Differential Gene Expression of Varroa-Tolerant and Varroa-Susceptible Honey Bees (*Apis mellifera*) in Response to *Varroa destructor* Infestation

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ABSTRACT

The honey bee is one of the most familiar insects in the world, and plays an important role in the global economy providing essential pollination services to crops, fruit trees and vegetables. However, honey bee health is severely threatened by the ectoparasitic mite *Varroa destructor*, which feeds on the hemolymph of pupal and adult bees, resulting in loss of nutrients and circulatory fluids, decreased overall body weight and eventually the death of the bees. To investigate the molecular defense mechanisms of the honey bee against varroa mite infestation, we employed DNA microarray analysis to compare gene expression of two contrasting honey bee colony phenotypes selected from the Saskatraz breeding program. One designated as G4 is susceptible to the varroa mite, while the other designated as S88 is highly tolerant to the varroa. Total RNAs were isolated from bees at two different stages, dark-eyed pupa and adult worker, infected or non-infected with varroa mites, and used for DNA microarray analysis. The results showed that distinct sets of genes were differentially regulated in the varroa-tolerant and varroa-susceptible honey bee phenotypes, with and without varroa infestation. In both phenotypes, there were more differentially-expressed genes identified at the pupal stage than at the adult stage, indicating that at the pupal stage honey bees are more responsive to the varroa infestation than adult bees. In the phenotype comparisons, substantially more differentially-expressed genes were found in the tolerant than susceptible line, indicating that the tolerant phenotype has an increased capacity to mobilize the expression of the genes in response to varroa mite infestation. Based on function, the differentially-expressed genes could be classified into groups that are involved in olfactory signal transduction, detoxification, metabolism and exoskeleton formation, implying several possible mechanisms for the host-parasite interaction and resistance. Quantitative RT-PCR was used to confirm the data obtained from the DNA microarray hybridization. Eleven out of twelve genes selected based on the microarray data showed consistent expression patterns measured by both methods. Overall, comprehensive evaluation of the gene expression of honey bees in response to the mite infestation by DNA microarray has revealed several possible molecular mechanisms for the host defense against the pest. Identification of highly differentially expressed genes between the two phenotypes provides potential biomarkers that can be used for breeding honey bees resistant to the varroa mite.

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ABPV: acute bee paralysis virus

AKI: acute-kashmir-israeli complex

AL: antennal lobe

AN: antennal nerve

ANOVA: analysis of variance

BQCV: black queen cell virus

CBPV: chronic bee paralysis virus

CCD: colony collapse disorder

cDNA: complementary DNA

CoA: Coenzyme A

CYP: cytochrome P450

DGE: digital gene expression

DWV: deformed wing virus

ESTs: expressed sequence tags

FDR: false discovery rate

$G4^{+}$: the susceptible honey bee phenotype (G4) with varroa mite infestation (+)

$G4^{-}$: the susceptible honey bee phenotype (G4) without varroa mite infestation (-)

GO: gene ontology

IAPV: israeli acute paralysis virus

KBV: kashmir bee virus

logFC: log-transformed fold change

MDR: multidrug resistance protein

MIAME: minimum information about microarray experiment

NADPH or $NADP^{+}$: Nicotinamide adenine dinucleotide phosphate

ORNs: olfactory receptor neurons

PN: projection neurons

qRT-PCR: quantitative reverse transcription PCR

QTL: quantitative trait locus

RpS5: ribosomal protein S5

SNPs: single nucleotide polymorphisms

$S88^{+}$: the tolerant honey bee phenotype (S88) with varroa mite infestation (+)

$S88^{-}$: the tolerant honey bee phenotype (S88) without varroa mite infestation (-)

SBV: sacbrood virus

SSH: suppression subtractive hybridization

VSH: Varroa sensitive hygiene

1.0 Project survey

1.1 Introduction

The honey bee is one of the most familiar insects in the world, and plays an important role in the global economy providing essential pollination services to crops, fruit trees and vegetables (Vanengelsdorp and Meixner, 2010). The honey bee-related activity worldwide contributes multi-billion dollars to the global economy annually (Vanengelsdorp and Meixner, 2010).

A healthy population of honey bees is essential for efficient pollination and honey production; however, like other insects, honey bees are subject to attack by a wide range of parasites and pathogens (Genersch *et al.*, 2010). Among all these disease-causing agents, the ectoparasitic honey bee mite *Varroa destructor* is the greatest threat for beekeeping. It has been implicated in the death of millions of bee colonies (colony collapse disorder, CCD), leading to great economic losses, and causing a serious concern for apiculture (Sammataro *et al.*, 2000).

V. destructor is a large ectoparasitic mite closely associated with its honey bee host and lacks a free living stage. The mother mite and her offspring feed on the hemolymph of pupal and adult bees, resulting in loss of nutrients and circulatory fluids (Sammataro *et al.*, 2000), leading to decreasing overall body weight and longevity (Martin *et al.*, 2001; Duay *et al.*, 2003) and eventually colony collapse (Amdam *et al.*, 2004). The varroa also acts as a vector for spreading numerous bacterial, fungal and viral diseases within and among colonies (Davidson *et al.*, 2003; Kanbar and Engels, 2003; Tsagou *et al.*, 2004).

The host-parasite relationship between the honey bee and varroa is complex, and is an interesting model for studying the mechanisms used by social insects to defend themselves against parasites (Gisder *et al.*, 2010). The Asian honey bee, *Apis cerana*, has co-evolved with the varroa mite for centuries and thus possesses traits which enable it to tolerate varroa infestations with minimal harm (Rosenkranz *et al.*, 2010). The hygienic behavior, grooming behavior and other so far unknown physiological activites, are all possible mechanisms the colonies can use to defend against varroa infestation (Peng *et al.*, 1987; de Guzman *et al.*, 2008). Unlike the Asian honey bee, the western honey bee *Apis mellifera* is more susceptible to varroa (Sammataro *et al.*, 2000). Initial gene expression studies suggest that differences in physiology and behavior, rather than in

the immune response, might underlie varroa tolerance (Navajas *et al.*, 2008). In addition, the host tolerance to the varroa may be characterized by different metabolic and nerve signaling processes (Zhang *et al.*, 2010). Current studies provide the first steps toward understanding the molecular mechanisms underlying host-parasite relationship in honey bees.

Genomic resources developed by The Honey Bee Genome Project (The Honeybee Genome Sequencing Consortium, 2006) and new technologies in gene expression analysis provide an integrated and comprehensive resource for molecular research on the honey bee and varroa mite interaction (Robinson *et al.*, 2006). Identification of differential gene expression in bees in response to the varroa infestation would help elucidate molecular mechanisms of defense against the varroa mite. DNA microarray analysis is a powerful tool for profiling gene expression (Xu *et al.*, 2010), and has proven to be a valuable approach to study various phenotypes in response to biotic stress (Yoo *et al.*, 2009). The information obtained by the microarray analysis could identify differentially expressed genes of the honey bee, and help explore the honey bee gene networks and regulation pathways for defense against the mite attack.

1.2 Hypothesis and objectives

If differential gene expression is involved in the honey bee response to the mite attack, DNA microarray analysis could be used to analyze the phenotypes that are differentially responsive and tolerant to the mite infestation. The information obtained by the analysis will identify genes that are differentially expressed in mite-susceptible and mite-tolerant lines, with and without mite infestation, which will help elucidate the possible mechanism underlying host tolerance to the parasite and aid the development of molecular markers for breeding mite-resistant honey bees.

The objectives of this research are (1) to identify differentially-expressed genes in mite-susceptible and mite-tolerant honey bee colony phenotypes using DNA microarray analysis, (2) to validate the microarray data by quantitative real-time PCR, and (3) to analyze the differentially-expressed genes *in silico* to elucidate possible defence mechanisms of the host against the parasite infestation.

2.0 Literature survey

2.1 Honey bee

2.1.1 Introduction

The honey bee is one of the most familiar insects in the world, and plays an important role in agriculture providing essential pollination services to crops, fruit trees and vegetables. Considering its indispensable role as an economically valuable pollinator, the honey bee is among the most important domestic livestock species and crucial for the maintenance of natural ecosystems and biodiversity (Vanengelsdorp and Meixner, 2010). Moreover, the honey bee produces honey, propolis, royal jelly, and other hive products. The value of honey produced by honey bees is far more than what most people perceive from its delicious taste; it also offers medical benefits for our health, such as healing for cuts, and curing of ailments and diseases. The bee venom, which contains various bioactive compounds, has been used to relieve pain and treat inflammatory diseases (Jang *et al.*, 2009). The honey bee-related economic activity worldwide contributes multi-billion dollars to the global economy annually (Vanengelsdorp and Meixner, 2010).

According to Statistics Canada, Canadian beekeepers produced 90.9 million pounds of honey in 2012, a 14 per cent increase compared to 2011 (Table 2.1). Canada had 8,126 beekeepers in 2012, 413 more than in 2011. In Saskatchewan, the increased number of colonies has contributed to an increase in honey production from 15.9 million pounds in 2011 to 23.1 million pounds in 2012.

Table 2.1 Annual production and value of honey in Canada (2008-2012).

Geography[1]	Estimates	2008	2009	2010	2011	2012
Canada[2]	Beekeepers[3]	6,931	7,028	7,403	7,713	8,126
	Bee colonies[3]	570,070	592,120	620,291	637,920	706,429
	Annual production of honey, total (pounds x 1,000)[4]	64,895	70,362	81,672	79,824	90,877
	Annual value of honey, total (dollars x 1,000)[5]	105,184	126,253	144,197	150,691	172,704
Saskatchewan	Beekeepers[3]	1,045	971	965	850	765
	Bee colonies[3]	90,000	85,000	86,000	90,000	125,000
	Annual production of honey, total (pounds x 1,000)[4]	16,560	17,000	18,404	15,930	23,125
	Annual value of honey, total (dollars x 1,000)[5]	24,840	25,500	28,526	24,692	38,156

Notes: 1. Figures were compiled by Statistics Canada from provincial data, except for New Brunswick and Prince Edward Island where data were collected through a Statistics Canada mail survey. 2. Does not include Newfoundland and Labrador. 3. Beekeepers and bee colony numbers may include pollinators that may not be used for extracting honey. 4. Production excludes inventory. 5. Value excludes inventory sales except for in Quebec. (Adopted from Statistics Canada, CANSIM database.)

2.1.2 Honey bee health

A healthy population of honey bees is essential for pollination and honey production; however, the beekeeping industry currently is experiencing world-wide large scale losses of honey bee colonies (Lebuhn *et al.*, 2013). Especially in recent years, this large-scale loss of honey bee colonies, named Colony Collapse Disorder (CCD), has been reported in the United States (Vanengelsdorp *et al.*, 2009). This syndrome remains a mysterious phenomenon characterized by an unexplained and rapid loss of a colony's adult bee population. The adult honey bee workers suddenly abandon their hives, leaving the brood and the queen poorly or completely unattended in the hive. Without the foraging adult bees, there would be no or very few bees remaining in the colonies.

Many studies have tried to unravel the causative factors of this enigmatic honey bee health problem, and several factors were hypothesized to be involved in the syndrome (Farooqui, 2013). These factors include sensitivity to numerous pesticides (Belzunces *et al.*, 2012), the widespread development of genetically modified crops (Duan *et al.*, 2008), electromagnetic radiation from mobile phones (Hsu *et al.*, 2007), nutritional stress (Alaux *et al.*, 2011), lack of genetic diversity due to mating with a single male (Mattila *et al.*, 2012), and combinational theory of interactions among multiple factors (Nazzi *et al.*, 2012).

It is generally believed that like other animals, honey bees are inevitably subject to attack by a wide range of pathogens (Genersch *et al.*, 2010). These pathogens include parasites, fungi, trypanosomes, nosema, and viruses such as deformed wing virus (DWV), acute bee paralysis virus (ABPV), black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), israeli acute paralysis virus (IAPV), kashmir bee virus (KBV), and sacbrood virus (SBV). All of these might be responsible for honey bee disease. In addition, a phorid fly *Apocephalus borealis* has been found recently to attack the honey bee (Core *et al.*, 2012). Among all of these disease-causing agents, the ectoparasitic honey bee mite *V. destructor* is the most serious concern for apiculture. It has been implicated in the deaths of millions of colonies (Sammataro *et al.*, 2000), leading to great economic losses.

2.2 Varroa mites

2.2.1 Introduction

V. destructor, the honey bee parasitic mite, has become the most serious problem of beekeeping worldwide. The varroa was first found by Jacobson on the Asian honey bee, *Apis cerana*, in 1904 in Java, Indonesia (Anderson and Trueman, 2000). Since then, it has been found in Russia, Japan, and China, as well as other countries in Europe and North America (Anderson and Trueman, 2000). The bee parasite has received scientific attention as it has become a major pest in *Apis mellifera* L. in Europe. The parasite has spread rapidly in recent years and now it has been reported in all continents except for Australia, and infests almost all honey bee colonies world-wide. According to the Canadian Honey Council, the varroa mite was first found in Canada in New Brunswick in 1989 (http://www.honeycouncil.ca/index.php). By 2002, the mite had spread across most beekeeping regions in Canada (Currie *et al.,* 2010), and it is the main culprit for the death and reduced populations of overwintered honey bee colonies in Ontario (Guzmán-Novoa *et al.,* 2010).

Although knowledge on *V. destructor* is still limited compared to some of the other honey bee pests and viruses, the information on *V. destructor* infestation of honey bee colonies has grown considerably over the last decade. A body of literature dealing with varroa identification, natural history, transmission, and pathology has been accumulating.

V. destructor is a large ectoparasitic mite of bees. It cannot survive without its bee host (Figure 2.1). The life cycle of the female mite is divided into two distinct phases: a phoretic phase on the adult bees and a reproductive phase inside the sealed drone and worker brood cells (Kuenen and Calderone, 1997). To reproduce, the female mite leaves the adult bee and enters a brood cell shortly before the cell sealing where she lays several eggs. Later the mother mite breaks the cuticle surface of the pupa in the cell (Kuenen and Calderone, 1997), and her offspring and herself then regularly feed on the hemolymph of the pupa and later the adult bee. This leads to loss of nutrients and circulatory fluids (Sammataro *et al.*, 2000), decrease of overall body weight (Martin *et al.*, 2001; Duay *et al.*, 2003) and eventually death of the host bees (Amdam *et al.*, 2004).

Besides the direct harm to bees, the varroa also acts as a vector for spreading bacterial, fungal and viral diseases (Davidson *et al.*, 2003; Kanbar and Engels, 2003; Tsagou *et al.*, 2004), within and among colonies. Significantly, *V. destructor* has been shown to transmit and amplify Deformed Wing Virus (DWV) (Boncristiani *et al.*, 2009) and Kashmir Bee Virus (KBV) in honey bee colonies (Chen *et al.*, 2004). The number of viral copies of Acute-Kashmir-Israeli complex (AKI) and Deformed Wing Virus (DWV) were correlated with the number of varroa mites (Francis *et al.*, 2013). In recent years, the large-scale loss of honey bee colonies named Colony Collapse Disorder (CCD) has been directly associated with and attributed to the varroa infestation (Vanengelsdorp *et al.*, 2009).

2.2.2 Varroa control

The varroa mite has spread rapidly worldwide within a short time period and now it is difficult to find a ‘‘varroa free” honey bee colony anywhere in the world, other than Australia. Without doubt, if the problem of the varroa is not resolved soon, this could have a major impact on the apiculture industry and agricultural productivity. As shown in Table 2.2, beekeepers currently utilize a wide range of methods to control mite populations (Rosenkranz *et al.*, 2010).

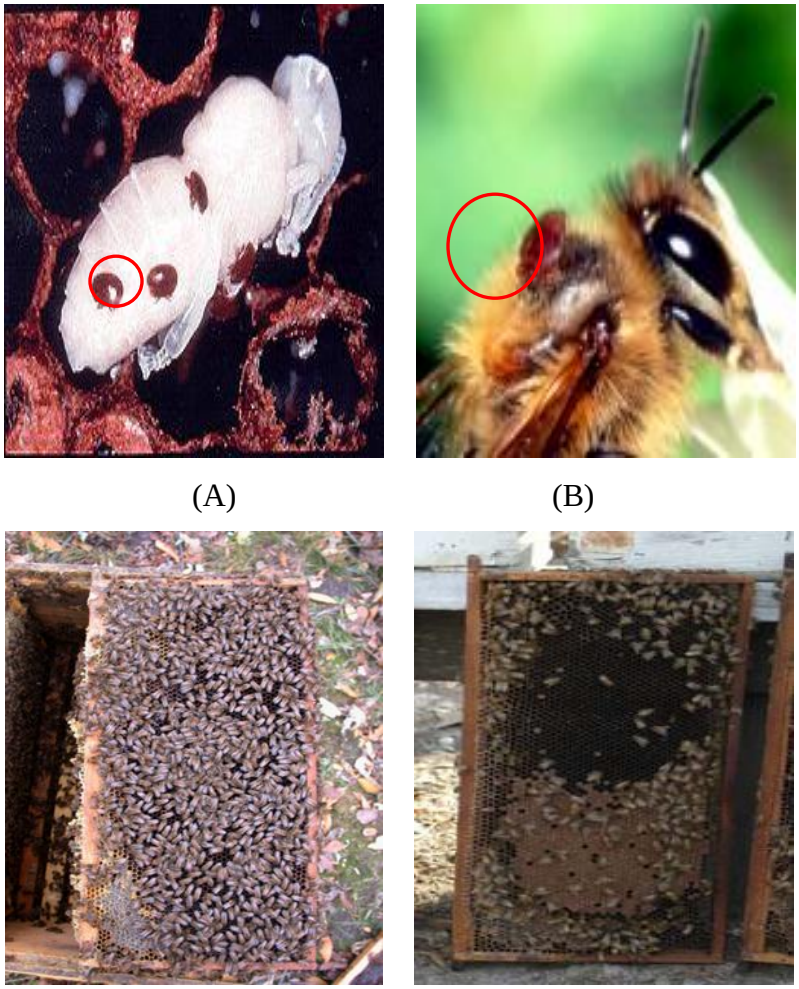


Figure 2.1 Varroa–infested honey bees.
A. Varroa attached to a pupal bee, B. Varroa attached to an adult bee, C. A frame from healthy honey bee colony, D. A frame from varroa-infested honey bee colony. The red circles highlight the varroa mites.

2.2.2.1 Miticide

The most effective and widely used method to control the varroa is probably chemical treatments, called miticides (Strange and Sheppard, 2001). The miticides essentially kill or retard the growth of the varroa in the hive. However, using miticides could pollute the honey and other honey bee products, like wax and pollen (Mullin *et al.*, 2010). Therefore, this method also carries a risk to the food industry for humans. Another negative aspect of using miticides is that the varroa mites could become resistant to the miticide following long term applications, leading to ineffectiveness of the miticide (Maggi *et al.*, 2011). In that case, the beekeepers will have to change miticides regularly. Therefore, use of miticides to control the mite increases not only the cost for beekeepers, but also the risk of chemical contamination in honey bee products and loss of effective miticide control.

2.2.2.2 Organic acids and essential oils

One way to reduce harmful chemical contamination in bee products from miticides is to use naturally occurring compounds that are safer. These products include organic acids and essential oils, such as formic acid, oxalic acid, lactic acid and thymol (Mahmood *et al.*, 2012; Rashid *et al.*, 2012). Organic acids and essential oils are sufficient to kill mites within the sealed brood cells, without causing serious harm to adult bees (Mert and Yucel, 2011). There is also a low risk of residue accumulation of these natural compounds in bee products (Bogdanov, 2006). However, there are some disadvantages in using these natural compounds. Lactic acid and oxalic acid have to be applied under broodless conditions (Emsen and Dodologlu, 2009). In addition, the efficacy of organic acids and essential oils is often more variable, as compared to registered miticides (Mert and Yucel, 2011).

2.2.2.3 Biological control

Another promising approach for varroa management is the use of biological agents to control the mite (Chandler *et al.*, 2001). The most effective agents are the fungal pathogens *Beauveria bassiana* (Steenberg *et al.*, 2010; Hamiduzzaman *et al.*, 2012), *Metarhizium anisopliae* (Kanga *et*

al., 2003; Kanga *et al.*, 2010), *Verticillium lecanii* (Shaw *et al.*, 2002), *Hirsutella thompsonii* (Kanga *et al.*, 2002), *Clonostachys rosea* (Hamiduzzaman *et al.*, 2012), as well as the bacterial pathogen *Serratia marcescens* (Tu *et al.*, 2010). Such methods of biological control are considered promising alternatives to the chemical control method. However, fungal pathogens often take time, as many as several days, to develop and kill varroa mites, and sometimes may not be able to effectively adapt to the local climatic and honey bee brood conditions (Chandler *et al.*, 2001).

In summary, where possible a combination of different treatments should be used to avoid development of mite resistance to miticides and to increase the overall efficacy of mite control. There are currently no mite control methods that fulfill all criteria, including safety, efficacy, and easy to apply. The synergistic effects of varroa and other pathogens are causing more serious problems for the beekeeping industry than ever before. The current methods to control varroa mites are not adequate, and it is critical to develop new measures such as breeding bees for resistance to the mite. This approach would be cost effective, environmentally friendly, and with little or no toxic residues for mammalian consumers.

Table 2.2 Comparison of the advantages and disadvantages of different varroa control methods

Treatment methods	Advantages	Disadvantages	References
Miticide	High efficiency Convenient to use	Pollute honey bee products Varroa mites can develop resistance to the miticides	Mullin *et al.*, 2010; Maggi *et al.*, 2011
Organic acids and essential oils	Sufficient to kill mites within the sealed brood cells Low risk of residue accumulation in bee products	Efficiency is variable	Mert and Yucel, 2011; Bogdanov, 2006
Biological methods	Effective; inexpensive	Fungal pathogens may not be adapted to the local climatic and honey bee brood conditions, and therefore not effective	Hamiduzzaman *et al.*, 2012; Chandler *et al.*, 2001
Resistance breeding	Longer-term solution Reduced chemical residues	Reduce genetic biodiversity	Buchler *et al.*, 2010; Rinderer *et al.*, 2010

2.3 Resistance breeding

2.3.1 Introduction

The selective breeding of varroa resistant bees is considered to be the only long-term solution to the varroa problem. The Asian honey bee, *Apis cerana*, co-evolved with the mite over centuries and possesses several features that enable it to resist varroa without serious harm (Rosenkranz *et al.*, 2010). Unlike the Asian honey bee, the western honey bee *Apis mellifera* is much more sensitive to the varroa (Sammataro *et al.*, 2000). Because the varroa is a new parasite to *A. mellifera*, a balanced host-parasite relationship is lacking and beekeepers do not have long-term experience in dealing with this pest. Recently, many different attempts have been made to use hybridization and natural selection to solve the varroa problem in various breeding programs from North America (Ward *et al.*, 2008; Rinderer *et al.*, 2010; Danka *et al.*, 2012), Europe (Buchler *et al.*, 2010) and Russia (Rinderer *et al.*, 2001; Bourgeois and Rinderer, 2009).

2.3.2 Resistance mechanisms

The host-parasite relationship between the honey bee and varroa is complex, and it is an interesting model for studying the mechanisms used by social insects to defend themselves against parasites (Gisder *et al.*, 2010). There are several possible mechanisms used by honey bees to defend themselves against varroa mites. Hygienic behavior is one of them, through which the bees can rapidly detect and remove the dead, diseased or parasitized pupa from the hive before the adults emerge (Peng *et al.*, 1987). These hygienic activities interrupt the reproductive cycle of the parasite, lead to a prolonged phoretic phase or even the death of the mites (Ibrahim and Spivak, 2006). Another one is grooming behavior, i.e., the removal of mites from adult bees by that individual bee (auto-grooming) or other hive mates (allo-grooming) (Peng *et al.*, 1987). A third mechanism may be related to an unknown physiological effect that can reduce mite reproduction (Moritz, 1994). The genetic variance and heritability of these defensive traits have not yet been determined. Using genomic technologies to identify differential gene expression of bees in response to *V. destructor* attack is important for elucidating the molecular mechanisms underlying the defense mechanisms against the pest.

2.4 Molecular biology of honey bee defence mechanisms against the pest

2.4.1 Introduction

Owing to their economic importance, honey bees have been targets for scientific research in recent years. Social behavior and individual bee development have been well-studied (Whitfield *et al.*, 2006). Over the past twenty years, with the help of molecular biology, bee research has focused on physical and genetic maps of the genome (Hunt and Page, 1995), collections of expressed sequence tags (ESTs) (Evans and Wheeler, 1999; Whitfield *et al.*, 2002) and transcript profiling (Kucharski and Maleszka, 2003).

With the recent advent of new sequencing technologies which can generate large amounts of sequencing data at a relatively lower cost than the traditional Sanger sequencing method, the honey bee *Apis mellifera* genome has been fully sequenced (Honey Bee Genome Sequencing Consortium, 2006). This is the fourth insect genome that has been sequenced so far and the first for an eusocial species, providing an integrated and comprehensive genetic resource for molecular research on bees and other insects.

2.4.2 Molecular biology studies on varroa parasitism

Genomic resources developed by The Honey Bee Genome Project (The Honeybee Genome Sequencing Consortium, 2006) and new technologies in gene expression analysis provide great opportunities for studying the defensive mechanisms of the honey bee against mite attack. Several methods have been developed for the measurement of transcript and protein changes during the pathogenesis of bees (Table 2.3). Quantitative real-time RT-PCR (qRT-PCR) reveals that expression of genes coding for three anti-microbial peptides (defensin, abaecin, hymenoptaecin) is either not significantly different between varroa-infested and uninfested bees or is significantly elevated in varroa-infested bees, varying with sampling date and bee developmental age (Aronstein *et al.*, 2012). In contrast, in the honey bee larvae, the varroa parasitism results in significantly higher abundance of the transcripts of the antimicrobial peptide genes (defensin1, abaecin, hymenoptaecin) and, as well, a pathogen recognition gene for peptidoglycan recognition protein (Gregorc *et al.*, 2012).

Using suppression subtractive hybridization (SSH) to compare the differential expression of genes between two honey bee species *Apis mellifera* and *Apis cerana* indicates that most of the differentially expressed genes in the libraries are involved in metabolic processes and nerve signaling (Zhang *et al.*, 2010). Digital gene expression (DGE) analysis on bee abdomens found that the varroa parasitism increases viral populations and decreases protein metabolism in bees (Alaux *et al.*, 2011).

One major quantitative trait locus (QTL) on chromosome 9 and a QTL on chromosome 1 were associated with the performance of varroa sensitive hygiene (Tsuruda *et al.*, 2012). Another QTL on chromosome 5 is related to the honey bee grooming behavior (Arechavaleta-Velasco *et al.*, 2012). Three QTLs located on chromosomes 4 (ranging from 2.1 to 4.3Mb), 7 (ranging from 3.6 to 8.5 Mb), and 9 (ranging from 1.0 to 3.5Mb) have significant impact on suppression of varroa reproduction (Behrens *et al.*, 2011). Another three QTLs were found to influence the hygienic behavior, two loci influenced the uncapping behavior, and one locus influenced the removal behavior (Oxley *et al.*, 2010).

In another study, proteome-wide correlation analyses in larval integument and adult antennae identified several proteins highly predictive of behavior and reduced hive infestation. In the larva, the response to wounding is identified as a key adaptive process leading to reduced infestation; specifically, chitin biosynthesis and immune responses appear to represent important disease resistant adaptations. At the adult stage, chemosensory and neurological processes also provide specificity for detection of the varroa by antennae (Parker *et al.*, 2012).

Microarrays have recently been used to examine differences in gene expression associated with varroa mite parasitism in both susceptible and tolerant colonies, and the data suggest that differences in physiology and behavior, rather than in the immune response, underlie varroa tolerance in honey bees (Navajas *et al.*, 2008). This result provides a first step towards understanding the molecular mechanisms for the host-parasite relationship. In addition, microarrays have also been utilized to compare the brain specific gene expression of bees selected for a high rate of hygienic behavior (VSH+) and a low rate of hygienic behavior (VSH-

). The set of genes identified are involved in the social immunity of highly varroa-hygienic bees that efficiently detect and remove broods infected with the varroa mite. The function of these candidate genes does not seem to support a higher olfactory sensitivity in hygienic bees, as previously hypothesized (Le Conte *et al.*, 2011).

In summary, molecular biology and genomics techniques are effective methods for detecting differentially expressed genes and have great potential in revealing defense mechanisms of bees against varroa attack. However, the exact resistance mechanisms of bees against the pest remains elusive, as the results published so far in this field are still limited. Therefore, further studies are needed to identify more genes that are differentially expressed between the tolerant and susceptible bee lines and to determine the biological function of these genes to better elucidate the molecular mechanism defining the honey bee-varroa relationship.

Table 2.3 Molecular biology and genomics of varroa-honey bee relationship.

Methods	Principal results	References
qRT-PCR	Three anti-microbial peptides are either not significantly different between varroa-infested and uninfested bees or are significantly elevated in varroa-infested bees, varying with sampling date and bee developmental age. In honey bee larvae, varroa parasitism resulted in significantly higher transcript abundances for the antimicrobial peptides.	Aronstein *et al.*, 2012; Gregorc *et al*., 2012
SSH	Most of the differentially-expressed genes between two honey bee species, *Apis mellifera* and *Apis cerana*, were involved in metabolic processes and nerve signaling.	Zhang *et al.*, 2010
Digital gene expression (DGE) analysis	Varroa parasitism caused a decrease in metabolism, specifically by inhibiting protein metabolism essential to bee health.	Alaux *et al*., 2011
QTL	QTLs on chromosome 1 and 9 were associated with the performance of the varroa sensitive hygiene. A QTL on chromosome 5 was found to be related to the honey bee grooming behavior. Three QTLs located on chromosomes 4, 7, and 9 had significant impact on suppression of varroa reproduction.	Tsuruda *et al*., 2012; Arechavaleta-Velasco *et al*., 2012; Behrens *et al.*, 2011
Proteomics	In the larva, response to wounding was identified as a key adaptive process leading to reduced infestation. Chitin biosynthesis and immune responses appear to represent important disease resistant adaptations. At the adult stage, chemosensory and neurological processes could also provide specificity for detection of varroa in antennae.	Parker *et al*., 2012
DNA microarray	Differences in physiology and behavior, rather than in the immune response, underlie varroa tolerance in honey bees; higher olfactory sensitivity did not appear in hygienic bees.	Navajas *et al*., 2008; Le Conte *et al*., 2011

2.5 Honey bee metabolism and development

2.5.1 Transcript profiling by microarray

A DNA microarray is a collection of thousands of short oligonucleotide probes deposited on a solid support. Through hybridization, it is used to assay the presence of fluorescent-labelled complementary DNAs that are derived from a RNA sample. DNA microarray analysis has been proven to be a powerful tool for large scale profiling of RNA transcripts (Gresham *et al.*, 2008). This technology can measure the relative mRNA abundances in the transcriptome under different conditions (Zhu *et al.*, 2006), and analyze the expression of thousands of genes at one time. DNA microarrays can also be used for infectious and genetic disease and cancer diagnostics, for detecting single nucleotide polymorphisms (SNPs), and provide valuable insights into various mutant phenotypes (Yoo *et al.*, 2009).

DNA microarrays have been used for profiling honey bee gene expression (Xu *et al.*, 2010). The information obtained by the microarray analysis identified differentially expressed genes of honey bees, and helped explore honey bee gene networks and regulation pathways. This information can be used to elucidate the possible molecular mechanisms for honey bee behavioral maturation, reproductive plasticity, and disease tolerance. Several microarray platforms for honey bee genomic analysis are reviewed (see below); and important advances in this research area are highlighted.

2.5.2 DNA microarray platforms for honey bee

The high throughput sequencing technologies generated large amounts of genomic data; with this available resource, it has been possible to develop mature DNA microarray platforms for honey bees (Table 2.4). In 2002, the first honey bee DNA microarray was developed at the University of Illinois, Urbana-Champaign (Whitfield *et al.*, 2002). The DNA microarray was fabricated with 7329 expressed sequence tag (EST) cDNAs representing unique transcripts. They were selected from over 20,000 cDNAs partially sequenced from a normalized library generated from the brain of adult *A. mellifera*, and 15,311 high-quality ESTs identified representing 8912 putative transcripts. The DNA microarray analysis demonstrated that genomic scale gene expression profiling was feasible in a single honey bee brain (Whitfield *et al.*, 2002).

In Japan, a cDNA microarray containing 480 differential display-positive candidate cDNAs was developed in 2002 (Takeuchi *et al.*, 2002). Afterwards, the microarray platform was improved by increasing the number of cDNAs in the array to over 5000 (Yamazaki *et al.*, 2006).

In 2006, after the completion of the honey bee genome sequencing, the University of Illinois research group created a new version of the honey bee microarray. Each array contains a total of 13,440 distinct 70-mer oligonucleotide probes including an "official gene set" of 10620 oligos recommended by Honey Bee Genome Sequencing Consortium, the oligos representing ESTs from other databases, and the honey bee viral pathogens. Since then, a number of experiments have been undertaken using this microarray platform. It is now commercially available for universities and academic institutes worldwide for bee research (http://www.life.illinois.edu/robinson/index.html).

In 2012, a 44K SNP assay array, a DNA microarray variant, was specifically designed for the analysis of hygienic behavior of individual worker bees directed against the varroa. Approximately 36,000 of these validated SNPs from the Honey Bee Genome Project and another 8000 SNPs were selected for the construction of the SNP assay, which provides access to genomic selection of several traits in honey bee breeding (Spotter *et al.*, 2012).

Table 2.4 Microarray platforms for honey bee studies.

Year	Microarray type	Number of oligos/ESTs	Manufacturer	References
2002	DNA microarray	7,329	University of Illinois, Urbana-Champaign, USA.	Whitfield *et al*., 2002
2002	DNA microarray	480	University of Tokyo, Japan	Takeuchi *et al*., 2002
2006	DNA microarray	13,440	University of Illinois, Urbana-Champaign, USA.	Whitfield *et al*., 2006
2006	DNA microarray	> 5,000	University of Tokyo, Japan	Yamazaki *et al*., 2006
2012	SNP assay	44,000	Research Institute for the Biology of Farm Animals (FBN), Germany	Spotter *et al*., 2012

2.5.3 Transcript analysis of honey bee behavior

The relationship between behavior and gene expression of bees is complex and poorly understood. The honey bee exhibits a wide variety of behaviours, such as behavioural maturation and socially-regulated division of labor. Therefore, since the first generation of the microarray was created, which had over 7000 ESTs represented, honey bee behavior has been a target for microarray analysis.

In 2002, brain gene expression of five behavior groups (genotype-matched full sisters, age-matched comb builders, guard undertakers, genotype-matched nurses and foragers of typical ages), were compared directly with cDNA microarrays (Whitfield *et al.*, 2002). In the next year, a highly replicated experimental design was employed involving 72 microarrays. Individual brain mRNA profiles correctly predicted the behavior of 57 out of 60 bees, indicating a robust association between brain gene expression and naturally occurring behavior in the individual (Whitfield *et al.*, 2003). However, at that time, the researchers did not apply gene ontology (GO) analysis for these differentially expressed genes, so limited information was obtained on what function these genes could have, and which regulatory pathways were involved in honey bee behavior.

Later on, microarray analysis was conducted to examine gene expression preceding the onset of foraging, the effects of physiological and genetic factors on the behavioral transition, and the effects of foraging experience. Gene Ontology was used to identify biological processes that might be particularly prominent in honey bee behavioral maturation. It was found that there were multiple pathways affecting behavioral maturation, and that gene expression in the brain provides a robust indicator of the interaction between hereditary and environmental factors (Whitfield *et al.*, 2006). Using eight sets of genes from the two microarray experiments, the cis-regulatory code was studied on the massive social regulation of gene expression. The results show that particular binding sites for the transcription factors are significantly associated with one or more gene sets, suggesting that there is a robust relationship between cis-elements, transcription factor and social regulation of brain gene expression (Sinha *et al.*, 2006).

The honey bee is a well-established model to study alterations in gene expression associated with age-related changes in behavior maturation. Microarrays were interrogated with cDNAs representing RNAs extracted from newly-emerged worker bees and experienced foragers. Compared with newly-emerged worker bees, experienced foragers over-express royal jelly proteins, a putative growth factor, a transcriptional regulator and several enzymes (Kucharski and Maleszka, 2002). To compare the behavior maturation across the genus, a microarray analysis was performed on brain gene expression for the western honey bee *A. mellifera* and three other key species found in Asia: *A. cerana*, *A. florea* and *A. dorsata*. For each species, brain gene expression patterns between foragers and one-day-old adult bees were compared. The results indicate that there is a widespread conservation of the molecular processes in the honey bee brain related to behavioral maturation in the genus (Sen Sarma *et al.*, 2007).

Similarly, a semiparametric approach was applied to study gene expression in the brains of *A. mellifera* raised in two colonies with consistent patterns across five maturation ages. The combination of microarray technology, genomic information and semiparametric analysis provided insights into the genomic plasticity and gene networks associated with behavioral maturation in the honey bee (Rodriguez-Zas *et al.*, 2006). In addition, meta-analysis approaches were used to integrate information from the two studies above to identify genes that are associated with behavioral maturation in honey bees. This not only reaffirmed the genes identified previously, but also identified novel gene ontology categories that were associated with behavior maturation in honey bees (Adams *et al.*, 2008).

In honey bees, two different phenotypes, a queen and a worker, have identical genotypes. Differential feeding of female larvae promotes the occurrence of the labor division. A cDNA microarray analysis identified a gene encoding a putative orphan receptor (HR38) homologue that mediates an ecdysteroid-signaling process. Expression of this gene is higher in forager brains, as compared to nurse bees and queens, suggesting that ecdysteroid-signaling in the mushroom bodies might be involved in the labor division of the workers (Yamazaki *et al.*, 2006).

In another cDNA microarray, 240 genes that were differentially-expressed between developing queens and workers were identified. Workers up-regulate more developmentally characterized

genes than queens, whereas queens up-regulate a greater proportion of metabolically characterized genes. Many of these differentially-expressed genes are likely involved in processes favoring the development of caste-biased structures, like brain, legs and ovaries, as well as cytoskeleton (Barchuk *et al.*, 2007).

2.5.4 Transcript analysis of honey bee hormone synthesis and neurosystem

Hormones are very important for honey bee communication. Using DNA microarray analysis, it was revealed that the queen mandibular pheromone transiently regulates the expression of several hundred genes and chronically regulates the expression of 19 genes, demonstrating the potential of transcript profiling techniques to trace the actions of a pheromone from perception to action (Grozinger *et al.*, 2003).

The exposure of young bees to brood pheromone causes a delay in the transition from working in the hive to foraging. The pheromone treatment up-regulated the genes in the brain of bees specialized in brood care, and down-regulated the genes that are up-regulated in foragers (Alaux *et al.*, 2009). In addition, exposing honey bees to alarm pheromone at the hive entrance for one minute altered expression of hundreds of genes in the brains. Among the genes significantly up-regulated, several were involved in biogenic amine signaling. This result demonstrates the strong effects of a very brief environmental stimulus on brain gene expression, which might be related to behavioral sensitization (Alaux *et al.*, 2009). Another cDNA microarray analysis was used to test if caffeine might induce changes in gene expression in the honey bee brain. The results provide initial evidence that the dopaminergic system and calcium exchange are the main targets of caffeine in the honey bee brain (Kucharski and Maleszka, 2005).

Honey bee colonies rely on diverse chemical and visual communication signals to coordinate activity in their neurosystem. Microarray analysis was used to compare brain gene expression between bees that performed vibration signaling persistently (V+) and carefully matched bees that never performed this activity (V-). 412 genes were up-regulated and 491 were down-regulated in V+ compared to V- bees. The results show that communication is characterized by distinct neurogenomic states in the brains of both senders and receivers (Alaux *et al.*, 2009).

Additionally, microarray analysis revealed that 1329 genes were differentially expressed in the brains of honey bees associated with foraging-related spatiotemporal memories. This result indicates that distinct spatiotemporal foraging memories in honey bees are associated with distinct neurogenomic signatures (Naeger *et al.*, 2011). Furthermore, new putative clock-controlled genes were identified by the microarray in the nurse and forager bees when sampling was done around the clock. Circadian rhythmicity is evident based on the expression in nurse bees that are active around the clock (Rodriguez-Zas *et al.*, 2012).

2.5.5 Transcript analysis of honey bee diseases

A healthy population of honey bees is essential for pollination of agricultural crops and the production of hive products; however, honey bees are inevitably subject to attack by a wide range of parasites and pathogens (Genersch *et al.*, 2010).

DNA microarray analysis was proven useful for determining genetic components behind honey bee immune response to specific natural pathogens. In recent years, the large-scale loss of honey bee colonies has come into focus with worldwide concern. Johnson *et al.* (2009) used whole-genome microarrays to compare gene expression in the guts of bees from colony collapse disorder (CCD) colonies and healthy colonies sampled before the emergence of CCD. The result reveals that unusual ribosomal RNA fragments are conspicuously more abundant in the guts of CCD bees (Johnson *et al.*, 2009).

In summary, with a growing interest in honey bees for both ecological and economic reasons, studying the role of genetic variation in honey bees will become increasingly important. The application of molecular biology and genomic techniques has provided new information about how gene expression varies in honey bees. DNA microarray, one of the most powerful genomic tools, has been used in the research on honey bees for several aspects, including social behavior, hormone regulation, neurologic response and pathogenesis.

3.0 Study 1: Identification of differentially expressed genes in honey bees in response to *Varroa destructor* infestation by microarray-based transcript profiling

3.1 Abstract

High-throughput DNA microarray analysis was employed to investigate the genome-wide gene expression of two honey bee colony phenotypes, the mite-tolerant S88 and the mite-susceptible G4. A total of 24 two-channel arrays in a replicated loop design were used in the hybridization of RNA samples isolated from pupa and adult bee heads of the two phenotypes. Comparison of the expression data revealed sets of genes that were differentially expressed between the two colony phenotypes. Further analysis of the genes by Gene Ontology and gene clustering unveiled biological processes that may be involved in the response of honey bees to the varroa mite infestation, and highlighted possible mechanisms underlying host defence against the parasite.

3.2 Hypothesis

If a mite-tolerant bee line responds to varroa infestation differently from a mite-susceptible line, then gene expression might show distinct patterns. Differences in mRNA profiles, detected by DNA microarray, might provide information on the molecular process required for host defense against the mite.

3.3 Experimental approach

3.3.1 Experimental design

Genetic material for this study was selected and supplied by the Saskatraz project: The Saskatchewan Honey Bee Breeding and Selection Program. A review of the breeding program (Robertson, Albert J 2010, The Saskatraz Project-A Review 2004-2009), and relevant references are available at www.saskatraz.com. The varroa tolerant colony S88 was selected in May 2007 while the varroa susceptible colony (G4) was selected in May 2010 by the Saskatraz research team (Figure 3.1).

Mite-susceptible and mite-tolerant bees at pupal and adult stages, with and without varroa infestation, were analyzed for differential gene expression using DNA microarrays (Figure 3.2).

At each developmental stage (pupal or adult), six biological replicates were conducted for each treatment group: susceptible with varroa mite infestation ($G4^+$); susceptible without varroa mite infestation ($G4^-$); tolerant with varroa mite infestation ($S88^+$) and tolerant without varroa mite infestation ($S88^-$). The sample comparisons were arranged into a loop comparison model (Figure 3.2). In general, there are two major comparisons: the mite infestation comparison and the honey bee phenotype comparison. The mite infestation comparison compares differential gene expression of the honey bees with or without mite infestation within the same honey bee phenotype. This includes the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$) ($G4^+/G4^-$), and the tolerant phenotype with the mite infestation ($S88^+$) relative to the tolerant phenotype without the mite infestation ($S88^-$) ($S88^+/S88^-$). The honey bee phenotype comparison compares differential gene expression between the two honey bee phenotypes, which are the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$) ($S88^+/G4^+$), and the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible without varroa mite infestation ($G4^-$) ($S88^-/G4^-$). All these comparisons are performed at pupal stage and adult stage, respectively. This design maximizes the direct comparisons between parasitized and non-parasitized, susceptible and tolerant bee lines.

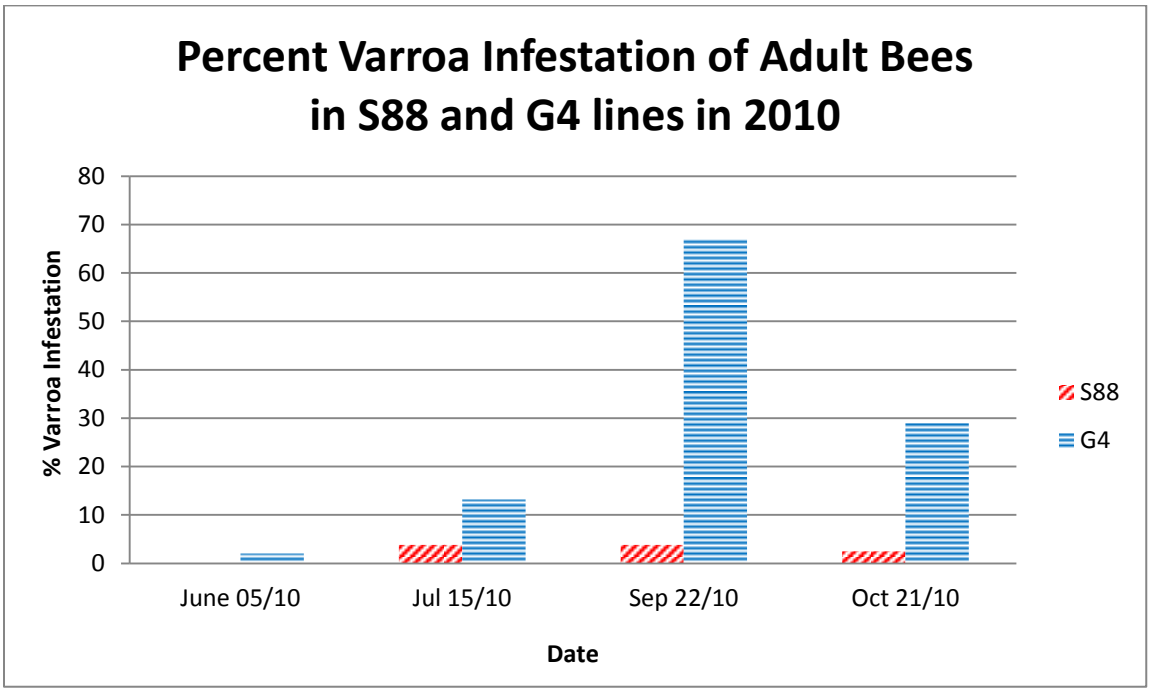


Figure 3.1 Comparison of the number of the varroa present in G4, a varroa-susceptible line and S88, a varroa-tolerant line, in the summer of 2010.

The varroa-tolerant line S88 was selected in May 2007 and the varroa-susceptible line G4 was selected in May 2010. The varroa-susceptible line G4 collapsed and died in October 2011, 17 months after selection, whereas the varroa tolerant line survived 52 months before death in September 2011. The varroa infestation rate (%) represents the number of varroa-infestated adult bees over the total number of the adult honey bees examined in the summer of 2010. Data provided by Saskatraz research project team (Robertson *et al.*, unpublished).

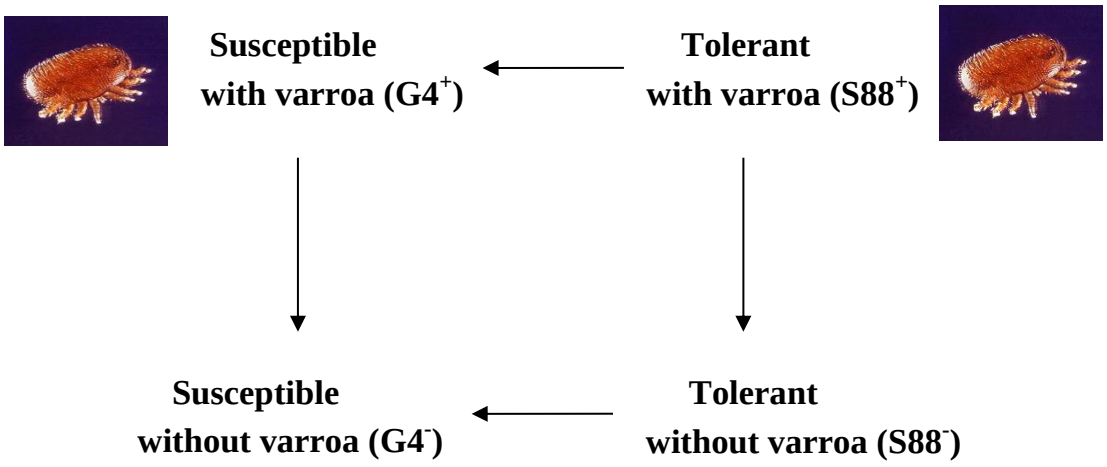


Figure 3.2 Loop design for the DNA microarray hybridization.

Two honey bee phenotypes differing in tolerance (S88 and G4), and infestation statuses (with and without the mite), at two developmental stages (pupal and adult), were employed in the microarray analysis. The pupal honey bees and the adult honey bees were analyzed separately. The arrows represent the mite infestation comparison ($G4^+/G4^-$ and $S88^+/S88^-$) and the phenotype comparison ($S88^+/G4^+$ and $S88^-/G4^-$).

3.3.2 Sample collection

A honey bee worker experiences four different developmental stages (Figure 3.3): egg, larva, pupa and adult (Kemp and Bosch, 2005). Samples for RNA extraction were collected between September 22 and 23 of the year 2010 from mite susceptible line G4 and mite tolerant line S88. The honey bees at two developmental stages were collected from Saskatraz natural selection apiaries operated by Meadow Ridge Enterprises LTD near the city of Saskatoon, Saskatchewan, Canada (52º11' N, 106º63' W) with the help of the Saskatraz research team. For pupa sampling, brood frames were removed from the hive and incubated in darkness at 32°C and 80% humidity in the field laboratory at Meadow Ridge Enterprises LTD or in the Lipid Laboratory of the University of Saskatchewan. Capped brood cells were carefully opened; the eye cuticle color of the brood was used to distinguish developmental stages of the pupa. Pupae at the dark eye stage were collected from the cells and frozen in liquid nitrogen before being stored at −80°C. Pupae from cells infested with mites were identified and separated from non-infested pupal honey bees before freezing. Adult bees were captured on the brood frame when they fly back to the hive, and frozen in liquid nitrogen before being stored at −80°C. A bee was considered to be parasitized if there was at least one mite attached to the bee, and bees with mites were separated from non-parasitized bees before freezing.

3.3.3 RNA extraction

Before RNA extraction, two honey bee heads of either dark-eye pupa or adult bees were separated from the body in liquid nitrogen. The heads were pulverized with a pestle while in liquid nitrogen in a 2 ml plastic tube. The total RNA of each sample was isolated using RNeasy Plant Mini kits (Qiagen, Valencia, California) and treated with DNase (RNase free Dnase I, also Qiagen) as described by the manufacturer. RNA purity and integrity were checked by spectrophotometer and agarose gel electrophoresis (1% agarose gels).

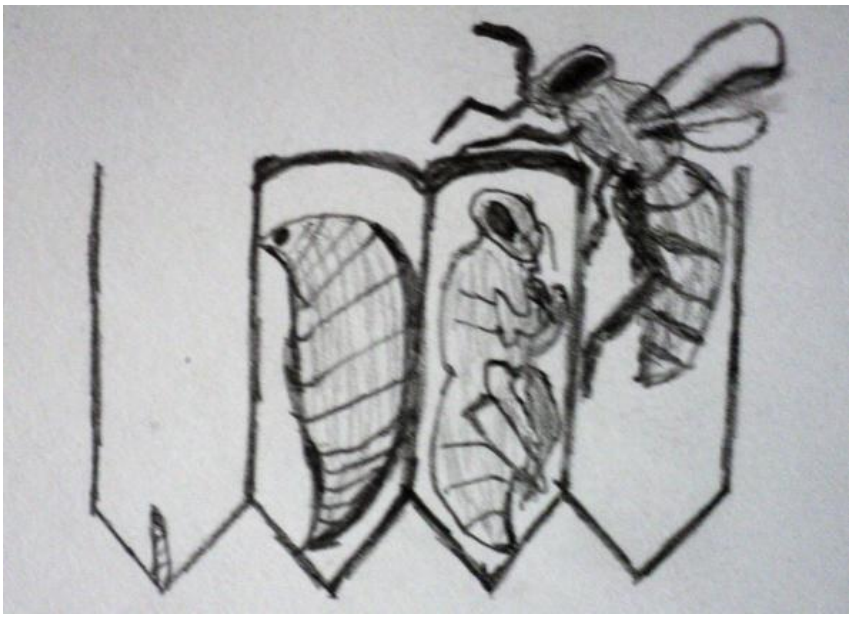


Figure 3.3 An illustration of the life cycle of honey bee workers.
Honey bee workers are non-reproducing females. The average life span of a worker honey bee is about one and a half months. The fertile queen lays eggs singly in cells of the honeycomb, worker bees develop from fertilized eggs and are diploid. Larvae are fed by worker bees with royal jelly, later switching to honey and pollen. Cells are capped by worker bees when the larva pupates. Pupae develop through several stages in the cells, and then emerge. During the time period, the worker honey bee develops from an egg to adult typically in 21 days. From left to right, egg, larva, pupa and adult. (Drawn by Sanjie Jiang.)

3.3.4 DNA microarray design and hybridization

DNA microarray hybridization was conducted at the Department of Entomology and Institute for Genomic Biology, University of Illinois at Urbana-Champaign. One μg of the total RNA from each sample was amplified using the Amino Allyl Message AmpII RNA Amplification kit (Ambion / Applied Biosystems, Austin, Texas) according to the manufacturer's instruction. Aliquots of the amplified RNA sample were independently labelled with either cyanine 3-dCTP (Cy3; 532 nm) or cyanine 5-dCTP (Cy5; 635 nm) fluorescent dyes. Dye swaps were conducted for replicate of each sample to avoid the effects of dye bias (Table 3.1). Labelled probes were hybridized to the bee whole-genome oligonucleotide arrays which were designed in 2006 (Whitfield *et al*., 2006).

Long oligos (70 mers) representing individual genes were synthesized and deposited on the arrays at the University of Illinois at Urbana-Champaign. Each array contains a total of 13,440 distinct oligonucleotides including an "official gene set" of 10620 oligos recommended by Honey Bee Genome Sequencing Consortium, the oligos representing ESTs from other databases, and the honey bee viral pathogens (http://www.biotech.uiuc.edu/functionalgenomics/services-equipment/honeybeeoligo). Hybridizations were carried out at 42^{o}C overnight using Agilent hybridization cassettes. Following incubation, slides were washed and fluorescence was measured on an Axon 4000B confocal laser scanner (Molecular Devices). Spot finding and image editing were performed using GenePix 6.1 software at the University of Illinois at Urbana-Champaign.

Table 3.1 Labeling and dye swap of pupa and adult honey bee samples on the microarray slides.

Microarray	Pupa Sample Dye Swap	Label		Microarray	Adult Sample Dye Swap	Label	
		Cy3	Cy5			Cy3	Cy5
Slide1	$G4^{+}1.G4^{-}1$	$G4^{+}$	$G4^{-}$	Slide13	$G4^{+}1.G4^{-}1$	$G4^{+}$	$G4^{-}$
Slide2	$G4^{+}5.\ G4^{-}5$	$G4^{+}$	$G4^{-}$	Slide14	$G4^{+}5.\ G4^{-}5$	$G4^{+}$	$G4^{-}$
Slide3	$G4^{+}4.\ S88^{+}4$	$G4^{+}$	$S88^{+}$	Slide15	$G4^{+}4.\ S88^{+}4$	$G4^{+}$	$S88^{+}$
Slide4	$G4^{-}3.G4^{+}3$	$G4^{-}$	$G4^{+}$	Slide16	$G4^{-}3.G4^{+}3$	$G4^{-}$	$G4^{+}$
Slide5	$G4^{-}2.S88^{-}1$	$G4^{-}$	$S88^{-}$	Slide17	$G4^{-}2.S88^{-}1$	$G4^{-}$	$S88^{-}$
Slide6	$G4^{-}6.\ S88^{-}5$	$G4^{-}$	$S88^{-}$	Slide18	$G4^{-}6.\ S88^{-}5$	$G4^{-}$	$S88^{-}$
Slide7	$S88^{-}3.\ G4^{-}4$	$S88^{-}$	$G4^{-}$	Slide19	$S88^{-}3.\ G4^{-}4$	$S88^{-}$	$G4^{-}$
Slide8	$S88^{-}2.\ S88^{+}1$	$S88^{-}$	$S88^{+}$	Slide20	$S88^{-}2.\ S88^{+}1$	$S88^{-}$	$S88^{+}$
Slide9	$S88^{-}6.\ S88^{+}5$	$S88^{-}$	$S88^{+}$	Slide21	$S88^{-}6.\ S88^{+}5$	$S88^{-}$	$S88^{+}$
Slide10	$S88^{+}2.G4^{+}2$	$S88^{+}$	$G4^{+}$	Slide22	$S88^{+}2.G4^{+}2$	$S88^{+}$	$G4^{+}$
Slide11	$S88^{+}6.G4^{+}6$	$S88^{+}$	$G4^{+}$	Slide23	$S88^{+}6.G4^{+}6$	$S88^{+}$	$G4^{+}$
Slide12	$S88^{+}3.\ S88^{-}4$	$S88^{+}$	$S88^{-}$	Slide24	$S88^{+}3.\ S88^{-}4$	$S88^{+}$	$S88^{-}$

Note: $G4^{+}$: the susceptible with varroa mite infestation, $G4^{-}$: the susceptible without varroa mite infestation, $S88^{+}$: the tolerant with varroa mite infestation, and $S88^{-}$: the tolerant without varroa mite infestation. Cy3 (cyanine 3-dCTP, 532 nm) and Cy5 (cyanine 5-dCTP, 635 nm) are the fluorescent dyes used for labeling. Numbers 1-6 represent the biological replicates.

3.3.5 Statistical analysis

Statistical analysis was performed using the R/Bioconductor package (R software, http://www.r-project.org/). For background subtraction, manually flagged spots (-100) were excluded, but auto-flagged spots (-50) were included. A print-tip loess normalization was performed using log2-transformed values on each array to even out the green dye bias. A scale normalization were performed between all arrays so that the distributions of M-values (log2 (Cy5/Cy3)) was approximately the same for all spots. Subsequently, a mixed-model analysis of variance (ANOVA) was fitted on the M-values that included a fixed term for dye (the same dyes always used), plus a random term for the duplicate spots for each oligo. A Bayesian correction was used to moderate the variance for each oligo. The raw p-values were adjusted separately for each comparison using the False Discovery Rate method. The microarray data obtained met Minimum Information about Microarray Experiment (MIAME) standards.

3.3.6 Functional analysis

BLAST searches of molecular databases at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) were carried out to identify homologies between probe cDNAs of interest and the honey bee genome, or genes from other organisms. Gene ontology analysis was used to explore the functional insights into differentially expressed genes using the FlyBase identification number. Each gene was assigned to the single "best hit" match in BLASTX searches of fruit fly *Drosophila melanogaster* predicted proteins. GO functional terms, and Drosophila gene GO annotations were downloaded from the GO website (www.geneontology.org, February 2012). Enrichment analysis was performed using GOToolBox (http://genome.crg.es/GOToolBox/) through a hypergeometric test followed by the Benjamini and Hochberg False Discovery Rate adjustment. The functional clustering of the genes was also conducted in GOToolBox using the WPGMA algorithm with a Bonferroni correction for multiple testing. Only categories that had more than 3 genes were selected for further analysis.

3.4 Results

3.4.1 Sample collection

Honey bee pupae at different developmental stages, with and without varroa mite infestation, were collected from brood combs removed from the two colony phenotypes (G4 and S88). The number of pupae at different developmental stages collected from each colony phenotype is listed in Table 3.2. Approximately 50 adult bees with and without phoretic mites were also collected from each colony phenotype.

The susceptibility and tolerance of the honey bees are based on measures of the infestation, including the proportion of bees infected with mites and the number of mites per infested bee (Table 3.3). As shown in Table 3.3, the varroa-tolerant phenotype S88 had fewer mites per dark-eyed pupa, than the susceptible phenotype G4.

3.4.2 RNAs isolated for the microarray hybridization

The total RNA was isolated from two heads of dark-eye pupae or two heads of adult bees. The initial concentration of the RNA sample isolated from the pupal and adult bees is shown in Table 3.4. The quality of RNA samples was confirmed by spectrophotometer and agarose gel electrophoresis. As shown in Figure 3.4, ribosomal RNA bands were clear, indicating that high-quality RNAs have been isolated.

Table 3.2 Sample collection from G4 (mite-susceptible) and S88 (mite-tolerant) honey bee colonies.

Pupal Stages	G4		S88	
	With varroa	Without varroa	With varroa	Without varroa
Pre-Pupa	0	0	12	5
White eye	14	2	7	9
Pink eye	129	24	13	28
Dark eye	**315**	**133**	**36**	**172**
Dark body	0	0	16	88
Pre-emergence	0	0	13	118
Total	458	159	97	420

Note: At the pupal stage, the sequential development of honey bees is differentiated by the color of the eye and body: pre-pupa (the stage between larval stage and pupal stage), white eye, pink eye, dark eye, dark body and pre-emergence (the pupal honey bee is going to break the capped brood cell). The dark-eyed pupal bees were used in the DNA microarray analysis (bolded). No honey bee was collected at the pre-pupa, dark body and pre-emergence stages in the susceptible phenotype G4.

Table 3.3 The number of mites per infested dark-eyed pupa.

Phenotype	G4	S88
Total number of infested dark-eye pupa*	315	36
Total number of mites	1090	82
Number of mites per infested pupa	3.46	2.28

Note: During sample collection, the number of infected dark-eyed pupa and the number of mites in each individual brood cell was recorded. The mean value of varroa mites per infested cell was then calculated. In the susceptible phenotype G4, there were more varroa mites per infested brood cell than the tolerant phenotype S88. *From Table 3.2

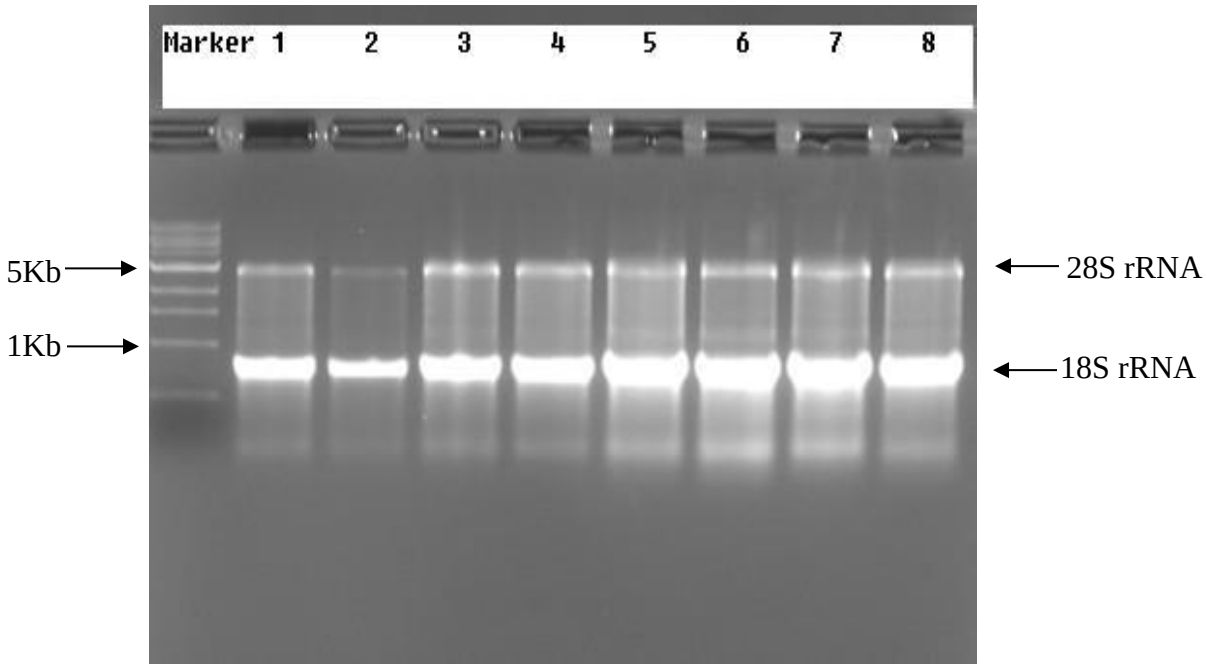


Figure 3.4 Representative agarose gel electrophoresis distributions of RNAs isolated from honey bees.

Five µl of RNA was denatured at 65 ^{o}C for 5 min, cooled down on ice for 2 min before loading onto a 1% agarose gel. Marker: 1 Kb DNA ladder. The concentration of the 5 Kb band was 80 ng/10µl, the other bands were 40 ng/10µl. Lanes 1-8: honey bee RNA samples.

Table 3.4 Initial RNA concentration of the pupal and adult honey bee samples.

Pupal sample	Concentration(µg/ml)	Adult sample	Concentration(µg/ml)
$G4^{+}1$	1520.2	$G4^{+}1$	572.0
$G4^{+}2$	870.6	$G4^{+}2$	291.8
$G4^{+}3$	556.6	$G4^{+}3$	683.5
$G4^{+}4$	591.6	$G4^{+}4$	360.1
$G4^{+}5$	1030.1	$G4^{+}5$	581.0
$G4^{+}6$	1450.5	$G4^{+}6$	509.1
$G4^{-}1$	665.4	$G4^{-}1$	569.5
$G4^{-}2$	745.4	$G4^{-}2$	991.1
$G4^{-}3$	695.5	$G4^{-}3$	738.8
$G4^{-}4$	1265.1	$G4^{-}4$	588.2
$G4^{-}5$	746.4	$G4^{-}5$	483.1
$G4^{-}6$	380.8	$G4^{-}6$	898.6
$S88^{+}1$	1028	$S88^{+}1$	377.4
$S88^{+}2$	736.7	$S88^{+}2$	650.9
$S88^{+}3$	446.3	$S88^{+}3$	820.7
$S88^{+}4$	596.1	$S88^{+}4$	694.3
$S88^{+}5$	617.7	$S88^{+}5$	570.9
$S88^{+}6$	1109.1	$S88^{+}6$	546.0
$S88^{-}1$	959.8	$S88^{-}1$	615.9
$S88^{-}2$	924.2	$S88^{-}2$	973.5
$S88^{-}3$	639.0	$S88^{-}3$	300.4
$S88^{-}4$	662.7	$S88^{-}4$	417.4
$S88^{-}5$	779.4	$S88^{-}5$	621.8
$S88^{-}6$	758.6	$S88^{-}6$	303.8

Note: $G4^{+}$: the susceptible phenotype with varroa mite infestation, $G4^{-}$: the susceptible phenotype without varroa mite infestation, $S88^{+}$: the tolerant phenotype with varroa mite infestation, and $S88^{-}$: the tolerant phenotype without varroa mite infestation. For each sample, 40 ul of the total RNA was obtained. Numbers 1-6 represent the biological replicates.

3.4.3 Primary results of the microarray hybridization

The DNA microarray we used consisted of 13,440 distinct 70-mer oligonucleotide probes including an "official gene set" of 10620 oligos recommended by Honey Bee Genome Sequencing Consortium. Representative primary microarray hybridization results are shown in Table 3.5. The fold change is the arithmetic ratio of the numerator over the denominator. For example, AM06878, a microarray oligo probe for Gene *GB14278* was located at block 24, row 1 and column 19 of the chip. The fold change in the comparison of $G4^{+}$ vs $G4^{-}$ was 9.25, while the false discovery rate (FDR)-corrected p-value (fdrPval) was 0.02. The fold change in the comparison of $S88^{+}$ vs $S88^{-}$ was 7.31, while the FDR corrected p-value was 0.02. Because the FDR corrected p-values here were less than 0.05, and the fold change is larger than 2, the gene expression differences in these comparisons were considered significant and could be used for the next analysis step.

Table 3.5 Sample of primary microarray hybridization results.

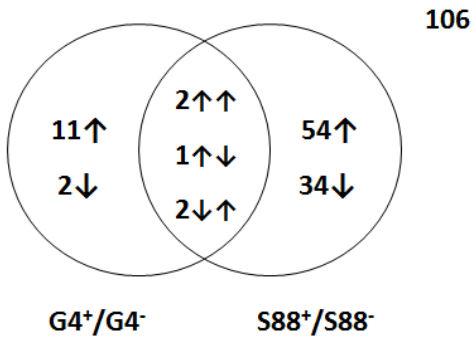
Name	ID	Location			FC.	fdrPval.	FC.	fdrPval.
		Block	Row	Column	$G4^+/G4^-$	$G4^+/G4^-$	$S88^+/S88^-$	$S88^+/S88^-$
GB11716	AM04335	6	15	15	1.42	**0.03**	1.33	**0.04**
GB11059	AM03683	1	1	11	1.49	**0**	1.37	**0**
GB13605	AM06205	35	11	9	1.26	**0.03**	1.26	**0.01**
GB13688	AM06286	14	11	13	1.47	**0**	1.37	**0**
GB14057	AM06656	25	5	23	2.43	**0.04**	2.08	**0.04**
GB14278	AM06878	24	1	19	9.25	**0.02**	7.31	**0.02**
GB15049	AM07640	20	16	19	1.68	**0**	1.25	0.05
GB10502	AM03128	29	9	7	1.20	0.38	1.45	**0.01**
GB11040	AM03664	23	14	1	1.20	0.08	1.32	**0**
GB11493	AM04113	5	11	21	1.68	0.16	2.25	**0.01**
GB11588	AM04208	2	16	9	1.27	0.34	1.74	**0**
GB11945	AM04562	9	16	1	1.18	0.06	1.19	**0.01**
GB12041	AM04658	33	16	15	4.41	0.16	6.02	**0.03**
GB12097	AM04718	43	10	5	2.48	0.15	3.36	**0.01**
GB12202	AM04822	38	5	13	1.14	0.63	1.61	**0**
GB12287	AM04906	48	8	19	2.10	0.15	2.31	**0.04**
GB12797	AM05412	33	12	1	1.37	0.06	1.77	**0**
GB12853	AM05467	46	3	19	1.51	0.13	2.10	**0**
GB13236	AM05844	2	19	13	1.39	0.05	1.51	**0**
GB13764	AM06363	27	13	9	3.14	0.16	4.14	**0.02**
GB14058	AM06657	7	2	5	3.61	0.22	6.50	**0.02**
GB14060	AM06659	43	4	19	1.08	0.56	1.23	**0**
GB14161	AM06761	19	7	1	1.11	0.54	1.37	**0**

Note: Name - the bee GB id for the oligo, if it exists. ID - the internal id for the oligo on the array. Block, Row, Column - the physical location of the oligo spot. FC.- the fold change for the comparison. fdrPval.- the False Discovery Rate p-value. Differential expression of genes at a false discovery rate (FDR)-corrected p-value of < 0.05 were considered significant (bolded).

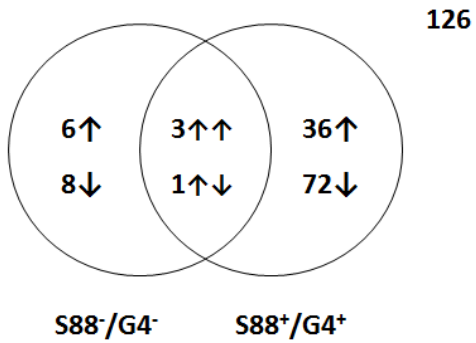
3.4.4 Differential gene expression in pupal and adult bees

DNA microarray analysis of bees at the pupal stage showed that there were 106 genes significantly differentially expressed in the mite infestation comparison, while there were 126 genes that were differentially expressed between the two honey bee phenotypes [False discovery rate (FDR), $P<0.05$; and fold-change >2, Figure 3.5]. As shown in the Venn diagram, the largest difference in gene expression was observed in the phenotype comparison with mite infestation ($S88^{+}/G4^{+}$) where 39 genes were up-regulated and 73 genes were down-regulated, indicating that mite-tolerant and mite-susceptible phenotypes responded to mite infestation with an extensive difference in gene expression (Figure 3.5B). Another noticeable comparison at the pupal stage was $S88^{+}/S88^{-}$, the tolerant line with and without the mite, showed 58 genes were up-regulated and 35 genes were down-regulated in expression (Figure 3.5A). This was in the contrast to the comparison of $G4^{+}/G4^{-}$, the susceptible colony with and without the mite, where only 14 genes were up-regulated and 4 genes were down-regulated, indicating that the tolerant colony S88 had a higher capacity to alter the gene expression in response to varroa mite infestation.

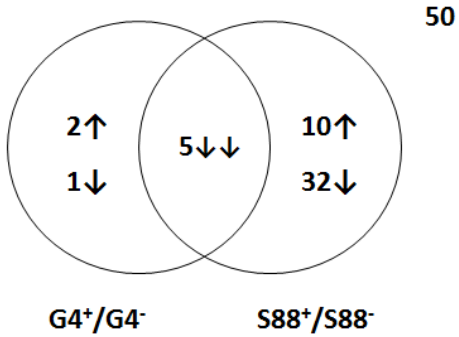
DNA microarray analysis at the adult stage showed that there were 50 genes that were differentially expressed in the mite infestation comparisons, while there were only 13 genes that were differentially expressed between the two honey bee phenotypes (Figure 3.5 C and D). Similar to the pupal stage, the $S88^{+}/S88^{-}$ comparison identified a larger number of differentially expressed genes with 10 genes being up-regulated and 37 genes being down-regulated. In contrast, the $G4^{+}/G4^{-}$ comparison had only 2 genes that were up-regulated and 6 genes that were down-regulated, indicating consistently that adult bees of the tolerant colony S88 also have a higher capacity to alter gene expression in response to varroa mite infestation when compared to the susceptible line G4. In addition, the phenotypic comparison with mites ($S88^{+}/G4^{+}$) showed 5 genes were up-regulated and 6 genes were down-regulated in expression, compared with the phenotypic comparison without the mite ($S88^{-}/G4^{-}$) where only a total of 4 genes were differentially expressed, indicating that different phenotypes respond to varroa mite infestation differently at the adult stage with the tolerant line being more highly responsive to the mite infestation.



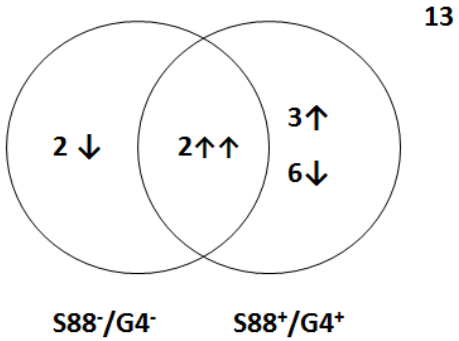
(A) Mite infestation comparison (Pupa)



(B) Phenotype comparison (Pupa)



(C) Mite infestation comparison (Adult)



(D) Phenotype comparison (Adult)

Figure 3.5 Venn diagram showing the number of differentially-expressed genes identified in mite infestation and phenotype comparisons at pupal and adult stages.

(A), The mite infestation comparison (pupa): comparison between presence (+) and absence (-) of the mite at the pupal stage. ↑ ↑ : up-regulation (↑) in $G4^{+}/G4^{-}$ and up-regulation (↑) in $S88^{+}/S88^{-}$; ↑ ↓ : ↑ in $G4^{+}/G4^{-}$ and down-regulation (↓) in $S88^{+}/S88^{-}$. (B), phenotype comparison (pupa): comparison between tolerant (S88) and susceptible (G4) colony phenotypes at the pupal stage. ↑ ↑ : ↑ in $S88^{-}/G4^{-}$ and ↑ in $S88^{+}/G4^{+}$; ↑ ↓ : ↑ in $S88^{-}/G4^{-}$ and ↓ in $S88^{+}/G4^{+}$. (C), mite infestation comparison (adult): comparison between presence (+) and absence (-) of the mite at the adult stage. ↓ ↓ : ↓ in $G4^{+}/G4^{-}$ and ↓ in $S88^{+}/S88^{-}$. (D), phenotype comparison (adult): comparison between tolerant (S88) and susceptible (G4) colonies at the adult stage. ↑ ↑ : ↑ in $S88^{-}/G4^{-}$ and ↑ in $S88^{+}/G4^{+}$. $G4^{+}/G4^{-}$: the susceptible phenotype with varroa mite infestation ($G4^{+}$) relative to the susceptible phenotype without varroa mite infestation ($G4^{-}$), $S88^{+}/S88^{-}$: the tolerant phenotype with varroa mite infestation ($S88^{+}$) relative to the tolerant phenotype without varroa mite infestation ($S88^{-}$), $S88^{+}/G4^{+}$: the tolerant phenotype with varroa mite infestation ($S88^{+}$) relative to the susceptible phenotype with varroa mite infestation ($G4^{+}$), and $S88^{-}/G4^{-}$: the tolerant phenotype without varroa mite infestation ($S88^{-}$) relative to the susceptible phenotype without varroa mite infestation ($G4^{-}$).

3.4.5 Genomic distribution of the differentially expressed genes

There are 16 pairs of chromosomes in the honey bee worker's genome (Beye *et al.*, 2006). The localization of the differentially expressed genes on these chromosomes could indicate the relative importance of individual chromosome and the potential mechanism for regulating these genes. As shown in Table 3.6, the differentially expressed genes identified by DNA microarray analysis from the four comparisons at the pupal stage were not evenly distributed on the chromosomes. Chromosomes 15 and 1 contained a higher number of differentially expressed genes which accounted for 30 and 29, respectively. Chromosome 2, 4, 5, 6, 7, 8, 12, 12, 13 and 16 each contained between 10 to 24 genes. Chromosome 3, 10 and 14 had the lowest number of differentially-expressed genes which was less than 10 (Table 3.6). Interestingly, some of the differentially expressed genes resided in clusters.

Among the genes that were differentially expressed, the majority of them were identified from the two comparisons at the dark-eyed pupal stage. 93 out of the 106 differentially expressed genes from the pupa mite infestation comparison were found to reside on 15 chromosomes and the rest of the genes were on unmapped scaffolds (Table 3.6, Figure 3.6A). Chromosomes 1, 4 and 15 contained the highest number of the differentially expressed genes identified from the pupa mite infestation comparison which accounted for 12, 10 and 12, respectively. Chromosome 8 and 13 each contained 8 genes, chromosome 2, 5, and 12 each had 6 genes, while chromosomes 9, 11 and 16 each had 5 genes. Interestingly, some of these genes were co-localized, for example, *apidermin-1* (*GB30202*), *apidermin-3* (*GB30203*), *uncharacterized LOC727131* (GB12449) and *apidermin-3* (*GB12636*) on chromosome 4, *serine protease 5* (*GB12300*), *serine protease homolog 51* (*GB13397*) and *serine protease 4* (*GB10646*) on chromosome 9, *putative polypeptide N-acetylgalactosaminyltransferase 9* (*GB13681*) and *alpha-amylase* (*GB18312*) on chromosome 13, *CYP6A14* (*GB11754*) and *CYP6A1* (*GB12136*) on chromosome 13 , *osiris 17* (*GB16817*), *osiris 19* (*GB16804*) and *osiris 20* (*GB15865*) on chromosome 15. The genes that were tightly linked together appeared to have related biological functions. Presumably, they would share similar expression patterns controlled by a similar transcriptional regulation mechanism.

For the phenotype comparisons at the pupal stage, 112 of the 126 differentially expressed genes were dispersed among all 16 chromosomes and 14 genes were found on unmapped scaffolds (Table 3.6). The number of genes on individual chromosomes ranged from 2 to 15 (Figure 3.6B). Chromosome 1, 8, 9 and 15 each contained 13, 13, 11 and 15 genes, respectively. Chromosome 4 had 8, chromosomes 2 and 7 each contained 7, while chromosome 6 and 13 each had 6 genes. Similarly, some of these genes were found to be co-localized together, for instance, *uncharacterized LOC725238* (*GB12700*) and *histone H2A* (*GB18806*) on chromosome 1, *apidermin-1* (*GB30202*) and *apidermin-3* (*GB30203*) on chromosome 4, *tubulin beta-1* (*GB10275*), *beta-Tubulin 60D* (*GB11920*) and *tubulin beta-1 chain* (*GB13049*) on chromosome 4, *UDP-glycosyltransferase* (*GB17015*) and *beta-glucosidase* (*GB18896*) on chromosome 6, *uncharacterized LOC409163* (*GB13457*), *uncharacterized LOC551089* (*GB14811*) and *retinoid-inducible serine carboxypeptidase* (*GB11273*) on chromosome 7, *uncharacterized LOC725454* (*GB15046*) and *uncharacterized LOC725804* (*GB10347*) on chromosome 8, *hexamerin 70b* (*GB10869*) and *hexamerin 70c* (*GB13613*) on chromosome 8, *serine protease 5* (*GB12300*), *serine protease homolog 51* (*GB13397*) and *serine protease 4* (*GB10646*) on chromosome 9, *uncharacterized LOC551133* (*XM_623529*) and *uncharacterized LOC725903* (*GB17322*) on chromosome 9, *uncharacterized LOC552190* (*GB13936*) and *uncharacterized LOC726758* (*GB17888*) on chromosome 12, *odorant binding protein 14* (*GB30365*), *odorant binding protein 17* (*GB11092*) and *odorant binding protein 18* (*NM_001040227*) on chromosome 15 as well as *osiris 18* (*GB16900*), *osiris 19* (*GB16804*) and *osiris 20* (*GB15865*) on chromosome 15.

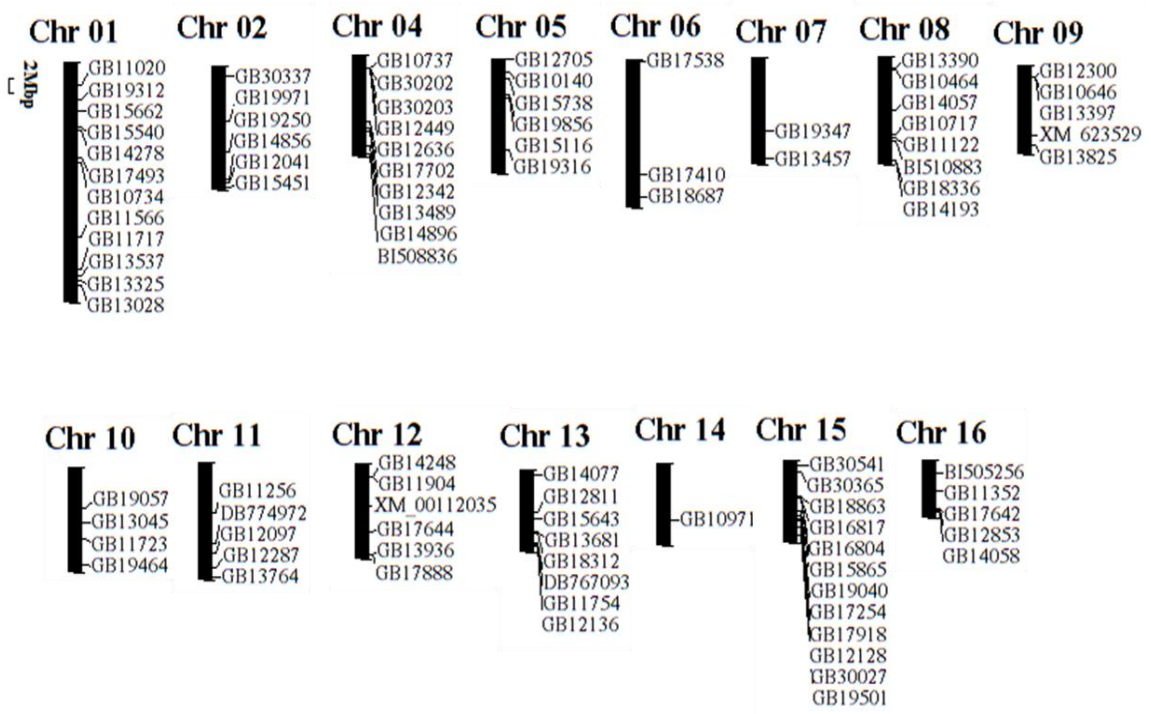
Fewer differentially expressed genes were identified in the two comparisons at the adult stage when compared to those at the pupal stage. For the adult mite infestation comparison, 43 of the 50 differentially expressed genes were dispersed among the 16 chromosomes and 7 genes were on unmapped scaffolds (Table 3.6). However, the number of genes ranged from only 1 to 5 on these chromosomes (Figure 3.6C). Chromosome 4 had the highest number of the genes with 5, followed by chromosome 13 and 16 each with 4, and chromosome 1, 2, 7, 8, 11 and 12 each with 3 genes. Among them, *apidermin-1* (*GB30202*) and *apidermin-3* (*GB30203*) were co-localized together on chromosome 4 , and two very long chain fatty acid elongation protein genes (*GB13264* and *GB12176*) were co-localized together on chromosome 16.

For the adult phenotype comparison, 13 differentially expressed genes were identified. They were dispersed on 9 chromosomes with no gene found on unmapped scaffolds (Table 3.6). Three genes were localized on chromosome 13, two genes each were on chromosome 3 and 12, and one gene each on chromosome 1, 2, 4, 7, 9 and 15 (Figure 3.6D). No differentially expressed genes from this comparison were found to reside together.

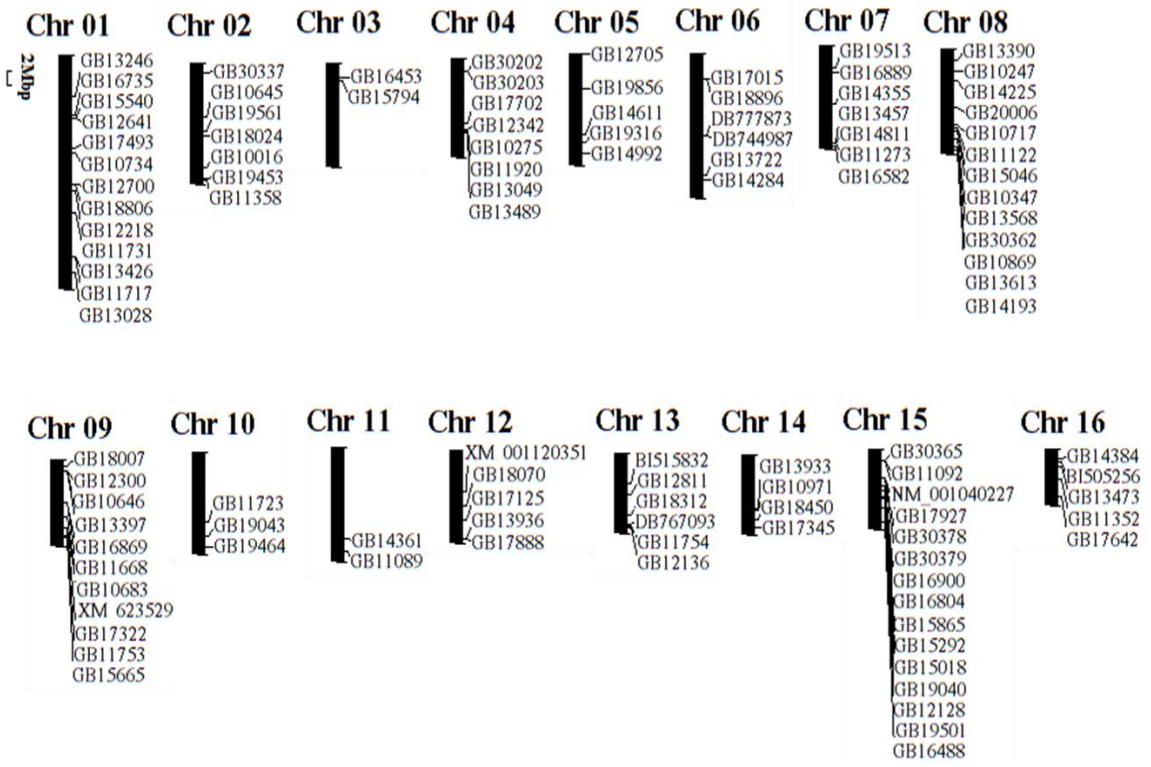
Table 3.6 Genomic distribution of differentially-expressed genes.

Chromosome	Pupa		Adult	
	Mite infestation comparison	Phenotype comparison	Mite infestation comparison	Phenotype comparison
1	12	13	3	1
2	6	7	3	1
3	0	2	2	2
4	10	8	5	1
5	6	5	2	0
6	3	6	2	0
7	2	7	3	1
8	8	13	3	0
9	5	11	1	1
10	4	3	1	0
11	5	2	3	0
12	6	5	3	2
13	8	6	4	3
14	1	4	2	0
15	12	15	2	1
16	5	5	4	0
Unmapped	13	14	7	0
Total	106	126	50	13

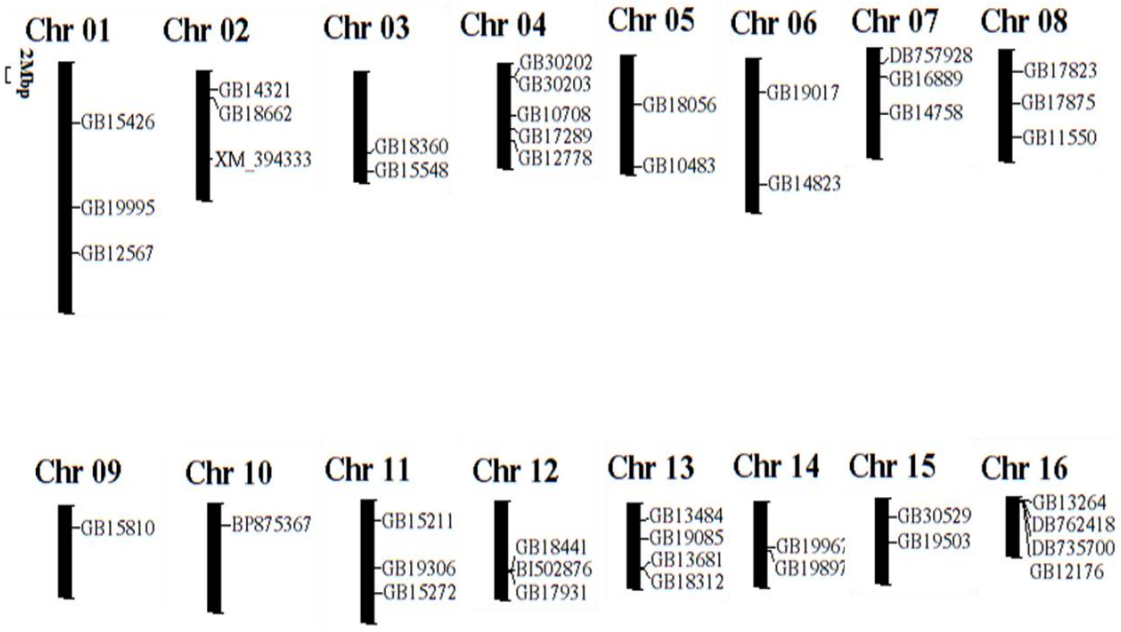
Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. The differentially-expressed genes are not equally distributed on each chromosome, and a few genes are not mapped on the chromosome. More detailed information about the genomic distribution of a single transcript is shown in Figure 3.6.



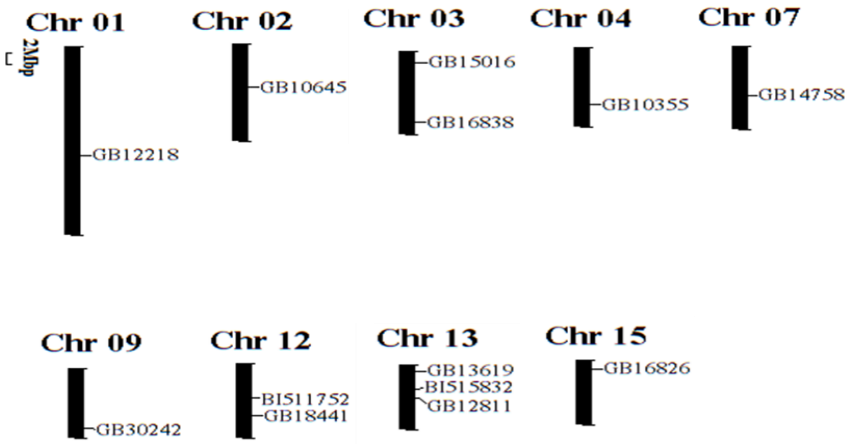
(A)Mite infestation comparison (pupa)



(B) Phenotype comparison (pupa)



(C) Mite infestation comparison (adult)



(D) Phenotype comparison (adult)

Figure 3.6 Genomic distribution of the differentially-expressed genes identified in the mite infestation and phenotype comparisons.

(A), The mite infestation comparison (pupa): comparison between presence (+) and absence (-) of the varroa mites at the pupal stage. (B), the phenotype comparison (pupa): comparison between tolerant (S88) and susceptible (G4) colony phenotypes at the pupal stage. (C), the mite infestation comparison (adult): comparison between presence (+) and absence (-) of the varroa mites at the adult stage. (D), the phenotype comparison (adult): comparison between tolerant (S88) and susceptible (G4) colonies at the adult stage. Refer to Figure 3.5 for each set.

3.4.6 Identification of overlapping genes between different comparisons

Of all the differentially expressed genes identified by DNA microarray analysis, nine were common among the comparisons at both developmental stages (Table 3.7), indicating these genes might be important in the host response to parasite infestation throughout life. It was particularly notable that a gene encoding dynein was significantly up-regulated in all the phenotype comparisons at both stages. A gene encoding esterase was up-regulated in the phenotype comparison with mite infestation at the pupal stage and the mite infestation comparison of the tolerant line at the adult stage. The genes encoding histone H1, apidermin 1, apidermin 3 and a hypothetical protein were all significantly down-regulated in the phenotype comparison with mite infestation at the adult stage. Apidermin genes were also down-regulated in the mite infestation comparison of the tolerant line at the adult stage. In addition, apidermin 3 gene was also up-regulated in the adult phenotype comparison. Histone H1 and the hypothetic protein genes were down-regulated in the phenotype comparison without mite infestation at the pupal stage.

Table 3.7 Differentially-expressed genes that were commonly found in phenotype and mite infestation comparisons at pupal and adult stages.

Gene	Phenotype comparison with and without mites				Mite infestation comparison within phenotypes				Honey bee protein
	Pupa		Adult		Pupa		Adult		
	Without mites $S88^-$ /$G4^-$	With mites $S88^+$ /$G4^+$	Without mites $S88^-$ /$G4^-$	With mites $S88^+$ /$G4^+$	$G4^+$ /$G4^-$	$S88^+$ /$S88^-$	$G4^+$ /$G4^-$	$S88^+$ /$S88^-$	
GB12218	Down			Down					Histone H1
GB12811	Down			Down			Up		Hypothetical protein
GB10645	Up	Up	Up	Up					Dynein-1-beta heavy chain, flagellar inner arm I1 complex
GB13681					Up			Up	N-acetylgalactosaminyltransferase 9
GB30529					Up			Up	Peroxisomal acyl-coenzyme A oxidase 1
GB30203			Up	Down		Up		Down	Apidermin 3
GB30202				Down		Up		Down	Apidermin 1
GB16889				Up		Down		Up	Esterase E4
GB18312			Up		Down	Down	Down		Alpha-amylase

Note: $G4^+$/$G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+$/$S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+$/$G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-$/$G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$).

3.4.7 Functional analysis of the differentially expressed genes

Gene ontology analysis was used to assign putative biological functions to differentially expressed genes using the FlyBase orthologs as references. At the pupal stage, 74 out of 106 differentially-expressed genes identified from the mite infestation comparison had identifiable fruit fly orthologs, and these genes clustered into 31 GO terms, while 85 out of 126 differentially-expressed genes identified from the phenotype comparison had fruit fly orthologs and clustered into 31 GO terms. These GO terms described a wide range of biological processes, molecular functions and cellular components (Table 3.8). Of the GO terms derived from two different types of comparisons, 8 of them (GO:0042302, GO:0048067, GO:0005576, GO:0004252, GO:0001726, GO:0008236, GO:0017171 and GO:0005198) were commonly found in both comparisons. They are related to the structural constituent of cuticle, cuticle pigmentation, extracellular region and ruffle, as well as serine-type protease activity. These biological functions might define the specific interaction of the host bee and the parasitic mite, therefore, they will be discussed in detail in the following sections.

At the adult stage, 35 out of the 50 differentially expressed genes identified from the mite infestation comparison had identifiable fruit fly orthologs and were clustered into 29 GO terms, whereas only 5 out of 13 differentially expressed genes identified from the phenotype comparison had fruit fly orthologs and these clustered into 18 GO terms (Table 3.9). There were no overlapping GO term clusters in the two adult comparisons of mite sensitivity phenotype and mite infestation.

Table 3.8 Enrichment analysis of GO terms in the mite infestation comparison and phenotype comparison at the pupal stage.

GO ID	Level	GO Term	R O	R F	D O	D F	P-value
Mite infestation comparison (Pupa)							
GO:0005214	4	structural constituent of chitin-based cuticle	115	0.0105	5	0.11	0.0001
GO:0042302	3	structural constituent of cuticle	120	0.011	5	0.11	0.0001
GO:0048067	5,4	cuticle pigmentation	7	0.0007	2	0.06	0.0002
GO:0005576	2	extracellular region	463	0.055	7	0.27	0.0003
GO:0009062	6,7,8	fatty acid catabolic process	13	0.0013	2	0.06	0.0009
GO:0016054	5	organic acid catabolic process	14	0.0014	2	0.06	0.0010
GO:0046395	6	carboxylic acid catabolic process	14	0.0014	2	0.06	0.0010
GO:0016339	5	calcium-dependent cell-cell adhesion	22	0.0022	2	0.06	0.0025
GO:0005887	8,7,6	integral to plasma membrane	198	0.0235	4	0.15	0.0027
GO:0031226	7,6,5	intrinsic to plasma membrane	201	0.0239	4	0.15	0.0028
GO:0007155	3	cell adhesion	180	0.0178	4	0.11	0.0029
GO:0044459	6,5,4	plasma membrane part	349	0.0415	5	0.19	0.0033
GO:0009450	7,6,9,10,11	gamma-aminobutyric acid catabolic process	1	0.0001	1	0.03	0.0035
GO:0046359	8,9,10	butyrate catabolic process	1	0.0001	1	0.03	0.0035
GO:0019626	7,8,9	short-chain fatty acid catabolic process	1	0.0001	1	0.03	0.0035
GO:0044242	5,6	cellular lipid catabolic process	27	0.0027	2	0.06	0.0038
GO:0022610	2	biological adhesion	194	0.0192	4	0.11	0.0038
GO:0004777	6	succinate-semialdehyde dehydrogenase activity	1	0.0001	1	0.02	0.0040
GO:0043874	7	acireductone synthase activity	1	0.0001	1	0.02	0.0040
GO:0004252	7,6	serine-type endopeptidase activity	279	0.0256	5	0.11	0.0042
GO:0007156	5	homophilic cell adhesion	30	0.003	2	0.06	0.0046
GO:0001726	5,4	ruffle	2	0.0002	1	0.04	0.0062
GO:0043102	6,5,7	amino acid salvage	2	0.0002	1	0.03	0.0069
GO:0019509	7,8,6,9	methionine salvage	2	0.0002	1	0.03	0.0069
GO:0008236	6,5	serine-type peptidase activity	319	0.0292	5	0.11	0.0072
GO:0017171	4	serine hydrolase activity	321	0.0294	5	0.11	0.0073
GO:0016042	4,5	lipid catabolic process	39	0.0039	2	0.06	0.0076
GO:0004523	9	ribonuclease H activity	2	0.0002	1	0.02	0.0080

GO:0005198	2	structural molecule activity	469	0.043	6	0.14	0.0083
GO:0005549	3	odorant binding	115	0.0105	3	0.07	0.0099
Phenotype comparison (Pupa)							
GO:0005214	4	structural constituent of chitin-based cuticle	115	0.0105	9	0.16	4.708E-09
GO:0042302	3	structural constituent of cuticle	120	0.011	9	0.16	6.853E-09
GO:0005198	2	structural molecule activity	469	0.043	14	0.25	4.565E-08
GO:0008236	6,5	serine-type peptidase activity	319	0.0292	10	0.18	3.16E-06
GO:0017171	4	serine hydrolase activity	321	0.0294	10	0.18	3.34E-06
GO:0042335	4	cuticle development	66	0.0065	5	0.12	7.73E-06
GO:0004252	7,6	serine-type endopeptidase activity	279	0.0256	9	0.16	8.20E-06
GO:0008010	5	structural constituent of chitin-based larval cuticle	43	0.0039	4	0.07	0.0001
GO:0005200	3	structural constituent of cytoskeleton	43	0.0039	4	0.07	0.0001
GO:0048067	5,4	cuticle pigmentation	7	0.0007	2	0.05	0.0004
GO:0045298	10,9,8,7,6,4,	tubulin complex	9	0.0011	2	0.07	0.0004
GO:0006508	7,6	proteolysis	709	0.0701	10	0.23	0.0005
GO:0030163	6,5	protein catabolic process	718	0.0709	10	0.23	0.0005
GO:0004175	6	endopeptidase activity	489	0.0448	9	0.16	0.0005
GO:0043285	5	biopolymer catabolic process	757	0.0748	10	0.23	0.0008
GO:0070011	5	peptidase activity, acting on L-amino acid peptides	645	0.0591	10	0.18	0.0009
GO:0009057	4	macromolecule catabolic process	801	0.0792	10	0.23	0.0012
GO:0008233	4	peptidase activity	667	0.0611	10	0.18	0.0012
GO:0043292	8,7,6,5	contractile fiber	19	0.0023	2	0.07	0.0017
GO:0005576	2	extracellular region	463	0.055	6	0.21	0.0030
GO:0016203	7,8,3	muscle attachment	20	0.002	2	0.05	0.0031
GO:0030421	6,7,5	defecation	1	0.0001	1	0.02	0.0042
GO:0007588	5,6,4	excretion	1	0.0001	1	0.02	0.0042
GO:0035017	5,6	cuticle pattern formation	25	0.0025	2	0.05	0.0048
GO:0009056	3	catabolic process	991	0.0979	10	0.23	0.0051
GO:0060538	6,7	skeletal muscle organ development	88	0.0087	3	0.07	0.0056
GO:0001726	5,4	ruffle	2	0.0002	1	0.04	0.0066
GO:0051258	7,6	protein polymerization	30	0.003	2	0.05	0.0068
GO:0006723	5,4	cuticle hydrocarbon biosynthetic process	2	0.0002	1	0.02	0.0085

GO:0048856	3	anatomical structure development	2013	0.1989	15	0.35	0.0091
GO:0007018	4	microtubule-based movement	106	0.0105	3	0.07	0.0091

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. Enrichment analysis was tested by GOToolBox through a hypergeometric test followed by the Benjamini Hochberg FDR correction. RO: The number of genes annotated for this term in the reference set. RF: The frequency of genes annotated for this term in the reference set. DO: The number of differentially expressed genes annotated for this term. DF: The frequency of differentially expressed genes annotated for this term. The GO ID is bolded if the GO term is commonly found in the mite infestation comparison and the phenotype comparison.

Table 3.9 Enrichment analysis of the GO terms at the adult stage.

GO ID	Level	GO Term	RO	R F	DO	D F	P-value
Mite infestation comparison (Adult)							
GO:0015645	5	fatty-acid ligase activity	8	0.0007	3	0.14	3.406E-07
GO:0004467	6	long-chain-fatty-acid-CoA ligase activity	8	0.0007	3	0.14	3.406E-07
GO:0016877	4	ligase activity, forming carbon-sulfur bonds	22	0.002	3	0.14	9.15E-06
GO:0006631	5,7,6	fatty acid metabolic process	49	0.0048	3	0.17	0.0001
GO:0032787	6	monocarboxylic acid metabolic process	75	0.0074	3	0.17	0.0003
GO:0006082	4	organic acid metabolic process	268	0.0265	4	0.22	0.0010
GO:0019752	5	carboxylic acid metabolic process	268	0.0265	4	0.22	0.0010
GO:0005329	7	dopamine transmembrane transporter activity	1	0.0001	1	0.05	0.0019
GO:0019811	4	cocaine binding	1	0.0001	1	0.05	0.0019
GO:0003868	6	4-hydroxyphenylpyruvate dioxygenase activity	1	0.0001	1	0.05	0.0019
GO:0005330	5,8,10	dopamine:sodium symporter activity	1	0.0001	1	0.05	0.0019
GO:0016942	5,4,3	insulin-like growth factor binding protein complex	2	0.0002	1	0.08	0.0028
GO:0051937	6,7	catecholamine transport	2	0.0002	1	0.06	0.0036
GO:0006723	5,4	cuticle hydrocarbon biosynthetic process	2	0.0002	1	0.06	0.0036
GO:0015872	7,8	dopamine transport	2	0.0002	1	0.06	0.0036
GO:0001676	6,8,7	long-chain fatty acid metabolic process	2	0.0002	1	0.06	0.0036
GO:0008471	6	laccase activity	2	0.0002	1	0.05	0.0038
GO:0005520	5	insulin-like growth factor binding	2	0.0002	1	0.05	0.0038
GO:0044255	4,5	cellular lipid metabolic process	198	0.0196	3	0.17	0.0045
GO:0009408	5,4	response to heat	60	0.0059	2	0.11	0.0048
GO:0015844	5,6	monoamine transport	3	0.0003	1	0.06	0.0053
GO:0003824	2	catalytic activity	4172	0.3821	14	0.67	0.0056
GO:0016401	7	palmitoyl-CoA oxidase activity	3	0.0003	1	0.05	0.0057
GO:0016682	5	oxidoreductase activity, acting on diphenols and	3	0.0003	1	0.05	0.0057
GO:0008504	6	monoamine transmembrane transporter activity	3	0.0003	1	0.05	0.0057
GO:0009266	4	response to temperature stimulus	66	0.0065	2	0.11	0.0058
GO:0016491	3	oxidoreductase activity	670	0.0614	5	0.24	0.0064
GO:0042811	5	pheromone biosynthetic process	4	0.0004	1	0.06	0.0071

GO:0016874	3	ligase activity	239	0.0219	3	0.14	0.0093
Phenotype comparison (Adult)							
GO:0006333	6	chromatin assembly or disassembly	169	0.0167	2	0.67	0.0008
GO:0016922	5	ligand-dependent nuclear receptor binding	4	0.0004	1	0.33	0.0011
GO:0035076	9,8,5,7,6	ecdysone receptor-mediated signaling pathway	6	0.0006	1	0.33	0.0018
GO:0031010	14,13,12,11,10,9,8,5,7	ISWI complex	5	0.0006	1	0.33	0.0018
GO:0016589	15,14,13,12,11,10,9,6,8	NURF complex	5	0.0006	1	0.33	0.0018
GO:0006325	5	establishment or maintenance of chromatin	260	0.0257	2	0.67	0.0019
GO:0030522	7,6	intracellular receptor-mediated signaling pathway	7	0.0007	1	0.33	0.0021
GO:0030518	8,7	steroid hormone receptor signaling pathway	7	0.0007	1	0.33	0.0021
GO:0042766	6,8,7,5	nucleosome mobilization	7	0.0007	1	0.33	0.0021
GO:0035073	7,6,3	pupariation	8	0.0008	1	0.33	0.0024
GO:0035210	6,5	prepupal development	9	0.0009	1	0.33	0.0027
GO:0051276	4	chromosome organization	400	0.0395	2	0.67	0.0045
GO:0030431	3,4	sleep	19	0.0019	1	0.33	0.0056
GO:0035075	6,5	response to ecdysone	23	0.0023	1	0.33	0.0068
GO:0048545	5	response to steroid hormone stimulus	23	0.0023	1	0.33	0.0068
GO:0016246	11,10,6,9	RNA interference	26	0.0026	1	0.33	0.0077
GO:0032870	4,5	cellular response to hormone stimulus	27	0.0027	1	0.33	0.0080
GO:0006334	5,7,6,8	nucleosome assembly	29	0.0029	1	0.33	0.0085

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. Enrichment analysis was tested by GOToolBox through a hypergeometric test followed by the Benjamini Hochberg FDR correction. RO: Number of genes annotated for this term in the reference set. RF: Frequency of genes annotated for this term in the reference set. DO: Number of differentially expressed genes annotated for this term. DF: Frequency of differentially expressed genes annotated for this term. The GO ID is bolded if the GO term is commonly found in the mite infestation comparison and the phenotype comparison.

3.4.8 Deep analysis of functionally grouped genes

3.4.8.1 Olfaction

The chemical interactions in honey bee colonies occur in a complex environment (Carroll and Duehl, 2012). This interaction can be initiated by the action of chemosensory and odorant binding proteins. At the pupal stage, three genes (*GB30365, GB11092* and *NM_001040227*) encoding odorant binding proteins and one gene *GB19453* encoding chemosensory protein showed differential expression in the phenotype comparison with mite infestation ($S88^+/G4^+$), while without the varroa infestation, these genes did not show significantly different expression ($S88^-/G4^-$) (Table 3.10). This is in accordance with a previous study which demonstrated that honey bee could detect varroa-emitted odor by odorant-binding proteins (Schoning *et al.*, 2012). Interestingly, all the genes encoding odorant binding proteins and chemosensory protein were significantly down-regulated in $S88^+$ relative to $G4^+$. Our previous observations indicated that there were more mites present in cells of the susceptible line G4 (Table 3.3) and consequently a higher odor concentration in the susceptible brood cell would induce the higher expression of odor-binding protein genes. This result might imply that honey bees, regardless of their phenotype, are sensitive to the odor emitted by varroa mites.

In the mite infestation comparison at the pupal stage, of five olfactory genes which were differentially expressed, *GB30365* was up regulated in $G4^+$ relative to $G4^-$, while *GB11904*, *GB14248* and *GB13325* encoding putative odorant receptor 13a and chemosensory protein 6 were up-regulated in $S88^+$ relative to $S88^-$. This data confirmed that the bees were particularly sensitive to odor stimuli emitted by the mite, and in presence of the mite, olfactory genes were highly expressed regardless of the phenotypic response to mite infestation.

Table3.10 Differentially expressed genes related to olfaction.

Phenotype comparison (Pupa)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
NM_001040227	–	0.42	Odorant binding protein 18
GB11092	–	0.32	Odorant binding protein 17
GB30365	–	0.41	Odorant binding protein 14
GB19453	–	0.38	Chemosensory protein 2
Mite infestation comparison (Pupa)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB30365	2.21	–	Odorant binding protein 14
GB11904	–	4.32	Putative odorant receptor 13a
GB14248	–	2.23	Putative odorant receptor 13a
GB13325	–	2.10	Chemosensory protein 6
GB11092	–	0.39	Odorant binding protein 17
Phenotype comparison (Adult)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
GB16826	–	0.47	Odorant binding protein 16 precursor
GB30242	–	2.23	Odorant binding protein 3 precursor
Mite infestation comparison (Adult)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB10729	–	2.33	Putative odorant receptor 85b

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$). The number in the table represents the fold change in each comparison.

At the adult stage, three olfactory genes were differentially expressed in the phenotype and mite infestation comparisons. *GB10729* encoding a putative odorant receptor 85b was up regulated in the mite infestation comparison of $S88^+$ versus $S88^-$, which confirmed the result at the pupal stage that in presence of the mite, the olfactory genes were higher expressed in both phenotypes. However, while *GB16826* was down-regulated in $S88^+$ relative to $G4^+$, *GB30242* was up-regulated in $S88^+$ compared with $G4^+$. This observation indicates *GB16826* and *GB30242* may be important for differentiating the two phenotypes in terms of olfactory responses to the mite odor stimuli.

3.4.8.2 Signal transduction

Another set of differentially expressed genes identified by DNA microarray analysis are involved in signal transduction. According to the olfactory networking system, odor stimuli could induce a complex spatio-temporal activity within the honey bee brain (Figure 3.7).

In the pupa mite infestation comparisons, the signal-transduction related genes in the G4 susceptible line did not show any difference in expression, however, four of these genes were differentially expressed in the tolerant S88 line (Table 3.11). The gene *GB17254* encoding neuronal nicotinic acetylcholine receptor (nAChR) Apisα7-2, was expressed five times higher in $S88^+$ relative to $S88^-$. Previous immunocytochemical and electrophysiological studies have shown that the olfactory pathway in the insect brain is mainly cholinergic, the mushroom bodies receive cholinergic input from the antennal lobe for olfactory learning and memory formation (Kreissl and Bicker, 1989), implying that this gene might play an important role in transducing signals during bee sensory perception of the mite infestation. In the same comparison, the other three proteins, cadherin-87A (*GB17702*), neural-cadherin (*GB12853*) and neurogenic protein big brain (*GB12287*), were also highly expressed in $S88^+$. Cadherins constitute a family of multi-domain membrane glycoproteins which mediate initial calcium-dependent cell adhesion. Neural-cadherin, named for its initial identification in neural tissues, affects neural development and cell adhesion. The neurogenic protein big brain is also involved in the biological process of cell adhesion (Tatsumi *et al.*, 2009). Cell adhesion has been shown to play critical roles in

cytoskeletal reorganization and activation of multiple signal transduction pathways that influence cell survival, growth and differentiation (Parsons *et al.*, 2010).

Differential expression of neurological signal-transduction related genes coincides with that of the olfactory genes in the $S88^{+}$ versus $S88^{-}$ comparison at the dark-eyed pupal stage. This finding might imply that the tolerant pupa have a higher capacity to detect the varroa mite and relay the information through subsequent neural cascade processes.

In the pupa phenotype comparison with the mite, cadherin-87A (*GB17702*) was up regulated in the tolerant line S88 relative to G4. This gene might be important to differentiate the neurological signal transduction process in these two phenotypes following mite infestation.

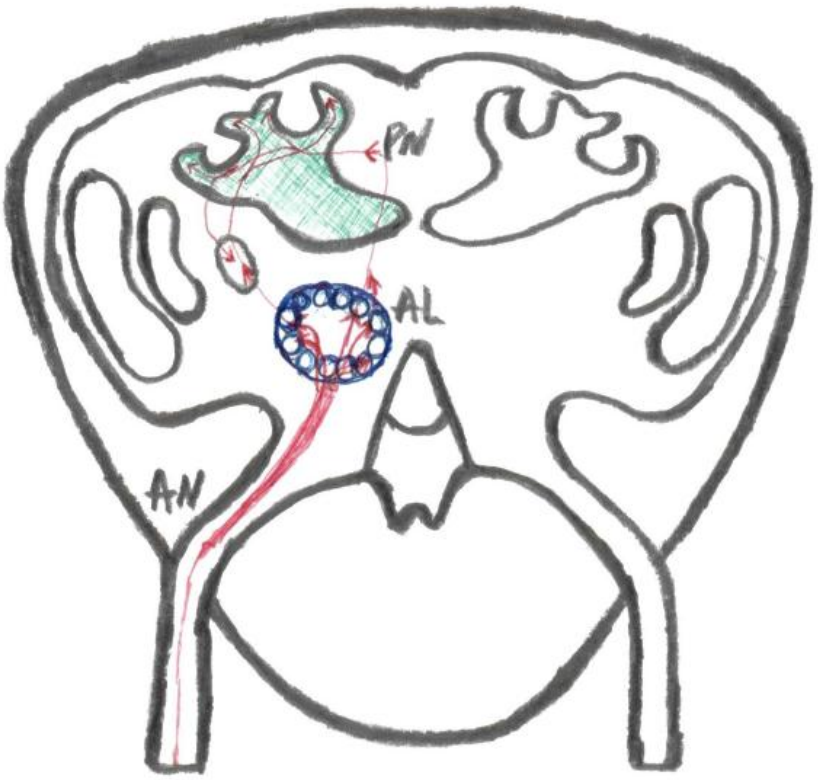


Figure 3.7 Schematic representation of the honey bee brain highlighting the olfactory network.

Odor molecules are received by olfactory receptor neurons (ORNs) in sensory epithelia of antenna, and olfactory information enters primary olfactory centers, the antennal lobe (AL, blue) in the brain via the antennal nerve (AN, red) where the information is processed and relayed to projection neurons (PN, red), finally to the lateral protocerebral lobe and the mushroom bodies (green). Abbreviations: AL, antennal lobe; AN, antennal nerve; PN, projection neuron. (Drawn by Sanjie Jiang).

Table 3.11 Differentially expressed genes related to signal transduction.

Phenotype comparison (Pupa)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
GB17702	–	2.40	Cadherin-87A
Mite infestation comparison (Pupa)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB17254	–	4.87	Neuronal nAChR Apisα7-2 subunit
GB12287	–	2.31	Neurogenic protein big brain
GB17702	–	2.12	Cadherin-87A
GB12853	–	2.11	Neural-cadherin
Mite infestation comparison (Adult)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB14823	–	0.35	Neurotrimin

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$). The number in the table represents the fold change in each comparison.

3.4.8.3 Exoskeleton formation

The insect exoskeleton is the outer physical structure surrounding an insect body, providing protection for the insect. It is also a site for deposition of exocrine gland secretions, thereby mediating chemical communication for mating, defence and kin recognition (Figure 3.8).

In the pupa phenotype comparison with mite infestation, seven genes encoding the cuticle protein and apidermin were identified that were more highly expressed in the susceptible G4 line relative to the tolerant S88 line (Table 3.12). Two of these genes, *GB19234* and *GB14193* encoding tweedle motif cuticular protein 1 and tweedle motif cuticular protein 2, respectively, were up-regulated in $G4^+$ relative to $S88^+$. Interestingly, tweedle motif cuticular protein 2 gene was also up-regulated in the $G4^+$ versus $G4^-$ comparison. In fruit fly, tweedle proteins are expressed in the epidermis and have an important effect on body shape. However, over-expression of these proteins would result in a squat body shape and reduction of the length/width ratio (Guan *et al.*, 2006). Therefore, a significantly increased tweedle protein production in the susceptible G4 line following mite infestation could result in a deformed exoskeleton. In addition to the tweedle protein genes, two genes, *GB15203* encoding larval cuticle protein A3A and *GB12600* encoding another cuticle protein, were up-regulated in $G4^+$ relative to $S88^+$. Similarly, *GB30337* encoding endocuticle structural glycoprotein SgAbd-2, one of the two parts that constitute procuticle, was more highly expressed in $G4^-$ relative to $S88^-$, while the same gene was differentially expressed in the $S88^+/S88^-$ comparison. Presumably, over-expression of this gene in the susceptible bee line, similar to that of tweedle protein genes, would have negative effects on the optimal formation of exoskeleton. The mite may exploit the differences in the cuticular composition of its host for a refined selection that allows it to reach a brood cell and start reproduction (Del Piccolo *et al.*, 2010). Over-expression of cuticular genes in the susceptible G4 line may make the bees more attractive to the varroa.

Another important finding relevant to exoskeleton formation was the differential expression of the apidermin gene family. *GB30202* and *GB30203* encoding apidermin 1 and apidermin 3 showed lower expression in $S88^+$ relative to $G4^+$, as well as in $S88^+$ relative to $S88^-$, at the dark-eyed pupal stage. However, the expression of the apidermin 1 gene was up-regulated in $S88^-$

relative to $G4^-$ at the pupal stage. It appeared that the expression pattern of this family of genes was complex. Increased expression of apidermin genes after the mite parasitism may be required for repairing the epidermis damage made by the mite attack. Wounding by the mites may induce higher expression of apidermin genes for repairing cuticular damage. In addition, repairing damaged exoskeleton may also involve many other genes for the synthesis of new cuticle and epidermis proteins. This assumption is consistent with previous studies showing that insects can prevent haemolymph loss by mobilizing wound healing proteins (Theopold *et al.*, 2002).

It is noteworthy that the expression of two epidermis genes *GB30202* and *GB30203* in $S88^+$ was more than seven times higher than in $S88^-$ at the adult stage, which was exactly opposite in the same comparison at the pupal stage. This indicates that the expression of these two genes in the tolerant line changes with the developmental stage. This might indicate possibly different roles for these genes in exoskeleton formation at the different developmental stages.

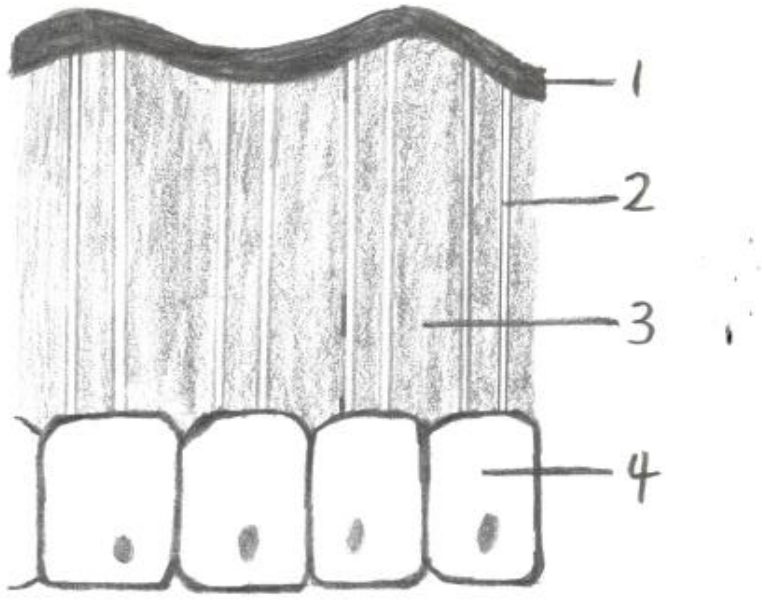


Figure 3.8 A diagram illustrating the main features of the integument of an insect.
The bee exoskeleton is made up of two physical layers: the single epidermis layer of living cells that secrete chitin and proteins as well as other components, and the cuticle layer with two highly organized sub-layers, an outer thin epicuticle rich in lipids and proteins, and the inner thick procuticle consisting of proteins and chitin. 1.epicuticle, 2.pore canal, 3.procuticle, 4.epidermis. (Drawn by Sanjie Jiang).

Table 3.12 Differentially expressed genes related to exoskeleton formation.

Phenotype comparison (Pupa)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
GB30337	0.42	–	Endocuticle structural glycoprotein SgAbd-2
GB15203	–	0.18	Larval cuticle protein A3A
GB12600	–	0.14	Cuticle protein
GB19234	–	0.33	Tweedle motif cuticular protein 1
GB14193	–	0.40	Tweedle motif cuticular protein 2
GB30202	–	0.31	Apidermin 1
GB30203	2.20	0.33	Apidermin 3
Mite infestation comparison (Pupa)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB14193	2.01	–	Tweedle motif cuticular protein 2
GB12600	2.74	0.31	Cuticle protein
GB12636	–	3.06	Apidermin 2
GB30337	–	2.02	Endocuticle structural glycoprotein SgAbd-2
GB30202	–	0.36	Apidermin 1
GB30203	–	0.23	Apidermin 3
Mite infestation comparison (Adult)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB30202	–	7.45	Apidermin 1
GB30203	–	12.64	Apidermin 3

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$). The number in the table represents the fold change in each comparison.

3.8.4.4 Detoxification process

The varroa mites feed on haemolymph of both pupal and adult bees (Rosenkranz *et al.*, 2010). Salivary gland secretions of the varroa mite inhibit honey bee heamocytes from extending pseudopods, causing their rupture and aggregation (Richards *et al.*, 2011). Therefore, secretions synthesized in the mite salivary gland contain compounds that are detrimental to the honey bee. After introduction into the bee heamoymph during exoskeleton penetration, these salivary toxicants can inhibit the host defense system to maintain the activities (Richards *et al.*, 2011). Honey bee colonies harbor a complex mixture of volatile compounds produced by different bee castes and hive materials (Trhlin and Rajchard, 2011), as well as chemical scents from the varroa mites (Schoning *et al.*, 2012). Toxic volatile compounds emitted by the mite can be detected by the honey bees. In addition, the mite also acts as a transmission vector for bacterial, fungal and viral pathogens within and among colonies (Davidson *et al.*, 2003; Kanbar and Engels, 2003; Tsagou *et al.*, 2004). These factors may work synergistically to threaten honey bee health.

Detoxification is a physiochemical process which removes toxic substances from honey bee cells. This process is divided into three phases: modification, conjugation and excretion (Xu *et al.*, 2005). Phase I is to use cytochrome P450, esterase or other enzymes to modify the toxicants (oxidation, reduction, hydrolysis and/or hydration). Generally it is the first defensive biochemical activity that can inactivate foreign compounds (Iyanagi, 2007). Phase II involves conjugation reactions which transform toxicants into water-soluble compounds through glucuronidation and sulfation, as well as glutathione and amino acid ligation (Trinh *et al.*, 2008). In phase III, a variety of membrane transporters of the multidrug resistance protein family (MDR) transport conjugated toxicants to the extracellular medium where they are further metabolized or excreted (Suzuki *et al.*, 2001).

3.4.8.4.1 Cytochrome P450

The cytochrome P450 superfamily (abbreviated as CYP) constitutes a large and diverse group of enzymes, which are widespread in vertebrates, invertebrates and plants. Cytochrome P450s have diverse functions involving lipid metabolism, sensory perception, and biosynthesis of juvenile

hormone (Baldwin *et al.*, 2009). They can catalyze xenobiotic compounds by oxidizing exotic organic substances, thus are considered an essential component of the detoxification system.

Similar to the genes related to signal transduction, differentially expressed genes encoding cytochrome P450 displayed complex expression patterns associated with the bee phenotypes at both pupal and adult stages (Table 3.13). Of five differentially expressed cytochrome P450 genes, *GB19306* encoding cytochrome P450-9E2, and *GB19306* and *GB14612* encoding P450-6K1 in $S88^+$ at the adult stage, and *GB11754* encoding P450-6A14 in $S88^+$ at the pupal stage were down-regulated relative to $S88^-$. However, *GB12136* encoding cytochrome P450-6A1 was up-regulated in both the phenotype comparison with mite infestation ($S88^+/G4^+$) and mite infestation comparison of the tolerant $S88^+/S88^-$ line at the pupal stage. The P450-6A1 gene showed a 4 fold difference in transcript abundance in the $S88^+/G4^+$ comparison, and a 6 fold difference in the $S88^+/S88^-$ comparisons at the pupal stage. On the other hand, the P450-6A14 gene was down-regulated in $S88^+$ relative to both $G4^+$ and $S88^-$ at the pupal stage. The distinct expression patterns for cytochrome P450-6A14 and 6A1 at the pupal stage could be used to differentiate susceptible and tolerant bee phenotypes in response to varroa infestation.

Previous studies show that the CYP6 and CYP9 cytochrome P450 families in insects are responsible for pyrethroid resistance, an insecticide produced by the flowers of *Chrysanthemum cinerariaefolium* (Claudianos *et al.*, 2006). Honey bee CYP9Q1, CYP9Q2, and CYP9Q3 were demonstrated to be able to metabolize tau-fluvalinate to a form suitable for further cleavage by the carboxylesterases contributing the tolerance to tau-fluvalinate (Mao *et al.*, 2011). All five genes encoding P450s identified by our DNA microarray analysis were differentially expressed in the phenotype and mite infestation comparisons belong to these two families. In the pupa phenotype comparison, cytochrome P450-6A1 was highly expressed in the mite infested tolerant S88 phenotype relative to the mite infested susceptible G4 phenotype. Previous studies showed that CYP6A1 was highly expressed in insecticide-tolerant strains of house flies and that purified recombinant CYP6A1 was able to detoxify diazinon with a high efficiency (Carino *et al.*, 1994). Thus, this gene might be important in host tolerance to both mites and insecticides. It may be an effective biomarker for breeding bees with a varroa tolerant trait.

3.4.8.4.2 Esterase

Esterase hydrolyzes ester compounds by cleaving ester bonds thereby splitting esters into acids and alcohols. These enzymes work in Phase I detoxification processes (Iyanagi, 2007). Interestingly, a gene *GB16889* encoding esterase E4 was identified that was differentially expressed in three comparisons, indicating this gene may play an important role in mite tolerant bee's response to varroa mite infestation (Table 3.14). In the phenotype comparison with mite infestation at the pupal stage, the expression of this gene was three times higher in the tolerant S88 line than in the susceptible G4 line. In the mite infestation comparison at the pupal stage, expression of this gene in $S88^{+}$ was approximately 4 fold higher than in $S88^{-}$. On the other hand, in the mite infestation comparison at the adult stage, the expression of esterase E4 gene was down-regulated in $S88^{+}$ relative to $S88^{-}$.

As described above, the varroa mites attack their host by cutting the exoskeleton, secreting toxic compounds into the host body and sucking heamolymph from the bee. The toxic substances comprise a complex group of bioactive compounds that include various organic esters. The higher expression of the esterase E4 gene in $S88^{+}$ relative to $G4^{+}$ as well as in $S88^{+}$ relative to $S88^{-}$ would provide the tolerant bees with an increased capacity to cope with the toxic esters. Increased esterase activity in tolerant bees may also be beneficial in dealing with the toxic side effects of miticides used to treat honey bee colonies for mite infestations. With the extensive distribution of mite infestation in North American bees, insecticide application for controlling mites has become a general practice in the apiculture industry. Esterase might be able to help hydrolyze the insecticide compounds and reduce the harm the insecticide causes in bees, as this esterase belongs to the carboxyl/cholinesterase family that have structural and functional diversity with broad substrate specificities (Oakeshott *et al.*, 1999). Indeed, the up-regulation of the esterase gene was previously found to be associated with insecticide resistance in a variety of insects. For example, insecticide resistance in aphids could result from the increased synthesis of esterase E4 that hydrolyses insecticidal esters (Devonshire and Moores, 1982).

Table 3.13 Differentially expressed genes encoding cytochrome P450s.

Phenotype comparison (Pupa)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
GB11754	–	0.31	Cytochrome P450-6A14
GB12136	–	4.08	Cytochrome P450-6A1
Mite infestation comparison (Pupa)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB11754	–	0.34	Cytochrome P450-6A14
GB12136	–	6.58	Cytochrome P450-6A1
Mite infestation comparison (Adult)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB19967	–	0.46	Cytochrome P450-9E2
GB19306	–	0.45	Cytochrome P450-6K1
GB14612	–	0.48	Cytochrome P450-6K1

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$). The number in the table represents the fold change in each comparison.

Table 3.14 Differentially expressed genes encoding esterase.

Phenotype comparison (Pupa)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
GB16889	–	3.41	Esterase E4
Mite infestation comparison (Pupa)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB16889	–	3.92	Esterase E4
Mite infestation comparison (Adult)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB16889	–	0.47	Esterase E4

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$). The number in the table represents the fold change in each comparison.

3.4.8.5 Metabolism

Parasite-induced responses in insects are dependent on the nutritional status of the host (Schneider and Ayres, 2008). In honey bees, nutritional status has a critical influence on the expression of genes affecting production of defensive compounds (Alaux *et al.*, 2011). The varroa mite feeds on the hemolymph of pupal and adult bees, resulting in loss of nutrients and circulatory fluids (Sammataro *et al.*, 2000), leading to severe disease (Martin, 2001; Duay *et al.*, 2003). In addition, varroa parasitism also disturbs the host's overall protein and lipid metabolism (Alaux *et al.*, 2011). This has a profound effort on the health and longevity of infected bees (Yang and Cox-Foster, 2007). Nevertheless, the molecular mechanisms underlying the effect of varroa parasitism on the host metabolic process has yet to be defined.

3.4.8.5.1 Protein metabolism

The effect of varroa mite infestation on host protein metabolism is complex. Previous studies showed that the varroa-infested pupa had significantly elevated free amino acid content and decreased protein content, suggesting that protein synthesis is inhibited or protein catabolism is increased in the infested bees (Aronstein *et al.*, 2012). The inhibition of protein synthesis resulted in a detrimental effect on bee health (Alaux *et al.*, 2011).

A total of eight differentially expressed genes involved in protein catabolism were identified in the two comparisons at the pupal stage (Table 3.15). No differentially expressed genes were detected at the adult stage. In the pupa phenotype comparison without mite infestation, three genes (*GB30379*, *GB17927* and *GB30378*) encoding serine proteases were expressed at lower level in the tolerant versus the susceptible phenotype, and one gene (*GB15018*) encoding a chymotrypsin inhibitor was more highly expressed in the tolerant versus susceptible phenotype. Chymotrypsin is a type of serine protease, and increased expression of chymotrypsin inhibitor would slow protein catabolism in the host cells of the tolerant phenotype. These results suggest that the varroa susceptible phenotypes may have a higher rate of protein catabolism when compared to the tolerant line. A high rate of protein catabolism could result in high levels of free amino acids in the susceptible bees. This is consistent with previous studies showing that the

protein density was decreased in varroa-infested pupa (Aronstein *et al.*, 2012) and newly-emerged adult bees (Bowen-Walker and Gunn, 2001).

In the phenotype comparison with varroa mite infestation, the expression patterns were more complex (Table 3.15). Two genes (*GB11273* encoding Retinoid-inducible serine carboxypeptidase and *GB18450* encoding transmembrane protease serine 6) were down-regulated, and two genes (*GB10646* encoding Trypsin-7 and *GB13489* encoding serine protease 34) were up-regulated in the tolerant relative to the susceptible phenotype. Interestingly, the same gene *GB10646* was also down-regulated in the $G4^{+}$ relative to $G4^{-}$, while *GB13489* was also up-regulated in $S88^{+}$ relative to $S88^{-}$ at the pupal stage. Increased levels of expression of two serine proteases, trypsin 7 and serine protease 34 in the tolerant phenotype $S88^{+}$ might provide protection for these bees from the toxic proteins transmitted by varroa mite saliva during initial infestation.

Table 3.15 Differentially expressed genes related to protein metabolism.

Phenotype comparison (Pupa)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
GB30379	0.29	–	Serine protease
GB17927	0.33	–	Serine protease
GB30378	0.40	–	Serine protease
GB11273	–	0.41	Retinoid-inducible serine carboxypeptidase
GB18450	–	0.44	Transmembrane protease serine 6
GB10646	–	3.12	Trypsin-7
GB13489	–	4.88	Serine protease 34
GB15018	2.11	–	Chymotrypsin inhibitor
Mite infestation comparison (Pupa)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB10646	0.48	–	Trypsin-7
GB13489	–	2.96	Serine protease 34

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$). The number in the table represents the fold change in each comparison.

3.4.8.5.2 Lipid metabolism

Lipids, a group of hydrophobic compounds in cells, serve as carbon storage, signalling molecules, and components of cellular membranes. Maintenance of the homeostasis of lipid metabolism is imperative for bee health. However, few studies have looked at the effect of varroa parasitism on lipid metabolism in the honey bee.

DNA microarray analysis identified a set of genes involved in lipid metabolism which were differentially expressed in varroa susceptible and tolerant colony phenotypes (Table 3.16). A phenotype comparison with mite infestation revealed expression of *GB11723* encoding apolipoprotein D, and *GB18070*, encoding delta-11 acyl-CoA desaturase were approximately seven and two times higher in the tolerant versus susceptible pupa, respectively. Conversely, expression of *GB13246* encoding phospholipase A1 was about two times lower in the tolerant versus susceptible pupa. Apolipoproteins are a special type of proteins which bind lipids, forming lipoproteins. In animals, lipoproteins are used to transport neutral lipids throughout the lymphatic and blood circulatory systems. Apolipoprotein D participates in the formation of high-density lipoprotein particles. Apolipoprotein D transcripts were also increased by 2.6 fold in $S88^{+}$ relative to $S88^{-}$ (Table 3.16). Therefore, increased levels of apolipoprotein D expression in the tolerant S88 phenotype may enhance lipid metabolism, and possibly protect bees from detrimental effects associated with varroa infestation.

Phospholipase A1 is a phospholipase enzyme which removes fatty acids from the sn-1 position of cell membrane phospholipids. Increased expression of phospholipase A1 may affect membrane stability and integrity in the varroa susceptible G4 phenotype after mite infestation. Delta 11 acyl-CoA desaturase introduces a double bond into the 11th position of long-chain acyl-CoA producing Δ11 unsaturated fatty acid. Unsaturated fatty acids are essential components of membrane lipids and serve as signal molecules in response to the environmental changes. The increased expression of this desaturase gene in the tolerant, $S88^{+}$ phenotype suggests the desaturase may play a role in promoting membrane dynamics and fluidity following varroa mite infestation.

One differentially expressed gene *GB30529* encoding peroxisomal acyl-CoA oxidase 1 was identified in two mite infestation comparisons, $S88^{+}/S88^{-}$ at the pupal stage and $G4^{+}/G4^{-}$ at the adult stage. Peroxisomal acyl-CoA oxidase catalyzes the reaction from acyl-CoA to 2-trans-enoyl-CoA, in the β-oxidation of very long chain fatty acids in peroxisome. Increased expression of peroxisomal acyl-CoA oxidase in the two mite infestation comparisons suggests that regardless the honey bee phenotype, mite infestation may cause enhanced oxidation of very long chain fatty acids.

In the adult mite infestation comparison of the tolerant line, six genes (*GB11969*, *GB17931*, *GB12567*, *GB12176*, *GB13264*, and *GB19070*) related to the metabolism of long chain fatty acid were differentially expressed. Among them, three genes (*GB12176*, *GB13264* and *GB19070*) encode fatty acid elongases that are involved in the elongation of long chain fatty acids (Table 3.17). *GB17931* codes for fatty acyl-CoA reductase that catalyzes the reaction of acyl-CoA to fatty alcohol which is a precursor for wax ester, a component of cuticles. *GB12567* encodes long chain fatty acyl-CoA ligase that catalyzes ligation of free fatty acid to CoA. All these genes were down-regulated in the tolerant phenotype following mite infestation, but not in the counterpart comparison of the susceptible line G4. This observation indicates the biosynthesis of long chain fatty acids may be inhibited in the tolerant line as a result of the mite attack.

Table 3.16 Differentially expressed genes related to lipid metabolism.

Phenotype comparison (Pupa)			
Gene	**$S88^-/G4^-$**	**$S88^+/G4^+$**	**Honey bee protein**
GB11723	–	6.88	Apolipoprotein D
GB18070	–	2.23	Delta 11 acyl-CoA desaturase
GB13246	–	0.47	Phospholipase A1
Mite infestation comparison (Pupa)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB11723	0.44	2.58	Apolipoprotein D
GB30529	–	3.04	Peroxisomal acyl-coenzyme A oxidase 1
GB11256	–	2.97	Pancreatic lipase 2
Mite infestation comparison (Adult)			
Gene	**$G4^+/G4^-$**	**$S88^+/S88^-$**	**Honey bee protein**
GB30529	2.71	–	Peroxisomal acyl-CoA oxidase 1
GB11969	–	0.34	Delta 11 acyl-CoA desaturase
GB17931	–	0.35	Fatty acyl-CoA reductase 1
GB12176	–	0.29	Elongation protein
GB13264	–	0.50	Elongation protein
GB19070	–	0.45	Elongation protein
GB12567	–	0.46	Long-chain fatty-acid-CoA ligase

Note: The mite infestation comparison: comparison between presence (+) and absence (-) of the varroa mites. The phenotype comparison: comparison between tolerant (S88) and susceptible (G4) colony phenotypes. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$). The number in the table represents the fold change in each comparison.

Table 3.17 Catalytic reactions associated with lipid metabolism for genes differentially expressed in adult bees.

Gene	Enzyme	Reaction
GB17931	Fatty acyl-CoA reductase	Long-chain acyl-CoA + NADPH + H^+ ⇌ Long-chain alcohol + CoA + $NADP^+$
GB12567	Long chain fatty acid-CoA ligase	Long-chain carboxylate + CoA+ ATP ⇌ Long-chain acyl-CoA +AMP + Diphosphate
GB11969	Delta 11 acyl-CoA desaturase	Long-chain acyl-CoA + Reduced acceptor + O_2 =Delta11-acyl-CoA + Acceptor + 2 H_2O
GB12176 *GB13264* *GB19070*	Elongase	Long-chain acyl-CoA+ CoA ⇌ Long-chain 3-oxoacyl-CoA
GB30529	Peroxisomal acyl-CoA oxidase	Long-chain acyl-CoA = 2-trans-enoyl-CoA

Note: ⇌ represents the reaction is reversable. NADPH or $NADP^+$: Nicotinamide adenine dinucleotide phosphate. CoA: Coenzyme A.

3.5 Discussion

V. destructor sucks the blood from both the adult bee and the developing brood, which severely harms honey bees and transmits diseases among bees. Based on a 10 year selective breeding program, two phenotypes of honey bees (*Apis mellifera*) were identified. The mite-sensitive G4 line and the mite-tolerant S88 line, were selected for the DNA microarray analysis. This analysis identified a large number of genes that were differentially expressed when comparing these two honey bee phenotypes in the presence/absence of varroa infestation. Brain RNA abundance was used to distinguish gene expression in these two contrast phenotypic honey bee lines.

3.5.1 Comparisons between the tolerant and susceptible honey bee phenotypes

The phenotype comparisons for pupa revealed genes encoding the cuticle protein and apidermin protein were highly expressed in the susceptible G4 phenotype relative to the tolerant S88 phenotype. It was previously suggested that mites may exploit difference in the cuticular composition of its host, allowing it to reach a brood cell and start reproduction (Del Piccolo *et al.*, 2010). Higher expression of these cuticular genes in the susceptible phenotype G4 could result in a composition and structure of cuticles that is more attractive to the varroa mites.

At the pupal stage, three genes encoding odorant binding proteins and one gene encoding chemosensory protein show differential expression when comparing phenotypes with mite infestation ($S88^{+}/G4^{+}$). While without varroa infestation, these genes were not significantly different in expression. This result indicates that the tolerant line may be more responsive to mite odorant compounds. *GB12136* encoding cytochrome P450-6A1 was up-regulated in the phenotype comparison following mite infestation ($S88^{+}/G4^{+}$). In an insecticide-tolerant strain of house flies, CYP6A1 was highly expressed and purified recombinant CYP6A1 was able to detoxify the insecticide (Carino *et al.*, 1994). *GB16889* encoding esterase E4, an enzyme working in the detoxification process, was three times higher in the tolerant line S88 than in the susceptible line G4. High expression of these genes would provide the tolerant phenotype with an increased capacity to cope with toxic compounds emitted by the mite, or the toxic side effects of miticides used to treat honey bee colonies against the mite infestation.

Genes encoding serine proteases were more highly expressed in the susceptible G4 phenotype than in the tolerant S88 phenotype. This implies that the varroa susceptible phenotype has a higher rate of protein catabolism when compared with the tolerant line, and this may result in higher levels of free amino acids. On the other hand, genes encoding Apolipoprotein D and delta-11 acyl-CoA desaturase were approximately seven and two times higher in the tolerant versus the susceptible phenotype. The increased expression of these genes in the tolerant $S88^{+}$ phenotype may equip bees with high energy for fighting the mite.

3.5.2 Comparisons between the presence and absence of the varroa mite

In the pupa mite infestation comparison, out of five olfactory genes that are differentially expressed, *GB30365* was up-regulated in $G4^{+}$ relative to $G4^{-}$, while *GB11904*, *GB14248* and *GB13325* encoding putative odorant receptor 13a and chemosensory protein 6 were up-regulated in $S88^{+}$ relative to $S88^{-}$. This is in agreement with the notion that regardless of the phenotype, the odor associated with the mite would induce the expression of these olfactory genes.

GB17254 encoding neuronal nicotinic acetylcholine receptor (nAChR) Apisα7-2 shows 5 times higher expression in $S88^{+}$ relative to $S88^{-}$. The olfactory regulatory process in the insect brain is mainly cholinergic (Kreissl and Bicker, 1989). The high expression of this gene would promote an olfactory learning and memory in response to the mite odor in the tolerant bees. In the same comparison, cadherin-87A (*GB17702*), neural-cadherin (*GB12853*) and neurogenic protein big brain (*GB12287*), are also highly expressed in $S88^{+}$ relative to $S88^{-}$. High expression of these cell adhesion genes would be beneficial for healthy cell growth and differentiation in the tolerant line (Parsons *et al.*, 2010).

Of five differentially expressed cytochrome P450 genes, *GB19306* encoding cytochrome P450-9E2, and *GB14612* and *GB14612* encoding P450-6K1 in $S88^{+}$ at the adult stage, and *GB11754* encoding P450-6A14 in $S88^{+}$ at the pupal stage were down-regulated relative to $S88^{-}$. However, at the pupal stage *GB12136* encoding cytochrome P450-6A1 was up-regulated in $S88^{+}$ relative to $S88^{-}$. Interestingly, *GB16889* encoding esterase E4 showed a similar expression pattern as

GB12136 at the pupal stage where expression of the gene in $S88^+$ is approximately four times higher than that in $S88^-$. However, at the adult stage, this gene was down-regulated in $S88^+$ relative to $S88^-$. Both cytochrome P450 and esterase are involved in the detoxication process. High expression of cytochrome P450-6A1 and esterase E4 in the tolerant pupa bees would help remove the toxic compounds the mite introduces.

One differentially expressed gene *GB30529* encoding peroxisomal acyl-CoA oxidase 1 was identified in two mite infestation comparisons, $S88^+/S88^-$ at the pupal stage and $G4^+/G4^-$ at the adult stage. Peroxisomal acyl-CoA oxidase catalyzes the reaction from acyl-CoA to 2-trans-enoyl-CoA in the β-oxidation of very long chain fatty acids in peroxisome. The increased expression in the two mite infestation comparisons indicates regardless of the phenotype, the mite infestation could cause enhanced oxidation of very long chain fatty acids. In the adult mite infestation comparison of the tolerant line, six genes (*GB11969*, *GB17931*, *GB12567*, *GB12176*, *GB13264*, and *GB19070*) related to the metabolism of long chain fatty acid were differentially expressed. All these genes were down-regulated in the tolerant phenotype with the mite infestation, but not in the corresponding comparison of susceptible line G4, indicating the biosynthesis of long chain fatty acids is inhibited in the tolerant line as a result of the mite attack. The reason for this is currently unknown.

3.5.3 Comparison between pupa and adult stages

A larger number of genes show differential expression at the pupal than at the adult stage. At the pupal stage, 126 genes were differentially expressed in the phenotype comparison. At the adult stage, however, only 63 genes showed differential expression among all the comparisons. Among them, 50 genes arise from the phenotype comparison. The greater number of differentially expressed genes at the pupal stage indicates that the pupa is more sensitive and responsive to the varroa attack. Therefore, the pupal stage may be a critical period for detecting differentially expressed genes that can be used to distinguish the varroa tolerant from the susceptible bee phenotype.

There may be several reasons for this phenomenon. At the pupal stage, the living space of a bee is limited to the sealed brood cell shared with the varroa mite. During the summer, 90% of the mite population is in the brood (Rosenkranz and Renz, 2003). Within a sealed brood cell infected by the mite, the concentration of the odor emitted by the mite in the cell may be very high, which can effectively induce expression of genes related to host defence against the pest (Del Piccolo *et al.*, 2010). In addition, the adult honey bee itself can carry out behavioral actions to eliminate the varroa mites, while pupae are unable to escape infestation. The honey bee hygienic behavior can also detect and remove mites from the hive, efficiently interrupting the reproductive cycle of the parasite, leading to a prolonged phoretic phase or even death of the mite (Ibrahim and Spivak, 2006). Furthermore, at the adult stage, defences against the mites can be mounted at the group level, referred to as social immunity. The grooming behavior between bees can effectively remove mites from adult bees (Peng *et al.*, 1987). In contrast, without hygienic and grooming behavior at the pupal stage, the pupa must rely on manipulation of gene expression to respond to mite parasitism.

3.5.4 Comparison between our study and previous studies

DNA microarray analysis was previously used in a study (Navajas *et al.*, 2008) to examine gene expression associated with varroa mite parasitism in both susceptible and tolerant colonies. Although our research and the previous study share a similar experimental design, only three genes are commonly identified differentially expressed in the two microarray studies. *GB18056* encoding DnaJ protein homolog 1 for protein folding, *GB19503* encoding heat shock protein Hsp70 and *GB19995* encoding an essential protein were down-regulated in the tolerant line when compared to the susceptible line in the previous study. However, our data show these three genes were down-regulated in the tolerant S88 bees with varroa infestation relative to S88 without varroa infestation. The difference in results might be caused by the source of tissue used in the two experiments, which might possess different defensive mechanism against the pest. The tissue we used were heads from S88 and G4 bees, which were produced from a local breeding program in Saskatchewan. In contrast, the previous experiment used the whole body of bees bred in French. The previous study emphasized the main effect of the genotypes by combining the infested and uninfested bees, and the main effect of varroa infestation by combining the two

genotypes for microarray analysis. Our study separates the possible effects of phenotype and mite infestation during the comparison. Therefore, our experiment provides more detailed analysis of the genes differentially expressed in the different lines in response to mite infestation.

Like the previous study, we did not identify many differentially expressed genes involved in immune responses, thought to play a role in defense against parasite infestation. This might be because the honey bee possesses only one-third the number of immune response genes of other social insects (The Honeybee Genome Sequencing Consortium, 2006). Therefore, these genes might not be differentially expressed in the current comparisons. In another words, mechanisms other than immune response, such as olfactory signal transduction, detoxification process, metabolisms and exoskeleton formation discussed above, might play more important roles in the varroa tolerance. In addition, a previous qRT-PCR study reveals that expression of genes coding anti-microbial peptides (defensin1, abaecin, hymenoptaecin) was significantly elevated in varroa-infested bees, albeit varying with sampling date and bee developmental age (Aronstein *et al.*, 2012). At the honey bee larva stage, the varroa parasitism results in significantly higher expression of antimicrobial peptides and peptidoglycan recognition proteins (Gregorc *et al.*, 2012).

The brain specific gene expression profiles of two adult bee lines with a high rate of hygienic behavior (VSH+) and a low rate of hygienic behavior (VSH-) were compared in another DNA microarray study (Le Conte *et al.*, 2011). Out of 39 genes identified, *GB16453* encoding fluoxetine resistant protein 6 and *GB30242* encoding odorant binding protein 3 were expressed at higher levels in VSH- compared to VSH+. Our result indicates *GB16453* was more highly expressed in $G4^{+}$ (susceptible line with a low rate of VSH) compared to $S88^{+}$ (tolerant line with a high rate of VSH) at the pupal stage, while at the adult stage, *GB30242* was more highly expressed in adult S88+ compare to $G4^{+}$.

A comparison of gene expression between the western honey bee, *Apis mellifera,* and the eastern honey bee, *Apis cerana,* identified many differentially expressed genes that were involved in metabolic processes (Zhang *et al.*, 2010). Our result indicates that genes involved in protein and lipid metabolism were differentially expressed in pupa when comparing between the

two bee lines. Previous digital gene expression (DGE) analysis on bee abdomens also found that the varroa parasitism results in decreased metabolism, particularly inhibition of protein anabolism (Alaux *et al.*, 2011).

Three quantitative trait loci (QTLs) located on honey bee chromosomes 4 (ranging from 2.1 to 4.3Mb), 7 (ranging from 3.6 to 8.5 Mb), and 9 (ranging from 1.0 to 3.5Mb) were found to have a significant impact on suppression of varroa reproduction (Behrens *et al.*, 2011). *GB14758* at the 7.7 Mb position of chromosome 7 encodes a heat shock protein 90 responsible for protein folding (Neckers, 2007). Our analysis indicates that the expression of this gene was 4 times lower in adult $S88^{+}$ compared to $G4^{+}$, and 4 times lower in adult $S88^{+}$ compared to $S88^{-}$. *GB14355* at the 7.5 Mb position of chromosome 7 encodes anosmin required for normal development (Endo *et al.*, 2012). Expression of this gene was 3 times higher in the tolerant S88 line compared to the susceptible line G4, regardless of the presence and absence of mite infestation ($S88^{-}/G4^{-}$ or $S88^{+}/G4^{+}$). *GB15810* encodes a protein of unknown function at the 2.7 Mb position of chromosome 9 and the expression was 3 times less in $G4^{+}/G4^{-}$ and $S88^{+}/S88^{-}$. Three closely linked genes at the 1.5 Mb position of chromosome 9, *GB12300*, *GB10646* and *GB13397* encoding proclotting enzyme, trypsin-7 and vitamin K-dependent protein C were also differentially expressed between the bee lines in our analysis. QTLs are phenotypically defined genomic regions associated with variation in a phenotypic trait; differentially expressed genes identified in these regions may provide more direct guidance that they are involved in defense against the varroa infestation.

4.0 Study 2: Real-time qRT-PCR validation of gene expression in honey bees in response to *Varroa destructor* infestation

4.1 Abstract

Real time qRT-PCR was performed to validate the differential expression of selected genes identified by DNA microarray hybridization. The result shows that 11 out of 12 genes shared similar expression patterns when measured by both methods. This agreement in results supports the conclusion that data from microarray hybridization provided reliable profiling of transcripts from two different honey bee phenotypes, with or without the mite. In addition, qRT-PCR analysis was also used to evaluate the relative infestation rate of deformed wing virus (DWV) among bee samples.

4.2 Hypothesis

DNA microarray analysis is a sensitive genomics tool to quantitatively analyze expressions of large numbers of genes in a specific cell, tissue or organ. However, DNA microarray, like any other genomic tool is inevitably subject to experimental errors associated with biological materials and the measurement of gene expression. Therefore, the data generated by DNA microarray need to be validated by a second independent method such as real-time qRT-PCR. If the mite-tolerant and mite-susceptible honey bee phenotypes respond differently to varroa infestation, then the genes in these distinct phenotypes might show differential expression. Differences in mRNA abundance within bee lines, with or without mite infestation should be detectable by real-time qRT-PCR. Furthermore, the DNA microarray data and the real-time qRT-PCR data should be consistent with each other. In addition, qRT-PCR analysis of selected genes initially identified in DNA microarray could facilitate development of DNA markers for the selective breeding of bees resistant to mite infestation.

4.3 Experimental approach

4.3.1 Genes and primers

Six genes with large fold changes and six genes with low fold changes in differential expression identified by the DNA microarray as well as a gene from the honey bee virus DWV were chosen

for real-time qRT-PCR analysis (Table 4.1). Two housekeeping genes, *actin* and *ribosomal protein S5* (*RpS5*) were used as internal standards. Primers were developed using primer3plus online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesized by Sigma-Aldrich (www.sigmaaldrich.com).

4.3.2 Reaction system

One microgram of a RNA sample used for the microarray analysis was reverse transcribed to the first strand cDNA using qScript cDNA SuperMix (Quanta). The reaction was heat-inactivated and diluted fivefold with water. Four µl of a diluted sample was used in a 16-µl Real Time qRT-PCR reaction containing 4µl of each of the two primers (10 ng), and 8µl SYBR Supermix (BIORAD). Amplifications were carried out in 96-well plates in the CFX96 System (BIORAD), using the following thermo cycling conditions: an initial denaturation at 95°C for 30-s, followed by 45 cycles of 5-s denaturation at 95°C, 30-s annealing and elongation at 60°C. For each sample, triplicate reactions were performed.

4.3.3 Data analysis

Data obtained by the iCycler software was subsequently analyzed with custom-designed Excel spreadsheets. The relative expression ratios of target genes were calculated using the Comparative CT Method (ΔΔCt).

$$\text{Fold Change} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = \Delta Ct \text{ sample1} - \Delta Ct \text{ sample2}$$

ΔCt sample = Ct value for the sample1 normalized to the housekeeping genes

ΔCt control = Ct value for the sample2 normalized to the housekeeping genes

Statistical analysis was performed using the SPSS v.18.0 software package for Windows (SPSS Inc., USA). General Linear Model univariate analysis, and multiple comparisons were conducted using Duncan post hoc test. When the p-value was less than 0.05, the difference was regarded as statistically significant.

4.4 Results

4.4.1 Reliability of qRT-PCR amplification

Quantitative RT-PCR was first performed to validate expressions of six differentially expressed genes with large fold changes determined by the DNA microarray. A representative example of the qPCR amplification curves is shown in Figure 4.1.

4.4.2 Comparison of expression profiles of the genes with large fold changes generated by qRT-PCR and microarray

Expression profiles of six genes with large fold changes verified by qRT-PCR indicated that except for one comparison for the gene *GB14278* ($G4^-/G4^+$), the remaining 11 comparisons of six genes (*GB12600, GB19316, GB30203, GB14355, DB744987*) showed similar expression patterns as measured by the two methods (Table 4.2 and Figure 4.2). This result indicates that the initial DNA microarray data on differential expression of the genes in two different bee phenotypes with and without the varroa mite infestation was reliable.

Table 4.1 Primers used in qRT-PCR.

Genes	Forward primer sequence	Reverse primer sequence
Actin	F: GTACCACCATGTATCCTGGAATC	R: GAGATCCACATCTGTTGGAAGG
RPS5	F: CCGCAATGTCCTATAGTCGAAC	R: GATGATAGCAGTCACAAGAACCTG
GB30203	F: TGCTGGACCAACACTAGTTGC	R: CAATGGTGAGCGAGTACAGATG
GB14355	F: CTTGGGCCCAGGTATATAGAATC	R: GGTCTGGACGGTTGAGAATATC
DB744987	F: GGCAGCACCGTATATTTCTACAC	R: CGTGGAAATACACACAGTTTAGTTG
GB14278	F: GACGTCAGGAATGATACTGCAC	R: ATGATGTACTCCCTCTCCTCCTTC
GB12600	F: CTTATGCTCCTGGTGTACCCTTAG	R: GCATAGCTGTATTGAGGATGAGG
GB19316	F: CGATCGTTCTGATGACTTACCG	R: CCTGACGCTTATTCTCCAGTTC
GB12136	F: GCCCACTTGGAACTCTATAATACG	R: CCTGAACACGTTTCTCTCTTTCC
GB11723	F: GATGGGAAATTCCGTGTCAG	R: TTTATCTCGCCCTCCAACAC
GB16889	F: ACCATATTCCCCGTGTATCG	R: TGTATGCCGTATCGTTGCTC
GB14612	F: CGAAAGGAACTTGCATAGCC	R: TCTTCGGAAAATCGTTCTGG
GB19306	F: TCCTCCGACTCCAATTATCG	R: AAACGGAGAGGATCTGGATG
GB19967	F: TGTTCGGCTTGAGATTCCTC	R: ATCTGTTGGTGCCCAACTTC
DWV	F: GAGATTGAAGCGCATGAACA	R: TGAATTCAGTGTCGCCCATA

Note: *Actin* and *Ribosomal protein S5* (*RpS5*) were used as internal standards. 12 transcripts from the honey bee were tested as well as a honey bee virus DWV. The size of amplification products was in the range of 70-150 base pairs.

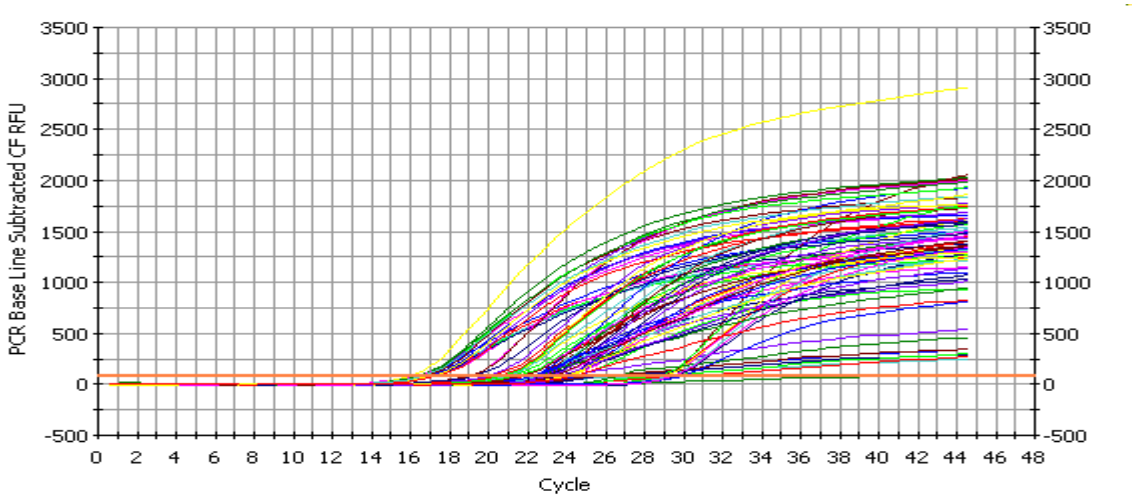


Figure 4.1 Representative curves of the qRT-PCR amplification.

The horizontal axis stands for the number of cycles of amplification, the vertical axis stands for the relative fluorescence units (RFU). Each curve represents an amplification reaction.

Table 4.2 Tabulated comparison of the gene expression fold change as measured by qRT-PCR and DNA microarray.

Gene	Array $G4^+/G4^-$	qPCR $G4^+/G4^-$	Array $S88^+/S88^-$	qPCR $S88^+/S88^-$
GB14278	9.09	0.74	7.14	1.28
GB12600	2.78	14.29	0.31	0.20
GB19316	0.31	0.13	3.33	2.08
Gene	Array $S88^+/G4^+$	qPCR $S88^+/G4^+$	Array $S88^-/G4^-$	qPCR $S88^-/G4^-$
GB30203	0.33	0.07	2.20	2.91
GB14355	2.69	1.22	4.45	1.06
DB744987	3.40	33.22	2.02	19.13

Note: Array: the fold change from the DNA microarray analysis. qPCR: the fold change from the qRT-PCR analysis. $G4^+/G4^-$: the susceptible phenotype with varroa mite infestation ($G4^+$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$), $S88^+/S88^-$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the tolerant phenotype without varroa mite infestation ($S88^-$), $S88^+/G4^+$: the tolerant phenotype with varroa mite infestation ($S88^+$) relative to the susceptible phenotype with varroa mite infestation ($G4^+$), and $S88^-/G4^-$: the tolerant phenotype without varroa mite infestation ($S88^-$) relative to the susceptible phenotype without varroa mite infestation ($G4^-$).

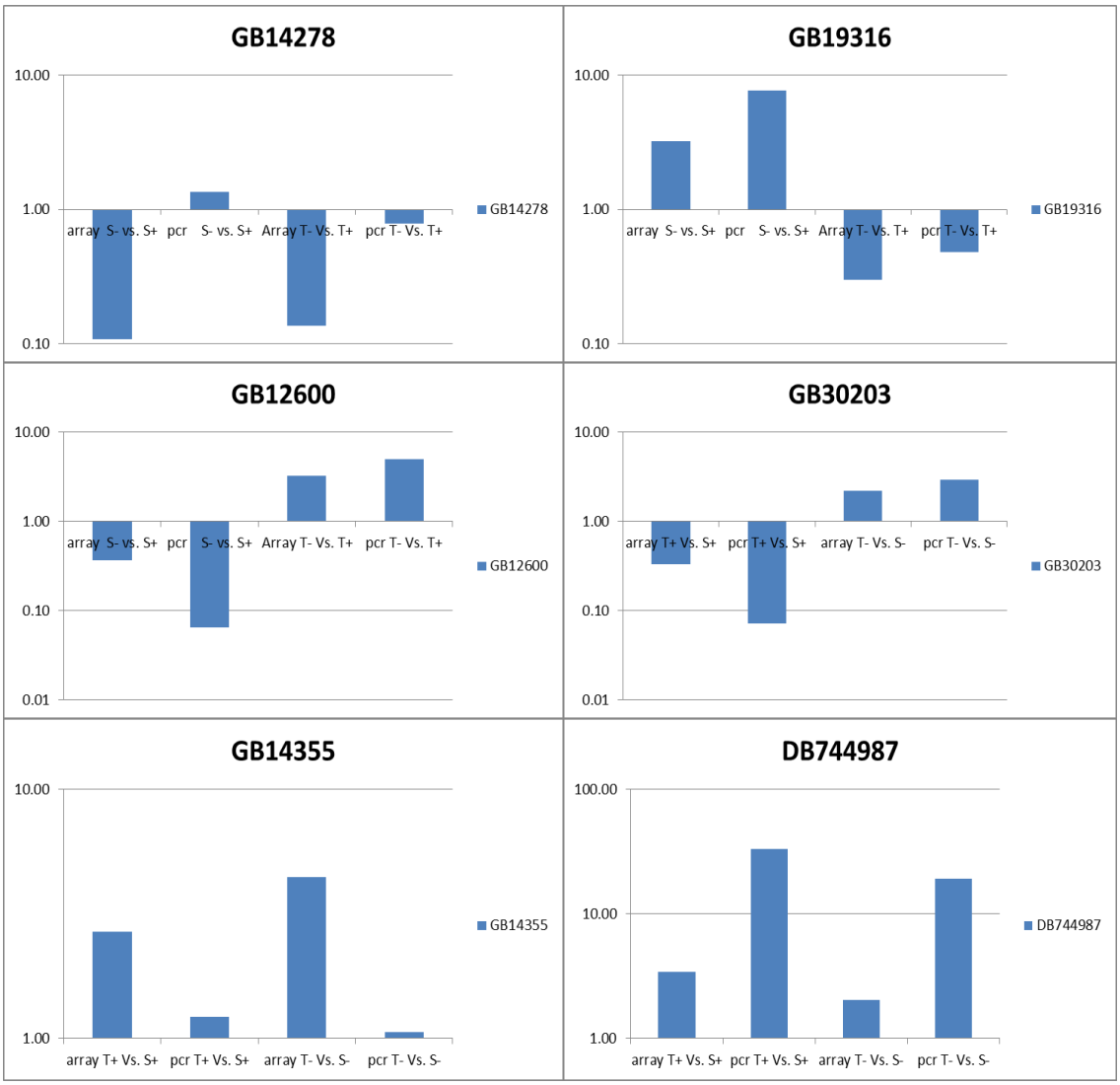


Figure 4.2 Comparison of expression patterns as measured by qRT-PCR and DNA microarray.

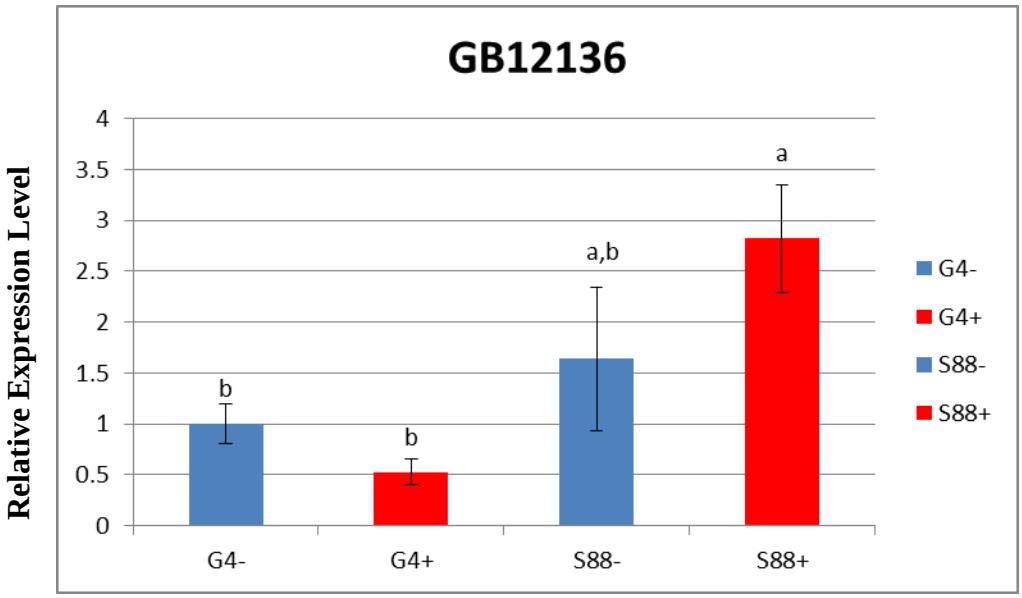
The data was transformed by Log10. If both the values of DNA microarray data and qRT-PCR data were above 1 or less than 1, the expression patterns of DNA microarray and qRT-PCR were considered "similar". If the values of DNA microarray data and qRT-PCR data were not above 1 or less than 1, the expression patterns of DNA microarray and qRT-PCR were considered "different" from each other.

4.4.3 Analysis of differentially expressed genes by qRT-PCR

Expression profiles of six other differentially expressed genes identified by the DNA microarray were also analyzed by qRT-PCR, although their differential expressions were not as pronounced as the first six genes analysed. These genes, however, might be important for mite tolerance based on predicted function. The result showed that all the comparisons of these genes had similar expression patterns (Figure 4.3-4.8), as measured by the two methods. The qRT-PCR results give a more quantitative measure of gene expression for each sample, which was the susceptible phenotype with mite infestation ($G4^+$), the susceptible without mite infestation ($G4^-$), the tolerant with mite infestation ($S88^+$) and the tolerant without mite infestation ($S88^-$).

Of the six genes, four encoded cytochrome P450 proteins. *GB12136* (cytochrome P450-6A1) and *GB14612* (cytochrome P450-6K1) had significantly higher expression in pupa of the tolerant line following mite infestation ($S88^+$) when compared to the susceptible line (G4) with or without mite infestation. In particular, the expression level of *GB12136* was more than five times higher in $S88^+$ than $G4^+$. However, the situation was very different at the adult stage when both genes had significantly higher expression in the susceptible line without the mites ($G4^-$) relative to the other samples (Figure 4.3 and Figure 4.4). The high expression of the cytochrome P450-6A1 and cytochrome P450-6K1 in S88 with the mite at the pupal stage might equip the tolerant bee with better capacity to detoxify the compounds introduced by the varroa. The unique expression patterns of the two cytochrome P450 genes in the phenotype comparison at the pupal stage could be used to differentiate the two bee lines in response to the varroa infestation.

On the other hand, the two cytochrome P450 genes *GB19306* (cytochrome P450-6K1) and *GB19967* (cytochrome P450-9E2) were more highly expressed in the tolerant line at the adult stage without mites ($S88^-$) relative to the same line with mites ($S88^+$) (Figure 4.5 and Figure 4.6), while at the pupal stage there was no significant difference in the expression levels of the two genes. The unique expression pattern indicates this group of P450 genes might have distinct roles in adult bees responding to mite infestation.



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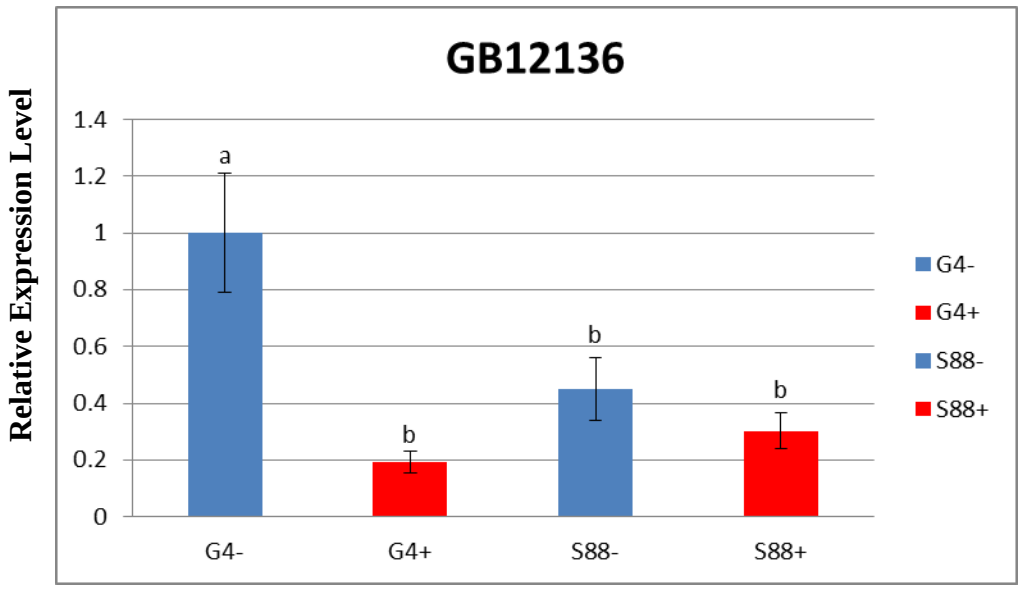
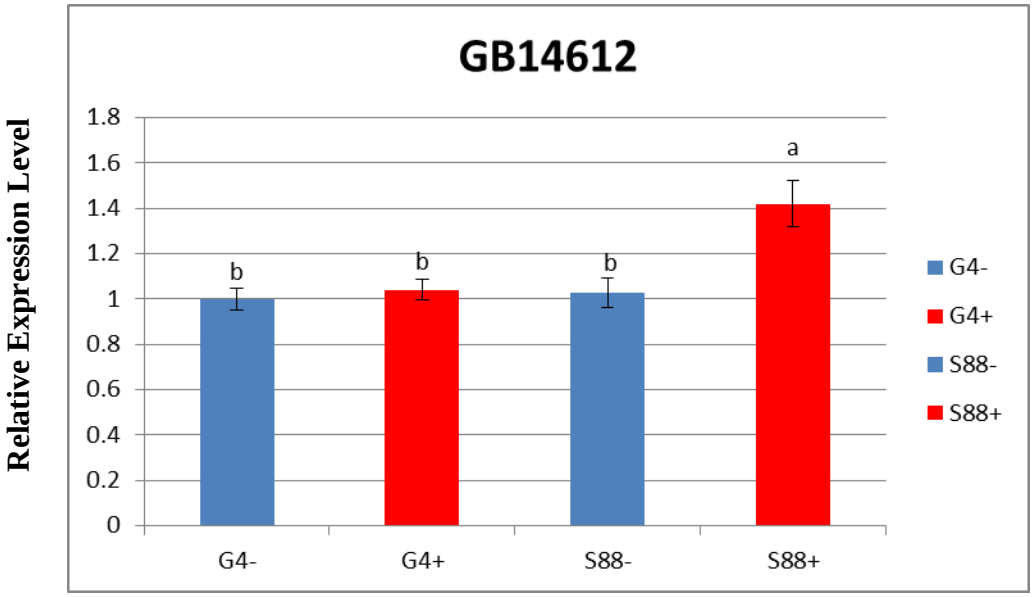


(B)

Figure 4.3 Relative expression levels of *GB12136* (Cytochrome P450-6A1) at the pupal (A) and adult (B) stages measured by qRT-PCR.

$G4^+$: the susceptible phenotype with varroa mite infestation, $G4^-$: the susceptible phenotype without varroa mite infestation, $S88^+$: the tolerant phenotype with varroa mite infestation, and $S88^-$: the tolerant phenotype without varroa mite infestation. The y-axis represents the relative expression level. Values followed by a different letter (a or b) are significantly different using Duncan post hoc test ($P < 0.05$). All the values shown are mean ± SE.



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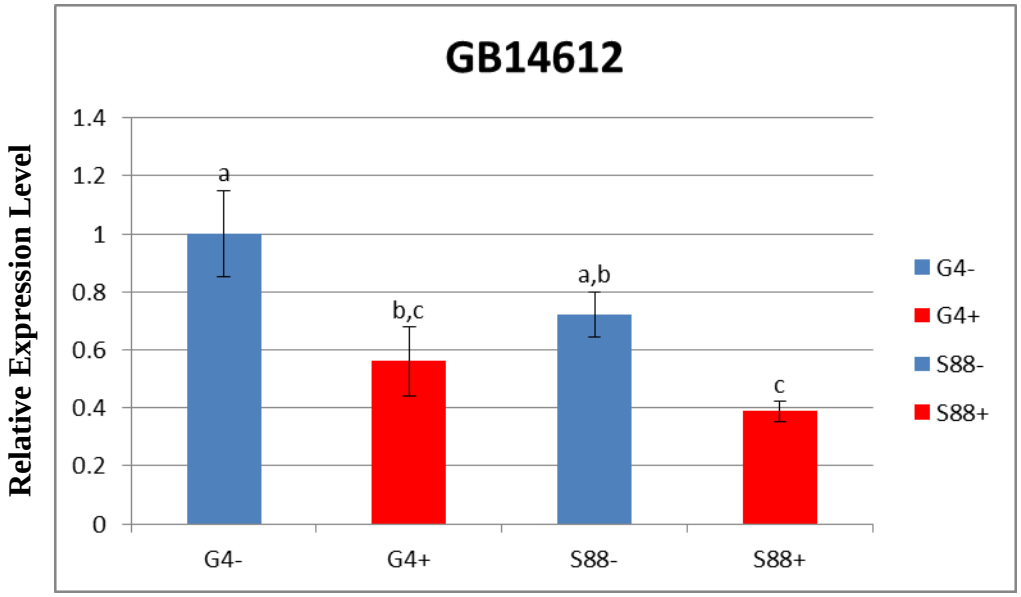
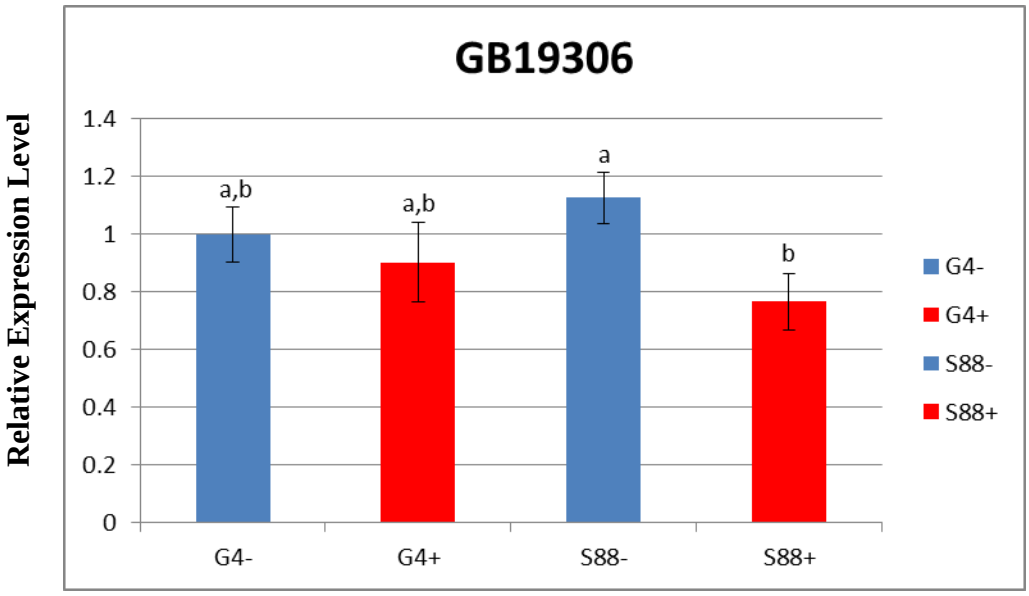


(B)

Figure 4.4 Relative expression levels of *GB14612* (Cytochrome P450-6K1) at the pupal (A) and adult (B) stages measured by qRT-PCR.

$G4^+$: the susceptible phenotype with varroa mite infestation, $G4^-$: the susceptible phenotype without varroa mite infestation, $S88^+$: the tolerant phenotype with varroa mite infestation, and $S88^-$: the tolerant phenotype without varroa mite infestation. The y-axis represents the relative expression level. Values followed by a different letter (a, b or c) are significantly different using Duncan post hoc test ($P < 0.05$). All the values shown are mean ± SE.



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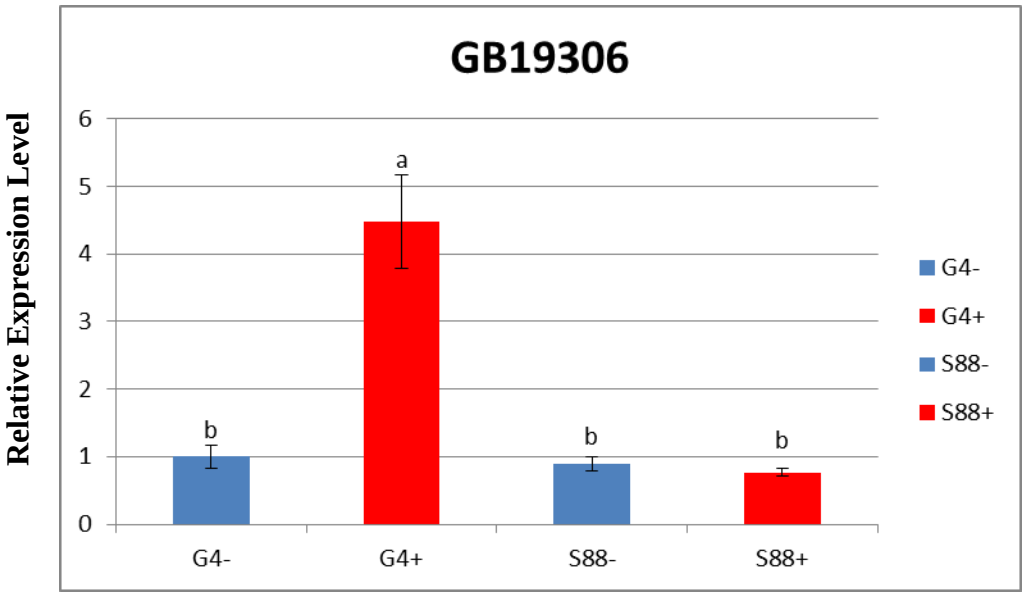
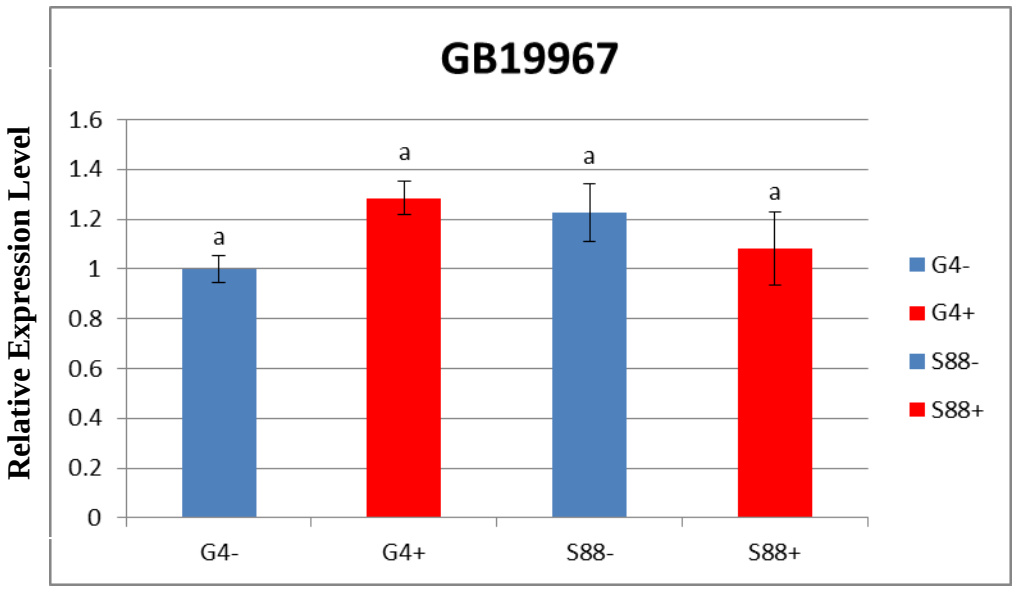


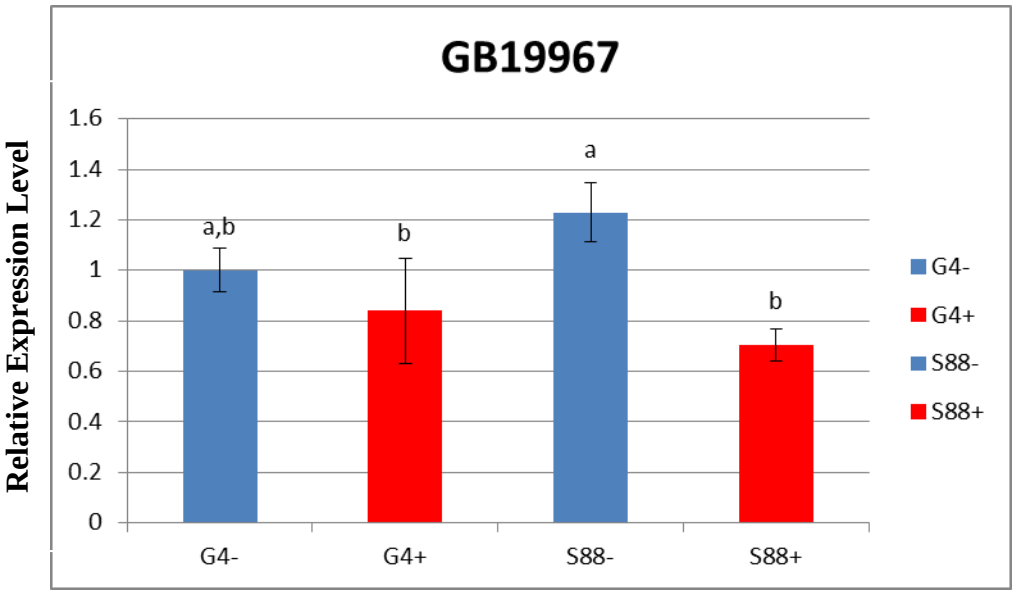
(B)

Figure 4.5 Relative expression levels of *GB19306* (Cytochrome P450-6K1) at the pupal (A) and adult (B) stages measured by qRT-PCR.

$G4^+$: the susceptible phenotype with varroa mite infestation, $G4^-$: the susceptible phenotype without varroa mite infestation, $S88^+$: the tolerant phenotype with varroa mite infestation, and $S88^-$: the tolerant phenotype without varroa mite infestation. The y-axis represents the relative expression level. Values followed by a different letter (a or b) are significantly different using Duncan post hoc test ($P < 0.05$). All the values shown are mean ± SE.



(A)



(B)

Figure 4.6 Relative expression levels of *GB19967* (Cytochrome P450-9E2) at the pupal (A) and adult (B) stages measured by qRT-PCR.

$G4^+$: the susceptible phenotype with varroa mite infestation, $G4^-$: the susceptible phenotype without varroa mite infestation, $S88^+$: the tolerant phenotype with varroa mite infestation, and $S88^-$: the tolerant phenotype without varroa mite infestation. The y-axis represents the relative expression level. Values followed by a different letter (a or b) are significantly different using Duncan post hoc test ($P < 0.05$). All the values shown are mean ± SE.

GB16889 encoding esterase E4 had significantly higher expression in the tolerant line with mites ($S88^+$), relative to the rest samples. It was noted that the expression level was ten times higher in the tolerant line with mites ($S88^+$) than that in the susceptible line with mites ($G4^+$) at the pupal stage (Figure 4.7A). However, at the adult stage this gene was more highly expressed in the tolerant line without mite $S88^-$. This expression pattern implies that the role of this gene varies with the developmental stages. At the pupal stage, it might function in the detoxification process to cope with toxic esters and protect the bee from the toxicity. At the adult stage, it might be associated with insecticide resistance as suggested by Devonshire and Moores (Devonshire and Moores, 1982).

The expression pattern of the gene *GB11723* encoding Apolipoprotein D was quite different from those of the genes involved in the detoxification process. At the pupal stage, this gene was significantly down-regulated in the susceptible line with mite $G4^+$, relative to the other samples. In particular, the expression level of this gene was 14 times lower in the susceptible line with mite $G4^+$ than that in the tolerant line with mites $S88^+$ (Figure 4.8). However, at the adult stage the expression difference was observed only between the two lines regardless of the presence or absence of the mite. The expression pattern at the pupal stage was consistent with its positive role in lipid transport, conferring the tolerant line with a higher rate of lipid metabolism to fight mite infestation. Therefore, the expression pattern of this gene differentiates readily the two different lines and could be used as a biomarker for breeding the resistant bees.

4.4.4 qRT-PCR analysis of deformed wing virus

Deformed wing virus (DWV) is a prevalent honey bee virus that causes wing deformity and mortality in honey bees worldwide (Chen and Siede, 2007). DWV infections were often reported to be associated with the varroa mites (Schoning *et al.*, 2012). Virus infection in honey bees can be detected and quantified by real-time qRT-PCR, providing rapid and accurate information for virus epidemiology, pathogenesis and diagnosis (Chen *et al.*, 2005).

Quantitative RT-PCR analysis of the virus in the samples demonstrated that at the pupal stage, the highest amount of DWV was detected in the susceptible line with mites $G4^+$. This indicates

that the susceptible line is not only susceptible to the varroa mite infestation, but also vulnerable to the DWV infection (Figure 4.9). This is consistent with previous reports that DWV infections were often associated with the mites, and varroa mites might have a role in spreading the virus (Schoning *et al.*, 2012). As such, both the tolerant lines ($S88^{+}$) and the susceptible line ($G4^{+}$) with mite infestation had higher DWV load than bees without mite infestation. In addition, it was also noted that DWV was abundantly detected in the susceptible line without mites $G4^{-}$, however, it was hardly detected in the tolerant line without mites $S88^{-}$ at the pupal stage. This result indicates that the mite susceptible bees are more prone to the DWV infection.

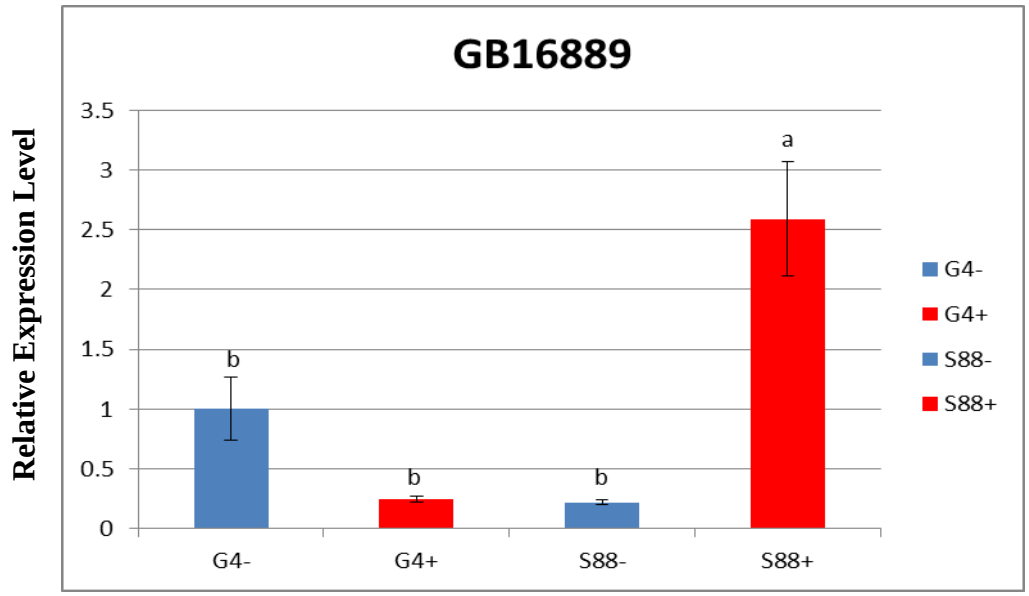
At the adult stage, the amount of DWV RNA did not vary significantly among the bee samples, although the virus RNA in $G4^{+}$ was slightly higher relative to the other samples. This result indicates that the virus spreads among the adult bees, no matter the phenotype or level of the mite infestation. DWV could be transmitted between bees through contact or varroa mite infestation (Bowen-Walker *et al.*, 1999).

4.5 Discussion

Quantitative RT-PCR is a method that enables both detection and quantification of transcriptional expression of genes. It is widely used for analysis of gene expression and validation of DNA microarray data. Differential expressions of eleven out of the twelve genes selected from the microarray analysis were confirmed by qRT-PCR, indicating that DNA microarray is a reliable method for genomic profiling of transcripts in these two honey bee phenotypes, with or without mite infestation. Quantitative RT-PCR analysis of genes in the two lines, with or without mite infestation, indicates that several genes may play important roles in mite tolerance and could be used as biomarkers for future honey bee breeding programs.

Our result also confirms that that varroa mite infestation is associated with increased deformed wing virus (DWV) infection. Thus, the combination of mite infestation and viral infection poses a serious threat to honey bee health and survival (Schoning *et al.*, 2012). At the vulnerable pupal stage, the susceptible line with mites ($G4^{+}$) had the highest DWV RNA among the four samples, indicating that the susceptible line is not only susceptible to the varroa mite infestation, but also

had increased DWV infection. It has been suggested that the parasitizing mites can carry replicating DWVs and consequently transmit virulent DWVs among the bees. Even at the adult stage, DWVs could be transmitted between bees through direct contact as well as varroa mite infestation (Bowen-Walker *et al.*, 1999).



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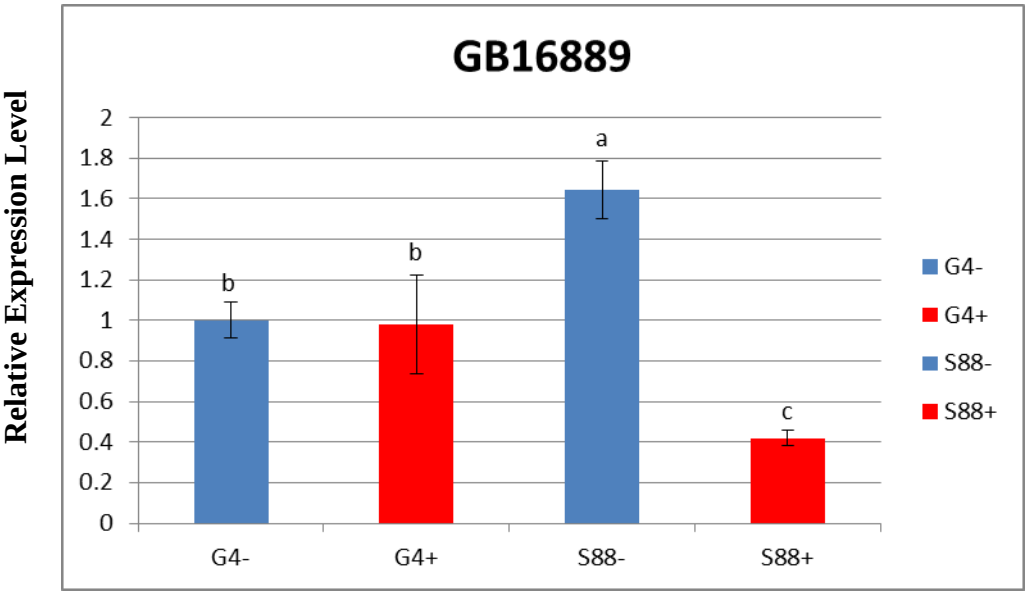
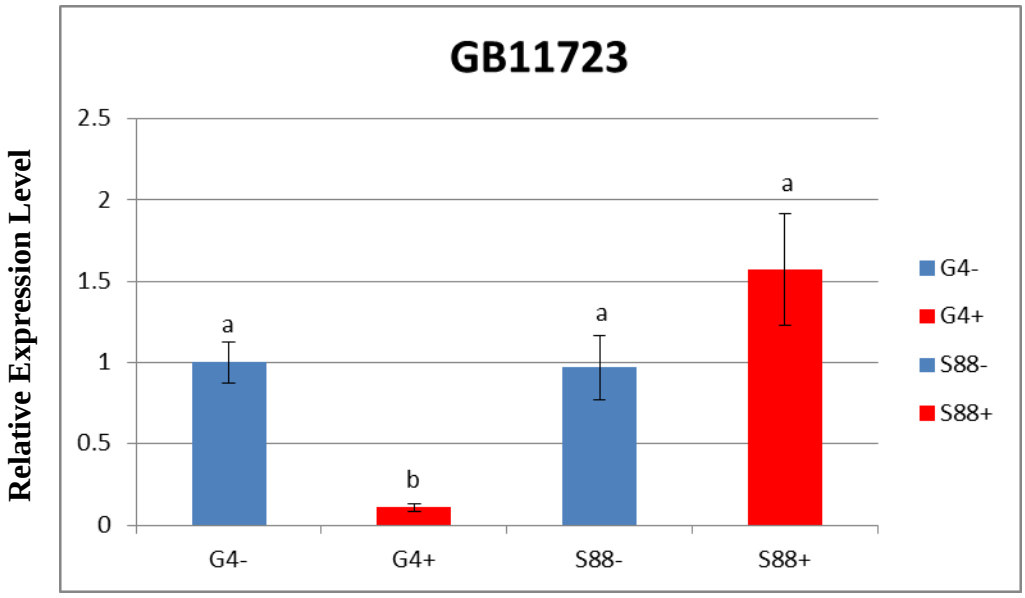


(B)

Figure 4.7 Relative expression levels of *GB16889* (Esterase E4) at the pupal (A) and adult (B) stages measured by qRT-PCR.

$G4^+$: the susceptible phenotype with varroa mite infestation, $G4^-$: the susceptible phenotype without varroa mite infestation, $S88^+$: the tolerant phenotype with varroa mite infestation, and $S88^-$: the tolerant phenotype without varroa mite infestation. The y-axis represents the relative expression level. Values followed by a different letter (a, b or c) are significantly different using Duncan post hoc test ($P < 0.05$). All the values shown are mean ± SE.



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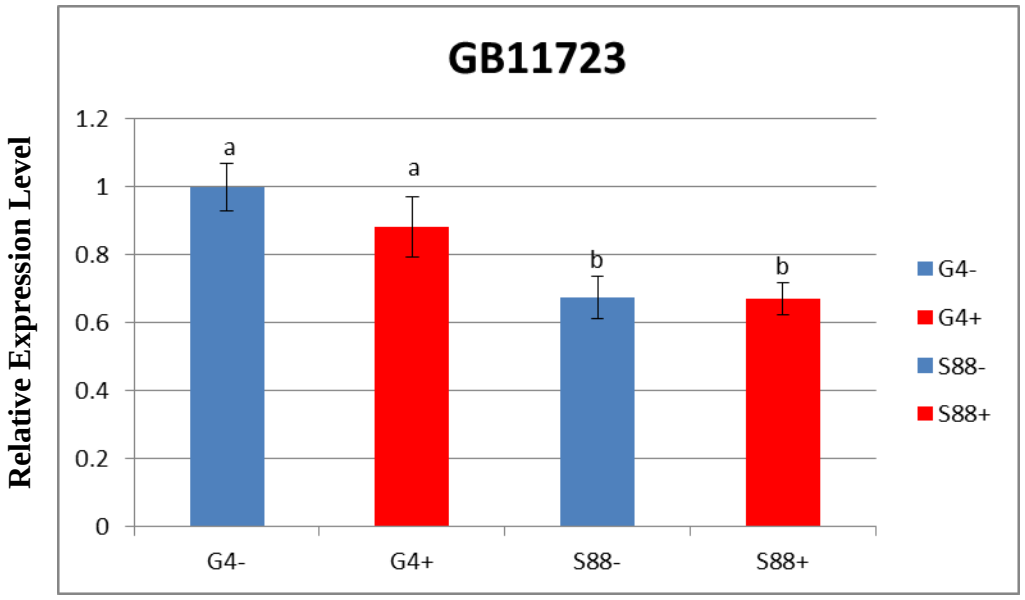
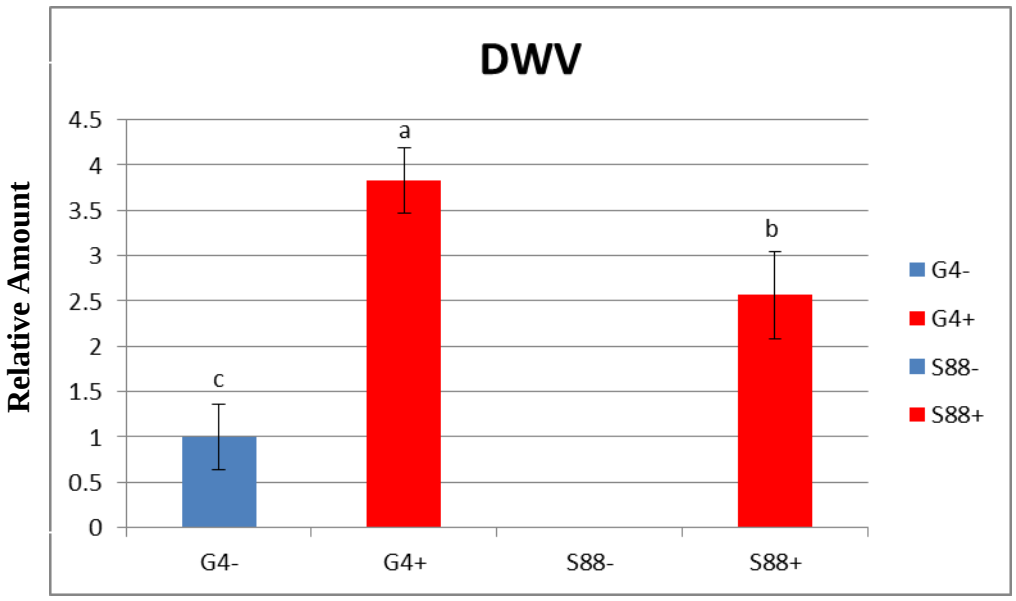


(B)

Figure 4.8 Relative expression levels of *GB11723* (Apolipoprotein D) at the pupal (A) and adult (B) stages measured by qRT-PCR.

$G4^+$: the susceptible phenotype with varroa mite infestation, $G4^-$: the susceptible phenotype without varroa mite infestation, $S88^+$: the tolerant phenotype with varroa mite infestation, and $S88^-$: the tolerant phenotype without varroa mite infestation. The y-axis represents the relative expression level. Values followed by a different letter (a or b) are significantly different using Duncan post hoc test ($P < 0.05$). All the values shown are mean ± SE.



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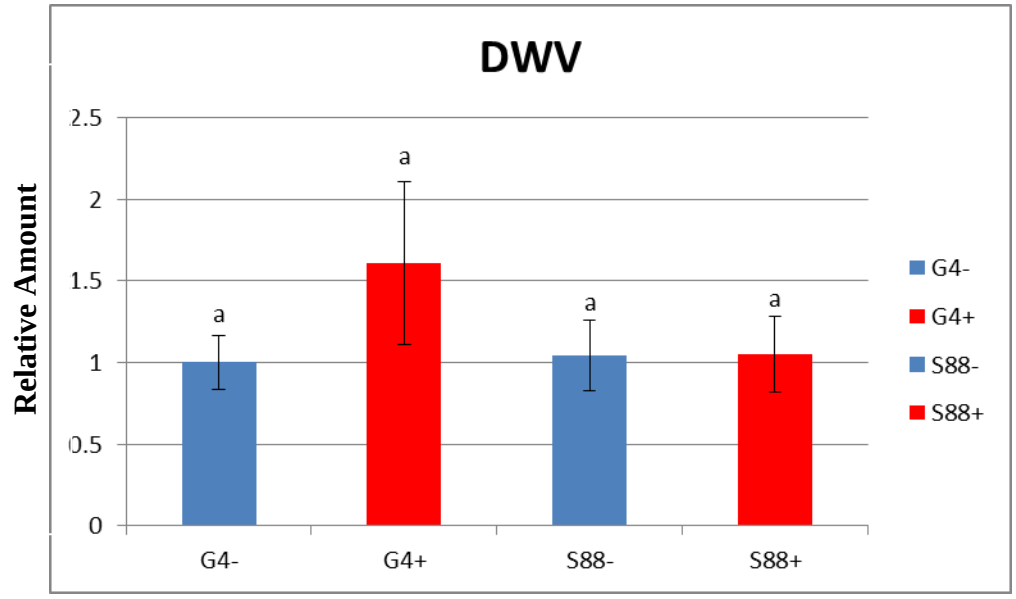

(B)

Figure 4.9 Relative amount of DWV (Deformed wing virus) at the pupal (A) and adult (B) stages measured by qRT-PCR.

$G4^+$: the susceptible phenotype with varroa mite infestation, $G4^-$: the susceptible phenotype without varroa mite infestation, $S88^+$: the tolerant phenotype with varroa mite infestation, and $S88^-$: the tolerant phenotype without varroa mite infestation. The y-axis represents the relative amount. Values followed by a different letter (a, b or c) are significantly different using Duncan post hoc test ($P < 0.05$). All the values shown are mean ± SE.

5.0 General conclusion and future prospects

In this study, high-throughput DNA microarray analysis was employed to investigate genome-wide gene expression in two varroa-tolerant and varroa-susceptible honey bee colony phenotypes, with or without varroa infestation. Gene expression data was independently confirmed by qRT-PCR analysis of 12 genes with high or low differential expression. Among these 12 genes, 11 retain similar expression patterns when measured by the DNA microarray. Comparison of the microarray expression profiles revealed that more than two hundred genes were differentially expressed when comparing the two bee lines in response to mite infestation. More differentially expressed genes were found at the pupal stage than at the adult stage, indicating that the pupae are more responsive to varroa attack than adult bees. More differentially expressed genes were identified when comparing phenotypes than comparing responses to mite infestation, regardless of the developmental stage. These data confirm that the two bee lines respond very differently to mite infestation. According to the predicted function, the differentially expressed genes can be classified into groups that are involved in olfactory signal transduction, detoxification process, protein and lipid metabolisms as well as exoskeleton formation, implying that these processes underlie the defensive mechanisms of honey bees against the varroa mite.

This study highlights differential expression of genes associated with distinct phenotypes and developmental stages of honey bees. Gene expression data provides possible molecular mechanisms for bee tolerance to mite infestation. This information not only strengthens our knowledge about the interaction between bees and parasitic mite, but also provides potential molecular markers that can be used for selecting honey bees resistant to the varroa mite.

As for future direction, the key genes that are highly differentially expressed between the selected phenotypes can be cloned, and functionally expressed in the model systems such as *E. coli* and yeast. The biochemical activity and substrate specificity of these proteins *in vitro* will provide direct information on the biochemical roles they may play in defending against varroa. In addition, RNA interference could be used to knockout or knockdown the genes, providing information on the biological function of these genes *in vivo*. For example, *GB16889* encoding esterase E4 and *GB12136* encoding cytochrome P450-6A1 which were highly differentially

expressed between the susceptible and tolerant phenotypes, are of great interest due to their possible detoxification function for the varroa tolerance. Detailed biochemical and genetic analysis of these two genes would help elucidate their exact roles in honey bee defense against the parasite.

Furthermore, investigation of host responses to multiple disease agents could be performed to identify the authentic cause of the colony collapse syndrome. This would offer a better understanding of the disease pathogenesis in bees, including secondary infections and possible synergistic effects of more than one pathogen. In this regard, the varroa mite as the most serious pathogen of honey bees deserves more research to identify virulent factors, which may give a broader view of the varroa-honey bee relationship.

Finally, the long-term approach of selective breeding for varroa resistant honey bee should be explored through the use of molecular biology techniques. In this regard, molecular markers could be developed based on the differential expression of key genes identified when comparing the tolerant and susceptible lines. Application of these markers in independent colonies would facilitate more effective selection of bees tolerant to the mite. With the application of both traditional selective breeding and modern molecular biology, varroa infestation of honey bees could be controlled in the near future.

6.0 References

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7.0 Appendix. Differentially expressed genes that were identified by DNA microarray analysis

	Comparisons at the pupal stage				Comparisons at the adult stage				
	The phenotype comparison		The mite infestation comparison		The phenotype comparison		The mite infestation comparison		
Gene	$S88^-$ /$G4^-$	$S88^+$ /$G4^+$	$G4^+$ /$G4^-$	$S88^+$ /$S88^-$	$S88^-$ /$G4^-$	$S88^+$ /$G4^+$	$G4^+$ /$G4^-$	$S88^+$ /$S88^-$	Honey bee protein
GB30379-RA	0.29								Serine protease snake
BB160015A20H04	0.48								NA
XM_623529	0.42			2.56					Hypothetical protein LOC551133
GB17927-RA	0.33								Serine protease snake
GB10527-RA	0.41								dTDP-glucose 4,6-dehydratase-like
GB30337-RA	0.42			2.02					endocuticle structural glycoprotein SgAbd-2-like
GB30378-RA	0.40								serine protease snake
GB19464-RA	0.42			2.37					yellow-y
GB12700-RA		0.31							hypothetical protein LOC725238
GB13457-RA		0.05		0.14					hypothetical protein LOC409163 isoform 1
GB19040-RA		0.08		0.25					transmembrane protein 161B-like
GB15203-RA		0.19							larval cuticle protein A3A
GB15203-RA		0.18							larval cuticle protein A3A
GB18626-RA		0.24	3.35						structural maintenance of chromosomes protein 6-like
GB18626-RA		0.20	3.26						structural maintenance of chromosomes protein 6-like
GB12600-RA		0.13	2.74	0.34					cuticle protein
GB12600-RA		0.15	2.74	0.29					cuticle protein
GB19856-RA		0.23	3.57						tripartite motif-containing protein 2-like
GB17345-RA		0.24							hypothetical protein LOC408494
GB10734-RA		0.21	2.50						hypothetical protein LOC725882
GB14811-RA		0.28							hypothetical protein LOC551089 isoform 2

GB14225-RA	0.27						hypothetical protein LOC100577189
GB17322-RA	0.24						hypothetical protein LOC725903
GB11681-RA	0.26						hypothetical protein LOC725305
GB12218-RA	0.44			0.36			histone H1-like
GB10347-RA	0.41						hypothetical protein LOC725804
GB17125-RA	0.49						prostaglandin reductase 1-like
GB14225-RA	0.30						hypothetical protein LOC100577189
GB19234-RA	0.33						tweedle motif cuticular protein 1
XM_001123192	0.42						Apis mellifera odorant binding protein 18 (Obp18), mRNA
GB17768-RA	0.41						mitochondrial glutamate carrier 1-like, partial
GB14193-RA	0.40	2.01					tweedle motif cuticular protein 2
GB11412-RA	0.36		0.43			0.38	hypothetical protein LOC411983
GB18806-RA	0.46						histone H2A-like
BI515832	0.37				0.45		NA
GB19453-RA	0.38						chemosensory protein 2
GB12811-RA	0.40	2.18		0.08	2.71		hypothetical protein LOC409962
GB10016-RA	0.39						hypothetical protein LOC100576118
GB17015-RA	0.46						ecdysteroid UDP-glucosyltransferase-like
GB15046-RA	0.46						hypothetical protein LOC725838
GB18024-RA	0.50						hypothetical protein LOC726793
GB11092-RA	0.32		0.39				odorant binding protein 17
GB15292-RA	0.43						NA
GB18896-RA	0.47						lactase-phlorizin hydrolase-like
GB18007-RA	0.47						hypothetical protein LOC409465 isoform 1
GB11089-RA	0.41						probable multidrug resistance-associated protein lethal(2)03659-like
GB14384-RA	0.50						hypothetical protein LOC726864
GB11358-RA	0.48						LOW QUALITY PROTEIN: twitchin
GB19013-RA	0.11		0.19				hypothetical protein LOC551905

GB17384-RA	0.34			hypothetical protein LOC409345
GB20006-RA	0.25			bluestreak
GB15865-RA	0.17		0.17	hypothetical protein LOC726229
GB17493-RA	0.30		0.46	hypothetical protein LOC725683
GB13390-RA	0.12		0.09	NA
GB16900-RA	0.22			hypothetical protein LOC726185
GB17278-RA	0.24		0.30	hypothetical protein LOC100578587
GB11273-RA	0.41			retinoid-inducible serine carboxypeptidase-like
GB20132-RA	0.29		0.31	hypothetical protein LOC100578587
GB12705-RA	0.31		0.45	zinc finger protein 512B
BB170019B10C10	0.41	2.68		NA
XM_001120351	0.40		0.47	hypothetical protein LOC725309
GB16453-RA	0.47			nose resistant to fluoxetine protein 6-like
GB11122-RA	0.34		0.40	bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2-like
GB14611-RA	0.46			hypothetical protein LOC410736
GB13246-RA	0.47			phospholipase A1 member A-like isoform 1
GB15665-RA	0.40			2-hydroxyacylsphingosine 1-beta-galactosyltransferase-like isoform 1
GB18450-RA	0.44			transmembrane protease serine 6
DB767093	0.41		0.28	talin-1-like
GB11754-RA	0.31		0.34	probable cytochrome P450 6a14 isoform 1
GB16804-RA	0.23		0.19	hypothetical protein LOC726206
GB16735-RA	0.40			glucose dehydrogenase [acceptor]
GB13936-RA	0.31		0.34	hypothetical protein LOC552190
GB10717-RA	0.42		0.48	muscle-specific protein 20
GB12300-RA	0.36		0.34	proclotting enzyme isoform 1
GB19501-RA	0.40		0.47	myophilin-like
GB16582-RA	0.46			hypothetical protein LOC725175
GB17888-RA	0.34		0.25	hypothetical protein LOC100578613

GB12342-RA	0.40		0.36		hypothetical protein LOC100578368
GB30365-RA	0.41	2.21			odorant binding protein 14
GB19561-RA	0.49				hypothetical protein LOC100578730 isoform 1
GB12641-RA	0.44				alpha-tocopherol transfer protein-like
GB15540-RA	0.47		0.33		SET and MYND domain-containing protein 4-like, partial
GB30202-RA	0.31		0.36	7.45	apidermin 1
GB16889-RA	3.41		3.92	0.47	esterase E4-like
GB10683-RA	2.31				hypothetical protein LOC726950
GB13426-RA	2.34				hypothetical protein LOC100577043
GB18070-RA	2.23				acyl-CoA Delta(11) desaturase-like
GB13933-RA	2.36				hypothetical protein LOC100576814
GB10971-RA	2.37		2.05		collagen alpha-1(IV) chain-like
GB14992-RA	2.32				NA
GB12128-RA	3.08	0.46			hypothetical protein LOC552836 isoform 2
GB17702-RA	2.40		2.12		cadherin-87A-like
GB11717-RA	3.25		2.28		hypothetical protein LOC725273
GB12136-RA	4.13		6.67		cytochrome P450 6A1
GB12136-RA	4.04		6.49		cytochrome P450 6A1
GB19513-RA	2.03				hypothetical protein LOC100577098
GB11920-RA	2.53				tubulin beta-3 chain-like isoform 2
GB14361-RA	2.32				hexamerin 110
GB16488-RA	2.58				hypothetical protein LOC408508
GB17642-RA	3.76		2.57		pro-resilin
GB15794-RA	2.41				hypothetical protein LOC410975
GB11668-RA	2.17				circadian clock-controlled protein-like
GB16869-RA	2.12				circadian clock-controlled protein-like
GB11352-RA	3.39		2.32		hypothetical protein LOC552100
GB13473-RA	2.07				apidaecins type 73
GB10646-RA	3.12	0.48			trypsin-7-like

GB13613-RA		2.34							hexamerin
GB10869-RA		2.32							hexamerin 70b
GB19316-RA		6.25	0.31	3.32					hypothetical protein LOC100578085
GB30362-RA		3.17							hexamerin
GB10247-RA		2.03							hypothetical protein LOC551717
GB13568-RA		2.94							monocarboxylate transporter 12-like
GB19043-RA		2.03							hypothetical protein LOC725082
DB777873		2.83							neurobeachin-like, partial
GB13489-RA		4.88		2.96					venom serine protease 34
GB13028-RA		5.16		2.41					hypothetical protein LOC724993
GB11723-RA		6.88	0.44	2.58					apolipoprotein D-like isoform 2
GB13397-RA		10.23		6.74					vitamin K-dependent protein C
GB13049-RA		3.12							tubulin beta-1 chain-like
GB10275-RA		3.16							tubulin beta-1 chain
GB30203-RA	2.20	0.33		0.23				12.64	apidermin 3
GB15018-RA	2.11								chymotrypsin inhibitor
GB14284-RA	2.03								sorbitol dehydrogenase-like isoform 2
GB13722-RA	2.44								glucosylceramidase-like
GB18312-RA	2.22		2.05				0.11	0.10	alpha-amylase
GB11753-RA	2.14								hypothetical protein LOC408981
GB11731-RA	2.32								hypothetical protein LOC551319
DB744987	2.02	3.40							NA
GB10645-RA	3.33	2.95			2.22	2.28			NA
GB14355-RA	4.45	2.69							anosmin-1-like
GB14278-RA			9.27	7.33					putative inorganic phosphate cotransporter-like
GB14057-RA			2.43	2.08					ribonuclease H2 subunit A-like isoform 1
GB17644-RA			2.18						CUE domain-containing protein 2-like
GB10140-RA			2.00						hypothetical protein LOC411809
GB19347-RA			2.06						hypothetical protein LOC724749

GB18593-RA	10.24	NA
GB14896-RA	6.74	NA
GB14058-RA	6.51	na
GB12041-RA	6.01	band 7 protein AAEL010189-like isoform 1
GB17254-RA	4.87	neuronal nicotinic acetylcholine Apisa7-2 subunit
GB17918-RA	4.35	protein phosphatase PP2A 55 kDa regulatory subunit isoform 1
GB11904-RA	4.32	putative odorant receptor 13a-like
GB13764-RA	4.14	biglycan-like
GB19312-RA	4.01	vacuolar protein sorting-associated protein 37B-like
GB14856-RA	3.85	protein JTB precursor
GB12097-RA	3.36	SHC SH2 domain-binding protein 1 homolog B-like
NW_001260424	2.70	NA
GB17538-RA	2.38	hymenoptaecin preproprotein
GB12287-RA	2.31	neurogenic protein big brain
GB17410-RA	8.02	palmitoyltransferase ZDHHC17 isoform 1
GB10464-RA	4.37	s-adenosylmethionine mitochondrial carrier protein-like
GB15643-RA	4.30	hypothetical protein LOC408435
GB19971-RA	4.05	zinc finger protein 214-like
GB15451-RA	4.05	polynucleotide 5'-hydroxyl-kinase NOL9-like
GB18687-RA	3.88	WD repeat-containing protein 74
GB13045-RA	3.12	succinate-semialdehyde dehydrogenase, mitochondrial
GB11566-RA	3.06	homeobox protein OTX2-B-like
GB12636-RA	3.06	apidermin 3
GB11256-RA	2.97	pancreatic lipase-related protein 2-like
GB19057-RA	2.65	putative ATP-dependent RNA helicase DHX57-like
GB15116-RA	2.47	endochitinase-like
GB18336-RA	2.32	PHD finger-like domain-containing protein 5A-like [Nasonia vitripennis]
GB11020-RA	2.31	uncharacterized protein KIAA1841-like isoform 1

GB10737-RA	2.30				NA
GB30541-RB	2.29				hypothetical protein LOC411466
GB11493-RA	2.25				hypothetical protein LOC100578552
GB14248-RA	2.23				putative odorant receptor 13a-like
BB160003B20E11	2.16				NA
GB15738-RA	2.15				protein YIF1B-like
GB12853-RA	2.11				neural-cadherin
GB13325-RA	2.10				chemosensory protein 6
GB12449-RA	2.06				hypothetical protein LOC727131
GB30027-RA	2.02				NA
DB774972	0.49				NA
GB16817-RA	0.49				hypothetical protein LOC726155
GB15662-RA	0.49				dehydrogenase/reductase SDR family member 11-like
BB170013A20G10	0.46				NA
GB18863-RA	0.46				hypothetical protein LOC726155
GB13825-RA	0.45				hypothetical protein LOC100578770
GB13537-RA	0.43				hypothetical protein LOC725724
GB14077-RA	0.32				hypothetical protein LOC410402 isoform 1
GB19250-RA	0.20				hypothetical protein LOC725507
GB13681-RA	3.91			3.41	putative polypeptide N-acetylgalactosaminyltransferase 9-like
GB30529-RA	3.04				probable peroxisomal acyl-coenzyme A oxidase 1-like
AF092924	2.75				Sacbrood virus complete genome
GB16826-RA			0.47		odorant binding protein 16 precursor
GB15016-RA			0.50		heat shock protein cognate 3
GB10355-RA			0.40		melittin precursor
GB18441-RA			0.50	0.47	actin-binding Rho-activating protein-like isoform 2
GB14758-RA			0.28	0.26	heat shock protein 90
GB13619-RA		2.19	3.43		pyridoxine/pyridoxamine 5'-phosphate oxidase-like
GB30242-RA			2.23		odorant binding protein 3 precursor

GB16838-RA	2.27	hypothetical protein LOC100577512
BB160006B20F09	2.06	hypothetical protein [Plasmodium berghei strain ANKA]
GB17289-RA	4.65	n-acetylneuraminate lyase-like
GB17885-RA	3.31	hypothetical protein LOC100651411 [Bombus terrestris]
GB11550-RA	4.06	hypothetical protein LOC552685
GB14975-RA	2.44	hypothetical protein LOC552154
GB10729-RA	2.33	Putative odorant receptor 85b-like [Apis florea]
BP875367	2.02	na
GB12778-RA	2.40	acyl-CoA synthetase family member 2, mitochondrial precursor
GB13484-RA	2.15	troponin C type IIb
BB170020B20F11	0.31	na
GB17875-RA	0.36	chemosensory protein 1 precursor
GB11969-RA	0.34	acyl-CoA Delta(11) desaturase-like
GB17931-RA	0.35	fatty acyl-CoA reductase 1 >gb|ADJ56408.1| fatty acyl-CoA reductase 1
GB14823-RA	0.35	neurotrimin-like
GB15211-RA	0.36	MRJP5
GB12176-RA	0.29	elongation of very long chain fatty acids protein AAEL008004-like
GB10483-RA	0.08	venom acid phosphatase Acph-1-like
NW_001253288	0.43	ATP synthase subunit b, mitochondrial-like [Bombus impatiens]
GB19967-RA	0.46	cytochrome P450 9e2 isoform 4
GB18056-RA	0.32	dnaJ protein homolog 1-like isoform 1
GB12567-RA	0.46	long-chain-fatty-acid--CoA ligase ACSBG2
GB19085-RA	0.45	probable 4-coumarate--CoA ligase 3-like
GB19306-RA	0.45	cytochrome P450 6k1
antisense XM_394333.3	0.48	protein lethal(2)essential for life-like isoform 1
GB15426-RA	0.46	dopamine transporter
DB762418	0.44	na

GB14612-RA		0.48	cytochrome P450 6k1
GB13264-RA		0.50	elongation of very long chain fatty acids protein AAEL008004-like
DB735700		0.43	na
DB757928		0.44	na
GB10708-RA		0.49	reticulon-4 receptor-like
GB19897-RA		0.37	hypothetical protein LOC727486 isoform 1
GB19995-RA		0.41	protein lethal(2)essential for life-like isoform 1
GB19503-RA		0.33	heat shock protein Hsp70Ab-like
GB18662-RA		0.49	protein lethal(2)essential for life-like isoform 1
GB15272-RA		0.44	alpha-tocopherol transfer protein-like isoform 2
GB19070-RA		0.45	elongation of very long chain fatty acids protein 6-like
GB18360-RA	0.31		4-hydroxyphenylpyruvate dioxygenase-like
GB15810-RA	0.41	0.30	na
GB17823-RA	0.35	0.28	hypothetical protein LOC724570
GB19017-RA	0.28	0.17	alpha-glucosidase precursor
GB15548-RA	0.45	0.42	laccase-5-like