miranda, JR (2012) Acaricide treatment affects viral dynamics in *Varroa destructor*-infested honey bee colonies via both host physiology and mite control. *Applied and Environmental Microbiology* **78**: 227-335.

Lodesani, M and Costa, C (2005) Limits of chemotherapy in beekeeping: development of resistance and the problem of residues. *Bee World* **86**: 102-109.

Longo, MC, Berninger, MS and Hartley, JL (1990) Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* **93**: 125-128.

Maggi, M, Peralta, L, Ruffinengo, S, Fuselli, S and Eguaras, M (2012) Body size variability of *Varroa destructor* and its role in acaricide tolerance. *Parasitology research* **110**: 2333-2340.

Mao, W, Rupasinghe, SG, Johnson, RM, Zangerl, AR, Schuler, MA and Berenbaum, MR (2009) Quercetin-metabolizing CYP6AS enzymes of the pollinator *Apis mellifera* (Hymenoptera: Apidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **154**: 427-434.

Mao, W, Schuler, MA and Berenbaum, MR (2011) CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). *Proceedings of the National Academy of Sciences* **108**: 12657-12662.

Maori, E, Paldi, N, Shafir, S, Kalev, H, Tsur, E, Glick, E and Sela, I (2009) IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect molecular biology* **18**: 55-60.

Mariani, F, Maggi, M and Porrini, M (2012) Parasitic interactions between *nosema* spp. and *varroa destructor* in *apis mellifera* colonies. *Zootecnia Trop* **30**: 81-90.

Markussen, MD and Kristensen, M (2010) Cytochrome P450 monooxygenase-mediated neonicotinoid resistance in the house fly *Musca domestica* L. *Pesticide Biochemistry and Physiology* **98**: 50-58.

Martel, A-C, Zeggane, S, Aurières, C, Drajnudel, P, Faucon, J-P and Aubert, M (2007) Acaricide residues in honey and wax after treatment of honey bee colonies with Apivar® or Asuntol® 50. *Apidologie* **38**: 534-544.

Martin, SJ, Highfield, AC, Brettell, L, Villalobos, EM, Budge, GE, Powell, M, Nikaido, S and Schroeder, DC (2012) Global honey bee viral landscape altered by a parasitic mite. *Science* **336**: 1304-1306.

McGregor, SE (1976) *Insect pollination of cultivated crop plants*. Agricultural Research Service, US Department of Agriculture.

Meikle, WG, Sammataro, D, Neumann, P and Pflugfelder, J (2012) Challenges for developing pathogen-based biopesticides against *Varroa destructor* (Mesostigmata: Varroidae). *Apidologie* **43**: 501-514.

Milani, N and Iob, M (1998) Plastic strips containing organophosphorous acaricides to control *Varroa jacobsoni*: a preliminary experiment. *American bee journal* **138**: 612-615.

Mondet, F, Alaux, C, Severac, D, Rohmer, M, Mercer, AR and Le Conte, Y (2015) Antennae hold a key to Varroa-sensitive hygiene behaviour in honey bees. *Scientific reports* **5**.

Montella, IR, Schama, R and Valle, D (2012) The classification of esterases: an important gene family involved in insecticide resistance - A Review. *Memórias do Instituto Oswaldo Cruz* **107**: 437-449.

Moore, AJ, Breed, MD and Moor, MJ (1987) The guard honey bee: ontogeny and behavioural variability of workers performing a specialized task. *Animal Behaviour* **35**: 1159-1167.

Moritz, RF and Erler, S (2016) Lost colonies found in a data mine: Global honey trade but not pests or pesticides as a major cause of regional honeybee colony declines. *Agriculture, Ecosystems & Environment* **216**: 44-50.

Navajas, M, Migeon, A, Alaux, C, Martin-Magniette, M, Robinson, G, Evans, J, Cros-Arteil, S, Crauser, D and Le Conte, Y (2008) Differential gene expression of the honey bee *Apis mellifera* associated with *Varroa destructor* infection. *BMC Genomics* **9**: 301.

Nazzi, F, Brown, SP, Annoscia, D, Del Piccolo, F, Di Prisco, G, Varricchio, P, Della Vedova, G, Cattonaro, F, Caprio, E and Pennacchio, F (2012) Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathogens* **8**: e1002735.

Nazzi, F and Le Conte, Y (2015) Ecology of *Varroa destructor*, the major ectoparasite of the western honey bee, *Apis mellifera*. *Annual review of entomology* **61**: 417-432.

Nazzi, F and Pennacchio, F (2014) Disentangling multiple interactions in the hive ecosystem. *Trends in parasitology* **30**: 556-561.

NOH, MY, Beeman, RW and Arakane, Y (2012) RNAi-based functional genomics in Tribolium castaneum and possible application for controlling insect pests. *Entomological Research* **42**: 1-10.

Oakeshott, J, Claudianos, C, Russell, R and Robin, G (1999) Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. *Bioessays* **21**: 1031-1042.

Oakeshott, JG, Claudianos, C, Campbell, PM, Newcomb, R and Russell, R (2010) *Biochemical genetics and genomics of insect esterases*. London: Academic.

Onstad, DW (2013) *Insect resistance management: biology, economics, and prediction*. Academic Press.

Page, RE and Peng, CY-S (2001) Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. *Experimental gerontology* **36**: 695-711.

Pankiw, T, Huang, Z, Winston, ML and Robinson, GE (1998) Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) foraging ontogeny and juvenile hormone titers. *Journal of Insect Physiology* **44**: 685-692.

Papadopoulos, AI, Polemitou, I, Laifi, P, Yiangou, A and Tananaki, C (2004) Glutathione S-transferase in the insect *Apis mellifera macedonica*: Kinetic characteristics and effect of stress on the expression of GST isoenzymes in the adult worker bee. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **139**: 93-97.

Perdomo, G, Kim, DH, Zhang, T, Qu, S, Thomas, EA, Toledo, FG, Slusher, S, Fan, Y, Kelley, DE and Dong, HH (2010) A role of apolipoprotein D in triglyceride metabolism. *Journal of lipid research* **51**: 1298-1311.

Pirali-Kheirabadi, K, Teixeira-da-Silva, JA, Razzaghi-Abyaneh, M and Nazemnia, M (2013) A field experiment to assess the rate of infestation in honey bee populations of two *Metarhizium anisopliae* isolates on *Varroa destructor* (Acari: Mesostigmata). *Journal of arthropod-borne diseases* **7**: 15-22.

Porrini, C, Sabatini, AG, Girotti, S, Ghini, S, Medrzycki, P, Grillenzoni, F, Bortolotti, L, Gattavecchia, E and Celli, G (2003) Honey bees and bee products as monitors of the environmental contamination. *Apiacta* **38**: 63-70.

Rademacher, E and Harz, M (2006) Oxalic acid for the control of varroosis in honey bee colonies-a review. *Apidologie* **37**: 98-120.

Ramakers, C, Ruijter, JM, Deprez, RHL and Moorman, AF (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience letters* **339**: 62-66.

Rashid Mahmood, SA, Sarwar, G and Ahmad, W (2013) Control of *Varroa destructor* mite by using oxalic acid, formic acid in honey bee *Apis mellifera* L. colonies in Pakistan. *World Applied Sciences Journal* **26**: 1574-1578.

Rassart, E, Bedirian, A, Do Carmo, S, Guinard, O, Sirois, J, Terrisse, L and Milne, R (2000) Apolipoprotein d. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* **1482**: 185-198.

Razavi, SM, Asadpour, M, Jafari, A and Malekpour, SH (2015) The field efficacy of *Lepidium latifolium* and *Zataria multiflora* methanolic extracts against *Varroa destructor*. *Parasitology research* **114**: 4233-4238.

Robertson, AJ, Trost, B, Scruten, E, Robertson, T, Mostajeran, M, Connor, W, Kusalik, A, Griebel, P and Napper, S (2014) Identification of developmentally-specific kinotypes and mechanisms of Varroa mite resistance through whole-organism, kinome analysis of honeybee. *Frontiers in Genetics* **5**: 139.

Rosenkranz, P, Aumeier, P and Ziegelmann, B (2010) Biology and control of *Varroa destructor*. *Journal of Invertebrate Pathology* **103 Suppl 1**: 96-119.

Ryabov, EV, Wood, GR, Fannon, JM, Moore, JD, Bull, JC, Chandler, D, Mead, A, Burroughs, N and Evans, DJ (2014) A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or in vitro, transmission. *PLoS Pathogens* **10**: e1004230.

Sammataro, D, Gerson, U and Needham, G (2000) Parasitic mites of honey bees: life history, implications, and impact. *Annual review of entomology* **45**: 519-548.

Sanchez, D, López-Arias, B, Torroja, L, Canal, I, Wang, X, Bastiani, MJ and Ganfornina, MD (2006) Loss of glial lazarillo, a homolog of apolipoprotein D, reduces lifespan and stress resistance in *Drosophila*. *Current Biology* **16**: 680-686.

Sanders, SS, Mui, KK, Sutton, LM and Hayden, MR (2014) Identification of binding sites in Huntingtin for the Huntingtin Interacting Proteins HIP14 and HIP14L. *PloS one* **9**: e90669.

Schmehl, DR, Teal, PE, Frazier, JL and Grozinger, CM (2014) Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). *Journal of Insect Physiology* **71**: 177-190.

Schmittgen, TD and Livak, KJ (2008) Analyzing real-time PCR data by the comparative CT method. *Nature protocols* **3**: 1101-1108.

Schöning, C, Gisder, S, Geiselhardt, S, Kretschmann, I, Bienefeld, K, Hilker, M and Genersch, E (2012) Evidence for damage-dependent hygienic behaviour towards *Varroa destructor*-parasitised brood in the western honey bee, *Apis mellifera*. *Journal of Experimental Biology* **215**: 264-271.

Seeley, TD (2007) Honey bees of the Arnot Forest: a population of feral colonies persisting with *Varroa destructor* in the northeastern United States. *Apidologie* **38**: 19-29.

Serra Bonvehí, J, Ventura Coll, F and Ruiz Martínez, J (2016) Residues of essential oils in honey after treatments to control *Varroa destructor*. *Journal of Essential Oil Research* **28**: 22-28.

Sewify, G, Ibrahim, Y and El-Deen, MS (2015) Beauveria bassiana (Balsamo), a potential mycopesticide for efficient control of the honey bee ectoparasitic mite, *Varroa destructor* Anderson and Trueman. *Egyptian Journal of Biological Pest Control* **25**: 333-337.

Sharma, R, Chisti, Y and Banerjee, UC (2001) Production, purification, characterization, and applications of lipases. *Biotechnology advances* **19**: 627-662.

Shimanuki, H, Calderone, NW and Knox, DA (1994) Parasitic mite syndrome: the symptoms. *American bee journal (USA)* **134**: 117-119.

Smart, M, Pettis, J, Rice, N, Browning, Z and Spivak, M (2016) Linking measures of colony and individual honey bee health to survival among apiaries exposed to varying agricultural land use. *PloS one* **11**: e0152685.

Snodgrass, RE (1984) *Anatomy of the honey bee*. Cornell University Press.

Sogorb, MA and Vilanova, E (2002) Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicology letters* **128**: 215-228.

The Honey bee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **443**: 931-949.

Tsuruda, JM, Harris, JW, Bourgeois, L, Danka, RG and Hunt, GJ (2012) High-resolution linkage analyses to identify genes that influence Varroa sensitive hygiene behavior in honey bees. *PLoS One* **7**: e48276.

Tu, S, Qiu, X, Cao, L, Han, R, Zhang, Y and Liu, X (2010) Expression and characterization of the chitinases from *Serratia marcescens* GEI strain for the control of *Varroa destructor*, a honey bee parasite. *Journal of invertebrate pathology* **104**: 75-82.

Van der Geest, L, Elliot, S, Breeuwer, J and Beerling, E (2000) Diseases of mites. *Experimental & applied acarology* **24**: 497-560.

vanEngelsdorp, D and Meixner, MD (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology* **103 Suppl 1**: 80-95.

vanEngelsdorp, D, Steinhauer, N and Rennich, K (2014) Colony Loss 2013-2014. http://beeinformed.org/2014/05/colony-loss-2013-2014/.

Villalobos, EM (2016) The mite that jumped, the bee that traveled, the disease that followed. *Science* **351**: 554-556.

Wang, LL, Huang, Y, Lu, XP, Jiang, XZ, Smagghe, G, Feng, ZJ, Yuan, GR, Wei, D and Wang, JJ (2015) Overexpression of two α-esterase genes mediates metabolic resistance to malathion in the oriental fruit fly, *Bactrocera dorsalis* (Hendel). *Insect molecular biology* **24**: 467-479.

Watanabe, K, Rahmasari, R, Matsunaga, A, Haruyama, T and Kobayashi, N (2014) Anti-influenza viral effects of honey in vitro: potent high activity of manuka honey. *Archives of medical research* **45**: 359-365.

Whitfield, CW, Behura, SK, Berlocher, SH and Clark, AG (2006) Thrice out of Africa: ancient and recent expansions of the honey bee, *Apis mellifera*. *Science* **314**: 642-645.

Williams, NM, Crone, EE, Roulston, TaH, Minckley, RL, Packer, L and Potts, SG (2010) Ecological and life-history traits predict bee species responses to environmental disturbances. *Biological Conservation* **143**: 2280-2291.

Winston, ML (1991) *The Biology of the Honey Bee*. Harvard University Press.

Zhang, J, Li, D, Ge, P, Guo, Y, Zhu, KY, Ma, E and Zhang, J (2014) Molecular and functional characterization of cDNAs putatively encoding carboxylesterases from the migratory locust, *Locusta migratoria*. *PLoS One* **9**: e94809.

Zhang, Y, Liu, X, Zhang, W and Han, R (2010) Differential gene expression of the honey bees *Apis mellifera* and *A. cerana* induced by *Varroa destructor* infection. *Journal of Insect Physiology* **56**: 1207-1218.