

Mitocidal resistance in the ectoparasitic mite, *Varroa destructor*, and the relationship with its host *Apis mellifera*



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Declaration

This Thesis has not been submitted in whole or in part to this or any other university for any degree, and is the original work of the author except where otherwise stated.

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Abbreviations

ABPV	Acute Bee Paralysis Virus
ACN	Acetonitrile
AFB	American Foulbrood
AmBic	Ammonium Bicarbonate
AMP	Anti-microbial Peptide
APS	Ammonium persulphate
ATP	Adenosine triphosphate
ATP	Adenosine triphosphate
BP	Biological Process
bp	Base Pair
BSA	Bovine serum albumin
CBPV	Chronic Bee Paralysis Virus
CCD	Colony Collapse Disorder
cm	Centimeter
CoA	Coenzyme A
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
DWV	Deformed Wing Virus
EDTA	Ethylene diamine tetra acetic acid
EFB	European Foulbrood
EST	Expressed Sequence Tag

FDR	False Discovery Rate
FIBKA	Federation of Irish Beekeepers
GO	Gene Ontology
GST	Glutathione s-Transferase
GTP	Guanidine tri-phosphate
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HSP	Heat Shock Protein
IAA	Iodoacetamide
IAPV	Israeli Acute Paralysis Virus
IEF	Isoelectric Focussing
IMD	Immunodeficiency pathway
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC MS	Liquid Chromotography Mass Spectrometry
LFQ	Label Free Quantification
LPS	Lipopolysaccharide
M	Molar
MF	Molecular Function
MgCl ₂	Magnesium chloride
mm	Millimeter
mM	MilliMolar
M _r	Molecular weight
N	Sample Number
NADH	Nicotinamide Adenine Dinucleotide Hydrogen

P value	Probability
PAMP	Pathogen Associated Membrane Pattern
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
<i>pI</i>	Isoelectric point
PIPES	Piperazine
PO	Phenoloxidase
proPO	proPhenoloxidase
PRR	Pattern Recognition Receptor
RNase	Ribonuclease
RT	Reverse transcriptase
SDS PAGE	sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis
TAE	Tris acetate buffer
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
tRNA	Transfer Ribonucleic Acid
UV	UltraViolet
V	Volt
v/v	Volume per Volume
w/v	Weight per Volume

Abstract

The Western honeybee, *Apis mellifera*, is an economically important insect, responsible for a large portion of global pollination services. They live in densely populated colonies, which give the optimum chance for opportunistic pathogens and parasites to spread. Honeybees, like all organisms, are subject to a wide range of threats, from viruses to parasites. The immune response of *A. mellifera* to these threats relies on a fast acting non-adaptive immunity, with effectors such as cellular defences and the release of antimicrobial peptides. The defence against invading pathogens and parasites is important not only for the individual bee, but for the colony as a whole. Many bee diseases have been shown as capable of causing collapse of honeybee colonies.

The first section of this thesis examines the occurrence and prevalence of four honeybee viruses (Deformed wing virus, Chronic bee paralysis virus, Acute bee paralysis virus, Israeli acute paralysis virus), the microsporidian parasite *Nosema*, and the parasitic tracheal mite, *Acarapis woodi*. The results from all seasons indicated a very low prevalence of the tracheal mite, with only 2% of the colonies testing positive in the Spring sampling, with none positive in the Summer or Autumn samples. Deformed wing virus was detected at very high levels throughout the year, with Israeli acute paralysis virus detected in the Autumn round of sampling in 3% of the tested colonies. The other two viruses were not detected. Levels of *Nosema* were also high throughout the year, at 18%, 6% and 12% of the colonies testing positive in the Spring, Summer and Autumn respectively. The results indicated no obvious disease variations present in the colonies tested from apiaries that lost more than 20% of their hives during the Winter post sampling than those that had lost less than 20%.

The next section was to examine the mechanisms by which one of the most serious threats to the honeybee, the parasitic mite *V. destructor*, has developed resistance to pyrethroid chemicals. *Varroa* are thought to have a negative impact in the overall health and vitality of the bee, transmitting viruses through haemolymph feeding and possibly weakening the immune response of the bee. Proteomic analysis was used to compare the proteomic profile of sensitive and resistant mites, in order to observe any variations that may be conferring the resistant phenotype. The comparison showed that a number of proteins were detected at higher levels of abundance in the resistant mites, such as heat shock proteins and detoxifying enzymes such as aldehyde dehydrogenase.

A number of proteins present at lower levels include cuticle proteins involved in cuticle structure. The altered levels of these proteins in the resistant *Varroa* could be conferring resistance through decreased penetration and increased metabolism of the pyrethroid.

In the final section, the full effect that parasitization by *Varroa* has on the bee was examined. Parasitized Winter bees were compared to unparasitized and the proteomic profiles were analysed for changes. Hexamerin was present at lower levels in the bees that were parasitized, as was enolase-like protein. The decreased level of these proteins indicates *Varroa* parasitisation could lead to insufficient energy metabolism. Drone pupae that were parasitized by *Varroa* were compared to unparasitized drones using proteomic analysis. Cuticle proteins decreased in abundance which could indicate a compromised healing response following parasitization. A number of proteins involved in energy and nutrition such as hexamerin were also present at lower levels of abundance in the parasitized drone pupae. Similar proteins decreased in abundance in parasitized workers. Cuticle structure proteins were present at lower levels of abundance, with proteins involved in the stress response present at higher levels in the parasitized workers. Quantitative PCR analysis of parasitized drone and worker pupae indicated a reduced level of two immune genes – *Abaecin* and *Defensin*, with two other immune related genes increased in expression: *Phenoloxidase* and *Hymenoptaecin*. Changes in the expression of immune related genes following parasitization indicates that *Varroa* are affecting how the immune response functions. To identify whether or not this change in the immune response was caused by salivary effectors secreted by the mite during feeding, the haemolymph from parasitized pupae was compared using label free proteomics to haemolymph from unparasitized pupae. A number of proteins were found exclusive to the parasitized haemolymph, including a metalloendopeptidase which is found in other blood feeding insects and could be functioning in the digestion of haemolymph. Sox 14, a regulator of transcription, was also exclusively present in the parasitized haemolymph.

The work presented throughout this thesis offers a comprehensive analysis of the diseases found in honeybee colonies, the effect that parasitization by *Varroa* has on adult and developing pupae, and analysis of the pyrethroid resistant phenotype. The results offer an explanation as to why *Varroa* are considered one of the most serious

honeybee threats, and highlights the importance of controlling infestation levels in colonies.

Chapter One

Introduction

1.1 *Apis mellifera*

The Western honeybee, *Apis mellifera* is an insect of huge economic importance, responsible for the pollination of a wide range of agricultural crops globally (Klein *et al.*, 2007; McMahon *et al.*, 2015). *A. mellifera* is a highly social insect, living in colonies comprising of three main castes (Figure 1.1) (Barchuk *et al.*, 2007). Each colony has two distinct castes of females - a single functioning fertile queen and tens of thousands of infertile female workers, phenotypically different in reproductive ability, size, job roles and life span (Weinstock *et al.*, 2006). The colony will also support 200-1000 drones (male bees) during the active mating season (May to August in Ireland), but in late Autumn they will be removed by the workers (Evans and Wheeler 1999). The queen and the workers develop from fertilized eggs, and are diploid with 32 chromosomes, while the drones develop from unfertilized eggs, have half the number of chromosomes and are haploid (Evans and Wheeler 1999). The size of the cell may be an important stimulus on how the occupant in a specific cell is reared, and it is the quantity and quality of food given to the larva which determines its caste. Nurse bees, which are young workers with developed mandibular and hypopharyngeal glands, have the ability to differentiate between worker and queen cells, and the larvae are fed with either worker jelly or royal jelly after 3 days, depending on the decision reached (Li *et al.*, 2010). By comparing the proteomes of worker and queen larvae, studies have shown that the fate of the larvae is determined by 72 hours, which is thought to be due mostly to the effect of the different food on the larval hormone system, inducing changes in hormones such as the Juvenile hormone (Li *et al.*, 2010). Expression of genes between the workers and queens during developmental larval stage is thought to vary substantially, with one study identifying 240 differentially expressed genes during development (Barchuk *et al.*, 2007). On a proteomic level, when examined at 72 hours; queen intended larvae over-expressed transketolase, aldehyde reductase, and enolase proteins which are involved in carbohydrate metabolism and energy production, imaginal disc growth factor 4 which is a developmental related protein, long-chain-fatty-acid CoA ligase and proteasome subunit alpha type 5 which metabolize fatty and

amino acids (Li *et al.*, 2010). Worker intended larvae over-expressed ATP synthase beta subunit, aldehyde dehydrogenase, thioredoxin peroxidase 1 and peroxiredoxin 2540, lethal (2) 37 and 14-3-3 protein epsilon, fatty acid binding protein, and translational controlled tumor protein (Li *et al.*, 2010).

The constituent of royal jelly that causes the phenotypic differences is largely unknown, however one study found that a 57kDa protein in royal jelly named Royalactin increased overall body size and ovary development in worker pupae fed with the substance (Kamakura 2012). It is thought to initiate the differentiation into queen larvae by activating the p70 S6 kinase through the Egfr-mediated signaling pathway (Kamakura 2012).

Pheromones are produced by the queen to maintain the function and integrity of this very complex social structure (Trhlin and Rajchard 2011). Queen Retinue Pheromone is a blend of nine different compounds; 9-oxo-(E)-2-decenoic acid, (R)- and (S)-9-hydroxy-(E)-2-decenoic acid, methyl p-hydroxybenzoate, 4-hydroxy-3-methoxyphenylethanol, methyloleate, coniferyl alcohol, palmityl alcohol, and linolenic acid, which has been shown to induce many aspects of control of the colony, including the suppressed development of ovaries in the female workers (Trhlin and Rajchard 2011). An aging or non-laying queen however can be replaced by worker bees, in a process known as supersedure, where a new queen will be reared to kill the old queen, in order to keep the colony functioning (Page and Peng 2001).



Figure 1.1 The three caste systems within an *A. mellifera* colony.

Image shows a worker on the left, a drone bee in the middle, with the largest on the right being the queen.

Source: http://www.devbio.biology.gatech.edu/?page_id=2744

1.2 The insect immune response

Insects are the most successful and numerous group of higher organisms on the planet, but like all others, must deal with the constant pressure of potential pathogenic and parasitic attack (Samways 1993). The immune system of mammals consists of two subsets: the adaptive immune response and the acquired immune response. Insects lack the acquired immune response and must rely on a fast detection and fast action system instead, with no evidence to suggest immune memory (Strand 2008). This type of immunity is remarkably similar to the innate immunity of mammals, with many of the ancient features homologous between the two species (Krautz *et al.*, 2014). The immune response of insects is subdivided into cellular immunity and humoral immunity (Strand 2008; Azzami *et al.*, 2012; Falabella *et al.*, 2012; Krautz *et al.*, 2014). The fat body of insects is comparable in function to the liver of mammals, responsible for secreting immune effector molecules into the haemolymph as part of the humoral immune response (Gobert *et al.*, 2003; Azzami *et al.*, 2012). Cellular immunity comprises circulating immune cells, comparable to human neutrophils, capable of recognizing and engulfing foreign invaders in the haemocoel (Satyavathi *et al.*, 2014). Barrier systems are also in place, to halt an invader before it has the chance to enter the haemocoel. The insect cuticle is similar in function to mammalian skin, acting as a first line of defense against potential threats (Falabella *et al.*, 2012). Wounding of the cuticle also elicits an immune response within the insect, to ensure that the barrier is replaced as quickly as possible after the insult to avoid breach (Christensen *et al.*, 2005; Krautz *et al.*, 2014). In *Galleria mellonella*, histochemical analysis of the healing process of a wound showed that firstly, a plug is formed to cover the wound, made from fat body fragments, haemolymph, and haemocytes, followed by the condensing of a compact layer of cells glued together by haemolymph to fully close the wound (Krautz *et al.*, 2014). The lining of the midgut and the trachea are also barriers to avoid pathogenic insult through feeding and breathing. The peritrophic matrix is a sieve like structure in the midgut, made up of chitin and protein, which acts as a barrier system to pathogens and parasites entering through the digestive system (Azzami *et al.*,

2012; Agrawal *et al.*, 2014). If an opportunistic pathogen or parasite happens to breach these barrier systems, the rapid acting immune cellular and humoral immune response aim to neutralize the attacker before infection takes place (Hoffmann 1995; Gillespie *et al.*, 1997; Azzami *et al.*, 2012).

1.2.1 Humoral immune response of insects

The humoral immune response of insects relies on soluble molecules that circulate in the haemolymph such as antimicrobial peptides and enzymatic cascades responsible for the melanization and clotting response (Strand 2008). Recognition of non-self is carried out by Pattern Recognition Receptors (PRRs) which are capable of detecting motifs on the outer surfaces of invading organisms, known as Pathogen Associated Molecular Patterns (PAMPSs) and initiating the appropriate response (Ratcliffe *et al.*, 1984, Siva-Jothy *et al.*, 2005; Evans *et al.*, 2006). The insect Toll and Toll-like receptors are transmembrane signal transducers, and are a critical component of the immune response (Evans *et al.*, 2006; Kurata 2014). They are activated in *Drosophila*, when the cytokine-like molecule *spätzle* binds to the extracellular domain of the transmembrane receptor Toll (Medzhitov 2001; Evans *et al.*, 2006). The IMD pathway is activated by peptidoglycan recognition protein via *relish* in *Drosophila*, which is an NF- κ B-like transcription factor (Kurata 2014). The signaling then initiates the release of antimicrobial peptides and proteins (AMPs) dependent on which pathogen was detected and which pathway was initiated. The Toll pathway is associated with AMPs specific to Gram-positive bacteria and fungal cells, whereas the IMD pathway initiates AMPs effective against Gram-negative bacteria (Gobert *et al.*, 2003; Evans *et al.*, 2006; Azzami *et al.*, 2012; Kurata 2014).

A vast array of antimicrobial peptides (AMPs) have been identified across the classes of insects, with a variety of antimicrobial effects (Hoffmann 1995). Lysozyme, homologous in structure and function to mammalian lysozyme, is effective against bacteria. It works by hydrolyzing β -acetylglucosamine and n-acetylmuramic acid in the peptidoglycan layer of

bacterial cell walls (Gillespie *et al.*, 1997). A group of small peptides around 4kDa in size have also been found in insects following immune aggravation and are named cecropin. These have been shown to be effective against both Gram-negative and Gram-positive bacteria, with variations to their amino acid sequence possibly conferring their adaption toward the various bacterial challenges (Hoffmann 1995; Gillespie *et al.*, 1997). Defensin is a small AMP effective against Gram-positive bacteria, and similar in structure and function to mammalian defensin (Hoffmann 1995). Defensin mediates the death of invading bacterial cells by causing the formation of a voltage gated channel in the cell wall of the bacteria, leading to leakage of ions (Hoffmann 1995). A proline-rich class of AMP has also been identified across a wide range of insect species, of which proline makes up more than 25% of the total amino acids. They are between 18-34 amino acids in size and have been found in many species, and include abaecin in *A. mellifera*, which is active against bacterial cells (Gillespie *et al.*, 1997; Jefferson *et al.*, 2013).

1.2.2 Cellular immune response of insects

The cellular immune response of insects involves immune cells called haemocytes that function while circulating in the haemolymph, carrying out defensive roles such as encapsulation and phagocytosis of foreign invaders (Figure 1.2) (Lavine and Strand 2002; Schmid *et al.*, 2008; Satyavathi *et al.*, 2014). Haemocytes have been studied most thoroughly in *Drosophila* where three distinct types of haemocytes have been identified (Lavine and Strand 2002). Plasmatocytes are capable of phagocytosing small foreign invaders such as bacteria cells (Lavine and Strand 2002). The phagocytosing cells bind to the target via a receptor on the surface of the cell (Strand 2008; Jiang *et al.*, 2010). This binding triggers the haemocyte to form a phagosome, which engulfs the target via actin polymerization-dependent mechanisms (Marmaras *et al.*, 1996; Strand 2008; Marmaras and Lampropoulou 2009). The phagosome then matures into a phagosome by a series of fusion and fission events that take place with endosomes and lysosomes, and the invading cell is destroyed (Strand 2008; Satyavathi *et al.*, 2014).

Another defense mechanism involves the use of the immune cells following the infection of a large foreign threat such as a parasitoid egg, which causes them to join together to cover the invader and render it harmless in a process known as melanotic encapsulation (Hoffmann 1995; Strand 2008). This is mainly carried out by plasmatocytes, but also sometimes by granulocytes depending on the species of insect (Marmaras and Lampropoulou 2009; Jiang *et al.*, 2010). The distribution of cells across the surface of invading cell is sometimes random, such as in the case of *Manduca sexta* (Strand 2008). In other insect species, such as *Chrysodeixis includens*, the attachment of immune cells to the surface of the invader is very organized. First, a number of granulocytes bind to the surface of the target, followed by a large number of plasmatocytes which then bind all over the outside of the invader (Strand 2008). Finally, a large number of plasmatocytes adhere to the build-up of cells with a very strong adhesion, to form a multilayer structure entrapping the invader, where a layer of melanin is formed through the melanization cascade (See section 1.2.3) (Lavine and Strand 2002; Gandhe *et al.*, 2007). Haemocytes can also bind to larger numbers of targets such as a group of invading bacterial cells, in a process known as nodulation, where they aggregate to form a multilayer structure, sequestering the foreign invaders (Gandhe *et al.*, 2007; Satyavathi *et al.*, 2014, Charles and Killian 2015). This process is very similar in function to encapsulation, and can occur with or without an accompanying melanization deposit (Strand 2008; Satyavathi *et al.*, 2014).

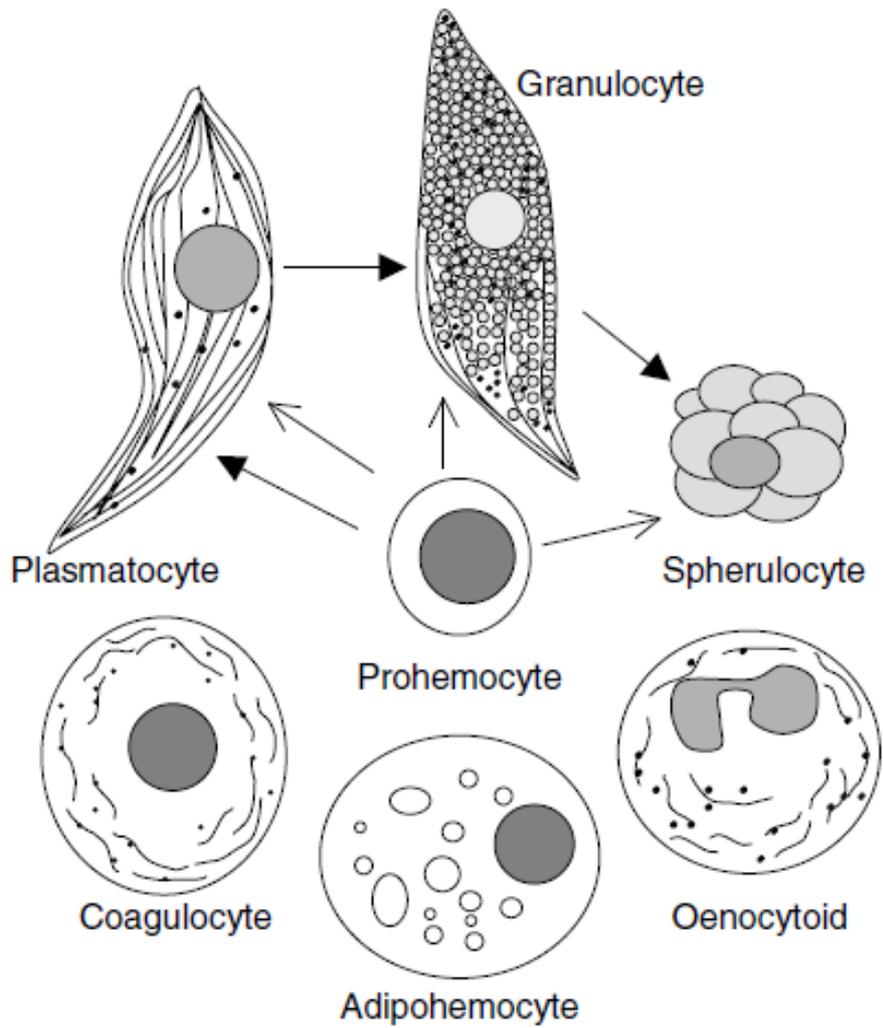


Figure 1.2 Insect haemocytes

Image shows an example of insect immune cells. The ones depicted are cells found in *G. mellonella*.

(Kavanagh and Reeves 2004).

1.2.3 Prophenol oxidase cascade in insects

Melanin is involved in cuticular melanization and sclerotization of insects, but is also involved in insect immunity against invaders as one of the most important immune responses (Marmaras *et al.*, 1996; Yassine *et al.*, 2012). The melanization cascade involves the activation of prophenoloxidase (proPO) (a zymogen present in the haemolymph of insects), which through proteolytic activation becomes the active form, phenoloxidase (PO) (Gillespie *et al.*, 1997; Kanost 1999; Kan *et al.*, 2008). Two types of PO have been identified from insects: Tyrosine type, found in the haemocytes, haemolymph, and integument, and laccase type PO found only in the integument (Marmaras *et al.*, 1996). Activation of proPO takes place in the haemolymph, carried out by proteolysis by a cascade of clip domain serine proteases, which are released following activation of the insect immune response (Figure 1.3). Active PO catalyzes the production of quinones, which can non-specifically cross link neighbouring molecules to form melanin at the site of injury or over the surface of an invading organism (Marmaras *et al.*, 1996; Kan *et al.*, 2008; Yassine *et al.*, 2012). Melanin appears to be involved in the defense response during both pathogenic invasion and also wound healing, playing an important role in cellular defense reactions such as phagocytosis, nodulation and melanotic encapsulation (Gillespie *et al.*, 1997; Yassine *et al.*, 2012). The formation of melanin must be tightly regulated within the host however, as the formation of excessive amounts of quinones and systemic melanization could damage the host (Marmaras *et al.*, 1996). Its activation is tightly regulated by the serine proteases, which only cleave to activate following a wound or pathogenic insult (Marmaras and Lampropoulou 2009; Yassine *et al.*, 2012)

1.2.4 Social immunity in *A. mellifera*

Social insects, due to their close interaction and dependence on each member's survival to benefit the entire colony, are thought to also exhibit social

immunity in order to protect the whole colony. Chronic increases in individual mortality may lead directly to hive collapse (Jefferson *et al.*, 2013). Colony members of species such as bees and ants, take part in a social group defense against pathogenic invaders, such as grooming, cleaning the hive and removal of infected individuals (Evans *et al.*, 2006). Nest material can be made from antimicrobial materials such as wax combs, and young can also be reared in sterile nurseries, in the case of termites and ants (Evans *et al.*, 2006). The innate immune system of *A. mellifera*, through the sequencing of the honeybee genome (2006), has shown to lack a number of immune genes when compared to other sequenced insects such as *Drosophila* (Weinstock *et al.*, 2006). Their immune repertoire consists of six AMPs- abaecin, hymenoptaecin, apidaecin, apisimin, defensin 1 and defensin 2 (Jefferson *et al.*, 2013). Through genome sequencing, Toll-like and IMD orthologues of the humoral immune response have also been found in *A. mellifera* (Evans *et al.*, 2006). *A. mellifera* also possess a variety of haemocytes, including those capable of phagocytosis, although circulating haemocytes are believed to exist mainly in the larvae stage, disappearing from circulation in adult *A. mellifera* most likely as a social colony level strategy to reserve energy (Schmid *et al.*, 2008). The activation of the immune response through pathogenic or parasitic invasion is thought to not only affect the individual due to the weakening effects caused by virulence factors in the invader, but is also thought to have a negative effect on the capability of other life systems to function adequately (Kralj *et al.*, 2007). The activation of the immune response is thought to have a negative impact on successful foraging in *A. mellifera* and also on the rate of return to the colony (Kralj *et al.*, 2007). Social interactions are also affected during the infection of an individual bee by a pathogen, as demonstrated in LPS injected bees (Richard *et al.*, 2008). Healthy workers no longer interacted with the LPS treated workers normally, which could affect the survival of these individuals, but in turn could have a positive affect on the health of the whole colony due to the halting of the spread of the pathogen (Richard *et al.*, 2008).

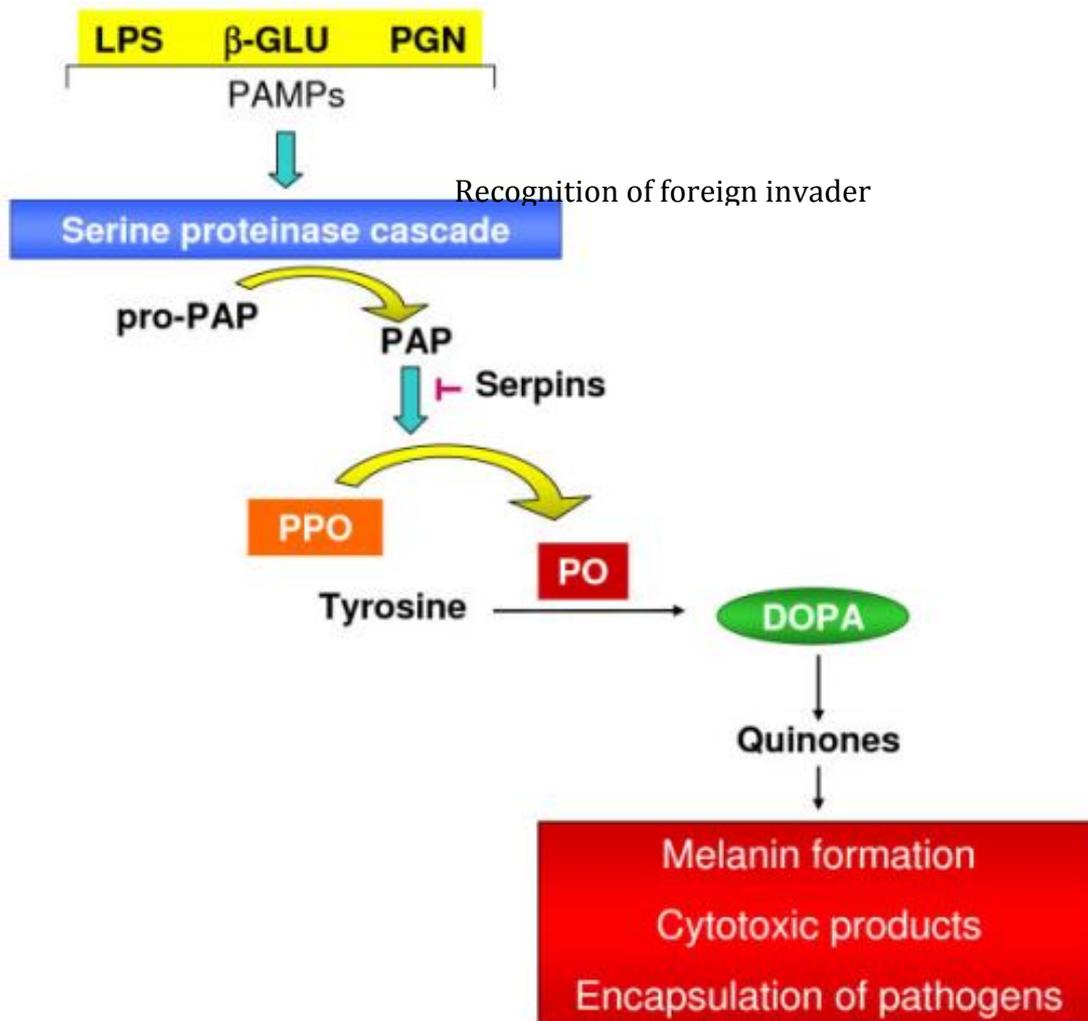


Figure 1.3 Schematic representation of the melanization cascade.

Representative image showing the activation of the various steps along the melanization cascade through specific enzymatic reactions.

(Adapted from Garcia *et al.*, 2009)

1.3 Diseases of the honeybee

1.3.1 *Melissococcous plutonius*

M. plutonius is a lanceolate Gram-positive bacterium that is the causative agent of European foulbrood (EFB) (Roetschi *et al.*, 2008). It was first described in 1885 and has since become widespread across all geographic areas (Budge *et al.*, 2010). The occurrence of EFB has risen over the past number of years, coinciding with the increased prevalence of most other honeybee diseases. In Switzerland for example, one of the countries with a high number of commercially kept bees, there were 20-30 EFB cases per year the early 1990s, which had risen to 184 by 2003 (Forsgren *et al.*, 2005). EFB infects the developing *A. mellifera* larva with symptoms such as color change in the larvae from cream to grayish/black, and displacement from the normal position to the bottom of the cell (Forsgren *et al.*, 2005). The bacterium can be treated with antibiotics, but left untreated, whole colonies can be lost (Budge *et al.*, 2010).

1.3.2 *Paenibacillus larvae*

P. larvae is the causative agent of American foulbrood (AFB), a serious disease of honeybee colonies. It is a very difficult disease to manage as it produces very resilient spores which are hard to eradicate (Fries *et al.*, 2006). It is common and widespread, which is a major problem for beekeeping as it can cause complete colony loss (Qin *et al.*, 2006). As with EFB, young larvae are most susceptible, with ingestion of as few as ten bacterial spores from the more virulent strains enough to cause death (Qin *et al.*, 2006). Larvae are typically infected before they reach 24 hours old and once infected, they do not recuperate (Lindström *et al.*, 2008). The infected larvae are characterized by a brown color and a mucilaginous appearance during the sporulation phase of the infection (Lindström *et al.*, 2008). Treatment by oxytetracycline is sometimes effective but resistant strains of *P. larvae* have also emerged in recent years

(Kochansky *et al.*, 2001; Fernández *et al.*, 2014) . Affected hives are burned to avoid spread in some countries such as in Ireland (Coffey, 2007).

1.3.3 *Nosema*

Honeybees are susceptible to two different strains of *Nosema* which cause nosemosis in infected bees, *Nosema apis* and *Nosema ceranae* (Figure 1.4). *Nosema* are intracellular opportunistic microsporidia, which are widespread globally in honeybee colonies (Chen *et al.*, 2009). Nosemosis of *A. mellifera* was originally attributed to *N. apis*, but due to the globalization of beekeeping, *N. cerana* has been found to also infect colonies of the western honeybee, at an increasing rate (Chen *et al.*, 2009). *N. ceranae* has been linked with Colony Collapse Disorder (CCD), most likely due to its recent appearance in *A. mellifera* and the lack of an evolutionary history of developed tolerance (Chen *et al.*, 2009). *N. apis* is thought to infect the epithelial layer of ventriculus and midgut of adult bees, leading to a shortened lifespan and a decrease in overall colony numbers, especially after the Winter period (Higes *et al.*, 2006). *N. ceranae* is thought to infect in the same way but with a much higher level of mortality, demonstrated using caged bees infected with the same load of *N. apis* and *N. ceranae* which resulted in a higher mortality in the bees infected with the latter strain (Mayack and Naug 2009b) (Chen *et al.*, 2009). Parasites like *Nosema* compete with their host for nutrition and are known to cause energetic stress in their host (Mayack and Naug 2009b). Bees suffering from nosemosis have lower levels of protein in the haemolymph which results in a reduced hypopharyngeal gland (Mayack and Naug 2009b). Infected workers show an elevated appetite and increased hunger, most likely due to a reduced rate of metabolic efficiency caused by degradation of ventricular epithelium and a lower secretion rate of digestive enzymes (Mayack and Naug 2009b). This increased appetite could lead to higher trophallactic rates within the colony, aiding the spread of the microsporidian (Mayack and Naug 2009b). The replacement of *N. apis* by *N. ceranae* in *A. mellifera* colonies could be a contributing factor to the occurrence of colony collapse disorder, as the higher rates of mortality could lead to rapid decline in numbers of adults bees (Higes *et al.*, 2006; Mayack and Naug 2009b, Natsopoulou *et al.*, 2015).

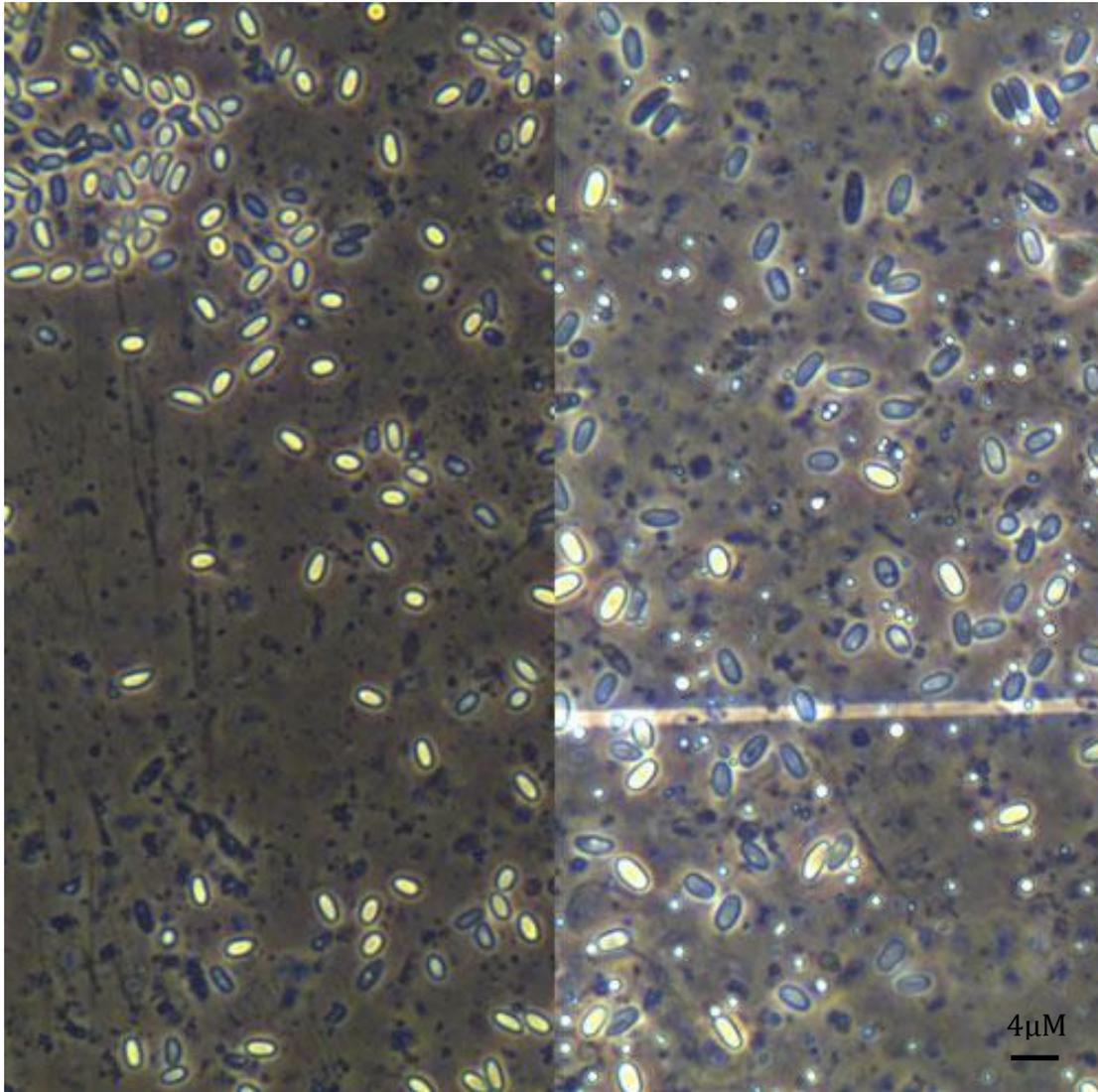


Figure 1.4 Image of *N. ceranae* (left) and *N. apis* spores (right).

Image shows the similar structure of both *Nosema* spores under compound microscope.

N. ceranae spores are slightly more oval in shape.

Image adapted from (Huang 2011)

1.3.4 Parasitic mites of *A. mellifera*

1.3.4.1 *Acarapis woodi*- The tracheal mite

The tracheal mite is one of the least serious parasitic mites that infects the honeybee, with little effect on mortality, except during the Winter months (Harrison *et al.*, 2001). It was first discovered in 1920 and named “Isle of Wight” disease, which affected the UK and mainland Europe (McMullan and Brown 2005). During the search for the causative agents of this loss of bees, the tracheal mite was discovered (McMullan and Brown 2005). They infect *A. mellifera* primarily in the first few days of its life, by entering the trachea via the first thoracic spiracle (Ellis and Baxendale 1997; Harrison *et al.*, 2001). Here, they reproduce rapidly, with higher levels of mites found in older bees (Harrison *et al.*, 2001). The full life cycle from egg to egg takes around 20 days, with female mites developing to sexual maturity within 10 days (Royce *et al.*, 1991). Mated females leave the bee they were born in, to infect another bee, moving between members of the hive via pleural hair (McMullan and Brown 2005). Originally, they were thought to cause a high level of mortality, but in recent years, this mortality that was once blamed on them has been attributed to other disease causing agents such as viruses (McMullan and Brown 2005). They do, however, cause a higher level of mortality in the Winter than at other times of the year (Harrison *et al.*, 2001; McMullan and Brown 2005). The higher rates of mortality in the Winter could be due to the additional stress Winter causes such as lower temperature, or due to the older average age of bees present in overwintering colonies, in which higher levels of the mite are typically found. They may also cause stress due to the heightened immune response in bees that are affected by any parasite or pathogen. They feed on the haemolymph of their host, through the walls of the trachea, and can transmit viral particles (Harrison *et al.*, 2001). They are thought to interfere with the gas exchange needs of the flight muscles and may affect the return to colony after foraging (Harrison *et al.*, 2001). Treatment of tracheal mites involves the use of

acaricides such as monoterpenoids, which show good effectiveness against the mites (Ellis and Baxendale 1997). Indirect treatment of tracheal mites also occurs due to the use of varroacides, which has had a major impact on the numbers found in colonies (Watkins 2003).

1.3.3.1 *Varroa destructor*

V. destructor is possibly the most serious threat to the survival of *A. mellifera*, with infected colonies collapsing in 2-3 years without treatment (Rosenkranz *et al.*, 2010). It is an obligate ectoparasite of the larvae and pupae of the honeybee, and also feeds on adults if no brood cells are present, with the close interaction of the two making transport around the hive possible in a relatively short space of time via horizontal transmission (Le Conte *et al.*, 2010). *V. destructor*, first discovered in Java, was originally confined to the eastern honeybee, *A. cerana*, with the shift to *A. mellifera* most likely occurring at the start of the twentieth century, as a result of the close proximity of the two species when *A. mellifera* were transported to East Russia and to the Far East (Sammataro *et al.*, 2000; Rosenkranz *et al.*, 2010; Garrido *et al.*, 2013). One of the main reasons for the susceptibility of the western honeybee *A. mellifera* towards *V. destructor* is that the relationship of parasite and host between the two is relatively new (Rosenkranz *et al.*, 2010). *A. mellifera* lacks the history of coevolution seen in the relationship of *A. cerana* and the mite (Rosenkranz *et al.*, 1993, Rosenkranz *et al.*, 2010). *A. cerana* display a better means of dealing with the parasite due to this co-evolution, and intensive grooming during infestation is one effective means of control (Büchler *et al.*, 1993). Also, the damage incurred by the bees is not at total devastation level because of the tendency and ability of *V. destructor* to only reproduce in the drone brood larvae in *A. cerana* (Fries *et al.*, 2006). The spread of *V. destructor* has been rapid through *A. mellifera*, with their incidence first reported in the Western Europe in the late 1970s, with increase in international commerce and travel first facilitating dispersal, and the colony to colony spread helped by tendency of hives to swarm and rob (Thompson *et al.*, 2002; Sammataro *et al.*, 2005; Le Conte *et al.*, 2010).

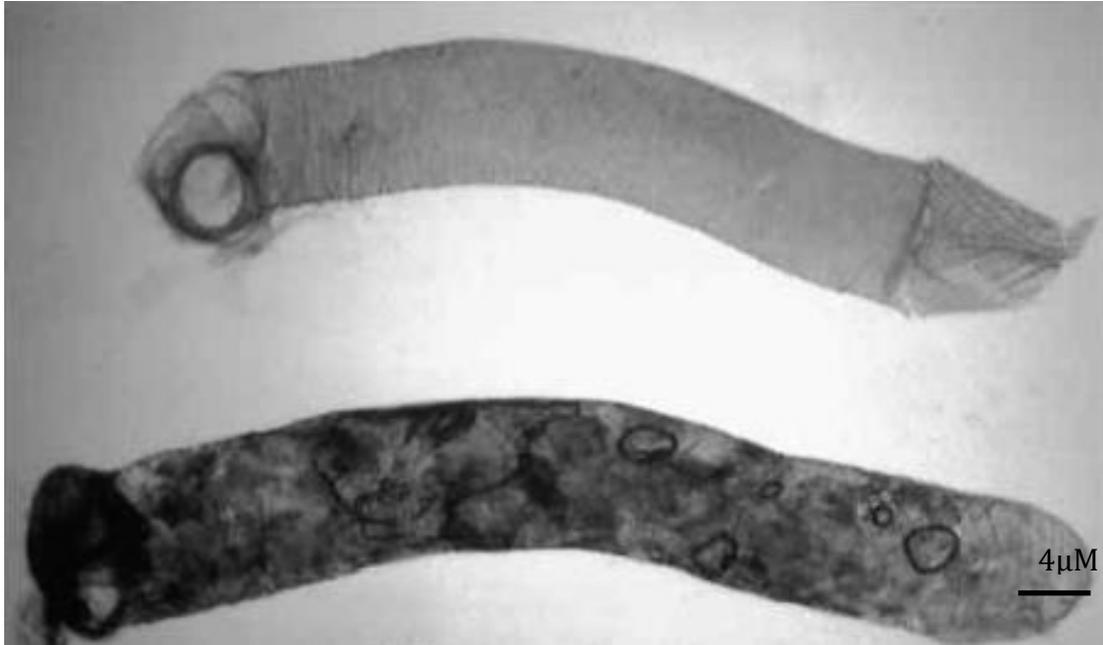


Figure 1.5 Light micrographs of healthy trachea (top) and *A. woodi* infested trachea (bottom).

Image shows the enlargement and the darkening due to melanization of a tracheal mite infested trachea.

Image adapted from (Harrison *et al.*, 2001)

Biology of the Varroa mite

The *V. destructor* mite has many morphological features that contribute to its success as an ectoparasite of *Apis* species, both its natural and adopted hosts (Sammataro *et al.*, 2000; Rosenkranz *et al.*, 2010). The body of the mite is divided into two distinct parts - The idiosoma which contains the dorsal shield and the gnathosoma which forms the mouthparts which consist of two sensory structures known as pedipalps and two Chelicerae with functions in the feeding process (Sammataro *et al.*, 2005; Rosenkranz *et al.*, 2010). The female mite in particular has evolved in a specific manner adapted to the host. It measures about 1.1 mm in length and 1.6 mm wide, with an oval, flattened body to enable the parasite to escape the natural grooming behavior of the host, and also to remain unseen in the brood cell prior to capping (Sammataro *et al.*, 2000). They also have a much higher degree of sclerotisation to the idiosoma for protection and have a specially modified gnathosoma for the piercing and tearing of the developing *Apis* larvae's cuticle to facilitate both personal feeding and that of its progeny (Sammataro *et al.*, 2000; Rosenkranz *et al.*, 2010).

Host finding by *V. destructor* seems to be attributed to a number of different morphological features, with chemical orientation playing a crucial role (Aumeier *et al.*, 2002; Rosenkranz *et al.*, 2010). The entire body of the mite is covered by tiny hairs (Figure 1.6), some of which have been shown to have chemo-receptive functions, in particular those on the front legs, which are used mostly as antennae and rarely for movement (Figure 1.6) (Rosenkranz *et al.*, 2010). *V. destructor* have been shown to respond to certain organic molecules in the process of host finding, with attractiveness to fatty acid esters observed in experimental studies, and also have been shown to be attracted to aldehydes and aliphatic alcohol (Sammataro *et al.*, 2000). Additionally, situated on the tarsi of the front legs is a sensory organ similar to the Haller's organ in tick species, which has been implicated in having a role in olfactory sensing (Rosenkranz *et al.*, 2010). *V. destructor* has also been shown to possess an

ability to perceive light and temperature, but has not been proven to have a role in host orientation (Rosenkranz *et al.*, 2010). Aliphatic esters and certain hydrocarbons produced by the last instar of a developing *A. mellifera* larva before capping have also been shown to help direct the female *V. destructor* towards a brood cell (Aumeier *et al.*, 2002). The size and height of the brood cell affect the decision of the female mite in choosing the desired one in which to enter and reproduce, with an apparent preference to choosing drone brood cells due to their larger size and the longer period of capping (Rosenkranz *et al.*, 2010).

Life cycle of *Varroa*

The life cycle of *V. destructor* is closely linked to that of its host species *A. mellifera*, and is completely dependent with no free-living stage (Sammataro *et al.*, 2000; Rosenkranz *et al.*, 2010). All reproduction occurs in the brood cells of *A. mellifera*, with the female *V. destructor* entering the cell shortly before the larva undergoes an ecdysis, resulting in her being left undisturbed (Contzen *et al.*, 2004; Kanbar and Engels 2004; Shen *et al.*, 2005). The mite passes between the larva and the cell wall and moves to the bottom of the brood cell avoiding detection by hygienic bees by hiding in the larval food until the cell is capped between 20-40 hours later, using specially modified peritremes as a snorkel-like structure to breath (Sammataro *et al.*, 2005; Shen *et al.*, 2005; Rosenkranz *et al.*, 2010).

Roughly 60 hours after the brood cell is capped, the mature female mite will lay her first egg, usually near the top of the cell wall to protect from damage while the young *A. mellifera* larva is molting (Oldroyd 1999). *V. destructor* are haplodiploid organisms, meaning that unfertilized eggs develop into males and fertilized eggs develop into females (Figure 1.8a) (Oldroyd 1999; Sammataro *et al.*, 2000). A haploid male hatches from the unfertilized first egg of the female and reaches sexual maturity in 5-6 days (Figure 1.7) (Sammataro *et al.*, 2000). All subsequent eggs are produced at an average rate of 30 hour intervals and contain diploid females, which are mated by the male

at the fecal accumulation point which is comprised mostly of guanine and is deposited on the cell wall (Figure 1.8b) (Sammataro *et al.*, 2000).



Figure 1.6 Adult female *Varroa* on drone *A. mellifera* pupa.

Image shows adult female *Varroa* mite feeding on a drone pupa. The chemosensitive hairs can be seen covering the body surface and the front legs, typically used as antennae, can be seen to the front near the head (indicated by arrows).

One of the most interesting aspects of the activity of the mature female *Varroa* and her progeny inside the brood cell of the developing *A. mellifera* larvae is the use of a communal, single feeding wound which all the family use throughout the time in which the brood cell remains capped (Figure 1.8b) (Kanbar and Engels 2004; Gregory *et al.*, 2005). The feeding site is usually located on the fifth segment of the developing larva, near the fecal accumulation site (Rosenkranz *et al.*, 2010). As a haemophagous feeder, *V. destructor*, like other haemolymph or blood-sucking insects, aims to ensure adequate and easy feeding. This is achieved by preventing the blood from clotting and not allowing the puncture wound to seal, particularly for the reproductive cycle, as oogenesis and subsequent oviposition depend on the female *V. destructor* having repeated haemolymph meals (Kanbar and Engels 2004).

The usual healing methods are not activated in the cuticle of the *A. mellifera* larva, and analyzing the properties of *V. destructor* saliva found that it possibly contains a suppressor of the normal haemocyte mediated healing properties of *A. mellifera*, explaining why artificial wounds healed normally but those made by *V. destructor* to the cuticle did not (Richards *et al.*, 2011b).

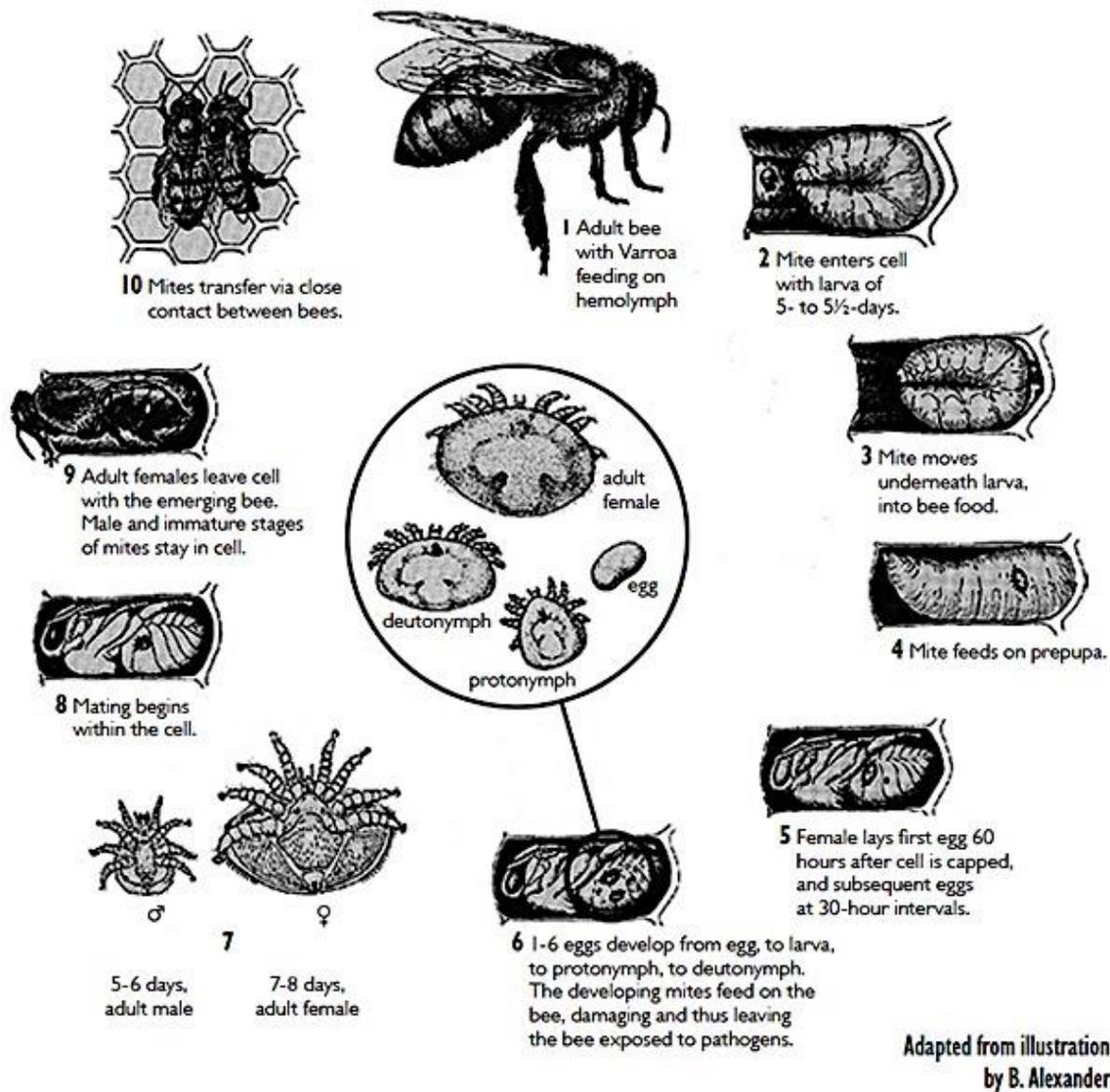


Figure 1.7 Life cycle of *V. destructor* in the brood cell of *A. mellifera*.

Image shows the life cycle that takes place within the capped brood cell of a developing *A. mellifera* pupa. The adult female mite enters the cell shortly before capping, and hides in the larval food. She then produces a haplodiploid male, which is the father to all subsequent female offspring. Female daughters sexually mature and are also mated by the male. Females leave the cell upon uncapping to infect others and reproduce. Image highlights how quick reproduction process is and how quickly numbers can increase if untreated.

Image adapted from (Huang 2012)

a)



b)



Figure 1.7(a) Various life stages of *V. destructor*. Clockwise from bottom: Protonymph mite, Deutonymph mite, recently molted female, adult female mite. **Figure 1.7(b) Family of *V. destructor* present in a single cell upon uncapping** Image shows the high number of mites that can parasitize a single pupa during development. Image shows various aged developing mites and a number of adult females.

Effect of parasitization by *V. destructor* on *A. mellifera*

The first effect of parasitization of the developing larva is a noticeable loss of weight of the larva after emergence, most likely due to the loss of haemolymph and the nutrients contained in it that are used by the developing larva during the transition from larvae to adult (Figure 1.9a and 1.9b) (Rosenkranz *et al.*, 2010). Parasitized drones, for example, had an average decrease in body weight of 11-19%, with worker bees showing an average weight loss of 7% (Rosenkranz *et al.*, 1993). The effect of developing under parasitization on the larva also causes behavioral changes to the bee, including increased foraging time, reduced incidence of returning safely to the colony, and a reduced life span, showing possible evidence of an immunological trade off to other aspects of survival (Rosenkranz *et al.*, 2010). Parasitized *A. mellifera* foragers also showed decreased ability to remember the location of their own colony and often returned to another, contributing to the spread of the *V. destructor* (Aronstein *et al.*, 2012). An example of a reason for the cognitive impairment of *A. mellifera* due to parasitization by *V. destructor* has been suggested by one study to be due to the down regulation of *pale*, a gene responsible for Dopamine synthesis, which is a nervous system stimulant (Navajas *et al.*, 2008). One of the most serious side effects of parasitization by *V. destructor* on the vitality of *A. mellifera* is the transmission of viral particles (Shen *et al.*, 2005; Yang and Cox-Foster 2005; Navajas *et al.*, 2008; Le Conte *et al.*, 2010; Di Prisco *et al.*, 2011). Before *V. destructor* became an established parasite of *A. mellifera*, bee viruses were considered only as a minor problem (Rosenkranz *et al.*, 2010). The decline in immunogenic capability in *A. mellifera* harboring *V. destructor* mites seems to induce the proliferation of certain bee viruses, both those carried in *V. destructor* as it acts as a vector for their transmission, and also those found only in bees (Navajas *et al.*, 2008). The immunosuppressive qualities of the stress involved in having a parasite leeching nutrients seems to create the optimum environment for viruses and other microbes, and the saliva of *V. destructor* may possess certain compounds that have a potential immunosuppressive role (Richards *et al.*, 2011a).

a)



b)



Figure 1.9(a) Adult *A. mellifera* worker heavily parasitized by *V. destructor*. Image highlights the high numbers of mites that can be present on a single adult bee.

Figure 1.9(b) Drone purple eye stage pupa heavily parasitized by *V. destructor* Five adult female mites can clearly be seen on a single pupae upon uncapping of the cell. Both images also highlight the relative size of the mite to the bee.

Treatment for *V. destructor* infestations

When *Varroa* first arrived to Europe in 1970s, efficient control was rapidly deployed, using a variety of mitocides, namely Amitraz, Coumaphos, Fluvalinate and Bromopropylate (Le Conte *et al.*, 2010). By 1995 however, the first occurrence of resistance to fluvalinate was observed in Europe, also conferring cross resistance to other pyrethroid chemicals such as flumethrin (Gregorc and Poklukar 2003; Le Conte *et al.*, 2010). No chemical treatment is ever 100% effective, and if there are a number of individuals following treatment, they are the sole source of genes for the next generation and so resistance can develop rapidly (Gregorc and Poklukar 2003; Le Conte *et al.*, 2010).

Resistance can occur by a number of different means. Behavioral changes can lead to avoidance of the threat, the organism could decrease penetration through its cuticle, to stop the chemical entering its system, it could change or desensitize the target site that the chemical is trying to act on, or it could up-regulate detoxifying mechanisms, such as the production of a certain metabolic enzyme (Sammataro *et al.*, 2005). The reproductive mechanism of *V. destructor* as haplodiploid is also one that favors the spread of a resistance gene, through its sibling mating habits (Sammataro *et al.*, 2005). A study to evaluate the types of gene knockouts present in pyrethroid resistant *V. destructor* found a number of mutations in the *para* family of sodium channel genes which are the target site of the pyrethroid mitocides (Wang *et al.*, 2002; Wang and Wang 2003).

The need to explore other non-chemical means of controlling *V. destructor* has driven research into the use of essential oil compounds found in plants as natural acaricides (Imdorf *et al.*, 1999; Sammataro *et al.*, 2005). The main components of essential oils are Terpenes and although they provide some attractive advantages over the chemical acaricides, such as a much lower cost to the beekeeper, they have been found to leave possible residues in the honey and wax rendering it unsuitable for use in other applications (Imdorf *et al.*, 1999). One however, Thymol, which is a volatile from the common thyme plant, has

shown good potential, and today there are a wide range of thymol based varroacides available to beekeepers. Although they are less effective than the chemical based varroacides, they play a crucial role in many integrated pest management strategies and in conjunction with oxalic acid. Oxalic acid is a naturally occurring organic acid and has been widely used since the 1980s (Gregorc and Planinc 2002). The method of delivery involves either of the two main methods, tricking or vaporization. Oxalic acid kills only the phoretic stage of the mite thus is more effective if administered under brood-less conditions (Gregorc and Planinc 2002). In the Northern hemisphere this usually occurs during the Winter months.

There are other methods of controlling *Varroa* infestation that do not involve the use of either synthetic or natural chemicals but are not effective as stand alone treatments. Dusting colonies of *A. mellifera* with powdered icing sugar to induce the natural grooming behavior of the bees has proven effective against mite populations. One study found that the numbers of mites in the colonies fell by about 78% following the application of the dusting sugar (Ellis *et al.*, 2009). Another method which has proven to remove large numbers of mites is drone brood comb removal, which exploits the natural tendency of the *V. destructor* female to seek drone brood for the reproductive stage of her life cycle (Sammataro *et al.*, 2000). The higher heat tolerance of *A. mellifera* in comparison to *V. destructor* can also be used as a method of control, as the *A. mellifera* brood can survive but the mite succumbs at 44° C (Sammataro *et al.*, 2000). In studies performed using the Asian bee, *A. cerana*, caging the queen and separating the brood frames helped to lower the mite numbers by interrupting the life cycle of the female *V. destructor* between the phoretic and reproductive stages (Sammataro *et al.*, 2000). Simple methods such as placing wire or adhesive mesh along the bottom of the hive has also proven effective in trapping mites that have accidentally slipped or fallen off bees, and also gives the beekeeper a chance to observe mite levels present in the hives at any given time (Rosenkranz *et al.*, 2010).

Other biological control methods have also been tried and tested since the appearance of pyrethroid resistance in *V. destructor* mites. The use of biological methods for pest control has been widely used for the protection of food crops for example, and a number of different possibilities have been assessed over recent years in order to see if these methods could be used to control infestation of colonies by *V. destructor*. Predatory mites, nematodes, viruses, fungi and bacteria have all been tested, including a bacteria that is already widely used for crop protection, *Bacillus thuringiensis* (Chandler *et al.*, 2001). *B. thuringiensis* is a spore forming bacteria, with the sporulation process causing the formation of a toxic proteinaceous crystal that has insecticidal activities, and was shown to cause no adverse reactions in *A. mellifera* (Chandler *et al.*, 2001). Entomopathogenic fungi, including some members of the Mitothecium and Zygomycetes families, have also shown very promising results, (Chandler *et al.*, 2001). Two species in particular have shown extra promise as potential agents for the biological control of *V. destructor* in colonies, *Neozygites floridana* and *Hirsutella thompsoni*, both of which are acari specific (Chandler *et al.*, 2001). Under laboratory conditions, *H. thompsoni* demonstrated the ability to infect *V. destructor* through the membranous arolium of the leg sucker part of the mite simply by walking over a carpet of the fungus (Peng *et al.*, 2002). Also, the fungus failed to infect or cause any adverse effects to *A. mellifera*, at larval or adult stage (Peng *et al.*, 2002). The idea of breeding *A. mellifera* that exhibit a naturally higher tolerance towards *V. destructor* has also been examined. The natural behavior of the original host of *V. destructor*, *A. cerana* such as the grooming behavior means that the problem of infestation never reaches a critical level to the colony, and the idea of selecting *A. mellifera* individuals that show signs of extra grooming capacity is a promising novel idea (T. E. Rinderer *et al.*, 2010). Researchers have bred for increased grooming tendencies in *A. mellifera* and have made a commercially available species that shows increased auto and allo-grooming, leaving them less susceptible to the threat of *V. destructor* (Rinderer *et al.*, 2010).

1.3.4 Viruses of the honeybee and the link with *V. destructor*

A total of twenty five viruses have been isolated so far from *A. mellifera*, many of which have also been seen in *V. destructor* (Rosenkranz *et al.*, 2010; Yañez *et al.*, 2012). Evidence for the role of *V. destructor* in the proliferation of viruses can be seen when viral loads in *A. mellifera* are examined, with the incidence of infections rapidly rising after introduction of *V. destructor* in 1987 in the USA (Shen *et al.*, 2005). The presence of one virus in particular is closely correlated to the number of mites present in the colony; Deformed Wing Virus (DWV). DWV is an iflavivirus, and has been shown to infect both *A. mellifera* and *V. destructor*, and produces symptoms in bees which include seriously deformed *A. mellifera* adults, rendered flightless by deformed wings and shortened abdomens (Figure 1.10) (Shen *et al.*, 2005; de Miranda and Genersch 2010; Yañez *et al.*, 2012). When *A. mellifera* are parasitized *V. destructor* combined with exposure to other microbes, it has been shown that DWV can replicate at levels of up to 10^5 times higher than in unparasitized bees (Yang and Cox-Foster 2005). One enzyme, polyphenol oxidase, that has immune functions through the Prophenol Oxidase cascade involved in certain immune reactions such as those triggered during the melanization process and is a widely used measure of immune activation, is found to be down-regulated at the level of transcription in *A. mellifera* that are under parasitization by *V. destructor* (Yang and Cox-Foster 2007). This lowered immune reponse could lead to a lower ability to withstand viral infections. Acute bee paralysis virus, which is another virus that has been shown to be replicative competent in *V. destructor*, is capable of wiping out colonies at high levels of viral load (Govan *et al.*, 2000). The virus normally spreads through salivary interaction of adult bees and is also found in food stores, but has been shown to be present in much higher levels in hives that are also heavily parasitized by *Varroa* (Govan *et al.*, 2000). *V. destructor* have also been shown to act as a vehicle for another virus of *A. mellifera*, Israel Acute Paralysis virus and also as a vector for Kashmir Bee Virus, which have both been linked with colony collapse disorder (Shen *et al.*, 2005; Cox-Foster *et al.*, 2007; Di Prisco *et al.*, 2011). An additional virus found to be replication competent in *V. destructor* is Chronic Bee Paralysis

virus, which causes chronic paralysis, first noticeable by a greasy appearance on infected bees (Figure 1.10). Using RT-PCR techniques, Celle *et al.*, (2008) found it to be present in *V. destructor* in its replicative RNA from infected *A. mellifera*.

The increase in the incidence of these viruses following the introduction of the *V. destructor* mite shows that they are likely to act as vectors in the spread and proliferation (Le Conte *et al.*, 2010). A large-scale survey conducted by a group of six bee viruses in France and their prevalence and seasonal variations in accordance with *V. destructor* levels and found a direct putative role of *V. destructor* in the transmission of these viruses (Tentcheva *et al.*, 2004). This increase in the virulence of these viruses since the arrival of *Varroa* has also been shown in a number of other studies, indicating that *Varroa*, in combination with viruses could lead to the collapse of colonies (Sumpter and Martin 2004; Chen *et al.*, 2006; Martin *et al.*, 2012; Gajger *et al.*, 2014).

1.4 Colony Collapse Disorder (CCD)

CCD is the name given to the phenomenon of the mass disappearance of bees since approximately 2006 (Cox-Foster *et al.*, 2007). CCD is characterized by a seemingly healthy colony with plenty of food and brood, but no workers or in some cases, very small amounts clustered around the brood (Le Conte *et al.*, 2010). Over the past century, there has been a 50% increase in the numbers of honeybee stocks around the world, but a staggering >300% increase in the number of pollinator dependent crops (Williams *et al.*, 2010). This extra stress on bees due to the extra pressure to feed on monocrop landscapes has possibly influenced the large scale losses recorded worldwide, but most research seems to agree that there are probably many factors influencing the disappearance of bee populations, and no single one can be blamed (Cox-Foster *et al.*, 2007; Williams *et al.*, 2010). There are however, some cases of CCD where no cause for the loss can be identified and the disappearance of the bees from the

colonies is genuinely unexplainable (Williams *et al.*, 2010). The idea that a microbe or group of microbes may be to blame for the losses has been shown to be a possibility, as colonies affected by CCD from the same apiary are usually neighbours, and irradiated comb from a CCD hive can be re-colonized by naïve bees, suggesting that a disease causing agent could be to blame (Cox-Foster *et al.*, 2007; vanEngelsdorp *et al.*, 2009). A large-scale survey into the microbes that were present following the collapse of CCD affected colonies found that two viruses, Israeli acute paralysis virus (IAPV), and Kashmir bee Virus (KBV) were present in none of the control colonies and present in all of the CCD affected colonies in the case of IAPV, and three quarters of the CCD affected colonies in the case of KBV (Cox-Foster *et al.*, 2007). IAPV is a recently discovered bee virus, and is characterized by shivering bees, eventually leading to paralysis and death (Cox-Foster *et al.*, 2007). This may not fully explain how CCD affected colonies share the trademark characterization of no visible workers, either in the hive or dead outside. There may be a clue in the social immunity patterns of honeybees, to leave the area of the hive when they are infected by a pathogen such as a virus, in order to maintain overall health of the hive (van Engelsdorp *et al.*, 2009). A study of the microbes in CCD affected colonies along with levels of certain pesticides and parasitic mites found no overall link between any of the tested factors (van Engelsdorp *et al.*, 2009). This gives further proof to the theory that it is probably due to a multitude of reasons, from stress of commercial beekeeping, to *Varroa*, and other microbes which when combined can have a detrimental effect on the vitality of the colony and lead to its collapse.



Figure 1.10 Adult *A. mellifera* displaying symptoms of DWV

Source: http://entnemdept.ufl.edu/creatures/misc/bees/varroa_mite.html



Figure 1.11 Adult *A. mellifera* displaying symptoms of CBPV

The shiny, greasy appearance of the bees affected by CBPV is clearly visible.

Source: <http://www.extension.org/pages/71172/honey-bee-viruses-the-deadly-varroa-mite-associates>

1.5 Use of proteomic analysis for identifying resistance mechanisms in *V. destructor* and the effect of parasitization by *V. destructor* on *A. mellifera*

Proteomic studies allow the observation of the actual functional molecules within an organism, rather than the observation of alterations in expression rate of genes. This type of study is useful when dealing with an overall view of variations between two organisms that exhibit alterations in their phenotype such as the observation of an individual with a higher tolerance for an insecticide such as pyrethroid, or an unparasitized insect compared to a parasitized insect. Expression proteomics such as 2-Dimensional studies allow for comparison of the total proteome of one phenotype versus another, with the differences visible and identifiable using densitometrical software such as Progenesis SameSpots (Rosengren *et al.*, 2003; Bantscheff *et al.*, 2012). Advances in molecular and proteomic techniques and the vast range of data made available by genomic and transcriptomic projects have made it possible to look at overall changes in organisms rather than a small section of select genes or proteins (Ozsolak and Milos 2011). Here, label free “shotgun” proteomics was also employed to identify resistance mechanisms in the resistant *Varroa* and in the comparison of the proteomes of parasitized and unparasitized *A. mellifera* pupae. It also enabled the observation of quantitative differences between certain proteins that could be leading to a more efficient means of dealing with xenobiotic metabolism in *Varroa* that were resistant to Bayvarol.

This analysis gives a larger picture and allows unprecedented insights into the processes that may be at play in differentiating the resistant phenotype (Bantscheff *et al.*, 2012). Label free proteomic analysis takes into account the intensity of three parameters: peptide count, spectral count and fragment ions, giving the chance for quantitative analysis of potentially thousands of proteins between sample sets (Griffin *et al.*, 2010).

1.6 Aims of this study

1. To analyze the occurrence and prevalence through seasonal variations of diseases in Irish honeybee colonies.
2. To identify the mechanisms by which *V. destructor* become resistant to pyrethroid using proteomic analysis to compare the proteome of pyrethroid sensitive and resistant *Varroa*.
3. To gain a full understanding of the total effect that parasitization by *Varroa* has on adult and pupae *A. mellifera* using both proteomic methods and molecular methods.

Chapter Two

Materials and Methods

2.3 Chemicals

All chemicals and reagents were of the highest purity and were purchased from Sigma Aldrich Chemical Co. Ltd., Dorset, United Kingdom, unless otherwise stated.

2.4 Sterilisation Procedures

All liquids were sterilised prior to use by autoclaving in a Systec 3170 ELV autoclave at 121°C and 15 lb/sq.in. for 15 minutes. Any chemicals unsuitable for autoclaving were filter-sterilised using a filter with a pore size of 0.2 µm (Sarstedt, Nümbrecht, Germany).

2.5 *Apis mellifera* Sampling and Experimental Conditions

2.5.1 Adult *A. mellifera* sampling conditions

All adult *A. mellifera* for proteomic analysis were sampled from apiaries in Counties Meath, Galway or Carlow. Samples for viral analysis were from apiaries throughout the Republic of Ireland (Figure 2.1a). Adult bees were sampled from the same area of the hive near the centre brood boards to ensure the same age of bee was taken (Figure 2.1b). A container of bees was sampled and kept frozen until further use. Adults workers were sampled from colonies where the level of parasitization by *V. destructor* was known. This was determined by examining the sticky inserts placed in the floor of the hive for the level of natural mite drop per daBees were chosen for analysis from very mildly parasitized and very heavily parasitized colonies for comparison. Individual bees were subsequently examined for the presence of *Varroa* mites before use also. Samples were also chosen from mildly parasitized and heavily parasitized Winter colonies and Summer colonies for comparison.

a)



b)



Figure 2.1a: Example of an apiary showing the hives. **Figure 2.1b:** Image shows the inside of hive. Each section is a brood board, and adults were sampled from the centre of these.

2.5.2 Worker *A. mellifera* sampling conditions at defined pupal age

Worker *A. mellifera* were collected from an apiary in Kilmessan, County Meath, using a queen trapping method. A queen excluder box was placed around an empty brood comb and the queen was placed inside. This was carried out in three different hives. The queen excluder comb allows the worker bees to travel through to tend to the queen but due to her larger size, she remains in place on the brood comb (Figure 2.2). This ensures that all of the eggs that are laid in the hive are contained on this piece of brood comb, helping to ensure that all of the pupae are of a similar age. The containment of all brood onto a single piece of brood comb means that any *Varroa* present in the colony will all move into this area, increasing natural infestation levels for experimental use of the pupae. Each cell was uncapped and the number of adult female *Varroa* present upon uncapping was recorded, along with the number of progeny present. A pupa was considered as unparasitized and used as a control sample if there were no female *Varroa* observed upon uncapping of the cell.



Figure 2.2: Example of a queen excluder (metal) being placed over a brood board (wood).

The size of the holes in the excluder means that workers can pass freely but the larger queen is secured in place.

To correctly match the age of the pupae taken for experiments, only pupae in the purple eye phase with no darkening of the cuticle were used. During development from larvae to adult, eye colour changes from white to purple as the pupae develops and so this can be used as an accurate guide to the age of the pupae (Figure 2.3).

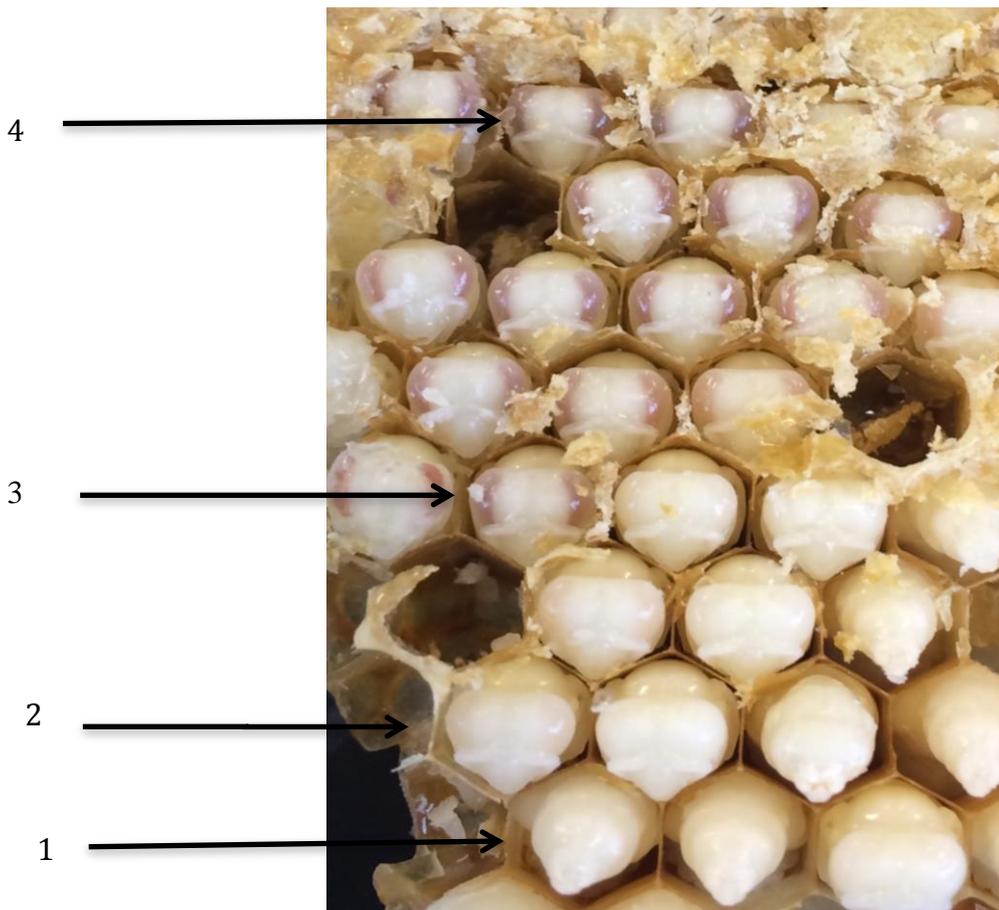


Figure 2.3: Image shows the distinct eye type for the various developmental stages of the *A. mellifera* pupae.

At the bottom of the image, the headless larval stage can be seen (1) followed on top by the white eye stage of the pupae (2), then the pink eye stage (3), finally followed by the purple eye stage (4).

2.5.3 Drone *A. mellifera* sampling conditions at determined pupal age

Drone pupae were received from apiaries in Counties Meath, Carlow and Galway. Brood comb containing drone cells was removed and frozen until use. All pupae used for proteomic and molecular analyses were chosen by age, based on the eye colour and cuticle darkness (See section 2.3.3). As for the worker pupae, each cell was uncapped prior to use and the number of adult and young *Varroa* inside each cell was enumerated. A pupa was considered to be unparasitized and used as a control sample if no adult female *Varroa* was observed upon uncapping.

2.5.4 Artificial infestation of *A. mellifera* pupae by adult female *V. destructor* mites

A brood comb was obtained from a heavily infested colony in County Carlow and maintained at optimum temperature in an insulated box to be brought back to the laboratory. Upon arrival, the lids were removed from the brood cells piece by piece and the *Varroa* adult females that ran from each cell were captured and secured in a petri dish using a fine paintbrush. Four worker and two drone pupae were removed from the brood cells using a blunt tweezers to avoid damage, and each was placed in a sterile 1.5ml eppendorf containing a small piece of moist cotton wool to maintain humidity (Figure 2.4). Between eight and twelve adult *Varroa* were then placed into each tube using the fine paintbrush and the lid closed to secure. The eppendorf tubes were placed in an incubator at 35°C for 48 hours. Pupa were inspected following removal from the incubator to ensure that they were still in a viable condition following 48 hours and only those that looked healthy and had no signs of melanisation were used for subsequent analysis.



Figure 2.4: Method used for artificial infestation of *A. mellifera*.

Image shows an eppendorf tube containing purple eye stage worker pupa, a number of adult female *Varroa*, and a piece of moist cotton wool for natural humidity replication.

2.6 *V. destructor* Sampling and determination of susceptibility to Bayvarol

2.4.1 Beltsville Method of determining resistance to Bayvarol

Bees were sampled by shaking one or two brood combs into a basin and scooping them up into a container. The container had a mesh lid and a slit in the bottom through which the Bayvarol strip was placed. To prevent the strip from falling through into the container, a small nail was pierced through the end of it to suspend it above the slit (Figure 2.5). A layer of sticky tape was placed under the mesh lid to catch any mites that fell from the bees overnight (Figure 2.6). The container was then left upturned in the dark for 24 hours in order to obtain maximum effectiveness of the Bayvarol. After 24 hours, the number of mites that had fallen onto the sticky tape through the mesh were counted and placed to one side. These mites were deemed sensitive to the

Bayvarol. The container was then placed in the freezer for a number of hours to kill the bees before washing in a soapy water solution to dislodge any remaining mites. The bees were washed over a sieve with a pore size of 2mm to trap bees but allow any mites to pass through. This process was repeated until all mites were dislodged. The number of mites that were dislodged by washing was enumerated. The percentage efficacy was then calculated as the number of mites that fell off bees during washing divided by the total number of mites (mites that fell off plus mites that were washed off). Mites from the colony were then deemed resistant to Bayvarol if the number was less than 50% of the total number of mites originally on the bees sampled.

Sample number:	Location:	Resistant/Sensitive?
1	Mallow, Cork	Sensitive
2	Castlebar, Mayo	Sensitive
3	Dundalk, Louth	Sensitive
4	Mallow, Cork	Sensitive
5	North Tipperary	Sensitive
6	Clonmel, Tipperary	Sensitive
7	Naas, Kildare	Sensitive
8	Tralee, Kerry	Sensitive
9	Kilmessan, Meath	Resistant
10	Kildare	Resistant
11	Cahir, Tipperary	Sensitive
12	Cabra, Dublin 7	Sensitive
13	Connemara, Galway	Resistant
14	Fingal, Dublin	Resistant
15	Tipperary	Resistant

Table 2.1: List of various locations that *Varroa* samples were received from. Susceptibility was determined either by Beltsville method or by the history of chemicals used, i.e. if the colony had never been in contact with Bayvarol it was deemed sensitive.



Figure 2.5: Container of bees showing the Bayvarol strip secured by nail. Container held approx. 200 bees and was used to sample for Bayvarol susceptibility testing.



Figure 2.6: Mesh lid of the container used for the Beltsville resistance test. A layer of sticky tape was placed over this mesh and the container left upturned to trap mites that fell off during the 24hour incubation with the Bayvarol strip.

2.7 National survey of the occurrence and prevalence of diseases in Irish honeybee colonies –Sample collection

2.7.1 Method of sampling and postage

Samples were collected in Spring, Summer and Autumn of each year by filling a small container with approximately 100-150 bees. Samples were posted immediately and then frozen on arrival, or frozen prior to postage.

2.7.2 Sampling locations Nationwide

To ensure that the selection of beekeepers invited to participate in this survey was representative of Irish beekeeping/beekeepers a stratified random sampling method was used. The entire beekeeping population was divided into groups using criteria such as geographical location or local beekeeping association. Then, from within each group a random sample was selected which was representative of that group. Due to Data Protection Issues, FIBKA was not in a position to make the list of members available. Therefore a list of beekeepers who themselves confirmed their willingness to be included in the initial selection list was compiled. During April and early May 2014 a total of 750 beekeepers confirmed their willingness to participate. The required sample was weighted by the number of beekeepers affiliated per county and random numbers were used to select 200 beekeepers, thus ensuring a good geographical spread of participants. Each selected beekeeper was then individually invited and given the option to decline the invitation if circumstances had changed. A total 161 beekeepers confirmed their willingness. To ensure the anonymity, each beekeeper was given a Participant number which was known only to the administrator and the beekeeper.

Participant No.	Address	County	Participant No.	Address	County
2	Ballyhackett, Tullow	Carlow	105	Beaufort Bar, Beaufort, Killarney	Kerry
5	Gortnaleck, Bawnboy, ballyconnell	Cavan	109	Baurearagh, Bonane, Kenmare	Kerry
6	24 Carrickfern Cavan	Cavan	113	8 Tower View Park	Kildare
7	Mullaghboy, Ballyhealon, Kilnaleck	Cavan	114	Newtown Bagnelstown	Kildare
8	Crimlin Craggagh, Fanore	Clare	115	Balscott, Celbridge	Kildare
9	Dromin, Miltown Malbay	Clare	116	Tullabrin, Johnswell	Kilkenny
10	Six-mile-bridge	Clare	117	Jeninstown Park	Kilkenny
11	Ballyconry, Ballyvaughan	Clare	118	Bramblestown, Gowran	Kilkenny
12	Fennagh, Clooney, Quin	Clare	119	Riverview Ennisnag, Stonyford	Kilkenny
13	Moses Bridge House, Greenane, Kanturk	Cork	120	Killoshulan, Barna, via Thurles	Kilkenny
14	Railway House Midleton	Cork	121	Levally, Rathdowney	Laois
15	Ait na Greine, Coolbay, Cloyne	Cork	124	Creevymore, Cliffoney, Sligo	Leitrim
16	Ballinure, Bartlemy, Fermoy	Cork	127	Coolanoran, Ardagh	Limerick
18	Oast Cottage, Castlemartyr	Cork	128	Clonrollagh, Longford	Longford
19	Kilmeedy West Kinsalebeg, Youghal	Cork	129	The Lock House, Coolnahinch, Kenagh	Longford
20	Scart, Kildorrey	Cork	130	Smear, AUGHNACLIFFE	Longford
22	Sanctoire, Lotamore, Glanmire	Cork	131	Ballymakellett, Ravensdale, Dundlak	Louth
25	Ring House An Ring, Clonakilty	Cork	132	Trean, Dunleer	Louth
26	Parkana, Bantry	Cork	133	Castletowncooley, Riverstown, Dundalk	Louth
30	Cuil Aodha, Maghchromtha	Cork	134	Killineer House, Drogheda	Louth
33	Discart, Toames West, Macroom	Cork	135	Castle View, Darver, Readypenny, Dundalk	Louth
34	Ardagh Bere Island	Cork	136	Belcarra, Castlebar	Mayo
36	Enterprise Ireland, Rossa Avenue, Bishopstown	Cork	137	Carra Lodge, Kilkeeran, Partry	Mayo
37	Kilbarry, Bandon	Cork	138	14 Pinewoods Westport	Mayo
38	Coolmore, Carrigaline	Cork	140	Westport (club hives)	Mayo
39	Collatrim, Bandon	Cork	142	Kilmena Hill, Westport	Mayo
40	Lee View, Macroom	Cork	143	7 Summerfield, Castlebar, Mayo	Mayo
41	Lee mount cottage, Coachford	Cork	146	Knockeragh, Kilbride Newport	Mayo
42	34 Willsbrook, Dooradoyle, Limerick	Cork	147	Saliesian House Drumree, Navan	Meath
43	Ballinskeha, Leamlara	Cork	149	Fraine, Athboy	Meath
44	Cuil Rathain, Corbally, Leamlara	Cork	150	Peterstown, Trim	Meath

45	castlewhite, waterfall	Cork	154	4 Meadowlands, Athboy	Meath
46	Costa Maningi, Derrymihan East, Castletownbere	Cork	157	Ballagh, Carrig, Birr	Offaly
47	Ballyshannon	Donegal	158	High view, Kileen, Birr	Offaly
48	Chruchtown, Kilcar	Donegal	160	Kilbride, Tullamore	Offaly
49	Carrig West, Laghey	Donegal	161	Newtown, Stokestown	Roscommon
51	Church Brae, Greencastle	Donegal	162	17 Greenhillsm, knockcroghery	Roscommon
53	Lissahully, Ballyshannon	Donegal	163	Bealnamulla, Athlone	Roscommon
54	Mullaghmore Rd., Cliffoney	Sligo	164	Cloonfinlough Strokestown	Roscommon
55	81 Seafiled Court, Killiney	Dublin	165	Snee, Carha, Coolaney	Sligo
56	Brackenhurst, Nashville Rd Howth	Dublin	166	Shiralees, Montiagh, Curry	Sligo
57	19 Villarea Park, Glenageary	Dublin	167	Creggconnell, Rosses Point	Sligo
58	Courtlands. Carysfort Ave. Blackrock	Dublin	168	Deerpark, Dundrum	Tipperary
59	Sliding Rock, Blackglen Rd, Sandyfort	Dublin 18	169	Tincurry, Cahir	Tipperary
60	Burrow Road Portrane, Donabate	Dublin	170	Mylertown, Clonmel	Tipperary
62	4 Fairbrook Terrace, Rathfarnham	Dublin 14	171	Lissaniskey Toomevara	Tipperary (N)
64	Craigmore, Nashville Road, Howth	Dublin	172	Clonegannagh, Dunkerrin, Birr	Offaly
65	31 Carrickhill Close, Portmarnock	Dublin	173	Currabaha, Templederry	Tipperary (N)
66	Arbored Lawns New Road, Donabate	Dublin	174	Lissenhall, Nenagh	Tipperary (N)
68	171 Killester Avenue, Killester	Dublin 5	176	Garnafana, Toomevara, Nenagh	Tipperary (N)
69	85 Ashcroft, Raheny	Dublin	177	Oldtown, Templemore	Tipperary (N)
70	34 Ard Na Mara Crescent, Malahide	Dublin	178	Doire na hAbhann, Tickincor, Clonmel, Co Tipperar	Tipperary (S)
71	222 lr Kilmacuddy Road Goatstown	Dublin 14	179	Rathloose, Powerstown, Clonmel	Tipperary (S)
72	St Mary's Luttrellstown Road, Portertown	Dublin 15	180	Dangan, Kilmacow, Kilkenny, via Waterford	Waterford
73	30 Woodview Grove, Blanchardstown	Dublin 15	181	Rose Cottage, D'Loughane, Youghal	Waterford
75	19 Upper Beechwood Avenue, Ranelagh	Dublin 6	182	Kilnafarna, Dungarvan	Waterford
76	27 Annadale Crescent, Drumcondra	Dublin 9	183	Ballycurrane North, Clashmore	Waterford
77	8 The Haven, Mobhi Boithrin, Glasnevin	Dublin 9	184	Coolishael Ballyduff Upper	Waterford
78	Ballynew, Moyard	Galway	185	4 Davis Street, Dungarvan	Waterford
81	Derrylough, Renvyle	Galway	187	Holly Cottage, Bunowen, Glasson, Athlone	Westmeath
82	Doonwood Mountbellew, Ballinasloe	Galway	188	Weston, 38 Elton Park, Sandycove, Dublin	Wexford

83	Doirebhanbh, Rosmuc	Galway	189	8 Granville Road, Blackrock, Dublin	Wexford
84	Derryrush, Rosmuc	Galway	190	Carrigbeg House Carrigbeg, Gorey	Wexford
85	Seana Mhoinin, Spideal	Galway	191	Oldross, Clonroche	Wexford
86	Carrowmore, Oughterard	Galway	192	Castlepaliser, Carne	Wexford
87	Derrymoyle, Oughterard	Galway	193	Churchtown, Fethard on Sea, New Ross	Wexford
88	Clonmore, Rosschail	Galway	194	6 The Court Clonattin Village, Gorey	Wexford
89	Fermoyle East, Barna	Galway	195	Lissadell Little Newtown, Enniskerry	Wicklow
90	Drumharsna South, Ardrahan	Galway	199	Tomriland, Annamore, Roundwood	Wicklow
91	Reasgarriff, Ardrahan	Galway	200	Idiro Technology, 34 Clarendon St. Dublin 2	Wicklow
92	Glinsk, Castlerea, co Roscommon	Galway	201	34 Landsdowne Park, Templeogue, Dublin 16	Wicklow
96	Maam West, Maam	Galway	202	Shanakiel House, Drumnagran, Tullyvin	Cavan
97	Canuig Mastergeehy, Cahersiveen	Kerry	203	Carnakib, Headford	Galway
98	Moybella, Lisselton	Kerry	204	Ballywilliam, Rathkeale	Limerick
99	Guhard, Lisselton, Lisotowel	Kerry	205	The Duffery Cottage, Hacketstown	Carlow
100	Gortnaminch, Listowel	Kerry	206	Old Road, Old Kildine	Limerick
101	Bawn Luskaha, College Rd, Castleisland	Kerry	207	Riverstown Kilmessan	Meath
102	Glencuttane, Kilgobnet, Beaufort	Kerry	208	Ballylusk, Ballyfin, Portlaois	Laois
103	Gortback, Ballyseedy, Tralee	Kerry	209	Laois	Laois
104	Carnahone, Beaufort, Killarney	Kerry	210	Feevaghmore, Dysart, Ballinasloe	Roscommon

Table 2.2: Table shows locations of all bee samples received for the survey
Each participant was chosen at random according to section 2.5.2.

2.8 Analysis of the presence of *Nosema* in Irish honeybee colonies

2.8.1 Checking for *Nosema* using light microscopy

A sample size of approximately 30 bees was used to check for the presence of *Nosema* in each sample received for each sampling set. The bees were crushed in 30mls sterile water and 10µl of the homogenate was placed on a haemocytometer for examination under the light microscope. The sample was diluted 1 in 10 if needed. The spores of *Nosema* are clearly visible under light microscope (Figure 2.7).

<i>Nosema</i> spp.	Primer Sequence	Amplicon length (bp)	Reference
<i>Nosema Apis</i>	5' - GGGGGCATGTCTTTGACGTACTATGTA - 3'	218	(R. Martín-Hernández <i>et al.</i> , 2007)
	5' - GGGGGGCGTTTAAAATGTGAAACAACACTATG - 3'		
<i>Nosema ceranae</i>	5' - CGGCGACGATGTGATATGAAAATATTAA - 3'	321	(R. Martín-Hernández <i>et al.</i> , 2007)
	5' - CCCGGTCATTCTCAAACAAAAAACCG - 3'		

Table 2.3: Species specific primers used for detection of *Nosema* species present in the positive samples.

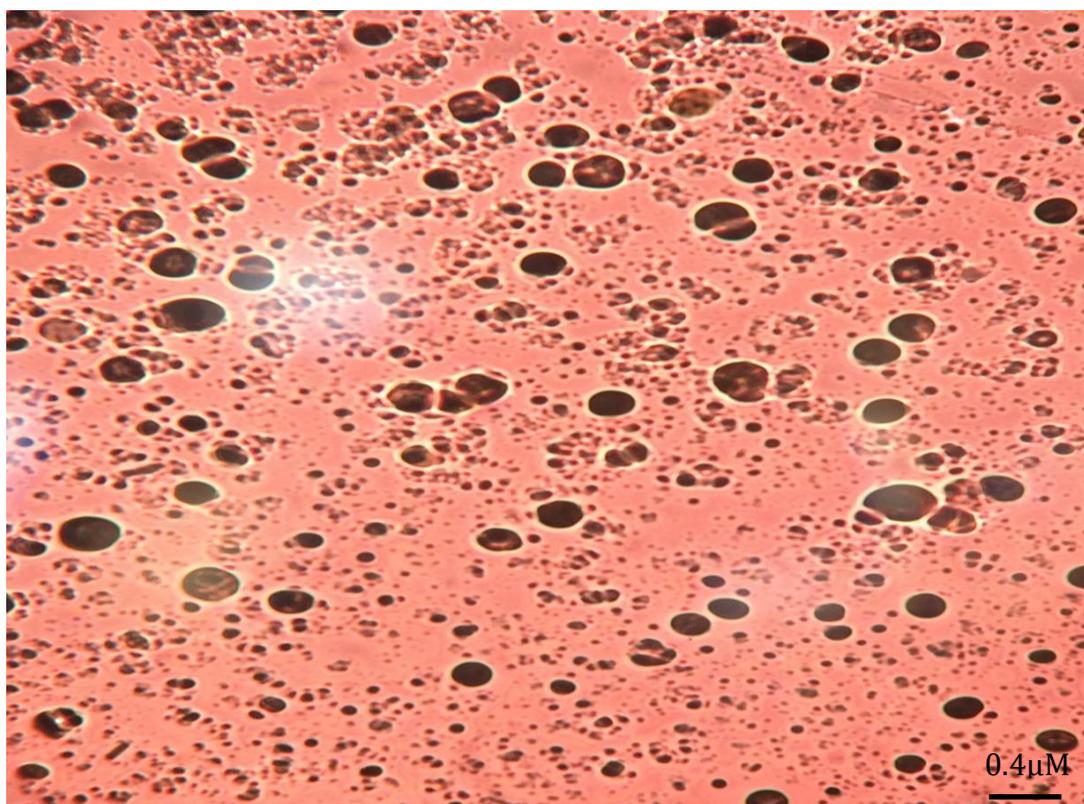


Figure 2.7: *Nosema* spores under the light microscope.
 The spores are the dark circles visible under 40x magnification, which were isolated from a sample of adult bees according to section 2.6.1.

2.8.2 DNA extraction and PCR reaction conditions for determination of *Nosema* species

Forty-one of the samples were determined to be positive for the microsporidian *Nosema* out of 381. Each sample was crushed with a mortar and pestle in the presence of liquid nitrogen and homogenised in Diethylpyrocarbonate treated water. An aliquot of 100µl liquid homogenate was transferred to an Eppendorf tube and centrifuged at 16,100 g for 5 min. The supernatant was discarded followed by repeated freeze-thaw cycles of the pellet in liquid nitrogen. The pellet was resuspended in lysis buffer and RNase, and incubated at 37°C for 20minutes. DNA was extracted using the DNeasy Plant mini kit (Qiagen), according to the manufacturer's instructions. DNA was quantified and the purity checked using a Nanodrop 1000 spectrophotometer (Thermo Scientific). DNA for each sample was used for uniplex PCR reactions using primers specific to *Nosema apis* and *Nosema ceranae*. Primers were dissolved in molecular grade water to give a stock solution of 100 µM initially, which was diluted to a working solution of 10 µM (See Table 2.3 for primer sequences). Five microliters of DNA was added to MyTaq (Bioline) PCR master mix, 10µl nuclease free H₂O, 1µl 10µM Forward primer, 1µl 10µM reverse primer and 0.2µl Mytaq Taq polymerase (Bioline) in a 200µl nuclease free PCR tube and mixed. PCR reaction was carried out in a Techne Flexigene thermocycler (Figure 2.7) by initial denaturation at 95°C 5 minutes, followed by 35 cycles of 95°C for 30seconds, annealing at 62°C for 30seconds, extension at 72°C for 30seconds, followed by a final 5 minute extension at 72°C. Each trial also included a positive control; 5µl of a *Nosema* standard received from ANSES Sophia Antipolis laboratory, and a negative control, which contained water instead of cDNA. A positive band was only considered to be a true positive if the positive standard also showed as positive and if the negative sample remained blank.

2.8.3 Visualization of PCR product

PCR product (8µL) was mixed with 2µl of Blue Orange 6X loading dye (Promega) and loaded onto a 1.2% agarose gel, which contained Ethidium bromide at a concentration of 4%. Gels were ran at 70volts in a BioRad SubCell GT rig using a BioRad powerpac300 until the dye had reached

approximately half the length of the gel. Gels were visualized under UV light using Genesnap software (SYNGENE) to examine for the presence of bands, and the size of any bands present were referenced to the lane containing Hyperladder (Bioline).

2.9 Analysis for the presence of the Tracheal mite, *Acarapis woodi*, in Irish *A. mellifera* colonies

2.9.1 Method for examination for the presence of Tracheal mites in *A. mellifera* samples

To assess samples for the presence or absence of *A. woodi*, 30 bees were individually tested by removing the head, forelegs and collar to expose the trachea. A positive diagnosis was noted if the tracheae had lost the smooth white or fleshy appearance and showed any signs of bronzing, black specks or streaks in either tracheal trunk. This work was carried out by Dr. Mary Coffey as part of collaboration on a national disease survey.

2.10 Determination of the occurrence and prevalence of four honeybee viruses in *A. mellifera* colonies in Ireland

2.10.1 Preparation of RNase free buffers and equipment

All glassware and all pestles and mortars were baked at 220°C for 12 hours prior to use to reduce contamination by RNases. Diethyl pyrocarbonate (DEPC) is a strong inhibitor of RNases and was used at a concentration of 0.1% (v/v) to treat water, which was left stirring overnight followed by incubation at 37°C for a minimum of 4 hours prior to sterilization by autoclaving. This DEPC-treated water was used in the preparation of all buffers needed for RNA extraction. All bottle lids, O-rings and magnetic stirrers were soaked overnight in DEPC water and autoclaved before use. All chemicals were weighed without the use of a spatula. Gloves were worn at all times and changed regularly. Pipette tips and tubes were taken from freshly opened bags and autoclaved twice prior to use. Inside of laminar flow cabinets

and all pipettes, eppendorf racks etc that were used were cleaned with RNase Zap (Ambion) before use.

2.8.2 RNA extraction for *A. mellifera* samples

2.8.2(a) Sample Set 1 and 2 (Spring 2014 and Summer 2014)

Approximately 30 bees were placed in a nuclease free pestle and mortar and crushed into a fine powder using liquid nitrogen. To this, 5 mL TRI[®]-Reagent was added, mixed thoroughly, and left to defrost at room temperature. Four mls of the homogenate was collected and placed in sterile 1.5 mL eppendorf tubes and centrifuged at 12,000 *g* for 10 minutes. The pellets were discarded and the supernatants collected in a new tube. Chloroform (200 μ L-molecular grade) was added and mixed vigorously by vortexing. The solution was allowed to stand at room temperature for 10 minutes and centrifuged at 12,000 *g* for 10 minutes at 4°C. The top clear layer was collected and placed in a new tube and 500 μ L of 2-propanol (molecular grade) was added. The tube was inverted several times, left to stand for 10 minutes and centrifuged at 12,000 *g* for 10 minutes at 4°C. The supernatant was discarded and the resulting pellet was washed in 75% (v/v) ethanol (50 μ L, molecular grade) and centrifuged at 12,000 *g* for 10 minutes at 4°C. Supernatant was removed completely and the resulting pellet was air-dried and re-suspended in RNase-free water, aliquoted and stored at -70°C or used immediately.

2.8.2(b) Sample Set 3 (Autumn 2014)

A larger samples size of approximately 60 bees was taken for the third sample set in Autumn 2014 to increase the chance of detecting the less prevalent viruses. Bees were crushed in sterile pestle and mortars as before, in the presence of liquid nitrogen, and homogenised in 10 mM Tris-400 mM NaCl buffer (pH 7.5). Samples were spun at 5000 *g* for 15mins, after which an aliquot of 400 μ l was removed to a nuclease free eppendorf and mixed with

400µl of lysis buffer from NucleoSpin[®] RNA extraction kit (Macherey-Nagel). Samples were passed through a sterile 23g needle approximately 20 times to ensure complete homogenization of the sample, in order to prevent blockage of the Nucleospin filter during the first spin step of the RNA extraction kit method. The remaining steps of the RNA extraction were then carried out according to manufacturer's instructions, taking care to keep all equipment sterile and regularly changing gloves to avoid contamination. RNA was suspended in nuclease free water once isolated and either carried forward for immediate cDNA synthesis or frozen at -70⁰C until future use.

2.9.3 RNA gel electrophoresis method and preparation

Prior to use, the gel rig and tank were washed with 0.5% (w/v) SDS, rinsed with DEPC-treated water followed by ethanol and allowed to air dry. A 1% (w/v) agarose gel was prepared (1g agarose, 10 ml 10X FA buffer (200 mM 3-[N-morpholino]propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7) and DEPC water to make 100 mL). The mixture was heated until dissolved and allowed to cool to hand-hot. One point eight millilitres of 37% (v/v) formaldehyde plus 1 µL of a 10-mg/mL ethidium bromide solution was added prior to pouring the gel. Prior to running, the gel was equilibrated in 1X FA running buffer for at least 30 minutes. RNA (4 µL) was added to 4 µL of 5X RNA gel loading dye; stock: 500 mM EDTA (80 µL, pH 8), 37% (v/v) formaldehyde (720 µL), glycerol (2 mL), formamide (3.084 mL), 10X FA buffer (4 mL) and 16 µL saturated aqueous bromophenol blue solution), were heated to 65°C for 5 minutes and chilled on ice. Samples were loaded into the wells of the gel and run at 6 V/cm in 1X FA buffer. The gel was visualised using UV light (Figure 2.8).

Virus	Primer Sequence	Amplicon length (bp)	Reference
DWV	5' - GGTCCGCGGCTAAGATTGTA - '3	420	(Cox-foster <i>et.</i> , 2007)
	5' - CGGCTGTTTGATGGAAGAAGTT - '3		
CBPV	5'-TCAGACACCGAATCTGATTATTG-3'	570	(Blanchard <i>et.</i> , 2008)
	5'-ACTACTAGAAACTCGTCGCTTCG-3'		
ABPV	5' - CATATTGGCGAGCCACTATG - '3	398	(Cox-foster <i>et.</i> , 2007)
	5' - CCACTTCCACACAACACTATCG - '3		
IAPV	5' - CGATGAACAACGGAAGGTTT - '3	767	(Cox-foster <i>et.</i> , 2007)
	5' - ATCGGCTAAGGGGTTTGT - '3		

Table 2.4: Primers used for the uniplex PCR reactions to detect for the four honeybee viruses in all of the samples.

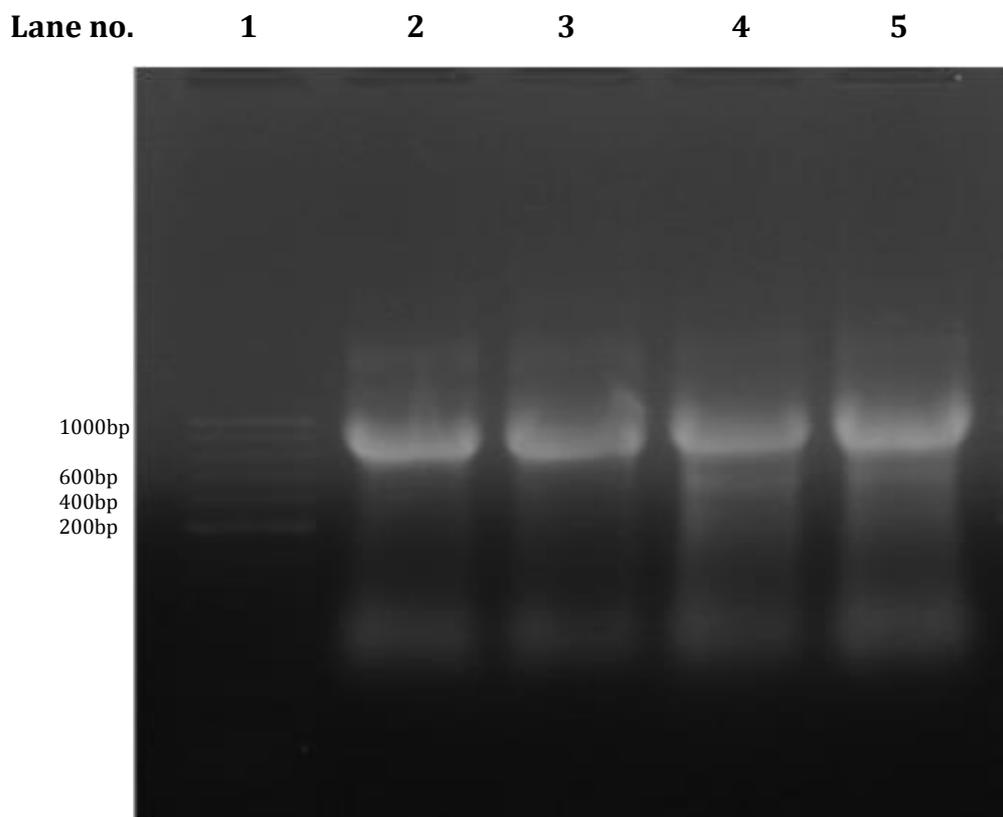


Figure 2.8: RNA gel image under UV light.
RNA gel showing electrophoresis results of four lanes of total RNA extracted from four *A. mellifera* samples to check for the quality of RNA.

2.9.4 cDNA synthesis from total extracted RNA

cDNA was synthesised using the Superscript III First-Strand Synthesis System for RT-PCR kit from Invitrogen (CA, USA). RNA concentration was determined and all samples contained equal amounts of RNA prior to cDNA synthesis. RNA was added to an RNase and DNase-free tube and RNase-free water was added to bring the volume up to 8 μ L. To this, 1 μ L of 10 mM dNTP mix (provided) and 1 μ L of 50 μ M oligo (dT) (provided) were added. The solution was incubated at 65°C for 5 minutes, and placed on ice for at least 1 minute. A master mix was prepared (Per reaction: 2 μ L 10X RT buffer (supplied), 4 μ L 25 mM MgCl₂, 2 μ L 0.1 M DTT, 1 μ L RNaseOUT (supplied) according to the number of reactions needed. Master mix (9 μ L) was added to each of the RNA/primer mixes, mixed gently, incubated at 42°C for 2 minutes and held on ice. One microlitre of SuperScript III RT (200 U/ μ L, supplied) was added to each reaction tube and incubated as follows: 42°C for 50 minutes, 70°C for 5 minutes and held on ice. One microlitre of RNaseH (supplied) was added to each tube and further incubated at 37°C for 20 minutes. cDNA was aliquoted and stored at -20°C.

2.9.5 PCR protocol and product visualization for the four bee viruses

PCR amplification was carried out using four separate reactions for each sample, for each of the four viruses: Deformed Wing Virus (DWV), Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV), and Israeli Acute Paralysis Virus (IAPV). The Primers were dissolved in molecular grade water to give a stock solution of 100 μ M initially, which was diluted to a working solution of 10 μ M (See table 2.4 for primer sequences). Five microliters of cDNA was added to MyTaq (Bioline) PCR master mix, 10 μ l nuclease free H₂O, 1 μ l 10 μ M Forward primer, 1 μ l 10 μ M reverse primer and 0.2 μ l Mytaq Taq polymerase (Bioline) in a 200 μ l nuclease free PCR tube and mixed. PCR reaction was carried out in a Techne Flexigene thermocycler by initial denaturation at 95°C 5 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds, followed by a final 5 minute extension at 72°C. The positive control used consisted of 5 μ l of a viral standard at a concentration of 10⁻⁶, received from

ANSES Sophia Antipolis laboratory, and a negative control, which contained water instead of cDNA. A positive band was only considered to be a true positive if the positive standard also showed as positive and if the negative sample remained blank.

2.10 Protein extraction for gel electrophoresis

2.9.1 Protein extraction from *A. mellifera* adults and pupae

Three adult/pupal stage *A. mellifera*, depending on experiment, were crushed in a sterile pestle and mortar to a fine powder using liquid nitrogen. Care was taken before hand to ensure the samples chosen were of the same age and the same level of parasitization. Samples were resuspended in 3mls of ice cold lysis buffer (20mM PIPES, 5mM NaCl, 0.2% Triton X 100, pH 7.2) and a protease inhibitor cocktail (Pepstatin A (10µg/ml), Aprotinin (10µg/ml), Leupeptin (10µg/ml), Tosyl-lysylchloromethane (10µg/ml), and left mixing slowly at 4°C for two hours to achieve maximum lysis of all remaining cellular structures and breakdown of the large storage proteins that are present in the pupal *A. mellifera*. The samples were spun at 9000 g for 5 minutes to pellet cellular debris, and the supernatants removed to fresh tubes. For the pupal *A. mellifera* samples, the samples were allowed to rise to room temperature before centrifugation. The spin step at 9000 g for 5 minutes was carried out at room temperature also. This allows the large amount of lipids present in the young *A. mellifera* to rise to the top of the supernatant, allowing it to be removed using a fine, sterile spatula. Protein samples were then diluted 1 in 10 in sterile PBS, and kept on ice until quantification by Bradford assay (2.9.4). Proteins samples were quantified to a concentration of 1µg/1µl for 1 dimensional electrophoresis and placed in solubilisation buffer or acetone precipitated according to section 2.9.5 to a concentration of 400µg for 2-Dimensional electrophoresis.

2.9.2 Protein extraction from *A. mellifera* pupal stage haemolymph

Haemolymph was extracted from six purple eye stage *A. mellifera* pupae per sample set. Haemolymph was removed using a microcapillary tube inserted laterally along the abdominal section of the bee. Approximately 2-3µl of haemolymph was obtained from each pupa and pooled together in 50µl of ice cold PBS per sample set. Once all six pupae were bled per sample set, the samples were centrifuged at 4°C and 8000g for 5 minutes, brakes off, to pellet haemocytes and other cellular debris. Supernatant was removed to a fresh tube. A further 100µl of ice cold PBS was added per sample, and set aside on ice for quantification by Bradford assay.

2.9.3 Protein extraction from *V. destructor*

V. destructor adult female mites (0.04 grams) were used per sample set, from colonies with a known level of resistance to Bayvarol. Samples were collected following Autumn treatment from the sticky inserts on the floor of the hive (Figure 2.8). *Varroa* were isolated from the inserts using a fine tip tweezers and frozen until further use. *Varroa* were crushed in a miniature pestle and mortar using liquid nitrogen to a fine powder, before resuspension in lysis buffer containing a cocktail of protease inhibitors (see section 2.10.1). Samples were left mixing at 4° C for two hours, before centrifugation at 9000 g for 5 minutes to pellet cellular debris. Supernatant was then removed to a fresh tube, and kept on ice until quantification by Bradford assay. Protein samples were quantified to a concentration of 1µg/1µl for 1-Dimensional electrophoresis and placed in solubilisation buffer or acetone precipitated according to section 2.10.5 to a concentration of 400µg for 2-Dimensional electrophoresis.

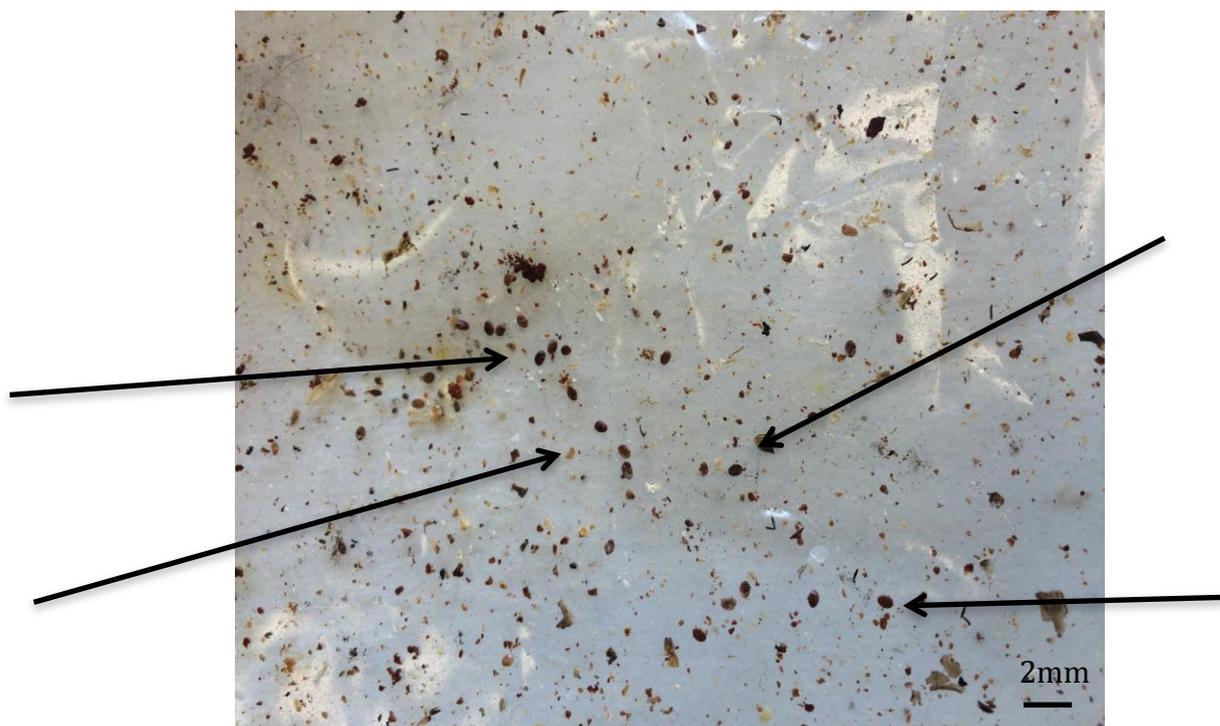


Figure 2.8: Example of a sticky insert from the floor of a hive for the collection of *Varroa* mites.

The mites are indicated by the arrows, and were collected using a fine tip tweezers.

2.9.4 Bradford assay for protein quantification

Sterile PBS was used to make a serial dilution of bovine serum albumin and this was used to make a range of standards (0.05-1.5mg/mL). All samples used were diluted in sterile PBS. Twenty μL of sample were placed in a 1mL cuvette. To this 980 μL of Biorad Bradford protein assay reagent (Bio-Rad, Hercules, California, USA) was added. Samples were inverted to mix and then allowed to incubate for 5 minutes at room temperature before being read in an Eppendorf Bio-photometer. The quantity of protein was based on the OD590nm readings of the standards as in Figure 2.9.

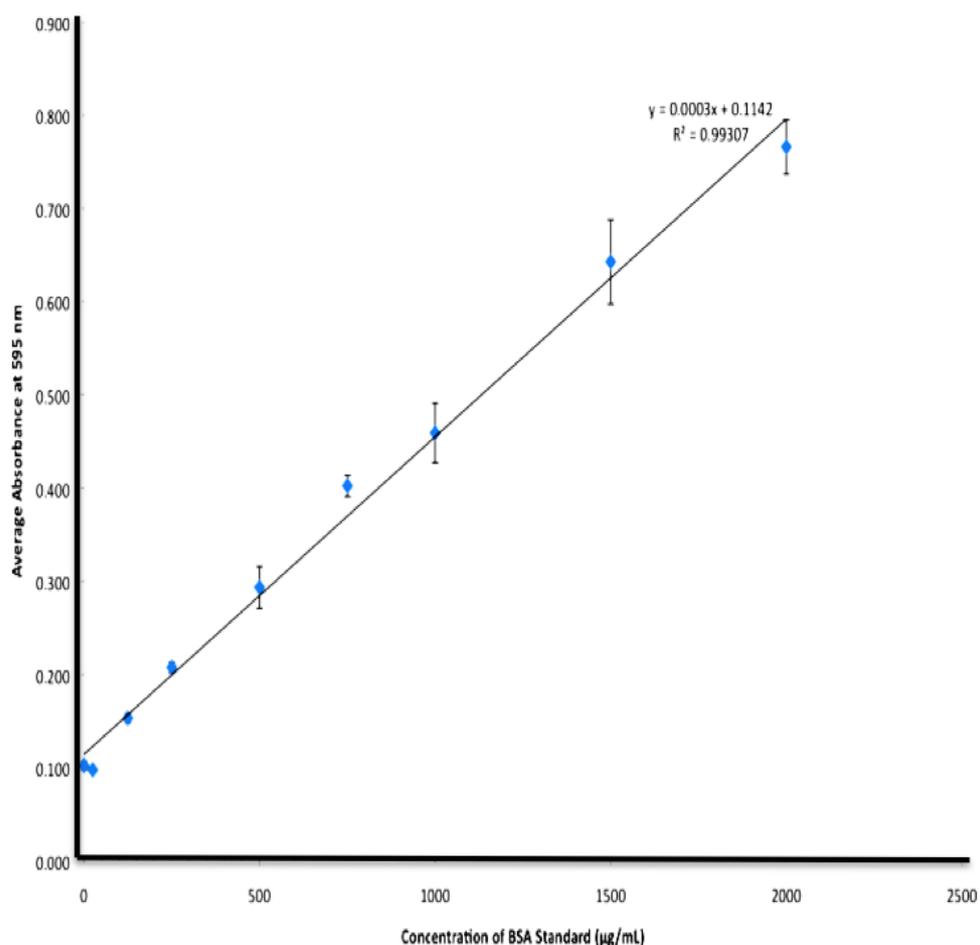


Figure 2.9: Standard curve of Bovine serum albumin used to determine unknown protein concentrations

2.9.5 Acetone precipitation of protein samples

Acetone precipitation was used to concentrate protein from a dilute sample volume and also to help purify. The required volume of protein which corresponded to a calculated quantity of protein from Bradford assay quantification (section 2.10.4) was aliquoted to a pre-chilled eppendorf tube and 100% ice-cold acetone was added to the tube at a ratio of 1:3 (sample volume: 100% Acetone). Protein was left to precipitate overnight at -20°C and centrifuged at 13000g for 10 minutes to pellet protein. All pellets were placed upside down to air dry for 5 minutes following removal of acetone.

2.10 1-Dimensional and 2-Dimensional SDS PAGE gel electrophoresis

2.10.1 0.5M Tris-HCl

Tris-HCl (1.5 M) was prepared by dissolving 36.3 g Trizma Base, (Tris Base) in 170ml deionised water and adjusted to pH 8.9 using HCl. Following pH adjustment, 1.5 M Tris – HCl was filter sterilised through a 0.22 µm cellulose filter (Millipore) and stored at 4°C.

2.10.2 1.5M Tris-HCl

Tris-HCl (0.5 M) was prepared by dissolving 12.1 g Trizma Base in 175 ml deionised water and adjusted to pH 6.8 using HCl. Following pH adjustment 0.5 M Tris – HCl was filter sterilised through a 0.22 µm filter (Millipore) and stored at 4°C

2.10.3 10% Ammonium Persulphate

APS (10% w/v) was prepared by adding 0.1 g APS placed into microcentrifuge tube and filled up to 1ml with deionised water and vortexed briefly to achieve solubility. APS (10% w/v) stocks were frozen.

2.10.4 10x running buffer for electrophoresis

Running buffer (10X), (electrode buffer), was prepared by adding Tris Base 30 g/l, Glycine 144 g/l and SDS 10 g/l to distilled water filled up to 1000ml mark and the mixture was stirred until the solution was solubilised. Electrode running buffer (10X) stock was diluted to 1X concentration by making 1/10 dilution with distilled water when required.

2.10.5 10% Sodium Dodecyl Sulphate

Sodium Dodecyl Sulphate (SDS), (10% w/v) was prepared by placing 5 g SDS into a 50ml falcon and filling up to 50 ml mark with deionised water and vortexed until all SDS had been solubilised. The solution was stored at room temperature.

2.10.6 5X Solubilisation Buffer for 1-Dimensional gel electrophoresis

Solubilisation buffer was prepared by dissolving the following constituents:

- Glycerol 8ml
- Deionised water 4ml
- 10% (w/v) SDS 1.6ml
- 0.5 M Tris – HCl 1ml
- Bromophenol Blue (0.5% w/v) 200 µl
- 2 – Mercaptoethanol 400 µl

The buffer was gently mixed for 1 hour at 4° C and aliquoted in 500 µl volumes prior to storage at -20° C.

2.10.7 Preparation of SDS-PAGE mini gels

All glass plates were washed in warm soapy water and cleaned thoroughly with 70% (v/v) ethanol prior to use. SDS–PAGE minigels (cast using the Mini- Protean II gel casting apparatus), were made of acrylamide with 12.5% Bis-acrylamide in all experiments.

Separating gel composition

12.5% Bis-Acrylamide solution

- 1.5 M Tris-HCl (pH 8.9) 3ml
- Deionised water 3.8ml
- 30% Bis-Acrylamide 5ml
- 10% v/v SDS 120µl
- 10% v/v APS 75µl
- TEMED 3µl

These volumes were sufficient to make 3 minigels and volumes were adjusted accordingly where a larger volume was required.

Stacking gel composition

The following components were mixed together and applied on top of the separating gel;

- Deionised water 3.4ml
- 30% Bis-Acrylamide 830µl
- 0.5 M Tris–HCl (pH 6.8) 630µl
- 10% v/v SDS 50µl
- 10% v/v APS 50µl
- TEMED 5µl

Combs were placed in the gel matrix before it set to create wells for sample loading. These volumes were sufficient to make 2 minigels and volumes were adjusted accordingly where a larger volume was required.

2.10.8 Electrophoresis of 1-Dimensional minigels

SDS-PAGE gels (12.5% Acrylamide) were immersed in 1X running buffer. Samples at a concentration of 20 μ g/20 μ l were loaded to each lane using a sterile tip. The gels were electrophoresed at 40V initially and the voltage was increased to 60 V once the protein had travelled sufficiently through the stacking gel. Once the blue tracking dye had moved to the bottom of the gel, the gels were transferred to a clean staining dish.

2.11 2-Dimensional Isoelectric focussing and SDS-PAGE electrophoresis preparation and execution

2.11.1 Isoelectric Focussing Buffer (IEF)

IEF was prepared by dissolving the following constituents:

- Urea 8M
- Triton X-100 1% (v/v)
- CHAPS 4% (w/v)
- Tris HCL 10mM
- Thiourea 2nM

DTT, 0.2 μ g per ml of IEF and 2 μ l of pH 4-7 ampholytes were added per sample prior to use

2.11.2 Equilibration Buffer

Equilibration buffer was prepared by mixing the following constituents:

- Tris Base 50nM
- Urea 6M
- SDS 2% (w/v)

- Glycerol 30% (v/v)

The solution was adjusted to pH 6.8 and aliquoted in 40 ml volumes prior to storage at -20⁰ C. For equilibration the buffer was modified as either reducing or alkylating. For reduction, DTT (0.01 g/ml) was added and dissolved thoroughly. For alkylation, IAA (0.025 g/ml) was dissolved thoroughly in the buffer.

2.11.3 Agarose sealing solution

- Agarose 1% (w/v)
- 1X running Buffer 100ml
- Bromophenol blue 0.5% (w/v)

Components were mixed together and microwaved until molten, allowing to cool to hand warm before using on gels.

2.11.4 Gel preparation for 2-Dimensional electrophoresis

Glass plates were washed thoroughly with warm soapy water, rinsed and dried with 70% (v/v) ethanol to remove any residual contamination left on the glass. Front glass plates were 200 mm wide and were 200 mm in length, and 223 mm in length at the back. Gels (12.5 % acrylamide) were poured to approximately 190 mm x 160 mm in surface area and 1.5mm thick.

- Tris – HCl (1.5 M) 60ml
- Deionised water 76ml
- Bis-Acrylamide (30% w/v) 100ml
- SDS (10% w/v) 2.4ml
- APS (10% w/v) 1.5ml
- TEMED 0.5ml

2.11.5 2-Dimensional gel electrophoresis

Protein samples (extracted as described in section 2.9.2 and 2.9.3) were acetone precipitated (as per section 2.10.5), spun down at 13000 g for

10mins and air dried in preparation for isoelectric focussing. Pellets were resuspended in 100µl of IEF buffer (containing 0.2µg DTT per ml of IEF, and 2µl of pH 4-7 ampholytes per sample) and allowed to resuspend fully by using both vigorous vortexing and sonication in the water bath for 5 minutes. A further 50µl of IEF, coloured blue using a few grains of bromophenol blue, was added per sample. The sample was briefly vortexed and applied to a 13cm Immobline DryStrip pH 4-7 (G.E. Healthcare). Prior to isoelectric focusing the strips were covered in Plus One drystrip cover fluid, (GE Healthcare). Focussing was performed on an Ettan IPGphor II (Amersham Biosciences, NJ, USA) (Figure 2.10) system using the following programme:

- | | | |
|--------------|---------------|------------|
| 1. 50volts | Step and Hold | 10 hours |
| 2. 250volts | Step and Hold | 15 minutes |
| 3. 8000volts | Gradient | 5 hours |
| 4. 8000volts | Step and Hold | 8 hours |

The second dimension of protein separation was achieved by placing the gels in a Protean Plus™ Dodeca™ Cell gel rig with horizontal electrophoresis cells. The chamber was filled to mid-way on the top grey glass plate spacer of all the gel glass plates with 1X electrode running buffer to ensure the buffer covered the acrylamide gel level for proper running. Gels were initially electrophoresed for 1 hour at 1 Watt per gel, and then increased to 1.5 Watts per gel if left overnight provided the gel rig coolant system was kept below 10°C to prevent faster protein migration. The following day gels were increased from 1.5-2.5Watts per gel depending on how far and fast the blue tracking dye line migrated. Gels were monitored at regular intervals to assess the level of electrophoresis and once complete were transferred to a staining dish which was previously washed with soap and 70% ethanol to prevent any contamination to the gels during staining.

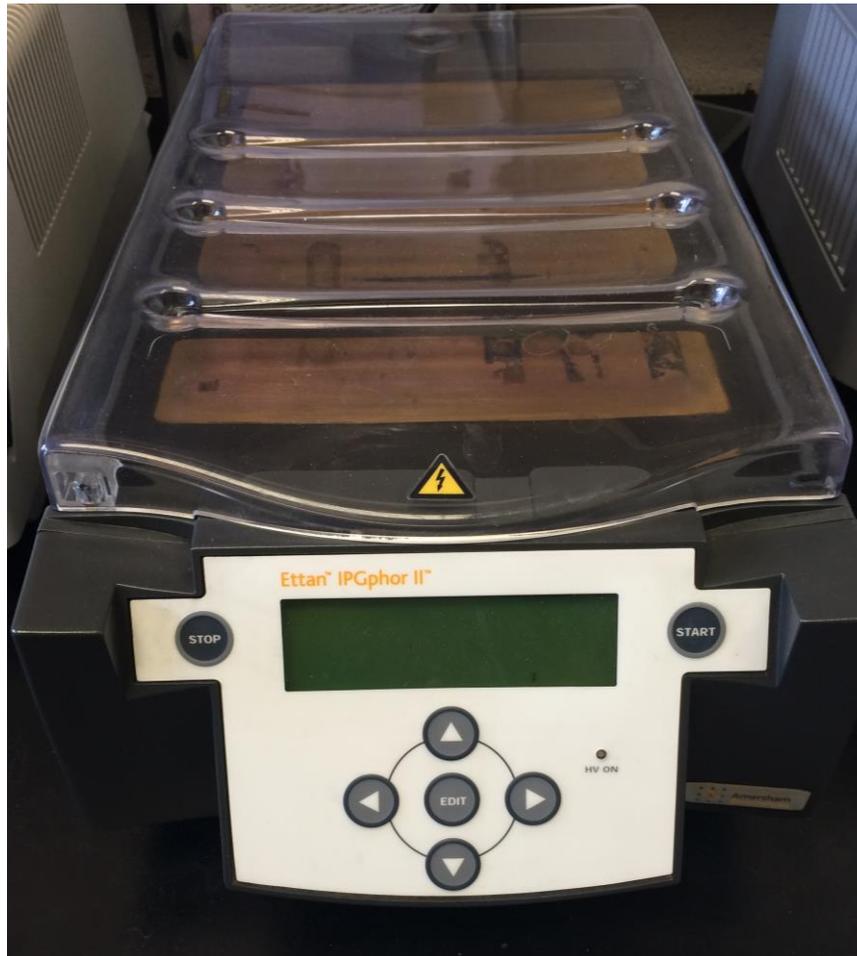


Figure 2.10 Isoelectric focussing machine used for all first dimension separation during 2-Dimensional SDS-PAGE gel electrophoresis

2.12 Staining of gels and analysis of differential expression of proteins

2.12.1 Colloidal Coomassie staining of gels

Gels were placed in fixing solution directly after electrophoresis, and remained fixing for at least 4 hours in a 13cm petri dish. Following fixing, the gels were rinsed in ddH₂O and left to hydrate for an hour until the gels had returned to their original size. Water was removed and Colloidal stain solution added to the gels. To this, 0.1g of Serva blue was added per gel. Gels were left to stain for between 2-5 days, depending on the size. All stain was rinsed off before scanning.

Colloidal Coomassie Fixing Solution:

- Ethanol 50% (v/v)
- Phosphoric acid 3% (v/v)
- Distilled water 47% (v/v)

Colloidal Coomassie Stain Solution:

- Methanol 34% (v/v)
- Phosphoric acid 3% (v/v)
- Ammonium Sulphate 17% (w/v)
- SERVA Blue G (SERVA) 0.1 g
- Distilled water 4% (v/v)

2.12.2 Image J densitometrical analysis of 1-Dimensional Gels

Image J software was used to identify differential expression of bands in 1-Dimensional gels. All gels were carried out on at least three separate occasions, densitometrical values for bands showing differential expression were the average of all replicates achieved using the Image J programme.

2.12.3 Progenesis SameSpots software for analysis of 2-Dimensional Gels

2-D electrophoresis gel images (in triplicate) were analysed using Progenesis SameSpots software (Nonlinear Dynamics) in order to assess the fold change in protein expression in larvae subjected to different treatments. The level of differential expression was analysed by ANOVA with p-values of ≤ 0.05 considered statistically significant for changes in expression.

2.13 Identification of proteins from SDS-PAGE gels

2.13.1 Preparation for spot/band excision

All micro-centrifuge tubes were fresh from the bag or placed in a sealed container and autoclaved before use. Tips were autoclaved and cut to varied lengths for spot cutting and subsequently tips and all other utensils in contact with the gel, scalpels, and blunt needles were soaked in acetonitrile prior to use

to eliminate keratin contamination.

2.13.2 Trypsin Digest of peptides from 1-Dimension and 2-Dimensional SDS-PAGE gels

Processing of bands and spots for LC/MS analysis was achieved by following the method of Shevchenko *et al.*, (2006). Gel pieces were cut and transferred to sterile micro-centrifuge tubes and gel pieces were cut if too large (approximately 1-2 mm) using a scalpel when required to ensure full saturation of gel piece for de-staining and digestion process. Gel pieces were de-stained by addition of 100 μ l of 100 mM Ammonium bicarbonate: Acetonitrile 1:1 ratio and subsequent vortexing every 10- 15min (3x).

Acetonitrile (500 μ l) was added to dehydrate and shrink gel pieces to the point they became white. Acetonitrile was removed and the samples were stored at -20° C or processed further immediately. Tryptic digestion was achieved with the addition of approximately 20-30 μ l of trypsin buffer (~10 ng/ μ l, trypsin enzyme prepared in trypsin reconstitution buffer, 10 mM ammonium bicarbonate, 10% (v/v) acetonitrile). Samples were placed at 4° C to prevent trypsin auto-digestion and allow for the penetration of trypsin buffer into the gel piece. Gel pieces were checked after 30 minutes to ensure they were sufficiently covered or if more trypsin buffer was required. Samples were rechecked after a further 30 min for adequate coverage of gel pieces. If required gel pieces were topped up with 10 mM ammonium bicarbonate/10% (v/v) acetonitrile to prevent drying out. The samples were transferred to the 37° C orbital incubator overnight and set to 160-180 revolutions per minute.

Digested samples were centrifuged on a desk top Tomy centrifuge and the supernatant was transferred to clean micro-centrifuge tube. For a double extraction the original gel piece was extracted further with 5% (v/v) Formic Acid in HPLC grade water. Samples were incubated at 37° C in the orbital shaker and all supernatant was transferred to the tryptic digest supernatant. The samples were lyophilised in a vacuum centrifuge and stored at -20° C or immediately re-suspended in 20 μ l 0.1% formic acid and sonicated. All samples were filtered through 0.22 μ m cellulose acetate spin filter tubes (Spin-X®,

Costar) and transferred to mass spectrometry vials. Analysis of digested peptides was achieved using an Agilent 6340 Ion Trap LC/MS using acetonitrile elution.

2.13.3 Bioinformatic analysis of peptide identification results

The fragmented protein samples were eluted by LC/MS (Agilent 6340 Ion Trap) which determines the relative charge to mass ratio from detected ionized particles. These data were analysed using the mascot search engine to identify the protein (www.matrixscience.com) or via Spectrum Mill MS Proteomics. MASCOT scores above 67 were deemed to have a significant match ($p < 0.05$). The mass error tolerance was 1 Da allowing for a maximum of no more than two missed cleavages. Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov) websites.

2.14 Protein Methodology for shotgun label free proteomics

2.14.1 Protein extraction from *A. mellifera* worker and drone pupae for label free shotgun proteomics

A single pupa was taken per replicate, with the eye and body colour used to estimate the age of the pupae as closely as possible. Only pupae that had an identical level of parasitization were used, i.e. the same number of adult female *Varroa* plus the same number of offspring. At least four replicates were carried out per sample set. Pupae were placed into a sterile eppendorf tube and 500 μ l of buffer added (7M Urea, 2M Thiourea, 1 protease inhibitor cocktail tablet). Using a pellet pestle, the pupae were homogenised to a liquid consistency, resting on ice occasionally to avoid overheating. A further 500 μ l of buffer was added per sample. The tubes were spun at room temperature for 5 minutes at 9000g and the lipid layer removed from the top of the supernatant using a sterile, fine tip spatula. The supernatant was removed to a fresh eppendorf tube and kept on ice before being quantified. A

Bradford assay was carried out as described in section 2.9.4, and acetone precipitated (section 2.9.5) at a concentration of 300µg. Following overnight acetone precipitation, the samples were spun at 13000 g for 10 minutes and allowed to air dry upside down. Urea (8M) was added to each pellet, 100µl in total, and the samples vortexed for approximately 30 seconds. Samples were sonicated in a water bath for 5 minutes and the pellet examined. If the pellet, or pieces of the pellet, remained visible, the vortexing and sonicating was repeated until the sample was successfully resuspended. Requantification was carried out using a Qubit fluorometer (Figure 2.11) and the Qubit protein assay kit (Thermo scientific) to a concentration of 75µg, using urea (8M) to bring up to a volume of 100µl per sample. Protein samples were digested in solution overnight according to section 2.14.3.

2.14.2 Protein extraction from *V. destructor* mites for label free shotgun proteomics

A total of ten adult female mites were used per replicate, with four replicates in total. Mites were homogenized in a sterile eppendorf tube using a pellet pestle in 50µl of buffer (7M Urea, 2M Thiourea, 1 protease inhibitor cocktail tablet) and then a further 50µl of buffer was added per sample. The samples were quantified by Bradford analysis as described in 2.9.4, and acetone precipitated overnight at a concentration of 100µg (see section 2.9.5). Samples were spun for 10 minutes at 13000 g the following day, and allowed to air dry upside-down for 5 minutes. Urea (8M) was added to each sample each sample (100µl) and the pellet resuspended as described in section 2.14.1. Re-quantification was carried out using the Qubit Fluorometer and the protein assay kit and re-quantified to 75µg as in section 2.14.1. Protein samples were digested in solution overnight according to section 2.14.3.



Figure 2.11 Image shows the Qubit fluorometer used for quantification of proteins for label free proteomics

2.14.3 In solution digest protocol for overnight peptide digestion for label free proteomics

The list of buffers used for the in solution digest of proteins in preparation for label free proteomics are presented below. Buffers must be made fresh directly before use. Protease Max (Promega) must be used in order for the trypsin (Promega) to digest the protein when using Urea and Thiourea. All water was deionised and was taken fresh before use from the deionised dispenser.

200mM Ammonium Bicarbonate (AmBic)

- 0.394g Ammonium bicarbonate
- 25ml water

50mM Ambic

- 2.5ml 200mM AmBic

- 7.5ml water

0.5M DTT

- 0.077g DTT
- 1ml 50mM AmBic

0.55M IAA (Protect from light)

- 0.102g IAA
- 1ml 50mM AmBic

Ammonium bicarbonate (200mM) was added per sample (30µl) and mixed using pipette. DTT (0.5M) was added to each sample (1µL), following brief mix by vortexing, samples were placed at 56°C for 20 minutes. After the incubation period samples were cooled to room temperature, and 2.7µl of 0.55M IAA was added. Samples were incubated at room temperature for 15 minutes in the dark. Protease Max (1.3µl) and 1.8µl of Trypsin (Promega reconstituted in 40µl of trypsin reconstitution buffer to a concentration of 0.5µg/µl) were added to each sample. Samples were incubated at 37° C overnight for maximum peptide recovery. Samples were cleaned up according to section 2.11.4.

2.14.4 Sample clean-up prior to loading on Q-exactive

The list of buffers used for the sample clean-up for use on Q-exactive using C18 spin columns (Thermo Scientific) are listed below. Buffers were made fresh directly before use. All water was deionised and was taken fresh before use from the deionised dispenser. The C18 columns are designed to trap only 30µg of protein and so is the final step of re-quantification before loading on the Q-exactive OrbyTrap. Care was taken to ensure the resin did not reach any flow through, as shown in Figure 2.12.

Sample buffer (2% TFA, 20% Acetonitrile)

- 200µL Acetonitrile
- 20µl TFA
- 780µl water

Equilibration Buffer (0.5% TFA, 5% Acetonitrile)

- 4.3ml water
- 25µl TFA
- 250µl Acetonitrile

Wash buffer (Same as equilibration buffer)

Elution buffer (70% Acetonitrile, 30% water)

Activation buffer (50% Acetonitrile, 50% water)

Loading buffer (0.05% TFA, 2% Acetonitrile)

- Taken straight from the Q-exactive buffer reservoirs

Columns were tapped briefly to settle the resin, and the protective caps were removed from either end. Columns were placed in sterile 1.5 ml eppendorf tubes as shown in Figure 2.12. Resin was activated using 200µl of activation buffer, added to the top of the resin, and spun at 1500g for 1 minute. Flow through was discarded and the process repeated. Equilibration buffer (200µL) was added to the column, spun for 1 minute 1500 g and the flow through discarded, and repeated once more. Digested protein samples (following digestion according to section 2.11.3) were briefly spun in a microfuge to collect any condensate, straight from the 37° C incubator following overnight peptide digestion. TFA to a concentration of 0.75% of the total volume of sample was added (approximately 0.75µl), vortexed briefly, and incubated at room temperature for 5 minutes. The sample may appear cloudy at this stage but this is normal. Samples were then spun at 13000 g for 10 minutes to remove any debris that may have formed overnight, and the supernatant transferred to a fresh tube. Samples were mixed at a ratio of 3 parts sample:1 part sample buffer, and added to the top of the resin in the C18 column, and a fresh receiver tube placed underneath. Tubes were spun at 1500g for 1 minute, flow-through collected, and placed back onto the resin. This was repeated three times to ensure complete peptide binding to the C18 resin. C18 columns were placed in a fresh receiver eppendorf tube again, and

200 μ l of wash buffer added. This was then spun at 1500g for 1 minute, flow-through discarded, and the process repeated a total of three times to remove contaminants such as Urea and AmBic. Column was placed over a fresh receiver tube, this time with the lid open and no hole pierced through the lid, and 20 μ l of elution buffer added to the top of the resin bed. The tubes were spun at 1500g for 1 minute, and the flow-through untouched. This was repeated a total of three times to obtain a final volume of 60 μ l in the receiver eppendorf tube. This is now the cleaned peptide sample, and the column can be disposed of. Samples were then dried down in a SpeedyVac and stored at -20 μ l until running on the Qexactive.

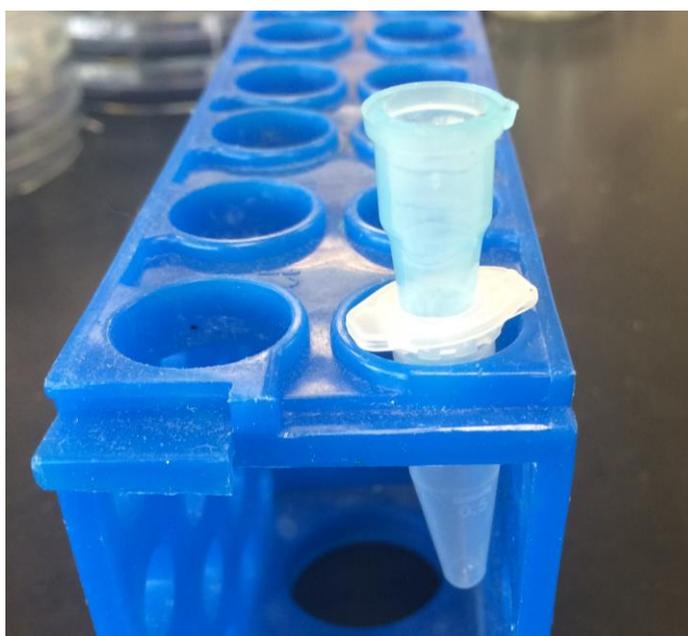


Figure 2.12 Image shows the C18 spin column placed in a 1.5ml eppendorf tube. The lid of the tube is closed with a hole large enough to fit the C18 column through in order to keep the bottom part of the C18 spin column which contains the resin away from any liquid during the clean up which could cause fluctuations in results.

2.14.5 Preparation of sample prior to loading on Q-exactive

Peptides were resuspended in 32 μ l of sample loading buffer (0.05% TFA, 2% Acetonitrile, taken from Q-Exactive buffer reservoir) and vortexed

for approximately 30 seconds. The samples were placed in a water bath for sonication for 5 minutes. Following resuspension of the peptide pellet, samples were spun at 13000 g for 5 minutes at room temperature to pellet any insoluble material, and 30µl of the supernatant transferred to vials (VWR).

2.14.6 Parameters for running samples on Qexactive

One microliter of peptide mix was eluted onto the QExactive, a high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 Picofrit™ column (100 mm length, 75 mm ID), using a 120 mins reverse phase gradient at a flow rate of 250 nL /min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

2.14.7 Parameters for analysing quantitative results and statistical analysis

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; <http://maxquant.org/>) to correlate the data against a database for *V. destructor* or *A. mellifera*, depending on the experiment. A combined database for the two organisms was also used. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with minimum length of seven amino acid length were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed.

Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.0.31. LFQ intensities were \log^2 -transformed

and ANOVA of significance and t-tests between the proteomes of Bayvarol sensitive and Bayvarol resistant mites was performed using a p-value of 0.05 and significance was determined using FDR correction (Benjamini-Hochberg). Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were included in the study only when they were completely absent from one group and present in at least three of the four replicates in the second group (referred to as qualitatively differentially abundant proteins). The Blast2GO suite of software tools was utilized to assign gene ontology terms (GO terms) relating to biological processes, molecular function and cellular component. Enzyme commission (EC) numbers and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping was performed as part of the Blast2GO annotation pipeline.

2.15 Quantitative analysis of four immune genes from pupal stage *A. mellifera* worker and drone

2.15.1 Preparation and sample selection

All preparation for the RNA extractions was carried out as per section 2.9.1. RNA was extracted from one pupa per replicate, and all extractions were carried out on at least four separate occasions. Pupae of a similar age were chosen and the level of parasitization recorded. The extractions were carried out using the Trizol and chloroform method, discussed in section 2.9.2(a).

2.15.2 DNase treatment of RNA

Contaminating DNA was removed by DNase treating the RNA extract with a Deoxyribonuclease I (AMP-D1) kit. RNA (4 µg) quantified by spectrometry (A260/A280), was brought up to a volume of 8 µl using DEPC treated water. This was followed by the addition of 1 µl 10X reaction buffer and 1 µl DNase 1 (1 unit/µl) to the micro centrifuge tube containing the RNA mixture, prior to incubation at room temperature for 15 min. The enzyme activity was stopped by adding 1µl of Stop Solution (50 mM EDTA) to the RNA mixture and mixed by pulsing on the micro centrifuge. Samples were

incubated at 70°C for ten minutes and cooled on ice. RNA was further processed for cDNA synthesis or frozen at -70°C.

2.15.3 cDNA synthesis of total extracted RNA

cDNA synthesis was carried out using the Superscript III First-Strand Synthesis System for RT-PCR kit from Invitrogen (CA, USA), as described in Section 2.9.4.

2.15.4 Primers used for detection of quantitative differences in four immune genes of *A. mellifera* worker and drone pupae

Primer	Sequence	Amplification Target	Reference
bActin	5'-ATGCCAACACTGTCCTTTCTG-3'	B-Actin (Reference gene)	(Antúnez <i>et al.</i> , 2009)
	5'-GACCCAATCCATACGGA-3'		
Abaecin	5'-CAGCATTTCGCATACGTACCA	Antibacterial peptide Abacein	(Antúnez <i>et al.</i> , 2009)
	5'-GACCAGACGTTGGAAAC-3'		
Defensin	5'-TGTCGGCCTTCTCTTCATGG-3'	Antibacterial peptide Defensin	(Yang and Cox-Foster 2005)
	5'-TGACCTCCAGCTTTACCCAAA-3'		
Phenoloxidase	5'-AATCCATTACCTGAAATTGATGCTTAT-3'	Phenol Oxidase	(Yang and Cox-Foster 2005)
	5'-TAATCTTCCAACCTAATTCATACGCTCTT-3'		

Hymenoptaecin	5'-GCGTCTCCTGTCATTCCATT-3'	Antibacterial peptide	(Antúnez <i>et al.</i>, 2009)
	5'-CTCTTCGCCGTTGCATA-3'	Hymenopterlin	

Table 2.5 Primers used for relative quantification of four immune related genes in control pupae and those parasitized by *Varroa*

All primers were reconstituted to a 100mM concentration upon arrival, in nuclease free water. They were diluted further by 1 in 10 before use, to a working concentration of 10mM. Primers were stored at -20°C.

2.15.5 Real time PCR

Real Time PCR was performed on a Light Cycler 480 system®(Roche) using the highest quality reagents obtained from Sigma and Roche. Cycle conditions were optimised for each primer set. The primers were obtained from literature (Yang and Cox Foster, 2005; Auyunez *et al.*, 2009) and ordered from Integrated DNA technologies. Primers were analyzed for secondary structures including dimer, hairpin-loop and palindrome formation using the Netprimer website available at <http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html> prior to ordering. Table 2.5 represents primer sequences for each primer set.

Primers were dissolved in molecular grade water to give a stock solution of 100 µM initially, which was diluted to a working solution of 10 µM. Each PCR reaction took place in a sterile 96 well plate (Roche) (9 µL volume) which contained 1 µg/µL cDNA, 5µL LightCycler® 480 SYBR Green, 1 µL water (molecular grade), 1 µL forward primer, 1 µL reverse primer. Solutions were mixed by gentle centrifugation and placed into the Light Cycler immediately. SYBR Green fluoresces as it associates with DNA. It forms bonds with the minor groove of dsDNA (Figure 2.14). SYBR green emits a 1000 greater fold signal when it is bound to DNA than when it is free in solution. The greater the quantity of DNA, the greater the emission spectra

(Figure 2.13). The calculation was done by the delta-delta Ct method, whereby the Ct (Cycle time) is the time at which fluorescence reaches the threshold, the difference in Ct values between samples allows the software to quantify the DNA present (Valasek and Repa 2005). As a standard curve, PCR amplification was performed with several dilutions of DNA template from control pupae (S1=1000ng, S2= 250ng, S3=62.5ng, S4=15.625ng, S5=3.9ng). The cDNA prior to Real Time PCR amplification was of high quality as the RNA purity was assessed prior to synthesis. All RNA purity A260/A280 was above 1.88. All genes were standardized on the Roche Light Cycler 480® with efficiency values of 2.0 ± 0.4 .

All values and standardisations were performed on at least three occasions. The standard curves of these genes can be seen in Figures 2.14-2.18.

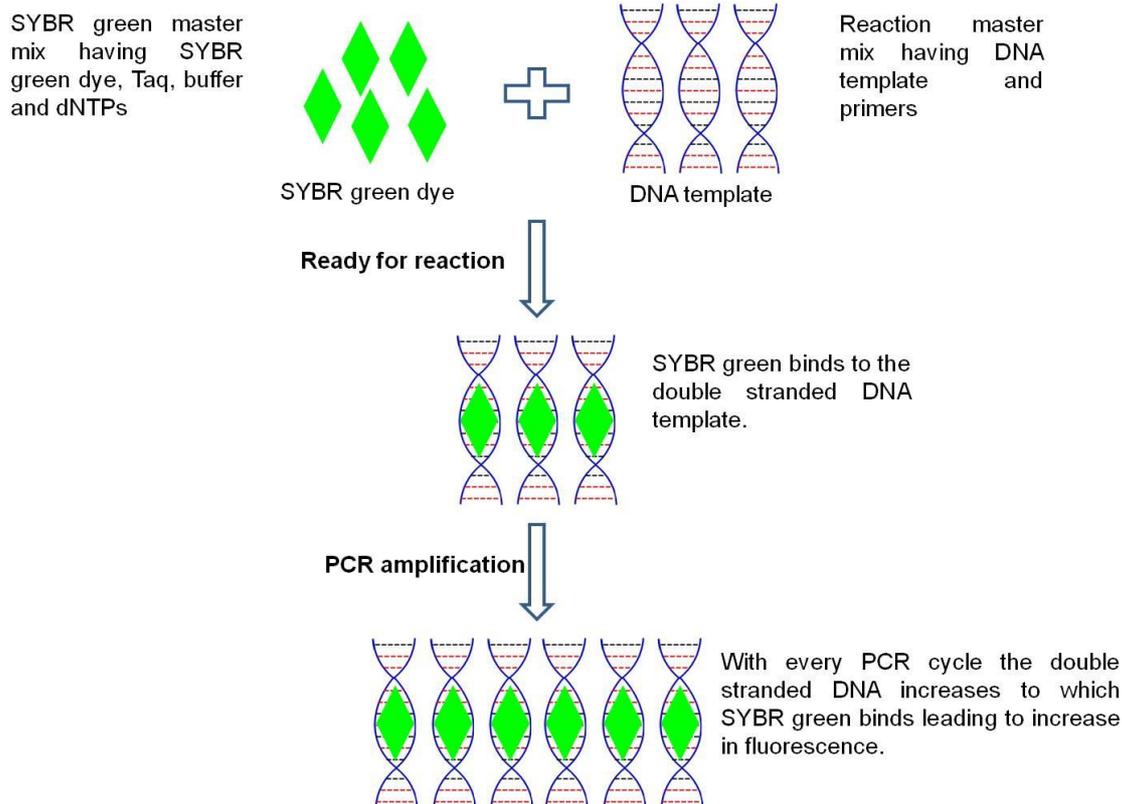


Figure 2.13: The intercalation of SYBR Green with DNA and the fluorescent emission

Source <http://www.intechopen.com/books/gene-therapy-developments-and-future-perspectives/differential-gene-expression-and-its-possible-therapeutic-implications>

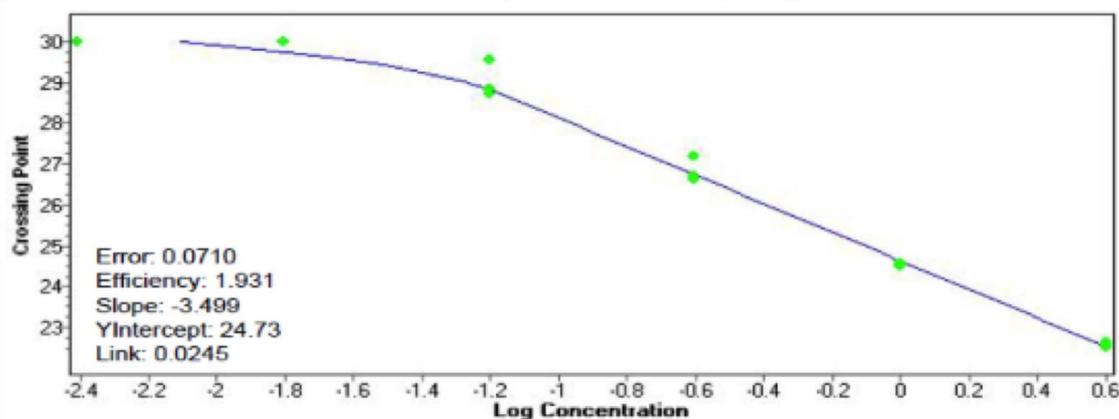


Figure 2.14: Standard curve of b-Actin reference gene in control *A. mellifera*

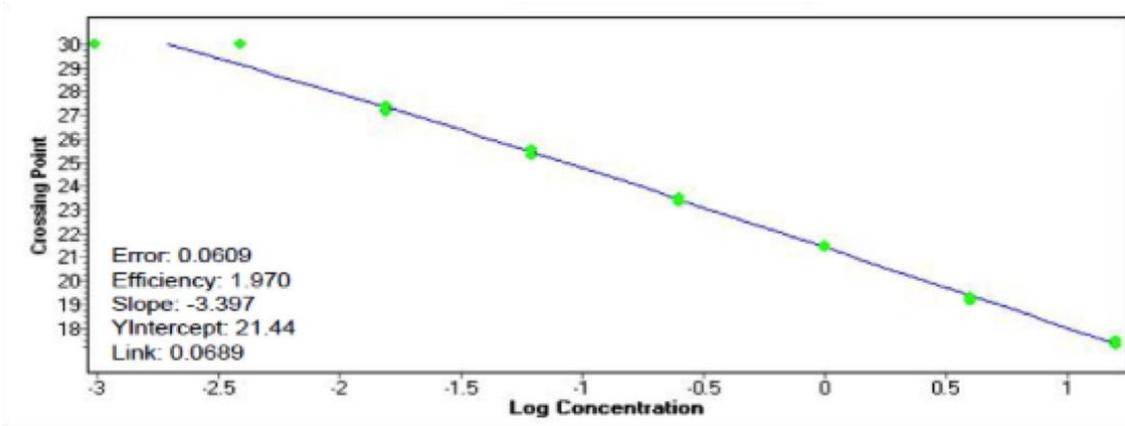


Figure 2.15: Standard curve of Abaecin in control *A. mellifera*

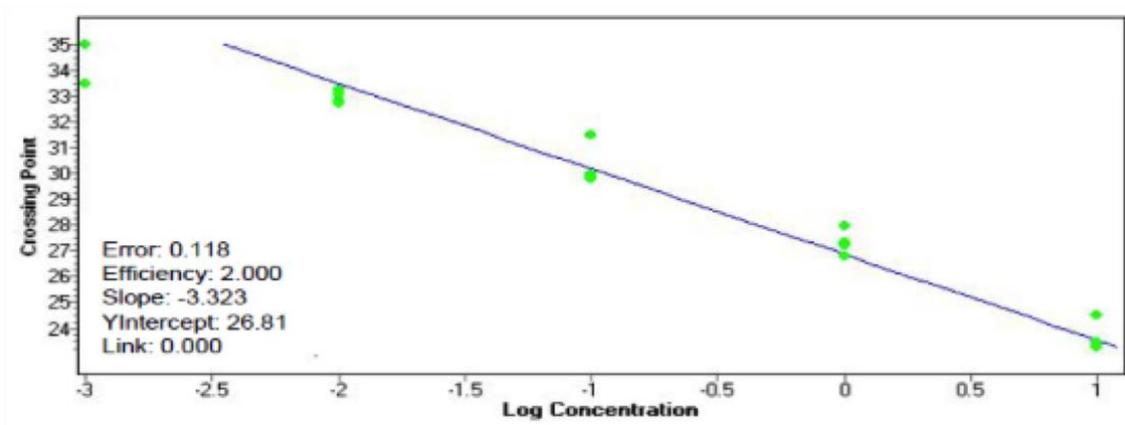


Figure 2.16: Standard curve of Defensin in control *A. mellifera*

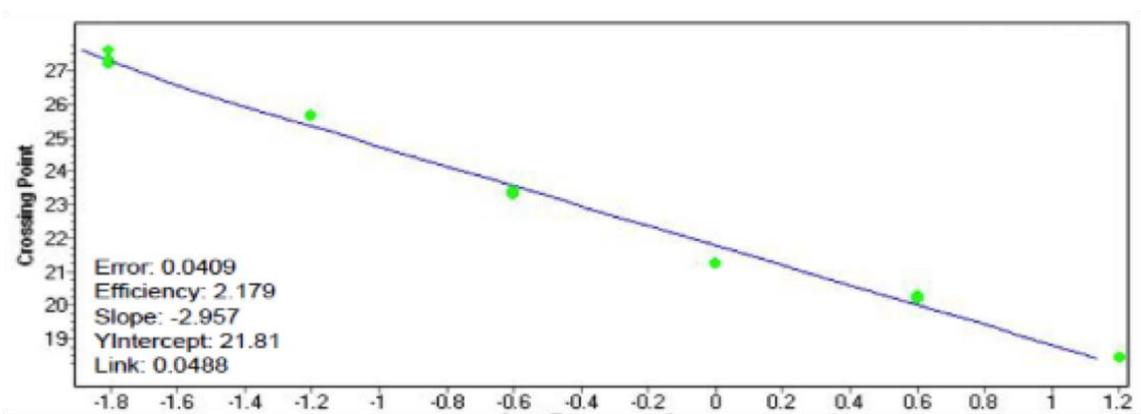


Figure 2.17: Standard curve of Phenoloxidase in control *A. mellifera*

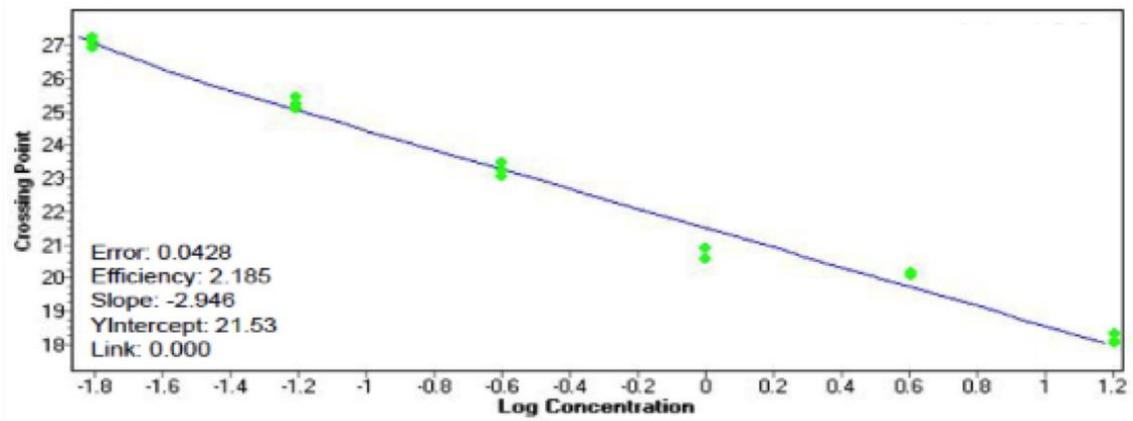


Figure 2.18: Standard curve of Hymenoptaecin in control *A. mellifera*

Chapter Three

Occurrence and prevalence of honeybee diseases and parasites in Irish colonies

3.1 Introduction

Honeybees, like all other organisms, are subject to a wide range of foreign threats, from viruses to parasites. The close proximity and level of interaction of individuals in the bee colony means that the spread of diseases and parasites can be rapid and have serious consequences. There are many diseases and parasites known to pose a threat to honeybee colonies, and the aim of this Chapter was to identify the occurrence and the prevalence of some of the most common bee diseases in Irish apiaries. A random list of 161 beekeepers from the Republic of Ireland was generated from the list of beekeepers in the Country, to send samples in the Spring, Summer and Autumn. The aim was to identify the presence and the seasonal variations of four viruses, the tracheal mite and the microsporidian *Nosema*. Samples were taken from the same colony with the same queen, unless the colony initially used died, in which case another from the same apiary was used.

Four bee viruses were chosen for analysis of occurrence and seasonal variation; Deformed wing virus (DWV), Acute bee paralysis virus (ABPV), Chronic bee paralysis viruses (CBPV), and Israeli acute paralysis virus (IAPV). These viruses were chosen as they have previously been found in colonies throughout Europe and the UK (Tentcheva *et al.*, 2004; McMahon *et al.*, 2015). These viruses have also been shown to be replicative competent in *Varroa* and have reached new levels of virulence when they exist alongside *Varroa* in a colony (Tentcheva *et al.*, 2004; Le Conte *et al.*, 2010; Francis *et al.*, 2013).

The occurrence and prevalence of the tracheal mite, *A. woodi*, was also analyzed. The tracheal mite is a parasite of *A. mellifera*, colonizing the trachea making it difficult for normal respiration to occur (Harrison *et al.*, 2001; McMullan and Brown 2005). Though not a very serious threat to the honeybee, the tracheal mite may cause an increase in mortality during the Winter months, and so their occurrence was examined in conjunction with colony losses in apiaries to see if they influenced losses (Harrison *et al.*, 2001). The tracheal mite affects normal respiration due to its colonization of the trachea, which

can negatively impact the fitness of the bee during times of long flight such as during foraging (Harrison *et al.*, 2001)

Lastly, the prevalence of *Nosema* microsporidia in Irish honeybee colonies was examined as *Nosema* is one of the main threats to honeybee colonies, particularly since the introduction of *N. ceranae* (Chen *et al.*, 2009; Mayack and Naug 2009a). A study into the causative agents of colony loss found *Nosema* as the third most prevalent cause of 7.3% of the total colonies lost during an examined period (Büchler *et al.*, 2014). The level of *Nosema* throughout Ireland was firstly analyzed by light microscopy, with samples that showed the presence of spores further analyzed to identify *Nosema* species by PCR.

The aim of this Chapter was to provide a report on the occurrence and prevalence of a number of honeybee diseases in Irish honeybee colonies, and to identify any link that the prevalence of these diseases may have on the collapse of colonies in the Winter.

3.2 Analysis of occurrence and prevalence of four honeybee viruses in Irish honeybee colonies and the seasonal variations of these viruses

There are approximately 25 different viruses known to infect *A. mellifera*, and since the introduction of *Varroa*, they have become one of the major contributing factors in the collapse of colonies worldwide (Francis *et al.*, 2013; Furst *et al.*, 2014). The four viruses chosen for the survey were Deformed wing virus (DWV), Acute bee paralysis virus (ABPV), Chronic bee paralysis virus (CBPV), and Israeli acute paralysis virus (IAPV). The total RNA from the Spring and Summer samplings was extracted using the method described in section 2.8.2(a), with the RNA from the Autumn samples extracted as described in section 2.8.2(b). cDNA was synthesized and the specific primers were used in uniplex PCR reactions as described in section 2.8.5 (Figure 3.2).

3.2.1 Prevalence of viruses during Spring 2014 period in Irish honeybee colonies

A total of 141 beekeepers from the 161 randomly sampled beekeepers returned samples for testing for the Spring dataset. The results from these can be seen in appendix Table A3.1. In total 114 of the samples tested positive for DWV, with 27 testing negative. This meant that during the Spring period, 81% of the colonies samples had DWV (Figure 3.1). All of the samples tested negative for CBPV, ABPV and IAPV. The locations of all of the samples that tested positive for DWV can be seen on a map in Figure 3.3.

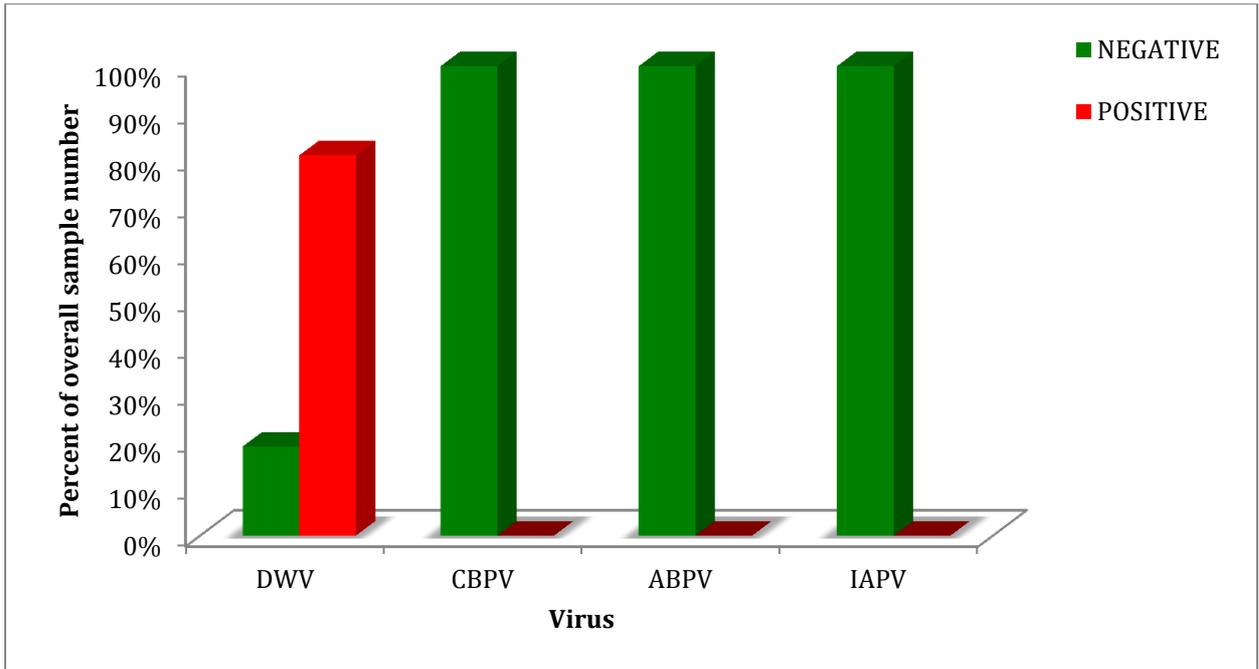


Figure 3.1 Viruses detected in Irish colonies during Spring 2014 period. Results showing presence of DWV in 81% of colonies samples during Spring period. No other virus was detected.

Lane no. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

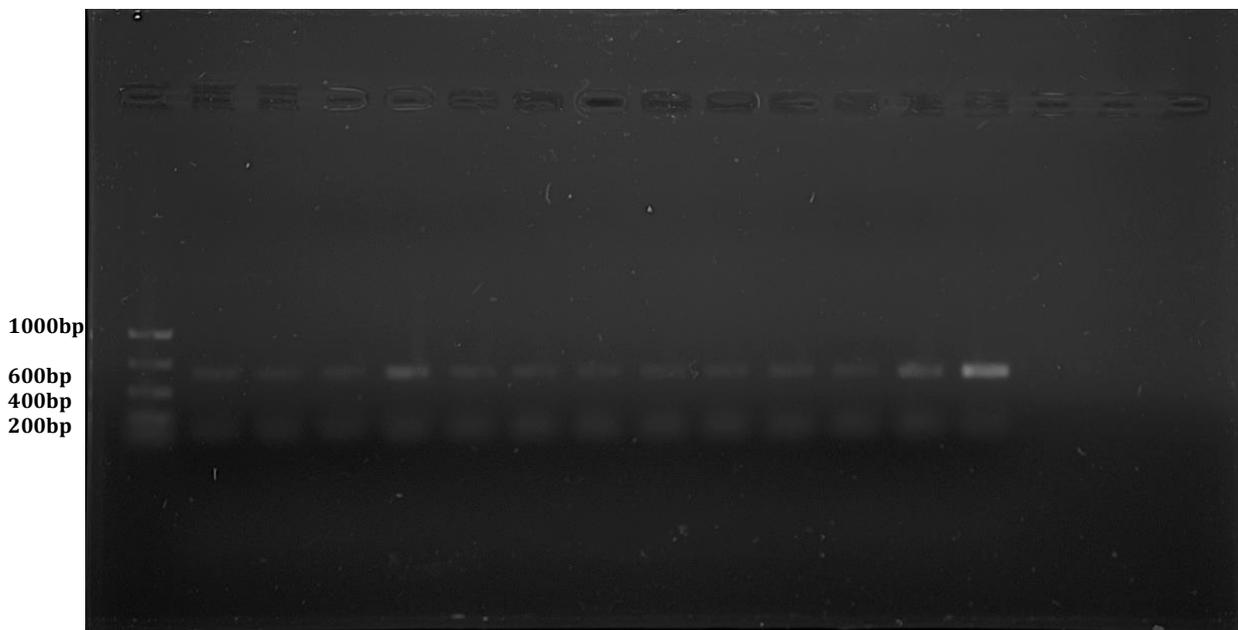
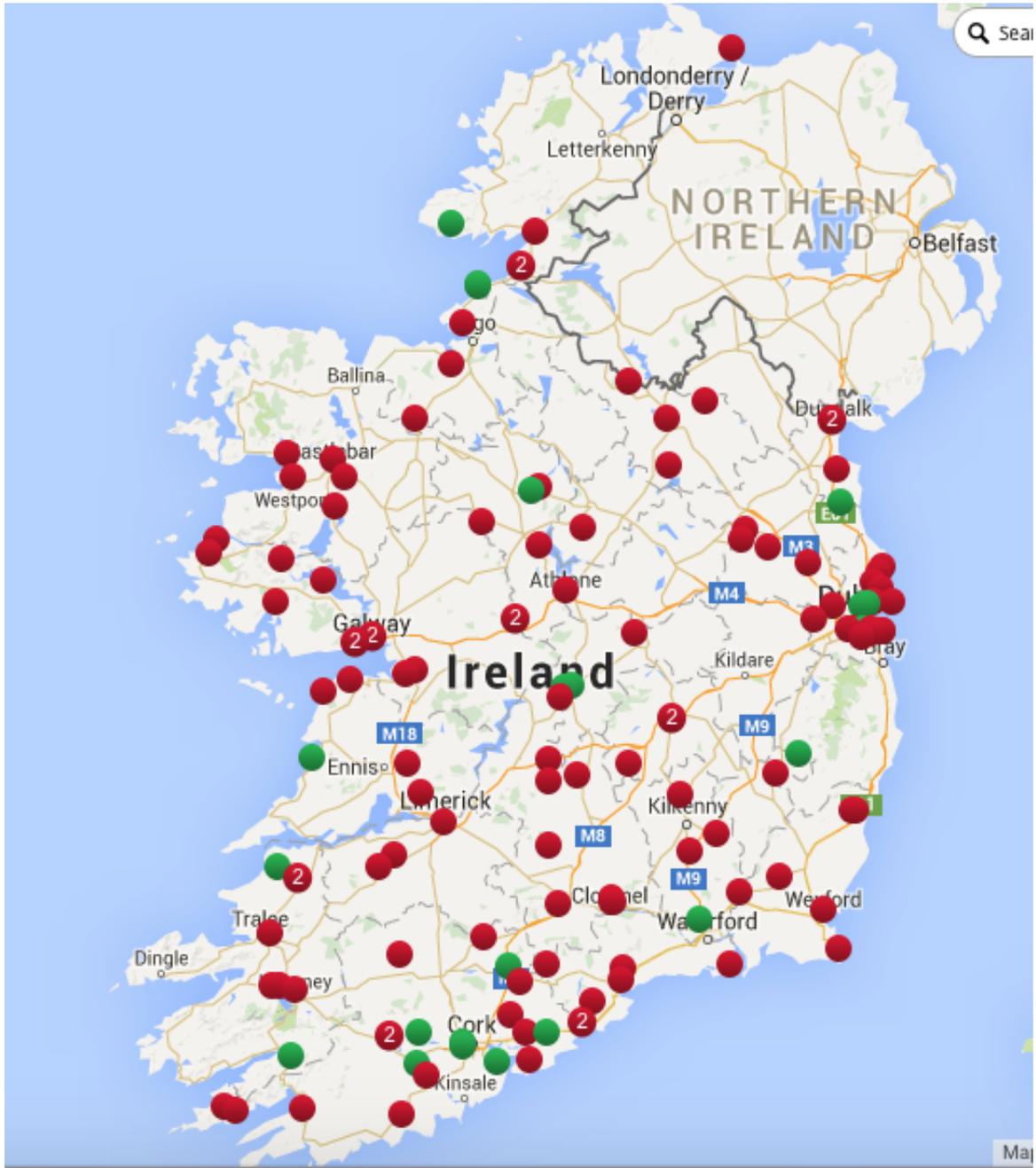


Figure 3.2 Example of PCR gel image from viral detections survey Image shows DWV standard in lane 2, followed by positive detection for lanes 3-14, and negative detection for lane 15.



DWV	81% +	
	19% -	

Figure 3.3 Distribution of samples testing positive for DWV in Spring 2014. Map shows all of the locations from which samples were collected. Green on the map indicates an area that tested negative for DWV, and a red circle indicates a sample that tested positive for DWV.

3.2.2 Prevalence of viruses during Summer 2014 period in Irish honeybee colonies

Samples were collected again in the Summer period from the same colonies of same list of beekeepers that were originally asked to provide samples, in order to track seasonal variations in the types of viruses present. A total of 128 beekeepers from around Ireland returned the samples for the Summer period. The results again showed no detection of ABPV, CBPV, and IAPV (Figure 3.4). For DWV, a total of 13 colonies tested negative for DWV, with the remaining 115 testing positive (94%). These results can be seen in Table A3.2. The location of the colonies that tested positive for DWV can be seen on a map in Figure 3.5.

3.2.3 Prevalence of viruses during Autumn 2014 period in Irish honeybee colonies

The methodology was changed for the final sampling in the Autumn, with a larger sample of bees taken through to extraction in order to optimize the chances of detecting the less prevalent viruses. RNA was extracted according to section 2.9.2(b). For the final sampling, a total of 113 beekeepers provided samples for viral analysis. From this, no samples tested positive for CBPV or ABPV. For DWV, 109 tested positive out of 113, which was a positive result for 96% of samples. For IAPV, 3 samples tested positive, which was 2.7% of the overall total. The results can be seen in Table 3.3a. The distribution of the samples can be seen on a map in Figure 3.7. The results are also shown depicting the percentage of positive samples for each virus in Figure 3.6.

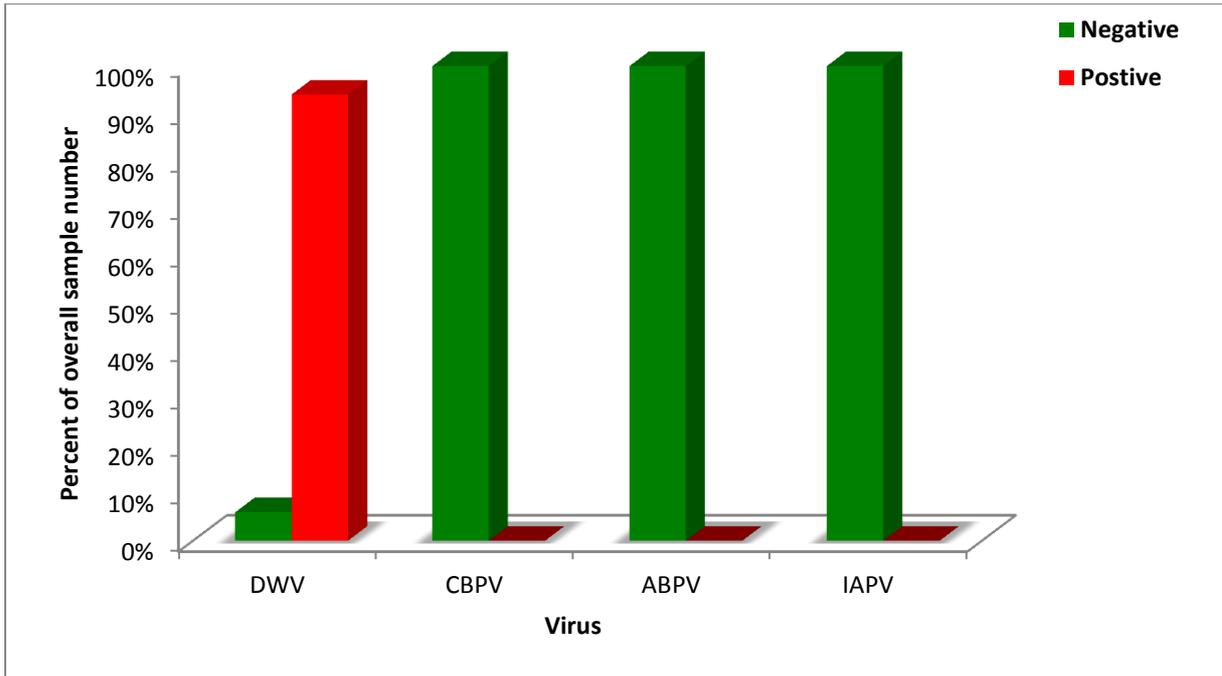
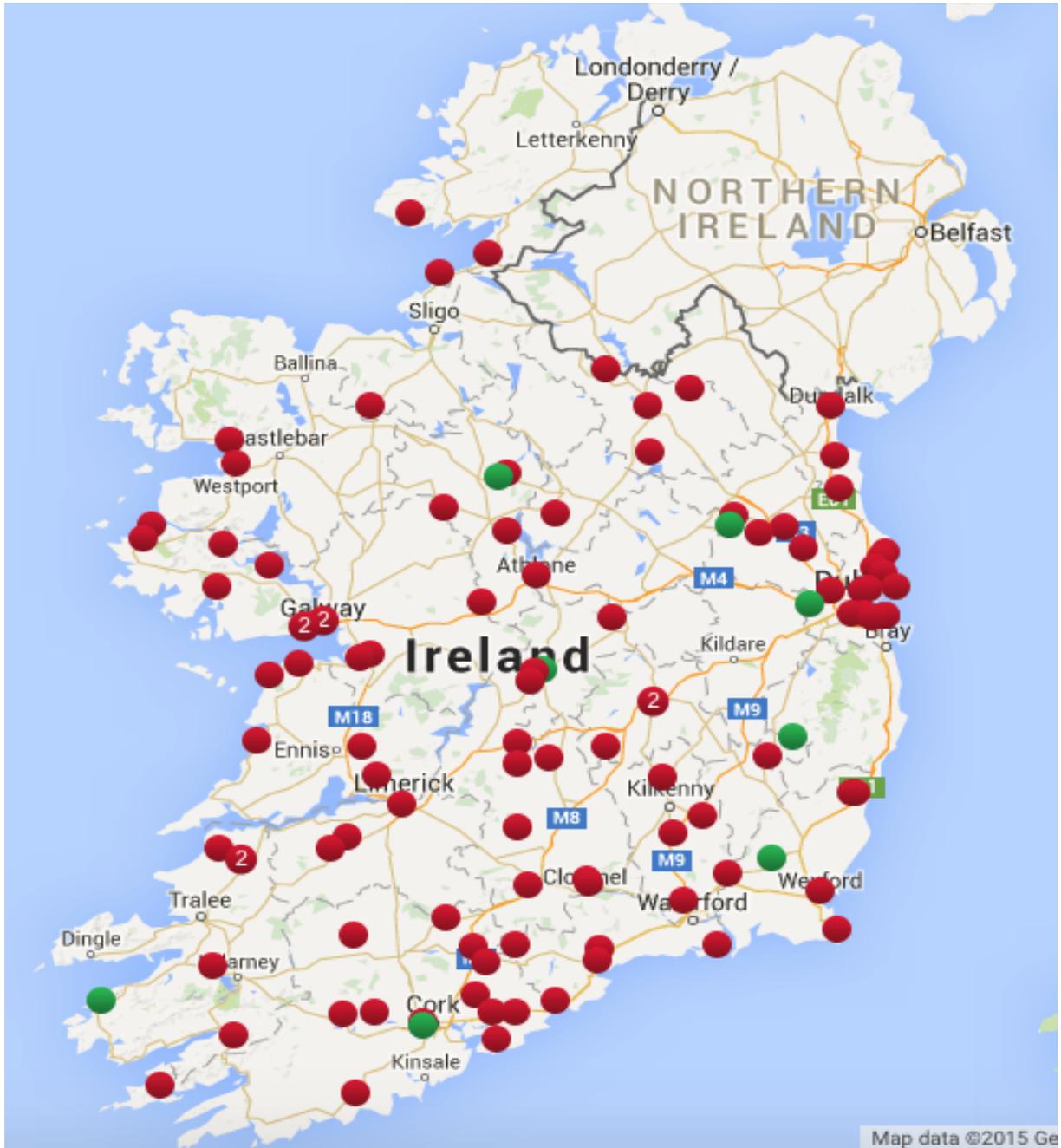


Figure 3.4 Viruses detected in Irish colonies during Summer 2014 period.
 Results showing presence of DWV in 94% of colonies samples during Summer period.
 No other virus was detected.



DWV	94% +	
	6% -	

Figure 3.5 Distribution of samples testing positive for DWV in Summer 2014

Map shows all of the locations which samples were collected from. Green on the map indicates an area that tested negative for DWV, and a red circle indicates a sample that tested positive for DWV in Summer samples.

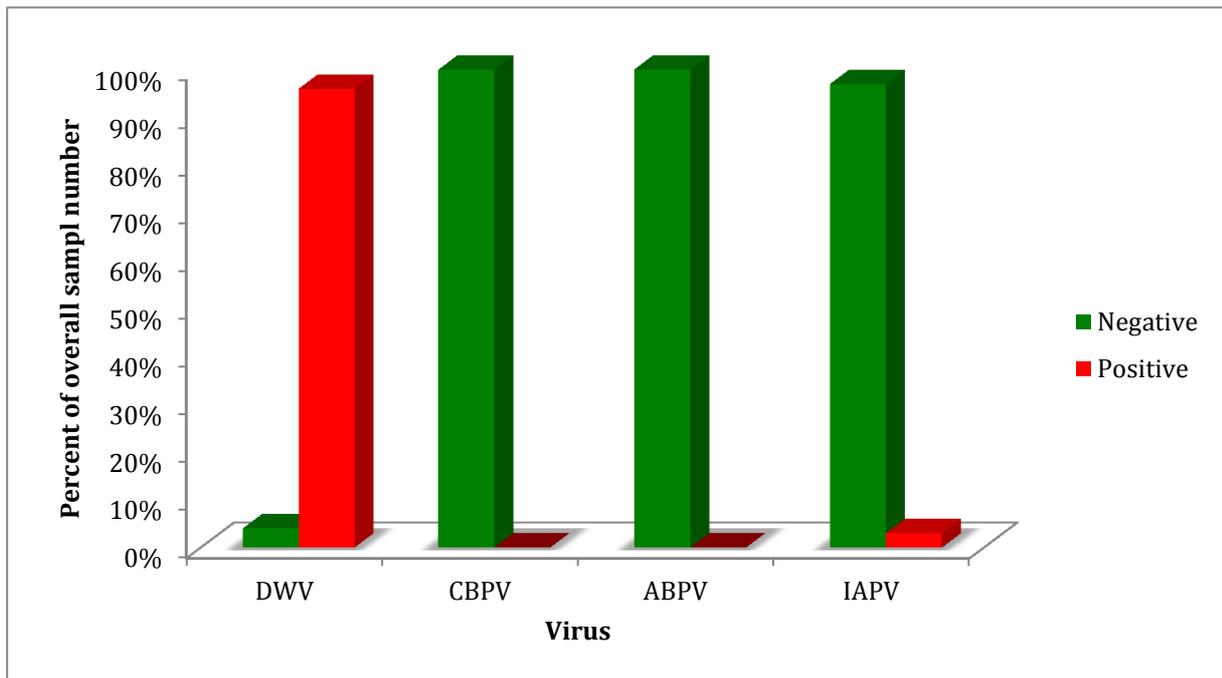
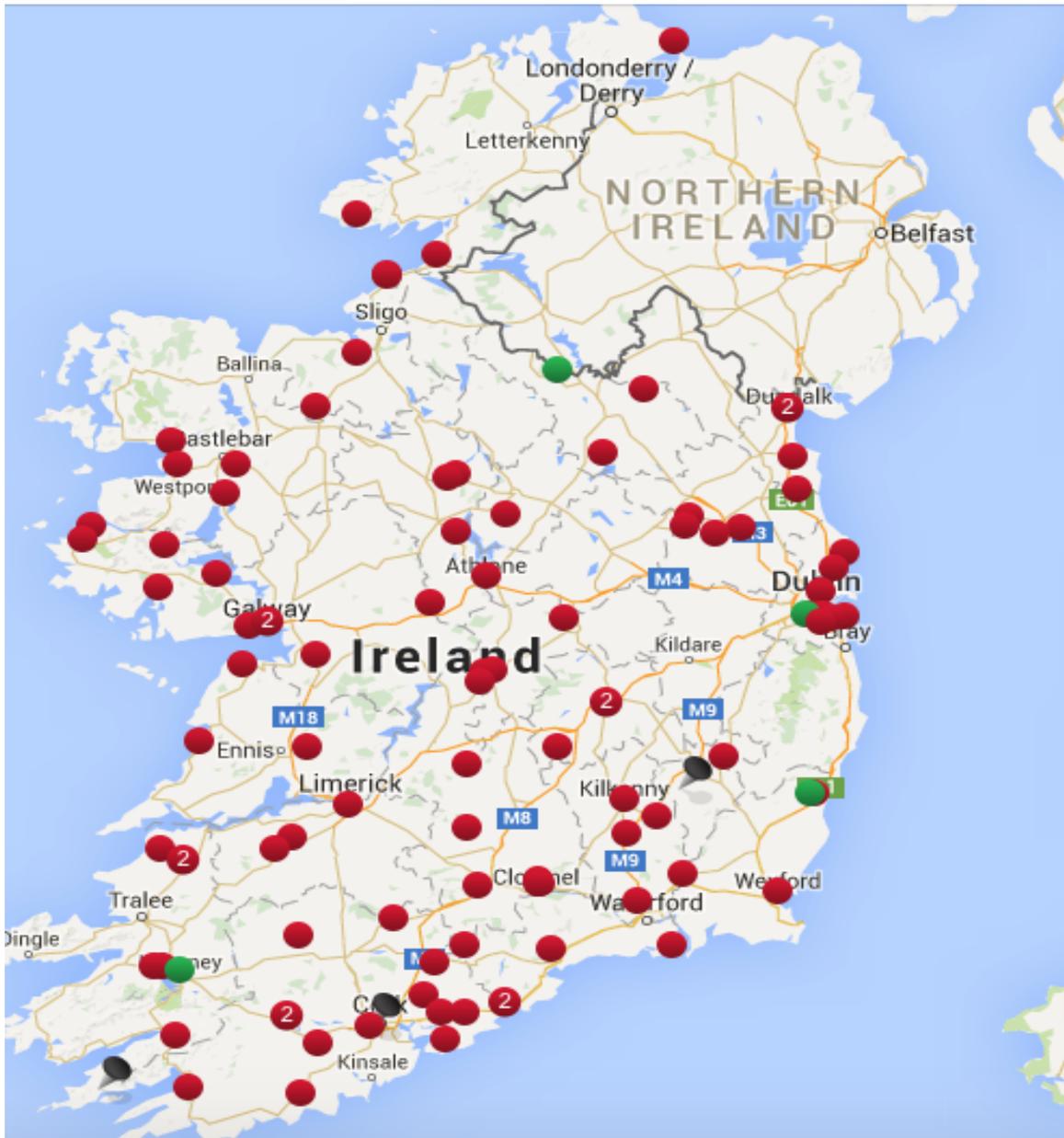


Figure 3.6 Viruses detected in Irish colonies during Autumn 2014 period. Results showing presence of DWV in 96% of colonies samples during Autumn period. IAPV was detected in 3% of Irish colonies.



DWV	96% +	●
	4% -	●
IAPV	3% +	●
	97% -	●

Figure 3.7 Distribution of samples testing positive for DWV and IAPV in Autumn 2014.

Map shows all of the locations from which samples were collected. Green on the map indicates an area that tested negative for all viruses. Red circle indicates a sample that tested positive for DWV in Summer samples, and black indicates a positive result for IAPV in Autumn samples

3.3 Analysis of occurrence and prevalence of tracheal mites in Irish honeybee colonies

The tracheal mite is one of the least threatening pests of the honeybee, but due to its higher prevalence and killing rate in colonies throughout the Winter period, and its possible ability to transmit viruses through haemolymph feeding, it must still be monitored (Harrison *et al.*, 2001; McMullan and Brown 2005). Samples collected from around Ireland were analyzed by dissection and observation of the trachea, as described in section 2.7.1. The number of samples that were received for each of the sampling dates amounted to 141 in Spring, 128 in Summer, and 113 in the Autumn sampling round. The prevalence of tracheal mite during each of the sampling rounds was very low (Figure 3.8). Only 2 colonies tested positive for tracheal mite during the Spring sampling, with no colonies positive in the Summer or in the Autumn sampling periods.

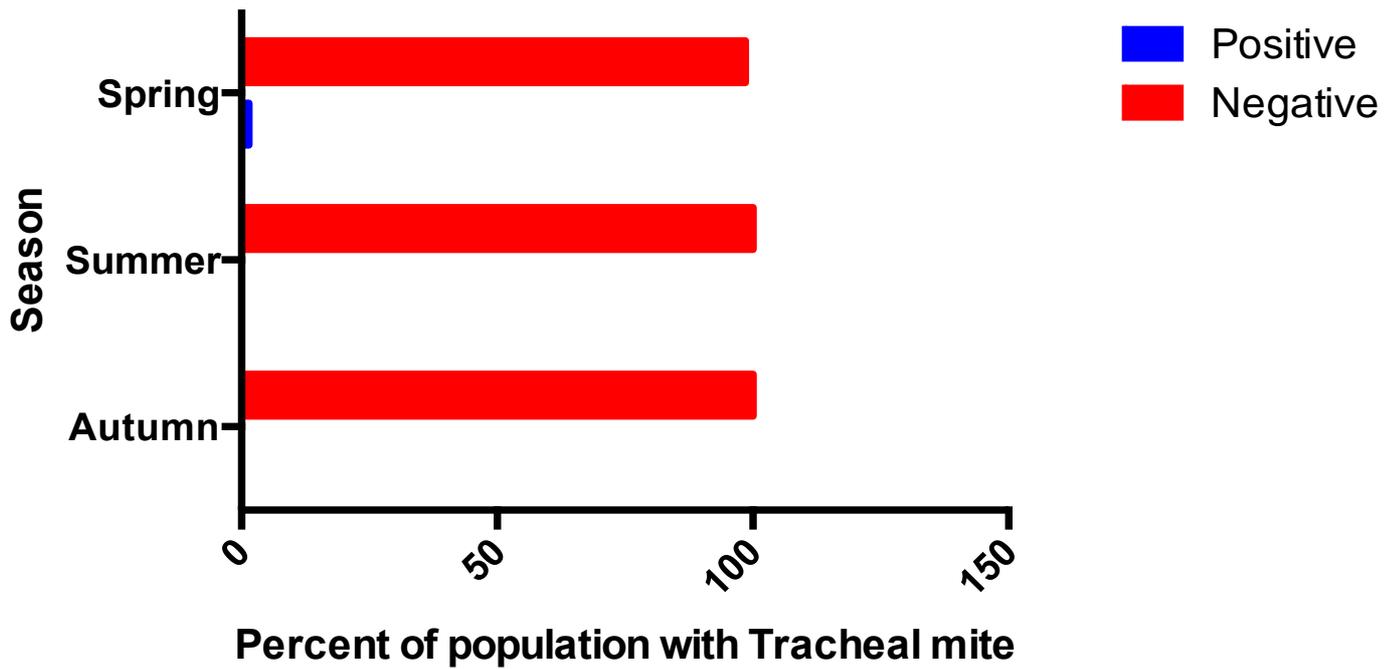


Figure 3.8 Percent of the sampled population positive for Tracheal mite in Spring, Summer and Autumn samples.

Graph shows the low prevalence of tracheal mite throughout the year in Irish honeybee colonies.

3.4 Analysis of occurrence and prevalence of *Nosema* in Irish honeybee colonies

The microsporidian *Nosema* is a serious pest of honeybee colonies worldwide, particularly since the hypothesized progressive replacement of *N. apis* with *N. ceranae*, which is not the natural strain that Western honeybees evolved with (Higes *et al.*, 2006; Natsopoulou *et al.*, 2015). To analyze the occurrence of *Nosema* spores in Irish colonies, samples taken during each seasonal sampling were first checked for the presence of *Nosema* spores through observation by light microscopy (See section 2.6.1). The total number of samples that tested positive for any type of *Nosema* spores is shown in Figure 3.10. A total of 25 colonies showed spores present out of the total number of 141 samples received in the Spring (18% positive). In total, 8 colonies out of the 128 samples received for the Summer round of sampling showed spores present in the Summer (6% positive), with 13 colonies out of 113 testing positive in the Autumn (12% positive) (Figure 3.11b). The samples that showed the *Nosema* spores present under observation by light microscope were then analyzed using PCR with specific primers to check for the presence of either *N. apis* or *N. ceranae* (Figure 3.9). A total of 46 colonies tested positive for *Nosema* microsporidia across the three sample dates, and were used for further analysis by reverse transcriptase PCR. The occurrence of the different *Nosema* species can be seen in Figure 3.11a. A total of 21% of the colonies tested positive for *N. apis*. The number of colonies that tested positive for *N. ceranae* was 28%, with 51% of the colonies testing positive for the presence of both species of *Nosema* (Figure 3.11a). The fluctuations in the occurrence of *N. apis* and *N. ceranae* throughout the seasons can be seen in Figure 3.11b. The prevalence of *N. apis* stayed relatively constant throughout the year, it was found in 73%, 78% and 67% of infected colonies in Spring, Summer and Autumn respectively (Figure 3.11b). *N. ceranae* was present at the highest levels of prevalence of infected colonies in Spring (91%) and Autumn (92%), falling to 67% during the Summer round of sampling.

Lane No. 1 2 3 4 5 6 7 8 9

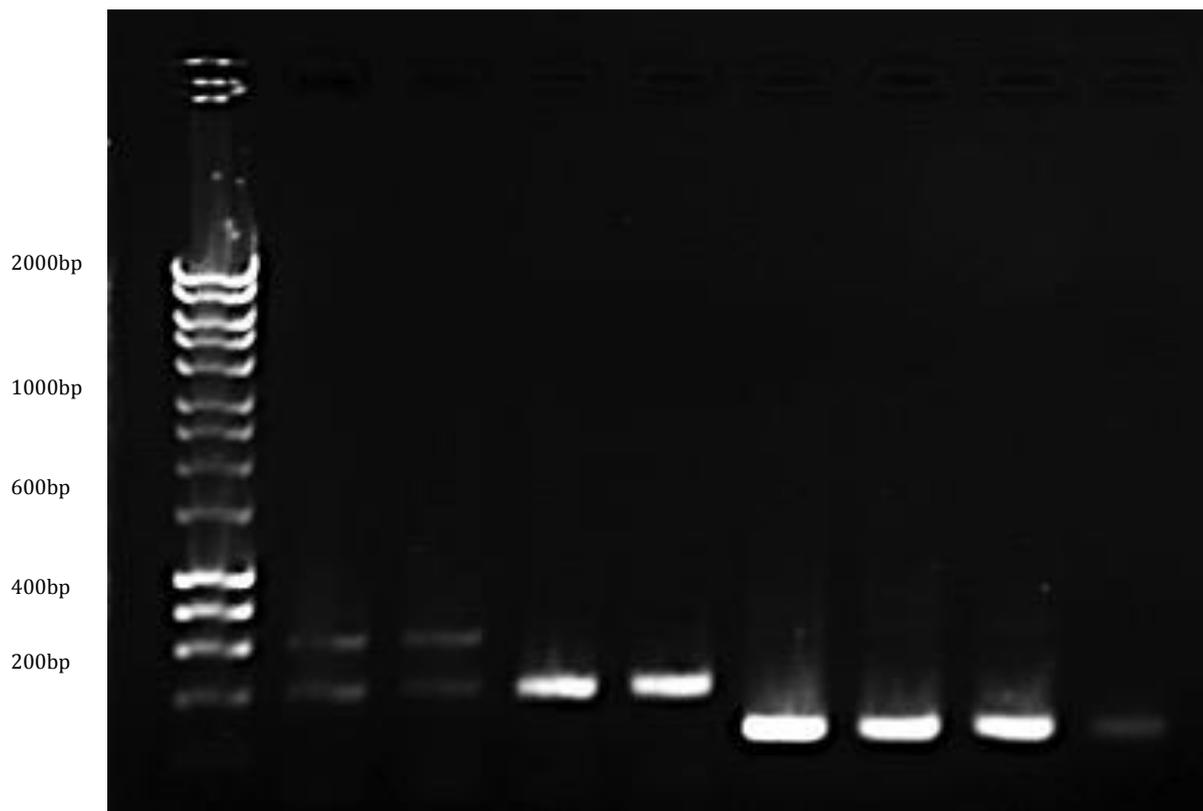


Figure 3.9 Example of PCR gel image from *Nosema* species detection. Image shows *N. apis* standard in lane 1 and 2, split due to overloading, followed by lane 4 and 5, positive for *N. apis*, and 6-9 positive for *N. ceranae*.

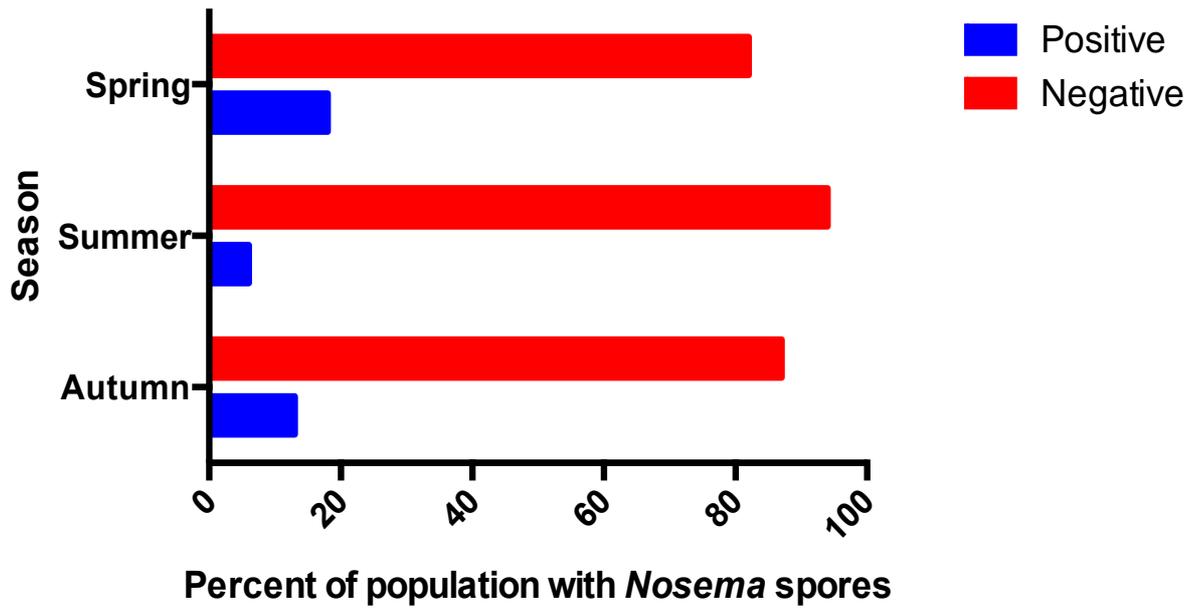


Figure 3.10 Percentage of sampled Irish honeybee colonies that tested positive for *Nosema* spores under observation by light microscope. Image shows the prevalence of *Nosema* spores in Irish colonies using light microscopy techniques throughout the seasons.

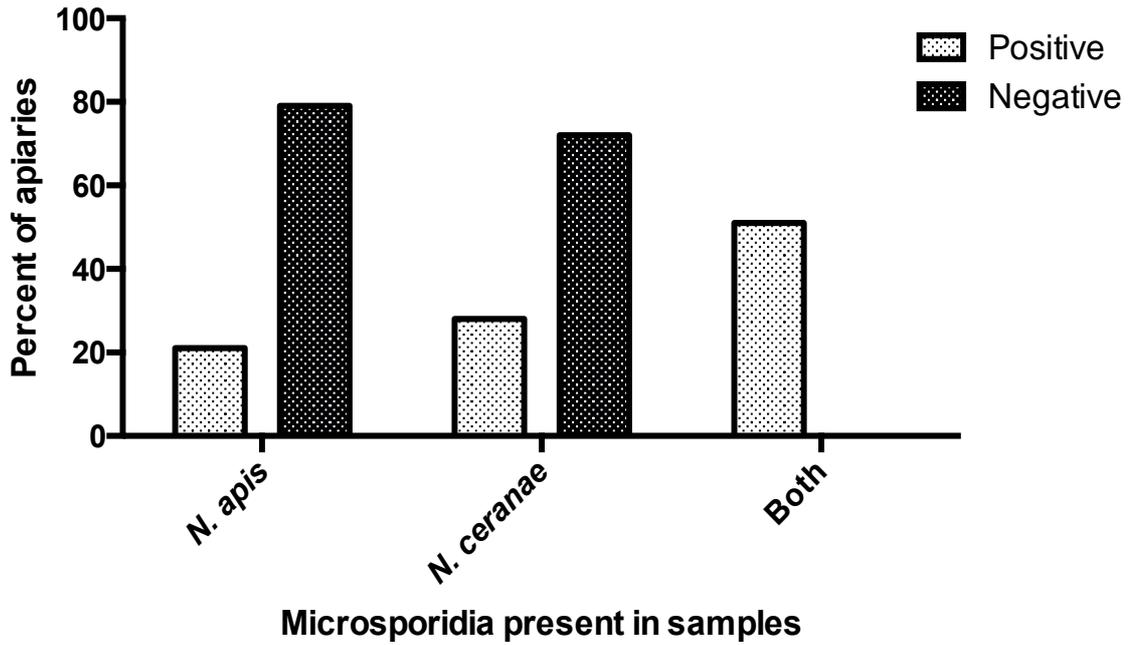


Figure 3.11a Occurrence of *N. apis* and *N. ceranae* in apiaries across Ireland in samples that were identified as *Nosema* positive by light microscopy

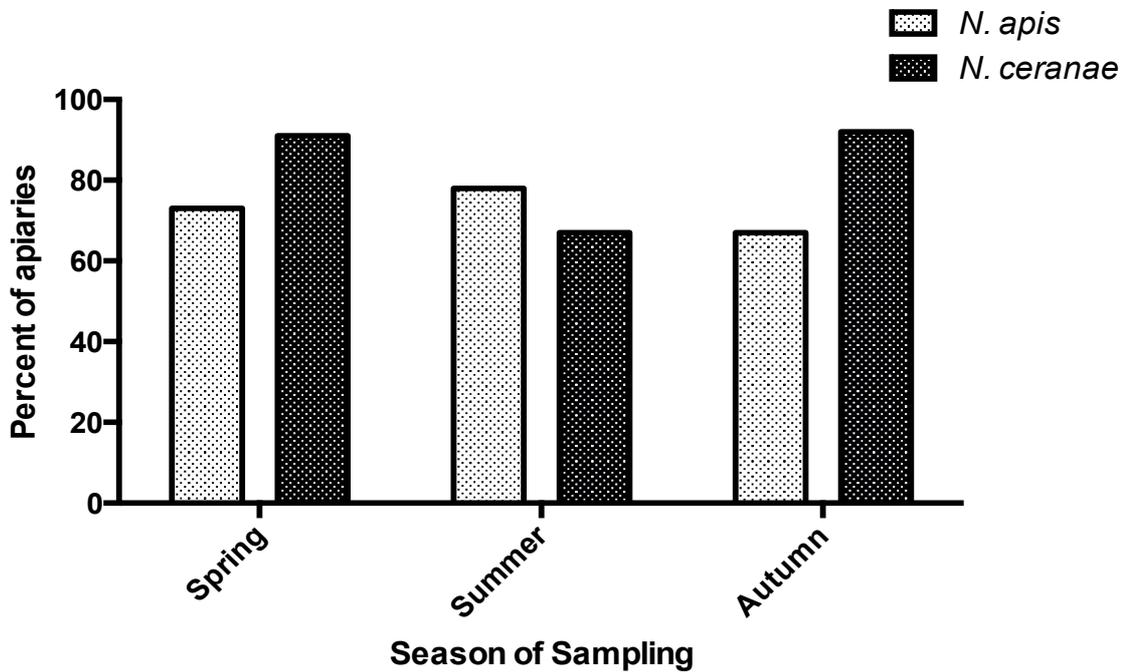


Figure 3.11b Seasonal variations in *N. apis* and *N. ceranae* in apiaries across Ireland. Image highlights occurrence of either *N. apis* or *N. ceranae* in positive colonies



Figure 3.12 Geographic distribution of *Nosema* species across Ireland

Image shows the locations of all samples that tested positive for *Nosema* by microscopic examination and were tested using specific primers. Green dots indicate areas which showed the presence of *N. ceranae*. Blue indicates the presence of *N. apis*. Red dots indicate areas where both *N. apis* and *N. ceranae* were detected. Black dots indicate samples that did not test positive for either *N. apis* or *N. ceranae* using PCR.

3.5 Colony loss and the link to honeybee diseases

The beekeepers that were chosen at random to provide samples for the disease analysis were also asked to provide a survey of the losses that occurred in the Winter prior to sampling (Winter 2013/2014), and the losses that occurred after sampling for disease (Winter 2014/2015). From the total list of beekeepers, 95 provided loss information and disease samples for all sampling periods, and were taken through for further analysis. The colonies were examined for losses during Winter 2014/2015 with losses of less than 20% and losses of more than 20%. The occurrence of disease in the seasons leading up to Winter, through Spring, Summer and Autumn were all examined, to identify a possible link between the occurrence of honeybee diseases in colonies that end up dying.

A total of 45 out of the 95 beekeepers experienced colony losses of more than 20% in the Winter following disease sampling and analysis. The diseases found in apiaries can be seen in Figure 3.13. In the Autumn immediately before the Winter that the losses occurred 67% of the apiaries, which lost more than 20% of their hives in Winter that followed, tested positive for DWV. A further 15% of these apiaries tested positive for both DWV and *Nosema*, with 9% of colonies found to be free from all of the tested diseases. The results from these apiaries that experienced losses greater than 20% were relatively unchanged in the Spring and Summer sampling (Figure 3.14). The prevalence and occurrence of diseases found in the apiaries that experienced less than 20% overall colony loss in Winter 2014/2015 throughout the seasons of 2014 are shown in Figure 3.14. A total of 44 beekeepers experienced losses of less than 20% in the Winter following disease analysis. The disease occurrence and prevalence in the apiaries that experienced less than 20% loss were not noticeably different to the rate of disease in the colonies that experienced more than 20% loss (Figure 3.13). A total of 84% of the apiaries tested positive for DWV, which was higher than the amount of apiaries that tested positive for DWV in the apiaries that experienced more than 20% loss. The number of apiaries that tested positive for *Nosema* and DWV was lower than in the apiaries that experienced a total colony loss of less than 20% (8% testing positive for both diseases) compared to apiaries that lost over 20% of their colonies (15% testing positive for both diseases). The number of apiaries which tested negative for all diseases was also lower, with 6% testing negative in the

apiaries that suffered less than 20% total colony loss compared to 9% of the colonies in the apiaries that lost more than 20% of their colonies. Interestingly, the colonies that tested positive for IAPV in the Autumn sampling (Table 3.3) lost 0% of their colonies in the Winter following the detection of the virus. Sample 36 and 46 tested positive for DWV and IAPV, with the third colony, sample 114, testing positive for Nosema, DWV and IAPV.

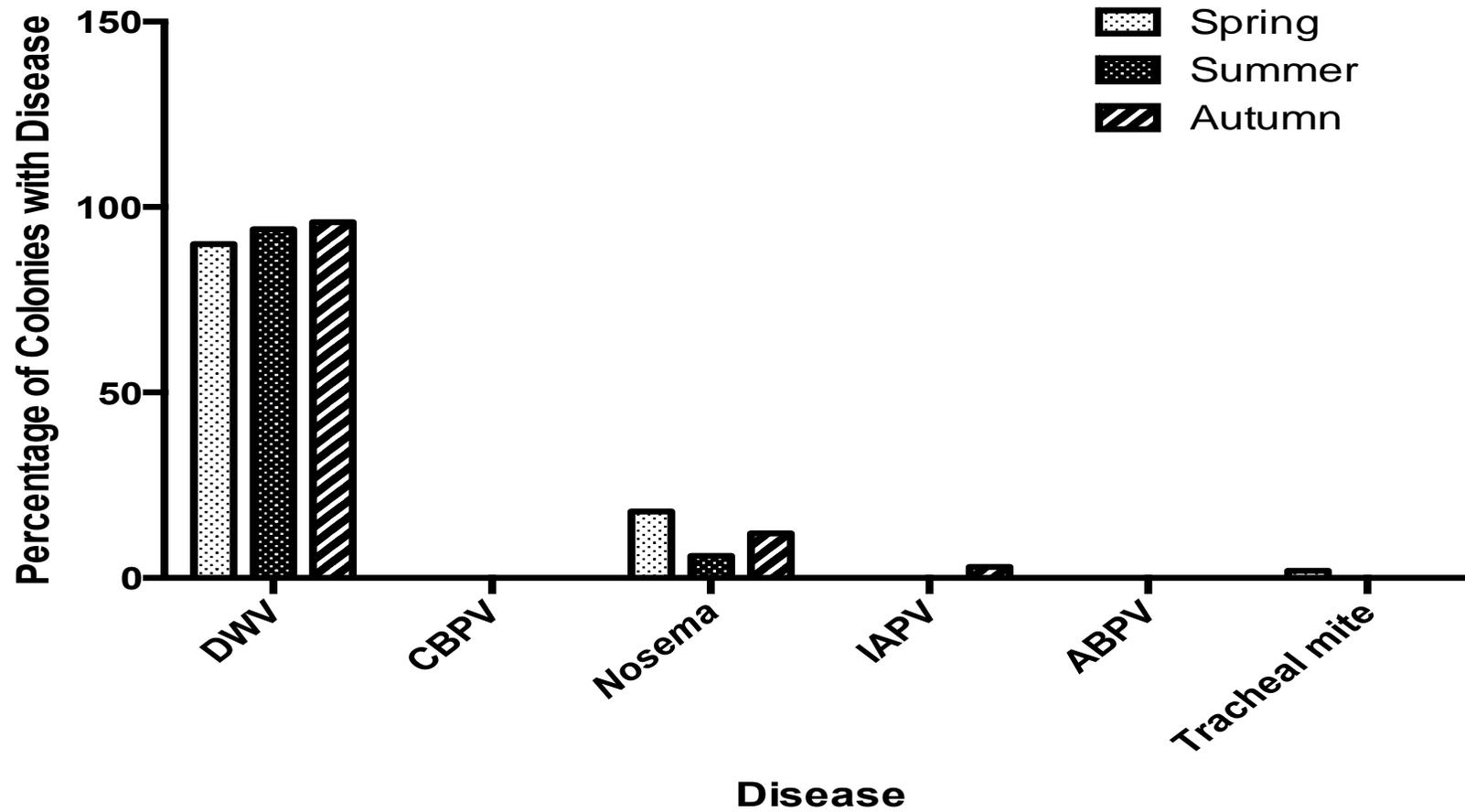


Figure 3.13 Overall percent of colonies positive for each disease in each season sampled.

The total number of colonies that tested positive for each disease though the different sampling periods is shown as a percent of the overall samples received.

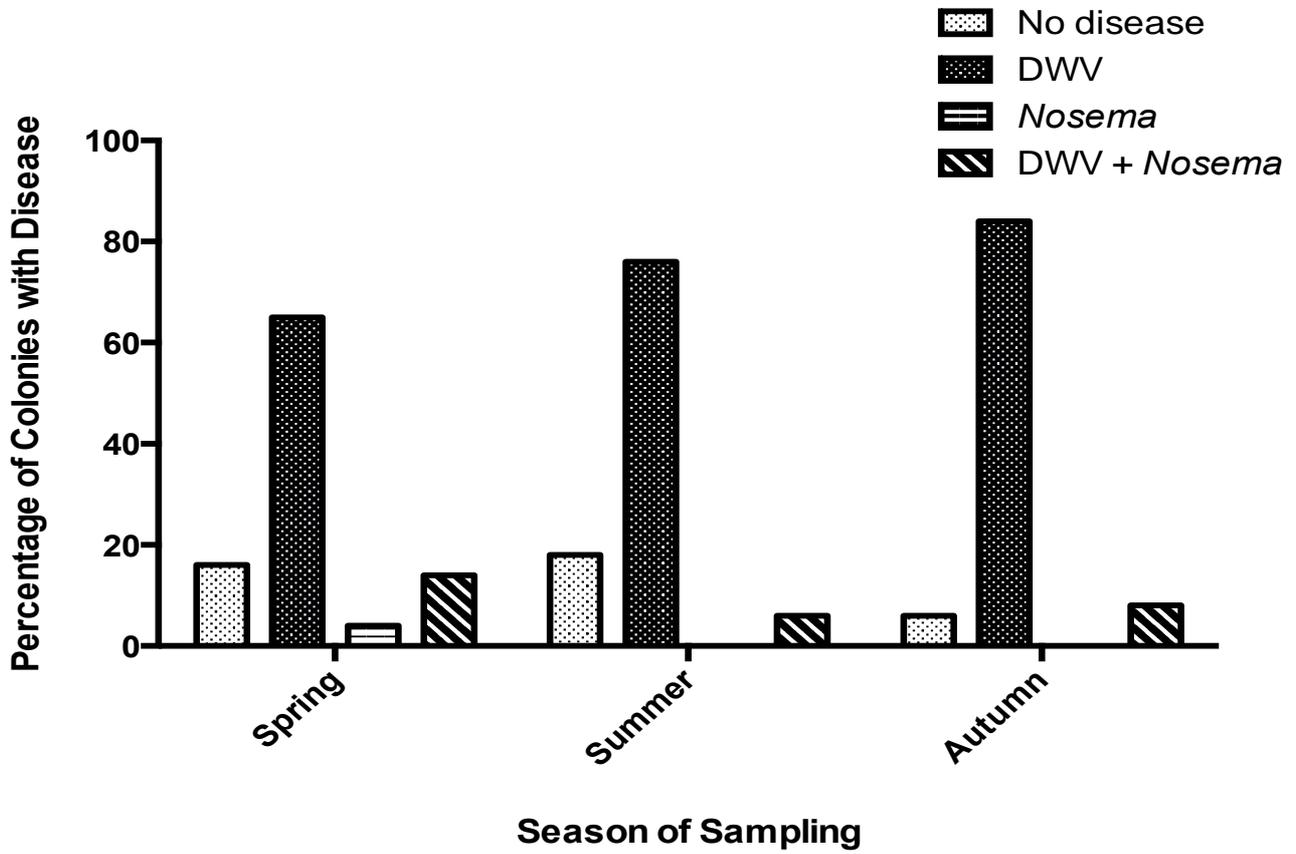


Figure 3.14 Percent of colonies with losses of less than 20% during the Winter following disease analysis.

From the 95 beekeepers that provided samples and colony loss information, 44 of these experienced colony losses less than 20% in Winter 2014/2015 post sampling for disease analysis.

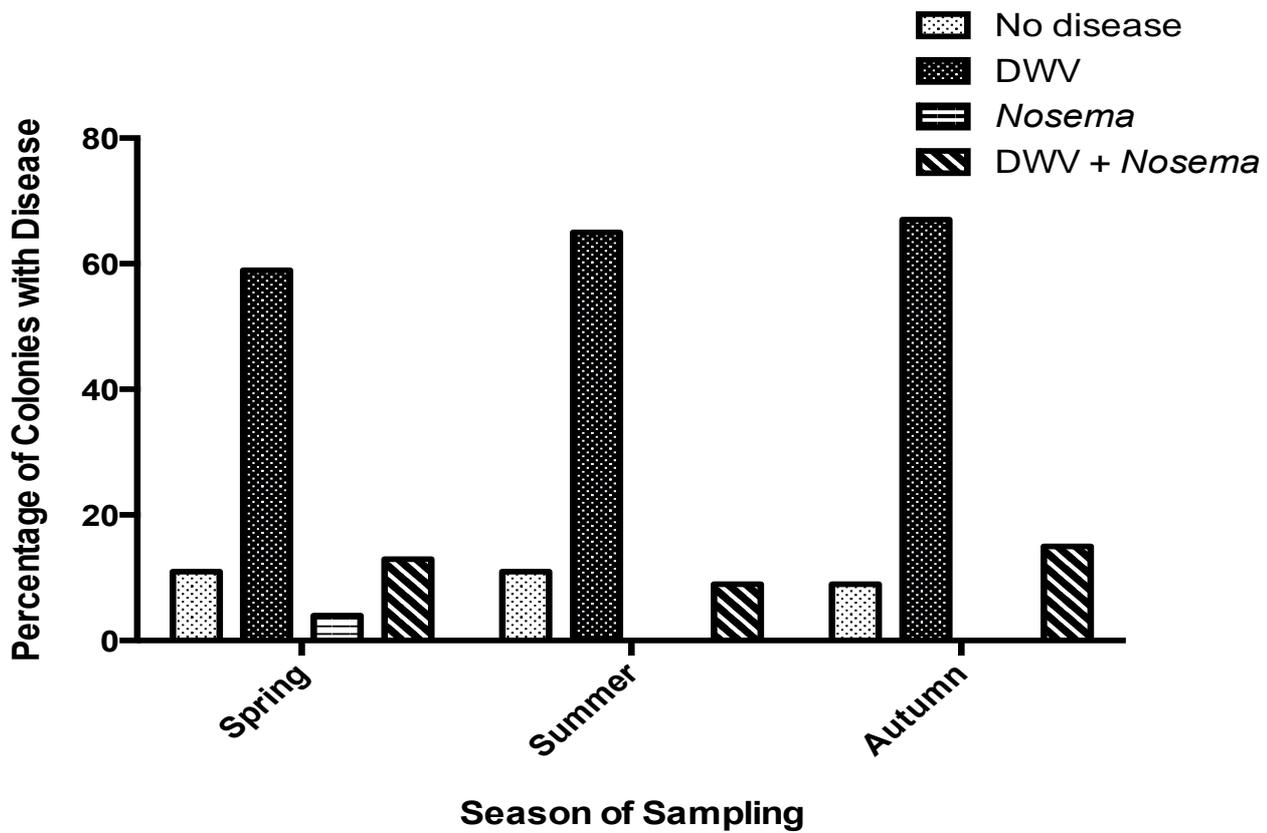


Figure 3.15 Percent of colonies with losses of more than 20% in the Winter following disease analysis.

From the 95 beekeepers that provided samples and colony loss information, 45 of these experienced colony losses more than 20% in Winter 2014/2015 post sampling for disease analysis.

3.6 Discussion

Knowledge of the diseases found in Irish honeybee colonies is very important, in order to understand why losses may occur some years at high rates. The geographic distribution of diseases is also important, as a cluster occurrence of a certain disease in one area may indicate spread of the disease between colonies or may indicate that a certain climate in one part of the county, for example, is causing proliferation of disease.

The prevalence of four honeybee viruses was examined using RT-PCR with primers specific to DWV, CBPV, ABPV and IAPV. The Spring and Summer samples were extracted using a total number of approximately thirty bees per sample, according to section 2.9.2a. This relatively small sample size did not allow for the most effective detection means for ABPV, CPBV and IAPV, which are less prevalent in colonies and also, due to their high virulence, may cause death too rapidly to appear in a sample of bees taken from the hive (Shen *et al.*, 2005; Francis *et al.*, 2013; Chen *et al.*, 2014). In the Autumn sampling, a larger sample size of 60 bees was taken for total RNA extraction, in order to maximize that chance of detecting the less prevalent viruses as was also done in other viral detection studies (Tentcheva *et al.*, 2004; Blanchard *et al.*, 2008; Daughenbaugh *et al.*, 2015). A total sample size of thirty bees was taken for detection of both *Nosema* and tracheal mite using observational techniques, with a sample size of thirty bees also used for detection of *Nosema* by molecular methods.

The first round of sampling was carried out during Spring in May 2014, when the colonies had returned to full function following Winter hibernation. A total of 141 beekeepers returned samples from the random list of 161, with sufficient samples returned to carry out analysis of all of the chosen diseases. DWV was the only virus detected during this sampling round, with 81% of the colonies tested showing a positive result for its presence during RT-PCR. DWV is a single stranded RNA virus that, in the absence of *Varroa*, fails to produce visible symptoms (de Miranda and Genersch 2010). In colonies infected by *Varroa* however, which is almost every colony in countries where *Varroa* have

reached, the symptoms of DWV can be very debilitating, resulting in deformed bees with crippled wings and severely shortened and bloated abdomens (Lanzi *et al.*, 2006) (Martin *et al.*, 2013). Three of the samples tested in the Spring showed positive results for tracheal mite, which is to be expected as the levels of tracheal mite are known to peak through the Winter period (McMullan and Brown 2005). The levels of infection overall, at just over 2% of the total sample number, is very low. The levels of tracheal mites in recent years has dropped, leading to an overall low rate of mortality attributed to the mites (Harrison *et al.*, 2001). The level of tracheal mites had fallen to no detected cases by the Summer sampling. This could be due to the short lived Summer bees, where only one generation per host is possible. *Nosema* was positively detected in 25 colonies out of the total sample number 141 (18% positive) (Figure 3.11b). The 25 colonies were further analyzed to identify the species of *Nosema* that was infecting the sampled colony, with 16 of these testing positive for *N. apis* and 20 testing positive for *N. ceranae*, with the two species co-existing in 12 of the colonies (Figure 3.11a). *N. ceranae* and *N. apis* are microsporidian species with similarities to fungus, and are known to infect *A. mellifera* (Paxton 2010; Martin *et al.*, 2013). *N. apis* was originally the only species to infect the Western honeybee, and rarely lead to the death of the infected colony (Paxton 2010). In 1994 however, *N. ceranae* was discovered and the first confirmed case of its existence in the Western honeybee within its natural habitat in Spain was diagnosed in 1995 (Paxton 2010). During the wide scale survey carried out to identify the causative agents of CCD in honeybee colonies, *N. ceranae* was identified as a possible causative agent of CCD, but was not shown as being significantly present in all collapsed colonies (Cox-Foster *et al.*, 2007). The mode by which *Nosema* infects the host could potentially leave it open to the possibility of additional infection by more spores or by other pathogens, due to the damage caused in the gut (Martin *et al.*, 2013). The high level seen here during the Spring sampling indicates that the colony had a high level of *Nosema* during the overwintering period, which could be problematic for the colony recovering after the hibernation period. Nosemosis is known to cause an increase in hunger in infected bees, which could influence foraging effectiveness in the early Spring when food is already scarce (Higes *et al.*, 2009).

The level of DWV in the Summer had risen to a positive result for 94% of sampled colonies (Figure 3.4). This rise is to be expected, as the level of DWV is correlated with the level of *Varroa*, and Summer is when the levels of *Varroa* peak due to the rise in brood (Martin *et al.*, 2002). This high level of DWV could pose very problematic for a colony, as the higher the viral load within the colony, the higher the prevalence of symptomatic workers unable to carry out foraging effectively due to deformities and reduced mortality (Lanzi *et al.*, 2006; Highfield *et al.*, 2009; de Miranda and Genersch 2010). The level of infection by *Nosema* had fallen however, from 18% to 6% (Figure 3.14). This could be due to the overall healthier conditions within the colony during the Summer (Higes *et al.*, 2006; Huang 2011).

In the final sample, taken in the Autumn of 2014, the RNA was extracted from the larger sample size of approximately 60 bees, as described in Section 2.9.2b for the viral analysis. This larger samples size was taken in the hope of detecting the other viruses that are not as prevalent (Tentcheva *et al.*, 2004). DWV was detected at the highest levels during the Autumn period, with a positive result detected in 96% of the colonies sampled (Figure 3.5). The increased level throughout the year is correlated with the increased level of brood within the colonies, which is to be expected, as increased brood leads to increased levels of *Varroa* infestation (Shen *et al.*, 2005; Yañez *et al.*, 2012). The higher prevalence of DWV in colonies directly before they enter into Winter is worrying, as the health of the colony during this time directly impacts on the survival of the hive through the Winter into full function the following Spring (Roetschi *et al.*, 2008). Interestingly, neither ABPV nor CBPV was detected in the Autumn samples, despite the larger sample size. This could be due to the fact that they were not present in the colonies sampled. It could also be due to the fact that bees infected by these high virulence viruses leave the colony to die, and so are not sampled (Morimoto *et al.*, 2012). IAPV, interestingly, was detected in three out of the 113 colonies (Figure 3.6). This detection, though at a low level, is quite significant, as IAPV is believed to be one of the main causative agents of CCD (Tentcheva *et al.*, 2004; Cox-Foster *et al.*, 2007). IAPV is a dicistrovirus, first identified in Israel in 2004 (Formato *et al.*, 2011). Infected bees first display abnormal shivering of the wings, which progresses into

paralysis and eventual death (Blanchard *et al.*, 2008; Formato *et al.*, 2011). Ninety percent of haemocoel injected bees die within one week under laboratory conditions, with 70 – 80% mortality in bees that are fed with IAPV (Maori *et al.*, 2009). This high mortality rate may help to explain its low detection rates.

The level of *Nosema* had also risen in the Autumn sampling, compared with the Summer prevalence, with 12% of all colonies sampled testing positive for the disease (Figure 3.13). The species detection within this Autumn group showed that *N. ceranae*, the species most associated with the collapse of colonies in the Winter, was detected in 91% of the samples, either alongside *N. apis* or alone. Such a high occurrence of *N. ceranae* during the overwintering period may have detrimental effects on the health of the colony, and may negatively impact its survival through Winter (Higes *et al.*, 2009; Paxton 2010). The damage caused by *Nosema* to the gut, and its effect on the bees which includes infective dysentery, means that its presence in the colony during Winter may lead to further infection by other pathogens, or a spread of large numbers of *Nosema* spores around the colony (Higes *et al.*, 2009; Martin *et al.*, 2013).

The relationship between the diseases found in Irish honeybee colonies and colony loss was also examined. A total of 95 beekeepers gave samples for each of the sampling dates and also provided colony loss information for the year. This meant that the seasons leading up to the Winter of 2015 could be examined for any pattern of disease that may lead to a higher loss of colonies. The samples were split into two groups - apiaries that had experienced more than 20% loss in Winter 2014/2015 (Figure 3.15) and apiaries that had experienced less than 20% loss in Winter 2014/2015 (Figure 3.14). There was no major difference between any of the disease groups that had lost more than 20% of their colonies compared to the diseases found in the apiaries that had lost less than 20% of their colonies. The diseases detected in the Autumn immediately before the Winter of the recorded colony losses showed no significant difference between the types or levels of diseases found in healthy apiaries versus apiaries that experienced a high level of loss. The level of DWV was actually higher in the group of colonies that experienced less loss, at a rate of 84% prevalence compared to 67% in the colonies that experienced a higher loss. The number of

colonies free from diseases in the apiaries that experienced more than 20% loss was 9% of the total number, compared to 6% of the colonies that experienced less than 20% loss, which is unusual. The number of colonies infected by *Nosema* alone was 0% for both the low colony loss and the high loss groups in Autumn (Figure 3.13 and Figure 3.14). The only increase in disease prevalence in the group that experienced a higher loss of colonies was with regard to samples that had both DWV and *Nosema* infections (Figure 3.14). The colonies that experienced a low loss had a double infection in 8% of apiaries sampled, whereas the colonies that experienced a loss of more than 20% had a double infection in 15% of the colonies. DWV has been linked with CCD, found at higher levels in colonies which has collapsed than in healthy colonies (Highfield *et al.*, 2009). DWV has also been linked with CCD when found existing alongside other honeybee diseases, such as ABPV (Roetschi *et al.*, 2008). A synergistic effect of a combined DWV and *N. ceranae* was not proven during a study of the combined infection of the diseases on a colony however, when bees were inoculated with *Nosema* spores and examined for DWV levels to see if there was an increase (Martin *et al.*, 2013; Zheng *et al.*, 2015). As previously mentioned however, *Nosema* causes damage to the bee gut, and could facilitate the spread of DWV and other pathogens as the lining of the gut is one of the main barrier defenses in the bee immune system (Evans *et al.*, 2006). A double infection could also simply weaken the individual bee at a higher rate than infection by a single disease, which could impact on the vitality of the whole colony, as individual fitness is important for the recovery of the complete colony in the Spring (Higes *et al.*, 2009).

Overall, there were no significant disease markers from the study, which indicated a possible link with colony loss, but other external factors that can influence the survival of a colony must also be taken into consideration. The variations in weather could also be a major factor for the high losses of colonies in Winter 2014/2015, which was not taken into consideration here. A late Spring means later foraging for bees, which can be detrimental to the survival of the colony, as it takes much longer for it to reach full function again. This may help to explain the much higher losses that occurred in Winter 2014/2015 (Schmickl and Crailsheim 2002). Variations in the food supply to sustain the colonies over

Winter could also be a factor in larger losses seen in colonies, which vary from year to year. This, when combined with a long Winter and late flowering in the Spring could be detrimental to a colony, regardless of disease presence or absence in the colony.

The results presented here are a snapshot of the diseases found in a single year, and could not provide any concrete evidence towards understanding the factors affecting colony loss. The prevalence of the various diseases through the season is interesting, as it show that, for example, during times of high levels of brood, diseases such as DWV flourish. It was also interesting to see the high levels of *N. ceranae* present in Irish colonies which further supports the theory that *N. ceranae* is replacing *N. apis* in *A. mellifera* colonies worldwide (Milbrath *et al.*, 2015). The detection of IAPV was the first recorded incidence in the Republic of Ireland, which is also a significant finding. Further work taking an even larger sample size, or perhaps just sampling visibly deformed bees from the colony could be preformed to detect the other viruses.

Chapter Four

Assessment of Bayvarol
resistance in *V. destructor*
through analysis of
differential proteomic
profiles of sensitive and
resistant mites

4.1 Bayvarol resistance in *V. destructor* - implications for the control of *V. destructor* and examination of possible mechanisms for the development of resistance.

Varroa destructor (Anderson and Trueman 2000) is one of the most serious threats to the Western honeybee, *Apis mellifera*, which is a pollinator of great economic importance. *Varroa* are not a natural parasite of *A. mellifera* but shifted host from *Apis cerena* in the early years of the twentieth century (Gómez-Moracho *et al.*, 2015). Due to this recent host shift, the host-parasite relationship is imbalanced with the result that *Varroa* has negatively impacted on *A. mellifera* populations. *Varroa* are obligate parasites, feeding on the haemolymph of both larvae and adults leading to a weakening of the health and vitality of the individual bee and colony due to the transmission of pathogens and viruses (Shen *et al.*, 2005; Yang and Cox-Foster 2005; Navajas *et al.*, 2008; Di Prisco *et al.*, 2011).

The most widely used chemical acaricides against *V. destructor* are the pyrethroids tau-fluvalinate and flumethrin, licensed under the trade names Apistan (Mavrik) and of Bayvarol (Bayer), respectively (Rosenkranz *et al.*, 2010). First introduced in the 1980s they were very effective in controlling *Varroa* infestation with relatively few deleterious effects on the colony (Thompson *et al.*, 2002). Resistance in *V. destructor* to pyrethroid acaricides has emerged in recent years (Mozes-Koch *et al.*, 2000; Tan *et al.*, 2007; Maggi *et al.*, 2009). The main mechanisms employed by an organism in order to become resistant to a chemical are grouped into three main methods: Target site alteration of the chemical to prevent binding (e.g. alteration to the shape of sodium channel), an increased ability to deal with the effects of the chemical at a cellular level (such as an increased ability to deal with the oxidative stress caused by the chemical), and finally, structural alterations to reduce penetration of the chemical through the cuticle or epithelial lining of the digestive tract, which is the least understood mechanism and is thought to often act in combination with the other two mechanisms (Kasai *et al.*, 2014).

The aim here was to compare the proteomic profile of Bayvarol resistant mites to the profile of Bayvarol sensitive mites and look for any changes to the proteome that may be conferring resistance.

4.2 Analysis of variations in the proteomic profile of Bayvarol sensitive and resistant mites resolved using 1-Dimensional SDS-PAGE

Mites were collected from various areas in Ireland from colonies that were sensitive to Bayvarol, or with a level of observed resistance above 50% (Table 2.2). Protein was extracted from mites as described (Section 2.10.5) and resolved by 1-Dimensional SDS PAGE. A total of seven bands were identified by Image J densitometry as being differentially expressed (2.12.2), and these bands were cut and digested for peptide identification by LC-MS (2.13). 1-Dimensional analysis comparing the proteomic profile of resistant and sensitive *Varroa* was used initially to give a preliminary overview of any changes and whether they might be significant in conferring the resistant phenotype. All of the differentially expressed bands were shown to be present in higher abundance in Bayvarol resistant *Varroa* (Table 4.1). Heat shock protein 70kDa was identified as being present at higher abundance in the resistant *Varroa* (Band 3; 2.82 fold increase; Figure 4.2). This protein is involved in protein folding as a molecular chaperone (Becker and Craig 1994). An additional heat shock protein, a 90kDa heat shock protein was also present at higher levels of abundance in the resistant *Varroa* (Band 5; 3.62 fold increase; Figure 4.2). Actin 5C, which is a protein involved in structural arrangement within the cell (Kabsch and Vandekerckhove 1992), was present at higher abundance in the mites that were deemed resistant to Bayvarol (Band 7; 1.82 fold increase; Figure 4.2). Histamine release factor (Band 1; 1.80 fold increase; Figure 4.2) was identified from band 1, and showed an increased level of abundance in the mites that were resistant to Bayvarol. Histamines release factors release histamine from receptors that are chloride channels, which function as inhibitors of neurons (Bartley *et al.*, 2009). Glutamate receptor 1-like was also at a higher level of abundance in the Bayvarol resistant *Varroa* (Band 2; 4 fold increase; Figure 4.2). This protein is involved in the transmembrane transfer of an ion by a channel that opens when extracellular glutamate has become bound by the channel complex (Pin and

Duvoisin 1995). A protein which exhibits oxidoreductase activity, glyceraldehyde 3-phosphate dehydrogenase, was also present at higher abundance in the Bayvarol resistant *Varroa* (Sirover 1999) (Band 6; 2.18 fold increase; Figure 4.2). Finally, a mitochondrial processing peptidase was identified from band 4 was also present at higher levels in the resistant *Varroa*. This exhibits hydrolase activity and is also involved in protein processing (Gakh *et al.*, 2002).

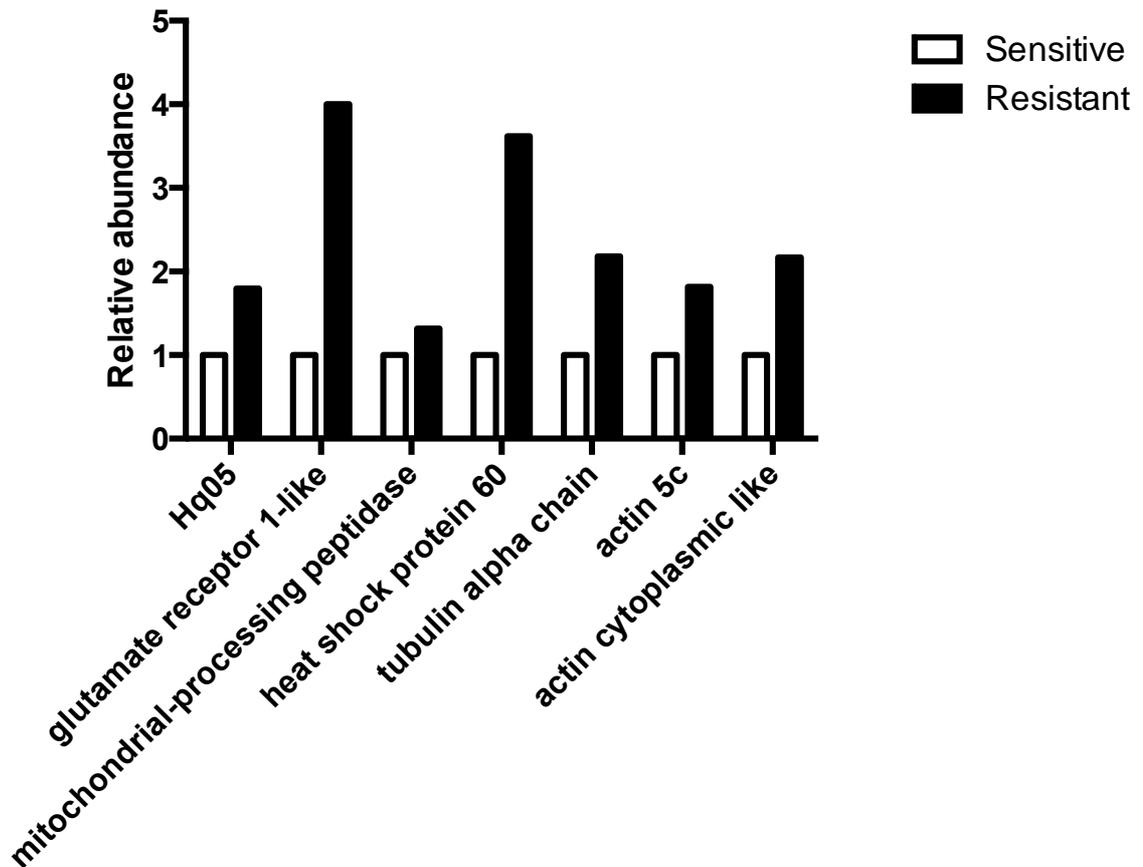


Figure 4.2 Relative fold changes in abundance of protein bands identified as being differentially abundant in Bayvarol resistant *Varroa*.

Bar chart showing the relative fold expression of the proteins that were identified as differentially expressed between the proteomic profile of Bayvarol sensitive and resistant *Varroa*. All proteins shown were significantly differentially abundant at a level of $p < 0.05$.

Band Number	Protein annotation	M _r	pI	% Coverage	Protein Identity	Relative abundance of protein		Function
						Sensitive	Resistant	
1	Histamine release factor	10968	4.60	8%	gi 195972560	1	1.80	Histamine release factors release histamine from receptors that are chloride channels that function as inhibitors of neurons.
2	Glutamate receptor 1-like	26981	9.75	38%	Vdkk00112310_940	1	4.00	Transmembrane transfer of an ion by a channel that opens when extracellular glutamate has been bound by the channel complex or one of its constituent parts.
3	Heat shock protein 70	70563	5.64	19%	gi 380014538	1	2.82	Chaperone involved in protein folding.
4	Mitochondrial-processing peptidase	54182	5.61	21%	gi 241043304	1	1.32	Hydrolase activity Also protein processing involved in protein targeting to mitochondrion.
5	Heat shock protein 90	92666	5.32	10%	gi 241998026	1	3.62	Chaperone involved in protein folding.
6	Glyceraldehyde 3-phosphate dehydrogenase	44571	9.62	8%	gi 442753619	1	2.18	Has oxidoreductase activity.
7	Actin 5c	42218	5.29	34%	gi 39133468	1	1.82	Cytoskeletal arrangement and tubule formation.

Table 4.1 Identities of proteins that showed differential abundance in expression between Bayvarol resistant and sensitive *Varroa*.

Table shows the list of proteins that were identified from 1-Dimensional SDS-PAGE gel electrophoresis of the proteomic profile of resistant versus sensitive *Varroa* (Figure 4.1). Fold changes for bands were calculated using Image J software (2.12.2).

4.3 Characterization of *Varroa* proteome using 2-Dimensional SDS-PAGE analysis and LC-MS

In order to see what changes may be happening at the proteomic level in Bayvarol resistant mites that could be contributing to the resistant phenotype, the proteome produced using 2-Dimensional resolution was identified. Protein was extracted from sensitive *Varroa* (2.9.3). The protein extractions were carried out in triplicate and 400µg was separated in the first dimension by isoelectric focusing before resolution by size on 13cm 12.5% acrylamide gels (2.11.5). Visible spots were cut from the gel, destained and trypsin digested before identification by LC-MS (2.13). A total of 67 spots were successfully identified from the gel (Figure 4.3). Some identities appear repetitively due to the presence of isoforms of the same protein appearing at different size or pH points following isoelectric focusing and separation by size on an SDS-PAGE gel (Table 4.2). A range of various types of proteins were identified from the gel, such as a group of heat shock proteins involved in the refolding of proteins during times of stress and unraveling (Becker and Craig 1994) (Spot 4, 5, 6, 7). A number of detoxification enzymes were also identified from the 2-Dimensional proteome which are involved in cell detoxification and normal metabolic process within the cell such as aldehyde dehydrogenase (Spot 14), succinyl CoA ligase beta (Spot 18), and enolase-like (Spot 25) (Hannaert *et al.*, 2003; Rothacker and Ilg 2008). A number of structural proteins involved in cell structure and muscle structure were also identified from the 2-Dimensional proteome such as actin (Spot 24), and tubulin beta (Spot 22) (Kilmartin and Adams 1984, Kabsch and Vandekerckhove 1992). Proteins involved in the structure of the cuticle of *Varroa* were also identified from excised spots from the 2-Dimensional gel, such as spot 59 (Cuticle protein 10.9) (Andersen *et al.*, 1995).

4.4 Comparative 2-Dimensional gel electrophoresis of proteomic profile of Bayvarol sensitive mites versus Bayvarol resistant mites

Resistant and sensitive mites were collected from apiaries with a known level of resistance to Bayvarol (Table 2.2). Protein was extracted from samples in triplicate (2.9.5) and 400µg was resolved using SDS-PAGE on the second dimension (2.13). Spots of interest that showed differential expression between the sensitive and the resistant mites were identified using Progenesis SameSpots software (2.12.3). These spots of interest were excised, destained and trypsin digested for analysis by LC-MS (2.14). A total of 11 spots were identified as being differentially expressed between the Bayvarol sensitive (Figure 4.4) and resistant *Varroa* (Figure 4.5). These proteins were all found to be present at higher levels of abundance in the resistant mites (Table 4.3). An increase in abundance of proteins involved in detoxification was observed in the Bayvarol resistant mites including Glutathione-s-transferase (Spot 3; 2.8 fold increase; $p = 0.020$), aldehyde dehydrogenase (Spot 2; 5.9 fold increase; $p = 0.002$), and retinal dehydrogenase (Spot 1; 6.1 fold; $p = 0.01$) (Claudianos *et al.*, 2006; Rothacker and Ilg 2008). Proteins with transferase activity were also shown to be present at significantly higher levels of abundance: spermidine synthase-like (Spot 8; 3.9 fold; $p=0.029$), Glutathione-s-transferase mu 1-like (Spot 8; 2.8 fold; 0.025) (Claudianos *et al.*, 2006; Wen *et al.*, 2010; Gomes *et al.*, 2012). Inorganic pyrophosphatase (Spot 6; 6.6 fold; 0.037) was present in substantially higher levels in resistant *Varroa*. Inorganic pyrophosphatase has been shown to have transferase activity and is involved in ion binding (Gomes *et al.*, 2012).

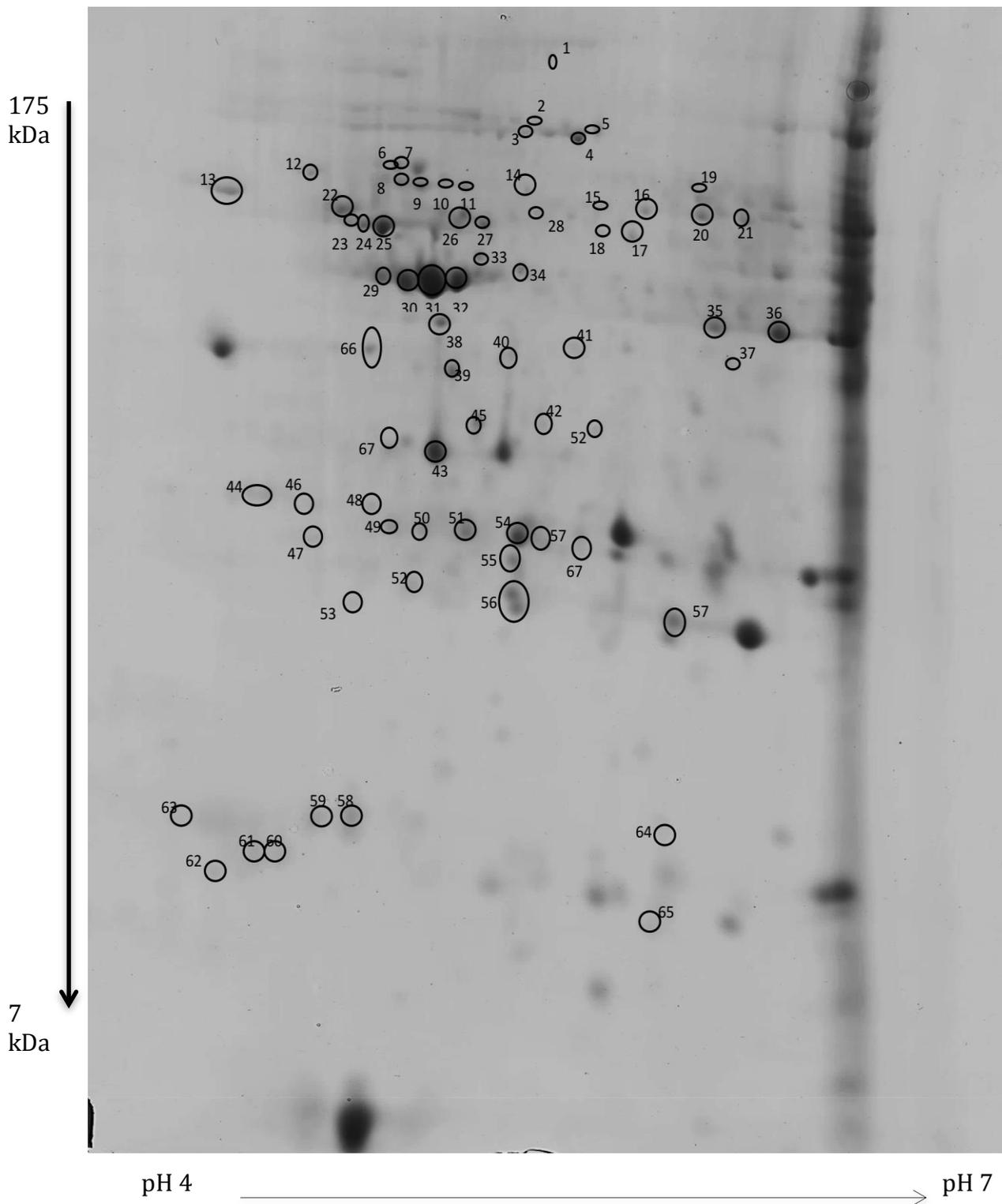


Figure 4.3: Representative gel image highlighting protein spots identified from *Varroa* proteome. 2-Dimensional SDS-PAGE gel showing spots that were identified by LC-MS. Protein was extracted from *Varroa* that were sensitive to Bayvaol as described and the spots excised and identified by LC-MS (Table 4.2).

Spot	Protein Annotation	Coverage (%)	M _r	pI	Protein Identity
1	Myosin heavy chain, muscle like isoform 2	10.2	15801.9	10.05	VDK00104201-1037_4
2	Heat shock 70kDa protein isoform 1	20.4	20488.3	9.6	VDK00109667-971_2
3	Aldehyde dehydrogenase	35.4	13788.8	9.19	VDK00178282-436_2
4	Heat shock cognate 71kDa protein-like	35.6	23811.5	9.3	VDK00085495-1293_2
5	Heat shock cognate 71kDa protein-like	62.6	14006.7	10.15	VDK00072177-1513_1
6	HSP 60kDa	33.1	25236.6	5.02	VDK00038595-2347_2
7	Hsp 60 chaperonin subunit	25.8	30316.2	5.47	VDK00038595-2347_1
8	Vacuolar h+ ATPase subunit B-like	13.8	20857.8	6.84	VDK00038901-2336_3
9	Tubulin alpha 1-c like	13.2	12738.1	5.37	VDK00026505-2846_1
10	V type protein ATPase subunit B-like	19.1	20857.8	6.84	VDK00038901-2336_3
11	Alpha tubulin	8.7	42430.2	5.14	VDK00073905-1481_1
12	Protein disulphide isomerase like	11.9	40773.8	5.29	VDK00090792-1216_1
13	Beta tubulin	13.2	21654.7	4.58	VDK00004132-5341_1
14	Aldehyde dehydrogenase	7.1	43780.3	9.38	VDK00013090-3792_2
15	Retinal dehydrogenase 1-like	38.4	21008.5	5.59	VDK00013090-3792_3
16	RAB GDP dissociation inhibitor beta	8.7	33002.3	6.75	VDK00028364-2755_3
17	Dihyrolipoyllysine residue succinyltransferase component of 2 oxoglutarate dehydrogenase complex rab GDP	26.6	32019.8	9.12	VDK00002106-6249_5
18	Succinly CoA ligase subunit beta	21.1	10268.9	10.75	VDK00016774-3448_3
19	Chaperonin subunit putative	31.1	46025.4	6.08	VDK00035897-2442_1
20	Thioredoxin reductase 2	35.4	55788.8	9.19	VDK00008303-4400
21	Dihyrolipolyllsine residue succinyltransferase compact of 2 oxoglutarate dehydrogenase complex, mitochondrial like	13.9	32019.8	9.12	VDK00002106-6249_5
22	Tubulin beta variant 1	46.9	17032.5	10.16	VDK00086014-1286_1
23	ATP synthase subunit beta	24.8	25683.1	6.26	VDK00075236-1459_1
24	Actin	5.8	42086.5	5.39	VDK00021889-3094_3
25	Enolase-like	6.6	41101.5	6.5	VDK00029529-2704_4

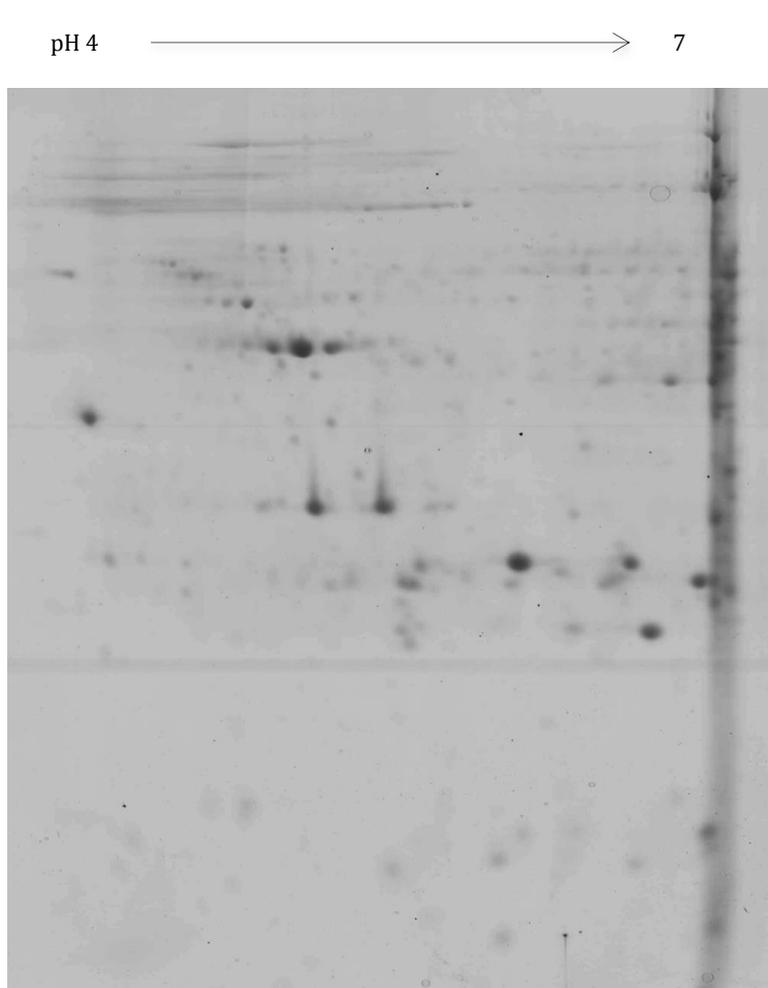
26	60 kDa HSP	36.4	10283.9	10.65	VDK00002992-5780_5
27	Mitochondrial processing peptidase subunit beta like	27.8	15245	11.31	VDK00053186-1918_7
28	Retinal dehydrogenase 1-like	23.9	43780.3	9.38	VDK00013090-3792_2
29	Actin	22.7	42086.5	5.39	VDK00021889-3094_3
30	Actin	53.1	42086.5	5.39	VDK00021889-3094_3
31	Actin	23	42086.5	5.39	VDK00021889-3094_3
32	Actin	41.7	42086.5	5.39	VDK00021889-3094_3
33	Actin	14.5	42086.5	5.39	VDK00021889-3094_3
34	Stress 70 protein, mitochondrial like	21.1	62406.9	8.69	VDK00024521-2947_1
35	Glutamate receptor 1-like	30.7	26981.7	9.75	VDK00112310-940_1
36	Arginine kinase receptor	13.9	16327.3	6.38	VDK00064495-1661_3
37	Glyceraldehyde-3-phosphate dehydrogenase 2-like	11.2	13966.6	5.23	VDK00031278-2628_1
38	Isocitrate dehydrogenase subunit alpha	34.4	19339.7	4.68	VDK00000572-7968_7
39	Isocitrate dehydrogenase subunit alpha, mitochondrial	8.4	19339.7	4.68	VDK00000572-7968_7
40	Inorganic pyrophosphate like	23.1	16026.8	6.51	VDK00046011-2113_1
41	Aldehyde dehydrogenase	14.2	42086.5	5.39	VDK00021889-3094_3
42	Inorganic pyrophosphate like	32.6	16026.8	6.51	VDK00046011-2113_1
43	Stress 70 protein, mitochondrial like	21.1	62406.9	8.69	VDK00024521-2947_1
44	14-3-3 protein zeta-like	46.8	11171.6	8.94	VDK00029768-2692_5
45	Spermidine synthase like	16.4	14147.8	4.7	VDK00000751-7624_4
46	14-3-3 protein zeta-like	24.3	17774.2	9.66	VDK00029295-2713_2
47	Glutamate receptor (also arginine kinase)	7.7	26981.7	9.75	VDK00112310-940_1
48	Proteosome subunit alpha type 5-like	52.6	10660.2	5.91	VDK00025009-2923_3
49	Phosphatidylethanolamine-binding protein homolog f40a33	12.6	11445.7	9.19	VDK00004857-5136_4
50	Actin cyto 2 like	10.8	42086.5	5.39	VDK00021889-3094_3
51	Glutathione-s-transferase mu 1-like	10	13060.5	5.86	VDK00010469-4095_3
52	Actin cyto 2 like	18.2	42086.5	5.39	VDK00021889-3094_3
53	Peroxioredoxin	23.9	16361.2	7.2	VDK00006432-

					4757_5
54	Glutathione-s-transferase mu 1-like	10	13060.5	5.86	VDK00010469-4095_3
55	Glutathione-s-transferase mu 1-like	29	13060.5	5.86	VDK00010469-4095_3
56	Peroxiredoxin-6-like	29.8	11310.1	4.9	VDK00052934-1925_1
57	Uncharacterised protein loc100903770	44.2	26556.6	6.31	VDK00015350-3569_2
58	Thymosin repeated protein 1	11.6	12861.6	9.11	VDK00005248-5037_6
59	Cuticle protein 10.9 like	7.4	18272.8	4.7	VDK00052526-1935_5
60	Myosin light chain alkali-like	25.5	10691.4	9.1	VDK00054017-1897_2
61	Cuticle protein 10.9 like	14.3	18272.8	4.7	VDK00052526-1935_5
62	Sex regulated protein janus like	18.8	12371.9	7.24	VDK00004901-5122_4
63	Myosin regulatory light chain 2 like	41.3	10293.5	4.27	VDK00011581-3955_3
64	LOC100907454 Secreted protein putative	20.1	18963.3	7.11	VDK00000732-7662_12
65	Ubiquitin conjugating enzyme e2 n-like	11.8	14620.7	8.18	VDK00116680-893_1
66	Conserved hypothetical protein	11	39129	4.73	gi 170596716
67	Hypothetical protein CAEBREN-23827	4	80515	8.37	gi 341900744

Table 4.2 Identified proteome from *Varroa* resolved by 2-Dimensional SDS PAGE.

Table showing the identities of all spots that were positively identified by LC-MS, and the coverage, PI, molecular weight and accession number for each spot.

Sensitive (a)



Resistant (b)

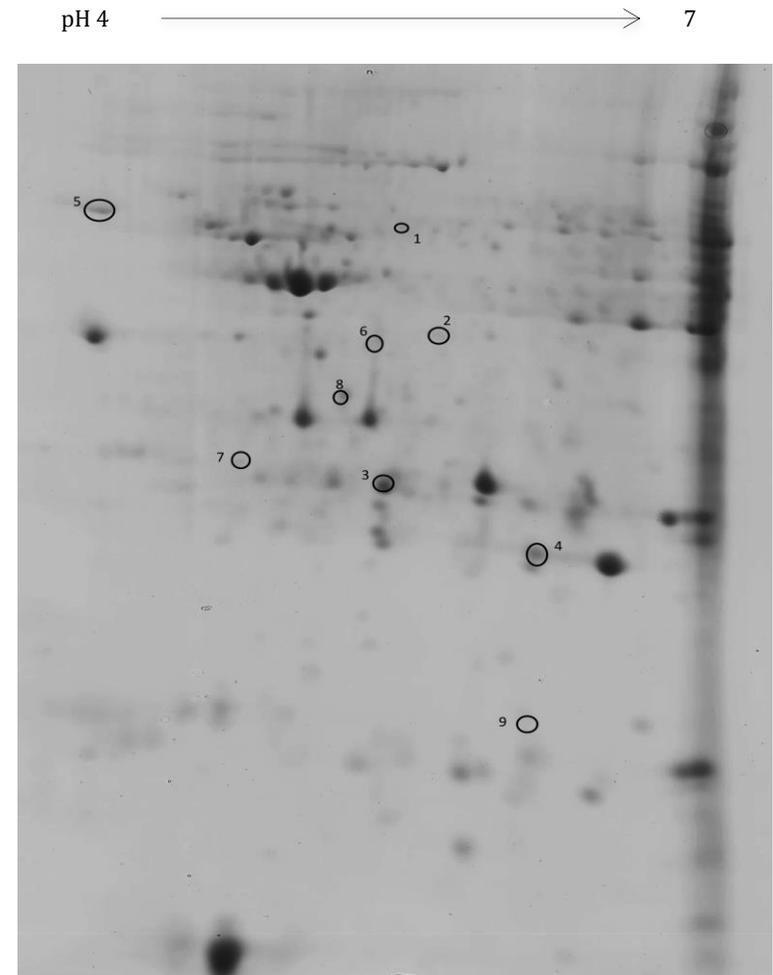
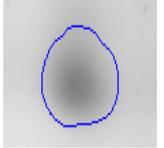
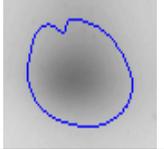
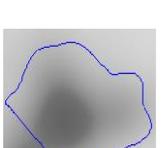
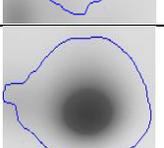
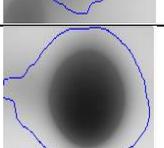
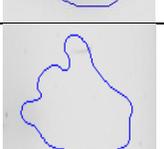
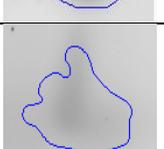
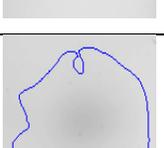
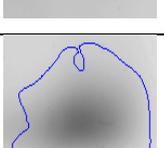


Figure 4.4: 2-Dimensional comparison of Bayvarol sensitive and resistant *Varroa*.

Proteomic profile of Bayvarol sensitive (A) and Bayvarol resistant (B) *Varroa* mites. Proteins were extracted from *Varroa* as described and resolved by 2D SDS-PAGE (n=3). Proteins showing alterations in abundance between the sensitive and resistant mites were excised and identified by LC-MS (2.14).

Spot number	Protein Annotation	M _r	pI	Progenesis Images		% Coverage	Protein Identity	Fold change in abundance		ANOVA p value (<0.05)	Protein function
				Sensitive	Resistant			Sensitive	Resistant		
1	Retinal dehydrogenase 1-like	43780.3	9.38			23.9	VDK0001309 0-3792_2	1	6.1	0.017	Oxidoreductase with xenobiotic metabolism role
2	Aldehyde dehydrogenase	42086.5	5.39			14.2	VDK0002188 9-3094_3	1	5.9	0.003	Oxidoreductase. Overexpressed in pyrethroid resistant <i>Aedes aegypti</i>
3	Glutathione-s-transferase mu 1-like	13060.5	5.86			10	VDK0001046 9-4095_3	1	2.8	0.025	Transferase activity Expressed at higher levels in many insecticide resistant species
4	Uncharacterized protein LOC100907454	18963.3	7.11			35.4	VDK0000073 2-7662_12	1	1.8	0.035	Function Unknown
5	Beta Tubulin	21654.7	4.58			13.2	VDK0000413 2-5341_1	1	3	0.024	Constituent of cytoskeleton involved in microtubule process
6	Inorganic pyrophosphatase	16026.8	6.51			20.1	VDK0004601 1-2113_1	1	6.6	0.037	Hydrolase activity and ion binding.

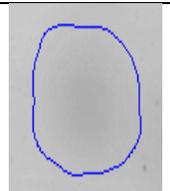
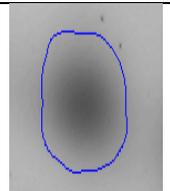
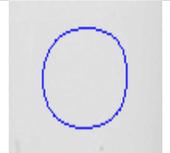
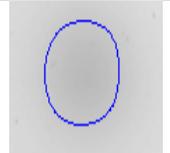
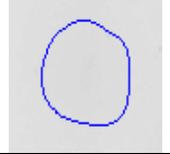
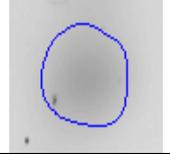
7	Proteasome subunit alpha type 5-like	10660.2	5.91			11	VDK0002500 9-2923_3	1	2.3	0.040	Involved in Ubiquitin dependant protein catabolic process
8	Spermidine synthase like	14147.8	4.7			52.6	VDK0000075 1-7624_4	1	3.9	0.029	Transferase involved in small molecule metabolism
9	Uncharacterised protein loc100903770	26556.6	6.31			16.4	VDK0001535 0-3569_2	1	1.4	0.013	Function Unknown

Table 4.3 Protein identities of differentially abundant spots from comparison of 2-Dimensional proteomic profile of Bayvarol sensitive and resistant mites.

Table of proteins that were identified as being differentially expressed by Progenesis SameSpots. Spots were excised for identification by LC-MS as described (2.14). Relative fold expression of identified spots is also shown in Figure 4.5.

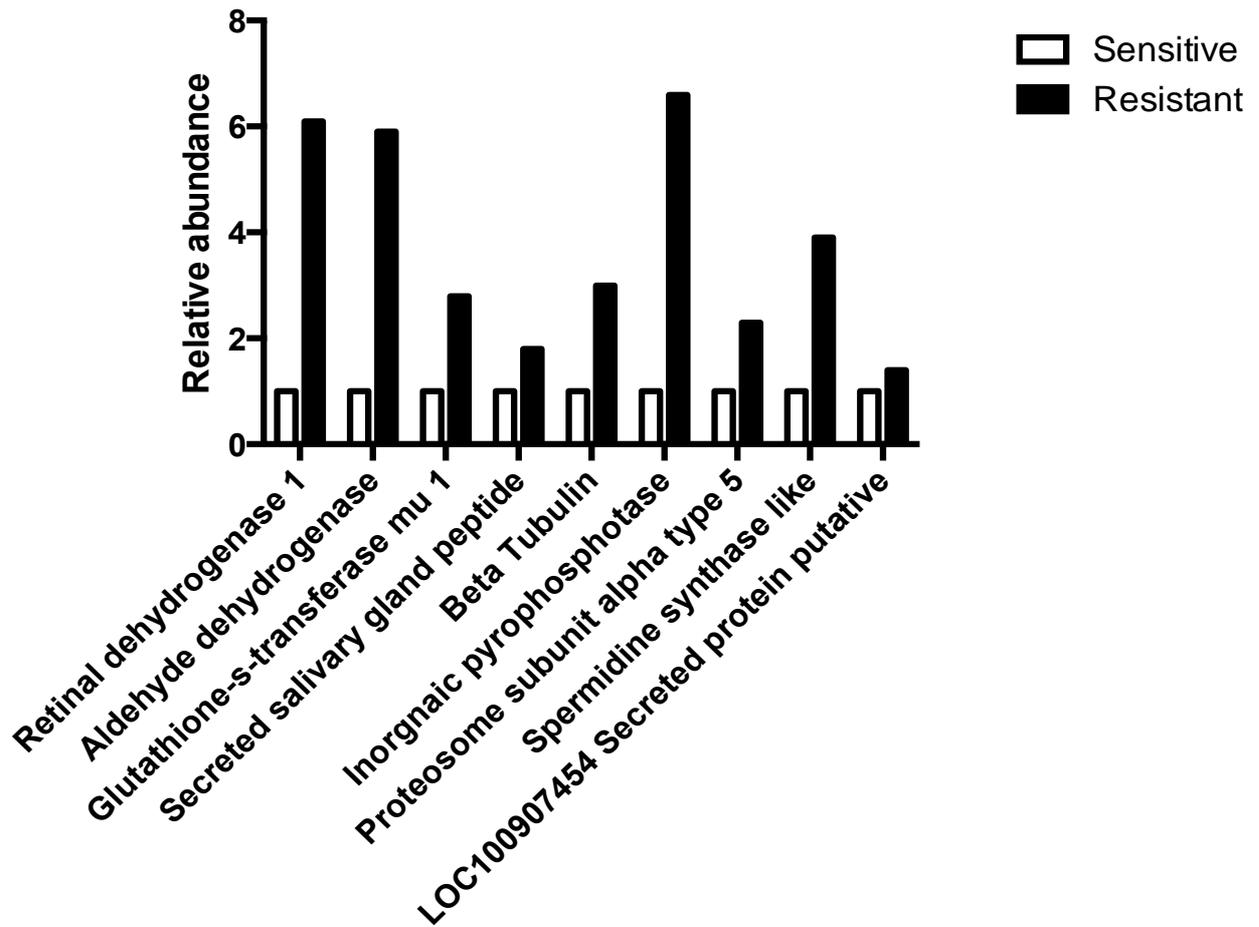


Figure 4.5 Relative fold expression of proteins identified as differentially abundant in resistant *Varroa*.

The relative fold expression of the nine spots that showed significant variation in abundance between the sensitive and resistant proteomic profile resolved by 2-Dimensional SDS-PAGE.

4.5 Comparative analysis of the proteomic profiles of Bayvarol sensitive and Bayvarol resistant *Varroa* using Label free shotgun proteomics

Recent advances in mass spectrometry-based proteomics and the increasing availability of genomic and transcriptomic resources are now making it possible to investigate global system level changes in an organism's proteome (Ozsolak and Milos, 2011). Quantitative expression proteomics permits the comparison of the proteomes of phenotypically different or experimentally treated organisms to identify either proteins, molecular processes or biological pathways that may regulate or even contribute directly to the phenotype or characteristic itself. Label free mass spectrometry measures the relative abundances of thousands of proteins across multiple sample groups in single mass spectrometry runs (Bantscheff *et al.*, 2012). Here, label free proteomics was used to identify variations in expression of proteins between the Bayvarol sensitive and resistant *Varroa* (2.14).

In total, 3757 peptides were identified representing 650 proteins with two or more peptides and 137 proteins were determined to be differentially abundant (ANOVA $p < 0.05$) at a fold change of >1.5 . Eighty nine proteins found in higher abundance in the resistant mites (Table 4.4) and forty five were found in higher abundance in the sensitive mites (Table 4.5). A total of 41 proteins were present in all three resistant samples and absent in all three sensitive mites samples, and a further 4 were present in each replicate of the sensitive mites and absent in all three of the resistant samples. These proteins were termed as “exclusively expressed proteins” (Table 4.6). These protein hits were also used in the statistical analysis of the total differentially expressed group following imputation of the zero values using a number close to the lowest value of the range of proteins plus or minus the standard deviation. After data imputation these proteins were included in subsequent statistical analysis (Tables 4.4 and 4.5).

The top proteins changed in abundance in the comparison of the profile of sensitive and resistant *Varroa* are shown in Figure 4.6. The top twenty proteins present in the highest abundance and the bottom twenty present in the lowest

abundance in resistant *Varroa* are highlighted in the volcano plot, with the proteins appearing on the right hand side of the red box present in higher levels in resistant mites, at a fold difference $\text{Log}(2)$. The proteins appearing on the left hand side of the plot (Figure 4.6) are present in lower levels of abundance in resistant mites.

A number of related proteins were observed at similar abundance levels including a number of aldehyde dehydrogenases, myosin, heat shock proteins, and Na^+/K^+ subunit proteins which were of higher abundance in the resistant *Varroa* (Figure 4.7). Numerous proteins involved in cuticle structure and ribosomal associated proteins were found at higher abundance in Bayvarol sensitive *Varroa* (Figure 4.7).

The Blast2GO annotation software (www.blast2GO.com) was used to group proteins based on conserved gene ontology (GO) terms in order to identify processes and pathways potentially associated with Bayvarol resistance or sensitivity. GO terms were categorized by biological processes (BP; Figure 4.8a) and molecular function (MF; Figure 4.8b). No major changes were evident between the biological process profiles of resistant and sensitive mites, with the most noticeable change being in the proportion of proteins involved in single-organism metabolic processes, which accounted for 3% of the overall amount of proteins in the sensitive mites but 12% of the protein composition of resistant mites. A comparison of the overall proportion of proteins found in sensitive and resistant mites, grouped based on molecular function assignment is also presented (Figure 4.8a). The largest groups in resistant *Varroa* compared to sensitive *Varroa* were the proteins collectively involved in carbohydrate derivative binding, small molecule binding, ion binding, and oxidoreductase activity. Proteins involved in cuticle and ribosome structure were present at lower levels in the resistant mites (Figure 4.8b).

Using KEGG pathway analysis, pathways involving proteins of differential abundance between the sensitive and resistant mites were examined for possible association with the resistant phenotype. The P450 metabolic pathways involved in xenobiotic detoxification was one pathway which showed differences between

the sensitive and the resistant mites (Figure 4.9) with a number of enzymes found at higher levels in the resistant mites (Figure 4.10).

Protein annotation	Protein Identity	Peptides	Sequence coverage [%]	PEP	Overall Intensity	Abundance in resistant <i>Varroa</i>
Myosin-9	VDK00026364-2854_3	3	46.2	7.71E-110	6.39E+09	18.9
Methylmalonate-semialdehyde dehydrogenase	VDK00104201-1037_3	5	47.6	7.23E-99	2.73E+09	12.5
Isocitrate dehydrogenase	VDK00122566-831_1	4	65.2	1.48E-50	1.62E+09	7.2
Myosin-9	VDK00008068-4440_1	5	51.9	4.82E-49	1.60E+09	6.6
Alpha tubulin	VDK00049208-2024_1	6	42	1.62E-254	1.52E+09	6.5
Myosin-9	VDK00036026-2438_2	8	40.9	1.04E-120	2.45E+09	6.4
Ankyrin 2 3/unc44	VDK00019466-3251_1	8	36.3	2.49E-106	9.77E+08	6.0
Myosin heavy chain	VDK00038284-2357_8	9	46.7	2.36E-167	1.91E+09	5.8
Methylenetetrahydrofolate dehydrogenase	VDK00034029-2514_6	9	26.3	6.77E-162	1.29E+09	5.4
T-complex protein 1 subunit beta	VDK00057971-1803_3	6	38.9	2.62E-70	9.77E+08	5.1
Actin-5C	VDK00036770-2410_2	6	22.6	5.32E-39	1.86E+10	5.0
Aldehyde dehydrogenase	VDK00058502-1791_1	4	44.8	2.34E-54	1.99E+09	5.0
Myosin-9	VDK00014752-3623_3	10	24.8	2.03E-42	8.28E+08	4.4
Spermidine synthase	VDK00053241-1917_1	2	20.8	5.17E-09	1.07E+09	4.4
Quinone oxidoreductase	VDK00090651-1219_5	3	30.8	2.62E-51	1.08E+09	4.3
Chaperonin subunit	VDK00013155-3784_6	10	35	2.08E-95	9.34E+08	4.1
Translation elongation factor	VDK00090156-1226_2	4	38.4	1.63E-91	2.02E+09	4.1
Paxillin	VDK00066785-1616_1	9	43.2	3.60E-114	1.32E+09	3.8
Myosin heavy chain skeletal muscle	VDK00073615-1486_1	13	55	4.82E-194	3.78E+09	3.6
Cystathionine beta-lyase	VDK00005734-4912_2	2	67.7	1.03E-58	5.42E+08	3.6
Alcohol dehydrogenase	VDK00041240-2258_4	5	37.3	1.96E-142	1.56E+09	3.4
Succinate dehydrogenase	VDK00021117-3143_2	4	24.3	2.23E-37	8.40E+08	3.4
Spermatogenesis associated factor	VDK00134345-717_1	4	49.7	9.82E-49	8.28E+08	3.3
Peptidyl-prolyl cis-trans isomerase	VDK00057545-1813_1	3	61.5	1.15E-80	9.63E+08	3.3
Paramyosin	VDK00053691-1905_2	16	36	6.58E-274	9.38E+09	3.3
Sumo-1-activating enzyme E1a	VDK00137318-691_3	12	28	3.37E-146	1.69E+09	3.3

Aconitase	VDK00102107-1062_2	9	63.8	6.87E-234	5.56E+09	3.2
Vinculin	VDK00003663-5513_4	4	13.9	1.55E-22	6.61E+08	3.2
Myosin heavy chain skeletal muscle	VDK00001154-7071_12	14	64.2	1.81E-138	2.89E+09	3.1
Iron-containing alcohol dehydrogenase	VDK00000751-7624_3	5	40.5	2.27E-65	2.60E+10	3.0
Flavin-containing monooxygenase	VDK00030409-2665_2	4	36.3	3.64E-62	9.60E+08	2.8
Alpha-actinin-4	VDK00035897-2442_1	6	22.3	2.58E-45	7.48E+08	2.7
Myosin-9	VDK00026299-2857_6	6	51.6	5.00E-172	8.62E+09	2.7
Myosin heavy chain	VDK00003490-5577_1	16	50.7	6.15E-164	4.06E+09	2.7
Tubulin alpha-3 chain	VDK00039125-2328_4	4	55.4	5.66E-77	1.95E+09	2.7
60S ribosomal protein L19	VDK00013155-3784_1	2	17.9	9.75E-06	1.11E+09	2.6
Myosin-9	VDK00061196-1731_1	9	69.3	0.00E+00	8.33E+09	2.6
GOT2 aspartate aminotransferase	VDK00041754-2241_3	2	25.2	4.70E-07	6.74E+08	2.5
Myosin-9	VDK00002274-6145_4	7	67.9	0.00E+00	5.13E+08	2.5
Conserved protein (Transmembrane)	VDK00104201-1037_4	2	23.6	3.70E-12	4.58E+08	2.5
6-phosphogluconolactonase	VDK00004127-5343_1	3	39.3	2.96E-33	1.32E+09	2.5
Processing peptidase beta subunit	VDK00022713-3046_1	2	30.1	2.01E-15	6.20E+09	2.4
Glycogenin-1	VDK00009181-4267_3	3	61.8	1.33E-20	4.96E+08	2.3
ADP/ATP translocase	VDK00030728-2651_5	9	46.8	1.72E-57	2.86E+09	2.2
Citrate synthase	VDK00024252-2961_6	6	26.9	2.56E-35	2.60E+09	2.2
Aminotransferase	VDK00000249-9060_6	2	14.5	3.76E-45	5.81E+08	2.2
LIM domain-containing protein	VDK00021254-3134_1	4	52.7	1.49E-162	8.41E+09	2.2
Aconitase	VDK00018031-3356_3	2	43.9	3.73E-11	3.70E+08	2.2
ADP/ATP translocase	VDK00010026-4153_6	7	64.1	1.33E-25	3.79E+09	2.2
Myosin-9	VDK00038284-2357_7	19	56.2	1.05E-147	1.51E+10	2.2
Ornithine aminotransferase	VDK00004127-5343_3	3	32.2	7.07E-33	4.70E+08	2.2
Glycerol-3-phosphate dehydrogenase	VDK00020401-3188_3	4	18.5	4.50E-72	6.73E+08	2.1
Phosphoglucomutase	VDK00022279-3071_5	4	40.6	1.34E-40	7.89E+08	2.1
Paramyosin	VDK00160148-521_1	4	26.8	3.16E-235	4.99E+09	2.1
Formiminotransferase-cyclodeaminase	VDK00031542-2616_2	4	45.5	2.86E-16	4.62E+08	2.1

ADP/ATP translocase	VDK00012733-3828_4	8	48.5	1.23E-35	3.11E+09	2.1
Filamin	VDK00001557-6655_8	6	53.6	3.00E-130	3.52E+09	2.0
Acetyl-CoA hydrolase	VDK00164891-498_1	8	49	2.72E-68	5.51E+09	2.0
Hsp70	VDK00071124-1533_2	13	31.8	1.90E-198	9.70E+09	2.0
E1-E2 ATPase	VDK00024521-2947_1	14	38.8	6.70E-273	7.92E+09	2.0
Hsp70	VDK00073477-1489_1	8	44.2	1.77E-113	4.56E+09	2.0
GARS/AIRS/GART	VDK00109667-971_2	5	15.3	4.74E-15	3.38E+08	1.9
NADH:ubiquinone oxidoreductase NDUFS2/49 kDa subunit	VDK00039978-2299_4	3	33.3	2.77E-15	4.36E+08	1.9
Filamin-C	VDK00116268-897_2	8	65.1	7.52E-63	5.93E+09	1.9
ATP-binding cassette subfamily E member 1	VDK00017018-3429_1	3	40	1.53E-120	4.08E+09	1.9
Vacuolar H ⁺ -ATPase V1 sector subunit B	VDK00013480-3750_1	9	79.8	2.48E-88	3.14E+09	1.9
Paxillin	VDK00038901-2336_3	5	38.4	5.15E-71	8.50E+09	1.8
Spectrin alpha chain	VDK00114599-915_1	4	73	6.07E-39	4.02E+08	1.8
Tubulin beta-1 chain	VDK00004655-5191_2	11	66	5.43E-192	1.11E+10	1.8
Aldo-keto reductase	VDK00086014-1286_1	3	34.5	4.50E-68	8.63E+09	1.8
THO complex subunit	VDK00089524-1235_1	3	28.4	4.27E-29	5.55E+08	1.8
THO complex subunit	VDK00010723-4058_6	33	71.6	0.00E+00	4.28E+10	1.8
Arginine kinase	VDK00001475-6735_4	4	33.7	0.00E+00	1.04E+10	1.7
Phosphoribosylamidoimidazole- succinocarboxamide synthase	VDK00019483-3250_2	5	48.4	7.59E-117	4.93E+09	1.7
4-aminobutyrate aminotransferase	VDK00038440-2352_1	2	38.5	1.53E-15	3.79E+08	1.7
Acetyl-CoA acetyltransferase	VDK00008146-4427_1	4	35.6	4.38E-67	4.15E+09	1.7
Na ⁺ /K ⁺ ATPase beta subunit	VDK00023879-2981_1	3	22.2	9.35E-08	1.26E+09	1.7
Na ⁺ /K ⁺ ATPase alpha subunit	VDK00027978-2774_1	8	33.5	4.48E-87	3.75E+09	1.7
Hsp90 protein	VDK00010723-4058_5	14	34.2	1.36E-203	1.41E+10	1.7
Aldehyde dehydrogenase	VDK00010909-4033_2	7	46.4	1.81E-59	1.13E+09	1.6
Aminoimidazole-4-carboxamide ribonucleotidetransformylase	VDK00037510-2383_4	3	46.2	1.50E-84	3.82E+09	1.6
Chaperonin subunit	VDK00002992-5780_5	2	34.4	3.16E-45	8.08E+09	1.6

Translation elongation factor EF-1 alpha/Tu	VDK00024136-2967_7	6	41.7	3.78E-63	1.35E+10	1.6
Phosphoglycerate kinase	VDK00052433-1937_2	4	41.7	2.85E-56	5.54E+09	1.6
Heat shock protein	VDK00085495-1293_2	12	53.1	0.00E+00	3.83E+10	1.6
Glutamine synthetase 2 cytoplasmic	VDK00006812-4673_2	5	38.5	9.27E-189	1.05E+10	1.5
Phosphoglycerate mutase	VDK00033074-2553_3	2	18.6	4.93E-27	8.57E+08	1.5
Heat shock protein	VDK00072177-1513_1	10	71.5	1.42E-283	1.12E+10	1.5
Chaperonin subunit	VDK00038595-2347_2	14	63	5.73E-207	1.47E+10	1.5

Table 4.4 Identities of proteins present at higher abundance in the resistant *Varroa*.

Proteins identified as being present in higher levels in Resistant *Varroa* compared to the levels present in sensitive mites. Fold abundance values are expressed as log₂ fold change.

Protein annotation	Protein Identity	Peptides	Sequence coverage [%]	PEP	Overall Intensity	Abundance in resistant <i>Varroa</i>
Glyceraldehyde 3-phosphate dehydrogenase	VDK00031278-2628_1	7	72	0.00E+00	1.77E+11	-1.5
Ribosomal protein L13	VDK00010478-4094_2	9	47.2	8.31E-70	8.07E+09	-1.5
40S ribosomal protein S2	VDK00009175-4268_2	5	36	3.37E-32	1.75E+09	-1.5
Cuticular protein	VDK00065490-1640_1	5	45.5	1.76E-89	1.21E+10	-1.5
Conserved hypothetical protein	VDK00004857-5136_4	4	43.2	3.51E-21	4.44E+09	-1.5
Heat shock protein 20.6	VDK00009236-4260_3	7	44.4	3.43E-304	3.57E+10	-1.5
Ribosomal protein L3	VDK00016364-3481_2	4	48.5	4.35E-45	5.07E+09	-1.5
60S ribosomal protein L30	VDK00071016-1535_3	5	49.2	1.46E-68	6.57E+09	-1.5
60S ribosomal protein L10	VDK00057135-1822_3	3	27.6	1.04E-44	7.92E+09	-1.5
Ribosomal protein L15	VDK00090385-1223_4	4	27.9	7.39E-32	2.54E+09	-1.6
Cuticular protein	VDK00030664-2654_5	5	53.5	7.36E-241	1.35E+11	-1.6
Histone H4	VDK00101972-1064_2	12	55.3	2.62E-92	3.38E+10	-1.6
Transformer-2 sex-determining protein	VDK00000419-8375_6	3	46.2	4.55E-65	3.80E+09	-1.6
60S ribosomal protein L3	VDK00060758-1741_4	3	21.2	4.59E-25	3.39E+09	-1.6
60S ribosomal protein rpL7a	VDK00017338-3405_2	6	26.6	1.37E-56	2.56E+09	-1.6
Calmodulin-A	VDK00020188-3202_5	15	94.6	9.25E-287	2.75E+10	-1.6
40S ribosomal protein S13	VDK00097517-1122_1	5	33.3	1.29E-12	3.91E+09	-1.6
Beta-galactosidase precursor	VDK00056033-1848_3	6	59.6	7.44E-31	1.16E+09	-1.6
60S ribosomal protein L9	VDK00011189-4001_3	5	38.6	4.95E-94	3.96E+09	-1.8
Secreted salivary gland peptide	VDK00013948-3705_3	4	32	2.82E-54	6.78E+10	-1.8
Cuticular protein	VDK00085734-1290_1	5	70.6	0.00E+00	7.12E+10	-1.8
Transferrin receptor	VDK00018994-3286_3	4	47.2	2.10E-100	5.34E+09	-1.8
Rho guanine dissociation factor	VDK00039112-2328_6	2	37.3	1.25E-83	2.62E+09	-1.8
Alternative splicing factor SRp20/9G8	VDK00021195-3138_1	2	11.9	8.10E-58	7.50E+09	-1.8
Secreted protein	VDK00003463-5588_2	2	16.2	5.15E-11	3.39E+08	-1.9
Angiotensin-I converting enzyme	VDK00005516-4974_3	4	26.5	4.69E-94	6.63E+08	-1.9
Cuticular protein	VDK00047820-2060_1	4	52.1	6.18E-259	3.19E+10	-1.9

Ribosomal protein S18	VDK00155877-545_1	5	54.5	1.12E-172	8.42E+09	-1.9
Cuticle protein	VDK00145336-624_1	2	35.4	3.32E-34	8.54E+08	-1.9
60S ribosomal protein L23	VDK00193306-379_1	4	38.8	1.93E-82	5.25E+09	-2.0
Structural constituent of cuticle	VDK00052080-1945_3	6	51.6	5.20E-278	4.80E+10	-2.0
Cuticular protein	VDK00052526-1935_5	7	46	0.00E+00	7.82E+10	-2.0
Ribosomal protein L8	VDK00121631-842_2	4	19.3	1.42E-08	2.74E+09	-2.0
Transferrin receptor	VDK00053515-1910_1	4	57.9	2.86E-127	5.34E+09	-2.1
Cuticular protein	VDK00000578-7948_5	7	25.5	3.94E-150	8.29E+09	-2.1
Cuticular protein	VDK00087687-1262_3	15	75.1	0.00E+00	1.62E+10	-2.3
Cuticular protein	VDK00002797-5866_5	6	45	3.94E-129	3.84E+09	-2.4
Cuticular protein	VDK00110134-966_1	8	65.1	5.60E-220	2.99E+10	-2.4
Ubiquitin carboxyl-terminal hydrolase	VDK00039887-2302_1	2	22.1	3.82E-07	1.82E+08	-2.5
Cytochrome B5	VDK00066125-1628_4	2	19.4	1.01E-06	5.22E+08	-2.9
Ferritin	VDK00093932-1171_1	6	36.4	1.94E-200	5.21E+10	-3.2
Cold-inducible RNA-binding protein	VDK00015608-3548_4	2	32.7	9.66E-41	1.12E+09	-3.9
Gastric triacylglycerol lipase	VDK00075502-1454_2	3	21.5	8.82E-13	6.63E+08	-4.0
Ribosomal protein L37A	VDK00022997-3031_3	3	28.5	7.14E-11	1.01E+09	-5.0
Conserved hypothetical protein	VDK00000892-7401_5	2	48.5	1.22E-28	1.64E+09	-13.3

Table 4.5 Identities of proteins present at lower levels of abundance in the resistant *Varroa*.

Proteins identified as being present in lower levels in Resistant *Varroa* compared to the levels present in sensitive mites. Fold abundance values are expressed as log₂ fold change.

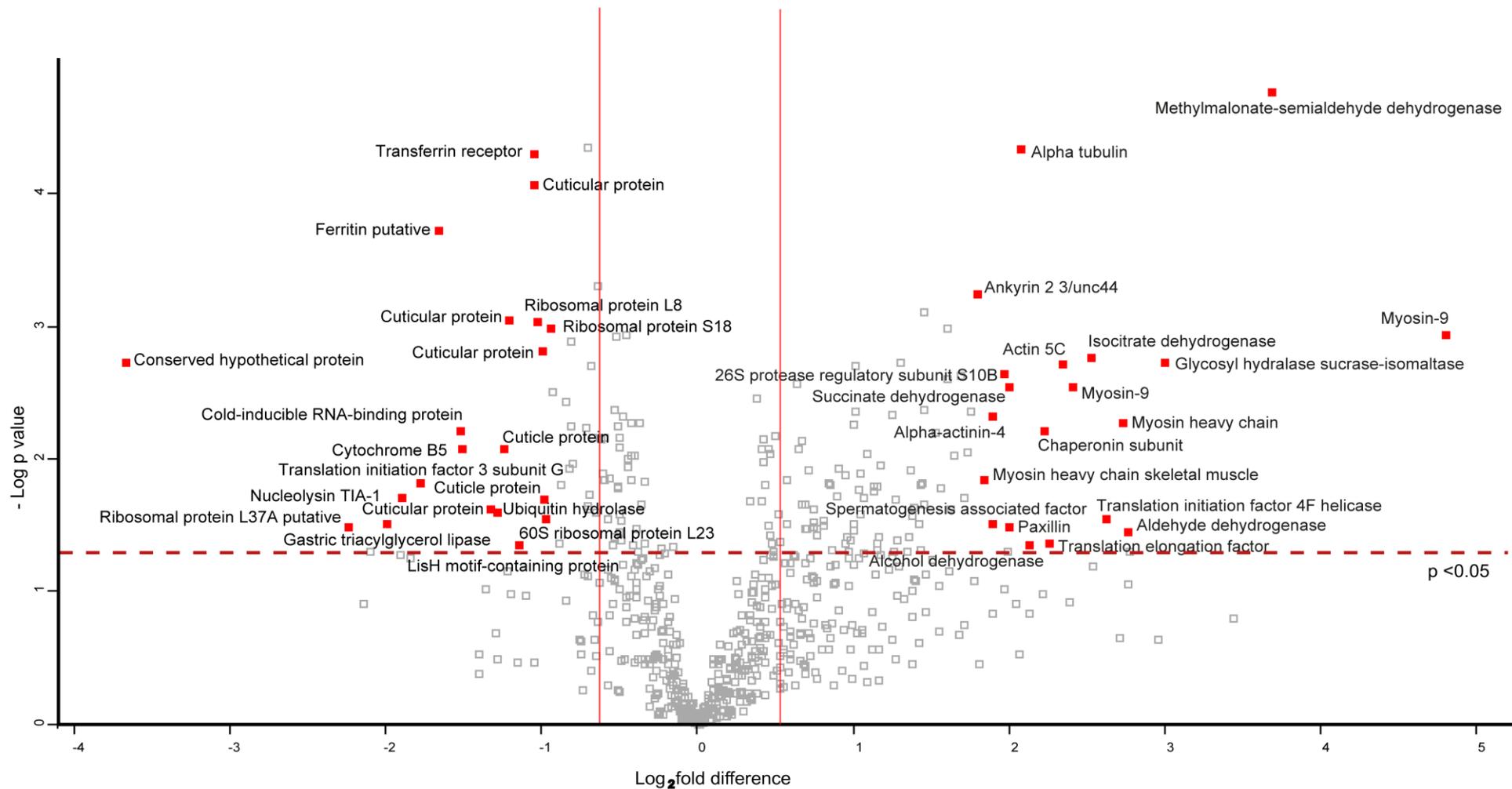


Figure 4.6 Volcano plot of top differentially expressed proteins in Bayvarol sensitive and resistant *Varroa*

Image of Volcano plot obtained using Perseus software (2.14.7). Proteins above the line are statistically significant (p value < 0.05) and those to the right and left of the vertical lines indicate fold changes greater than 1.5 fold positive and 1.5 fold negative in the resistant and sensitive mites respectively.

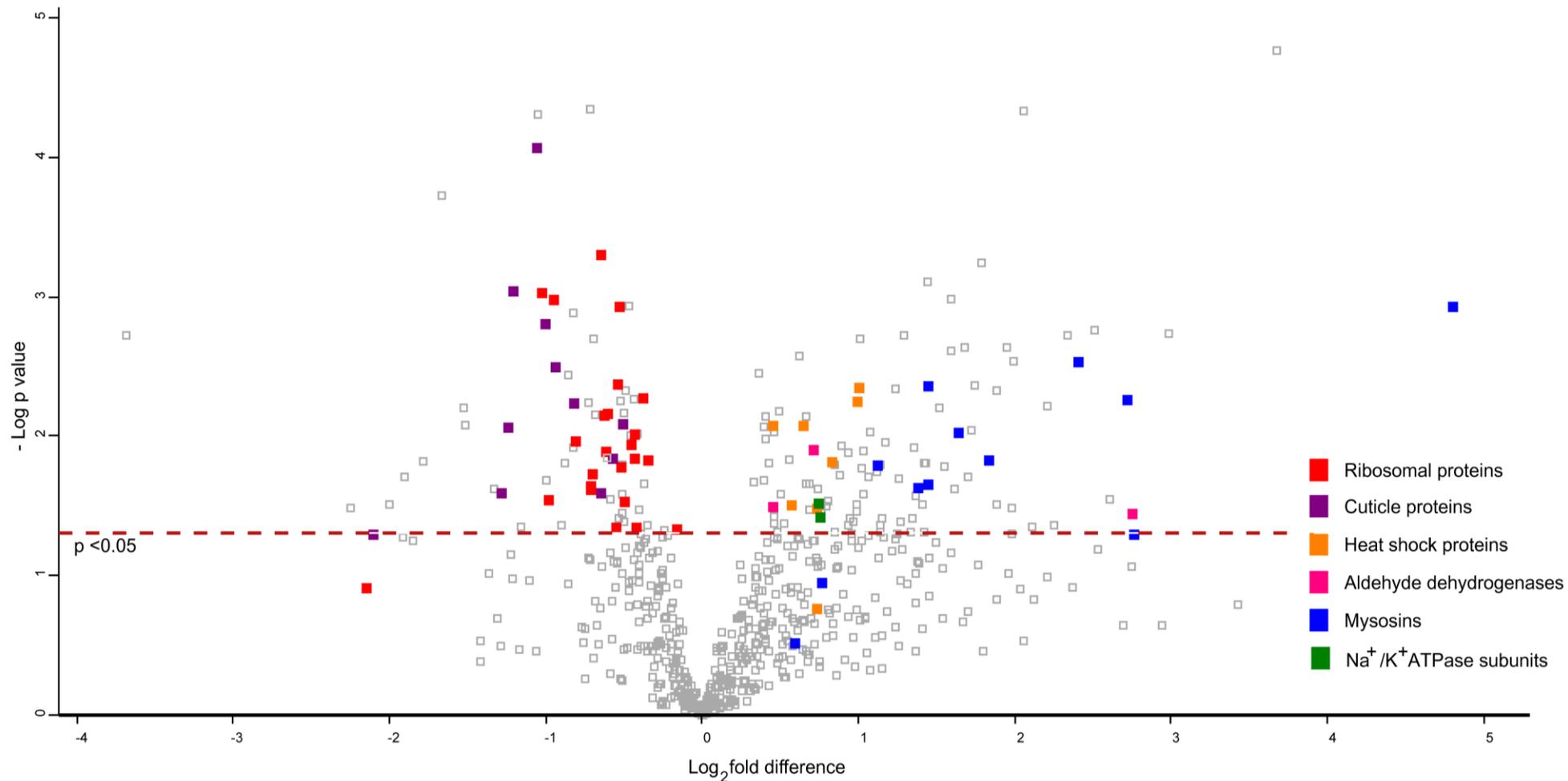


Figure 4.7 Volcano plot representing a number of possible resistance associated protein groups.

A number of associated proteins showed similar and grouped levels of abundance, differentially expressed between the proteomic profiles of the mites deemed sensitive to Bayvarol and those that were deemed resistant. The expression of proteins above the line is statistically significant (p value <0.05).

Protein annotation	LFQ Intensity Sensitive_1	LFQ Intensity Sensitive_2	LFQ Intensity Sensitive_3	LFQ Intensity Resistant_1	LFQ Intensity Resistant_2	LFQ Intensity Resistant_3
Conserved hypothetical protein	5.6E+08	1.2E+09	1.0E+09	0	0	0
Translation initiation factor 3	1.5E+08	2.6E+08	1.4E+08	0	0	0
Sulfotransferase	1.0E+08	9.7E+07	9.8E+07	0	0	0
Trailer hitch, isoform D	4.5E+07	4.8E+07	5.2E+07	0	0	0
Spermidine synthase	0	0	0	2.7E+08	3.0E+08	3.4E+08
Coronin-6	0	0	0	8.5E+07	1.3E+08	7.6E+07
Myosin-9	0	0	0	2.3E+08	3.6E+08	2.0E+08
Alpha tubulin	0	0	0	4.6E+08	4.0E+08	4.1E+08
Dipeptidyl peptidase III	0	0	0	1.2E+08	1.9E+08	1.2E+08
Myosin-9	0	0	0	2.8E+08	6.3E+08	3.4E+08
Spectrin alpha chain	0	0	0	1.4E+08	1.5E+08	1.3E+08
Peptidyl-prolyl cis-trans isomerase	0	0	0	3.4E+08	2.1E+08	3.9E+08
Glycosyl hydralase sucrose-isomaltase	0	0	0	4.2E+08	8.6E+08	5.9E+08
aconitase	0	0	0	9.2E+07	1.7E+08	1.3E+08
26S proteasome subunit S9	0	0	0	1.0E+08	1.6E+08	1.1E+08
Formiminotransferase-cyclodeaminase	0	0	0	1.6E+08	2.0E+08	1.3E+08
DEAD box ATP-dependent RNA helicase	0	0	0	5.7E+07	7.7E+07	4.6E+07
Glycogenin-1	0	0	0	1.2E+08	1.9E+08	1.7E+08
Ornithine aminotransferase	0	0	0	1.5E+08	1.6E+08	1.6E+08
Aminotransferase	0	0	0	1.6E+08	1.6E+08	2.2E+08
Isocitrate dehydrogenase	0	0	0	4.8E+08	4.3E+08	4.9E+08
Myosin-9	0	0	0	1.5E+09	3.5E+09	1.2E+09
60S ribosomal protein L19	0	0	0	2.8E+08	2.1E+08	2.1E+08
Methylmalonate-semialdehyde dehydrogenase	0	0	0	1.2E+08	2.6E+08	1.3E+08
Methylmalonate-semialdehyde dehydrogenase	0	0	0	8.8E+08	1.1E+09	8.6E+08
THO complex subunit	0	0	0	1.4E+08	1.6E+08	1.8E+08
Vinculin	0	0	0	2.2E+08	2.5E+08	2.0E+08
Chaperonin subunit	0	0	0	2.8E+08	4.3E+08	2.0E+08
T-complex protein 1 subunit beta	0	0	0	2.6E+08	3.9E+08	2.9E+08
GARS/AIRS/GART	0	0	0	9.6E+07	1.4E+08	1.3E+08
Ankyrin 2 3/unc44	0	0	0	2.4E+08	3.2E+08	3.0E+08
Alpha-actinin-4	0	0	0	1.6E+08	3.4E+08	1.9E+08
Spectrin alpha chain	0	0	0	8.5E+07	1.7E+08	8.8E+07
Flare, isoform A	0	0	0	1.4E+08	1.7E+08	2.3E+08
Nucleolysin TIA-1	0	0	0	1.6E+07	2.8E+07	3.6E+07
Succinate dehydrogenase	0	0	0	1.8E+08	3.4E+08	2.5E+08
Enoyl-CoA hydratase	0	0	0	1.0E+08	1.6E+08	8.3E+07
Spermatogenesis associated factor	0	0	0	2.7E+08	3.0E+08	3.2E+08
26S protease regulatory	0	0	0	1.5E+08	1.2E+08	1.3E+08

Subunit 6A						
Phosphofructokinase	0	0	0	1.3E+08	2.2E+08	1.7E+08
GOT2 aspartate aminotransferase	0	0	0	1.8E+08	2.4E+08	1.5E+08
Myosin-9	0	0	0	1.2E+08	2.1E+08	1.6E+08
Polyadenylate-binding protein	0	0	0	1.4E+08	1.5E+08	1.5E+08
Phosphoglucomutase	0	0	0	2.2E+08	3.4E+08	2.4E+08
Prolylcarboxypeptidase	0	0	0	3.7E+07	3.9E+07	4.7E+07

Table 4.6 LFQ intensities of proteins from the sensitive and resistant *Varroa* mite samples.

A zero value indicates a protein that was absent or undetected in the sample. Only proteins that were present or absent in all three samples of each group were considered exclusive protein hits. These proteins were termed as being “Exclusively expressed”.

a)

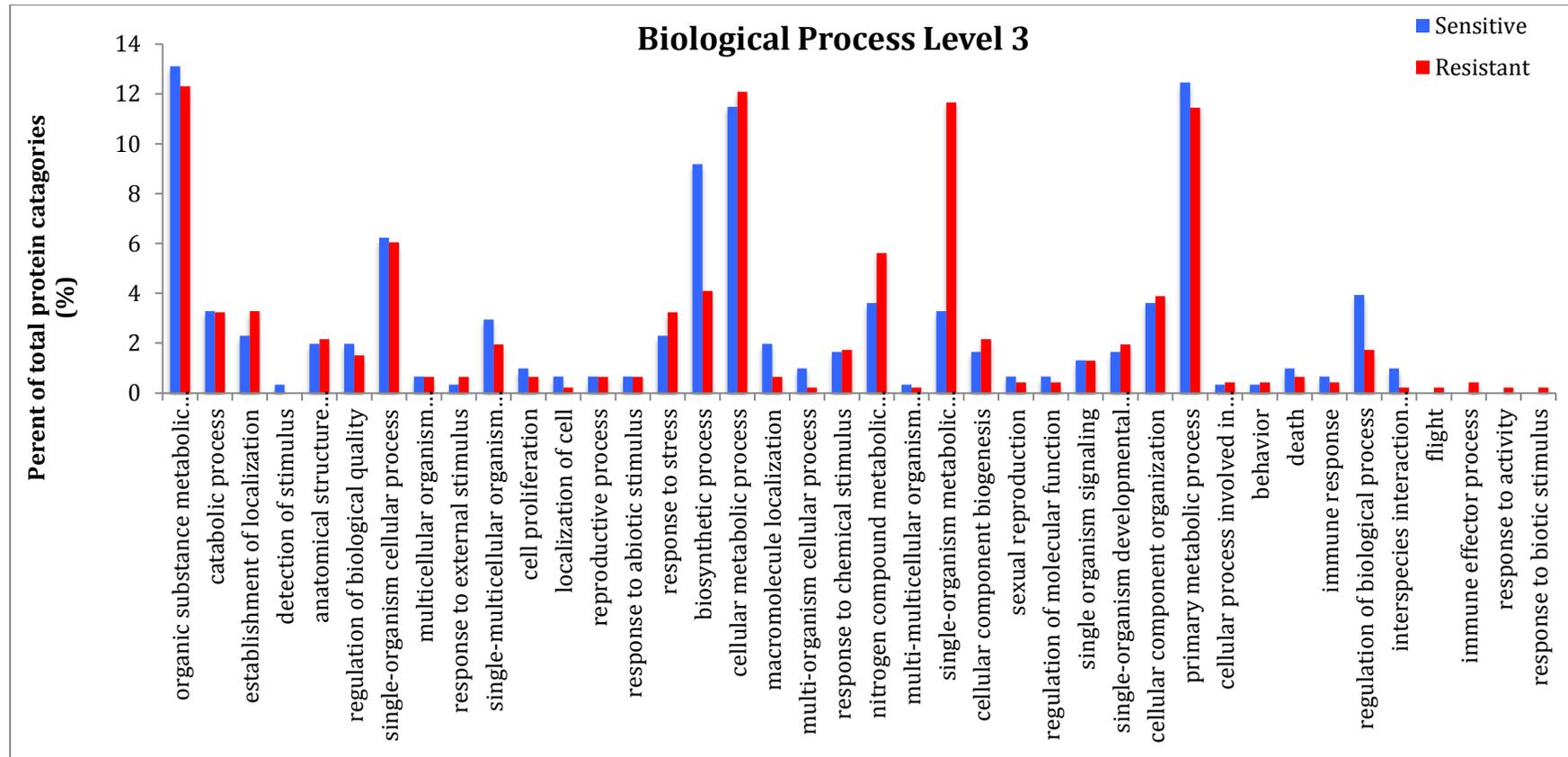


Figure 4.8(a) Bar chart showing changes to proportion of proteins involved in various Biological Processes at level 3 ontolog

Bar chart shows the grouping of proteins based on Biological process annotation using Blast2Go. Proteins were assigned groups based on involvement in Biological processes for the resistant and the sensitive *Varroa* samples. Each group was assigned a percentage proportion of the total proteins found in the proteomic profile of each sample group and graphed on a comparative bar chart.

b)

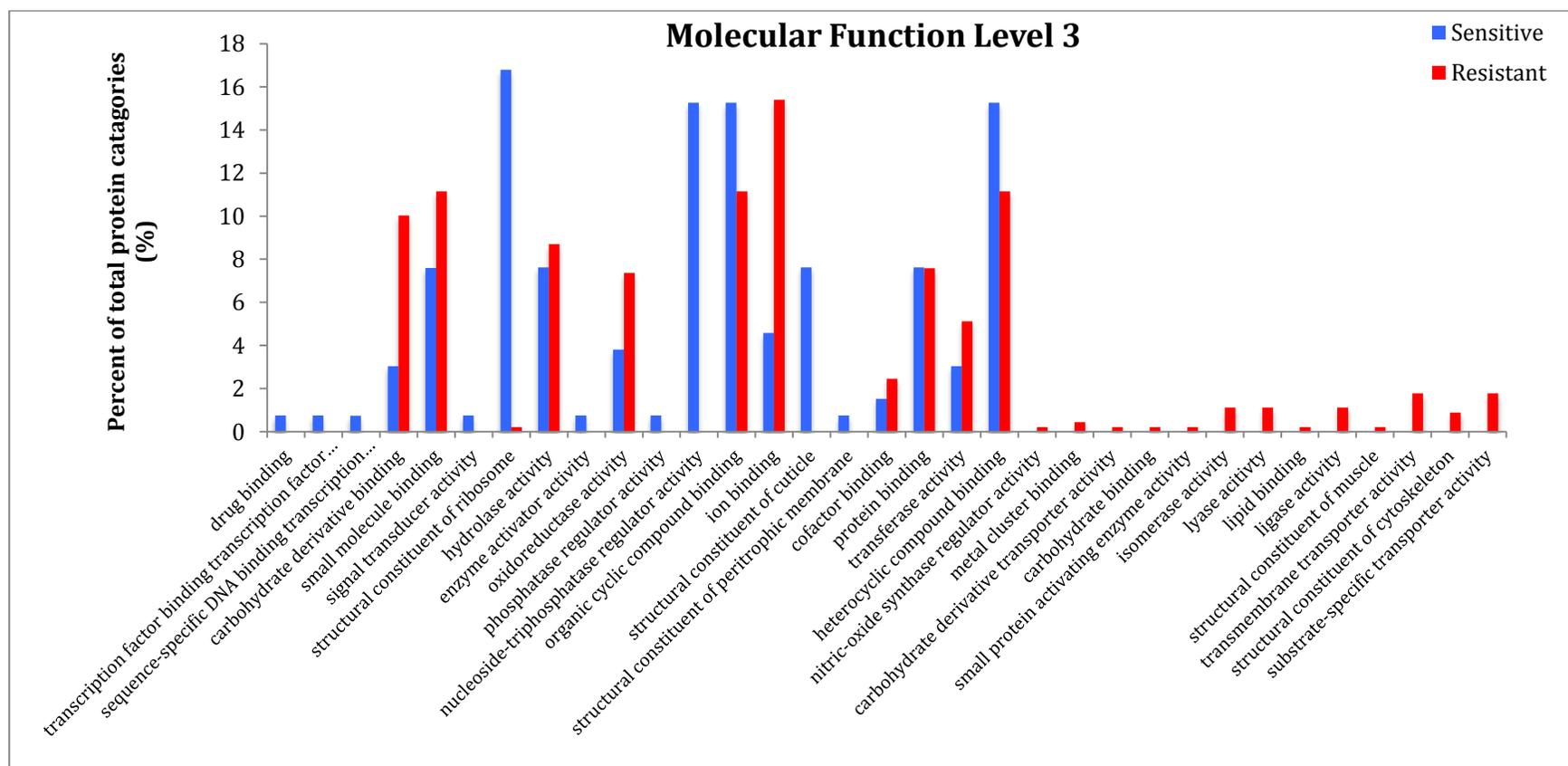
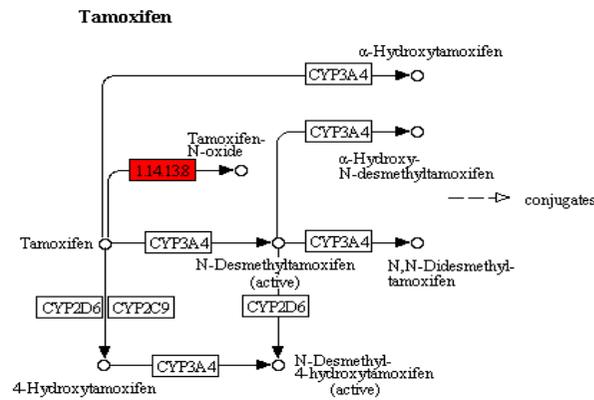
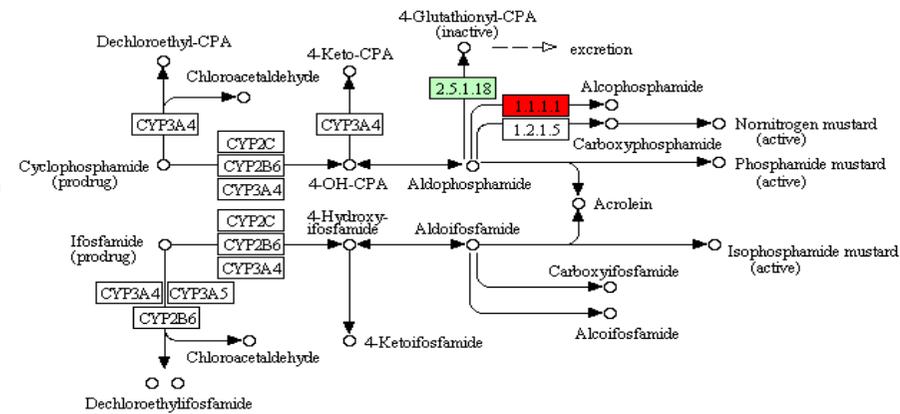


Figure 4.8(b) Bar chart showing changes to proportion of proteins involved in various Molecular function at level 3 ontology.

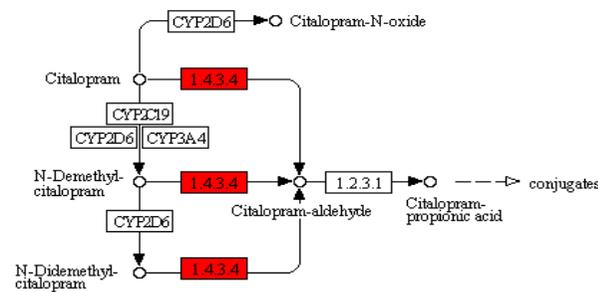
Bar chart shows the grouping of proteins based on functional annotation using Blast2Go. Proteins were assigned groups based on involvement in Molecular function for both the resistant and the sensitive mite samples. Each group was assigned a percentage proportion of the total proteins found in the proteomic profile of each sample group and graphed on a comparative bar chart.



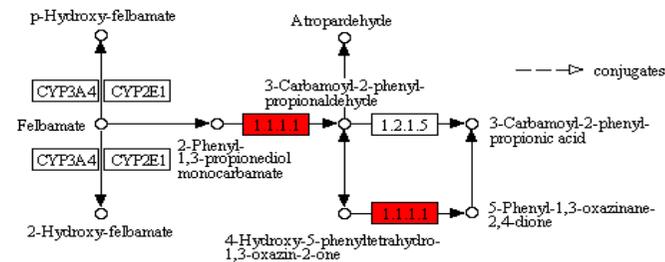
Cyclophosphamide & Ifosfamide



Citalopram



Felbamate



00982 11/11/13
(c) Kanehisa Laboratories

Figure 4.9 KEGG pathway analysis of a drug detoxification pathway showing a number of proteins increased in abundance in Resistant *Varroa*.

Figure shows various parts of the cytochrome P450 drug metabolism pathway that were found to be present at significantly higher levels in mites that showed resistance to Bayvarol (Red indicates up regulation) using KEGG pathway analysis. The average LFQ intensities for these proteins are indicated in Figure 4.10, which shows the variation in expression patterns of the components that were shown present in higher levels.

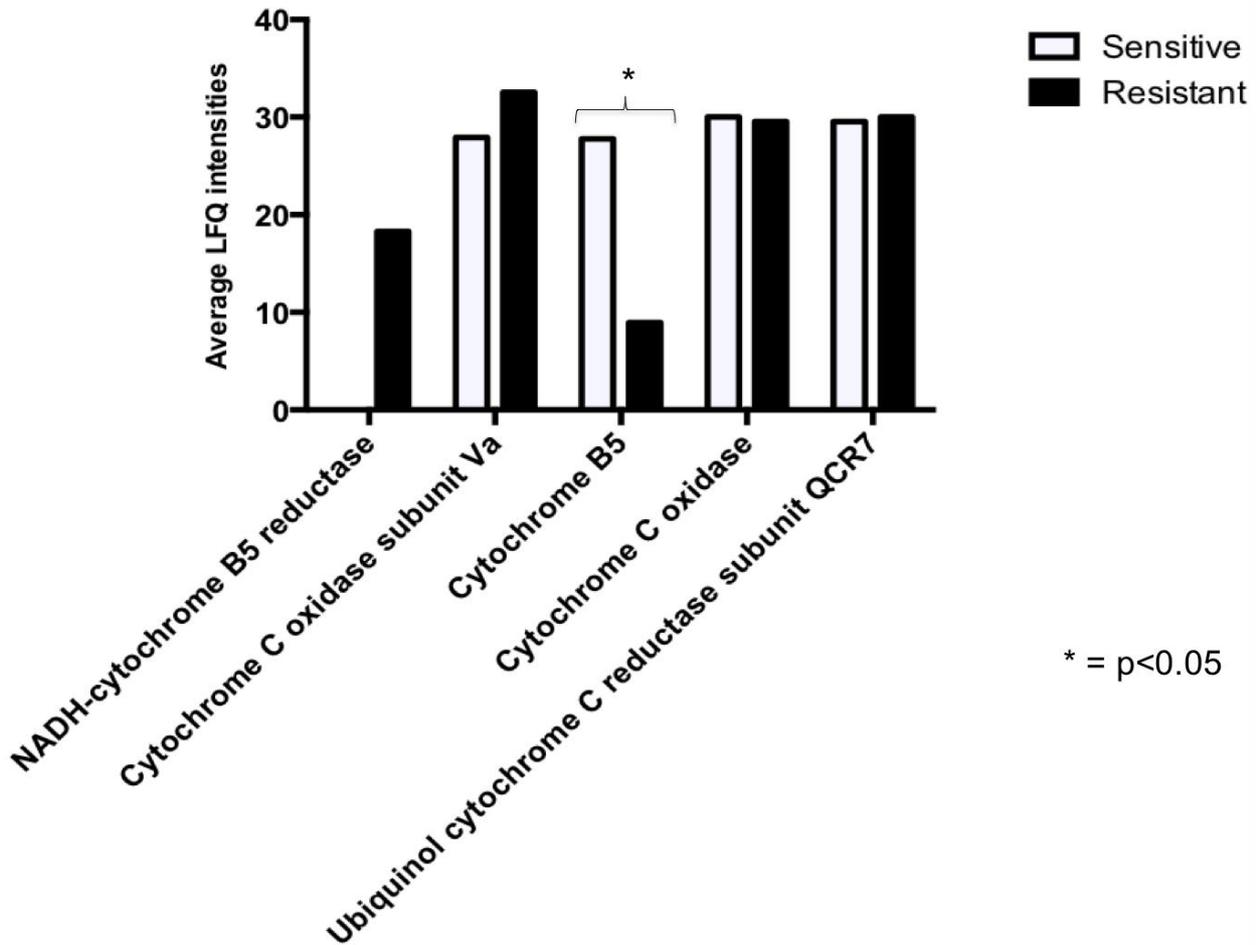


Figure 4.10 Proteins from the cytochrome P450 superfamily differentially expressed between Bayvarol sensitive and resistant *Varroa*.

Average LFQ intensities of a number of cytochrome p450 components differentially expressed in the proteomic profile of Bayvarol sensitive and resistant *Varroa*. Figure shows a number of cytochrome P450 components that were found to be differentially expressed between the sensitive and resistant *V. destructor* mites. * Indicates a statistically significant change in expression at $p < 0.05$.

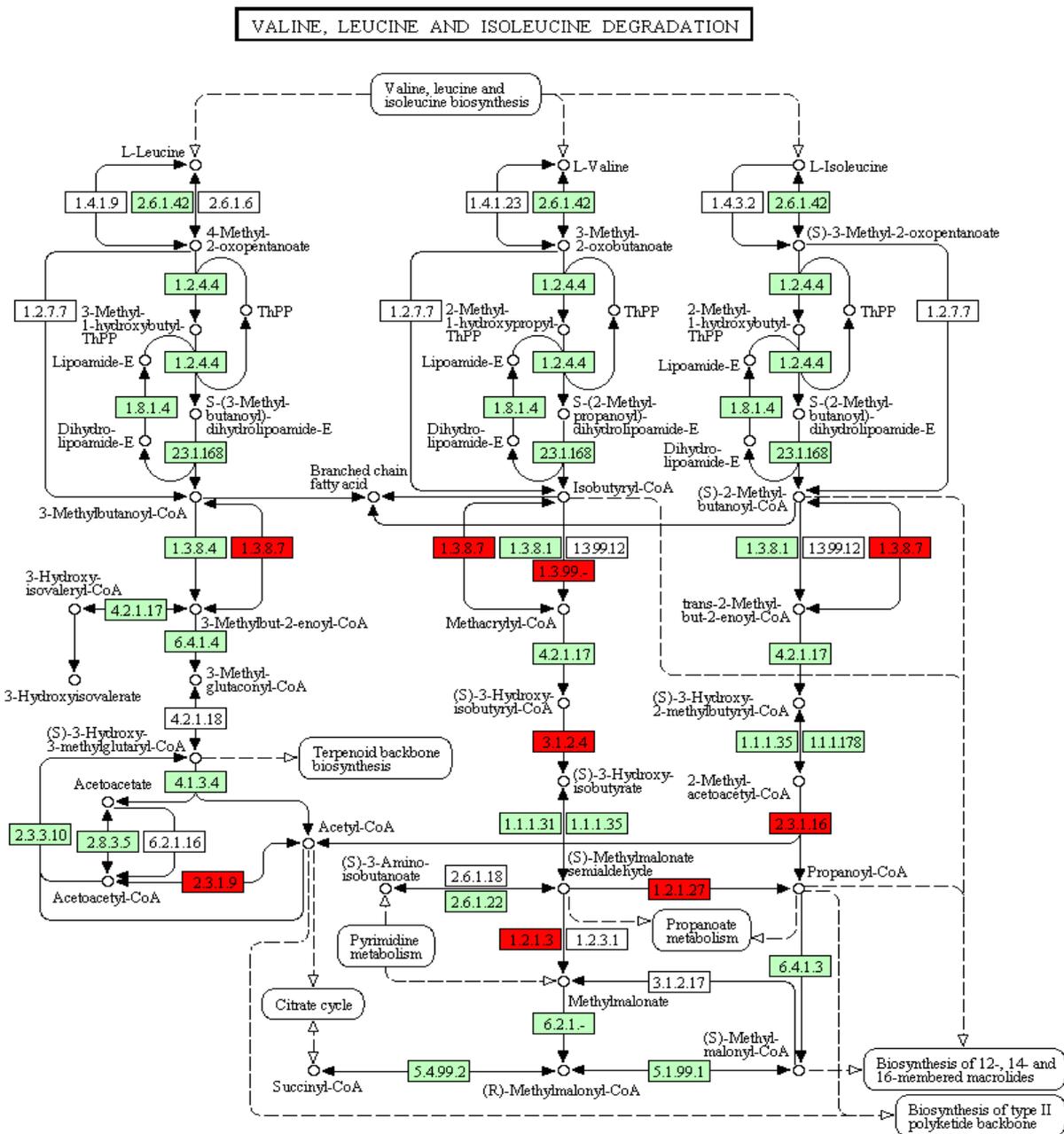


Figure 4.11 KEGG pathway analysis of a protein degradation pathway showing a number of proteins increased in abundance in resistant *Varroa*.

KEGG pathway analysis of a protein degradation pathway that was shown to be significantly altered in the comparison of the Bayvarol sensitive and Bayvarol resistant *Varroa*. The red sections in the pathway show areas that were expressed at higher abundance in resistant mites.

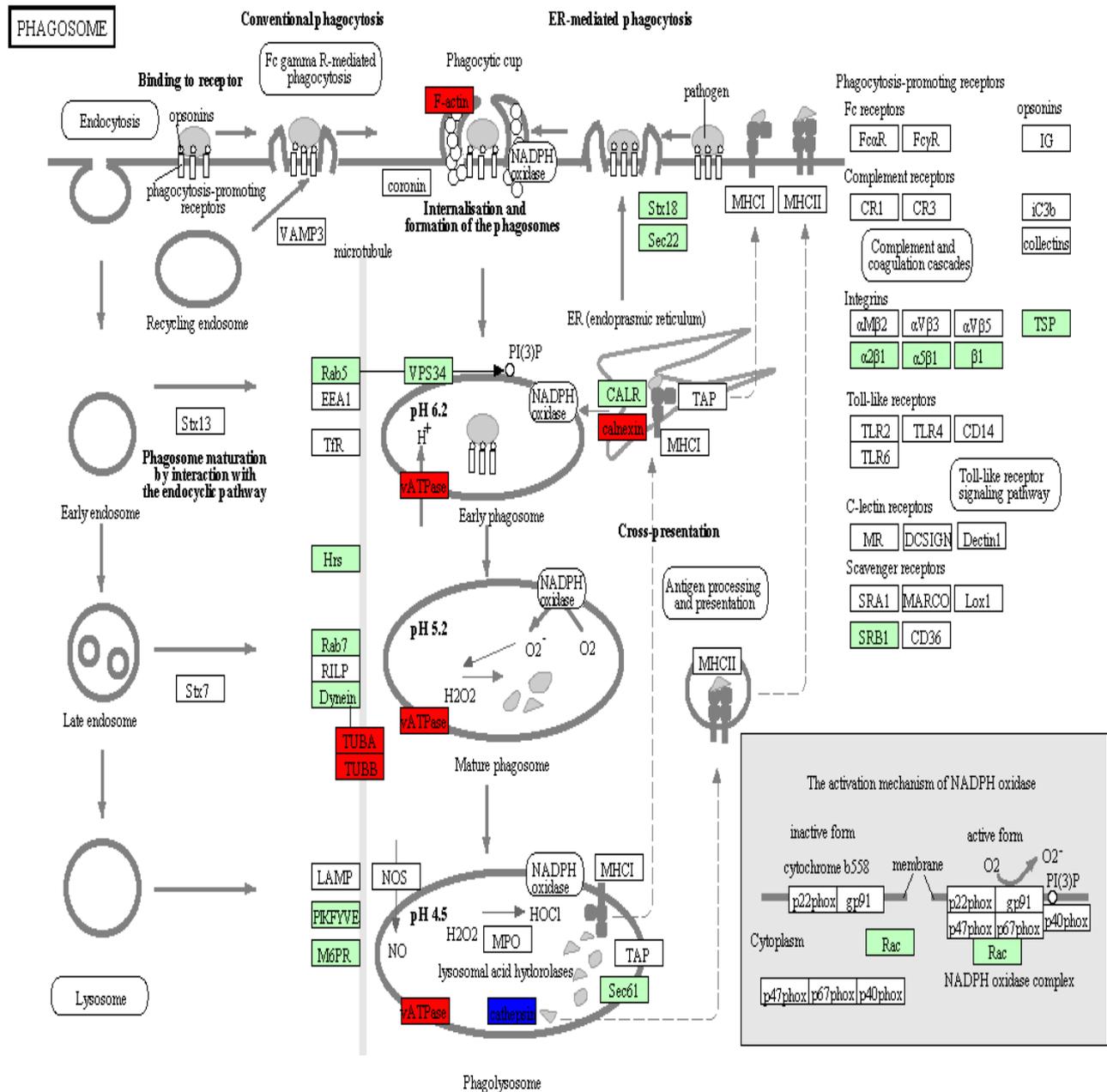


Figure 4.12 KEGG pathway analysis of a phagosome pathway showing a number of proteins increased in abundance in resistant *Varroa*.

KEGG pathway analysis of a phagosome assembly pathway that was shown as significantly altered in the comparison of the Bayvarol sensitive and Bayvarol resistant *Varroa*. The red sections in the pathway show areas that were expressed at higher levels of abundance in resistant mites, with the blue box showing an area that was expressed at higher levels in the sensitive mites.

4.6 Discussion

The results presented in this Chapter give a proteomic comparison of the proteomic profile of *V. destructor* mites that were deemed to be sensitive and resistant to Bayvarol. The aim of this work was to identify any changes in the profiles of the mites that could be conferring the resistant phenotype. Initially, 1-Dimensional gel electrophoresis was performed in order to identify any changes in banding patterns between the resistant and sensitive mites. The total proteome were resolved using pH 4-7 isoelectric focusing and SDS PAGE electrophoresis under 2-Dimensional separation, and subsequently, quantitative shotgun proteomics of the total proteome of both *Varroa* phenotypes under label free conditions was employed.

The active agent in Bayvarol® flumethrin is a member of pyrethroid group of insecticides which acts upon the sodium channel of the cell membrane resulting in the loss of the ability to close correctly, leading to over saturation of potassium and sodium within the cell and subsequent cell death (Martin 2004; Van Leeuwen *et al.*, 2010). Resistance to pyrethroid may be due to knockdown resistance of the target site of the chemical, but evidence suggests that this type of resistance does not exist independently and that other resistance mechanisms such as increased detoxification can also operate (Wang *et al.*, 2002; Martin 2004; Van Leeuwen *et al.*, 2010). The cross resistance of *Varroa* mites to unrelated chemical acaricides gives further strength to the theory that metabolic resistance could play a role in the development of resistant populations (Martin 2004; Van Leeuwen *et al.*, 2010). Resistance due to changes in the rate or efficacy of metabolism is thought to be mainly due to up-regulation of metabolic enzymes such as esterases, glutathione-S transferases, and P450 mono-oxygenases (Shen *et al.*, 2005; Puinean *et al.*, 2010).

Proteins extracted from sensitive and resistant *Varroa* gave differential banding intensities for seven protein bands in 1-Dimensional SDS PAGE. Two of these proteins, histamine release factor (1.8 fold increase in resistant mites; Table 4.1) and glutamate receptor 1-like (4 fold increase in

resistant mites; Table 4.1) have functions in the nervous system, acting on the neurotransmitter synapses (Raymond-Delpech *et al.*, 2005). The increase in these proteins in the Bayvarol resistant *Varroa* could indicate an increased ability to overcome the effects of the increase in neurotransmitter signals which lead to the eventual paralysis in pyrethroid treated organisms (Vijverberg and Van den Bercken 1990). The resistant *Varroa* also showed increased expression of two heat shock proteins: heat shock protein 60 (2.82 fold increase; Table 4.1) and heat shock protein 90 (3.82 fold increase; Table 4.1). Heat shock proteins are molecular chaperones which function by preventing protein aggregation during stressful conditions (Becker and Craig 1994, Sørensen *et al.*, 2003). A variety of heat shock proteins have previously been shown to be present at higher levels of abundance in organisms that are resistant to pyrethroid. Heat shock protein 70 in particular has been shown to be increased in many pyrethroid resistant organism, such as *A. gambiae* and *Myzus persicae* (Vontas *et al.*, 2005; Silva *et al.*, 2012). Overexpression of heat shock protein such as heat shock protein 70 and 90 could potentially play a role in the development of the resistant phenotype observed in the Bayvarol resistant *Varroa* by helping the cells deal with the stress caused by the chemical. The up regulation of glyceraldehyde-3-phosphate dehydrogenase (2.18 fold increase; Table 4.1) was unexpected as the gene encoding for this protein is frequently used as a reference gene, however it does have oxidoreductase activity and so could be increased in an effort by the *Varroa* to deal with the oxidative stress caused by the pyrethroid (Park *et al.*, 2007; Silva *et al.*, 2012).

The next step in identifying proteins that were differentially expressed between the profiles of Bayvarol resistant and sensitive mites was to resolve the total proteome of both by 2-Dimensional SDS PAGE. The second separation step allows for a clearer view of the proteins that are expressed as they appear spread out across a larger surface area on the gel (Bunai and Yamane 2005). The complete proteomic profile achievable using SDS-PAGE was first identified (Figure 4.3). An identity was achieved for a total of 67 spots (Table 4.2) which were used to identify proteins of interest that were differentially expressed in resistant mites. A total of 9 proteins were identified

as being differentially abundant between the proteome of the sensitive (Figure 4.4a) and resistant mites (Figure 4.4b), all of which were present at higher levels of abundance in the resistant *Varroa* (Table 4.3). Protein spots which were found to be present at higher levels of abundance in the proteomic profile of the Bayvarol resistant *Varroa* included some with proposed oxidoreductase functions in the cell e.g. retinol dehydrogenase 1-like (6.1 fold increase; p value 0.017; Table 4.3) and aldehyde dehydrogenase (5.9 fold increase; p value 0.003; Table 4.3). Aldehyde dehydrogenase expression was increased in pyrethroid resistant *A. gambiae* after exposure to permethrin (Vontas *et al.*, 2005). Pyrethroid/DTT resistant *A. gambiae* also showed similar change in levels of aldehyde dehydrogenase with two members of the family expressed at higher levels in resistant mosquitoes at both the genetic and proteomic level (Lumjuan *et al.*, 2014). Retinol dehydrogenase is another member of the dehydrogenase superfamily, and has been linked with xenobiotic detoxification in mammals (Vasiliou *et al.*, 2000). The up regulation of both of these dehydrogenases in the resistant mites could indicate that an up-regulation of certain proteins could be leading to increased efficacy of the detoxification system in the resistant mites. This could be a potential resistance mechanism. Thioredoxin reductase-2 has been shown to have a role in the oxidoreductase response of a cell to stress and was increased by 5.9 fold in resistant mites compared to the levels in susceptible mites (Table 4.3). Thioredoxin reductase was also found to be up-regulated in pyrethroid resistant *A. gambiae* as part of an overall up-regulation of a number of detoxification proteins (David *et al.*, 2005). The comparison of the proteomic profiles using 2-Dimensional separation of both sensitive and resistant *Varroa* mites also showed a significant increase in the abundance of glutathione-s-transferase (GST) in Bayvarol resistant mites compared to the level in sensitive mites (2.8 fold increase; p = 0.025; Table 4.3) GST has previously been associated with detoxification against insecticides (Kostaropoulos *et al.*, 2001; Fragoso *et al.*, 2003) and has been linked to the metabolic resistance to pyrethroids in the spotted mite *Tetranychus urticae* (Stumpf and Nauen 2002). Pyrethroid exposure leads to oxidative stress by inducing lipid peroxidation and by depletion of reduced glutathione, and superoxide dismutases, catalases and glutathione-s-transferases provide defense against this stress (Nardini *et al.*,

2013). The elevated levels of these detoxification enzymes in resistant *Varroa* mites could confer a greater ability to withstand the oxidative stress caused by the chemical and lead to the organism becoming more tolerant to the effects of the chemical.

Similar results to those observed using 2-Dimensional SDS PAGE were seen in the label free quantitative proteomic analysis comparing the whole proteome of Bayvarol resistant and sensitive mites. A total of 89 proteins were expressed at higher levels in the Bayvarol resistant mites (Table 4.4) with 45 present at a lower abundance (Table 4.5). The exclusively expressed hits showed 41 proteins only expressed in the resistant mites and 4 exclusive to the sensitive mites (Table 4.6). A large number of proteins from the dehydrogenase family can be seen present at much higher levels in the Bayvarol resistant *Varroa*. Some of these were also present in the exclusive to resistant protein group and were included in statistical analysis after imputation (Table 4.6 and Table 4.4 respectively). This group of dehydrogenases included two alcohol dehydrogenases (5 fold and 1.6 fold increase), methylmalonate semialdehyde dehydrogenase (12.5 fold increase), isocitrate dehydrogenase (12.5 fold) and methylenetetrahydrofolate dehydrogenase (5.4 fold increase). Dehydrogenases have been previously linked to insecticide resistance due to their esterase activity (Kedishvili *et al.*, 2000). Aldehyde dehydrogenase expression was increased in pyrethroid resistant *A. gambiae* after exposure to permethrin (Vontas *et al.*, 2005). Pyrethroid/DTT resistant *A. gambiae* also showed similarly changes in the abundance of aldehyde dehydrogenase expression with two members of the family shown to be expressed at higher levels in resistant mosquitoes at both the genetic and proteomic levels (Lumjuan *et al.*, 2014). Methylmalonate-semialdehyde dehydrogenase was found expressed exclusively in the proteomic profile of the Bayvarol resistant *Varroa* (Table 4.6). It exhibits esterase activity, typical of all dehydrogenases, but is unique among the members of this family because coenzyme A is required for the reaction and a CoA ester is produced (Kedishvili *et al.*, 2000). The presence of these dehydrogenases in the resistant mites at such increased levels of expression indicate that they may be playing a role in conferring the resistant

phenotype. The higher levels of these proteins could potentially allow for a more efficient means of breaking down a chemical, such as Bayvarol.

The expression of some protein groups that are present in higher or lower abundance in the resistant mite profile when compared to the sensitive mites profile is evident in Figure 4.7. The various colors highlight proteins that share a similar expression pattern in the LFQ intensities. A number of cuticle proteins were present at lower abundance in the proteomic profile of resistant *Varroa* (Figure 4.7). One possible means by which organisms are thought to become resistant is by alteration of the route of entry of the chemical such as a thickening or hardening of the cuticle or other epithelial barriers (Hemingway *et al.*, 2004). A number of cuticular proteins were present in lower amounts in resistant mites (Figure 4.7), which partly contradicts the trend observed in previous reports (Vontas *et al.*, 2005; Puinean *et al.*, 2010; Silva *et al.*, 2012; Wang *et al.*, 2015). The protein that showed the lowest abundance in the resistant mites, exclusively expressed in the profile of sensitive mites, was a conserved hypothetical protein (Table 4.6). This protein is possibly also a cuticular protein as it shows homology to *gasp*, a protein from *D. melanogaster*, which is a chitin binding cuticle constituent (bLINK BLAST resource <http://www.ncbi.nlm.nih.gov/sutils/blink>). Some research has found however, that a down-regulation of some cuticle proteins alongside an up-regulation of others does occur, suggesting that a change to the components of the cuticle may be taking place such as replacing some more penetrable cuticle components with others less penetrable, leading to decreased rate of entry of the chemical (Vontas *et al.*, 2005; Wang *et al.*, 2015). Here any up-regulation of cuticle proteins was not found and this could mean there was some up-regulated at a non-significant level, or it could mean that the cuticular changes in the resistant mites involved the removal of some proteins without their replacement.

A number of proteins with structural roles were found significantly increased in abundance in mites that showed resistance to Bayvarol® (Table 4.5). Some of these were also present in the exclusive list indicating presence and absence in all three replicates of the resistant and sensitive mites

respectively (e.g. coronin, alpha tubulin) (Table 4.6). Proteins involved in cytoskeleton structure have previously been shown to be changed in abundance in deltamethrin resistant mosquitoes (Bonizzoni *et al.*, 2012). Here, a large group of myosins were also shown to be present at much higher levels in resistant mites and some were exclusively present in the resistant mite population. Similar results have previously been reported in deltamethrin resistant *Culex pipiens pallens* which were found to have higher levels of a light chain regulatory myosin which also conferred resistance in a cell line when over-expressed (Yang *et al.*, 2008). Changes in the structure of cells and of the junctions between cells may confer resistance to a chemical, in particular chemicals in which the mode of entry is through the skin, such as the pyrethroid insecticides.

A number of other groups of proteins were also differentially expressed in the Bayvarol sensitive and the resistant *Varroa* mites including a group of ribosomal proteins the majority of which were present at lower levels in the mites that were deemed resistant (Figure 4.7). Deltamethrin resistant mosquitoes demonstrated a 23 fold increase in the expression of the L39 gene (Tan *et al.*, 2007), but this was not the case in the Bayvarol resistant mites studied here. One study carried out demonstrated that another ribosomal protein S29 regulates metabolic resistance by binding to CYP6N3, so perhaps the unbound versions of certain ribosomal proteins were more abundant during proteomic analysis of the sensitive mites (Yu *et al.*, 2014). A number of proteins involved in ion transport were also greater in abundance in resistant *Varroa*. NADH ubiquinone oxidoreductase NDUF52/49 kDa subunit is thought to act as a proton pump for sodium and potassium and was present at levels 1.9 fold higher in resistant mites (Brandt *et al.*, 2003) (Table 4.5). Mosquitoes that were resistant to pyrethroids had similar elevated levels of this protein post-treatment with permethrin (Vontas *et al.*, 2005). E1-E2 ATPase putative, vacuolar H⁺-ATPase V1 sector subunit B, Na⁺/K⁺ ATPase beta subunit and alpha subunit were all expressed at higher levels in the proteome of resistant *Varroa*, (2, 1.9, 1.7, and 1.7 fold, respectively) (Table 4.5). These proteins act as proton pumps for detoxification of the cell, and higher levels of these proteins in the cell could help combat the extra ions due to

malfunctioning sodium transport channel (Emery *et al.*, 1998). Pyrethroid is metabolized by the hydrolysis of ester bonds, oxidation at the acid and alcohol moieties and several conjugation reactions such as hydrophilic and lipophilic conjugates (Kaneko 2012; Strachecka *et al.*, 2013). Here, a number of proteins involved in hydrolysis (processing peptidase beta subunit putative, acetyl-CoA hydrolase putative, aminoimidazole-4-carboxamide ribonucleotidetransformylase/IMP) were present at higher levels in pyrethroid resistant *Varroa* (Table 4.5) and these could be potential effectors of the more efficient breakdown of the chemical in resistant mites.

Through analysis of comparative proteomics comparing the profile of Bayvarol resistant and sensitive mites, alterations to the expression pattern can be observed and possible resistance mechanisms identified. This Chapter highlights the complete analysis of the alterations in expression profiles using 1-Dimensional, 2-Dimensional and label free quantitative mass spectrometry. A number of common themes emerged through all the methods employed for proteomic analysis, the main observations being the overall increase of a variety of detoxification proteins and oxidative stress proteins in the Bayvarol resistant mites. These could potentially be leading to the phenotype observed in the resistant mites by helping to overcome the oxidative stress induced by the synthetic pyrethroid. In addition, the ion transport and transferase proteins were also expressed at higher levels in the resistant mites and this could also be contributing toward the resistant phenotype by helping to combat the influx of ions induced by the pyrethroid. The observed alteration to the cuticle structure could imply a change to the target entry site for the chemical, and though additional proteins were not detected to imply a thickening of the cuticle, the alteration could have been through the upper non proteinaceous layers of the barrier. The alteration of the cytochrome p450 components was an interesting observation as this complex is widely accepted to be one of the main mechanisms for acquiring metabolic resistance to a chemical.

In conclusion, the work presented here offers some insight into the metabolic resistance mechanisms that could play a role in the resistance of

Varroa to Bayvarol and may open the possibility of identifying targets to circumvent this phenotype.

Chapter Five

Analysis of the effect of
parasitization
by *Varroa destructor* on the
Western Honeybee, *Apis
mellifera*

5.1 Introduction

A. mellifera, the Western honeybee, is an insect of huge economic importance, as the world's most important global pollinator (Klein *et al.*, 2007). They live in densely populated colonies, which gives the optimum chance for pathogens and parasites to thrive. The most threatening of these parasites is the ectoparasitic mite, *Varroa destructor*. Chapter four described the effect that the development of resistance is having on the control of *Varroa*, and this Chapter will discuss the findings into the relationship that has developed between *A. mellifera* and *Varroa* through various life stages. The host-parasite relationship between the two is new, as *Varroa* are not a natural parasite of the Western honeybee. The original host, *A. cerana*, has developed a level of tolerance to *Varroa* that develops from a natural host-parasite relationship. *A. cerana* for example, exhibits a greater tendency to groom itself, knocking off *Varroa* (Boecking and Spivak 1999). *Varroa* also display a much more pronounced tendency to infest drone brood cells, only rarely infesting worker brood cells, resulting in a much lower infestation level as the amount of drone brood in a colony is much lower than worker brood (Rath 1999). *Varroa* parasitized both adult and young *A. mellifera*, meaning that no life stage is free from the effects of parasitization and the mites are completely dependent on the life cycle of the honeybee and will only reproduce in the capped brood cells (Corrêa-Marques *et al.*, 2003). The effect of parasitization on the bee is not fully understood, and has not yet been fully investigated or documented. The aim of this Chapter was to analyze the effect that parasitization has on different life stages of the bee, from pupa to adult. The effect that *Varroa* parasitization has on adult *A. mellifera* could have serious implications for the health and vitality of the colony, and weakening due to parasitization could result in collapse of the entire population. Proteomic analysis was used to examine the effect of parasitization on adult *A. mellifera*, during both the summer and the over wintering period. Harboring parasites over winter could adversely affect the vitality of the colony in the spring. If parasitization during development occurs, the bee is not free from *Varroa* until emergence when the cell is uncapped (Sammataro *et al.*, 2000). Proteomic analysis was used to observe any changes that may be happening during parasitization, in order to see any

effects on both drone and worker pupae. Molecular analysis was also used to analyze the expression of genes involved in the immune response of the bee to identify any immune suppression that may be occurring during parasitization.

5.2 Analysis of variations in the proteomic profile of adult *A. mellifera* during overwintering and during parasitization

5.2.1 Analysis of variations in the proteomic profile of adult *A. mellifera* in summer bees compared to bees that were overwintering

In order to identify the effect of parasitization by *Varroa* on adult bees, the differences between unparasitized summer and winter bees was first examined, to see what proteomic alterations naturally occur in the overwintering period. The total whole body proteome was extracted from adult bees (2.9.1). Proteins were first resolved by 1-Dimensional SDS PAGE (Figure 5.1). Image J software was used to identify any changes in intensity of bands (2.12.2) and the 8 bands that were identified as present in variable abundance between the summer and winter control samples were excised for identification by LC-MS (2.13). The identities of each band are shown in Table 5.1, and the relative fold change in abundance of each protein band is shown in Figure 5.2. A number of hexamerins were present at higher levels in the summer bees, ranging from 1.2-1.54 fold increase in abundance compared to levels in winter bees. Hexamerins are involved in the storage of nutrients such as proteins in the haemolymph as a nutritional source during periods of non-feeding (Burmester 1999). Proteins that are thought to play a role in the stress response were present at higher levels in the winter bees: Glutathione s-transferase, which is thought to play a role in oxidative stress prevention (1.82 fold increase) and 3-ketoacyl-CoA thiolase, involved in negative regulation of apoptosis (1.48 fold increase) (Claudianos *et al.*, 2006; Vickers 2009). Heat shock protein 60kDa was present in higher abundance (1.32 fold) in the bees that were sampled from an overwintering colony. HSP60 is a chaperone protein involved in preventing protein aggregation of unfolded proteins that can occur under stress or following damage to the organism (Bukau and Horwich 1998).

Protein was extracted from summer and winter adult *A. mellifera* for 2-Dimensional analysis (2.11) and the resulting gels were analyzed by Progenesis SameSpots to identify differences in abundance of protein spots (2.12.3). Representative gel image from the summer bees can be seen in Figure 5.3a and gel image from winter bees can be seen in Figure 5.3b. Spots of interest were excised for identification by LC-MS, with the results presented in Table 5.2.

The relative abundance of the spots of interest is shown in Figure 5.4. ATP synthase was found at lower abundance in the profile of winter bees (4.4 fold decrease; $p = 0.001$), with thioredoxin dependent peroxide reductase also found at lower levels in the profile of overwintering bees (2.6 fold, $p = 0.045$). These proteins are involved in normal metabolism and energy production, and their lower abundance could have a negative impact on overall vitality (Chae *et al.*, 1994, Müller and Grüber 2003). Odorant binding protein 14 precursor was found also at lower levels of abundance in the overwintering bees (1.5 fold; $p = 0.018$). This protein is involved in the sensing of chemical and smell stimulus (Vogt *et al.*, 1991). Peroxiredoxin-6-like, involved in redox regulation in the cell, was found at higher levels of abundance in the profile of the bees that were overwintering which could indicate that the organism is under stress (2.9 fold; $p = 0.018$) (Wang *et al.*, 2003). Major royal jelly protein 1 precursor was present in lower abundance in the winter bees (0.5 fold decrease; $p = 0.023$), which is a developmental protein, also implicated in the immune response (Tamura *et al.*, 2009). 14-3-3 zeta-like protein, involved in development also, was found present at higher abundance in the overwintering bees (2.6 fold increase; $p = 0.037$) (Van Hemert *et al.*, 2001).

5.2.2 Analysis of the variations in the proteomic profile of unparasitized and parasitized overwintering adult *A. mellifera*

In order to identify any changes to the proteomic profile of winter bees when parasitized by *Varroa* during the overwintering period, proteins were extracted from unparasitized winter bees and compared to proteins extracted from parasitized bees (2.9.1). Proteins were extracted on at least three separate occasions, focused on a gradient of pH 4-7 and resolved by SDS PAGE (2.11).

Spots which showed a variation in density through analysis by Progenesis SameSpots software were excised for identification by LC-MS (2.13). Succinate dehydrogenase was present at higher levels in the parasitized winter bees (2.1 fold increase; $p = 0.030$), as was heat shock protein beta 1-like (1.4 fold increase; $p = 0.033$). Heat shock proteins are involved in protection of proteins from stress, and the presence of a heat shock protein at higher levels in the parasitized bees may indicate stress (Becker and Craig 1994, Bukau and Horwich 1998). Succinyl CoA ligase was present at higher levels in parasitized winter bees, which is involved in ATP and metal ion binding (1.4 fold increase; $p = 0.048$) (Rothacker and Ilg 2008). Hexamerin 110 precursor, a storage protein involved in providing nutrition, was identified at lower levels in the winter bees that were parasitized (1.4 fold decrease; $p = 0.028$) (Burmester 1999). Enolase-like protein, which is a protein involved in magnesium binding and glycolysis was also present at lower abundance levels in the parasitized winter bees (2.6 fold decrease; $p = 0.008$) (Hannaert *et al.*, 2003). Myosin heavy chain muscle protein was present at lower abundance in the parasitized winter bees (1.6 fold decrease; $p = 0.016$). This protein is involved in movement through muscle contraction and calcium binding (Lehman and Szent Gyorgyi 1975).

5.2.3 Summary

The aim of this section was to examine the effect that parasitization by *V. destructor* has on adult worker *A. mellifera*. The first aim was to examine the changes that take place at a proteomic level during the normal overwintering period, so that any additional changes due to parasitization during this period could be identified. A number of proteins involved in nutrition were present at lower levels in the overwintering bees, such as hexamerin, which is a lipid storage and transport protein. The lower abundance of these proteins is to be expected during the winter in adult *A. mellifera*, as there would be a much more limited supply of food than during the foraging months.

The effect of parasitization on overwintering *A. mellifera* was examined, in order to establish if harboring a parasite during the winter could have a

negative effect on the vitality of a healthy worker when foraging commenced in the spring. The abundance of proteins involved in stress response were at a higher level in the parasitized overwintering *A. mellifera*, which indicates that parasitization by *Varroa* is causing the bee to become stressed at the cellular level. This could have a negative impact on the health of the worker. Proteins involved in normal cell metabolism were also affected by parasitization by *Varroa* with proteins such as enolase-like present at lower levels of abundance in the parasitized winter bees. This could effect energy production in the bee and could have a negative effect on energy levels for foraging in the spring.

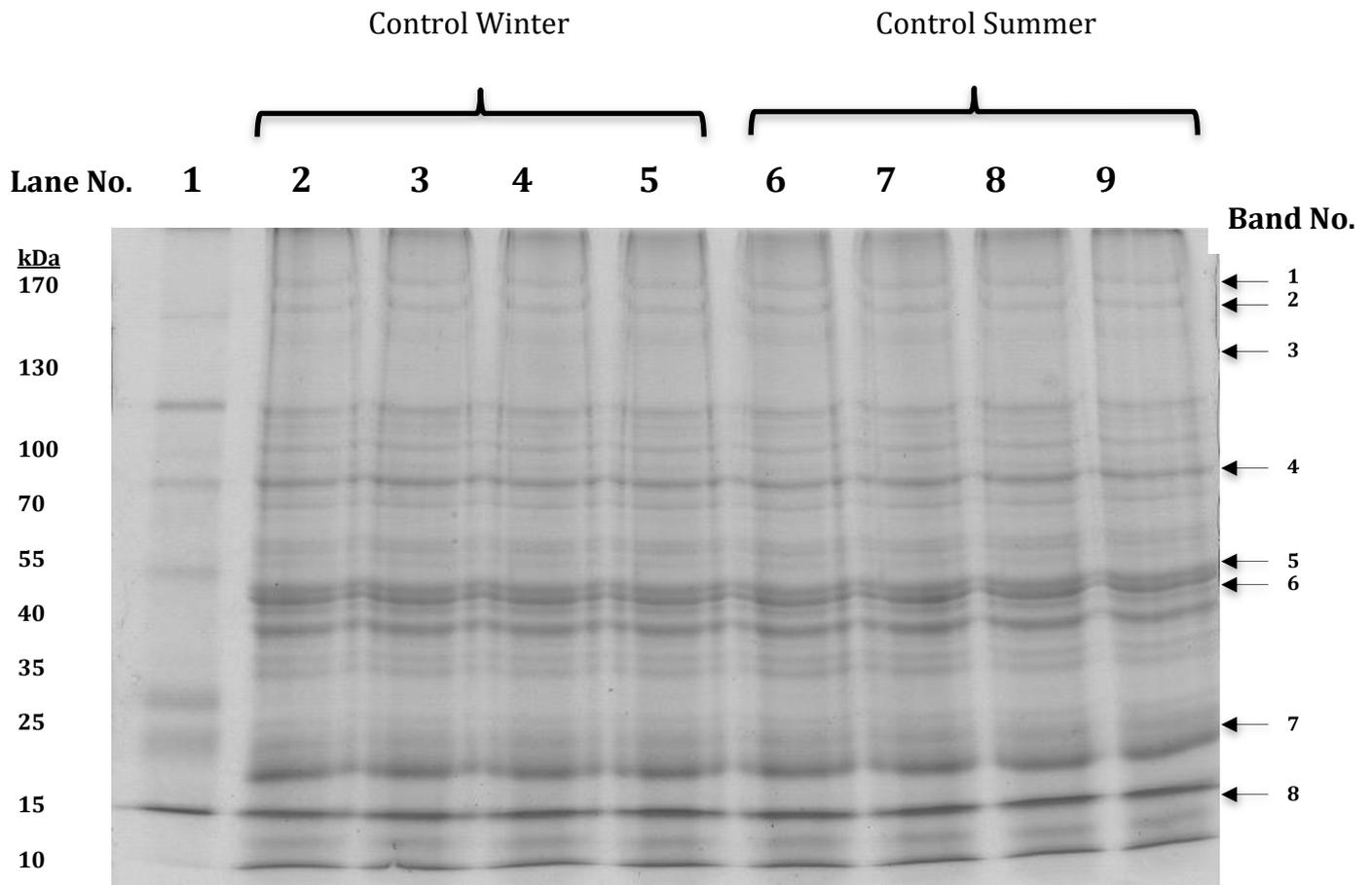


Figure 5.1 1-Dimensional SDS PAGE gel of adult winter and summer *A. mellifera*. Lane 1 shows molecular weight ladder, lanes 2-5 show protein extracted from summer adult *A. mellifera* (n=4) and lanes 6-9 are proteins from winter adult *A. mellifera* (n=4) Numbers 1-8 indicate protein bands that were identified as being differentially abundant and were identified by LC-MS (Table 5.1). Protein extractions were carried out separately on independent occasions (n=3).

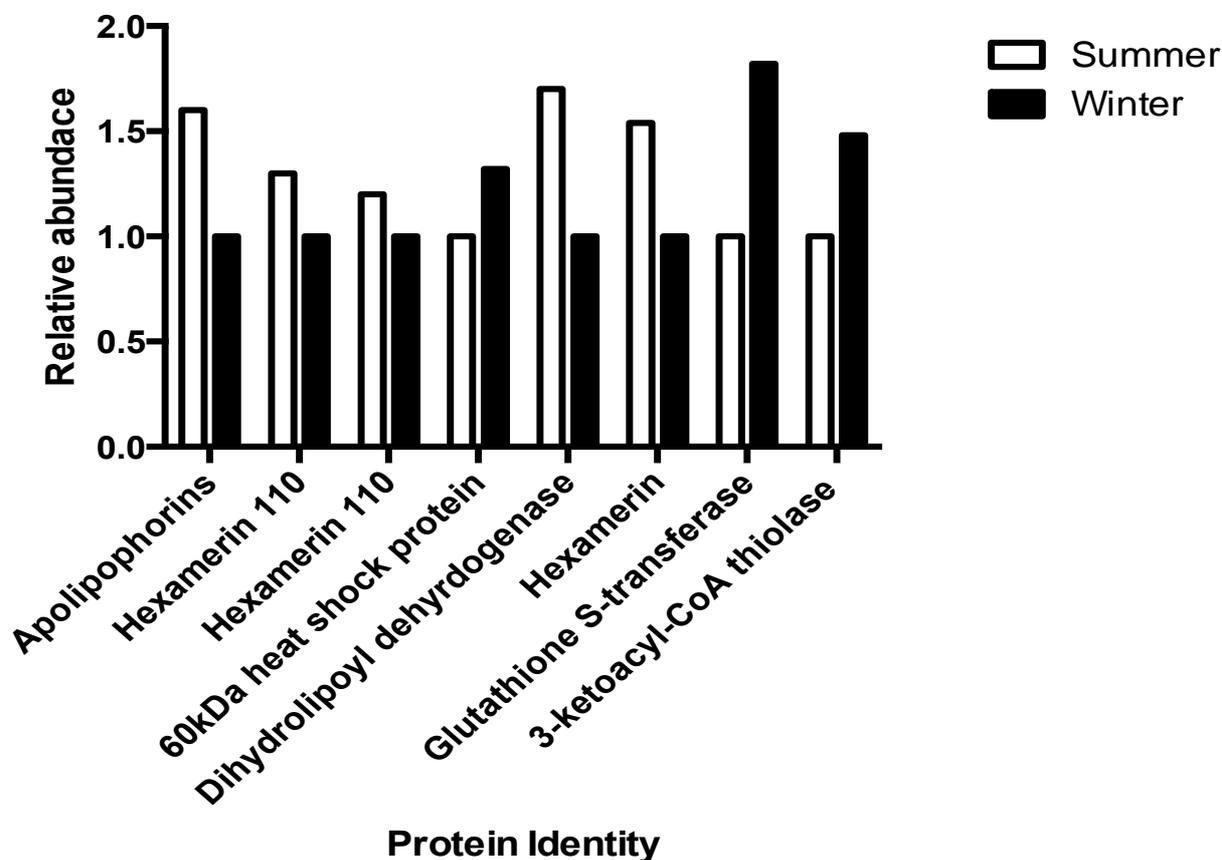


Figure 5.2 Relative fold abundance of proteins identified as significantly differentially expressed between summer and winter adult *A. mellifera*.

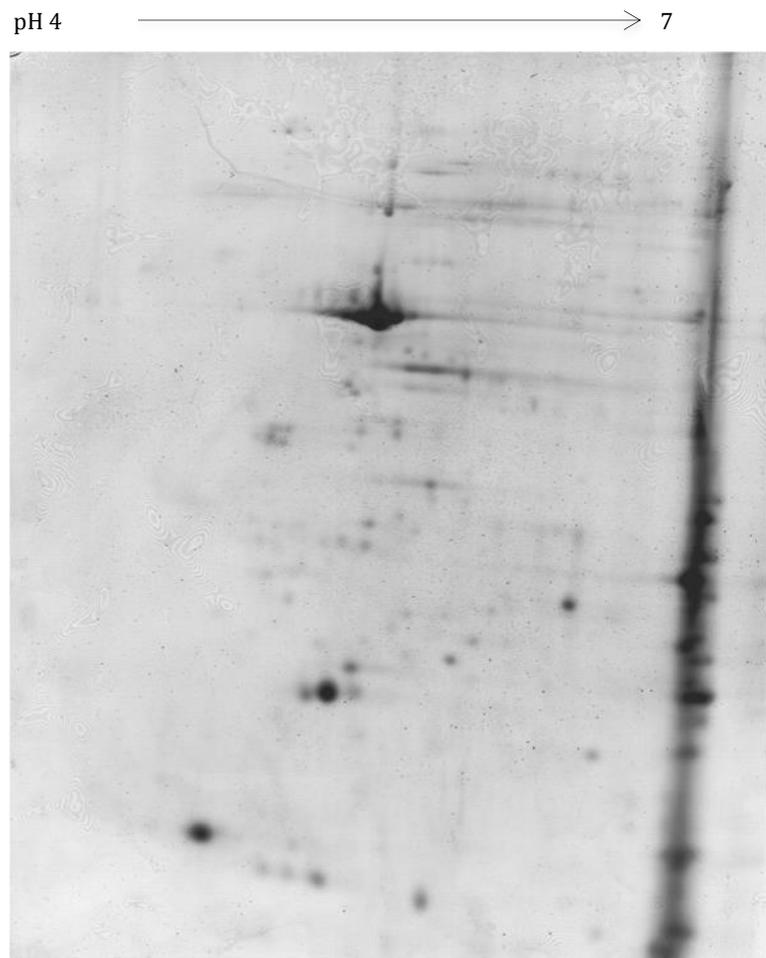
The relative change in abundance of the 8 differentially expressed protein bands comparing summer and overwintering adult *A. mellifera* by 1-Dimensional SDS PAGE (Table 5.1). All 8 proteins were differentially expressed at a level of $p < 0.05$ ($n=3$).

Band Number	Protein Annotation	M _r	pI	% Coverage	Protein Identity	Abundance of protein		Function
						Summer	Winter	
1	Apolipoproteins isoform 1	202142	8.65	11%	gi 328780884	1.6	1	Involved in fatty acid binding and heme binding
2	Hexamerin 110	111977	6.43	37%	gi 156637469	1.3	1	Storage protein that stores amino acids
3	Hexamerin 110	111977	6.43	34%	gi 156637469	1.2	1	Storage protein that stores amino acids
4	60kDa heat shock protein	54182	5.61	19%	gi 380014538	1	1.32	Chaperone involved in protein folding
5	Dihydrolipooyl dehydrogenase mitochondrial like	655129	5.64	20%	gi 66526646	1.70	1	Oxidative stress and cell redox stability
6	Hexamerin	81493	8.19	32%	gi 149939405	1.54	1	Storage protein that stores amino acids
7	Glutathione S-transferase	22983	5.49	36%	gi 380020933	1	1.82	Oxidoreductase involved in stress response
8	3-ketoacyl-CoA thiolase mitochondrial like	42710	8.89	28%	gi 48097100	1	1.48	Involved in negative regulation of apoptosis and stress response to oxidation

Table 5.1 Identities of differentially expressed proteins from 1-Dimensional analysis of adult summer and winter *A. mellifera*

Identities, molecular weight, pI, sequence coverage, accession number and relative abundance for the 8 protein bands identified as differentially abundant between summer and winter adult *A. mellifera* and identified by LC-MS (2.14).

Summer (a)



Winter (b)

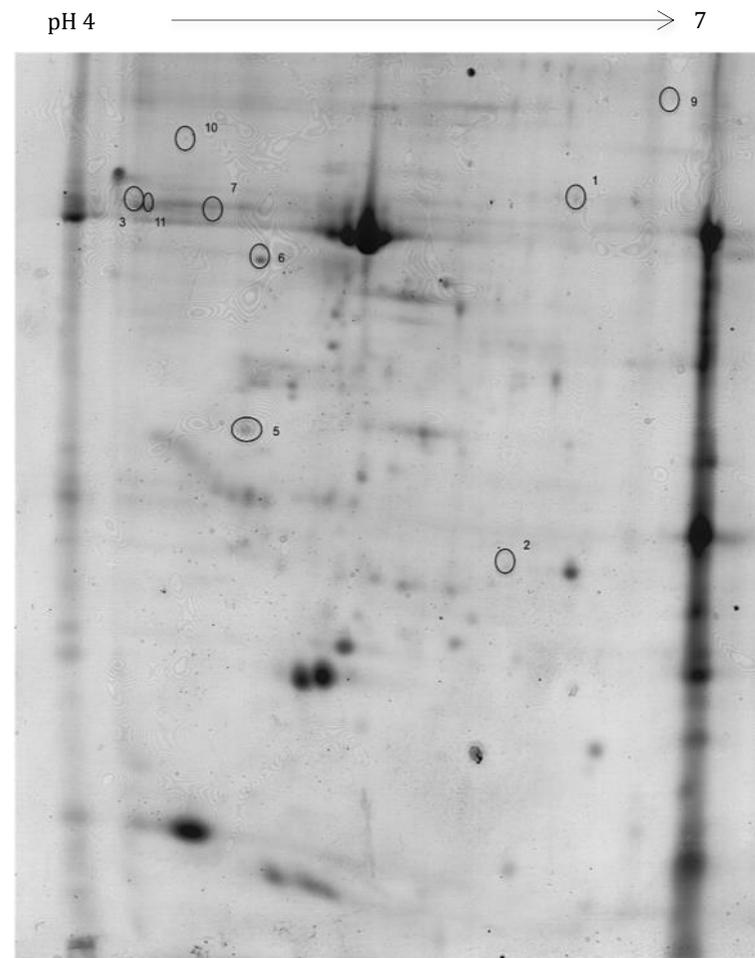


Figure 5.3: 2-Dimensional SDS PAGE electrophoresis of summer and winter adult *A. mellifera*

Proteomic profiles of summer (A) and winter (B) *A. mellifera* adults. Proteins were extracted as described and resolved by 2D SDS-PAGE, replicates (n=3). Proteins showing alterations to expression were excised and identified by LC-MS (2.14)

Spot number	Protein Annotation	Mr	pI	% Coverage	Protein Identity	Relative abundance		ANOVA p value (<0.05)	Protein function
						Summer	Winter		
1	Major royal jelly protein 1 precursor	49311	5.10	8%	gi 58585098	1	0.5	0.023	Involved in caste differentiation. Also involved in defence against bacteria and fungus.
2	Odorant binding protein 14 precursor	49311	5.71	32%	gi 94158822	1.5	1	0.002	Olfactory protein involved in behaviour and pheromone response
3	Cofilin/actin depolymerizing factor homolog 1	13060.5	5.95	27%	gi 391328000	1	2.8	0.025	Involved in actin binding and development of various systems e.g. gonad development
4	Thioredoxin-dependant peroxide reductase	25845	9.10	10%	gi 391347975	2.6	1	0.040	Redox regulation of the cell. Protects radical-sensitive enzymes from oxidative damage by a radical-generating system
5	peroxiredoxin-6-like	24941	5.31	10%	gi 391347975	1	2.9	0.018	Involved in redox regulation of the cell. Can reduce H ₂ O ₂ and short chain organic, fatty acid, and phospholipid hydroperoxides
6	14-3-3- protein zeta-like	27776	4.75	10%	gi 391330375	1	2.6	0.037	Developmental protein involved in protein folding, olfactory learning and nervous system development
7	cuticular protein precursor	6.82	6.82	12%	gi 399158374	1	2.3	0.040	Structural constituent of cuticle
8	ATP synthase	56113	4.12	18%	gi 391342624	4.4	1	0.001	Cellular metabolic process and energy production
9	Tropomyosin	32545	4.61	10%	gi 391342624	1.8	1	0.011	Protein involved in muscle regulation

Table 5.2 Identities of differentially expressed proteins from 2-Dimensional analyses of summer and winter adult *A. mellifera*
Identities of protein spots that were identified by LC-MS showing differential abundance between summer and winter adult *A. mellifera*. The relative fold abundance of the protein spots is also shown in Figure 5.4.

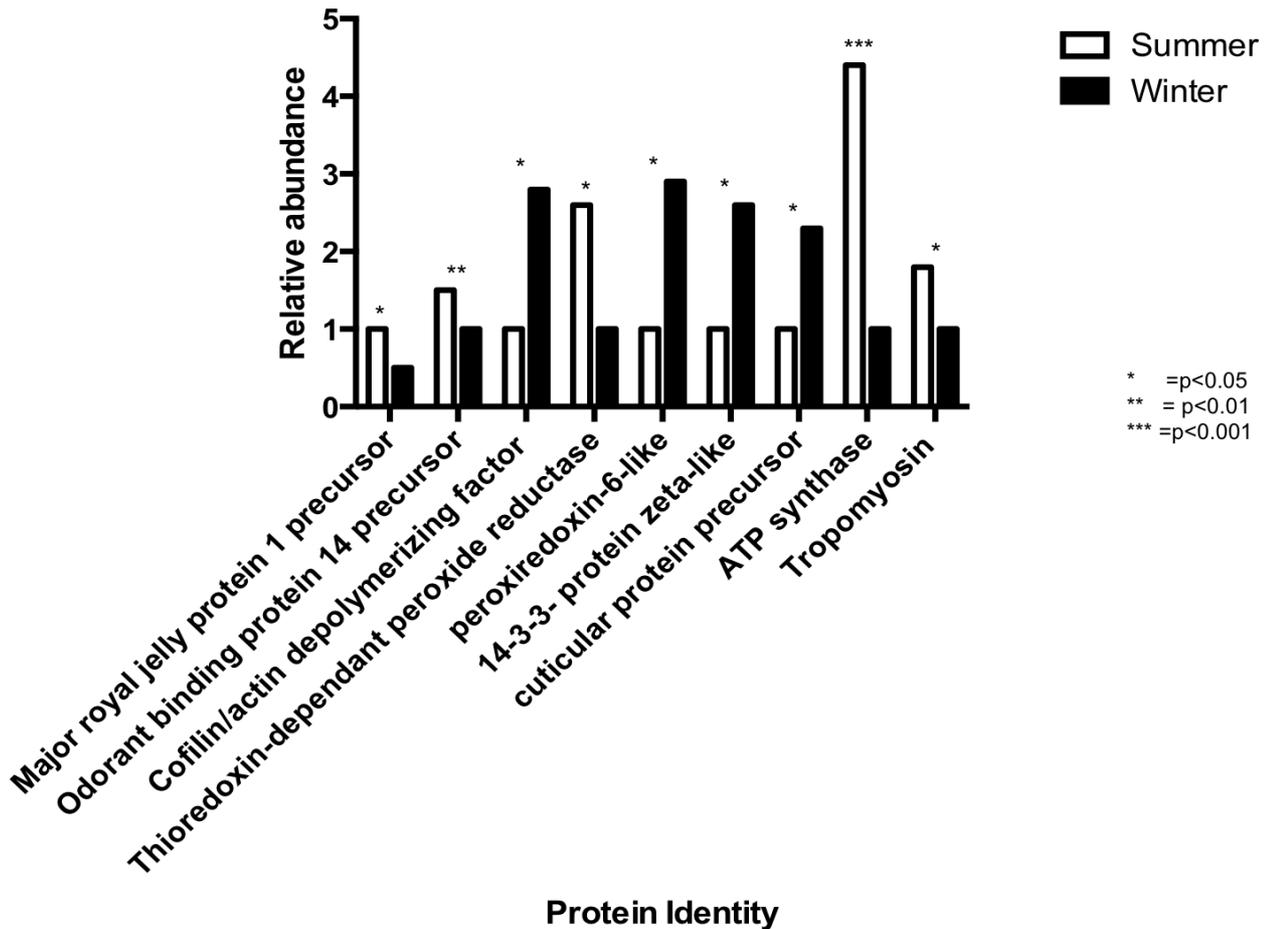
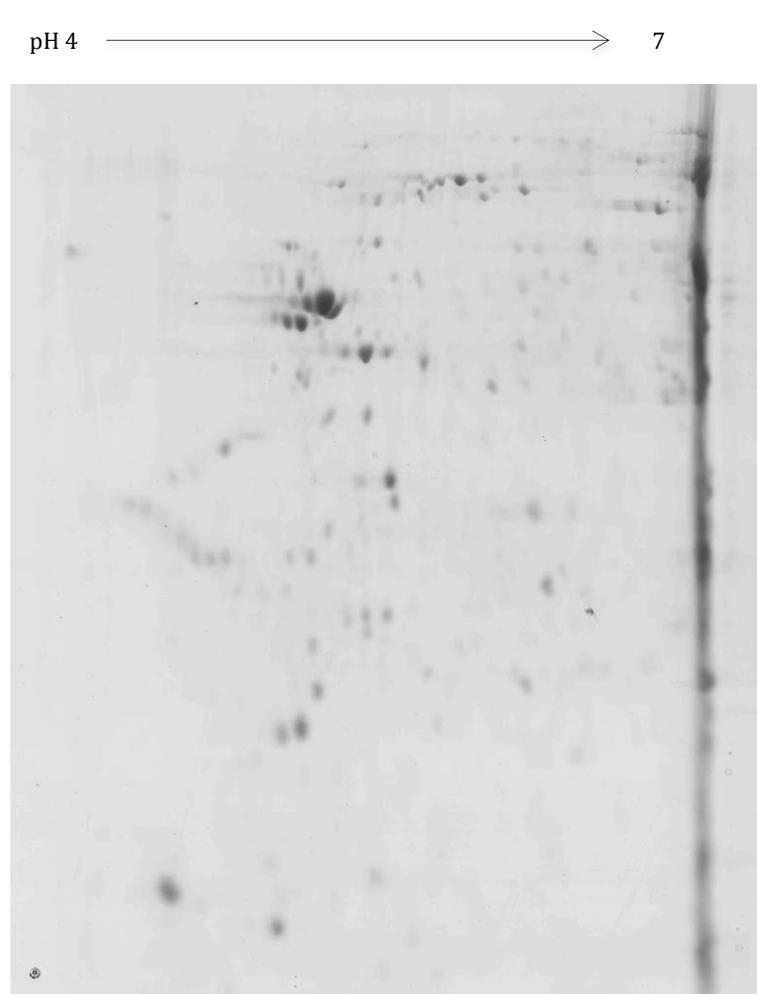


Figure 5.4 Relative abundance of significantly differentially expressed proteins from 2-Dimensional gel electrophoresis of summer and winter adult *A. mellifera*
 Graph shows the relative change in abundance of the 9 differentially abundant protein spots identified during comparative 2-Dimensional SDS-PAGE of summer period and winter period adult *A. mellifera* (Table 5.2). Protein extractions were carried out on independent occasions (n=3).

Unparasitized Winter(a)



Parasitized Winter (b)

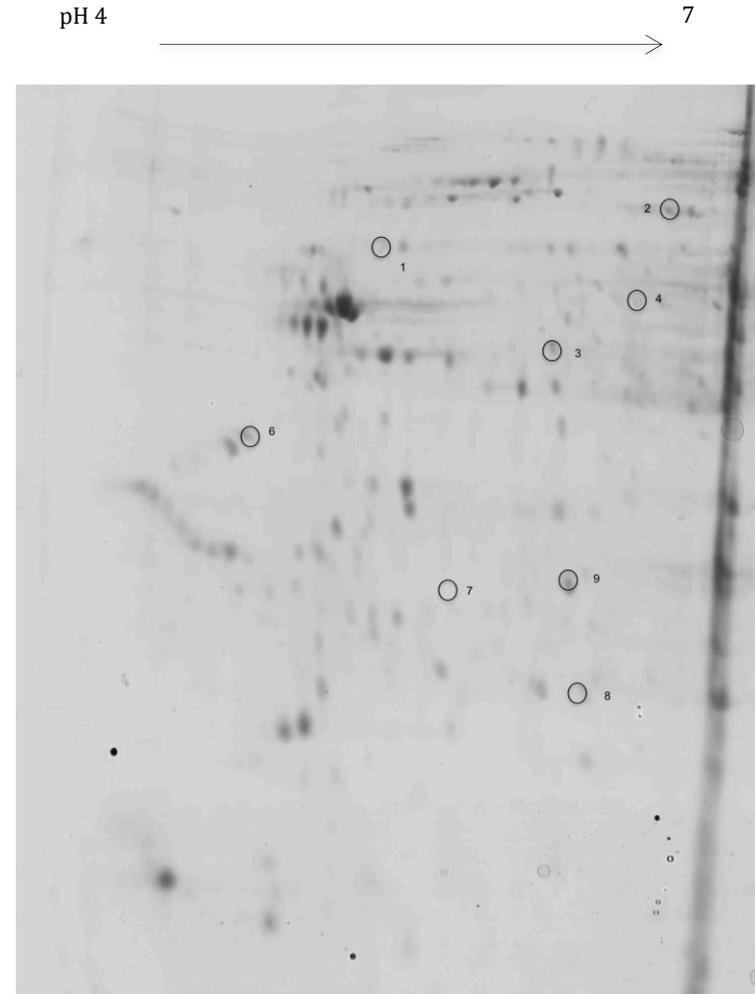


Figure 5.3: 2-Dimensional SDS PAGE gel electrophoresis of unparasitized and parasitized winter adult *A. mellifera*

Proteomic profiles of unparasitized (A) and parasitized (B) *A. mellifera* overwintering adults. Proteins were extracted as described and resolved by 2D SDS-PAGE, replicates (n=3). Proteins showing alterations to expression were excised and identified by LC-MS (2.13).

Spot number	Protein Annotation	Mr	pI	% Coverage	Protein Identity	Relative abundance		ANOVA P value (<0.05)	Protein function
						Unparasitized	Parasitized		
1	Translational endoplasmic reticulum ATPase TER94 isoform 1	90390	5.42	19%	gi 328783535	1	1.3	0.006	Hydrolase with ATPase activity
2	Succinate dehydrogenase flavoprotein subunit	73286	6.75	42%	gi 665005480	1	2.1	0.030	Oxidoreductase with electron carrier activity
3	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial like	48984	6.97	15%	gi 66548261	1	1.4	0.048	ATP and metal ion binding activity involved in small molecule metabolic process and cellular metabolism.
4	Enolase-like	39280	6.24	46%	gi 328776580	2.6	1	0.008	Magnesium binding protein involved in glycolysis
5	Lysosomal aspartic protease	42659	5.90	45%	gi 66560290	1	2.2	0.022	Aspartic-type endopeptidase activity
6	Tropomyosin 1 isoform b	32682	4.73	55%	gi 229577296	1	3.6	0.040	Muscle protein involved in muscular contraction
7	Hexamerin 110 precursor	112177	6.43	12%	gi 155369750	1.4	1	0.028	Storage protein that stores amino acids

8	Myosin heavy chain, muscle isoform 1	251217	5.60	7%	gi 328790595	1.6	1	0.016	Muscle protein involved in movement
9	Heat shock protein beta 1 like isoform 1	25608	6.01	16%	gi 110757651	1	1.4	0.033	Chaperone activity involved in negative regulation of apoptosis and response to growth factor signalling

Table 5.3 Identities of differentially expressed proteins from 2-Dimensional analyses of unparasitized and parasitized winter adult *A. mellifera*.

Identities of protein spots that were identified by LC-MS showing differential abundance between unparasitized and parasitized adult winter *A. mellifera*. The relative fold abundance of the protein spots is also shown in Figure 5.6.

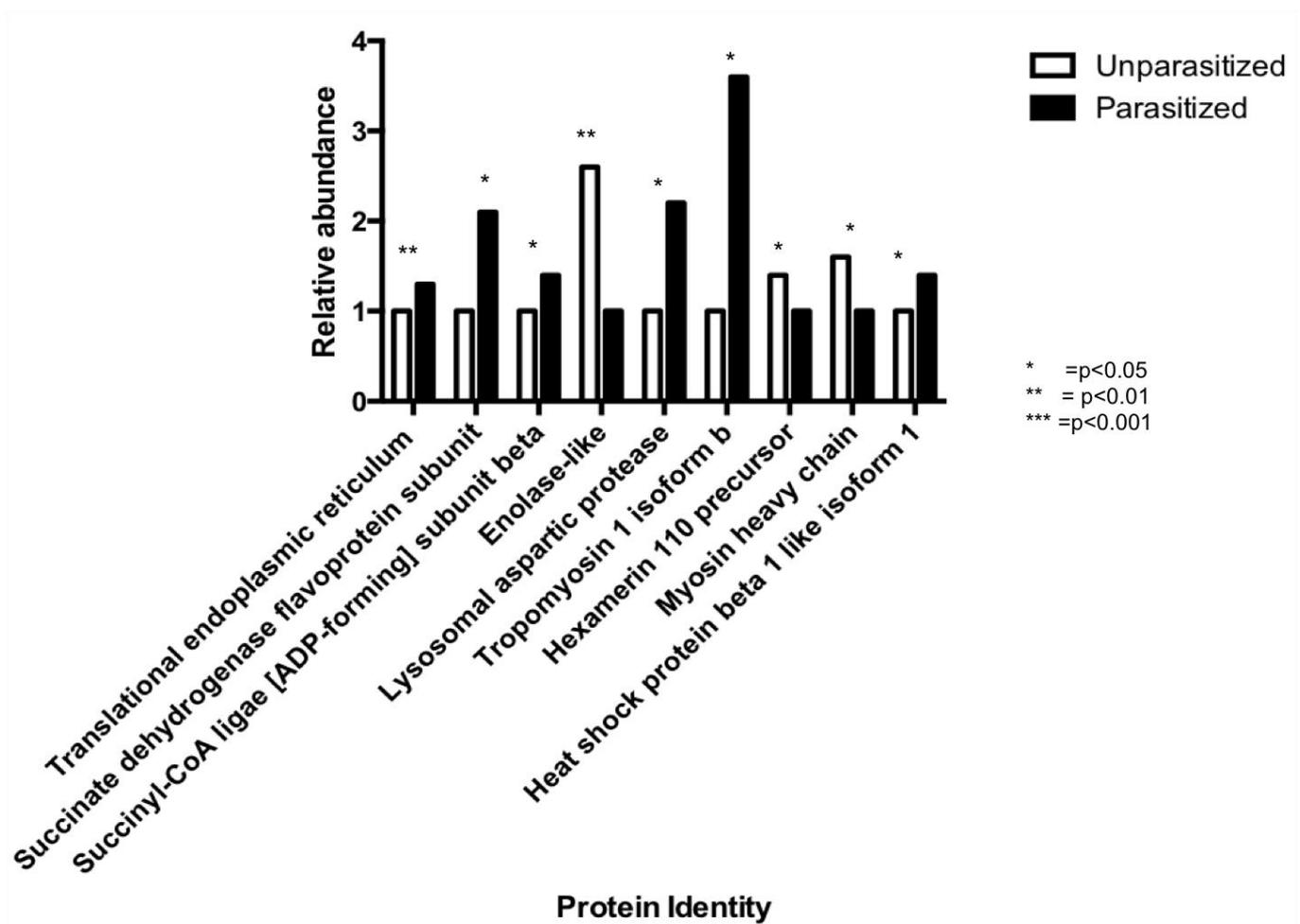


Figure 5.6 Relative abundance of differentially expressed proteins from 2-Dimensional gel electrophoresis of unparasitized and parasitized winter adult *A. mellifera*.

Relative abundance of the 9 proteins spots that were identified as being differentially expressed between the unparasitized winter and parasitized winter adult *A. mellifera* (Table 5.3). Protein was extracted on independent occasions (n=3).

5.3 Analysis of variations in the proteomic profile of *A. mellifera* pupae during parasitization by *Varroa*

5.3.1 Analysis of variations in the proteomic profile of *A. mellifera* drone pupae during parasitization by *Varroa*

Purple eye stage drone pupae were selected from an apiary in County Carlow and were analyzed for the presence of *Varroa* upon uncapping the cells. Protein was extracted from pupae that were found to be free from *Varroa* and pupae that were parasitized by between 1 and 3 adult female mites (2.19.1). Proteins were resolved by 2-Dimensional SDS PAGE gel electrophoresis and the protein spots showing variation in density by Progenesis SameSpots were excised for identification by LC-MS (Table 5.4). The majority of proteins identified as being differentially expressed between the unparasitized and parasitized drone pupae were found to be present at lower levels upon parasitization. Endocuticle structural glycoprotein was expressed at lower levels of abundance in the pupae that were parasitized by *Varroa* (2.5 fold decrease, $p = 0.023$). Cuticle proteins are involved in the structure of a healthy cuticle, which is one of the main barriers against infection (Vincent and Wegst 2004). ATP synthase (3.2 fold decrease, $p = 0.048$), and Hexamerin (2.1 fold decrease, $p = 0.05$) were present at lower levels in the pupae that were parasitized by *Varroa*. Hexamerin is a haemolymph storage protein and is important for nutrition of the developing pupae (Burmester 1999). ATP synthase is involved in the synthesis of ATP within the developing pupae (Müller and Grüber 2003). Arylphorin was present at higher levels of abundance in the parasitized drone pupae (5.4 fold increase, $p = 0.008$). Arylphorin is a storage protein that may have a role in the invertebrate immune response (Banville *et al.*, 2012). Fatty acid binding protein was also present at higher levels of abundance upon parasitization (7.5 fold increase, $p = 0.001$). Fatty acid binding protein is involved in lipid binding and has also been implicated in the stress response, in particular oxidative stress (Canavoso *et al.*, 2001).

5.3.2 Analysis of variations in the proteomic profile of *A. mellifera* drone pupae across varying levels of parasitization by *Varroa*

After establishing that parasitization by *Varroa* had an effect on the proteomic profile of drone pupae during development, the next step was to identify whether or not the level of parasitization also had an effect. Pupae were chosen based on the level of parasitization by *Varroa* upon uncapping of the cell, with the number of adult female mites per cell enumerated. Pupae were grouped for protein extraction based on being unparasitized (0 *Varroa*), mildly parasitized (1 *Varroa*), moderately parasitized (2 *Varroa*) and heavily parasitized (3+ *Varroa*). Following identification of the level of parasitism, protein was extracted from 3 pupae per replicate, with at least 3 replicates carried out on independent occasions. Whole body proteins were separated on the first and second dimension by SDS PAGE gel electrophoresis, any spots of interest showing varied expression by Progenesis SameSpots were excised and identified by LC-MS (2.13). A total of 17 protein spots were identified as being significantly different in their expression between the unparasitized and the pupae of different levels of parasitization (Table 5.5). The expression patterns can be seen in Figure 5.10. There was an increase of short chain dehydrogenase/reductase depending on the level of parasitization; rising steadily with *Varroa* numbers, to a total fold increase of 2.3. Hexamerin and Hexamerin 110 precursor also fell in abundance and this correlated with the level of parasitization, to total fold change in abundance of 2.33 fold and 1.4 fold decrease respectively. Hexamerin is a haemolymph storage protein and the fall in abundance dependent on the level of parasitization could be a result of haemolymph loss (Burmester 1999). Malate dehydrogenase, a protein with oxidoreductase properties, was present at much higher levels in the pupae that were parasitized by *Varroa*, with its total increase of 3.9 fold rose steadily with the rise in *Varroa* in each cell (Figure 5.10) (Minárik *et al.*, 2002). 3-ketoacyl-thiolase was present at higher levels in the parasitized pupae also, however its abundance is lowest in the pupae that were parasitized at a level of 2 *Varroa* per pupae, having fallen from a 1.9 fold increase in the pupae, parasitized by a single *Varroa*, before rising again to a 2.2 fold increase in the pupae parasitized by 3 *Varroa* (Figure 5.10). The 60kDa heat shock protein showed a total

increase of 2.14 fold. Apolipophorin increased in abundance to the highest level in the pupae parasitized by 2 *Varroa* (1.85 fold). Apolipophorin is a storage protein with a possible immune role and the drop when the parasitization becomes high could indicate a drop in immune function (Whitten *et al.*, 2004). Glutathione s-transferase, an oxidoreductase with stress related function within the cell, was found to increase under parasitization, rising sharply when two or more *Varroa* were present in the cell (Claudianos *et al.*, 2006). This increase could indicate that the pupae are under stress due to the presence of the *Varroa*.

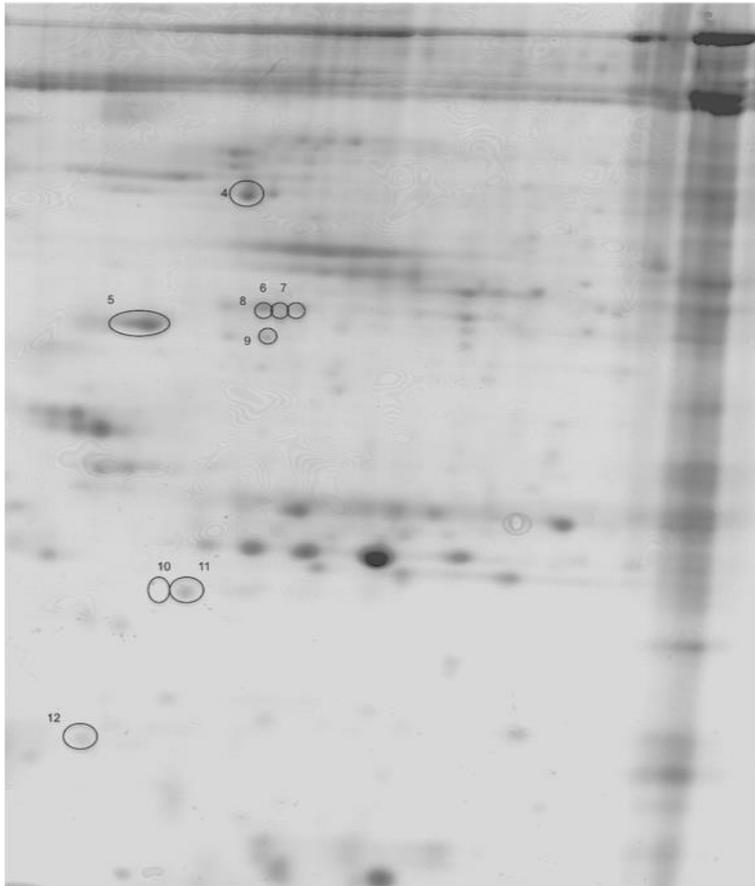
5.3.3 Summary

The host-parasite relationship of *Varroa* with its original host is almost exclusively built on interaction with drone brood. The aim of this section was to examine the variations to the proteomic profile that take place during parasitization by *Varroa* in drone pupae. Unparasitized drone pupae were first compared to pupae that had been parasitized by *Varroa*, with a number of proteins exhibiting variations in the level of abundance. Tropomyosin was present at lower levels in the drone pupae that were parasitized, which could indicate impaired growth of normal muscle due to parasitization by *Varroa*. The abundance of proteins involved in cuticle structure also fell in the parasitized drone pupae, which could be indicative of an impaired ability to heal following parasitization by *Varroa*.

The effect of different levels of *Varroa* on a developing drone pupa was then examined. The relative fold change in the proteins altered in abundance between the parasitized and unparasitized drone pupae was dependent on the level of parasitization. Hexamerin, for example, fell in abundance in the drone pupae that were parasitized by the highest number of *Varroa*. Stress proteins such as glutathione S-transferase increased in abundance dependent on the level of *Varroa*. These changes to the proteomic profile could indicate that a higher level of *Varroa* infestation could be problematic for a colony.

Unparasitized drone pupae (a)

pH 4 → 7



Parasitized drone pupae (b)

pH 4 → 7

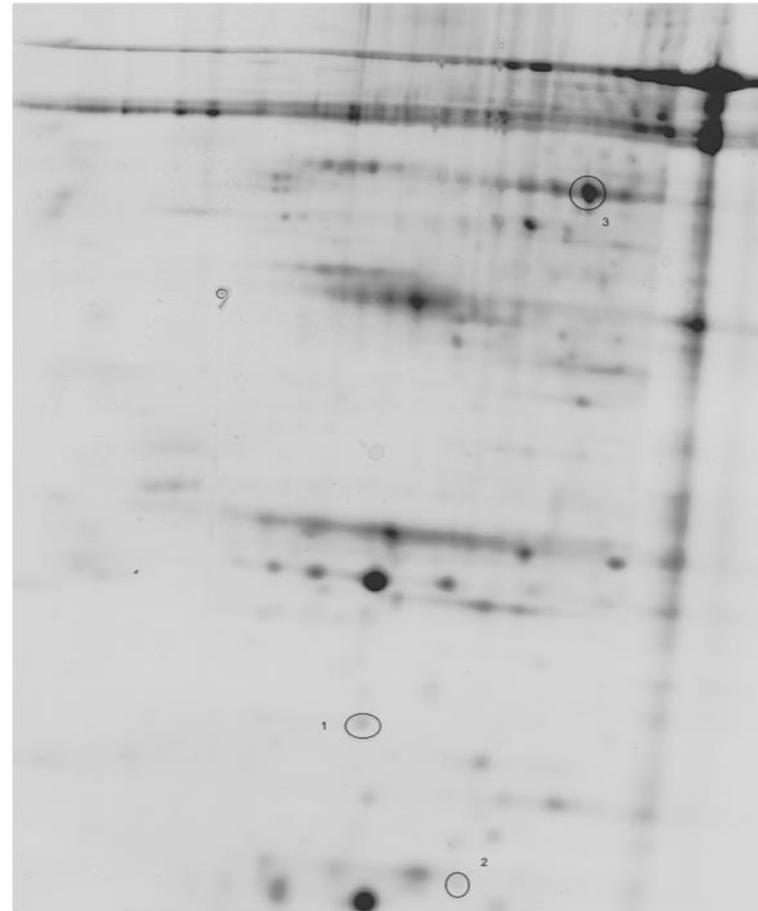


Figure 5.7 2-Dimensional SDS PAGE of unparasitized and parasitized drone pupal stage *A. mellifera*.

Proteomic profile of unparasitized (A) and parasitized (B) *A. mellifera* drone pupae. Proteins were extracted as described and resolved by 2D SDS-PAGE, replicates (n=3). Proteins showing alterations to expression were excised and identified by LC-MS (2.13)

Spot number	Protein Annotation	M _r	pI	% Coverage	Protein Identity	Relative abundance		ANOVA p value (<0.05)	Protein function
						Unparasitized	Parasitized		
1	Lysosomal aspartic protease	42659	5.90	16%	gi 66560290	3.3	1	0.008	Aspartic type endopeptidase activity
2	Endocuticle structural glycoprotein	20293	4.94	29%	gi 383851062	2.5	1	0.023	Component of cuticle
3	Tropomyosin 2-like	33052	5.13	39%	gi 48129583	5.7	1	0.023	Muscle protein involved in regulation
4	Tropomyosin 2-like	33052	5.13	29%	gi 48129583	5.4	1	0.001	Muscle protein involved in regulation
5	Tropomyosin 1 isoform B	32682	4.73	44%	gi 229577296	1	4.5	0.04	Muscle protein also involved in dendrite morphogenesis
6	Fatty acid binding like protein	15540	5.46	57%	gi 5858214	1	7.5	0.001	Lipid binding protein also implicated in stress response
7	ATP synthase subunit beta, mitochondrial	23632	5.30	65%	gi 48129583	3.2	1	0.048	Involved in ATP synthesis
8	APD-3-like protein	23632	6.49	11%	gi 2905786564	3.2	1	0.017	Component of the cuticle
9	60s ribosomal protein P1	11674	4.43	55%	gi 297591983	2.1	1	0.009	Structural component of ribosome involved in translational elongation
10	Tropomyosin 2-like	33052	5.13	29%	gi 48129583	4.6	1	0.004	Muscle protein involved in regulation
11	Arylphorin subunit alpha-like	81390	6.70	6%	gi 380018990	1	5.4	0.008	Storage protein with possible immune role

12	Hexamerin 110 precursor	112177	6.43	20%	gi 155369750	2.1	1	0.05	Storage protein for storing of nutrients
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Table 5.4 Identities of differentially expressed proteins from 2-Dimensional analyses of unparasitized and parasitized drone pupal stage *A. mellifera*

Identities of protein spots that were identified by LC-MS showing differential abundance between unparasitized and parasitized purple eye stage drone *A. mellifera*. The relative fold abundance of the protein spots is also shown in Figure 5.8.

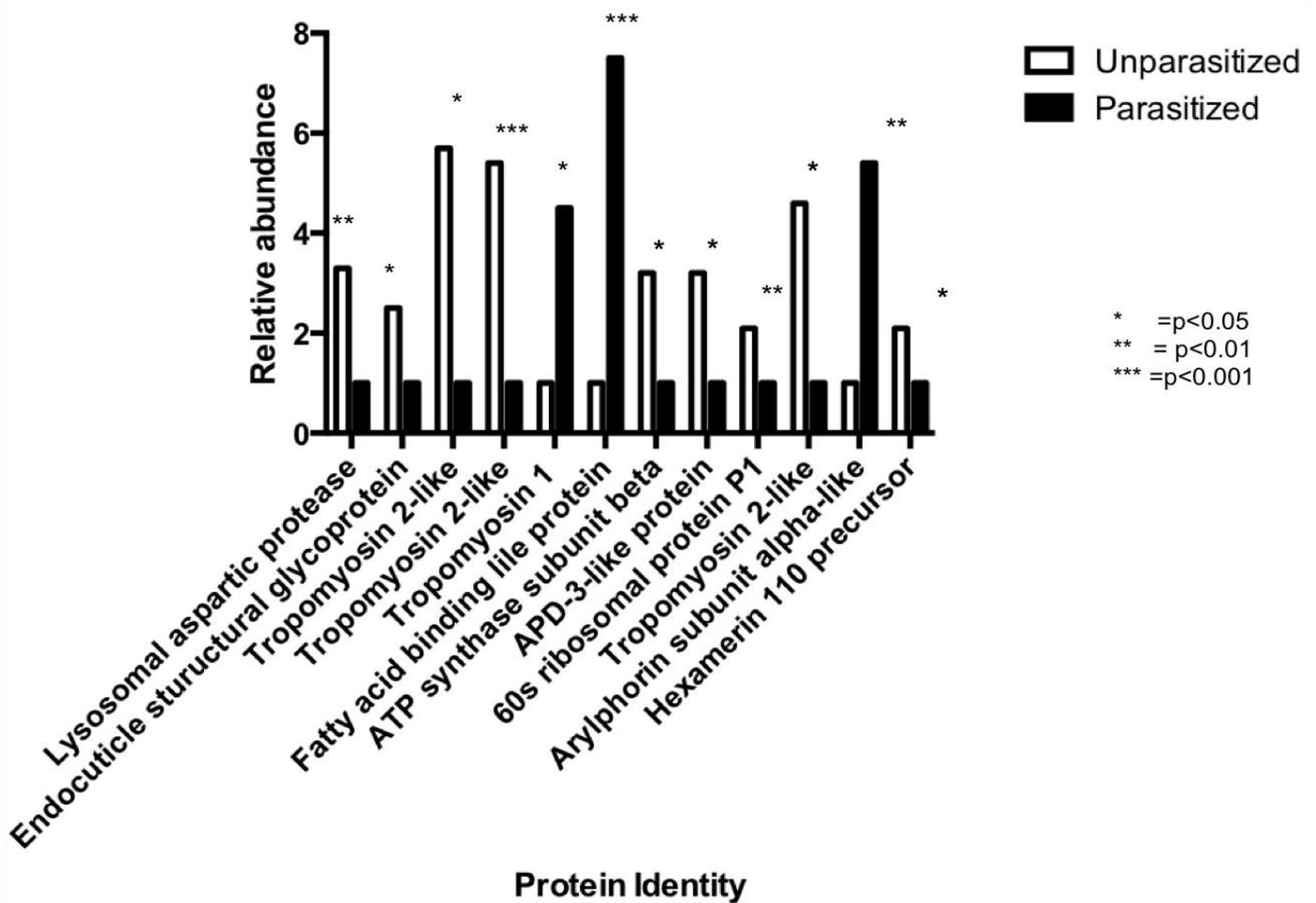


Figure 5.8 Relative abundance of differentially expressed proteins from 2-Dimensional SDS PAGE of unparasitized and parasitized drone *A. mellifera* pupae.

Relative abundance of the 12 proteins spots that were identified as being differentially expressed between the unparasitized and parasitized drone purple eye stage *A. mellifera* (Table 5.4).

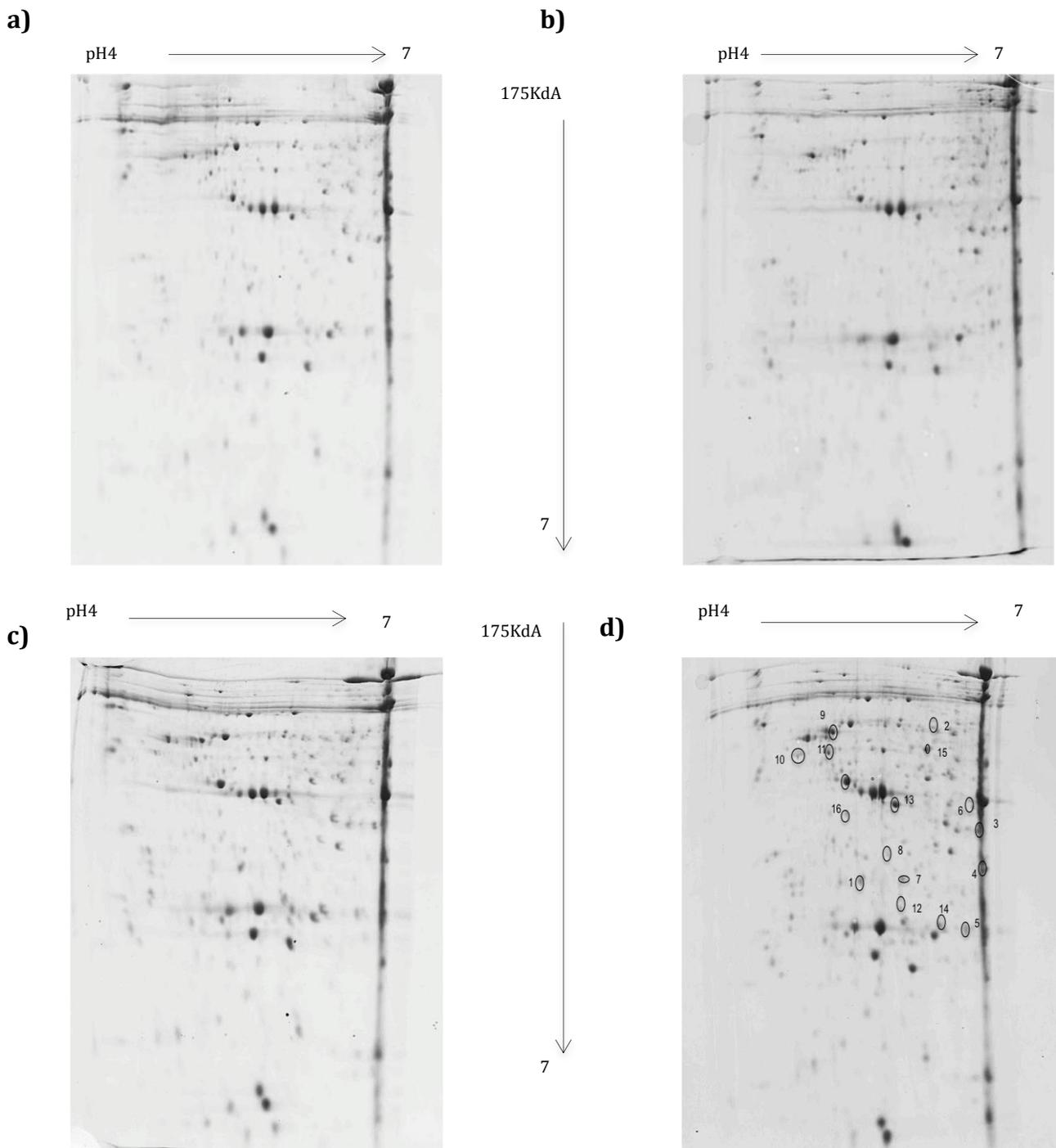


Figure 5.9 2- Dimensional SDS PAGE of drone pupal stage *A. mellifera* under varying levels of parasitization.

The proteomic profile as resolved by 2-Dimensional SDS PAGE gel electrophoresis of unparasitized (a) parasitized by 1 *Varroa* (b), parasitized by 2 *Varroa* (c) and parasitized by 3+ *Varroa* (d) pupae were compared and the spots of interest that showed significant alteration in density were excised for identification by LC-MS, Table 5.5.

Spot	Protein Annotation	Mr	pI	% Coverage	Protein Identity	Protein function
1	Short chain dehydrogenase/reductase	27638	5.24	57%	gi 58585184	Has oxidoreductase activityInvolved in cellular metabolism
2	Hexamerin	81551	6.43	14%	gi 149939403	Storage protein used for storage of nutrients
3	N-acetylneuraminase lyase-like	33052	5.13	39%	gi 48129583	Lyase involved in carbohydrate binding
4	Malate dehydrogenase	35953	5.22	52%	gi 66513092	Carbohydrate binding and oxidoreductase properties
5	Phosphatidylethanolamine-binding protein homolog	20646	8.84	45%	gi 66524882	Involved in lipid binding
6	3-ketoacyl-CoA-thiolase	42710	8.89	69%	gi 48097100	Transferase involved in fatty acid beta oxidation
7	Actin-2a-like	41894	5.48	40%	gi 328707799	Actin type protein involved in cell structure
8	Proteasome subunit alpha type 1-like	60549	5.78	34%	gi 66530404	Part of proteasome core complex
9	60kDa heat shock protein	11674	4.43	48%	gi 66547450	Chaperonin involved in stress response of cell
10	ATP synthase subunit beta	55316	5.30	42%	gi 48129583	Protein involved in energy production
11	Protein disulfide isomerase A6 like	47688	5.12	29%	gi 66551889	Involved in ubiquitin dependent protein catabolic process
12	Short chain dehydrogenase/reductase	27638	5.24	71%	gi 5858184	Has oxidoreductase activityInvolved in cellular metabolism
13	Apolipoproteins-like	375895	8.68	10%	gi 380014988	Fatty acid transport and lipid binding. Could have novel immune role
14	Arginine Kinase	40331	5.66	45%	gi 5858146	Transferase involved in phosphorylation
15	Hexamerin 110 precursor	112177	6.43	13%	gi 155369750	Storage protein involved in nutrition
16	Kynurenine-oxoglutarate	13664	9.73	14%	gi 328789112	Aminotransferase involved in metabolism
17	Glutathione S-transferase	23721	6.12	32%	gi 3283353	Oxidoreductase involved in stress response

Table 5.5 Proteins identified as being differentially abundant between the unparasitized and parasitized drone pupae

Identities of protein spots that were identified by LC-MS showing differential abundance between unparasitized and parasitized purple eye stage drone *A. mellifera*. The relative fold abundance of the protein spots is also shown in Figure 5.10.

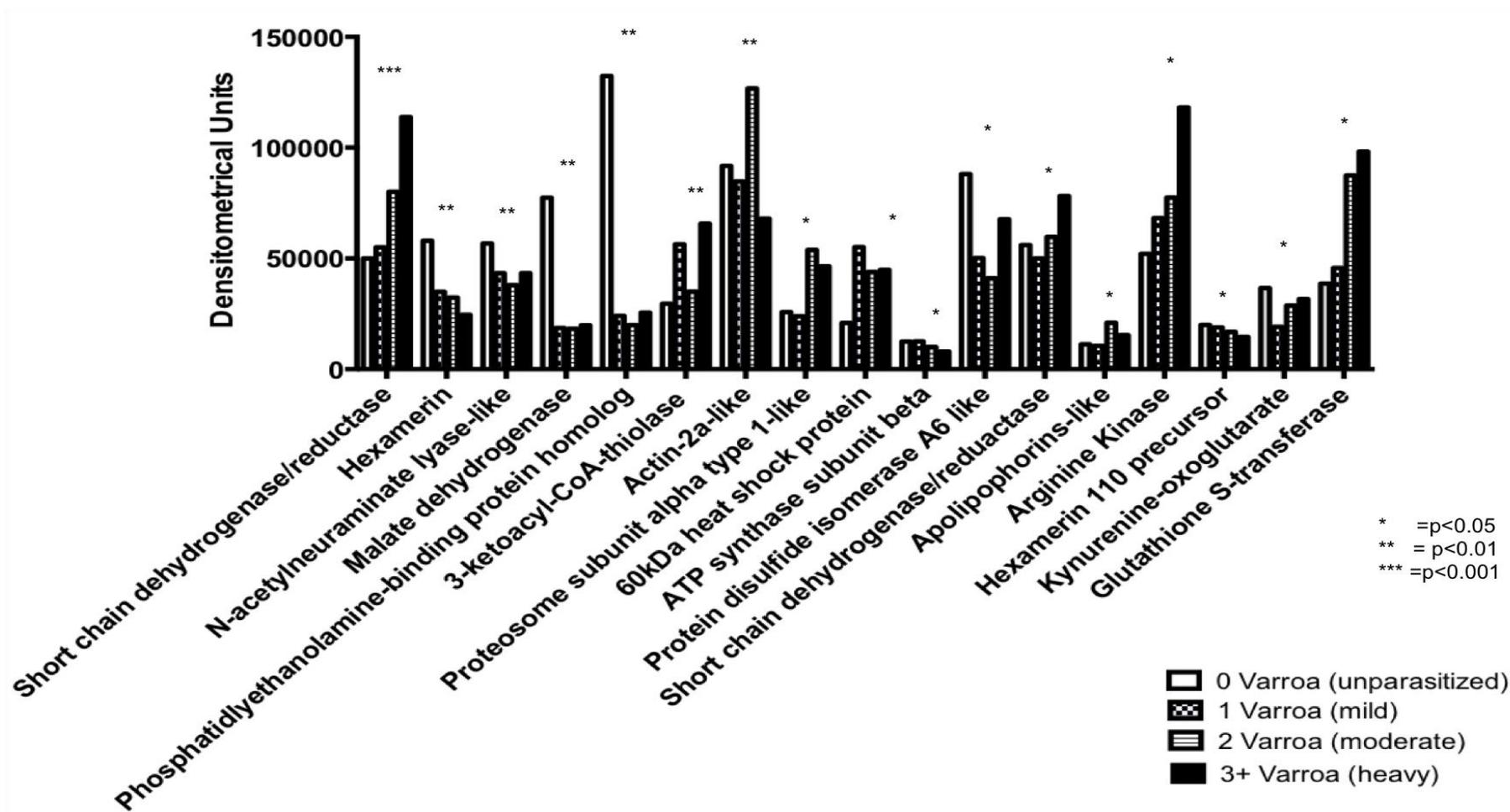


Figure 5.10 Densitometrical variations in identified proteins from comparative 2-Dimensional SDS PAGE analysis on the effect of *Varroa* numbers on developing *A. mellifera* drone pupae.

Relative abundance of the 17 proteins spots that were identified as being differentially expressed between the unparasitized and parasitized drone purple eye stage *A. mellifera* (Table 5.5). Unparasitized= 0 mites, Mild=1 mite, Moderate=2 mites, Heavy= 3+ mites.

Protein extractions were carried out independently on separate occasions (n=3).

5.3.4 Analysis of variations in the proteomic profile of drone pupal stage *A. mellifera* during parasitization using label free mass spectrometry

In order to determine the full effect of parasitization by *Varroa* on the developing drone pupa, the proteomic profile of unparasitized pupae was compared to heavily parasitized pupae using label free quantitative proteomics. Protein was extracted from a single pupa per replicate, with a known level of parasitization of 4 adults and 8 young *Varroa* exactly per cell, to minimize variability between samples. Whole body protein extraction was carried out on a total of four replicates per sample, cleaned up using c18 columns, and trypsin digested overnight to achieve maximum peptide recovery (Section 2.14). Label free quantitative proteomics gives an excellent overview of all of the proteomic changes that are occurring within the organism and enables a comparison of the abundance of these proteins between two or more sample sets, such as unparasitized versus parasitized drone pupae (Bantscheff *et al.*, 2012).

A total of 2518 peptides were identified, 139 proteins were determined to be differentially abundant proteins (ANOVA $p < 0.05$) at a fold change greater than ± 1.5 , of which 80 were found in higher abundance in the parasitized pupae (Table 5.6) and 59 were found in higher abundance in the unparasitized pupae (Table 5.7). There were also a total of 51 proteins that were identified as being exclusively expressed between the two sample sets, with 21 exclusive to the unparasitized pupae and 30 exclusive to the pupae that were parasitized. The proteins were only determined to be exclusively expressed in the control or parasitized pupal profile if they were absent from all four replicates and present in at least three of the four replicates in the control or parasitized pupae respectively. These proteins were termed as “exclusively expressed proteins” (Table 5.8). These protein hits were also used in the statistical analysis of the total differentially expressed group following imputation of the zero values using a number close to the lowest value of the range of proteins plus or minus the standard deviation. After data imputation these proteins were included in subsequent statistical analysis (Tables 5.6 and 5.7). The overall proteomic profile of expression can be seen in Figure 5.11 in a heat map derived from hierarchical clustering using the Perseus software (2.14.7), representing overall expression of all of the differentially abundant proteins. The proteins showing

the highest and lowest levels of changes in abundance between the parasitized and the control pupae are highlighted in a scatter volcano plot, also obtained using Perseus software (2.14.7), and this can be seen in Figure 5.12. The proteins highlighted on the right hand side of the red box present in higher levels in parasitized pupae at a fold difference $\text{Log}(2)$. The protein that was present at the highest level of abundance in the parasitized pupae was apidermin 2 precursor, present at 48 times higher level upon parasitization (Table 5.6, Figure 5.12). Myosin regulatory light chain 2 was present at levels of 13.5 times higher in the parasitized pupae, and it is a calcium binding protein involved in muscle contraction, with a novel role in the immune response (Al-Khayat *et al.*, 2003; Han *et al.*, 2010). MD-2 related lipid recognition protein-like was found exclusively expressed in the parasitized pupae (Table 5.8) and appeared at a level of 5.73 fold higher abundance post-imputation when included in statistical analysis. MD-2 is a lipid recognition protein and an essential co-receptor in the LPS/Toll like signaling pathway (Inohara and Nuez 2002). Girdin-like protein was also present in the exclusively expressed group of protein (Table 5.8), and post-imputation and statistics, it showed a 6.68 fold increase in abundance in the parasitized pupae. Girdin is a positive regulator of cell growth and proliferation and a negative regulator of apoptosis (Enomoto *et al.*, 2006). Ornithine aminotransferase was exclusively expressed in the parasitized pupae (Table 5.8) and showed a 17.54 fold increase in abundance in the parasitized pupae. Ornithine aminotransferase is involved in cellular metabolism, and is found expressed at higher levels in queen larvae during differentiation into queen in the larval stage (Li *et al.*, 2010).

The proteins appearing on the left hand side of the plot (Figure 5.12) are present in lower levels of abundance in parasitized pupae. The protein present at the lowest levels of abundance in the parasitized pupae was mantle protein-like, at a fold change of 14.56 lower during parasitization. Little information is known about this protein, but due to its hydrophobic proline rich motifs it could potentially be a component of the cuticle (Selkirk *et al.*, 1991). A number of proteins involved in cuticle formation and structure were present at much lower levels of abundance in the pupae that were parasitized (Pupal cuticle protein-like; 8.41 fold decrease; Table 5.7, Glycine rich cuticle protein; 6.11

fold decrease, Table 5.7, Cuticle protein 18.7 like; 2.50 fold decrease; Table 5.7). A number of uncharacterized proteins were found present at lower levels of abundance upon parasitization also in the parasitized drone pupae (LOC726920; 6.57 fold decrease; Table 5.7, LOC100577362; 2.33 fold decrease; Table 5.7, LOC102653832; 2.32 fold decrease; Table 5.7). These proteins are possible cuticle proteins as they show homology to already characterized cuticular components (bLINK BLAST resource <http://www.ncbi.nlm.nih.gov/sutils/blink>). Two proteins involved in development and sex determination (Protein double-sex like; 11.66 fold decrease, Table 5.7; Maternal protein exuperantia; 5.28 fold representative decrease post imputation; Table 5.7 and Table 5.8) were found at significantly lower levels of expression following parasitization of the drone pupae. These proteins are involved in development and signaling for development, and also involved in sex and caste determination (Amrein *et al.*, 1988, Guerrero *et al.*, 2009). Flocculation protein FLO11 protein was found at 2.51 fold lower abundance in the parasitized pupae. This protein has been shown to be involved in aggregation and flocculation of cells in *Saccharomyces cerevisiae* (Bayly *et al.*, 2005; Goossens and Willaert 2010). Lower abundance here could indicate impaired haemocyte function or impaired immune response. Two zinc finger binding proteins were also present at lower levels of abundance in the parasitized drone pupae (Zinc finger 512b like; 4.83 representative fold decrease post imputation; Table 5.7 and Table 5.8, Zinc finger 512b like; 3.82 fold decrease; Table 5.7). Zinc finger proteins are involved in metal ion binding and DNA binding (Laity *et al.*, 2001).

The Blast2GO annotation software (www.blast2GO.com) was used to group proteins based on conserved gene ontology (GO) terms in order to identify processes and pathways altered between the unparasitized and parasitized drone pupae. GO terms were categorized by biological processes (BP; Figure 5.13) and molecular function (MF; Figure 5.14). Grouping the proteins into BP level 3 showed a number of protein groups that were altered in overall proportion (Figure 5.13). The percent of overall proteins involved in cellular component biogenesis increased from 1.6% to 3.4% of the overall proportion of protein in the parasitized and the unparasitized pupae, respectively (Figure 5.13). This group contains structural proteins such as

actin, and calmodulin, and also contains a number of proteins involved in transcription such as transcription factors and histone proteins.

Proteins grouped into the cellular metabolic process GO term were also changed significantly between the unparasitized and the parasitized drone pupae, from 7.8% to 14.9% in the unparasitized versus the parasitized pupae, respectively (Figure 5.13). Proteins assigned this GO term included enzymes involved in metabolism such as NADH dehydrogenase and also ribosomal proteins such as 40s and 60s ribosomal subunit components. Grouping the proteins according to molecular function level 3 present in the unparasitized pupae and compared to the parasitized pupae also showed certain groups that had changed significantly in proportion in the pupae that were parasitized by *Varroa* (Figure 5.14). The most noticeable change was in the group termed Hydrolase activity (Figure 5.14). There was a 6 fold increase in proportion of proteins with this GO term in the parasitized drone pupae, from 1.5% of the overall protein content in the unparasitized pupae to 9% in the parasitized pupae (Figure 5.14). Proteins grouped into the hydrolase category included fatty acid synthase, esterase, and serine proteases, which play a role in the insect immune response through the melanization cascade (Gorman and Paskewitz 2001; Ligoxygakis *et al.*, 2002). There was also a substantial increase in the heterocyclic compound binding GO group, from zero to 12% of the overall proportion of proteins. This group contains proteins such as those involved in transcription and translation, for example histone proteins, transcription factors, and elongation factors.

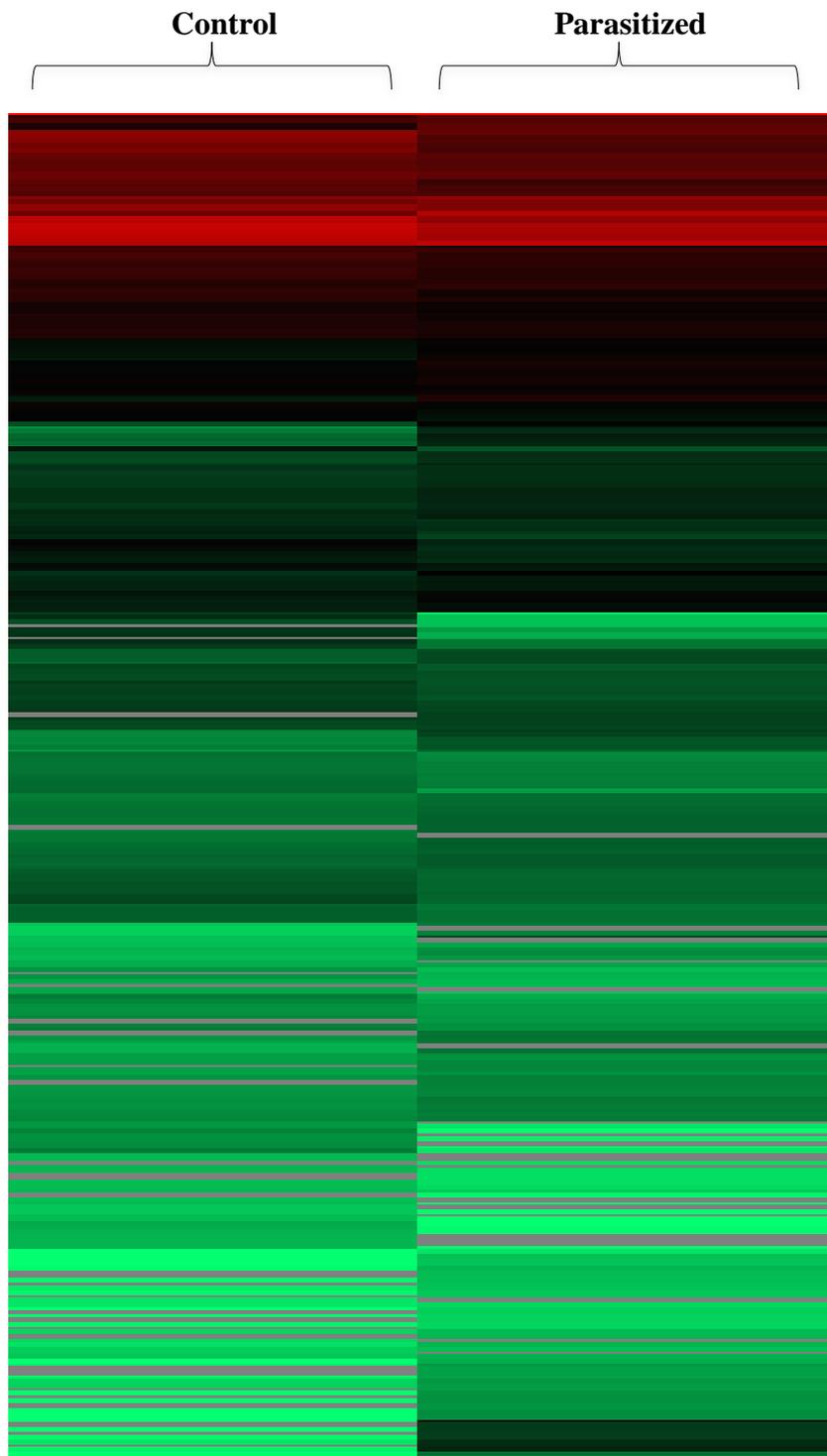


Figure 5.11 Hierarchical clustering of the quantitative differences in the proteomic profile of unparasitized and parasitized drone pupae

The heat map shows the variation in abundance of the proteins from the profile of control and heavily parasitized drone pupae obtained using Perseus software (2.14.7). The grey sections indicate an area of no detected expression, red areas indicate high level of abundance, and the green areas indicate low levels.

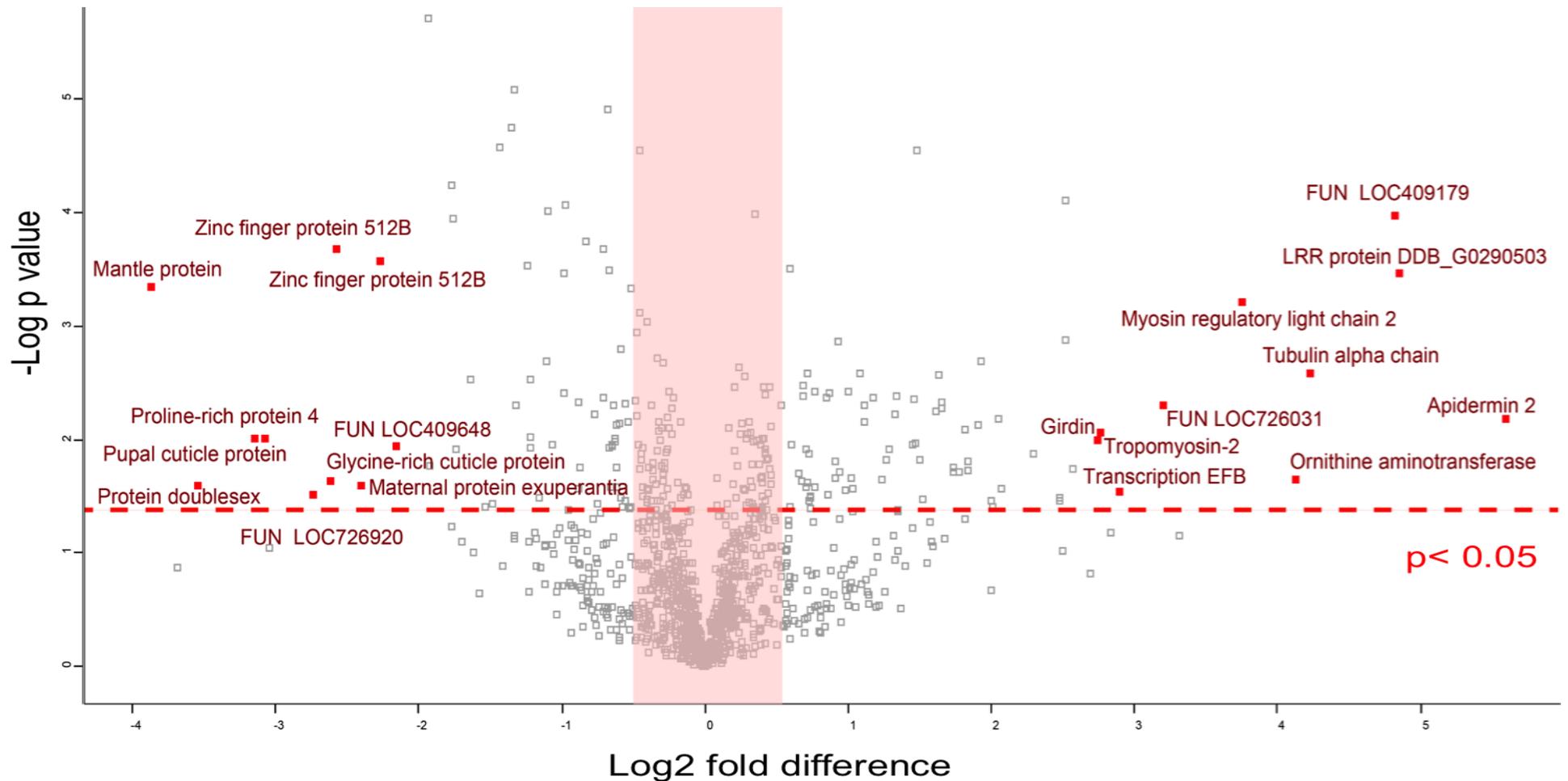


Figure 5.12 Volcano plot highlighting the twenty proteins present in the highest and lowest abundances between the unparasitized and parasitized drone pupae.

Image of Volcano plot obtained using Perseus software (2.14.7). Proteins above the line are statistically significant (p value <0.05) and those to the right and left of the vertical lines indicate fold changes greater than 1.5 fold positive and 1.5 fold negative in the parasitized and unparasitized drone pupae respectively. The red box indicates proteins that were not differentially expressed over a fold change of +/- 1.5 between the unparasitized and parasitized drone pupae so were not deemed significant.

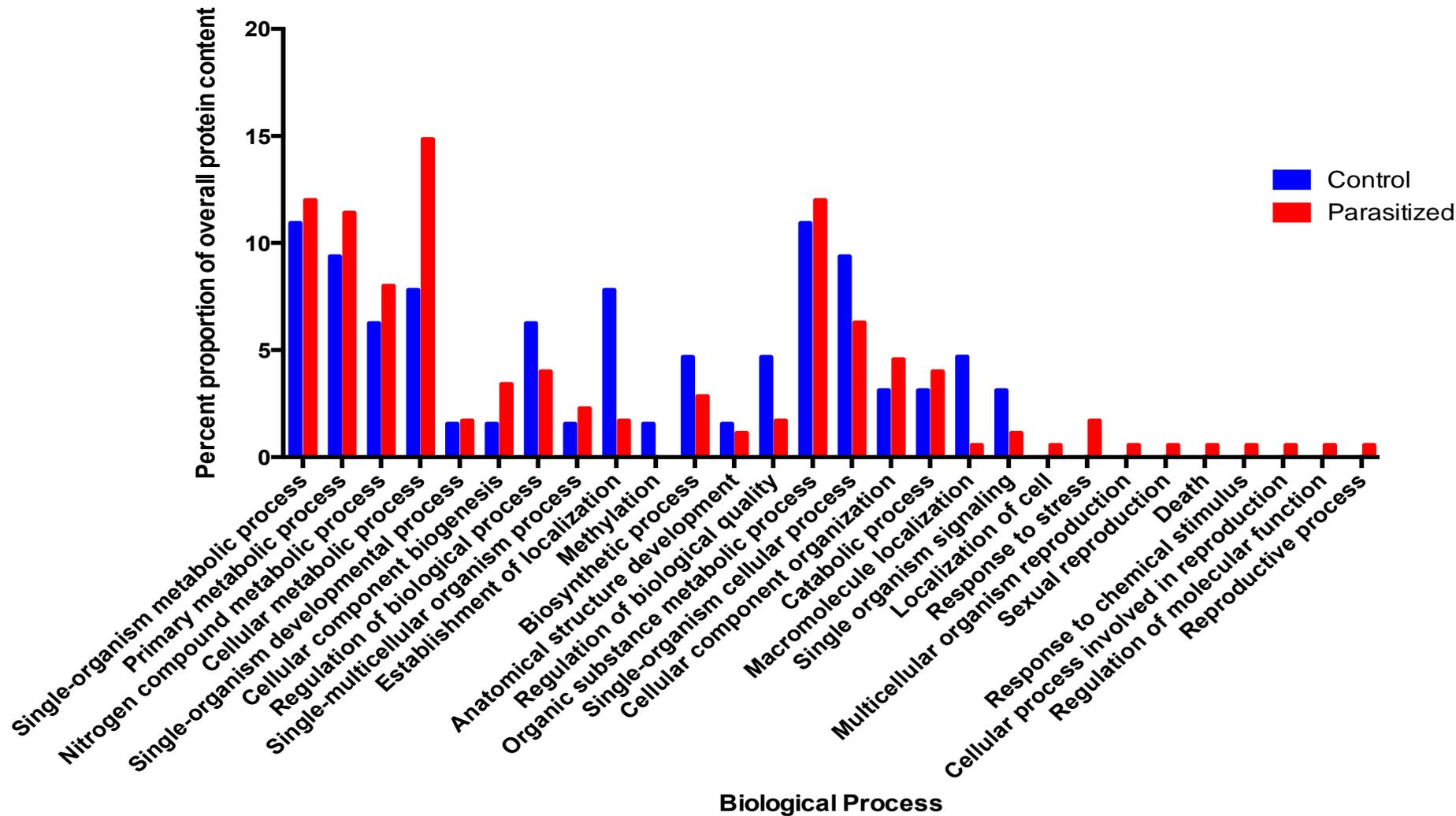


Figure 5.13 Biological Process grouping of proteins found in unparasitized and parasitized drone pupae
 Bar chart showing changes to proportion of proteins involved in various Biological Processes at level 3 ontology. Bar chart shows the grouping of proteins based on Biological process annotation using Blast2Go. Proteins were assigned groups based on involvement in Biological processes for both the unparasitized and the parasitized samples. Each group was assigned a percentage proportion of the total proteins found in the proteomic profile of each sample group and graphed on a comparative bar chart. (Y-axis is the group's percentage of overall proteins found in sample).

Protein annotation	Protein Identity	Peptides	Sequence coverage [%]	PEP	Overall Intensity	Expression in Parasitized drone
Apidermin 2	gi 145386569 ref NP_001078815.1	3	37.7	1.06E-08	2.41E+09	48.22
Putative leucine-rich repeat-containing protein DDB_G0290503	gi 571543335 ref XP_006559699.1	28	42.3	7.15E-173	5.63E+08	28.82
Acetyl-CoA hydrolase	gi 66531438 ref XP_392704.2	25	64.3	5.26E-150	6.99E+08	28.37
Tubulin alpha chain	gi 571574764 ref XP_006563567.1	23	67	1.76E-239	8.78E+08	18.73
Ornithine aminotransferase	gi 571575364 ref XP_006563788.1	22	66.5	2.53E-111	1.02E+09	17.55
Myosin regulatory light chain 2	gi 571563419 ref XP_006558384.1	8	37.1	2.53E-38	3.92E+08	13.46
Repetitive proline-rich cell wall protein	gi 292494895 ref NP_001167610.1	5	43	1.20E-12	2.66E+08	9.17
Transcription elongation factor B polypeptide 2	gi 66507736 ref XP_623908.1	5	60.7	7.14E-28	4.17E+08	7.47
Tropomyosin-2	gi 571532587 ref XP_006558182.1	22	68	2.06E-115	1.21E+09	6.78
Girdin	gi 571501093 ref XP_006562021.1	2	2.6	5.41E-08	2.01E+08	6.69
Uncharacterized protein LOC725683	gi 110749186 ref XP_001121500.1	7	10.3	7.16E-19	1.57E+08	5.94
MD-2-related lipid-recognition protein	gi 110764750 ref XP_392711.3	2	27	2.02E-07	1.51E+08	5.74
Hexaprenyldihydroxybenzoate methyltransferase	gi 66560294 ref XP_395548.2	5	25.5	2.81E-30	1.30E+08	5.71
Troponin I	gi 571514045 ref XP_006568754.1	11	35.4	3.79E-98	8.58E+08	5.56
Uncharacterized protein LOC725148	gi 571528885 ref XP_006567675.1	17	70.1	1.89E-81	5.32E+08	5.55
Mitochondrial inner membrane protei	gi 328779076 ref XP_392094.3	15	27.2	4.51E-50	1.54E+08	4.92
Uncharacterized protein LOC100578511	gi 328779643 ref XP_003249683.1	8	52.2	1.36E-34	1.31E+08	4.20
Transcription elongation regulator 1	gi 571577515 ref XP_006572093.1	19	72.5	0	9.93E+09	4.13

Gamma-glutamylcyclotransferase CG2811	gi 66522157 ref XP_623476.1	11	72.8	3.64E-25	1.23E+08	4.03
Ferric-chelate reductase 1	gi 328775989 ref XP_003249096.1	6	46.3	6.06E-59	2.32E+08	3.99
Troponin T	gi 571532274 ref XP_006557549.1	24	45.8	8.15E-145	1.71E+09	3.82
Neurochondrin	gi 571529028 ref XP_006567699.1	17	28.7	9.57E-82	6.62E+08	3.76
2-oxoglutarate dehydrogenase	gi 571554595 ref XP_006566130.1	20	28.3	1.53E-73	3.53E+08	3.58
Titin	gi 571539391 ref XP_006564370.1	92	7.8	0	1.03E+09	3.57
Uncharacterized protein LOC725283	gi 328792556 ref XP_001121151.2	3	27.8	8.09E-15	7.83E+07	3.53
NADH dehydrogenase [ubiquinone] iron-sulfur protein 2	gi 328775921 ref XP_397330.3	13	41.5	3.14E-55	1.74E+08	3.43
Cytochrome b-c1 complex subunit 8	gi 110751008 ref XP_001122467.1	3	34.6	6.05E-09	9.45E+07	3.32
Pupal cuticle protein	gi 571546137 ref XP_006568334.1	8	47.7	3.42E-55	1.13E+09	3.32
Troponin C type I	gi 571559082 ref XP_006566313.1	12	71.7	1.70E-65	1.23E+09	3.15
Myosin light chain alkali	gi 571563560 ref XP_006558909.1	13	88.3	5.70E-74	3.44E+09	3.13
Myosin heavy chain, muscle	gi 571546923 ref XP_006569863.1	201	71.2	0	4.02E+10	3.10
Paramyosin, long form	gi 66510482 ref XP_393281.2	88	71	0	1.02E+10	3.05
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	gi 328778513 ref XP_396785.4	10	35.9	1.71E-26	1.54E+08	2.87
Hexokinase type 2	gi 571573226 ref XP_006557649.1	24	61	1.13E-108	5.87E+08	2.84
Long-chain-fatty-acid--CoA ligase 3	gi 571548426 ref XP_006561674.1	9	13.4	6.53E-25	9.60E+07	2.78
Small nuclear ribonucleoprotein G	gi 66525153 ref XP_625205.1	3	38.2	4.75E-09	6.03E+07	2.76
Carboxylesterase	gi 571539116 ref XP_006564307.1	15	36.2	2.63E-63	2.39E+08	2.74

Uncharacterized protein LOC100577080	gi 571543606 ref XP_006561488.1	4	16.2	9.70E-14	5.38E+07	2.74
Yorkie	gi 571554364 ref XP_006566090.1	4	15.3	3.91E-19	7.63E+07	2.55
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9	gi 48107260 ref XP_393089.1	6	46.9	2.46E-24	1.73E+08	2.54
Inosine triphosphate pyrophosphatase	gi 571567059 ref XP_006559389.1	5	31.1	7.83E-12	8.86E+07	2.53
Arginase-1	gi 328786638 ref XP_624935.2	14	45.6	2.11E-60	4.43E+08	2.51
Lon protease	gi 571533607 ref XP_006562528.1	26	36.4	4.98E-96	3.22E+08	2.44
Putative leucine-rich repeat-containing protein DDB_G0290503	gi 571515949 ref XP_006569117.1	10	15.7	2.91E-37	1.27E+08	2.41
Serine/threonine-protein phosphatase 4 catalytic subunit	gi 110774832 ref XP_001123151.1	5	33.7	4.48E-22	3.72E+07	2.35
Tropomyosin-1	gi 571567292 ref XP_006560146.1	17	53.5	6.11E-122	5.05E+08	2.25
Probable GDP-L-fucose synthase	gi 571565660 ref XP_006566989.1	6	26.4	1.86E-21	8.20E+07	2.17
Motile sperm domain-containing protein 2	gi 66500091 ref XP_393236.2	8	29.4	1.08E-42	7.90E+07	2.16
Calponin homology domain- containing protein DDB_G0272472	gi 571507346 ref XP_006571119.1	30	82.8	0	3.26E+09	2.12
Alpha-tocopherol transfer protein	gi 66499088 ref XP_624151.1	23	77.1	1.06E-107	1.99E+09	2.04
Leucine--tRNA ligase	gi 571501205 ref XP_006562583.1	10	11.2	7.23E-31	1.03E+08	2.04
ADP-ribosylation factor GTPase- activating protein 2	gi 110766623 ref XP_393119.3	3	6.9	3.71E-11	1.91E+07	2.02
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	gi 66505480 ref XP_623065.1	14	35.6	2.44E-117	4.69E+08	2.01
NADH dehydrogenase [ubiquinone] iron-sulfur protein 4	gi 328793182 ref XP_001123307.2	4	30.1	3.92E-40	1.55E+08	1.97
Probable isocitrate dehydrogenase [NAD] subunit alpha	gi 571534141 ref XP_006564183.1	20	50.1	2.84E-90	5.44E+08	1.92
FUN14 domain-containing protein 1	gi 328781625 ref XP_396316.4	4	29.1	4.91E-25	7.97E+07	1.92
Alpha-actinin, sarcomeric	gi 571563087 ref XP_006570549.1	55	63.8	0	2.53E+09	1.90

Endocuticle structural glycoprotein SgAbd-8	gi 571576913 ref XP_006571888.1	5	29.5	5.61E-49	1.03E+09	1.88
Uncharacterized protein LOC724521	gi 571549654 ref XP_001120395.3	3	25	7.04E-37	8.54E+09	1.88
Protein lethal(2)essential for life-like	gi 110750766 ref XP_001120194.1	14	73	2.06E-225	3.49E+09	1.83
Myb-like protein X	gi 328779160 ref XP_625237.3	4	11.1	2.17E-11	3.27E+07	1.81
Cytochrome c oxidase subunit 6A1	gi 110755333 ref XP_001121817.1	6	57	2.10E-26	3.08E+08	1.80
Heat shock protein 75 kDa	gi 66511337 ref XP_623366.1	22	41.3	7.49E-109	4.37E+08	1.70
Uncharacterized protein LOC408365	gi 48097532 ref XP_391915.1	21	41.5	4.73E-204	2.21E+09	1.70
Cuticular protein 27 precursor	gi 399220318 ref NP_001257758.1	8	34.2	8.11E-41	1.92E+08	1.67
Uncharacterized protein DDB_G0283357	gi 571576342 ref XP_006561464.1	7	7.7	6.46E-19	3.48E+07	1.66
Uncharacterized protein LOC552154	gi 328793329 ref XP_624536.2	12	80.6	1.05E-81	9.90E+08	1.66
Glucose dehydrogenase [FAD, quinone]	gi 571502113 ref XP_006563332.1	12	28.8	1.43E-41	1.15E+08	1.66
Thioredoxin-2	gi 328784195 ref XP_003250408.1	7	82.9	8.59E-58	3.24E+09	1.65
NADH dehydrogenase [ubiquinone] iron-sulfur protein 8	gi 66524760 ref XP_623480.1	7	45.8	2.57E-30	2.00E+08	1.65
Tubulin alpha-2/alpha-4 chain	gi 571563641 ref XP_006559304.1	25	61.1	0	2.56E+09	1.64
Microtubule-associated protein 1B	gi 328791437 ref XP_003251564.1	10	22	7.66E-87	6.86E+08	1.63
Serine/arginine repetitive matrix protein 2	gi 571567009 ref XP_006559380.1	15	18.7	1.09E-52	2.84E+08	1.63
Pentatricopeptide repeat-containing protein 2	gi 571570118 ref XP_006560291.1	22	70.7	7.36E-148	1.26E+09	1.61
Glutamate dehydrogenase	gi 66523390 ref XP_392776.2	31	62.4	4.45E-200	2.19E+09	1.61
Methylmalonate-semialdehyde dehydrogenase [acylating]	gi 571568825 ref XP_006568110.1	14	40	2.76E-42	3.09E+08	1.60

NADH-ubiquinone oxidoreductase 75 kDa subunit	gi 288872187 ref NP_001165875.1	31	55.6	7.10E-167	1.01E+09	1.57
Histone H1B	gi 328791749 ref XP_003251627.1	7	32.1	2.55E-34	2.70E+08	1.52
Dynein light chain 2	gi 328783818 ref XP_003250346.1	10	69.7	5.51E-66	2.71E+09	1.51
Apolipoprotein D	gi 66555021 ref XP_623335.1	6	39.6	1.50E-53	2.20E+08	1.51

Table 5.6 Proteins identified as being present in higher levels in parasitized drone pupae

Table of proteins identified as being more abundant in the drone pupae that were parasitized by *Varroa*. Protein identities, number of peptides, PEP, sequence coverage, overall intensity and fold expression in the parasitized pupae are all listed. Fold changes are expressed as $-\text{LOG P}$ value.

Protein annotation	Protein Identity	Peptides	Sequence coverage [%]	PEP	Overall Intensity	Expression in Parasitized drone-
Mantle protein	gi 571549859 ref XP_006561945.1	7	67.1	2.82E-24	5.21E+08	-14.56
Protein doublesex	gi 571549850 ref XP_006561944.1	8	33.6	1.37E-26	7.66E+08	-11.66
Proline-rich protein 4	gi 110759642 ref XP_001121782.1	6	62.8	1.51E-34	6.35E+08	-8.85
Pupal cuticle protein	gi 571546121 ref XP_001121640.3	5	29.5	7.18E-28	4.68E+08	-8.42
Histidine-rich glycoprotein	gi 571505512 ref XP_006570179.1	10	67	7.96E-31	6.83E+08	-6.66
Glycine-rich cuticle protein	gi 217330652 ref NP_001136109.1	4	25.6	1.46E-52	7.91E+08	-6.12
Zinc finger protein 512B	gi 571553142 ref XP_006562940.1	5	18.9	3.96E-12	1.09E+08	-5.98
Maternal protein exuperantia	gi 571569692 ref XP_006560074.1	8	28.6	1.40E-32	1.41E+08	-5.29
Zinc finger protein 512B	gi 328781403 ref XP_003249973.1	3	18.3	6.24E-08	1.25E+08	-4.83
Uncharacterized protein LOC409648	gi 48112268 ref XP_393146.1	6	40.3	2.13E-20	2.33E+08	-4.45
Zinc finger protein 512B	gi 328781268 ref XP_001121697.2	5	20.7	1.12E-09	1.01E+08	-3.83
Uncharacterized protein LOC100578085	gi 328788333 ref XP_003251110.1	15	64.6	1.42E-153	1.34E+09	-3.79
Uncharacterized protein LOC408570	gi 571508855 ref XP_006571420.1	8	15.8	2.43E-23	7.77E+07	-3.42
Nucleolar and coiled-body phosphoprotein 1	gi 571554217 ref XP_006566059.1	6	10.5	1.16E-15	8.05E+07	-3.39
enolase-phosphatase E1	gi 328778928 ref XP_003249566.1	8	25.2	3.60E-20	1.04E+08	-3.33
Zinc finger MYND domain-containing protein 10	gi 571578547 ref XP_006572434.1	7	22.7	3.09E-19	5.99E+07	-3.11
Regulator complex protein LAMTOR2	gi 571577997 ref XP_006572245.1	2	34.4	8.76E-17	9.49E+07	-2.90
Myb-like protein X	gi 571516244 ref XP_006569191.1	10	26.6	2.19E-28	1.93E+08	-2.79

	1					
Uncharacterized protein LOC100577680	gi 328777526 ref XP_003249362. 1	27	78.6	0	1.90E+10	-2.69
Uncharacterized protein LOC100576738	gi 328779180 ref XP_003249603. 1	4	14.1	6.77E-14	6.42E+07	-2.54
Flocculation protein FLO11	gi 328780407 ref XP_001122189. 2	6	10.3	3.82E-20	6.03E+07	-2.51
Cuticle protein 18.7	gi 110759098 ref XP_001120418. 1	10	75.1	3.19E-116	1.59E+10	-2.50
Fibrillin-2	gi 571574244 ref XP_006561116. 1	14	7.3	1.00E-51	3.34E+08	-2.35
Uncharacterized protein LOC100577362	gi 328786011 ref XP_003250694. 1	5	36.8	1.23E-35	6.81E+08	-2.33
Uncharacterized protein LOC100576126	gi 328783156 ref XP_003250245. 1	26	53.6	0	4.34E+09	-2.33
Uncharacterized protein LOC102653832	gi 571573763 ref XP_006560156. 1	13	48.3	8.37E-161	1.36E+10	-2.32
Secretory carrier-associated membrane protein 1	gi 571568001 ref XP_006560471. 1	6	30.8	2.45E-79	1.61E+08	-2.23
Ice-structuring glycoprotein	gi 571573192 ref XP_001122923. 2	2	16.6	3.86E-25	5.04E+10	-2.16
Transferrin 1	gi 58585086 ref NP_001011572.1	69	86.9	0	4.06E+10	-2.14
Cytochrome P450 12a5	gi 571549750 ref XP_006561922. 1	29	64.6	3.35E-177	2.22E+09	-2.09
Pupal cuticle protein G1A	gi 328780861 ref XP_003249871. 1	10	59.6	5.28E-85	2.23E+10	-2.02
Cuticular protein CPF1 precursor	gi 399220322 ref NP_001257760. 1	3	20.9	6.77E-68	1.00E+10	-1.98
Apolipoprotein III	gi 166795901 ref NP_001107670. 1	14	83.9	1.89E-264	1.71E+10	-1.98
Cuticular protein 1 precursor	gi 399158374 ref NP_001257746. 1	19	80.1	0	1.37E+11	-1.96
Slit homolog 2 protein	gi 66507096 ref XP_397526.2	6	17.2	1.78E-16	7.11E+07	-1.94
Neurofilament heavy polypeptide	gi 110755329 ref XP_001121746. 1	21	57.8	0	6.24E+10	-1.85
Apoptosis-inducing factor 3	gi 110758058 ref XP_625035.2	5	15.7	5.32E-45	1.17E+08	-1.85

Cuticular protein 16 precursor	gi 399220312 ref NP_001257755.1	20	62.1	1.04E-115	1.10E+09	-1.84
Cytosolic 10-formyltetrahydrofolate dehydrogenase	gi 571544151 ref XP_006563851.1	40	54.7	1.59E-204	1.73E+09	-1.79
Guanine nucleotide-binding protein G(q) subunit alpha	gi 66512822 ref XP_623369.1	6	18.7	2.55E-20	5.74E+07	-1.71
Odorant binding protein 17	gi 94158711 ref NP_001035297.1	6	57.8	2.11E-45	4.62E+08	-1.68
Importin-4	gi 328790151 ref XP_396270.4	22	28.8	1.14E-57	2.23E+08	-1.66
Cuticular protein 2 precursor	gi 399220296 ref NP_001257747.1	14	57.2	0	6.55E+10	-1.64
Uncharacterized protein LOC725078	gi 571507451 ref XP_006571147.1	4	10.8	2.54E-18	7.44E+07	-1.64
Sorbitol dehydrogenase	gi 571536203 ref XP_392401.2	27	69.6	0	7.25E+09	-1.60
Fructose-bisphosphate aldolase-like isoform	gi 571502064 ref XP_006563323.1	38	83.6	0	5.63E+10	-1.59
Antithrombin-III	gi 571552510 ref XP_006562425.1	17	38.8	1.66E-64	6.11E+08	-1.59
Glyceraldehyde-3-phosphate dehydrogenase 1	gi 571555339 ref XP_006566262.1	17	64.9	5.11E-65	6.03E+08	-1.57
Adenylate cyclase, terminal-differentiation specific	gi 328782467 ref XP_003250150.1	15	58.8	1.92E-74	7.16E+08	-1.57
Small ubiquitin-related modifier	gi 48102954 ref XP_392826.1	5	52	3.74E-41	9.33E+08	-1.57
Histone H4	gi 571506616 ref XP_006570863.1	4	41.7	8.94E-18	1.62E+08	-1.56
Transmembrane protein 214-A	gi 571549174 ref XP_006561802.1	7	13.5	2.21E-27	3.05E+08	-1.55
Heterogeneous nuclear ribonucleoprotein R	gi 571526365 ref XP_006569431.1	45	75.1	1.39E-278	5.25E+09	-1.54
Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	gi 66530373 ref XP_623497.1	19	66.2	2.39E-244	4.92E+09	-1.54
Transcriptional activator protein Pur-beta-B	gi 571537256 ref XP_006557370.1	19	65.3	2.23E-154	2.05E+09	-1.54
Odorant binding protein 14 precursor	gi 94158822 ref NP_001035313.1	18	77	2.81E-287	5.36E+10	-1.53
Uncharacterized protein LOC551528	gi 571563070 ref XP_006570545.1	22	89.1	1.65E-275	6.28E+09	-1.51

Copper transport protein ATOX1	gi 110772198 ref XP_001121022.1	3	44.4	5.25E-30	2.72E+08	-1.51
Aldose reductase	gi 571574163 ref XP_624401.4	34	90.3	2.24E-255	5.96E+09	-1.50

Table 5.7 Proteins identified as being present in lower levels in parasitized drone pupae.

Table of proteins identified as being less abundant in the drone pupae that were parasitized by *Varroa*. Protein identities, number of peptides, PEP, sequence coverage, overall intensity and fold expression in the parasitized pupae are all listed. Fold changes are expressed as $-\text{LOG P}$ value.

Protein Identity	LFQ Intensity Unparasitized1	LFQ Intensity Unparasitized2	LFQ Intensity Unparasitized3	LFQ Intensity Unparasitized4	LFQ Intensity Parasitized1	LFQ Intensity Parasitized2	LFQ Intensity Parasitized3	LFQ Intensity Parasitized4
Maternal protein exuperantia	1.41E+08	1.34E+08	1.54E+10	4.43E+08	0	0	0	0
Zinc finger protein 512B	1.09E+08	1.20E+09	1.09E+09	3.22E+08	0	0	0	0
Zinc finger protein 512B	1.25E+08	1.45E+08	1.33E+09	1.27E+08	0	0	0	0
Zinc finger protein 512B	1.01E+08	1.10E+08	1.03E+09	3.72E+07	0	0	0	0
Enolase-phosphatase E1	1.04E+08	1.09E+08	1.20E+09	5.05E+08	0	0	0	0
Uncharacterized protein LOC408570	7.77E+07	6.65E+07	6.66E+08	8.20E+07	0	0	0	0
Threo-3-hydroxyaspartate ammonia-lyase	6.58E+07	5.55E+07	5.63E+07	7.90E+07	0	0	0	0
Brahma associated protein 55kd	6.87E+07	6.55E+07	6.57E+08	7.83E+07	0	0	0	0
Nucleolar and coiled-body phosphoprotein 1	8.05E+07	8.80E+07	7.47E+08	1.74E+08	0	0	0	0
Fasciclin-2	7.28E+07	6.48E+07	7.46E+07	9.45E+07	0	0	0	0
Slit homolog 2 protein	7.11E+07	7.48E+07	8.37E+08	1.13E+09	0	0	0	0
Translation machinery-associated protein 7	8.53E+07	4.35E+07	5.46E+07	1.23E+09	0	0	0	0
33 kDa inner dynein arm light chain	5.65E+07	5.65E+07	7.46E+08	1.01E+09	0	0	0	0
Zinc finger MYND domain-containing protein 10	5.99E+07	7.64E+07	6.47E+08	2.70E+08	0	0	0	0
Uncharacterized protein LOC100576738	6.42E+07	4.54E+07	7.46E+08	2.71E+09	0	0	0	0
Guanine nucleotide-binding protein G(q) subunit alpha	5.74E+07	6.47E+07	9.87E+08	2.20E+08	0	0	0	0

Flocculation protein FLO11	6.03E+07	5.53E+07	8.40E+08	5.96E+09	0	0	0	0
Uncharacterized protein DB_G0282133	3.12E+07	3.24E+07	5.37E+07	2.72E+08	0	0	0	0
Scavenger receptor class B member 1	4.73E+07	4.78E+07	7.16E+08	6.28E+09	0	0	0	0
Fatty acyl-CoA reductase CG8306	4.18E+07	5.46E+07	4.02E+10	5.36E+10	0	0	0	0
Signal peptidase complex subunit 1	4.19E+07	5.37E+07	1.23E+09	2.05E+09	0	0	0	0
Tubulin alpha chain	0	0	0	0	4.35E+07	5.01E+07	4.89E+07	0
Leucine-rich repeat-containing protein DDB_G0290503	0	0	0	0	0	2.70E+08	3.00E+08	3.40E+08
Ornithine aminotransferase	0	0	0	0	8.53E+07	8.50E+07	1.30E+08	7.60E+07
Pupal cuticle protein C1B	0	0	0	0	5.65E+07	7.11E+07	7.48E+07	2.00E+08
Girdin	0	0	0	0	1.41E+08	8.53E+07	0	4.10E+08
Uncharacterized protein LOC725683	0	0	0	0	1.41E+08	5.65E+07	5.65E+07	1.20E+08
Uncharacterized protein LOC100578511	0	0	0	0	1.09E+08	1.41E+08	1.34E+08	3.40E+08
Venom carboxylesterase-6	0	0	0	0	1.25E+08	1.40E+08	1.50E+08	1.30E+08
Hexaprenyldihydroxybenzoate methyltransferase	0	0	0	0	1.01E+08	3.40E+08	2.10E+08	3.90E+08
MD-2-related lipid-recognition protein	0	0	0	0	0	4.20E+08	8.60E+08	5.90E+08
IQ domain-containing protein E	0	0	0	0	7.77E+07	9.20E+07	1.70E+08	1.30E+08
Cytochrome b-c1	0	0	0	0	6.58E+07	1.00E+08	1.60E+08	1.10E+08

complex subunit 8									
Alpha-glucosidase precursor	0	0	0	0	6.87E+07	1.60E+08	7.48E+07	8.37E+08	
Uncharacterized protein LOC725283	0	0	0	0	8.05E+07	5.70E+07	4.35E+07	5.46E+07	
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9	0	0	0	0	7.28E+07	0	5.65E+07	7.46E+08	
Uncharacterized protein LOC100577080	0	0	0	0	7.11E+07	1.50E+08	1.34E+08	1.54E+10	
Mitochondrial import receptor subunit TOM70	0	0	0	0	8.53E+07	1.60E+08	1.60E+08	2.20E+08	
Yorkie	0	0	0	0	5.65E+07	4.80E+08	4.30E+08	4.90E+08	
Inosine triphosphate pyrophosphatase	0	0	0	0	5.99E+07	0	3.50E+09	1.20E+09	
Long-chain-fatty-acid-CoA ligase 3	0	0	0	0	7.11E+07	2.80E+08	0	2.10E+08	
Serine/threonine-protein phosphatase 4 catalytic subunit	0	0	0	0	8.53E+07	1.20E+08	2.60E+08	1.30E+08	
Probable GDP-L-fucose synthase	0	0	0	0	0	6.64E+07	7.48E+07	8.37E+08	
motile sperm domain-containing protein 2	0	0	0	0	1.41E+08	6.58E+07	4.35E+07	5.46E+07	
Small nuclear ribonucleoprotein G	0	0	0	0	7.48E+07	6.64E+07	5.65E+07	7.46E+08	
Myb-like protein X	0	0	0	0	4.35E+07	5.53E+07	1.34E+08	0	
uncharacterized protein DDB_G0283357	0	0	0	0	5.65E+07	7.11E+07	7.48E+07	8.37E+08	
40S ribosomal protein S9	0	0	0	0	1.34E+08	8.53E+07	4.35E+07	5.46E+07	
Gamma-glutamylcyclotransfera	0	0	0	0	8.37E+08	0	5.65E+07	7.46E+08	

se								
Uncharacterized protein LOC102656497	0	0	0	0	5.46E+07	1.41E+08	1.34E+08	1.54E+10
M7GpppX diphosphatase	0	0	0	0	7.46E+08	1.41E+08	1.01E+08	1.04E+08

Table 5.8 LFQ intensities of exclusively expressed proteins unparasitized and parasitized drone pupae.

A zero value indicates a protein that was absent or undetected in the sample. Only proteins that was absent in all four samples of one group and present in three of the four in the other group.

5.3.5 Analysis of variations in the proteomic profile of *A. mellifera* worker pupae during parasitization using label free mass spectrometry

In *A. mellifera*, *Varroa* frequently infest and reproduce in worker brood cells, at almost the same rate as they infest drone brood (Medina *et al.*, 2002). Developing from larvae to adult while harboring one or more *Varroa* in the cell, could potentially have an effect on the vitality of the worker bee upon emergence. The main roles within the colony, from foraging to maintenance, are all carried out by worker bees, so their individual fitness affects the fitness of the colony as a whole (Johnson 2010). Previous reports have suggested that parasitization by *Varroa* on worker *A. mellifera* has a serious effect on the health of the bee, through loss of haemolymph, transmission of disease, and overall loss of body weight (Bowen-Walker and Gunn 2001). Here, using label free mass spectrometry, the aim was to compare the proteomic profile of worker pupae that were unparasitized during development with workers that were heavily parasitized by a total of 4 adult and 8 young *Varroa*. Proteins are the effector molecules of all process within an organism and any changes to the proteomic profile could potentially give insight into the effect that parasitization is having on the vitality of the bee.

Protein was extracted from four replicates for each sample set using a single pupa per replicate of a known age and known parasitization level to ensure minimal variability. A low level of variability between samples ensures that any changes observed in the proteome were due to parasitization by *Varroa* and not due to variation from sample to sample (2.15.1). A total of 5210 peptides were identified, 221 proteins were determined to be differentially abundant proteins (ANOVA $p < 0.05$) with a fold change of >1.5 , of which 130 were found in higher abundance in the parasitized pupae (Table 5.9) and 91 were found in higher abundance in the unparasitized pupae (Table 5.10) There were also a total of 120 proteins that were identified as being exclusively expressed between the two sample sets, with 26 exclusive to the unparasitized pupae and 94 exclusive to the pupae that were parasitized. The proteins were determined to be exclusively expressed in the control or parasitized pupal profile if there were absent from all four replicates and present in at least three of the four replicates in the control or parasitized pupae respectively. These proteins were termed as “exclusively expressed proteins”

(Table 5.11). These protein hits were also used in the statistical analysis of the total differentially expressed group following imputation of the zero values using a number close to the lowest value of the range of proteins plus or minus the standard deviation (Tables 5.9 and 5.10). The overall expression in each proteomic profile of the unparasitized and the parasitized worker pupae can be seen in Figure 5.15, depicted using a hierarchical clustering heat map. The column on the right hand side shows the expression of a large number of proteins switched on, compared to the column on the left, which shows a map of the proteomic expression in the parasitized pupae (grey bands in the left column become green bands in the right column Figure 5.15). This indicates at a glance that there is a large number of proteins changed completely in expression following parasitization by *Varroa*. The top twenty proteins expressed at the highest level of abundance in the unparasitized and the parasitized pupae are highlighted in Figure 5.16. The protein that was present at the highest level of abundance in the parasitized worker pupae was fatty acid synthase-like protein, present at levels of 52.70 fold higher. Leucine rich repeat neuronal protein 1 like was present at 30.15 fold higher in the parasitized worker pupae, and is involved in protein recognition and synapse activity (Kobe and Kajava 2001). An uncharacterized protein LOC413264 was found exclusively expressed in the worker pupae that had been parasitized by *Varroa*, and after statistical analysis post imputation was at 8.97 fold higher in abundance compared to the control (Table 5.9 and Table 5.11). This protein showed homology to proteins from the major royal jelly family, involved in caste specification. An additional protein involved in sex determination was present at higher levels upon parasitization by *Varroa*, protein double-sex like (Amrein *et al.*, 1988). It was present at 7.13 fold higher in the parasitized worker pupae. Protein croquemort, which is involved in the immune response and phagocytosis, was 4.67 fold higher in the parasitized worker pupae (Table 5.9) (Franc *et al.*, 1999; Jiravanichpaisal *et al.*, 2006). Serine/threonine phosphatase PP1 beta, which is another immune related protein involved in cell-to-cell adhesion, was exclusively expressed in the parasitized worker pupae (Larsen *et al.*, 2003). This protein is also involved in the cell growth pathway as a transforming growth factor receptor. Additional proteins involved in cell growth and proliferation were found more abundantly expressed in the parasitized worker pupae when they were compared to unparasitized workers. These include Long chain fatty acid CoA ligase (8.4 fold increase; Table 5.9),

Carnithine O-palmitoyltransferase 1 (4.49 fold increase; Table 5.9) and sortilin-related receptor (4.27 fold increase; Table 5.9) (Tomoda *et al.*, 1991, Akil *et al.*, 2011; Yuan *et al.*, 2013; Yang and Chen 2014).

Of the proteins that were found at higher abundance in the control pupae that had not been parasitized during development, the most abundant protein was myosin regulatory light chain, which was found at 25.91 fold lower in the parasitized worker pupae than in the control (Table 5.10). Myosins are muscle proteins and are involved in movement and calcium binding (Lehman and Szent Gyorgyi 1975). Another protein present at much lower levels in the parasitized worker pupae was chymotrypsin inhibitor, at a level of 25.69 fold lower (Table 5.10). This is a serine protease inhibitor, and the decrease in its abundance during parasitization may indicate activation of the immune system (Kanost 1999). Transmembrane protein serine 9, an additional protein from the same family, was found completely absent in expression in the parasitized worker pupae (Table 5.11). This protein is involved in the immune response through the melanization cascade (Kanost 1999; Gorman and Paskewitz 2001). Apolipoprotein D-like, which has a proposed immune role, was expressed at lower levels upon parasitization by *Varroa* (2.13 fold decrease, Table 5.10) (Sanchez *et al.*, 2006). Proteoglycan 4-like, was 2.44 fold lower in the parasitized pupae (Table 5.10), and has a proposed role in the immune response of arthropods (Johansson 1999). Similar to the drone pupae under parasitization, there were a number of cuticle associated proteins found at decreased levels of abundance following parasitization by *Varroa* (Figure 5.16). These include cuticle protein 18.7-like (2.97 fold decrease; Table 5.10) and endocuticle structural glycoprotein (2.98 fold decrease; Table 5.10). Another cuticle protein was completely absent in all replicates of the parasitized worker pupae (cuticle protein 28 precursor; Table 5.11). Prefolding subunit 5-like and prefolding subunit 2-like were also at higher levels in workers that were not parasitized by *Varroa*, 2.42 and 2.13 fold respectively. These are involved in protein folding and cytoskeletal formation (Vainberg *et al.*, 1998; Miyazawa *et al.*, 2011). Two translation initiation factor subunits were completely absent in workers that were parasitized (eukaryotic translation initiation factor subunit h and g, Table 5.1). The protein that these subunits are part of is involved in gene expression and in protein synthesis (Krauss and Reuter 2000) .

The Blast2GO annotation software (www.blast2GO.com) was used to group proteins based on conserved gene ontology (GO) terms in order to identify processes and pathways altered between the proteomic profile of the unparasitized and parasitized worker pupae. GO terms were categorized by biological processes (BP; Figure 5.17) and molecular function (MF; Figure 5.18). Grouping the proteins into BP level 3 showed a number of protein groups that were altered in overall proportion (Figure 5.17). The percent of overall proteins involved in single organism metabolic process rose from 4.07% in the unparasitized pupae to 11.4% in the parasitized (Figure 5.17). Proteins grouped under this GO term include a range of tubulins, glucose dehydrogenase and proteins involved in ATP processing and synthesis (Kilmartin and Adams 1984, Müller and Grüber 2003). Another biological process group which changed significantly between the unparasitized and the parasitized workers is the catabolic process group (Figure 5.17). There was a 2.23 fold increase in the percent of total proteins involved in catabolic process, from 1.36% in the unparasitized to 3.03% in the parasitized. Proteins in this group include transcriptional related proteins such as elongation factors and transcription initiation factors (Krauss and Reuter 2000). Methylation was another GO group changed significantly between the unparasitized and parasitized pupae, with 0% of proteins involved in methylation under normal unparasitized conditions, which rises to 1.26% of the overall protein content following parasitization (Figure 5.17).

Grouping the proteins based on molecular function in Blast2GO also showed some variations between the proteomic profile of the unparasitized workers and the parasitized. Small molecule binding proteins rose in abundance by 1.61 fold in the parasitized workers, from 6.10% to 9.84%, and this included structural proteins such as tubulin, and enzymes such as succinyl dehydrogenase. Proteins grouped into the GO term structural constituent of the ribosome also rose in the parasitized pupae by 3 fold, from 1.53% of the overall total protein content to 4.59%. This group contains proteins that make up the ribosome such as the 40S and 60S subunits. Oxidoreductase activity rose in the parasitized pupae also, by an average of 3.86 fold, from 2.29% to 8.85% of the overall protein content. This group has stress related proteins such as fatty acid synthase, aldehyde dehydrogenase and glucose dehydrogenase (Kletzien *et al.*, 1994, Howe and Schillmiller 2002). Proteins involved in cofactor binding rose in the parasitized pupae by 5.17 fold from 0.76% of the total protein content in unparasitized to 3.93 % in the parasitized pupae

(Figure 5.18). This GO grouping include proteins such as acyl binding protein and trifunctional enzyme mitochondrial subunit. Proteins with transferase activity also rose in abundance in the parasitized pupae from 3.82% to 8.52% in the parasitized workers, which was a rise of 2.23 fold (Figure 5.18). Enzymes such as hexokinase and carnothine O-palmitoyltransferase were grouped under this GO term. The GO term group of proteins involved in ligase activity also rose in the parasitized pupae, with 0% of the overall protein content involved in ligase activity in the unparasitized pupae, rising to 2.26 % of the overall content in the pupae that were parasitized by *Varroa* (Figure 5.18). Proteins involved in ligase activity include pyruvate, serine tRNA ligase, serine tRNA logase, and long chain fatty acid ligase.

5.3.6 Summary

Label free analysis of the complete proteomic profile allows for an excellent overview of variation between the profiles of two phenotypes. The aim of this section was to identify any variations in the proteomic profile of *A. mellifera* pupae during parasitization by *Varroa*. The preferred host of *Varroa* is drone, but the mite will also choose worker brood in the Western honeybee. Both were chosen under normal and under heavy parasitization conditions for proteomic analysis. Some similarities exist between the worker and drone response to parasitization by *Varroa*. Cuticle proteins are present at lower levels of abundance in the parasitized pupae of both worker and drone, which could indicate a compromised healing ability. Worker pupae showed increased expression of proteins involved in the stress response during parasitization by *Varroa*, which was not evident in the parasitized drone, such as glucose dehydrogenase. The abundance of proteins with roles in insect immunity also changed when parasitized by *Varroa* in both worker and drone brood, more noticeably in the worker brood however. The overall effect of parasitization on the proteomic profile of workers was more severe than drone brood, which is to be expected due to the history of *Varroa* on their original host *A. cerana*.

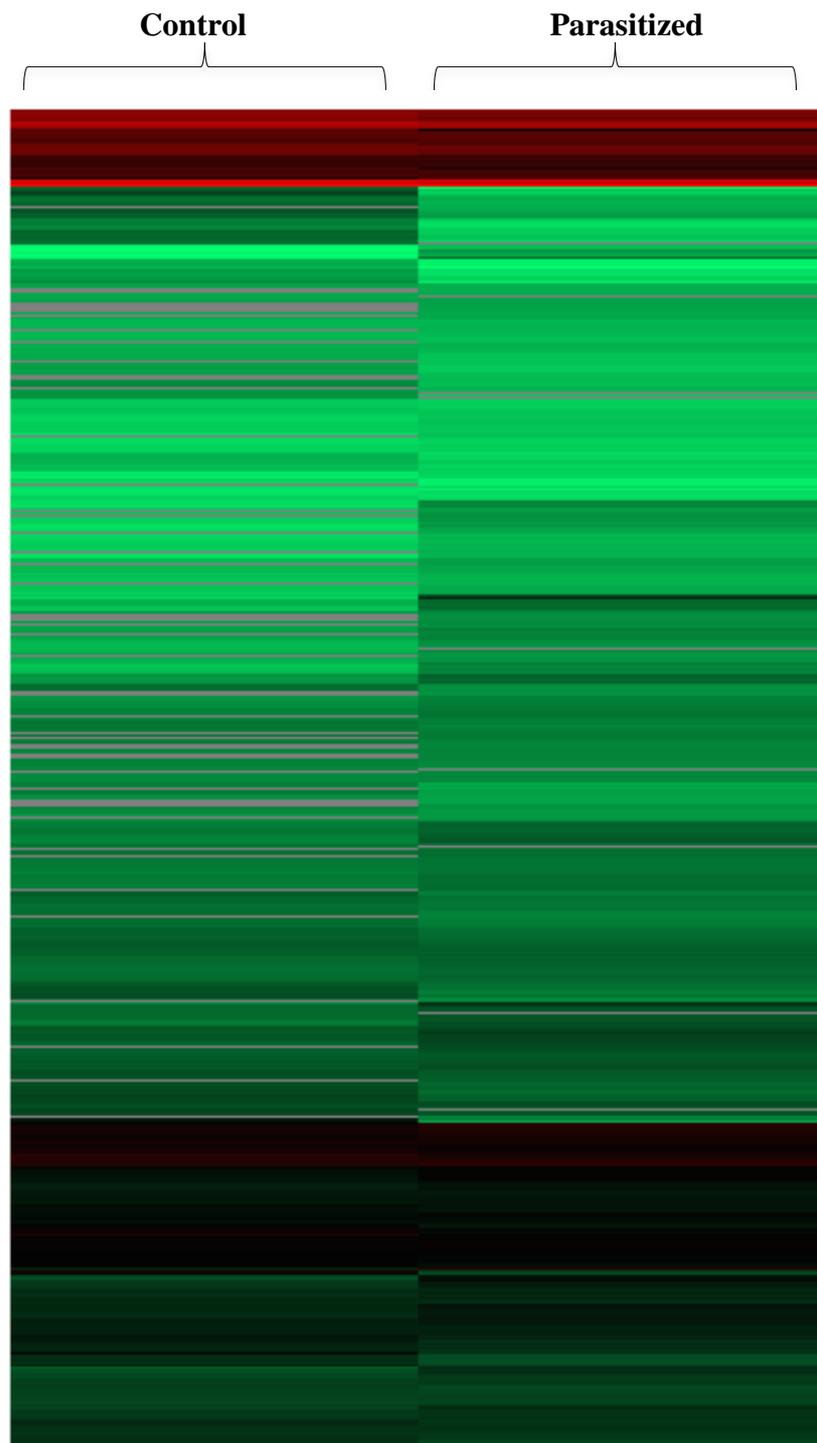


Figure 5.15 Hierarchical clustering of the quantitative differences in the proteomic profile of unparasitized and parasitized worker pupae

The heat map shows the variation in expressional abundance of the proteins from the profile of control and heavily parasitized worker pupae obtained using Perseus software (2.14.7). The grey sections indicate an area of no detected expression, red areas indicate high level of abundance, and the green areas indicate low levels.

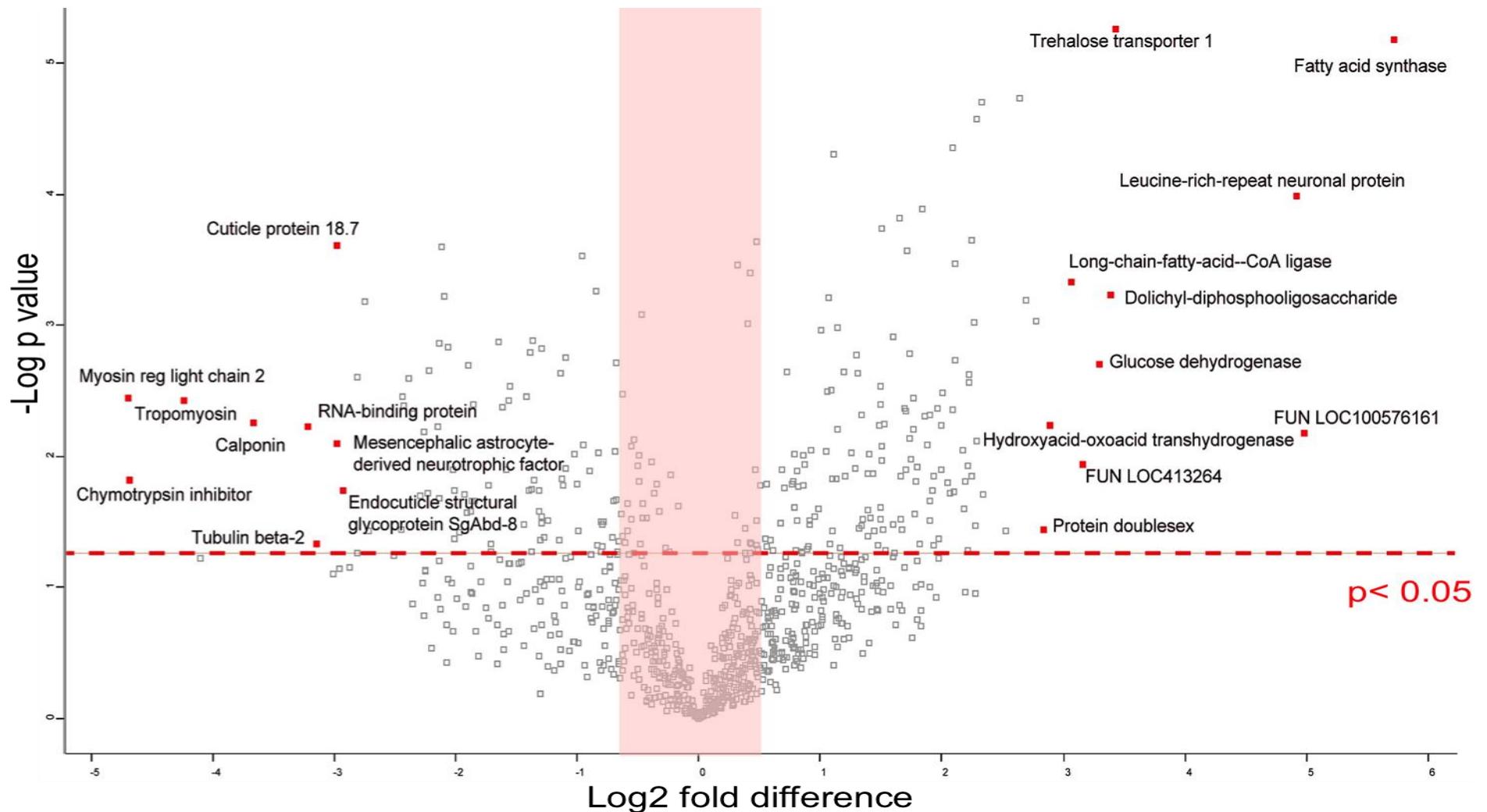


Figure 5.12 Volcano plot highlighting the twenty proteins present in the highest and lowest abundances between the unparasitized and parasitized worker pupae.

Image of Volcano plot obtained using Perseus software (2.14.7). Proteins above the line are statistically significant ($p < 0.05$) and those to the right and left of the vertical lines indicate fold changes greater than 1.5 fold positive and 1.5 fold negative in the parasitized and unparasitized drone pupae respectively. The red box indicates proteins which were not differentially expressed over a fold change of +/- 1.5 between the unparasitized and parasitized worker pupae so were not deemed significant.

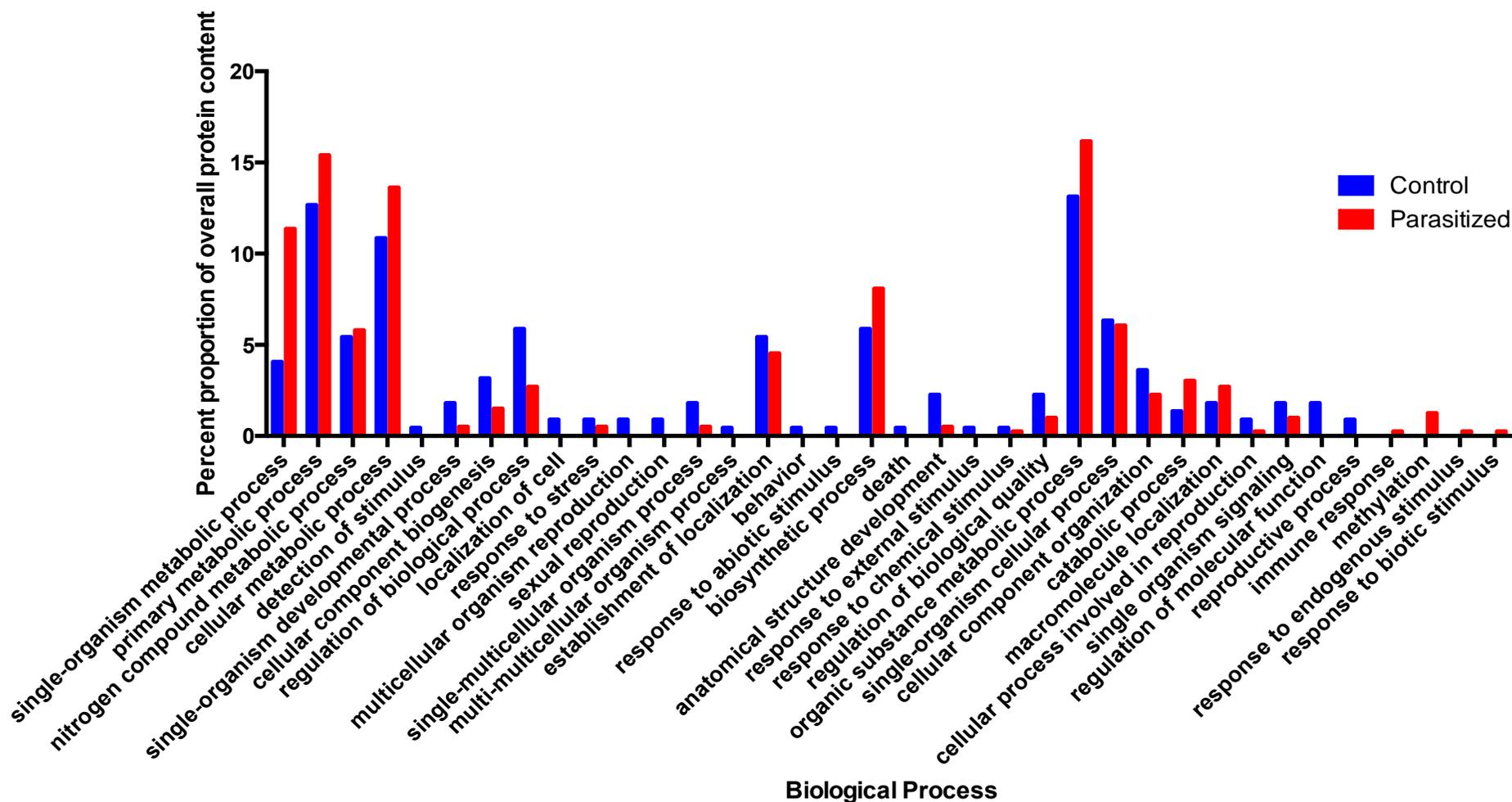


Figure 5.17 Biological Process grouping of proteins found in unparasitized and parasitized worker pupae.

Bar chart showing changes to proportion of proteins involved in various Biological Processes at level 3 ontology. Bar chart shows the grouping of proteins based on Biological process annotation using Blast2Go. Proteins were assigned groups based on involvement in Biological processes for both the unparasitized and the parasitized samples. Each group was assigned a percentage proportion of the total proteins found in the proteomic profile of each sample group and graphed on a comparative bar chart. (Y-axis is the group's percentage of overall proteins found in sample).

Protein annotation	Protein Identity	Peptides	Sequence coverage [%]	PEP	Overall Intensity	Expression in Parasitized worker
Fatty acid synthase	gi 571550343 ref XP_006567467.1	84	11.2	0	8.71E+09	52.71
Uncharacterized protein LOC100576161	gi 571549821 ref XP_003249952.2	1	9.3	4.69E-22	7.50E+09	31.66
Leucine-rich repeat neuronal protein 1	gi 328788840 ref XP_001119831.2	18	49.6	4.72E-197	2.98E+09	30.15
Trehalose transporter 1	gi 571505770 ref XP_006570642.1	7	0.8	4.83E-50	1.24E+09	10.76
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	gi 335892843 ref NP_001229451.1	31	47.6	3.80E-174	4.03E+09	10.48
Glucose dehydrogenase [FAD, quinone]	gi 66499547 ref XP_392145.2	15	10.4	8.86E-115	1.21E+09	9.86
Protein yellow	gi 571547098 ref XP_006569902.1	10	4.9	6.07E-52	1.08E+09	8.97
Long-chain-fatty-acid--CoA ligase ACSBG2	gi 571508759 ref XP_006571405.1	11	14.3	5.47E-51	7.37E+08	8.40
Hydroxyacid-oxoacid transhydrogenase	gi 571555594 ref XP_003250818.2	17	32.2	9.40E-194	1.90E+09	7.42
Protein doublesex	gi 571549850 ref XP_006561944.1	8	11.3	2.45E-22	1.90E+09	7.13
Uncharacterized protein LOC100577527	gi 328782411 ref XP_003250139.1	8	10.9	4.51E-166	1.43E+09	6.85
NADPH--cytochrome P450 reductase	gi 571546441 ref XP_006569769.1	18	47.3	1.69E-75	8.08E+08	6.44
3-hydroxyisobutyryl-CoA hydrolase	gi 571575119 ref XP_006563706.1	12	12.3	9.06E-43	5.04E+08	6.22
Histone H2A	gi 328791683 ref XP_003251613.1 0186.1	8	12.8	2.39E-39	6.61E+09	5.77
Trifunctional enzyme subunit alpha	gi 66519936 ref XP_393806.2	30	4.9	3.84E-150	2.91E+09	5.05
Importin-5	gi 571531264 ref XP_001120827.3	17	22.3	4.12E-54	5.10E+08	5.03
Twinfilin	gi 571541899 ref XP_006558914.1	8	9.8	7.58E-47	8.78E+08	4.89
Uncharacterized protein LOC100576760	gi 328790524 ref XP_003251427.1	10	3.9	4.61E-46	3.92E+08	4.89

40S ribosomal protein S23	gi 110771708 ref XP_624651.2	11	5.6	1.33E-95	2.29E+09	4.86
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	gi 571576863 ref XP_006571875.1	4	24.9	2.34E-12	4.20E+08	4.81
Succinyl-coa ligase [GDP-forming] subunit beta	gi 571525636 ref XP_006567341.1	8	38.4	3.98E-48	7.00E+08	4.75
Calcium-transporting atpase sarcoplasmic/endoplasmic reticulum	gi 571564914 ref XP_006565284.1	16	2.4	1.28E-75	1.21E+09	4.73
Protein croquemort	gi 571525343 ref XP_006567284.1	7	27	2.26E-17	3.62E+08	4.68
Threo-3-hydroxyaspartate ammonia-lyase	gi 66508985 ref XP_624902.1	11	25.2	1.89E-63	6.13E+08	4.66
Trna (guanine(26)-N(2))-dimethyltransferase	gi 571574242 ref XP_006561115.1	16	12.1	1.34E-116	1.21E+09	4.64
Histone H3.3	gi 66565026 ref XP_624499.1	6	7.6	2.33E-62	4.32E+09	4.57
6-phosphogluconate dehydrogenase, decarboxylating	gi 571565639 ref XP_006566986.1	23	29.6	0	6.90E+09	4.53
Carnitine O-palmitoyltransferase 1	gi 571510808 ref XP_006557681.1	9	60.2	5.37E-32	9.53E+08	4.50
AP-1 complex subunit beta-1	gi 571512170 ref XP_006561243.1	16	58.5	2.04E-65	5.65E+08	4.31
Serine/threonine-protein kinase clka	gi 328792196 ref XP_001121241.2	19	33.8	6.58E-261	2.45E+09	4.30
Sortilin-related receptor	gi 571550277 ref XP_006567452.1	27	4.2	5.01E-94	6.24E+08	4.28
Acyl-coa synthetase family member 2	gi 332801003 ref NP_001193902.1	9	26.4	2.81E-42	3.92E+08	4.26
6-phosphofructokinase	gi 571554778 ref XP_006566154.1	15	44.5	8.92E-107	4.60E+08	4.25
60S ribosomal protein l23a	gi 66549799 ref XP_393135.2	15	15.7	6.78E-52	2.38E+09	4.21
Phosphate carrier protein	gi 66525867 ref XP_396960.2	8	13.3	9.18E-33	1.57E+09	4.12
Bifunctional purine biosynthesis protein PURH	gi 66516304 ref XP_395924.2 ;gi 571547954 ref XP_006560830.1	16	3.3	3.11E-80	6.93E+08	4.00
40S ribosomal protein S26	gi 328787488 ref XP_001122510.2	7	3.7	3.78E-18	1.23E+09	3.99
Uncharacterized protein LOC726658	gi 328784779 ref XP_001122379.2	15	3.1	5.32E-118	1.67E+09	3.99

Protein lethal(2)essential for life	gi 110750754 ref XP_001119884.1	11	7.3	2.82E-60	6.89E+08	3.92
Aminomethyltransferase	gi 571573087 ref XP_394029.3	12	11.1	2.38E-51	5.00E+08	3.90
4-aminobutyrate aminotransferase	gi 328790551 ref XP_392483.3	9	45.4	5.77E-82	4.03E+08	3.82
Acetyl-coa carboxylase	gi 571564723 ref XP_006565244.1	21	26.1	1.95E-77	4.17E+08	3.81
Suppressor of variegation 3-9	gi 283837917 ref NP_001164640.1	14	18	1.72E-86	7.67E+08	3.78
Glycerol-3-phosphate dehydrogenase	gi 62526114 ref NP_001014994.1	18	72.1	2.33E-70	1.36E+09	3.74
ADP-ribosylation factor 1	gi 66549728 ref XP_392990.2	14	10.6	2.93E-85	1.43E+09	3.74
Long-chain fatty acid transport protein 4	gi 571576551 ref XP_006563203.1	20	10.8	2.51E-162	1.50E+09	3.73
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	gi 571562115 ref XP_003250858.2	7	26.1	1.92E-48	7.31E+08	3.68
C-1-tetrahydrofolate synthase	gi 571526768 ref XP_006559841.1	47	11.6	1.03E-231	5.01E+09	3.64
Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	gi 328784765 ref XP_624408.2	29	2.5	0	2.17E+10	3.57
Uncharacterized protein LOC552106	gi 571529312 ref XP_006567755.1	3	20.9	3.89E-59	1.08E+09	3.52
Ras-related protein Rab-11A	gi 571542355 ref XP_006559507.1	8	15.5	4.93E-44	1.05E+09	3.47
Pyridoxal kinase	gi 571554543 ref XP_006566118.1	10	2.8	5.10E-43	7.93E+08	3.45
Endochitinase	gi 66511507 ref XP_623995.1	28	52	6.56E-178	3.06E+09	3.42
Serine-tRNA ligase	gi 66509916 ref XP_396462.2	16	57.9	6.70E-111	1.19E+09	3.41
Protein transport protein Sec31A	gi 571519001 ref XP_006563951.1	21	32.1	2.48E-125	7.10E+08	3.41
ATP synthase subunit g	gi 571536514 ref XP_006570969.1	5	5.1	1.54E-12	6.76E+08	3.36
Citrate synthase 1	gi 66521738 ref XP_393545.2	20	9	1.76E-212	3.24E+09	3.33
Translocon-associated protein	gi 66555386 ref XP_624607.1	6	17.3	2.08E-46	4.96E+08	3.32
Carnitine O-palmitoyltransferase 2	gi 571506170 ref XP_394948.5	10	9.5	2.12E-27	2.91E+08	3.29
Very long-chain specific acyl-coa	gi 571549122 ref XP_006561795.1	21	36.8	1.93E-184	1.56E+09	3.27

dehydrogenase	1					
ATP-citrate synthase	gi 66530142 ref XP_623083.1	70	31	0	1.29E+10	3.25
Aldehyde dehydrogenase family 3 member B1	gi 571550579 ref XP_006567513.1	7	6.9	5.80E-19	3.93E+08	3.24
Mitochondrial inner membrane protein	gi 328779076 ref XP_392094.3	14	5.6	1.62E-57	6.23E+08	3.19
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kda subunit	gi 328784835 ref XP_624433.2	15	7.9	2.24E-212	2.20E+09	3.17
Ubiquitin carboxyl-terminal hydrolase 5	gi 328788537 ref XP_624702.2	14	3.8	1.36E-42	5.34E+08	3.16
Eukaryotic translation initiation factor 3 subunit F	gi 48126476 ref XP_396596.1	8	1.5	7.53E-33	4.91E+08	3.14
UDP-glucuronosyltransferase 2A3	gi 571558837 ref XP_006564460.1	12	11.5	2.11E-86	6.45E+08	3.04
Aldehyde dehydrogenase	gi 66530423 ref XP_623084.1	37	0.6	0	1.92E+10	3.03
Fatty acyl-coa reductase CG5065	gi 300116407 ref NP_001177850.1	8	10	6.37E-31	2.72E+08	2.88
Juvenile hormone esterase precursor	gi 58585076 ref NP_001011563.1	41	45	0	2.43E+10	2.86
Glutamyl aminopeptidase	gi 571576925 ref XP_006571893.1	16	55.3	1.45E-59	6.89E+08	2.85
Serine/threonine-protein phosphatase PP1-beta	gi 110751312 ref XP_392943.3	8	4.2	1.66E-34	6.84E+08	2.84
40S ribosomal protein S2	gi 48103127 ref XP_392843.1	24	21.9	3.56E-140	6.51E+09	2.83
60S ribosomal protein L32	gi 571558447 ref XP_006564379.1	15	58	2.38E-65	2.49E+09	2.83
Fatty acyl-coa reductase CG8306	gi 335892852 ref NP_001229455.1	8	74.2	4.15E-21	3.24E+08	2.83
Peroxisomal multifunctional enzyme type 2	gi 571537192 ref XP_006571053.1	41	0.8	0	7.26E+09	2.82
Chloride intracellular channel exc-4	gi 48098249 ref XP_392027.1	11	16.1	1.20E-73	9.62E+08	2.81
Ice-structuring glycoprotein	gi 571573192 ref XP_001122923.2	2	4	6.70E-36	9.63E+10	2.79
40S ribosomal protein S7	gi 571576123 ref XP_624943.3	18	31.8	1.17E-141	2.67E+09	2.79
Isocitrate dehydrogenase [NAD] subunit alpha	gi 571534141 ref XP_006564183.1	14	3.5	8.02E-77	6.82E+08	2.72
Poly(A) RNA polymerase gld-2	gi 571562153 ref XP_393329.4	6	8.1	2.98E-97	3.27E+08	2.71

homolog A						
Coatomer subunit gamma	gi 571575903 ref XP_006559437.1	17	12.5	6.21E-85	7.76E+08	2.67
Prolyl endopeptidase	gi 571519898 ref XP_006566860.1	10	2.9	1.54E-48	4.03E+08	2.63
Actin-like protein 87C	gi 66547891 ref XP_623834.1	5	35.7	7.26E-51	5.24E+08	2.63
Uncharacterized protein LOC726080	gi 571522413 ref XP_006558822.1	23	11.3	1.24E-146	6.13E+09	2.62
60S ribosomal protein L27	gi 328788609 ref XP_003251156.1	12	2.3	2.84E-79	3.16E+09	2.62
Peroxisomal hydratase-dehydrogenase-epimerase	gi 571551848 ref XP_006560185.1	22	3.5	4.57E-235	7.75E+09	2.56
Coatomer subunit alpha	gi 66504662 ref XP_623198.1	18	8.6	1.48E-120	7.49E+08	2.54
Delta-1-pyrroline-5-carboxylate dehydrogenase	gi 571559142 ref XP_006566327.1	11	16	3.84E-46	3.37E+08	2.53
Beta-galactosidase	gi 110764149 ref XP_001121565.1	19	55.1	4.17E-86	1.03E+09	2.49
Coatomer subunit delta	gi 66551478 ref XP_624725.1	11	31.8	7.22E-58	9.20E+08	2.47
Sorbitol dehydrogenase	gi 571536203 ref XP_392401.2	33	9.7	0	1.59E+10	2.46
Maleylacetoacetate isomerase 2	gi 48095935 ref XP_394562.1	4	15.4	4.17E-32	3.48E+08	2.44
Neutral and basic amino acid transport protein rbat	gi 571528517 ref XP_006564818.1	7	28.2	9.69E-28	2.45E+08	2.39
Cytosolic 10-formyltetrahydrofolate dehydrogenase	gi 571544151 ref XP_006563851.1	50	5.7	0	1.26E+10	2.30
40S ribosomal protein S4	gi 66517407 ref XP_623050.1	24	57	4.97E-244	7.89E+09	2.30
Alanine--trna ligase,	gi 571548485 ref XP_006561680.1	15	37.4	2.11E-54	4.73E+08	2.21
Aldehyde dehydrogenase family 7 member A1	gi 48096130 ref XP_394614.1	34	44.4	0	1.71E+10	2.20
40S ribosomal protein S25	gi 571528797 ref XP_006565645.1	12	2.4	2.74E-90	4.72E+09	2.19
Enoyl-coa hydratase	gi 328778689 ref XP_001123353.2	28	20.5	2.30E-182	1.46E+10	2.17
Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP	gi 571575322 ref XP_397138.5	34	7.4	0	1.37E+10	2.14

lyase						
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	gi 48099278 ref XP_394885.1	7	5.7	1.03E-44	4.17E+08	2.14
60S ribosomal protein L14	gi 571500903 ref XP_006561989.1	19	21	7.83E-138	6.84E+09	2.13
MATH and LRR domain-containing protein PFE0570w-	gi 571508626 ref XP_397478.3	24	33.8	5.66E-134	1.42E+09	2.12
Microsomal triglyceride transfer protein large subunit	gi 571568566 ref XP_006565094.1	37	14.7	5.18E-236	3.54E+09	2.11
Poly(U)-specific endoribonuclease	gi 110760204 ref XP_625112.2	11	9.7	1.27E-58	3.29E+08	2.08
Importin subunit beta-1	gi 571567906 ref XP_006560453.1	17	4.3	1.46E-92	1.61E+09	2.07
Transketolase	gi 328789361 ref XP_623196.3	54	5.6	0	2.44E+10	2.01
60S ribosomal protein l10a	gi 571501696 ref XP_006563278.1	14	12.5	3.75E-101	4.91E+09	1.97
GTP:AMP phosphotransferase AK3	gi 66519534 ref XP_624890.1	8	3.2	2.15E-31	3.70E+08	1.93
Staphylococcal nuclease domain-containing protein 1	gi 328785330 ref XP_624638.3	59	46.5	0	9.15E+09	1.89
Lambda crystallin	gi 209180477 ref NP_001129207.1	26	1.4	0	1.54E+10	1.89
Uncharacterized protein LOC100577486	gi 328794151 ref XP_003252003.1	8	5.4	5.18E-26	2.08E+08	1.88
Mitochondrial-processing peptidase subunit alpha	gi 571577068 ref XP_006571935.1	5	10.5	6.57E-42	2.53E+08	1.88
Fumarylacetoacetase	gi 571568097 ref XP_006560490.1	20	0.9	1.41E-151	3.66E+09	1.87
40S ribosomal protein SA	gi 66524293 ref XP_393965.2	14	15.1	3.31E-271	9.27E+09	1.83
Pyruvate carboxylase	gi 571568806 ref XP_006568106.1	74	10.8	0	1.33E+10	1.81
Trifunctional enzyme subunit beta	gi 66507594 ref XP_624164.1	30	10.4	4.81E-169	5.78E+09	1.81
Serine hydroxymethyltransferase	gi 571558150 ref XP_395263.4	15	1	2.40E-50	6.34E+08	1.76
Serine protease K12H4.7	gi 571557018 ref XP_006558107.1	7	3.3	7.53E-36	4.02E+08	1.75
D-arabinitol dehydrogenase 1	gi 66546988 ref XP_393651.2	25	21.6	1.28E-190	4.15E+09	1.75
GTP-binding protein SAR1b-	gi 571575114 ref XP_006563704.1	9	4.3	1.83E-58	1.89E+09	1.73

	1					
Glutathione S-transferase S1	gi 571577567 ref XP_006572106.1	23	19.6	3.19E-104	5.98E+09	1.73
40S ribosomal protein S14	gi 335057540 ref NP_001229398.1	17	4.1	5.59E-189	5.04E+09	1.71
GTP-binding nuclear protein Ran	gi 571562837 ref XP_006570503.1	20	26.8	5.41E-75	5.08E+09	1.71
60S ribosomal protein L6	gi 571500086 ref XP_006558672.1	26	15	2.29E-185	5.01E+09	1.65
Medium-chain specific acyl-coa dehydrogenase	gi 66499429 ref XP_392111.2	28	2.5	8.73E-284	8.93E+09	1.63
Phosphoglucomutase	gi 66561330 ref XP_395366.2	31	9.2	1.13E-141	2.77E+09	1.61
Tubulin alpha-1 chain	gi 571565862 ref XP_006567025.1	45	1.3	0	1.20E+11	1.60
Importin-4	gi 328790151 ref XP_396270.4	14	1.2	3.41E-47	2.57E+08	1.50

Table 5.9 Proteins identified as being present in higher levels in parasitized worker pupae

Table of proteins identified as being more abundant in the worker pupae that were parasitized by *Varroa*. Protein identities, number of peptides, PEP, sequence coverage, overall intensity and fold expression in the parasitized pupae are all listed. Fold changes are expressed as $-\text{LOG P}$ value.

Protein annotation	Protein Identity	Peptides	Sequence coverage [%]	PEP	Overall Intensity	Expression in Parasitized worker
Myosin regulatory light chain 2	gi 571563419 ref XP_006558384.1	19	55.6	0	7.96E+09	-25.91
Chymotrypsin inhibitor	gi 571574384 ref XP_006563422.1	5	24.3	2.11E-62	8.73E+09	-25.69
Tropomyosin-1	gi 571567292 ref XP_006560146.1	35	7.4	0	5.59E+09	-18.86
Mesencephalic astrocyte-derived neurotrophic factor	gi 66533332 ref XP_625023.1	17	7.9	1.32E-113	2.04E+09	-12.70
Plasminogen activator inhibitor 1 RNA-binding protein-	gi 571537972 ref XP_006559219.1 ;gi 328778152 ref XP_392925.3	15	9.3	1.30E-165	3.11E+09	-9.32
Tubulin beta-2 chain	VDK00138938-677_1	3	5.9	7.56E-25	2.86E+09	-8.85
Endocuticle structural glycoprotein sgabd-8	gi 571578843 ref XP_001120797.3 ;gi 399154150 ref NP_001257743.1	3	1.2	7.34E-11	6.04E+09	-7.89
Cuticle protein 18.7	gi 571546127 ref XP_006568331.1	4	9.9	1.61E-17	1.06E+09	-7.88
Calponin homology domain-containing protein DDB_G0272472	gi 571507346 ref XP_006571119.1	46	7.4	0	2.28E+10	-7.61
SH3 domain-binding glutamic acid-rich protein homolog	gi 328778804 ref XP_624305.2 ;gi 571517839 ref XP_006559826.1	11	7.8	3.66E-251	1.14E+09	-7.03
Reticulocalbin-2	gi 66517554 ref XP_393699.2	10	3.7	2.10E-65	9.04E+08	-6.72
Cuticular protein 18 precursor	gi 399220316 ref NP_001257757.1	1	5.7	5.27E-19	1.73E+09	-6.58
Proteoglycan 4	gi 571545198 ref XP_006564678.1	16	1.7	4.66E-120	8.24E+08	-5.46
UPF0587 protein v1g245604	gi 571554823 ref XP_006566165.1	6	3.3	3.86E-30	6.4E+08	-5.42
Prefoldin subunit 5	gi 48102645 ref XP_395405.1 ;	13	4.9	3.19E-99	1.08E+09	-5.36
Lish domain-containing protein C1711.05	gi 110763879 ref XP_625293.2	7	5.2	1.28E-58	1.07E+09	-5.23
Thyroglobulin	gi 328784279 ref XP_001120452.2	16	4.9	7.91E-115	5.2E+08	-5.11
Tubulin-specific chaperone A	gi 571533174 ref XP_006561337.1	9	1.3	2.47E-34	1.02E+09	-4.90

Glutamic acid-rich protein	gi 66506802 ref XP_625265.1	5	8	4.31E-31	8.6E+08	-4.81
Reticulon-4	gi 571549008 ref XP_006561769.1	11	0.6	1.90E-71	1.09E+09	-4.69
Microtubule-associated protein Jupiter	gi 571522894 ref XP_006560653.1	12	1.9	0	6.31E+08	-4.65
Sorting nexin-12	gi 66551610 ref XP_392658.2	9	12	4.32E-35	3.92E+08	-4.44
Prefoldin subunit 2	gi 571512124 ref XP_006561235.1	11	7	2.67E-39	7.5E+08	-4.40
Uncharacterized abhydrolase domain-containing protein DDB_G0269086	gi 571574500 ref XP_006563471.1	47	1.9	0	4.74E+09	-4.38
Neurofilament heavy polypeptide	gi 110755329 ref XP_001121746.1	35	6.2	0	3.35E+11	-4.34
Coiled-coil-helix-coiled-coil-helix domain-containing protein 6B-	gi 66524513 ref XP_624132.1	5	4.7	2.59E-18	5.21E+08	-4.28
Peptidyl-prolyl cis-trans isomerase FKBP14	gi 571532858 ref XP_006560563.1	17	3.3	2.47E-219	9.16E+09	-4.17
Titin-	gi 328783026 ref XP_001119962.2	61	5.8	0	7.65E+09	-4.05
Peptidyl-prolyl cis-trans isomerase	gi 66534750 ref XP_393381.2	27	4.1	0	3.96E+10	-4.03
ATP synthase subunit e	gi 66547758 ref XP_624249.1	6	3.4	2.28E-22	8.93E+08	-4.02
Serine-/arginine-rich splicing factor 4	gi 571560138 ref XP_6566505.1	26	4.3	3.03E-147	2.69E+09	-3.91
Pterin-4-alpha-carbinolamine dehydratase	gi 571576460 ref XP_006563164.1	8	3.6	3.83E-148	3.15E+09	-3.80
Cuticular protein 28 precursor	gi 399220320 ref NP_001257759.1	2	8.2	5.38E-96	9.04E+08	-3.76
Odorant binding protein 13 precursor	gi 94158810 ref NP_001035314.1	27	12.8	0	1.18E+11	-3.73
Prefoldin subunit 1	gi 328784669 ref XP_003250481.1	11	5.4	8.11E-34	6.06E+08	-3.67
Heterochromatin protein 1-binding protein 3	gi 328784720 ref XP_003250488.1	8	1.9	4.34E-22	6.64E+08	-3.65
Eukaryotic translation initiation factor 3 subunit H	gi 335892839 ref NP_001229449.1	9	6.1	3.21E-52	5.07E+08	-3.62
F-actin-capping protein subunit alpha	gi 571527542 ref XP_006562207.1	14	6.2	1.14E-146	1.79E+09	-3.59
Uncharacterized protein LOC408365	gi 48097532 ref XP_391915.1 ;gi 571559852 ref XP_006566457.1	26	7.7	0	5.63E+09	-3.32

	VDK00054443-1886_1					
Eukaryotic translation initiation factor 3	gi 66532125 ref XP_397589.2	14	9.7	1.09E-89	9.15E+08	-3.27
RNA-binding protein lark	gi 571572673 ref XP_006565678.1	27	18.9	1.56E-200	7.94E+09	-3.14
Small ubiquitin-related modifier	gi 48102954 ref XP_392826.1	7	4.5	2.28E-73	2.3E+09	-3.07
Enhancer of rudimentary	gi 110748988 ref XP_001119900.1	6	4.1	1.80E-31	7.84E+08	-2.99
Atpase inhibitor mai-2	gi 571573480 ref XP_006559096.1	8	6.6	4.10E-173	8.37E+09	-2.96
Fatty acid binding protein	gi 58585202 ref NP_001011630.1 ;gi 571513390 ref XP_006568213.1	21	3.6	0	1.49E+10	-2.94
Calponin homology domain-containing protein DDB_G0272472	gi 571507350 ref XP_006571120.1 ;gi 571507263 ref XP_006571110.1.1	43	14.4	0	1.82E+09	-2.70
Dehydrogenase/reductase SDR family member 7	gi 571561581 ref XP_006566738.1 ;gi 328787123 ref XP_395330.3	1	4.3	6.69E-05	51685000	-2.68
Neurofilament heavy polypeptide	gi 571526055 ref XP_006569367.1	22	4.9	0	8.53E+09	-2.63
Calmodulin	gi 571565086 ref XP_006565317.1	18	3.2	0	1.51E+10	-2.61
Actin 5C	VDK00026299-2857_6	12	1.2	5.39E-213	1.12E+10	-2.59
Calumenin	gi 66509518 ref XP_624357.1	31	2.4	0	8.78E+09	-2.58
Adenylate kinase	gi 66556661 ref XP_623921.1	24	9.3	1.63E-141	2.98E+09	-2.56
Fatty acid binding protein	gi 571513386 ref XP_006568212.1	20	5.1	0	6.73E+08	-2.49
Titin	gi 571548383 ref XP_006561665.1	3	7.2	2.24E-17	52666000	-2.48
Bile salt-activated lipase	gi 328784556 ref XP_392696.4	9	2.8	1.30E-38	4.29E+08	-2.47
Elongation factor 1-alpha	gi 58585198 ref NP_001011628	34	2	0	6.33E+09	-2.45
Uncharacterized protein LOC413256	gi 571547531 ref XP_006558888.1	37	8.4	0	2.05E+10	-2.45
ATP synthase-coupling factor 6	gi 328785946 ref XP_001121882.2	10	6.9	1.91E-40	2.62E+09	-2.44
Cylicin-2	gi 66525165 ref XP_392065.2	26	10.5	1.68E-198	6.69E+09	-2.41

Intracellular protein transport protein USO1	gi 571549128 ref XP_006561796.1	24	7.4	8.72E-227	5.44E+09	-2.36
Eukaryotic translation initiation factor 4E-1A	gi 571577643 ref XP_006572136.1	7	14	1.20E-61	6.07E+08	-2.22
Tumor protein D53	gi 571552571 ref XP_006562560.1 ;gi 328782269 ref XP_001121953.2	23	2.1	8.62E-133	4.04E+09	-2.19
Kinesin-like protein K39	gi 328786800 ref XP_625029.2 ;gi 571559643 ref XP_006566427.1 ;gi 571559637 ref XP_006566426.1	26	7.8	0	8.5E+09	-2.19
Mitochondrial-processing peptidase subunit beta	gi 66500205 ref XP_393509.2	28	3.6	0	6.44E+09	-2.17
Golgin subfamily B member 1	gi 571555579 ref XP_006566301.1	16	13.1	1.25E-226	1.71E+09	-2.13
Apolipoprotein D	gi 66555021 ref XP_623335.1 ;gi 66536388 ref XP_392799.2	9	2.2	8.69E-47	4.86E+08	-2.13
Uncharacterized protein LOC551323	gi 110760993 ref XP_623723.2 ;V DK00122370-834_1	33	15.5	0	6.53E+09	-2.03
Moesin/ezrin/radixin	gi 571539203 ref XP_006564324.1	45	2.4	0	8.29E+09	-2.01
Spectrin alpha chain	gi 571516901 ref XP_006558458.1	156	3.3	0	1.58E+10	-1.99
14-3-3 protein zeta	gi 571554799 ref XP_006566160.1	30	4.4	0	4.63E+10	-1.94
Glutaredoxin-C4	gi 328786065 ref XP_003250702.1	13	3.2	1.71E-190	4.36E+09	-1.93
Odorant binding protein 14 precursor	gi 94158822 ref NP_001035313.1	26	6.1	0	9.86E+10	-1.86
Acyl-coa-binding protein	gi 571544763 ref XP_006564597.1	12	10	8.44E-67	5.04E+09	-1.79
Uncharacterized protein LOC552217	gi 110759415 ref XP_624597.2	29	3.6	0	9.47E+09	-1.79
Protein lethal(2)essential for life	gi 571513519 ref XP_006568238.1	9	0.9	9.23E-36	1.18E+09	-1.77
V-type proton atpase subunit G	gi 66553147 ref XP_624346.1	12	6.2	2.29E-116	2.75E+09	-1.75
40S ribosomal protein S20	gi 110766823 ref XP_001120524.1	12	4.4	5.33E-76	7.86E+09	-1.74

Lamin Dm0	gi 571564543 ref XP_006565215.1	67	5.5	0	1.07E+10	-1.73
Circadian clock-controlled protein	gi 328781008 ref XP_001122724.2	15	9	2.35E-250	2.89E+09	-1.72
OV-16 antigen	gi 110763663 ref XP_001122227.1	16	3.8	1.31E-174	6.18E+09	-1.63
Cuticular protein 12 precursor	gi 399220308 ref NP_001257753.1	5	6	4.55E-141	4.75E+09	-1.62
Rab escort protein	gi 507418852 ref NP_001265259.1	30	3.4	5.41E-287	3.67E+09	-1.62
Protein DJ-1	gi 571571489 ref XP_006563123.1	17	18.9	3.51E-234	3.92E+09	-1.61
60S acidic ribosomal protein P1	gi 297591983 ref NP_001172073.1	15	11.3	4.79E-145	2.27E+10	-1.60
Hsp90 co-chaperone Cdc37	gi 335892823 ref NP_001229443.1	14	18.6	1.21E-84	1.28E+09	-1.57
Alpha-glucosidase	gi 94400901 ref NP_001035349.1	21	9.3	5.22E-271	7.86E+09	-1.56
Proteasome subunit alpha type-5	gi 571526489 ref XP_006557286.1	14	4.5	4.70E-193	5.24E+09	-1.55
Ras gtpase-activating protein-binding protein 2	gi 571538909 ref XP_006562630.1.3	22	4.1	2.76E-260	2.5E+09	-1.54
Glucosidase 2 subunit beta	gi 328789473 ref XP_625125.2	29	0.5	0	3.53E+09	-1.52
Importin-7	gi 571508598 ref XP_006571373.1	6	7.9	1.54E-16	1.07E+08	-1.51
Apolipoprotein D	gi 571555598 ref XP_003250823.2	29	8.7	1.00E-188	1.51E+10	-1.50

Table 5.10 Proteins identified as being present in lower levels in parasitized worker pupae

Table of proteins identified as being less abundant in the worker pupae that were parasitized by *Varroa*. Protein identities, number of peptides, PEP, sequence coverage, overall intensity and fold expression in the parasitized pupae are all listed. Fold changes are expressed as $-\text{LOG P}$ value.

Protein Identity	LFQ Intensity Unparasitized 1	LFQ Intensity Unparasitized 2	LFQ Intensity Unparasitized 3	LFQ Intensity Unparasitized 4	LFQ Intensity Parasitized 1	LFQ Intensity Parasitized 2	LFQ Intensity Parasitized 3	LFQ Intensity Parasitized 4
Chymotrypsin inhibitor	0	7.49E+09	6.48E+08	5.28E+08	0	0	0	0
Cuticular protein 18 precursor	1.73E+09	9.79E+09	1.00E+11	5.65E+08	0	0	0	0
Cuticle protein 18.7	1.06E+09	9.95E+09	3.98E+08	6.76E+08	0	0	0	0
Prefoldin subunit 2	7.50E+08	7.47E+09	5.54E+09	3.92E+08	0	0	0	0
Proteoglycan 4	8.24E+08	6.48E+09	7.75E+08	4.03E+08	0	0	0	0
Cuticular protein 28 precursor	9.04E+08	1.01E+09	0	5.10E+08	0	0	0	0
Glutamic acid-rich protein	8.60E+08	6.77E+09	9.84E+09	7.49E+09	0	0	0	0
Microtubule-associated protein Jupiter	6.31E+08	0	5.55E+09	6.37E+08	0	0	0	0
Thyroglobulin	5.20E+08	4.36E+07	6.37E+08	8.74E+08	0	0	0	0
Reticulocalbin-2	9.04E+08	6.46E+08	8.41E+09	8.71E+09	0	0	0	0
Heterochromatin protein 1-binding protein 3	0	7.74E+08	5.37E+09	8.96E+08	0	0	0	0
Synapse-associated protein of 47 kda	6.80E+08	8.84E+08	1.21E+09	7.37E+08	0	0	0	0
Sorting nexin-12	3.92E+08	4.41E+08	1.24E+09	8.08E+08	0	0	0	0
Coiled-coil-helix-coiled-coil-helix domain-containing protein 6B	5.21E+08	6.48E+09	0	5.12E+08	0	0	0	0
UPF0587 protein v1g245604	6.40E+08	0	8.08E+08	6.13E+08	0	0	0	0
Mitochondrial import receptor subunit TOM22	5.11E+08	6.54E+09	5.12E+08	6.24E+08	0	0	0	0
Eukaryotic translation initiation factor 3 subunit H	5.07E+08	9.77E+08	6.13E+08	0	0	0	0	0
Eukaryotic translation initiation factor 3 subunit G	5.21E+08	3.87E+08	6.24E+08	6.93E+08	0	0	0	0
Prefoldin subunit 3	3.59E+08	4.26E+08	5.54E+07	4.17E+08	0	0	0	0
Bile salt-activated lipase	0	6.66E+08	4.56E+08	7.00E+08	0	0	0	0
Dynactin subunit 3	2.94E+08	4.35E+09	6.58E+10	5.04E+08	0	0	0	0
Microtubule-associated protein futsch	7.93E+08	6.76E+08	5.47E+10	5.65E+08	0	0	0	0
Omega-conotoxin	7.96E+08	3.92E+08	5.48E+09	6.76E+08	0	0	0	0
Armadillo repeat-containing protein 6	2.26E+08	4.03E+08	0	5.47E+10	0	0	0	0
Transmembrane protease serine 9	2.34E+08	4.42E+09	5.47E+09	3.53E+08	0	0	0	0

Nucleoprotein TPR	3.03E+08	1.47E+09	1.47E+09	6.48E+08	0	0	0	0
Fatty acid synthase	0	0	0	0	8.71E+09	2.26E+08	3.92E+08	1.49E+08
Protein doublesex	0	0	0	0	1.90E+09	4.07E+08	4.91E+08	1.91E+08
Protein yellow	0	0	0	0	1.08E+09	3.94E+09	6.45E+08	4.61E+08
Glucose dehydrogenase	0	0	0	0	1.21E+09	2.26E+09	5.16E+08	1.60E+09
Trehalose transporter 1	0	0	0	0	1.24E+09	0	1.26E+10	1.40E+08
Long-chain-fatty-acid--coa ligase	0	0	0	0	3.72E+07	3.94E+09	4.35E+10	2.23E+08
NADPH--cytochrome P450 reductase	0	0	0	0	8.08E+08	8.50E+08	9.40E+09	7.09E+07
Cytochrome P450 4G11	0	0	0	0	5.12E+08	8.58E+09	6.49E+08	0
Threo-3-hydroxyaspartate ammonia-lyase	0	0	0	0	6.13E+08	5.48E+09	4.61E+10	5.92E+08
Sortilin-related receptor	0	0	0	0	0	5.92E+09	5.10E+09	1.89E+09
Aminomethyltransferase	0	0	0	0	5.00E+08	6.58E+07	9.44E+07	9.44E+07
Bifunctional purine biosynthesis protein PURH	0	0	0	0	6.93E+08	4.12E+08	3.88E+08	8.05E+07
Acetyl-coa carboxylase	0	0	0	0	4.17E+08	2.15E+08	1.58E+08	1.38E+08
Succinyl-coa ligase [GDP-forming] subunit beta	0	0	0	0	7.00E+08	1.09E+09	0	9.09E+07
3-hydroxyisobutyryl-coa hydrolase	0	0	0	0	5.04E+08	5.16E+08	4.50E+08	1.53E+08
AP-1 complex subunit beta-1	0	0	0	0	5.65E+08	6.07E+08	8.68E+08	5.88E+08
ATP synthase subunit g	0	0	0	0	6.76E+08	1.33E+09	1.39E+09	1.14E+09
Acyl-coa synthetase family member 2	0	0	0	0	3.92E+08	5.47E+08	4.96E+08	3.94E+08
4-aminobutyrate aminotransferase	0	0	0	0	0	1.14E+09	3.08E+08	2.33E+08
Importin-5-like	0	0	0	0	5.10E+08	2.43E+08	3.24E+08	1.33E+09
Uncharacterized protein LOC100576760	0	0	0	0	3.92E+08	1.82E+08	5.89E+08	2.26E+08
Eukaryotic translation initiation factor 3 subunit F	0	0	0	0	4.91E+08	6.87E+08	2.65E+08	5.47E+08
Phosphoglycolate phosphatase	0	0	0	0	6.45E+08	1.42E+09	8.71E+08	5.35E+08
Partner of Y14 and mago-like isoformx2	0	0	0	0	5.16E+08	2.30E+08	3.46E+08	3.05E+08
Serine/threonine-protein phosphatase PP1-beta	0	0	0	0	6.84E+08	4.64E+08	7.13E+08	0
Hydroxyacid-oxoacid transhydrogenase	0	0	0	0	3.48E+08	2.26E+08	2.33E+08	2.43E+08
Gephyrin isoform 2	0	0	0	0	2.74E+08	0	4.35E+09	5.46E+07
Protein transport protein Sec61 subunit	0	0	0	0	2.69E+08	6.79E+08	3.78E+09	2.28E+09

alpha									
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	0	0	0	0	4.20E+08	3.78E+09	9.40E+09	5.78E+09	
Aldehyde dehydrogenase family 3 member B1	0	0	0	0	3.93E+08	1.38E+09	3.19E+09	2.34E+09	
Protein croquemort	0	0	0	0	0	1.05E+09	1.41E+09	1.51E+09	
Transmembrane emp24 domain-containing protein bai	0	0	0	0	4.57E+08	1.76E+08	0	2.33E+08	
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	0	0	0	0	4.17E+08	5.55E+08	6.48E+09	6.46E+08	
Alanine--trna ligase	0	0	0	0	4.73E+08	1.46E+09	3.76E+08	3.68E+08	
Translocon-associated protein subunit alpha	0	0	0	0	4.31E+08	2.13E+10	3.00E+09	3.03E+09	
Poly(A) RNA polymerase gld-2 homolog A	0	0	0	0	3.27E+08	3.10E+09	1.14E+09	7.87E+08	
Fatty acyl-coa reductase CG8306	0	0	0	0	3.24E+08	1.33E+09	1.39E+09	1.14E+09	
Carnitine O-palmitoyltransferase 2	0	0	0	0	2.91E+08	0	2.33E+08	2.43E+08	
Translocon-associated protein subunit delta	0	0	0	0	4.96E+08	6.58E+08	5.47E+09	4.36E+08	
Casein kinase II subunit alpha	0	0	0	0	3.08E+08	5.79E+08	3.93E+08	4.51E+08	
ATP-dependent RNA helicase me31b	0	0	0	0	5.40E+08	1.02E+09	9.30E+08	1.20E+09	
Barrier-to-autointegration factor B	0	0	0	0	5.89E+08	8.87E+08	5.42E+08	0	
Very-long-chain enoyl-coa reductase	0	0	0	0	2.65E+08	3.40E+09	3.45E+09	3.82E+09	
Mitochondrial import receptor subunit TOM40 homolog 1	0	0	0	0	0	4.65E+08	5.27E+08	5.70E+08	
Proline synthase co-transcribed bacterial homolog protein	0	0	0	0	2.18E+08	2.12E+09	1.06E+09	6.57E+08	
Small nuclear ribonucleoprotein Sm D1	0	0	0	0	1.95E+08	5.36E+08	2.69E+08	1.22E+09	
Neutral and basic amino acid transport protein rbat	0	0	0	0	2.45E+08	5.16E+08	2.53E+08	3.09E+08	
Plexin domain-containing protein 2	0	0	0	0	2.66E+08	1.33E+09	1.86E+08	4.70E+08	
Sequestosome-1	0	0	0	0	1.78E+08	4.78E+08	3.29E+08	1.52E+09	
Fatty acyl-coa reductase CG5065	0	0	0	0	2.72E+08	4.04E+10	2.22E+08	3.95E+08	
Vesicular integral-membrane protein VIP36	0	0	0	0	1.74E+08	4.89E+09	2.39E+08	3.85E+10	
Signal peptidase complex subunit 2	0	0	0	0	3.22E+08	1.44E+09	0	3.99E+09	
Importin-4	0	0	0	0	2.57E+08	3.95E+08	3.42E+08	7.22E+08	
Isovaleryl-coa dehydrogenase	0	0	0	0	2.69E+08	4.67E+08	2.02E+08	1.65E+10	

Transmembrane emp24 domain-containing protein 5	0	0	0	0	2.53E+08	1.30E+09	2.08E+08	1.43E+10
Syntaxin-12	0	0	0	0	1.86E+08	9.46E+08	2.82E+08	1.69E+08
Poly(U)-specific endoribonuclease	0	0	0	0	3.29E+08	5.03E+10	1.95E+08	1.17E+09
Transmembrane protein 214-A	0	0	0	0	2.22E+08	4.80E+09	2.53E+08	3.24E+08
Asparagine--trna ligase,	0	0	0	0	2.39E+08	4.68E+08	0	4.32E+08
Glutaredoxin-related protein 5	0	0	0	0	0	3.55E+09	1.52E+08	4.86E+08
Alpha-tocopherol transfer protein	0	0	0	0	3.42E+08	3.70E+08	2.69E+08	5.49E+08
ATP-binding cassette sub-family E	0	0	0	0	2.02E+08	2.18E+08	5.12E+09	2.66E+08
Uncharacterized protein LOC100577486	0	0	0	0	2.08E+08	1.95E+08	7.58E+08	1.77E+08
Tropomodulin	0	0	0	0	2.82E+08	2.45E+08	2.92E+09	7.77E+08
Endoplasmic reticulum oxidoreductin-1	0	0	0	0	1.95E+08	2.66E+08	3.96E+08	1.41E+09
Mitochondrial-processing peptidase subunit alpha	0	0	0	0	2.53E+08	1.78E+08	8.39E+07	0
Pontin protein	0	0	0	0	3.11E+08	2.72E+08	1.19E+08	2.44E+08
Tweedle motif cuticular protein 2	0	0	0	0	1.52E+08	0	1.06E+09	7.04E+08
Hexokinase-1	0	0	0	0	2.69E+08	3.22E+08	6.41E+08	1.32E+09
Mitochondrial import inner membrane translocase subunit TIM50	0	0	0	0	1.92E+08	2.57E+08	1.32E+09	7.34E+08
IGF-II mrna-binding protein	0	0	0	0	2.86E+08	2.69E+08	4.92E+09	5.08E+10
Dehydrogenase/reductase SDR family member 4	0	0	0	0	1.22E+08	2.53E+08	0	4.96E+08
FUN14 domain-containing protein 1	0	0	0	0	1.73E+08	0	4.44E+09	3.08E+08
NADH dehydrogenase [ubiquinone] iron-sulfur protein 7	0	0	0	0	1.65E+08	3.29E+08	5.71E+08	3.24E+08
Dnaj homolog subfamily C member 3	0	0	0	0	7.34E+07	5.23E+07	5.56E+07	5.89E+08
PRA1 family protein 3	0	0	0	0	1.49E+08	1.55E+09	1.43E+09	2.65E+08
Ubiquitin	0	0	0	0	1.91E+08	1.81E+09	2.92E+09	3.06E+09
Kinesin light chain	0	0	0	0	4.61E+08	1.52E+09	3.07E+09	2.49E+09
Mannose-1-phosphate guanyltransferase alpha-A	0	0	0	0	1.60E+08	2.43E+10	2.16E+10	3.81E+10
Isoleucine--trna ligase	0	0	0	0	1.40E+08	1.29E+09	1.47E+09	0
Glutamate decarboxylase 1	0	0	0	0	2.23E+08	2.18E+09	1.44E+09	1.44E+09
Alcohol dehydrogenase [NADP(+)]	0	0	0	0	3.04E+08	1.62E+09	1.22E+09	1.21E+09

Importin subunit alpha-7	0	0	0	0	1.94E+08	3.07E+10	1.96E+10	2.84E+10
Mitochondrial import receptor subunit TOM20	0	0	0	0	0	2.93E+09	3.00E+09	2.83E+09
Succinyl-coa ligase [GDP-forming] subunit beta	0	0	0	0	1.89E+08	2.03E+09	3.45E+09	2.94E+09
Alpha-L-fucosidase	0	0	0	0	9.44E+07	8.74E+08	1.10E+09	6.07E+08
Epidermal retinol dehydrogenase 2	0	0	0	0	8.05E+07	1.22E+09	1.41E+09	1.44E+09
Phenylalanine--trna ligase beta subunit	0	0	0	0	1.38E+08	1.77E+09	1.45E+09	1.52E+09
Derlin-1	0	0	0	0	9.09E+07	5.03E+08	3.39E+08	3.97E+08
Glutamine--trna ligase	0	0	0	0	1.53E+08	1.50E+09	1.65E+09	1.93E+09
Importin-7	0	0	0	0	1.07E+08	1.12E+09	6.30E+08	7.01E+08
Sushi, von Willebrand factor type A	0	0	0	0	2.78E+08	4.53E+07	4.81E+07	5.24E+07
Titin	0	0	0	0	5.27E+07	4.76E+08	0	4.20E+08
Dehydrogenase/reductase SDR family member 7	0	0	0	0	5.17E+07	3.78E+08	2.31E+08	2.51E+08

Table 5.11 LFQ of exclusively expressed proteins in unparasitized and parasitized worker pupae.

A zero value indicates a protein that was absent or undetected in the sample. Only proteins that were absent in all four samples of one group and present in three of the four in the other group.

5.4 Quantitative analysis of the expression of four immune related genes in parasitized worker and drone pupae using Real Time PCR

The immune response of the honeybee, like all arthropods, is non-adaptive with no strong evidence to suggest capacity for memory of previous insults (Gillespie *et al.*, 1997). This type of immunity means that a rapid detection system and fast acting immune effectors are necessary in order to protect against foreign threats (Evans *et al.*, 2006). Analysis of parasitization of developing *A. mellifera* by *Varroa* mites during development has indicated that there is a negative effect on the health of the adult bee upon emergence, through physical damage and through the spread of diseases. Viral loads for example, correlate with increased levels of *Varroa* (Francis *et al.*, 2013). The aim of this section was to identify any changes in the transcript levels of four immune genes in parasitized worker and drone brood of *A. mellifera*. The immune genes that were analysed for changes in expression in comparison to control pupae were *abaecin*, *defensin*, *phenoloxidase* and *hymenoptaecin*. All genes were normalised against the β -*actina* gene, and the parasitized worker and drone brood were compared to unparasitized worker and drone pupae respectively.

Abaecin encodes for the antimicrobial peptide abaecin, and is involved in the defence against bacteria and is also an important effector of haemolymph clotting (Vilmos and Kurucz 1998; Yamakawa and Tanaka 1999). The expression of *abaecin* was down-regulated significantly in both parasitized worker and parasitized drone pupae, when compared to their controls (Figure 5.19 and 5.20 respectively). Expression was lower by an average of 60% in the parasitized worker pupae when compared to the level of expression in control, unparasitized worker pupae ($p < 0.05$). Expression in the parasitized drone pupae was lower by an average of 56% ($p < 0.01$) compared to the level present in the unparasitized drone pupae.

Defensin encodes another antimicrobial peptide, defensin, which is active against bacteria, fungi and some viruses (Hoffmann 1995; Muta and Iwanaga 1996; Lourenço *et al.*, 2013). Here, the expression of *defensin* in both

worker and drone pupae was analyzed, from unparasitized and heavily parasitized worker and drone pupae. In the drone pupae we see a decrease in the level of expression of *defensin* relative to the control (Figure 5.19). The overall expression in the parasitized drone pupae was lower by 48% compared to the unparasitized pupae ($p < 0.05$). The worker pupae also showed a similar decrease in the expression of *defensin*, with an overall decrease of 52%, but the difference was not deemed significant (Figure 5.20).

The prophenoloxidase cascade is one of the major components of the immune response in insects, which catalyses the synthesis of melanin when triggered by elicitors (Marmaras *et al.*, 1996; Yang and Cox-Foster 2007; González-Santoyo and Córdoba-Aguilar 2012). *Phenoloxidase* expression was analysed in both worker and drone pupae under normal conditions and compared to pupae that were found to be heavily parasitized by *Varroa* upon uncapping. In drone pupae, the expression of *phenoloxidase* was found increased by an average of 30%, however the increase was not deemed significant at $p < 0.05$. In worker pupae that had been parasitized the expression was also increased in the parasitized workers, by an average of 52% when compared to unparasitized worker ($p < 0.05$).

The final gene that was analysed using Real time PCR for variations in expression in worker and drone when parasitized by *Varroa* was *Hymenoptaecin*. This is another antimicrobial peptide gene, encoding for hymenoptaecin, an antibacterial protein (Casteels *et al.*, 1993, Lourenço *et al.*, 2013). Expression of *hymenoptaecin* in parasitized drone pupae was 34% higher than in the control drone pupae, but this was not deemed significant at $p < 0.05$. The expression of the gene was also increased in the parasitized worker pupae by an average of 43% ($p < 0.01$).

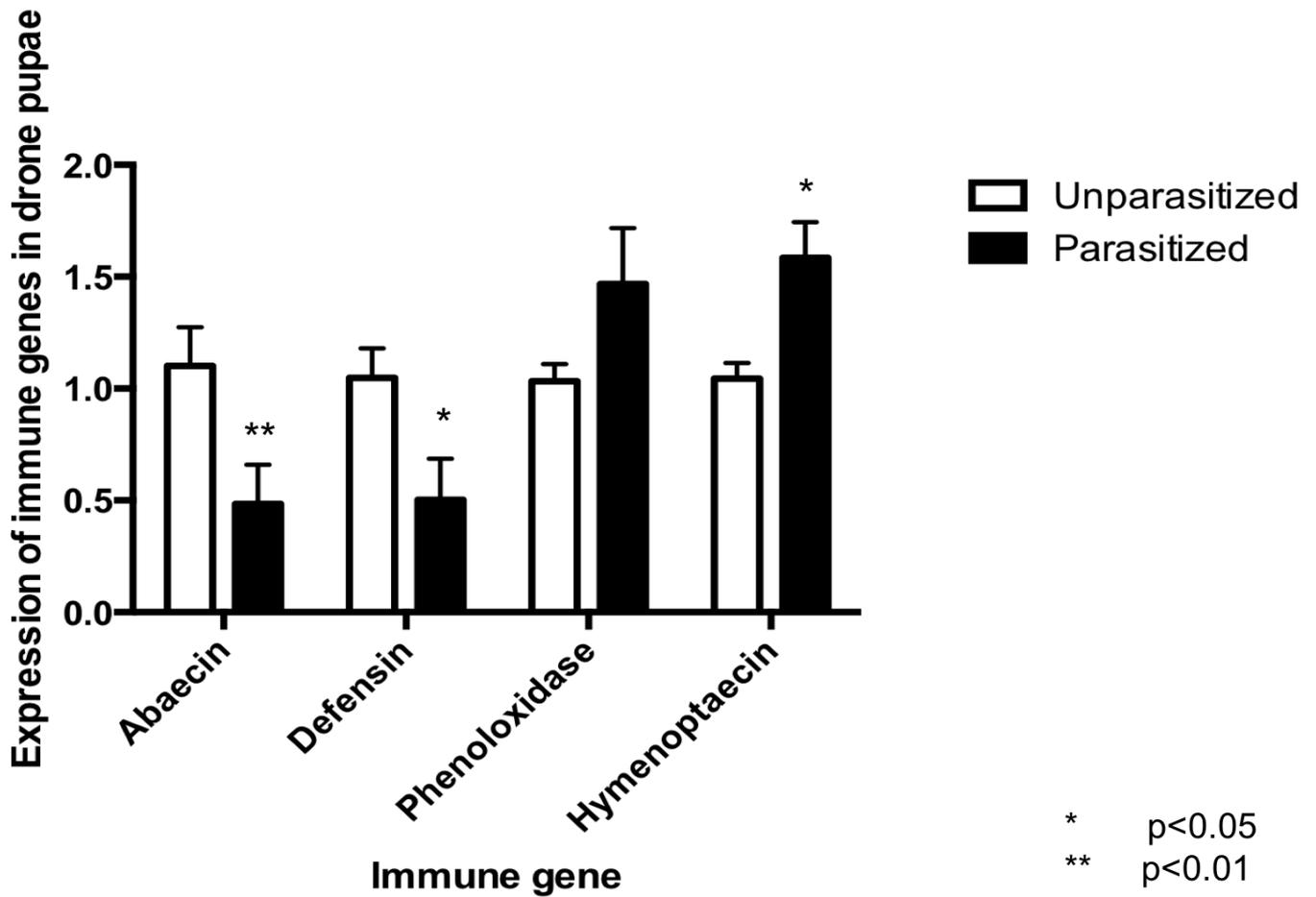


Figure 5.19 Expression of immune genes in parasitized *A. mellifera* drone pupae.

Genes were normalized against the β -actin references gene, and the expression of each gene in unparasitized control pupae was compared to parasitized pupae (n=3). Two way ANOVA was used to determine statistical significance in expression of each gene in parasitized drone compared to the control pupae.

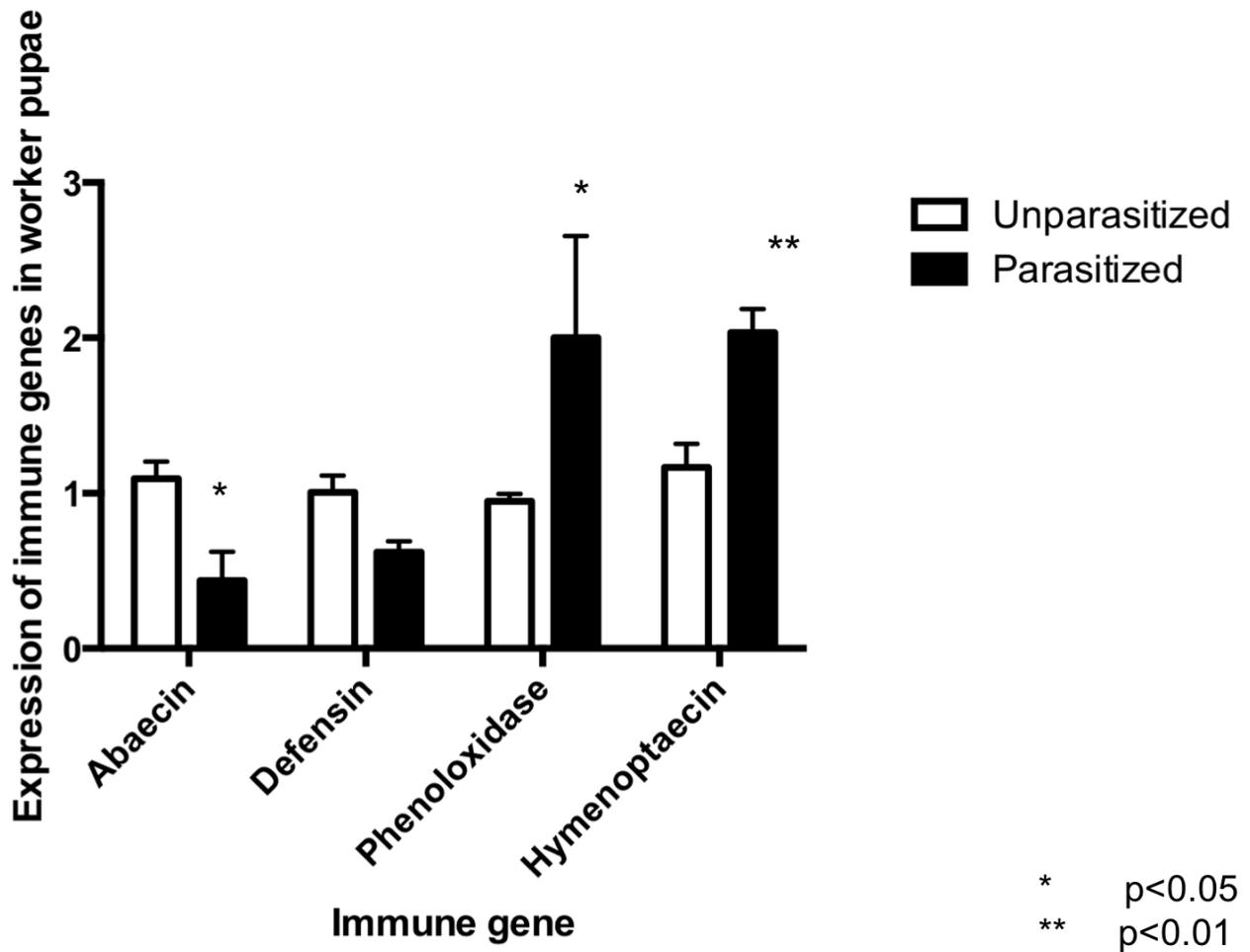


Figure 5.19 Expression of immune genes in parasitized *A. mellifera* worker pupae.

Genes were normalized against the β -actin reference gene, and the expression of each gene in unparasitized control pupae was compared to parasitized pupae (n=3). Two way ANOVA was used to determine statistical significance in expression of each gene in parasitized worker compared to the control pupae.

5.5 Examining the haemolymph of parasitized *A. mellifera* for the presence of salivary effectors from *Varroa*

There is evidence from research on many blood feeding parasites suggesting that their saliva may contain certain effectors which function as immune suppressors and anti-coagulants, in order to feed successfully and without detection (Wang and Nuttall 1999; Wikel 1999). Through proteomic and genetic analysis of the variations in the vitality of parasitized pupae and adults of *A. mellifera* throughout this Chapter, it is evident that *Varroa* have a detrimental effect on the health of the honeybee. There is also some evidence of immunosuppression.

To identify salivary effectors inducing these symptoms on the bees, haemolymph was extracted from both artificially infested (2.3.4) and naturally infested *A. mellifera* pupae (See section 2.9.2). The haemolymph was extracted from six pupae per sample and the haemolymph pooled for maximum concentration of any *Varroa* proteins (2.9.2). Haemolymph was analysed using label free proteomics, searching all resulting proteins through a combined *A. mellifera* and *V. destructor* database. The proteins that matched *Varroa* hits only in the database can be seen in Table 5.12. There were 23 proteins identified as matched only to the *Varroa* database. The LFQ value of expression can be seen for each protein in the three samples: Control (Unparasitized), Artificial (Manually infested, See section (2.3.4) and Natural (From heavily infested colony). From the list generated of matched *Varroa* hits, there were two proteins absent from the control pupal haemolymph and present in both the artificially and naturally *Varroa* parasitized pupal haemolymph samples (Table 5.12). T complex protein 1 subunit b is a molecular chaperone involved in protein binding, protein folding, toxin transport, phagocytosis and tubulin complex assembly (Trent *et al.*, 1991, Waldmann *et al.*, 1995). It was detected only in the haemolymph samples from pupae that had been parasitized by *Varroa* (Table 5.12). Alpha actinin 4 was an additional protein undetected in the control haemolymph samples and present in both of the haemolymph samples that had been parasitized by *Varroa* (Table 5.12). Alpha actnin 4 is involved in the crosslinking of actin, and in calcium

ion binding. It is also thought to play a role in platelet degranulation in mammals (Sixma *et al.*, 1989, Xu *et al.*, 2000).

Some proteins were absent from the control and present in one of the sample sets. These were still considered as possible candidates for salivary effectors from *Varroa* extracted during parasitization and the absence in one of the parasitized sample sets could be due to differential feeding habits of mites directly before the haemolymph extraction. Also, mites that were artificially infested may not have fed as they normally would in a capped brood cell. A total of 6 proteins were absent in the control uparasitized haemolymph and present in the artificially infested pupal sample (Table 5.12). Metalloendopeptidase OMA1 is a stress related protein found in mitochondria. It protects the mitochondria from fusion due to stress following its activation by cleavage (Quirós *et al.*, 2015). Another stress related oxidoreductase, glyoxylate/ hydroxypyruvate reductase was detected in the artificially infected pupae only (Cartwright and Hullin 1966, Corpas *et al.*, 2013). Sox 14, a negative regulator of transcription was also found only in the artificially infected pupal haemolymph (Table 5.12) (Bowles *et al.*, 2000). A zinc finger protein involved in metal ion and nucleic acid binding was detected only in the artificially infected also (Table 5.12) (Laity *et al.*, 2001). Alpha tocopherol transfer protein and beta tubulin were also found expressed in the artificially infected pupal haemolymph (Table 5.12). They are involved in intermembrane transport and in microtubule formation respectively (Naruse *et al.*, 1998; Yamauchi *et al.*, 2001).

A number of proteins were only detected in the naturally infected pupal haemolymph samples. GTP binding ADP ribosylation factor AFR6 was expressed exclusive to the naturally infected, which is involved in protein trafficking, cell adhesion, and cytoskeletal remodeling (Radhakrishna and Donaldson 1997; Kondo *et al.*, 2000). Vacuolar sorting protein, an additional protein involved in protein transport and transmembrane trafficking was also found absent from the control samples and only detected in the naturally infected pupae. Nesprin 1, exclusively expressed in the naturally parasitized pupae and completely absent from the control haemolymph, is an actin binding

protein involved in cell structure. Acotinase 1, which binds to iron sulphur clusters, was also only detected in the naturally parasitized pupae (Table 5.12). Lethal (3) 78BDm was also detected exclusively in the naturally parasitized pupal haemolymph, but a functional assignment for this protein is hard to make as the information is limited. Patched 1, which is a regulator of cell growth and is also a player in cell fate determination, was found exclusively detected in the artificially infected pupae (Yao *et al.*, 2002; Lai *et al.*, 2003).

Protein Identity	LFQ intensity Control	LFQ intensity Artificial	LFQ intensity Natural	Peptides	Intensity	PEP	Sequence coverage [%]	Unique peptides
Actin 5C	2.75E+09	7.53E+09	0	8	1.19E+10	3.15E-123	33.8	3
T-complex protein 1 subunit beta	0	6.46E+08	5.39E+08	8	1.41E+09	2.45E-104	29.4	2
26S protease regulatory subunit 4	2.46E+09	0	0	6	7.71E+09	0	35.2	1
Enolase	9.63E+07	1.36E+08	0	5	2.60E+08	5.85E-66	20.7	2
Heat shock protein	3.49E+08	4.90E+08	0	2	1.09E+09	5.66E-05	2.4	2
Metalloendopeptidase OMA1	0	3.10E+07	0	2	1.01E+08	7.95E-06	5.1	2
Serine/threonine-protein phosphatase PP-V	5.03E+08	0	1.07E+09	2	1.59E+09	1.07E-05	4.1	2
GTP-binding ADP-ribosylation factor ARF6	0	0	4.04E+07	2	4.10E+07	3.11E-03	0.9	2
Long chain fatty acid CoA ligase	1.90E+08	0	0	2	1.90E+08	1.48E-09	13.1	1
Alpha-actinin-4	0	6.78E+08	7.30E+08	1	1.79E+09	1.02E-10	5.3	1
Glyoxylate/hydroxypyruvate reductase	0	6.55E+07	0	1	7.93E+07	9.81E-03	4	1
Sox14	0	4.92E+07	0	1	5.96E+07	6.88E-05	7.3	1
Zinc finger	0	3.45E+07	0	1	4.18E+07	6.51E-03	10.1	1
Alpha-tocopherol transfer protein	0	3.27E+07	0	1	3.96E+07	6.22E-03	6.6	1
Beta tubulin	0	2.39E+07	0	1	2.90E+07	3.04E-04	4.3	1
Nesprin-1	0	0	2.29E+08	1	2.32E+08	5.97E-03	5.3	1
Vacuolar sorting protein	0	0	1.55E+08	1	3.59E+08	1.17E-04	9.5	1
Zinc finger protein	0	0	7.80E+07	1	9.70E+07	6.07E-03	8	1
Aconitase	0	0	7.37E+07	1	3.03E+08	2.92E-03	8.6	1
Electron transfer flavoprotein alpha subunit	0	0	5.78E+07	1	1.11E+08	6.49E-03	5.1	1
Lethal (3) 76BDm	0	0	4.76E+07	1	4.83E+07	1.13E-02	2.6	1
Patched 1	0	0	5.12E+06	1	2.31E+07	1.09E-02	3.6	1
Heat shock protein	5.02E+07	0	0	1	5.02E+07	1.32E-10	6.3	1

Table 5.12 Identities of *Varroa* proteins present in haemolymph samples of *A. mellifera* pupae

Table of identities, peptides, intensity, sequence coverage and unique peptides of the proteins found in haemolymph of *A. mellifera* pupae that matched a *Varroa* hit on the combined database.

5.6 Discussion

The vitality of the *A. mellifera* hive depends on individual fitness, cooperation, and shared resources. The eusocial behavior of the members in the hive means that almost all roles are shared between the unfertile workers, and their health is vital for successful foraging and maintenance of the hive (Barchuk *et al.*, 2007). The aim of this Chapter was to analyze the effect that parasitization by the mite *V. destructor* has on *A. mellifera*. The aim was to examine adult worker bees and also developing pupae, as the workers are arguably the most important group within the colony, carrying out all work with the exception of reproduction. The effect of *Varroa* on drone pupae parasitized during development was also examined in detail, as in their natural host, *A. cerana*, *Varroa* almost exclusively parasitize the male brood (Rosenkranz *et al.*, 2010).

The first step into the analysis of the effect of *Varroa* parasitization on the health and vitality of the bee was to examine the effect that they have on adult worker bees. The aim was to identify changes in the proteomic profile of bees that were parasitized during the overwintering period to see what effect this might have on overall vitality of the hive over winter, which may affect recovery in the spring. First, the proteomic variation in unparasitized worker bees during the winter period was compared to the profile of unparasitized summer bees in order to see what changes to the proteomic profile normally occur. Using 1-Dimensional gel electrophoresis, proteins from unparasitized summer workers were compared to proteins from unparasitized winter workers and the bands showing variations in abundance were excised for identification. A total of 8 bands were identified as differentially abundant (Figure 5.1). Apolipoporphins isoform 1 was present at lower levels of abundance in the winter bees, which is a protein involved in lipid transport. Apolipoporphins also has a proposed role in the insect immune response, as they have been shown to bind the cell wall of Gram-negative and Gram-positive bacteria, and also the cell wall of fungal cells. It is considered therefore to be a pattern recognition receptor (PRR) (Zdybicka-Barabas and Cytrynska 2013). The lower abundance that naturally occurs over the winter could be due to nutrient depletion and could

possibly have a negative effect on the survival of overwintering bees if they were under stress such as parasitization by *Varroa*, as lower abundances of proteins involved in the immune response such as PRR could result in a higher susceptibility to infection from other pathogens. A number of hexamerins were also present at lower levels of abundance in the overwintering bees compared to the summer bees. Hexamerin is a storage protein that provides nutrition to the bee through the haemolymph during non-feeding periods (Burmester 1999). Therefore it is expected to see the lower abundance during the overwintering period as the bees do not have as much access to food. A number of stress related proteins were also differentially expressed in the proteomic profile of overwintering bees compared to summer bees, which is also expected as the overwintering period can be very stressful due to temperature changes and limited resources within the hive (Amdam *et al.*, 2004). Using 2-Dimensional analysis, the proteome of the unparasitized winter and unparasitized summer bees was examined, as 2-Dimensional SDS PAGE leads to better overall separation of individual proteins and may show additional changes in the profile not visible by 1-Dimensional analysis. A number of proteins involved in normal cell function such as energy production and muscle contraction were present at statistically significantly lower abundance in the winter bees compared to those sampled in the summer (ATP synthase; 1.8 fold decrease; $p = 0.001$, Tropomyosin; 1.8 fold decrease; $p = 0.011$). This reduction in proteins involved in normal activities in the bee could have a negative effect on the vitality of bees in the spring, when foraging is possible again (Bosch and Kemp 2004). Interestingly, cuticular protein precursor was present at higher levels of abundance in the overwintering bees (2.3 fold higher). This could possibly be due to the fact that in the summer period, some of the bees sampled could have been younger bees that had just recently emerged and had not yet developed a fully hardened cuticle, whereas all of the winter bees would have been emerged for some time prior to sampling. A protein involved in redox cycling and protection against oxidative damage, thioredoxin-dependent peroxide reductase, was decreased significantly at a level of 2.9 in the overwintering bees. Previous research has shown that some thioredoxin reductases implicated in the response to temperature stress in *Bombyx mori*, and the lower level observed here in the

overwintering bees may be indicative of a temperature change over winter (Lee *et al.*, 2011).

The variations in the proteomic profile of winter bees that were unparasitized during the overwintering period was then compared to the profile of bees that were parasitized by *Varroa* at a high level of infestation. The aim was to see any differences between the profiles compared to normal variations that occur during the overwintering period due to natural stresses such as lower amounts of available food. The first observation when comparing the proteomic profiles of the unparasitized and the parasitized overwintering *A. mellifera* was that the reduction of the storage protein hexamerin had not increased further in the parasitized winter bees than the drop in abundance that had been observed when comparing winter to summer bees. This is interesting as the assumption could be made that the loss of haemolymph would in turn lead to a loss of storage proteins. The hexamerin that was present at higher levels in the parasitized winter bees was hexamerin precursor however, so perhaps the increased abundance in the precursor form was due to the bee attempting to make up for lost hexamerin due to the parasitization by *Varroa*. A heat shock protein was present at higher levels of abundance in the parasitized winter bees, which is indicative of an activated stress response. A stressed bee could potentially be more susceptible to infections from pathogens as the immune system is thought to act in a trade off system with other systems within the body (Cotter *et al.*, 2004; Yang and Cox-Foster 2007). Enolase-like protein, which is involved in glycolysis, was found at decreased abundance in the parasitized winter bees, which could affect the effective release of energy in the bee. A drop in the amount of available energy through metabolism could negatively affect the emergence of healthy worker bees in the spring, which in turn could affect the vitality of the whole colony due to inefficient foraging (Mayack and Naug 2009b). Tropomyosin 1 was interestingly present at higher levels of abundance in the parasitized winter *A. mellifera* than in the unparasitized. Tropomyosin 1 is a muscle protein and is involved in movement. Its presence at higher levels of abundance in the parasitized winter bees is not fully understood, as another muscle protein, myosin heavy chain, was seen present at lower levels in the winter *A. mellifera*.

The next section in the analysis of the effect that parasitization has on the vitality of *A. mellifera* was to examine the proteomic profile of parasitized drone brood and unparasitized drone brood. In their original host, *A. cerana*, *Varroa* almost exclusively parasitize male brood, and so may have a unique host-parasite relationship (De Jong 1988). The proteomic profile firstly of unparasitized drone brood was compared to parasitized drone brood using 2-Dimensional electrophoresis. The drone brood used were either *Varroa* free upon uncapping or had either one or two adult *Varroa* present. The comparison of the proteomic profiles of the parasitized and the unparasitized showed a total of 12 spots that were identified as being statistically significantly different in abundance. Tropomyosin 2 was identified for three of these spots, and was present at lower levels of abundance (between 4.6 to 5.7 fold in the parasitized winter bees). The substantial decrease in tropomyosin during development could indicate impaired growth, which could affect the mobility of the drone upon emergence. Interestingly, tropomyosin 1-like was present at higher levels of 4.5 fold increased abundance in the parasitized drone brood. This increase in the abundance of tropomyosin 1 was also found in the parasitized winter adult *A. mellifera*. Proteins involved in cuticle formation and structure were present at lower levels in the parasitized drone pupae compared to levels in the unparasitized drone, such as endocuticle structural glycoprotein. This reduction in the amount of cuticle structure proteins could be indicative of a reduced healing response of the puncture wound made in order to feed by *Varroa* (Richards *et al.*, 2011a). The reduction in the abundance of cuticular proteins in the parasitized pupae could be due to the suppression of cuticular healing by effectors in the salivary secretions of *Varroa* during feeding. Arlyphorin, which is a storage protein proposed to have an immune function, was found decreased dramatically in abundance in the parasitized drone pupae. Arlyphorin has been shown previously as an effector of the humoral immune response, capable of causing damage to the outer surface of bacteria (Beresford *et al.*, 1997; Zhu *et al.*, 2013). A lower abundance of this critical immune protein could have serious implications for the immune response to other pathogenic threats during the development of a healthy adult bee. Hexamerin was also present at lower levels of abundance in the drone pupae that were parasitized by *Varroa*, which could have implications on the vitality of the growing pupae due to the lack of

nutrients available for energy production. Another protein involved in normal energy production, ATP synthase, was decreased in abundance by over three fold in the parasitized drone pupae compared to the unparasitized, and this could also mean that requirements for energy could potentially fall short, leading to insufficient metabolism therefore affecting vitality.

Following the results on the analysis of how *Varroa* can potentially affect the health and vitality of drone pupae through proteomic analysis, the next aim was to analyze whether these variations were dependent on the level of parasitization or if the reaction by the bee was constant irrespective of the level of parasitization. Drone pupae with varying levels of parasitization were chosen and their relative proteomic profiles resolved by 2-Dimensional gel electrophoresis. A total of 71 protein spots were identified as being statistically significantly different between the unparasitized, mildly parasitized, moderately parasitized, and severely parasitized. Hexamerin 110 precursor and hexamerin were decreased in abundance from the unparasitized pupae through to the heavily parasitized pupae. This correlated decrease could be due to the fact that extra *Varroa* feeding on the heavily parasitized pupae means a greater loss of haemolymph and its associated proteins. Glutathione s-transferase and short chain dehydrogenase/reductase both increased in abundance and this correlated with the amount of *Varroa* that were found to be parasitizing the pupae upon uncapping. Both of these proteins are involved in the stress response with oxidoreductase activity, and a rise in the level of their abundance could indicate that the level of parasitization determines the levels of stress on the pupae. There was a steady increase in the abundance of arginine kinase dependent on the level of parasitization also. Arginine kinase is a transferase that has also been shown to have a role in the immune response of *Drosophila* to fungal pathogens. Its increase in abundance here could indicate activation of the immune response due to the presence of *Varroa*. (Vierstraete *et al.*, 2004b). Apolipoporphins, previously described in this section as having a role in the immune response, showed an increase in abundance in the moderately parasitized pupae, which dropped again when the pupae were heavily parasitized. This type of pattern indicates that possibly a low level of parasitization reduces the abundance of the protein, but that under higher levels

of *Varroa*, which would be more stressful, the pupae must try to combat the loss of certain proteins by expressing more of the protein. This initial drop in abundance levels in the mildly parasitized pupae and the steady rise again in the moderately and severely parasitized pupae is also seen in a number of other proteins. Proteosome disulfide isomerase 6-like shows this pattern of abundance as the level of parasitization increases. This protein is involved in the stress response by preventing other proteins from aggregating during times of protein unraveling (Vargas-Albores *et al.*, 2009). It is also thought to play a role in cell adhesion, and was shown to be increased in abundance in *Litopeneus vannamei* haemocytes when challenged with bacteria (Vargas-Albores *et al.*, 2009). The most notable drop in abundance during parasitization by *Varroa* was on the spot identified as containing phosphatidylethanolamine-binding protein homolog. The role of this protein is poorly understood, but it has recently been associated with the immune response (Hengst *et al.*, 2001). It has been described in mice as the prototype of a novel family of serine protease inhibitors, with an increased abundance also seen in immune challenged *Drosophila* (Vierstraete *et al.*, 2004a). It has also been shown to be a probable antimicrobial compound in *Musca domestica*. The sharp drop in its abundance in the parasitized pupae is not dependent on the level of parasitization and stays at an almost constant level of abundance over the various parasitization stages, which further supports the theory it is a response to an immune threat. A heat shock protein 60kDa was present at higher levels in the parasitized pupae at an almost constant level also, which is indicative of a stress response. The constant level of abundance is suggestive that the stress felt under parasitization is not dependent upon the amount of *Varroa* present.

Label free analysis was employed in order to look at the highest levels of proteomic changes possible in the unparasitized and parasitized drone pupae. The most differentially abundant protein in the parasitized drone pupae, present at levels of over 48 times higher than the levels in the unparasitized pupae, was apidermin 2. The function of this protein is largely unknown, but it has been linked with a possible role in the structure of the cuticle, particularly the inner cuticle linings of the trachea and the gut (Kucharski *et al.*, 2007). The huge increase seen here, along with some trademark characteristics such as its small

sequence length, indicate that possibly the proteins may also have a novel role in the immune response. Apidermin-2 was found present at higher levels following *Crithidia bombi* infection of *Bombus terrestris* (Barribeau *et al.*, 2014). MD-2 related lipid recognition protein, another immune related protein, was present at higher levels in the parasitized drone pupae. MD-2 is a lipid recognition protein involved in the Toll-like signaling pathway, a hugely important feature of the humoral immune response. Its presence at higher levels in the parasitized drone pupae indicates an activation of an immune response to the presence of the feeding *Varroa*. A leucine rich repeat protein was the second most abundant protein in the parasitized drone pupae, which could possibly have a role in the immune response. Certain toll-like receptors of the toll-like signaling pathways are thought to contain leucine rich motifs and so this could potentially be a receptor protein involved in recognition (Nürnberg *et al.*, 2004). Its presence at a level of over 28 times higher in the parasitized drone pupae presented here could further reinforce this theory. Tropomyosin 2, which has previously been shown through 2-Dimensional analysis of parasitized adults and pupae present in higher levels, is also found at higher levels here. This could indicate some evidence for the response of the host to the parasite, possible through calcium binding or cell adhesion, but more work will have to be carried out in order to support this theory.

A number of proteins involved in growth and cell proliferation were also present at much higher levels in the drone pupae that were parasitized by *Varroa*, such as girdin-like. This is suggestive of a growth response of the pupae by *Varroa*. Possibly the mites, due to the host parasitize relationship on drone pupae in *A. cerana* have deciphered a means of ensuring that the pupae undergo extra growth during the feeding period to ensure they benefit from extra haemolymph. The large number of myosins and tropomyosins present at higher levels in the parasitized pupae could also potentially indicate support for this hypothesis, as extra muscle mass due to larger size would give a higher abundance LFQ for these proteins. Certain proteins normally associated with the process of caste differentiation from worker to queen were also interestingly present at much higher levels in the parasitized drone pupae, some of which were also present in the exclusive hits list, completely absent from the

unparasitized drone such as ornithine aminotransferase. Tubulin alpha chain-like, ornithine aminotransferase and long chain fatty acid-Coa ligase 3-like are all normally present at higher levels of abundance in queen larvae than workers or drone (Barchuk *et al.*, 2007). One of the main differences, aside from reproductive potential between queens and members of the other castes, is their larger size (Evans and Wheeler 1999) This potentially supports the hypothesis that *Varroa* during feeding attempt to manipulate the size of the pupae in order to benefit themselves. During analysis of the variations in the drone pupae that were parasitized by *Varroa*, major royal jelly protein one was also exclusively expressed in the parasitized pupae, but is not shown as it was absent from all four unparasitized replicates but only present in 2 out of the 44 parasitized replicates so was not considered significantly exclusive. Together with the changes in the abundance of the other caste differentiation associated protein described here however, it may be a significant find.

The protein showing the lowest differential abundance in the parasitized drone pupae compared to the unparasitized pupae was mantle protein-like. This protein is largely function unknown also, but due to its small size and its proline rich motifs could possibly be a component of the cuticle. Proline rich protein 4-like, another protein present at much lower levels of abundance in the drone pupae that were parasitized by *Varroa* could also be involved in cuticle formation for the same reasons, as otherwise it also is largely function unknown. A number of proteins with already defined roles in the cuticle were also found present in lower levels of abundance in the parasitized drone pupae, such as pupal cuticle protein-like. The lower level of these proteins following parasitization by *Varroa* could indicate a compromised healing response of the cuticle in order to allow the *Varroa* to feed without the need to open a new hole in the cuticle each time (Richards *et al.*, 2011a). Maternal protein exuperantia was found exclusively expressed in the control pupae, and post imputation for statistical analysis was found at over 5 fold higher abundance in the unparasitized pupae. Its role here is unclear, as the maternal isoform is largely associated with localization of RNA in the developing oocytes (Marcey *et al.*, 1991). It is also reported however to play a role in sex determination through alternative splicing, which could further support the hypothesis that the *Varroa*

induce growth increase in parasitized pupae by activation of caste specific queen differentiation pathways (Hazelrigg and Tu 1994). A protein involved in cell-cell adhesion during flocculation in *S. cerevisiae* was found present exclusive to the unparasitized pupae, and at a fold change of 2.51 following imputation. This protein could possibly be involved in the flocculation of haemocytes during the normal healing response and its presence at lower levels of abundance during parasitization by *Varroa* could be indicative of an impaired healing response. A number of zinc finger domain containing proteins were present at lower levels of abundance in the parasitized drone pupae, including zinc finger protein 512B. All were exclusively expressed in the control pupae, and were all significantly changed in abundance post imputation also. Zinc finger proteins are involved mainly in DNA binding and also in protein binding and recognition (Gamsjaeger *et al.*, 2007). The lower abundance of the zinc finger protein presented here in the parasitized pupae is not completely clear, but if the hypothesis that *Varroa* aim to take control over certain functions such as the growth response, the suppression of these protein is needed to make the manipulation possible. A number of uncharacterized and function unknown proteins were present at lower levels of abundance in the parasitized drone pupae, such as LOC726920. Using bLINK analysis, three of these were linked to cuticle proteins (LOC726920, LOC100577362, LOC102653822), which further supports the claim that parasitization by *Varroa* leads to lower levels of proteins involved in cuticle structure and formation and may lead to an impaired healing response. LOC1026576126 (2.33 fold decrease in parasitized) and LOC100578085 (3.78 fold decrease in parasitized) showed no obvious homology to any other proteins and so their function can only be hypothesized as having a possible immune role or role in cuticle formation. Transferrin 1 and apolipophorin III were present at lower levels of abundance in the parasitized drone pupae -2.14 fold decrease and -1.98 fold respectively. These two proteins have been shown to be involved in the immune response of arthropods, with their expression is usually upregulated following pathogenic insults such as in bacterially challenged *Drosophila* (Yoshiga *et al.*, 1999). The presence of these proteins in lower levels in the pupae that were parasitized by *Varroa* provides more support of the hypothesized that *Varroa* possess means by which to suppress their hosts' immune response in order to feed unaffected.

The next step to fully analyze the effect that *Varroa* have on *A. mellifera* was to look at the relationship between the developing worker bees and their host. Proteomic analysis was employed to look at the overall effect on the proteomic profile using label free mass spectrometry on the whole body proteome of unparasitized and parasitized worker pupae. The protein which showed the highest level of abundance in the parasitized worker pupae compared to the unparasitized was fatty acid synthase, present at levels of 53 fold higher abundance. This increased in abundance is substantial, but the reason remains largely unclear. The synthesis of fatty acids has no obvious role in immune protection from the parasite. Queen pupae however overexpress many proteins, including those involved in fatty acid synthesis and metabolism (Begna *et al.*, 2011). The large increase in abundance here could suggest that the effect of *Varroa* on the developing worker pupae is similar to the proposed effect of *Varroa* on drone pupae, with attempts made to manipulate size in order to benefit through extra haemolymph. Two additional proteins found at higher levels of abundance in the parasitized worker pupae further support this hypothesis; LOC413264 (8.97 fold increase) and long chain fatty acid CoA ligase (8.4 fold increase). These proteins are normally expressed at higher levels in queen pupae, and the uncharacterized protein, LOC413264 is actually a yellow protein from the major royal jelly superfamily, heavily involved in caste differentiation. Preventative tactics employed by the *Varroa* to stop the loss of haemolymph reducing the size of the pupae could benefit the parasite as it would increase the amount of available haemolymph. An additional protein involved in sex development was also increased in abundance in the parasitized worker pupae, protein double sex like. Its role here is not completely clear however, as it is normally involved in sex determination through alternative splicing (Waterbury *et al.*, 1999). An immune protein, protein croquemort was present in the parasitized pupae at 4.68 fold higher than the control. This protein is involved in phagocytosis (Franc *et al.*, 1999). The increase in abundance could be indicative of an activated immune response to the presence of *Varroa*. Proteins involved in the melanization cascade, serine tRNA ligase and serine/threonine protein phosphatase were also present at higher levels of abundance in the parasitized worker pupae, which also indicates an immune

response elicited by the parasitization by *Varroa* (Bachmann and Möröy 2005). A number of uncharacterized proteins showing no homology to known proteins were also found at higher levels in the workers that were parasitized by *Varroa* (LOC100576161; 31.65 fold increase, LOC100577527; 6.85 fold increase, LOC100576770; 4.89 fold increase,). Their lack of homology to any known proteins means that their functions can only be hypothesized as possible immune proteins due to their increased abundance following parasitization by *Varroa*.

A large number of proteins were found at lower levels of abundance in the parasitized worker pupae when compared to the unparasitized pupae using label free analysis. The least abundant protein in the comparison of unparasitized worker to parasitized worker pupae was myosin regulatory light chain 2, at 25.91 fold lower levels in the parasitized worker pupae. This is a muscle protein involved in calcium binding but it was suggested previously with regard to the drone results that myosin may have a role in the immune response of organisms, due to its change in expression so frequently in the drone results. Tropomyosin was also present at lower levels in the parasitized worker pupae, by 18.85 fold. A chymotrypsin inhibitor was also decreased in abundance upon parasitization of worker pupae by *Varroa*, with a 25.69 fold drop. This protein is a serine protease inhibitor and its lower levels in parasitized workers is suggestive of an activated immune response (Kanost 1999). A group of cuticle proteins involved in the structure and formation of the cuticle were present at lower levels in the parasitized worker pupae, similar to the drop in abundance in the parasitized drone pupae. This could be indicative of a reduced healing response due to suppression of the expression of cuticle components by the feeding *Varroa*. A number of proteins with a chaperone role in the folding of proteins such as prefoldin subunit 5-like were present at lower levels in the parasitized worker pupae than in the unparasitized. Heat shock proteins and other protein chaperones are thought to act with the immune response of organisms such as during the receptor mediated protein signaling cascades (Pockley 2003). This could be the case here, as suppression of proteins that enable the correct signaling following detection of an immune threat could dampen the immune response. Another reason for the lower abundance of these

proteins involved in proteins folding and protein-protein interactions could be the *Varroa* attempting to manipulate the normal process within its host in order to benefit itself by larger volumes of haemolymph for example. A number of proteins involved in cell structure were also found decreased in abundance in the parasitized worker pupae compared to the unparasitized workers, such as tubulin and calponin. The reason why these proteins would be present at lower levels following parasitization by *Varroa* remains unclear, but it could be hypothesized that a lower presence of these proteins forming structure could allow for the growth to occur that the *Varroa* could be inducing.

Real time PCR analysis was employed in order to see if there were any notable changes in the expression of four immune genes in the parasitized worker and drone pupae when compared to their relative controls. Overall, the expression of the genes was similar in the drone and worker pupae, indicating that the immune response to *Varroa* of both with regard to these four genes was comparable. There was no obvious host-parasite differences in the drone and worker pupae under parasitization. Parasitization by *Varroa* resulted in a drop in the relative expression of *abaecin* in both worker and drone pupae at a significant level. *Abaecin* is involved in the immune response as an antimicrobial peptide (Bull *et al.*, 2012). Its presence at lower levels in the pupae of both workers and drones that were parasitized by *Varroa* could have serious implications for the protection against other pathogens such as *Nosema*. Similar results were shown by Gregory *et al.*, (2005) with a drop in the transcript levels of *abaecin* following parasitization by *Varroa*. Transcript levels of *defensin* also fell in the drone and worker pupae after parasitization by *Varroa*, with the drop in the drone pupae deemed significant. *Defensin* is also an antimicrobial peptide, and a similar drop in the level of its transcription has also been previously demonstrated (Gregory *et al.*, 2005; Klaudiny *et al.*, 2005). The lower level of transcription of *defensin* in *Varroa* parasitized bees could potentially indicate immune suppression and a compromised defense system. The level of transcripts of *phenoloxidase* in the parasitized worker and drone pupae were higher than their respective control significantly so in the worker pupae. This is an immune related enzyme involved in the response to microbes and also implicated in wound healing (Gregor *et al.*, 2012). The increased level

of expression here is interesting as the healing response of *A. mellifera* to *Varroa* is generally quite poor, so perhaps the level of transcripts is increased but the suppression of the response by *Varroa* happens further downstream at the proteomic level. The final gene used to identify changes in the level of its transcription was *hymenoptaecin*. This is another gene which encodes for an antimicrobial peptide (Casteels *et al.*, 1993). The level of transcription of *hymenoptaecin* in drone and worker when parasitized by *Varroa* was similar, with an increase in the level of transcription seen in both at a significant level. This increase in the level of *hymenoptaecin* has previously been shown in bees that were parasitized by *Varroa* and is indicative of an immune response to the threat (Gregorc *et al.*, 2012). The changes in the level of these transcripts could ultimately affect the vitality of the bee upon emergence, especially with the lower levels of two of the immune genes seen upon parasitization. This could leave the bee more susceptible to the many opportunistic pathogens that readily parasitize *A. mellifera*. It is interesting to see also that the levels of transcription in the parasitized worker and drone brood are quite similar, as in the proteomic analysis many differences in the response to parasitization are evident between drone and worker response. It must be noted however, that the proteomic analysis of the parasitized versus unparasitized *A. mellifera* did not factor into account the presence or absence of viruses. A recent study by Kuster *et al.*, (2014), suggested that the immune suppression seen in parasitized *Apis* are due to the presence of viruses and not due to the mite. Though a possibility, the results present throughout this chapter are a global study of all of the proteins present in varied abundance following parasitization, including proteins involved in sex determination, unlikely to be due to infection by viruses and are almost certainly a direct result of parasitization by *Varroa*.

The final step into analyzing the full relationship of *Varroa* and *A. mellifera* was to try to identify any effectors of the responses seen in the parasitized *A. mellifera* by identifying salivary effectors. Haemolymph from parasitized pupae was used and the results searched against an *A. mellifera* and *V.destructor* database, with the hits that matched only *Varroa* proteins considered as possible salivary effectors. The aim was to identify if any of these could potentially be causing the immunosuppression and changes in the protein

expression of the parasitized bees. Haematophagous arthropods cause immune reaction in the host, not thought to be from the initial perforation of the cuticle alone. Blood feeding parasites can feed for days at length without the wound healing over, up to ten days for example in some tick species (Nuttall 1998; Fontaine *et al.*, 2011). The saliva of blood feeding parasites is thought to contain the effectors, which enable this ability to suppress the host's immunity and allow feeding to happen uninterrupted. Salivary proteins are the most likely candidate and are probably secreted into the host to carry out a systematic effect. One protein found in *Rhodnius prolixus* for example, showed anti-aggregation ability of platelets, preventing normal blood clotting allowing blood to flow freely (Fontaine *et al.*, 2011). The composition of the saliva is thought to be complex, with a variety of different proteins thought to work in conjunction. In the aphid *Acyrtosiphon pisum*, mass spectrometry of the salivary proteome identified a total of nine protein from a silver stained 1-Dimensional gel, including an oxiodorecutase, a regucalin, and a metalloprotease (Carolan *et al.*, 2009). With recent advance using label free analysis, this figure could potentially be a lot bigger. The analysis identified two proteins that were only present in the haemolymph that was extracted from parasitized pupae. One of these was T complex protein 1 subunit b. This protein is part of an 8 protein complex which makes up a molecular chaperone essential for correct protein folding and correct actin folding (Miyata *et al.*, 2014). Interestingly, it is also responsible for cooperation of prefoldin in actin/tubulin formation, which were a group of proteins found present at lower levels in the parasitized worker pupae. This could potentially have significance. The second protein found only in the haemolymph from parasitized pupae was alpha actinin 4. Alpha actinin 4 cross link actin filaments, with this crosslinking activity regulating the formation of focal adhesions and cell migration (Shao *et al.*, 2010). It has been shown to be present at different areas of platelets depending on the state that the platelet is in, and is localized during coagulation (Sixma *et al.*, 1989). Due to its role in platelets in mammals and due to its cell migration ability, perhaps it is involved in an anticoagulation response during blood feeding. A metalloendopeptidase was present in the artificially infected pupal haemolymph, and not detected at all in the control pupae. Metallopeptidases have been previously identified from various parasites, including blood feeding

nematodes, aphids, and the cattle tick (Williamson *et al.*, 2003; Carolan *et al.*, 2009; Kongsuwan *et al.*, 2010). They are thought to play an important role in the digestion of blood in parasitic arthropods, and the finding here in the haemolymph could provide evidence for the enzymes as part of the digestive repertoire of *Varroa* (Williamson *et al.*, 2003; Kongsuwan *et al.*, 2010). Patched 1 protein, only detected in the artificially infected pupae and not in the control, is thought to play a role as a regulator of cell growth and also cell fate determination (Sun *et al.*, 2014). This could potentially be an effector of the extra growth rate hypothesis of the parasitized pupae. An oxidoreductase, glyoxylate/hydroxypyruvate was also absent from detection in the control and only present in the artificially infected pupae. The saliva of the aphid *A. pisum* also showed an oxidoreductase present upon analysis. This could also be a significant finding, as oxidoreductases tend to be one of the common proteins identified from either the saliva or the midgut of blood feeding parasites (Carolan *et al.*, 2009; Kongsuwan *et al.*, 2010). A number proteins with transfer activity were present in only the parasitized pupal haemolymph, such as beta tubulin, alpha tocopherol and vacuolar sorting protein. These proteins are all involved in the intermembrane transport of proteins, which could highlight a method for the salivary proteins entering into the parasitized bee. These findings, though preliminary, show a variety of potential salivary effectors that could be employed during the host-parasite battle during feeding by *Varroa*.

This Chapter highlights a complete investigation into the relationship that *Varroa* have with their host *A. mellifera*. The relationship between the two is complex, with the effect that the *Varroa* have on the bee also varying on the proteomic level between drone and worker, most likely due to the evolution of the mite on its natural host, *A. cerana*. The effect that the mites have on drone pupae as worker and with more specific targets, with a widespread systemic effect seen in the worker pupae, is indicative of a stressful response. Both adults and young show some immunosuppression following parasitization. The molecular effects on *Varroa* on the transcript levels of the immune genes showed similar results on both the worker and the drone, with clear immunosuppression of two immune genes taking place. This could have a serious knock-on effect on the vitality of the bee upon emergence. The

investigation into the salivome of the mites also gave some interesting results, with many potential salivary effectors identified. The effect that *Varroa* have on the bee, both adult and young, is detrimental, clearly highlighted here using a variety of investigation means.

Chapter Six

General discussion

6.0 General discussion

The honeybee is an economically and ecologically important insect as one of the principle pollinators (Klein *et al.*, 2007). Pollinators collectively are responsible for around 35% of global food production, with their services valued at around \$153 billion per annum (Blacquière *et al.*, 2012). Large-scale losses of *A. mellifera* numbers have occurred in recent years, through a phenomenon termed Colony Collapse Disorder (CCD). CCD affected hives are characterized by absent workers with enough resources to discount starvation (Cornman *et al.*, 2012). In 2006; CCD was first reported in the USA, with 23% of beekeeping operations losing up to 45% of their hives during the Winter of that year (Cox-Foster *et al.*, 2007). Many factors have been blamed, including the parasitic mite, *V. destructor* (Francis *et al.*, 2013). Due to its relatively recent host shift onto *A. mellifera* from its original host *A. cerana*, it is one of the most serious threats to the survival of the honeybee (Oldroyd 1999). The effect that parasitization by *Varroa* has on the honeybee is not fully understood, despite the knowledge that it leads to haemolymph loss and can transmit viruses through feeding (Gisder *et al.*, 2009; Le Conte *et al.*, 2010). The aim of this project was to examine the effect that *Varroa* has on *A. mellifera*, both on the young and the adults, in order to gain a better understanding into the threat that *Varroa* pose to honeybee populations.

The first objective was to complete a nationwide disease survey of the types of disease found in Irish honeybee colonies, with their seasonal prevalence, as this has never before been documented. Understanding the types of diseases found in colonies in Ireland throughout the year could help to offer some explanation towards the variations in colony survival levels from year to year. The sample colonies were chosen at random with a good geographical spread across the Republic of Ireland. Samples were taken from the colonies in the Spring, Summer and Autumn, and analyzed for the presence of four common bee viruses (DWV, CBPV, ABPV and IAPV), the microsporidian parasite *Nosema*, and the parasitic mite, *A. woodi*. These diseases were chosen as they are common to *A. mellifera* and have been suggested as possible contributors to the collapse of colonies (Tentcheva *et al.*, 2004; McMullan and Brown 2005;

Shen *et al.*, 2005; Chen *et al.*, 2014, Gómez-Moracho *et al.*, 2015). The prevalence of DWV rose steadily through the seasons, as was to be expected as brood numbers rise, due to the correlation of DWV to levels of *Varroa* (Francis *et al.*, 2013). The levels of DWV throughout the country were extremely high, which is possibly an indicator of the corresponding levels of *Varroa* infestation. DWV can be detrimental to the survival of honeybee colonies when it occurs with high levels of *Varroa*, possibly due to the extra stress on the immune response and the additional ability of *Varroa* to transmit viruses from bee to bee during feeding (Bowen-Walker *et al.*, 1999). CBPV and ABPV were undetected in any of the samples throughout the year, despite the larger sample size taken in the Autumn round of sampling. This is possibly due to the lower rate of occurrence of these viruses, or could also be due to their higher virulence, meaning that only bees free from the viruses were sampled as infected bees died quickly (Tentcheva *et al.*, 2004; Nielsen *et al.*, 2008). IAPV was detected using the larger sample size which was a significant finding as IAPV has been linked with being a possible causative agent of CCD (Cox-Foster *et al.*, 2007). The colonies were also analyzed for the presence of the microsporidian parasite *Nosema*, which is a significant threat to honeybee populations worldwide (Genersch 2010). In recent years, the replacement of the original honeybee strain, *N. apis* by *N. ceranae* has been reported, and the analysis of the species present in Irish honeybee colonies confirmed that this is potentially the case. *N. ceranae* was identified as being the sole species infecting 28% of the tested samples, with *N. apis* the sole species infecting 21% of the samples. Both species were found to co-infect in 51% of samples that tested positive for noseiosis by microscopy. This is significant for the health of the bee as *N. ceranae* is a more virulent species, capable of wiping out whole colonies if untreated (Raquel Martín-Hernández *et al.*, 2007). Lastly in this survey, the prevalence of the tracheal mite, *A. woodi*, was examined. Only 2 samples in the Spring tested positive for tracheal mite, with none detected during the other seasons. The tracheal mite is one of the least serious threats to the honeybee in recent years, possibly due to competition by other parasites such as *Varroa*, and so this was expected (Doebler 2000).

The next section of the research was to examine the pyrethroid resistance mechanism of *V. destructor*, with the aim of identifying possible proteomic changes in the resistant mites that could be leading to the conferred resistance. The main methods for the control of *Varroa* are chemical based, with only two licensed for use in Ireland, one of which is a synthetic pyrethroid under the trade name of Bayvarol (Coffey and Breen 2013). Organisms are known to acquire resistance by three main mechanisms - behavioral changes to avoid the chemical, changes to the target of the chemical such as the sodium channel, or changes in the amount and efficacy of metabolic enzymes that enable a more efficient mechanism of breakdown (Sammataro *et al.*, 2005). The aim of the work presented here was to compare the proteomic profile of Bayvarol resistant mites to Bayvarol sensitive mites and observe if any variations between the two could be conferring resistance to the chemical. The proteomic profile was compared using 1-Dimensional, 2-Dimensional and label free proteomic approaches. There were various alterations in the proteomic profile of the Bayvarol resistant mites evident, even through the 1-Dimensional analysis which is the most basic means of separating proteins from a sample. The main changes to the proteomic profiles of resistant mites compared to the sensitive was a clear increase in abundance of certain proteins that could potentially be helping to metabolize the chemical more efficiently, such as glutathione s-transferase, and a number of dehydrogenases. Heat shock proteins were also found to be increased in abundance in the resistant mites. Heat shock proteins are molecular chaperones, involved in the stress response of organisms, helping proteins that have become unfolded due to an external or internal stress, by preventing them from aggregating and allowing them to form a correct structure again. This higher abundance of heat shock proteins was also observed in a number of other pyrethroid resistant species (Vontas *et al.*, 2005; Silva *et al.*, 2012). A number of structural proteins such as myosin were also present at higher levels of abundance in the pyrethroid resistant *Varroa*. Changes in the structure of internal cells or the cell underlying the cuticle could possibly inhibit the passing of the chemical into the mite, and could be a potential resistance mechanism (Wood *et al.*, 2010; Koganemaru *et al.*, 2013). The sensitive mites showed an increased abundance of proteins involved in cuticle structure, which could indicate a change in the structure of the cuticle in the resistant phenotype. One of

the three methods used by an organism to avoid the toxic effects of a chemical is to change the structure of the barrier systems which allow the chemical to enter (Sammataro *et al.*, 2005). The altered protein content between the two phenotypes may indicate that this is a factor in conferring the resistance in the Bayvarol resistant mites. Understanding how an organism gains resistance towards a chemical is important in developing new chemicals for their control. As pyrethroid based chemicals are one of only two methods for the control of *Varroa* currently allowed in Ireland, the need to develop a better control method is pressing (Coffey 2007; Coffey and Breen 2013). Proteomic approaches give an excellent overview of the effector molecules of all processes within an organism, and can be used to compare two phenotypically different organisms from the same species (Griffin *et al.*, 2010). Here, proteomic analysis was used to identify variations in the proteome of Bayvarol resistant mites compared to the proteome of sensitive mites. Gel based techniques were first used to look for any variations to protein intensity, followed by label free shotgun proteomics. Label free proteomics gives a much more accurate and wider range of proteins and their relative fold abundance, which allows for a global observation of the changes taking place.

The final Chapter deals with the host –parasite relationship between *Varroa* and *A. mellifera*, examining in detail the effect that harboring a parasite has on both the developing and the adult bee. The first aim was to identify the effect that parasitization has on the adult stage workers, which are the most valuable groups within the colony, responsible for all foraging and maintenance within the hive. The proteomic profile of unparasitized workers during the overwintering period was compared to the profile of workers that had been parasitized. The revival of a colony to full function in the Spring is dependent upon the health of the worker bees, and harboring parasites during this already stressful time may have a serious impact on the health of the entire colony (Yves Le Conte *et al.*, 2010). The parasitized workers showed lower abundance levels of certain proteins involved in maintaining normal metabolism such as enolase-like protein. There was an increase in abundance of certain proteins which indicate a stress response during parasitization such as heat shock protein and glutathione S-transferase (Sørensen *et al.*, 2003). Hexamerin was present at

lower levels in the workers that were parasitized by *Varroa*, which could be an indicator for loss of haemolymph, as hexamerin is a haemolymph storage protein (Burmester 1999). The change to the proteomic profile of workers when parasitized by *Varroa* is indicative of stress response and a lower metabolic efficiency. This could potentially harm the vitality of the worker over the Winter period and affect its emergence in the Spring, which could have severe consequences for the health of the entire colony.

Due to the almost exclusive host-parasite relationship that *Varroa* has with drone *A. cerana* brood, the next section of this Chapter examined the effect that parasitization has on developing drone brood. Firstly, the proteomic profile of drone brood that were unparasitized was compared to the profile of drones that were parasitized by either 1 or 2 adult female mites. The abundance levels of a number of proteins fell in the drone brood that were parasitized during development, such as tropomyosin 2, which is involved in muscle movement and calcium binding (Lehman *et al.*, 1974). Hexamerin was found at lower levels of abundance in the parasitized drone pupae, which could indicate loss of haemolymph as with the parasitized workers. An additional storage protein, arylphorin, was found at increased levels of abundance in the drone brood that were parasitized by *Varroa* during development to purple eye stage, which could indicate an activation of the immune response (Banville *et al.*, 2012). Activation of such an energy consuming system could severely affect development of a healthy viable drone upon emergence (Cotter *et al.*, 2004; Yang and Cox-Foster 2007). The next step was to identify if the changes to the proteome of the parasitized drone was dependent on the level of *Varroa* that were parasitizing the developing drone, or if a base line alteration occurred regardless of the amount of *Varroa* present in the cell upon uncapping. The drone were chosen based on having no *Varroa* present in the cell upon uncapping as the control, and then chosen based on being parasitized by either 1, 2 or 3+ adults female mites, regarded as being mildly, moderately and severely parasitized respectively. The abundance of all of the proteins that were significantly changed between the unparasitized and the parasitized drone brood were changed in a manner that correlated to the amount of *Varroa* present in the cell parasitizing the drone brood. Heat shock protein 60kDa for example, was present at the highest level of

abundance in the drone pupae that were parasitized by the highest number of *Varroa*, increasing in a manner dependent on the level of parasitization. This was also the case for glutathione S-transferase, which reached the highest level of abundance in the drone pupae parasitized by the highest number of *Varroa*. These stress related proteins increased in a manner correlated with the level of *Varroa*, which could indicate that the higher the level of infestation within a colony, the worse the effect on the vitality of the colony (Pockley 2003; Enayati *et al.*, 2005). The levels of hexamerin, a nutritional protein during phases of non-feeding, fell in abundance in a manner dependent on the level of *Varroa* parasitization (Burmester 1999). This drop in abundance correlated to the increased level of *Varroa* could also be indicative of extra volumes of haemolymph being lost due to extra feeding. ATP synthase, a protein involved in normal energy production within the organism was decreased in a manner dependent on the level of parasitization (Younis *et al.*, 2002). The lower rate of energy production in the pupae that are parasitized by *Varroa* could negatively impact on the emergence of a healthy adult bee. Lastly, in order to examine the effect that parasitization by *Varroa* has on the whole proteome of developing drone pupae, label free quantitative proteomics analysis was used. The proteomic profiles of drone pupae that were unparasitized were compared profile of drone pupae that were heavily parasitized at a level of 4 adult female mites and 8 developing progeny. The abundance of a group of cuticle protein fell significantly in abundance when *Varroa* parasitized the pupae, which is most probably due to the suppression of the healing response. It was also hypothesized that *Varroa* could be attempting to manipulate the overall size of the pupa in order to benefit from a healthier pupa with larger volumes of haemolymph due to the suppression of expression of proteins involved in normal developmental response, such as protein double- sex and protein maternal exuperantia (Marcey *et al.*, 1991, Waterbury *et al.*, 1999). The lower abundance of these cuticular proteins could be linked with this hypothesis, as suppressing the synthesis of cuticular proteins would allow the pupa to swell to a larger size. The abundance of a novel cuticle protein, apidermin, with a possible role in the immune response was much higher in the pupae that were parasitized by *Varroa* than in the control pupae (Barribeau *et al.*, 2014). This could be indicative of immune initiation following parasitization.

Label free quantitative analysis was employed to look for changes in the proteomic profile of parasitized worker pupae compared to these that were unparasitized during development, as viable worker bees are crucial to a colony's survival. The same alterations to the abundance of cuticle proteins were evident in the parasitized worker pupae with much lower expression when compared to the level present in the unparasitized workers. The opposite expression of the two developmental proteins, protein double-sex and protein maternal exuperantia was observed in the parasitized worker pupae than in the drone, as they were present in lower levels of abundance (Marcey *et al.*, 1991, Waterbury *et al.*, 1999). This could be indicative of a drone specific response during parasitization by *Varroa* from their evolutionary host-parasite relationship. The worker pupae showed a much more obvious activation of the immune response following parasitization by *Varroa* than the drone pupae, with a serine protease inhibitor present at lower levels of abundance following parasitization, and protein croquemort, an immune protein with a role in phagocytosis present at higher levels of abundance (Franc *et al.*, 1996; Franc *et al.*, 1999). This activation of the immune response was not as evident in the parasitized drone pupae, which makes sense, as through the history of host-parasite evolution, *Varroa* would have mastered feeding unnoticed on the pupae, as the goal of haemophagous parasite is to feed unnoticed and undisturbed (Takken and Verhulst 2013). Label free analysis offers the chance to observe the whole proteome of a given organism, compared to another phenotypically different. Here, it gives an excellent overview into what is happening on a proteomic level when *A. mellifera* is parasitized by *Varroa*. It gives the opportunity to observe smaller peptides also, which is invaluable when examining changes to the abundance of immune peptides.

qPCR was used to identify changes in the level of transcript expression of four immune genes in unparasitized pupae compared to parasitized pupae. The four genes chosen for analysis were *abaecin*, *hymenoptaecin*, *defensin* and *phenoloxidase*. These genes are involved directly in the immune response of *A. mellifera* and were chosen with the aim of determining if any level of immune suppression was taking place when worker and drone pupae were parasitized.

The level of expression of all four genes was very similar between the drone and worker pupae when parasitized, with the level of *abaecin* and *defensin* lower in the pupae that were parasitized by *Varroa*. The levels of expression of *phenoloxidase* and *hymenoptaecin* rose when the pupae of both worker and drone were subject to parasitization by *Varroa*. This similar response is interesting to see as the proteomic responses of the drone and worker were quite varied. The altered levels of these immune genes could ultimately leave the developing pupae more susceptible to infection (Gregorc *et al.*, 2012; Garrido *et al.*, 2013).

The final step in examining the host parasite relationship was determining the method by which *Varroa* cause these changes within the organism, such as immune suppression, by identifying potential salivary effectors. The haemolymph was collected from unparasitized pupae and from pupae that had been either manually infected by *Varroa* or naturally infected within the colony. A number of interesting proteins were identified as either exclusive to one or both of the parasitized pupal haemolymph and completely absent from the control, unparasitized haemolymph. These proteins included alpha actinin, which was found in both parasitized haemolymph samples, and is thought to have a role in platelet aggregation in mammals (Fontaine *et al.*, 2011). A metalloendopeptidase was also detected exclusively in the haemolymph from parasitized pupae, which is interesting as similar proteins to this have previously been implicated in having a role in the feeding by other external parasites (Williamson *et al.*, 2003; Carolan *et al.*, 2009; Kongsuwan *et al.*, 2010). A number of proteins involved in protein trafficking and transmembrane transport were detected only in the haemolymph of pupae that had been parasitized by *Varroa*, such as vacuolar sorting protein, which could offer an explanation for how the *Varroa* alters internal functions within its host from an external position.

The results presented throughout this thesis offer a global view of the complete relationship that the parasitic mite *V. destructor* has with its host, *A. mellifera*. The knowledge of the occurrence of disease within the colony gives an insight into other stresses and threats that honeybees must deal with on a

daily basis throughout the year, and how these diseases could be influencing the survival of colonies from year to year. The development of resistance to pyrethroid has serious implications for the health of honeybees, as through proteomic and molecular analysis, the devastating effect that *Varroa* have on all life stages of the bee is evident. The apparent metabolic resistance of the mite evident through proteomic analysis of Bayvarol resistant mites highlights the need for other means of control, as they clearly have the capacity to overcome chemicals through an adaptive metabolic system, with the spread of resistance made easier through their haplodiploid nature. The detrimental effect that *Varroa* infestation has on honeybee colonies, particularly when they are present in high numbers is highlighted here, offering proof that *Varroa* have a probable role in the colony collapse phenomenon. The evidence of immune suppression in the bee when parasitized by *Varroa* gives a possible initiative for future work, to prevent this immune suppression by boosting the immune response of individuals within colony, possibly through their food source. The evidence of metabolic resistance through proteomic alteration in the Bayvarol resistant mites also gives new reason to examine other means of control, as metabolic resistance to pyrethroid may also confer cross-resistance to other chemicals. The apparent specific response of the mite on drone brood shows that it still holds some of the evolutionary history that it shared with *A. ceranae* and due to their higher tolerance for parasitization by the mite, should be an important future goal for bee breeding with the attempt of obtaining a more *Varroa* resistant phenotype. Research is already underway with the main focus of breeding bees that show a higher grooming tolerance and this may be a possible target in securing the future of *A. mellifera* (Ibrahim and Spivak 2006; Rinderer *et al.*, 2010).

Chapter Seven

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Chapter Eight

Appendices

Sample no.	DWV	CBPV	ABPV	IAPV	Sample no.	DWV	CBPV	ABPV	IAPV
2	Positive	Negative	Negative	Negative	109	Negative	Negative	Negative	Negative
5	Positive	Negative	Negative	Negative	113	Positive	Negative	Negative	Negative
6	Positive	Negative	Negative	Negative	114	Negative	Negative	Negative	Negative
7	Positive	Negative	Negative	Negative	115	Negative	Negative	Negative	Negative
8	Positive	Negative	Negative	Negative	117	Positive	Negative	Negative	Negative
9	Negative	Negative	Negative	Negative	118	Positive	Negative	Negative	Negative
10	Positive	Negative	Negative	Negative	119	Positive	Negative	Negative	Negative
11	Positive	Negative	Negative	Negative	120	Positive	Negative	Negative	Negative
12	Positive	Negative	Negative	Negative	121	Positive	Negative	Negative	Negative
13	Positive	Negative	Negative	Negative	124	Negative	Negative	Negative	Negative
14	Positive	Negative	Negative	Negative	127	Positive	Negative	Negative	Negative
15	Positive	Negative	Negative	Negative	128	Positive	Negative	Negative	Negative
16	Negative	Negative	Negative	Negative	129	Positive	Negative	Negative	Negative
18	Negative	Negative	Negative	Negative	131	Positive	Negative	Negative	Negative
19	Positive	Negative	Negative	Negative	132	Positive	Negative	Negative	Negative
20	Positive	Negative	Negative	Negative	133	Positive	Negative	Negative	Negative
25	Positive	Negative	Negative	Negative	134	Negative	Negative	Negative	Negative
26	Positive	Negative	Negative	Negative	135	Positive	Negative	Negative	Negative
30	Positive	Negative	Negative	Negative	136	Positive	Negative	Negative	Negative
33	Positive	Negative	Negative	Negative	137	Positive	Negative	Negative	Negative
34	Positive	Negative	Negative	Negative	140	Positive	Negative	Negative	Negative
36	Negative	Negative	Negative	Negative	143	Positive	Negative	Negative	Negative
37	Negative	Negative	Negative	Negative	146	Positive	Negative	Negative	Negative
38	Negative	Negative	Negative	Negative	147	Positive	Negative	Negative	Negative
39	Positive	Negative	Negative	Negative	149	Positive	Negative	Negative	Negative

82	Positive	Negative	Negative	Negative	190	Positive	Negative	Negative	Negative
83	Positive	Negative	Negative	Negative	191	Negative	Negative	Negative	Negative
84	Positive	Negative	Negative	Negative	192	Positive	Negative	Negative	Negative
85	Positive	Negative	Negative	Negative	193	Positive	Negative	Negative	Negative
87	Positive	Negative	Negative	Negative	194	Positive	Negative	Negative	Negative
89	Positive	Negative	Negative	Negative	195	Positive	Negative	Negative	Negative
90	Positive	Negative	Negative	Negative	199	Positive	Negative	Negative	Negative
91	Positive	Negative	Negative	Negative	200	Positive	Negative	Negative	Negative
92	Positive	Negative	Negative	Negative	201	Positive	Negative	Negative	Negative
96	Positive	Negative	Negative	Negative	202	Positive	Negative	Negative	Negative
98	Negative	Negative	Negative	Negative	204	Positive	Negative	Negative	Negative
99	Positive	Negative	Negative	Negative	205	Negative	Negative	Negative	Negative
100	Positive	Negative	Negative	Negative	206	Positive	Negative	Negative	Negative
102	Positive	Negative	Negative	Negative	208	Positive	Negative	Negative	Negative
103	Positive	Negative	Negative	Negative	209	Positive	Negative	Negative	Negative
104	Positive	Negative	Negative	Negative	210	Positive	Negative	Negative	Negative
105	Positive	Negative	Negative	Negative					

Table A3.1 Spring viral detection results.

Table shows all of the samples received for viral analysis during the spring sampling period. Locations corresponding to each sample number are in section 2.2.

Sample	DWV	CBPV	ABPV	IAPV	Sample	DWV	CBPV	ABPV	IAPV
2	Positive	Negative	Negative	Negative	109	Negative	Negative	Negative	Negative
5	Positive	Negative	Negative	Negative	113	Positive	Negative	Negative	Negative
6	Positive	Negative	Negative	Negative	114	Negative	Negative	Negative	Negative
7	Positive	Negative	Negative	Negative	116	Negative	Negative	Negative	Negative
8	Positive	Negative	Negative	Negative	118	Positive	Negative	Negative	Negative
9	Positive	Negative	Negative	Negative	119	Positive	Negative	Negative	Negative
10	Positive	Negative	Negative	Negative	120	Positive	Negative	Negative	Negative
11	Positive	Negative	Negative	Negative	121	Positive	Negative	Negative	Negative
12	Positive	Negative	Negative	Negative	124	Positive	Negative	Negative	Negative
13	Positive	Negative	Negative	Negative	127	Positive	Negative	Negative	Negative
14	Positive	Negative	Negative	Negative	128	Positive	Negative	Negative	Negative
15	Positive	Negative	Negative	Negative	126	Positive	Negative	Negative	Negative
16	Positive	Negative	Negative	Negative	129	Positive	Negative	Negative	Negative
18	Positive	Negative	Negative	Negative	132	Positive	Negative	Negative	Negative
19	Positive	Negative	Negative	Negative	133	Positive	Negative	Negative	Negative
20	Positive	Negative	Negative	Negative	134	Positive	Negative	Negative	Negative
21	Positive	Negative	Negative	Negative	135	Positive	Negative	Negative	Negative
25	Positive	Negative	Negative	Negative	140	Positive	Negative	Negative	Negative
30	Positive	Negative	Negative	Negative	146	Positive	Negative	Negative	Negative
34	Positive	Negative	Negative	Negative	147	Positive	Negative	Negative	Negative
36	Positive	Negative	Negative	Negative	149	Negative	Negative	Negative	Negative
37	Positive	Negative	Negative	Negative	150	Positive	Negative	Negative	Negative
38	Negative	Negative	Negative	Negative	154	Positive	Negative	Negative	Negative
40	Positive	Negative	Negative	Negative	157	Positive	Negative	Negative	Negative
41	Positive	Negative	Negative	Negative	158	Positive	Negative	Negative	Negative
42	Positive	Negative	Negative	Negative	160	Positive	Negative	Negative	Negative
43	Positive	Negative	Negative	Negative	161	Positive	Negative	Negative	Negative
44	Positive	Negative	Negative	Negative	162	Positive	Negative	Negative	Negative

92	Positive	Negative	Negative	Negative	206	Positive	Negative	Negative	Negative
97	Negative	Negative	Negative	Negative	207	Positive	Negative	Negative	Negative
98	Positive	Negative	Negative	Negative	208	Positive	Negative	Negative	Negative
99	Negative	Negative	Negative	Negative	209	Positive	Negative	Negative	Negative
100	Positive	Negative	Negative	Negative	208	Positive	Negative	Negative	Negative
102	Positive	Negative	Negative	Negative	209	Positive	Negative	Negative	Negative
105	Positive	Negative	Negative	Negative					

Table A3.2 Summer viral detection results.

Table shows all of the samples received for viral analysis during the summer sampling period. Locations corresponding to each sample number are in section 2.2.

Sample	DWV	CBPV	ABPV	IAPV	Sample	DWV	CBPV	ABPV	IAPV
2	Positive	Negative	Negative	Negative	118	Positive	Negative	Negative	Negative
5	Negative	Negative	Negative	Negative	119	Positive	Negative	Negative	Negative
7	Positive	Negative	Negative	Negative	120	Positive	Negative	Negative	Negative
9	Positive	Negative	Negative	Negative	121	Positive	Negative	Negative	Negative
11	Positive	Negative	Negative	Negative	124	Positive	Negative	Negative	Negative
12	Positive	Negative	Negative	Negative	127	Positive	Negative	Negative	Negative
13	Positive	Negative	Negative	Negative	128	Positive	Negative	Negative	Negative
14	Positive	Negative	Negative	Negative	129	Positive	Negative	Negative	Negative
15	Positive	Negative	Negative	Negative	132	Positive	Negative	Negative	Negative
18	Positive	Negative	Negative	Negative	133	Positive	Negative	Negative	Negative
19	Positive	Negative	Negative	Negative	134	Positive	Negative	Negative	Negative
19	Positive	Negative	Negative	Negative	135	Positive	Negative	Negative	Negative
20	Positive	Negative	Negative	Negative	136	Positive	Negative	Negative	Negative
25	Positive	Negative	Negative	Negative	137	Positive	Negative	Negative	Negative
26	Positive	Negative	Negative	Negative	140	Positive	Negative	Negative	Negative
33	Positive	Negative	Negative	Negative	146	Positive	Negative	Negative	Negative
36	Positive	Negative	Negative	Positive	149	Positive	Negative	Negative	Negative
37	Positive	Negative	Negative	Negative	150	Positive	Negative	Negative	Negative
40	Positive	Negative	Negative	Negative	154	Positive	Negative	Negative	Negative
42	Positive	Negative	Negative	Negative	157	Positive	Negative	Negative	Negative
43	Positive	Negative	Negative	Negative	158	Positive	Negative	Negative	Negative
44	Positive	Negative	Negative	Negative	160	Positive	Negative	Negative	Negative
45	Positive	Negative	Negative	Negative	161	Positive	Negative	Negative	Negative
46	Positive	Negative	Negative	Positive	162	Positive	Negative	Negative	Negative
48	Positive	Negative	Negative	Negative	164	Positive	Negative	Negative	Negative
51	Positive	Negative	Negative	Negative	165	Positive	Negative	Negative	Negative
53	Positive	Negative	Negative	Negative	166	Positive	Negative	Negative	Negative
54	Positive	Negative	Negative	Negative	168	Positive	Negative	Negative	Negative

58	Positive	Negative	Negative	Negative	169	Positive	Negative	Negative	Negative
59	Positive	Negative	Negative	Negative	170	Positive	Negative	Negative	Negative
60	Positive	Negative	Negative	Negative	172	Positive	Negative	Negative	Negative
64	Positive	Negative	Negative	Negative	173	Positive	Negative	Negative	Negative
65	Positive	Negative	Negative	Negative	176	Positive	Negative	Negative	Negative
68	Positive	Negative	Negative	Negative	178	Positive	Negative	Negative	Negative
70	Positive	Negative	Negative	Negative	179	Positive	Negative	Negative	Negative
71	Positive	Negative	Negative	Negative	180	Positive	Negative	Negative	Negative
76	Positive	Negative	Negative	Negative	181	Positive	Negative	Negative	Negative
78	Positive	Negative	Negative	Negative	182	Positive	Negative	Negative	Negative
81	Positive	Negative	Negative	Negative	184	Positive	Negative	Negative	Negative
82	Positive	Negative	Negative	Negative	187	Positive	Negative	Negative	Negative
83	Positive	Negative	Negative	Negative	188	Positive	Negative	Negative	Negative
84	Positive	Negative	Negative	Negative	189	Positive	Negative	Negative	Negative
85	Positive	Negative	Negative	Negative	190	Negative	Negative	Negative	Negative
86	Positive	Negative	Negative	Negative	193	Positive	Negative	Negative	Negative
91	Positive	Negative	Negative	Negative	194	Positive	Negative	Negative	Negative
96	Positive	Negative	Negative	Negative	195	Positive	Negative	Negative	Negative
98	Positive	Negative	Negative	Negative	199	Positive	Negative	Negative	Negative
99	Positive	Negative	Negative	Negative	200	Positive	Negative	Negative	Negative
100	Positive	Negative	Negative	Negative	201	Negative	Negative	Negative	Negative
102	Positive	Negative	Negative	Negative	202	Positive	Negative	Negative	Negative
104	Negative	Negative	Negative	Negative	204	Positive	Negative	Negative	Negative
105	Positive	Negative	Negative	Negative	205	Positive	Negative	Negative	Negative
109	Positive	Negative	Negative	Negative	206	Positive	Negative	Negative	Negative
113	Positive	Negative	Negative	Negative	207	Positive	Negative	Negative	Negative
114	Positive	Negative	Negative	Positive	208	Positive	Negative	Negative	Negative

115	Positive	Negative	Negative	Negative	209	Positive	Negative	Negative	Negative
116	Positive	Negative	Negative	Negative					

Table A3.3 Autumn viral detection results.

Table shows all of the samples received for viral analysis during the autumn sampling period. Locations corresponding to each sample number are in section 2.2.



Figure A3.1 ABPV PCR images Spring 2014

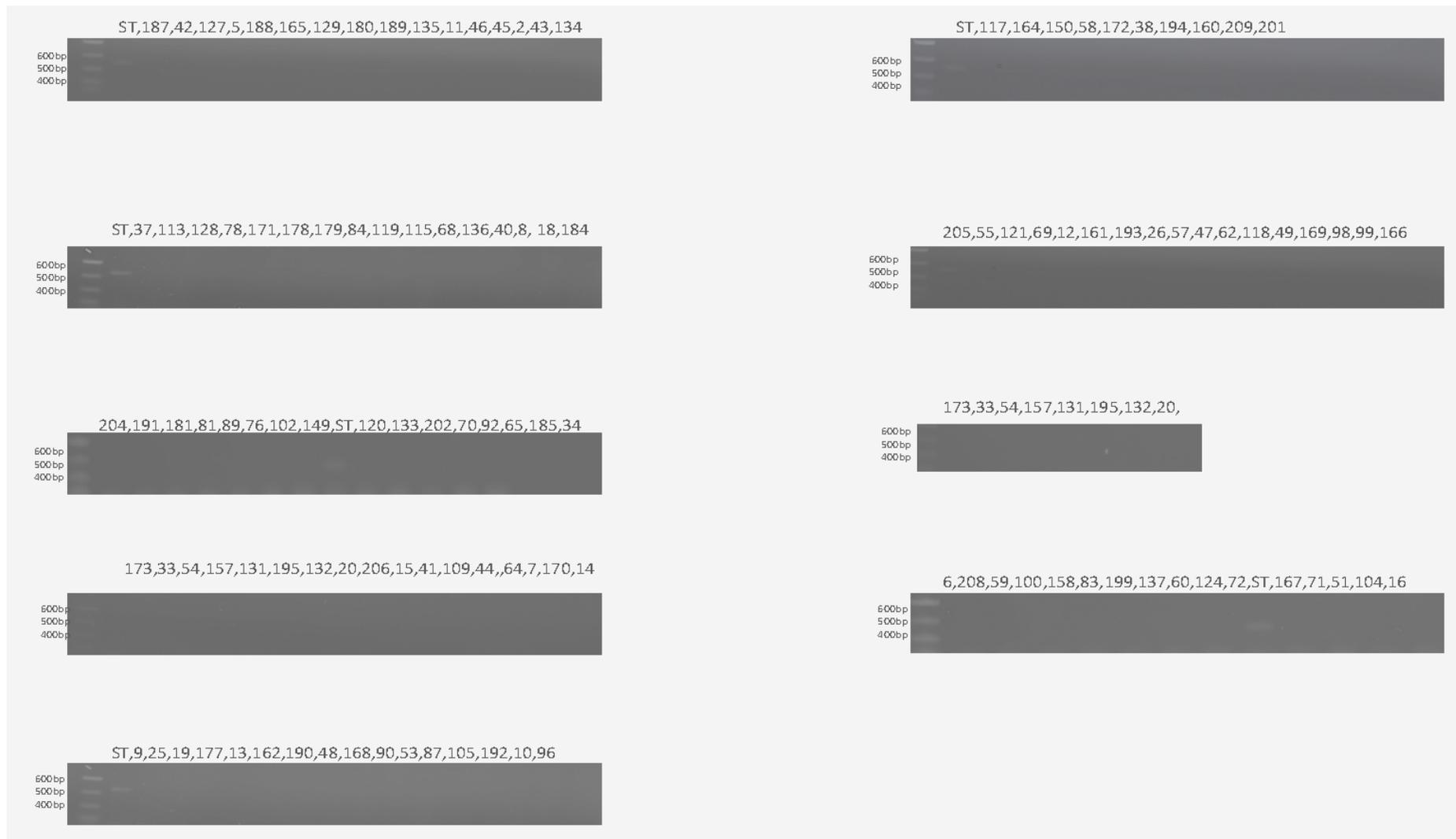


Figure A3.2 CBPV PCR images Spring 2014

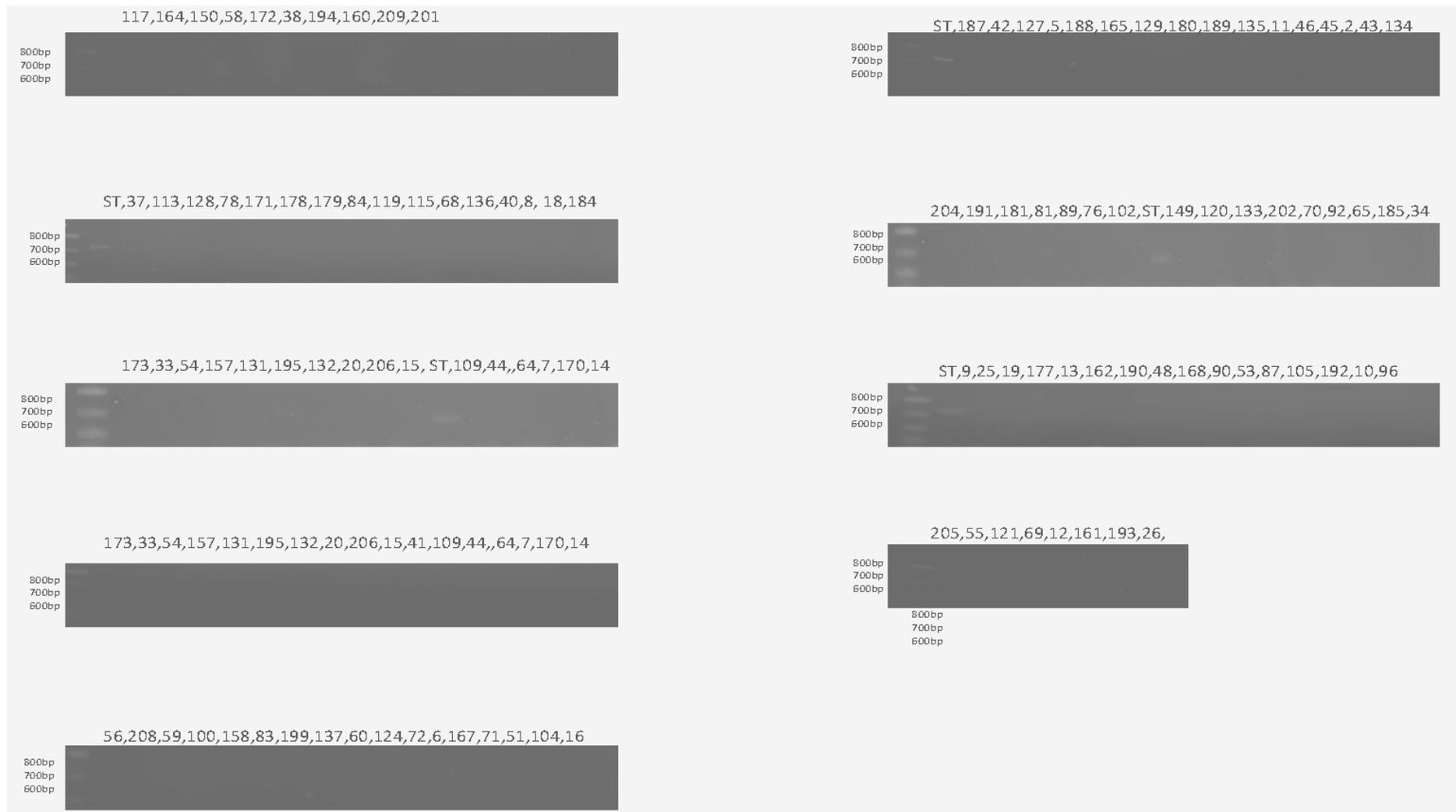


Figure A3.3 IAPV PCR images Spring 2014

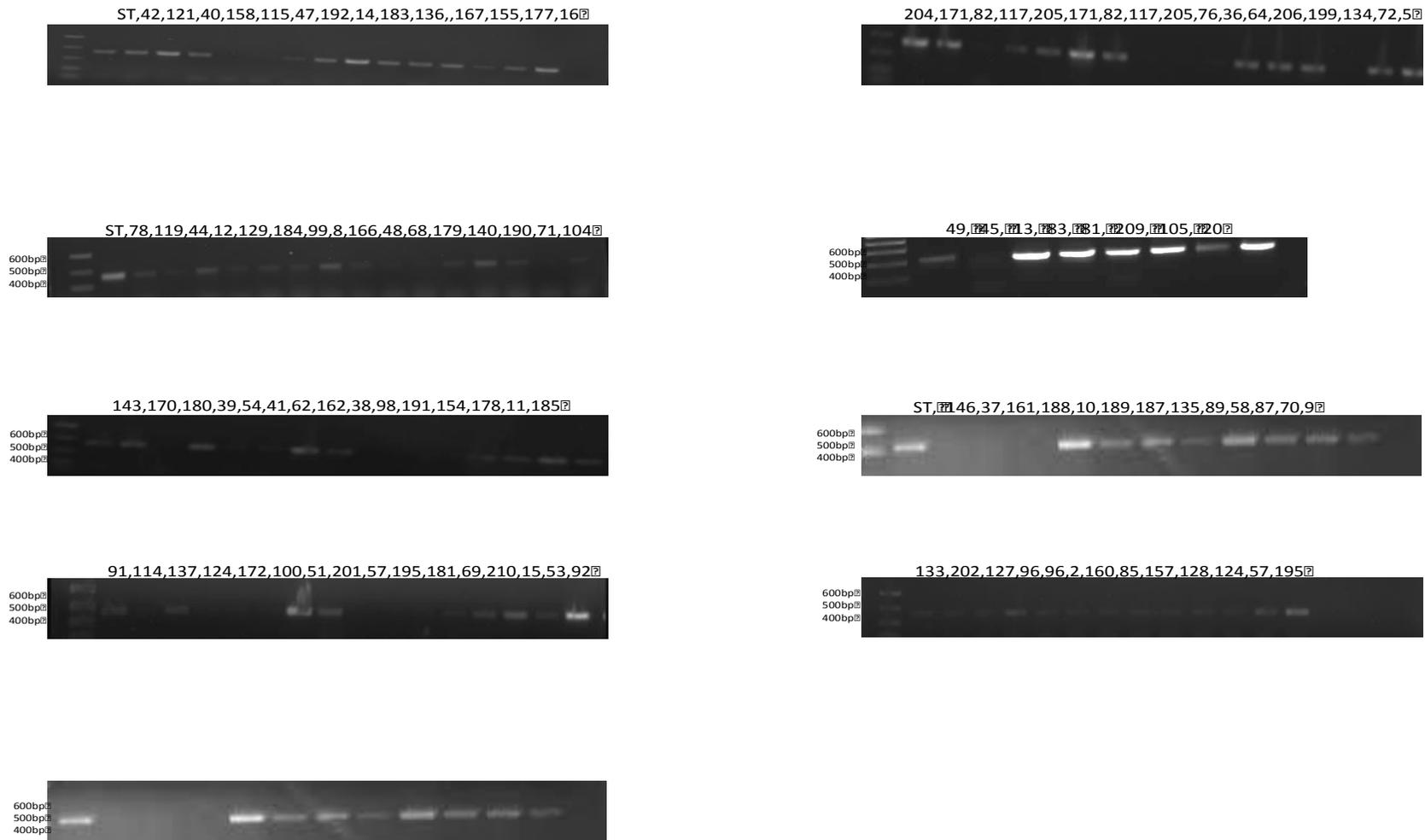


Figure A3.4 DWV PCR images Spring 2014

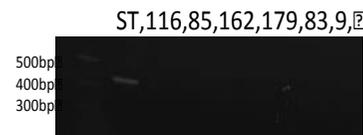
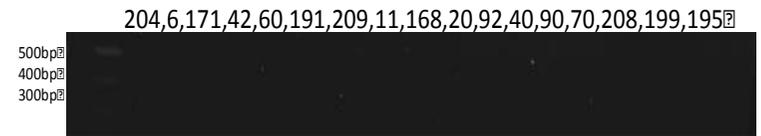
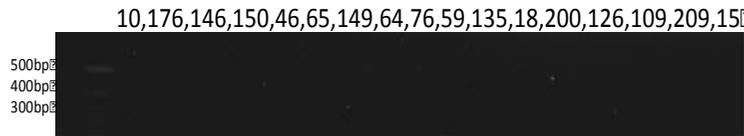
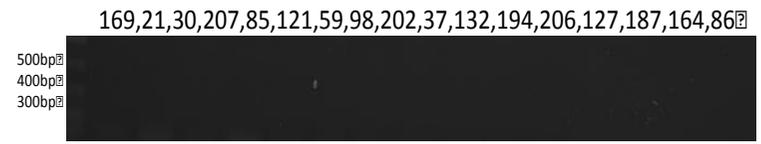
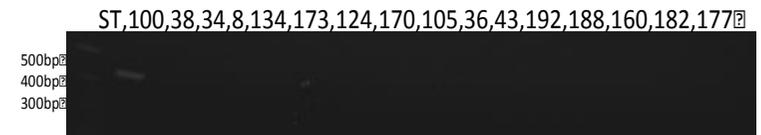
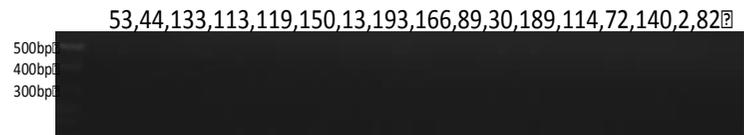


Figure A3.5 ABPV PCR images Summer 2014

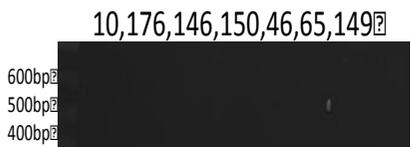
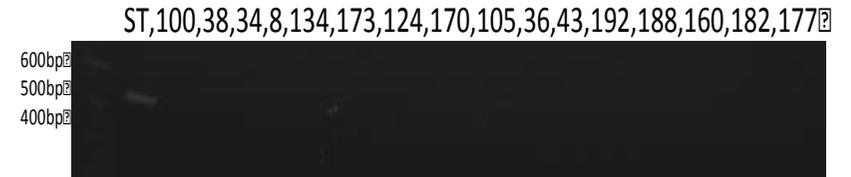
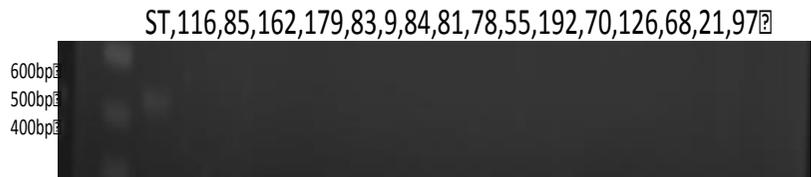
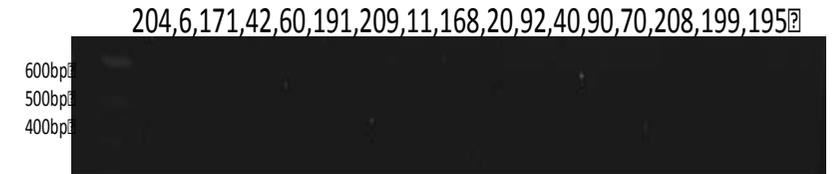
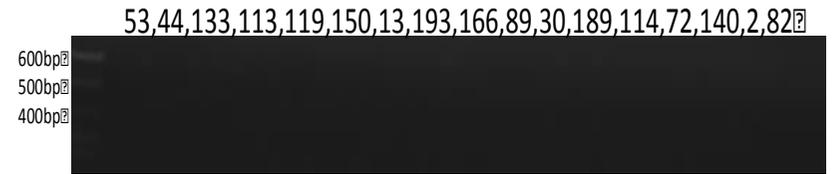
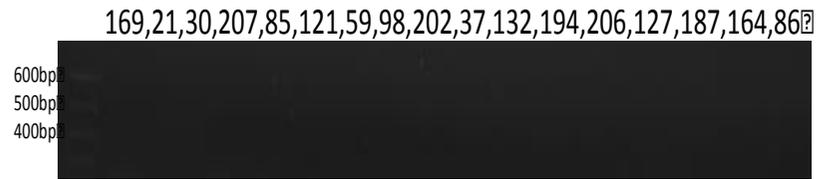


Figure A3.6 CBPV PCR images Summer 2014

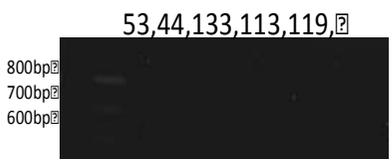
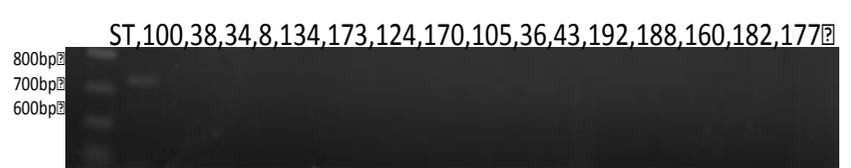
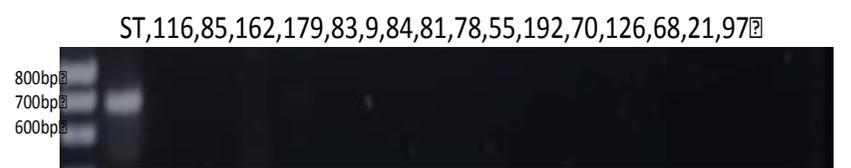
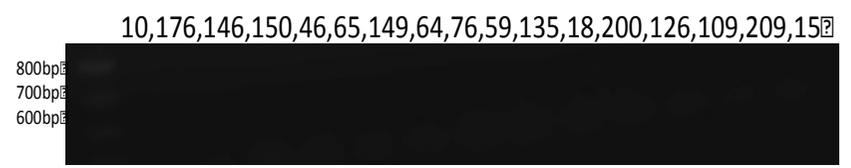
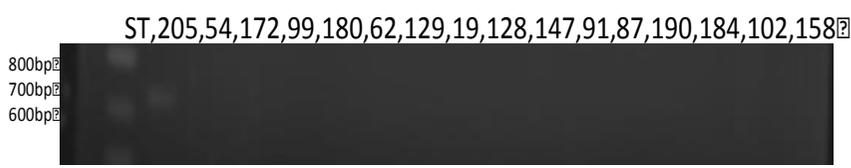


Figure A3.7 IAPV PCR images Summer 2014

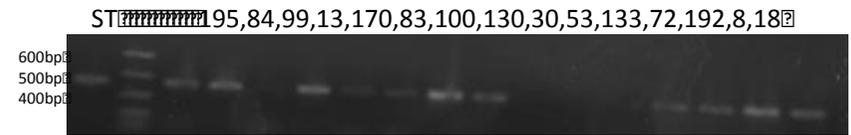
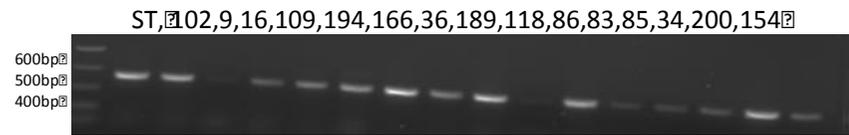
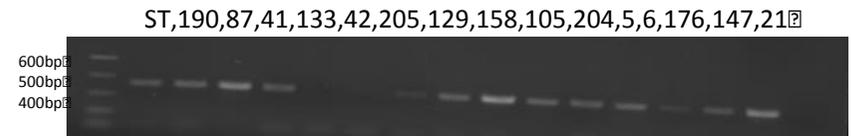
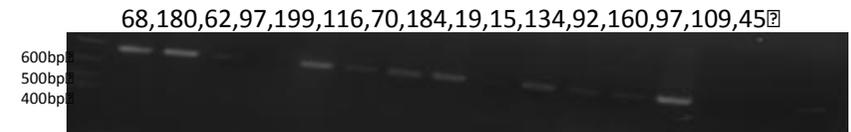
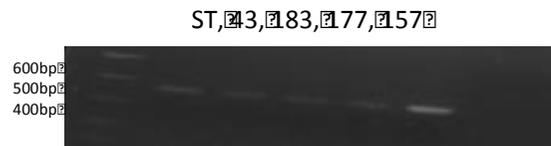
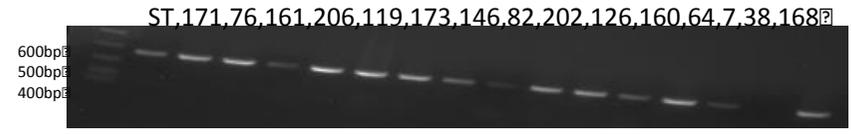
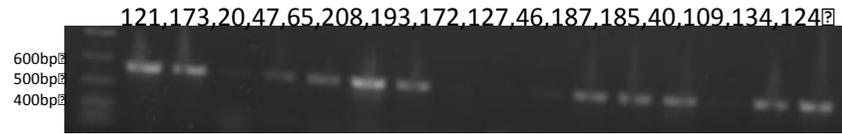


Figure A3.8 DWV PCR images Summer 2014

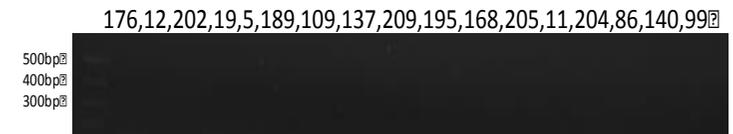
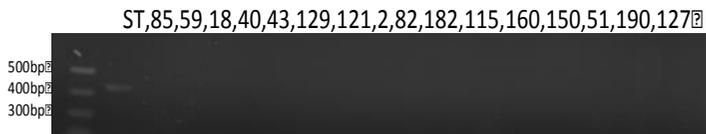
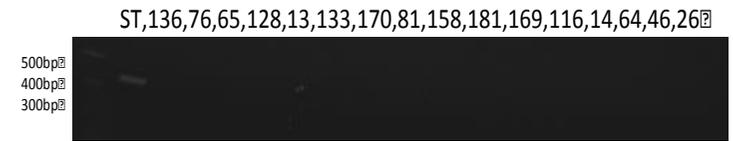
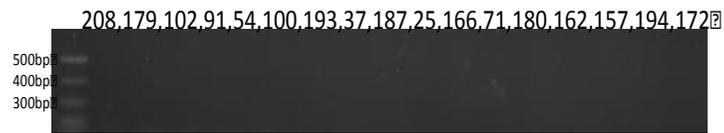
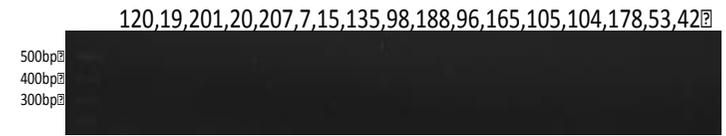
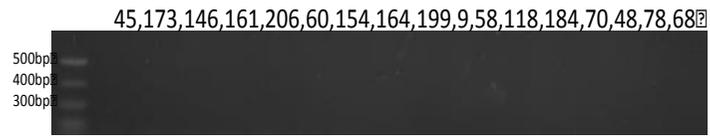


Figure A3.9 ABPV PCR images Autumn 2014

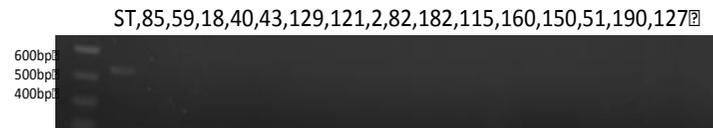
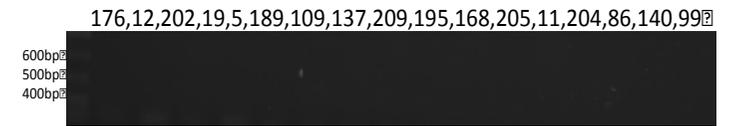
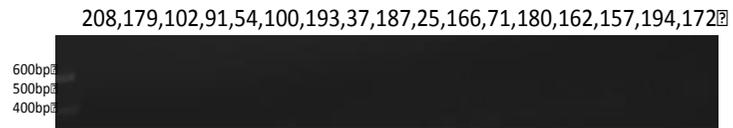
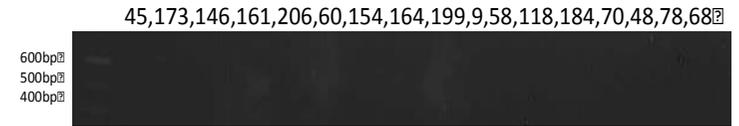
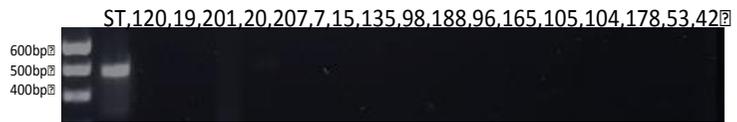


Figure A3.10 CBPV PCR images Autumn 2014

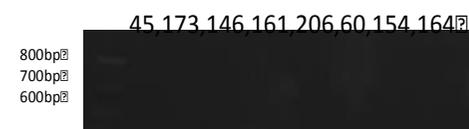
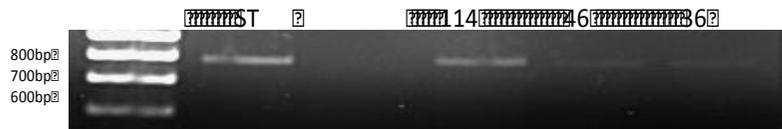
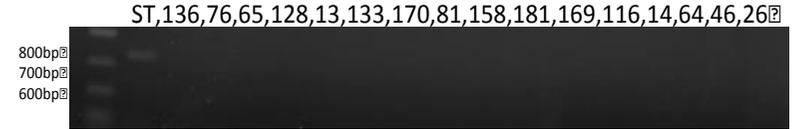
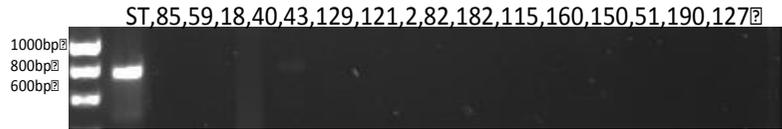
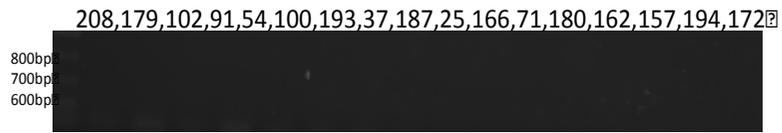


Figure A3.11 IAPV PCR images Autumn 2014

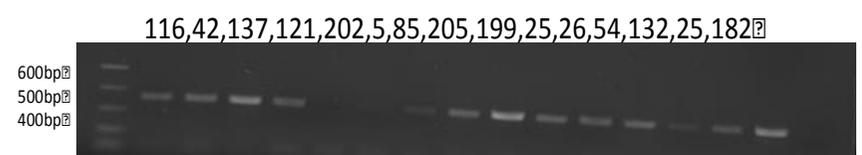
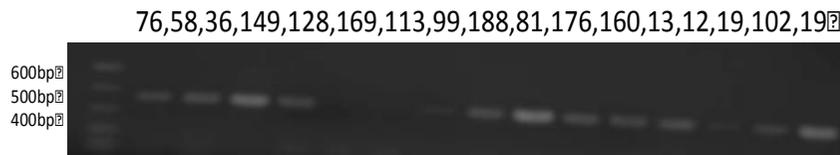
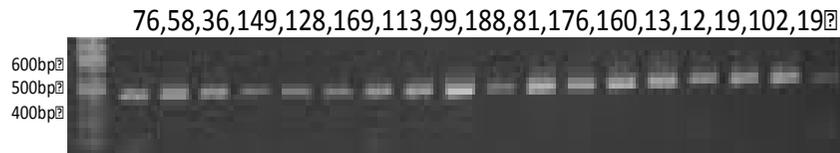
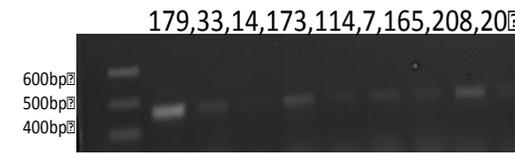
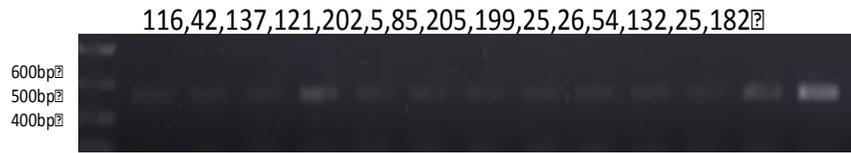


Figure A3.12 DWV PCR images Autumn 2014