DEVELOPMENT AND APPLICATION OF HONEY BEE *IN VITRO* SYSTEMS

by

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Abstract

The honey bee, *Apis mellifera*, has become tightly linked to human agriculture as one of the most important pollinators. The recent honey bee population decline has raised global concerns of a pollination crisis, yet honey bee research lags far behind in available research tools compared to other model organisms, limiting the pace we can hope to advance our knowledge of honey bee biology and improve bee health. Therefore, the major goal of this thesis was to establish new tools and to improve some of the existing tools for honey bee research and then to demonstrate that these tools can be combined with other genetic and proteomic techniques to help us address questions on honey bee biology. First of all, honey bee primary cell cultures from various tissues could be established and maintained for at least four months. Embryonic cultures could be cryopreserved and also transduced with lentivirus to express EGFP. Proteomic analysis revealed biological pathways related to glucose metabolism and oxidative stress were significantly altered in primary cells during two weeks of cultivation. In addition to cell culture, *in vitro* larval rearing was also established and the use of various artificial diets was compared for ability to sustain growth. Basic larval diet was by far the most efficient formulation and it was applied to study honey bees' response to American foulbrood (AFB) infection. RNA interference (RNAi) was used to silence prophenoloxidase, a gene implicated in bees' resistance to AFB and a multiple reaction monitoring mass (MRM) spectrometry assay was developed to assess degree of knockdown. Although dosage response was observed in *in vitro* rearing for AFB infection, significant gene silencing could not be achieved. Overall, we established several *in vitro* systems, including cell cultures and *in vitro* larval rearing, for honey bee research and these systems in combination with lentiviral transduction, RNA interference, proteome analysis, and MRM assay could form a thorough analysis platform for future studies to improve our knowledge of honey bee biology.

Preface

A version of chapter 2 has been published. Chan, M.M., Choi, S.Y., Chan, Q.W., Li, P., Guarna, M.M. and Foster, L.J. 2010, "Proteome profile and lentiviral transduction of cultured honey bee (Apis mellifera L.) cells", Insect molecular biology, vol. 19, no. 5, pp. 653-658. I was the co-first author with SYC for this manuscript. I established the methods for sample collection, culture establishment and cryopreservation. SYC performed most of the culture maintenance and conducted the proteome comparison experiment. LJF, QWC, and I analyzed the data from proteome comparison experiment. Lentiviral transduction was done by LP at Applied Biological Materials Inc.. QWC and MMG provided valuable advices to improve culturing techniques and media composition. The manuscript was primarily written by me, with editing help and minor written contributions from my supervisor LJF and other co-authors.

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List of Abbreviations

LT_{100}	100% mortality
4E-BP	eIF4E Binding Protein
ABPV	Acute Bee Paralysis Virus
ACT5C	Actin 5C
AFB	American foulbrood
Ago-2	Argonaute-2
ALT	Alternative Lengthening of Telomeres
Am18w	Toll-related receptor 18W
BLD	Basic larval diet
bp	base-pair
BQCV	Black Queen Cell Virus
BSA	Bovine serum albumin
$Ca(ClO)_2$	Calcium hypochlorite
CBPV	Chronic Bee Paralysis Virus
cfu	Colony-forming unit
CMV	Cytomegalovirus immediate-early
CO_2	Carbon dioxide
COPIA	Copia transposon promoter
CrPV	Cricket Paralysis Virus
csd	Complementary sex determination
CSP5	Chemosensory protein 5
ddH_2O	Distilled-deionized water
DTT	Dithiothreitol
DWV	Deformed Wing Virus
dsGFP	Double-stranded RNA targeting GFP gene
dsPPO	Double-stranded RNA targeting PPO gene
dsRNA	Double-stranded RNA
EGFP	Enhanced green fluorescent protein
eIFs	Eukaryotic translational initiation factors
FBS	Fetal bovine serum
fem	Feminizer
Fmoc	9-fluorenylmethoxycarbonyl

βGRP	β-glucan recognition proteins
GNBPs	Gram-negative binding proteins
HBTU	O-(Benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium hexafluorophosphate
HCl	Hydrochloric acid
HOBT	1-hydroxybenzotriazole
KBV	Kashmir Bee Virus
IAPV	Israeli Acute Paralysis Virus
Imd	Immune deficiency
JAK	Janus kinase
m/z	Mass-to-charge ratio
MRM	Multiple reaction monitoring
NHS	N-hydroxysuccinimide
PBS	Phosphate saline buffer
PGRPs	Peptidoglycan recognition proteins
PO	Phenoloxidase
PPAE	Prophenoloxidase-activating enzyme
PPO	Prophenoloxidase
PTEN	Phosphatase and tensin
QRP	Queen retinue pheromone
rcf	Relative centrifugal force
RISC	RNA-induced silencing complex
RJ	Royal jelly
RNAi	RNA interference
SBV	Sacbrood Virus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small-interfering RNA
STAGE	STop And Go Extraction
UBC	University of British Columbia

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I offer my greatest gratitude to my parents who have worked very hard for all their lives to provide me with love and support. It is their hard work that allows me to come to study in Canada and to pursue a career of my interest. I also thank my grandma, sister, uncles, and aunts who have always been here for me and give me such a sweet home in a foreign place. Lastly, I would like to also thank my dearest friend Eric, who has always given me endless love and patience to face every challenge in life.

Dedication

To my dearest family

1 Introduction

1.1 Importance of honey bee research

The honey bee, *Apis mellifera*, has long been managed by humans for its honey production and has also become tightly linked to human agriculture as one of the most important pollinators. In the past 50 years, human agriculture had become more and more pollinator-dependent. Cantaloupe, kiwi, macadamia nut, and rowanberry are just some of the crops for which honey bee pollination is essential for crop production (Aizen et al. 2008, Klein et al. 2007). The economic value of insect pollination is estimated at $14.4 billion per annum in North America and the annual crop value attributed to honey bee pollination in Canada is over $2.5 billion (Gallaia et al. 2009, Lee 2011). The recent increase in over-winter honey bee colony losses in different parts of the world (34.4% - 42.2% in USA (vanEngelsdorp et al. 2011), 36% in Canada (Currie, Pernal & Guzmán-Novoa 2010), 25% in Europe (Potts et al. 2010b) has raised concerns about the global pollination crisis. Pests and pathogens, environmental stressors, genetic diversity and vitality are three important drivers for pollinator declines (Potts et al. 2010a). Therefore, there is an urgent need for the development of honey bee research tools in order to enhance our understanding of honey bee biology and prevent the bee population from undergoing further decline.

1.2 Honey bee biology

Honey bees, ants, wasps, and termites are eusocial organisms, characterized by an advanced level of colonial existence: Adult colonial members belong to two or more overlapping generations, care cooperatively for the young, and are divided into reproductive and non-reproductive castes (Wilson, Holldobler 2005). In the case of honey bees, there are three different castes: Queens, workers and drones (Figure 1.1), with each caste having distinct roles in the colony.

Figure 1.1 Honey bee castes.
Queens (left) have long and enlarged abdomen, and the cavity inside the abdomen is mainly taken up by their developed ovaries. Drones (center) are characterized by having a more muscular thorax and abdomen. They have the biggest pair of eyes among the three castes. The workers (right) are the smallest in size but the most abundant in number among the three castes. Photo credits: Queenie Chan.

Typically, there is only one queen per colony; she is the only fertile female within the hive. The major task of the queen is to lay eggs for the whole colony, up to 200,000 eggs per year. In addition to reproduction, the queen also controls and organizes many colony functions through secretion of pheromones (Ambrose et al. 1992). Honey bee queens can live for 2-3 years, although they are often replaced after one year in commercial hives (Page, Peng 2001). Workers are the sterile females in the hive, numbering from 20,000 – 40,000 per colony (Page, Peng 2001). As their name implies, workers are the work force for the colony and their task distribution depends on their age and colony conditions. Young workers typically stay in the hive and perform tasks such as cleaning, brood and queen tending, comb building and food handling. Older workers perform outside hive tasks such as ventilating, guarding and foraging (Ambrose et al. 1992). The life span of the workers varies from three to six weeks in the summer and about four months in the winter (Page, Peng 2001). Drones are the males of the colony, typically ranging from 660-3960 in number. Drones are produced in the summer (Lee, Winston 1987), and have only one task in the colony, which is to mate with the queen. As their abdomen bursts during copulation, drones die after mating and they have no other known function (Ambrose et al. 1992). Drones are reported to live for an average of 21 days; they either die

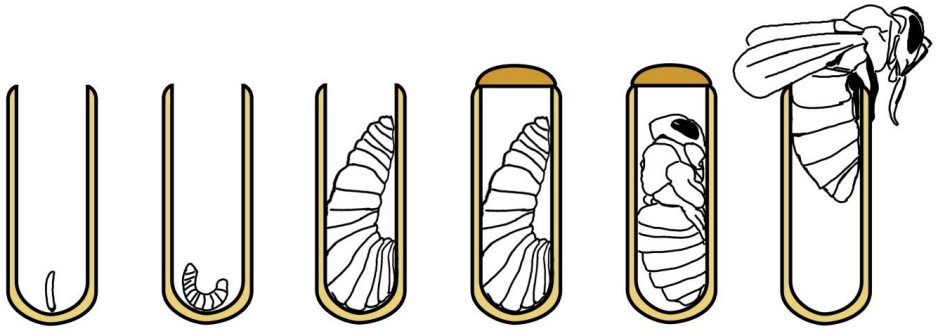
from mating or are expelled from the nest and killed by other workers by the end of summer (Page, Peng 2001).

Sex determination in honey bees follows a haplodiploidy system, which is a common sex determination system in the insect order Hymentoptera where males are haploid and females are diploid (Heimpel, de Boer 2008). Honey bee sex determination was first described by Dzierzon, who discovered that an unmated queen could only lay unfertilized eggs, hence, producing male drones exclusively (Dzierzon 1845). It is true that in most cases, the queen controls the fertilization of the honey bee eggs, only releasing sperm to worker or queen eggs. As each caste develops from hexagonal wax cells of different sizes, locations, and orientations in the hive, the queen determines the caste of the cell by inspecting the size with her forelegs and/or the angle of her abdomen during oviposition (Ambrose et al. 1992). However, fertilization is not the only sex determinant in honey bees. The discovery of diploid drones from inbreeding studies (Woyke 1963) points to the presence of a sex determination locus. The isolation of the sex determination locus leads to the discovery of a complementary sex determination (*csd*) gene, which induces alternative splicing in the gene *feminizer* (*fem*), determining the ultimate sex of the honey bee. Bees that are heterozygous at *csd* will have a functional *fem* gene and develop as females; meanwhile, bees that are homozygous or hemizygous at *csd* will have alternative splicing that introduces a premature stop codon in the *fem* gene and develop as males (Beye et al. 2003, Hasselmann et al. 2008). Diploid drones are either inviable or sterile; they are usually cannibalized by worker bees shortly after hatching (Herrmann et al. 2005).

1.2.1 Honey bee development

Understanding the developmental biology of honey bees is essential for honey bee research because various life stages have very different physiology and respond to

environmental stress in drastically different ways. Like many other insects, honey bees go through four major stages in development: Egg, larva, pupa, and adult (Figure 1.2).



	Egg	Larva	Pupa	Total
Worker	3 days	5.5 days	11-12 days	21 days

Figure 1.2 Honey bee worker developmental stages
Honey bees go through four developmental stages: Egg, larva, pupa, and adult. First of all, the queen lays an egg at the bottom of the cell. Once, the egg hatches into a larva, the nurse bees will feed the larva for 5-6 days and cap the cells. The larva turns into a pupa in the capped cell and eventually emerges as an adult.

The formation of an egg begins with the female germ cells differentiating into oocytes, which become the egg cells and the nurse cells inside the queen's ovary (Guizeit, Zissler & Fleig 1993). As the egg cells mature, they absorb the nurse cells as nutriments. Lastly, the follicles secrete the chorion over the egg cells to complete egg formation (Fleig 1995). The mature eggs are pearly white in color, approximately 1.3 – 1.8 mm in length, and have an elongated oval shape with the end to develop into the head thicker than the abdominal end. Then, the queen will deposit the egg vertically at the bottom of the honey bee comb cell (Winston 1987).

After oviposition, the larva will slowly form inside the egg over the next three days. During the first 14 hours, cleavage cells develop and migrate to the surface of the egg, forming the blastoderm. In the next 10 hours, the blastoderm undergoes several divisions and clear

space can be observed at both ends of the egg. At about 35 hours after oviposition, the blastoderm thickens at the ventroanterior region, marking the beginning of gastrulation. The head becomes visible by 49 hours after oviposition, followed by segmentation of the body (Milne, Phillips & Krell 1988). About two hours before hatching, liquid can be seen at the surface of the egg, and the chorion slowly dissolves, and the tracheal system becomes visible. Finally at 72-76 hours old, the larva fully hatches and bends into a C-shape (Collins 2004).

During the larval stage, the cells are uncapped and the nurse bees feed the larvae with a large quantity of food. The larvae go through five stages of development, known as instars, signaled by the molting of its outer skeleton approximately once a day, allowing them to grow rapidly in size. By the sixth day, the adult workers will seal the cell with wax capping and the larvae will uncurl, spin the cocoon, and defecate into the base of the cell. The duration of the larval uncapped stage varies between castes, with an average of 5.5 days for workers, 4.6 days for queens, and 6.3 days for drones. The pupal stage is the last stage of development, in which the head, eyes, antennae, mouthparts, thorax, legs, and abdomen form. The cuticle will slowly darken and can be used to determine pupal stage. Typically, it takes eight to nine days for workers and drones pupae to fully develop; meanwhile it takes only four to five days for the queens. Lastly, the pupae undergo the final molt and emerge from the cells by chewing through the wax capping. The total developmental time for the queens, workers, and drones are 16, 21, and 24 days respectively (Winston 1987).

One of the most interesting areas of honey bee research is female caste determination during larval development. In queenright colonies, the queen is the only fertile female and less than 0.01% of workers have full-sized eggs in their ovaries (Ratnieks 1993). The queen controls the workers through queen retinue pheromone (QRP), which consists of secretions from several glands such as the mandibular gland, Dufour’s gland, and tergal glands. QRP attracts workers to feed and groom the queen; further contact with other workers distributes the chemicals throughout the colony. With this rapid dispersal of the queen's signal in the colony, the queen

can prevent workers from rearing new queens and also inhibit the workers' ovarian development and reproduction (Keeling et al. 2003, Hoover et al. 2003, Le Conte, Hefetz 2008). Therefore, most of the female larvae are reared as workers in queenright situations. Interestingly, the female larvae remain totipotent for the first three days of their lives, meaning they can change their caste development in response to their diets and environment. As there is one queen per colony, the whole colony relies on a single female for reproduction and the totipotent mechanism becomes very important for honey bee colony survival. The workers can sense a queen loss within 24 hours and a new queen can be reared from remaining female eggs or larvae to ensure the colony can continue to reproduce (Hatch, Tarpy & Fletcher 1999). It is generally accepted that female castes are determined by the quality and quantity of food fed to the female larvae, although other factors such as genetics and cell sizes also contributes to caste differentiation (Winston 1987, Kucharski et al. 2008, Shi et al. 2011). Worker larvae transferred to queen cells within the first three days can fully develop into queens and vice versa; however, if transferred beyond three days old, the castes are not reversible or the larvae can at most develop into intercaste (Woyke 1971, Weaver 1957). Queen larvae are provided with a much greater quantity of royal jelly (RJ) than worker larvae. Early studies show that queen larvae consume 13% more food than worker larvae in the first three days and the difference increases to approximately 40% more food by the sixth day (Dietz, Lambremont 1970). Electrophoretic analysis also revealed different protein components in larval food for queens and workers (Patel, Haydak & Gochnauer 1960). After the third day of larval life, workers are provided with a mixture of royal jelly, honey, and pollen; meanwhile, queen larvae remain on a diet of pure RJ. This nutritional switch is believed to be the key event in caste differentiation (Winston 1987). Factors in larval food that can affect caste differentiation have been further investigated using laboratory rearing. Two to three days old larvae fed with worker jelly supplemented with 8% glucose and 8% fructose developed into workers; however, if larvae are fed with 20% glucose and 20% fructose, they can develop into queens, intercastes, and workers

(Asencot, Lensky 1976). Moreover, when a highly hydrophilic low molecular weight fraction of RJ extract was added to an artificial diet without queen inducing power, up to 59% of larvae emerged as queens (Rembold, Lackner & Geistbecka 1974). Later, it was discovered that this nutritional switch acts on the endocrine system of honey bees. Topical application of a juvenile hormone to three to five days old larvae induces queen-like individuals (Asencot, Lensky 1976, Wirtz, Beetsma 1972, Rembold, Czoppelt & Rao 1974). Further investigation showed that juvenile hormone prevented ovary programmed cell death in fifth instar worker larvae (Capella, Hartfelder 1998). Nutritional sensing pathways, IIS (insulin/insulin-like signalling) and TOR (target of rapamycin) are shown to control caste differentiation via a juvenile hormone. Rearing on a queen-inducing diet, RNAi knockdown of components in either pathway resulted in worker phenotype (Patel et al. 2007, Wolschin, Mutti & Amdam 2011, Mutti et al. 2011a). Topical application of juvenile hormone can rescue the queen phenotype in knockdown larvae (Mutti et al. 2011a). Recently, another 57-kDa protein (royalactin) from royal jelly was also shown to induce queen differentiation. Young larvae reared on a royalactin-depleted diet emerged as workers; when supplemented with recombinant royalactin, the larvae emerged as queens. Royalactin acts through the epidermal growth factor receptor (EGFR) pathway, which induces queen characteristics through the elevation of juvenile hormone titer, and activation of both mitogen-activated protein kinase and p70 S6 kinase (Kamakura 2011). Other effects of differential feeding with less defined mechanisms include the alteration of gene expression (Barchuk et al. 2007) and DNA methylation levels (Kucharski et al. 2008) in different castes.

1.2.2 Honey bee nutrition

Nutrition is tightly linked to the honey bee's development, caste differentiation, overall colony health, and susceptibility to various diseases. Basic nutrients required for normal growth in honey bees include proteins, carbohydrates, minerals, fats, vitamins, and water. These nutrients come from nectar, pollen, and water collected by forager bees. Pollen serves as the

major protein source for the bees, while nectar, which is ripened into honey, serves as the major carbohydrate source (Ambrose et al. 1992). Adult queens are fed by nurse bees, while adult workers and drones need to feed themselves. On the contrary, all larvae are fed by the workers. During the larval period, larvae are inspected an average of 1926 times, but are only fed during 143 visits (Lindauer 1952). The number of inspections and feedings vary with the ratio of larvae to nurse bees, going up to 7200 visits or 1140 feedings per larva (Lineburg 1924, Kuwabara 1947). Inspection and feeding frequencies are also caste dependent: Up to 1600 feedings have been observed for queen larvae (Haydak 1970).

The quantity and quality of food fed to each larva are also caste and age dependent. Throughout the whole larval stage, queen larvae are supplied with an abundance of royal jelly, which is a secretion from both the hypopharyngeal gland and mandibular gland of nurse bees. The RJ consists of 1:1 ratio of clear and milky-white secretions. Each has a high protein content of 110.5 mg/g and 140.5 mg/g respectively. The total amount of food fed to a queen larva is about 1.5 g. In contrast to queen larvae, worker larvae are not provided with RJ during the entire larval stage. Young worker larvae up to 2.5 days old are also fed with a mixture of clear and milky-white secretions, but in a proportion of about 3:1 or 4:1, and the amount fed to the worker larvae is only 1.7 mg. Worker larvae older than three days are fed with pollen and honey, in addition to the clear secretion. The composition of drone food is similar to that of worker food, and it also begins with a mixture of clear and milky-white secretions, with the addition of pollen and honey for older drone larvae. However, drone food has more diverse proteins, more carbohydrates, and other nutrients than worker food, probably due to the increased quantity of honey and pollen fed to older larvae. Drone larvae receive more food from the nurse bees, an average of 9.6 mg, almost six times more than worker larvae. Feeding stops when the cells are capped, and only resumes when the bees emerge as adults. Only adult queens will continue to be fed with RJ by the nurse bees for the rest of their lives. Young drones are fed by the workers with brood food for the first few days and they feed themselves from honey cells afterwards. For

adult workers, they mostly feed themselves with honey as an energy source. Younger workers also consume pollen, which is essential for post-emergence glandular development and growth of internal structures during the first eight to ten days of their lives (Winston 1987, Haydak 1970).

1.3 Honey bee innate immunity

During development, honey bees are constantly exposed to different pathogens. Therefore, understanding how honeybees respond to pathogens at both a social and individual level becomes an interesting area of study. Social responses against pathogens include hygienic behavior, grooming, and undertaking. These approaches reduce disease risk of nestmates through removal of the pathogen or infected individuals from the hive. In addition to such social responses, individual innate immunity is also important for honey bees' survival, and individual innate immunity can be divided into cellular and humoral responses (Evans et al. 2006, Evans, Spivak 2010).

1.3.1 Cellular response

The cellular immune response is mediated by hemocytes, which are insect blood cells circulating in the hemolymph. In insects, prohemocytes can differentiate into three primary kinds of hemocytes: Crystal cells, plasmatocytes, and lamellocytes (Lemaitre, Hoffmann 2007). These cell types or analogs are present in honey bees as well (De Graaf et al. 2002). Plasmatocytes consist of 90-95% of all mature larval hemocytes, and they are responsible for phagocytosis of dead cells and pathogens. Lamellocytes are large, flat, adherent cells rarely found in healthy individuals, but often prohemocytes differentiate into lamellocytes upon infection. Lamellocytes are responsible for encapsulation and neutralization of objects too large to be phagocytosed. Crystal cells are non-phagocytic hemocytes, mature crystal cells are fragile and readily disrupt to release propheonoloxidase, an enzyme involved as a key player in the melanization process (Lemaitre, Hoffmann 2007). Honey bees also possess granulocytes, which is a class of

hemocytes needed for phagocytosis in wasps (Strand et al. 2006). Cellular responses mediated by the above classes of hemocytes can be subcategorized into phagocytosis, nodulation, and melanization, which are further discussed in the sections below.

1.3.1.1 Phagocytosis

Plasmatocytes can engulf a wide variety of particles within minutes, such as bacteria, yeast, double-stranded RNA (dsRNA), or even ink particles (Lemaitre, Hoffmann 2007). The phagocytosis process involves the phagocyte attaching to the target, undergoing cytoskeleton modification, internalization, and destruction of the engulfed target. Epidermal growth factor domain protein Eater and Immunoglobulin superfamily domain protein Dscam have been shown to be important for phagocytosis. Eater is expressed on plasmatocytes, and binds to and helps to internalize bacteria (Kocks et al. 2005). Similarly, Dscam is also involved in binding of bacteria and hemocytes-specific loss of Dscam has reduced efficiency in phagocytic uptake of bacteria (Watson et al. 2005). Phagocytosis of *Nosema apis* spores is well documented by Gilliam (1967), although the debate remains whether this is an effective immunity defense or simply reflects an invasion strategy used by the microsporidia (Evans, Spivak 2010).

1.3.1.2 Nodulation

Nodule formation happens when foreign substances cannot be removed from circulation by phagocytosis. Hemocytes loaded with bacteria are entrapped by coagulum produced by granular cells and less pathogenic bacteria are melanized in the nodules while pathogenic bacteria may subsequently break from the nodules and return to hemolymph (Gliński, Jarosz 2001). Noduler, a novel protein discovered in silkmoth *Antheraea mylitta*, was found to be a key player in nodulation. Noduler binds to a wide range of bacteria, yeast, lipopolysaccharides (LPS), lipotechoic acid, and β-1,3 glucan components of microbial cell walls, and insect hemocytes. RNA interference (RNAi)-mediated knockdown of Noduler resulted in a reduction in number of

nodules and also an increased bacterial load in hemolymph (Gandhe, John & Nagaraju 2007). Eicosanoids also mediate nodulation in newly emerged worker honey bees, with eicosanoid biosynthesis inhibitors impairing the formulation of nodules upon bacterial infection. Interestingly, nodule formation was not observed in older honey bee foragers, implying nodulation does not happen in all phases of insect life cycles (Bedick et al. 2001).

1.3.1.3 Encapsulation

Encapsulation response has mostly been studied in *Drosophila* when parasitoid wasps lay their eggs into the hemocoel of the *Drosophila* larvae (Meister 2004, Russo et al. 1996). Lamellocytes are released to form a multilayered capsule around the invader, and eventually the invader is killed by cytotoxic products or melanization (Nappi et al. 1995). Unlike nodulation, encapsulation response is not developmental stage dependent in honey bees (Wilson-Rich, Dres & Starks 2008).

1.3.2 Humoral response

The other subdivision of innate immunity is the humoral response, which works closely together with the cellular response for insects to combat pathogens. Humoral response is generally defined as the pathogen-recognition/defense events that happen in the hemolymph. Humoral defenses include production of antimicrobial peptides, reactive intermediates of oxygen or nitrogen, and the enzymatic cascades that regulate coagulation or melanization of hemolymph. Although innate immunity is subdivided into two categories, humoral and cellular responses have considerable overlaps as many humoral factors are of hemocytes origin. It is believed that the two sides of innate immune response work closely together in processes, such as pathogen recognition, and are essential for insect survival (Lavine, Strand 2002).

1.3.2.1 Pathogen recognition and immune pathways

The first step of humoral response is pathogen recognition, which triggers the activation of other immune pathways in response to the immune challenge. In honey bees, this step requires a pattern recognition receptor (PRR) to come in contact with microbial molecules. Two families of PRR working upstream of Toll and Imd pathways are peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) (Lemaitre, Hoffmann 2007). Other pathogen recognition gene families in bees include Class B scavenger receptors, β-glucan recognition proteins (βGRPs), galectins, and fibrinogen-related proteins. Once pathogens are recognized, various immune-related signaling pathways are activated to produce antimicrobial effectors to combat infection. Honey bees possess almost all the orthologs for the core members of four pathways implicated in inducible host defense: Toll, Immune deficiency (Imd), Janus kinase (JAK)/STAT, and JNK (Figure 1.3). Overall, honey bees possess the major immune pathways and immune-gene families, but the number of paralogous members in each family is reduced compared to the *Drosophila and Anopheles* genomes. This is probably due to the honey bees' strong social barrier to diseases or that the bees are more likely to be attacked by a limited set of highly co-evolved pathogens (Evans et al. 2006).

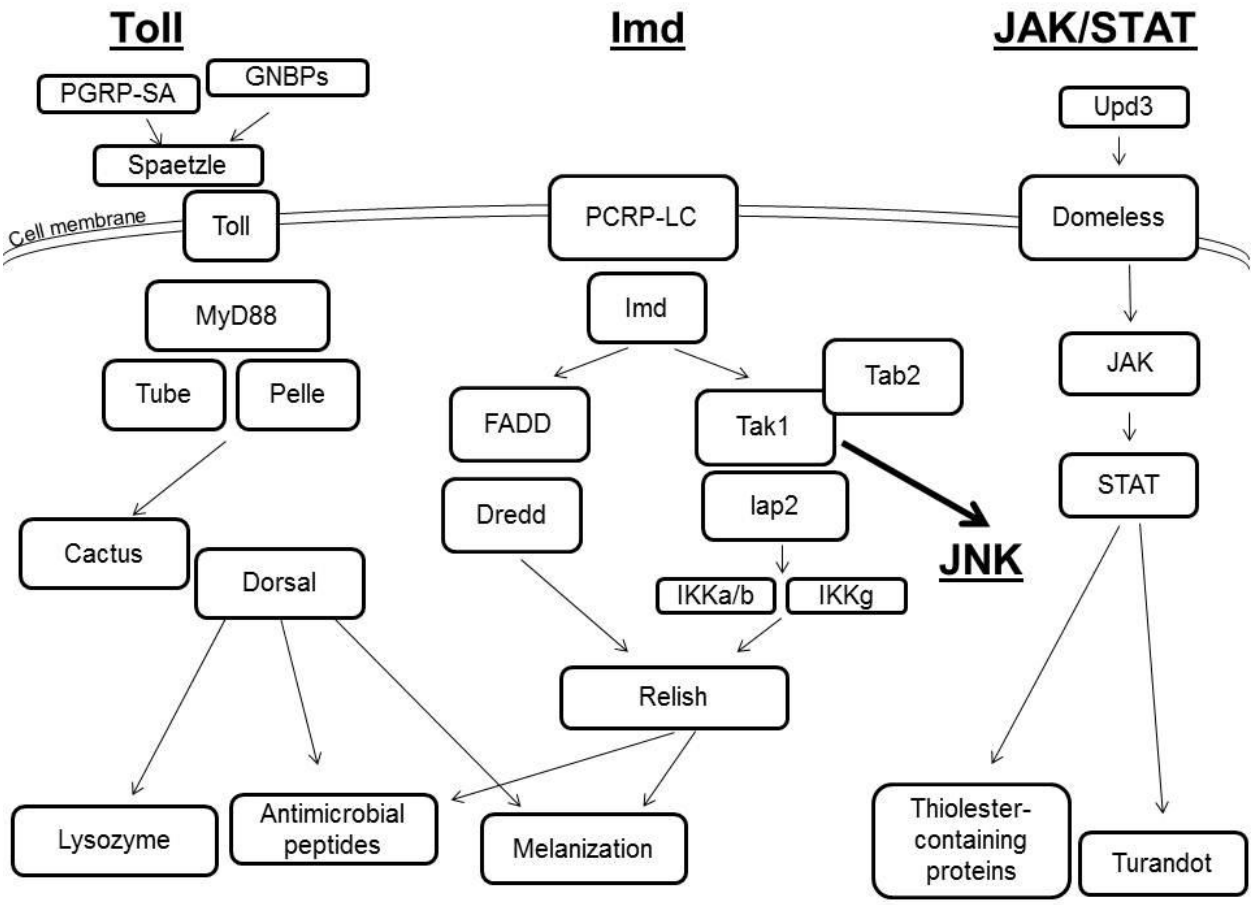


Figure 1.3 Components of three of the primary immune pathways expressed in bees.

1.3.2.2 Toll pathway

The Toll pathway is evolutionarily conserved and plays critical roles in both immunity and development. The extracellular cytokine Spaetzle, binds to the transmembrane receptor Toll, which recruits the Tube and MyD88 adaptors and the Pelle kinases, to form a receptor complex. Activation of this complex leads to degradation of NF kappa B inhibitor Cactus and nuclear transportation of the NF-κB transcription factor Dorsal. Effectors of the Toll pathway include: A complement of antimicrobial peptides, the melanizing agent phenoloxidase and three lysozymes (Evans et al. 2006). The Toll pathway is implicated in immune response against fungal and Gram-positive bacterial infection (Lemaitre et al. 1996, Rutschmann, Kilinc & Ferrandon 2002).

1.3.2.3 Immune deficiency (Imd) pathway

Unlike the Toll pathway, the Imd pathway is specific for immunity and is not essential for normal development (Lemaitre et al. 1995). The Imd pathway responds mainly to Gram-negative bacterial infection, but Gram-positive bacteria with diaminopimelic acid-type peptidoglycans can also act as strong elicitors (Evans et al. 2006). Other peptidoglycan and fungi can also trigger a weak response (Stenbak et al. 2004). Acting through the NF-κB transcription factor Relish, the Imd pathway induces production of all major antimicrobial peptides in *Drosophila* (Hedengren et al. 1999). In addition, the Imd pathway also activates the JNK pathway, providing positive and negative feedbacks for the expression of antimicrobial peptides (Boutros, Agaisse & Perrimon 2002, Wojda, Kowalski & Jakubowicz 2004). Interaction of the Toll and Imd pathway have a synergistic effect in immune response, potentially through forming heterodimers of transcription factors induced by both pathways (Tanji et al. 2007).

1.3.2.4 JAK/STAT pathway

The JAK/STAT pathway can be induced by complement-like factors, over-proliferation of hemocytes, and recently has also shown to participate in antiviral response in *Drosophila* (Agaisse, Perrimon 2004, Dostert et al. 2005). Although the major ligand, Upd, of JAK/STAT pathway is not present in honey bee, the presence of the gp130 cytokine receptor homologue Domeless and other components of the signaling pathway suggest that the JAK/STAT remains intact in honey bees as part of the innate immunity system (Evans et al. 2006).

1.3.2.5 Antimicrobial peptides

Antimicrobial peptides are major effectors in the humoral innate immunity response against pathogens. They are small proteins (typically cationic and <100 amino acids in length) often synthesized by the fat bodies (an organ similar to the mammalian liver) in insects and are circulated in hemolymph freely (Bulet, Stocklin 2005). Honey bees have only six antimicrobial

peptides (AMPs), much less than the 20 and the nine found in *Drosophila* and *Anopheles* respectively. The six AMPs include two conventional defensins, two proline-rich forms known as abaecin and apidaecin, and two honey bee specific AMPs apisimin and hymenoptaecin (Evans et al. 2006). Defensins are found in all insects and are classified into two sub families: antibacterial and antifungal. Antibacterial defensins are efficacious against Gram-positive bacteria; meanwhile, antifungal defensins are predominantly effective against filamentous fungi (Bulet, Stocklin 2005). Unlike the other insect defensins, the two 51 amino acid long honey bee defensins are amidated and have an extra stretch of 11 amino acids at their C-terminus. They have been shown to be effective against both fungus and also *Paenibacillus larvae*, the Gram-positive bacteria causing American foulbrood disease of honey bees (Klaudiny et al. 2005). Abaecin and apidaecin belong to another common class of proline-rich AMPs; abaecin (34 amino acids long) targets both Gram-positive and Gram-negative bacteria through permeabilizing the outer bacterial membrane (Casteels et al. 1990), and conversely, apidaecin (18 amino acids long) targets mostly Gram-negative bacteria by binding irreversibly to the bacterial periplasmic space or inner membrane (Li, Ma & Zhou 2006). Hymenoptaecin is 93 amino acids long with a characteristic 2-pyrrolidone-5-carboxylic acid at the N-terminus; it inhibits both Gram-positive and Gram-negative bacteria by sequential permeabilization of the bacterial outer and inner membrane (Casteels et al. 1993). Lastly, apisimin is a 54 amino acids long peptide that has been shown to associate with the oligomericapalbumin (previously named MRJP1) (Bilikova et al. 2002). Apisimin in complex with apalbumin can stimulate cell proliferation in human lymphoid cells, suggesting apisimin alone or in complex with apalbumin can activate different cellular process in honey bees as well (Tamura et al. 2009).

1.3.2.6 Melanization

In addition to the secretion of antimicrobial peptides, melanization is another important effector of the humoral innate immunity response, particularly in the processes of wound healing, nodulation, and encapsulation. In the process of melanization, tyrosine is converted to dopa and then dopaquinone by activated phenoloxidase (PO). Dopaquinone is then non-enzymatically converted to dopachrome, which is further converted to 5,6-dihydroxyindole by dopachrome-conversion enzyme. 5,6-dihydroxyindole oxidizes and polymerizes to form melanin (Figure 1.4). PO was found to exist as inactive propheonoloxidase (PPO), which can be activated by a serine protease (PPO-activating enzyme, or PPAE) in both hemolymph and cuticle (Marmaras, Lampropoulou 2009). Honey bees have only one copy of the PPO gene, and like the PPOs in other insects, it lacks a signal peptide and has the consensus sequence NRFG around the activation site (Zou et al. 2006). PPO was not detected in one or two day old honey bee larvae, but was found to be expressed starting from day 3. Increased expression was detected as pupae developed into adults (Chan et al. 2009, Zufelato et al. 2004). PPO and PPAE were both found to be activated upon *P.larvae* infection (Evans et al. 2006, Chan et al. 2009).

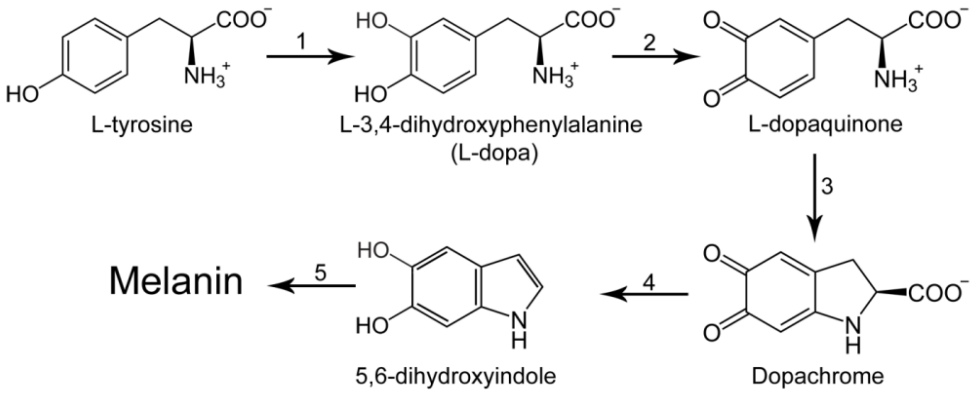


Figure 1.4 Conversion of L-tyrosine to Melanin.
Step 1 and 2 are catalyzed by activated prophenoloxidase. Step 3 is non-enzymatic reaction. Step 4 is catalyzed dopachrome conversion enzyme. Step 5 involves oxidation and polymerization of 5,6-dihydroxyindole to form melanin.

1.3.3 RNA interference

While innate immunity against bacterial and fungal infections is mediated by toll, imd, and the other immune pathways mentioned above, innate immunity against viral infections is mediated by the RNA interference (RNAi) pathway in insects. The presence of dsRNA is generally sensed as a danger signal by the cells, and an antiviral RNAi response is often triggered. The RNAi pathway begins with the cleavage of long dsRNA into 21-nt small interfering RNA (siRNA) by ribonuclease Dicer. Dicer and the R2D2 heterodimer will load the siRNA into the RNA-induced silencing complex (RISC) containing Argonaute-2 (Ago-2). The thermodynamically less stable passenger of siRNA is cleaved and degraded by Ago-2 and the adaptor molecule C3PO. The remaining siRNA strand acts as the guide strand and is 2'-*O*-methylated at the 3' terminal end by S-adenosylmethionine-dependent methyltransferase Hen-1 (DmHen-1), forming the mature RISC. The mature RISC uses the guide strand to bind complementary RNA sequence; upon full complementary, target RNA will be cleaved by Ago-2. However, if central mismatches exist between the guide strand and target RNA, Ago-2 will induce translation repression of the target RNA instead of RNA cleavage (van Mierlo, van Cleef & van Rij 2011). Evidence supporting RNAi employed as an antiviral response in insects include: RNAi-deficient mutant flies were more susceptible to positive strand RNA viruses (Zambon, Vakharia & Wu 2006, Campbell et al. 2008), accumulation of viral siRNA was observed in both *Drosophila* and *Anopheles* during viral infection (Sanchez-Vargas et al. 2004, Wu et al. 2010), and viruses encode for viral suppressors of RNAi as counterdefense against the insect RNAi system (van Rij et al. 2006, Nayak et al. 2010). In the case of honey bees, the effective treatment of bees with dsRNA against Israeli Acute Paralysis Virus (IAPV) shows that honey bees also possess the machinery needed for RNAi antiviral response (Hunter et al. 2010).

1.4 Honey bee pathogens

As pathogens are important drivers behind the population decline of honey bees, there is a strong need for researchers to understand the honey bee pathogens and also their interactions with the host. The major honey bee pathogens can be divided into three groups: viruses, fungus, and bacteria.

There are approximately 20 positive-strand RNA viruses that infect honey bees, primarily from the families of *Dicistroviridae* and *Iflaviridae* (Evans, Schwarz 2011). These viruses affect the morphology, physiology, and life span of the bees. Some viral infections have defined disease symptoms. For example: deformed wings virus (DWV) infected bees often have shrunken, crumpled wings, decreased body size, and discoloration in adult bees; chronic bee paralysis virus (CBPV) infected bees are characterized by abnormal trembling of the body and wings, causing them to crawl on the ground due to their flight inability, bloated abdomens, and dislocated wings. Nonetheless, bees can also be infected without apparent symptoms (Chen, Siede 2007). Therefore, it is very difficult to establish direct causal relationship of viruses with particular diseases, although there was evidence that Israeli Acute Paralysis Virus was associated with the bee population decline in the U.S (Cox-Foster et al. 2007). In studies of prevalence of honey bee viruses, multiple viruses could be detected in a single bee or multiple viruses could be detected from various bees in a single colony (Chen et al. 2004, Chen, Pettis & Feldlaufer 2005, Baker, Schroeder 2008). Interestingly, the prevalence of the viruses appeared to be temporal. From the study monitoring US commercial bee keeping operations for over 10 months, most viral infections occurred in the summer. Typically, a single virus was detected in multiple colonies, but infection did not persist over time (Runckel et al. 2011).

Little is known about the virulence mechanism of honey bee viruses. However, insights can be gained from Cricket Paralysis Virus (CrPV), a close relative of honey bee viruses in the *Discistroviridae* family. During viral infection, viruses hijack the host translational machinery for

viral replication. There is always a competition between the virus to shut off host translation and the host cell antiviral response to shut off viral translation. Often, viruses inhibit host translation through targeting eukaryotic translational initiation factors (eIFs) (Walsh, Mohr 2011). In the case of poliovirus infection, eIF4G isoforms are cleaved by viral 2A protease, and the cleavage of eIF4GII coincides with the shutoff of host protein synthesis (Gradi et al. 1998). Poly(A)-binding protein is also cleaved by viral protease 3C during poliovirus infection, inhibiting translation at later times of infection (Joachims, Van Breugel & Lloyd 1999, Kuyumcu-Martinez et al. 2004). In addition to protein cleavage, poliovirus and encephalomyocarditis virus infection also induce dephosphorylation and activation of eIF4E Binding Protein (4E-BP), inhibiting eIF4G recruitment in cap-dependent translation (Gingras et al. 1996, Connor, Lyles 2002). During viral infection, the host counteracts by releasing an antiviral response. One antiviral response is to phosphorylate eIF2α, resulting in global protein synthesis inhibition, inhibiting both host and viral translation (McInerney et al. 2005, Berlanga et al. 2006). To get around the antiviral response, viruses such as poliovirus and hepatitis C virus contain internal ribosome entry sites (IRES), which can recruit ribosomes in a cap-independent manner and requires only a subset of eIFs (Hellen, Sarnow 2001, Doudna, Sarnow 2007). The intergenic region (IGR) IRES found in the *Dicistroviridae* family can recruit ribosome without translation initiation factor by mimicking tRNA structures, mediating translation even when functions of multiple eIFs are compromised (Jan 2006). As many bee viruses belong to the *Dicistroviridae* family, it is likely that shutting off host translation and IGR IRES might play important roles in viral infection in honey bees.

Honey bee viruses employ both horizontal and vertical modes of transmission. Foodborne and fecal-oral transmission are two forms of direct horizontal transmission. In honey bee colonies, all larvae are fed by worker bees and trophallaxis (inter-adult feeding) is also common. Individuals could easily be infected by ingesting virus-contaminated food. Previous studies have detected Kashmir Bee Virus (KBV) and Sacbrood Virus (SBV) in honey, pollen, and royal jelly, as well as in all developmental stages of bees (Shen et al. 2005a). Additional

viruses such as Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), CBPV, and DWV had also been detected in honey and pollen and BQCV and DWV were detected in over 80% of brood and adult workers (Chen, Evans & Feldlaufer 2006). This evidence suggests that virus-contaminated food could transmit viruses, although successful infection could still be dependent on viral load in the contaminated food (Chen, Siede 2007). Additionally, fecal-oral transmission may also spread viruses among bees. BQCV and DWV have been detected in the feces of honey bee queens, and KBV has also been detected in worker feces (Chen et al. 2006, Hung 2000). The feces may contaminate bee food and infect other bees through oral ingestion. Indirect horizontal transmission of viruses happens through the parasitic mite *Varroa destructor,* which feeds on hemolymph of the developing bee larvae and pupae. *Varroa* mite infected bees often have decreased body weight and shorter life spans (Rosenkranz, Aumeier & Ziegelmann 2010). Moreover, these mites also act as a vector for many honey bee viruses, transmitting virus by injecting viral particles into hemocoel of honey bee pupae (Gisder, Aumeier & Genersch 2009) and activating latent virus infections (Shen et al. 2005b). Vertical transmission of viruses is also possible, and this is supported by the detection of viruses in the ovaries of queens, the semen of drones, and also on the surface sterilized eggs (Chen, Evans & Feldlaufer 2006, Yue et al. 2007, Yue et al. 2006). The virulence of viruses is governed by the competition between the two transmission pathways. For horizontal transmission, virulence increases with the production of high numbers of pathogens, as this can increase the chance of host exploitation and rate of transmission. On the contrary, virulence decreases with vertical transmission as the hosts' survival and reproduction are essential for pathogen reproduction. Therefore, both horizontal and vertical transmissions are essential for viruses' survival. When honey bee colonies are under a non-competitive healthy state, viruses often use vertical transmission and exist as a latent infection. However, when under stress such as co-infection with other pathogens, viruses may switch to horizontal transmission to replicate to higher numbers,

resulting in host death or possibly colony collapse (Chen, Siede 2007, Lipstich, Siller & Nowak 1996).

The second category of honey pathogens is fungi belonging to the phylum Microsporidia, namely *Nosema apis* and *Nosema cerane*. Both infect adult honey bee midgut epithelial cells and spread throughout the colony in the form of infectious spores (Forsgren, Fries 2010). *Nosema* infection often occurs through the ingestion of spores with food or water; the spores germinate and multiply inside the bee midgut cells and eventually break through the midgut cells and are released with the feces. Hence, fecal-oral transmission is a prominent route for *Nosema* infection to be spread in the colony. In addition to midgut tissues, *Nosema cerane* can also spread to other tissues such as hypopharyngeal glands, malpighian tubules, and fat bodies. (Chen et al. 2009). *Nosema* infected colonies were found to be under higher energetic stress, with positive correlation of parasite loads and sugar consumption. The increased nutritional demand is probably due to degeneration of midgut cells in infected bees, diminishing nutrient absorption. Eventually, the infected colonies suffer from higher mortality, probably due to poor thermoregulation and starvation (Higes et al. 2007, Martin-Hernandez et al. 2011).

In addition to viral and fungal infection, bacterial diseases also affect bee health significantly. Honey bee larvae are infected by two firmicute bacteria, *Paenibacillus larvae* and *Melissococcus plutonius*, causative agents of American foulbrood and European foulbrood respectively. These two brood diseases are highly infectious and often kill the larvae before pupation (Forsgren 2010, Genersch 2010). In addition, two mollicute bacteria, *Spiroplasma apis* and *Spiroplasma melliferum* parasitize adult honey bees, causing systemic infection when they breach the gut barrier and invade hemolymph, often leading to lethality as in May disease and spiroplasmosis (Mouches et al. 1983, Clark et al. 1985). Although these diseases are under better control nowadays compared to a decade ago, occasional outbreaks still happen and continuous efforts are given to develop new treatments to these diseases (Ministry of Agriculture 2010a, Ministry of Agriculture 2010b).

1.4.1 American foulbrood

One of the bacterial diseases particularly of interest in this thesis is American foulbrood (AFB), caused by Gram-positive, spore-forming bacteria, *Paenibacillus larvae*. The spores of *P. larvae* are the only infectious form of the bacteria (Hornitzky 1998). Larvae less than three days old are highly susceptible to the disease, and a dosage of 10 spores per larvae is sufficient to cause mortality (Brødsgaarda, Ritterb & Hansena 1998, Genersch, Ashiralieva & Fries 2005). However, the spores hardly affect older larvae and adult bees (Wilson 1971, Riessberger-Galle, von der Ohe & Crailsheim 2001). In most cases, infected larvae died in 7-12 days as engorged larvae, just at the onset of metamorphosis, but occasionally infected dead pupae could also be found (Genersch 2010). Diseased larvae will turn into ropy masses and eventually form a hard scale at the bottom of the cell, containing billions of spores (Shimanuki, Knox 2000). *P. larvae* spores are resistant to heat, cold, and desiccation (Forsgren, Stevanovic & Fries 2008). These properties make them highly infectious and are effectively transmitted horizontally within and between colonies. *P. larvae* can be classified into four subtypes of different virulence, ERIC I to ERIC IV. All four subtypes could induce AFB clinical symptoms, but only ERIC I and ERIC II were isolated from AFB diseased colonies (Genersch et al. 2006). ERIC I can induce 100% mortality (LT_{100}) in 12 days, meanwhile ERIC II induces LT_{100} of seven days. Therefore, ERIC II was considered the more virulent genotype on an individual larval level (Genersch, Ashiralieva & Fries 2005). However, at the colony level, AFB infected larvae were often removed by nurse bees due to hygienic behavior. In this case, ERIC II infected larvae were cleaned out four times more often than ERIC I infected larvae, probably due to ERIC II killing the larvae before the onset of metamorphosis and nurse bees could detect the disease progression more easily. Hence, ERIC I was more virulent at a colony level, likely to persist in a colony for a longer time and produce more spores for disease transmission (Rauch et al. 2009).

American foulbrood could be transmitted by both horizontal and vertical transmission. The major routes of horizontal transmission include rearing new larvae in cells previously containing diseased larvae for intra-colony transmission, and robbing disease infected hives or establishing a new site in disease-infected hives in swarming for inter-colony transmission (Fries, Camazine 2001). Although less effective compared to horizontal transmission, vertical transmission is also possible for AFB, from mother colony to daughter colony. It was shown that swarming of clinically diseased colonies could transmit spores to a new colony, decreasing spore load initially after swarming but returning to a detectable level 13 weeks post swarming (Fries, Lindstrom & Korpela 2006). Young larvae often ingest AFB spores from contaminated food and the spores germinate in the gut epithelium into rod-shaped vegetative states. It was initially thought that the bacteria entered the gut epithelium by phagocytosis (Gregorc, Bowen 1998). However, recent studies showed that the bacterium enter the epithelium paracellularly (Yue et al. 2008). It has been proposed that secreted proteases were used by *P. larvae* to degrade host epithelial cell junction (Antunez et al. 2011, Dancer, Chantawannakul 1997) and additional potential virulence factors such as toxins, hemolysins, and proteins conferring antibiotic resistance were also found in the updated *P. larvae* genome assembly (Chan et al. 2011). A definite role of these virulence factors has yet to be shown.

Currently, the mechanism behind the three days old honey bee's resistance to AFB remains unknown. Early electron microscopic studies have shown that *P. larvae* spores ingested by honey bee larvae less than three days old germinated in the midgut and entered the hemocoel through phagocytosis. It was suggested that the increasing thickness of peritrophic membrane acted as a barrier hindering bacterial penetration of the midgut cells (Davidson 1970, Davidson 1973). Later fluorescence *in situ* hybridization (FISH) analysis demonstrated that vegetative cells of *P. larvae* penetrated midgut epithelium paracellularly instead of via phagocytosis. Nonetheless, the study supported that peritrophic membrane indeed restrained most of the bacteria in the midgut lumen (Yue et al. 2008). In addition to a physical barrier, other

factors may also help to confer resistance in older honey bee larvae. This is supported by the fact that the rate of germination was lower when spores were fed to two days old larvae compared to just hatched larvae (Bamrick 1967). Extracts from adults and larvae older than four days contained a substance that inhibited *P. larvae* germination and growth (Riessberger-Galle, von der Ohe & Crailsheim 2001, Crailsheim, Riessberger-Galle 2001). Previous studies showing elevated level of propheonoloxidase (PPO) activation upon AFB infection and its parallel timing to PPO's increased expression in third instar larvae suggest that PPO may play an important role in AFB resistance (Evans et al. 2006, Chan et al. 2009). Further experiments are needed to validate the definitive role of PPO in honey bees' resistance against American Foulbrood infection.

1.5 *In vitro* systems for other model organisms

Much of our knowledge on insect biology has come from studying model insects such as *Drosophila* using various *in vitro* systems. *In vitro* is the Latin word for "in glass", often used to describe experiments conducted in a test tube or other glass or plastic vessels in the laboratory (Alberts et al. 2002). Maintenance of life by a living organism is a complex process involving its interactions with the environment externally and internally. In order for us to study these interactions in a controlled manner, *in vitro* systems offer techniques with reduced complexity through ways such as isolating the cells from the organism to minimize biological variations and taking the organism away from its natural environment to reduce interactions with external environmental factors. Two common *in vitro* systems used in model organisms are cell cultures and *in vitro* rearing.

1.5.1 Cell cultures

The history of primary cell culture can be traced back to Harrison (1907) who studied frog nerve cells under a hanging drop culture. The next hallmark of human cancer cell line

development was Earle (1943) who generated a rodent continuous cell line. Not long after, Gey *et. al* (1952) produced the first human immortalized cell line – HeLa cells. With the development of cell culture medium (Eagle 1955), cell cultures became one of the most widely used *in vitro* systems used in research. Most cells isolated from tissues from different organisms can be maintained in a tissue culture dish given the right environment, generally defined by incubation temperature, atmospheric carbon dioxide concentration, humidity, and the cell culture media used. Typical culture media are composed of various amino acids, vitamins, salts, serum or other proteins, and antibiotics. Cells isolated from tissue without any cell proliferation are called primary cell cultures; any subsequent cultures from cell proliferation are called secondary cultures (Alberts et al. 2002). Early continuous cell lines were often derived from embryos or tumors and these lines often automatically proliferate indefinitely by continuous passaging (Todaro, Green 1963, Fogh, Fogh & Orfeo 1977, Fogh, Wright & Loveless 1977). However, continuous proliferation is not often observed in cells isolated from normal tissue and immortality has to be induced by various techniques such as exposure to chemical carcinogens or X-ray (Endo et al. 1990, Bols et al. 1992, Kino et al. 1997) and transfection with oncogenes and viral proteins (Darnbrough et al. 1992, Thenet et al. 1992, Sugimoto et al. 2004). The limited lifespan for primary cultures was later attributed to the continuous shortening of their telomeres (Counter et al. 1992). Over 70% of human cell lines achieved immortality *in vitro* by activation of telomerase and approximately 25% of the remaining immortalized cell lines have acquired the mechanism for Alternative Lengthening of Telomeres (ALT) (Bryan, Reddel 1997). Interestingly, introduction of telomerase alone can also induce cell immortality without the lengthening of telomeres (Zhu et al. 1999). Hence, introduction of telomerase alone or in combination of an oncogene gained popularity as a tool for cell immortalization (Cascio 2001). Currently, there are more than 3600 cell lines from over 150 different species available, consisting of over 950 cancer cell lines and other collections such as stem cells (ATCC). Some commonly used immortalized cell lines include: HeLa (human epithelial cells), 3T3 (mouse fibroblast), S2

(*Drosophila* macrophage-like cells), Sf9 (fall armyworm ovary cells) (Alberts et al. 2002). Although immortalized cells are convenient for us to conduct research in a controlled environment, proteome profiling reveals that immortalized cell lines can have significant rearrangement of metabolic pathways, up-regulation in cell cycle-associated functions, and down-regulation of enzymes characteristic of origin tissues (Craven et al. 2006, Pan et al. 2009, Merkley et al. 2009). Therefore, it is important to note the altered characteristic of immortalized cells, and validation *in vivo* may be needed when referring to cell functions in specific tissue origin.

1.5.2 *In vitro* rearing

In addition to cell cultures, *in vitro* rearing is another technique commonly used in research for understanding insect biology without performing field studies. Insect *in vitro* rearing or laboratory rearing is a valuable tool for biological, physiological, and behavioral studies; it is also useful for mass production of organisms for practical uses, such as in biological pest controls and pesticide testing (Grenier 2009). Early records of the laboratory reared yellow fever mosquito (*Aedesaegypti L.*) can be traced back to the mid-1930s (Kuno 2010). Protocols for *in vitro* rearing of model insects such as fruit flies (Stocker, Gallant 2008), mosquitos (Coluzzi 1964), and beetles (Beeman, Haas & Friesen 2009) are easily available. Efforts are continuously made to improve production and quality of *in vitro* reared insects for research and other purposes (Boller, van Lenteren & Delucchi 2006, van Lenteren 2003)

1.6 Available honey bee *in vitro* systems

The eusocial nature of honey bee posed a particular challenge for honey bee research because their behavior and physiology are heavily influenced by the hivemates and external environment. Hence, the lack of *in vitro* systems makes many highly controlled experiments done in other model organisms unfeasible for honey bees in the laboratory. Even though there

are over 500 insect cell lines (Lynn 2001), there are only a few reports on primary honey bee cell cultures and there is currently no immortalized honey bee cell line available. In addition to the lack of a cell line, individual organism study is also difficult due to the slow development of artificial diets for *in vitro* larval rearing. Although some success has been made in rearing using a partially defined diet, there is yet to be a chemically defined diet that we can fully manipulate for asking questions on honey bee nutrition and its effect of honey bee susceptibility to various environmental stresses.

1.6.1 Previous reports on honey bee primary cells

Although an immortalized cell line is not available, there are several reports on short or long term maintenance of primary honey bee cells. One of the earliest established primary honey bee cell cultures is neurons from the antennal lobe. These cells were maintained for up to four weeks for various studies including morphological analysis (Gascuel, Masson & Beadle 1991, Devaud et al. 1994) and characterization of olfactory receptors (Laurent, Masson & Jakob 2002, Barbara et al. 2008, Barbara et al. 2005, Gisselmann et al. 2003). In addition to antennal lobe cells, Kenyon cells from mushroom bodies have also been cultured for studying cellular and electrophysiological properties and neurogenesis (Kreissl, Bicker 1992, Bicker, Kreissl 1994, Malun, Moseleit & Grunewald 2003). Nonetheless, these cultures were short-lived and only limited experiments could be done in a few weeks. The first report of long-term maintenance of embryonic cultures appeared in 2006, in which undifferentiated cultures could be obtained from honey bee eggs and maintained for more than three months (Bergem, Norberg & Aamodt 2006). In addition, midgut epithelial cells from *Apis cerana* have also been cultured for over 20 weeks (Zhang et al. 2009). Recently, a honey bee immortalized cell line developed from an embryonic culture transfected with human c-myc proto-oncogene has been reported to live for six months (Kitagishi et al. 2011). Although the new bee cell line is yet to be disclosed to other researchers,

the successful immortalization of bee cells using human oncogene opens up future opportunities of bee cell lines from other bee tissues.

1.6.2 Methods for *in vitro* larval rearing

In vitro larval rearing is particularly difficult due to the eusocial nature of honey bees; bee larvae are heavily tended and fed by worker bees with brood food consisting of royal jelly. As the brood food content changes with the age and caste of the larvae and the exact composition of royal jelly is still unknown, reconstitution of a completely chemically defined diet remains as a huge challenge for *in vitro* larval rearing. Early *in vitro* larval rearing was done by raising larvae on brood food obtained from worker or queen cells, and 40-50% of female larvae can be reared to adulthood by transferring larvae three times daily into fresh royal jelly (Jay 1964a). Subsequently, several artificial diets have been reported for rearing queens or workers with improved survival rates (Rembold, Lackner & Geistbecka 1974, Rembold, Lackner 1981, Shuel, Dixon 1986, Vandenberg, Shimanuki 1987) , and most of the artificial diets used nowadays are based on a diet reconstituted with a mixture of lyophilized royal jelly, glucose, fructose, and yeast extract (Peng et al. 1992). Improvements on the artificial diet continue in recent years, including adjustment of diet according to the age of larvae (Aupinel et al. 2005), variation in interval between food replenishment (Kaftanoglu, Linksvayer & Page 2010), and development of diet for rearing drones (Behrens et al. 2007). Although success has been made in rearing with artificial diet, the question of the feasibility of a chemically defined artificial diet remains. A partially defined diet was reported by Shuel and Dixon (1986); this diet has most of its components known except the metabolites and proteins extracted from royal jelly.

1.6.3 RNA interference in honey bees

RNA interference (RNAi) is a relatively new field in insect science research and limited RNAi research has been done in honey bees. Five factors largely influencing efficiency of RNAi

in honey bees and other insects include: nucleotide sequence, length of the dsRNA fragment, concentration of dsRNA, the life stage of the target organism, and persistence of the silencing effect (Huvenne, Smagghe 2010). Nucleotide sequence determines the possible off-target effects in the host. With the honey bee genome being published (Honeybee Genome Sequencing Consortium 2006), gene-specific dsRNA sequence can be selected easily and hence minimize off-targets possibility. Typically, a length between 300-520 bp is chosen for dsRNA knockdown in insects (Huvenne, Smagghe 2010).This is also the usual range for honey bee dsRNA treatment (Patel et al. 2007, Amdam et al. 2003, Aronstein, Saldivar 2005, Nunes, Simoes 2009), although shorter length dsRNA down to 170-180 bp can also achieve significant knockdown (Maleszka et al. 2007, Jarosch, Moritz 2011). For each gene, optimal concentration has to be determined to conduct experiments in a cost-efficient manner. It is shown that administration of dsRNA exceeding optimal concentration cannot increase the effect of gene silencing in other insects (Meyering-Vos, Muller 2007, Shakesby et al. 2009). The amount for dsRNA needed is gene dependent and also varies with the life stages of the insect and the methods for delivery. For honey bee eggs, injection of 20-25 ng for chemosensory protein 5 (CSP5) and 90 ng for vitellogenin is sufficient to achieve silencing effect (Amdam et al. 2003, Maleszka et al. 2007). Meanwhile for fourth to fifith instar larvae, injection of 0.77–1.28 µg of mixed dsRNA targeting Toll-related receptor 18W (Am18w) can achieve 33% complete silencing and 67% partial silencing (Aronstein, Saldivar 2005). Injection of honey bee pupae and adults are more popular due to their large size and ease for manipulation, and injection can be done with a microsyringe with a 30G needle instead of a microinjector (Amdam et al. 2006). Injecting 5-10 µg can induce vitellogenin and *Amjhe-like* juvenile hormone esterase gene silencing in newly emerged adults, meanwhile 30 µg is needed for silencing insulin receptor substrate gene (Amdam et al. 2003, Amdam et al. 2006, Mackert et al. 2008, Wang et al. 2010). As an alternative to injection, feeding is also possible as a non-invasive method for dsRNA delivery. Feeding dsRNA is most commonly done by mixing dsRNA into an artificial diet and rearing the

larvae *in vitro.* Concentration as low as seven µg dsRNA per ml of diet can silence the Am18w gene by feeding approximately 180 µl of diet to young larvae over 24 hours (1.26 µg dsRNA per larva) (Aronstein, Pankiw & Saldivar 2006). However, the concentration needed is once again gene-dependent. For phosphatase and tensin (PTEN), a concentration of 450 µg/ml of diet over four days of feeding is needed to achieve knockdown meanwhile 150 µg/ml does not affect gene expression (Mutti et al. 2011b). Due to the huge difference in dsRNA concentration needed for different gene knockdown, the recommended starting concentration is 200 µg per ml of diet, feeding every 12 hours over two days, and further optimization is needed for different genes (Amdam et al. 2011). In addition to *in vitro* rearing, feeding can also be done in a natural setting. Vitellogenin can be knocked down by adding 0.5 – 3 µg dsRNA to just-hatched honey bee larvae in their comb followed by returning the larvae to be reared naturally (Nunes, Simoes 2009). Lastly, persistence of the silencing effect can be closely related to the turnover rate of the proteins. In aphids, the silencing effect for aquaporin lasted for five days and was reduced thereafter (Shakesby et al. 2009). Transient effect is also observed in knockdown of pheromone binding protein in the light brown apple moth, probably due to the high expression level and turnover rate of the protein (Turner et al. 2006). The same reason may also account for ineffective or incomplete silencing in some of the honey bee RNAi experiments. Therefore, protein turnover rate and expression level should be taken into consideration for the amount of dsRNA needed for the experiment. To conclude, RNAi gene knockdown can be achieved efficiently, however, concentration and method of delivery have to be chosen in a gene and life stage dependent manner.

1.7 Project overview

In this thesis, we will explore the potential for using cell culture and the *in vitro* larval rearing assay to study honey bees. In chapter two, we will compare the establishment of honey bee cells from eggs and pupae using various cell culture media. Cryopreservation of embryonic culture, proteome profile changes during cultivation, lentiviral transduction of EGFP in embryonic cultures will also be investigated. In chapter three, we will attempt to reconstruct the partially artificial diet and look into the potential of making a chemically complete and defined diet. Using the *in vitro* larval rearing assay and RNA interference, we will investigate the role of propheonoloxidase in the ability of honey bees to resist American foulbrood infection.

2 Proteome profile and lentiviral transduction of cultured honey bee (*Apis mellifera L.)* cells

2.1 Introduction

Most of honey bee research is done in whole colonies or at the single animal level; with some notable exceptions, honey bees have not been studied at the biochemical, molecular or cell biological level in the way that other systems have been. One of the main reasons for this is the lack of a well-controlled experimental system for molecular-level work. Virtually everything we know about subcellular events, from DNA replication to vesicle trafficking, comes from studies in cultured cells, whether they are bacteria or human cells. While thousands of established cell lines are now available from humans and other organisms, including about 500 insect cell lines; there are only a few reports of cultured primary honey bee cells from various tissues (Gascuel, Masson & Beadle 1991, Kreissl, Bicker 1992, Bicker, Kreissl 1994, Bergem, Norberg & Aamodt 2006, Zhang et al. 2009, Hunter 2010). At the time of this study, there was no report of immortalized bee cells but recently, Kitagishi *et al.* (2011) reported a human oncogene transformed bee cell line; however, the authors have not yet released this cell line for use by other research groups. Many studies, particularly on viral and bacterial infections in honey bees are limited by the lack of an immortalized honey bee cell line (Grabensteiner et al. 2001). Therefore, the need for a bee cell line still remains and the availability of an immortalized cell line would definitely facilitate biochemical analyses and allow for further genetic manipulation; at the same time avoid the limitations on the availability of honey bee materials and bee cell lifespan.

Although there were many reports of primary honey bee cell cultures, the characterization of these cultures has been limited. In this study, we investigated the changes that bee cells undergo in culture using mass spectrometry based quantitative proteomics. We hereby report the first proteome profiling of honey bee cells in culture, using freshly collected

embryonic cells as a reference. With these primary bee cells, we used lentivirus to introduce DNA into bee cells since it can infect both dividing and non-dividing cells by active nuclear import and integration of viral DNA (De Rijck et al. 2007). Using enhanced green fluorescent protein (EGFP) as an indicator, we demonstrated the successful delivery of a functional foreign gene into honey bee cells by lentivirus transduction. The success of DNA introduction into bee cells reinforces the idea of introducing constitutively active oncogenes as an approach for obtaining immortalized honey bee cell lines.

2.2 Experimental procedures

2.2.1 Eggs harvesting

Honey bee worker eggs were collected from honey bee colonies maintained at the University of British Columbia (UBC) Farm (Vancouver, BC, Canada). Frames were taken out of the colonies and available eggs were transferred from honeycomb cells into either microcentrifuge tubes with 500 µl phosphate buffered saline (PBS) or 40 µm cell strainers (BD Biosciences, ON) in a 5 cm Petri dish with 5 ml of PBS using a toothpick. A Zeiss Discovery V8 stereomicroscope with an achromat 0.63x lens was used to aid in retrieving the eggs.

2.2.2 Embryonic cell collection and culture conditions

Twenty to thirty eggs were collected per well in a six-well plate and the cultures were initiated as described by Lynn (2001) with minor modifications. Briefly, the outside of the eggs (or eggs together with the cell strainer) were sterilized by submerging them briefly in 2.5% calcium hypochlorite. The eggs (and strainer) were then rinsed twice in PBS and the culture media was added after the second rinse. Embryonic cells were released from the eggs by crushing them using a pipette tip against the bottom of microcentrifuge tubes in 500 µl media or against the mesh of the cell strainer in 2 ml of culture media, then the cell suspension was

transferred into a six-well plate with volume topped up to 3 ml per well. Cultures were maintained at 32 °C with 5% CO_2 in Grace's Insect Medium, Schneider's Drosophila Medium, or a mixture of Schneider's Drosophila Medium and Eagle's Basal Medium (at a ratio of 1:4). All media were supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 µg/mL). All media and supplements were purchased from Invitrogen (Burlington, ON). Culture media was refreshed every three to four days.

2.2.3 Modified media, pupal cell collection and culture conditions

An M-WH2 medium was constructed with modification to the WH2 medium (Hunter 2010). M-WH2 medium (approximately 1 L) consisted of 350 ml Schneider's Drosophila Medium (Invitrogen,ON), 50 ml Medium 199 (10X) with Hank's salt without glutamin (Sigma, ON), 500 ml 0.06 M L-histidine (Sigma, ON) pH 6.5-6.6, 25 ml Medium CMRL 1066 (1X) (Invitrogen,ON), 10 ml MEM Amino Acids Solution (50X) (Invitrogen,ON), 5 ml Insect Medium Supplement (10X) (Sigma, ON), 5 ml Hank's Balanced Salt Solution (10X) (Sigma, ON), 100 ml FBS (Invitrogen,ON), 1 ml Gentamycin Reagent Solution (50 mg/ml) (Invitrogen,ON), 5 ml Nystatin Suspension (Sigma, ON), 10 ml penicillin-streptomycin (100X) (Invitrogen,ON), and 10 ml Fungizone Antimycotic (Invitrogen,ON). This media was developed after first publication of embryonic cell cultures (Chan et al. 2010). Subsequent preparation of honey bee cells from eggs, larvae, and pupae were established in M-WH2 medium.

In vitro reared honey bee pupae were used for pupal cell preparations. First, midguts of pupae were removed by pulling at the last integument with a pair of sterile forceps. The remaining body was washed three times with 5 ml of PBS containing penicillin-streptomycin (50 µg/mL), gentamicin (50 µg/mL), and fungizone (0.25 µg/ml) in a 15 ml conical tube rotated with a MACSmix^{TM} tube rotator (Miltenyi Biotech Inc, CA). Bodies were then dissected into head, thorax, and abdomen using a sterile scalpel. Each body part was cut into smaller pieces on a 5 cm Petri dish. 5 ml of M-WH2 medium was added to pieces and further disintegrated by

pipetting up and down through a 40 µm cell strainer into. Cultures were again maintained at 32 °C with 5% CO_2.

2.2.4 Larval cell collection and culture conditions

Fourth instar larvae were obtained from UBC farm and disinfected by soaking in either 70% ethanol or 0.25% calcium hypochlorite for 2 min. Larvae were then rinsed once in PBS and the midguts were removed by pulling out at the anal end of the larvae. Remaining tissues were rinsed twice with PBS and cut into small pieces with sterile scalpel on a 5 cm Petri dish. Five millilitres of M-WH2 medium was added to tissues pieces and further disintegrated by pipetting up and down through a 70 µm cell strainer into 1-2 wells in a 6-well plate. Cultures were again maintained at 32 °C with 5% CO_2.

2.2.5 *In vitro* eggs hatching and rearing in sterile conditions

A honey bee queen from the UBC Farm was excluded using a Jenter Kit with removable mini cups for 24 h. Ninety-one cups were removed from colony and temporarily stored in 10 cm Petri dish lined with wet Kimwipes in a 34 °C humidified incubator for another 24 h. The eggs together with the cups were disinfected by briefly soaking in 1.5% calcium hypochlorite, followed by rinsing once with water and twice with PBS. All solutions were shaken off between transfers. Disinfected cups with eggs were put open-side up inside a 5 cm Petri dish and stored in 32 °C incubator. All eggs were processed in batches of 8 to 10 cups to avoid prolonged exposure to room temperature.

Semi-sterile diet was prepared by mixing 4.2 g of lyophilized royal jelly with filter-sterilized Yeast-dextrose-fructose solution (1% yeast extract, 3% dextrose, 3% fructose) in an autoclaved 50 ml beaker. Hatched larvae were transferred onto semi-sterile diet with curved sable 00 paintbrush disinfected with 70% ethanol.

2.2.6 Cryopreservation

Cells were pelleted and washed with PBS, resuspended in freezing medium (45% conditioned medium, 45% fresh medium, 10% DMSO) at a density of $9.9x10^5$ cells/ml and stored at -80 °C for 24 h before being transferred to liquid nitrogen for long term storage. Cells were revived in Grace's Insect Medium supplemented with 20% FBS, and gentamicin (50 µg/mL) nine months later. Alternatively, direct cryopreservation without prior culturing was done by collecting 250 eggs in a cell strainer and disinfecting as described in Section 2.2.2. Cells were released into 3 ml of M-WH2 medium, transferred to a 15 ml conical tube, and centrifuged at 100 g for 5 min at 4 °C. Supernatant was removed and cells were resuspended in 2 ml of freezing medium (M-WH2 medium with 10% DMSO). Cells were stored at -80 °C for 24 h before being transferred to liquid nitrogen for long-term storage.

2.2.7 Lentivirus transduction

Embryonic cells were released into six-well plates in Schneider's Drosophila Medium supplemented with 20% FBS and gentamicin (50 µg/mL) and transferred to Applied Biological Materials Inc. (Richmond, BC) for transduction following an established protocol (Wong et al. 2009, Kim et al. 2010). HEK293 cells cultured in DMEM with 10% FBS, 1% L-glutamin, and 1% penicillin-streptomycin were used as a control. Briefly, a mix of attached and suspension cells in 2 ml of culture medium were infected with 2 ml of EGFP lentiviral vector ($1x10^6$ cfu/ml; 1:1 dilution) per well in the presence of 2 µg of polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) overnight. The following day, suspension cells were harvested by centrifugation at 1,500 relative centrifugal force (rcf) in a 15 ml conical tube and resuspended in 2 ml of fresh medium, while 2 ml of fresh medium was also added to the attached cells. Finally, the suspension cells were combined with the attached cells in the original corresponding six-well plate. The lentiviral vector used in this study was the HIV-1 based 3rd generation lentiviral

vector (Lenti-His, Applied Biological Materials Inc. Richmond, BC). The reporter gene EGFP was subcloned through Kpn/EcoR I sites to generate lenti-EGFP. The lentiviral vectors were produced using the 3-plasmid expression system reported earlier (Naldini et al. 1996).

2.2.8 Microscopy

Cells were imaged directly in six-well culture plates in Schneider's Drosophila Medium supplemented with 20% FBS and gentamicin (50 µg/mL) for embryonic cells or M-WH2 medium for pupal cells. Micrographs were acquired with an Axio Observer Z1 microscope and a LD Plan-Neofluar 20x objective using an AxioCamHR3 Camera.

2.2.9 Proteomics

The proteome profiles of freshly collected embryonic cells and cultured embryonic cells were compared as follows: Cells were obtained from 80 eggs as described above and were split into four tubes: two of them were centrifuged, the cell pellets were washed twice in PBS, and then stored at -70 °C until analysis; cells in the other two were transferred to wells of a six-well plate and cultured as above. After two weeks, the cells were harvested with a cell scraper, centrifuged, washed twice in PBS, and the cell pellet was stored at -70 °C for 30 min. All four samples (fresh and cultured cells) were then thawed and lysed by boiling for 5 min in 1% sodium deoxycholate in 50 mM ammonium bicarbonate. Subsequent steps for preparation and analysis of the samples were adapted from previously described procedures (Rogers, Foster 2007). Briefly, proteins were precipitated in 800 µl of ethanol, 20 µl of 2.5 M sodium acetate (pH 5.5) and 2 µl of glycogen (10 mg/ml) at room temperature for 90 min. After centrifugation at 16,100 rcf, pellets were dried, and the proteins were solubilized and reduced in urea buffer (6 M urea, 2 M thiourea, 100 mM Tris-Cl at pH 8.0, 20 mM DTT). After removing insoluble material by centrifugation at 16,100 rcf, protein concentration was measured by a micro Bradford assay in a 96 well plate. Proteins were then alkylated with iodoacetamide and digested with

endoproteinase LysC and trypsin as described (Foster, De Hoog & Mann 2003). Digested peptides of each sample were isolated using C18 STop And Go Extraction (STAGE) tips. Eluted peptides were dried, resuspended in 2.5 M sodium acetate (pH ~8.0), labelled with formaldehyde isotopologues, and the mixed peptide samples were analyzed by liquid chromatography-tandem mass spectrometry as described in Rogers and Foster (2007). Averages and standard deviations of the protein ratios from the two replicates of each condition were calculated.

2.3 Results

2.3.1 Establishment of suspension and attached primary embryonic cell culture and comparison of culture media.

The pluripotency of most cells in bee embryos should make them a good source of culturable cells and, indeed, multiple honey bee embryonic cell cultures were successfully initiated from eggs collected from honey bee combs (Figure 2.1A, 2.1B). Cultures contained both suspension and fibroblast-like adherent cells and both types were easily separated and independently maintained (Figure 2.1C, 2.1D). Cultures established by crushing eggs in microcentrifuge tubes had relatively more cell clumps and egg chorion debris compared to cultures established by releasing cells with cell strainers. These cell cultures could be maintained for at least four months, although almost no obvious division was observed. In addition, the cells were grown in different media and conditions that have been used for primary bee cell cultures in previous reports to assess effect of media (Barbara et al. 2008, Gisselmann et al. 2003, Bergem, Norberg & Aamodt 2006). In particular, we evaluated Grace's Insect Medium, Schneider's Drosophila Medium, and a mixture of Schneider's Drosophila Medium and Eagle's Basal Medium (at a ratio of 1:4). Cultures appeared healthier and could be maintained

longer in insect medium (Grace's or Schneider's) than in medium based mainly on Eagle's Basal Medium.

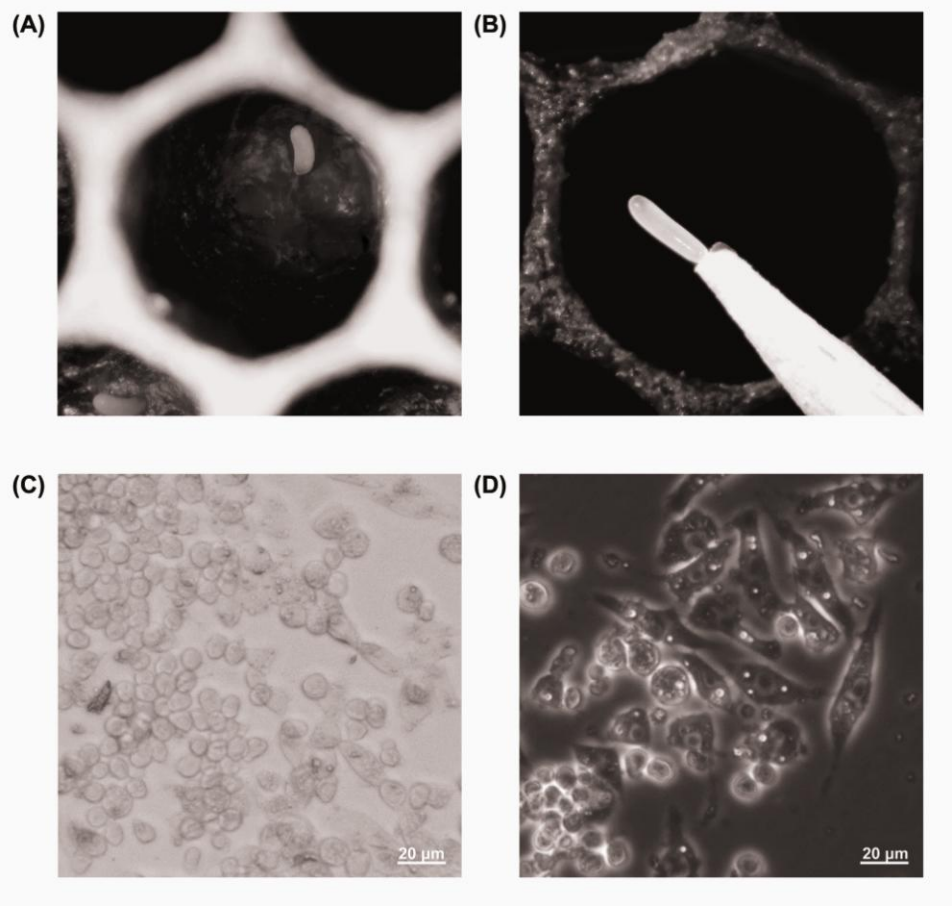


Figure 2.1 Establishing suspension and attached primary embryonic cultures.
(A) Image of a honey bee egg (1.6 mm) at the bottom of a comb cell (6 mm diameter). (B) Eggs were transferred from the bottom of a cell to a test tube using a toothpick. (C) Suspension culture of embryonic honey bee cells cultured for three days. (D) Embryonic honey bee cell culture with predominantly attached cells cultured for three days.

Embryonic cultures with sufficient density could be easily established; however, as honey bee eggs were not available during the winter, preparation of primary cultures could only be done in the summer. In order to investigate if cells could be stored for future use when eggs were not available, we applied a standard method for cryopreservation to embryonic bee cells and stored them for nine months in liquid nitrogen. The cryopreserved cells were successfully revived and maintained for a further four months, demonstrating the ability of long term

embryonic cell storage. In addition, embryonic cells could be cryopreserved without prior culturing by first releasing cells in culture medium, followed by pelleting the cells with centrifugation, and resuspending the cells in freezing medium. This freezing medium contained 10% DMSO but not conditioned medium, hence, did not require prior culturing for making freezing medium. There was no observable difference between embryonic cells cryopreserved with a standard cryopreservation method or direct cryopreservation.

2.3.2 Establishment of cultures from different parts of pupae and larvae

In addition to embryonic cells, several cell cultures from different body parts of honey bee pupae could also be established and maintained for a short period of time. After removing the midgut, honey bee pupae were disinfected and then dissected into head, thorax, and abdomen. Cell morphology was significantly different between cultures of different body parts. Abdominal culture consisted mainly of lose and round cells (Figure 2.2A). Thoracic cells were a mixture of suspension and attached cells in various sizes; a special type of long, thread-like cells was usually obtained from thorax cultures (Figure 2.2B, arrow). Lastly, pupal head cultures usually resulted in the most diverse mixture of cell types, but the amount of cells obtained was also less than that from either abdominal or thorax cultures (Figure 2.2C). Attempts were also made to establish cultures from honey bee larvae; however, debris was severe in these cultures and they were discontinued shortly after.

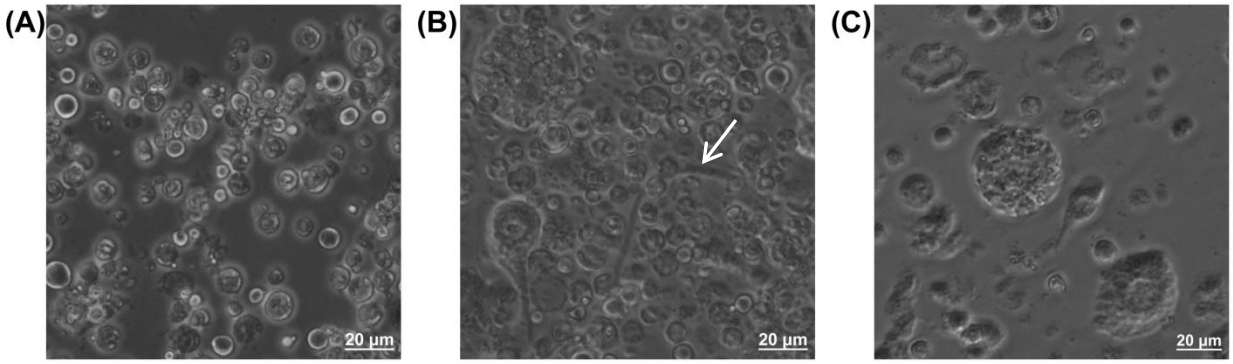


Figure 2.2 Primary cell cultures from honey bee pupae.
Honey bee pupal cells cultured for one day. (A) Cells from abdomen. (B) Cells from thorax. Arrow indicates thread-like cells. (C) Cells from head.

2.3.3 Comparison of various sterilization methods of honey bee tissues

As honey bees were reared in non-sterile environment, disinfection was a crucial step in preparation of primary cultures. Sterility of cultures was indicated by no observable growth of bacteria or fungi over culturing period. Honey bee eggs could be easily disinfected with bleach (2.5% calcium hypochlorite) followed by rinsing with water and PBS. No damage from sterilization was observed in embryonic culture, probably due to the egg chorion protecting the cells from the disinfectants. Conversely, the presence of melanized debris in larval cultures suggests that sterilization steps caused direct damage to the cells, thereby initiating the melanization cascade, although it is also conceivable that it was simply initiated by the oxidizing potential of the bleach. Therefore, we investigated various methods to obtain sterile honey bee tissues for cell cultures.

First, we compared the effect of sterilizing with 70% ethanol versus bleach (0.25% calcium hypochlorite). Fourth instar larvae were disinfected by soaking larvae in either solution for two minutes, followed by washing twice in PBS. Cells were then released into culture medium by breaking the larval tissue mechanically. Ethanol-treated larvae resulted in cultures with mixed cell types, including small granular spherical cells and large oenocyte-like cells,

occasionally melanized cells could be observed. On the other hand, bleach-treated larvae resulted in cultures with some round cells but lots of floating debris; the debris was often from melanized cells. Hence, 70% ethanol seems to be a milder disinfectant for sterilizing larval tissue.

Although 70% ethanol disinfection was milder than bleach to the larval cultures, there was still a significant damage from the sterilization steps. Therefore, we attempted to obtain sterile larvae by disinfecting the eggs and allowing the larvae to hatch and be raised in a sterile environment. As honey bee eggs were very fragile and easily damaged if removed using a toothpick or paintbrush, Jenter Kits with removable cups were chosen to minimize handling of the eggs. A honey bee queen was excluded to lay into mini cups in a Jenter Kit, if eggs in these cups were simply removed from the hive into incubator without further manipulation, more than 50% of eggs could hatch into larvae (data not shown). For obtaining sterile larvae, each individual cup was disinfected with bleach together with the egg inside it. After washing with water and PBS, eggs were allowed to hatch in the cups in the cell culture incubator; hatched larvae were raised on semi-sterile diet. Out of the 91 eggs obtained using the Jenter Kit, only eight hatched successfully after disinfection. Various abnormalities were observed in eggs that failed to hatch, often partial development of larvae inside the eggs with clear space present at both ends could be seen (Figure 2.3). Four of the hatched larvae were transferred by first covering larvae with PBS and then picking them up using a paint brush; the other four hatched larvae were transferred dry with a paint brush. Only two larvae transferred by first covering in PBS survived for two days and neither survived for more than three days. Even though sterile larvae could be reared for a short time, the high mortality rate in the preparation process limited the establishment of cell cultures from these larvae.

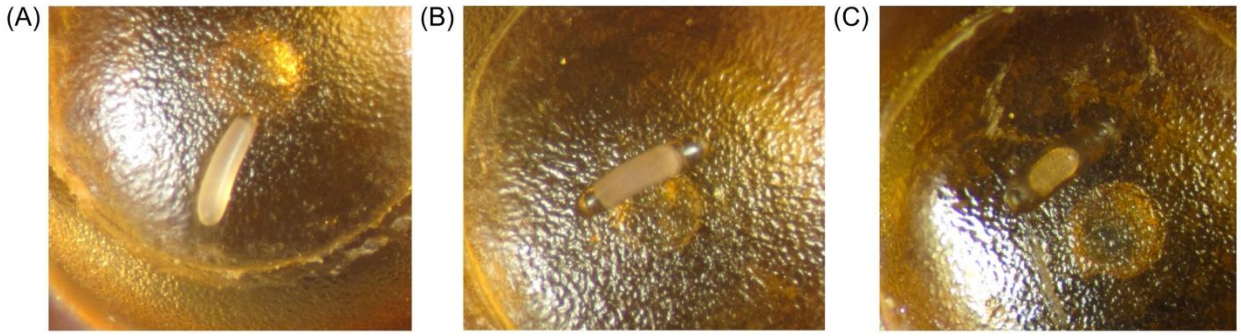


Figure 2.3 Abnormalities of honey bee eggs after disinfection.
(A) A honey bee egg obtained in mini-cup before disinfection. (B) A honey bee egg with clear space visible at both ends, two days post disinfection. (C) A honey bee egg with partially developed larvae inside, two days post disinfection.

2.3.4 Differential protein expression in honey bee embryonic cells before and after culturing

In order to evaluate how bee cells adapt to culture conditions, we used a quantitative proteomics approach to compare protein expression of freshly harvested embryonic cells versus embryonic cells cultured for two weeks. Chemical derivatization of peptides from the two different conditions with formaldehyde isotopologues allowed differences in protein expression to be analyzed by LC-MS/MS (see experimental procedures). On a gross level, about two-thirds of all detected proteins were observed to be up-regulated during culturing (Figure. 2.4) and no proteins were found to be down-regulated more than ten-fold. Four of the ten most up-regulated proteins were chaperone/heat-shock proteins; other up-regulated proteins included catalase, phospholipid hydroperoxide glutathione peroxidase, lactate dehydrogenase, aldolase, and Barrier to Autointegration Factor (Table 2.1).

Table 2.1 The 10 most up-regulated and down-regulated proteins in cultures embryonic cells.

Up-regulated during culturing

Accession number[1]	Description[2]	Remarks[3]	Cultured/Fresh[4]
gi|110758428	Lactate dehydrogenase	Anaerobic respiration	>10[5]
gi|66504546	Similar to Lethal(2)essential for life, isoform 1	Heat shock protein	>10
gi|110750764	Similar to Lethal(2)essential for life	Heat shock protein	>10
gi|66521514	Similar to CG7380-PA	83% identity to Barrier to autointegration factor (XP_001844471.1, *Culex quinquefasciatus*)	>10
gi|66546646	Mitochondrial phospholipid hydroperoxide glutathione peroxidase	Protection from oxidative stress	>10
gi|110777113	Catalase, partial sequence	Protection from oxidative stress	>10
gi|66504551	Sequestosome 1	-	>10
gi|110750758	Similar to Protein lethal(2)essential for life (Protein Efl21) isoform 1	Heat shock protein	>10
gi|110748949	Aldolase, isoform F	Glycolysis	>10
gi|110750756	Similar to Lethal(2)essential for life, isoform 1	Heat shock protein	>10

Down-regulated during culturing

Accession number	Description	Remarks	Fresh/Cultured
gi|66503776	Similar to CG8036-PB, isoform B isoform 2	Transketolase activity	4.4 ± 3.3
gi|110762101	Similar to CG6621-PA	-	4.4
gi|58585098	Major royal jelly protein 1	-	4.7
gi|110750857	ALY	RNA binding protein	4.8
gi|110758113	Mapmodulin, isoform B	Protein phosphatase inhibitor	5.1
gi|110760247	Nopp140, isoform B	Nucleolar and coiled-body phosphoprotein	5.3 ± 3.7
gi|110749582	similar to CG10472-PA	Trypsin-like serine protease	6.6
gi|110749616	Histone H1	-	6.8
gi|110759433	La	Ribonucleoprotein	7.8 ± 11
gi|110762287	Hypothetical protein	-	9.9 ± 5

[1]GenBank accession numbers.
[2]Common or annotated name
[3]Additional functional information, if known
[4]The measured expression ratio ± 1 standard deviation. In the top section the ratios represent the fold-overexpression during culturing, while in the bottom the ratios represent the fold-down-regulation during culturing.
[5]Ratios greater than 10 are truncated to 10 so no standard deviations are reported.

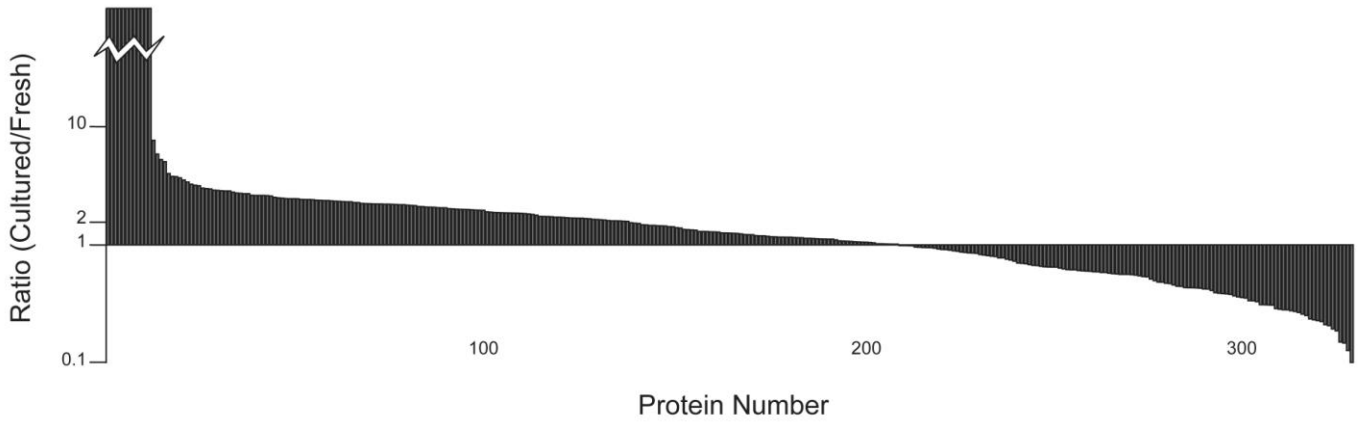


Figure 2.4 Differential protein expression in honey bee embryonic cells before and after culturing.
Fold differences in protein expression for cells cultured for two weeks versus cells obtained directly from eggs. Protein ratios quantified by MSQuant were plotted from highest to lowest. Additional information on protein ratios could be found in supplementary Table S1.

2.3.5 EGFP expression of honey bee embryonic cells transduced with lentivirus

We also investigated the use of lentivirus transduction to introduce foreign genes into honey bee cells. Lentivirus was an ideal tool for this purpose since these cells did not divide rapidly. We successfully transduced bee embryonic cells with lentivirus encoding EGFP, and obtained cultures with approximately 20 % of cells showing green fluorescence as the result of EGFP expression (Figure 2.5). The transduction efficiency was comparable to lentiviral transduction in the control HEK293 cells (data not shown).

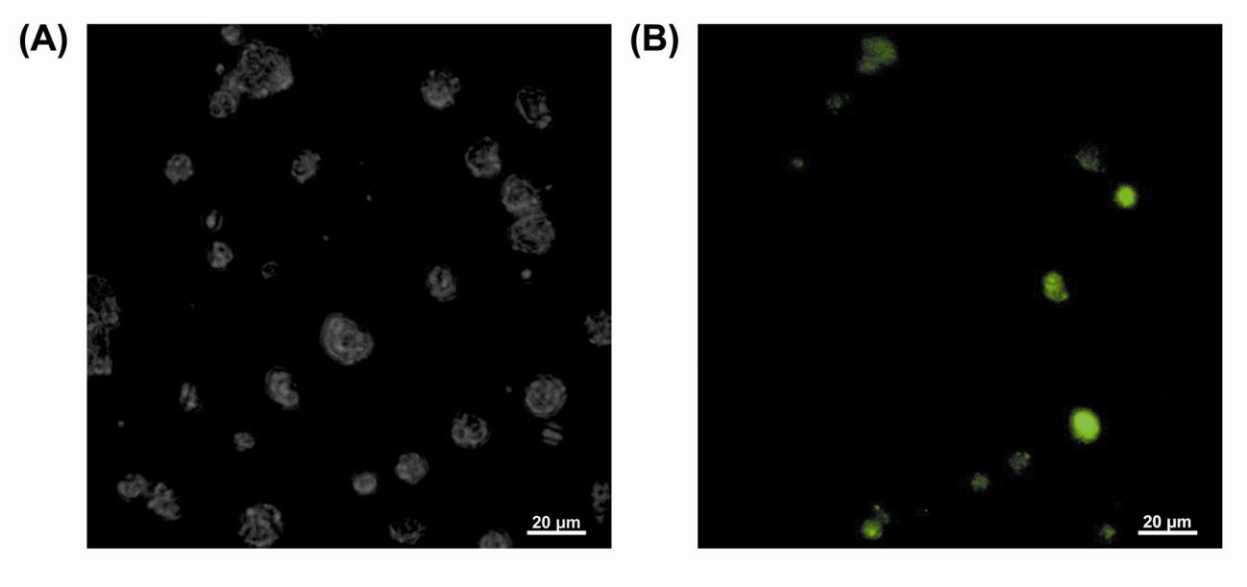


Figure 2.5 EGFP expression of honey bee embryonic cells transduced with lentivirus. (A) Image taken under phase contrast showing honey bee cells in the field of view. (B) Image taken of the same field with a GFP filter showing cells expressing EGFP.

2.4 Discussion

Honey bee embryonic cell cultures could be easily established and the success in cryopreservation allowed interruptions in their continual maintenance and transportation between laboratories. The appearance of both suspension and adherent cells was consistent with the previous report that embryonic cell cultures from eggs less than 36 hours old were mostly round and semi-adherent, meanwhile cultures from 36-44 hours old eggs showed a differentiated, elongated morphology (Bergem, Norberg & Aamodt 2006). As we obtained honey bee eggs without queen exclusion, age of the eggs could vary from 0-76 hours old. Hence, mixed morphologies of undifferentiated and more differentiated cells were expected. Comparing pipetting against microcentrifuge tubes versus pipetting against cell strainers, the latter resulted in cleaner cultures with less cell clumps and egg chorion. Cell strainers were often employed in primary cell preparation for dissociation of cells from other primary tissues and isolation of single cells from cell suspensions (Crawford, Pilling & Gomer 2010, Harris et al. 2007, Mohanty et al. 2011). In the case for honey egg cells preparation, the use of cell strainers was particularly useful for shortening preparation process. First of all, cell strainers provided us with a bigger

deposition surface than microcentrifuge tubes during egg collection. More than 300 eggs could be processed in a single batch with a cell strainer meanwhile only 30 eggs could be processed in a microcentrifuge tube. Additionally, eggs in microcentrifuge tubes would float and crushing of eggs became inefficient and time consuming. Cell strainers would not retain medium and hence eggs would stay on nylon mesh and were easily crushed for releasing cells efficiently. Lastly, cultures prepared with microcentrifuge tubes often contained big cell clumps or even partially broken eggs floating on the surface due to inefficient egg crushing. Cell strainers served as a filter for removing such debris, providing us with cleaner cultures. Therefore, the use of cell strainers for obtaining primary cultures was recommended for future cell preparation from honey bee eggs and tissues.

Using these embryonic cultures, we compared the effect of different culture media used by previous reports. Many of the early insect primary cells were cultured in hemolymph, and resemblance to insect hemolymph physiological conditions was one of the major factors in developing insect culture medium (Day, Grace 1959). Both Grace's and Schneider's insect culture medium resulted in healthier cultures than the mixture of Eagle Basal Medium and Schneider's medium. The result was expected because both Grace's and Schneider's insect media were designed to simulate insect hemolymph (Grace 1962, Schneider 1966), and thus, should support the growth of honey bee cells better than Eagle's Basal Medium designed for mammalian cultures (Eagle 1955). Even though the mixture of Eagle's Basal Medium and Schneider's Drosophila Medium should contain most of the insect specific nutrients, the pH of mammalian media is still higher than that of honey bee larval hemolymph pH (~6.8) (Bishop 1923), hence may not be as suitable for bee embryonic culture as medium designed for insect cell cultures. The recently-described WH2 medium for larval honey bee cells, which contains higher levels of histidine and other nutrients (Hunter 2010), may be an even better medium for honey bee cells. Therefore, a modified WH2 medium (M-WH2) (modified according to personal

communication with Dr. Wayne Hunter) was used in the later preparations of the honey bee larvae and pupae cell cultures.

Although embryonic cultures could easily be established, the process of egg harvesting was still the limiting factor for establishing culture because each egg must be handpicked and the number of cells obtained from eggs was relatively low. Approximately 70 eggs were needed to obtain 70% cell confluence in a well of a six-well plate; therefore, a large number of eggs must be sacrificed for any experiments. Hoping to obtain more cells from a single organism, we attempted to obtain cultures from both honey bee larvae and pupae. When we established cultures, midguts were first removed because they were under constant exposure to non-sterile food and might harbour a lot of bacteria, which may contaminate our cells. Separation of head, thorax, and abdomen was used as a systematic way to reduce the amount of cells being released into a small amount of culture medium; this also allowed us to see if any tissue specific cell types were present. As a significant volume of the abdomen was occupied by the intestinal system of the honey bee, it was not surprising to see a less diverse cell mixture from abdominal cultures when the midgut was removed. Moving up to the thorax where the thoracic glands and the flight muscle located, we observed a slightly more diverse cell types and the thread-like cells could likely be muscle cells from flight muscle. Lastly, the head cultures had the most diverse cell types, which were expected because of the presence of various organs such as brain, eyes, and salivary glands. The lower amount of cell obtained could probably due to the relatively small size of the heads and the tougher exoskeleton might have prevented cells from being released efficiently.

The failure in establishing cultures from larval cells was probably due to the damage caused by disinfection. Meanwhile, honey bee eggs had chorion and pupae had the hardened cuticle, honey bee larvae had neither of those to protect their tissue from the disinfectants and more melanized cells resulted from larval cultures. Therefore, the choice of disinfectant became crucial in establishment of tissue cultures. Both bleach and ethanol have been used as

disinfectant for cell cultures (Lynn 2001), but the two disinfectants had different mode of actions. Bleach used in this study was calcium hypochlorite; it was a kind of chlorine-releasing agents which acted against bacteria as strong oxidizing agents that destroyed cellular activity of proteins and also inhibited DNA synthesis. Ethanol was a known dehydrating agent; however, it did not kill the bacteria by dehydration, instead, its bactericidal power came from damaging cell membranes and denaturing proteins (McDonnell, Russell 1999). Bleach was a better disinfectant for eggs because it helped to weaken the chorion and facilitated the release of embryonic cells. However, the strong oxidizing power of bleach induced a more severe damage to the honey bee larvae and hence was less suitable for use in the case for preparation of larval tissue culture compared to disinfection with 70% ethanol.

Although 70% ethanol caused less damage to the larval cultures compared to bleach, the resulting cultures still had some melanized cells, which was not desirable for primary cell culture preparation. Therefore, we attempted to obtain sterile larvae, which moved the disinfection steps to the egg, minimizing the cells' direct contact with disinfectants. As 50% of the eggs could hatch if not subjected to disinfection but only 10% of the eggs could hatch if disinfected, the high mortality rate in obtaining sterile larvae was likely due to the disinfection steps. During disinfection, the chorion was weakened by the bleaching step, and subsequent incubation might have resulted in severe dehydration and residual disinfectant might have also affected embryo development. Although we could successfully hatch the larvae in the laboratory after disinfection, alternative approach such as using milder disinfectant such as 70% ethanol or PBS with antibiotics should be used for obtaining viable eggs in future experiments. Regarding those larvae successfully hatched *in vitro,* the high mortality rate was probably due to damage in transferring steps as just hatched larvae were extremely fragile. Nonetheless, we could not exclude the fact that sterile larvae may not be viable as symbiotic microbes are often needed for insect survival (Evans, Armstrong 2006, Hamdi et al. 2011, Shi et al. 2010). Several methods for collection of larvae after hatching *in vitro* could achieve >70% survival, typically by placing

honey bee eggs on cotton string or tissue paper on top of larval food (Wegener, Al-Kahtani & Bienefeld 2009). However, these methods all required transferring honey bee eggs with specialized forceps, meaning additional training and also significant modifications would be needed before they could be applied for obtaining sterile larvae.

Proteome comparison of freshly harvested egg cells versus cultured cells allowed us to understand how the bee cells adapted to the culturing condition. Four of the ten most up-regulated proteins were chaperone/heat-shock proteins involved in the protein folding quality control machinery, in fitting with the cultured cells' need to adapt to the unnatural environment they suddenly found themselves in. Two other of the most up-regulated proteins, catalase and a phospholipid hydroperoxide glutathione peroxidase, were involved in protecting cells from apoptosis and oxidative stress, which also fitted with the cells having to deal with an oxygen-rich environment without the benefit of being in a tissue, and their co-regulation with sequestosome was also in fitting with other observations of cells under oxidative stress (Sandstrom, Buttke 1993, Zhang et al. 1997, Mathew et al. 2009, Hu et al. 2010). The up-regulation of lactate dehydrogenase and aldolase, two key enzymes in anaerobic energy production from hexoses, suggested that the cells were altering fundamental metabolic pathways in order to take advantage of the suddenly unlimited supply of glucose. Lastly, the up-regulation of Barrier to Autointegration Factor, which was involved in chromatin condensation/opening, supported the overall increased expression of proteins observed. Regarding the down-regulated proteins, histone H1 was responsible for maintaining and stabilizing higher order of chromatin structure, its down-regulation might open up chromatin for transcription (Thoma, Koller 1981, Raghuram et al. 2009). Interestingly, one of the functions of La was shown to prevent histone mRNA from exonuclease degradation; its down-regulation might contribute to decreased level of histone protein in cultured cells. (Wolin, Cedervall 2002, McLaren, Caruccio & Ross 1997, Boelens, Palacios & Mattaj 1995, Simons et al. 1996). Mapmodulin was also known as ANP32a, which was a potent inhibitor for protein phosphatase 2A, hence implicated to be involved in regulation

of cell differentiation and proliferation pathways through other protein kinases (Matilla, Radrizzani 2005). Additionally, ANP32a was also a component of the INHAT complex, which down-regulates transcription by inhibiting histone acetylation (Seo et al. 2002, Kadota, Nagata 2011). Therefore, decreased level of histone H1, La and mapmodulin also supported overall increased expression of proteins. The other two proteins Nopp140 and Aly were important for cell proliferation. Nopp140 was known to be a molecular chaperone delivering for small nucleolar ribonucloprotein complexes and shown to be involved in nucleolar assembly. (Miau et al. 1997, He, DiMario 2011). Aly is a known mRNA export factor and a downstream target for nuclear PI3K signalling cascade involved in cell growth regulation (Okada, Jang & Ye 2008). Hence, the down-regulation of Nopp140 and Aly corresponded to the fact that these bee cells were not actively dividing. Lastly, transketolase was an enzyme catalysing the non-oxidative branch of pentose phosphate pathway, synthesizing precursors for biosynthesis and metabolites for glycolysis (Schenk, Duggleby & Nixon 1998). However, as the bee cells were already provided with unlimited amount of glucose, down-regulation of transketolase might reflect the decreased need for the products from non-oxidative branch of the pentose phosphate pathway. It would be interesting to validate the proposed functions of the above proteins by knockdown experiments using RNA interference (RNAi) technology. So far, we had only tested RNAi in honey bee larvae; however, it is possible to apply RNAi as early as the egg stage and proteins' functions could be assessed from primary cells isolated from these eggs. It would be even better if an immortalized cell line were available since the functions of some proteins may only be relevant in actively dividing cells, such as Nopp140 and Aly on cell growth regulation, can be confirmed with RNAi-mediated knockdown.

Last but not least, the ability to express EGFP in honey bee cells could be a helpful tool for cell immortalization and tracing the progeny of subsequent generation of cells. Cell senescence was induced by continue shortening of telomerase and introduction of human telomerase or other oncogenes with lentivirus had been shown to be an useful tool to

immortalize mammalian primary cells (Akimov et al. 2005, Salmon et al. 2000) Further, co-expression of a GFP gene and an oncogene under the same upstream activation sequence might be used to identify potentially immortalized cells, as had been reported while establishing *Drosophila* cell lines (Simcox et al. 2008). To our knowledge, this is the first report describing the transduction and expression of a recombinant gene in cultured honey bee cells. Our success suggests that it is possible to hijack the cell's machinery to suppress apoptosis and encourage continual cell proliferation using DNA as an immortalization reagent. It also opens the potential for injecting viral genetic material into bee cells to study viral diseases in honey bees. However, optimization of the current protocol might be needed as the transduction efficiency remains relatively low. Recent reports have shown that increasing concentration of the cationic polyer polybrene could increase lentiviral transduction efficiency; however, precautions need to be made because polybrene also adversely affects proliferation of human stem cells (Lin et al. 2011). Additionally, the choice of a constitutive promoter also had an effect on transduction efficiency. The current lentiviral system used a cytomegalovirus immediate-early (CMV) promoter, which was a fairly strong promoter in mammalian cell lines. However, it was shown to be less effective compared to copia transposon promoter (COPIA) and actin 5C promoter (ACT5C) in *Drosophila* cell transfection (Qin et al. 2010). Therefore, alternative promoter might also help to increase lentiviral transduction efficiency in honey bee cells. The recent publication by Kitagishi *et. al.* (2011) took this approach further and introduced the human oncogene c-myc into honey bee cells to obtain the first reported honey bee cell line; hopefully this cell line will be made available to the honey bee research community in the near future.

2.5 Conclusions

Cell culture techniques have not been well established in honey bees and most of the previous studies were only performed in short term tissue cultures. Particularly, a lack of a widely available immortalized cell line limited honey bee research on the molecular level. In this study, various primary honey bee cell cultures from eggs and pupae were established and maintained for up to four months. The disinfection process had a direct impact on the subsequent cell cultures and proteome comparison indicated that cultured cells altered their cellular mechanisms in response to oxidative stress and unlimited nutrients. Cryopreservation of these cells allowed us to continue experiments when honey bee eggs or pupae were not available in the winter. Embryonic honey bee cells could be transduced by lentivirus to express EGFP; further development with this technique opens up the possibility of an immortalized embryonic cell line and allows us to study other viral diseases *in vitro* by introducing foreign genetic materials into honey bee cells.

3 *In vitro* larval rearing and RNA interference of prophenoloxidase to assess honey bees' susceptibility to American foulbrood disease

3.1 Introduction

During the developmental process of honey bees, the larvae are exposed to various pathogens and different environments in their habitat. The social structure of a honey bee colony, particularly the progressive brood rearing by nurse bees, limits our ability to manipulate honey bees' nutrition and to study the influence of their diet on important aspects of their biology such as caste differentiation and pathogens exposure. Although various diets have been developed for *in vitro* rearing of queens, workers, and drones (Patel et al. 2007, Peng et al. 1992, Behrens et al. 2007), these diets depend heavily on either fresh or lyophilized royal jelly. Royal jelly's quality is highly variable depending on source and freshness (Li et al. 2008, Isidorov et al. 2009, Isidorov, Bakier & Grzech 2011, Genc, Aslan 1999) and this in turn affects the nutritional power of the resulting diet. Therefore, having a chemically completely defined artificial diet would be beneficial as it would allow us to manipulate individual components to investigate their effect on honey bee's development. In this study, we first establish the *in vitro* larval rearing method in our laboratory and then explore the potential of furthering the partially defined artificial diet developed by Shuel and Dixon (1986) to a completely defined artificial diet. We then use this progress to test a hypothesis developed out of previous work (Chan et al. 2009) regarding the role of prophenoloxidase (PPO) in resistance to a major bacterial disease of honey bees.

One of the many food borne diseases affecting honey bee larvae is American foulbrood (AFB). The major route of infection is by ingesting contaminated food but interestingly only larvae younger than three days old are susceptible. Previously, Chan *et. al.*(2009) identified a potential role for prophenoloxidase, an immune-related protein, in honey bees' ability to resist

AFB infection after they are three days old. PPO is activated in AFB infected larvae, and its expression greatly increased by day three, paralleling the time when honey bees became resistant to the disease. In this chapter, we will apply the established *in vitro* larval rearing in combination with RNAi technology to investigate the potential role of PPO in AFB resistance. Here, we will report the effect of RNAi-mediated knockdown of PPO on honey bee's susceptibility to AFB disease using *in vitro* rearing.

3.2 Experimental procedure

3.2.1 Honey bee larvae

Worker honey bee larvae were obtained from a honey bee colony designated for research in University of British Columbia Farm. The queen was excluded to lay on one fully drawn, empty comb for 24 h. After 24 h of confinement, the comb containing freshly deposited eggs was returned to the brood chamber to be provisioned by the workers after hatching. On the fourth day, the comb was removed from hive and brought into the laboratory for grafting.

3.2.2 Basic larval diet and general rearing procedure

Honey bee larvae were reared according to Peng *et. al* (1992). Basic larval diet (BLD) was constructed by mixing 4.2 g of lyophilized royal jelly, 0.6 g of dextrose, 0.6 g of fructose, 0.2 g of Difco yeast extract, and 14.4 g of distilled-deionized water (ddH_2O) using a magnetic stir bar. Diet was pre-warmed to 35 °C before grafting, and unused diet could be stored at 4 °C for up to 2 days. Larvae from worker cells were grafted onto non-tissue culture treated 24-well plate using a Sable 00 paintbrush with a bent tip. Ten first instar larvae were grafted onto 300 µl BLD and reared in an incubator at 35 °C, >85% relative humidity. Larvae were transferred to fresh food on the 3^{rd}, 5^{th}, and 6^{th} day in groups of 5, 2, and 1 larva per well using a paintbrush or a bent spatula. As soon as signs of defecation were observed (around day 7 to 9), larvae were

removed from the feeding well, gently rolled on Kimwipe tissue to remove excess food, and transferred to the same type of 24-well plate with wells lined with autoclaved Kimwipe tissue (3 x 2 cm). The pupation tray was then returned to the same incubator.

3.2.3 Partially defined artificial diet preparation and honey bee rearing

Partially defined artificial diet was constructed following Shuel and Dixon (1986) with slight modifications. Lyophilized royal jelly (25 g) was extracted with 60 ml of anhydrous, peroxide-free ether in a 125 ml Erlenmyer flask covered by aluminum foil and stirred with a magnetic stir bar for 40 h. Ether extract was filtered through a Pasteur pipette stuffed with a piece of glass filter paper. The filtered ether extract and the royal jelly residue were air-dried until the ether had completely evaporated. Dried ether extract was used as a metabolite source and stored at -20 °C. Air-dried royal jelly residue was resuspended in 60 ml of ddH_2O, and dialyzed against ddH_2O using a dialysis tube with 10000 MWCO. Dialyzed royal jelly was freeze-dried and stored at -20 °C before use.

Stock solutions were made for constructing artificial diet as show in Table 3.1. Proteins, amino acids, mineral and water-soluble vitamin solutions in proportions shown in Table 3.2 were blended for 2-3 min in a 30 ml beaker using a magnetic stir bar, and then frozen overnight. The mixture was thawed the next day, the remaining ingredients were added and the complete diet was stirred for another 2-3 min in a water bath kept just below 50 °C. Unused diet could be refrigerated and stirred for 2-3 min again at just below 50 °C before the next feeding. Larvae were grafted onto 250 µl of AD as described in the previous section and diet was refreshed daily. For day 2 and day 3, 40 mg of glucose-fructose (1:1) was added per gram of diet each day to increase diet concentration. Diet composition remained the same as day 3 for the rest of the rearing until defecation. Larvae were cleaned with Kimwipe tissue and transferred to new well lined with autoclaved Kimwipe tissue for pupation.

Table 3.1 Stock solutions used in partially defined artificial diet for rearing honey bees.

Stock solutions	Component weight	Final volume (in ddH_2O)
Amino acid supplement		
Proline	110 mg	4.6 ml
Lysine	80 mg	
Leucine	40 mg	
Isoleucine	40 mg	
Serine	66 mg	
Tryptophane	22 mg	
Mineral solution I		
Monopotassium phosphate	8.8 g	250 ml
Postassium chloride	8.6 g	
Calcium Chloride, Dihydrate	2.14 g	
Mineral solution II		
Sodium chloride	2.5 g	250 ml
Magnesium sulfate heptahydrate	7.00 g	
Copper(II) sulfate pentahydrate	0.110 g	
Ferric citrate	0.250 g	
Manganese(II) chloride tetrahydrate	0.065 g	
Zinc acetate	0.232 g	
Vitamin solution I		
Thiamine hydrochloride	36 mg	500 ml
Pyridoxine hydrochloride	11 mg	
Biotin	7.4 mg	
Folic acid	8.0 mg	
Carnitine	7.0 mg	
Vitamin solution II		
Choline chloride	1800 mg	25 ml
Acetyl choline chloride	200 mg	
Ca pantothenate	300 mg	
Nicotinamide	107 mg	
Cyanocobalamin	1.5 mg	
meso-Inositol	107 mg	
Vitamin solution III		
Flavin mononucleotide	3.5 mg	50 ml
Flavin adenine dinucleotide	6.0 mg	
Ascorbic acid solution		
Ascorbic acid	8 mg	5 ml
Vitamin A/E solution		
Vitamin A acetate	2.2 mg	10 ml
α-Tocopherol acetate	12.5 mg	
Sterols		
β-sitosterol	200 mg	50 ml
Cholesterol	50 mg	

Table 3.2 Formulation of partially defined artificial diet used for rearing honey bees.

Component	Quantity
Protein	1000 mg
Amino acid solution	0.46 ml
Mineral solution I	0.66 ml
Mineral solution II	0.66 ml
Vitamin solution I	0.61 ml
Vitamin solution II	0.23 ml
Vitamin solution III	0.61 ml
Ascorbic acid solution	50 µl
Glucose-Fructose (1:1)	500 mg
Sucrose	500 mg
Sterols	2 ml
Metabolites	130 mg
Glycogen	46 mg
Vitamin A/E solution	100 µl
Adenosine triphosphate	22 mg
Creatine phosphate	15 mg
Gluconic acid (40%)	0.09 ml
ddH_2O	1.9 ml

** Addition of 40 mg of Glucose-Fructose (1:1) per gram of diet on day 2 and again on day 3.

3.2.4 Assessment of royal jelly integrity after ether extraction and dialysis

For each of the following samples: 1) untreated royal jelly, 2) residue from ether extraction (ether-extracted royal jelly), and 3) the residue after dialysis and freeze-drying (freeze-dried royal jelly), 20 µg of sample was resuspended in 9 µl of ddH_2O, 3 µl of 4XLDS loading buffer, and 0.24 µl 1 M DTT. Samples were boiled for 10 min at 70 °C and resolved on 10% SDS-PAGE for analysis.

3.2.5 Royal jelly digestion with pepsin coupled to NHS-sepharose beads

Pepsin was coupled to N-hydroxysuccinimide (NHS) activated sepharose beads according to the manufacturer's protocol. Briefly, pepsin from porcine gastric mucosa powder (400-800 unit/mg protein) was purchased from Sigma (Oakville, ON) and dissolved in coupling

buffer (0.2 M sodium acetate, 0.5 M sodium chloride, pH 6.0) to a final concentration of 40 mg/ml. NHS-activated sepharose bead (1 ml of 50% slurry) was washed with 6 ml of cold 1 mM hydrochloric acid (HCl) and spun down at 2000 rcf for 1 min to pellet beads. Beads were washed again with 1 ml of coupling buffer and spun down at 2000 rcf for 1 min again to remove supernatant. Lastly, 1 ml of pepsin solution was added to beads and allowed to couple overnight at 4 °C. After coupling, unreacted groups on beads were blocked by incubating in 0.1 M Tris-HCl, pH 6.0 for 4 h at 4 °C. Beads were washed 3 times with 0.5 ml of 0.1 M sodium acetate, 0.5 M sodium chloride, pH 4.0 and stored as 50% slurry in 20% ethanol at 4 °C.

Lyophilized royal jelly was dissolved in 10 mM and 50 mM HCl to 50 mg/ml, boiled at 95 °C for 10 min, and cooled to room temperature for digestion. Twenty microlitres of pepsin bead slurry was washed 3 times with either 10 mM or 50 mM HCl. Volumes of 200 µl or 400 µl of royal jelly solution were added to the pepsin beads and incubated at 37 °C shaking at 200 rpm overnight. One microliter of digested royal jelly was loaded onto a 10% SDS-PAGE for analysis. Digested royal jelly solution was spun down at 2000 rcf for 5 min and filtered through a folded glass fiber filter paper over a 1.5 ml microfuge tube. Ten microliters of filtrate was mixed with 5 µl of BSA (10 µg/µl in 10 mM HCl, denatured by boiling at 95 °C for 10 min) and incubated overnight at 37 °C to detect for residual proteolytic activity. Half of the reaction volume (25 µg of BSA) was analyzed by 10% SDS-PAGE.

3.2.6 Royal jelly digestion with pepsin in solution

Royal jelly (10 g) was dissolved in 200 ml of 20 mM HCl in an autoclaved 250 ml Erlenmyer flask. The solution was boiled at 95 °C for 10 min in a water bath and allowed to cool to room temperature. Pepsin (0.2 g) was dissolved in 5 ml of 20 mM HCl and added to the royal jelly solution. Samples were incubated at 200 rpm 37 °C for 48 h. A 10 µl aliquot was taken out at 24 h and 48 h and resolved by 10% SDS-PAGE to monitor digestion. Digested royal jelly was boiled at 95 °C for 10 min in a water bath to inactivate pepsin. Five microliters of digested royal

jelly was mixed with 5 µl of bovine serum albumin (BSA) (10 µg/µl in 10 mM HCl, denatured by boiling at 95 °C for 10 min) and incubated overnight at 37 °C to detect any residual proteolytic activity. The whole reaction (50 µg of BSA) was then resolved on 10% SDS-PAGE for analysis. Digested royal jelly solution was lyophilized and stored in -20 °C for use to reconstitute diet. Control royal jelly was treated the same way without adding pepsin.

3.2.7 Reconstitution of larval diet with digested royal jelly

Diets were then reconstituted with either pepsin-digested royal jelly, or control royal jelly. Firstly, a diet was constructed by mixing 1.05 g of royal jelly, 0.15 g of dextrose, 0.15 g of fructose, and 0.05 g of yeast extract, with 3.6 ml of ddH_2O but the texture of this larval diet was too thick to be used for rearing. Therefore, an extra 0.16 g of royal jelly, 0.022 g of dextrose, 0.022 g of fructose, 0.003 g of yeast extract, and 200 µl of ddH_2O was added to adjust the diet's texture to better resemble BLD. Larvae were grafted and reared with this reconstituted diet as described in section 3.2.2.

3.2.8 American foulbrood infection in *in vitro* reared larvae

Paenibacillus larvae spores isolated from naturally occurring AFB scale were obtained from Andony Melathopoulos (Apiculture Biotechnician, Agriculture and Agri-Food Canada) at $2.53x10^8$ spores/mg of ground scale. From this stock, 28.24 mg of spores were resuspended in 1 ml of ddH_2O and boiled at 85 °C for 20 min. Spores were diluted to an estimated 709 spores/µl and stored in 4 °C to be used as inoculum to make AFB diet.

BLD was constructed as in section 3.2.2. Rearing was also done as described in section 3.2.2 but with slight modifications to the diet and transferring intervals. Various volume of inoculum was added to BLD to make AFB diets with final spore concentrations of 1000, 2500, or 10000 spores/ml of diet. For Day 0 infections, larvae (N=24) less than 24 hours old were grafted directly onto 300 µl AFB diet for each spore concentration. For Day 1 infection, larvae (N=24)

less than 24 hours old were first grafted onto BLD for 24 hours and then transferred to either 1000 or 2500 spore/ml AFB diet for subsequent rearing. Twelve larvae were reared in the same well for the first three days. On Day 4, larvae were transferred to one larva per well with 300 µl freshly made AFB diet. Larvae were replenished with 50 to 100µl of AFB diet added near the mouth of the larvae if all diet in the well was consumed. Upon defecation, larvae were cleaned on Kimwipe tissues and transferred to pupation trays. Death attributed to American Foulbrood was inspected by appearance of engorged larvae with characteristic pinkish-brown color, which eventually would turn into ropy mass and dried to the bottom of the well.

3.2.9 Preparation of double-stranded RNA

Total RNA was isolated from honey bee 5th instar larvae using Trizol reagent (Invitrogen). First-strand reverse transcription was initiated with random primers using SuperScript® III Reverse Transcriptase (Invitrogen) following manufacturer's protocol. After digestion with RNaseH (Fermentas), cDNA was amplified with primers designed from NCBI Reference Sequence NM_001011627.1 of *Apis mellifera* phenoloxidase subunit A3 (PPO), mRNA (forward primer: 5'- CCAGAGGGTGCTAGAGTTCC-3' and reverse primer: 5'-GCCTCGTAAATACGATCACG-3'). Further, 1 µl of cDNA was added to a PCR reaction and amplified with initial incubation of 95 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min, 52 ºC for 1 min, 72 ºC for 1 min, and a final extension at 72 ºC for 7 min. Template for double-stranded RNA (dsRNA) synthesis for PPO was made by amplifying 1 µl of the PPO PCR product to a new PCR reaction using PPO primers fused with T7 promoter sequence (forward primer 5'- TAATACGACTCACTATAGGGCGACCAGAGGGTGCTAGAGTTCC-3' and reverse primer 5'- TAATACGACTCACTATAGGGCGAGCCTCGTAAATACGATCACGC-3'). Template for dsRNA synthesis for GFP was made by PCR reaction from pcDNA3-EGFP plasmid using GFP primer sequences fused with T7 promoter sequence (forward primer 5'-TAATACGACTCACTATAGGGAGATGGTGAGCAAGGGCGAG and reverse primer 5'-

TAATACGACTCACTATAGGGAGCGGTTCACCAGGGTGTCG). PCR reactions were done with initial incubation of 95 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min, 60 ºC for 1min, 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. PCR product was purified using the QIAquick™ PCR purification kit (QIAGEN). The dsRNA was prepared using the Promega RiboMax ™ T7 system (Promega) with sense and antisense strands transcribed from DNA template in the same reaction. Resulting RNA was purified with RNeasy Plus Universal Mini Kit (QIAGEN) and resuspended in nuclease free water, heated at 95 ºC for 1 min and let cool to room temperature. A total of 300 ng of dsRNA was loaded to 1% denaturing formaldehyde-agarose gel for assessing dsRNA integrity. Final concentration of dsRNA of both PPO and GFP were normalized with nuclease free water and stored at -20 ºC as 40 µg aliquots.

3.2.10 Prophenoloxidase RNA interference in American foulbrood infected larvae

AFB diet with 2500 spores/ml was made and 200 µl of diet was added per well in 24-well plate. BLD without any spores was used as the diet for the no spore control. For each condition, 40 µg of dsRNA was added to each well to make a final dsRNA concentration of 200 µg/ml of diet; equivalent volume of ddH_2O was added to no dsRNA and no spore controls. For each condition, larvae less than 24 hours old (N=24, 3 replicates) were obtained from the UBC Farm and grafted onto the corresponding diets with 12 larvae per well. At 24 hours intervals, larvae were transferred to new wells containing diet with dsRNA or water added on the day. Larvae were then reared on dsRNA diet for four days and subsequently reared on AFB diet (or BLD for no spore control) with one larvae per well until defecation. Upon defecation, larvae were cleaned on Kimwipe and transferred to a pupation tray. Each of the dead larvae or pupae was homogenized in 1 ml of ddH_2O, boiled at 85 °C for 20 min, centrifuged at 1000 rcf for 10 min, and 5 µl of supernatant was plated on MYPGP agar (1% Mueller-Hinton broth, 1.5% yeast extract, 0.3% potassium monophosphate, 0.2% glucose, 0.1% sodium pyruvate, and 2% agar).

Death was attributed to AFB if small, mostly rough, flat or raised and whitish to beige coloured colonies appeared after incubating at 37 ºC for two days.

3.2.11 Hemolymph protein collection in dsRNA treated larvae

To assess for effects of dsRNA knockdown, larvae less than 24 hours old (N=20) were reared in each of the four treatments (dsPPO, dsGFP, no dsRNA, and no spores) as described in the previous section. On the 8th day, nine larvae of similar size from each condition were selected and randomly divided into three groups of three larvae. Hemolymph was collected by making an incision along the ventral side of the larvae with care taken to avoid puncturing the midgut. Hemolymph from each group was pooled into 1 sample and centrifuged at 16,100 rcf for 15 min at 4 ºC. Proteins in the supernatant were subjected to ethanol/acetate precipitation (Foster, De Hoog & Mann 2003). Proteins precipitated after 1.5 hour incubation at room temperature were collected by centrifugation at 16,100 rcf. The protein pellet was resolubilized in 6 M urea, 2 M thiourea, 100 mM Tris-Cl (pH 8.0) and any insoluble material was subsequently removed by centrifugation at 16,100 rcf. Protein concentration from each sample was measured by Bradford assay. For each sample, 300 µg of resolubilized proteins were resolved on a 10% SDS-PAGE and 3 bands of molecular weight of 37 – 50 kDa, 50 – 75 kDa, and 75 – 150 kDa were excised for in-gel digestion with trypsin. The resulting peptides were stored on STAGE (STop And Go Extraction) tips with extra WP C18 Prep HPLC Bulk Packing beads until analysis, as described previously (Chan, Howes & Foster 2006).

3.2.12 MRM assay for measuring prophenoloxidase levels

A total of five most likely detected proteotypic peptides from *Apis mellifera* phenoloxidase subunit A3 (GI: 58585195) were selected based on the honey bee PeptideAtlas (Chan et al. 2011). These five peptides were synthesized at a 5 µmol scale using Fmoc (9-fluorenylmethoxycarbonyl) chemistry with a MultiPep 96-well plate peptide synthesizer (Intavis

AG, Germany). Fmoc amino acids (EMD4Bioscience, USA) were conjugated to Fmoc - Arg(Pbf) - HMP resin (Anaspec Inc. , USA). Subsequent amino acid residues (in 600 mM stock solutions) were double-coupled with 20% piperidine as the deprotector and 1-hydroxybenzotriazole (HOBT), O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) as the activator. The cleavage was performed with 92.5% (v/v) trifluoroacetic acid, 5% (v/v) triisopropyl silane, and 2.5% (v/v) water. The peptides were ether-precipitated and resolubilized in 200 µl of 0.5% acetic acid. Each synthesized peptide was diluted 1000-fold using 0.5% acetic acid and multiple injections of 1 µl each were used to optimize the fragmentor voltage (0 - 180 V, 20 V increments) and subsequently the collision energy (5 - 25 V, 5 V increments; 13 -18 V, 1 V increments). Fragment ions with a mass-to-charge ratio (*m/z*) greater than the precursor ion *m/z* were prioritized, leading to 3-4 transitions selected for the MRM assay (Table 3.3).

All peptide samples were analyzed on a triple quadrupole mass spectrometer (6460, Agilent Technologies) equipped with an HPLC-Chip Cube system (Agilent Technologies) operated in selected ion and product ion mode (generation of the MRM assay) or MRM mode (honey bee samples). In all experiments, Q1 and/or Q3 were operated at unit resolution (0.7 *m/z* full width at half height). For the generation of the MRM assay, a ProtID-Chip-43 (II) containing a 40 nL trap column and a 43 mm analytical column was used (G4240-62005, Agilent Technologies) and the trapping of the peptides was performed at 4 µL/min using solvent A (3% acetonitrile in 0.1% formic acid) only. Analysis was performed using a flow rate of 600 nL/min with a gradient of 10-40% solvent B (90% acetonitrile in 0.1% formic acid) in 2.5 min. Honey bee samples were eluted from STAGE tips with 50 µl of 80% acetonitrile in 0.5% acetic acid, vacuum-dried, and reconstituted in 40 µl of 0.5% acetic acid. A volume 0.5 – 1 µl (equivalent to 3.75 µg of protein) were injected (4 µL/min, solvent A) onto a Large Capacity Chip (II) consisting of a 160 nL trap column and 150 mm analytical column (G4240-62010, Agilent Technologies). Chromatographic separations were performed using a flow rate of 300 nL/min with a gradient of 5 - 20% solvent B in 30 min and 20 - 35% in 24 min. Dynamic MRM was set up to monitor each

transition for at least 67.93 ms, leading to in a duty cycle of 1 s for 6 min before and after the probably retention time. Validation of the peptide identity was performed by comparing the relative peak area of each of the transitions to the relative area of the transitions of the synthetic peptide.

Table 3.3 Parameters and transitions selected for each peptide used in dynamic MRM-assay of PPO for honey bee larval hemolymph sample analysis.

Peptide Sequences	Fragmentor voltage (V)	Transitions	Collision Energy (V)	Retention time (min)
SVATQVFNR	120	511.5→835.5 511.5→764.4 511.5→535.3 511.5→436.2	11 11 14 14	15.0
SSVTIPFER	120	518.5→762.5 518.5→661.4 518.5→548.3	12 15 11	22.7
IYEAIHTGSVINTR	120	525.5→847.5 525.5→731.1 525.5→649.6	19 10 10	17.7
FSDTIVPR	120	468.0→787.5 468.0→700.5 468.0→484.4 468.0→371.2	11 10 11 10	15.0
NILNTFWTK	120	569.0→909.5 569.0→796.4 569.0→341.0	14 14 10	39.2

3.3 Results

3.3.1 Rearing with basic larval diet resulted mostly in the worker caste

Rearing with our general procedure could sustain growth of female honey bee larvae with an average survival rate of 99% throughout the larval stage and up to 85% of larvae could successfully develop as adult. Most of the female larvae developed as workers, with approximately 3% larvae developed into queens. In this general rearing procedure, young worker larvae less than 24 hours old were obtained from the UBC Farm and carefully grafted

onto BLD using a bent paintbrush. It was important not to put too many larvae into one well as larvae could push against each other, leading to some being drowned and an overall reduced survival rate. Surviving young larvae had a slightly rounded, light-reflecting surface and respiratory movements could be observed under a stereomicroscope; on the contrary, drowned or dead larvae would appear dull-white and flat. Larvae were allowed to eat *ad libitum* for the eight to nine days before defecation. As larvae developed they grew in size, respired more frequently and often moved in their wells (Figure 3.1). Sometimes, molt shed by the larvae could be seen floating on the diet near the larvae. By the eighth or ninth day, larvae would defecate (Fig. 3.1E), indicated by the appearance of yellow-brownish feces and uric acid crystals, and spin the cocoon. At this stage, it was important to clean excessive food and feces off the larvae by gently rolling larvae on tissues and transferring them to new wells for pupation. This was determined empirically as initial trials without cleaning and transferring larvae to clean wells resulted in some larvae developing into pupae but none could survive to adulthood. After defecation and transferring to the pupation tray, the larvae often changed from a yellowish color to a whiter color. On days ten or eleven, larvae would usually metamorph into pupae, following which they started to develop colouring, beginning with the eyes, moving to the upper body and lastly to the abdomen in the subsequent days. The general procedure for rearing was to transfer larvae onto the new diet every other day until defecation. However, altering the transfer interval to only on the fourth day and upon defecation could also sustain larval development without a significant difference in their survival rate. One critical precaution was to maintain moisture of the larval food, achieved by adding water to the spaces between wells used for rearing. This method served as a great alternative to the general method as it significantly reduced the amount of handling and hence reduced damage to larvae during transfer. Overall, *in vitro* larval rearing was efficient using the BLD, although a slightly slowed growth was observed compared to their normal development in the colony. For bees from the same hive, it took approximately

17 days for workers to develop completely in natural hives, whereas it took approximately 16 days for queens and 21 days for workers to become fully developed *in vitro.*

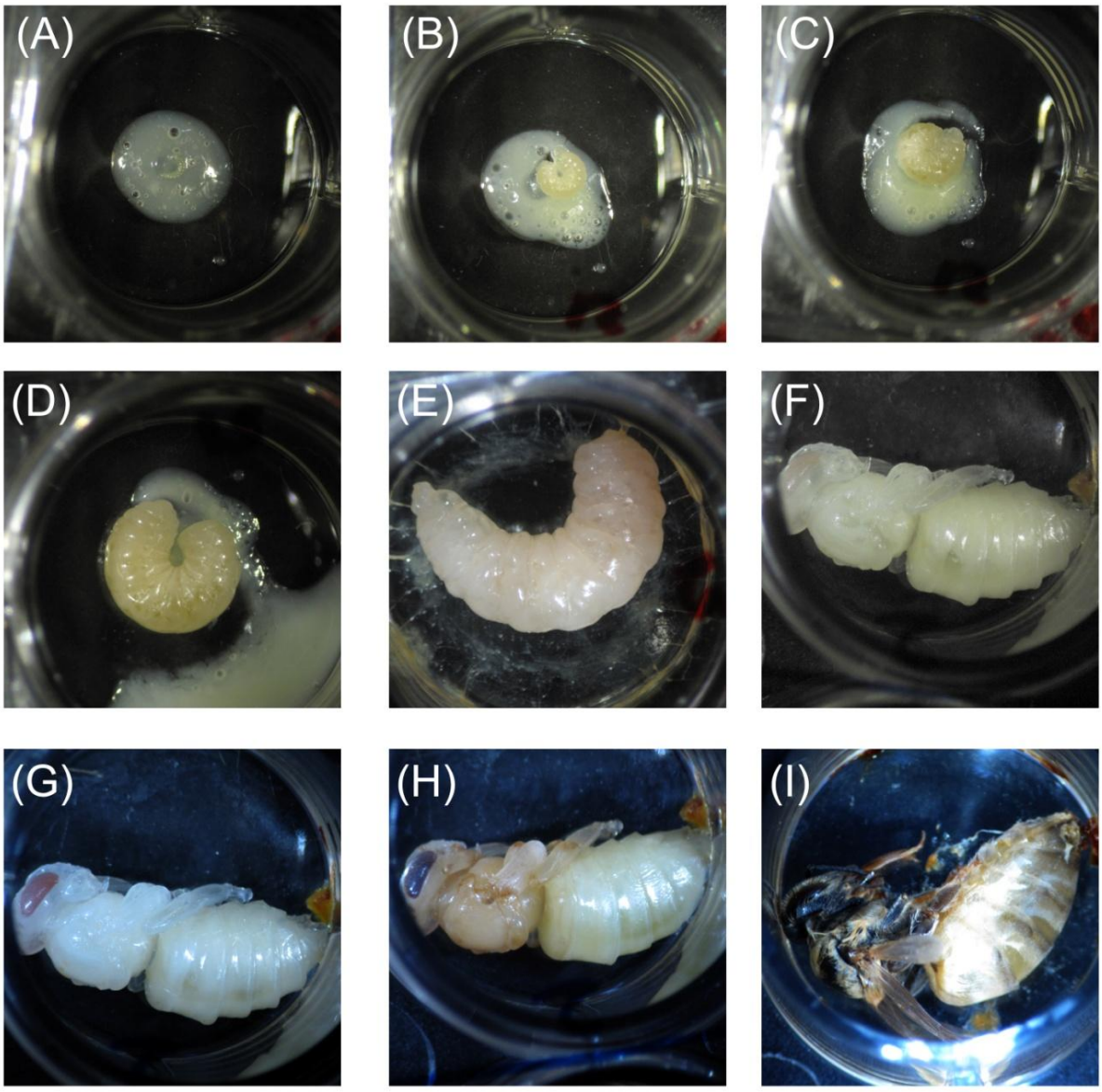


Figure 3.1 *In vitro* larval rearing assay.
Larvae <24 h were obtained from UBC Farm and reared on BLD in 24-well plate until defecation. Defecated larvae were cleaned on kimwipe and transferred to clean well for pupation. Pictures taken on: (A) Day 1 (B) Day 3 (C) Day 5 (D) Day 7 (E) Day 9 (F) Day 12 (G) Day 15 (H) Day 18 (I) Day 21

3.3.2 Rearing with a partially defined artificial diet

The BLD, as explored above, constitutes a reasonable in vitro system that eliminates many of the uncontrollable variables in a colony situation. However, we have little control over the quality of royal jelly and, hence, the final resulting caste of bees; so this is still suboptimal for further experiments on caste differentiation. From a survey of the literature, the diet developed by Sheul and Dixon (1986) seemed to be the best chemically defined diet with only proteins and metabolites sources derived from royal jelly. Therefore, we explored the possibility on furthering the partially defined artificial diet used by Sheul and Dixon (1986) to develop a fully defined diet where all individual components could be controlled to answer any specific question about caste differentiation or nutrition.

Three trials were done using the partially defined artificial diet described above and the best survival rate obtained was 67% of larvae making it to the defecation stage, 23% turning into pupae and 6% emerging as adults. In the first trial, more larvae survived but none of them defecated. It was observed that pre-warming of the diet to 45 ºC caused the diet to become too thick and it dried easily. Therefore, we modified the protocol to limit pre-warming to only two minutes for the next two trials. Additionally, larvae were also transferred to new diet every day for the two subsequent trials to ensure diet quality. And these two changes seemed to improve defecation and survival rate slightly although viability remained low. For trials two and three, most of the larvae appeared normal throughout the larval stage, except that their body sizes were more variable. Interestingly, we often observed more dark coloration remaining in the midgut of larvae reared on the partially defined artificial diet compared to larvae reared on BLD after defecation. Defecated larvae often turned black and failed to pupate (Figure 3.2A). Those who reached pupation continued to develop normally until day 16 or 17, when their body turned abnormally black and pupae died quickly thereafter (Figure 3.2B). Out of the small number of

emerged adults, most of them appeared to be slimmer than normal and one of them had a malformed membrane between her thorax and abdomen (Figure 3.2C).

Figure 3.2 Abnormal phenotype of larvae reared on partially defined artificial diet.
(A) A 14 days old defecated larva turned black. (B) A 17 days old pupa with abnormal pigmentation. (C) A 22 day old developed bee with malformed membrane.

In this partially defined artificial diet, royal jelly was first extracted with ether to obtain the metabolite fraction followed by extensive dialysis of the remaining material to remove any remaining small molecules. As there were many processing steps involved for the royal jelly before the diet reconstitution, royal jelly at various manipulation steps was analyzed by SDS-PAGE to determine if there was any major protein loss or degradation due to processing. Compared to the same amount of residue resolved on SDS-PAGE, there was no significant band missing or severe protein degradation (Figure 3.3). However, a more intense staining was observed in the royal jelly residue after freeze-drying, indicating that the proteins were becoming much more concentrated by the ether extraction.

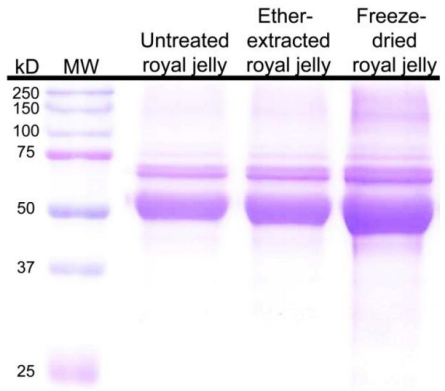


Figure 3.3 SDS-PAGE analysis of processed royal jelly.
Twenty microgram of untreated royal jelly, ether-extracted royal jelly, and freeze-dried royal jelly were run on 10% SDS-PAGE and stained with Coomassie Brilliant Blue stain.

3.3.3 Rearing with digested royal jelly

To further the development of a chemically completely defined diet, we investigated the potential for rearing with protease digested royal jelly, in hopes of confirming that royal jelly residue from the partially defined artificial diet only served as an amino acid source. However, to avoid potential side effect from feeding active protease to honey bee larvae, extensive efforts were required ensure that the protease could be removed after digestion. As royal jelly was intrinsically acidic (pH ~4), pepsin was selected as the desired protease as it had optimal activity from pH 1.5 - 3.5 (Christensen 1955, Schlamowitz, Peterson 1959). Pepsin could be coupled to NHS-sepharose beads without significant losses and 10 µl of pepsin conjugated to beads could digest 50 µg of BSA within 30 min (data not shown). In the case of royal jelly, resuspending in water and boiling for five minutes were not sufficient to allow pepsin to digest the proteins. Therefore, two concentrations of hydrochloric acid were added to acidify proteins for a more efficient digestion. When 10 mM HCl was added, no significant digestion was observed with 10 µl of pepsin-bead conjugate was used to digest 200 or 400µl of 50 µg/ml of royal jelly (volume ratios of 1:20 and 1:40). When 50 mM HCl was added, both 1:20 and 1:40 ratios resulted in complete digestion of royal jelly (Figure 3.4A). To check if pepsin remained coupled to the beads after royal jelly digestion, digested royal jelly solution was filtered and

incubated with BSA to see if there was any residual proteolytic activity. When royal jelly was digested in 10 mM HCl, no significant proteolytic activity was observed; however, when digestion was done in 50 mM HCl, residual proteolytic activity could be detected in supernatants from both volume ratios used for royal jelly digestion (Figure 3.4B). Hence, coupling pepsin to NHS-sepharose beads was not feasible as 50 mM HCl was needed for digestion but pepsin did not remain coupled to beads under those conditions.

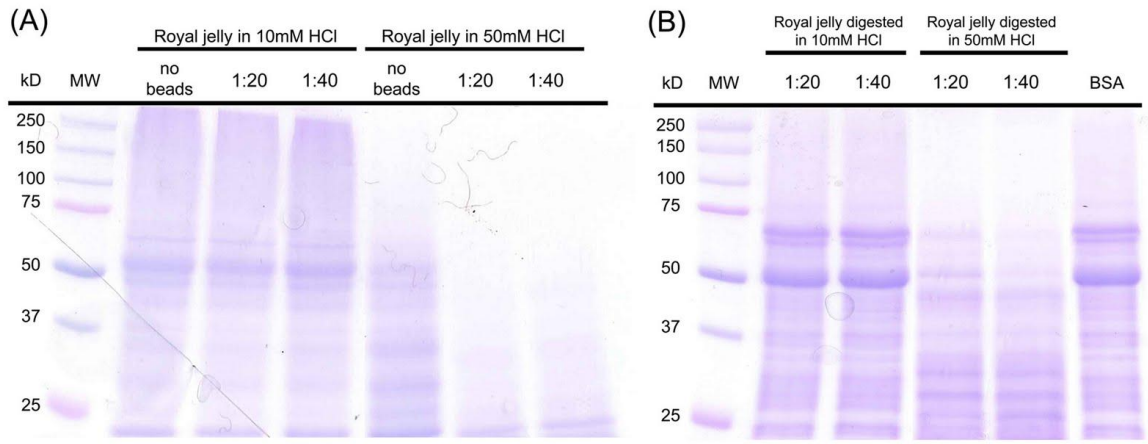


Figure 3.4 Royal jelly digested with pepsin beads.
Pepsin (40 mg/ml) was linked to NHS-sephorose beads. Royal jelly was resuspended in either 10 mM HCl or 50 mM HCl to 50 mg/ml and denatured by boiling at 95 °C for 10 min. (A) Overnight digestion of royal jelly using 10 µl of pepsin beads. Digestion was set up at volume to volume pepsin bead to royal jelly solution ratios of 1:20 or 1:40 in both 10 mM and 50 mM HCl conditions. (B) Pepsin beads were filtered out from digested royal jelly and the filtrates were tested for residual pepsin activity. 50 µg of BSA was incubated with 10 µl of filtrate overnight. Half of the reaction product was loaded onto the gel. Control: 25 µg BSA

As pepsin-bead conjugates could not be used for digestion, in-solution digestion was chosen as an alternative approach to eliminate protease activity after royal jelly digestion. First, 10 g of royal jelly was dissolved in 20 mM HCl and 0.4 g of pepsin was added to royal jelly for digestion. By 24 hours, most of the royal jelly was digested and incubating for another 24 hours did not result in further digestion (Figure 3.5). Instead of physically removing pepsin from royal jelly, pepsin was heat inactivated after digestion. After heat inactivation, the pepsin digest was

incubated with BSA to check for proteolytic activity (Figure 3.5, Lane 5 and 6). As no proteolytic activity was observed, digested royal jelly was lyophilized and used for reconstituting diets.

Lyophilized, digested royal jelly was used to reconstitute diets in the same proportions as in BLD. However, lyophilized royal jelly absorbed more water than untreated royal jelly, resulting in a diet that was much thicker than BLD and extra water was added to adjust the diet to similar viscosity as BLD. Nonetheless, when this new diet was used to rear honey bee larvae, none of the larvae survived more than three days, indicating digested royal jelly had lost nutrients that were essential for growth and development. Thus, we were unable to ascertain whether the protein residue used in partially defined artificial diet provided nutrients other than just amino acids. As both partially defined artificial diet and the digested royal jelly diet could not sustain growth as efficiently as BLD, all future rearing was done with BLD.

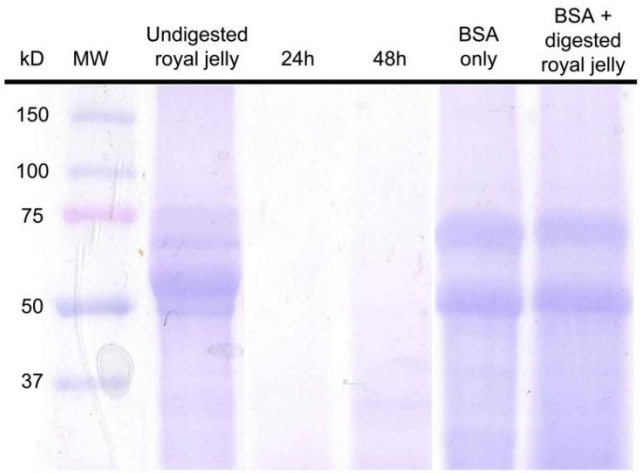


Figure 3.5 In-solution pepsin digestion of royal jelly.
Ten gram of royal jelly was dissolved in 200 mL of 20 mM HCl and digested with 0.4 g of pepsin for 48 h. Pepsin was inactivated by boiling the digested royal jelly solution for 10 min. Five microliters of digested royal jelly was incubated with 50 µg of BSA overnight to check for enzyme activity. Lane 1: 50 µg of undigested royal jelly. Lane 2 and 3: 2 µl of digestion product. Lane 4: 50 µg of BSA. Lane 5: 50 µg of BSA and 5 µl of digested royal jelly.

3.3.4 American foulbrood infection and prophenoloxidase knockdown using *in vitro* larval rearing

With the general *in vitro* larval rearing technique having been established, we then applied this technique to study the potential role of PPO in honey bees' resistance to American foulbrood after they have reached three days old. Three dosages of spores (1000, 2500, and 10000 spores/ml of diet) were fed to honey bee larvae less than 24 hours old to establish the dosage sufficient to induce approximately 10% AFB death; this minimal mortality would then allow us to see increased death if larvae became more susceptible to AFB in later experiments. Also, larvae were grafted onto AFB diet either immediately (Day 0) or reared on BLD for one day and then transferred to AFB diet (Day 1) to investigate whether we could have an additional 24 hours to knockdown PPO first before infecting the larvae. In these *in vitro* infected larvae, most of the AFB deaths started to appear when larvae were seven days old and most deaths occurred after defecation similar to the reports from natural infection. However, some AFB deaths appeared earlier when AFB diet with 10000 spores/ml was fed to the larvae. For larvae grafted onto AFB diet immediately, 1000, 2500, and 10000 spores/ml resulted in an average of 12.6%, 20.6%, and 38.3% AFB deaths respectively (Figure 3.6). Comparing infection of Day 0 and Day 1 larvae, there was no significant difference in average AFB deaths but variation between trails was much larger when infection was carried out on Day 1. Comparing the three concentrations for infecting day 0 larvae, 1000 spores/ml resulted in AFB death rate closest to the desired 10% deaths; however, variation between trials was higher than that of 2500 spores/ml. Therefore, grafting larvae onto AFB diet with 2500 spores/ml immediately at day 0 was selected as the method for AFB infection in this study.

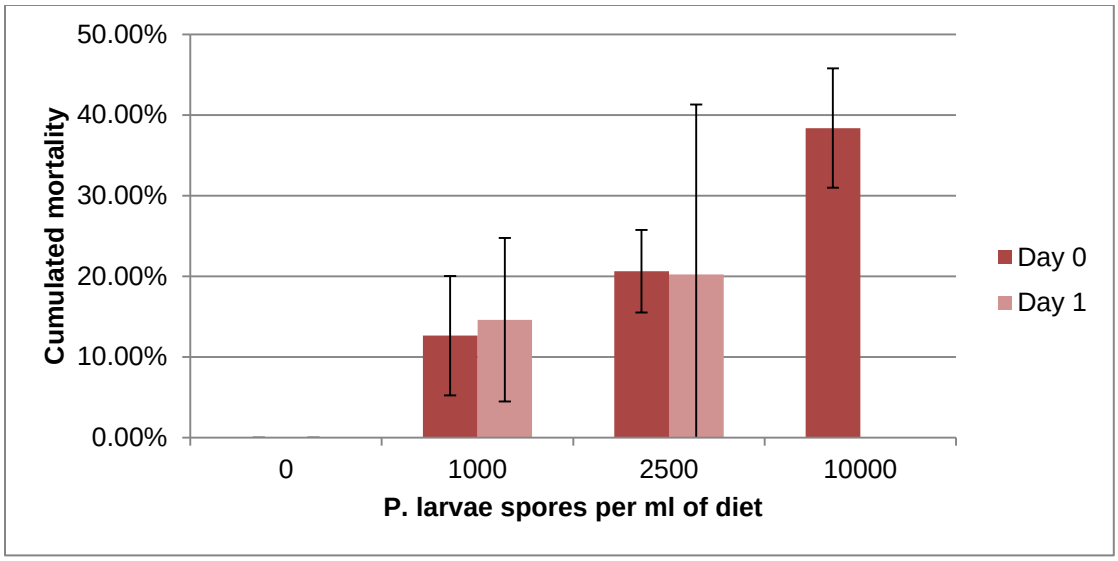


Figure 3.6 Dosage-mortality relationships of honey bee larvae fed with various spores concentrations.
Honey bee larvae <24 h old were obtained from UBC farm. Day 0: larvae were transferred onto diet containing *P.larvae* spores immediately upon grafting. Day 1: larvae were reared with BLD for 24 h and then transferred onto diet containing *P.larvae* spores the day after grafting. (N=24) 10000 spores/ml were only done to Day 0 larvae due to limitation in total number of bees obtained per frame.

Once the infection threshold for achieving 20% AFB deaths was established, dsRNA targeting PPO (dsPPO: 446 bp) and control dsRNA for GFP (dsGFP: 369 bp) were synthesized by *in vitro* transcription. Before each set of experiments, dsRNA was run on denaturing formaldehyde agarose gels to ensure its integrity (Figure 3.7) and 200 µg/ml of diet was selected as the feeding concentration following the recommendation from Amdam *et. al* (2011). When 200 µg/ml of dsRNA was fed to larvae younger than 24 hours old on BLD without spores, there were no visible phenotypic changes observed in honey bees at both the larval and pupal stages and also no differences in the overall survival rate (data not shown). Therefore, feeding dsPPO or dsGFP to larvae was not lethal for honey bee development. Next, we fed dsRNA to honey bee larvae less than 24 hours old together with AFB diet. Overall, there was no significant difference among dsPPO, dsGFP, and no dsRNA treatment (Figure 3.8). Nonetheless, there were a slightly higher number of deaths from AFB when dsRNA was fed to

the larvae and further experiments would be needed to confirm this unexpected trend. A typical, necessary control in any RNAi approach is the confirmation of knocked-down gene expression following the treatment. Towards this goal, an MRM assay was used to detect PPO levels in honey bee. Comparing the PPO levels in honey bee larvae fed with dsPPO, dsGFP, and no dsRNA, there was no significant difference among the three groups (Figure 3.9). Interestingly, comparing the relative intensities of the five peptides from PPO monitored by MRM assay, the peptide SVATQFNR was detected at a significantly higher level in AFB infected bees than non-infected bees, confirming the activation of PPO upon infection (Figure 3.10).

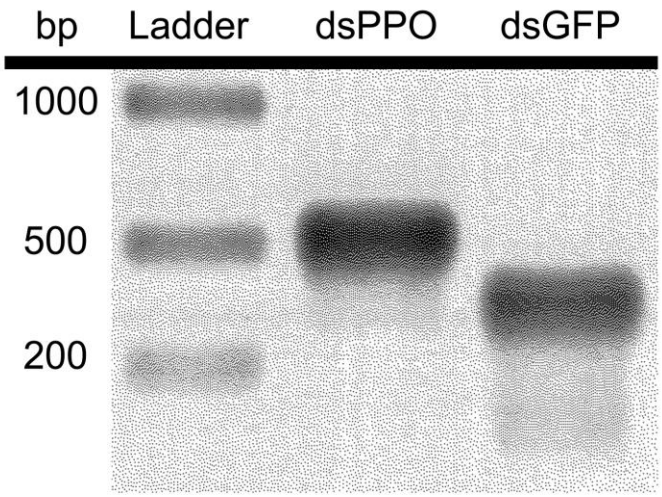


Figure 3.7 Assessment of dsRNA quality.
Synthesized dsRNA (300 ng) of dsPPO (446bp) and dsGFP (368bp) were run on 1% denaturing agarose gel.

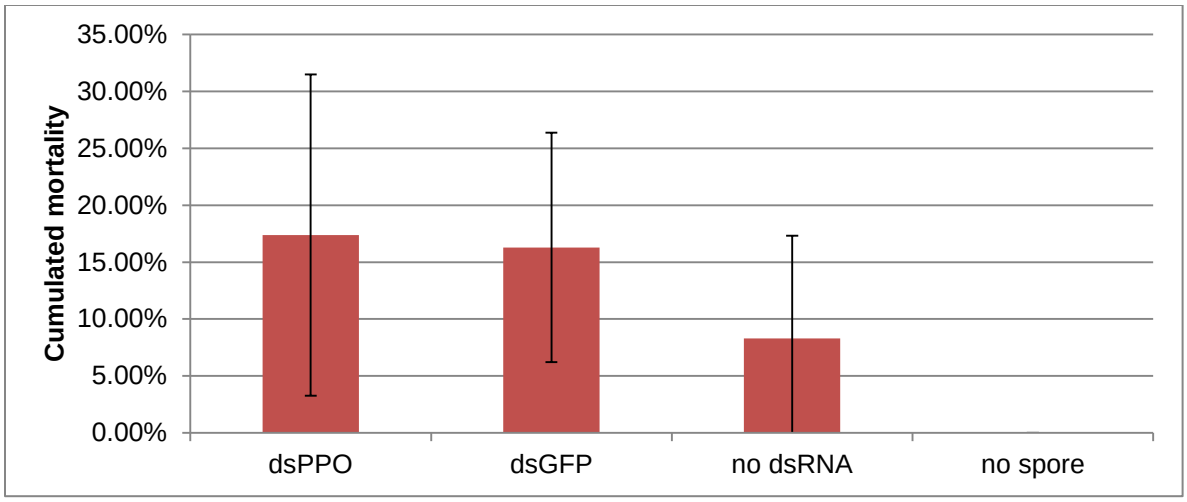


Figure 3.8 Cumulated AFB death rates in dsRNA knockdown.
Two hundred microgram per milliliter of dsPPO or dsGFP was fed to honey bee larvae <24 h old in AFB diet containing 2500 spores/ml. Controls were honey bee larvae <24 h old fed with AFB diet without dsRNA and honey bee larvae <24 h old fed BLD without spores. Average mortality rate from 4 trials were shown.

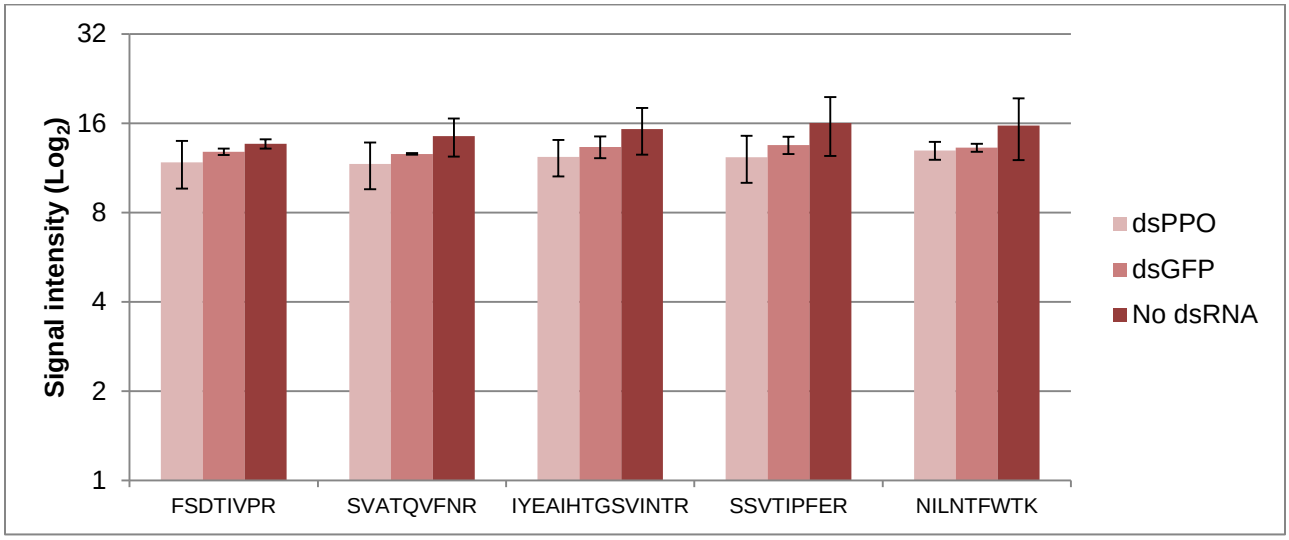


Figure 3.9 Expression levels of PPO in hemolymph of bee larvae fed with dsPPO, dsGFP, and no dsRNA.
PPO levels in hemolymph of honey bee larvae fed with dsPPO, dsGFP, and no dsRNA were assayed by MRM. Signal intensity for each peptide was measured by the sum of areas under the curve of all transitions monitored for that peptide.

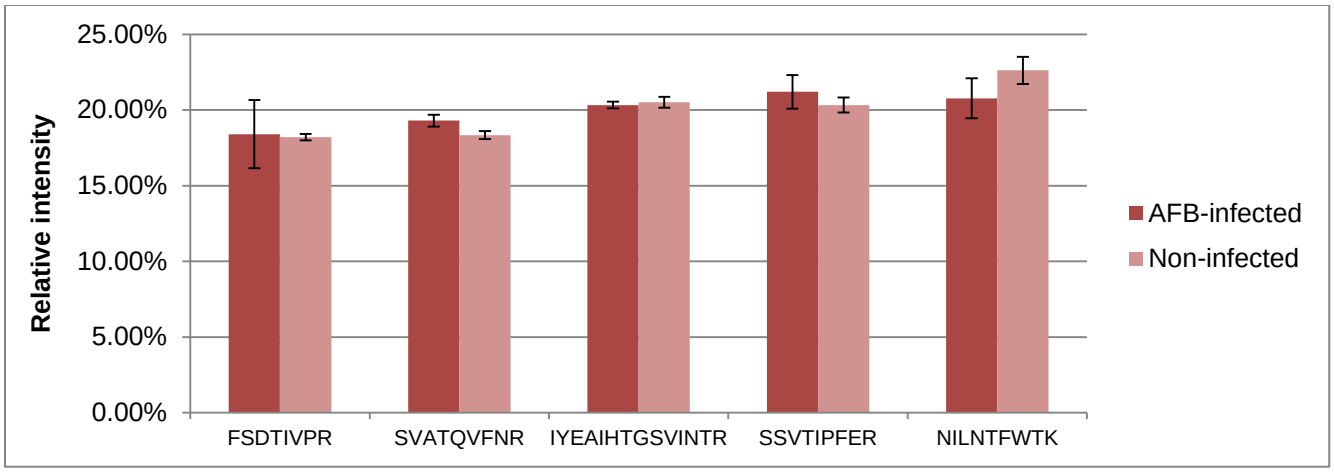


Figure 3.10 Relative intensities of peptides from PPO from AFB-infected and non-infected larvae.
PPO levels in hemolymph from AFB infected and non-infected larvae were assayed by MRM. Signal intensity for each peptide was measured by the sum of areas under the curve of all transitions monitored for that peptide. Relative intensity was calculated by signal intensity of each peptide compared to total signal intensity detected from all five peptides.

3.4 Discussion

BLD was efficient for sustaining honey bee larval growth in *in vitro* larval rearing with several important factors affecting survival rate. Food replenishment intervals were found to be adjustable as long as the food remained moist. This was probably due to the fact that dried food was also thickened, which inhibited movement of larvae and also increased the difficulty for a larva to consume its food. Previous reports on *in vitro* rearing with only one feeding during larval development reported no significant decrease in larval weight (Kaftanoglu, Linksvayer & Page 2010). However, our incubator could only achieve relative humidity around 80% instead of the controlled 90-95% used in other studies so replenishing once on the fourth day seemed to work the best for us with minimal handling and no decrease in survival rates. The second important factor in *in vitro* rearing was the cleaning of feces and transferring to a clean pupation tray. In the natural hive, honey bee larvae would turn and deposit feces at the bottom of the cells; however, for our *in vitro* setting, larvae remained in contact with the feces all over the body and hence cleaning was necessary to prevent fungal contamination (Peng et al. 1992). Also, in other

adopted protocols, defecated larvae were often transferred from 95% relative humidity to 70% relative humidity (Peng et al. 1992, Aupinel et al. 2005, Kaftanoglu, Linksvayer & Page 2010), indicating a lower humidity might be beneficial for pupation. Although survival rate was not significantly different in our case for not transferring to lower humidity, constant contact with excess food and feces might create an environment too humid for pupation to occur. Therefore, although it was not absolutely necessary for complete development, adding lining the bottom of each pupation well with a tissue could increase post-defecation survival rate by decreasing contact with feces and also by maintaining a dryer surface for pupation. It is important to note that *in vitro* reared larvae defecated approximately one to two days later and required three days longer development time overall compared to natural reared larvae, which was also reported by Peng (1992) when she developed this BLD diet. Therefore, there might be a slight life stage deviation in *in vitro* reared larvae compared to their age during their time of record in further experiments. Overall survival rates for rearing with BLD was similar to that reported by others who employed the same technique (Peng et al. 1992, Aupinel et al. 2005, Kaftanoglu, Linksvayer & Page 2010) and it served as a good starting diet for future experiments.

Using a partially defined diet, a few larvae could successfully develop into adults but the overall survival rate was very low compared to BLD. Ingredients used in this partially defined diet might not be as stable compared to the original ingredients in BLD as partially defined diet required refreshment every day to increase survival rate. Incomplete defecation of larvae might contribute to higher mortality, as defecation was an important way to excrete wastes and feces remaining inside larvae might be harmful for development. Other abnormalities in the late stages of pupation indicated important nutrients needed during metamorphosis might be missing in the partially defined diet. Jay (1964b) provided detailed documentation of developmental abnormalities of starved honey bees, classified to five stages in prepupal ecdysis and five stages in pupal ecdysis. Comparing our pupae to his documentation, most of our pupae had completed prepupal ecdysis as head and legs were usually visible and free of skin.

Interestingly, white-head worker pupae were observed if larvae failed stage two prepupal ecdysis due to a blockage of thoracic tracheae during molting (Jay 1964b). Similarly, our pupae with abnormal pigmentation in figure 3.2B might also indicate failed development in the tracheal system. Lastly, the pupa in figure 3.2C might be of similar pupae failed pupal ecdysis stage 0-1 with skin failed to move beyond head and hence antenna and wings were not extended. Overall, the above observed phenotypes correlated with malnutrition during larval stages, confirming extra nutrients were needed for partially defined artificial diet to sustain development beyond the larval stage. Assessing the integrity of processed royal jelly, it seemed like the missing nutrient might not be proteins, as no obvious protein bands were missing from SDS-PAGE analysis. The more intense staining from the freeze-dried royal jelly was probably due to extensive removal of water during lyophilisation, including even the proteins' hydration sphere, increasing the amount of proteins in the same weight of royal jelly. Other than proteins, important small molecules might also be lost in the ether extraction and/or dialysis steps. As detailed analysis on volatile and extractable compounds had been done on royal jelly (Isidorov et al. 2009, Isidorov, Bakier & Grzech 2011), metabolomics analysis comparing metabolites in ether extract and compounds found in previous studies might be helpful for identifying missing nutrients in partially defined artificial diets.

The role of royal jelly residue was investigated by reconstituting diet with digested royal jelly in the same manner as BLD. To avoid potential side effects from feeding proteases to honey bee larvae, pepsin was first coupled to NHS-sepharose beads so that it could be removed after digestion. As the optimal pH for pepsin was pH 1.0, royal jelly had to be resuspended in 50 mM HCl to allow for digestion. However, as ligand esters with NHS-sepharose bead are only stable between pH 3 and 13, and even then in a ligand-dependent manner (GE Healthcare), the pH of 50 mM HCl is likely too low and leads to hydrolysis of the ester, resulting in proteolytic activity in supernatant. The alternative in-solution digestion was successful; however, it still failed to sustain growth of honey bee larvae. Compared to the

preparation of partially defined artificial diet, preparation for digested royal jelly eliminated ether extraction and dialysis steps, which should result in less opportunity for loss of nutrients. Nonetheless, two boiling steps were need to denature royal jelly and eliminate pepsin activity, heat sensitive components other than proteins might have been destroyed and hence reconstituted diet could not sustain growth more than three days. Therefore, we could not investigate whether the proteins in royal jelly serve more functions than simply providing a dense source of amino acids as neither the partially defined artificial diet nor digested royal jelly diet sustained honey bee growth in this study.

In establishing the spore dosage response for AFB infection, 2500 spores/ml of diet was chosen to give an approximately AFB mortality rate of 20%. The dosage response and earlier AFB symptoms observed in the higher dose were consistent with previous studies on the lethal infection threshold in honey bee workers (Behrens et al. 2010). The dosage per larvae could be estimated from previous observation that 6 to 12 hours old larvae consumed 1.94 mg of larval food (Haydak 1970). In the construction of BLD, 20 g of ingredients resulted in approximately 20 ml of diet. Hence, young larvae consuming 1.94 mg of diet would have ingested approximately 4.85 spores. This dosage was comparable to the results from Brødsgaard (1998) where three *P. larvae* spores fed to 24 to 28 hours old larvae led to 10% mortality. However, it should be noted that the total number of physical spores was used here instead of colony-forming units (cfu) so 4.85 spores is likely an overestimation as non-viable spores would also have been counted in this study (Behrens et al. 2010). When larvae were transferred to AFB diet after one day of *in vitro* rearing, the average AFB death rate was not significantly different, but the variation between trials were much higher compared to grafting onto AFB diet immediately. The average 21% death rate when 2500 spores/ml diet was used was similar to that reportedly obtained from feeding six spores to 48 to 52 hours larvae in a previous study (Brødsgaarda, Ritterb & Hansena 1998), however, Brødsgaard et al. did not observe an increase in variation, probably because their larvae were reared in natural hives by nurse bees until the age of infection. On

the contrary, our larvae were reared *in vitro* before infection; as *in vitro* reared larvae showed delayed growth to various extends, our larvae might be of more diverse developmental stages compared to previous studies, resulting in higher variation in susceptibility to AFB.

Although there was no significant difference in susceptibility to AFB when comparing knockdown and control dsGFP fed groups, an increase in susceptibility to AFB was observed in both of the dsRNA fed groups compared to the no dsRNA control. This indicates that honey bee larvae fed with dsRNA might have impaired immune systems. Previous studies had shown that injection of dsGFP as a control increased tsetse flies' susceptibility to trypanosome infection (Nayduch, Aksoy 2007) and also mosquitoes' susceptibility to both gram-positive and gram-negative bacterial infection (Dong, Taylor & Dimopoulos 2006). Interestingly, another study on the role of defensin using RNAi discovered that while injection of dsDEF resulted in complete knockdown of defensin, injection control dsGFP could also result in partial suppression of antimicrobial peptide defensin level upon bacterial challenge in mosquitoes (Blandin et al. 2002). Alternatively, the immune system of dsRNA treated bees might not be impaired but indeed activated. It had been shown that Small interfering RNA (siRNA) can activate Toll like receptor 3 (TLR3) in mouse models and human cells (Cho et al. 2009, Kleinman et al. 2008). Innate immune responses could also be induced by dsRNA in marine invertebrate (Robalino et al. 2004). As dsRNA is an important intermediate formed in many viruses' life cycles, it is perhaps not surprising that the presence of dsRNA may activate antiviral responses in innate immune systems in a sequence non-specific manner. Therefore, feeding dsRNA might induce prolonged activation of bees' immune system and these bees might not be able to invoke the same level of immune response when challenged by other pathogens, resulting in increased susceptibility. To investigate the potential side effects of dsRNA treatment on honey bees' immune systems, it would also be interesting to see if feeding dsRNA would decrease expression of immune factors or provoke a generally suppressed immune response. Due to our limited honey bee season in Canada, only one concentration of dsRNA was tested for RNAi knockdown of PPO in this study.

As excess amount of dsRNA usually would not increase RNAi silencing effect (Meyering-Vos, Muller 2007, Shakesby et al. 2009), optimal concentration of dsRNA should be used in RNAi studies to minimize potential off-target effects or other side effects such as impact on immune system (Huvenne, Smagghe 2010). With the MRM assay established, future experiments on PPO could be done by investigating the optimal dsRNA concentration needed on efficient knockdown and hence explore further the role of PPO in honey bees' immunity against AFB infection.

3.5 Conclusion

In vitro larval rearing is a useful and important tool for studying honey bee development and effects of pathogen exposure in a controlled environment. In this study, *in vitro* rearing was successful with a BLD but important nutrients for pupal stage development might be lost in the preparation of partially defined artificial diets and hence prevent us from fully defining a diet. Nonetheless, we could still study the effects of pathogen exposure using the BLD. Feeding dsRNA for PPO to larvae did not result in significant increase in AFB infection mortality but interestingly, oral ingestion of dsRNA to honey bee larvae might have impaired the bee's immune system as a side effect and increased their susceptibility to AFB infection. Future experiments are needed to optimize RNA interference in honey bee larvae and to validate PPO's potential role in resistance against AFB infection.

4 Conclusion

In light of recent world-wide declines in honey bee populations, there is a considerable need for more research into bee health. And yet, our knowledge of honey bee biology lags far behind that in other model organisms limiting by tools available for us to conduct experiments in a controlled environment. Most of our knowledge about honey bee biology comes from early observations in the bee's natural habitat: the hive. With the strong interactions between individuals in a honey bee colony, results from experiments can be highly variable and thus complicate the molecular-level, functional interpretations of any experiments. Additionally, honey bees behaviour is highly dependent on the time of year and in Canada there is a considerable period in the winter when no experiments can be done at all, severely limiting the possibilities for research. At this time, there is as yet no available honey bee cell line for research purposes. With much of our current knowledge of molecular biology coming from studying cell cultures and other *in vitro* systems in other organisms, there is a huge need to make more tools available to apply to honey bee research.

4.1 Addressing the project aims

The major goal of this thesis was to address the lack of tools for honey bee research and to establish and improve some of the available research tools for use in our laboratory. Particularly, multiple techniques could be aligned and employed to answer some of our specific research questions. In Chapter 2, the major breakthrough in the current field of research was the additional cryopreservation and lentivirus transduction of primary honey bee cell cultures. Previous research had used primary cultures for short term analysis; however, their use was limited as these cultures had to be freshly prepared. With the success of cryopreservation, large amount of cells could be stored in the summer and used for analysis when bees were not available. Additionally, the success of lentiviral transduction of GFP indicated the potential of

transduction and expression of exogenous genes in these primary cultures, providing us the power to manipulate cellular machinery to test specific hypotheses. As these cultures could survive for at least four months, this provided enough of a time window for us to be able to ponder previously inaccessible questions about bee molecular biology and to observe changes in the cells. In combination with proteomic analysis, future experiments with semi-quantitative analysis of proteome changes after lentiviral transduction of various genes could be monitored over time, giving us valuable insights on regulations of life processes at the molecular level. Meanwhile cell cultures provided us with simplified systems to study honey bee biological processes; *in vitro* larval rearing provided us with a bigger picture of honey bee biology in a whole organism level. Although development of a completely defined artificial diet was not successful, rearing with a basic larval diet gave us the opportunity to isolate individual bees and have complete control over their environment, including exposure to pathogens. This is already a great advantage over studies in natural hives as results would not be affected by weather and progressive rearing of nurse bees. Particularly, *in vitro* rearing and infection allowed us to visually monitor phenotypic changes of honey bee larvae and pupae, which would be very difficult when cells were capped in natural hives and observation of disease symptoms might not be possible as sick or dead bees would be removed from the hive quickly by nurse bees. With further optimization, RNA interference might be used in combination with *in vitro* rearing. Large scale RNAi experiments have been done on the red flour beetle to provide functional genomic information on development and metamorphosis (Bucher 2012). With the maturation of RNAi technologies and the continual reduction in costs for producing dsRNA, similar projects could be done on honey bees to provide direct evidence of gene functions. Additionally, as many bee viruses are RNA viruses, dsRNA treatment had been developed for improving bee health (Hunter et al. 2010). *In vitro* larval rearing in combination with dsRNA feeding would definitely provide a good starting point for testing the efficacies of such treatments, as well as their potential side effects. Lastly, the addition of multiple reaction monitoring (MRM) mass

spectrometry assays to measure the degree of gene silencing was a unique alternative to the commonly used real-time polymerase chain reaction (qPCR) as it measuresdirectly at the ultimate product of gene expression – the protein. Both qPCR and MRM are more sensitive methods than Northern or Western blots and therefore they should be the better choices for assaying effectiveness of knockdown. Compared to qPCR, an MRM assay provides an arguably more relevant read-out of RNAi effects at protein expression level. Since transcript levels do not always correlate with protein levels due to various translational regulations (Selbach et al. 2008), MRM assays would be a better approach to confirming RNAi effects once they become more routine. An additional advantage provided by MRM assays is that it allows one to monitor levels of multiple peptides from the same protein. In the case of PPO, this is useful as it allows one to assay enzyme activation by monitoring one signature peptide. In AFB infected larvae, the tryptic peptide SVATQVFNR, whose C-terminus is the predicted propeptide cleavage site (Lourenco et al. 2005), was elevated by about 10-fold compared to tryptic peptides found in the remainder of the protein (Chan et al. 2009), indicating an increased phenoloxidase response due to proteolytic activation of PPO pool already present in the larvae. Therefore, by monitoring this signature peptide and other peptides from PPO, we could gain additional about both the expression and activation levels of PPO present in honey bees. Overall in this thesis, we have demonstrated the feasibility and value of cell culture and *in vitro* rearing as tools for honey bee research at cellular and whole organism levels, connecting these techniques with various tools opens up great opportunities for more thorough studies to improve our current knowledge of honey bee biology.

4.2 Addressing the project hypothesis

In this thesis, we started with the hypothesis that prophenoloxidase played an important role in honey bees' immunity against American foulbrood infection and PPO knockdown would result in increased susceptibility to AFB. Although a dosage response could be observed from AFB infection of *in vitro* reared honey bees, we could not confirm the knockdown of PPO and hence the role of PPO in AFB susceptibility. One limitation of the *in vitro* technique was a potential difference in immunity in *in vitro* reared bees upon RNAi treatment. Feeding dsRNA rendered the honey bees more susceptible to AFB infection, as shown in chapter 3 of this thesis. This might also be the case when *in vitro* reared bees were exposed to other pathogens for experiments. Therefore, non-sequence specific dsRNA must be used as a control in future RNAi experiment and concentrations must be optimized to minimize other potential side effects. In addition, variation in diets for rearing might also be considered as there was still a 3% chance for queens and some intercastes to come out from basic larval diet and immunity of bees varies between castes (Chan, Howes & Foster 2006), increasing the biological variation in our experiments.

4.3 Future experiments

4.3.1 Immortalization of honey bee primary cells

Although an embryonic cell line from honey bees has been reported, the authors have so far refused all requests to share this valuable resource with other researchers. Therefore, lentiviral transduction of various oncogenes into honey bee primary cell cultures from eggs and other culturable tissues to establish one or more immortalized cell lines would be beneficial for future studies.

4.3.2 Comparisons of proteomic differences of *in vitro* reared bees to natural bees

During prophenoloxidase MRM assay development, it was observed that *in vitro* reared honey bee larvae did not melanize to the same level compared to naturally reared bees at a similar age, indicating a potentially impaired melanization pathway in *in vitro* reared bees. At the early stage of establishing *in vitro* larval rearing technique in our laboratory, three sets of *in vitro* reared and natural reared honey bees were collected at the same age every other day from day 0 to day 15 (day of emergence in natural bees) and stored in -80°C for future analysis. Using this set of samples, relative PPO levels using MRM assay or quantitative hemolymph proteins comparison using mass spectrometry could be conducted to examine whether proteins involved in innate immune responses might be expressed in different levels in *in vitro* reared bees.

4.3.3 Optimization of RNA interference of prophenoloxidase in *in vitro* reared bees

With the previous experience of *in vitro* rearing and the well-developed MRM assay for prophenoloxidase, RNAi of PPO could be further optimized in several ways. First of all, various concentrations of dsPPO and dsGFP, such as 10 ug/ml, 100 ug/ml, and 450 ug/ml of diet, could be fed to honey bee larvae to assess the optimal concentration of dsRNA to be used without significant side effects. Secondly, feeding intervals could also be shortened to feed dsRNA every 12 hours for two days as recommended by Amdam *et al.*(2011). Once the optimal concentration for PPO dsRNA treatment and feeding intervals are established, we could reassess PPO's role in honey bees' ability to fight AFB infection.

4.4 Closing

Our molecular knowledge of honey bees has lagged far behind other model organisms and one of the greatest obstacles to honey bee research is the lack of available tools. As shown in this thesis, cell culture and *in vitro* larval rearing are two of the valuable tools that can be adopted in honey bee research and combination with other mass spectrometry-based proteomics techniques can rapidly advance of our understanding of honey bee biology at the molecular and whole organism levels. Hopefully, in the near future, more *in vitro* tools can be made available for bee research and these will be useful for solving the problem of honey bees' population decline.

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Appendix A Supplementary Materials

Table A.1 Expression ratios of honey bee proteins in cultured vs. fresh cells.
Column A: protein accession number, Column B: description of protein name/function, Column C: number of unique peptide sequences identified for each protein, Column D: relative ratio of protein levels in cultured cells vs. cells freshly prepared from eggs, Column E: standard deviation of ratios in Column D.

Accession	Description	No. Of peptides	Cultured /fresh	StDev
gi_110755329	XP_001121746.1 PREDICTED: hypothetical protein [Apis mellifera]	2	High	n/a
gi_66504546	XP_394333.2 *heat shock protein* PREDICTED: similar to Protein lethal(2)essential for life (Protein Efl21) isoform 1 [Apis mellifera]	2	High	n/a
gi_110750764	XP_001120137.1 PREDICTED: similar to Protein lethal(2)essential for life (Protein Efl21) [Apis mellifera]	2	High	n/a
gi_66521514	XP_624019.1 PREDICTED: similar to CG7380-PA [Apis mellifera]	3	High	n/a
gi_66546646	XP_623095.1 *phospholipid-hydroperoxide glutathione peroxidase* PREDICTED: similar to Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor (PHGPx) (GPX-4) [Apis mellifera]	4	High	n/a
gi_110777113	XP_001123234.1 *catalase* PREDICTED: similar to Catalase, partial [Apis mellifera]	4	High	n/a
gi_66504551	XP_392222.2 PREDICTED: similar to sequestosome 1 isoform 1 [Apis mellifera]	4	High	n/a
gi_110758259	XP_623576.2 *metalloprotease* PREDICTED: similar to CG14516-PA, isoform A, partial [Apis mellifera]	11	High	n/a
gi_110748949	XP_623342.2 *aldolase* PREDICTED: similar to Aldolase CG6058-PF, isoform F [Apis mellifera]	12	High	n/a
gi_110750756	XP_393575.3 *HSP* PREDICTED: similar to Protein lethal(2)essential for life (Protein Efl21) isoform 1 [Apis mellifera]	13	High	n/a
gi_110750758	XP_001120006.1 PREDICTED: similar to Protein lethal(2)essential for life (Protein Efl21) [Apis mellifera]	13	High	n/a
gi_110758428	XP_394662.3 *lactate dehydrogenase* PREDICTED: similar to Ecdysone-inducible gene L3 CG10160-PA [Apis mellifera]	16	High	n/a
gi_66504786	XP_624456.1 *saccharopine dehydrogenase* PREDICTED: similar to CG2604-PA, isoform A isoform 1 [Apis mellifera]	6	7.7	2.9
gi_66522931	XP_623191.1 *lectin* PREDICTED: similar to ergic53 CG6822-PA, isoform A isoform 1 [Apis mellifera]	4	5.9	
gi_48095181	XP_392253.1 PREDICTED: similar to visgun CG16707-PC, isoform C [Apis mellifera]	2	5.3	
gi_110756611	XP_623072.2 *Sodium pump subunit* PREDICTED: similar to Sodium/potassium-transporting ATPase alpha chain (Sodium pump) (Na+/K+ ATPase) isoform 1 [Apis mellifera]	4	5.0	
gi_66558612	XP_394922.2 *juvenile hormone epoxide hydrolase* PREDICTED: similar to Juvenile hormone epoxide hydrolase 1 CG15101-PA [Apis mellifera]	2	4.0	2.8

Accession	Description	No. Of peptides	Cultured /fresh	StDev
gi_110756613	XP_623145.2 PREDICTED: similar to Sodium/potassium-transporting ATPase alpha chain (Sodium pump) (Na+/K+ ATPase) isoform 2 [Apis mellifera]	14	3.8	
gi_66530527	XP_623582.1 *annexin* PREDICTED: similar to Annexin IX CG5730-PC, isoform C isoform 2 [Apis mellifera]	11	3.8	0.1
gi_110756533	XP_392358.3 PREDICTED: similar to Dystroglycan CG18250-PA, isoform A [Apis mellifera]	2	3.7	1.1
gi_48094341	XP_392114.1 *calponin, inhibitor of ATPase activity in smooth muscle myosin* PREDICTED: similar to Chd64 CG14996-PB [Apis mellifera]	8	3.5	1.0
gi_66538476	XP_624247.1 *calmodulin* PREDICTED: similar to Calmodulin CG8472-PA, isoform A [Apis mellifera]	6	3.4	0.4
gi_66534008	XP_392616.2 *ATPase inhibitor-like protein* PREDICTED: similar to apontic CG5393-PB, isoform B [Apis mellifera]	3	3.3	
gi_66521459	XP_623725.1 *porin* PREDICTED: similar to porin CG6647-PA, isoform A [Apis mellifera]	26	3.2	1.9
gi_48141870	XP_393570.1 *superoxide dismutase* PREDICTED: similar to SOD (superoxide dismutase) family member (sod-2) [Apis mellifera]	11	3.2	1.5
gi_66566395	XP_623926.1 *thymosin (actin monomer sequestering)* PREDICTED: similar to ciboulot CG4944-PB, isoform B [Apis mellifera]	12	3.0	1.1
gi_66525522	XP_624159.1 *chitin-binding protein* PREDICTED: similar to Roe1 CG6155-PA [Apis mellifera]	12	3.0	1.9
gi_66534655	XP_624662.1 *glutathione S transferase* PREDICTED: similar to Glutathione S transferase S1 CG8938-PA, isoform A, partial [Apis mellifera]	17	3.0	1.7
gi_110749420	XP_624548.2 *zinc metalloprotease* PREDICTED: similar to CG6512-PB, isoform B [Apis mellifera]	3	2.9	
gi_110749921	XP_392112.3 *mitochondrial outer membrane translocase* PREDICTED: similar to CG2713-PA [Apis mellifera]	2	2.9	
gi_48140590	XP_393517.1 *thioredoxin reductase* PREDICTED: similar to Thioredoxin reductase-1 CG2151-PA, isoform A isoform 1 [Apis mellifera]	11	2.9	1.2
gi_66551889	XP_395981.2 *disulfide isomerase* PREDICTED: similar to CaBP1 CG5809-PA isoform 1 [Apis mellifera]	19	2.9	2.2
gi_66515272	XP_623495.1 *ATP synthase component* PREDICTED: similar to Vacuolar ATP synthase catalytic subunit A, osteoclast isoform (V-ATPase subunit A 2) (Vacuolar proton pump alpha subunit 2) (V-ATPase 69 kDa subunit 2) (Isoform HO68) isoform 1 [Apis mellife	7	2.9	2.3
gi_48096523	XP_392479.1 *14-3-3 protein* PREDICTED: similar to 14-3-3 CG31196-PC, isoform C isoform 1 [Apis mellifera]	18	2.8	0.8
gi_110761202	XP_625222.2 PREDICTED: similar to CG9380-PA, isoform A [Apis mellifera]	8	2.7	2.6
gi_48097086	XP_391841.1 *14-3-3 protein, isoform zeta* PREDICTED: similar to 14-3-3-like protein (Leonardo protein) (14-3-3 zeta) isoform 1 [Apis mellifera]	22	2.7	0.7

Accession	Description	No. Of peptides	Cultured /fresh	StDev
gi_66523390	XP_392776.2 *glutamate dehydrogenase* PREDICTED: similar to Glutamate dehydrogenase CG5320-PF, isoform F isoform 1 [Apis mellifera]	9	2.7	1.5
gi_58585104	NP_001011578.1 *vitellogenin* vitellogenin [Apis mellifera]	148	2.7	2.5
gi_110756705	XP_624758.2 *solute carrier* PREDICTED: similar to CG2791-PA [Apis mellifera]	7	2.6	0.2
gi_66535742	XP_395851.2 *glutamate carboxypeptidase* PREDICTED: similar to CG17337-PA isoform 1 [Apis mellifera]	7	2.6	1.8
gi_66515469	XP_393284.2 PREDICTED: similar to CG17271-PA [Apis mellifera]	3	2.6	
gi_66556180	XP_392967.2 *Rab1* PREDICTED: similar to Rab-protein 1 CG3320-PA, isoform A [Apis mellifera]	20	2.6	1.2
gi_110777801	XP_001120025.1 *Rab7* PREDICTED: similar to RAB7, member RAS oncogene family [Apis mellifera]	3	2.6	1.6
gi_66524882	XP_392060.2 *phosphatidylethanolamine-binding protein* PREDICTED: similar to CG6180-PA isoform 1 [Apis mellifera]	9	2.6	0.7
gi_66513649	XP_623691.1 *spectrin* PREDICTED: similar to Spectrin alpha chain [Apis mellifera]	28	2.5	1.2
gi_110768018	XP_623323.2 *smooth muscle myosin* PREDICTED: similar to zipper CG15792-PD, isoform D [Apis mellifera]	11	2.5	1.7
gi_110766687	XP_001121359.1 PREDICTED: hypothetical protein, partial [Apis mellifera]	9	2.5	2.2
gi_66511001	XP_394645.2 *Hsc70 interacting protein* PREDICTED: similar to CG2947-PA, isoform A isoform 1 [Apis mellifera]	2	2.5	
gi_66504249	XP_625125.1 *protein kinase c substrate* PREDICTED: similar to CG6453-PA [Apis mellifera]	4	2.5	1.4
gi_48097857	XP_391959.1 *prohibitin* PREDICTED: similar to lethal (2) 37Cc CG10691-PA, isoform A [Apis mellifera]	31	2.5	1.4
gi_66531474	XP_624271.1 *glutamine amidotransferase* PREDICTED: similar to dj-1 CG1349-PA [Apis mellifera]	4	2.5	0.8
gi_110762076	XP_625049.2 *protein kinase c inhibitor* PREDICTED: similar to CG2862-PA, isoform A [Apis mellifera]	6	2.4	1.6
gi_48097144	XP_393701.1 *signal peptidase complex subunit 2 homolog* PREDICTED: similar to Signal peptidase complex subunit 2 (Microsomal signal peptidase 25 kDa subunit) (SPase 25 kDa subunit) [Apis mellifera]	6	2.4	0.8
gi_66548684	XP_624390.1 *pyruvate kinase* PREDICTED: similar to Pyruvate kinase CG7070-PB, isoform B [Apis mellifera]	4	2.4	1.6
gi_66522163	XP_624571.1 *protein disulfide isomerase family protein* PREDICTED: similar to CG9911-PA, isoform A [Apis mellifera]	3	2.4	
gi_110751310	XP_001120162.1 *endoplasmic reticulum protein (windbeutel)* PREDICTED: similar to windbeutel CG7225-PA [Apis mellifera]	8	2.4	1.7
gi_48142692	XP_393605.1 *glyceraldehyde 3-phosphate dehydrogenase* PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase 2 (Glyceraldehyde-3-phosphate dehydrogenase II) (GAPDH II) isoform 1 [Apis mellifera]	39	2.4	1.2

Accession	Description	No. Of peptides	Cultured /fresh	StDev
gi_48098315	XP_394036.1 *ATP synthase component* PREDICTED: similar to ATP synthase D chain, mitochondrial [Apis mellifera]	5	2.4	0.7
gi_66513092	XP_392478.2 *malate dehydrogenase* PREDICTED: similar to mitochondrial malate dehydrogenase precursor isoform 1 [Apis mellifera]	42	2.4	1.1
gi_110762902	XP_624156.2 *ATP synthase component* PREDICTED: similar to ATP synthase- CG11154-PA, isoform A [Apis mellifera]	74	2.4	1.4
gi_66546799	XP_624781.1 *ribosomal protein* PREDICTED: similar to 60S acidic ribosomal protein P1 (RP21C) (Acidic ribosomal protein RPA2) [Apis mellifera]	15	2.3	1.4
gi_66526635	XP_623252.1 *aldehyde dehydrogenase* PREDICTED: similar to CG31075-PA [Apis mellifera]	3	2.3	0.9
gi_110766509	XP_001122345.1 *bicaudal* PREDICTED: similar to bicaudal CG3644-PA, isoform A [Apis mellifera]	15	2.3	0.3
gi_48094441	XP_392125.1 *tropomyosin* PREDICTED: similar to Tropomyosin 1 CG4898-PD, isoform D isoform 1 [Apis mellifera]	4	2.3	0.3
gi_66526646	XP_623438.1 *dihydrolipoamide dehydrogenase * PREDICTED: similar to CG7430-PA isoform 1 [Apis mellifera]	3	2.3	1.3
gi_110765226	XP_001120364.1 *ribosomal protein* PREDICTED: similar to 60S acidic ribosomal protein P2 (Acidic ribosomal protein RPA1) [Apis mellifera]	15	2.3	0.4
gi_66510928	XP_623962.1 *catalase* PREDICTED: similar to Catalase, partial [Apis mellifera]	3	2.3	
gi_110773271	XP_001123353.1 *enoyl-CoA hydratase* PREDICTED: similar to CG6543-PA, isoform A [Apis mellifera]	19	2.2	1.4
gi_48124643	XP_393266.1 *alcohol dehydrogenase* PREDICTED: similar to alcohol dehydrogenase 5 [Apis mellifera]	2	2.2	1.7
gi_66509780	XP_623619.1 PREDICTED: similar to Actin-87E isoform 1 [Apis mellifera]	66	2.2	0.8
gi_66505984	XP_393141.2 *failed axon connections* PREDICTED: similar to failed axon connections CG4609-PC, isoform C isoform 1 [Apis mellifera]	3	2.2	1.6
gi_66531601	XP_397411.2 *lipid storage droplet surface binding protein* PREDICTED: similar to Lipid storage droplet-2 CG9057-PA [Apis mellifera]	3	2.2	0.5
gi_66560290	XP_392857.2 *cathepsin D* PREDICTED: similar to cathD CG1548-PA [Apis mellifera]	7	2.2	1.4
gi_48137684	XP_393368.1 *actin* PREDICTED: similar to Actin-5C isoform 1 [Apis mellifera]	71	2.2	1.2
gi_66499293	XP_623552.1 *glucose-6-phosphate isomerase* PREDICTED: similar to Glucose-6-phosphate isomerase (GPI) (Phosphoglucose isomerase) (PGI) (Phosphohexose isomerase) (PHI) [Apis mellifera]	2	2.2	0.9
gi_48138819	XP_393426.1 *vesicle-associated membrrane protein* PREDICTED: similar to VAMP (vesicle-associated membrane protein)-associated protein A [Apis mellifera]	6	2.2	2.3
gi_66513527	XP_392913.2 *superoxide dismutase* PREDICTED: similar to Superoxide dismutase CG11793-PA isoform 1 [Apis mellifera]	6	2.2	1.3

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gi_110760134	XP_624153.2 *heat shock protein 70* PREDICTED: similar to CG2918-PA [Apis mellifera]	5	2.2	0.3
gi_66522335	XP_391960.2 *proteasome subunit* PREDICTED: similar to Mov34 CG3416-PA [Apis mellifera]	6	2.2	0.9
gi_66530257	XP_396670.2 *lamin* PREDICTED: similar to Lamin CG6944-PA, partial [Apis mellifera]	56	2.2	1.0
gi_48099735	XP_392593.1 *annexin* PREDICTED: similar to Annexin IX CG5730-PC, isoform C isoform 1 [Apis mellifera]	14	2.2	0.8
gi_66565249	XP_625027.1 *elongation factor 1b* PREDICTED: similar to Probable elongation factor 1-beta (EF-1-beta) [Apis mellifera]	8	2.1	1.4
gi_110754998	XP_393090.3 *heat shock protein cognate 3* PREDICTED: similar to Heat shock protein cognate 3 CG4147-PA, isoform A [Apis mellifera]	75	2.1	0.5
gi_66548188	XP_393445.2 *thioredoxin peroxidase* PREDICTED: similar to thioredoxin peroxidase 1 CG1633-PA, isoform A isoform 1 [Apis mellifera]	45	2.1	1.1
gi_66545506	XP_392689.2 *calreticulin* PREDICTED: similar to Calreticulin CG9429-PA isoform 1 [Apis mellifera]	24	2.1	0.5
gi_66546657	XP_623282.1 *disulfide isomerase* PREDICTED: similar to ERp60 CG8983-PA, isoform A isoform 2 [Apis mellifera]	51	2.1	0.8
gi_58585198	NP_001011628.1 *eEF-1 alpha chain* translation elongation factor eEF-1 alpha chain [Apis mellifera]	7	2.1	
gi_110761214	XP_623682.2 *elongation factor 1 gamma * PREDICTED: similar to translation elongation factor 1-gamma isoform 2 [Apis mellifera]	16	2.1	1.7
gi_110768510	XP_001121905.1 *disulfide isomerase* PREDICTED: similar to Protein disulfide-isomerase precursor (PDI), partial [Apis mellifera]	20	2.1	0.8
gi_66536893	XP_623743.1 *proteasome subunit* PREDICTED: similar to Rpt1 CG1341-PA [Apis mellifera]	2	2.1	
gi_66566109	XP_624801.1 *NADH dehydrogenase* PREDICTED: similar to Pdsw CG8844-PA, isoform A [Apis mellifera]	3	2.0	
gi_110760701	XP_001120471.1 *hydroxyacyl-CoA dehydrogenase* PREDICTED: similar to 3-hydroxyacyl-CoA dehydrogenase type-2 (3-hydroxyacyl-CoA dehydrogenase type II) (Type II HADH) (3-hydroxy-2-methylbutyryl-CoA dehydrogenase) (Scully protein) [Apis mellifera]	30	2.0	1.8
gi_48097100	XP_391843.1 *acetyl-CoA acyltransferase* PREDICTED: similar to yippee interacting protein 2 CG4600-PA [Apis mellifera]	15	2.0	0.7
gi_66504360	XP_392760.2 *ATP synthase component* PREDICTED: similar to ATP synthase O subunit, mitochondrial precursor (Oligomycin sensitivity conferral protein) (OSCP) [Apis mellifera]	5	2.0	1.7
gi_66499429	XP_392111.2 *acyl-CoA dehydrogenase* PREDICTED: similar to CG12262-PA [Apis mellifera]	6	2.0	0.1
gi_66514614	XP_396769.2 *imaginal disc growth factor* PREDICTED: similar to Imaginal disc growth factor 4 CG1780-PA, isoform A [Apis mellifera]	3	2.0	

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gi_110760871	XP_623512.2 *aldehyde dehydrogenase* PREDICTED: similar to CG7145-PA, isoform A [Apis mellifera]	7	2.0	1.2
gi_110751158	XP_001120072.1 *actin depolymerizing factor* PREDICTED: similar to Cofilin/actin-depolymerizing factor homolog (Protein D61) (Protein twinstar) [Apis mellifera]	20	2.0	1.4
gi_66506786	XP_394487.2 *malate dehydrogenase* PREDICTED: similar to CG5362-PA isoform 1 [Apis mellifera]	8	2.0	1.4
gi_58585146	NP_001011603.1 *arginine kinase* arginine kinase [Apis mellifera]	20	2.0	1.4
gi_66509518	XP_624357.1 *DNA supercoiling factor* PREDICTED: similar to supercoiling factor CG9148-PA, isoform A [Apis mellifera]	2	1.9	
gi_66559541	XP_392731.2 *vesicle amine transport protein* PREDICTED: similar to Synaptic vesicle membrane protein VAT-1 homolog, partial [Apis mellifera]	15	1.9	0.7
gi_48100966	XP_392639.1 *ATP synthase component* PREDICTED: similar to bellwether CG3612-PA isoform 1 [Apis mellifera]	38	1.9	1.3
gi_110763326	XP_392086.2 *thioredoxin peroxidase* PREDICTED: similar to peroxiredoxin 3 isoform 1 [Apis mellifera]	6	1.9	0.6
gi_66549818	XP_396175.2 *fascin* PREDICTED: similar to singed CG32858-PA, isoform A [Apis mellifera]	6	1.9	0.5
gi_110762877	XP_624330.2 *prohibitin* PREDICTED: similar to B-cell receptor-associated protein 37 [Apis mellifera]	25	1.9	1.5
gi_66552871	XP_624979.1 PREDICTED: similar to coated vesicle membrane protein [Apis mellifera]	5	1.9	0.1
gi_66508940	XP_397201.2 *glycoprotein, mitochondrial* PREDICTED: similar to CG6459-PA [Apis mellifera]	19	1.9	0.8
gi_66501507	XP_392147.2 *heat shock protein cognate 5* PREDICTED: similar to Heat shock protein cognate 5 CG8542-PA [Apis mellifera]	67	1.9	0.6
gi_66526846	XP_623729.1 PREDICTED: similar to belphegor CG6815-PA [Apis mellifera]	5	1.8	1.1
gi_110759783	XP_395212.3 *beta-spectrin* PREDICTED: similar to Spectrin beta chain [Apis mellifera]	9	1.8	0.2
gi_110758921	XP_395614.3 *heat shock protein 90* PREDICTED: similar to Glycoprotein 93 CG5520-PA isoform 1 [Apis mellifera]	68	1.8	1.1
gi_66547447	XP_624910.1 *heat shock protein 10* PREDICTED: similar to CG11267-PA [Apis mellifera]	27	1.8	0.6
gi_66525858	XP_394112.2 *fumarylacetoacetate hydrolase* PREDICTED: similar to fumarylacetoacetate hydrolase domain containing 2A isoform 2 [Apis mellifera]	6	1.8	0.8
gi_48121613	XP_393238.1 *Rho GDP dissociation inhibitor* PREDICTED: similar to RhoGDI CG7823-PA isoform 1 [Apis mellifera]	2	1.7	1.2
gi_66518451	XP_391855.2 *nuclear migration protein* PREDICTED: similar to Nuclear migration protein nudC (Nuclear distribution protein C homolog) (Silica-induced gene 92 protein) (SIG-92) [Apis mellifera]	3	1.7	
gi_110762610	XP_391995.3 *esterase or lipase * PREDICTED: similar to Neurotactin CG9704-PB, isoform B [Apis mellifera]	2	1.7	

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gi_110760210	XP_001121882.1 *ATP synthase component* PREDICTED: similar to ATPase coupling factor 6 CG4412-PA [Apis mellifera]	4	1.7	1.1
gi_66531851	XP_623831.1 *disulfide isomerase* PREDICTED: similar to Protein disulfide-isomerase precursor (PDI), partial [Apis mellifera]	19	1.7	0.3
gi_110757651	XP_392405.3 *heat shock protein* PREDICTED: similar to CG14207-PB, isoform B isoform 1 [Apis mellifera]	6	1.7	2.4
gi_48095525	XP_392313.1 *tubulin* PREDICTED: similar to -Tubulin at 56D CG9277-PB, isoform B [Apis mellifera]	69	1.7	1.1
gi_110757102	XP_001121258.1 PREDICTED: similar to Actin 88F CG5178-PA [Apis mellifera]	4	1.7	
gi_110762620	XP_624452.2 PREDICTED: hypothetical protein [Apis mellifera]	18	1.7	1.5
gi_48094929	XP_392209.1 *apoptosis-linked gene 2* PREDICTED: similar to CG40410-PA.3 [Apis mellifera]	4	1.7	1.2
gi_66531434	XP_624112.1 *vacuolar and lysosomal proton pump* PREDICTED: similar to Vacuolar H+-ATPase 55kD B subunit CG17369-PB, isoform B [Apis mellifera]	4	1.7	
gi_48109763	XP_393115.1 *sar1, ER export* PREDICTED: similar to sar1 CG7073-PA, isoform A isoform 1 [Apis mellifera]	3	1.7	1.0
gi_110761561	XP_395280.3 *malic enzyme (not malate dehydrogenase)* PREDICTED: similar to Malic enzyme CG10120-PB, isoform B isoform 1, partial [Apis mellifera]	3	1.7	
gi_66549222	XP_624603.1 PREDICTED: similar to CG4164-PA [Apis mellifera]	3	1.7	
gi_66525882	XP_392094.2 *motor protein* PREDICTED: similar to CG6455-PA, isoform A [Apis mellifera]	6	1.6	
gi_66513257	XP_392280.2 *acyl carrier protein, mitochondria* PREDICTED: similar to mitochondrial acyl carrier protein 1 CG9160-PB, isoform B isoform 1 [Apis mellifera]	5	1.6	0.6
gi_48104680	XP_392963.1 *thioredoxin* PREDICTED: similar to thioredoxin-2 CG31884-PA, isoform A [Apis mellifera]	3	1.6	
gi_110761968	XP_625056.2 *enolase* PREDICTED: similar to Enolase CG17654-PA, isoform A, partial [Apis mellifera]	30	1.6	0.8
gi_66526470	XP_624102.1 *electron transfer flavoprotein* PREDICTED: similar to walrus CG8996-PB, isoform B isoform 1 [Apis mellifera]	7	1.6	1.5
gi_110756311	XP_001120521.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein L40 CG2960-PA [Apis mellifera]	6	1.6	1.3
gi_110764717	XP_623673.2 *isocitrate dehydrogenase* PREDICTED: similar to Isocitrate dehydrogenase CG7176-PC, isoform C isoform 2 [Apis mellifera]	9	1.6	1.2
gi_48101907	XP_392722.1 *proteasome subunit* PREDICTED: similar to Tat-binding protein-1 CG10370-PA [Apis mellifera]	4	1.6	
gi_66550890	XP_625114.1 *phosphoglyceromutase* PREDICTED: similar to Phosphoglyceromutase CG1721-PA, isoform A [Apis mellifera]	12	1.6	0.9

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gi_66547450	XP_392899.2 *heat shock protein 60* PREDICTED: similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) [Apis mellifera] MFIXED: MFIXED: MFIXED:	136	1.6	1.0
gi_66557994	XP_395853.2 *cytochrome c oxidase subunit* PREDICTED: similar to CG10664-PA, isoform A [Apis mellifera]	3	1.5	1.2
gi_66500205	XP_393509.2 *mitochondrial processing peptidase* PREDICTED: similar to CG3731-PB, isoform B [Apis mellifera]	8	1.5	1.2
gi_66500045	XP_392780.2 *eIF3-2b* PREDICTED: similar to Trip1 CG8882-PA [Apis mellifera]	5	1.5	
gi_66530247	XP_397478.2 *protein disulfide isomerase family protein* PREDICTED: similar to CG5554-PA [Apis mellifera]	10	1.5	0.4
gi_66499475	XP_624070.1 *receptor expression enhancing protein* PREDICTED: similar to CG8331-PA [Apis mellifera]	2	1.5	
gi_66512737	XP_396252.2 *moesin ezrin radixin* PREDICTED: similar to Moesin CG10701-PD, isoform D [Apis mellifera]	7	1.5	0.6
gi_66534286	XP_392892.2 *AAA+ ATPase with CDC48 domain* PREDICTED: similar to TER94 CG2331-PA, isoform A isoform 1 [Apis mellifera]	16	1.5	0.9
gi_66517317	XP_625060.1 *ATP synthase component* PREDICTED: similar to lethal (1) G0230 CG2968-PA [Apis mellifera]	7	1.5	0.0
gi_66560172	XP_392660.2 *T-complex chaperonin* PREDICTED: similar to T-complex protein 1 subunit alpha (TCP-1-alpha) (CCT-alpha) isoform 1 [Apis mellifera]	8	1.4	1.3
gi_66547531	XP_625090.1 *6-phosphogluconate dehydrogenase* PREDICTED: similar to 6-phosphogluconate dehydrogenase, decarboxylating, partial [Apis mellifera]	8	1.4	0.9
gi_66534660	XP_624682.1 *glutathione S transferase* PREDICTED: similar to Glutathione S transferase S1 CG8938-PA, isoform A [Apis mellifera]	3	1.4	1.2
gi_66512107	XP_395163.2 *proteasome subunit* PREDICTED: similar to Proteasome subunit beta type 1 (Proteasome 26 kDa subunit) [Apis mellifera]	7	1.4	0.9
gi_66518963	XP_393260.2 PREDICTED: similar to casein kinase II, alpha 1 polypeptide isoform 1 [Apis mellifera]	3	1.4	1.1
gi_66547758	XP_624249.1 *ATP synthase component* PREDICTED: similar to CG3321-PA, isoform A [Apis mellifera]	3	1.4	0.1
gi_66521433	XP_393851.2 *endoplasmic reticulum calcium pump* PREDICTED: similar to Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (Calcium pump) isoform 1 [Apis mellifera]	2	1.3	
gi_66522349	XP_393315.2 *chaperonin, T-complex* PREDICTED: similar to T-complex Chaperonin 5 CG8439-PA, isoform A [Apis mellifera]	5	1.3	1.0
gi_66505007	XP_623199.1 *HSC70Cb* PREDICTED: similar to Hsc70Cb CG6603-PA, isoform A isoform 1 [Apis mellifera]	11	1.3	1.0

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gi_66521545	XP_391936.2 *tubulin* PREDICTED: similar to Tubulin alpha-1 chain [Apis mellifera]	42	1.3	0.7
gi_110757102	XP_001121258.1 PREDICTED: similar to Actin 88F CG5178-PA [Apis mellifera]	7	1.3	
gi_48141950	XP_397284.1 PREDICTED: similar to translocase of outer mitochondrial membrane 20 homolog [Apis mellifera]	2	1.3	
gi_48104453	XP_395784.1 *odorant binding protein ASP5* PREDICTED: similar to CG2970-PA isoform 1 [Apis mellifera]	5	1.3	0.9
gi_66525867	XP_396960.2 *solute carrier* PREDICTED: similar to CG9090-PA isoform 1 [Apis mellifera]	13	1.3	0.5
gi_66530192	XP_623217.1 *nucleosome assembly protein* PREDICTED: similar to Nucleosome assembly protein 1-like 1 (NAP-1-related protein) (hNRP) isoform 1 [Apis mellifera]	8	1.3	0.9
gi_66500170	XP_623603.1 *alpha-glucosidase* PREDICTED: similar to CG14476-PB, isoform B isoform 2 [Apis mellifera]	2	1.3	
gi_110756003	XP_001119835.1 *heat shock protein 40* PREDICTED: similar to DnaJ homolog subfamily A member 1 (Heat shock 40 kDa protein 4) (DnaJ protein homolog 2) (HSJ-2) (HSDJ) [Apis mellifera]	13	1.3	0.9
gi_66517761	XP_392905.2 *cAMP-dependent protein kinase R2* PREDICTED: similar to cAMP-dependent protein kinase R2 CG15862-PA, isoform A isoform 1 [Apis mellifera]	2	1.3	
gi_66519233	XP_395866.2 *inner mitochondrial membrane translocase* PREDICTED: similar to CG11779-PA, isoform A isoform 1 [Apis mellifera]	5	1.3	0.9
gi_48142173	XP_393583.1 *proteasome subunit* PREDICTED: similar to Proteasome subunit alpha type 7-1 (Proteasome 28 kDa subunit 1) (PROS-Dm28.1) isoform 1 [Apis mellifera]	5	1.3	0.7
gi_66511337	XP_623366.1 *heat shock protein 90* PREDICTED: similar to Trap1 CG3152-PA isoform 1 [Apis mellifera]	6	1.2	0.7
gi_66525050	XP_625145.1 PREDICTED: similar to tubulin, alpha 1, partial [Apis mellifera]	10	1.2	0.6
gi_110749015	XP_001120613.1 *oxidoreductase* PREDICTED: similar to CG10962-PB, isoform B [Apis mellifera]	4	1.2	1.3
gi_66520256	XP_393789.2 *electron transfer beta polypeptide* PREDICTED: similar to CG7834-PA, isoform A [Apis mellifera]	9	1.2	
gi_110759540	XP_392544.3 *elongation factor 1d* PREDICTED: similar to Probable elongation factor 1-delta (EF-1-delta) [Apis mellifera]	7	1.2	1.0
gi_66520497	XP_393351.2 *nucleoside diphosphate kinase * PREDICTED: similar to Nucleoside diphosphate kinase (NDK) (NDP kinase) (Abnormal wing disks protein) (Killer of prune protein) [Apis mellifera]	10	1.2	0.6
gi_66534766	XP_624987.1 *cytochrome C oxidase* PREDICTED: similar to CG11015-PA [Apis mellifera]	5	1.2	0.6
gi_66534750	XP_393381.2 *prolyl cis-trans isomerase* PREDICTED: similar to Cyclophilin 1 CG9916-PA [Apis mellifera]	10	1.2	0.8

Accession	Description	No. Of peptides	Cultured /fresh	StDev
gi_66537377	XP_624620.1 *ribosomal protein* PREDICTED: similar to CG17489-PA.3, partial [Apis mellifera]	7	1.2	0.9
gi_66558818	XP_623504.1 *myosin-2 light chain* PREDICTED: similar to Myosin-2 essential light chain (Myosin II essential light chain) (Nonmuscle myosin essential light chain) isoform 1 [Apis mellifera]	6	1.2	
gi_66547340	XP_396741.2 *ribosomal protein* PREDICTED: similar to 40S ribosomal protein S3a (C3 protein) [Apis mellifera]	10	1.2	1.0
gi_110761874	XP_393878.2 *insulin-like growth factor mRNA binding protein* PREDICTED: similar to IGF-II mRNA-binding protein CG1691-PA, isoform A [Apis mellifera]	33	1.2	0.7
gi_66518848	XP_623051.1 *elongation factor, Tu (mitochondrial)* PREDICTED: similar to Elongation factor Tu mitochondrial CG6050-PA isoform 2 [Apis mellifera]	9	1.2	0.9
gi_66544546	XP_623093.1 *Rad23a* PREDICTED: similar to RAD23a homolog isoform 2 [Apis mellifera]	2	1.2	
gi_66537940	XP_392933.2 *heat shock protein 8* PREDICTED: similar to heat shock protein 8 isoform 1 [Apis mellifera]	72	1.2	0.6
gi_110755553	XP_396131.3 *aspartate aminotransferase* PREDICTED: similar to aspartate aminotransferase 2 precursor isoform 1 [Apis mellifera]	12	1.2	0.8
gi_48105889	XP_393034.1 *ribosomal protein* PREDICTED: similar to ribosomal protein L7a [Apis mellifera]	2	1.2	
gi_110764763	XP_001123191.1 *mitochondrial ATP synthase epsilon chain* PREDICTED: similar to stunted CG9032-PA, isoform A [Apis mellifera]	2	1.1	
gi_66558122	XP_396907.2 PREDICTED: similar to Nhp2 non-histone chromosome protein 2-like 1 [Apis mellifera]	3	1.1	0.8
gi_66532597	XP_624433.1 *oligosaccharide transferase* PREDICTED: similar to Oligosaccharyltransferase 48kD subunit CG9022-PA isoform 1 [Apis mellifera]	4	1.1	
gi_66540596	XP_623090.1 *chaperonin subunit* PREDICTED: similar to CG8351-PA isoform 1 [Apis mellifera]	8	1.1	0.8
gi_66499627	XP_393417.2 *ribosomal protein* PREDICTED: similar to mitochondrial ribosomal protein L12 CG5012-PA [Apis mellifera]	3	1.1	
gi_66500174	XP_394187.2 *nucleoplasmin* PREDICTED: similar to Nucleoplasmin CG7917-PA isoform 1 [Apis mellifera]	9	1.1	0.6
gi_62526112	NP_001014993.1 *elongation factor 1a* elongation factor 1-alpha [Apis mellifera]	57	1.1	0.7
gi_110751295	XP_623197.2 *GDP dissociation inhibitor* PREDICTED: similar to GDP dissociation inhibitor CG4422-PA [Apis mellifera]	4	1.1	0.5
gi_110764909	XP_001122985.1 *proliferating cell nuclear antigen* PREDICTED: similar to Proliferating cell nuclear antigen (PCNA) (Cyclin) (Mutagen-sensitive 209 protein) [Apis mellifera]	7	1.1	0.7
gi_58585250	NP_001011626.1 *profilin* profilin [Apis mellifera]	6	1.1	
gi_66507623	XP_393267.2 *nucleosome remodeling factor* PREDICTED: similar to Nucleosome remodeling factor - 38kD CG4634-PA [Apis mellifera]	4	1.1	

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gi_66533395	XP_393300.2 *chaperonin* PREDICTED: similar to CG7033-PA, isoform A isoform 1 [Apis mellifera]	20	1.1	0.4
gi_66512625	XP_623939.1 *heat shock protein 90* PREDICTED: similar to heat shock protein 90-alpha isoform 2 [Apis mellifera]	73	1.1	0.6
gi_66551115	XP_623285.1 *initiation factor, eIF4A* PREDICTED: similar to Eukaryotic initiation factor 4A (ATP-dependent RNA helicase eIF4A) (eIF-4A) isoform 2 [Apis mellifera]	5	1.1	0.8
gi_66513205	XP_623697.1 *chaperonin* PREDICTED: similar to lethal (1) G0022 CG8231-PA, partial [Apis mellifera]	8	1.1	0.6
gi_66558942	XP_623672.1 *chaperonin* PREDICTED: similar to CG5525-PA isoform 1 [Apis mellifera]	14	1.1	0.7
gi_66547760	XP_392684.2 *dihydropteridine reductase* PREDICTED: similar to Dihydropteridine reductase CG4665-PA, isoform A [Apis mellifera]	4	1.1	
gi_110763102	XP_394113.2 PREDICTED: similar to CG1458-PA [Apis mellifera]	3	1.1	0.8
gi_110761019	XP_391860.3 PREDICTED: similar to B52 CG10851-PB, isoform B [Apis mellifera]	3	1.0	
gi_66501020	XP_623784.1 *proteasome subunit* PREDICTED: similar to Rpt4 CG3455-PA [Apis mellifera]	2	1.0	0.7
gi_110751360	XP_001121454.1 *ribonucleoprotein* PREDICTED: similar to squid CG16901-PC, isoform C [Apis mellifera]	5	1.0	0.8
gi_66532824	XP_624894.1 *RNA helicase* PREDICTED: similar to Helicase at 25E CG7269-PA, isoform A [Apis mellifera]	12	1.0	0.8
gi_66541426	XP_624341.1 *proteasome subunit* PREDICTED: similar to Proteasome subunit alpha type 5 (Proteasome zeta chain) (Macropain zeta chain) (Multicatalytic endopeptidase complex zeta chain) [Apis mellifera]	5	1.0	0.5
gi_66563290	XP_392814.2 *chaperonin, T-complex* PREDICTED: similar to T-complex protein 1 subunit gamma (TCP-1-gamma) (CCT-gamma) [Apis mellifera]	9	1.0	0.6
gi_110756123	XP_623766.2 *HSP70/90 organizing protein* PREDICTED: similar to Hsp70/Hsp90 organizing protein homolog CG2720-PA isoform 1 [Apis mellifera]	11	1.0	0.6
gi_66508439	XP_392691.2 *elongation factor, EF2* PREDICTED: similar to Elongation factor 2 (EF-2) isoform 1 [Apis mellifera]	23	1.0	0.6
gi_66565444	XP_623506.1 *ribosomal protein* PREDICTED: similar to 60S ribosomal protein L9 [Apis mellifera]	5	1.0	0.6
gi_48102814	XP_392811.1 *isocitrate dehydrogenase* PREDICTED: similar to lethal (1) G0156 CG12233-PA, isoform A [Apis mellifera]	5	1.0	0.6
gi_66519842	XP_391905.2 *proteasome subunit* PREDICTED: similar to Proteasome 2 subunit CG3329-PA [Apis mellifera]	2	1.0	
gi_66521738	XP_393545.2 *citrate synthase* PREDICTED: similar to lethal (1) G0030 CG3861-PA, isoform A [Apis mellifera]	14	1.0	0.7
gi_66507594	XP_624164.1 *thiolase* PREDICTED: similar to Thiolase CG4581-PA [Apis mellifera]	7	1.0	0.2
gi_110755784	XP_001119886.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein S28b CG2998-PA [Apis mellifera]	5	1.0	

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gi_110765224	XP_623111.2 *clathrin heavy chain* PREDICTED: similar to Clathrin heavy chain CG9012-PA, isoform A isoform 1 [Apis mellifera]	4	1.0	0.5
gi_66536233	XP_395535.2 *glucose-1-phosphate uridylyltransferase* PREDICTED: similar to UGP CG4347-PA, isoform A isoform 1 [Apis mellifera]	4	1.0	
gi_110775155	XP_001122302.1 *enolase* PREDICTED: similar to Alpha-enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1), partial [Apis mellifera]	2	0.9	
gi_66547365	XP_392692.2 *proteasome subunit* PREDICTED: similar to proteasome (prosome, macropain) 26S subunit, non-ATPase, 13 [Apis mellifera]	2	0.9	0.1
gi_110761364	XP_001121589.1 *alpha-actinin* PREDICTED: similar to Alpha-actinin, sarcomeric (F-actin cross linking protein) [Apis mellifera]	2	0.9	
gi_110750119	XP_001121935.1 PREDICTED: similar to no on or off transient A CG4211-PB, isoform B [Apis mellifera]	9	0.9	0.5
gi_66515987	XP_395299.2 *translationally controlled tumor protein* PREDICTED: similar to Translationally controlled tumor protein CG4800-PA isoform 1 [Apis mellifera]	8	0.9	0.5
gi_48126476	XP_396596.1 *initiation factor 3f* PREDICTED: similar to CG9769-PA [Apis mellifera]	3	0.9	
gi_66525576	XP_624353.1 *aldo-keto reductase* PREDICTED: similar to CG6084-PA, isoform A isoform 1 [Apis mellifera]	20	0.9	0.6
gi_66566113	XP_624821.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein L3 CG4863-PA, isoform A [Apis mellifera]	12	0.9	0.4
gi_48103506	XP_392870.1 *thioredoxin* PREDICTED: similar to thioredoxin-like 2 [Apis mellifera]	3	0.9	
gi_66513627	XP_623997.1 *Rab11* PREDICTED: similar to Rab-protein 11 CG5771-PB, isoform B [Apis mellifera]	6	0.9	0.5
gi_110767655	XP_624951.2 PREDICTED: similar to eIF-5A CG3186-PA, isoform A [Apis mellifera]	2	0.9	
gi_110762180	XP_393451.3 *ribonucleoprotein* PREDICTED: similar to Heterogeneous nuclear ribonucleoprotein at 27C CG10377-PA, isoform A [Apis mellifera]	6	0.9	0.6
gi_110758628	XP_392556.3 PREDICTED: similar to baiser CG11785-PA [Apis mellifera]	3	0.9	
gi_110758129	XP_392465.3 *ribonucleoprotein* PREDICTED: similar to squid CG16901-PC, isoform C isoform 1 [Apis mellifera]	12	0.8	0.6
gi_66552230	XP_393559.2 *proteasome subunit* PREDICTED: similar to Rpn11 CG18174-PA isoform 1 [Apis mellifera]	2	0.8	
gi_48106363	XP_393059.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein S10b CG14206-PC, isoform C isoform 1 [Apis mellifera]	7	0.8	0.7
gi_110749824	XP_395168.3 *heat shock protein 1* PREDICTED: similar to heat shock protein 1, alpha [Apis mellifera]	47	0.8	0.2
gi_66530423	XP_623084.1 *aldehyde dehydrogenase* PREDICTED: similar to Aldehyde dehydrogenase CG3752-PA isoform 1 [Apis mellifera]	3	0.8	

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gi_66547438	XP_624846.1 *co-chaperone p23* PREDICTED: similar to CG16817-PA [Apis mellifera]	2	0.8	
gi_58531215	NP_001010975.1 *translocase, ATP* ADP/ATP translocase [Apis mellifera]	16	0.8	0.5
gi_66519936	XP_393806.2 *hydroxyacyl-CoA dehydrogenase* PREDICTED: similar to CG4389-PA, isoform A [Apis mellifera]	13	0.8	0.4
gi_66499419	XP_394245.2 *aminopeptidase, puromycin-sensitive* PREDICTED: similar to Puromycin sensitive aminopeptidase CG1009-PC, isoform C isoform 1 [Apis mellifera]	20	0.7	0.4
gi_66544702	XP_623832.1 *chaperonin subunit* PREDICTED: similar to CG8258-PA [Apis mellifera]	4	0.7	0.3
gi_66559310	XP_623106.1 *ribosomal protein* PREDICTED: similar to 60S acidic ribosomal protein P0 (DNA-(apurinic or apyrimidinic site) lyase) (Apurinic-apyrimidinic endonuclease) isoform 1 [Apis mellifera]	11	0.7	0.4
gi_110768698	XP_001121785.1 *ribosomal protein* PREDICTED: similar to ribosomal protein L5, partial [Apis mellifera]	2	0.7	
gi_66505480	XP_623065.1 *succinate dehydrogenase* PREDICTED: similar to succinate dehydrogenase complex, subunit A, flavoprotein (Fp) isoform 1 [Apis mellifera]	7	0.7	0.5
gi_110749389	XP_397439.3 PREDICTED: similar to eIF3-S10 CG9805-PA [Apis mellifera]	2	0.7	
gi_66517407	XP_623050.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein S4 CG11276-PA, isoform A isoform 1 [Apis mellifera]	6	0.7	0.4
gi_48102954	XP_392826.1 *SUMO1* PREDICTED: similar to SUMO (ubiquitin-related) homolog family member (smo-1) [Apis mellifera]	3	0.7	0.3
gi_66524293	XP_393965.2 *ribosomal protein* PREDICTED: similar to stubarista CG14792-PA, isoform A [Apis mellifera]	3	0.7	
gi_110763884	XP_001122537.1 PREDICTED: similar to RuvB-like 2 (p47 protein) [Apis mellifera]	3	0.7	
gi_110758819	XP_001120015.1 *dynein light chain* PREDICTED: similar to Dynein light chain 2, cytoplasmic (8 kDa dynein light chain) [Apis mellifera]	7	0.7	0.3
gi_66534191	XP_624580.1 PREDICTED: similar to Chromatin assembly factor 1 subunit CG4236-PA [Apis mellifera]	2	0.7	
gi_74271822	NP_001011604.2 *ribosomal protein* ribosomal protein S8 [Apis mellifera]	8	0.6	0.4
gi_110775909	XP_001122661.1 *aspartate aminotransferase* PREDICTED: similar to Glutamate oxaloacetate transaminase 1 CG8430-PA, isoform A, partial [Apis mellifera]	2	0.6	
gi_48106137	XP_393051.1 *DNA helicase* PREDICTED: similar to RuvB-like protein 1 isoform 1 [Apis mellifera]	3	0.6	
gi_48110113	XP_396259.1 *Got-1 superfamily domain protein* PREDICTED: similar to CG32576-PB, isoform B [Apis mellifera]	2	0.6	

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gi_48104663	XP_392962.1 *protein kinase C receptor* PREDICTED: similar to Guanine nucleotide-binding protein subunit beta-like protein (Receptor of activated protein kinase C homolog) isoform 1 [Apis mellifera]	3	0.6	
gi_66513481	XP_394434.2 *ubiquitin-activating enzyme E1* PREDICTED: similar to Ubiquitin activating enzyme 1 CG1782-PA isoform 1 [Apis mellifera]	6	0.6	
gi_48109133	XP_393102.1 PREDICTED: similar to Ribosomal protein L35A CG2099-PA isoform 1 [Apis mellifera]	2	0.6	
gi_110755382	XP_623524.2 *ribosomal protein* PREDICTED: similar to 40S ribosomal protein S15Aa [Apis mellifera]	3	0.6	0.3
gi_66506802	XP_625265.1 PREDICTED: hypothetical protein [Apis mellifera]	4	0.6	0.6
gi_110748820	XP_001120026.1 PREDICTED: similar to 40S ribosomal protein S17 [Apis mellifera]	2	0.6	
gi_66525285	XP_392071.2 *ribosomal protein* PREDICTED: similar to Ribosomal protein L4 CG5502-PA isoform 1 [Apis mellifera]	5	0.6	
gi_110762823	XP_624958.2 *proteasome subunit* PREDICTED: similar to Proteasome subunit beta type 3 (Proteasome theta chain) (Proteasome chain 13) (Proteasome component C10-II) [Apis mellifera]	2	0.6	
gi_110761176	XP_625241.2 PREDICTED: hypothetical protein [Apis mellifera]	2	0.6	
gi_66533950	XP_623800.1 *succinyl-CoA synthetase* PREDICTED: similar to Succinyl coenzyme A synthetase subunit CG1065-PA [Apis mellifera]	3	0.6	
gi_110763826	XP_395047.3 *phosphoglycerate kinase* PREDICTED: similar to Phosphoglycerate kinase isoform 1 [Apis mellifera]	4	0.6	
gi_66519917	XP_625029.1 *stathmin* PREDICTED: similar to stathmin CG31641-PA, isoform A [Apis mellifera]	10	0.6	0.2
gi_110773801	XP_001123335.1 *Aldose 1-epimerase.* PREDICTED: similar to CG9008-PA, isoform A, partial [Apis mellifera]	4	0.6	0.5
gi_66506394	XP_624700.1 *Histone 2A* PREDICTED: similar to CG31618-PA [Apis mellifera]	7	0.6	0.5
gi_48097815	XP_393894.1 PREDICTED: similar to CG4849-PA isoform 1 [Apis mellifera]	2	0.6	
gi_66512039	XP_392432.2 *sterol carrier* PREDICTED: similar to Sterol carrier protein X-related thiolase CG17320-PA isoform 1 [Apis mellifera]	4	0.6	
gi_66551967	XP_624896.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein L13 CG4651-PA, isoform A isoform 1 [Apis mellifera]	8	0.6	0.2
gi_48136057	XP_393356.1 PREDICTED: similar to eIF4AIII CG7483-PA isoform 1 [Apis mellifera]	2	0.6	
gi_48107653	XP_393092.1 *ribosomal protein* PREDICTED: similar to Qm CG17521-PA, isoform A isoform 1 [Apis mellifera]	2	0.5	0.5
gi_66504343	XP_623731.1 *ribosomal protein* PREDICTED: similar to 40S ribosomal protein S3 [Apis mellifera]	4	0.5	
gi_48103127	XP_392843.1 *ribosomal protein* PREDICTED: similar to string of pearls CG5920-PA [Apis mellifera]	5	0.5	0.2

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gi_66510594	XP_624428.1 *RNA binding protein* PREDICTED: similar to zinc finger RNA binding protein [Apis mellifera]	5	0.5	
gi_66509032	XP_623527.1 *proteasome subunit* PREDICTED: similar to Proteasome 26S subunit subunit 4 ATPase CG5289-PA isoform 1 [Apis mellifera]	3	0.5	
gi_66530142	XP_623083.1 *ATP citrate lyase* PREDICTED: similar to ATP citrate lyase CG8322-PA, isoform A isoform 1 [Apis mellifera]	2	0.5	
gi_48098918	XP_392565.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein L18 CG8615-PA [Apis mellifera]	2	0.5	
gi_66524972	XP_394061.2 *ornithine aminotransferase* PREDICTED: similar to Ornithine aminotransferase precursor CG8782-PA [Apis mellifera]	3	0.5	
gi_110761627	XP_001119826.1 PREDICTED: similar to Ribosomal protein L24 CG9282-PA [Apis mellifera]	2	0.5	0.4
gi_110756656	XP_001121222.1 *arginine methyltransferase* PREDICTED: similar to Arginine methyltransferase 1 CG6554-PA [Apis mellifera]	2	0.5	
gi_66516797	XP_397567.2 *splicing factor* PREDICTED: similar to RNA binding motif protein 4 [Apis mellifera]	3	0.4	
gi_66559208	XP_392988.2 *ribosomal protein* PREDICTED: similar to Ribosomal protein L6 CG11522-PB, isoform B [Apis mellifera]	2	0.4	
gi_110758613	XP_001120143.1 PREDICTED: similar to small nuclear ribonucleoprotein D3 [Apis mellifera]	2	0.4	
gi_66499186	XP_395748.2 *prolyl cis-trans isomerase* PREDICTED: similar to FK506-binding protein FKBP59 CG4535-PA [Apis mellifera]	2	0.4	
gi_66510755	XP_395436.2 *myelin protein expression factor* PREDICTED: similar to CG9373-PA [Apis mellifera]	5	0.4	0.0
gi_110763879	XP_625293.2 PREDICTED: hypothetical protein [Apis mellifera]	2	0.4	
gi_48098427	XP_392059.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein L26 CG6846-PA isoform 1 [Apis mellifera]	3	0.4	
gi_110749616	XP_001121111.1 PREDICTED: similar to Histone H1 [Apis mellifera]	2	0.4	
gi_66522612	XP_393927.2 *karyopherin* PREDICTED: similar to karyopherin beta 1 isoform 1 [Apis mellifera]	3	0.4	
gi_110772200	XP_392611.3 *GPI-anchored protein* PREDICTED: similar to CG18811-PA [Apis mellifera]	2	0.4	
gi_66547845	XP_623500.1 PREDICTED: similar to CG9586-PB [Apis mellifera]	13	0.4	0.1
gi_66531196	XP_624830.1 PREDICTED: similar to CG5641-PA isoform 1 [Apis mellifera]	3	0.4	
gi_110759996	XP_396057.3 *polyA-binding protein* PREDICTED: similar to poly A binding protein, cytoplasmic 1 isoform 1 [Apis mellifera]	3	0.4	
gi_48098039	XP_391994.1 *aconitase* PREDICTED: similar to Aconitase CG9244-PB [Apis mellifera]	4	0.4	
gi_110764347	XP_395276.3 *enolase-phosphatase E1 (methionine salvage pathway)* PREDICTED: similar to E-1 enzyme [Apis mellifera]	12	0.4	

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gi_110768336	XP_001121930.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein L11 CG7726-PA [Apis mellifera]	2	0.4	
gi_110758131	XP_623996.2 *Ras-GTPase activating protein* PREDICTED: similar to Ras-GTPase-activating protein-binding protein 2 (GAP SH3-domain-binding protein 2) (G3BP-2) [Apis mellifera]	3	0.4	
gi_110764999	XP_397274.3 PREDICTED: similar to Rbp1-like CG1987-PA [Apis mellifera]	2	0.4	0.2
gi_110763051	XP_623841.2 PREDICTED: similar to lark CG8597-PA, isoform A isoform 1 [Apis mellifera]	10	0.4	
gi_48097825	XP_393899.1 PREDICTED: similar to Deoxyuridine 5-triphosphate nucleotidohydrolase (dUTPase) (dUTP pyrophosphatase) (PPAR-interacting protein 4) (PIP4) isoform 1 [Apis mellifera]	2	0.3	
gi_110767333	XP_001122685.1 *farnesyl diphosphate synthase 1* PREDICTED: similar to Farnesyl pyrophosphate synthase CG12389-PA, partial [Apis mellifera]	31	0.3	0.2
gi_110749566	XP_001120988.1 PREDICTED: similar to germinal histone H4 [Apis mellifera]	13	0.3	0.1
gi_66525165	XP_392065.2 *chromatin-associated protein* PREDICTED: similar to CG1240-PA [Apis mellifera]	10	0.3	0.2
gi_110763684	XP_393211.3 PREDICTED: similar to CG3902-PA [Apis mellifera]	2	0.3	
gi_110767467	XP_396224.3 PREDICTED: similar to Farnesyl pyrophosphate synthase CG12389-PA, partial [Apis mellifera]	2	0.3	
gi_110761338	XP_396542.3 PREDICTED: similar to Arsenite-resistance protein 2 homolog [Apis mellifera]	2	0.3	
gi_110762382	XP_001120306.1 *spermidine synthase* PREDICTED: similar to spermidine synthase [Apis mellifera]	4	0.3	0.1
gi_110767982	XP_624411.2 PREDICTED: similar to Heterogeneous nuclear ribonucleoprotein at 87F CG12749-PB, isoform B [Apis mellifera]	5	0.3	
gi_110763187	XP_001119828.1 *ribosomal protein* PREDICTED: similar to Ribosomal protein L19 CG2746-PA, isoform A [Apis mellifera]	3	0.3	
gi_110749558	XP_001120889.1 *Histone 2B* PREDICTED: similar to Histone H2B [Apis mellifera]	13	0.3	0.2
gi_110756983	XP_001121384.1 PREDICTED: similar to High mobility group protein DSP1 (Protein dorsal switch 1), partial [Apis mellifera]	3	0.3	
gi_110763831	XP_393344.3 *Y-box protein* PREDICTED: similar to ypsilon schachtel CG5654-PA [Apis mellifera]	2	0.3	
gi_66543347	XP_623586.1 *farnesyl pyrophosphate synthase* PREDICTED: similar to Farnesyl pyrophosphate synthase CG12389-PA, partial [Apis mellifera]	3	0.3	
gi_110764283	XP_001122949.1 PREDICTED: hypothetical protein [Apis mellifera]	2	0.3	
gi_110749568	XP_001121026.1 PREDICTED: similar to CG31613-PA [Apis mellifera]	7	0.3	
gi_66505329	XP_392968.2 *translation regulator GCN1* PREDICTED: similar to CG17514-PA.3 [Apis mellifera]	9	0.2	

Accession	Description	No. Of peptides	Cultured /fresh	StDev
gi_110759058	XP_393065.3 PREDICTED: similar to maelstrom CG11254-PA, isoform A [Apis mellifera]	5	0.2	
gi_66503776	XP_623357.1 *transketolase* PREDICTED: similar to CG8036-PB, isoform B isoform 2 [Apis mellifera]	5	0.2	0.2
gi_110762101	XP_001121860.1 PREDICTED: similar to CG6621-PA [Apis mellifera]	4	0.2	
gi_58585098	NP_001011579.1 *major royal jelly protein 1* major royal jelly protein 1 [Apis mellifera]	4	0.2	
gi_110750857	XP_001121250.1 PREDICTED: similar to Aly CG1101-PA [Apis mellifera]	2	0.2	
gi_110758113	XP_396725.3 *mapmodulin* PREDICTED: similar to Mapmodulin CG5784-PB, isoform B [Apis mellifera]	2	0.2	
gi_110760247	XP_001120943.1 *nucleolar and coiled-body phosphoprotein* PREDICTED: similar to Nopp140 CG7421-PB, isoform B [Apis mellifera]	6	0.2	0.1
gi_110749582	XP_001120029.1 PREDICTED: similar to CG10472-PA [Apis mellifera]	3	0.2	
gi_110749616	XP_001121111.1 PREDICTED: similar to Histone H1 [Apis mellifera]	4	0.1	
gi_110759433	XP_395300.3 *ribonucleoprotein* PREDICTED: similar to La protein homolog (La ribonucleoprotein) (La autoantigen homolog) [Apis mellifera]	3	0.1	0.2
gi_110762287	XP_001120535.1 PREDICTED: hypothetical protein [Apis mellifera]	2	0.1	0.1