Using Proteomic Analysis to investigate the Effects of Aging, Seasonality, Treatments and Disease Presence Within *Apis mellifera* Colonies

A thesis submitted to the Maynooth University, for the degree of Doctor of Philosophy by

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Dissemination of research

Publications

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Presentations

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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.

Signed _____

Rachel Kathryn Ward B.Sc

Date _____

Abbreviations

ABA	Abscisic acid
ACNS	Allatoregulatory Central Nervous System
AMPs	Antimicrobial Peptides
CCD	Colony Collapse Disorder
CCE	Carboxylesterases
DH^2O	Distilled Water
DLG	Discs Large
DTT	Dithiothreitol
DWV	Deformed Wing Virus
ER	Endoplasmic reticulum
FDR	False discovery rate
GO	Gene ontology
GOBP	Gene Ontology Biological Process
GOCC	Gene Ontology Cellular Component
GOMF	Gene Ontology Molecular Function
GST	Glutathione-S-transferases
HEX	Hexamerins
HB	Hygienic Behaviour
HpG	Hypopharyngeal glands
HSP	Heat Shock Proteins
HVA	Hymenoptera venom allergy
IAA	Iodoacetamide
JH	juvenile hormone
KEGG	Kyto Encyclopedia of Genes and Genomes
LFQ	Label Free Quantification
MAGUKs	Membrane-associated guanylate kinases
MAQs	Mite Away Quick Strips
ml	Millilitre
MRJP	Major Royal Jelly Proteins
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OBP	Odorant Binding Proteins

ODA	9-oxo-(E)-2-decenoic acid
OXPHOS	Oxidative Phosphorylation
PCA	Principal Component Analysis
Pfam	Protein family
PO	Phenoloxidase
proPO	Pre-prophenoloxidase
ROS	Reactive Oxygen Species
SCDR	Short Chain Dehydrogenase Reductase
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SSDA	Statistically Significant Differentially Abundant Proteins
STRING	Search tool for the retrieval of interacting genes
TCA	Tricarboxylic acid cycle
VSH	*Varroa* Sensitive hygienic

Abstract

Apis mellifera colonies are a complex, highly organised society that are essential pollinators across the world for both wild flora and cultivated crops. *A. mellifera* colonies face a myriad of challenges that can be detrimental to their survival – the spread of diseases and pests, poor bee husbandry, loss of essential flora, exposure to commercial insecticides and migratory beekeeping. These daily challenges and exposures have caused a worrying depletion of *A. mellifera* colonies which is resulting in an increasing concern in the environmental and scientific community. Research is now focused on the relationship honeybee colonies have with the changing environment and the presence of diseases. Female worker honeybees make up the majority of bees within *A. mellifera* colonies and are essential for hive survival. Worker bees undergo changes to their anatomy and gland secretions as they age to optimise their productivity. Furthermore, worker bees can be divided based on seasons – summer and winter worker bees, both age and seasonality can affect the proteome and therefore the immune response of worker bees. Label-free quantitative (LFQ) proteomics was performed on worker bees at various stages of their lifecycle, between winter and summer, in response to exposure to anti-*Varroa* treatments and finally in response to the presence of *Varroa destructor* and *Nosema* spores within colonies. The work presented in this thesis has identified key age, seasonal, and virus-infected differences within hives at a proteomic level. These results show both age-dependent evolution and environmental adaptions experienced by a hive highlighting their complex relationship and vulnerability to external threats. Importantly, this work has highlighted the impact of anti-Varroa treatments and the potential side effects towards colonies. Finally, work conducted on the effects of *Varroa* mites and *Nosema* spores on bees highlighted the need to better understand how these pathogens interact when both are infecting colonies simultaneously. The results presented here provide a novel insight into the proteomic changes that occur in worker bees when exposed to different external factors. However, it also highlights the need for further research into how honeybee workers adapt to stresses such as insecticide exposure and dual infections by *Varroa* and *Nosema.*

Chapter 1

Introduction

1.1 General Information

1.1.1 *Apis mellifera* General Introduction

Honeybees are classified in the genus *Apis* which contains five main species and are part of the insect order Hymenoptera, which also includes wasps, ants and sawflies (Weinstock *et al.*, 2006). Hymenoptera insects are defined as having two sets of wings, a pronounced waist between the 2nd and 3rd abdominal segments and display haplodiploid sex determination, honeybee's eggs are parthenogenetic- eggs will develop regardless of fertilization (Weinstock *et al.*, 2006; Lawrence, 2016). The queen can lay both fertilized and non-fertilized eggs, the specificities of which depend on the cell shape. If the cell is a large hexagon the queen will lay non-fertilized eggs will develop into drones and if the cell is a small hexagon (**Figure 1.2**) or shaped like the top of an acorn, the queen will lay fertilized eggs which can emerge into workers or a queen, with nutrition being the determining factor (Hooper, 2010). Honeybees are eusocial insects, a term which is defined by three characteristics: cooperative brood care, the overlap of two or more generations of offspring and the division of labour based on fertility (Zablotny, 2009).

The Western honeybee, *Apis mellifera* are medium sized bees that when wild will build their combs in cavities in trees or in the ground but have adapted to live within a man-made hive and can be considered semi-domesticated (Coffey, 2007). *A. mellifera* can develop into short lived summer bees or long lived *diutinus* bees during the winter (Amdam and Omholt, 2002; Aurori *et al.*, 2014). Summer bees live for approximately six weeks with worker castes lifespan ranging between 18 to 28 days and foragers for 7-14 days, whereas *diutinus* bees can survive for six to eight months (Omholt and Amdam, 2004). Ireland has a native subspecies of *A. mellifera* called *Apis mellifera mellifera*, commonly known as the black honeybee (Coffey, 2007).

Bees are essential pollinators in both natural and commercial ecosystems, the need for pollinators is becoming more demanding each year. Honeybees, bumblebees and flies have been attributed to pollinate the top 48 crops in the world, contributing significantly to the world economy (Khalifa *et al.*, 2021). Honeybee pollination services have been suggested to account for 34% of pollination demands in the United Kingdom, however, this level of pollination has fallen from 70% since 1984 (Breeze

et al., 2011). Studies have shown how important honeybee pollination services are to both the crop yields and crop quality, with one study finding an increase in fruit weight by 20% when pollinated by high quality colonies (Geslin *et al.*, 2017). One essential factor to consider with honeybee pollination services that has to be considered when calculating their economic value is how the presence of these colonies effects wild pollinators such as hover flies and bumblebees and also how the intense pollination practices of honeybees may affect the health of the hive itself (Colwell *et al.*, 2017; Simone-Finstrom *et al.*, 2022). The practice of migratory beekeeping, although profitable for farmers can have a negative effect on the hive and can contribute to the spread of pathogens between colonies, the high number of colonies usually located in farms for this practice also disrupts the natural balance of pollinators (Colwell *et al.*, 2017; St Clair *et al.*, 2020; Simone-Finstrom *et al.*, 2022).

1.1.2 Structure of the Hive

Colonies of *A. mellifera* are composed of three castes; drones, workers and the queen. The number of bees belonging to the drone and worker cast change drastically between the seasons, this greatly effects the activity and productive value of the hive. During the active mating season (May to August) the hive can support between 200-1000 drones but come late Autumn the drones will be removed from the hives by the workers (Evans and Wheeler, 1999). Worker numbers can reach 50,000 in the summer but then drop to 10,000 during the winter (Hooper, 2010).

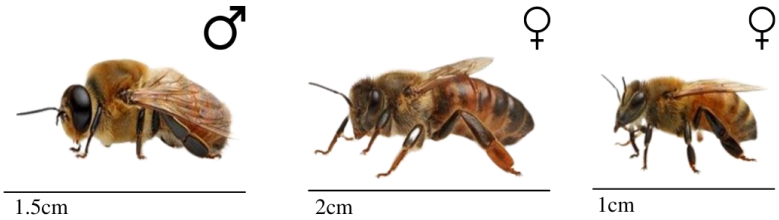


Figure 1.1: Honeybee castes: Drones, Queen, and Worker respectively.

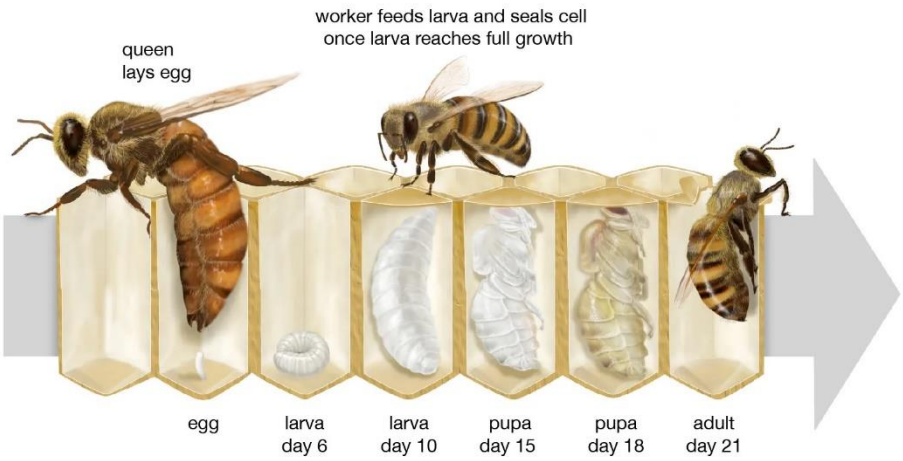


Figure 1.2: Development of worker bees within the colony. Image adapted from Encyclopaedia Britannica Inc. 2013.

1.1.3 The Queen

A queen will emerge from her queen cup after approximately 16 days once the egg has been laid (Coffey, 2007). While in her larval stage she is suspended in a large quantity of royal jelly, a food source secreted from the hypopharyngeal glands of nurse bees (Hooper, 2010; Buttstedt *et al.*, 2014; Eduardo Serrão *et al.*, 2021). On the 5th day the queen cup is capped, the larva will then proceed through a complete metamorphosis, developing into a new virgin queen 11 days after being capped (Coffey, 2007). Anatomically the queen is the longest bee out of the three castes, her wings span half-way down her pointed abdomen as seen in **Figure 1.1** (Hooper, 2010). A queen's lifespan is approximately 1-2 years, which is currently attributed to the amount and nutritional value of the food she will receive (Remolina and Hughes, 2008; Aurori *et al.*, 2014).

Once the virgin queen has emerged, she will spend the first period of her life killing potential rivals. She will then complete several orientation flights to establish the hives surroundings before starting her mating flights (Coffey, 2007). While on her mating flights the queen will release a pheromone from her mandibular glands called 9-oxo-(*E*)-2-decenoic acid (ODA) leaving a trail on her flight pattern for drones to locate her (Slessor *et al.*, 2005; Hooper, 2010). The queen will mate with up to 15 drones and will store viable sperm within her spermatheca for her lifetime (Coffey,

2007; Hooper, 2010). The spermatheca is an enlarged sac, capable of storing and nurturing sperm via the spermathecal glands, which join to the vagina via a small tube (Hooper, 2010:25).

When the queen is approximately three weeks old, she is no longer able to mate so will return to the hive and begin laying. Inside the hive, the queen secretes a pheromone that alters workers behaviour, causing them to face and lick her forming a retinue around her (Coffey, 2007; Hooper, 2010). Workers feed via trophallaxis which aids in communicating the queen's pheromones such as ODA throughout the colony as it is passed amongst the workers (Coffey, 2007). ODA inhibits the activation of ovaries and follicle development in workers thereby preventing workers laying haploid eggs (Hartfelder, 2000).

1.1.4 Drones

Drones are haploid, incapable of stinging, and exist solely for mating (Coffey, 2007:8). Drones will emerge 24 days after laying and will be ready to mate by day 37 (Hooper, 2010:31). Physically drones are the same weight as the queen but have a square end to their abdomen. Drones have a large spherical head and their wings completely cover the abdomen. Two large compound eyes broadly meet at the top of the head, leaving drones having a small 'face', the large eyes allows them to spot virgin queens while flying as seen in **Figure 1.1** (Hooper, 2010).

Drones will leave the hive and fly at around thirty to ninety feet above the ground, to the 'mating zone' (Hooper, 2010). Virgin queens will fly to this zone and mate with several drones. Upon mating the drone's genitalia explode resulting in the drone dying almost immediately after mating. The drones' sperm contains the same genes as those of their queen (due to haploid genetic make-up), this ensures genetic diversity in apiaries, as all the drones genes will be used by a virgin queen who will establish herself in a new hive (Coffey, 2007; Hooper, 2010). It is believed that haplodiploid-induced variations seen within *A. mellifera* are involved in the evolution and success of the eusocial structure of the hives (Weinstock *et al.*, 2006).

1.1.5 Workers

Workers make up the majority of the hive with their numbers reaching 50,000 during the summer months (Hooper, 2010). Physically workers are the smallest caste,

approximately half the weight and size of a queen or drone approximately 1cm in length (**Figure 1.1**). Honeybee workers will emerge from their cells 21 days after the egg has been laid (**Figure 1.2**). Worker larvae receive a large quantity of *bee milk* for the first three days, but then experience a reduction in the quantity and the quality of food. This results in the emergence of infertile workers as food depletion causes the ovaries to shrink, thereby stunting development (Coffey, 2007; Hooper, 2010). Studies have shown the quality of the bee milk given to worker and queen eggs differs, with worker bee milk containing around half the amount of sugar compared to the bee milk given to queen larvae. This variation in sugar levels causes hormonal differences between the larvae further contributing to larval caste determination (Hooper, 2010).

Workers typically live between 4 to 6 weeks in summer, but can live up to 22-24 weeks in winter. (Aurori *et al.*, 2014). The long lived worker bees in winter have been termed diutinus bees and they show a distinct difference in their age-related patterns to summer bees. Summer worker bees display slow senescence in early life stages (nursing and builders) which is followed by rapid senescence in later life (guard bees and foragers). Winter workers display almost negligible senescence in comparison (Aurori *et al.*, 2014).

The roles of workers alter as they age in a process called age-polyethism or temporal polyethism **(Section 1.4)**. The tasks of the workers are influenced by age, and both internal and external signals in their surrounding environment (Johnson, 2010; Wright *et al.*, 2017; Rodriguez Messan *et al.*, 2018). Workers possess adaptions for the various roles within the hive such as: their mandibles are spoon shaped without teeth, allowing them to mould wax, their third pair of legs can carry pollen loads in pollen baskets and a long proboscis allows them to access nectar in flowers (Hooper, 2010).

1.1.6 Diutinus Worker

Winter workers are called *Diutinus* bees, characterised by longevity and their tolerance to oxidative stresses, of which their name is derived from Latin. (Omholt and Amdam, 2004; Amdam and Page, 2005; Aurori *et al.*, 2014). It is thought that diutinus workers emerge when workers late in the year remain in the hive instead of

transitioning into foragers. Diutinus workers experience negligible senescence and can survive for up to 280 days, however, if prevented from entering the nurse and forger roles (in the spring) diutinus workers could possibly survive for 2 years (Omholt and Amdam, 2004; Amdam and Page, 2005).

One of the most defining characteristics of workers is the division of labour between summer and winter bees. In the spring and summer, the workers strive to maximize hive growth and accumulate stores of honey, pollen and propolis. Winter colonies are broodless and the role of workers shifts to thermoregulation, feeding and to survival until the following Spring so they can rear the young and are the first foragers (Omholt and Amdam, 2004; Amdam and Page, 2005; Rodriguez Messan *et al.*, 2018). Western honeybee species are adapted to survive the unfavourable winter conditions by maintaining a warm temperature within the hive through thermoregulation. The 'superorganism model' hypothesises of thermoregulation suggests workers cluster tightly together, regulating their metabolism within the inner core and workers within the core and on the outskirts of the core shiver their thoracic muscles to control temperature levels between 20-35^{o}C which can be observed in **Figure 1.3** (Stabentheiner *et al.*, 2003; Stabentheiner *et al.*, 2010).

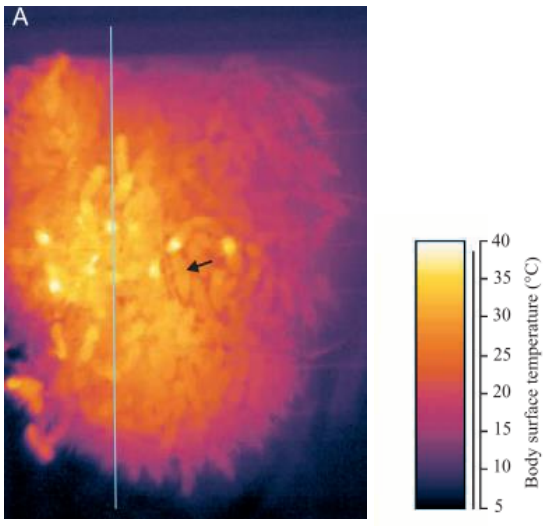


Figure 1.3: Infrared thermogram of workers within the central comb of a broodless winter cluster. Bees with warm thorax (yellow and white spots) are endothermic workers with shivering thermogenesis. The black arrow highlights the position of the queen. Image taken from Fig. 1. Endothermic heat production in honeybee winter clusters (Stabentheiner *et al.*, 2003).

Several aspects of the winter bees physiology have been investigated, with a recent study completing an in-depth analysis on the worker bees physiology and

immunity throughout the space of a year (Kunc *et al.*, 2019). Results from which have highlighted the increased content of proteins and lipids within winter workers in comparison to summer worker bees, one specific example was the increased amount of vitellogenin in winter workers in comparison to summer worker hemolymph titres (Kunc *et al.*, 2019). Other studies have investigated proteomic differences in winter worker bees in comparison to summer bees, these studies utilised 2D gel electrophoresis analysis which has provided initial insights into the workers proteomic changes (Erban *et al.*, 2013). Work in this thesis will use label free mass spectrometry techniques to complete an in-depth analysis on the proteomic differences between winter and summer honeybee workers.

1.2 Anatomy of *Apis mellifera*

1.2.1 Overview

The anatomy of adult *Apis mellifera* is categorized into the head, prothorax (separated from the mesothorax via a membranous area), a fused meso/meta-thorax and an abdomen. The upper segments of the abdomen have a slender peduncle fused to the metathorax giving the impression that it is part of the thorax (**Figure 1.4 & Figure 1.5**). This is a distinct characteristic of insects belonging to the Hymenoptera order (Snodgrass, 1910). Internal anatomy of the honeybee worker, outlining the position of the crop, rectum and thoracic musculature system is shown in **Figure 1.6**.

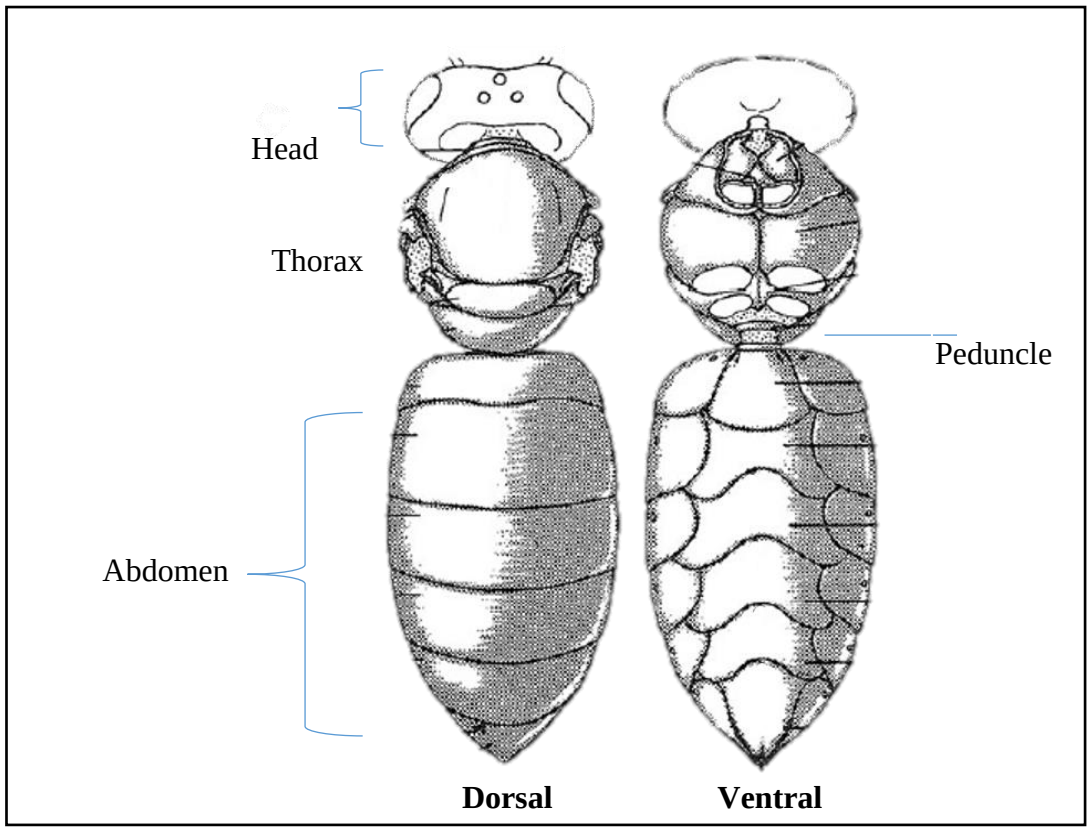


Figure 1.4: Dorsal and Ventral diagrams of a *Apis mellifera* worker. Adapted from Coloss Beebook 1. Standard methods for *Apis mellifera* anatomy and dissection.

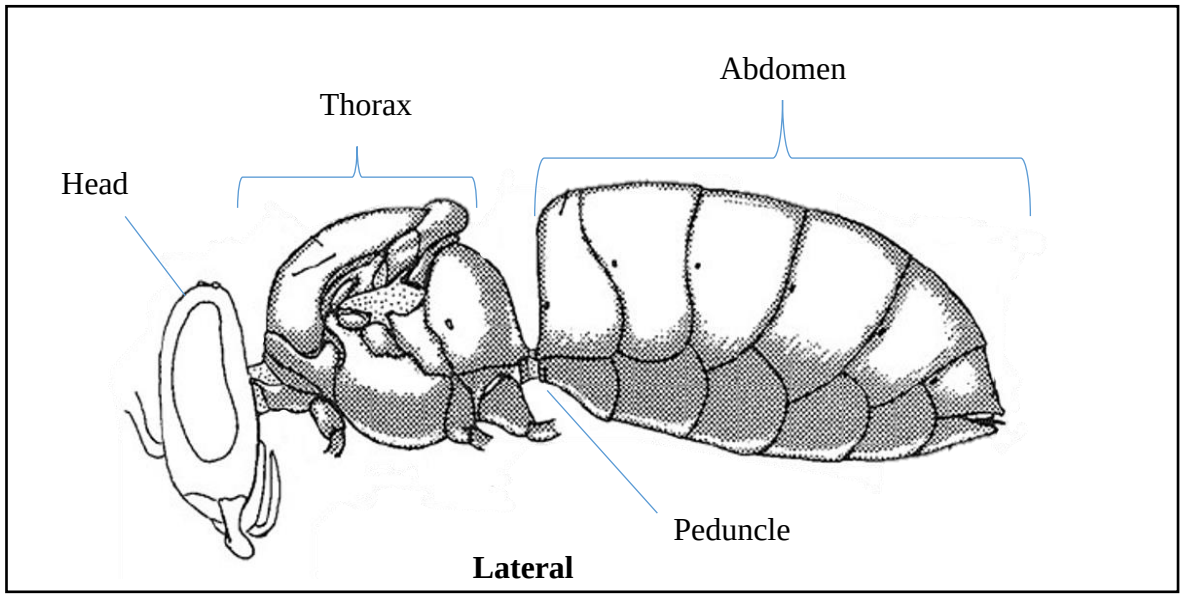


Figure 1.5: Lateral diagram of *Apis mellifera* worker. Adapted from Coloss Beebook 1. Standard methods for *Apis mellifera* anatomy and dissection.

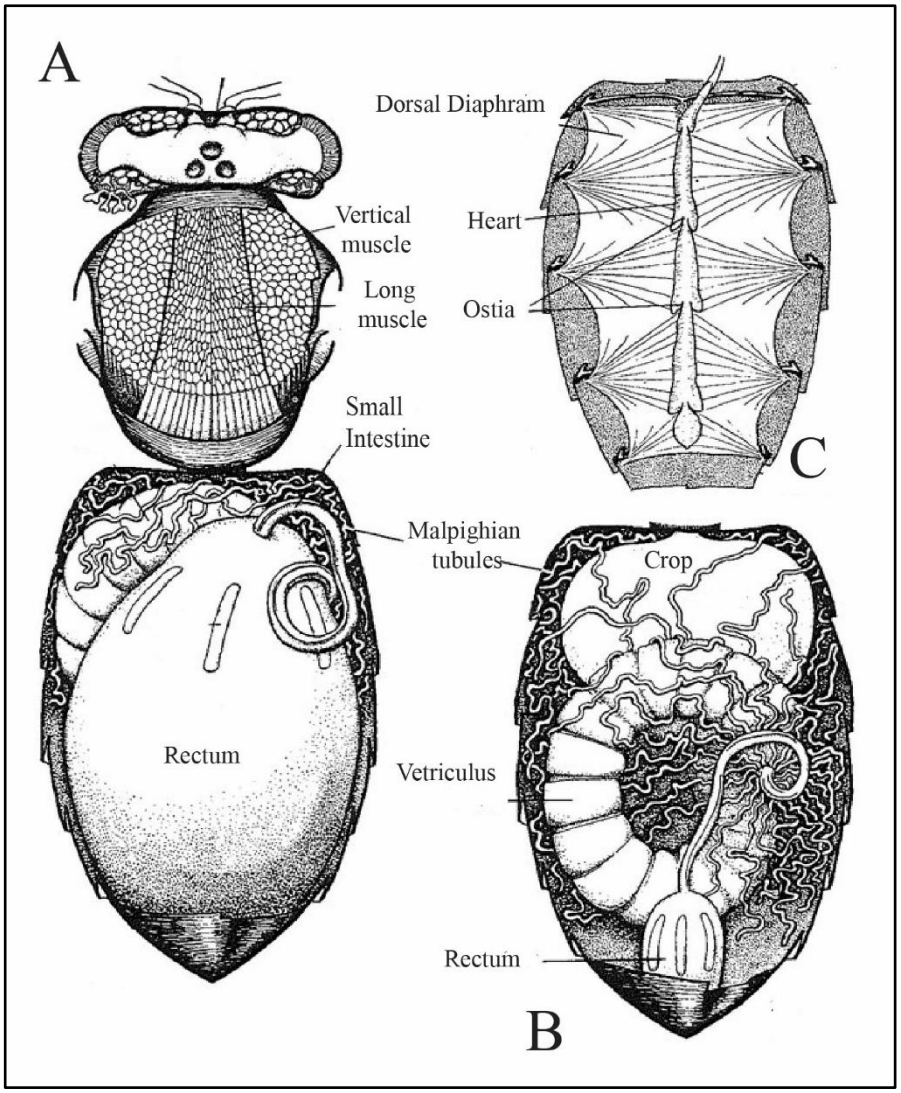


Figure 1.6: Internal diagram of *Apis mellifera* worker. Adapted from Coloss Beebook 1. Standard methods for *Apis mellifera* anatomy and dissection.

1.2.2 Hemolymph

Hemolymph in insects is the equivalent of blood in vertebrates. It is responsible for carrying nutrients to tissues and organs within the insects body, it's composition provides an insight into the physiology and immune system of the insects (Erban *et al.*, 2013). Hemolymph is composed of water, inorganic salts, carbohydrates, proteins, enzymes, hormones, lipids ad hemocytes (macrophage-like cells) (Bogaerts *et al.*, 2009; Erban *et al.*, 2013). The hemolymph is important for the recognition and defence against pathogens and is the major place of resistance during an infection (Bogaerts *et al.*, 2009). Hemocytes are found within the hemolymph and are part of cellular immune

response in insects (**Section 1.3.2**). Hemocytes can identify foreign bodies by direct interaction of surface receptors or indirectly using humoral receptors that bind to the surface of the invader (Lavine and Strand, 2002). Levels of hemocytes within the hemolymph decrease as the workers age (Negri *et al.*, 2016).

The most prevalent protein within the worker haemolymph is vitellogenin (30 to 50% of total protein) (see **Section 1.2.4** for synthesis details) (Amdam and Omholt, 2002; Amdam *et al.*, 2003; Omholt and Amdam, 2004). Hemolymph composition in worker honeybees varies during age polyethism for example: vitellogenin levels in the hemolymph are high in younger bees, these levels drop as the workers age and increased titres of juvenile hormone subsequently increase in the hemolymph (Bogaerts *et al.*, 2009; Erban *et al.*, 2013; Cervoni *et al.*, 2017). The changes in protein levels and immune cell presence in the hemolymph correspond with the changing roles the bees undertake within the colony and demonstrates how worker bees optimise their physiology and protein secretions to align with the roles they complete.

1.2.3 Hypopharyngeal Glands

Hypopharyngeal glands (HpG) are acinus glands composed of small spherical alveolar clusters of glandular cells. Each acinus contains 8-12 glandular cells which is connected to a central duct via duct cells which are specialised accessory cells (**Figure 1.7)** (Snodgrass, 1910; Naiem *et al.*, 1999; Amdam and Omholt, 2002; Deseyn and Billen, 2005; Feng *et al.*, 2009a; Hooper, 2010; Eduardo Serrão *et al.*, 2021). HpGs are packed around the brain and are connected to the inside of the mouth via a narrow tube (Hooper, 2010:18). HpGs are fully developed in workers 13 days post emergence during the early hive stage the glands synthesise Major Royal Jelly (MRJ) proteins used for bee milk or royal jelly fed to worker or queen larvae respectively (Feng *et al.*, 2009a; Wu *et al.*, 2017b; Ramanathan *et al.*, 2018). Vitellogenin is an egg yolk protein, workers begin the synthesis of vitellogenin 2-3 days after emerging as an adult (Rodriguez Messan *et al.*, 2018). Vitellogenin is secreted by the fat body (Section 1.2.4) and is used in the HpGs in the production of bee milk and royal jelly (Amdam and Omholt, 2003). Approximately 21 days post emergence the HPGs begin to decrease in size with no trace of the glands after 21 days (Feng *et al.*, 2009a). This is an anatomical example of the changes that occur within the honeybee workers as they age and adapt to the various roles within the colony.

Western blot assays completed by Feng *et al.*, (2009) have shown that on the day of emergence MRJ proteins 1, 2 and 3 could be detected and that by day 3 post emergence the expressions of these proteins are significantly higher than those levels seen in day 1. This indicates that the newly emerged bees are capable of secreting small amounts of MRJ proteins. It has also been observed that in the early stages of foraging duties the HPGs can still be well developed (Hrassnigg and Crailsheim, 1998). As workers age the HPGs begin to regress and shift in protein secretions. Initially workers secrete large amounts of MRJ proteins, however, the major 70-kDA protein α-glucosidase is specifically expressed in forager glands and provides one example on how age affects the biology of the bees (Feng *et al.*, 2009a).

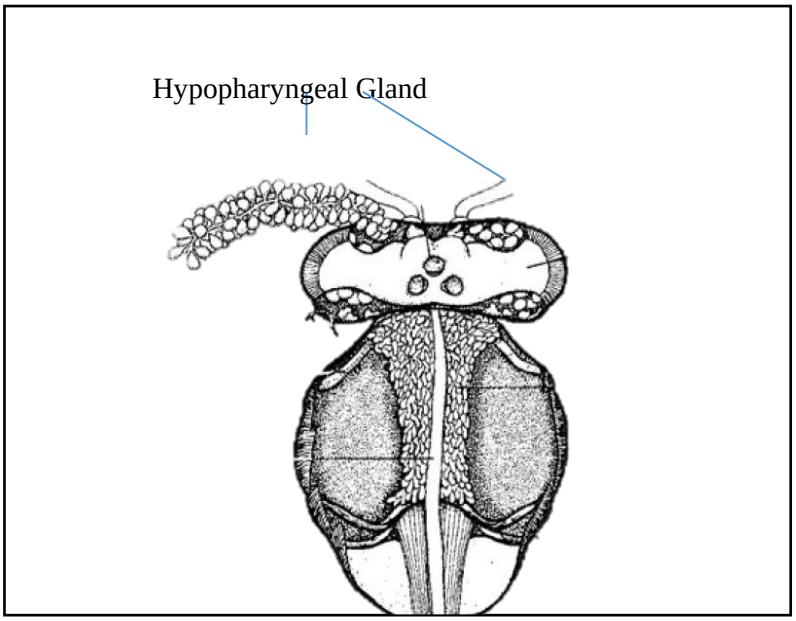


Figure 1.7: Location of hypopharyngeal glands in *Apis mellifera.*
Adapted from Coloss Beebook 1. Standard methods for *Apis mellifera* anatomy and dissection.

1.2.4 Fat Body

The fat body is composed of thin layers of cells loosely organised into thin lobes of highly tracheated tissues located against the wall of the abdomen **(Figure 1.8)** (Snodgrass, 1910; Amdam and Omholt, 2002; Omholt and Amdam, 2004). It plays an essential role in homeostasis, nutrient storage and protein synthesis (Amdam *et al.*, 2009; Arrese and Soulages, 2010). The fat body produces the maximum amount of proteins in workers after approximately 12 days post emergence, correlating with the age-caste of early hive bees (Amdam and Omholt, 2002). Protein production decreases

in the fat body during the winter and spring levels can be lower than those typically seen in foragers (Fluri *et al.*, 1977; Amdam and Omholt, 2002). The proteins synthesised in the fat body are either secreted into the haemolymph or stored within the fat body cells (Amdam and Omholt, 2002).

Vitellogenin is a female specific glycolipoprotein yolk precursor synthesised in the fat body and secreted into the hemolymph as a storage protein in worker honeybees (Amdam and Omholt, 2002; Omholt and Amdam, 2004). Vitellogenin's constitute a multigene superfamily that includes insect apolipophorin II/I, the large subunit of mammalian microsomal triglyceride transfer protein and human apolipoprotein B (Babin *et al.*, 1999; Amdam *et al.*, 2003; Amdam *et al.*, 2009). In honeybees vitellogenin is a 180kDa monomer synthesised by the adult fat body (Wheeler and Kawooya, 1990; Amdam *et al.*, 2003). Vitellogenin is the major zinc carrier in honeybee hemolymph and a reduction in zinc can trigger hemocytes pycnosis resulting in a reduction in immunity (Münch and Amdam, 2010).

Vitellogenin is a known antioxidant and studies have shown that vitellogenin was preferentially oxidized when compared to other hemolymph proteins (apolipoprotein 1 and hexamerin). This can be attributed to vitellogenin being strongly carbonylated by paraquat which is an oxidative stress agent and being a major zinc carrier (Seehuus *et al.*, 2006b). The study also demonstrated preferential carbonylation of Cu-Zn-superoxide dismutase, a key antioxidant enzyme, that shares metal-binding characteristics with vitellogenin (Seehuus *et al.*, 2006b). Vitellogenin knockout honeybees have a reduced lifespan and display an earlier shift from nurses to foragers compared to controls thereby demonstrating the inhibitory role vitellogenin has on the transition from nurse bees to foragers (Nelson *et al.*, 2007). Studies have also shown that low levels in vitellogenin early in life results in foragers collecting larger amounts of nectar whereas higher levels in early life prime foragers to collect more pollen (Nelson *et al.*, 2007; Münch and Amdam, 2010).

Vitellogenin levels within workers are affected by juvenile hormone (JH) titres which has been compiled into a model called "The double repressor hypothesis" (Amdam and Omholt, 2003). Juvenile hormone is an extremely versatile hormone in the animal kingdom that plays a role in multiple aspects of insect development, reproduction and behaviour (Omholt and Amdam, 2004). Juvenile hormone is

synthesised and secreted by the corpora allata glands located behind honeybee brains (Amdam *et al.*, 2009). During larval development juvenile hormone and ecdysteroids work together to control molts and in adults they govern reproductive behaviour (Hartfelder, 2000). Queen larvae have higher juvenile hormone synthesis compared to workers, who produce low titres of the morphogenic hormone (Hartfelder, 2000). In honeybees high Juvenile hormone levels have been associated with foraging behaviours (Hartfelder, 2000). The rise in Juvenile hormone titres are paired with a reduction in the size of HGs, a decrease in the production of vitellogenin and the reconstruction of protocerebral mushroom bodies (Hartfelder, 2000).

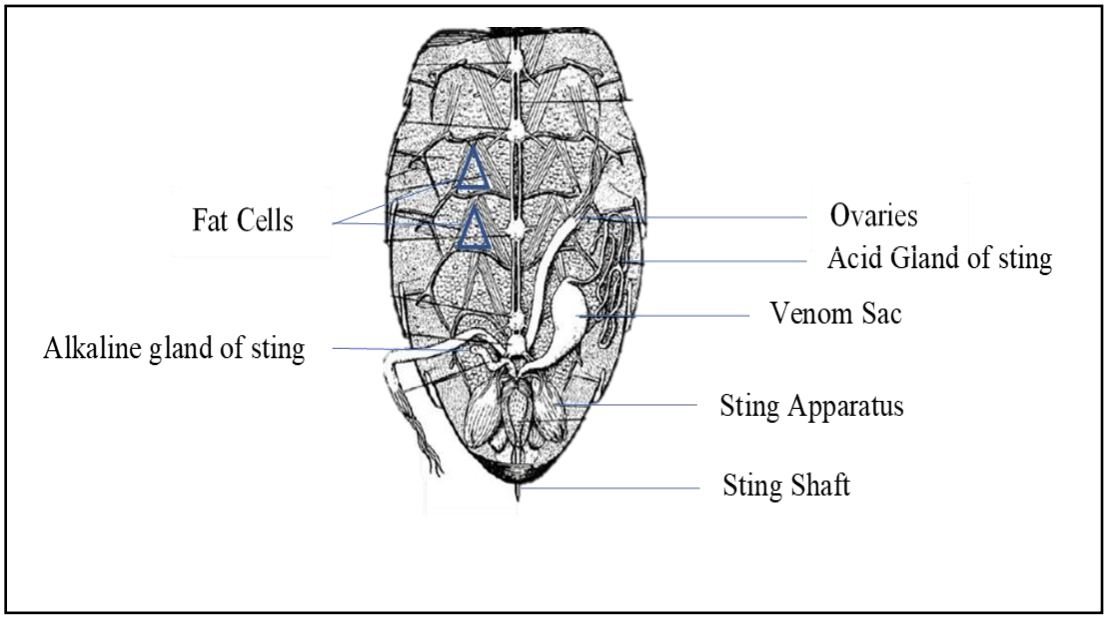


Figure 1.8 Fat Cells and Sting Apparatus in Apis mellifera Abdomen.
Adapted from Coloss Beebook 1. Standard methods for *Apis mellifera* anatomy and dissection.

1.2.5 Venom Sac

Insects which are part of the Hymenoptera order use venom as a chemical weapon for defence against threats such as wasps, mice and/or opposing colonies. The International Union of Immunological Studies have listed 12 proteins within *A. mellifera* venom that are allergens to humans (WHO/IUIS, 2018). Hymenoptera venom allergy (HVA) is the defined, systematic allergic or anaphylactic reaction that occurs in response to stings of insects of the Hymenoptera order (Spillner *et al.*, 2014). Venom glands are activated shortly after the adult bees emerge with the maximal production of venom occurring two to three weeks post emergence (Lima and Brochetto-Braga, 2003). Two glands - acid and alkaline, are associated with the sting

apparatus, labelled in **Figure 1.9.** The acid gland synthesises and secretes venom proteins to be stored in the venom sac and the alkaline gland secretes a lubricant solution for the sting mechanism (Hooper, 2010).

Venom is composed of a multitude of biogenic amines, peptides and proteins (Carpena *et al.*, 2020). Once released from venom glands proteins undergo a series of post-translational modifications such as phosphorylation, glycosylation and sulfation in the venom sac (Rosa *et al.*, 1992; Li *et al.*, 2013). Honeybee venom proteins activate systemic IgE mediated allergic reactions to inflict the most pain through their stings (Li *et al.*, 2013).

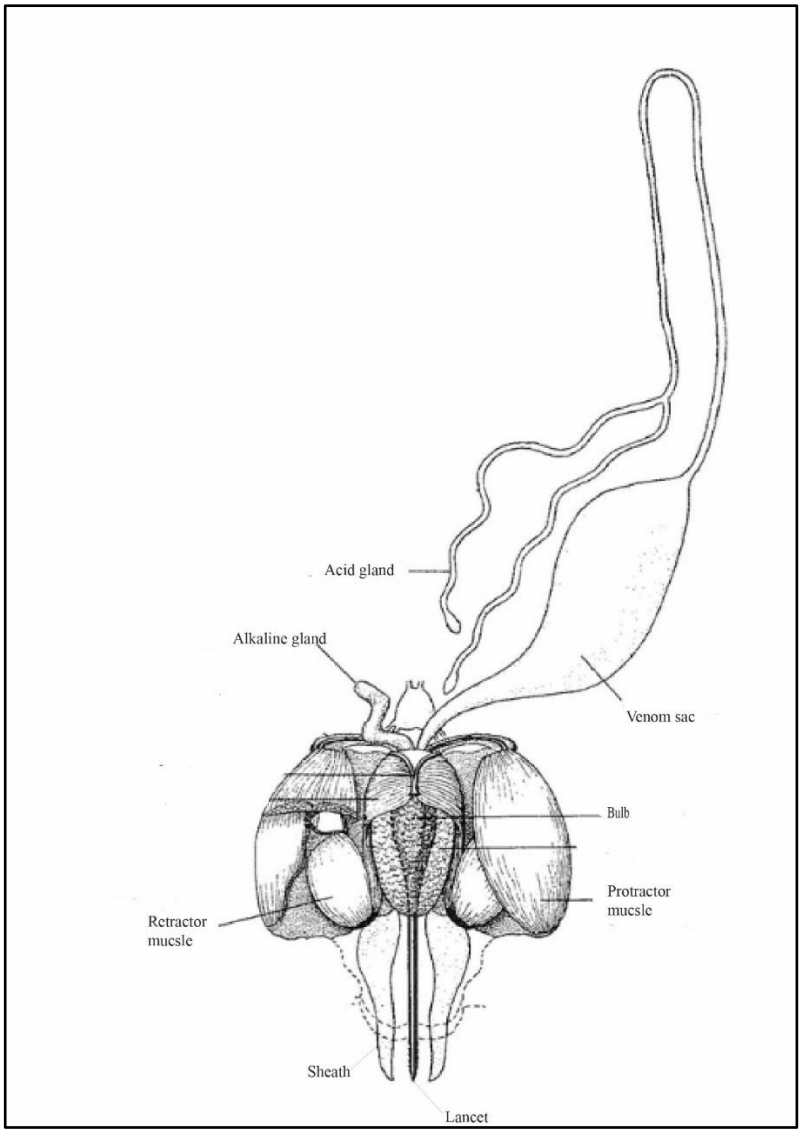


Figure 1.9 Sting Apparatus in *Apis mellifera*.
Adapted from Coloss Beebook 1. Standard methods for *Apis mellifera* anatomy and dissection.

1.3 Immune System of *Apis mellifera*

1.3.1 Overview

The first line of defence in both insects and mammals is the innate immune system. It is a non-specific response, widely distributed throughout the body that plays a crucial role in preventing disease and maintaining homeostasis (Riera Romo et al., 2016). Insects lack an acquired immune response, so they depend solely on a well-developed innate response in the defence against pathogens. Innate immunity is activated by cellular and humoral responses. Cellular responses in insects are mediated by hemocytes that circulate within the haemolymph and are involved in phagocytosis, nodulation and encapsulation (Strand, 2008; Negri et al., 2016). Nodulation refers to aggregation by hemocytes to structures that are too large for a single cell (Strand, 2008). Encapsulation is the process of multiple hemocytes binding to the surface of the invaders such as nematodes which cannot be phagocytosed by a single cell (Strand, 2008).

The humoral response in insects involves activating pathways resulting in melanisation, clotting and the secretion of antimicrobial peptides (AMPs). The immediate activation of the innate immune response in insects is driven by a combination of the cellular and humoral response. Hemocytes and the production of melanin work in conjunction to detect and activate the immune response of insects (Negri et al., 2016).

1.3.2 Cellular Immunity

Hemocytes are produced in two stages of insect development; during embryogenesis in the head or dorsal mesoderm and during larval development via mesodermally derived organs (Strand, 2008). Microorganisms found in the haemocoel, the main compartment of insect's body cavity, are recognized and subsequently removed by hemocytes, thereby preventing them spreading into the body cavity. Insects produce several hemocytes, the most common being; prohemocyte granular cells (granulocytes), plasmatocytes, sperulocytes and oenocytoids (Lavine and Strand, 2002; Strand, 2008). Hemocyte number, size and function vary depending on the worker bees age (Yelkovan *et al.*, 2021). Upon detecting a foreign surface,

hemocytes rapidly transform from non-adherent cells to activated adherent cells that will first attach to the foreign surface as a monolayer (Nardi et al., 2006; Negri et al., 2016). Once the "non-self" recognition response is activated the hemocytes will either promote phagocytosis (if the invader is small) or activate nodulation or encapsulation, this is the "spreading" stage of the innate response (Negri et al., 2016). When plasmatocytes encounter a small foreign invader through a cognate receptor, they are capable of phagocytosis in an actin polymerization-dependent manner, leading to the destruction of the invader (Lavine and Strand, 2002; Strand, 2008).

Negri et al. (2014) used nylon implants that trigger wound healing/encapsulation response, found a larger production of nitric oxide (NO) producing granulocytes suggesting their importance in wound healing or encapsulation responses. Nitric oxide (NO) is a highly reactive gas generated by the oxidation of L-arginine to L-citulline via nitric oxide synthase (NOS) (Negri et al., 2013). NO has a range of physiological functions especially in immune activation signalling, its rapid diffusion between cell membranes allows for efficient signalling (Davies and Dow, 2009). It has been demonstrated that NO plays a role in signalling between cells during wound healing or encapsulation responses within insects (Negri et al., 2013; Negri et al., 2014). Under inflammatory cellular conditions when NO is overproduced it inadvertently causes enzymatic and DNA damage in its attempt to ameliorate the immune response (Negri et al., 2014). Negri et al. 2013 suggested that NO has a key and initial role in activating the innate response in *A. mellifera* and that hemocyte spreading is dependent upon NO production. Abscisic acid (ABA) is a phytohormone in plants that regulates basic physiological functions (Negri et al., 2015). In animal cells ABA stimulates innate immune defences such as phagocytosis and can induce NO and reactive oxygen species production, both are used in cell signalling or as a direct defence against microorganisms (Bruzzone et al., 2012).

1.3.3 Humoral Immunity

Insect humoral immunity refers to soluble effector molecules such as; complement-like proteins, AMPs and enzymatic cascades involved in the regulation of melanin and clotting processes, produced via Toll and/or immune deficiency pathways (Lemaitre and Hoffmann, 2007; Negri et al., 2016). Antimicrobial peptides are essential in insect immunity as they provide a diverse range of actions such as;

altering transmembrane electrochemical gradients, inhibiting protein synthesis or causing membrane rupture that consequently results in cell death (Rahnamaeian et al., 2015). It has been shown that honeybees secrete four AMPs; apidaecins, apidaexin, defensin and hymenoptaecin (Casteels et al., 1989; Casteels et al., 1990; Casteels et al., 1993; Casteels-Josson et al., 1994; Badaoui et al., 2017). Apidaecins – are a group of inducible peptide antibiotics in insects, are the major components in the haemolymph during bacterial infection, however, it is suggested that they are bacteriostatic rather than bacteriolytic (Casteels et al., 1989). Defensin is similar in function and structure to mammalian defensin through its targeted approach to the bacterial cell wall to create a voltage-gated channel leading to the loss of ions from the cytoplasm as a means of imposing internal deterioration within bacteria (Hoffmann et al., 1996).

1.3.4 Melanisation

Pre-prophenoloxidase is a humoral protein that when activated triggers melanisation at the wound site or around invading pathogens (Lemaitre and Hoffmann, 2007). Melanisation results in a production of a dark pigment at the site of infection or injury. Melanin is a brown-black pigment that is produced at the site of infection and injury by the innate immune system (Eleftherianos and Revenis, 2011). The synthesis of melanin produces cytotoxic intermediates; quninone precursors, superoxides and hydroxy radicals all of which are thought to play an important role in killing invaders (Nappi and Christensen, 2005; Kan et al., 2008; Falabella et al., 2012). Prophenoloxidase (proPO), a zymogen is released by hemocytes upon a wound or pathogenic insult (Eleftherianos and Revenis, 2011). proPO is activated by clip-domain serine proteases called proPO-activating factors (PPAFs) or enzymes (PPAEs) to produce phenoloxidase (PO) (Kan et al., 2008). Melanisation is triggered when pattern recognition proteins recognize pathogen associated molecular patterns (PAMPs) such as peptidoglycan or bacterial lipopolysaccharide. Activation of the proPO triggers the transcription of genes encoding serine protease inhibitors (serpins) that prevent the premature and excessive activation of melanin via negative regulation (Eleftherianos and Revenis, 2011). The phenoloxidase system is activated when the prophenoloxidase-activating enzyme is converted from pro-ppA to its active form ppA. ppA can subsequently catalyse the proteolytic cleavage of prophenoloxidase to

phenoloxidase. PO is involved in a series of reactions resulting in the production of melanin. Firstly, the hydroxylation of monophenols to phenols, the phenols are then oxidised to form quinines. Quinines are subsequently catalysed by phenoloxidase-monophenyl-L-dopa to form melanin (Zhao et al., 2007; Lu et al., 2014).

1.3.5 Social Immunity

Honeybees are very social insects and have developed a group-level defence mechanism to prevent diseases being transmitted through the hive. (Negri et al., 2016). This social immunity relies mainly on hygienic behaviour or "antiseptic behaviour" which includes; grooming, undertaking, avoidance and glandular secretions and usage of resins within the nests (Wilson Rich *et al*., 2009). These behaviours can be triggered by chemical cues such as cuticular hydrocarbons, or through behavioural dances.

Grooming in honeybees is an important aspect of social immunity as it can be used to control the spread of ectoparasites which reside on the bees exoskeletal surface. Workers may groom themselves (auto-groom) or be groomed by another bee (allo-groom). Auto-grooming is used to remove pollen or foreign particles from the body and can be used as a defence against parasitic mites **(Figure 1.10**) (Evans and Spivak, 2010; Pritchard, 2016). Bees within the hive use their mouthparts as well as moving the pro and/or mesothoratic legs to remove mites or particles from their thorax (Danka and Villa, 2005). Allo-grooming is important as it removes foreign particles from areas of the body an individual cannot access through auto-grooming. Requests for allo-grooming are communicated to other bees through a jerking motion, surrounding bees will respond by cleaning the base of the wings (Evans and Spivak, 2010). For allo-grooming to be effective against ectoparasites bees need to both dislodge the mite and damage it by biting its idiosoma or legs (Aumeier *et al.*, 2000). One downfall of grooming is the possibility for an increase in transmission of honeybee parasites such as viruses or *Nosema*, if all-grooming involves licking or chewing other workers then it could increase the spread of the infection (Schmid-Hempel, 1998; Evans and Spivak, 2010).

Hygienic behaviour (HB) is a response by adult bees to diseased and parasitized worker brood (larvae and pupae). Workers that display hygienic behaviour are typically 15-20 days old (Spivak *et al.*, 2003). Hygienic behaviour is characterised

by individual worker bees identifying, uncapping and removing diseased brood rapidly thereby limiting the spread of the disease (**Figure 1.10**) (Woodrow and Holst, 1942). Non-hygienic behaviour by honeybee workers refers to the inability of workers to successfully uncap and remove infected brood from the colony which results in the spread of the diseased brood and therefore a weakening in hive strength (Woodrow and Holst, 1942; Masterman *et al.*, 2001).

Successful hygienic behavioural responses is linked to limiting the spread of several brood diseases (Woodrow and Holst, 1942). Studies have shown that successful hygienic behaviour requires early detection of diseased brood and that workers responsible for removing the brood have high olfactory sensitivity towards diseased brood (Masterman *et al.*, 2001; Spivak *et al.*, 2003; Swanson *et al.*, 2009; Evans and Spivak, 2010). Masterman et al (2001) determined that hygienic behaviour is genetically controlled and therefore there are some bees that will demonstrate more acute and efficient responses to diseased brood than non-hygienic bees. This study also demonstrated that hygienic bees possess primary chemosensory neurons that result in an increased sensitivity to the odour of chalkbrood at low concentrations, furthering their conclusion that hygienic behaviour is a genetic predisposition rather than a behavioural state (Masterman *et al.*, 2001).

Hygienic behaviour however, does not equate to successful removal of brood infected with *Varroa* mites. *Varroa* sensitive hygienic (VSH) behaviour is a subgroup of hygienic behaviour observed in honeybee colonies. Nurse bees have been observed to specifically remove brood infested cells (**Figure 1.10**) (Harbo and Harris, 2005; Mondet *et al.*, 2015b; Traynor *et al.*, 2020). It is suggested that the antenna are important in distinguishing between VSH and HB, as chemical sensing genes were observed to be expressed at a higher level in VSH bees in comparison to HB bees with specific interest on odorant binding proteins (Forêt and Maleszka, 2006; Le Conte *et al.*, 2011; Mondet *et al.*, 2015b). The role of olfactory signals cannot be directly linked with the presence or movement of the *Varroa* within the brood as it is likely the mites utilise their ability to camouflage their scent, mimicking the host to avoid detection (Aumeier and Rosenkranz, 2001; Le Conte *et al.*, 2011; Wagoner *et al.*, 2018). It is therefore more likely that the olfactory signals are originating from infected honeybee brood and an increase in brood cuticular hydrocarbons and brood ester pheromones

have both been linked with HB (Abdullah *et al.*, 2007; Navajas *et al.*, 2008; Jiang *et al.*, 2016; Wagoner *et al.*, 2018).

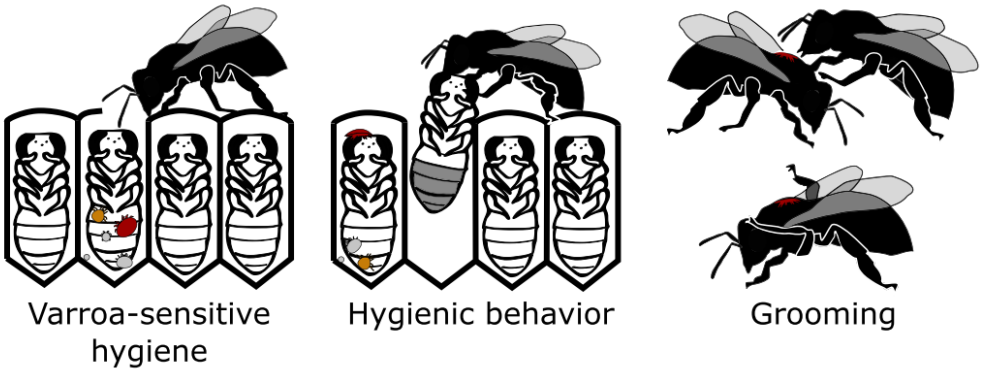


Figure 1.10: Social immunity includes grooming behaviour of worker bees to remove *Varroa* mites from both themselves and other workers. Hygienic behaviours are linked with the removal of infected brood identified through odours, however, this does not mean successful removal of *Varroa* infected brood. *Varroa* sensitive hygienic behaviour is the sub-group of hygienic behaviour that specifically targets the removal of *Varroa* infected brood. Image source (Traynor *et al.*, 2020).

1.4 Pathogens of *Apis mellifera*

Honeybees can be vulnerable to infection from a range of pests and pathogens. The most common threat to honeybee colonies in Ireland is the combination of the *Varroa destructor* and viruses such as Deformed Wing Virus (DWV) and/or acute bee paralysis virus. Combinations of *Varroa* mite presence and viruses is extremely common and if left untreated and monitored the colony can succumb to the parasitic load and subsequently fail (Grozinger and Flenniken, 2019). *Nosema* is another potential threat to the health of honeybee colonies, it's presence within hives is not as detrimental as the *Varroa* mites but can lead to a decline in honeybee health. This thesis will investigate proteomic changes that occur in Irish honeybee workers exposed to the presence of both *Varroa* mites and *Nosema* infections.

1.4.1 Varroa destructor

Varroosis is a very serious and unfortunately a very common disease caused by the parasitic mite, *Varroa destructor* (Di Prisco *et al.*, 2011). *Varroa* mites are ectoparasites of the honeybee and were originally confined to the eastern honeybee

Apis cerana but have since spread world-wide, reaching Ireland and the UK around the 2000s (Anderson, 2000; Rosenkranz *et al.*, 2010). This host shift was most likely a result of opportunistic infection during transport of *A. mellifera* colonies across Eastern Russia or the Far East during the early years of the twentieth Century (Rosenkranz *et al.*, 2010). Unlike *A. cerana, A. mellifera* has not had the history of co-evolution with the *V. destructor* which has resulted in the mite causing devastating effects to *A. mellifera* hives as the workers are not as efficient as removing the mites as *A. cerana* (Rosenkranz *et al.*, 2010). *Varroa* parasitization of *A. cerana* is mainly confined to the drone brood which limits the harmful effects of the mite's presence, compared to *A. mellifera* where both drones and workers are infected (Anderson, 2000; Rosenkranz *et al.*, 2010).

1.4.1.1 Morphology of the *Varroa destructor*

There are two defined body parts of the mite; the idiosoma and the gnathosoma. The idiosoma is the larger of the two body parts and is covered by the dorsal shell along with different ventral shields (**Figure 1.11**) (Rosenkranz *et al.*, 2010). Females have a larger, flattened ellipsoidal idiosoma that is highly sclerotised with a reddish-brown colour, whereas the males are pear shaped with weak sclerotization (Rosenkranz *et al.*, 2010). Females have shorter stronger legs with specialized adaptions that would enable stronger adherence to the bees compared to the longer legs of the males (Rosenkranz *et al.*, 2010). The gnathosoma forms the mouthparts which include two sensory pedipalps and two chelicerae (Rosenkranz *et al.*, 2010). The chelicerae are composed of three segments; the basal, middle and distal digit, the latter is movable in females and has two small teeth, in the males it is the spermatodactyl a hollow structure that transfers the sperm to the female genital tract, (Rosenkranz *et al.*, 2010). Therefore, the males cannot feed and are not found outside of the brood cells as their sole function is reproduction (Coffey, 2007). The female genitalia are divided into two systems; the first consists of the ovary, uterus and vagina, this system is situated between the second pair of legs. The second system's function is retaining, maturing and storing the sperm through sperm ducts, spermatheca and a camera spermatica which connects the spermatheca, ovary and uterus together (Rosenkranz *et al.*, 2010). Female varroa are larger than the males, females are approximately 1.1mm long and 1.5-1.6mm wide their male counterparts are 0.7mm by

0.7mm in size (Coffey, 2007). Females are covered in small hairs that act as sensory organs with some providing chemo- and mechanoreceptive signals, which are essential in finding the larvae or pupae (Rosenkranz *et al.*, 2010).

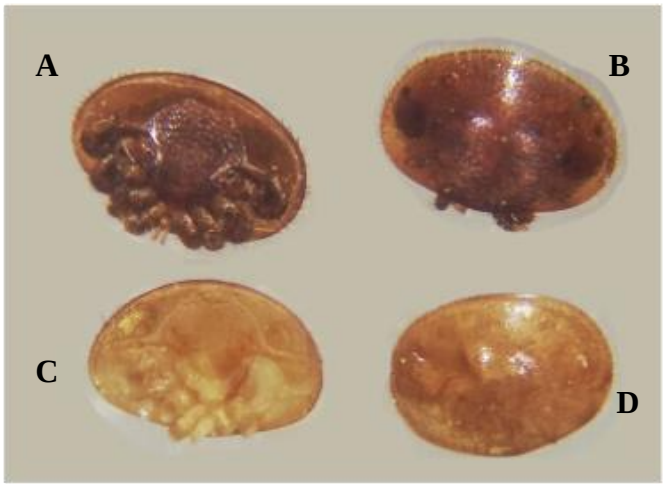


Figure 1.11: *Varroa destructor* – A) Ventral shields of adult mite , B) dorsal shell adult mite, C), ventral shields of juvenile mite D) dorsal shell of juvenile mite.

1.4.1.2 Mite Lifecycle

Varroa lack a free-living stage and are therefore completely dependent on the honeybees. The mites have two distinct phases in their life cycle: phoretic and larval (Coffey, 2007).

1.4.1.2.1 Phoretic phase:

This phase occurs on the adult bees that act as the intermediate host and as a means of transport for the mites (Coffey, 2007; Rosenkranz *et al.*, 2010). The females will consume the haemolymph/fat body from the adults, however, the mites need to feed on larval haemolymph/fat body to trigger egg laying (Coffey, 2007). Mites can spbioteend prolonged periods on adults with the majority of them attaching onto nurse bees, which provide access to the uncapped brood cells, a high nutritional food source and a prolonged life compared to foragers or newly emerged bees (Coffey, 2007; Xie *et al.*, 2016; Ramsey *et al.*, 2019).

1.4.1.2.2 Larval phase:

Female adults will enter a brood cell a few hours before the cells are capped, female mites prefer drone cells over workers, as their cells are larger and they remain capped for longer periods (Calderone and Kuenen, 2001; Coffey, 2007). The female mite will bury herself within the liquid brood food and move to the bottom of the cell to avoid detection by nurse bees, she will remain here until the cell has been caped (**Figure 1.12**, Day 10) (Coffey, 2007). The mite will feed on the larvae for a few days before laying her eggs, the eggs will hatch after 24hours (Coffey, 2007). *V. destructor* are haplodiploid, males emerge from unfertilized eggs and females from fertilized eggs. There are three stages of the mites development; firstly a protonymph will emerge, after 1-2 days the protonymph will molt to a deutonymph and feed for a further 3 days, finally emerging into an adult mite (Coffey, 2007). Females take 6-6.2 days to hatch and males take 6.8-6.9 days to hatch, however, the males hatch first followed by the females to which it will mate with (**Figure 1.12**, Day 17) (Coffey, 2007). The mother mite will puncture the larvae and create a single feeding hole to which her offspring will feed upon, this continuous and repeated feeding through this puncture delays the healing process (Rosenkranz *et al.*, 2010). Any immature females and the single male will die once the bee emerges (Martin, 2001).

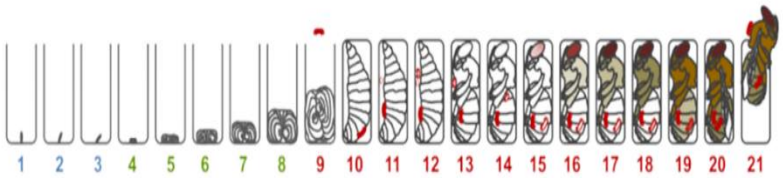


Figure 1.12: Larval phase of the *Varroa destructor* on *Apis mellifera* workers. The red dots represent female *Varroa* mites and the small pink dots represent male *Varroa* mites. Multiple *Varroa* mites can leave the cell on the newly emerged worker on day 21. Adapted from Varroa destructor: A Complex Parasite, Crippling Honey Bees Worldwide (Traynor *et al.*, 2020).

1.4.1.3 Parasitic effects from *Varroa destructor*

There are several negative effects from parasitization of the *Varroa* mite on *A. mellifera*, with a reduction in weight being the first identifiable indicator of the mites. Since the *Varroa* feed on the fat body there is a significant loss of weight in the emerging bees (Rosenkranz *et al.*, 2010). A single mother mite can cause an average

loss of 7% in workers weight and up to 12% in drones (De Jong *et al.*, 1982; Duay *et al.*, 2003). Emerging bees also have damaged fat bodies and underdeveloped hypopharyngeal glands and tend to enter forager stages much earlier (Schneider and Drescher, 1987; Janmaat and Winston, 2000; Amdam *et al.*, 2004a). These foragers also display abnormal behaviours compared to non-parasitized foragers; prolonged absences from the colony, lower return rate and a decrease in capability for non-associated learning (Kralj, 2004; Kralj *et al.*, 2007).

A recent study has reviewed the feeding habits of *V. destructor* and its damaging effects, Ramsey *et al* (2019) found that *V. destructor* feeds primarily on the fat body tissue in *A. mellifera* rather than the haemolymph. The study argues that the evolution of *V. destructor* is more closely associated with other mites that consume semisolid nutrients rather than a dilute liquid diet (Ramsey *et al.*, 2019). The fat body in insects is responsible for energy storage, protein and lipid synthesis (Arrese and Soulages, 2010; Ramsey *et al.*, 2019). Consumption of the fat body by *V. destructor* could explain the myriad of negative effects to the infected bees; reduction in protein titres, impaired metabolic function, decreased longevity, premature caste shift to foragers which have all previously been unexplained (Ramsey *et al.*, 2019). For example, with the fat body being the primary nutrition source for the mites would result in a diminished production of vitellogenins, which would subsequently cause a premature shift to foragers (Ramsey *et al.*, 2019).

Research has also emerged which provides an explanation as to why nurse bees are the preferred choice of adult bees in the hive by *Varroa* (Xie *et al.*, 2016; Ramsey *et al.*, 2019). Nurse bees have a larger and more nutritional fat body than foragers and newly emerged bees and are not subjected to allo-grooming to the same extent as newly emerged and forager bees (Xie *et al.*, 2016; Ramsey *et al.*, 2019). Xie *et al.* (2016) also found that mites that parasitize nurse bees have a higher fecundity than those on newly emerged or forager bees. Nurse bees play an essential role in the hive as they are responsible for rearing the larvae and pupae, therefore by parasitizing the nurse bees it results in a rapid spread in *Varroa* however, it causes a decrease in nurse bee health which can lead to a weak hive that could eventually fail. The preference of nurse workers over newly emerged and forager bees could be attributed to the amount of contact nurse bees have with brood (feeding and inspection) and nurses may also be

a better nutritional source for *Varroa* in comparison to foragers due to the size of the fat body (Xie *et al.*, 2016).

Primary effects of *Varroa* parasitization are not the only concern for beekeepers, *Varroa* are known vectors for honeybee viruses, there have been twenty-two viruses described in honeybees with eighteen of these associated with the presence of the mites with many of them being transmitted by the mite itself (Chen and Siede, 2007; Fanny *et al.*, 2014). The transmission of honeybee viruses is linked to the prevalence of *Varroa* mites in hives (Martin, 2001; Highfield *et al.*, 2009; Wilfert *et al.*, 2016). Viruses require a host to survive as they lack a metabolic and reproductive cellular system. In order for viruses to spread they need a vector, a complex living organism that is capable of transmitting virions to other organisms (Chen *et al.*, 2006; Chen and Siede, 2007). It is widely suggested that the presence of *Varroa* mites in hives is linked to the spread of viruses in honeybees with several studies finding 90-100% viral presence in *Varroa* (Tentcheva *et al.*, 2004; Berenyi *et al.*, 2006). *V. destructor's* success in being a viral vector is linked to its ability to induce immunosuppression in parasitized pupae, therefore, weakening the honeybee hosts allowing viruses to spread with less resistance in their new hosts (Yang and Cox-Foster, 2007).

1.4.2 Nosema

Nosema are obligate intracellular microsporidian parasites that infect the midgut epithelial cells of host adult honeybees. There are two subspecies of *Nosema, apis* and *cerana* which originally infected *Apis mellifera* and *Apis cerana* respectively (Fries *et al.*, 1996; Sinpoo *et al.*, 2018). Infection from *Nosema* occurs mostly through the ingestion of spores through food, water or passed on via trophallaxis (Chen *et al.*, 2009; Higes *et al.*, 2010). The conditions inside the midgut of workers (physically and chemically) trigger the germination of the rod shaped spores and begin the vegetative state resulting in the growth and multiplication of *Nosema* inside the cells (Chen *et al.*, 2009). The infection is initiated through the penetration of a polar tube into the cell membrane of the epithelial cells of the gut (Gisder *et al.*, 2011). This intracellular phase has two stages; the proliferation phase, the sporogonic phase, ending with the formation of spores, completing it's lifecycle in 3 days (Higes *et al.*, 2010). Spores are spread in the faecal matter of honeybees and are ingested by younger workers when

cleaning contaminated combs, this is more likely to occur in late winter when bees are confined to the hive (Bailey and Ball, 1930:69). It has been revealed that between 6.6 to 8.1 million spores could be found within a bee's midgut four days post infection (Bailey, 1955). The rate of infection decreases in summer as workers are able to fly freely and defaecate outside the colony (Bailey and Ball, 1930:69).

Infection from *Nosema* causes major health issues to honeybees which can be characterised by immune suppression, degradation of epithelial cells in the gut and a reduced lifespan (Bailey and Ball, 1930:65; Higes *et al.*, 2008; Antúnez *et al.*, 2009; Dussaubat *et al.*, 2012). *Nosema* infection in colonies also causes a decline in worker numbers within the hive as it is believed that foragers die far from the hive (Higes *et al.*, 2010). Studies have also shown that infection of *Nosema* causes a decrease in nitrogen levels and amino acids within the fat body and haemolymph respectively (Bailey and Ball, 1930:64).

Currently there is an absence of commonly used treatments against *Nosema* infections in hives. Fumagillin derived from *Aspergillus fumigatus* was used as a treatment against *Nosema* infections for decades until recently it has been discovered to have negative effects on bee health (Van Den Heever *et al.*, 2014; Burnham, 2019). It has been found to be toxic to bees causing chromosomal abbreviations and alterations to the ultrastructure of the hypopharyngeal glands (Liu, 1990; Van Den Heever *et al.*, 2014). The use of fumagillin has since been banned for use within the EU since 2010 (Burnham, 2019). There is a lack of treatment methods for *Nosema* and currently frame changes are suggested as a method to reduce the spread of spores within hives.

1.5 Anti-*Varroa* Treatments

1.5.1 Overview

Given the widespread presence of *Varroa* mites in hives across the world it is essential to monitor and control the spread and prevalence of the mites. Beekeepers have a range of acaricide applications that can be used on their hives. These applications can be grouped into mechanical or biotechnical methods, with a further

division of mechanical methods into soft (natural) and hard (synthetic chemicals) treatments. This study will investigate the effects the following treatments have on the workers within *A. mellifera* hives. The treatments used were one biotechnical method: Queen trapping and three soft mechanical treatments: Mite Away Quick strips (MAQs), Apiguard and Oxalic Acid with only one hard mechanical treatment Apivar being incorporated into the study.

1.5.2 Mode of Action

A mitocide/ acracide is a substance that is used to kill mites. Mitocides can be classified based on the mechanisms the compounds/chemicals target to kill the insects. The treatments used in this study each have different modes of action therefore have varying mechanisms of killing/paralysing the *Varroa* mites. Therefore, each could potentially have different adverse effects on the worker bee proteome.

1.5.2.1 Biotechnical Method: Queen Trapping

Queen trapping aims to control/reduce the number of *Varroa* in the hive only through human intervention of the hive arrangements. This method lures the mites that are dependent on the brood for reproduction, to reside in a specific frame. This is achieved by caging the queen on a frame for one week, subsequently removing this frame after a week has passed and by re-caging the queen on another frame for a week. This is repeated for another week. At the end of each week the frame is removed and analysed for the presence of *Varroa*. This method aims to firstly control the location of the brood within the hive. Secondly using the brood as bait for the *Varroa* currently in the hive of brood and finally removing both the infected brood and *Varroa* from the hive by taking the frames from the colony.

1.5.2.2 Mite Away Quick Strips

Mite Away Quick strips (MAQs) are single use saccharide gel strip applications that contain 46.7% formic acid and are placed along the top of the brood frames (**Figure 1.13**). It has been demonstrated that gel treatment of formic acid had a better efficacy (70.3%) than a liquid treatment (61.2%) in reducing *Varroa* populations (Feldlaufer et al. 1997). Formic acid (FA) is a volatile compound and is the active ingredient in MAQS. FA is known to inhibit cytochrome oxidase in the terminal

electron acceptor of the electron transport chain (Nicholls, 1975; Song and Scharf, 2009). Disruption to the respiratory system in the mitochondria is lethal and is the reason FA is used against mites. Cell death from cytochrome oxidase inhibition by FA, is another direct consequence of its presence in cells as apoptosis is triggered through mitochondrial damage or the generation of reactive oxygen species (ROS) (Song and Scharf, 2009). Although no evidence in insects has yet been found to suggest that FA also has a role in the generation of ROS within cells it is a known effect in mammalian cells (Dikalova *et al.*, 2001). FA in MAQs targets the cytochrome oxidase complex within the mitochondria resulting in the mites dying.

Figure 1.13: MAQ strips being placed on top of brood frames in a hive from the trial apiary in Oak Park Institute Co. Carlow.

1.5.2.3 Apiguard

Natural solutions for controlling *Varroa* populations are becoming more popular within beekeeping communities. Thymol a monoterpene extracted from *Thymus vulgaris* among various other plants, is a known biocide (Araújo *et al.*, 2015; Ferreira *et al.*, 2016). Thymol poses little risk to the environment or due to its rapid dissipation and low levels of remaining residues, so far there are no maximum limits on the thymol residues in honey (Floris *et al.*, 2009; Araújo *et al.*, 2015). Some evidence shows that thymol functions to cause membrane disruption, by penetrating

the phosphatidylcholine monolayers causing membrane expansion, increased fluidity and permeability (Trombetta *et al.*, 2005; Ferreira *et al.*, 2016).

A single Apiguard gel (25% thymol in 50g packets) packet is placed on the top of the brood frames for a two week period, after the two weeks a second packet replaces the first dose and is left in the hive until the gel packet is empty (10 weeks maximum) (**Figure 1.14**). It is a slow release treatment and monitoring of mite fall and brood production are recommended for the duration of the treatment.

Figure 1.14: Apiguard gel packet placed on top of brood frames in a hive from the trial apiary in Oak Park Institute Co. Carlow.

1.5.2.4 Apivar

Apivar treatment consists of polymer strips which contain amitraz as the active acaricide to be placed between the brood frames. Amitraz is a synthetic formamidine acaricide which is the active ingredient in Apivar (Gupta, 2018). Amitraz is a broad spectrum insecticide and acaricide that has been broadly used around the world since 1974 (Gupta, 2007) (**Figure 1.15**). It selectively blocks the octopamine receptor of the central nervous system through α2-adrenergic antagonist activity (Dalefield, 2017; Rinkevich, 2020). This paralyses the mouth parts on the female *Varroa* which prevents

them from feeding (Gupta, 2007). The paralysed mites fall below the frames onto the bottom boards of the hives and subsequently die of starvation (Semkiw *et al.*, 2013).

Figure 1.15: Apivar strips being placed between frames that mark the end of the brood frames within the hive in the trial apiary in Oak Park Institute Co. Carlow.

1.5.2.5 Oxalic Acid

Oxalic acid is a natural chemical that is found in low concentrations in honey, however when administered in high doses oxalic acid is used to control *Varroa* levels within hives. It has been used in hives since the end of the 20th century (Rademacher and Harz, 2006; Rademacher *et al.*, 2017). However, the exact mode of action against the *Varroa* and how oxalic acid effects the bees at a colony level has not yet been clarified.

1.6 Use of proteomic analysis for honeybee studies

'Omic' based research is a rapidly expanding and evolving field of study , the applications of which are far reaching and cutting edge. Proteomic studies on honeybees first began in 2005 which was quickly followed by the first release of the honeybee genome, this was subsequently followed by a myriad of 1-DE and 2-DE gel

analysis (Peiren *et al.*, 2005; Hora *et al.*, 2018). Over the past decade, analysis has moved away from in gel analysis and towards mass spectrometry techniques. Mass spectrometry analysis of honeybee proteomes allows for a more in-depth analysis of samples. There are two approaches to mass spectrometry analysis: "top down" and "bottom up" (Fornelli *et al.*, 2018). "bottom up" proteomics is the most common approach for studying complex samples, the basis of this technique is the characterisation of proteins through the analysis of peptides generated by the protein through proteolysis (Gao and Yates, 2019). When "bottom up" proteomics is performed on a mixture of proteins it is called shotgun proteomics, this provides indirect measurements of the proteins through the analysis of the peptides generated via the proteolysis process. The peptides are fractionated on a reverse phase C18 column prior to analysis by mass spectrometry (LC-MS) with tandem mass spectrometry (LC-MS/MS) (Gao and Yates, 2019; Wojtkiewicz *et al.*, 2021). Protein quantification can be performed with or without a label. Label free measures samples individually comparing the MS ion intensities of the peptides whilst labelling (isotope labels) allows for the mixture of samples at different experimental stages to be analysed (Gao and Yates, 2019). Label free allows the characterisation of sample compositions without focus on individual peptides, this makes label free mass spectrometry analysis a powerful tool for the characterisation of a proteome from a complex sample.

Numerous studies have been conducted on proteomic changes in the organs, hemolymph and glands of the honeybee (Jianke *et al.*, 2010; Iovinella *et al.*, 2011; Li *et al.*, 2013; Hu *et al.*, 2019; Kunc *et al.*, 2019; Altaye *et al.*, 2019). Despite research on specific glands or organs of the honeybee, only a small amount of research has been conducted on whole honeybee samples (Lee and Kim, 2017; Surlis *et al.*, 2018). This thesis looks to investigate proteomic changes in worker bees using whole cell lysate and label free quantitative proteomics through mass spectrometry analysis.

1.7 Aims of study

- Characterise the proteomic changes that occur within worker honeybees as they age. As workers progress in their life, their roles and responsibilities in the hives change, this correlation between age and role is called age-polyethism. Each role requires specific secretions or alterations in the bee's body. Here proteomic analysis will be used to characterise these changes in the bee's biology thereby gaining a more in-depth understanding into how the workers adapt to their roles as they age

- Comparison of the winter and summer honeybee proteomes. Proteomics will be applied to identify differences between honeybees that live in the summer and the winter. Focus will mainly be placed on observing variations in proteins that contribute to longevity. Winter workers live substantially longer lives than summer workers, so this work will investigate how proteins contribute to this lifespan difference.

- Investigating possible adverse effects anti-*Varroa* treatments have on the honeybee biology. Anti-*Varroa* treatments are known to be successful in reducing/eliminating the *Varroa* mite from hives, however, little is known about possible adverse effects the treatments may have on the bees. Several treatments have been selected and administered to hives and samples of worker bees have been collected for proteomic analysis to investigate if these treatments trigger any changes in the bee's proteome.

- Investigating the effects of both *Nosema* and *Varroa* on the proteome of honeybee workers. Proteomic analysis will be conducted on *Varroa* and *Nosema* infected worker bees to gain an insight into how the presence of mono and dual infections of these parasites affect the worker bees proteome.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Chemicals and Reagents

Double distilled water, ddH_2O was purified using a Millipore Milli-Q apparatus to obtain milli-Q water of 18MΩ. Complete mini protease inhibitor tablets were purchased from Roche Diagnostics (Mannheim, Germany). Qubit reagent for protein quantification was purchased from BioSciences, Invitrogen (Dun Laoghaire, Co Dublin, Ireland). Table 2.1 outlines the remaining solutions used in the protein extraction, digestion and clean-up.

2.1.2 Sterilisation Procedures

All micro-centrifuge tubes and pipette tips were stored in sealed containers and autoclaved at 121^{o}C for 15 minutes prior to use. All solutions were stored at 4^{o}C, pH checked before use and put through a 0.45 μm pore filter (Filtropur S Sarstedt).

2.1.3 One Dimensional Gel Electrophoresis

One-dimensional, sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS- PAGE) was used to separate proteins using the following reagents; Ultrapure Protogel, Resolving Buffer stock, acrylamide stock and tetramethyl ethylenediamine (TEMED) solution (National Diagnostics). Proteins were separated based on varying percentages of polyacrylamide. The compositions of the stacking and resolving gel are outlined below (**Table 2.2**) Gels were set following the manufacturer's instructions using 1.0mm plates. The molecular weight protein ladder was purchased from Thermo Fischer Scientific and was run with the samples in the first well of each gel (**Figure 2.1**). Gels scanned from this study can be accessed in **Supp. File 2.1**.

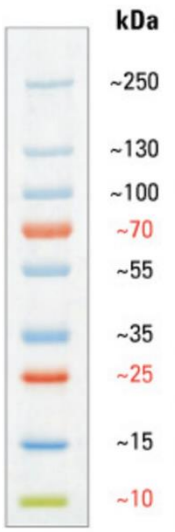


Figure 2.1: Pre-stained molecular weight ladder Thermo Scientific PageRuler Protein ladder contains 9 blue stained proteins ranging from 15kDa to 250kDa, two reference orange bands; 25kDa and 70kDa and a single green 10kDa band

Table 2.1: Stock solutions for 1-D SDS gels

Reagent	Separating Gel Stock	Stacking Gel Stock
30% Protogel	200ml	83ml
0.5M Tris Buffer	-	63ml
1.5M Tris Buffer	120ml	-
ddH_2O	152ml	340ml
10% SDS	4.8ml	5ml

Table 2.2: Composition of 1-D SDS gels

2 Gels	Volume	Volume
Gel Stock	10ml	5ml
10% APS	75µl	50µl
TEMED	4.5µl	5µl

2.1.4 Protein Digest Solutions

Table 2.3: Solutions and buffers used in protein sample digest

Material and Volume	Material and Volume
Resuspension Buffer (pH 8) Per 50ml ddH2O solution: 6M Urea 2M Thiourea 0.1M Tris-HCL	**Lysis Buffer (pH 8)** Per 50ml ddH2O solution: 6M Urea 2M Thiourea One Protease Inhibitor Cocktail Tablet (PIC; Complete Series Roche)
50mM Ammonium Bicarbonate 0.099g Ammonium Bicarbonate 25mls ddH^2O	**0.5M Dithiothreitol (DTT) (Sigma Aldrich)** 0.019g 250µl 50mM Ammonium Bicarbonate
Iodoacetamide (IAA) (Sigma Aldrich) 0.101g 1ml 50mM Ammonium Bicarbonate	**ProteaseMax (Promega)** 1mg ProteaseMax (Promega) 100µl 50mM Ammonium Bicarbonate
Trypsin (Promega) 20µg Sequence grade modified trypsin (Promega) 40µl trypsin reconstitution buffer (Promega)	

2.1.5 Protein Clean-up Solutions

Table 2.4: Buffers and solutions used in protein sample clean-up

Materials and Amount	Materials and Amount
Sample buffer (2%, TFA, 20% Acetonitrile) 200 µl Acetonitrile 20 µl TFA 780 µl ddH_2O	**Equilibration buffer/ Wash buffer (0.5% TFA, 5% Acetronitrile)** 150 µl TFA 1.5 ml Acetonitrile 25.8 ml ddH_2O
Activation buffer (50% Acetonitrile, 50% ddH_2O) 10 ml Acetonitrile 10 ml ddH_2O	**Elution buffer (70% Acetonitrile, 30% ddH_2O)** 700 µl Acetonitrile 300 µl ddH_2O
Loading buffer (0.05% TFA, 2% Acetonitrile) 500 µl TFA 20 ml Acetonitrile	

2.2 Methods

2.2.1 *Apis mellifera* Sampling Regime:

All samples of *A. mellifera* were collected from the apiary in Teagasc, Research Centre, Oak Park Co. Carlow, coordinates 52°51'53.3"N 6°54'09.7"W. Samples were initially collected during each stage of the lifecycle of the honeybee which is outlined in Table 2.4. Once collected, samples were stored at -20^{o}C on site until the month's sampling was completed. The samples were transported on ice and then stored at -80^{o}C in Maynooth University.

Obtaining bees of known ages was possible through caging the queen within the hive on a single frame. The queen was caged for two days after which, the cage was removed, and the queen was released back into the hive. The frame was kept within the hive for 13 days, allowing the cells to be filled with bee milk and subsequently capped. On the 13th day the frame was removed and placed in an incubator at 37^{o}C for 8 days until all the juvenile bees emerged. Initial work sampled workers at every stage of their development in the hive as outlined in **Table 2.5**. After analysis was conducted on these samples it was decided to reduce the sampling points as outlined in **Table 2.6.**

Bees were marked with a bee marker (specific marker) on day 21 on the top of their thorax. Each month was assigned a colour so as the sampling continued it was possible to determine the age of the bees (**Figure 2.2** & **Table 2.7**). Once bees were released back into the hive prior to marking they were sampled at specific time points determined by the above sampling regime. At least ten bees were collected per collection point.

Table 2.5: Initial sampling time points for age-polyethism study

Age	Duration of Role
Day 21	0 Days
Day 22-24	3 Days
Day 24- 32	7 Days
Day 32-40	7 Days
Day 40-43	3 Days
Day 43-50	7 Days
Day 50-57	7 Days
Day 57-65	7 Days

Table 2.6: Refined sample time points. Based on proteomic analysis

Age	Assigned Cast
Day 21	Emerged
Day 24- 32	Early
Day 40-43	Late
Day 50-57	Foragers

a)

b)

Figure 2.2: a) Example of newly emerged honeybees being marked, b) Queen cage being prepared for the hive

Table 2.7: Colour coordination of the months of sampling 2019/2020

Month	Colour
March	White
April	Yellow
May	Blue
June	White
July	Blue
August	Green
September	White
October	Blue

2.2.2 Anti-*Varroa* Treatment Trial Set-Up

Eighteen colonies were selected to be part of this trial. All selected hives had an adhesive insert placed under the frames which was left for three days. When collected the number of *Varroa* mites on each insert were counted, the hives were placed into groups of three based on the average of mite fall and each group was assigned a treatment. Samples were collected from the hives before treatments were implemented, these samples were labelled the 'pre-treatment bees'. Treatments were applied to hives as per manufacturer's instructions. If a secondary treatment was required for the overall health of the bees it too was applied as per manufacturer's instructions. Samples were taken from the hives at the end of each week and inserts remained within the hives for the duration of the treatment trials.

2.2.3 Determining Varroa populations on Adhesive Inserts

Varroa present on the adhesive inserts were enumerated on a large squared sheet and the results noted (**Figure 2.3**).

a) b)

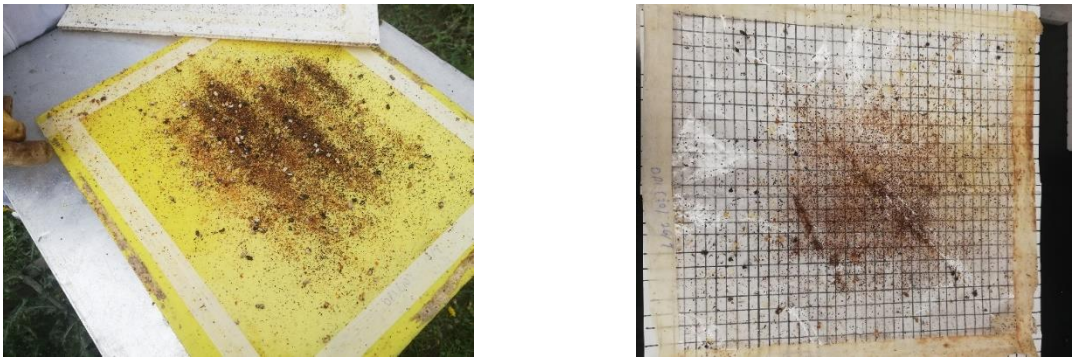


Figure 2.3: a) Insert from a hive in the treatment trial, b) Insert on gridded paper for counting

2.2.4 Disease Trial Set-up

Sixteen hives were selected to be part of this disease trial. Bees from all selected hives were sampled for *Nosema* levels (n=30 bees) and adhesive inserts were placed under the frames which were left for three days to catch dislodged *Varroa* mites. *Nosema* levels were determined by crushing 30 bees in 30ml ddH_2O and placing a drop of the solution onto a haemocytometer and calculating the approximate number of spores per bee. When both inserts and *Nosema* levels were counted, the hives were placed into groups of four based on the groups average of mite fall and *Nosema* levels**.** The four groups were: Control (treated for *Varroa* and controlled levels of *Nosema*), *Varroa* only (controlled for *Nosema*), *Nosema* only (treated for *Varroa*) and double infection (*Varroa* and *Nosema*) infected.

Six sample dates were used for this analysis: 30th July, 16th September, 25th September, 14th October, 23rd October, and 6th November 2019. Both *Varroa* and *Nosema* levels were monitored for the duration of the disease trial.

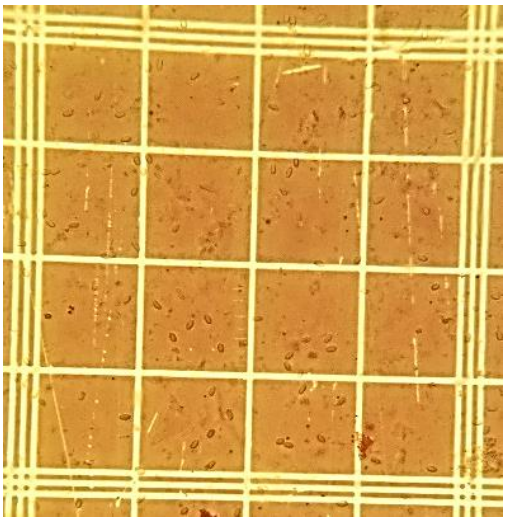


Figure 2.4: Image of *Nosema* spores on the haemocytometer under a light microscope at x40 magnification.

2.3 Protein Extraction Methodology

2.3.1 Head Extraction

Bees were decapitated using a sterile disposable scalpel and the heads collected in microcentrifuge tubes. Lysis buffer (300µl) **(Table 2.3)** was added and was agitated using a motorised hand-held pestle for approximately one minute to ensure the sample was homogenised sufficiently. Samples were centrifuged at 10000 x *g* for 5 minutes and the supernatant was collected into fresh microcentrifuge tubes. All the supernatant was precipitated overnight at a ratio of 1:5 with ice cold acetone (80%) at -20^{o}C.

2.3.2 Abdomen Extraction

The bee abdomen was separated from the remainder of the body by severing the connection between the thorax and abdomen on a sterile microscope slide. The abdomen was pinned on a sterile dissection board, the superior aspect of the abdominal exoskeleton was separated and pulled to one side. The stomach and venom sac were removed from the abdominal cavity. Lysis buffer (400µl) **(Table 2.3)** was added and the abdomen was homogenised using a motorised hand-held pestle for approximately 30 seconds. Samples were centrifuged at 12000 x *g* for 5 minutes. The supernatant was removed into fresh microcentrifuge tubes. All supernatant was precipitated overnight in 1.6ml of ice-cold acetone (80%) at -20^{o}C.

2.3.3 Venom Sac Extraction

The bee abdomen was removed from the remainder of the body by severing the connection between the thorax and abdomen on a sterile microscope slide. The abdomen was pinned on a sterile dissection board, the superior aspect the abdominal exoskeleton was separated and pulled to one side. The venom sac was removed from the abdominal cavity. Lysis buffer (200µl) **(Table 2.3)** was added and the venom sac was homogenised using a motorised hand-held pestle for approximately 30 seconds. Samples were centrifuged at 12000 x *g* for 5 minutes. The supernatant was removed into fresh microcentrifuge tubes. All supernatant was precipitated overnight at 4ºC in a 1:5 sample with ice-cold acetone ratio.

2.3.4 Protein Digestion

Samples were removed from the freezer, thawed and centrifuged at 13000 x *g* for 5 minutes. The acetone was gently poured off and microcentrifuge tubes left until the remainder of the acetone evaporated. Once dry, the samples were resuspended in 120 µl (head), 300 µl (abdomen) and 50 µl (venom sac) of resuspension buffer **(Table 2.3)** and pellets were aided in resuspension by two-minute sonication in a water bath. Samples were aliquoted into three amounts; 20 µl Digest, 10 µl 1D-Gel and Qubit quantification, and the remainder was stored. Using the 20 µl assigned for digest 105 µl of 50mM ammonium bicarbonate (**Table 2.3**) was added. Samples were then reduced with 1 µl of DTT for 20 minutes at 56ºC, allowed to cool to room temperature and then alkylated with 1 µl IAA (**Table 2.3**) for 15 minutes in the dark at room temperature. Finally, 1 µl ProteaseMax solution and 1 µl trypsin solution (**Table 2.3**) were added to the samples which were then incubated for 24 hours at 37ºC.

2.3.5 Qubit Protein Quantification

Protein quantification of all samples was preformed with Qubit Quant-IT™ protein assay kit on a Qubit fluorometer version 2.0 following the manufacturer's instructions. Sample (1 µl) was added to 199µl of working buffer (199µl of buffer B and 1µl dye A reagent). Samples were mixed with the working buffer and left to incubate in the dark at room temperature for 15 minutes. The protein concentration was measured by using a standard curve from three known Qubit standards: 10µl standard dye to 190µl of working buffer solution.

2.4 Workflow for LC-MS/MS Q-Exactive

2.4.1 Protein Clean-up

All buffers used in this procedure are outlined in **Table 2.4**. Samples were removed from the incubator and briefly centrifuged to collect any condensate. The samples were acidified with 1 µl TFA and vortexed briefly before being incubated at room temperature for 5 minutes. Samples were then centrifuged at 13000 x *g* for 10 minutes at room temperature to remove debris. The supernatant was added into a fresh microcentrifuge tubes containing 50 µl of sample buffer **(Table 2.4)**.

The lids of 2 ml microcentrifuge tubes were removed, one set for waste flow-through, a second for sample flow-through. Pierce C-18 spin columns (Thermo Scientific) were tapped gently to settle the resin and were then placed into the 2 ml microcentrifuge tubes. Columns were activated [200 µl activation buffer centrifuged at 1500 x *g* for 1 minute, flow through removed and repeated once more] and equilibrated [200 µl equilibration buffer centrifuged at 1500 x *g* for 1 minute, flow through removed and repeated once more]. Peptide sample (150 µl) was added and centrifuged at 1500 x *g* for 1 minute, flow through was collected and reapplied, this was repeated a total of three times. C-18 columns were placed in a fresh receiver tube and were washed [200 µl wash buffer centrifuged at 1500 x *g* for 1 minute, flow through removed and repeated a total of three times]. C-18 columns were placed in a new receiver tube (1.5 ml microcentrifuge tube) and 30 µl elution buffer was added and columns were spun at 1500 x *g* for 1 minute, after which elution buffer was added twice more, leaving the total volume of elution buffer at 90 µl. Samples were concentrated using a SpeedyVac concentrator (Thermo Scientific, Massachusetts, US) set to medium heat for 2 hours. Once samples were dried they were stored at 4^{o}C until needed.

2.4.2 Mass Spectrometry Analysis

Calibration of the QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer which was connected to a Dionex Ultimate 3000 (RSL Cnano) chromatography system was conducted using two HeLa cell lysate (250 ng/µl) samples.

Samples were resuspended with a QExactive loading buffer (2% v/v) acetonitrile and (0.05% v/v) TFA. Samples were sonicated in a sonication bath for 5 minutes and then centrifuged at 13400 x *g* for 10 minutes. The supernatant was transferred to MS/MS vials. Digested sample (1 µg) was loaded on a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSL Cnano) chromatography system. Peptides were separated over a 2-40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100mm length, 75 mm ID), using a 135 min reverse-phase gradient at a flow rate of 250nL min^{-1} for head samples. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode.

To ensure no drifting effect would occur between samples, blanks were run between each triplicate group. Blank samples were also analysed to confirm there was no overlap of experimental samples. A final quality assurance check was conducted once the mass spec run was completed by re-running two HeLa cell lysate (250 ng/µl) samples to confirm the MS parameters were maintained during the MS run time.

MaxQuant v 1.6.6.0 (https://www.maxquant.org/) was used for protein identification and label-free quantification (LFQ) normalization of all MS/MS data. The Andromeda search algorithm in MaxQuant was used to correlate all MS/MS data against the Honeybee Proteome fasta file which was generated by running a reciprocal BlastP search on the Uniprot and Genbank proteomes against one another. Proteins that were not assigned a function in the Uniprot file but had a function identified in the Genbank file were matched. Each protein still has its own Uniprot annotation. The resulting fasta file has 10,020 associated annotations. Search parameters were as follows: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm, carbamidomethylation of cysteines was set as a fixed modification, while oxidation of methionines and acetylation of N-terminals were set 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.

2.5 Proteomic Analysis

2.5.1 Data Analysis Workflow

Data analysis, processing and graphical interpretations were generated using Perseus v 1.6.6.0 (https://maxquant.net/perseus/). Normalised LFQ intensities were used as the quantitative measurement of protein abundance for further analysis. The dataset was first filtered for the removal of contaminants and peptides identified by site. LFQ intensities were log_2-transformed and each sample was assigned to a group. Further filtering excluded proteins that were not found repeatedly throughout the groups. Data imputation assigned values that simulate signals of low abundant proteins which were chosen randomly from a normal distribution to proteins with missing values.

ANOVA t-tests were applied for all relevant comparisons using $p<0.05$ as the cut-off on the dataset to identify statistically significant differentially abundant (SSDA) proteins. Volcano plots generated in Perseus plotted the negative log p- values on the y-axis and the log_2 fold-change values on the x-axis for each pair-wise comparison to visualise changes in protein expression. Intensity values were used for a principal component analysis (PCA) which highlights the degree of variation between all samples and enables a visualisation representation on the quality of the replicates. Proteins that are exclusively expressed in samples were identified from the pre-imputation dataset and were included in subsequent analysis. Hierarchical clustering was performed on Z-score normalised intensity values for all differentially abundant proteins by clustering both samples and proteins using Euclidean distance and complete linkage.

2.5.2 Functional Analysis

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) v 11 (https://string-db.org/) was used to map known and predicted protein: protein interactions. Uniprot protein codes (extracted from Perseus) were imputed and analysed in STRING using a confidence of medium to high (0.5 – 0.7) which generated interactive protein networks for each group in all comparisons. Biological processes, molecular function and cellular compartment were examined using KEGG pathway

analysis using the 'KEGG Mapper-Search & Colour Pathway' tool (https://www.genome.jp/kegg/tool/map_pathway2.html). Blast2GO analysis was utilised to assign gene ontology terms (GO terms)- biological processes (BP), molecular function (MF), and cellular component (CC) to SSDA proteins in comparative analysis. Proteins were also subjected to functional analysis online using Pfam and Gprofiler (https://biit.cs.ut.ee/gprofiler/gost) which classified proteins into families and providing insights into the functionality of the proteins and protein clusters.

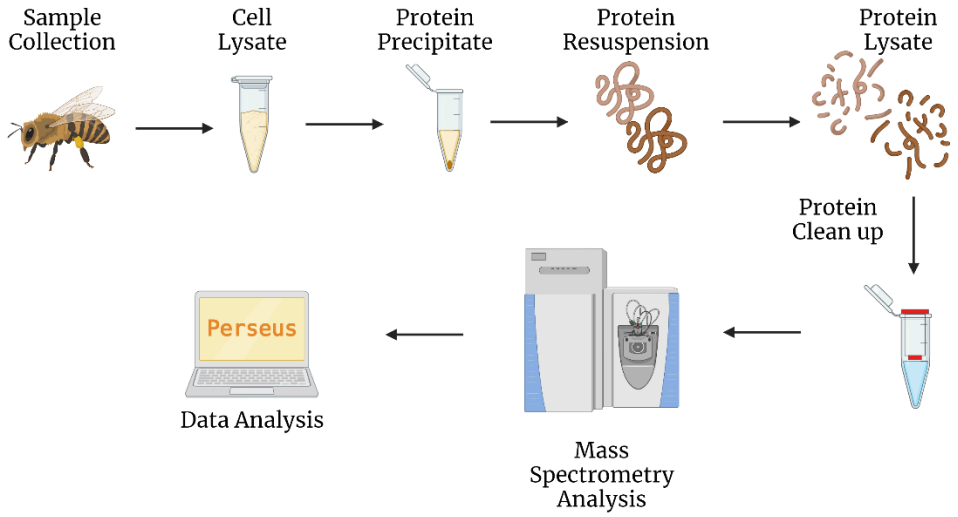


Figure 2.5: General workflow for proteomic analysis on *Apis mellifera* protein samples. Anatomical samples from worker bees are dissected and subjected to protein precipitate and resuspension (Section 2.3.1, 2.3.2, 2.3.3), lysate via trypsin (Section 2.3.4) and clean-up via C18 columns (Section 2.4.1) before being analysed on the mass spectrometer (Section 2.4.2). Data analysis is processed in Max Quant and Perseus along with several other annotative programmes (Section 2.5).

Chapter 3

Utilising Proteomic Analysis to Characterise the Changes in *Apis mellifera* During the Process of Age-Polyethism Throughout the Year

3.1 Introduction

Colonies of *Apis mellifera* act like a superorganism with thousands of bees working together to thrive as a colony and ensure reproduction. Colonies contain thousands of bees, which can be categorised into three castes: a queen, drones, and worker bees. In the summer, honeybee colonies expand both in size and productivity as the queen lays between 1,500 to 3,000 eggs per day (depending on the species), (Hooper, 2010). Worker bees in the summer are tasked with rearing brood, building comb for both brood and honey stores, guarding the hive and foraging for nutrients (Hooper, 2010:33). Drones are the males within the hive and are present in relatively low numbers. The role of drones within the colony is to mate with neighbouring virgin queens within apiaries, ensuring genetic diversity (Hooper, 2010).

Worker bees complete tasks around the hive in an age-related pattern, more formally termed age polyethism, which has been widely studied and analysed through the scope of behavioural changes and differences in anatomical organs (Lindauer and Watkin, 1953; Huang and Robinson, 1996; Amdam and Omholt, 2003; Deseyn and Billen, 2005; Amdam *et al.*, 2009; Johnson, 2010). Previous studies have identified changes to organs and their secretion profiles as worker bees age within the colony. For example, the hypopharyngeal glands found in the heads of honeybees, increase in size in the first 5-10 days post emergence but subsequently decline as worker bees age (Hrassnigg and Crailsheim, 1998; Deseyn and Billen, 2005). The difference in size of the hypopharyngeal glands is not the only change that occurs in worker bees as they age. The protein secretion of the glands also shift in correlation to the age of the worker: in nurse bees, the glands produce proteins (e.g. Major Royal Jelly Protein (MRJP)) used in the production of royal jelly, forager bees however, produce proteins (α-glucosidase and glucosidase oxidase) used for the conversion of nectar to honey (Deseyn and Billen, 2005; Dobritzsch *et al.*, 2019). Work in the first part of this chapter will utilise whole cell lysate protein extraction techniques to investigate changes in protein secretion in the heads of honeybee worker bees as they age and as the colony progresses through the seasons.

Changes to worker bees proteome and physiological state are not limited to age polyethism. Worker bees can be sub-divided into two categories: summer worker bees (display age-polyethism by transitioning from in-hive bees to foragers) and winter

worker bees (do not display age polyethism). Short-lived summer bees have a life span of approximately six weeks, and they undertake brood rearing, building and foraging (Johnson, 2010; Hooper, 2010). Long-lived winter bees (also known as diutinus bees) have a life span of up to six months and engage in thermoregulation over the winter (Maurizio and Hodges, 1950; Omholt and Amdam, 2004). There are several physiological attributes that distinguish diutinus worker bees from summer worker bees such as hypopharyngeal gland size and activity (Brouwers, 1983; Deseyn and Billen, 2005), organ composition, fat body activity (Fluri *et al.*, 1977), and titres of vitellogenin and hexamerin within the haemolymph (Amdam and Omholt, 2002; Lee and Kim, 2017).

3.1.1 Chapter Three Aims

- To determine if proteomic analysis can be used to identify shifts in the proteome of worker bees at different ages.
- Identify potential protein markers for specific ages of worker bees.
- To investigate how seasonal changes, affect the process of age-polyethism within worker bees.

3.2 Experimental Outline

In order to investigate the process of age-polyethism in worker bees, the exact age of the worker bees had to be determined. This was done by trapping the queen on one frame for three days. This frame was marked and left within the hive for a further 13 days. After 13 days the frame was removed and placed in an incubator for 8 days at 37^{o}C. The frames were left in the incubator until all the worker bees emerged from the cells and were subsequently collected into a basin where each bee was marked with a colour bee marker on the top of the thorax. The newly emerged bees were then released back into their respective hives. Initially a trial sampling regime was performed in March whereby bees were sampled at multiple time points (**Table 3.1**). However, after proteomic analysis across the numerous sample points, a future

collection regime was designed for subsequent months (**Table 3.2**). Specific sample dates from April to October is outlined in **Table 3.3**.

Table 3.1: Initial sampling time points for age-polyethism study

Age	Duration of Role
Day 21	0 Days
Day 22 – 24	3 Days
Day 24 - 32	7 Days
Day 32-40	7 Days
Day 40-43	3 Days
Day 43-50	7 Days
Day 50-57	7 Days
Day 57-65	7 Days

Table 3.2: Refined sample time points based on proteomic analysis

Age	Assigned Role
Day 21	Emerged
Day 24- 32	Early
Day 40-43	Late
Day 50-57	Foragers

A single head from each worker bee was used as a representative point for each colony and was subjected to mass spectrometric preparation i.e. protein extraction, clean-up, digestion, and peptide purification (C18). For mass spectrometric analysis 1µg of tryptic peptides were loaded onto a QExactive (ThermoFisher Scientific) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated over a 2-40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100mm length, 75mm ID), using a 135 min reverse-phase gradient flow rate of 250nL min^{-1}. Three hives were used for year-round sampling, each hive was treated as an individual replicate.

Protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.6.6.0 (https://www.maxquant.org/). Data processing and analysis was carried out using Perseus v 1.6.6.0 (https://maxquant.net/perseus/). Protein matrices were filtered for contaminants, reverse and single hit peptides. Proteins not identified in three of the replicas in at least one group were removed from the analysis. Using volcano plot analysis, proteins were deemed statistically significant differentially abundant (SSDA) if identified to have a

Log2 fold change greater than +/- 1 and a -Log p value greater than 1.3. To identify protein-protein interactions, functions, and pathway enrichments KEGG Mapper was utilised (https://www.genome.jp/kegg/tool/map_pathway2.html). To identify protein-protein interactions of SSDA proteins, functions, and pathway enrichment analysis was conducted using STRING v 11 (https://string-db.org/) and g:Profiler gGOst (https://biit.cs.ut.ee/gprofiler/gost) were utilised. g:Profiler term information used in this analysis are outlined as such: Term ID is the unique term identifier for each pathway/term. Term size refers to the number of genes that are annotated to the specific term. Query size is the number of genes that were included in the query run. Intersection size is the number of genes in the input query that are annotated to the corresponding term. The adjusted p-value is the hypergeometric p-value after correction for multiple testing. The user-threshold for each of the query runs is 0.05 (Raudvere *et al.*, 2019).

Table 3.3: Sampling dates for worker bees in each month from March to October 2019 that are included in the age-polyethism investigation.

Month	Caged Q	Release Q	Incubator	Emerged	Nurses	Builders	Foragers	Colour
March	15-Mar	17-Mar	02-Apr	19-Apr	26-Apr	03-May	17-May	White
April	16-Apr	18-Apr	03-May	07-May	17-May	24-May	31-May	Yellow
May	15-May	17-May	05-Jun	07-Jun	14-Jun	21-Jun	28-Jun	Blue
June	25-Jun	27-Jun	15-Jul	17-Jul	26-Jun	08-Aug	18-Aug	White
July	29-Jul	31-Jul	16-Aug	18-Aug	29-Aug	05-Sep	12-Sep	Blue
August	02-Sep	05-Sep	20-Sep	24-Sep	07-Oct	14-Oct	21-Oct	Green
September	23-Sep	26-Sep	11-Oct	15-Oct	25-Oct	01-Nov	08-Nov	White
October	14-Oct	16-Oct	03-Nov	05-Nov	15-Nov	22-Nov	29-Nov	Blue

3.3 Results

3.3.1 Investigating proteomic changes during the process of age polyethism

3.3.1.1 Initial Sampling

LC-MS/MS was performed with single head analysis on all samples outlined in **Table 3.1**. Initial sampling in March 2019 was performed on individual worker bees from a single hive and was therefore not completed in triplicate. This was to determine if proteomics could be used as a tool to identify age-related changes in worker bees. Principal Component Analysis (PCA) was conducted on all samples to determine the relationship between the proteomes of samples. Interestingly, samples were organised into groups of three based on the age of worker bees – emerged, early hive, late hive and foragers (**Table 3.2**) as seen in **Figure 3.1**. This provided evidence that whole cell lysate for proteomic analysis could be used to analyse worker bees at various ages to determine the shifts in the proteome. Sampling regime was adapted for the remainder of the trial to reduce the impact of sampling on the colonies, samples were collected from April 2019 to October 2019 as outlined in **Table 3.2.**

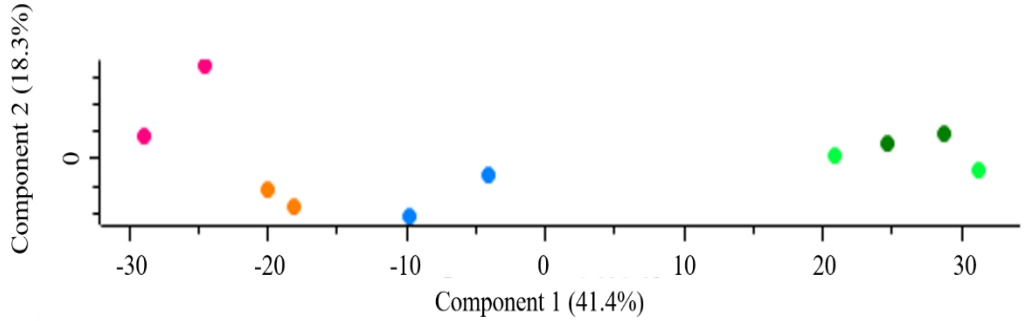


Figure 3.1: PCA graph from samples collected from a single hive in March 2019. Age categories are colour co-ordinated: Emerged - Pink, Early hive - Orange, Late hive – Blue, Forgers – Green. Total sample variation on the PCA is 59.7%

3.3.1.2 Mapping Age Polyethism

To map the process of age polyethism in *A. mellifera* samples were taken from three individual hives within one apiary at specific time points of worker bees lives (**Table 3.2**). This section will present the results of these sampling data points between April to October 2019.

LC-MS/MS data from each sample was uploaded into a data matrix in Perseus and subjected to several filtration steps, the size reduction for post filtration was logged (**Table 3.4**)**.** Filtration steps were completed in Perseus to remove contaminants, reverse hit proteins, and peptides identified by site. The data matrix is Log_2 transformed and proteins absent in all three replicates are removed, ANOVA test was then calculated (p-value = 0.1). This resulted in a reduced, filtered matrix that was used for further proteomic analysis. Characterisation of samples was conducted by comparing the four age categories, ranking the proteins based on relative fold change, identifying the most abundant proteins within each age group, analysing major protein pathways and tracking proteomic shifts throughout the months of the trial.

Table 3.4: Protein matrix numbers before and after filtration and ANOVA testing. Data collected is from each individual month analysis.

Months	April	May	June	July	August	September	October
Pre filtration	2,702	2,896	3,342	3,198	2,893	3,346	3,485
Post filtration	1,891	1,977	1,917	2,000	1,903	2,184	2,355
Post-ANOVA	760	683	500	456	374	1,018	1,125
SSDA	296	475	270	217	152	409	483

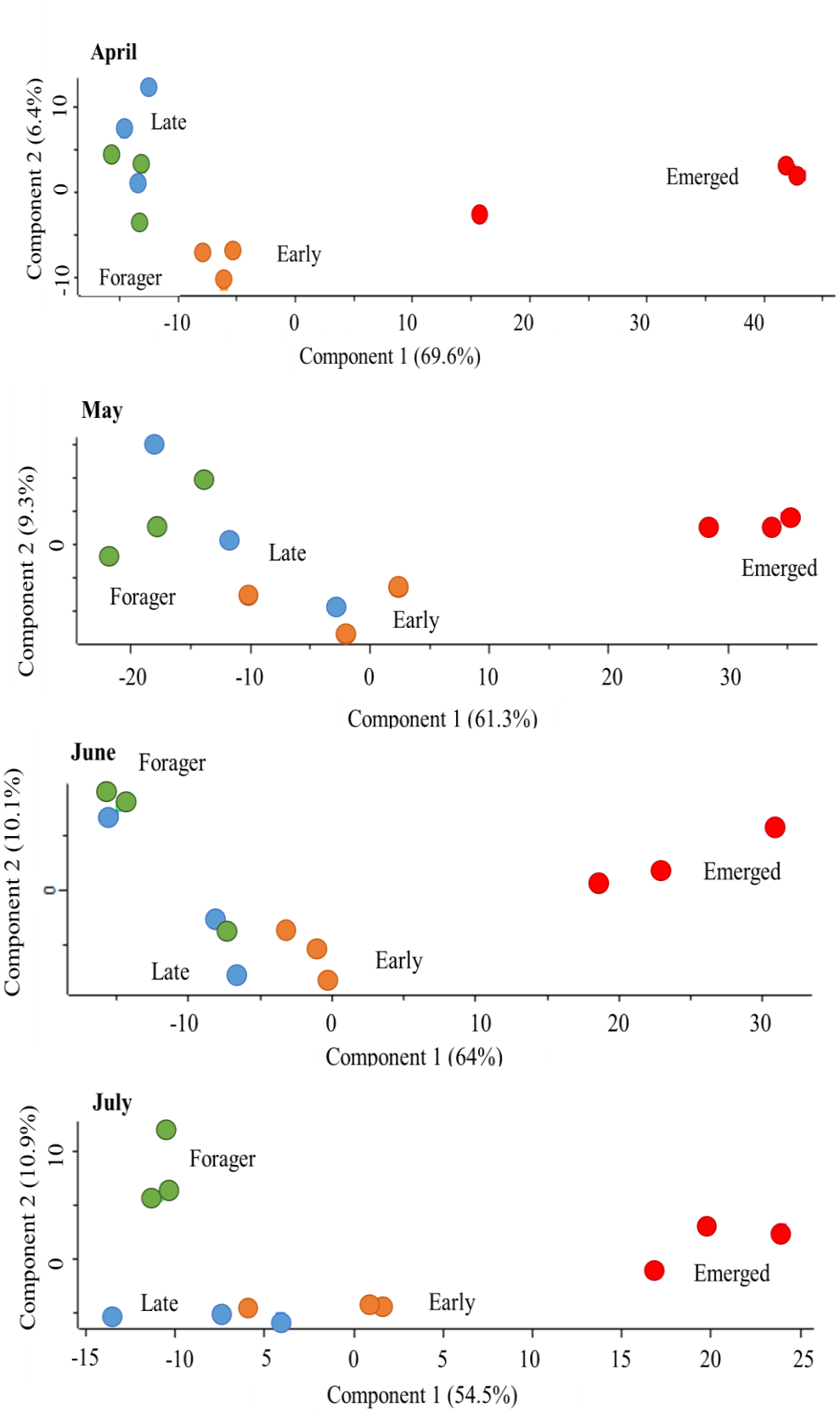
Principal component analysis was preformed to identify the relationship of the proteomes between each sample within the months (**Figure 3.2**). The proteomes of Emerged bees (highlighted in red, **Figure 3.2**) were consistently clustered separately from early hive (orange), late hive (blue), and forager (green) samples, demonstrating

a significant degree of proteomic separation. Hive and forager samples were arranged into two patterns;

1) The proteomes of hive (early and late) worker bees were clustered together and foragers were in a third separate cluster.
2) Early hive worker bees proteomes were in a defined cluster and late hive and forager samples were in a third cluster together.

There are two interesting exceptions to these patterns. The first was in June, a single forager sample was clustered alongside early hive and two late hive worker bees. The hive where this forager sample was collected experienced a queen loss during this month of sampling. Disruption due to the queen's absence in a colony could have caused the forager to return to the hive and shift behavioural tendencies to in-hive roles, subsequently affecting the natural progression of age-polyethism. However, a full investigative study would need to be completed to understand this further. The second exception to the arrangement patterns was October: hive (early and late) samples were both clustered with forager samples, in one large cluster, and separated from emerged bee samples (**Figure 3.2**).

The arrangement of the samples from April to July clearly grouped bees based on similar ages and their subsequent roles within the colony. In contrast August and September PCAs show that the proteome of the forager and the late hive bees share a similar proteome compared to forager and late hive bee proteomes in earlier month PCAs. The PCA from October samples clearly highlights a similarity in protein expression involving all hive and forager samples as they are clustered close together. The overall degree of separation in October's PCA is 73.7% demonstrating a large difference in protein profiles between emerged bee samples compared to hive and forager bee samples, as these samples are clustered separately. The high overall degree of separation in the October PCA highlights how similar the proteomes of hive and forager bee samples are to each other.


April
Late
Emerged
Early
Forager
Component 2 (6.4%)
Component 1 (69.6%)
May
Late
Emerged
Forager
Early
Component 2 (9.3%)
Component 1 (61.3%)
June
Forager
Emerged
Late
Early
Component 2 (10.1%)
Component 1 (64%)
July
Forager
Emerged
Late
Early
Component 2 (10.9%)
Component 1 (54.5%)

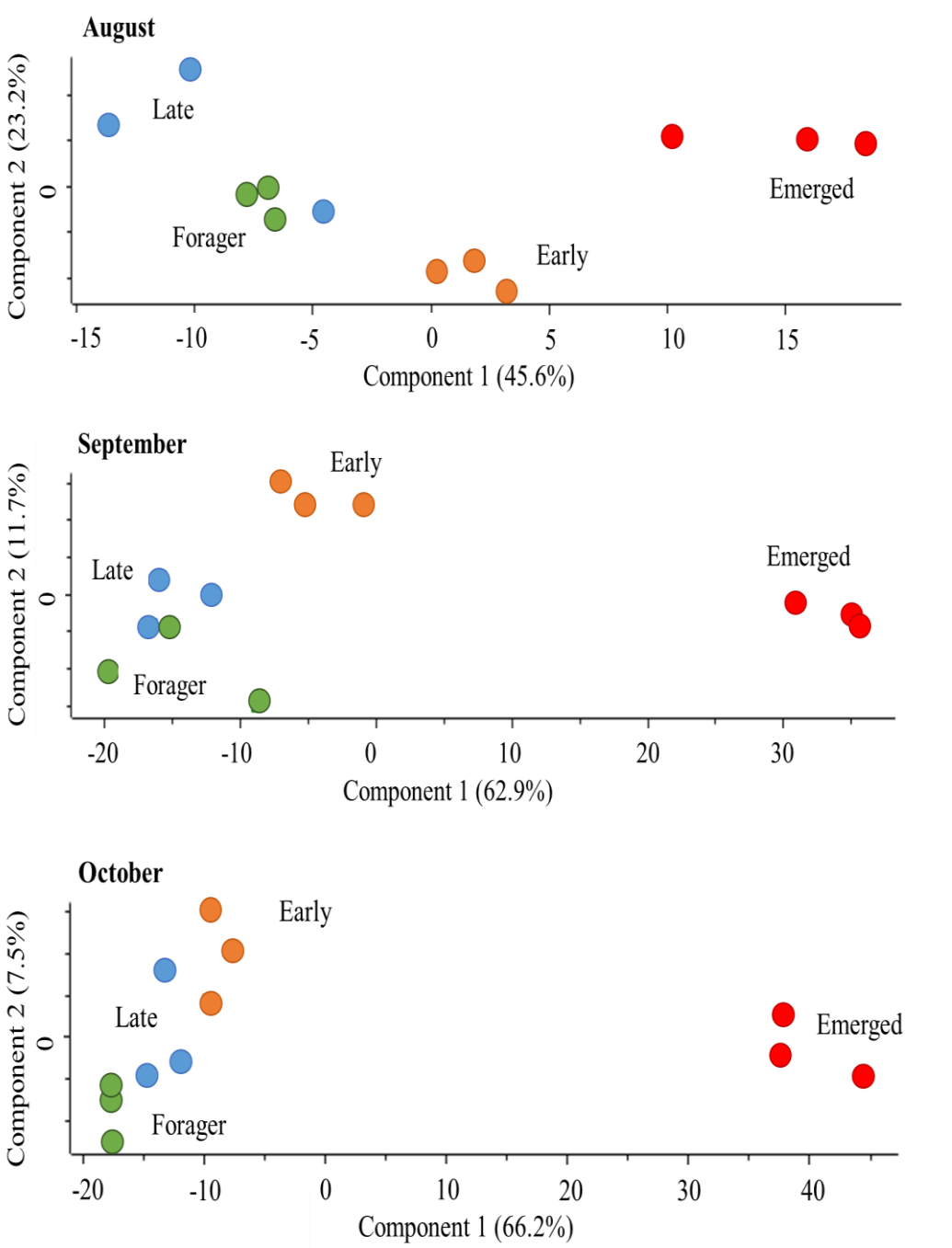


Figure 3.2: All PCA graphs from Perseus analysis from March to October in 2019. Age categories are colour co-ordinated: Emerged – Red, Early hive – Orange, Late hive – Blue, Forager – Green. The total explained variance of this PCA for the given datasets is the combined value of Component 1 and Component 2.

3.3.1.3 Protein expression

Analysis of the PCAs above highlight the relationship of proteome expression between samples by demonstrating how similar some samples are to the samples from other age groups. However, how the protein profile changes between ages needed to be investigated to gain an insight into what contributes to the separation in sample clustering in the PCAs. Protein LFQ intensity matrices were subjected to ANOVA significant t test (p value = 0.01), and significant proteins were analysed to characterise the shifts in protein expression. Proteins were identified using volcano plot comparisons as a guide to determine what proteins were higher in relative abundance between samples. This provided an insight into the expression of the proteins as the worker bees progress through the process of age polyethism. Samples were pooled together into two groups for ease of data analysis: April through to June and July through to October. This grouping provided further insight into the changes that occur due to age polyethism as the colonies progress through the seasons.

3.3.1.3.1 Short chain dehydrogenase

Short chain dehydrogenase reductase (SCDR) are enzymes found at high levels in honeybee larvae, are regulated through hormone expression, and play a role in metabolism of pollen rich food (Guidugli *et al.*, 2004). SCDR was identified as one of the most abundant proteins in emerged bee samples across all months in the analysis. Mean relative fold change in abundance of SCDR was 120.65 for all comparisons between emerged bee samples to hive (early and late) and forager bee samples. LFQ intensity values of SCDR were used to determine if there was a significant increase in protein expression in emerged bee samples (**Figure 3.3**). The data found that SCDR was significantly higher in emerged worker bees in April, May, July, September and October. There was no significant increase in SCDR expression in June and SCDR was not deemed SSDA in August analysis.

3.3.1.3.2 Hexamerin

Hexamerins (HEXs) are well-studied proteins which are abundantly expressed in larval fat body used for amino acid storage, insect development and JH binding (Lee and Kim, 2017). HEXs were identified as ANOVA significant ($p = 0.1$) in several months sampled between April and October (**Figure 3.4**). Overall, HEX protein LFQ intensity values were higher in emerged samples in comparison to hive and forager samples, but no significant changes in protein expression were observed. Three HEX proteins were identified in this analysis: 110, 70c and 70b. No HEX proteins were identified as SSDAs in June, July and August.

3.3.1.3.3 Major Royal Jelly Proteins

Several major royal jelly proteins were identified at high abundances in early hive worker bees in volcano plot comparisons (**Supp File 3.1 & 3.2**) The average of LFQ intensity values for MRJ proteins were analysed to determine the patterns of protein expression across samples collected (**Figure 3.5**). Intensity levels rise from the emerged to early bee samples and remain relatively high from early to forager bee samples. August MRJ protein intensities were highest in emerged samples and dropped in hive and forager samples which was not observed in other months.

3.3.1.3.4 Odorant Binding Protein

Several odorant binding proteins (OBPs) were identified as ANOVA significant throughout the multiple comparisons within each month (**Figure 3.6**). OBP 13 is higher in abundance in the majority of emerged bee samples than in hive and forager bee samples. Whereas, OBP 21 is identified in higher abundance in hive and forager bee samples compared to emerged worker bees. Interestingly, there was a shift in ANOVA significant proteins between the earlier months of the year (April to June) and the later months of the year (July to October). As the earlier months have more ANOVA significant OBPs than the later months.

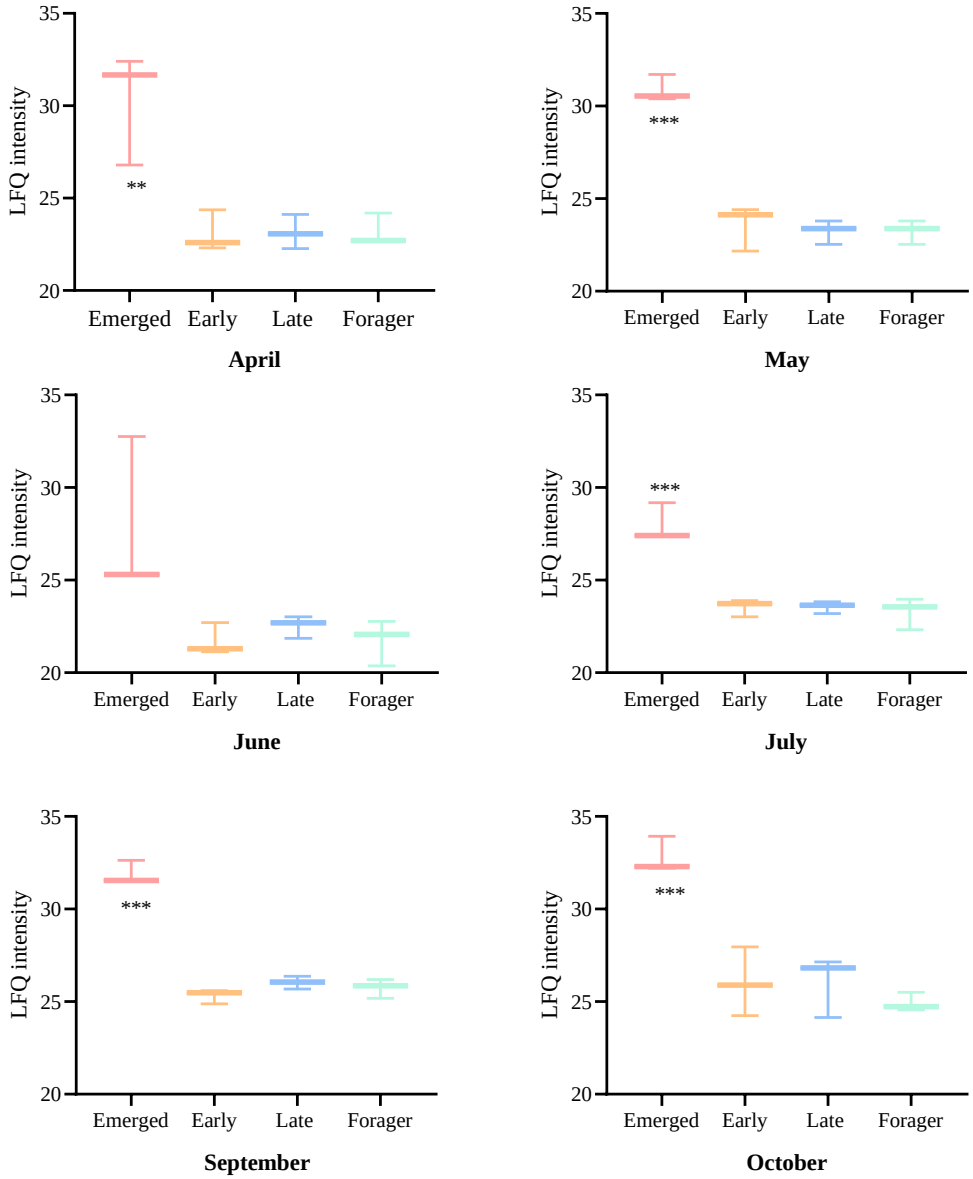


Figure 3.3: Protein intensities on short chain dehydrogenase reductase. Across the months from April to October excluding the month of August. These proteins are ANOVA significant (p-value >0.1). Significance is represented by 0 '***', 0.001 '**', 0.1 '* '.

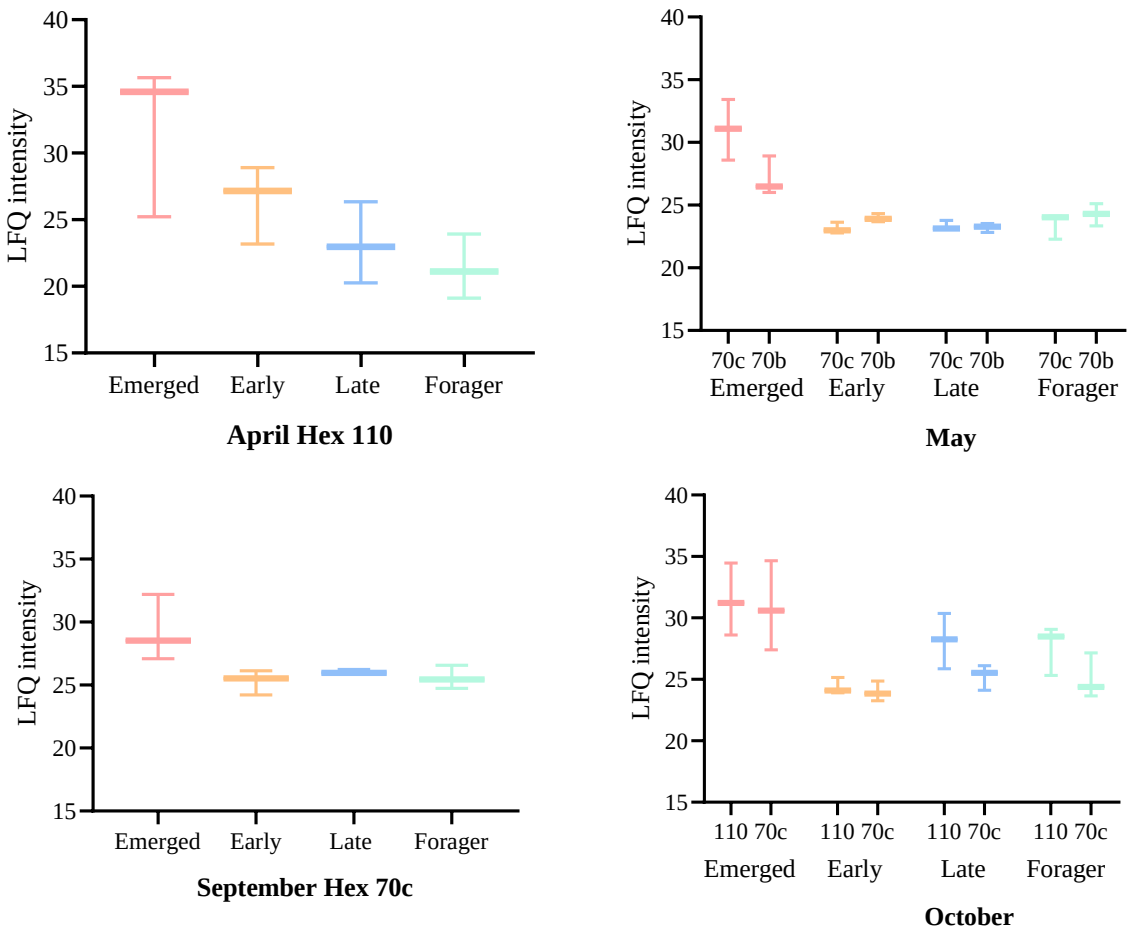


Figure 3.4: LFQ protein intensities on HEX proteins. HEX proteins were identified as SSDAs in April, May, September and October. These proteins are ANOVA significant (p-value >0.1). There is a general trend in increased protein intensity in emerged samples. Significance is represented by 0 '***', 0.001 '**', 0.1 '* '.

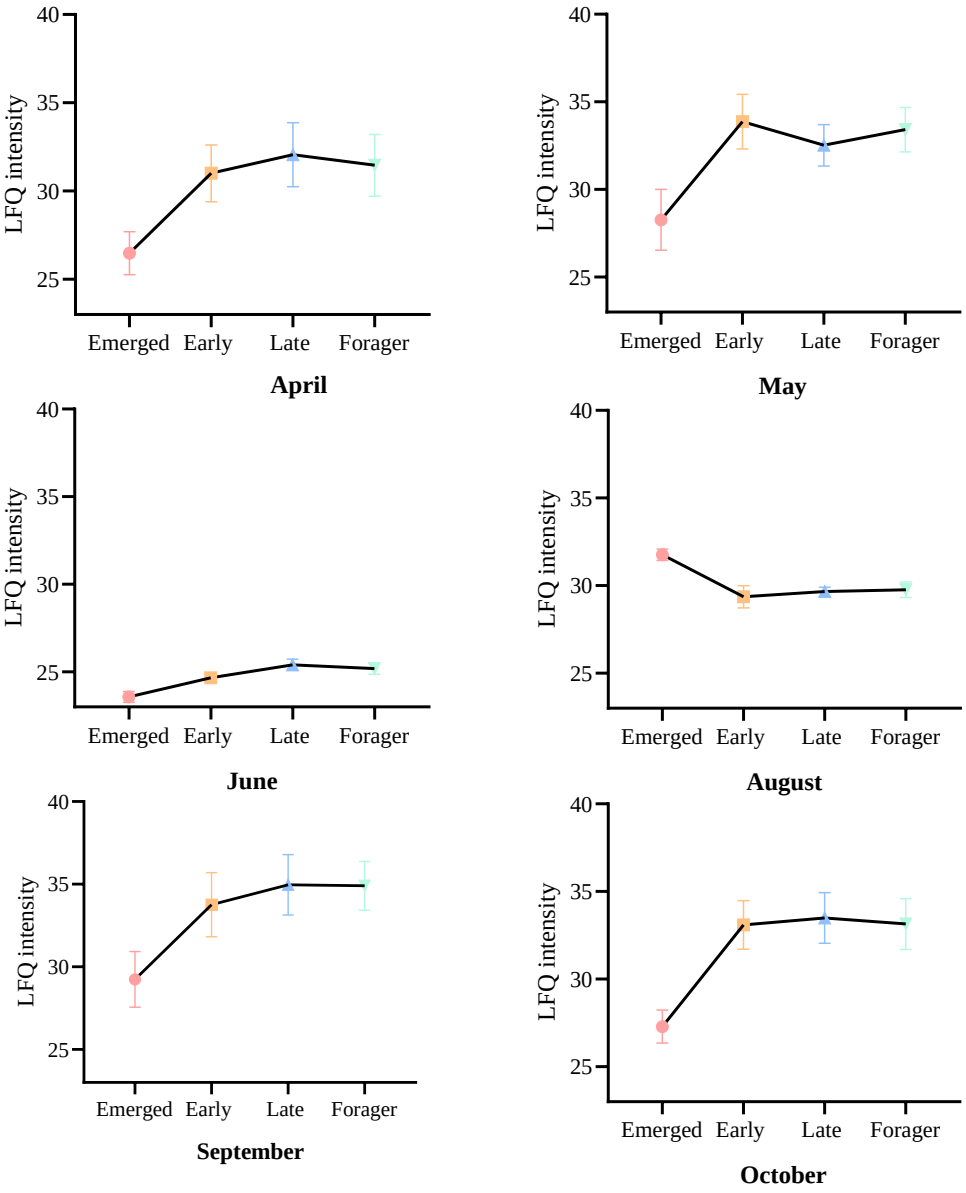


Figure 3.5: MRJ LFQ protein intensity values plotted on a line graph. All SSDA MRJ proteins were averaged and plotted.

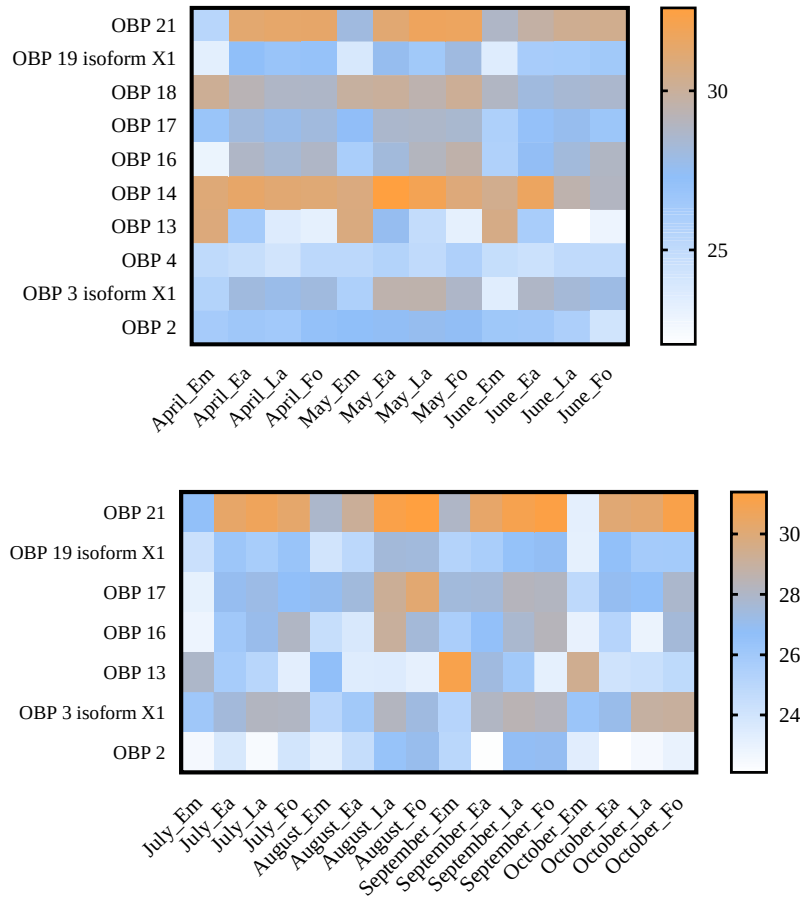


Figure 3.6: LFQ protein intensity values of all OBP proteins identified as ANOVA (p = 0.1). Samples coloured in orange have a high protein intensity value and samples in light blue/white have a low protein intensity value.

3.3.1.3.5 Vitellogenin

Volcano plot analysis (**Supp File 3.1 & 3.2**) did not identify vitellogenin as an SSDA in the majority of age comparisons of samples within the months. Vitellogenin was however identified as an SSDA in the samples from August to October. With the highest values of relative abundance being observed in October late hive samples when compared to October emerged bee samples.

3.3.1.3.6 Cuticular proteins

Across all the sample comparisons within the same months cuticular proteins were more abundantly expressed in emerged bees in comparison to hive and forager bees. A significant increase in cuticular proteins was observed in April emerged bee

samples in comparison to hive and forager bee samples (One-way ANOVA, Tukey's multiple comparison test, $p < 0.05$). No cuticular proteins were observed in August.

Apidermin 3 was identified as one of the most abundant proteins in the emerged bee samples in comparison to hive and forager bee samples across the comparisons within each month (**Supp File 3.1 & 3.2**). LFQ intensity values were used to plot the changes in protein abundance within each sample (**Figure 3.8**). Apidermin 3 was not identified as ANOVA significant in August or October. However, apidermin 2 was identified in October only and not in any of the other months. Apidermin 2 was increased in abundance hive and forager samples in comparison to emerged bee samples. The protein shift in expression between apidermin 3 to apidermin 2 provides an insight into the shift of the protein levels in workers emerging in later months.

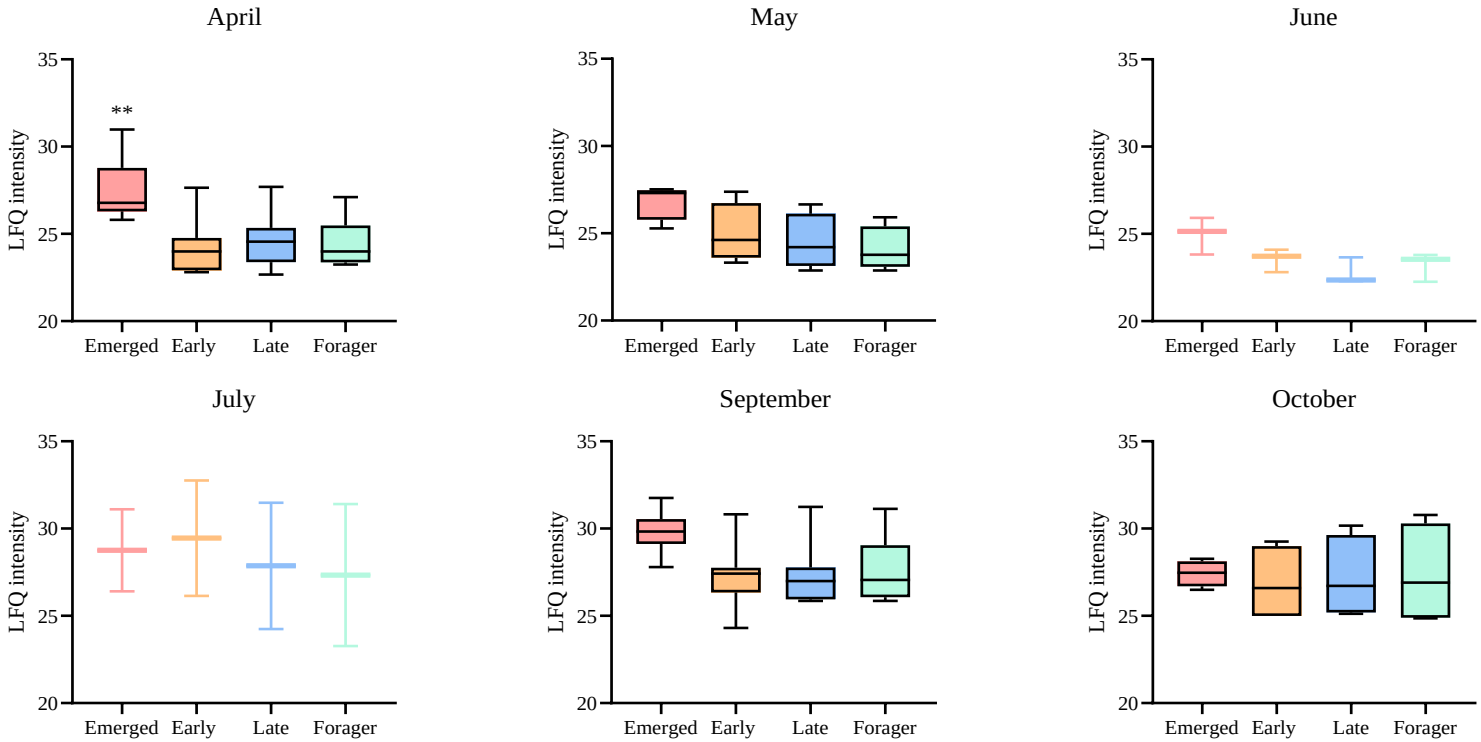


Figure 3.7: Boxplots of cuticular proteins LFQ intensity values. Significance calculated through Tukey's multiple comparison test: significance is represented by 0 '***', 0.001 '**', 0.1 '* '.

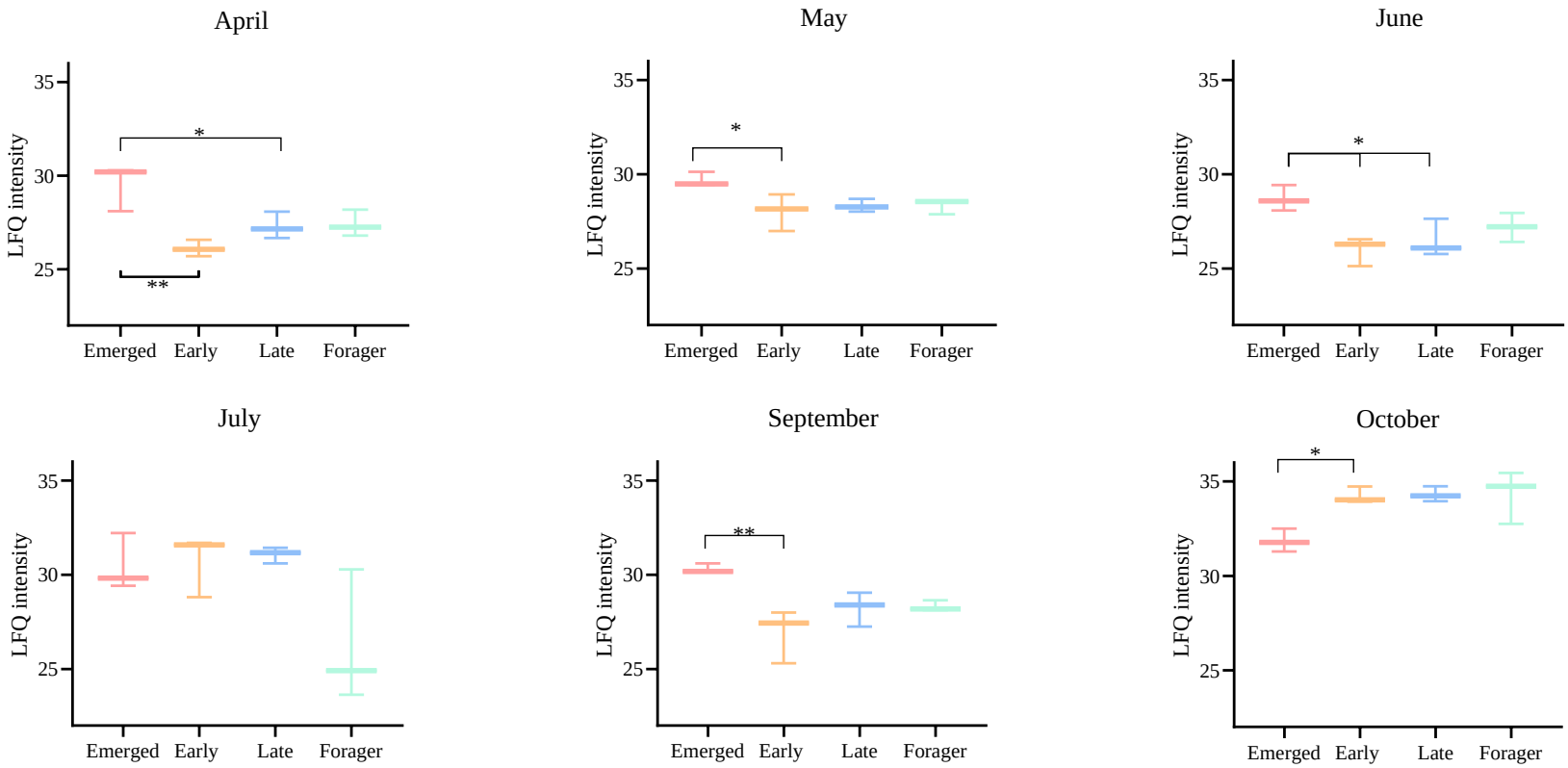


Figure 3.8: Boxplots of Apidermin 3 (April to September) and Apidermin 2 (October) LFQ intensity values. Significance calculated through Tukey's multiple comparison test: significance is represented by 0 '***', 0.001 '**', 0.1 '* '.

3.3.1.3.7 Honey Production Enzymes

As opposed to the cuticular protein levels, honey production enzymes such as alpha glucosidase, alpha amylase etc are expressed more abundantly in hive and forager samples in comparison to emerged worker bees. α-amylase, α-glucosidase and glucose oxidase proteins were observed as SSDAs throughout the months of April to October. An increasing trend of averaged LFQ intensity values of honey production enzymes identified within each month was observed from emerged worker bees up to forager bees, although no significant increase was observed (**Figure 3.9**). Honey production enzymes were also observed as increased in abundance in late hive and forager bee samples in comparison to emerged and early hive bee samples (**Supp file 3.1 & 3.2)**.

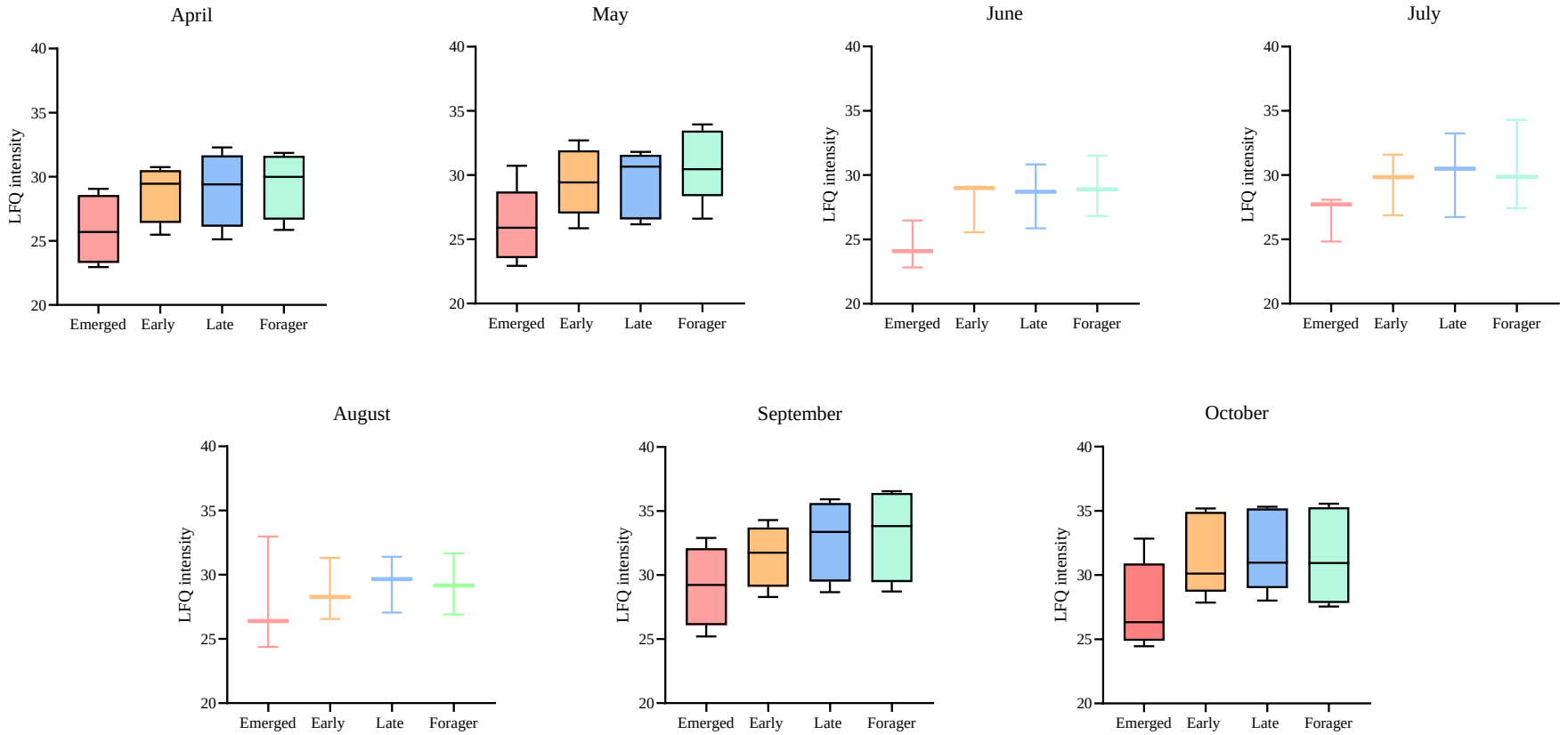


Figure 3.9: Boxplots on LFQ intensity values of honey production enzymes (α-glucosidase, α-amylase) identified through April to October.

To gain an insight into the protein expression profile proteins were collated into a table and numbered based on the abundance of the proteins in each sample age (**Table 3.5**). SCDR, OBP 13, Apidermin 2 and other cuticular proteins were most abundant in emerged worker bees. Major royal jelly proteins were more abundant in hive samples in comparison to emerged bees. Apidermin 3 and OBP 19 and 21 were most abundant in forager samples compared to hive and emerged bee samples.

Table 3.5: Potential protein markers as indicators to the life stages of age-polyethism. Annotations used to highlight the abundance of proteins with each age group: ‘1‘ low, ‘2’ medium, ‘3’ high, ‘4’ very high.

Protein	Protein ID	Emerged	Early	Late	Forager
Short chain dehydrogenase reductase	A0A088ARX6	4	1	1	1
alpha-glucosidase	Q17058	1	2	3	4
alpha-amylase	Q9U8X5	1	2	3	4
Major royal jelly protein 1	O18330	1	4	4	3
Major royal jelly protein 2	O77061	1	4	2	2
Major royal jelly protein 3	D3Y5T0	1	3	4	2
Major royal jelly protein 5	O97432	1	4	3	2
Major royal jelly protein 6 precursor	A0A088AU21	1	4	2	2
Major royal jelly protein 7 precursor	A0A088AU21	1	4	3	2
Major royal jelly protein 9 precursor	Q4ZJX1	1	2	2	2
Odorant binding protein 2	Q9U9J5	1	1	3	3
Odorant binding protein 3 isoform X1	Q1W647	1	2	2	2
Odorant binding protein 13	Q1W641	4	2	2	1
Odorant binding protein 14	A0A088A4K9	2	2	2	2
Odorant binding protein 16	Q1W638	1	2	2	2
Odorant binding protein 17	Q1W637	1	3	3	3
Odorant binding protein 18	A0A088A4L3	3	2	2	1
Odorant binding protein 19	A0A088AL4	1	2	3	4
Odorant binding protein 21	Q1W633	1	2	3	4
Cuticular proteins	NA	4	3	2	2
Apidermin 3	A0A088ANC9	4	3	3	1
Apidermin 2	A0A088AND4	1	2	3	4

3.3.2 Pathway expression pathway analysis on age polyethism samples

Volcano plot analysis (**Supp File 3.1 & 3.2**) was conducted on all samples within the same month (i.e. April emerged samples were compared to early, late and forager samples also collected in April). Proteins were deemed statistically significant differentially abundant (SSDA) if identified to have a relative Log_2 fold change greater than +/- 2 and a -Log p value greater than 1.3. All SSDAs were then used for pathways analysis using hierarchical clustering in Perseus, the number of SSDAs are outlined in **Table 3.4**. Across all months hierarchical clustering grouped samples in very similar patterns – emerged bees in one cluster and hive and foragers bees in a second. Protein clusters from each month were selected and used for pathway analysis to gain an overview to the protein expressions in each cluster. The top three pathways from each GO analysis (GOBP, GOMF, GOCC, and KEGG) were used to highlight proteomic expressions in sample groups (**Table 3.6 to Table 3.12**) (**Supp. File 3.3**).

Table 3.6: Hierarchical clustering Gene Ontology analysis of protein clusters using the SSDAs from the volcano plot comparisons from samples collected in April.

Age group	GO Term	Term Name	Term ID	Adjusted p-value	Term size	Query Size	Intersection Size
Emerged	GO:MF	Unfolded Protein Binding	GO:0051082	3.2E-04	26	150	7
	GO:MF	RNA Binding	GO:0003723	2.2E-04	305	150	21
	GO:MF	Chitin Binding	GO:0008061	5.3E-04	52	150	8
Hive and Forager	GO:MF	Catalytic Activity	GO:0003824	2.2E-09	2512	62	52
	GO:MF	Oxidoreductase Activity	GO:0016491	8.0E-09	414	62	22
	GO:MF	Isomerase Activity	GO:0016853	1.3E-03	72	62	7
	GO:BP	Generation of Precursor Metabolites and Energy	GO:0006091	4.1E-05	83	44	9
	GO:BP	Carbohydrate Metabolic Process	GO:0005975	8.3E-05	151	44	11
	GO:BP	Small Molecule Metabolic Process	GO:0044281	1.8E-04	330	44	15
	KEGG	Metabolic Pathways	KEGG:01100	4.7E-03	487	33	27
	KEGG	Biosynthesis of Nucleotide Sugars	KEGG:01250	7.7E-03	16	33	5
	KEGG	Galactose Metabolism	KEGG:00052	8.0E-03	10	33	4

Table 3.7: Hierarchical clustering Gene Ontology analysis of protein clusters using the SSDAs from the volcano plot comparisons from samples collected in May

Age group	GO Term	Term Name	Term ID	Adjusted p-value	Term size	Query Size	Intersection Size
Emerged	GO:MF	Guanyl Ribonucleotide Binding	GO:0032561	7.018E-05	157	227	21
	GO:MF	Guanyl Nucleotide Binding	GO:0019001	7.823E-05	158	227	21
	GO:MF	GTP Binding	GO:0005525	2.167E-04	154	227	20
Hive and Forager	GO:MF	Oxidoreductase Activity	GO:0016491	2.9E-14	113	37	414
	GO:MF	Catalytic Activity	GO:0003824	1.6E-09	113	83	2512
	GO:MF	Oxidoreductase Activity, Acting on the CH-OH Group Of Donors, NAD Or NADP as Acceptor	GO:0016616	8.6E-06	113	9	42
	GO:BP	Generation Of Precursor Metabolites And Energy	GO:0006091	3.4E-23	83	76	25
	GO:BP	ATP Metabolic Process	GO:0046034	1.0E-15	52	76	17
	GO:BP	Cellular Respiration	GO:0045333	1.9E-12	44	76	14
	GO:CC	Extracellular Region	GO:0005576	6.0E-10	177	49	18
	GO:CC	Mitochondrion	GO:0005739	1.7E-05	192	49	14
	GO:CC	Respirasome	GO:0070469	2.8E-04	31	49	6
	KEGG	Oxidative Phosphorylation	KEGG:00190	3.3E-10	73	60	19
	KEGG	Carbon Metabolism	KEGG:01200	1.3E-07	51	60	14
	KEGG	Biosynthesis of Amino Acids	KEGG:01230	4.6E-05	30	60	9

Table 3.8: Hierarchical clustering Gene Ontology analysis of protein clusters using the SSDAs from the volcano plot comparisons from samples collected in June

Age group	GO Term	Term Name	Term ID	Adjusted p-value	Term size	Query Size	Intersection Size
Emerged	GO:MF	Chitin Binding	GO:0008061	0.0071	52	114	7
	GO:MF	Unfolded Protein Binding	GO:0051082	0.0155	26	114	5
	GO:MF	Nucleoside-Triphosphatase Activity	GO:0017111	0.0161	231	114	14
Hive and Forager	GO:MF	Oxidoreductase Activity	GO:0016491	1.2E-09	414	84	27
	GO:MF	Catalytic Activity	GO:0003824	2.1E-07	2512	84	63
	GO:MF	Oxidoreductase Activity, Acting on the CH-OH Group Of Donors, NAD Or NADP as Acceptor	GO:0016616	2.3E-05	42	84	8
	GO:BP	Generation Of Precursor Metabolites and Energy	GO:0006091	4.3E-22	83	56	22
	GO:BP	ATP Metabolic Process	GO:0046034	2.8E-13	52	56	14
	GO:BP	Aerobic Respiration	GO:0009060	4.3E-13	42	56	13
	GO:CC	Mitochondrion	GO:0005739	9.4E-07	192	34	13
	GO:CC	Respirasome	GO:0070469	3.1E-05	31	34	6
	GO:CC	Proton-Transporting Two-Sector ATPase Complex	GO:0016469	4.2E-04	27	34	5
	KEGG	Metabolic Pathways	KEGG:01100	1.7E-10	487	44	37
	KEGG	Oxidative Phosphorylation	KEGG:00190	2.1E-08	73	44	15
	KEGG	Carbon Metabolism	KEGG:01200	2.9E-07	51	44	12

Table 3.9: Hierarchical clustering Gene Ontology analysis of protein clusters using the SSDAs from the volcano plot comparisons from samples collected in July

Age group	Term	Term Name	Term ID	Adjusted p-value	Term size	Query Size	Intersection Size
Emerged	GO:MF	Oxidoreductase Activity, Acting on the CH-CH Group Of Donors	GO:0016627	0.012	26	104	5
	GO:MF	Structural Molecule Activity	GO:0005198	0.034	204	104	12
	GO:MF	Collagen Trimer	GO:0005581	0.019	2	55	2
Hive and Forager	GO:MF	Oxidoreductase Activity	GO:0016491	0.00017	414	57	16
	GO:MF	Alpha-1,4-Glucosidase Activity	GO:0004558	0.01671	2	57	2
	GO:MF	Maltose Alpha-Glucosidase Activity	GO:0032450	0.01671	2	57	2
	GO:BP	Cellular Respiration	GO:0045333	0.00460	44	32	5
	GO:BP	Respiratory Electron Transport Chain	GO:0022904	0.00636	23	32	4
	GO:BP	Glutamine Family Amino Acid Biosynthetic Process	GO:0009084	0.00638	8	32	3
	GO:CC	Cytochrome Complex	GO:0070069	0.01024	18	21	3
	GO:CC	Mitochondrial Respirasome	GO:0005746	0.01024	18	21	3
	GO:CC	Mitochondrial Protein-Containing Complex	GO:0098798	0.01438	51	21	4
	KEGG	Longevity Regulating Pathway - Multiple Species	KEGG:04213	0.02275	21	30	4
	KEGG	Galactose Metabolism	KEGG:00052	0.02792	10	30	3
	KEGG	Metabolic Pathways	KEGG:01100	0.02828	487	30	19

Table 3.10: Hierarchical clustering Gene Ontology analysis of protein clusters using the SSDAs from the volcano plot comparisons from samples collected in August

Age	Term	Term Name	Term ID	Adjusted p-value	Term size	Query Size	Intersection Size
Emerged	GO:MF	acyl-CoA dehydrogenase activity	GO:0003995	0.0026	6	53	3
	GO:MF	Flavin Adenine Dinucleotide Binding	GO:0050660	0.0296	58	53	5
	GO:MF	5'-3' DNA helicase activity	GO:0043139	0.0463	3	53	2
	GO:CC	R2TP complex	GO:0097255	0.0034	2	23	2
	GO:CC	NuA4 histone acetyltransferase complex	GO:0035267	0.0499	6	23	2
Early Hive	GO:MF	Oxidoreductase Activity	GO:0016491	5.18E-05	414	17	9
	GO:MF	Oxidoreductase Activity, Acting on NAD(P)H	GO:0016651	4.29E-03	25	17	3
	GO:MF	FMN Binding	GO:0010181	2.13E-02	8	17	2
	GO:BP	Protein Localization to Endoplasmic Reticulum Exit Site	GO:0070973	1.12E-03	2	10	2
	GO:BP	Pyridine-Containing Compound Metabolic Process	GO:0072524	4.99E-02	10	10	2
	GO:BP	Pyridine-Containing Compound Biosynthetic Process	GO:0072525	4.99E-02	10	10	2
	GO:CC	Integral Component of Membrane	GO:0016021	2.26E-02	1430	18	15
	GO:CC	Intrinsic Component of Membrane	GO:0031224	2.30E-02	1432	18	15
	KEGG	Oxidative Phosphorylation	KEGG:00190	2.23E-02	73	10	4
Late hive and Foragers	GO:BP	Lipid Transport	GO:0006869	0.021	43	23	4
	GO:BP	Lipid Localization	GO:0010876	0.027	46	23	4
	GO:BP	Proline Biosynthetic Process	GO:0006561	0.050	4	23	2
	GO:CC	Extracellular Region	GO:0005576	0.000	177	13	8
	KEGG	Metabolic Pathways	KEGG:01100	0.002	487	14	12
	KEGG	Arachidonic Acid Metabolism	KEGG:00590	0.038	7	14	2

Table 3.11: Hierarchical clustering Gene Ontology analysis of protein clusters using the SSDAs from the volcano plot comparisons from samples collected in September

Age group	GO Term	Term Name	Term ID	Adjusted p-value	Term size	Query Size	Intersection Size
Emerged	GO:MF	Structural Molecule Activity	GO:0005198	0.0011	204	191	20
	GO:MF	Chitin Binding	GO:0008061	0.0054	52	191	9
	GO:MF	Extracellular Matrix Structural Constituent	GO:0005201	0.0500	5	191	3
Hive and Forager	GO:MF	Oxidoreductase Activity	GO:0016491	1.56E-09	414	74	25
	GO:MF	Catalytic Activity	GO:0003824	4.74E-05	2512	74	53
	GO:MF	Oxidoreductase Activity, Acting on CH-OH Group of Donors	GO:0016614	2.53E-03	65	74	7
	GO:BP	Generation of Precursor Metabolites and Energy	GO:0006091	6.49E-09	83	44	12
	GO:BP	Aerobic Respiration	GO:0009060	7.59E-08	42	44	9
	GO:BP	Cellular Respiration	GO:0045333	1.19E-07	44	44	9
	GO:CC	Extracellular Region	GO:0005576	4.14E-09	177	36	15
	GO:CC	Mitochondrion	GO:0005739	1.25E-03	192	36	10
	KEGG	Citrate Cycle (TCA Cycle)	KEGG:00020	7.58E-08	17	39	8
	KEGG	Carbon Metabolism	KEGG:01200	1.01E-05	51	39	10
	KEGG	Metabolic Pathways	KEGG:01100	3.27E-05	487	39	28

Table 3.12: Hierarchical clustering Gene Ontology analysis of protein clusters using the SSDAs from the volcano plot comparisons from samples collected in October

Age group	GO Term	Term Name	Term ID	Adjusted p-value	Term size	Query Size	Intersection Size
Emerged	GO:MF	RNA Binding	GO:0003723	0.0026	305	230	28
Hive and Forager	GO:MF	Oxidoreductase Activity	GO:0016491	3.57E-13	414	96	33
	GO:MF	Proton Transmembrane Transporter Activity	GO:0015078	4.14E-10	44	96	12
	GO:MF	Electron Transfer Activity	GO:0009055	9.86E-10	47	96	12
	GO:BP	Generation Of Precursor Metabolites And Energy	GO:0006091	4.73E-24	83	63	24
	GO:BP	Cellular Respiration	GO:0045333	1.40E-20	44	63	18
	GO:BP	Aerobic Respiration	GO:0009060	3.22E-19	42	63	17
	GO:CC	Mitochondrion	GO:0005739	1.99E-10	192	55	20
	GO:CC	Extracellular Region	GO:0005576	4.79E-10	177	55	19
	GO:CC	Cytochrome Complex	GO:0070069	7.06E-09	18	55	8
	KEGG	Metabolic Pathways	KEGG:01100	1.16E-10	487	54	43
	KEGG	Citrate Cycle (TCA cycle)	KEGG:00020	1.45E-06	17	54	8
	KEGG	Carbon Metabolism	KEGG:01200	3.13E-04	51	54	10

Chitin binding was the most frequent pathway expressed in emerged bee sample clusters from April to October. Mitochondrial and energy production associated proteins were most frequently expressed in hive and forager bee samples from April to October. Throughout all the months, August had a different sample clustering compared to the other months. There were three clusters in August: emerged, early hive, and late and forager clusters which were not identified in the other sample months. The change in the proteome of workers in August could be indicative to the change in the worker's phenotype – and therefore could suggest the emergence of the winter worker phenotype within the colonies.

3.3.3 Analysis of changes in proteomes of Winter bees from December to February

Analysis was conducted on samples from three hives over three months during the winter months of December, January and February. Samples were collected at the tops of the hives to reduce the risk of damaging the cluster in the hive. Initial data matrix contained 2,420 proteins, post data filtration and ANOVA ($p > 0.1$) testing resulted in 61 proteins for further analysis. Principal component analysis arranged samples in individual clusters, respective to the months sampled (**Figure 3.10**). Despite samples being clustered individually, there were little to no SSDAs generated between volcano plot sample comparisons.

This could be attributed to all samples experiencing similar environmental stimuli which do not contribute to heightened proteomic response in samples when compared together. No proteins identified with longevity, xenobiotic metabolism or oxidative phosphorylation were identified in the comparisons between the winter bee samples.

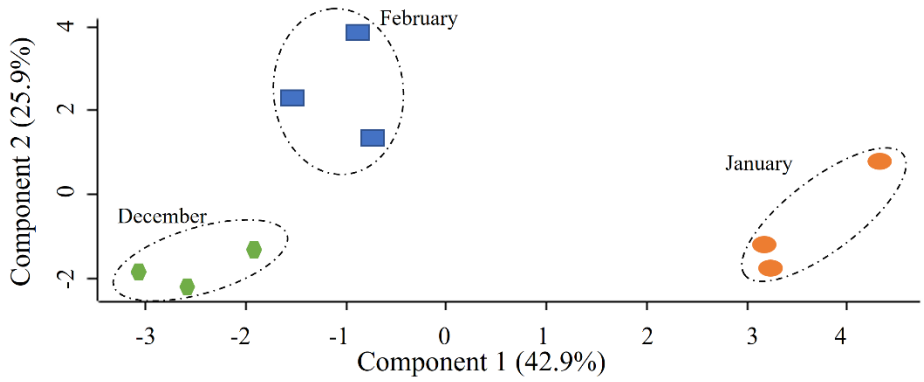


Figure 3.10: Principal component analysis on winter bees sampled from the hives in December through to February. The total explained variance of this PCA for the given dataset is 68.6%.

3.4 Discussion

3.4.1 Age Polyethism discussion

3.4.1.1 Overview on Sample Analysis

The roles of worker bees within colonies are strongly correlated to their age in a process known as age polyethism. This allows thousands of worker bees to function within the colony as a structured society therefore ensuring colony success. Each worker within each colony has a specific set of non-reproductive tasks to do, determined based on the age of the worker (Lindauer and Watkin, 1953; Beshers and Fewell, 2000). Age-polyethism is a flexible process, worker bees do not strictly follow a set progression of roles within the colony. Instead, age-polyethism is a diverse process that can be adapted based on environmental stimuli such as food shortages in the hive - hive worker bees can transition to foragers sooner than usual and if there a large amount of brood, foragers can reverse their roles back to the hive caste (Omholt and Amdam, 2004; Rueppell *et al.*, 2007; Herb *et al.*, 2012).

To investigate the process of age-polyethism, worker bees were collected at several timepoints in their lives starting with newly emerged worker bees and ending at foragers. The first part of the age-polyethism investigation sampled hives in March at multiple time points and completed proteomic analysis to characterise the proteomes

of worker bees at various ages. The results showed that worker bees close in age had a high degree of similarity, a pattern that decreased as worker bees got further apart in age. The initial sampling also identified how worker bees can be divided into four main groups: newly emerged, early hive, late hive and forager bees, as the PCA demonstrates in (**Figure 3.1**). This result allowed a smaller number of samples to be taken, thereby reducing the stress caused by opening a hive and limiting any proteomic changes that might be triggered by frequent interference.

The refined sampling regime confirmed that worker bees of the same age in different hives had similar proteomic profiles as they aged within their respective colonies. Samples from April to October were analysed on a month-by-month basis to investigate if age-polyethism is affected by the seasonal changes of the year (**Figure 3.2**). In each month sampled, PCA grouped the four age groups into separate clusters, with the highest degree of separation occurring between emerged and foragers and the lowest degree between the early and late hive caste. This demonstrates a large difference in the oldest and youngest worker bees, and a large overlap of protein expression between similarly age in-hive worker bees (early and late hive worker bees).

Analysis of samples taken in June highlighted the flexibility of age-polyethism as one of the hives in the trial had no queen for a period of time. The absence of a queen could have triggered a reversal of age-polyethism as the proteome of the forager in this hive was more closely associated with the early hive caste than the forager samples from different hives at this time (of which neither was experiencing queen issues). The queen secretes pheromones that have several essential functions: induce worker bees to create a retinue around her, suppresses the development of worker ovaries, encourages comb building and modulate the synthesis of juvenile hormone - a molecule in the hemolymph that works in a double repressor system with vitellogenin and influences worker bees transition through the hive (Robinson, 1992; Kaatz *et al.*, 1992; Slessor *et al.*, 2005). Therefore, an absence of a queen - even for a short time, could cause a regression in the process of age-polyethism. It is understood that bees from queen-less hives live longer than queen-right colonies but this could also be attributed to a reduction in brood pheromones as laying does not occur without a queen (Free and Spencer-Booth, 1959). A full investigation would need to be conducted to

determine the proteomic changes that occur in worker bees of various ages in a queenless colony.

3.4.1.2 Discussion on Proteomic Changes between Worker Bees at Different Ages

Proteomic analysis did reveal several changes to specific protein groups. Short chain dehydrogenase reductase was significantly increased in emerged worker bees. SCDR has been associated with a variety of cellular functions such as regulation of steroid formation, metabolism and alteration of active to inactive ligands when binding to their respective hormone receptors (Kallberg *et al.*, 2002; Guidugli *et al.*, 2004). SCDR expression has been hypothesised to be linked to a change in diet in honeybee larvae, with a further suggestion stating caste polyethism could be triggered by a nutritional signal, collectively these suggestions could provide an insight into the role of SCDR in emerged worker bees (Hunt and Nalepa, 1994; Evans and Wheeler, 1999; Guidugli *et al.*, 2004). It has been observed that SCDR is down-regulated by ecdysteroids but is up-regulated by JH tiers which further indicates a role in caste-specific changes in honeybees (Lago *et al.*, 2016).

Similar to SCDR, hexamerin proteins were also increased in abundance in emerged worker bee samples in comparisons from April to October. Hexamerins are abundantly expressed in larval fat body used for amino acid storage, insect development and JH binding (Lee & Kim, 2017). Hexamerin 70c was present at high levels in larval stages, but progressively declined in abundance in pupal development, but can still be detected on the 7th day of an adult (Cunha et al., 2005). Hexamerin 110 was identified in high abundance in honeybee larvae (Bitondi et al., 2006) and in newly emerged worker bees (Hu *et al.*, 2019). Hexamerins are in greater abundance in the diapausing *Anthonomus grandis* (boll weevil), functioning as a storage protein (Lewis et al., 2002), and hexamerin has also been identified in the adults of several ant species during the period of brood rearing (Wheeler & Martinez, 1995). Hexamerin protein levels decrease as bees age, with the abundance being lowest in foragers, and it has been suggested that the hexamerin levels are nutrient dependent (Bitondi et al., 2006; Martins et al., 2010). The abundance of hexamerin and short chain dehydrogenase proteins in emerged worker bees could provide an insight into possible markers for age polyethism in honeybees.

Major Royal Jelly (MRJ) proteins were found in high abundances in the early and late hive castes, but lower in foragers and emerged bees. However, in October samples MRJ protein intensities were higher in forager samples than in the hive castes. October samples also has a higher abundance of alpha-glucosidase in hive and forager castes compared to other months. These shifts in protein abundances suggest that the worker bees sampled in October have a slightly different proteome which could be attributed to the appearance of the diutinus caste (winter worker bees). Diutinus caste worker bees are characterised through longevity and their tolerance to oxidative stresses (Omholt and Amdam, 2004; Aurori *et al.*, 2014). An increase in MRJ proteins in foragers is unusual as the hypopharyngeal glands that secrete these proteins usually shrink about 21 days after emergence (Feng *et al.*, 2009b). Therefore, the presence of MRJ proteins in foragers in October indicate that these worker bees are not following the process of age-polyethism but instead are transitioning to diutinus caste worker bees.

Several odorant binding proteins were identified as ANOVA significant in the age polyethism analysis of the April to October samples. However, the protein profiles of the OBPs were not the same across the age groups of the worker bees. OBP13 was identified as more abundant in newly emerged worker bees in comparison to hive and forager worker bees. OBP21 was found in higher abundance in hive and forager worker bees in comparison to newly emerged worker bees. The pattern of OBP13 and OBP21 as increased and decreased respectively, in abundance in newly emerged worker bees respectively has been observed in other proteomic investigations of worker honeybees (Iovinella *et al.*, 2011). OBP13 binds to oleic acid with good specificity, oleic acid and β-ocimene have been linked to hygienic behaviour with brood removal (Iovinella *et al.*, 2011; McAfee *et al.*, 2018). Newly emerged worker bees usually take part in cell cleaning and have a close interaction with brood which could provide an insight into why OBP13 has been identified in these worker bees. OBP21 binds with some affinity to components of the queen's mandibular pheromones - 9-oxo-2-decenoic acid and methyl p-hydroxybenzoate, which are used to inhibit the activation of ovaries and follicle development in workers and aid in retaining the hierarchical structure within colonies (Hartfelder, 2000; Iovinella *et al.*, 2011). The increase in OBP21 in older worker bees in comparison to younger worker bees, could therefore be linked with the detection and exposure to queen pheromones.

Vitellogenin has been observed in fluctuating titres in honeybee workers as they age – increased in nurse bees and decreased in forager bee samples (Amdam and Omholt, 2002; Omholt and Amdam, 2004; Bogaerts *et al.*, 2009; Münch and Amdam, 2010; Erban *et al.*, 2013). However, it was not detected as an ANOVA significant (p value = 0.1) in samples from April to July. Vitellogenin LFQ intensity levels were highest in October samples in comparison to August and September samples. Vitellogenin levels have been observed to be increased in abundance in both nurse and winter worker bees, the increase of vitellogenin levels in October samples could indicate the emergence of winter bees colonies (Amdam and Omholt, 2002; Döke *et al.*, 2015). The presence of vitellogenin proteins in autumn months in this analysis correlate with previous findings, that also identified an increased abundance in vitellogenin titres in workers sampled in autumn when compared to winter workers (Fluri *et al.*, 1982). The absence of vitellogenin in the spring and summer samples and presence of vitellogenin in the autumn samples could be an indicator to the change of dynamic in the colonies from a summer to autumn and subsequently to winter colonies.

Cuticular proteins were increased in abundance in emerged bee samples across all months analysed in this chapter. Cuticular proteins, - cuticular, cuticle and apidermin proteins have previously been observed in increased abundance in emerged workers in comparison to hive and forager bees (Kucharski *et al.*, 2007; Soares *et al.*, 2007). One study identified a decrease in hydrocarbons on the surface of newly emerged worker bees in comparison to older hive bees (Breed *et al.*, 2004). Hydrocarbons function as hydrophobic barriers for arthropods and are also known to function as pheromones as a communication mechanism between nest mates (Van Zweden *et al.*, 2010; Chung and Carroll, 2015; Vernier *et al.*, 2019). Hydrocarbons are produced by oenocytes and transported to the cuticle (Chung and Carroll, 2015). This might provide reasoning to the low abundance of hydrocarbons previously identified in newly emerged workers and the high abundance of cuticular proteins identified in this study within newly emerged worker bees.

Apidermin proteins are expressed in cuticle formation with apidermin-1 having the broadest expression in the epidermis layer underlying the cuticle destined for sclerotisation (Willis, 2010). Apidermin 1 is highly expressed in late pupal and early adult development, coinciding with sclerotisation (Andersen *et al.*, 1995; Kucharski *et*

al., 2007). Apidermin 2 has been identified within the internal cuticle of the trachea and stomach (Kucharski *et al.*, 2007), which is unexpected considering apidermin 2 was identified in head samples from April to September. Apidermin 3 is expressed on the external epidermis including the eye (Kucharski *et al.*, 2007; Willis, 2010). It was surprising that apidermin 3 was only identified as ANOVA significant (p value = 0.1) in October samples and not in the samples collected from April to September. This could provide further evidence of the emergence of winter workers in honeybee colonies at the end of autumn.

Honey production enzymes were identified as increased in abundance in late hive and forager samples and decreased in abundance in emerged bee samples throughout the months April to October. Honey production enzymes are mainly α-amylase, α-glucosidase and glucose oxidase which are produced and secreted by the hypopharyngeal glands within worker bees (Kubo *et al.*, 1996; Deseyn and Billen, 2005). Amylase converts plant starch into glucose, and glucose oxidase which is needed to convert glucose to gluconic acid and hydrogen peroxide, both have been identified in higher abundance in forager bee samples in comparison to nurse bee samples (Ohashi *et al.*, 1999; Deseyn and Billen, 2005). α-glucosidase has also been identified as increased in abundance in forager hypopharyngeal glands in contrast to bee-milk proteins (MRJ proteins) which were identified within nurse bee samples (Ohashi *et al.*, 1999).

The shift in protein abundance in the hypopharyngeal glands within honeybee workers could be linked to the changes in the hypopharyngeal glands size and the subsequent role of the workers at specific age. Previous experiments that investigated changes in the hypopharyngeal glands anatomy and activity have identified similar findings to the proteomic changes identified here (Kubo *et al.*, 1996; Hrassnigg and Crailsheim, 1998; Ohashi *et al.*, 1999; Deseyn and Billen, 2005; Qi *et al.*, 2015). The peak size of the acini within the glands has been identified at 6 days old which corresponds to the nurse bee role of worker bees (Hrassnigg and Crailsheim, 1998; Shakeel *et al.*, 2020). The hypopharyngeal glands have then been observed to decrease in size from the age of 15 days (Deseyn and Billen, 2005; Qi *et al.*, 2015; Shakeel *et al.*, 2020). The increase in honey production enzymes in late hive and forager bees in

comparison to emerged and early hive bees provides a further insight into the proteomic shifts that occur within honeybee workers within summer colonies.

3.4.1.3 Discussion on Pathway Analysis from Protein Clusters Generated from Hierarchical Clustering

The chitin binding pathway was the most abundant pathway detected from hierarchical clustering in emerged worker bees from April to October. Chitin binding is the collection of proteins involved in the production of the exoskeleton which is composed of chitin (Tetreau *et al.*, 2015). As highlighted in the individual protein analysis emerged workers had an increased abundance of cuticular protein in comparison to hive and forager workers. This could be attributed to newly emerged workers being at the last stages of moulting during the development of pupae.

Other protein pathways that were observed as enriched in emerged samples include RNA binding, GTP binding and Structural Molecular Activity. These pathways indicate a growth and development proteome of newly emerged worker bees. RNA binding proteins are essential in regulating all aspects of RNA within the cell including transcription, splicing, intracellular trafficking and decay (Gebauer *et al.*, 2020). An increase in ribosomal proteins has previously been observed in the HPGs of newly emerged worker bees and was too suggested to aid in the development of the glands in worker bees (Liu *et al.*, 2014; Hu *et al.*, 2019).

GTP binding proteins are transducers what are linked with the opening and closing of signalling pathways and have been linked as a regulator for differentiation in *Streptomyces coelicolor* (Biochem *et al.*, 1991; Okamoto and Ochi, 1998; Bustelo *et al.*, 2007). Structural molecule activity proteins contribute to the structural integrity of a complex or its assembly within or outside the cell. An increase in structural protein is observed in insects entering diapause or from cold exposure (Xinxin *et al.*, 2020). Here there increase in cuticular and structural proteins in newly emerged workers may be indictive of their development to mature worker bees.

Mitochondrial and energy production proteins were the most abundant protein identified in the hierarchical clustering analysis from all hive and forager bee samples from April to October. The overall pathway enrichment across the months sampled was very similar: Catalytic activity, Oxidoreductase activity, Carbohydrate

metabolism process, and Metabolic Pathway proteins were all enriched in hive and forager bee samples from April to October. Carbohydrate and lipid metabolising enzymes catalyse reactions that aid in releasing energy from the break-down of food. Carbohydrate and galactose metabolism proteins have been observed to be increased in hive and forager bees in comparison to newly emerged (Hernández *et al.*, 2012; Ararso *et al.*, 2018; Hu *et al.*, 2019). Energy metabolism pathways are essential for the production of royal jelly in nurse bees and for the specific role of flight and honey production in foragers (Hu *et al.*, 2019). The demands of energy consumption on hive and forager bees are far greater than those of newly emerged worker bees and this is reflected through the upregulation of energy metabolism proteins in older workers.

Overall, there was a higher abundance of mitochondrial proteins in hive and forager samples in comparison to newly emerged worker bees. Increased mitochondrial proteins and activity has been recorded in nurse bees in comparison to foragers, and foragers had an increased abundance of antioxidant enzymes (Cervoni *et al.*, 2017). Carbonylation damage was observed in forager bees of both 20 and 200 days of age possibly because foragers have a reduction in mitochondrial proteins in comparison to nurse worker bees (Seehuus *et al.*, 2006a). Flying insects have the highest mass-specific rates of aerobic metabolism in the animal kingdom (Suarez *et al.*, 1996). The increase of mitochondrial proteins in hive and forager bees can be attributed to the energetic demand of the social roles (feeding, building foraging) older worker bees have within colonies.

3.4.2 Winter bee sample discussion

Analysis on worker bee samples collected from the tops of the frames in December to February showed very minimal deviation in the proteomes of winter bees when compared to each other. Each month was clustered separately in the PCA analysis, however, very few SSDAs were identified in the comparative analysis between samples. Winter worker bees are mostly confined to the hive over the winter and have very little exposure to external stimuli which could be a contributing factor to the lack of variation in proteome profiles. A reduction in immune protein abundance in winter honeybee workers has been previously observed, which could contribute to the winter worker bees longevity (Steinmann *et al.*, 2015). Proteomic comparisons between winter worker bees sampled at three different time points could be the reason

for the low protein abundance in the analysis. MaxQuant provides a LFQ intensity value to proteins detected from the mass spectrometer in relative abundance to other samples included in the MaxQuant analysis. As winter workers are all exposed to similar environments in this experiment the data resulted in similar proteomes and therefore very little SSDAs were generated.

3.5 Summary of Findings

Table 3.13: Overview of proteomic analysis from Chapter 3: Utilising proteomic analysis to characterise the changes in Apis mellifera during the process of age-polyethism throughout the year.

Age of Worker	Abundant Proteins	Pathway Enrichment	Notes
Emerged	Short chain dehydrogenase reductase Hexamerin Cuticular proteins, specifically apidermin proteins Odorant binding protein 13	Proteins associated with RNA and chitin binding, structural molecular activity were enriched in emerged worker bee samples.	The abundant proteins mentioned in this table were significantly increased in abundance in emerged bee samples in comparison to hive and forager bee workers within the months analysed. OBP13 was consistently increased in abundance in emerged workers in comparison to hive and forager samples. Cuticular proteins specifically apidermin 3 was identified as increased in emerged bee samples across the months in comparison to hive and forager samples.
Hive and Foragers	Major royal jelly proteins Obp21 Honey production proteins	Proteins associated with oxidoreductase, mitochondrial, metabolism (carbohydrate and galactose) and aerobic respiration were all enriched in hive and forager samples.	MRJ proteins were increased in abundance in hive workers in comparison to emerged bees. The level of abundance of MRJ proteins did not increase in foragers in comparison to hive workers. OBP21 was identified as increased in abundance in hive and forager samples in comparison to emerged workers. Proteins associated with the production of honey were increased in abundance in hive and forager samples in all months in comparison to emerged worker bees.

Winter Workers	Not a lot of singular protein abundance detected	No enrichment in pathways was detected in the winter worker analysis.	Winter worker proteomic analysis did not detect a large number of proteins or enrichment in protein pathway analysis in this study. This could be due to the lack of comparison with samples from a different environment.

Chapter 4

Utilising Proteomic Analysis to Identify Proteomic Changes in Worker Bees as they Transition from Summer to Winter

4.1 Introduction

As colonies of *A. mellifera* approach the winter months the activity, brood production and size of the colony begins to decrease (Mattila *et al.*, 2001; Döke *et al.*, 2015). Workers that emerge in late autumn will be the bees that will remain within the hive over the winter months (Mattila *et al.*, 2001; Steinmann *et al.*, 2015) and these bees are called winter or diutinus workers (a Latin word meaning long-lived) (Maurizio and Hodges, 1950; Omholt and Amdam, 2004; Yang *et al.*, 2017). Diutinus worker bees first emerge in late autumn and reside in the colony throughout the winter months, in spring diutinus workers will be replaced with the emergence of new brood that they will aid in rearing (Mattila *et al.*, 2001; Amdam and Omholt, 2002; Amdam *et al.*, 2004a; Münch and Amdam, 2010).

The appearance of diutinus worker bees in colonies allows *A. mellifera* to be one of only a few insect species to survive winter conditions without becoming dormant or migrating to warmer regions (Seeley and Visscher, 1985; Knoll *et al.*, 2020). The role of the diutinus worker bee is more simplistic in comparison to summer worker bees, diutinus bees are tasked with keeping the colony alive over the winter. Thermoregulation is the most important role of diutinus bees, it allows the core temperature of the cluster to range between 20 – 35^{o}C, and is regulated through reduction of heat loss by the mantle (surface) bees (Watmough and Camazine, 1995; Stabentheiner *et al.*, 2003).

Diutinus worker bees are characterised by a lower titre of juvenile hormone (Robinson and Huang, 1995; Mattila and Otis, 2007; Kunc *et al.*, 2019), hypertrophied hypopharyngeal glands with a low level of protein synthesis (Brouwers, 1983; Amdam and Omholt, 2002; Deseyn and Billen, 2005), enlarged fat bodies (Mattila and Otis, 2007; Smedal *et al.*, 2009; Kunc *et al.*, 2019), elevated levels of vitellogenin (Amdam and Omholt, 2002; Omholt and Amdam, 2004; Döke *et al.*, 2015; Lee and Kim, 2017; Kunc *et al.*, 2019) and storage proteins such as hexamerin (Lee and Kim, 2017).

Several conditions are thought to trigger the transition of summer to diutinus bees within the colony such as the absence of brood/brood pheromones, photoperiod, temperature and lack of nutritional availability (Free and Spencer-booth, 1959; Döke *et al.*, 2015). The exact time of the emergence of diutinus bees is still undefined.

However, previous work has observed worker bees that emerge in the first week of October, have a greater longevity than those emerging in August and September (Free and Spencer-Booth, 1959).

This chapter will investigate proteomic changes to worker bees in the autumn months of the year to identify shifts in protein expressions in order to identify the emergence of winter worker bees. This was completed by analysing proteomic data from worker bees of known ages from July to October.

4.1.1 Chapter Four Aims

1) Apply proteomic analysis to characterise changes in the protein expression in late autumn worker bees
2) Identify if any protein changes in autumn worker bees could be attributed to the emergence of winter workers

4.2 Experimental Design

Samples used in this analysis were age-polyethism samples used in chapter three. The sample collection of worker bees is outlined in **Table 3.3**. For this analysis samples were grouped into two categories: first half of the year (April to June) and second half of the year (July to October).

A single head from each worker bee was used as a sample point as a representative point for each hive and was subjected to mass spec preparation; protein extraction, clean-up, digestion, and peptide purification (C18). For mass spec analysis 1µg of tryptic peptides were loaded onto a QExactive (ThermoFisher Scientific) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated over a 2-40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100mm length, 75mm ID), using a 135 min reverse-phase gradient flow rate of 250nL min^{-1}. Three hives were used for year-round sampling, each hive was treated as an individual replicate.

Protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.6.6.0 (https://www.maxquant.org/). Data processing and analysis was carried out using Perseus v 1.6.6.0 (https://maxquant.net/perseus/). To identify protein-protein interactions, functions, and pathway enrichments STRING v 11 (https://string-db.org/) and KEGG Mapper (https://www.genome.jp/kegg/tool/map_pathway2.html) were utilised. The most differentially abundant proteins within each age category are investigated as potential biomarkers for age-polyethism. Proteins were deemed statistically significant differentially abundant (SSDA) if identified to have a Log2 fold change greater than +/- 1 and a -Log p value greater than 1.3.

g:Profiler term information used in this analysis are outlined as such: Term ID is the unique term identifier for each pathway/term. Term size refers to the number of genes that are annotated to the specific term. Query size is the number of genes that were included in the query run. Intersection size is the number of genes in the input query that are annotated to the corresponding term. The adjusted p-value is the hypergeometric p-value after correction for multiple testing. The user-threshold for each of the query runs is 0.05 (Raudvere *et al.*, 2019).

4.3 Results

4.3.1 Analysis of the Proteomic Changes Associated Seasonal Change in honeybees

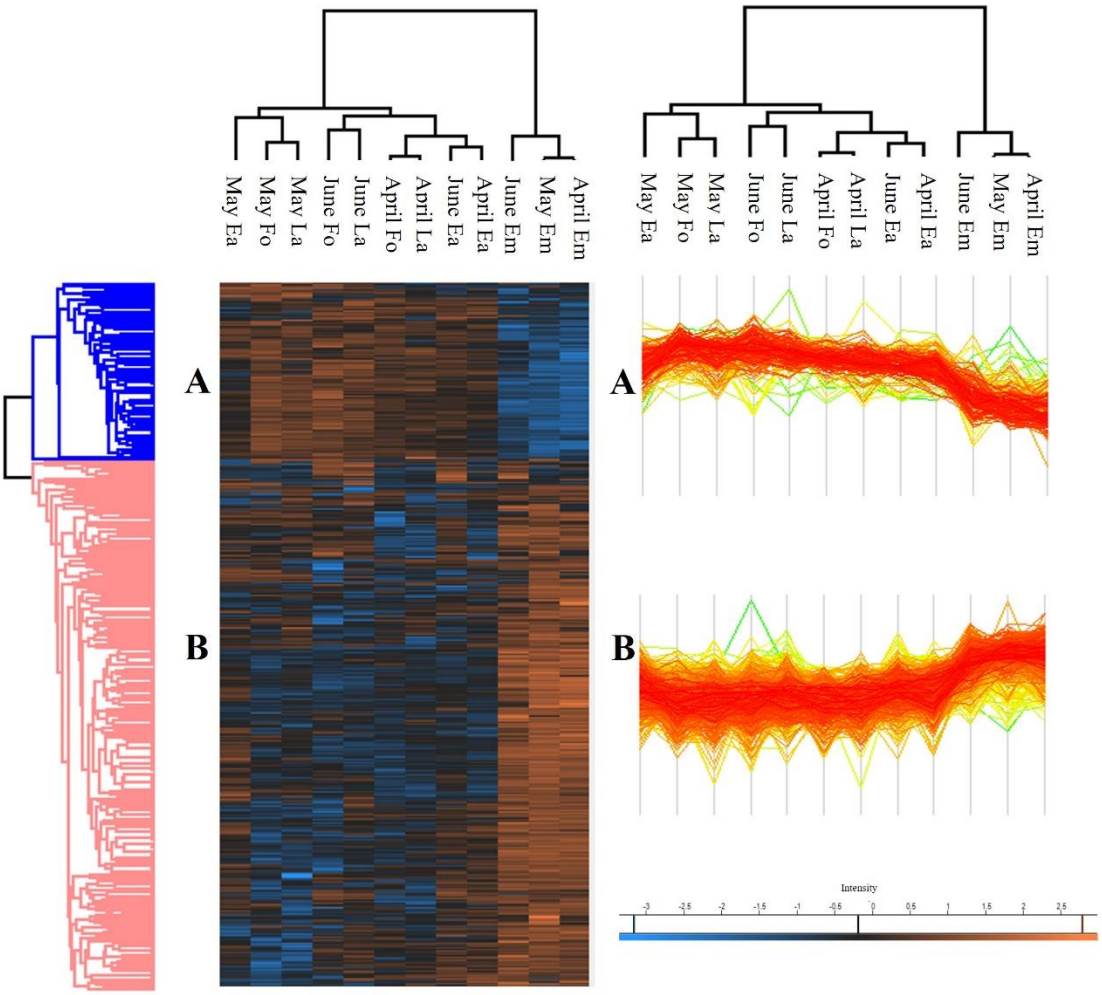
Samples collected from April to October 2019 were divided into two groups – first and second half of the year to allow for data handling analysis. The spring to summer batch consisted of samples from April to June, and summer to autumn batch consisted of samples from July to October. LFQ intensity values were Log_2 transformed and only proteins identified in at least three of the three replicates were included for further analysis. The matrix was further filtered through ANOVA significance testing ($p < 0.05$) as only ANOVA significant proteins were used for analysis. Size of matrices at the end of each analysis step are outlined in **Table 3.4.** In

total 785 and 984 SSDA proteins were identified in the first and second half matrices respectively.

Table 4.1: Protein matrix numbers before and after filtration and ANOVA testing. Data collected is from spring to summer and summer to autumn datasets

Months	Pre filtration	Post filtration	Post-ANOVA	SSDA
1	3,461	1,981	981	785
2	3,819	2,344	1,2951	984

The analysis on the data matrix from the first half of the year (April to June) contained 785 SSDAs. Hierarchical clustering on Z-scored normalised intensity values using Euclidean distance and complete linkage divided samples into two groups: group 1: emerged bees, and group 2: hive and forager bees (**Figure 4.1,** Cluster A & B) (**Supp. File 4.1**). Protein clusters were calculated based on protein abundance profile similarities, Gene Ontology (GO), Pfam and KEGG term enrichment analyses were performed on all clusters. Cluster A (n = 185), which consisted of hive and forager samples, had a high abundance of proteins associated with oxidative phosphorylation (22 proteins), glycolytic processes (12 proteins) and carbon metabolism (27 proteins) (**Figure 4.1 & Figure 4.2**). Cluster B (n = 548), consisting of emerged bee samples, had an abundance of proteins associated with the spliceosome (25 proteins) and RNA binding (36 proteins) (**Figure 4.1 & Figure 4.3**). The clear divide between the sample clustering highlights the differences between newly emerged worker bees to hive and forager bees from April to June.



Statistically enriched gene ontology terms identified in hierarchical clustering

Cluster	Name	p Value	Enrichment
A	Tricarboxylic Acid Cycle	5.02E-07	3.6574
	Glycolytic Process	5.99E-05	3.9622
	ATP synthesis coupled proton transport	0.000243	3.9622
B	RNA binding	0.000132	1.3376

Figure 4.1: Hierarchical clustering analysis on the median intensity values for 785 SSDA proteins from samples between April to June. Proteins were grouped via unsupervised clustering and subsequently divided into two groups (A & B). Heatmap colours represent the relative expression of proteins in the samples, relative abundances can be read from the intensity spectrum. Table: enrichment analysis on hierarchical clusters is derived from Perseus analysis.

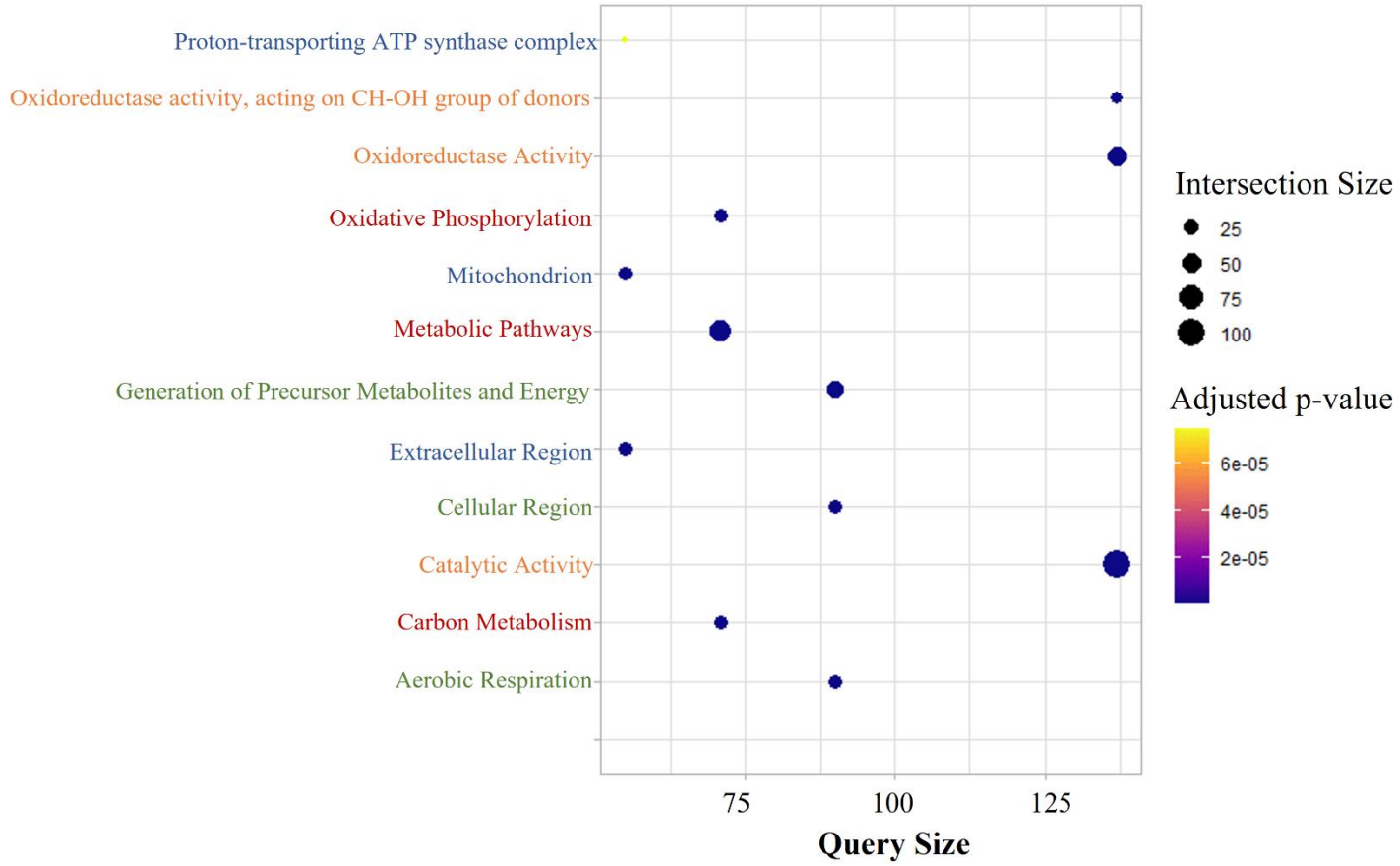


Figure 4.2: Gene ontology pathway analysis using g:Profiler on Cluster A (p value = 0.05) from Figure 4.1. Colour code for Term analysis: Blue – GOCC, Orange – GOMF, Green – GOBP and Red – KEGG.

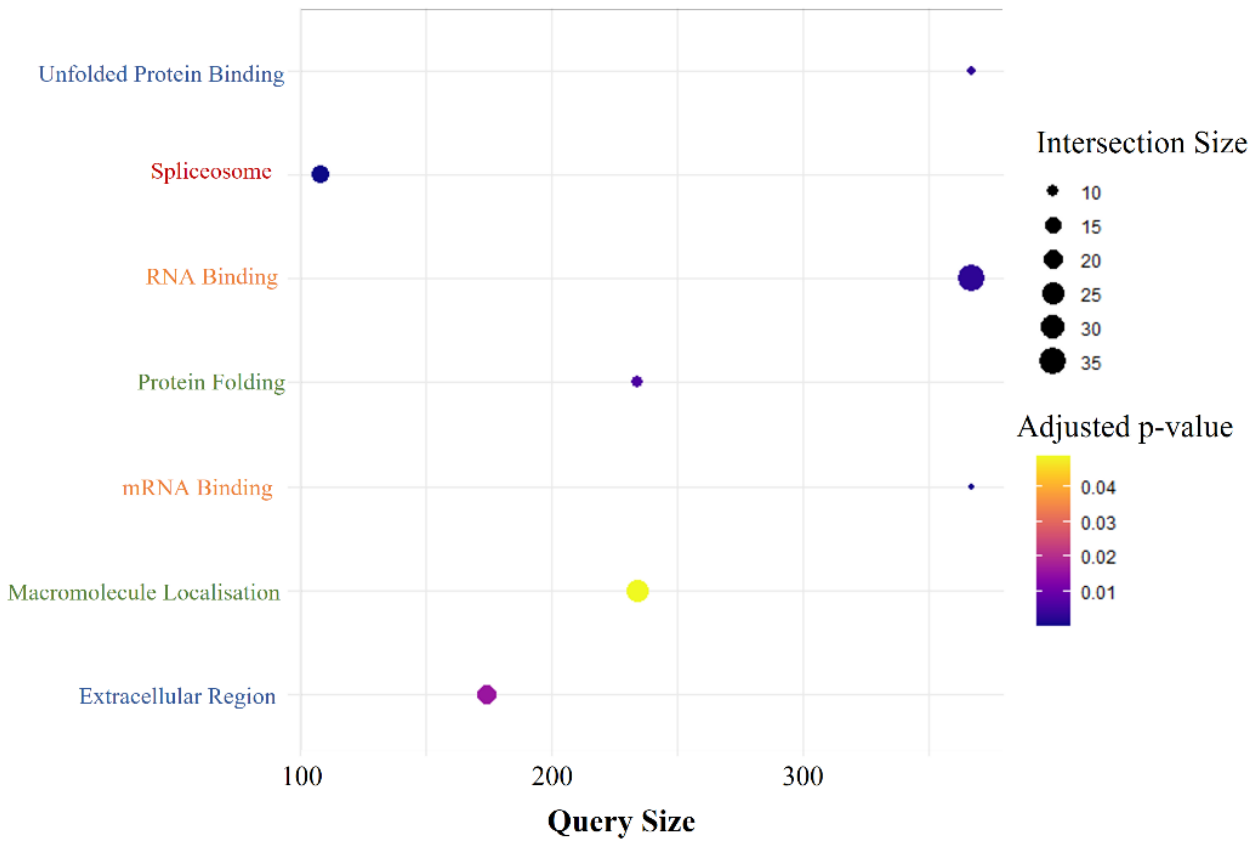


Figure 4.3: Gene ontology pathway analysis using g:Profiler on Cluster B (p value = 0.05) from Figure 4.1. Colour code for Term analysis: Blue – GOCC, Orange – GOMF, Green – GOBP and Red – KEGG.

Hierarchical clustering was performed on the z-scored normalised LFQ intensity values for the 984 SSDA proteins from the second half analysis. Samples were resolved into three distinct groups, July and August emerged bee samples, all hive (early and late) and forager bee samples, and September and October emerged bee samples (**Supp. File 4.2**). Three (A-C) protein clusters were identified based on protein abundance profile similarities (**Figure 4.4**). Gene Ontology (GO), Pfam and KEGG term enrichment analyses were performed on all clusters, of which cluster A and B showed protein enrichment, cluster C did not have any enriched protein pathways (**Table 4.2)**. This included proteins associated with tricarboxylic acid cycle (TCA), major royal jelly (MRJ) proteins, and extracellular region (**Figure 4.4 - Cluster A**). Integral component of membrane in cluster B. TCA and MRJ protein abundance was reduced in September and October emerged bee samples, which corresponded with what was observed in the volcano plot analysis. Interestingly, the integral component of membrane protein abundance was high in September and October emerged samples and in October hive (early and late) and forager samples.

Table 4.2: Statistically enriched gene ontology terms identified in hierarchical clustering (Figure 4.1). Cluster A and B are protein groups identified by calculating the Euclidean distance using Z-scored LFQ intensity values of SSDA proteins.

Cluster	Name	p value	Enrichment
A	Tricarboxylic acid cycle	2.26E-07	5.38
	MRJP	3.22E-07	4.3
	Extracellular region	1.17E-04	2.93
	HSP20	4.59E-05	4.70
B	Integral component of membrane	2.52E-07	1.16

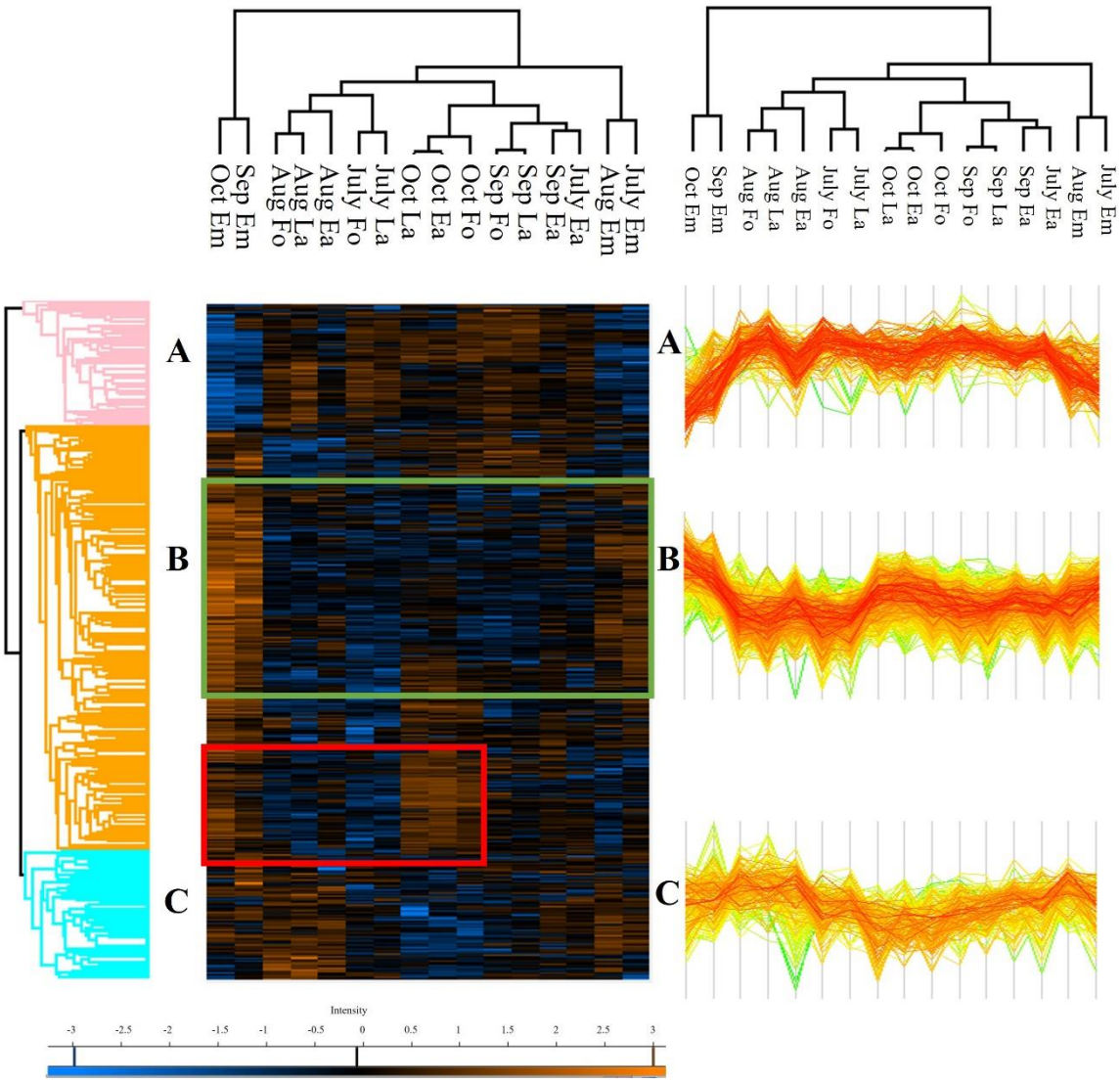


Figure 4.4: Hierarchical clustering analysis on 984 SSDA proteins from samples taken between July to October. Proteins were grouped via unsupervised clustering and subsequently divided into three groups (A-C). Heat map colours represent the relative expression of proteins in the samples, relative abundances can be read from the intensity spectrum.

Within Cluster B a group of 333 proteins were identified as upregulated in all newly emerged samples from July to October (**Figure 4.4**, **1, green box**), g:Profiler analysis identified proteins involved in the spliceosome and involvement in the basement membrane (**Figure 4.5**)

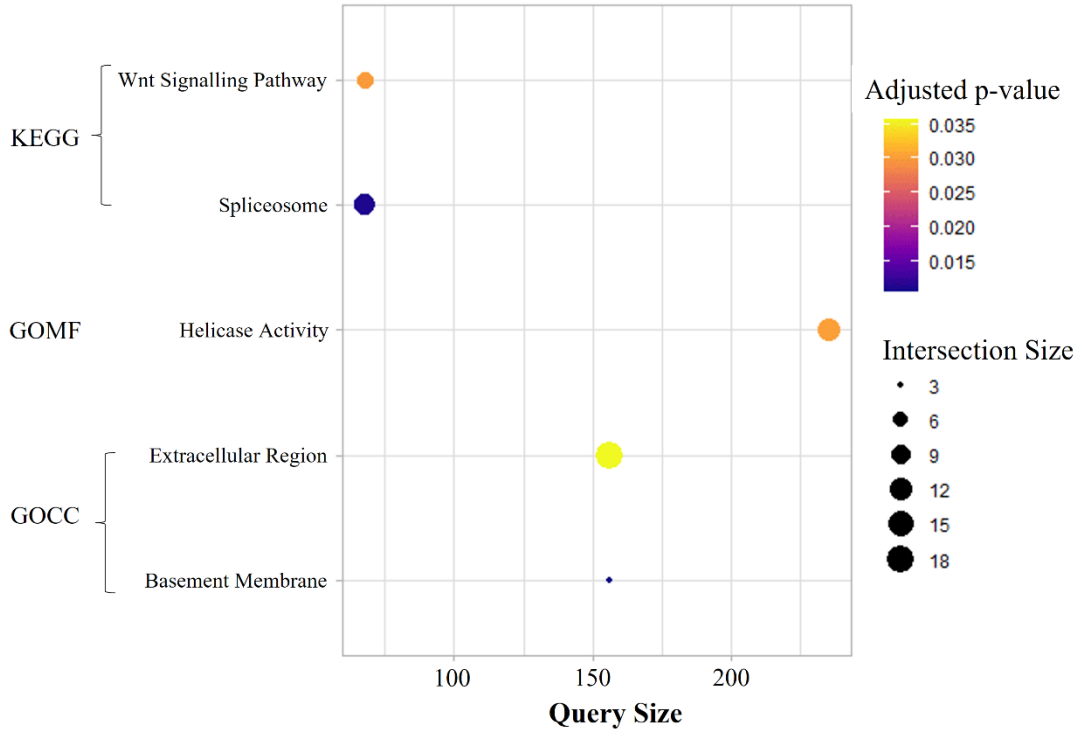


Figure 4.5: g:Profiler analysis on proteins identified in the green cluster from the second half analysis hierarchical analysis. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05

Also within Cluster B, a group of 157 proteins were identified as upregulated in October hive and forager bee samples, but downregulated in July, August and September (**Figure 4.4,** highlighted as the red box). These proteins were analysed using gprofiler to investigate the protein pathway distribution. A large number of proteins were identified to be associated with the mitochondrion and to have a role in ion transmembrane transporter activity, there was one single KEGG term identified as significant in protein abundance from the 157 proteins, oxidative phosphorylation (**Figure 4.6**).

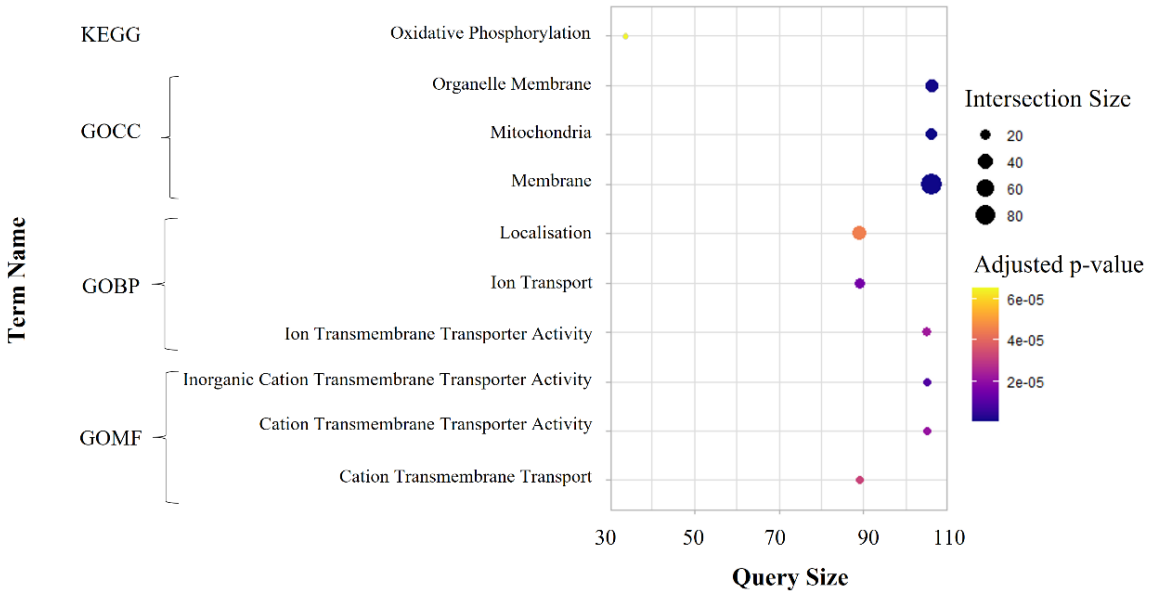


Figure 4.6: g:Profiler analysis on proteins identified in the red cluster from the second half analysis hierarchical analysis. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05

No enrichment terms were identified for cluster C in Perseus analysis (**Figure 4.4**). Protein IDs from the cluster were analysed in g:Profiler, which identified proteins involved in translation (GOMF), protein folding and cellular localisation (GOBP) and the cytoplasm prefoldin complex (GOCC) **Figure 4.7.**

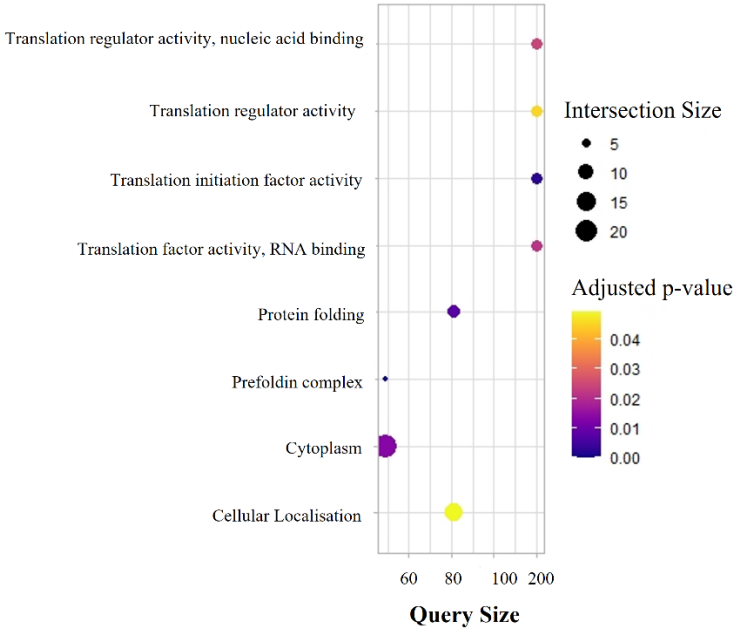


Figure 4.7: gprofiler analysis on proteins identified in cluster c of **Figure 4.4.** Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05

Hierarchical analysis on the second half of the sampling year (July to October) revealed differences between the newly emerged worker bees of July and August to September and October, which was not previously observed in the first half analysis. Further analysis was conducted on the second half of the year samples to investigate the proteomic shifts between worker bees of the same age from July to October (**Supp. File 4.3**). This was to provide an insight into how protein expression changes in worker bees of the same age as the months progress towards the winter season.

4.3.1.1 Analysis of Emerged Bees Proteomes

Comparisons of the proteomes of newly emerged bee samples revealed a significant shifts in protein abundance from July and August samples compared to September and October samples. Relative fold change (rfc) values for emerged bee samples in September and October were reduced in comparison to July and August samples, indicating a reduction in the proteome of September and October bee samples. July and August samples had a higher number of associated SSDAs in comparison to September and October (**Figure 4.8**).

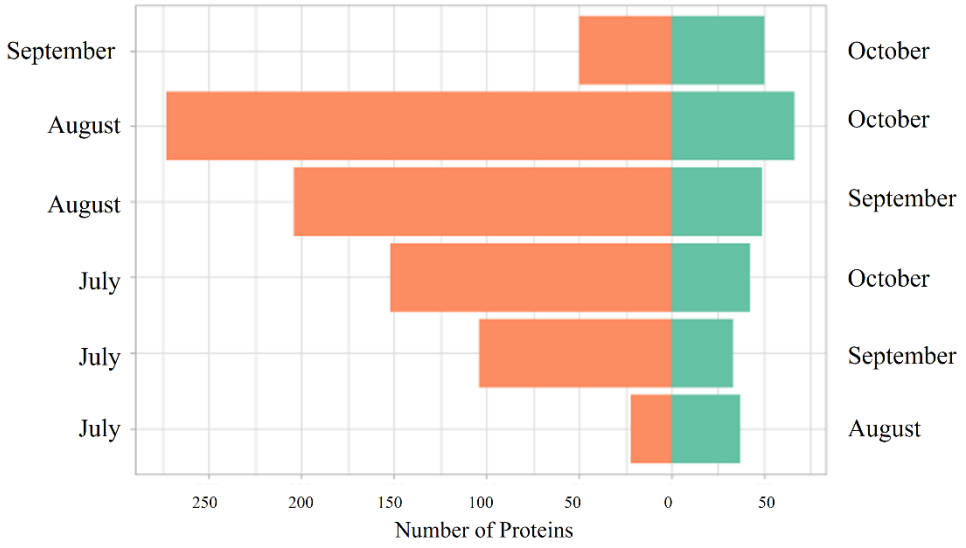


Figure 4.8: Bar chart of SSDAs generated from emerged bee volcano plot comparisons from samples collected between July to October.

Some interesting protein expression patterns were observed in the volcano plot comparisons between the emerged bee samples from the different months (**Supp. File**

4.2). Hexamerin 70c (A6YLP8) was identified as increased in abundance in October emerged bee samples when compared with July (rfc 75.6) and August (rfc 37.7) emerged bee samples (**Red star, Figure 4.9 & Figure 4.10**). LFQ intensity values of both hexamerin 70c and 110 were observed to be higher in September and October compared with July and August, however, no significant increase was observed.

A number of cuticular proteins, relating to bees exoskeleton were increased in abundance in September emerged samples in comparisons with July, August and October emerged samples (**Figure 4.9, Figure 4.10 & Figure 4.11**). Despite an increase in cuticular proteins in September emerged bee samples, no significant change in protein abundance was observed.

Major Royal Jelly Proteins (MRJPs) were consistently identified as highly expressed in July and August samples in comparison to September and October samples (**Table 4.3**, **Figure 4.9 & Figure 4.10**). Protein intensity values for MRJPs were averaged and plotted on a boxplot, which shows the significant increase in MRJP intensity values in July and August versus intensity values in September and October (**Figure 4.12**). ANOVA test also highlighted a further decrease in protein abundance in October in comparison to September.

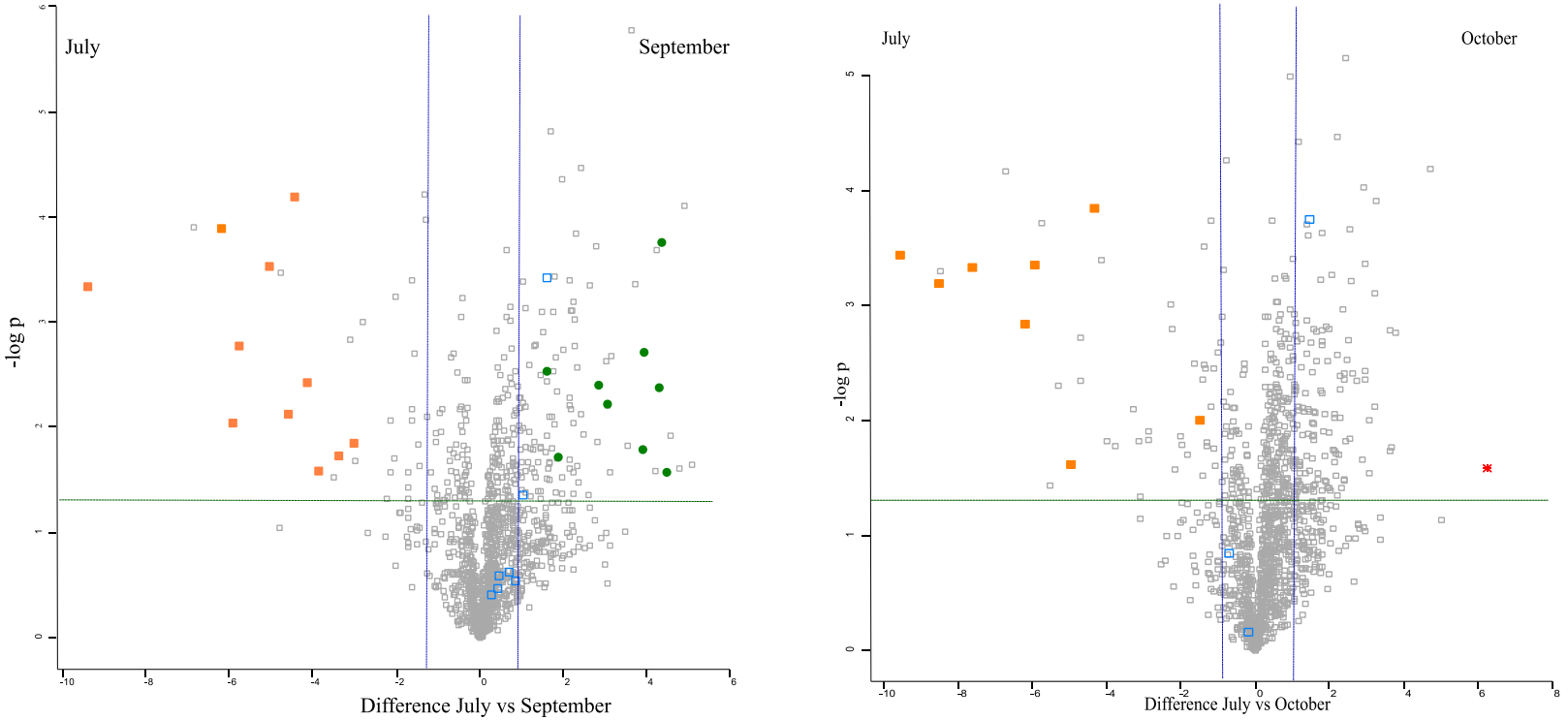


Figure 4.9: Volcano plots of emerged samples between July to September and October. Orange squares represent MRJ proteins and Blue squares represent cytochrome c oxidase proteins, Green circles represent cuticular proteins and Red star represents hexamerin proteins which were identified in the analysis. x-axis is the Log_2 fold difference, the y-axis is the -log p value of protein intensities. Blue vertical lines define a fold change value of 1 and the orange horizontal line is positioned at 1.3 -Log p value.

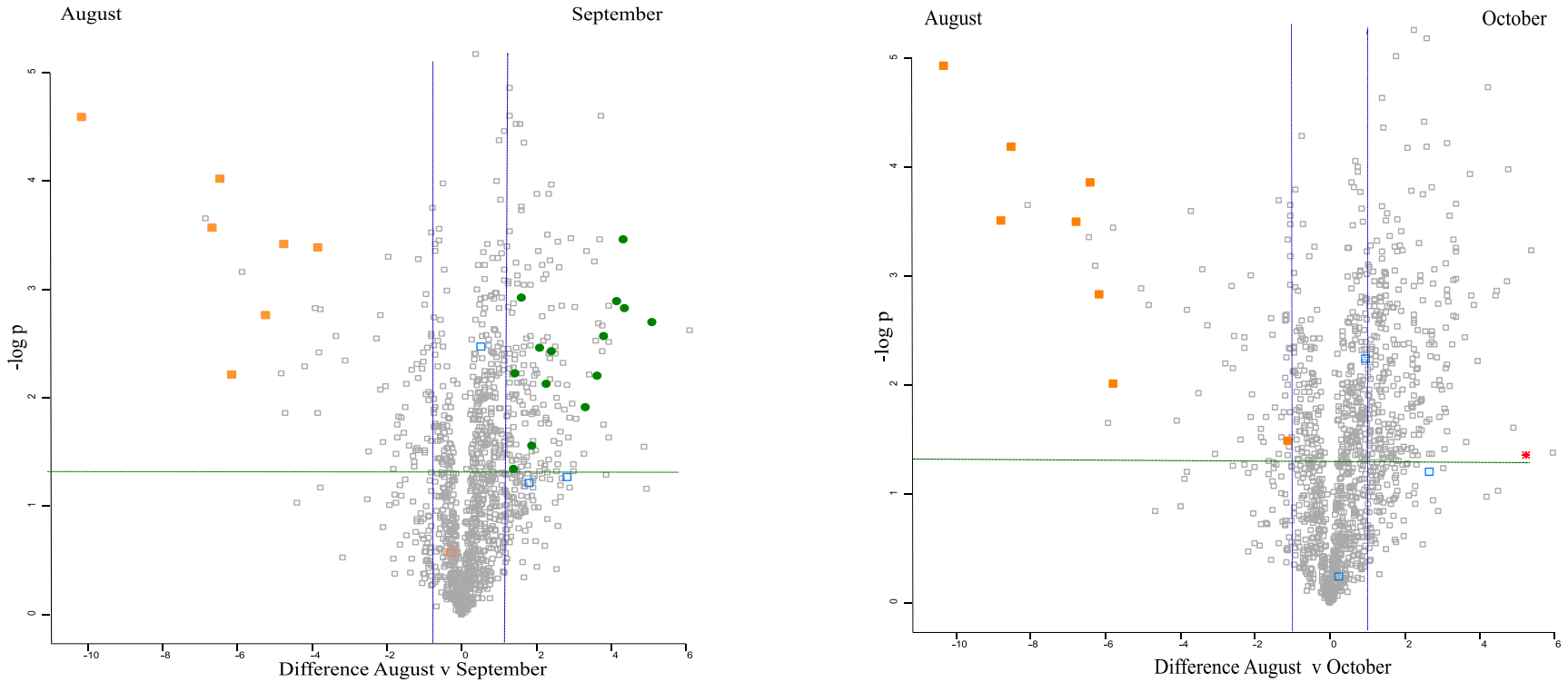


Figure 4.10: Volcano plots of emerged samples between August to September and October. Orange squares represent MRJ proteins and Blue squares represent cytochrome c oxidase proteins, Green circles represent cuticular proteins and Red star represents hexamerin proteins which were identified in the analysis. x-axis is the Log_2 fold difference, the y-axis is the -log p value of protein intensities. Blue vertical lines define a fold change value of 1 and the orange horizontal line is positioned at 1.3 -Log p value.

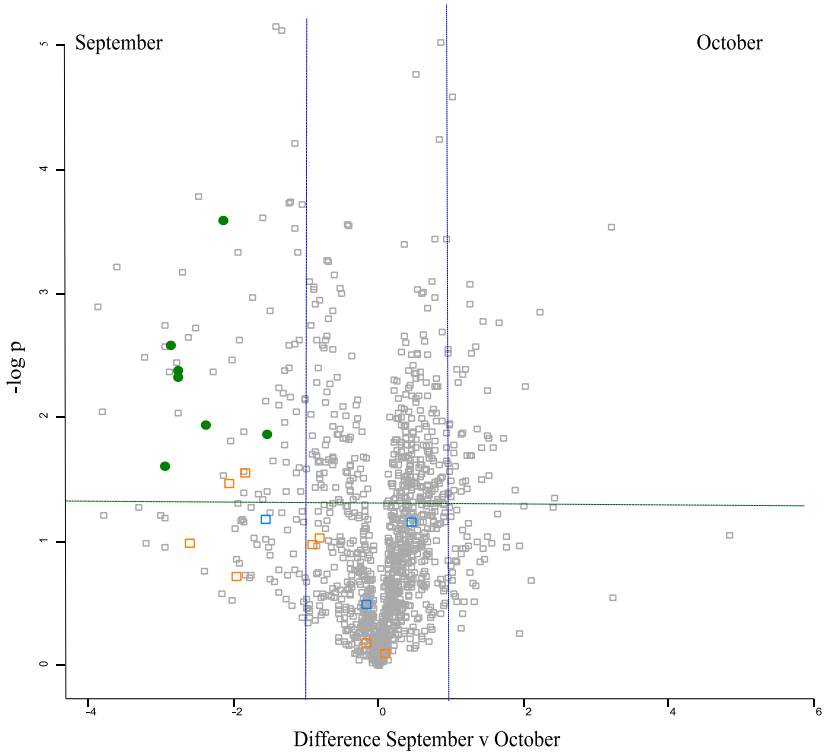


Figure 4.11: Volcano plots of emerged samples between September and October. Orange squares represent MRJ proteins and Blue squares represent cytochrome c oxidase proteins, Green circles represent cuticular proteins and Red star represents hexamerin proteins which were identified in the analysis. x-axis is the Log_2 fold difference, the y-axis is the -log p value of protein intensities. Blue vertical lines define a fold change value of 1 and the orange horizontal line is positioned at 1.3 -Log p value.

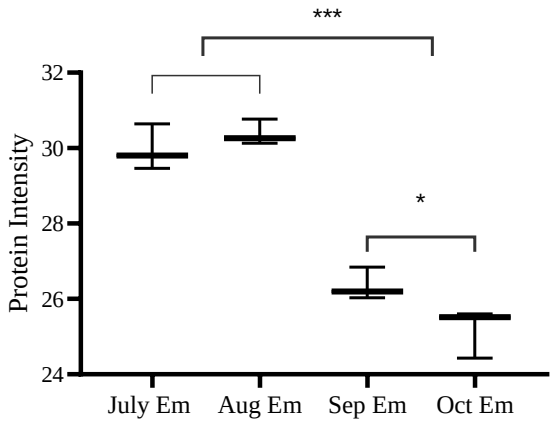


Figure 4.12: Averaged Major Royal Jelly Protein LFQ values from Emerged worker bees. Significance is represented by 0 '***', 0.001'**', 0.1'*.

Table 4.3: Relative fold change of MRJPs identified as increased in relative abundance in July and August emerged bee samples compared to September and October samples.

Months	September	October
July	8.04 – 672.10	2.83 – 755.506
August	14.36 – 1145.23	2.17 – 1287.34

4.3.1.2 Analysis of Early and Late hive Bees Proteomes

Overall, there was a reduced number of SSDAs generated from early and late hive bee volcano plot comparisons demonstrating a similarity in proteome composition and expression of hive bees across the months sampled (**Figure 4.13 & Figure 4.14**). The highest number of SSDAs, occurred in October early and late hive bee samples when compared to July and August samples, indicating a shift in protein expression in October worker bees.

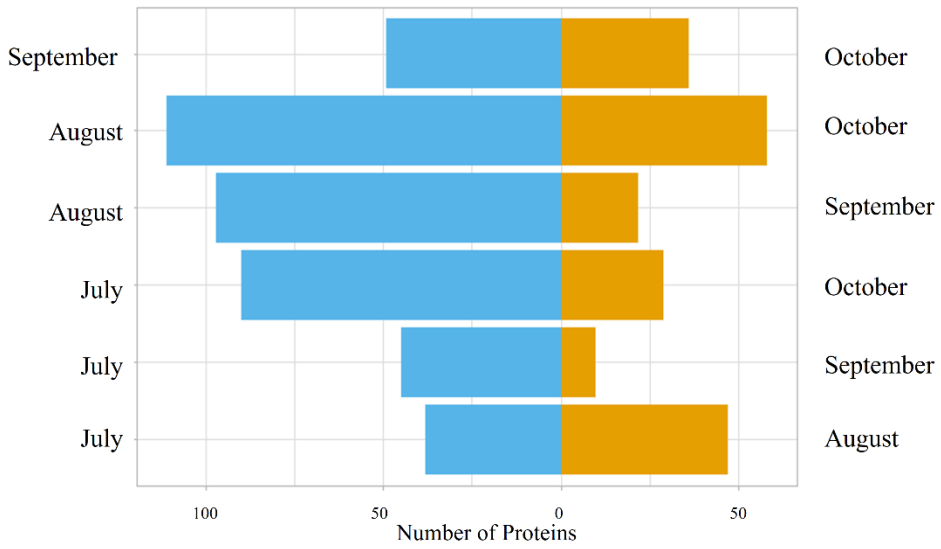


Figure 4.13: Bar chart of SSDA's generated from early hive bee volcano plot comparisons from samples collected between July to October.

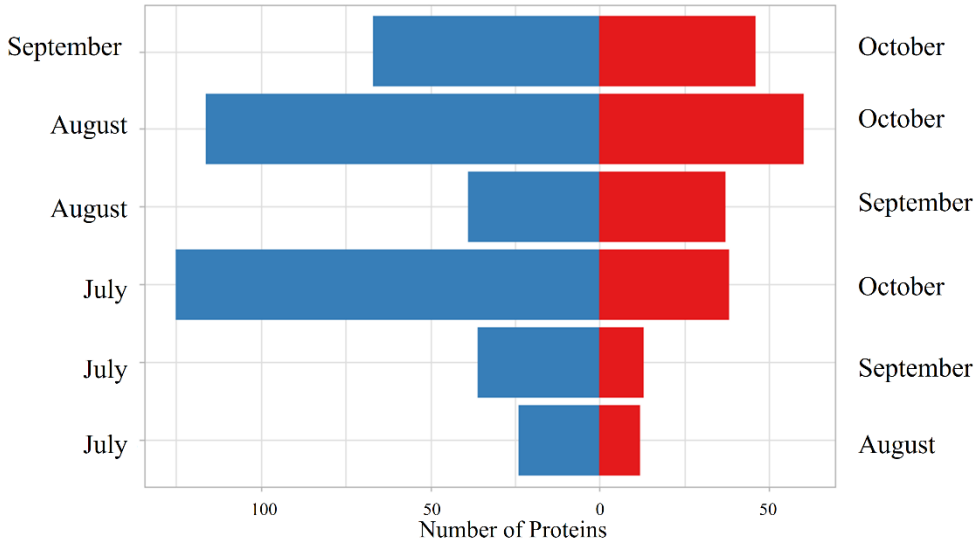


Figure 4.14: Bar chart of SSDA's generated from late hive bee volcano plot comparisons from samples collected between July to October.

Several mitochondrial and xenobiotic metabolism proteins were observed as SSDAs in September and October early and late hive bee samples. Specifically cytochrome P450 proteins (subunits: 9e2 and 6AQ1) and cytochrome c oxidase (4I1, 6a1, 6b1, 6c) were identified as SSDAs in October early and late hive bee samples in comparison to July and August samples (**Table 4.4**). Cytochrome c oxidase subunit

6c1 was observed as increased in abundance in September samples in comparison to July and August late hive bee samples (rfc, 2.27 and 2.41 respectively).

Table 4.4: October Early and Late relative protein intensity values from cytochrome P450 and cytochrome c oxidase SSDAs to July and August samples

Relative Fold Change	July Early	July Late	August Early	August Late
CYP9Q2	2.25	3.14	2.68	
CYP (A0A088AVB4)	2.09		2.01	
CYP6AQ1		5.49		
CYP6AS17		4.94	2.18	6.21
COX4I1	2.29	4.87	2.74	3.92
Cox6a1		3.86	2.61	3.64
Cox6c				2.52

Protocadherin Fat 4 isoform X1 (LOC552546) was amongst the top five most abundant SSDAs associated with July and August early and late hive bee samples when plotted against September and October worker bees (**Table 4.5**). Overall protocadherin Fat 4 isoform X1 LFQ values were significantly higher in abundance in July and August samples in comparison to September and October (**Figure 4.15**).

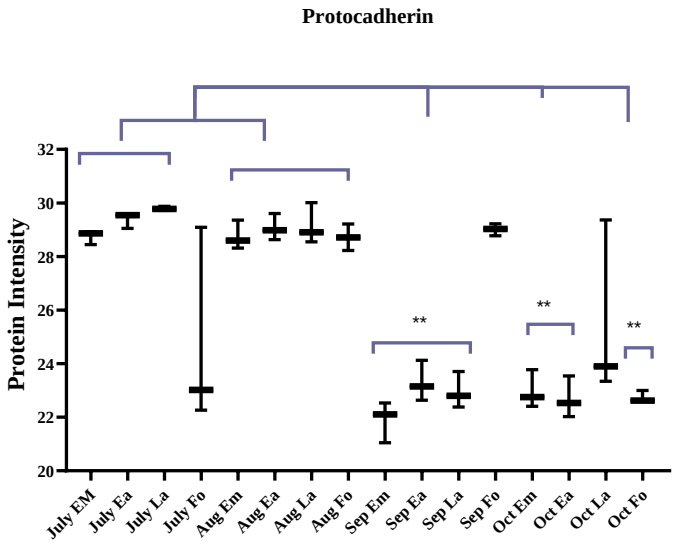


Figure 4.15: Boxplot of Protocadherin Fat 4 isoform X1 LFQ protein intensities from the July to October. Significant increase in protein intensity levels in July and August samples in comparison to September and October with the exception of July forager, September forager and October late samples. Significance is represented by 0 '***', 0.001'**', 0.1'*

Table 4.5: Relative fold change values for Protocadherin Fat 4 isoform X1 from to July and August early and late hive bee samples in comparison to September and October samples. Relative fold change values are increased in relative abundance in July and August samples.

Relative Fold Change	September Early	September Late	October Early	October Late
July Early	67.87		103.24	
July Late		113.59		NA
August Early	54.57		83	
August Late		73.05		NA

Several odorant binding proteins (OBPs) were identified in the early and late hive bee comparisons from July to October. July and August early comparison identified OPB 13 and 3 in July early hive samples (rfc, 8.66 and 3.88) and OBP 21 in August early hive sample (rfc, 2.08). August and September early comparison found OBP13, OBP3, OBP21 (rfc, 18.24, 5.2, 2.19) as SSDAs associated with September early hive samples. Late hive bee comparisons between July and September identified OBP2 and OBP17 (rfc, 31.81 and 2.19) as SSDAs associated with September samples. OBP16 was identified as an SSDA in both July and August late hive bee samples when compared to October late hive bee samples (rfc, 15.39, 32.94 respectively). Finally a volcano plot comparison between September and October late hive bee samples resulted in OBP16 (rfc, 23.78), OBP2 (rfc, 20.99) and OBP17 (rfc, 2.73) as SSDAs associated September late hive bee samples. No OBPs were identified as SSDAs in October early or late hive bee samples in the comparative analysis.

4.3.1.3 Analysis of Forager Bees Proteomes

Similar to early and late hive bee samples, a large number of SSDAs were generated when September and October forager bee samples were compared with July and August forager samples (**Figure 4.16**). This indicates a reduced protein expression in forager bees, which paired with early and late hive bee SSDAs suggests an overall decrease in protein expression for September and October samples.

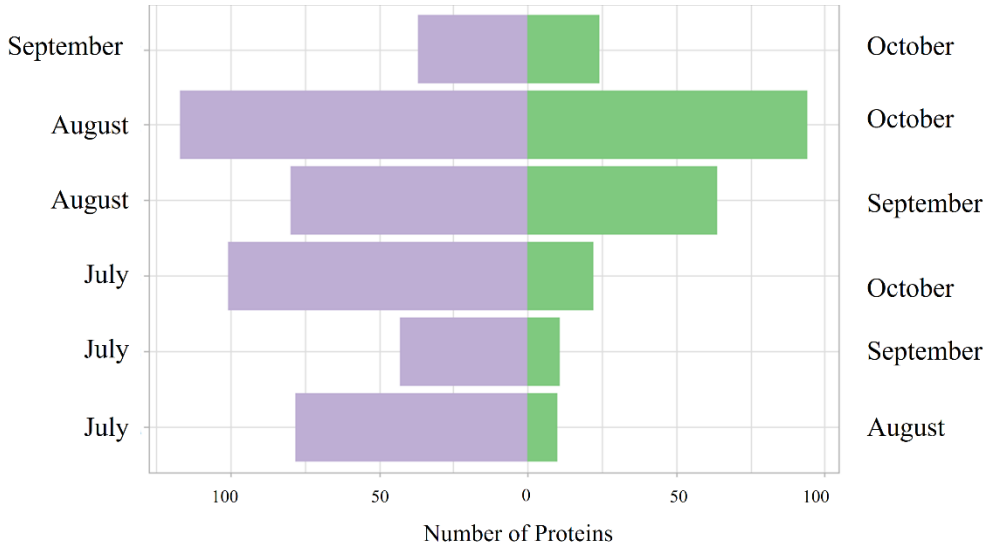


Figure 4.16: Bar chart of SSDA's generated from forager bees volcano plot comparisons from samples collected between July to October.

Mitochondrial proteins were observed to be increased in relative abundance in September and October forager samples, with the highest number of OXPHOS proteins identified in October samples across all comparisons (**Table 4.6**). g:Profiler analysis identified an increased abundance arginine and proline metabolism in August and September forager samples in comparison to October foragers (**Table 4.6**).

Volcano plot analysis of October samples compared to July, August and September samples revealed an increased abundance in cytochrome P450 proteins and cytochrome c oxidase proteins associated with October forager bees. Analysis with July revealed two cytochrome c oxidase proteins: Cox4I1 (rfc,4.17) and Cox6a1 (rfc,3.42) and two cytochrome P450 proteins: CYP9Q2 (rfc, 4.96) and CYP6a14 (rfc, 4.79). Comparison to August samples identified three cytochrome c oxidase (COX): Cox4I1 (rfc, 4.40), Cox6a (rfc, 3.51) and Coxfa4 (rfc, 2.81) and one cytochrome P450, CYP9Q2 (rfc, 2.35) as SSDAs in October. Finally, volcano plot analysis of September forager bee samples revealed one cytochrome P450: CYP6a14 (rfc, 2.99), and two cytochrome c oxidase proteins: COXfa4 (rfc, 2.15), COX (rfc, 2.15).

Protocadherin Fat 4 isoform X1 was the most abundant protein associated with September and August foragers in comparison to October forager samples (rfc, 63.09 and 77.06 respectively). It was not identified in other volcano plot analysis of forager

samples. Storage protein hexamerin 110 was only identified in the comparison between September and October and was one of the top 5 most abundantly expressed proteins in October forager samples (rfc, 6.89).

SSDAs from forager volcano plot analysis were further analysed in g:profiler for functional enrichment analysis (p-value of 0.05), and KEGG terms were used to identify enrichment in pathways (**Table 4.6**). No enrichment terms were identified as significant in the August forager samples when compared to September samples.

Table 4.6: gprofiler functional analysis term results from forager comparisons. Months with the same comparison number are a result of the direct comparison between those months. Term size is the number of genes that have been annotated to the term. Intersection size is the number of genes in the input query that are annotated to the corresponding term in the order of the input queries.

Test	Month	Gene Ontology Source	Term Name	Term ID	-log10 adjusted p-value	Term Size	Intersection Size
1	July	GOMF	MAP kinase activity	0004707	3.01	5	2
1	September	KEGG	Protein processing in endoplasmic reticulum	04141	1.79	70	6
2	September	KEGG	OXPHOS	00190	3.32	73	8
3	July	GOCC	Cytoplasm	0005737	2.36	1309	18
3	October	KEGG	Ribosome	03010	1.83	97	5
4	August	KEGG	Arginine and proline metabolism	00330	1.52	14	2
4	October	KEGG	OXPHOS	00190	3.46	71	8
5	September	KEGG	Arginine and proline metabolism	00330	0.35	14	1
5	October	KEGG	OXPHOS	00190	2.05	73	5

4.4 Emergence of Winter Bees from Summer Colonies Discussion

The time of the emergence of the diutinus or winter bee in *A. mellifera* populations is poorly characterised, which may be partly attributed to the lack of physical differences between summer and winter bees within colonies. Research has indicated that the chemical and environmental signals such as shorter days, reduced nutrient availability, brood absence and temperature decline may trigger the transition (Mattila and Otis, 2007; Döke et al., 2015). Previous studies have utilised immunohistochemistry and 'omic' methods such as genetics, proteomics and metabolomics to investigate the molecular composition of bees in order to better understand the physiological differences between summer and winter bees (Amdam and Omholt, 2002; Omholt and Amdam, 2004; Seehuus et al., 2006; Aurori et al., 2014; Kunc et al., 2019). Hierarchical analysis on both sets of data (first and second half of the sample year) revealed a shift in sample arrangement in the second half of the year that was not observed in the first half. September and October emerged bee samples were clustered separately from July and August emerged bee samples. Protein expression profiles in the first half analysis mirrored data that was observed in chapter three whereas the division in sample and protein profiles in the second half of the analysis needed further investigating. The shifts in sample arrangement within the hierarchical clustering in the second half of analysis could suggest the transitional period of summer to winter workers in the hives is approximately September as identified in previous work (Maurizio and Hodges, 1950; Döke et al., 2015; Kunc et al., 2019; Knoll et al., 2020).

Volcano plot analysis was performed to investigate the shifts in proteomic expression from samples of the same age taken in different months. This analysis identified an increase in MRJ proteins in July and August emerged bee samples in comparison to September and October emerged samples, with no MRJ proteins identified as SSDAs in September or October. MRJ proteins make up 80 – 90% w/w of the composition of royal jelly and are produced and secreted by the hypopharyngeal glands of worker bees (Jianke et al., 2010; Ramanathan et al., 2018). Newly emerged bees (Day 1) showed reduced production and secretion of MRJ proteins in comparison to both Day 3 and nurse bees (Day 5-7) (Qi et al., 2015), demonstrating that the development and activity of the glands is directly related to the age of the worker.

Hypopharyngeal glands in summer bees can become hypertrophied in response to broodless periods, much like the condition experienced by those of winter bee (Brouwers, 1983; Hrassnigg and Crailsheim, 1998). There is also a low rate of protein synthesis that occurs in the hypopharyngeal glands in both broodless summer and winter conditions (Brouwers, 1983; Huang et al., 1989; Amdam and Omholt, 2002). The hypopharyngeal glands of winter bees demonstrated the presence of MRJ proteins (Li et al., 2008). The current study did not find any MRJ proteins classified as SSDA in newly emerged September and October bees but highlights the abundance of MRJ proteins in newly emerged bees in July and August samples. This could provide some insight into what triggers the emergence of winter workers, as newly emerged bees sampled closer to summer months have a higher relative abundance of MRJ proteins than those sampled closer to winter months.

Protocadherin fat isoform X1 was increased in abundance in July and August samples in comparison to September and October samples. Protocadherins are members of the cadherins superfamily which are usually transmembrane proteins (Takeichi, 2006). The extracellular domain of these transmembrane proteins have cadherin repeats which contain sequences that are associated with calcium binding (Takeichi, 2006). In *Drosophila* protocadherin fat plays a role in regulating growth and for normal planar cell polarity of several *Drosophila* tissues including hair orientation (Clark et al., 1995; Casal et al., 2002; Hogan et al., 2011; Matakatsu and Blair, 2012; Bosch et al., 2014). Currently there is limited information about the role of protocadherin fat proteins function in bees and none associated with the emergence of winter bees. Cadherin proteins localised in the nuclear and cytoplasm of the gonads of both sexes and castes of bees, thereby suggesting alternative function of these proteins besides their characterised roles in cellular adhesion (Florecki and Hartfelder, 2012). Results presented here highlight the abundance of protocadherin fat isoform X1 in July and August hive and forager samples in comparison to September and October samples. Protocadherin fat isoform X1 may influence the development of bees through its role in the Hippo pathway and therefore a reduced expression in protocadherin fat isoform X1 may trigger the emergence of the winter bee phenotype, which are known to have a different structures and functions to summer workers in specific organs and glands such as hypopharyngeal glands and fat body (Snodgrass, 1910:121; Brouwers, 1983; Hrassnigg and Crailsheim, 1998).

Hexamerin 70c and 110 proteins were in higher relative abundance in September and October emerged and October forager samples. Hexamerins are abundantly expressed in larval fat body and used for amino acid storage, insect development and JH binding (Lee and Kim, 2017). Hexamerin 70c was present at high levels in larval stages, but progressively declined in abundance in pupal development, but can still be detected on the 7th day of an adult (Cunha et al., 2005). Hexamerin 110 was found to be high in bee larvae, to decrease in pupae, only to increase up to fivefold in 6 day old bees (Bitondi et al., 2006). Hexamerins are in greater abundance in the diapausing *Anthonomus grandis* (boll weevil), functioning as a storage protein (Lewis et al., 2002), and hexamerin has also been identified in the adults of several ant species during the period of brood rearing (Wheeler and Martinez, 1995). Hexamerin protein levels decrease as bees age, with the abundance being highest in newly emerged bees and lowest in foragers, and it has been suggested that the hexamerin levels are nutrient dependent (Bitondi et al., 2006; Martins et al., 2010; Hu et al., 2019). This suggests that the abundance of hexamerin proteins in winter bees has a dual function, to act as a storage protein in times of limited nutrient availability and to be used in late winter/early spring when brood rearing resumes.

The presence of Odorant Binding Proteins (OPBs) in early and late hive worker bee samples in July, August and September, but absence in October indicates a specific role of the proteins in the transition of bees from summer to winter. OBPs recognise chemical stimuli and have been revealed to have a role as pheromone carriers (Forêt and Maleszka, 2006; Iovinella *et al.*, 2011; Chertemps, 2017). Previous work using 2 Dimensional Gel Electrophoresis 2DGE and mass spectrometry on worker bee hemolymph, has identified a shift in odorant binding proteins between summer and winter workers, with summer worker bees expressing OBP13, 14 and 15 whereas OBP14 was only identified in winter workers (Erban *et al.*, 2013). Analysis in this chapter did not identify OBP14 as an SSDA in the comparative analysis between July to October. OBPs have different functions regarding cell signalling or pheromone detection for example: OBP13 binds to oleic acid with good specificity, oleic acid and β-ocimene have been linked to hygienic behaviour with brood removal (Iovinella *et al.*, 2011; McAfee *et al.*, 2018). Whereas, OBP21 binds with some affinity to components of the queen's mandibular pheromones - 9-oxo-2-decenoic acid and methyl p-hydroxybenzoate, which are used to inhibit the activation of ovaries and

follicle development in workers and aid in retaining the hierarchical structure within colonies (Hartfelder, 2000; Iovinella *et al.*, 2011). Work has also been completed on the change of protein expression levels of OBPs within worker bees according to their caste and age (Iovinella *et al.*, 2018). Some OBPs such as OBP 2 and 16 were intensely expressed in foragers antennae whereas OBPs 13, 17 and 21 are expressed more intensely within the mandibular glands of foragers (Iovinella *et al.*, 2011; Iovinella *et al.*, 2018). The presence of OBP 2, 3, 13, 16, 17, and 21 in July, August and September early and late hive bees and absence in October samples might be an indicator of the changes that occur within colonies as the seasons change from autumn to winter.

Several cytochrome c oxidase (4I1, 6a1, 6b1, 6c), cytochrome P450 (subunits: 9e2 and 6AQ1) and OXPHOS proteins were increased in abundance in October samples in hive and forager samples comparison to July, August and September samples. Collectively an increased abundance of OXPHOS and cytochrome P450 proteins in October samples indicates a better ability to deal with oxidative stress and metabolism of xenobiotics in comparison to summer months (July to September). An increased abundance of antioxidant enzymes in winter worker bees has been observed in comparison to summer worker bees and has been suggested to protect overwintering workers against ROS damages (Aurori *et al.*, 2014). However, little research has been conducted on the proteomic changes that occur with the transitional period of colonies from summer to winter, meaning that it is not possible to identify the specific role these proteins may have in the transition of colonies. The work here presents an initial start to characterising the changes in mitochondrial protein abundance in worker bees as the colony approaches the winter period.

Hierarchical clustering provided an overview of the relationship between the proteomes of bees of all ages from July to October. Interestingly, emerged bees from September and October samples were clustered separately from July and August emerged bee samples. This indicates a significant shift in proteome expression in September and October emerged bees and potentially the emergence of the winter bee phenotype within the hive. However, 333 proteins were upregulated in all emerged bee samples, which were characterised as being associated with the spliceosome and the basement membrane. The spliceosome and basement membrane are both essential pathways in the development and growth of organisms, responsible for removing

introns from pre-messenger RNAs and the ligation of remaining exons (Kahlscheuer et al., 2015; Stegeman et al., 2018), and the cellular polarity of cells ensuring cells are the correct size and shape in order to function normally (Khalilgharibi and Mao, 2021). The presence of these upregulated proteins in emerged bee samples could be indicative of the developmental process all emerged bees undergo as they mature to fully formed workers. Previous work has identified an increase in the abundance of ribosomal proteins in newly emerged worker bees (Liu et al., 2014) and suggests that the increase in genes associated with development in newly emerged bees could be linked with the rapid growth phase of the hypopharyngeal glands.

A cluster of 157 proteins was upregulated in only the October samples. Pathway characterisation of these proteins highlighted functionality in ion transport, mitochondrial activity, specifically oxidative phosphorylation. Organisms with an increased abundance of oxidative phosphorylation (OXPHOS) proteins have an increased tolerance to oxidative stress which can aid in the longevity of an organism (Harman, 1956; Cervoni et al., 2017). An increased tolerance of oxidative stress in winter bees has been characterised through increased levels of vitellogenin, OXPHOS and xenobiotic metabolism proteins (Omholt and Amdam, 2004; Amdam and Page, 2005; Münch and Amdam, 2010). The increased presence of mitochondrial proteins in the October samples in comparison to July – September samples, could identify the presence of the winter bees in the colonies. Typically foragers have a reduced OXPHOS capacity than nurse bees (Cervoni et al., 2017). However, hive and forager samples in October have an increased presence of OXPHOS proteins in comparison to hive and forager samples in July to September. This provides further evidence to the time frame when winter workers appear in the colonies, as it seems that October "foragers" are not typical summer foragers, but instead belong to the diutinus caste.

Previous work has identified autumn as the time for the emergence of winter workers, however the time frame can vary up to 4 weeks depending on the colony strength, nutrient availability and weather conditions (Maurizio and Hodges, 1950; Omholt, 1988; Mattila and Otis, 2007). Pollen stores can affect the production of long-lived winter bees relative to control colonies as an increase in pollen stores in colonies can delay the arrival of diutinus workers (Mattila and Otis, 2007). However, as the pollen and nectar vary in both availability and quality the effects nutrition has on the

transition of hives from summer to winter remains to be determined (Döke et al., 2015).

Weather data collected from the apiary location, identified a drop in temperature from July to September and subsequentially September to October. The drop in temperature could be a contributing factor to the change in proteome expression. However, future work would need to specifically investigate the changes in temperature as a factor to proteomic changes in worker bees.

4.5 Conclusion

The proteomic analysis conducted here demonstrates the pattern of age-polyethism in July and August months with the division of bee proteomes - between newly emerged bees through to foragers, and the emergence of winter bee phenotypes in colonies in late autumn – as observed in October's PCA. Specific changes in protein expression within samples taken between July and October provide evidence of how *A. mellifera* has adapted to unfavourable conditions in temperate climates. The increase in proteins associated with reducing oxidative stress (OXPHOS proteins) and of storage protein hexamerin in September and October samples, demonstrate some of the adaptions bees undergo to survive winter months. The shift of protein expression in newly emerged bees observed in the hierarchical clustering analysis, was particularly interesting as it indicates that bees emerging later in the year have a different protein profile to those that emerge in the summer or early autumn months. This work provides proteomic evidence of the time-frame when the winter bees emerges.

4.6 Summary of Findings

Table 4.7: Overview of protein analysis from Chapter 4: Utilising Proteomic Analysis to Identify Proteomic Changes in Worker Bees as they Transition from Summer to Winter

Protein	Expression Pattern
Major Royal Jelly Proteins	Major royal jelly proteins were increased in both July and August emerged bee samples
Protocadherin fat isoform X1	Protocadherin fat isoform X1 was increased in abundance in July and August samples in comparison to September and October.
Hexamerin	Hexamerin 70c and 10 proteins were identified in higher relative abundance in September and October samples.
Odorant binding proteins	Odorant binding proteins were identified as increased in abundance in early and late hive worker bee samples collected in July, August and September samples in comparison to October samples. Specifically OBP 2, 3, 13, 16, 17 and 21.
Mitochondrial proteins	Cytochrome c oxidase 4I1, 6a1, 6b1, 6c and were increased in abundance in October hive and forager samples in comparison to July, August and September samples.
Xenobiotic metabolism proteins	Cytochrome P450 subunits 9e2 and 6AQ1 were increased in relative abundance in October hive and forager samples in comparison to July, August and September samples.

Chapter 5

Proteomic Comparison of Honeybee Workers Sampled in Summer and Winter.

5.1 Introduction

Apis mellifera colonies are composed of three castes; the queen, the drones and the workers. Drones the males of the colony reside in the hive for the brood-laying period (Spring and Summer) and are pushed out of the hive in the Autumn. Workers make up the largest population of bees within the colony and carry out all housekeeping and foraging duties in the hive. It is the role of the workers to ensure the survival of the colony, in doing so they complete a myriad of tasks such as feeding larvae, building comb, guarding the hive and foraging for nutrients. In summer a colony can contain over 50,000 workers which live on average between 6 - 8 weeks. In winter the population reduces to 15,000 to 20,000 bees and winter colonies are characterised by long-lived winter worker bees known as ditunius bees, that survive for up to six months (Omholt and Amdam, 2004; Hooper, 2010; Aurori *et al.*, 2014). In contrast to summer bees, diutinus workers do not transition into foragers at the later stages of their development. Instead, they remain in the hive until the following spring (Omholt and Amdam, 2004). The emergence of the diutinus bee in the autumn correlates with the onset of unfavourable foraging conditions, reduced temperatures and brood rearing (Aurori *et al.*, 2014).

The role of diutinus bees in the hive varies to that of summer in-hive workers as there is no brood present within the colony. The main role of diutinus bees is thermoregulation (Omholt, 1987; Hooper, 2010; Steinmann *et al.*, 2015) which allows diutinus bees to regulate temperature between 32^{o}C – 36^{o}C by forming a cluster, generating heat through thoracic-muscular action and occupying empty cells (Gates, 1914; Omholt, 1987; Stabentheiner *et al.*, 2010; Hooper, 2010). The ‘superorganism model’ for thermoregulation agrees with the ‘heat entrapment model’ regarding reduction of heat loss in the mantle of the cluster (Lemke and Lamprecht, 1990; Heinrich, 1993), however the superorganism model adds that ‘shivering’ occurs in the core of the cluster (Fahrenholz *et al.*, 1989; Stabentheiner *et al.*, 2003). Thermoregulation is essential for the survival of the colony as honeybees are endothermic and can only generate small amounts of heat when in a cluster and have access to food, individually honeybees are not capable of long survival (Heinrich, 1993:292–293; Hooper, 2010:60). Winter clusters maintained through

thermoregulation allow colonies in temperate climates to survive unfavourable winter conditions.

During the following spring the diutinus bee populations will decline as their role transitions from thermoregulation to nursing newly laid summer workers and initiating foraging activities (Gates, 1914; Steinmann *et al.*, 2015). The re-emergence of the short-lived summer bee occurs in time for improved foraging conditions and increased brood production (Omholt and Amdam, 2004).

Summer bees have two stages of senescence, a slow in-hive stage (newly emerged, nurse and guard bees), and a rapid stage that correlates to workers transitioning to foragers (Rueppell *et al.*, 2007; Münch *et al.*, 2008). Increased senescence in older workers is linked with decreased titres of vitellogenin and an increase in juvenile hormone and insulin-like peptide 1(ilp1), both of which appear to be pro-aging factors in honeybees (Münch *et al.*, 2008). This leaves foragers more sensitive to stress such as starvation and heat, than younger in-hive workers (Remolina *et al.*, 2007). Unlike summer workers, diutinus workers have negligible senescence as a result of high tolerance to oxidative stress, a lack of foraging duties and no brood rearing which all contribute to their survival throughout winter (Omholt and Amdam, 2004; Amdam *et al.*, 2005b; Aurori *et al.*, 2014). Organisms exhibiting long lifespans commonly have a high abundance of antioxidant proteins, which reduce oxidative stresses by breaking down reactive oxygen species (ROS) that can cause oxidative damage to proteins, lipids and nucleic acids (Harman, 1956; Harman, 1981; Kodrík *et al.*, 2015). In-hive workers have a higher number of functional hemocytes and a better ability to deal with oxidative stress through increased protein titres such as vitellogenin, in comparison to foragers (Münch and Amdam, 2010; Havukainen *et al.*, 2013; Cervoni *et al.*, 2017).

Diutinus workers also have anatomical differences to their summer counterparts. Hypopharyngeal glands become hypertrophied in winter workers yet have a low rate of protein synthesis which is in contrast to summer workers who have a high rate of protein synthesis (Brouwers, 1983; Amdam and Omholt, 2002; Deseyn and Billen, 2005). Winter workers have lower protein content in their fat body cells than summer workers (Amdam and Omholt, 2002). The venom sac is one of the

honeybee workers defence strategies, however, little work has been completed investigating the potential proteomic differences between winter and summer workers.

During winter, hives are extremely vulnerable to failure due to limited food resources, poor foraging conditions and potential threats of pests and diseases within the confines of the clusters. The negative effect of pests and diseases is heightened during winter due to the close proximity of workers in the hive (Rosenkranz *et al.*, 2010). Despite the myriad of stresses that the diutinus bees face, their life span is significantly longer than short-lived summer bees (Münch and Amdam, 2010). The work presented here characterised proteomes of head, abdomen and venom sacs of late-hive caste summer workers and diutinus caste workers and examined the adaptations that may enable the diutinus bee to survive the stresses of winter.

5.1.1 Chapter Five Aims

- Conduct proteomic extraction and analysis on three anatomical samples from summer and winter worker honeybee samples.
- Investigate the proteomic differences between samples taken in summer versus winter.
- Characterise the proteome of winter honeybees from head, abdomen and venom sac samples.

5.2 Experimental Design

Head, abdomen and venom sac worker samples were prepared in triplicate from summer samples (June) and winter samples (December). Each sample was subjected to mass spectrometry preparation; protein extraction, clean-up, digestion, and peptide purification (C18) (Section 2.3 and 2.4.1). Sample volume for each anatomical sample load onto the mass spectrometer was 1 µg (head) and 0.75µg (abdomen and venom sac) of tryptic peptides were loaded onto a QExactive (ThermoFisher Scientific) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated

over a 2-40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100mm length, 75mm ID), using a 135 min reverse-phase gradient flow rate of 250nL min^{-1}. Three hives were used for the seasonal comparison, each hive was treated as an individual replicate.

Protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.6.6.0 (https://www.maxquant.org/). Data processing and analysis was carried out using Perseus v 1.6.6.0 (https://maxquant.net/perseus/). To identify protein-protein interactions, functions, and pathway enrichments STRING v 11 (https://string-db.org/) and KEGG Mapper (https://www.genome.jp/kegg/tool/map_pathway2.html) were utilised. Proteins were deemed statistically significant differentially abundant (SSDA) if identified to have a Log2 fold change greater than +/- 2 and a -Log p value greater than 1.3. Data from volcano plot comparisons can be seen in **Supp. File 5.1**.

5.3 Results

5.3.1 Proteomic Analysis of Bee Head Samples

Analysis of bee head samples revealed 433 and 66 exclusive proteins in summer and winter proteomes respectively, 1074 out of 1573 proteins were common between the samples. Seventeen cuticular and three major royal jelly proteins were identified as exclusive in the summer samples. Hexamerin 110 and five cytochrome P450 proteins were identified among the 66 exclusive proteins in the winter samples. Data matrix was further filtered through ANOVA t-test (p value = 0.05), 783 proteins were subsequently deemed as and were analysed in a Principal Component Analysis (PCA) graph (**Figure 5.1**). PCA clustered summer and winter samples separately with a total variance of the given dataset as 93.7%. This provides an initial insight into the distinct differences between winter and summer head proteomes.

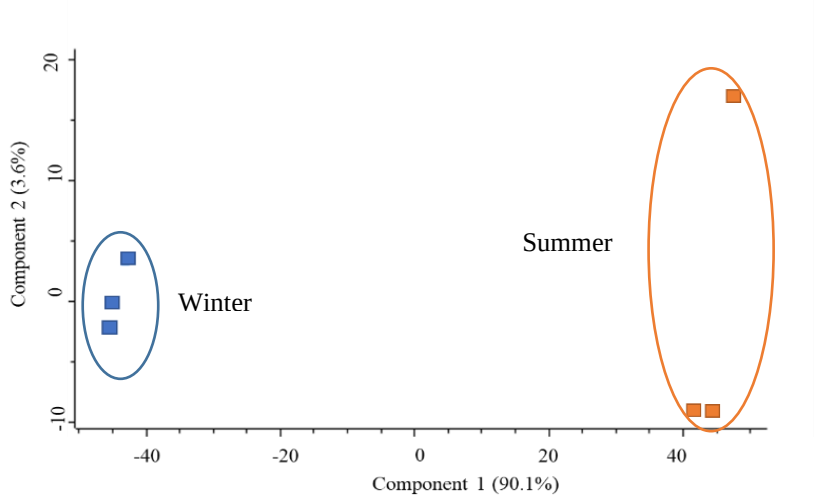


Figure 5.1: Principal component analysis on head samples from winter and summer bees. ANOVA significant head sample proteins (p value = 0.05) plotted on a PCA. Total explained variance of this PCA for the given dataset is 93.7%.

Volcano plot analysis was conducted to determine the distribution of proteins between the summer and winter samples (**Figure 5.2**). From this analysis, 522 proteins in summer samples and 197 proteins in winter samples were deemed SSDAs with a relative fold change (rfc) of ± 2. Summer bee samples had a larger rfc range of 2.02 to 4836.61 in comparison to winter samples rfc, 2.01 to 203.32, indicating reduced proteome abundance in winter bees compared to summer bee samples.

Summer samples had eleven cuticular proteins identified as SSDAs, of which nine were exclusively expressed in summer samples (**Table 5.1**). Relative fold change range of cuticular proteins was between 2.25 to 4836.61, cuticular protein 18.7 (A0A088ABR2) was the most abundant protein in this comparison. Cuticular protein intensity values were plotted and underwent Mann Whitney test ($p < 0.05$) to identify a significant increase in expression in summer workers versus winter workers (**Figure 5.3**).

Winter bee samples had an increased abundance of several xenobiotic proteins; Cyp4g11 (rfc, 92.35), Cyp9e2 (rfc, 28.27), Cyp6k1 (rfc, 26.42) in comparison to summer bee samples. LFQ intensity values of the cytochrome P450 proteins are significantly higher in winter samples versus summer samples (unpaired t-test $p > 0.05$) (**Figure 5.3**).

All major royal jelly proteins (MRJPs) identified in this analysis were increased in abundance in summer samples in comparison to winter samples. MRJP 6 was the most abundant MRJP identified in summer samples with a rfc of 332.03 (**Table 5.1**). The mean LFQ intensity values for all SSDA MRJPs identified were plotted (**Figure 5.3**) and tested for significance (Mann-Whitney $p < 0.05$). Summer bee samples were found to have a significantly higher expression of MJRPs in comparison to winter samples. Vitellogenin proteins (A0A088ADL8 and A0A088AUT9) were identified as SSDAs associated with winter samples (rfc, 17.03 and 9.25 respectively) and were significantly increased in abundance in winter samples (Tukey pairwise comparison 95% confidence) (**Figure 5.3**).

Summer samples had an increased abundance of three heat shock proteins (HSPs) (HSP70, HSP70Ab and Hsc70-5), however there was no significant increase in LFQ intensity values in HSPs in summer workers (**Table 5.1**) (**Figure 5.3**). One HSP was identified as a SSDA in the winter samples (HSP 83, rfc: 3.31) which was significantly increased in LFQ intensity value in winter samples (Unpaired t test $p < 0.05$).

All three honey production enzymes (alpha-amylase, alpha-glucosidase and glucose oxidase (rcf, 1049.77, 340.40, 75.76 respectively)), were identified as significantly increased in LFQ intensity values (Unpaired t test $p < 0.05$) in summer samples (**Figure 5.3**). Only two (alpha-glucosidase 2 precursor (rfc, 26.37), alpha-glucosidase precursor (rfc, 3.02)), were identified in winter samples but at a greatly reduced protein abundance and not significantly increased in LFQ intensity value.

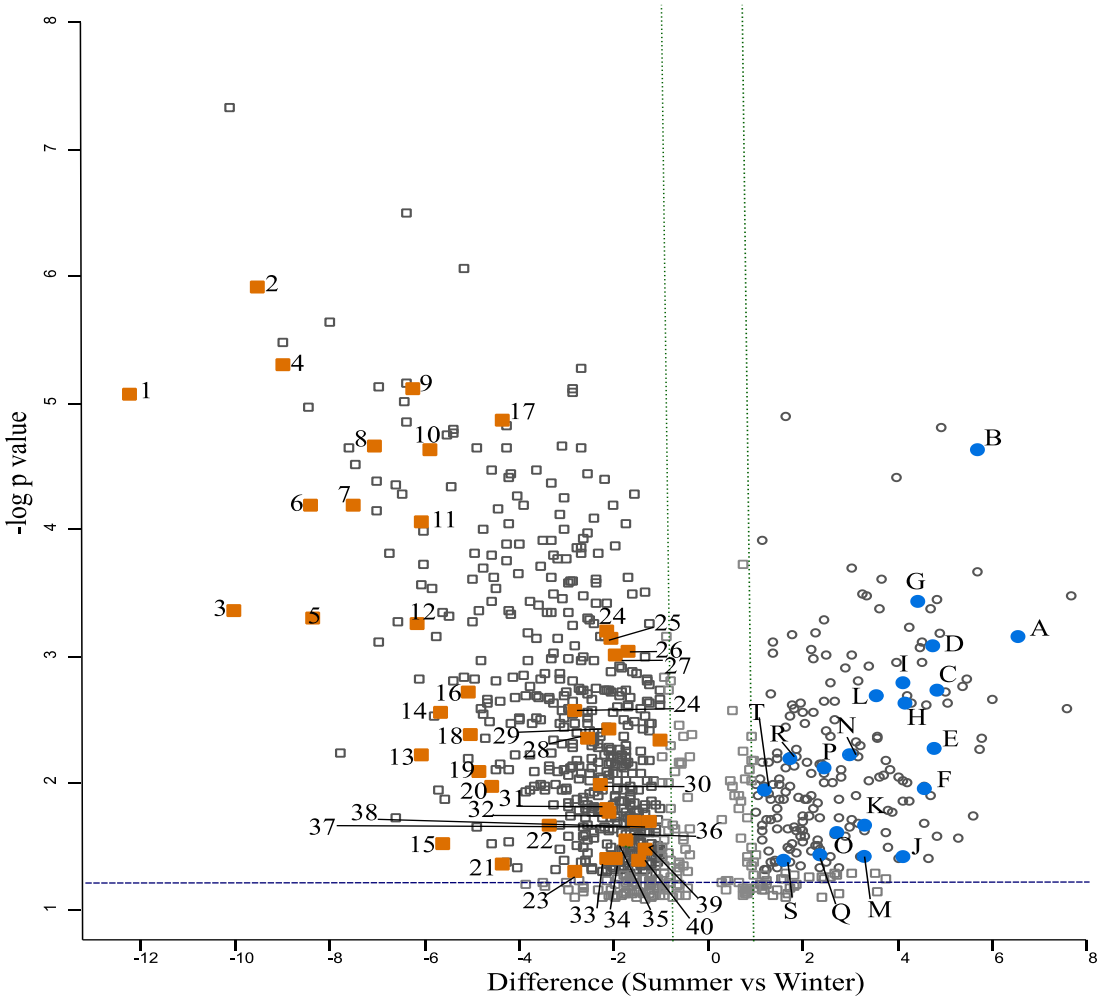


Figure 5.2: Volcano plot analysis of winter and summer head samples proteins above the green horizontal line and to the outside of the blue lines are considered SSDAs. Protein annotations are outlined in **Table 5.1.**

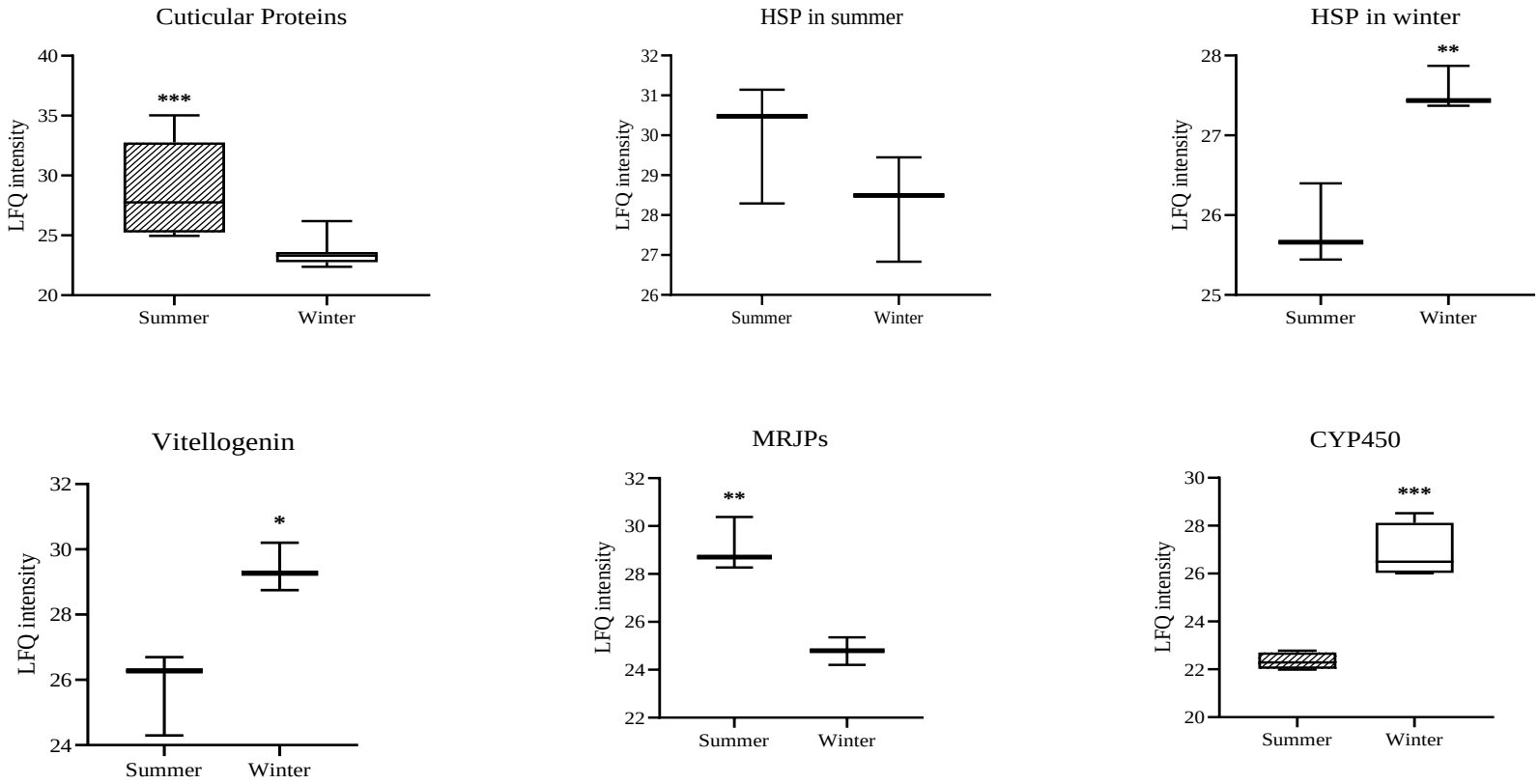


Figure 5.3: *Apis mellifera* head protein LFQ intensity values plotted on boxplots. Significance is marked as follows: 0 '***', 0.001'**', 0.01'*. Proteins labelled with the seasons identifies proteins identified as SSDAs to these seasons.

Table 5.1: Statistically significant proteins identified in the head samples of summer and winter bees. Proteins are categorised into three functional categories and labelled according to seasons - summer (S), winter (W). The real fold change highlights the variation in protein abundance between the sample sets. Proteins marked with * were identified as exclusively expressed in summer/winter

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
A0A088ABR2	Cuticle protein 18.7*	**Cuticular proteins**	S	1	4836.61
A0A087ZRG5	Cuticular protein CPF2 isoform 1 precursor*		S	2	733.41
A0A087ZNJ5	Cuticular protein 5*		S	4	512.28
A0A088AND4	Apidermin 2 precursor*		S	8	134.21
A0A088ANC9	Apidermin 3 precursor*		S	12	71.18
A0A088AKR9	Cuticular protein 28 precursor		S	13	67.70
A0A087ZR01	Cuticular protein analogous to peritrophins 3-C precursor*		S	16	34.11
A0A088ABN1	Cuticular protein 4 precursor*		S	30	4.86
A0A087ZT04	Cuticular protein analogous to peritrophins 3-D precursor*		S	31	4.37
A0A088ABN3	Cuticular protein 1 precursor		S	32	4.34
A0A088A955	Endocuticle structural glycoprotein		S	33	4.49
A0A087ZUS7	Cuticular protein precursor*		S	37	3.01
A0A088AG34	Cuticular protein 27 precursor		S	39	2.52
A0A088A3V5	Yellow-f precursor		S	10	58.66
A0A088AU21	Major royal jelly 6 precursor*	**Vitellogenin and MRJP**	S	5	332.03
O97432	Major royal jelly 5 precursor		S	15	49.09
A0A088AU20	Major royal jelly 4 precursor		S	21	20.73
O18330	Major royal jelly 1 precursor		S	23	7.01

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
Q4ZJX1	Major royal jelly 9 precursor*		S	24	7.15
A0A088AVG8	Major royal jelly protein 1*		S	34	3.96
A0A088ADL8	Vitellogenin		W	J	17.03
A0A088AUT9	Vitellogenin		W	M	9.25
P17722	Defensin-1 preprotein*	**Immune response**	S	7	183.61
A0A088AS56	Apolipophorins		W	Q	5.14
A0A088A3R5	Protein lethal(2) essential for life	**Heat Shock proteins**	S	14	50.76
A0A088A3R7	Protein lethal(2) essential for life		S	17	32.53
A0A088A3R6	Protein lethal(2) essential for life *		S	18	20.78
A0A088AGW8	Heat shock 70kDa protein		S	26	3.96
A0A087ZTY7	Heat shock protein cognate 5		S	27	3.24
A0A088AGJ8	Heat shock protein 70Ab-like		S	49	2.75
A0A088A2L4	Heat shock protein 83		W	R	3.31
A0A088A226	Glutaredoxin-related protein 5 mitochondrial	**Antioxidant Proteins**	S	42	2.49
A0A087ZYL4	Peroxiredoxin-5		S	20	23.95
A0A088ATH7	Methionine sulphoxide reductase		S	29	4.36
A0A088ABK0	Methionine-R-sulfoxide reductase		S	25	4.12
A0A088AGE7	Thioredoxin peroxidase 3 isoform 2		S	41	2.04
A0A088AV36	Microsomal glutathione S-transferase 1		W	I	17.33

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
A0A087ZUZ0	Cytochrome c oxidase subunit 6B1	**OXPHOS Proteins**	S	19	28.84
P00038	Cytochrome c		S	22	10.47
A0A088ALL7	Cytochrome b-c1 complex subunit 8		S	28	5.89
A0A087ZZU9	Cytochrome c oxidase subunit 5A		S	11	4.06
A0A088AI62	Cytochrome c oxidase subunit 6C1		S	36	3.37
A0A088A8C7	Cytochrome c oxidase subunit 5B		S	38	2.40
A0A088AIK9	Cytochrome P450 4G11*	**Xenobiotic Enzymes**	W	A	92.35
A0A088A7I0	UDP-glucuronosyltransferase 2C1*		W	B	51.75
A0A088AVB4	Cytochrome P450 9e2*		W	C	28.27
A0A088A7G1	Cytochrome P4506k1*		W	D	26.42
A0A087ZNX2	Beta-ureidopropionase		W	F	23.63
A0A088A7I2	UDP-glucuronosyltransferase 2A3*		W	G	21.78
A0A088AKD5	Cytochrome P4506AQ1 isoform X1*		W	K	11.54
A0A087ZXV8	Cytochrome P450 9e2		W	L	9.73
A0A088AJN6	beta-glucuronidase*		W	N	8.52
A0A087ZXU5	Cytochrome P450 9e2*		W	O	6.65
A0A088AEG8	Alpha-amylase	**Other**	S	3	1049.77
Q17058	Alpha-glucosidase		S	6	340.40
A0A088A031	Glucose oxidase		S	9	75.76
Q25BT8	Alpha-glucosidase 2 precursor		W	E	26.37

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
A0A087ZVX3	Glucose dehydrogenase		W	H	17.70
A0A0B4J2P5	Alpha-glucosidase precursor		W	S	3.02
A0A088A4K9	Odorant binding protein 14 precursor	**Protein folding and molecular transporters**	W	P	5.44
Q1W633	Odorant binding protein 21 precursor		W	T	2.25

SSDAs from both summer and winter bee samples were further analysed in BlastKOALA to identify the functional category assignment of proteins. Analysis of summer head proteome samples returned 391 proteins from 522 SSDAs and winter samples returned 147 of 197 SSDAs. Genetic information processing (messenger RNA biosynthesis, chaperones and folding catalysts, and chromosome and associated proteins), Energy metabolism (oxidative phosphorylation), and Environmental information processing (MAPK signalling pathway, Hippo signalling pathway) were increased in abundance in summer bee samples (**Figure 5.4**). Winter bee samples had a reduced abundance of proteins linked with the mitochondria 6 vs 31 involved in OXPHOS, 3 vs 7 in glycolysis and 0 vs 9 in TCA cycle. Carbohydrate metabolism had the smallest difference in protein abundance from the two sample groups (Summer 33: Winter 21). Three pathways associated with carbohydrate metabolism had a higher abundance of proteins increased in winter samples, galactose, ascorbate and aldarate metabolism, and pentose and glucuronate interconversions.

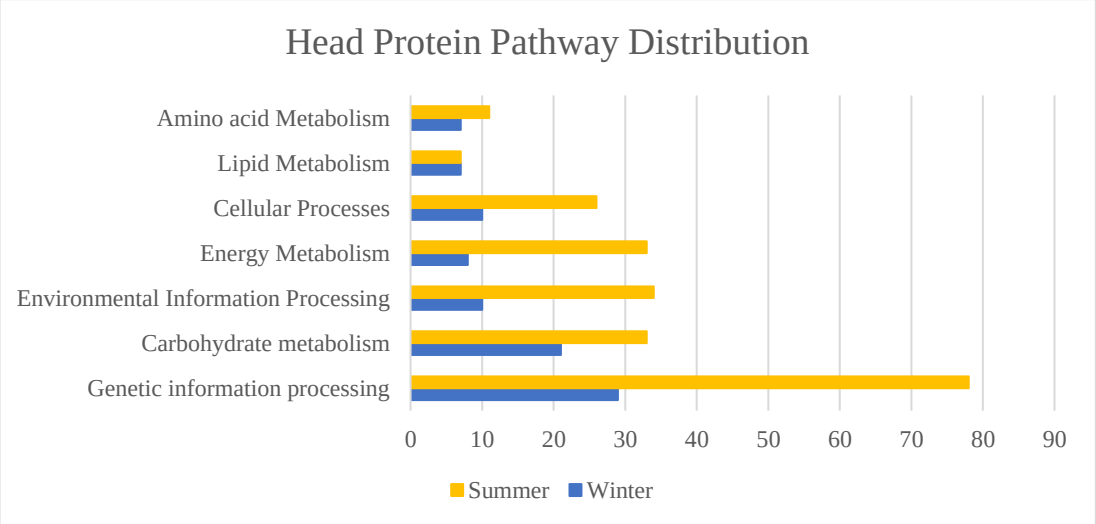


Figure 5.4: Pathway analysis on SSDA proteins identified in the summer and winter head samples. 147 of 522 entries in summer and 197 winter proteins of 391 were annotated and used in BlastKOALA analysis.

5.3.2 Proteomic Analysis of Abdomen Samples

Post filtration analysis of abdominal proteome samples resulted in 1,968 proteins, of which 130 and 27 were identified as exclusive in summer and winter honeybee samples respectively. Summer bee abdomen samples had defensin, alpha amylase and several cuticular proteins (7) identified as exclusive proteins. Winter bee abdomen samples had four venom-associated proteins and three xenobiotic proteins identified as exclusive proteins. ANOVA significant proteins (381) were analysed in a PCA, which revealed large differences between summer and winter proteomes (total explained variance of the PCA from this data equals 92.7%) demonstrates how different the sample proteomes are from each other (**Figure 5.5**).

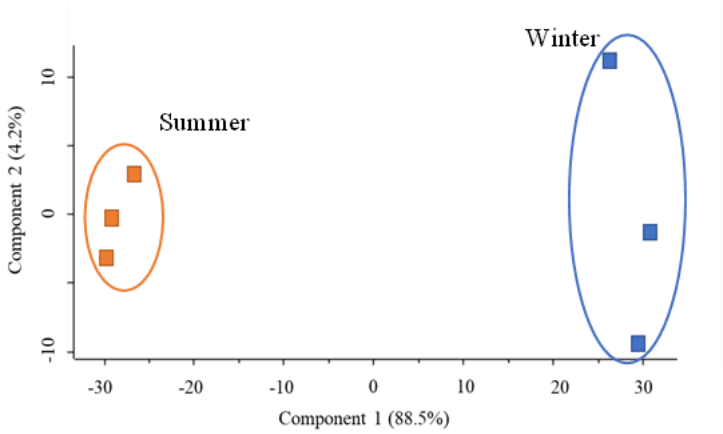


Figure 5.5: PCA on summer and winter abdominal samples. ANOVA significant abdomen proteins (p value = 0.05) plotted on a PCA. Total explained variance of this PCA for the given dataset is 92.7%.

ANOVA significant samples were analysed in a volcano plot which resulted in 197 and 104 SSDAs in summer and winter bee samples respectively. The relative fold change range for summer abdomen samples (2.03 – 1385.42) was larger than winter abdomen samples (2.02 – 1143.66). Cuticle protein 18.7 (rfc, 1385.42) and cuticular protein 5 (rfc, 432.82) were the two most abundance proteins in summer bee samples. Cuticle protein intensity values were significantly higher in summer samples in comparison to winter samples (unpaired t-test $p < 0.05$) (**Figure 5.7**).

Phospholipase A2 precursor (rfc, 1143.65) and allergen Api m 6 precursor (rfc, 270.42) were the most abundant proteins in winter bee samples (**Table 5.2**). Unusually

these proteins are associated with the venom sac. Odorant binding proteins 15 and 17 were identified as increased in abundance in winter samples (rfc, 66.66 and 4.04 respectively).

Following the same trend from the head proteome analysis, summer abdomen samples had a large abundance of proteins in several protein pathways, whereas winter samples had a reduced protein abundance and subsequently a reduced number of proteins associated with biological pathways (**Figure 5.6 & Table 5.2**). Two pathways were increased in abundance in winter abdomen samples, metabolism of cofactors and vitamins and carbohydrate metabolism pathway. As in the head samples three pathways associated with carbohydrate metabolism were increased in abundance in winter workers: galactose, ascorbate and aldarate metabolism, and pentose and glucuronate interconversions. Summer bee abdomen samples had a large abundance of proteins involved in genetic information processing, specifically in messenger RNA biogenesis (10), chromosome and associated proteins (8) and chaperones and folding catalysts (8). Metabolism of cofactors and vitamins were increased in winter samples in, one carbon pool by folate (4) and pantothenate and CoA biosynthesis (2) (**Figure 5.8**).

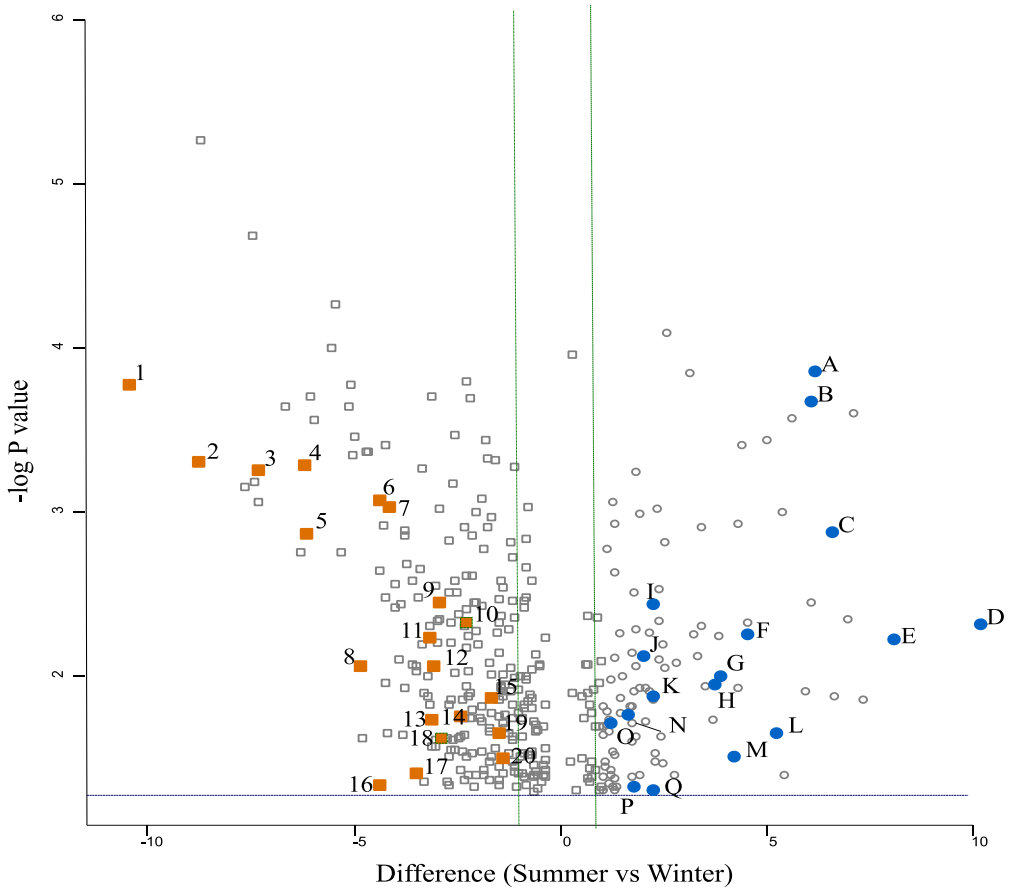


Figure 5.6: Volcano plot analysis on abdomen samples from summer and winter. Proteins above the horizontal dashed blue line and outside the two green lines are SSDAs.

Table 5.2: Statistically significant proteins identified in the abdomen samples of summer and winter bees. Proteins are categorised into three functional categories and labelled according to seasons summer (S), winter (W). The real fold change highlights the variation in protein abundance between the sample sets. Proteins identified with an * are exclusive to either summer or winter samples.

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
A0A088ABR2	Cuticle protein 18.7*	**Cuticular proteins**	S	1	1385.42
A0A087ZNJ5	Cuticular protein 5*		S	2	432.82
A0A087ZRG5	Cuticular protein CPF2 isoform 1 precursor*		S	3	161.39
A0A088ANC9	Apidermin 3 precursor		S	4	74.31
A0A088A3V5	Yellow-f precursor*		S	6	20.70
A0A088AND4	Apidermin 2 precursor*		S	13	8.67
A0A087ZR01	Cuticular protein analogous to peritrophins 3-C precursor*		S	9	7.71
A0A087ZZE9	Epidermal growth factor receptor		S	14	5.36
A0A088A4T4	Endocuticle structural glycoprotein		W	K	4.69
O97432	Major royal jelly 5 precursor	**Vitellogenin and MRJP**	S	12	8.40
Q76LA5	Juvenile hormone esterase precursor		W	H	13.39
A0A087ZVE8	Juvenile hormone epoxide hydrolase 1		W	P	3.39
P17722	Defensin-1 preprotein*	**Immune response**	S	7	17.54
A0A088A3R7	Protein lethal(2) essential for life	**Heatshock systems**	S	10	9.01
A0A088A3R6	Protein lethal(2) essential for life		S	11	7.45
A0A088A3R5	Protein lethal(2) essential for life		S	18	4.83
A0A087ZQD3	Heat shock protein 90		S	15	3.16

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
A0A088A7I0	UDP-glucuronosyltransferase 2C1*	**Xenobiotic Enzymes**	W	F	23.19
A0A088AIK9	Cytochrome P450 4G11*		W	G	14.77
A0A087ZNX2	Beta-ureidopropionase		W	Q	4.91
A0A088AFL5	Glutathione S-transferase D1 isoform X1		W	I	4.72
A0A087ZYL4	Peroxiredoxin-5	**Antioxidant and OXPHOS proteins**	S	19	2.36
P00038	Cytochrome c		S	20	2.60
A0A087ZRJ5	Peroxiredoxin-like protein		W	O	2.02
A0A088A031	Glucose oxidase	**Other**	S	5	70.35
A0A087ZVX2	Glucose dehydrogenase		S	8	29.21
Q17058	Alpha-glucosidase		S	16	20.54
A0A088AEG8	Alpha-amylase		S	17	11.43
P00630	Phospholipase A2 precursor		W	D	1143.66
P83563	Allergen Api m 6 precursor		W	E	270.42
P01501	Melittin precursor		W	C	96.50
A0A087ZUE7	Glucose dehydrogenase		W	A	71.06
Q25BT8	Alpha-glucosidase 2 precursor		W	L	37.31
P01499	Mast cell degranulating		W	M	18.30
A0A088A4L0	Odorant binding protein 15 precursor*	**Protein folding and molecular transporters**	W	B	66.66
Q1W637	Odorant binding protein 17 precursor		W	J	4.04

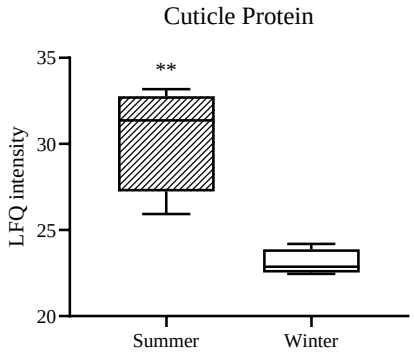


Figure 5.7: LFQ intensity values plotted of cuticle proteins plotted on a boxplot from abdomen winter and summer honeybee samples. Significance is marked as follows: 0 '***', 0.001'**', 0.01'*.

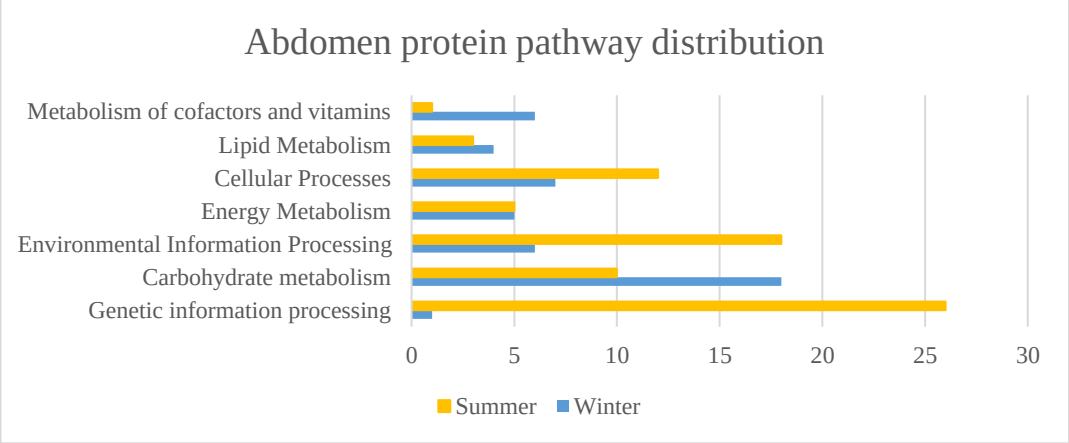


Figure 5.8: Pathway analysis on SSDA proteins identified in the Summer and Winter abdomen samples. 143 of 197 entries in summer and 73 winter proteins of 104 were annotated and used in BlastKOALA analysis.

5.3.3 Proteomic Analysis on Venom Sac Samples

Proteomic analysis on winter and summer bee venom sacs samples revealed significant differences in the proteomic profile of each sample as observed in the PCA graph (**Figure 5.9**). Analysis revealed 106 and 14 exclusive proteins in summer (rfc, 2.03 – 694.82) and winter bee (rfc, 2.02 – 46.60) proteomes respectively. Several proteins associated with bee venom activity were identified as exclusive in summer samples; major royal jelly proteins (MRJP) 8 and 9, mast cell degranulating peptide, and allergen Api m 6 precursor. No insect allergen proteins were identified in winter venom sac samples.

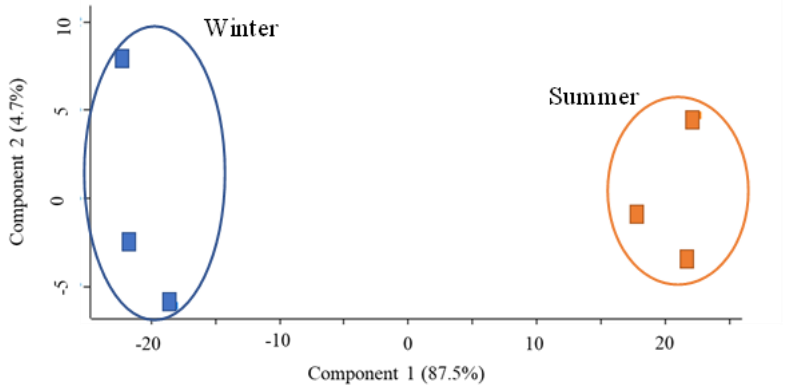


Figure 5.9: PCA on summer and winter venom sac samples. ANOVA significant venom sac proteins (p value = 0.05) plotted on a PCA. Total explained variance of this PCA for the given dataset is 92.2%.

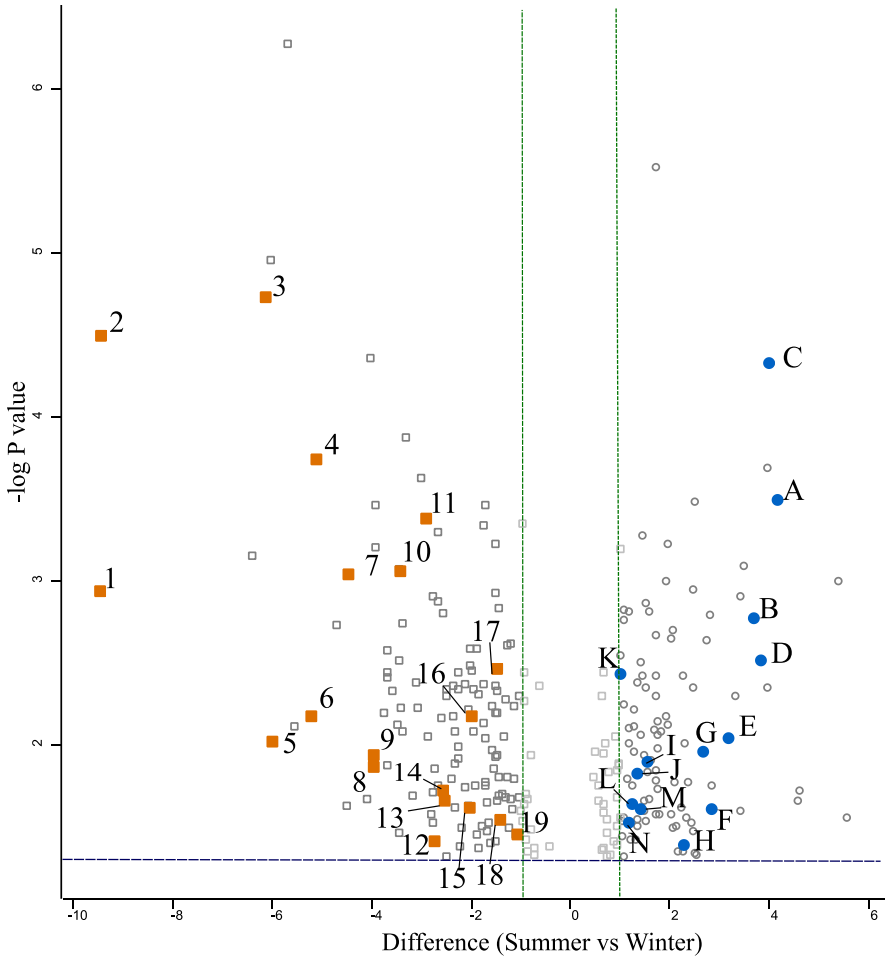


Figure 5.10: Volcano plot analysis on venom sac samples from summer and winter. Proteins above the horizontal dashed blue line and outside the two green lines are SSDAs.

Volcano plot analysis on venom sac protein samples from winter and summer bees revealed a small cluster of insect allergen related proteins in winter bee samples versus a larger number of insect allergen and venom proteins in summer worker bees (**Table 5.3, Figure 5.10**). Several previously identified venom proteins (venom serine proteases, dipeptidyl peptidases, carboxylesterases and acid phosphates) were observed in the data analysis of the samples, although were not deemed ANOVA significant or SSDA. Summer samples had a higher abundance of venom associated and antioxidant proteins in comparison to winter samples (**Table 5.3**). Phospholipase A2 precursor, Melittin precursor and Allergen Api m6 precursor were significantly increased in summer samples (LFQ intensities Tukey Pairwise Comparison 95% confidence) (**Figure 5.11)**.

Some MJRP were identified in the analysis, MRJP2 and MRJP3 were identified as SSDA proteins in winter samples. MRJP 2 & 3 were significantly increased in LFQ intensity values in winter samples versus summer samples. These MRJPs have not been associated with the venom sac in previous publications (Li *et al.*, 2013; Danneels *et al.*, 2015; Scieuzo *et al.*, 2021). MRJP9, a known venom sac protein was identified as significantly increased in LFQ intensity values in summer samples (unpaired-t test, p value > 0.05) (**Figure 5.11)**.

Gene ontology analysis identified 23 out of 91 proteins in summer venom sac samples which were involved in oxidative phosphorylation (**Figure 5.12**). No proteins associated with OXPHOS were identified in winter venom sac samples. Both samples had proteins involved in tricarboxylic acid process, summer samples had 8 proteins and winter had 2. Winter bee samples had an increase in ribosomal proteins (12) in comparison to summer worker bees (0). Lipid metabolism was the only other pathway that was more abundant in winter samples versus summer samples (7 vs 5). Overall there was an increase in proteins involved in energy and carbohydrate metabolism in summer samples, in comparison to an abundance of genetic information processing proteins in winter bee venom sac samples (**Figure 5.12**).

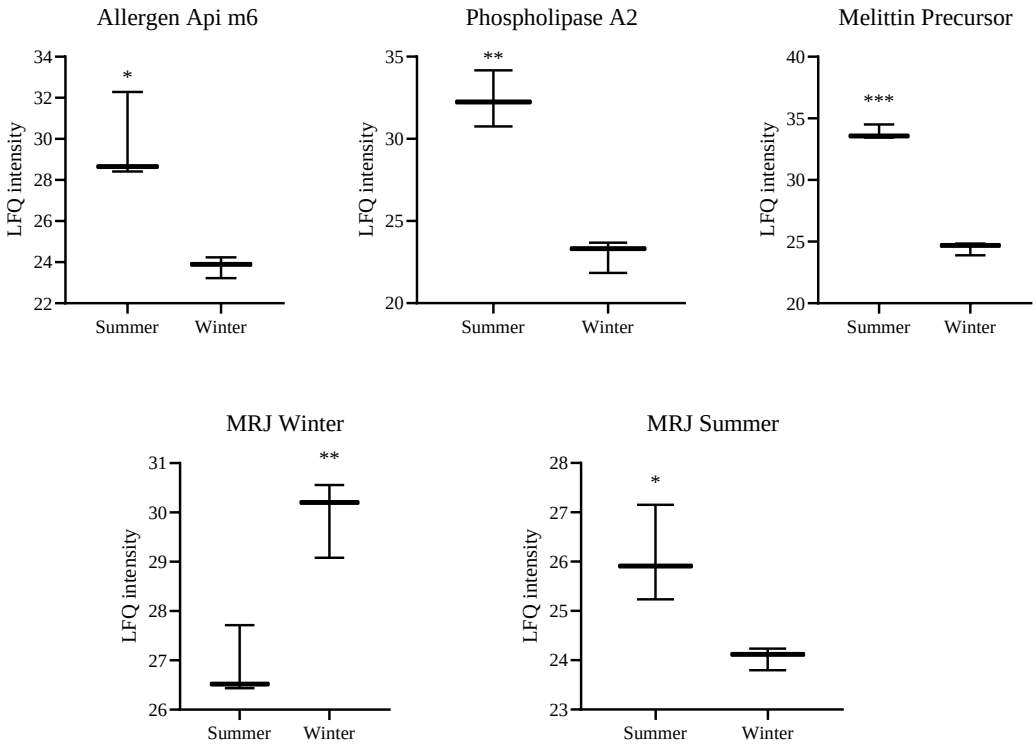


Figure 5.11: MRJP LFQ intensity values plotted on a boxplot from venom sac winter and summer honeybee samples. Significance is marked as follows: 0 '***', 0.001'**', 0.01'*. Proteins labelled with the seasons identifies proteins identified as SSDAs to those seasons.

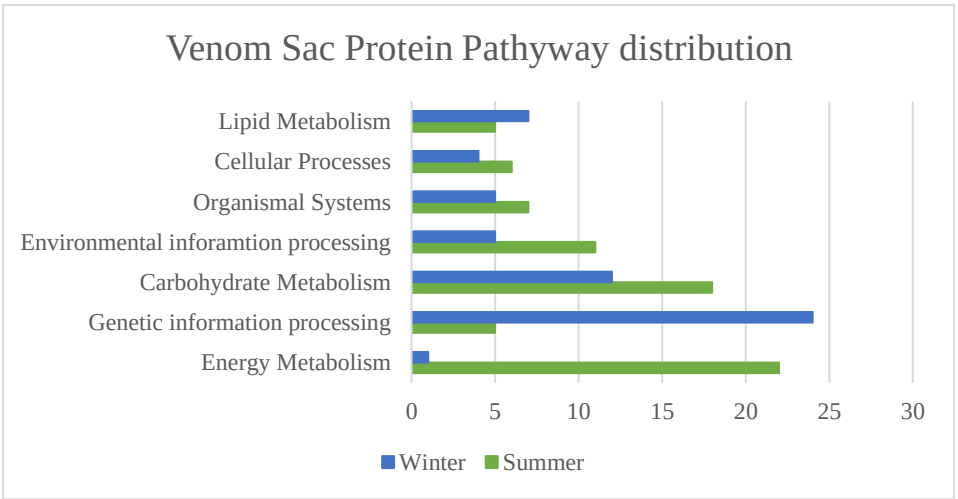


Figure 5.12: Pathway analysis on SSDA proteins identified in the Summer and Winter venom sac samples. Of the 129 summer SSDAs, 91 entries and 83 of 101 entries of winter samples were identified and classified into pathway groups.

Table 5.3: Statistically significant proteins identified in the venom sac samples of summer and winter bees. Proteins are categorised into three functional categories and labelled according to seasons summer (S), winter (W). The real fold change highlights the variation in protein abundance between the sample sets. Proteins marked with * were identified as exclusively expressed in summer/winter.

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
P00630	Phospholipase A2 (Api m1)	**Bee Venom Toxins**	S	1	694.82
P01501	Melittin (Api m4)		S	2	658.29
P83563	Allergen Api m6 (Api m 6)*		S	4	64
A0A088ARZ8	Arginine kinase isoform X2*		S	10	10.96
O61367	Arginine kinase		S	18	2.65
A0A088A9M1	Serine Protease 53		W	H	4.77
Q4ZJX1	Major royal jelly protein 9*	**Major Royal Jelly Proteins**	S	15	4.14
O77061	Major royal jelly protein 2		W	B	12.84
O18330	Major royal jelly protein 1		W	F	7.17
Q17060	Major royal jelly protein 3		W	G	6.26
A0A087ZUE7	Glucose dehydrogenase	**Antioxidant systems**	S	5	37.16
A0A088AAY9	Thioredoxin-containing protein 17-like		S	19	2.34
A0A088AQ13	Succinate dehydrogenase		S	17	2.75
A0A088AVB4	Cytochrome P450 9e2*		W	A	17.78
A0A088AJN6	Beta glucuronidase*		W	C	16.13
A0A088A5Z9	Hydroxyacid oxidase 1*		W	D	14.41
A0A088AIK9	Cytochrome P450 4G11		W	E	9.09
A0A088AE90	Protein lethal (2) essential for life		W	I	3.34

Protein Id Code	Protein Name	Functional Category	Sample	Label	Real fold change
C3VMN1	Glutathione S-transferase S1 isoform X1		W	L	2.34
A0A088AM69	Thioredoxin		W	N	2.23
A0A087ZZU9	Cytochrome c oxidase 5A	**Oxidative Phosphorylation Proteins**	S	3	70.06
A0A087ZUZ0	Cytochrome c oxidase 6b1		S	6	34.15
A0A088AQ34	Cytochrome c oxidase 6A1		S	16	3.93
A0A088A1K0	Superoxide dismutase 2		S	11	7.47
A0A088AFL5	Glutathione S-transferase D1 isoform X1		S	14	5.88
A0A088ACF4	Heat shock protein cognate 3	**Heat Shock Proteins**	W	J	2.51
A0A087ZQD3	Heat shock protein 90		W	K	2.02
A0A088A4L0	Odorant binding protein 15	**Protein folding and molecular transporters**	S	8	15.30
A0A088A4L4	Odorant binding protein 19*		S	13	5.82
A0A088A4L3	Odorant binding protein 18		W	M	2.68
A0A088ANY8	Protocadherin fat 4 isoform X1	**Cuticle Proteins**	S	7	21.93
A0A088AKR9	Cuticular protein 28 precursor		S	9	15.50

5.4 Discussion

Honeybee workers in temperate climates display different roles within the hive depending on the season: brood rearing and honey production in summer to survival and thermoregulation in winter (Münch and Amdam, 2010). Summer workers undertake a range of roles as they progress through the hive such as cleaners, nurses, builders, guards, and foragers, these roles are usually correlated with the workers age, a process known as age polyethism (Seeley, 1982; Robinson and Huang, 1995; Amdam *et al.*, 2004b; Münch and Amdam, 2010). Diutinus worker bees have a much narrower range of roles in the hive, thermoregulation during the winter and broodless periods, nursing duties, and ending with foraging activities in the following spring (Omholt, 1987; Hooper, 2010; Steinmann *et al.*, 2015).

Advances in whole cell lysate proteomic extraction techniques enables the identification of a large number of proteins, a higher level of sensitivity and accurate quantification of proteins in the comparison of winter and summer samples. This highlights a significant difference in the proteome of workers sampled in the summer and the winter. Summer worker bees have an increased abundance of cuticular proteins in the head and abdomens and a higher number of bee toxin proteins in the venom sac. In contrast workers sampled in winter have a greatly reduced proteome in all three anatomical sites. A reduction in immune protein abundance in winter honeybee workers has been observed previously, which suggests that the reduction is linked to longevity (Steinmann *et al.*, 2015).

Anatomical components of honeybee workers have been previously investigated to characterise changes/adaptions workers undergo to survive in their environment; changes in protein secretion within the hypopharyngeal glands in honeybee worker head samples have been correlated with age related changes (Brouwers, 1983; Ohashi *et al.*, 1999; Kubota *et al.*, 2004; Deseyn and Billen, 2005; Qi *et al.*, 2015; Dobritzsch *et al.*, 2019). Work has also been completed on specific protein changes that occur in workers during age-related changes or in comparisons of summer and winter workers (Amdam *et al.*, 2009; Smedal *et al.*, 2009; Steinmann *et al.*, 2015). A recent study highlighted physiological and immune parameters that change between winter and summer bees, identifying an increase in vitellogenin titres, lipid and a higher level of antibacterial activity in winter workers (Kunc *et al.*, 2019).

The work presented here sought to further explore the proteomic differences that occur in *Apis mellifera* workers between winter and summer.

Analysis of the bee head samples revealed a total of 6 MRJPs, all are more abundantly expressed in summer bees than in winter bees, including two exclusively expressed MRJPs. This is expected as winter honeybee workers hypopharyngeal glands are hypertrophied and no secretory cycle is visible in winter worker bees (Brouwers, 1983; Deseyn and Billen, 2005). Summer workers however, are exposed to brood rearing in the hive and have elevated MRJP levels to produce bee milk for larvae and royal jelly for the queen (Deseyn and Billen, 2005; Ramanathan *et al.*, 2018). It has been suggested that MRJPs that were secreted before workers enter the diutinus phase are stored within diutinus workers and have the potential to be used in the following spring, but little to no production of MRJPs occurs during winter (Deseyn and Billen, 2005).

Honeybees lack an adaptive immune system, so instead rely on their innate immune system which is composed of four lines of defence: hygienic behaviour, physical barriers, cellular immune and humoral immune responses (Evans *et al.*, 2006; Grzywnowicz *et al.*, 2009). Analysis on the head, abdominal and venom sac samples revealed an overall increase in cuticular proteins in summer samples in comparison to winter samples. A number of cuticular proteins were exclusively expressed in summer head (9 out of 11) and abdomen (6 out of 7) samples. Winter samples had a greatly reduced abundance of cuticular/chitin proteins in comparison to summer samples in both the head and abdomen analysis. It is suggested that winter workers have less "wear and tear" to their exoskeleton compared to nurse or forager summer workers as there is a reduction in water evaporation due to lower temperatures, little to no transport of external materials into the hive, no brood and a diminished level of hygienic behaviour involving "licking" or cleaning (Seehuus *et al.*, 2013; Pritchard, 2016).

Several xenobiotic metabolism proteins were identified as increased in abundance in winter worker head and abdomen samples. Several cytochrome P450s were identified as exclusively expressed in winter bee samples whereas no cytochrome P450 proteins were associated with summer samples. Cytochrome P450s are one of the main enzyme groups responsible for xenobiotic metabolism and catalysing

oxidation-reduction reactions in insects, however the stoichiometry of these enzymes has not been experimentally confirmed in insects (Feyereisen, 2012; Xu *et al.*, 2013). Cytochrome P450s can produce reactive oxygen species in their reactions, however, these enzymes are also involved in the production hormones, pheromones and other enzymes that break down ROSs (Xu *et al.*, 2013). Winter workers displayed a small number of antioxidant proteins upregulated in the head and abdomens - catalase isoform X1, microsomal glutathione S-transferase 1 and peroxiredoxin-like protein which break down hydrogen peroxide (Corona and Robinson, 2006).

Winter bee head samples also had an abundance of vitellogenin and apolipophorin-III, a known antioxidant with metal binding capacities and a lipid transporter linked with immune activation and secretion by hemocytes at the site of injury respectively (Wiesner *et al.*, 1997; Niere *et al.*, 1999; Whitten *et al.*, 2004; Kim *et al.*, 2004) which can preferentially oxidise hemolymph protein over other proteins such as apolipoprotein 1 (Seehuus *et al.*, 2006b; Corona and Robinson, 2006). A depletion in vitellogenin levels and subsequently a rise in juvenile hormone titres has been directly linked with the depletion of hemocytes in the hemolymph through nuclear pycnosis (Amdam *et al.*, 2004b; Amdam *et al.*, 2005b). This can lead to severe health issues with *Varroa* parasitisation as the mites are now known to feed on the fat body of workers (Ramsey *et al.*, 2019). Hemocytes play an important role in cellular immunity with immunological functions in phagocytosis, encapsulation, nodulation, wound repair and production in antibacterial peptides and prophenoloxidase enzymes (Amdam *et al.*, 2005b; Negri *et al.*, 2014; Yelkovan *et al.*, 2021). Collectively the increased abundance of xenobiotic metabolism proteins – cytochrome P450s, antioxidant proteins and abundance of hemolymph proteins - vitellogenin and apolipophorins, demonstrate a specialised immune system that aids in reducing stress in cells and aids in longevity.

Heat Shock Proteins (HSP) are involved in a wide range of protein processing pathways such as folding and assembly of new proteins and protect insects from environmental stresses (Rinehart *et al.*, 2000; Mayer and Bukau, 2005). In some species heat shock protein levels are influenced by diapause such as *Sarcophaga crassipalpis*, in which HSP levels are up regulated just before and after diapause but downregulated during diapause (Rinehart and Denlinger, 2000; Denlinger, 2002). In

other species HSPs are increased during diapause (Rinehart and Denlinger, 2000; Denlinger, 2002). This work found a decrease in heat shock protein levels in winter workers, these results align with previous work that completed a similar comparison with winter and spring honeybee workers (Lee and Kim, 2017). This could suggest that summer workers are exposed to environmental stresses that trigger an increase in heat shock proteins that is not observed in winter workers.

Carbohydrate metabolism proteins were increased in abundance in the abdomens of winter bees in comparison to summer bee samples. Three honey production proteins were identified as SSDAs in winter head and abdomen samples Galactose, ascorbate and aldarate, along with pentose and glucuronate interconversions all had an increased protein abundance in winter head and abdomen samples. Galactose metabolism is closely linked with energy metabolism (Coelho *et al.*, 2015). Ascorbate and aldarate metabolism forms UDP-glucose which is oxidised to UDP-glucuronic acid and is mainly used for detoxification of toxic compounds through conjugation (Chen *et al.*, 2019; Jiang *et al.*, 2019; Malka *et al.*, 2020). Ascorbate has also been observed to protect cells and organelles from oxidative damage by scavenging ROSs and aids in accumulating energy reserves in the hemolymph (Goggin *et al.*, 2010). Ascorbate can decrease the activity of respiratory enzymes and works to modulate humoral and cellular immune responses (Pristavko and Dovzhenok, 1974; Garg and Mahajan, 1994; Goggin *et al.*, 2010). In blood feeding insects such as *Anopheles gambiae* ascorbate was observed to lower the induction of superoxide radicals in the hemolymph in response to ingestion of iron-rich blood meals (Kumar *et al.*, 2003). Pentose and glucuronate metabolism generate NADPH and pentoses which are mainly used for maintaining/regenerating the cellular detoxifying and antioxidative defence systems, they also function in the synthesis of nucleotides and nucleic acids (Ceddia *et al.*, 2003; Agledal *et al.*, 2010; Malka *et al.*, 2020). An increased protein abundance in the three aforementioned pathways provides an insight into how the winter workers minimise the protein production in cells whilst reserving essential proteins with dual function such as energy metabolism and immune response.

Proteomic analysis on the venom sac samples revealed a significant difference between summer and winter bee samples. Volcano plot analysis highlighted the abundance of bee venom associated proteins expressed in summer samples with a rfc

range of 2.65 to 694.82. The three most abundant proteins associated with summer samples: Api m6, Api m1 and Api m4 may initiate an allergic response in humans, with Api m6 and Api m4 being the two most predominant proteins making up 10-12% and 50% of the dry weight of bee venom respectively (Hider, 1988; Peiren *et al.*, 2005). Several non-toxin proteins such as those with roles as antioxidant, energy metabolism, protein folding and molecular transporter proteins were also identified in high abundance in summer samples in contrast to winter samples. It is hypothesised that proteins involved in these pathways are necessary to protect the secretory cells from the damaging bee venom (Peiren *et al.*, 2008; Li *et al.*, 2013). The high abundance of bee venom proteins in summer samples in contrast to winter bee samples has been observed previously (Danneels *et al.*, 2015; Hossen *et al.*, 2017; Scaccabarozzi *et al.*, 2021).

Winter bee samples did not have any bee venom associated proteins identified in the venom sac analysis. Instead, winter workers had a higher abundance of genetic information processing proteins and a relatively high number of proteins involved in carbohydrate metabolism. Interestingly, there were two proteins identified in high abundance in winter samples that have not previously been identified in the venom sac: MRJP 2 (rfc 12.84) and MRJP 1 (rfc 7.17). MRJP9 was identified as SSDA in the summer samples and has been previously identified in bee venom analysis (Blank *et al.*, 2012; Danneels *et al.*, 2015; Ramanathan *et al.*, 2018). Allergen investigations on honey have identified major IgE-binding proteins that correspond to MRJP1 and MRJP2 (Rosmilah *et al.*, 2008). This could suggest that MRJP1 and MRJP2 could have an allergen role in the bee venom of winter workers, however further work would be needed to confirm the role MRJ1 and 2 have in bee venom.

Winter and summer bees experience different environments and have different levels of activity within the hive, with winter workers rarely leaving the hive in contrast to summer workers who frequently leave the hive to forage. The different environments also contribute to a change in the type of predator/pests encountered which could lead to the different levels of bee venom associated proteins in the summer and winter workers (Danneels *et al.*, 2015). Honeybee workers in winter may have evolved to reduce cellular energy cost and the chances of cell damage from storing bee venom while increasing longevity to aid survival during the winter.

5.5 Conclusion

This study provides an insight into the proteomic composition of honeybee workers in summer and winter. Winter bee samples had a reduced number of proteins identified as SSDAs in comparison to summer bee samples. This suggests that winter workers reduce the number of proteins synthesised and stored as a means to increase longevity. In all three anatomical components that were analysed, summer bees had higher abundance and number of SSDAs. Summer workers have a wide range of roles to complete within the hive, a varied and plentiful diet, and a constantly changing external environment to contend with. Adaption to these changes could be a possible explanation as to why summer workers have a wider range of proteins expressed. In contrast, winter bees experience a harsh environment, reduced nutrient availability and their main role is in thermoregulation. This study indicates that the winter bees display reduced protein abundance and expression as a means to limit energy consumption to aid in longevity while ensuring the hives survival until the following spring.

5.6 Summary of Findings

Table 5.4: Overview of proteomic analysis of Chapter five

Proteins and Pathways	Summer	Winter
Carbohydrate metabolism	Large abundance of carbohydrate metabolism proteins. However abundance of proteins associated with specific pathways differed between summer and winter samples.	Increase in specific carbohydrate metabolism proteins in winter worker proteomes specifically, galactose, ascorbate and aldarate, along with pentose and glucuronate interconversions.
Genetic and Environmental Information Processing	Summer workers had a greatly increased abundance of proteins associated with genetic and environmental information processing pathways. Specifically proteins associated with messenger RNA, chromosomal proteins, MAPK signalling pathway and Hippo signalling pathway.	Overall winter workers had a greatly reduced protein in comparison to summer workers.
Venom Associated Proteins	High abundance of venom associated proteins. MRJP 9 was also observed in summer venom sac samples	Major Royal Jelly proteins were identified in the venom sac of winter workers – MRJP 1 and MRJP2. Neither protein had previously been observed in venom sac proteomic analysis before.
Cuticle Proteins	Cuticular proteins were greatly increased in abundance in summer workers in both head and abdominal samples. Several proteins were identified as exclusively expressed in summer worker samples in comparison to winter workers.	Overall there was a decreased abundance and number of cuticular proteins in all anatomical samples in comparison to summer workers.
Vitellogenin and Major Royal Jelly Proteins	All Major Royal Jelly (MRJ) proteins were identified as increased in abundance in summer samples in comparison to winter workers in the head samples. Interestingly some MRJ proteins were identified as increased in the venom sac of winter workers and MRJP9 was increased in summer venom sac samples, something that has previously not been identified.	Vitellogenin was observed as increased in abundance in winter worker samples, specifically abdomen samples, in comparison to summer workers.

Anti-oxidant and Xenobiotic metabolism	Overall summer workers had an increase in proteins associated with antioxidant systems in comparison to winter workers. Specifically in head samples, summer bees had an increase in cytochrome c oxidase proteins.	Several cytochrome P450 proteins were observed increased in abundance in winter workers.

Chapter 6

Characterisation of the Effects of Anti-*Varroa* Treatments on *Apis mellifera*

6.1 Introduction

The ectoparasitic mite of the honey bee *Varroa destructor* (*Varroa*) (Anderson and Trueman, 2000) is the single greatest threat to honey bee colonies worldwide, with "*Varroa* free" hives being difficult to find (Rosenkranz *et al.*, 2010; Ramsey *et al.*, 2019). The lifecycle of *Varroa* mites consists of two phases: phoretic (spreading throughout the hive on adult bees) and reproductive (reproducing and mating in larval cells) (Rosenkranz *et al.*, 2010). *Varroa* mites were originally confined to *Apis cerana* colonies but have since expanded to parasitise *Apis mellifera* colonies, most likely when *A. mellifera* colonies were transported to Eastern Russia/ Far East (Buchler *et al.*, 1992; Oldroyd, 1999; Boecking and Genersch, 2008). There is a lack of a long term co-evolution relationship between *Varroa* mites and *A. mellifera* and this has resulted in insufficient levels of *Varroa*-sensitive hygienic behaviour in *A. mellifera*. The absence of *Varroa*'s reproductive preference for drone larval stages in *A. mellifera* colonies is also another factor contributing to the greater effect of *Varroa* parasitisation on *A. mellifera* colonies compared to that on *A. cerana* colonies (Ramsey *et al.*, 2019; Traynor *et al.*, 2020). Parasitisation by *Varroa* is termed varroosis and the effects are characterised by a reduction in the honeybee's body weight, immune responses, number of healthy larvae and adult workers in a hive and inhibition of protein and lipid production as a direct result of the *Varroa* feeding on the fat body (Ramsey *et al.*, 2019). *Varroa* mites are also successful viral vectors and are known to harbour 18 honeybee viruses and facilitate viral reproduction (Chen and Siede, 2007; Rosenkranz *et al.*, 2010).

As *Varroa* infestations are so widespread, treatment protocols are beneficial and aid in controlling the spread of *Varroa* in apiaries and reducing the damaging impact of *Varroa* mites on individual hives. Failure to treat hives can result in the rapid deterioration of hive health, due to the loss of healthy bees and an increase in viral diseases such as Deformed Wing Virus (DWV), and many hives will fail after two years in the absence of treatments (Gisder *et al.*, 2009; Rosenkranz *et al.*, 2010; Traynor *et al.*, 2020). There are several treatments available to beekeepers which can be divided into three groups: hard acaricides (synthetic compounds), soft acaricides (organic acid treatments) and biotechnical methods (beekeeper intervention). This Chapter investigates the effects of three anti-*Varroa* treatments on *A. mellifera*: Mite

Away Quick strips (MAQs) – an organic treatment, Apivar – a synthetic formamidine acaricide, and Apiguard - an organic treatment.

MAQ strips are a single use saccharide gel strip application containing 46.7% formic acid and are placed along the top of the brood frames for a 7-day period (as per manufacturer's instructions). Several factors may affect the efficacy of MAQs including the size of the colony, positioning of the strips above or below the brood chamber, and the ambient temperature (Underwood and Currie, 2003; Ostermann and Currie, 2009). Optimum efficacy of MAQs application is achieved when external temperatures range between 10^{o}C and 30^{o}C (Satta *et al.*, 2009). Variations in temperatures can affect the efficacy of the organic acid treatments such as formic acid as it influences the evaporation in the hive (Rosenkranz *et al.*, 2010). Organic acids such as formic, oxalic and lactic acid, are widely used to control Varroa populations as there is a low risk of the acids accumulating in the bee products and a low probability of *Varroa* developing resistance after repeated applications (Underwood and Currie, 2003; Rosenkranz *et al.*, 2010). Formic acid is the only organic acid treatment that can be applied when brood is present as it effectively kills mites both in brood cells and on adult honeybees(Fries, 1991; Boecking and Genersch, 2008; Rosenkranz *et al.*, 2010).

Formic acid is the simplest carboxylic acid, that can trigger metabolic acidosis by inhibiting cytochrome c oxidase at the sixth coordination position (Liesivuori, 2014). This causes disruption to the mitochondrial electron transport and energy production in the terminal electron acceptor of the electron transport chain, triggering histotoxic hypoxia (Liesivuori and Savolainen, 1991). Cell death from cytochrome c oxidase inhibition by formic acid is believed to result in a partial depletion of ATP reducing energy concentrations so essential cellular functions cannot be maintained (Liesivuori and Savolainen, 1991; Du *et al.*, 2008). Cytochrome c oxidase inhibition by formic acid can also cause cell death by increased production of cytotoxic reactive oxygen species (ROS) that cause damage to essential components of cells.

Apivar treatment involves polymer strips containing amitraz as the active acaricide and these are placed between the brood frames (Gupta, 2018). Amitraz is a broad spectrum insecticide that has been used internationally since 1974 (Gupta, 2007). The mode of action of amitraz is to selectively block the octopamine receptor of the central nervous system through α2-adrenergic receptor antagonist activity

(Dalefield, 2017; Rinkevich, 2020). α2-adrenergic receptors can inhibit neurotransmitter release from the presynaptic neuron and α2-adrenergic receptor antagonists are now used in clinical setting as anaesthesia adjuncts (Giovannitti *et al.*, 2015). Octopamine is a major biogenic amine present in insects and is elevated during stressful conditions and plays a role in lipid and carbohydrate metabolism (Evans and Maqueira, 2005). Octopamine has been linked to memory, learning process and the generation of flight in *A. mellifera* (Evans and Maqueira, 2005). Inhibition of octopamine in *Varroa* causes paralysis to the mouth parts on the female *Varroa* which prevents them from feeding (Gupta, 2007). The paralysed mites fall below the frames onto the bottom boards of the hives and subsequently die of starvation (Semkiw *et al.*, 2013).

Apiguard treatment is an organic or 'soft chemical' anti-*Varroa* treatment used in colonies (Rosenkranz *et al.*, 2010). Thymol is the active ingredient of Apiguard and is a monoterpenoid volatile plant oil secretion (De-Oliveira *et al.*, 1999; Fassbinder *et al.*, 2002; Escobar *et al.*, 2020). Monoterpenoids inhibit cytochrome P450 activity in human livers (De-Oliveira *et al.*, 1999). Thymol has no specific toxicity towards *Varroa* and therefore can affect exposed honeybees (Fassbinder *et al.*, 2002) and have a negative effect on colony development and behavioural responses (Floris *et al.*, 2009; Mondet *et al.*, 2011). It is suggested that the methyl functional group of thymol could be the contributing factor to its insecticidal mode of action (Park *et al.*, 2017). There are numerous studies completed on the toxicity of thymol acaricides (Da Silveira Novelino *et al.*, 2007; da Silva Matos *et al.*, 2014; Novato *et al.*, 2015; Wu *et al.*, 2017a), however, little is known about the mode of action of thymol products in mite populations.

Presently a limited amount of work has been published investigating the effects of anti-*Varroa* treatments on honeybees, instead focus has been placed on the residual amounts of the compounds (synthetic or organic) that can be detected within the hive after the treatment has been applied (Martel *et al.*, 2007). No pesticide used as apiary medicine is 100% effective and with the design of anti-*Varroa* treatment applications made to be easily appliable to hives, there has been a wide-spread and loosely controlled application of various acaricide to colonies worldwide (Rinkevich, 2020). This has led to *Varroa* mites developing resistance to some acaricides and therefore

combinatory treatment methods may be required to reduce *Varroa* numbers within hives. The application of acaricides to colonies has knock-on effects on the bees such as the increase in cytochrome P450 proteins, reduction of hydrophobic cuticle protease activity and increase in glutathione s-transferase activities (Johnson *et al.*, 2006; Loucif-Ayad *et al.*, 2008; Strachecka *et al.*, 2012a).

6.1.1 Chapter Six Aims

- To Investigate if MAQ strips affect the proteome of *A. mellifera.*
- To determine if Apivar treatments have an off-species effect on *A. mellifera* colonies.
- To establish if Apiguard treatments have an off-species effect on *A. mellifera.*

6.2 Experimental Design

All trial hives were monitored for the presence of *Varroa* using sticky inserts placed underneath a mesh floor in all the participating hives. Inserts remained in the hives for a week and were replaced at weekly intervals. Inserts were counted and results were used to provide an insight into the general health of the hives, this was particularly important in weeks eight and nine as mite fall was used to determine if hives needed to be given a secondary treatment (to prevent unnecessary hive loss).

A biotechnical method for reducing *Varroa* number within this study was conducted. Three frames ready for laying (containing sufficient stores of pollen and honey) was selected for this technique. The queen was identified, placed onto the first selected frame for laying and the frame was caged. She was left on the frame for a week allowing the brood cells to be capped. The queen was then moved onto the second of the selected frames and subsequently caged. The first frame was removed from the hive. The second and subsequent third frame were also removed after a week of the queen laying and the brood being capped. Three colonies were used for this biotechnical method. Unfortunately, none of the biotechnical colonies were successful in reducing *Varroa* numbers and therefore were not included in the proteomic analysis as a control to the treated colonies.

A single head sample from worker bees from the control and treatment groups was subjected to mass spectrometry preparation; protein extraction, clean-up, digestion, and peptide purification (C18). For mass spec analysis 1µg of tryptic peptides were loaded onto a QExactive (ThermoFisher Scientific) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated over a 2-40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100mm length, 75mm ID), using a 135 min reverse-phase gradient flow rate of 250nL min^{-1}. Three hives were used for both the control and treatment analysis. All remaining hives within the apiary that were not included in the study were also treated for *Varroa* and were monitored for parasitisation levels.

Protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.6.6.0 (https://www.maxquant.org/). Data processing and analysis was carried out using Perseus v 1.6.6.0 (https://maxquant.net/perseus/). Proteins were deemed statistically significant differentially abundant (SSDA) if identified to have a Log2 fold change greater than +/- 1 (unless specified otherwise) and a -Log p value greater than 1.3. To identify protein-protein interactions of SSDA proteins, functions, and pathway enrichment analysis was conducted using STRING v 11 (https://string-db.org/) and g:Profiler gGOst (https://biit.cs.ut.ee/gprofiler/gost) were utilised. g:Profiler term information used in this analysis are outlined as such: Term ID is the unique term identifier for each pathway/term. Term size refers to the number of genes that are annotated to the specific term. Query size is the number of genes that were included in the query run. Intersection size is the number of genes in the input query that are annotated to the corresponding term. The adjusted p-value is the hypergeometric p-value after correction for multiple testing. The user-threshold for each of the query runs is 0.05 (Raudvere *et al.*, 2019).

6.3 Results

6.3.1 Analysis of the effect of Mite Away Quick Strips on the proteome of *A. mellifera*

Control samples were collected prior to the application of MAQ strips (according to the manufacturer's instructions) to the hives. Treatment samples were collected at one, two and three weeks after treatment application (**Figure 6.1**). Weekly mite fall was monitored using adhesive inserts (**Figure 2.3**).

On average of 70 mites were collected from control colonies during the week prior to application of the treatment, 400 mites fell during Week 1 (treatment week) and 240 during Week 2. Thereafter the mite fall was approximately 80 mites per week. In order to characterise the effect of MAQs on *A. mellifera*, the proteome of control bees was compared to treated bees using label free quantitative (LFQ) mass spectrometry.

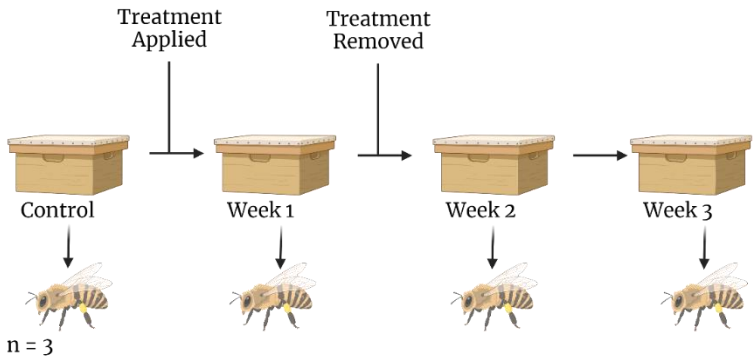


Figure 6.1. Schematic outlining the sample collection of worker bee samples for the MAQ treatment trial.

Protein extractions were performed on three replicates per sample group. Initially, a total of 3,070 proteins were identified, this was reduced through filtering steps to remove contaminant peptides, reverse hit peptides and peptides only identified by site. LFQ intensity values were Log_2 transformed and only proteins identified in at least three of the three replicates were included for further analysis. A further filtration step of ANOVA test (p-value = 0.05) reduced the matrix to 712 proteins.

Principal Component Analysis (PCA) highlighted a large separation between Week 1 samples versus Control samples and Weeks 2 and 3 samples (**Figure 6.2**). The PCA showed an overlap between Weeks 2 and 3 samples indicating a degree of similarity of the samples. The large degree of separation between Week 1 samples and samples from Weeks 2 & 3 could indicate an adverse effect of the anti-*Varroa* treatment. This is supported by the proximity of Control samples to Weeks 2 and 3 samples, therefore reducing the possibility of a disease effect causing the distance of Week 1 samples. However, the mite load present in the hive during Week 1 may have had an impact on the worker bees.

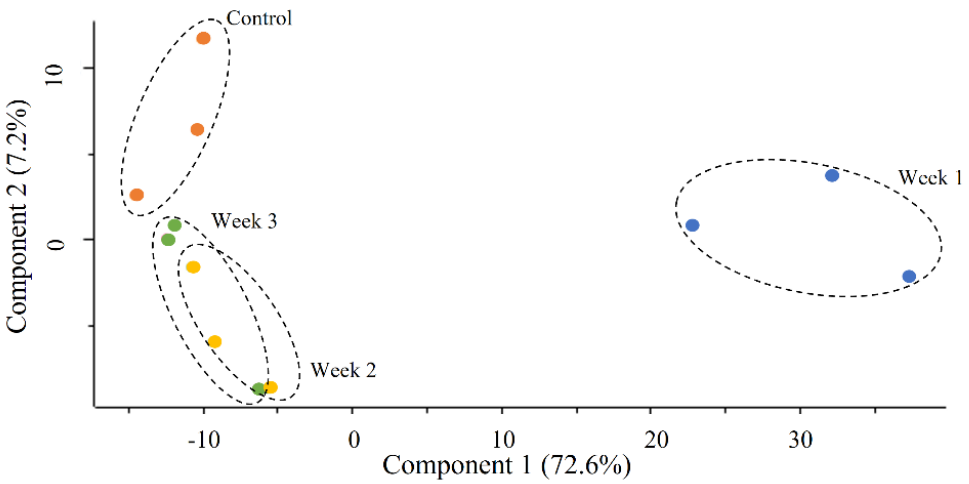


Figure 6.2: PCA on LFQ intensity values from proteins identified in Control and Treated samples. A clear distinction can be seen between Week 1 samples to Control and Weeks 2 & 3. Total explained variance of this PCA for the given dataset is 79.8%.

6.3.1.1 Control vs Week 1 of MAQs exposure

Volcano plot analysis between Control and Week 1 samples revealed the highest number of SSDA proteins with a relative-fold change (rfc) ranging between -26.28 to 7.20 fold respectively (**Figure 6.3**). The most prominent SSDA proteins increased in abundance in Week 1 samples were cuticular proteins (**Table 6.1**). Several xenobiotic proteins were increased in abundance in Control samples (**Table 6.1**). Some cytochrome c oxidase proteins were identified as SSDAs in this analysis, however,

there was a difference in the association with the samples. Subunits 5A &B and 6b1 were increased in abundance in Week 1 samples. Whereas, subunits Fa4, 4I1 and 6a1 were increased in abundance in Control samples (**Figure 6.3** & **Table 6.1**).

Table 6.1: Table of SSDA proteins identified between Control and Week 1 samples. Labels correlate to the volcano plot (**Figure 6.3**)

Protein ID	Protein Name	Functional Category	Sample	Label	Relative Fold Change
A0A088ABR1	Pupal cuticle protein	**Cuticular**	W1	A	6.64
A0A088AMM7	Protein obstructor-E		W1	C	5.99
A0A087ZRG5	Cuticular protein CPF2 isoform 1 precursor		W1	D	5.47
A0A087ZT04	Cuticular protein analogues to peritrophins		W1	E	5.17
A0A088A955	Endocuticle structural glycoprotein		W1	F	4.84
A0A088ANC5	Apidermin 3 precursor		W1	G	4.71
A0A088ABN3	Cuticular protein 1 precursor		W1	I	3.51
A0A088A4T4	Endocuticle Glycoprotein SsAbd-8-like		W1	J	3.46
A0A087ZNJ5	Cuticular protein 5		W1	K	2.66
A0A087ZUZ0	Cytochrome c oxidase 6b1	**Cytochrome c oxidase**	W1	H	3.90
A0A087ZZU9	Cytochrome c oxidase subunit 5A		W1	L	2.57
A0A088A8C7	Cytochrome c oxidase subunit 5B		W1	M	2.42
A0A087ZZ11	Cytochrome c oxidase subunit Fa4		Control	5	7.25
A0A087ZVP7	Cytochrome c oxidase subunit 4I1		Control	7	3.74
A0A088AQ34	Cytochrome c oxidase subunit 6a1		Control	12	2.43
A0A088AEJ2	Cytochrome P450 6a14	**Xenobiotic metabolism**	Control	1	11.38
A0A088AUI4	Multidrug resistance protein homolog 49 Ix1		Control	2	7.88
A0A088AS35	UDP-glucuronosyltransferase 1-3		Control	3	7.66
A0A087ZYR9	UDP-glucuronosyltransferase 1-3		Control	4	5.26

Protein ID	Protein Name	Functional Category	Sample	Label	Relative Fold Change
A0A088A7I2	UDP-glucuronosyltransferase 2A3		Control	6	7.66
A0A087ZP04	ATP-binding cassette sub-family F member 2		Control	8	4.04
A0A088AK15	ATP-binding cassette sub-family F member 1		Control	9	3.44
A0A088AV36	Microsomal glutathione S-transferase 1		Control	10	2.92
A0A087ZR09	ATP-binding cassette sub-family E member 1		Control	11	2.29
A0A088AEK4	Cytochrome P450 6AS5		Control	13	2.16
A0A088A4L4	Odorant binding protein 19	**Odorant Binding**	W1	B	7.20

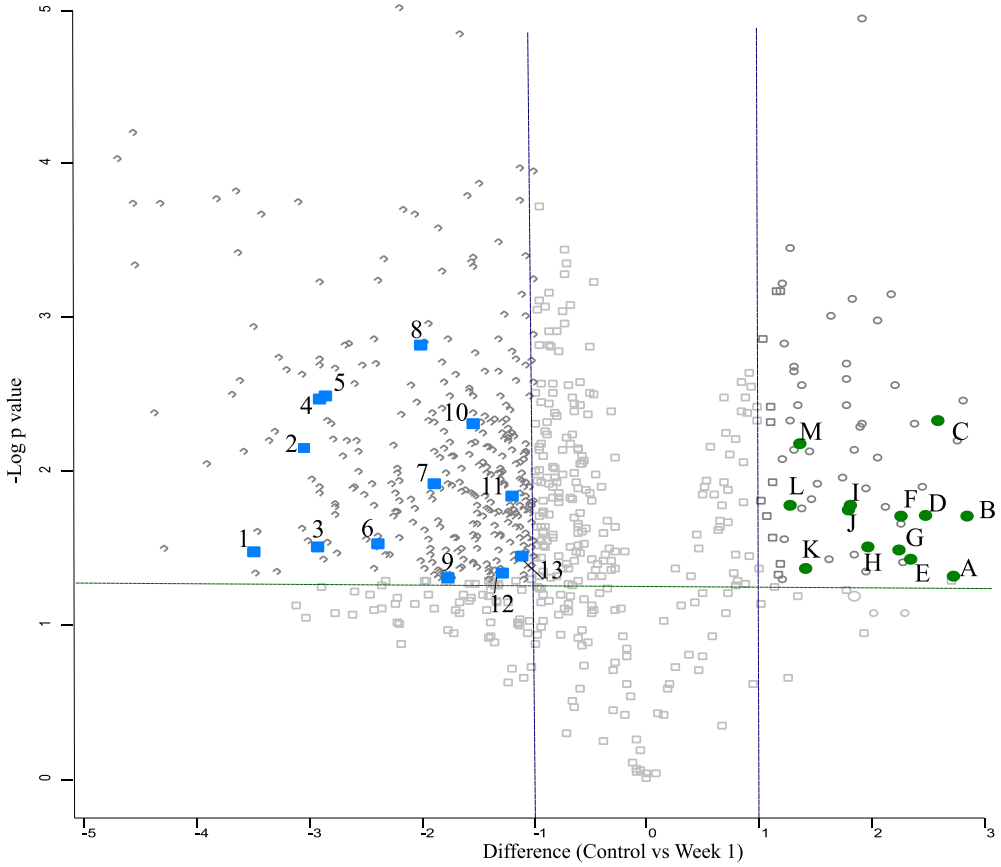


Figure 6.3: Volcano plot highlighting SSDAs identified from Control (left) vs Week 1 (right) of MAQs exposure samples. Samples above and to the outside of the dashed lines represent SSDAs: proteins with a -Log p value above 1.3 and a difference above or below 1. Proteins highlighted and labelled in green and blue represent proteins of interest as referred to in **Table 6.1**

6.3.1.2 Week 1 vs Week 2 of MAQs exposure

Comparative analysis between Week 1 and Week 2 samples yielded 150:276 SSDAs respectively with a relative fold range between -14.62 to 28.30 (**Figure 6.4 A**). Xenobiotic detoxification proteins were increased in abundance in Week 2 samples in comparison to Week 1 samples. Three cytochrome c oxidase proteins were identified as increased in abundance in Week 2 samples in comparison to Week 1 samples - Coxfa4, Cox4I1, and Cox6a. Whereas, Cox6b1 was increased in relative abundance in Week 1 samples (**Figure 6.4 A**). Similar to the previous comparison with the Control samples, cuticular proteins were increased in abundance in Week 1 samples relative to Week 2 samples (**Table 6.2**). There was also an increased abundance of three odorant

binding proteins associated with Week 1 in comparison to Week 2 samples (**Table 6.2**).

6.3.1.3 Week 1 vs Week 3 of MAQs exposure

Results from the Week 1 versus Week 3 comparative analysis resulted in 77:298 SSDAs with a relative fold change range of -10.15 to 30.88 respectively **(Figure 6.4 B).** Xenobiotic detoxification proteins were increased in relative abundance in Week 3 samples (**Table 6.3**). Cytochrome c oxidase proteins were increased relative abundance in Week 3 samples: Coxfa4, Cox4I1, and Cox6a1. Cytochrome c oxidase subunit Cox6b1 was however identified as in higher abundance in Week 1 samples. Cuticular proteins were increased in abundance in Week 1 samples in comparison to Week 3 samples (**Table 6.3**).

Table 6.2: Table of SSDA proteins identified between Week 1 (W1) and Week 2 (W2) of MAQs exposure samples. Labels correlate to **Figure 6.4 A**

Protein ID	Protein Name	Functional Category	Sample	Label	Relative Fold Change
A0A088AMM7	Protein obstructor-E	**Cuticular**	W1	2	8.79
A0A087ZT04	Cuticular protein analogues to peritrophins		W1	3	6.89
A0A088ABR1	Pupal cuticle protein		W1	4	5.84
A0A088ANC5	Apidermin 3 precursor		W1	5	4.96
A0A087ZRG5	Cuticular protein CPF2 isoform 1 precursor		W1	6	4.82
A0A088ABR2	Cuticular protein 18.7		W1	7	4.57
A0A087ZNJ5	Cuticular protein 5		W1	8	3.25
A0A088AG34	Cuticular protein 27 precursor		W1	10	3.01
A0A088A956	Endocuticle structural glycoprotein SgAbd-2 isoform X1		W1	12	2.96
A0A088A4T4	Endocuticle Glycoprotein SsAbd-8-like		W1	13	2.76
A0A087ZUZ0	Cytochrome c oxidase 6b1	**Cytochrome c oxidase**	W1	11	2.78
A0A087ZZ11	Cytochrome c oxidase subunit FA4		W2	A	11.14
A0A087ZVP7	Cytochrome c oxidase subunit 4I1		W2	F	5.39
A0A088AQ34	Cytochrome c oxidase subunit 6A1		W2	G	4.06
A0A088AEJ2	Cytochrome P4506A14	**Xenobiotic metabolism**	W2	B	10.09
A0A088AUI4	Multidrug resistance protein homolog 49 isoform X1		W2	C	8.73
A0A088A7I2	UDP-glucuronosyltransferase 2A3		W2	D	5.81
A0A087ZYR9	UDP-glucuronosyltransferase 1-3		W2	E	5.71
A0A088AF57	ABC transporter G family member		W2	H	4.04

Protein ID	Protein Name	Functional Category	Sample	Label	Relative Fold Change
A0A088AEK4	Cytochrome P450 6AS5 isoform X1		W2	I	3.39
A0A087ZP04	ATP-binding cassette sub-family F		W2	J	3.33
A0A087ZNI3	Probable Cytochrome P450 6a14		W2	K	3.05
A0A088AV36	Microsomal glutathione S-transferase 1		W2	L	2.56
A0A088ARD0	ATP-binding cassette sub-family B		W2	M	2.20
A0A088A4L4	Odorant binding protein 19 isoform X1	**Odorant Binding Proteins**	W1	1	9.57
A0A088AP29	Odorant binding 4 precursor		W1	9	3.56

Table 6.3: Table of SSDA proteins identified between Week 1 (W1) and Week 3 (W3) of MAQs exposure samples. Labels correlate to **Figure 6.4 B**

Protein ID	Protein Name	Functional Category	Sample	Label	Relative Fold Change
A0A087ZT04	Cuticular protein analogues to peritrophins	**Cuticular**	W1	1	5.23
A0A088ABR1	Pupal cuticle protein		W1	3	3.71
A0A088ANC5	Apidermin 3 precursor		W1	4	3.31
A0A087ZRG5	Cuticular protein CPF2 isoform 1 precursor		W1	5	2.85
A0A088ABR2	Cuticle protein 18.7		W1	6	2.09
A0A088AG34	Cuticular protein 27 precursor		W1	7	2.02
A0A087ZUZ0	Cytochrome c oxidase 6b1	**Cytochrome c oxidase**	W1	8	2.40
A0A087ZZ11	Cytochrome c oxidase subunit FA4		W3	B	12.06
A0A087ZVP7	Cytochrome c oxidase 4I1		W3	E	6.79
A0A088AQ34	Cytochrome c oxidase subunit 6A1		W3	J	3.48
A0A088AUI4	Multidrug resistance protein homolog 49 isoform X1	**Xenobiotic metabolism**	W3	A	14.19
A0A088AEJ2	Cytochrome P4506A14		W3	C	11.21
A0A087ZYR9	UDP-glucuronosyltransferase 1-3		W3	D	7.20
A0A088A7I2	UDP-glucuronosyltransferase 2A3		W3	F	6.58
A0A088AF57	ABC transporter G family member 20 isoform X1		W3	G	6.12
A0A087ZP04	ATP-binding cassette sub-family F member 2		W3	H	4.18
A0A088AEK4	Cytochrome P450 6AS5 isoform X1		W3	I	3.84
A0A087ZNI3	Probable Cytochrome P450 6a14		W3	K	2.56
A0A088AV36	Microsomal glutathione S-transferase 1		W3	L	2.36
A0A087ZR09	ATP-binding cassette sub-family E member 1		W3	M	2.04
A0A088A4L4	Odorant binding protein 19 isoform X1	**Odorant Binding Proteins**	W1	2	4.50

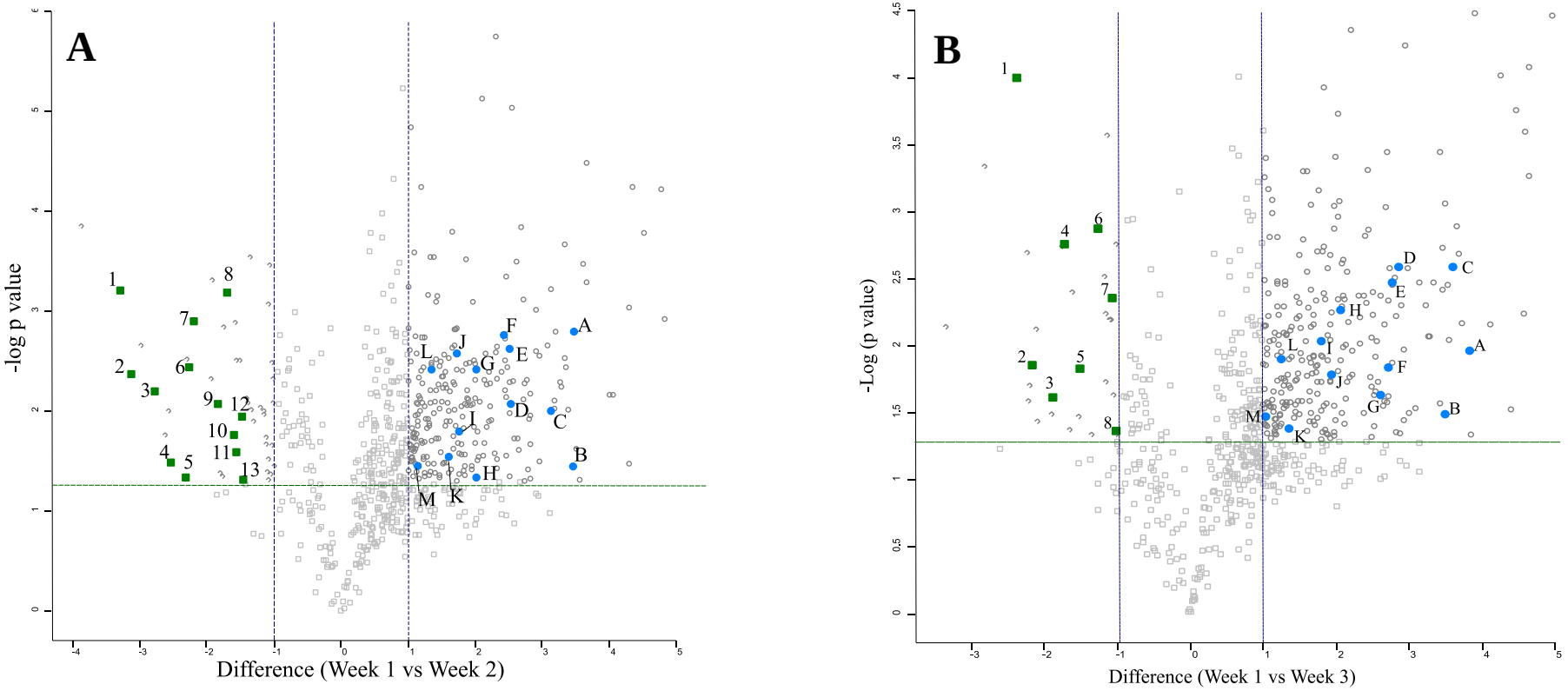


Figure 6.4: A: Volcano plot of ANOVA significant proteins ($p < 0.05$) in the comparison between Week 1 (left) and Weeks 2 and 3 (right) MAQs exposure samples. Samples above and to the outside of the dashed lines represent SSDAs. Proteins highlighted in green and blue represent proteins of interest as highlighted in **Table 6.2**. **B**: Volcano plot ANOVA significant proteins ($p < 0.05$) in the comparison between week 1 (left) and week3 (right) samples. Samples above and to the outside of the dashed lines represent SSDAs. Proteins highlighted in green and blue represent proteins of interest as highlighted in **Table 6.3.**

6.3.1.4 Volcano Plot Overview

Volcano plot analysis revealed an absence of oxidative phosphorylation proteins in Week 1 samples in comparison to Weeks 2, 3 and Control samples. There is also an overall absence of xenobiotic metabolism proteins in Week 1 samples. The reduction of oxidative phosphorylation proteins and the absence of xenobiotic proteins in Week 1 samples could indicate an extensive effect on bees exposed to MAQs in the seven days of treatment. Week 2 samples (7 days after treatment was removed), showed a significant increase in both oxidative phosphorylation and xenobiotic metabolism proteins. Comparisons between Control to Weeks 2 and 3 samples revealed very few SSDAs indicating that the samples share a large number of proteins in the proteomes of the samples (**Supp File 6.1**).

Cytochrome c oxidase (the target of formic acid) is the fourth complex in oxidative phosphorylation pathway and shows a dual response to the activity of MAQs; subunits Cox5a, Cox5b and Cox6b1 are in significantly higher abundance in Week 1 samples. Whereas subunits Coxfa1, Cox4I1 and Cox6a1 are significantly decreased in abundance in Week 1 samples (**Figure 6.5**). Cytochrome c oxidase was the only OXPHOS complex to have subunits both increased and decreased in abundance throughout the volcano plot analysis.

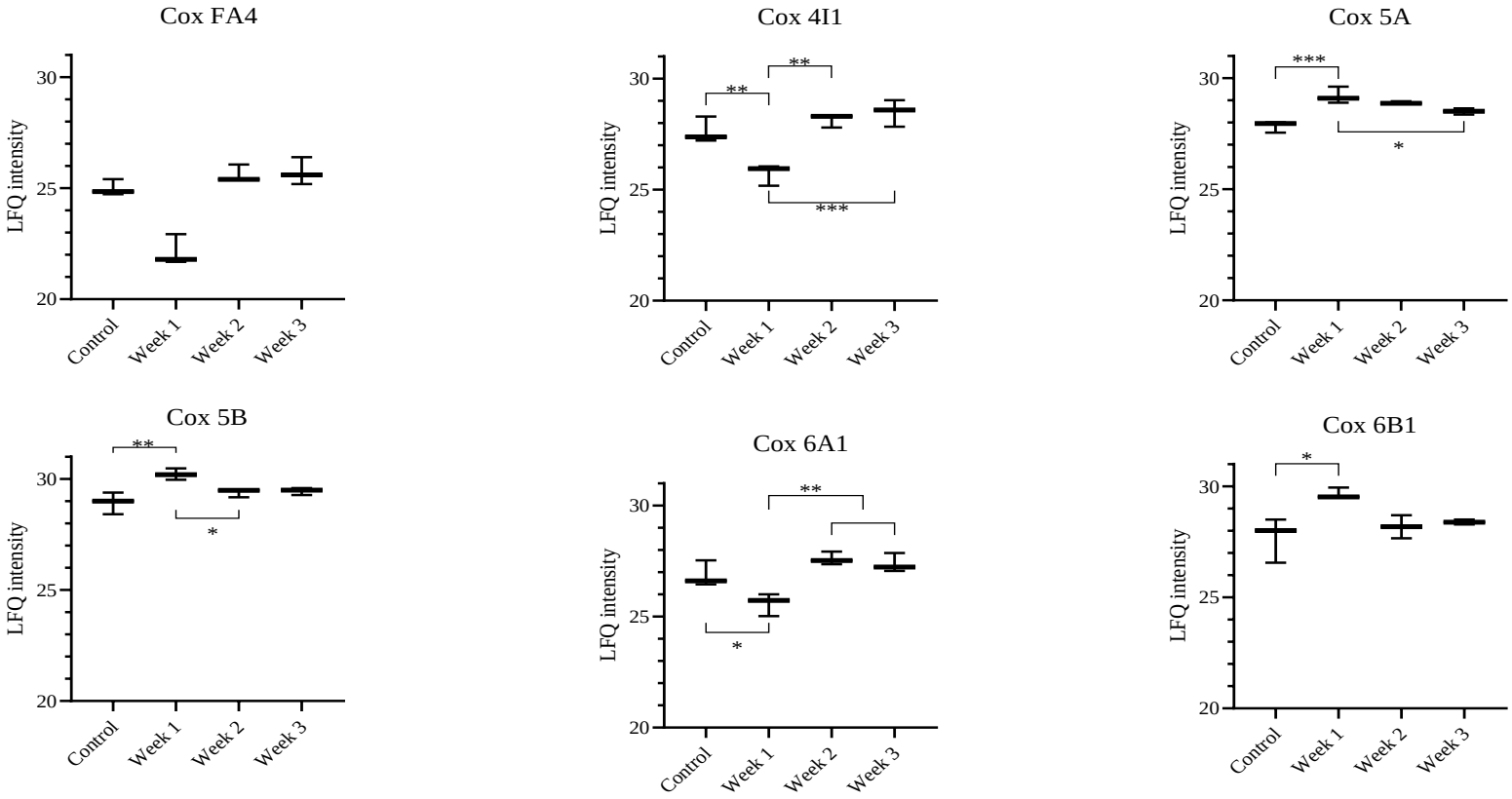


Figure 6.5: LFQ intensity values for Cytochrome c oxidase proteins identified as ANOVA significant ($p < 0.05$). Significance is calculated with One-way ANOVA ($p < 0.05$) and displayed as such 0 "***", 0.001 "**", 0.1 "*".

6.3.1.5 Hierarchical clustering analysis

Hierarchical clustering analysis was performed on 409 z-scored normalised, statistically significant differentially abundant proteins (SSDAs). Samples were clustered based on Euclidean distance analysis which grouped Week 1 samples as one cluster and the Control and Weeks 2 and 3 samples as another (**Figure 6.6**) (**Supp. File 6.2**). Protein clustering grouped the 293 SSDAs into two distinct protein clusters – Cluster A is mostly associated with proteins increased in Week 1 samples. Cluster B is associated with enriched protein profiles in Weeks 2, 3 and Control samples. Cluster A has an enrichment of proteins associated with the structural constitute of the cuticle (p value, 3.8901E-05) and also contains two cytochrome c oxidase subunits (Cox6b1 and Cox5a). Cluster B has an enrichment of proteins associated with the integral component of the membrane (p value = 2.2418E-07) and also contained 3 cytochrome c oxidase proteins (Cox6a1Coxfa4 and Cox4I1). g:Profiler analysis identified an enrichment of proteins associated with the Endoplasmic Reticulum (ER) (p value = 6.45E-08), ER sub compartment (p value = 1.07E-07) and ER membrane (p value = 1.07E-07).

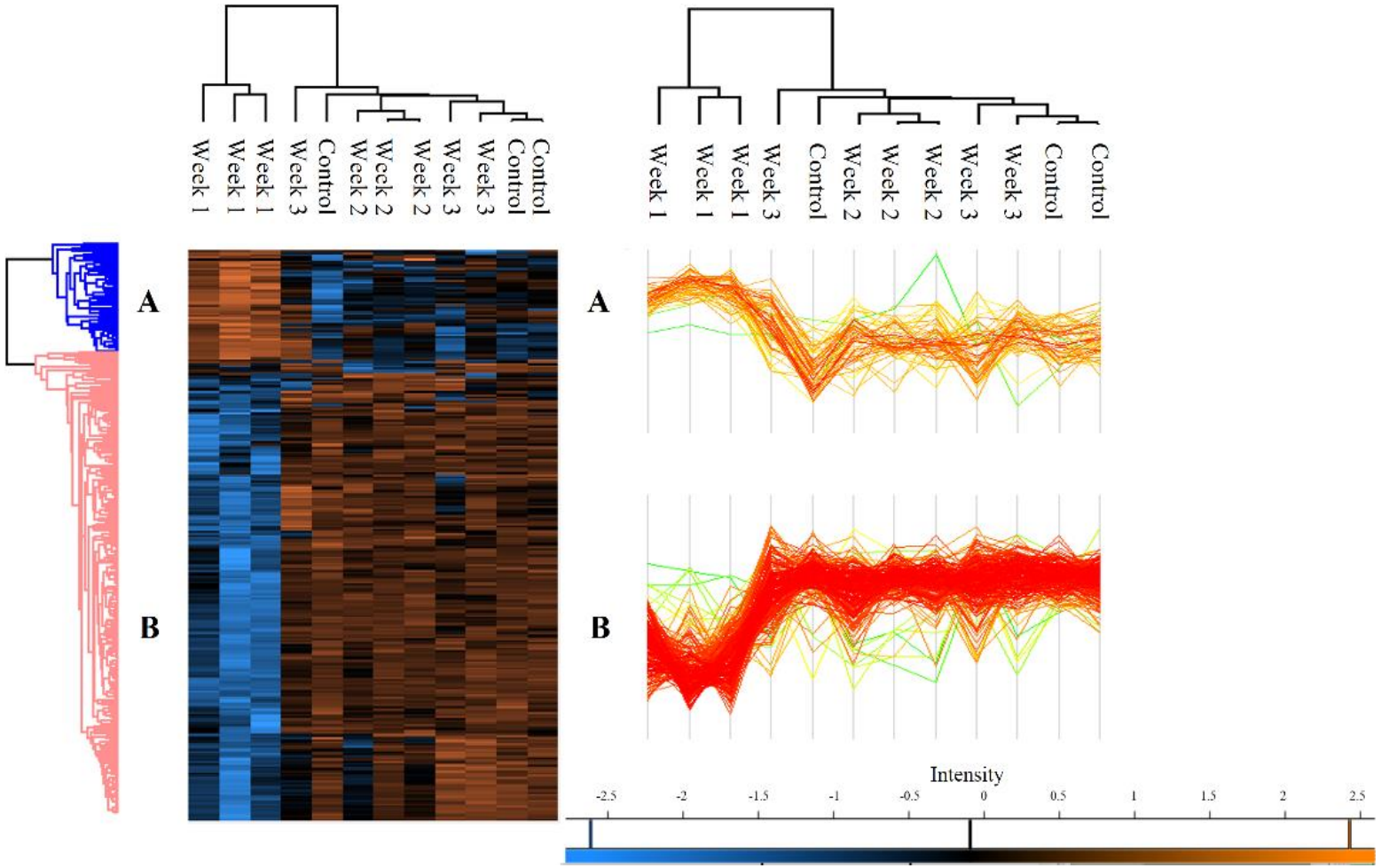


Figure 6.6. Hierarchical clustering analysis on 409 SSDA proteins identified from volcano plot analysis. Week 1 samples are least like the Control samples and are not clustered with Weeks 2 and 3 samples. Relative abundances (z-scored normalised) of individual proteins can be observed on the intensity spectrum.

6.3.1.6 Discussion of proteomic analysis on *A. mellifera* samples exposed to MAQ treatment

The work presented in this section concentrated on the use of Mite Away Quick strips and the potential proteomic alterations that may occur in the bees due to treatment exposure. MAQs contain formic acid (47.6%) as the active ingredient, which is known to affect the mite's ability to deal with oxidative stress and mitochondria leading to cell death (Nicholls, 1976; Liesivuori and Savolainen, 1991).

Proteomic analysis identified that the presence of MAQs within colonies induced a significant change in the proteomes of bees sampled on Week 1 (treatment week). Overall, there was an absence of proteins associated with xenobiotic metabolism and a reduced presence of oxidative phosphorylation proteins in Week 1 samples in comparison to Control and Weeks 2 and 3 samples. Formic acid targets cytochrome c oxidase which has been suggested to be the main regulation site for the oxidative phosphorylation pathway (Liesivuori and Savolainen, 1991; Li *et al.*, 2006). Interestingly, there was a significant decrease in the abundance of three cytochrome c oxidase subunits; Cox4I1, Cox6a1, and Coxa41, but a significant increase in the abundance of Cox5a, Cox5b and Cox6b1 subunits in Week 1 samples compared to the Control. Cytochrome c oxidase subunit 5 is the largest of the cytochrome c oxidase subunit, COX5a is expressed under normoxic conditions and COX5b is expressed in low oxygen conditions (Dodia *et al.*, 2014). These results suggests that the formic acid within MAQs inhibit/disrupts cytochrome c oxidase activity and subsequentially the oxidative phosphorylation pathway in *A. mellifera* samples. This response is limited to the time the treatment is within the hive as by Week 2 the abundance of Cox5b decreases and the abundance of COX5a rises. Cytochrome c oxidase 4 isoform 1 is essential for the assembly and respiratory function of the cytochrome c oxidase enzymatic complex (Li *et al.*, 2006), which was found at significantly lower intensities in Week 1 samples. The subunit that was identified to be least abundant in Week 1samples was cytochrome c oxidase subunit NDUFA4 which has recently been renamed as an additional cytochrome c subunit, FA4 (Pitceathly *et al.*, 2013; Pitceathly and Taanman, 2018). The deficiency of CoxFA4 in cells has been observed to cause a reduction in cytochrome c oxidase functionality and could potentially alter the protein complex synthesis (Pitceathly *et al.*, 2013; Pitceathly and Taanman, 2018). CoxFA4

has been associated with Cox5 subunit and may be essential for the structure of cytochrome c complex IV (Balsa *et al.*, 2012). A reduction in CoxFA4 and an abundance of Cox5a and Cox5b in Week 1 samples of MAQ treatment suggests that MAQ strips cause an adverse effect in honeybees by disrupting the function of cytochrome c oxidase and therefore altering the oxidative balance in cells. This effect seems to be temporary as samples from Weeks 2 and 3 more closely resembled Control samples, so disruption to cytochrome c oxidases may be limited to the presence of the MAQs within the hive.

Metabolism of xenobiotics in insects is associated with an increase in metabolic processes and drug metabolism enzymes (Li *et al.*, 2007). There are three phases of xenobiotic metabolism: Phase I – toxic structure is altered preventing it from interacting with target sites this is primarily completed with cytochrome P450 monooxygenases (P450) and carboxylesterases (CCE) (Berenbaum and Johnson, 2015). Phase II – conjugation of products from Phase I for solubilisation and transport carried out by glutathione-S-transferases (GST) (Ketterman *et al.*, 2011; Berenbaum and Johnson, 2015). Phase III – is transporting the products from Phase II for excretion this is mainly done by multi-drug resistant proteins and ATP-binding cassette transporters (Dermauw and Van Leeuwen, 2014; Berenbaum and Johnson, 2015; Poquet *et al.*, 2016).

Numerous proteins associated with xenobiotic metabolism and detoxification processes were identified as SSDAs in Control and Weeks 2 and 3 samples in comparison to Week 1 samples. Cytochrome P450 subunit 6A14 was identified as one of the most abundant xenobiotic metabolism proteins in Control, Week 2 and 3 samples when compared to Week 1 bees. This analysis did not identify any xenobiotic metabolism proteins as SSDAs in Week 1 samples in any of the volcano plot comparisons.

The role of cytochrome P450s in the metabolism of an acaricide (pyrethroid, another *Varroa* treatment) has been studied and was determined that pyrethroid is safe to use to reduce *Varroa* populations in hives as honey bees utilise cytochrome P450 activity for the metabolism of this treatment (Johnson *et al.*, 2006; Johnson *et al.*, 2012; Rand *et al.*, 2015). CYP9 and CYP6 subfamily members have been identified as those responsible for tau-fluvalinate and pyrethroid metabolism (Yang *et al.*, 2006; Mao *et*

al., 2011). This study has also identified proteins belonging to those cytochrome P450 gene clans that decreased in abundance in Week 1 samples. Although no significant changes in protein intensities were observed, there was an overall decrease in cytochrome P450 abundance in Week 1 samples which recovered to levels mirroring the control in Week 2 and 3 samples.

Several cuticular proteins were observed to be in higher abundance in Week 1 samples compared to Control and Weeks 2 and 3 samples. A previous study has identified the negative impacts formic acid has upon the proteolytic barrier, however, the impacts of formic acid on the workers' proteome is dependent upon the stage of development (Strachecka *et al.*, 2012b). The increase in these proteins in Week 1 samples may indicate a response to formic acid penetration of the cuticle, possibly as a means of slowing insecticide penetration into the internal organs thereby allowing metabolic detoxification to begin (Wood *et al.*, 2010; Yahouédo *et al.*, 2017). A study on the response of *Anopheles funestus* to short exposures of insecticides (pyrethroid) concluded that tolerant/resistant *A. funestus* was females are likely to have a thicker cuticle layer than susceptible *A. funestus* was females (Wood *et al.*, 2010; Yahouédo *et al.*, 2017).

6.3.2 Analysis of the effects of Apivar on the proteome of *A. mellifera*

Control samples were collected prior to the application of Apivar strips (according to the manufacturer's instructions) to the hives. Treatment samples were collected at one, three, six and eight weeks after treatment application (**Figure 6.7**). Weekly mite fall was monitored using adhesive inserts (**Figure 2.3**). An average of 71 mites were collected in the Control during the week prior to application of the treatment (Treatment application was at the start of week 1). Large mite fall was observed within the first 5 weeks after treatment was applied, after which mite fall dropped to 27 by Week 8 (**Table 6.4**).

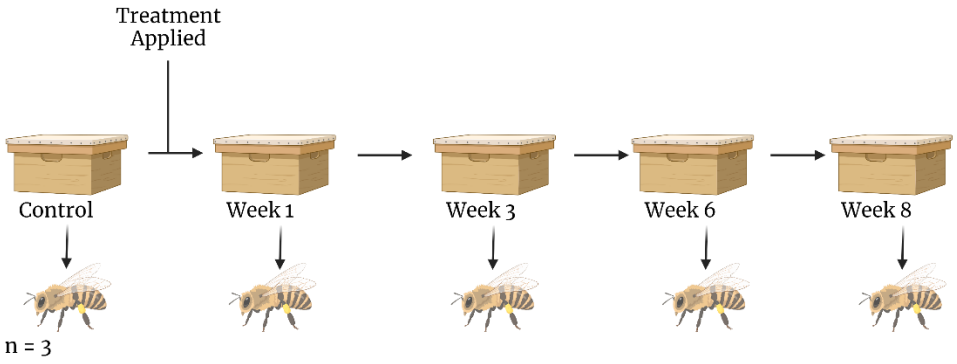


Figure 6.7. Schematic outlining sample collection for Apivar treatment trial.

Table 6.4: Average mite fall throughout the duration of the treatment trail. Treatment was applied at the start of Week 1.

Control	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
71	393	301	158	136	143	80	32	27

LFQ mass spectrometry was performed on single head samples from five time points in the Apivar treatment trial: Control, Weeks 1, 3, 6, and 8 post treatment application. Each group was collected in triplicate and all hives were part of the same apiary. Initial protein matrix contained 2,747 proteins, this was reduced significantly through filtering steps to remove contaminant peptides, reverse hit peptides and peptides only identified by site. LFQ intensity values were Log_2 transformed and only proteins identified in at least three of the three replicates were included for additional

analysis. ANOVA t test (p value = 0.1) was applied to further filter the matrix resulting a in 387 protein matrix.

Principal component analysis was completed on ANOVA significant samples, providing an overview of the proteomic relationship between samples. Proteomes from Weeks 3, 6 and 8 were clustered closely together away from the Control and Week one samples (**Figure 6.8**). Control and Week 1 samples were clustered independently (**Figure 6.8**). This demonstrates that the proteome of Weeks 3, 6 and 8 post treatment bees are similar whereas Control and Week 1 post treatment bees have a deviation in the proteome.

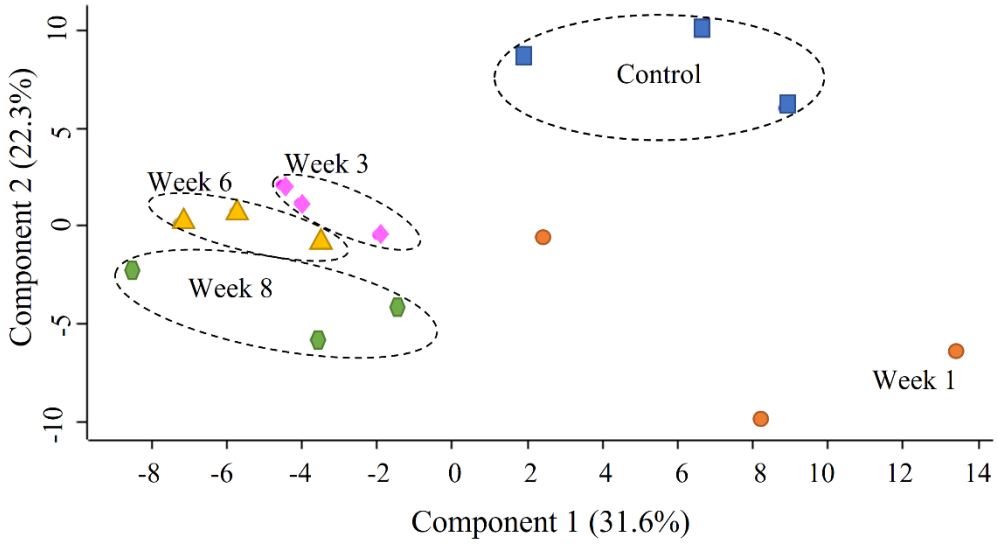


Figure 6.8: Principal component analysis of ANOVA significant proteins from Apivar study. Weeks 3, 6, and 8 samples are clustered away from Week 1 and Control samples, suggesting a level of separation in the proteomic profiles between the sample groups. The total explained variance of this PCA for the given dataset is 53.9%.

Student's t-test comparisons were conducted on all samples, and proteins with a significance (log_{10} p value) greater than 1.3 and a relative fold change (Log_2 LFQ intensity difference) greater than 1.5 were identified as statistically significant differentially abundant (SSDA) proteins (**Supp. File 6.3**). Analysis identified an increased abundance of cuticular proteins in Control and Week 1 samples in

comparison to Weeks 3, 6 and 8 (**Table 6.5** & **Table 6.6**) and (**Figure 6.13 to Figure 6.16** highlighted by green circles).

Ribosomal proteins were increased in abundance in Weeks 3 and 6 samples and in comparison to Control (**Figure 6.9 & Figure 6.10**) and Week 1 samples (**Figure 6.11 & Figure 6.12**). No ribosomal proteins were identified as SSDAs in the comparison of Control to Week 1 samples. Analysis of Week 8 samples did not identify an enrichment of ribosomal proteins when compared to Control and Week 1 samples.

Comparisons between Week 3 and 6 resulted in 7 and 4 SSDAs respectively. The low number of SSDAs generated from the comparison indicates a similar proteome between the two weeks. Two cuticular proteins were increased in abundance in Week 3 in comparison to Week 8 samples, cuticular protein 14 (rfc, 2.45) and pupal cuticle protein (rfc, 2.80). Week 3 and 6 comparisons to Week 8 resulted in a low number of SSDAs associated with Week 8 samples (10 and 6 SSDAs respectively). This low abundance of SSDAs in Week 8 in comparison to Weeks 3 and 6 samples was unexpected as Weeks 3 and 6 had an increased abundance of ribosomal proteins in comparison to Control and Week 1 samples that was not observed in Week 8 samples.

Table 6.5: Cuticular proteins increased in abundance (rfc) in Control samples in comparison to Weeks 3, 6 and 8 samples.

Protein name	Week 3	Week 6	Week 8
Cuticular protein 28	2.31	1.80	3.35
Apidermin 3	2.43	2.14	-
Cuticular protein 14	2.65	2.07	6.48
Cuticular protein 22	3.86	-	6.51
Cuticular protein 27	-	2.19	-
Pupal cuticle protein	-	-	3.70
Protein obstructor-E	-	-	4.00

Table 6.6: Cuticular proteins increased in abundance (rfc) in Week 1 samples in comparison to Weeks 3, 6 and 8 samples.

Protein name	Week 3	Week 6	Week 8
Cuticular protein 28	1.98	1.54	2.87
Apidermin 3	2.90	2.56	5.60
Cuticular protein 14	-	-	3.66
Cuticular protein 22	2.08	-	3.50

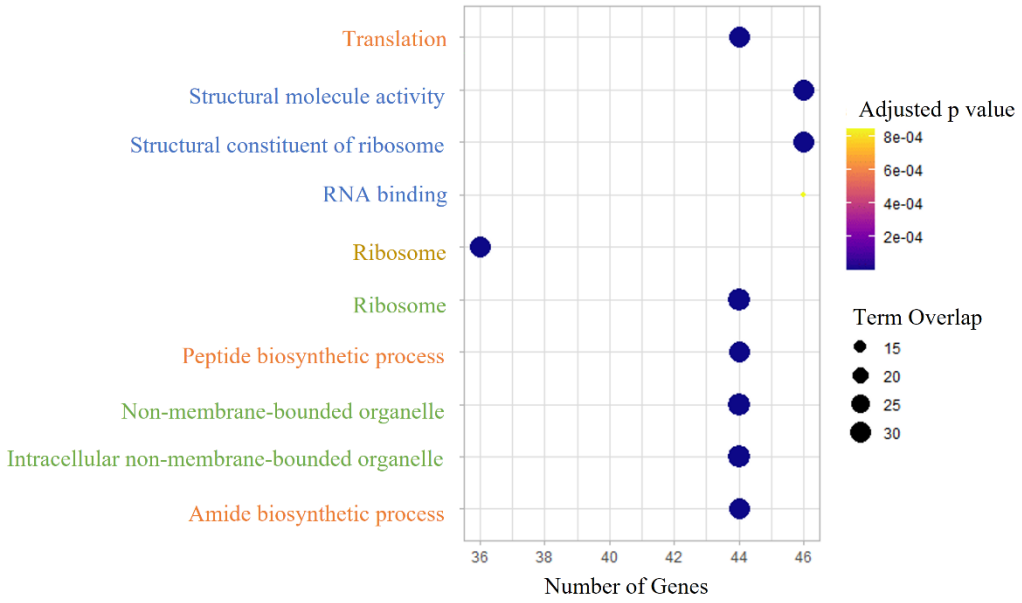


Figure 6.9: g:Profiler analysis on 56 SSDAs in Week 3 samples in comparison to Control samples. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, Green – GOCC and Yellow - KEGG.

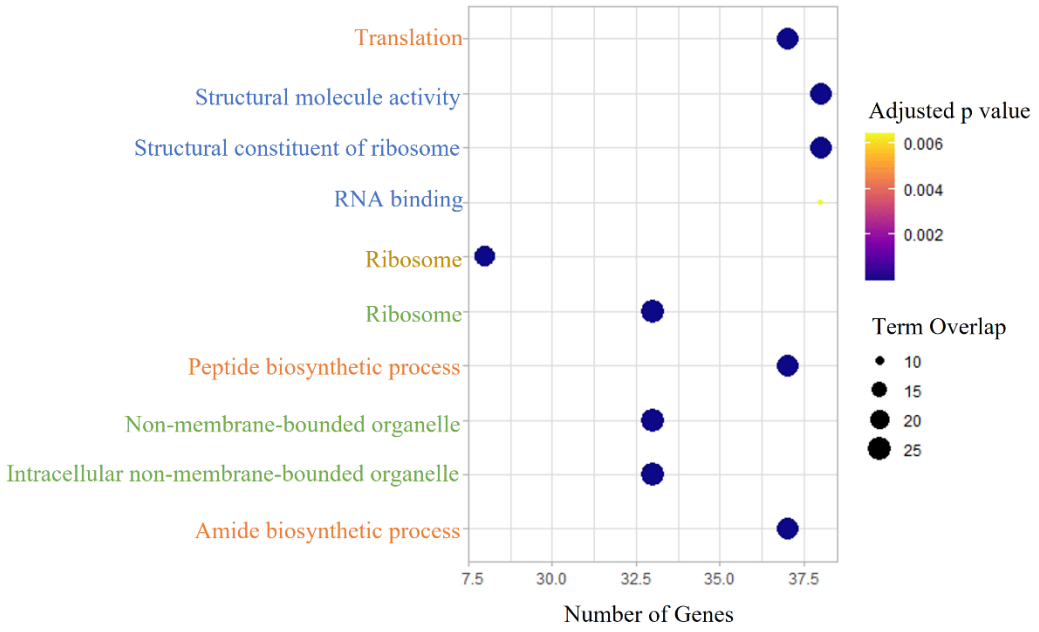


Figure 6.10: g:Profiler analysis on 44 SSDAs in Week 6 samples in comparison to Control samples. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, Green – GOCC and Yellow - KEGG.

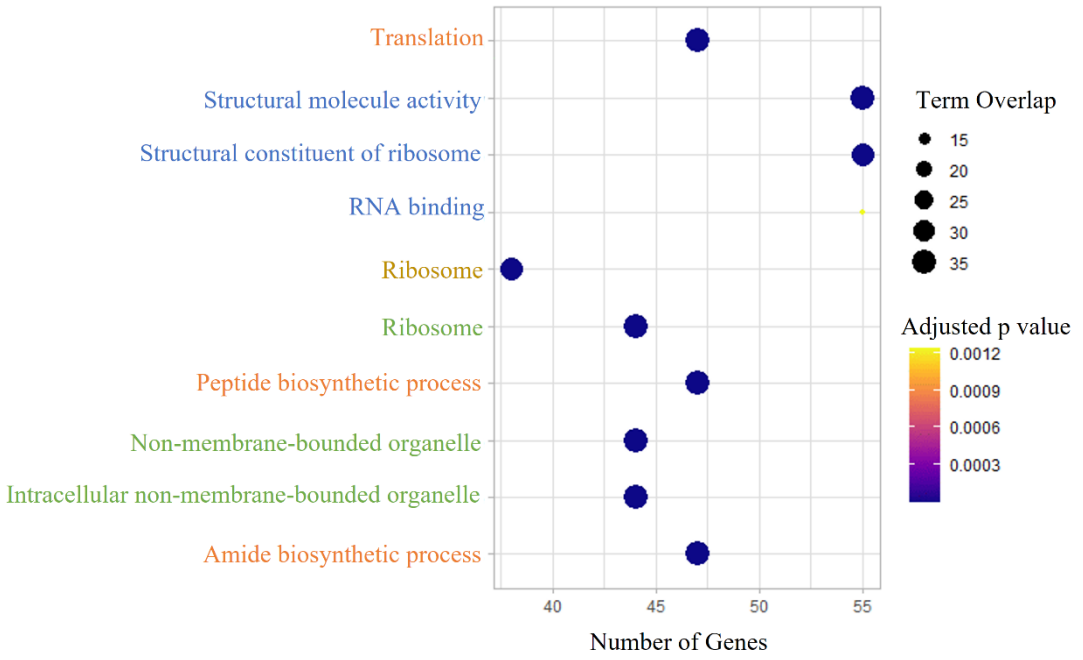


Figure 6.11: g:Profiler analysis on 60 SSDAs in Week 3 samples in comparison to Week 1 samples. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, Green – GOCC and Yellow - KEGG.

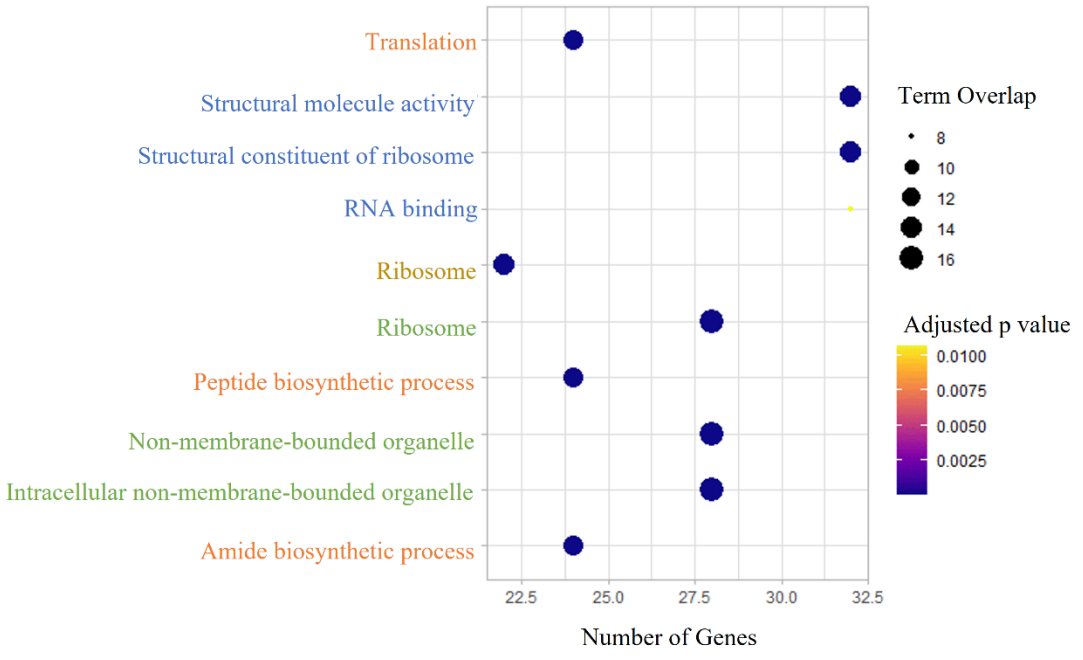


Figure 6.12: g:Profiler analysis on 39 SSDAs in Week 6 samples in comparison to Week 1 samples. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, Green – GOCC and Yellow - KEGG.

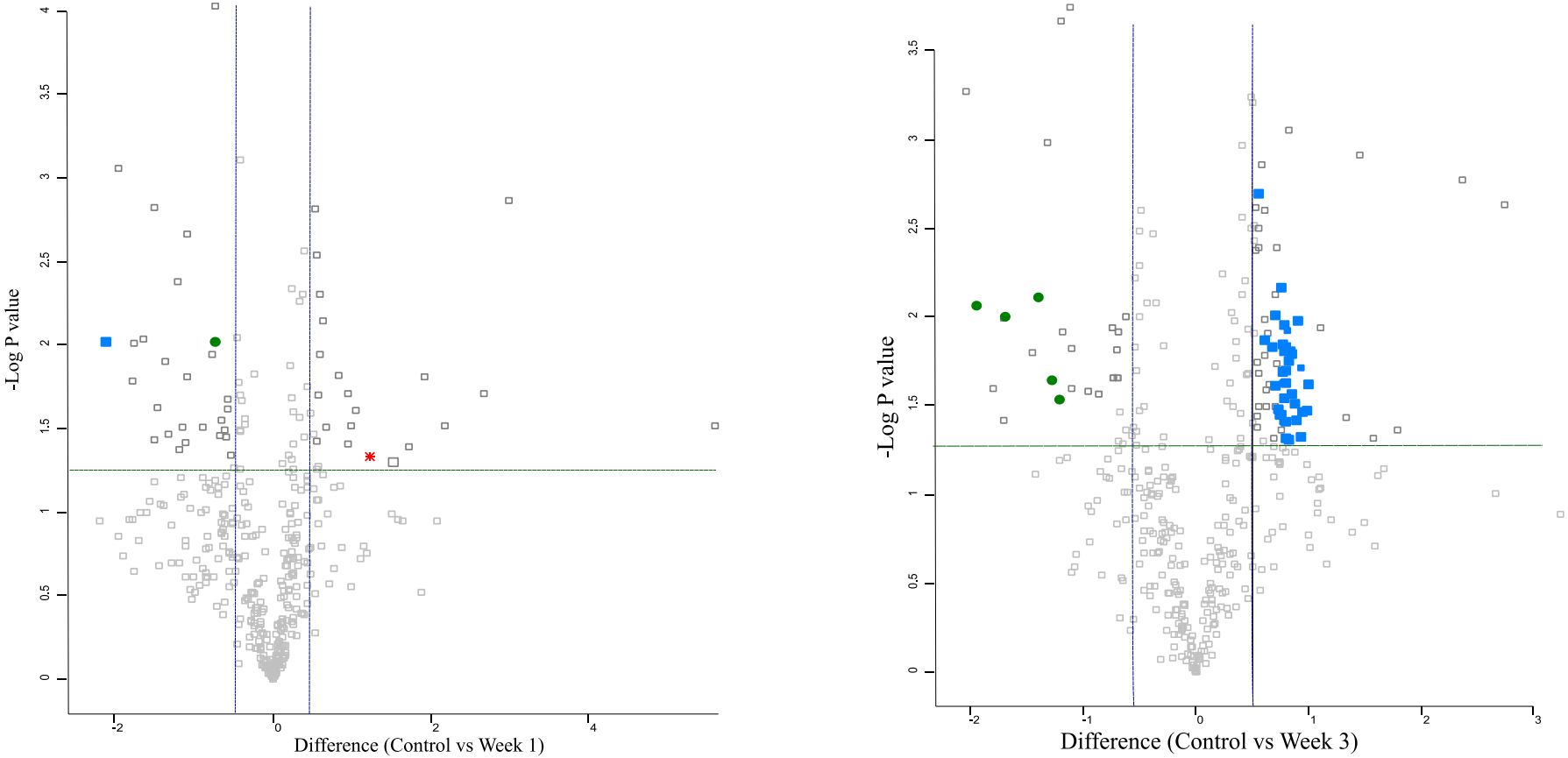


Figure 6.13: Volcano plots of ANOVA significant (p value = 0.1) in the comparison between Control (left) and Apivar treatment (right) groups. Protein groups are highlighted as such: cuticular proteins (green circles), translational proteins (blue squares) and major royal jelly proteins (orange star). Samples above and to the outside of the dashed lines represent SSDAs.

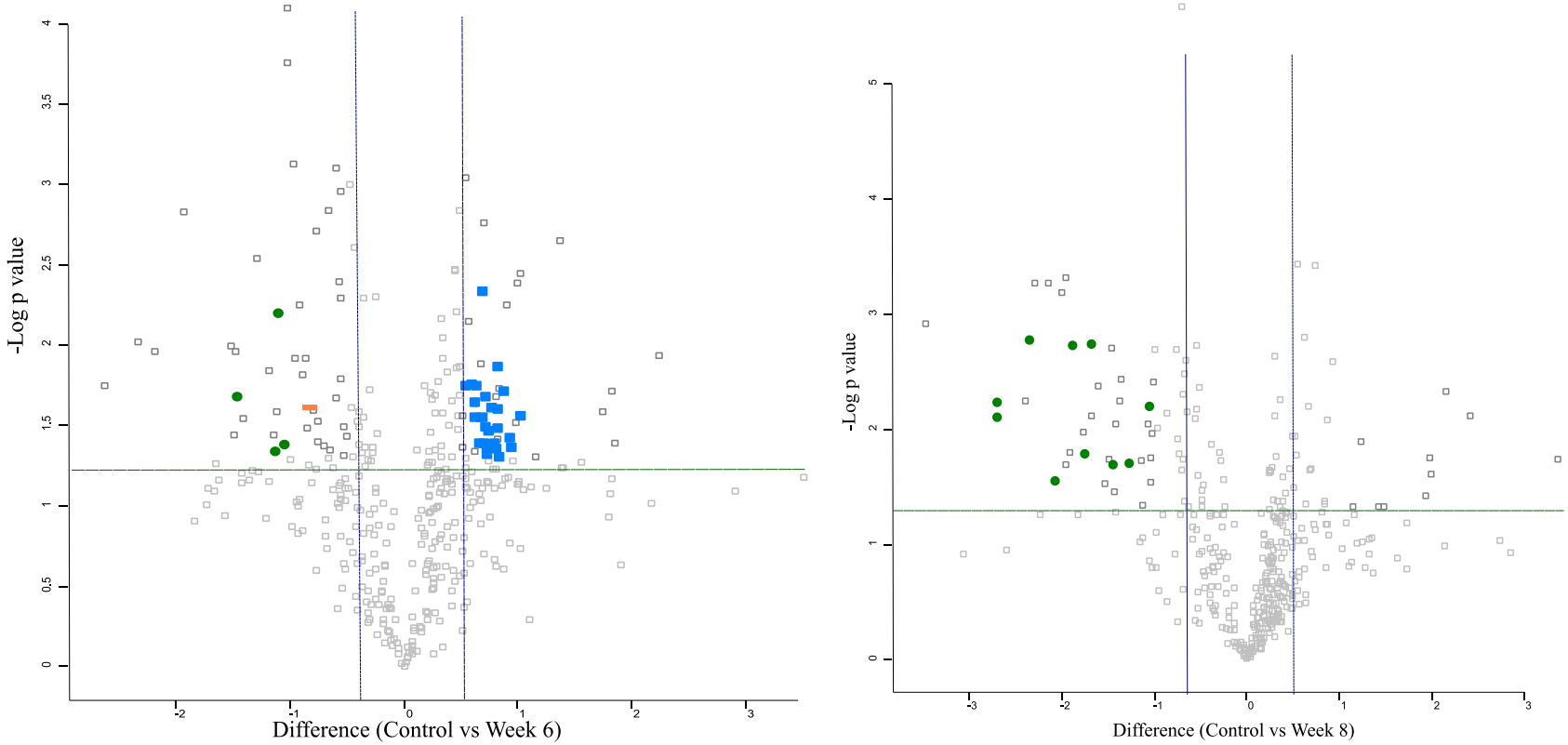


Figure 6.14: Volcano plots of ANOVA significant (p value = 0.1) in the comparison between Control (left) and Apivar treatment (right) groups. Protein groups are highlighted as such: cuticular proteins (green circles) and translational proteins (blue squares). Samples above and to the outside of the dashed lines represent SSDAs.

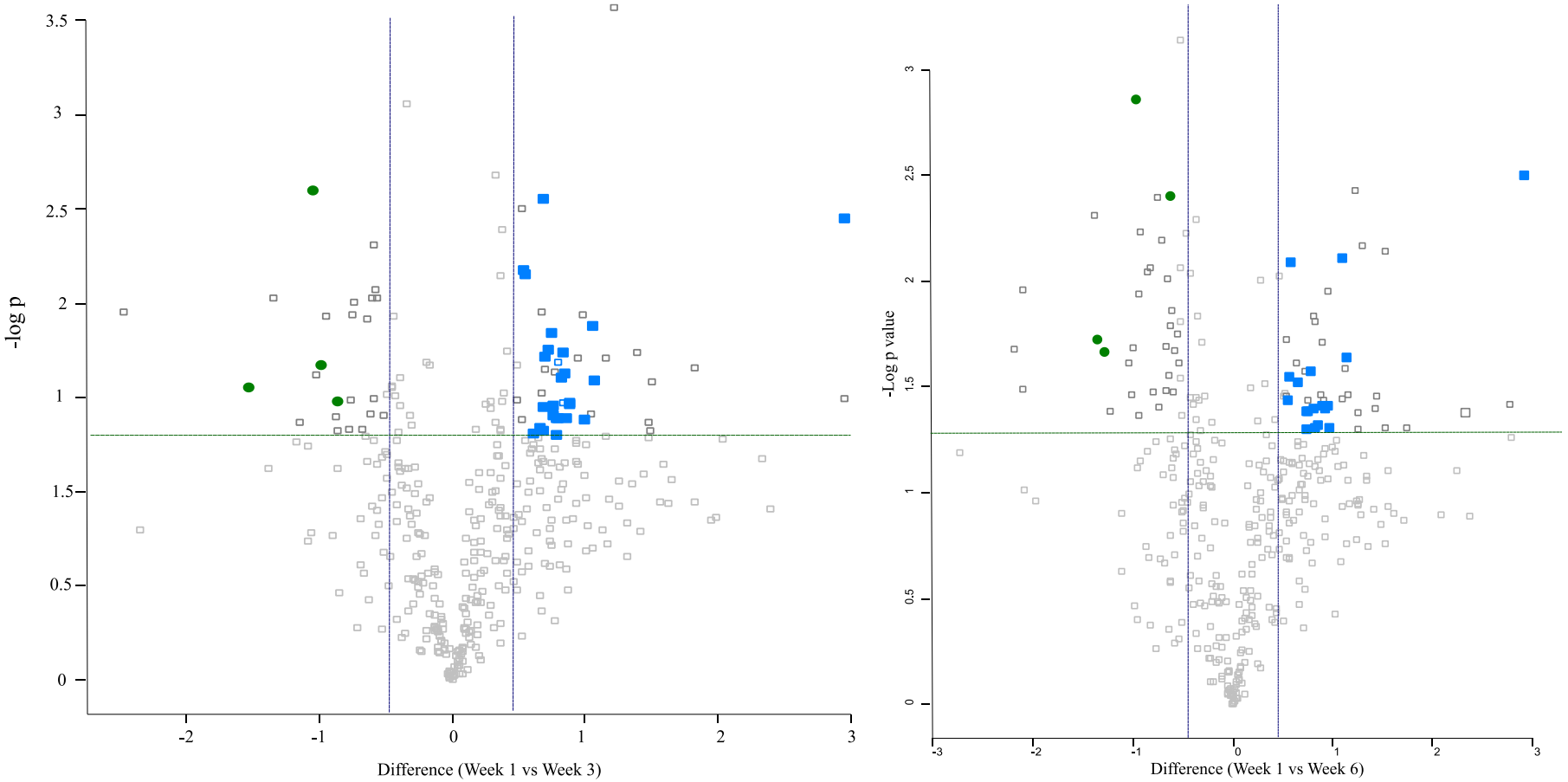


Figure 6.15: Volcano plots of ANOVA significant (p value = 0.1) in the comparison between Week 1 (left) and Weeks 3 and 6 samples (right). Protein groups are highlighted as such: cuticular proteins (green circles) and translational proteins (blue squares). Samples above and to the outside of the dashed lines represent SSDAs.

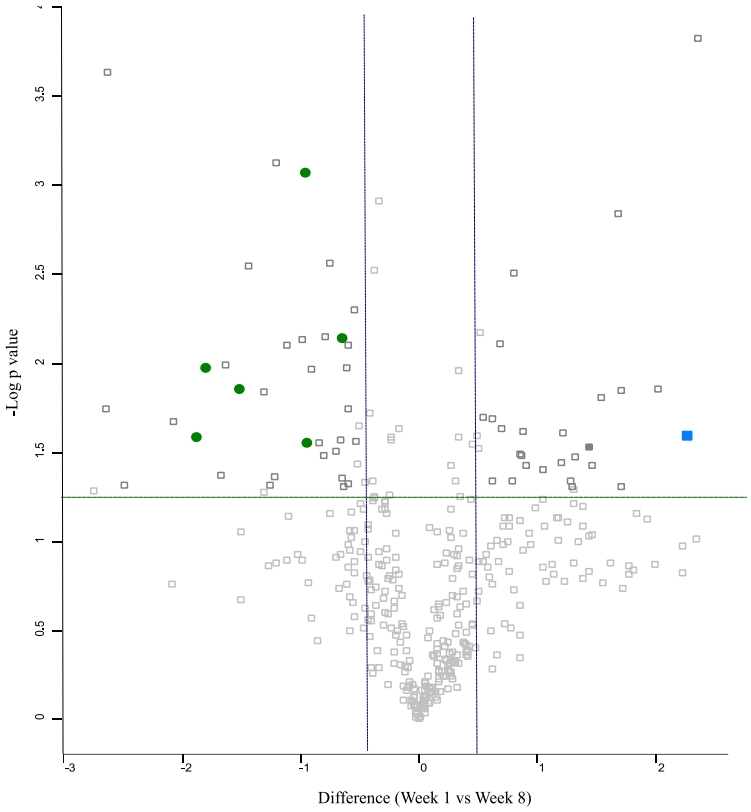


Figure 6.16: Volcano plots of ANOVA significant (p value = 0.1) in the comparison between Week 1 (left) and Week 8 (right) groups. Protein groups are highlighted as such: cuticular proteins (green circles) and translational proteins (blue squares). Samples above and to the outside of the dashed lines represent SSDAs.

A total 244 SSDA ANOVA significant proteins ($p < 0.1$) were Z-scored and clustered via Euclidean distancing (**Figure 6.17**) (**Supp. File 6.4**). Samples were clustered into their respective sample groups with Week 3 and 6 having a similar pattern of protein up and downregulation **(Figure 6.17 A, Cluster 2**). Pathway enrichment analysis identified ribosomal and translational proteins enrichment in Weeks 3 and 6 samples (**Figure 6.17 A**, **Cluster 3**) and cuticular proteins enrichment in Control samples (**Figure 6.17 A**, **Cluster 4**), the other clusters did not have protein pathway enrichment.

Proteins within each cluster were analysed further using g:Profiler (**Figure 6.17 B**). Cluster 1 has proteins involved in intramolecular oxidoreductase activity, which are upregulated in both Week 1 and Week 8 samples. Cluster 2 which is associated with Weeks 3, 6 and 8 samples has proteins upregulated in endopeptidase

regulator and inhibitor activity. Cluster 3 mirrors the pathway enrichment of the Perseus analysis, of translational proteins increased in abundance in Weeks 3 and 6. Cluster 4 which is mostly associated with the Control samples has proteins involved in the structural constitute of the cuticle, some of these proteins are also upregulated in Week 1 samples. Cluster 5 contains proteins involved in oxidoreductase activity which are largely associated with Week 1 samples (**Figure 6.17 B**).

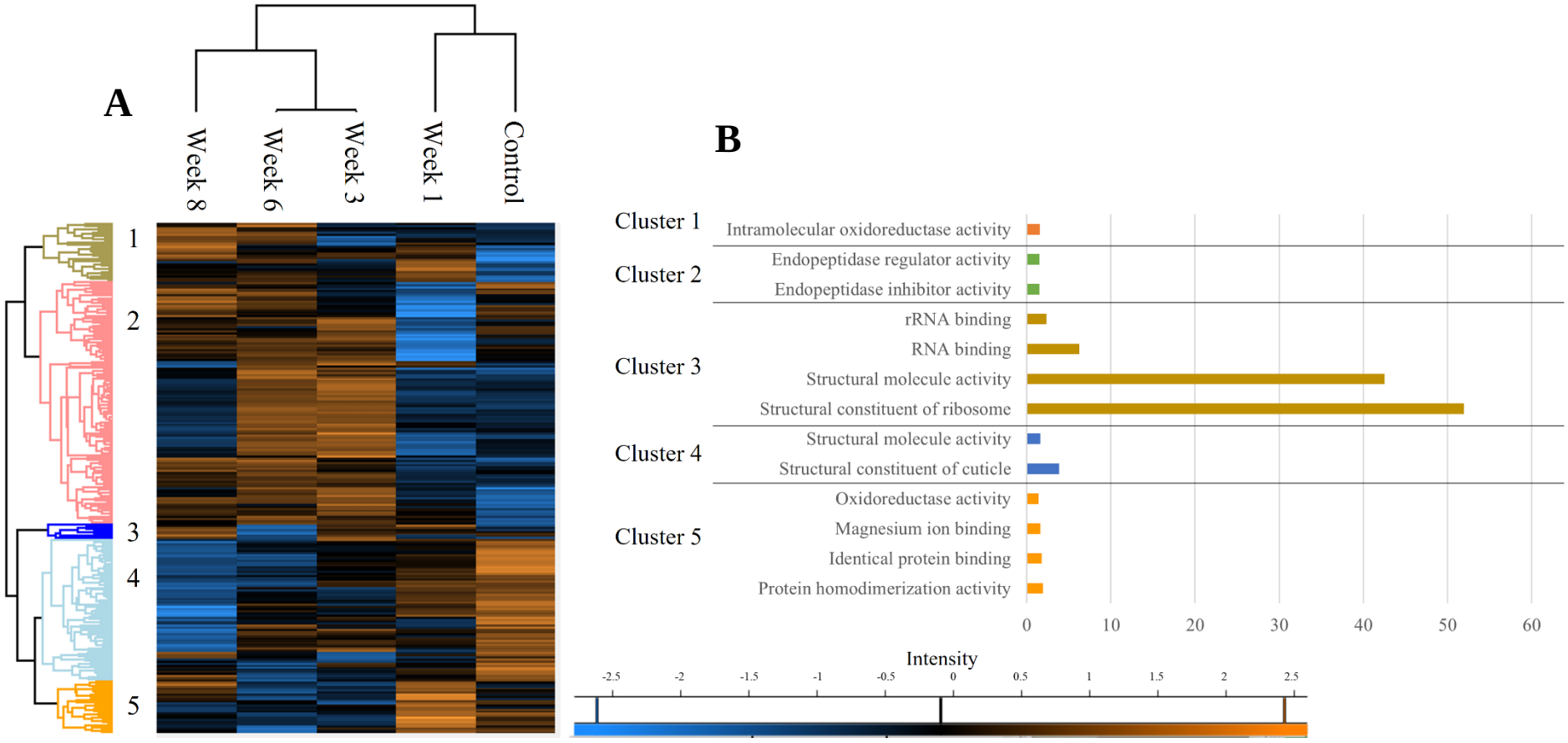


Figure 6.17: Hierarchical analysis of SSDA proteins from volcano plot analysis on Apivar samples. Clusters based on protein abundance profile similarities were determined by hierarchical clustering of mutisample comparisons between the five sample groups (A). In total six clusters were resolved containing proteins that display similar protein profiles. g:Profiler analysis of clusters identified in (A) the graph represents the number of proteins associated with the pathways (B).

6.3.2.1 Discussion of proteomic analysis on *A. mellifera* samples exposed Apivar treatment

This section investigated the impact Apivar treatment application had on honeybee workers in *A. mellifera* colonies. Amitraz is the active acaricide used in Apivar strips which are placed between the brood frames (Gupta, 2007; Gupta, 2018). Amitraz paralyses the mites which subsequently fall below the frames onto the bottom boards of the hives and die of starvation (Semkiw *et al.*, 2013). A large number of mites fell after the treatment was applied for the first three weeks, after which the numbers gradually decreased. Amitraz is readily absorbed through dermal and oral exposure, which could provide an insight into the large number of mites collected within the first three weeks (Filazi and Yurdakok-Dikmen, 2018). Despite the large number of mites collected from the sticky insert boards from Week 1 post treatment application, the proteome of the bees sampled in Week 1 were positioned closer to the Control samples than the remainder of the other treated samples. There are two possible reasons for the arrangement of the proteomes on the PCA graph: Control samples represent a diseased group with the parasitation of the *Varroa* affecting their proteome. Secondly, despite the effect of amitraz being observed in the mite fall data within the first week, the bees do not show detectable off-species effects until the third week post exposure, as the volcano plots suggest a stronger reaction to amitraz exposure in the third week in comparison to the first. It is difficult to identify the specific reason for the arrangement of the samples in the PCA as standardising *Varroa* levels within field trials is not possible. However, the dramatic increase in translational proteins in Weeks 3 and 6 does indicate a proteomic response to treatment exposure as upregulation in translational proteins is not observed in the Control samples.

The lack of cuticular proteins in Weeks 3, 6 and 8 samples in comparison to Control and Week 1 bee samples could be indicative of a damaging response to amitraz exposure or a response to the exposure and parasitisation of *Varroa* mites. *Varroa* mites have been linked with alterations in cuticular protein expression. A decrease in cuticular proteins has been observed in honeybee pupae (Salvy *et al.*, 2001; Surlis *et al.*, 2018). Whereas, an increase in cuticular proteins has been detected in adult worker bees parasitised by *Varroa* mites (Salvy *et al.*, 2001; Erban *et al.*, 2019). However, an increase in cuticular proteins has also been observed in previous work which has

identified a reduction in cuticular proteins in response to amitraz exposure, whereby both hydrophilic and hydrophobic cuticular protease activities were reduced (Strachecka *et al.*, 2012a). This study theorises that amitraz exposure causes deactivation of protease inhibitors or amitraz reacts with the protease/substrates therefore blocking cuticular protease activity. The findings of this study could add to the argument of Strachecka *et al.* (2012) as cuticular proteins were decreased in abundance in treatment Weeks 3, 6, and 8 in comparison to Control and Week 1 samples. However, to confirm the impact of Amitraz on the bees cuticular expression, further investigative work would need to be conducted, with the Control group being clear of both treatment and *Varroa* presence.

Cuticular protein reduction has been documented in insects in response to insecticide exposure: honeybees in response to formic acid (Strachecka *et al.*, 2012b) and *Ctenocephalides canis* when exposed to cyromazine (Page, 2008). Changes in cuticle composition is one of the methods of insecticide resistance and is usually paired with an increase in detoxification enzymes that collectively work to reduce the impact of the insecticide (Balabanidou *et al.*, 2018). The proteome of Weeks 3, 6 and 8 bee samples have a reduction in cuticular protein expression and overall there were very few detoxication enzymes detected as SSDAs.

The findings presented here suggest that the workers exposed to high levels of *Varroa* have an increased abundance of cuticular exposure, that then dramatically decrease when exposed to amitraz. The proteome of Week 8 bees provide an insight into the physiology of workers with a greatly reduced *Varroa* presence and workers that have 'recovered' from insecticide exposure. Despite the reduction in cuticular abundance in Week 8 samples in comparison to Control and Week 1 samples. There are two cuticular proteins that are increased in abundance in Week 8 samples when compared to Weeks 3 and 6 samples – cuticular protein 14 (A0A088A4T3) and pupal cuticular protein (A0A088ABR1). Despite only having two cuticular proteins increased in abundance in Week 8 samples, the presence of the proteins could indicate recovery from both *Varroa* and Apivar exposure. Apivar treatment is usually left within hives for 10 weeks, as amitraz is unstable and undergoes rapid biotransformation in the hive environment (Pohorecka *et al.*, 2018). Therefore, by

Week 8, the level of amitraz within the hive would be much lower than levels of amitraz in Weeks 3 and 6 and this could be a possible attribute to the shift in proteome.

A second difference observed between Control and Week 1 samples to Weeks 3, 6 and 8 was the increased abundance of translation proteins in the latter samples. Overall there was a large number of translational proteins identified in bees samples in Weeks 3, 6 and 8 in comparison to Control and Week 1 samples. An increase in translational proteins in response to amitraz exposure was observed in a previous study where most of the differentially expressed genes associated with biological processes (GO term) were translational proteins (Ye *et al.*, 2020). Other studies have identified an increase in translational processes within the transcriptome when insects were exposed to insecticides (Wei *et al.*, 2019; Ingham *et al.*, 2021), and when insects develop resistance to insecticides (Tan *et al.*, 2007; Sun *et al.*, 2011; Yu *et al.*, 2014). Bees sampled in Week 8 had a reduction in the number of translational proteins in comparison to Weeks 3 and 6 bees which again suggests a reduction in an off-species effect of Apivar in colonies.

6.3.3 Characterisation of the effect of Apiguard on *A. mellifera* proteome

Apiguard trays were used to treat three hives as per the manufacturer's instructions. Weekly mite fall was monitored using adhesive inserts (**Figure 2.3**). An average of 69 mites were collected in the control during the week prior to application of the treatment (Treatment application was at the start of week 1). An increase in mite fall was observed from Week 1 to Week 5, when after which mite fall decreased to Week 8 (**Table 6.7**).

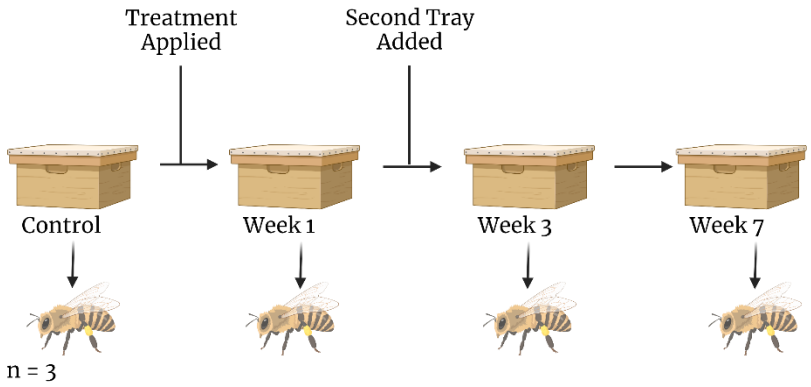


Figure 6.18: Schematic on sampling regime and treatment application for Apiguard trial

Table 6.7: Average mite fall throughout the duration of the treatment trail. Treatment was applied at the start of Week 1.

Control	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
69	263	354	313	355	727	502	220	281

LFQ mass spectrometry was performed on single head samples from four time points in the Apiguard treatment trial: Control, Weeks 1, 3 and 7 post treatment application. Each group was collected in triplicate and all hives were part of the same apiary. Initial protein matrix contained 3,106 proteins, this was reduced significantly through filtering steps to remove contaminant peptides, reverse hit peptides and peptides only identified by site. LFQ intensity values were Log_2 transformed and only proteins identified in at least three of the three replicates were included for additional analysis. A further filtration of an ANOVA t test (p value = 0.1) resulted in 202 protein matrix.

Principal component analysis was completed on ANOVA significant samples, providing an overview of the proteomic relationship between samples. Proteomes from all samples resolved into their respective groups. Week 3 and Control samples were clustered the closest together, whereas Weeks 1 and 7 were clustered at opposite sides of the PCA (**Figure 6.19).**

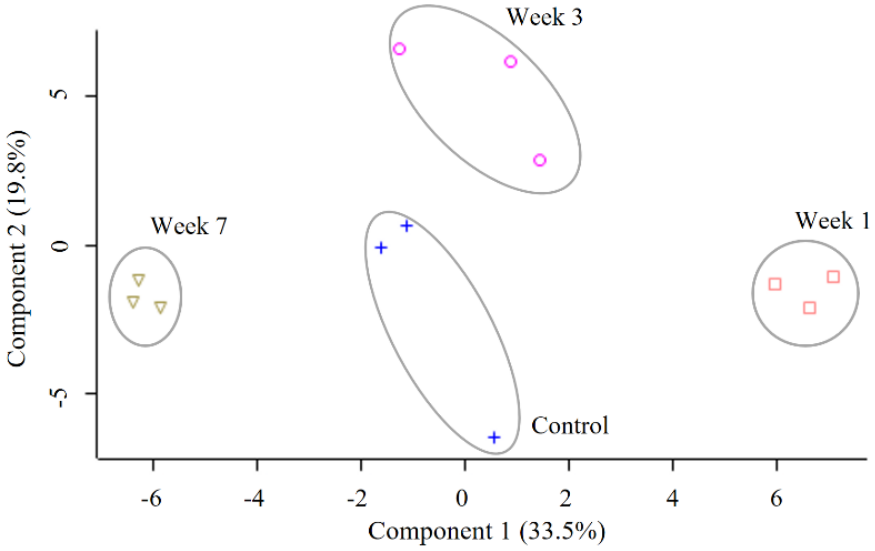


Figure 6.19: Principal component analysis on ANOVA significant Apiguard samples. All samples are clustered individually which suggests a level of separation in the proteomic profiles of the sample groups. . The total explained variance of this PCA for the given dataset is 53.3%.

ANOVA significant proteins were used for analysis in volcano plot comparisons. Proteins with a significance (log_{10} p value) greater than 1.3 and a relative fold change (Log_2 LFQ intensity difference) greater than 1.5 were identified as statistically significant differentially abundant (SSDA) proteins (**Supp. File 6.5**). Cytochrome P450 subunit 6AS5 (Cyp6AS5) was increased in abundance in both Week 1 and Week 3 bee samples in comparison to Control samples (rfc, 2.93 and 3.22 respectively). Cytochrome P450 protein, subunit 9e2 (Cyp9e2) was increased in Week 1 samples in comparison to Week 7 proteins (rfc, 1.77).

Control samples had two cuticular proteins increased in abundance in comparisons to Weeks 3 and 7 samples, cuticular protein analogous to peritrophins 3-C precursor (rfc 1.59 and 2.09 respectively) and cuticular protein 2 precursor was identified as upregulated in Control samples in comparison to Week 7 samples (rfc

4.14). Apidermin 3 precursor was identified as the most abundant protein in both Week 1 and Week 3 samples in comparison to Week 7 samples (rfc, 81.99 and 158.32 respectively). Glucose dehydrogenase was the most abundant protein in both Weeks 3 and 7 in comparison to Week 1 (rfc, 5.73 and 5.09).

A total of 65 SSDA proteins were Z scored and subjected to Euclidean distance hierarchical clustering analysis (**Figure 6.20**) (**Supp. File 6.6**). Samples resolved into their respective groups with all replicates grouping together. Proteins were divided into 5 clusters based on protein abundance profile similarities. Cluster 1 contained 2 proteins - dipeptidyl aminopeptidase-like protein 6 isoform X1 and an uncharacterised protein. Cluster 2 had two Cytochrome P450 (Cyp6AS5 and Cyp9e2) and cytochrome c oxidase (Cox6c). Cluster 3 consisted of three proteins - Hyaluronidase isoform X1, rho guanine nucleotide exchange factor 11 isoform X4 and Immune responsive protein 30. Cluster 4 had the three cuticular proteins – cuticular protein analogous to peritrophins, cuticular protein 2 and apidermin 3. Cluster 5 contained some translational proteins. No protein pathway enrichment was detected in the protein clusters.

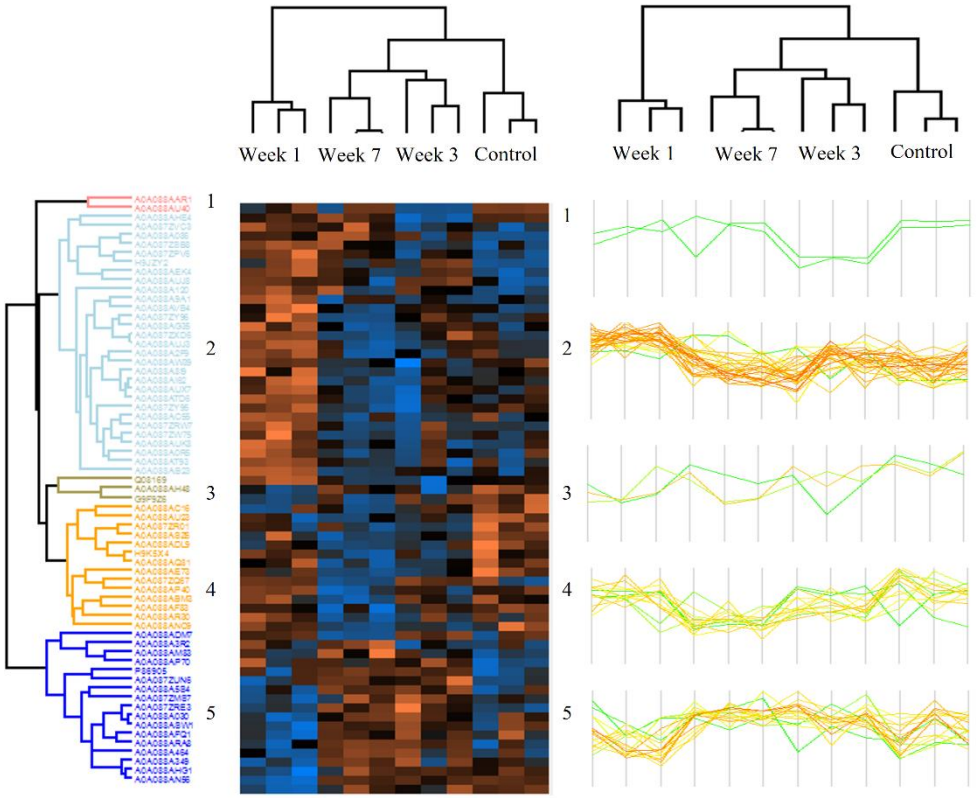


Figure 6.20: Hierarchical clustering on 65 SSDAs from Apiguard analysis. Clusters based on protein abundance profile similarities were determined by hierarchical clustering of mutisample comparisons between four sample groups. In total five clusters were resolved containing proteins that display similar protein profiles. No enrichment was identified in any of the clusters.

Overall, there was a low number of SSDAs generated from the Apiguard analysis, in all of the comparisons. Volcano plot analysis identified a small number of proteins that were increased in relative abundance in Control and Week 1 bee samples. However, no protein pathways were observed to be affected by the presence of the treatment as no enrichment was observed in the heatmap analysis.

6.3.3.1 Discussion on the proteomic effects Apiguard exposure has on *A. mellifera*

Numerous studies have been completed on thymol based treatments and their effectiveness against reducing/eliminating mite populations in agricultural and apicultural environments (da Silva Matos *et al.*, 2014.; Da Silveira Novelino *et al.*,

2007; Novato *et al.*, 2015; Wu *et al.*, 2017). However, little research has been conducted into how the presence of thymol based treatments such as Apiguard may effect species it is used to protect, such as *A. mellifera*. One study has found that Apiguard causes behavioural changes in workers of different ages within *A. mellifera* colonies, with foragers not previously exposed to Apiguard treatment actively avoiding close proximity to the gel (Mondet *et al.*, 2011). Avoidance of Apiguard gels was observed in a lesser extent in younger bees (2 days old) but increased in 4 day old bees, as they avoided gels and displayed fanning behaviour, similar to that of foragers (Mondet *et al.*, 2011).

Work presented here identifies changes to the worker bee's proteome as observed by the cluster separation in the PCA graph (**Figure 6.19**). The impact on the worker bees by Apiguard in this trial is difficult to interpret as the number of SSDAs generated in the comparisons are low, there are no large protein pathway changes induced by the presence of the Apiguard and lastly the age of the workers was unknown. What can be understood from the results is that there are small protein expression levels within each sample. Week 1 samples had an increased abundance of cytochrome P450 proteins (Cyp4506AS5 and Cyp4509e2) in comparison to the other samples. An increase in cuticular proteins in Control samples in comparison to treatment Weeks 3 and 7 samples was also observed. As the mode of action of thymol against mites or insects is not fully characterised and it is very difficult to understand the proteomic response to Apiguard within this experiment. More research is needed to investigate the mode of action of thymol containing acaricides and furthermore, how these treatments effect the host-species of the target pests.

6.4 Conclusion

Treatments for *Varroa* parasitisation on honeybee colonies has been a topic of debate in beekeeping associations as to whether or not to integrate treatments into beekeepers management practice. *Varroa* are known vectors for honeybee viruses, there have been twenty-two viruses described in honeybees with eighteen of these associated with the presence of the mites with many of them being transmitted by the mite itself (Chen and Siede, 2007; Fanny *et al.*, 2014). It is also suggested that the presence of *Varroa* in colonies could be linked with colony collapse disorder (VanEngelsdorp *et al.*, 2010; Rosenkranz *et al.*, 2010). The full parasitic effect of

Varroa on honeybees is still being investigated as only recently was it shown that *Varroa* feed on the fat body of workers as opposed to previous beliefs of the mite feeding on the hemolymph (Ramsey *et al.*, 2019). With this in mind it is important to understand the current lack of understanding on the effect of applying anti-*Varroa* treatments to honeybee colonies that have *Varroa* infestations. As seen above, commonly used anti-*Varroa* treatments do have significant effects on the honeybee workers. The exact mode of action for a lot of anti-*Varroa* treatments is not known and therefore the full impact of applying these treatments to honeybees is not fully understood. More investigative research needs to be conducted on how these treatments impact the honeybees that they are supposed to be aiding.

6.5 Summary of Findings

Table 6.8: Overview of proteomic analysis from Chapter Six – Characterisation of anti-*Varroa* treatments on *Apis mellifera*

Treatment Trail	Summary of Findings
MAQ	MAQ treatment was applied to hives immediately after Control samples were collected. It remained within the hive for one week and was removed after Week 1 samples were collected. Week 1 worker bee samples had the greatest changes to their proteomes in comparison to Control and Weeks 2 and 3 samples. Week 1 samples had a decrease in aerobic associated cytochrome c oxidase proteins (the known target of formic acid – the active ingredient of MAQ strips), in comparison to Control and Weeks 2 and 3 samples. A decreased abundance of xenobiotic metabolism proteins was also observed in Week 1 samples in comparison to Control and Weeks 2 and 3 samples. Cuticular proteins were increased in abundance in Week 1 samples in the analysis. A degree of similarity was observed between Control and Weeks 2 and 3 samples, indicating a recovery of the proteome after the exposure to MAQs within the colony.
Apivar	Apivar treatment was applied to colonies immediately after Control samples were collected. Apivar treatment remained within the colonies for the duration of the trail. Control samples were clustered alone in the PCA and also presented with a different proteome to the other samples analysed. This could be an effect of the presence of *Varroa* within the colony at the time of sampling so may represent a disease sample in contrast to a negative control to stress factors. Week 1 samples had a distinguishable proteome from Weeks 3, 6 and 8 treatment samples.

	Overall Control and Week 1 samples had an increase in cuticular proteins in comparison to Weeks 3, 6 and 8. However, Weeks 3, 6 and 8 had an increased abundance of translational proteins in comparison to Control and Week 1 samples.
Apiguard	Apiguard treatment was applied to colonies immediately after Control samples were collected and remained within the colony for the duration of the trial. Overall there was very little difference in the proteomes of the samples analysed in this study. Some small changes to cuticular proteins were observed in Control samples and Week 1 samples did present with two cytochrome P450 proteins increased in abundance in comparison to Control, Weeks 3 and 7. The lack of understanding to the active target of Apiguard treatments makes designing a treatment trail and analysing the results difficult.

Chapter 7

Proteomic Analysis of Response of *Apis mellifera* Workers to *Nosema apis* and/or *Varroa destructor* Mono and Dual Infections

7.1 Introduction

Varroosis is a very serious and unfortunately a very common disease caused by the parasitic mite, *Varroa destructor* (Di Prisco *et al.*, 2011). *Varroa* mites are ectoparasites of the honeybee and were originally confined to the eastern honeybee *Apis cerana* but have since spread world-wide, reaching Ireland and the UK around the early 2000s (Anderson, 2000; Rosenkranz *et al.*, 2010). This host shift was most likely a result of opportunistic infection during transport of *A. mellifera* colonies across Eastern Russia or the Far East in the first half of the past century (Rosenkranz *et al.*, 2010). Unlike *A. cerana, A. mellifera* has not had the history of co-evolution with the *V. destructor* which has resulted in the mite causing devastating effects to *A. mellifera* hives as the workers are not as efficient as removing the mites as *A. cerana* (Rosenkranz *et al.*, 2010). *Varroa* parasitisation of *A. cerana* is mainly confined to the drone brood which limits the harmful effects of the mite's presence, compared to *A. mellifera* where both drones and workers are affected (Anderson, 2000; Rosenkranz *et al.*, 2010).

Another common and widespread pathogen of honeybee colonies is the microsporidian *Nosema apis* and *Nosema cerana*. Unlike the *Varroa* mite, *Nosema* infections rarely cause the death of an infected colony (Paxton, 2010). *Nosema* prevalence has been reported to build up over a period of 18 months within a colony before becoming detrimental to the long term health of the colony (Higes *et al.*, 2008). The presence of *Nosema* within colonies not only causes harm to the individual but can also impact the size of the brood and subsequently the strength of the hive (Higes *et al.*, 2008). The infection rates at the time of the collapse of hives differs between winter and spring colonies. Infections from *Nosema* being more intense and the queens were also infected with *Nosema* spores in winter**,** in contract, in spring infection rates were lower and the queen was not infected, or infections could only be detected via PCR (Higes *et al.*, 2008). *Nosema* spores have been detected in both colony collapse disorder (CCD) colonies and dead non-CCD colonies, indicating that *Nosema* infection could be a contributing factor to CCD and hive collapse but not necessarily the sole reason for collapse (Cox-Foster, 2014).

Currently there is a small number of studies that have investigated the effect of dual infection by *Nosema* and *Varroa* on honeybee colonies. The results from these

studies are conflicting, with some suggesting a link between *Varroa* infections in the autumn to an increase in *Nosema* infection in the following spring (Hedtke *et al.*, 2011), however other studies have not identified a link between *Varroa* parasitisation and *Nosema* infections (Bahreini and Currie, 2015). There are difficulties investigating dual infection by *Varroa* and *Nosema* in honeybee colonies due to a myriad of reasons: *Varroa* sensitive hygienic (VSH) behaviour (Harbo and Harris, 2005; Rinderer *et al.*, 2010; Mondet *et al.*, 2015a; Traynor *et al.*, 2020), grooming- both auto and self-grooming (Pritchard, 2016), times of the year (Coffey *et al.*, 2010), and infection of *N. apis* or *N. cerana* (Sinpoo *et al.*, 2018). There is little research completed on dual infections of *Nosema* and *Varroa* in honeybee colonies using proteomic techniques.

The aim of this Chapter was to use proteomic analysis to characterise the response of *A. mellifera* to mono infection with *V. destructor* and *Nosema* and to compare this to the response of bees to dual infection with *V. destructor* and *Nosema* in order to establish whether dual infection produced a distinct proteomic response.

7.1.1 Aims of Chapter Seven

- Investigate proteomic changes that occur to honeybee workers when exposed to *Varroa* and *Nosema* individually within hives.
- Determine what proteomic changes occur when honeybee workers are infected with both *Varroa* and *Nosema.*

7.2 Experimental Design

All trial hives were within one apiary and each group (Control, single *Varroa,* single *Nosema* and dual infection) had three hives assigned to the group. Each hive was monitored for both the presence of *Varroa destructor* and *Nosema. Varroa* monitoring was implemented using sticky inserts placed underneath a mesh floor in all the participating hives. Inserts remained in the hives for a week and were replaced at weekly intervals. The number of mites on each insert were counted and results were used to provide an insight into the general health of the hives. When the inserts were

removed from the hives, a sample of workers was collected from the door of the hive for *Nosema* monitoring. Of the worker bees that were sampled, 30 were used for *Nosema* counts (determined by crushing 30 bees in 30ml ddH_2O and placing a drop of the solution onto a haemocytometer and calculating the approximate number of spores per bee).

A single head sample from worker bees from all disease trial groups were subjected to mass spec preparation; protein extraction, clean-up, digestion, and peptide purification (C18). For mass spec analysis 0.75µg of tryptic peptides were loaded onto a QExactive (ThermoFisher Scientific) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated over a 2-40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100mm length, 75mm ID), using a 135 min reverse-phase gradient flow rate of 250nL min^{-1}. Three hives were used for Control and all disease group analysis. All remaining hives within the apiary that were not included in the disease trail were also treated for *Varroa* and were monitored for parasitisation levels by the attending beekeeper. Apivar treatment was applied on the 6th August 2020 to Control and *Nosema* samples.

Protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.6.6.0 (https://www.maxquant.org/). Data processing and analysis was carried out using Perseus v 1.6.6.0 (https://maxquant.net/perseus/). Proteins were deemed statistically significant differentially abundant (SSDA) if identified to have a Log2 fold change greater than +/- 1 (unless specified otherwise) and a -Log p value greater than 1.3. To identify protein-protein interactions of SSDA proteins, functions, and pathway enrichment analysis was conducted using STRING v 11 (https://string-db.org/) and g:Profiler gGOst (https://biit.cs.ut.ee/gprofiler/gost) were utilised. g:Profiler term information used in this analysis are outlined as such: Term ID is the unique term identifier for each pathway/term. Term size refers to the number of genes that are annotated to the specific term. Query size is the number of genes that were included in the query run. Intersection size is the number of genes in the input query that are annotated to the corresponding term. The adjusted p-value is the hypergeometric p-value after

correction for multiple testing. The user-threshold for each of the query runs is 0.05 (Raudvere *et al.*, 2019)

7.3 Results

This Chapter describes the results obtained from a disease trial that was completed in summer 2020. The trial contained four groups each with three hives: control (treated for *Varroa* and controlled for *Nosema*), *Varroa* only (controlled for *Nosema*), *Nosema* only (treated for *Varroa*), double infection (infected with both *Varroa* and *Nosema*). Hives in the disease trial needed to be treated as all trial hives were in the same apiary, therefore drifting and subsequent spread of *Varroa* and *Nosema* could not be controlled. Hives were monitored for *Varroa* and *Nosema* levels throughout the trial (**Figure 7.1**). The number of *Varroa* present within the control and *Nosema* hives decreased as the weeks progressed. In contrast *Varroa* numbers increased in the *Varroa* only and double infected hives as the weeks progressed. Interestingly, a drop in mite fall was observed in *Varroa* only hives in Weeks 4 and 5, this could be attributed to a drop in colony activity and subsequent reduction in brood. A consistent decrease in *Varroa* numbers within the Control and *Nosema* groups provides an indication into the effectiveness of the treatments on these colonies. There was a slow increase in *Varroa* populations within the Double infection group, with the highest *Varroa* counts occurring in Week 3 and 5.

Nosema populations per were relatively consistent in *Nosema* and Doubly infected groups (**Figure 7.2**). However, Control and *Varroa* groups had inconsistent numbers of *Nosema* spores in samples. This could be attributed to the method of analysing samples for *Nosema* disease levels (30 bees were sampled and used to analyse the colony's level of *Nosema*). Workers that were sampled could have had low/small infections of *Nosema* some weeks in comparison to workers with high infections being sampled in later weeks. Overall the preventative methods of removing frames and replacing floors and foundation in the Control and *Varroa* hives seemed to have kept the *Nosema* levels lower than those detected in the *Nosema* and Double infection hives.

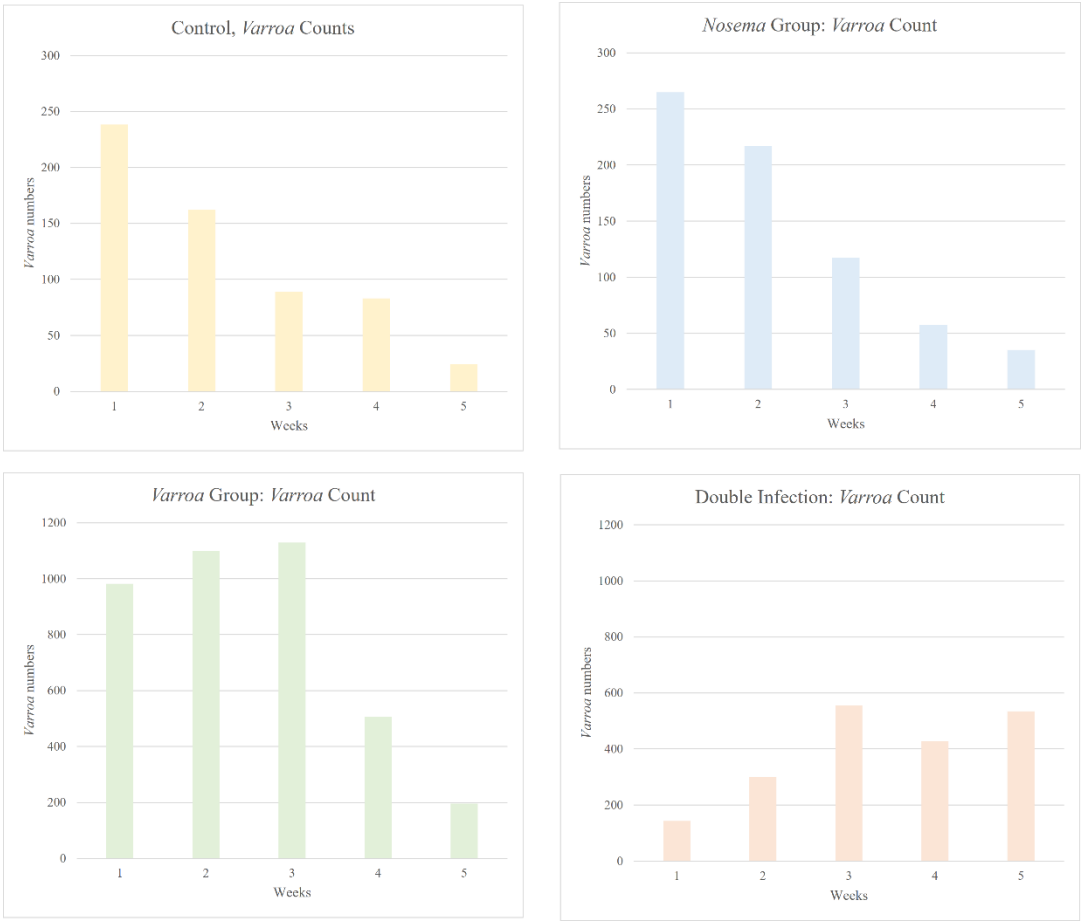


Figure 7.1: *Varroa destructor* populations counted from sticky inserts that were placed underneath the frames of the hives. The average mite fall per week was calculated between the three trial hives within each group and used to plot the above graphs.

Figure 7.2: *Nosema* levels per bee from all disease trial hives from Control, *Varroa* only, *Nosema* only and Double infections. The average *Nosema* population per bee were calculated between the three hives within each group and used to plot the graphs.

Proteomic analysis on disease trial samples was completed in three sets: Control vs *Varroa*, Control vs *Nosema*, Control vs Double infection. The following sections outlines the data analysed from each of the arms of the trial compared to the Control group. The work aims to characterise the changes in the proteome of worker bees when exposed to the presence of *Varroa*, *Nosema* in both a mono and dual infection environment.

7.3.1 Proteomic analysis of Control samples compared with *Varroa* infected colonies

A total of 3,394 proteins were initially identified in Perseus analysis of the dataset analysing Control and *Varroa* samples from Weeks 1, 3, and 5. This was significantly reduced through filtering steps to remove contaminant peptides, reverse hit peptides and peptides only identified by site. LFQ intensity values were Log_2 transformed and only proteins identified in at least three of the three replicates were included for further analysis. This resulted in a data matrix of 500 proteins. The matrix was further filtered through ANOVA significance testing ($p < 0.05$) as only ANOVA significant proteins were used for analysis.

In the Principal Component Analysis (PCA) Week 1 samples (Control and *Varroa*) were separated individually, Control, Weeks 3 and 5 samples were clustered together and *Varroa* Weeks 3 and 5 samples were clustered together (**Figure 7.3**). It was surprising to observe the arrangement of samples in the PCA as it was expected that there would be a more significant difference between Control and *Varroa* infected hives.

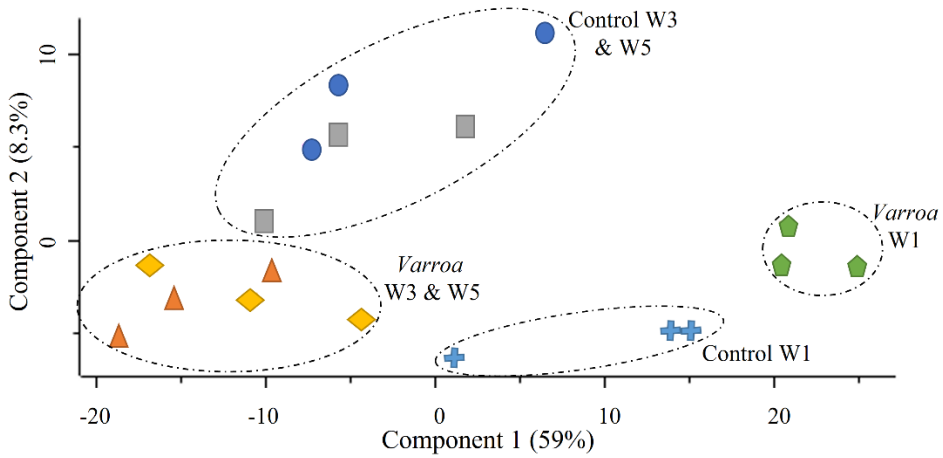


Figure 7.3: PCA graph on control and *Varroa* samples. Week 1 *Varroa* and Control samples are clustered separately from Week 3 and 5 *Varroa* and control samples. Weeks 3 and 5 Control samples are clustered together as are *Varroa* Week 3 and 5 samples. Blue cross – Control W1, Grey square – Control W3, Blue circles – Control W5, Green pentagons – *Varroa* W1, Orange triangle – *Varroa* W3, Yellow diamonds – *Varroa* W5. Total variance on this PCA with this dataset was 67.3%.

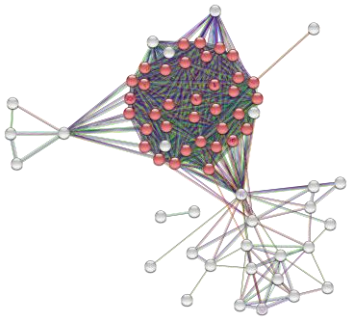
To investigate proteomic changes between samples respective to each other, Student's t-tests were implemented between Control and *Varroa* samples as the disease trial progressed through the weeks. Proteins with a relative fold change (rfc) > 2 and a -log p value > 1.3 were determined to be statistically significant differentially abundant (SSDA) proteins and were used for further analysis (**Supp file 7.1**).

7.3.1.1 Control and *Varroa* infected Student's t-test comparisons

Initial analysis was completed between Control and *Varroa* infected colonies from the respective sample weeks – 1, 3, and 5. Analysis between Control Week 1 samples and *Varroa* Week 1 samples revealed a large number of ribosomal proteins associated with the Control group. Of the 76 SSDAs that were upregulated with Control Week 1 samples, 31 proteins are linked with ribosomal and translational functions (**Figure 7.4**). Only three proteins were identified as upregulated in *Varroa* samples - 4-hydroxyphenylpyruvate dioxygenase isoform X1 (rfc, 2.04), leukocyte elastase inhibitor (rfc, 2.01), and alpha-tocopherol transfer protein-like (rfc, 1.76).

In Week 3, *Varroa* samples had a higher abundance of ribosomal proteins in comparison to Control samples, which was the opposite to what was observed in Week 1 sample analysis. Of 33 proteins upregulated in *Varroa* samples to results from Week 1 comparisons, 12 were ribosomal proteins. (**Figure 7.5**).

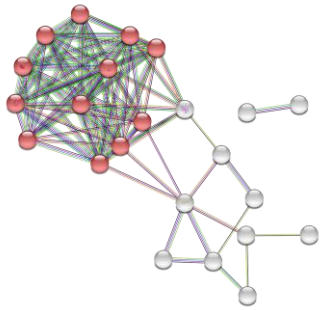
The final comparison between Control and *Varroa* samples collected in Week 5 showed in an absence of ribosomal proteins. However, a small number of proteins associated with protein processing in the endoplasmic reticulum (ER) were upregulated in *Varroa* samples (n=5) (**Figure 7.6**). Only seven proteins were identified as upregulated in Control samples, two of which were uncharacterised proteins - NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 3 (rfc, 2.29), short/branched chain specific acyl-CoA dehydrogenase (rfc, 2.63), D-beta-hydroxybutyrate dehydrogenase, mitochondrial isoform X1 (rfc, 2.75), ryanodine receptor isoform X1 (rfc, 3.95), and serine-arginine protein 55 isoform X11 (rfc, 6.03).



STRING enrichment analysis on Week 1 Control SSDAs generated via comparison with Week 1 *Varroa* samples. Minimum required interaction score set to 0.7.

KEGG pathway	Count in Network	Strength	False Discovery Rate	Colour
Ribosome	35	1.78	5.17e-49	Red

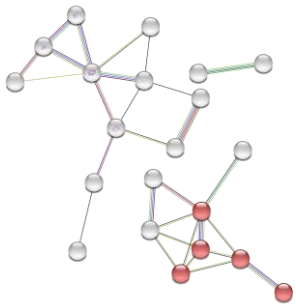
Figure 7.4: STRING enrichment analysis on SSDA proteins generated from Control Week 1 Student's t-test analysis to *Varroa* Week 1 samples. Non-connected nodes in the network were removed.



STRING enrichment analysis on Week 3 *Varroa* SSDAs generated via comparison with Week 3 Control samples. Minimum required interaction score set to 0.7.

KEGG pathway	Count in Network	Strength	False Discovery Rate	Colour
Ribosome	12	1.68	3.02e-15	Red

Figure 7.5: STRING enrichment analysis on SSDA proteins generated from *Varroa* Week 3 Student's t-test analysis to Control Week 3 samples. Non-connected nodes in the network were removed.



STRING enrichment analysis on Week 3 *Varroa* SSDAs generated via comparison with Week 3 Control samples. Minimum required interaction score set to 0.4.

KEGG pathway	Count in Network	Strength	False Discovery Rate	Colour
Protein processing in the ER	5	1.4	0.00025	Red

Figure 7.6: STRING enrichment analysis on SSDA proteins generated from *Varroa* Week 5 Student's t-test analysis to Control Week 5 samples. Non-connected nodes in the network were removed.

7.3.1.2 Control sample Student's t-test comparisons

Volcano plot comparisons were also completed between all Control samples collected from Weeks, 1, 3, and 5. Interestingly, apidermin 3 was the most abundant protein in Week 1 Control samples in comparison to both Weeks 3 and 5 Control samples (rfc 301.02 and 175.09 respectively). Two odorant binding proteins were also identified in increased abundance in Week 1 Control samples in comparison to Weeks 3 (OBP2, OBP4, rfc – 10.29 and 10.49 respectively) and 5 Control samples (OBP2, rfc – 7.86). Neither apidermin 3 nor odorant binding proteins were identified as SSDAs in the comparison between Weeks 3 and 5 Control samples. No pathway enrichment was identified in Weeks 1 and 3 comparison. However, two major royal jelly proteins (MRJP1 and MRJP5, rfc – 6.97 and 5.05 respectively) were upregulated in Week 3 Control samples in comparison to Week 1. Two cytochrome P450 proteins were upregulated in Week 1 Control samples - CYP6AS5 and CYP6a13 (rfc, 2.61 and 2.61 respectively) in comparison to Week 3. No MRJ or cytochrome P450 proteins were identified as SSDAs in the comparison between Week 1 and 5 Control samples. The differences between the Control samples, specifically the increased abundance of

cytochrome P450, apidermin 3 and odorant binding proteins in Week 1 Control samples could be an indicator of a response to anti-*Varroa* treatment that was applied to the colonies.

7.3.1.3 *Varroa* sample Student's t-test comparisons

The final set of comparisons on this dataset was between *Varroa* samples collected from Weeks 1, 3, and 5. This was to map the proteomic changes that might occur as colonies are exposed to an increasing presence of *Varroa* mites over time. Volcano plot analysis revealed large differences in protein expression between Week 1 to Week 3 and 5 samples. Apidermin 3 was identified as the most abundant protein in Week 1 in comparison to Week 3 *Varroa* samples (rfc, 145.16), however it was not identified as an SSDA in the comparison to Week 5 samples. Apidermin 3 was also not identified as an SSDA from the comparison of Week 3 and 5 *Varroa* samples.

A number of major royal jelly proteins were increased in abundance in Weeks 3 and 5 in comparison to Week 1 (**Table 7.1** & **Table 7.2)**. This was surprising as Week 3 samples had a higher number of *Varroa* mites present in the hives in comparison to Week 1, which would have been expected to affect the levels of major royal jelly proteins through the parasitisation effect of the *Varroa*.

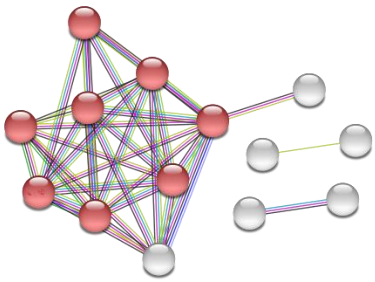
Samples from Weeks 3 and 5 also had a higher number of proteins associated with various biological pathways such as, translational, ribosomal, and energy metabolism in comparison to Week 1 *Varroa* samples (**Figure 7.7, Figure 7.8 & Figure 7.9**).

Table 7.1: Major royal jelly proteins identified as SSDAs in Week 3 *Varroa* samples in comparison to Week 1 *Varroa* samples.

Protein ID	Protein Name	Relative Fold Change
A0A088A3F4	major royal jelly protein 3	487.86
D3Y5T0	major royal jelly protein 3 precursor	243.77
A0A088AU27	major royal jelly protein 7	127.95
A0A088AU26	major royal jelly protein 2	108.65
A0A088AU20	major royal jelly protein 4	75.35
O18330	major royal jelly protein 1 precursor	30.69
O97432	major royal jelly protein 5 precursor	6.35

Table 7.2: Major royal jelly proteins identified as SSDAs increased in abundance in Week 5 *Varroa* samples in comparison to Week 1 *Varroa* samples.

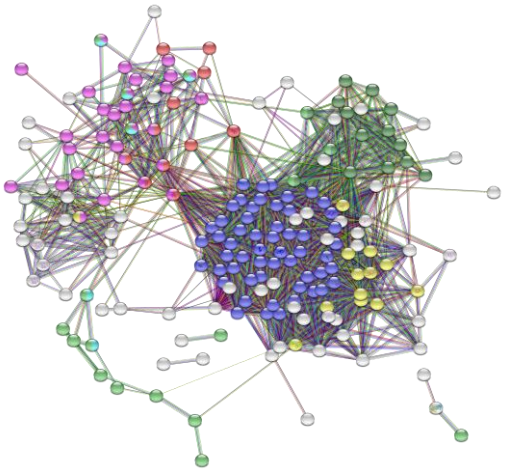
Protein ID	Protein Name	Relative Fold Change
A0A088A3F4	major royal jelly protein 3	361.14
D3Y5T0	major royal jelly protein 3 precursor	185.57
A0A088AU27	major royal jelly protein 7	95.05
A0A088AU26	major royal jelly protein 2	86.54
A0A088AU20	major royal jelly protein 4	56.30
O18330	major royal jelly protein 1 precursor	32.36



STRING Enrichment analysis on Week 1 *Varroa* SSDAs generated via comparison with Week 3 *Varroa* samples.

KEGG pathway	Count in Network	Strength	False Discovery Rate	Colour
Oxidative Phosphorylation	8 of 86	1.32	1.05e-06	Red

Figure 7.7: STRING enrichment analysis on SSDA proteins generated from *Varroa* Week 1 comparison to Week 3 *Varroa* sample. Minimum interaction score was high confidence (0.700). Non connected nodes in the network were removed.

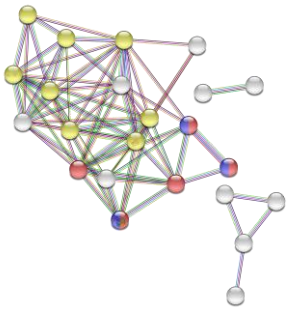


STRING Enrichment analysis on Week 3 *Varroa* SSDAs generated via comparison with Week 1 *Varroa* samples.

KEGG pathway	Count in Network	False Discovery Rate	Colour
Protein Export	11 of 19	5.7e-12	Red
Ribosome	55 of 117	3.99e-58	Dark Blue
Aminoacyl-tRNA biosynthesis	15 of 35	8.49e-15	Dark Green
Protein processing in the ER	30 of 106	1.32e-25	Pink
N-Glycan Biosynthesis	6 of 33	3.9e-04	Light Blue
RNA transport	13 of 117	1.17e-06	Yellow
Metabolic Pathways	25 of 820	7.6e-03	Light Green

Figure 7.8: STRING enrichment analysis on SSDA proteins generated from *Varroa* Week 3 comparison to Week 1 *Varroa* samples. Minimum interaction score was high confidence (0.700). Non connected nodes in the network were removed.

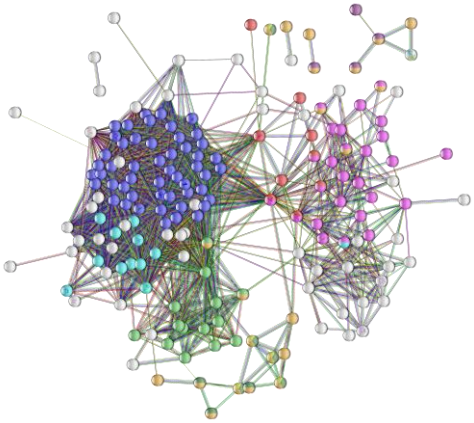
A



STRING Enrichment analysis on Week 1 *Varroa* SSDAs generated via comparison with Week 5 *Varroa* samples.

KEGG pathway	Count in Network	False Discovery Rate	Colour
Citrate cycle (TCA cycle)	5 of 32	5.92e-06	Red
Pyruvate metabolism	3 of 30	3.9e-03	Dark Blue
Oxidative Phosphorylation	8 of 86	9.97e-08	Yellow

B



STRING Enrichment analysis on Week 5 *Varroa* SSDAs generated via comparison with Week 1 *Varroa* samples.

KEGG pathway	Count in Network	False Discovery Rate	Colour
Protein Export	9 of 19	2.47e-09	Red
Ribosome	51 of 117	1.54e-53	Dark Blue
Aminoacyl-tRNA biosynthesis	14 of 35	9.44e-14	Light Green
Fructose and mannose metabolism	5 of 17	1.8e-05	Yellow
Protein processing in the ER	26 of 106	2.832-21	Pink
Amino sugar and nucleotide sugar metabolism	8 of 38	4.58e-06	Dark Green
Fatty acid degradation	4 of 23	6.1e-03	Purple
RNA transport	12 of 117	4.58e-06	Light Blue
Metabolic Pathways	28 of 820	2.0e-04	Orange

Figure 7.9: STRING enrichment analysis on SSDA proteins generated from *Varroa* Week 1 (A) and 5 (B) samples. Minimum interaction score was high confidence (0.700). Non connected nodes in the network were removed.

7.3.1.4 Hierarchical clustering analysis

Hierarchical clustering analysis was completed on all SSDAs generated from all volcano plot comparisons. Euclidean distance analysis was performed on 222 Z-scored proteins (**Figure 7.10**). Samples were resolved into three groups: Week 1 samples (Control and *Varroa* samples), Weeks 3 and 5 *Varroa* samples and finally Weeks 3 and 5 Control samples (**Supp. File 7.2**). Proteins were clustered based on similar expression profiles which resulted in four clusters: Cluster 1 contained 7 proteins upregulated in Control Week 1 and all *Varroa* samples, no pathway enrichment was detected with the proteins within cluster 1. Cluster 2 contained 21 proteins downregulated in *Varroa* Week 5 samples, no pathway enrichment was detected with the proteins within cluster 2. Cluster 3 consisted of 104 proteins upregulated in Control and *Varroa* Week 1 samples involved in oxidative phosphorylation, cellular respiration and proton transporting ATP pathway (**Figure 7.11**). Finally, Cluster 4 revealed 248 proteins upregulated in Control Weeks 3 and 5 samples, associated with ribosomal processes (**Figure 7.12**).

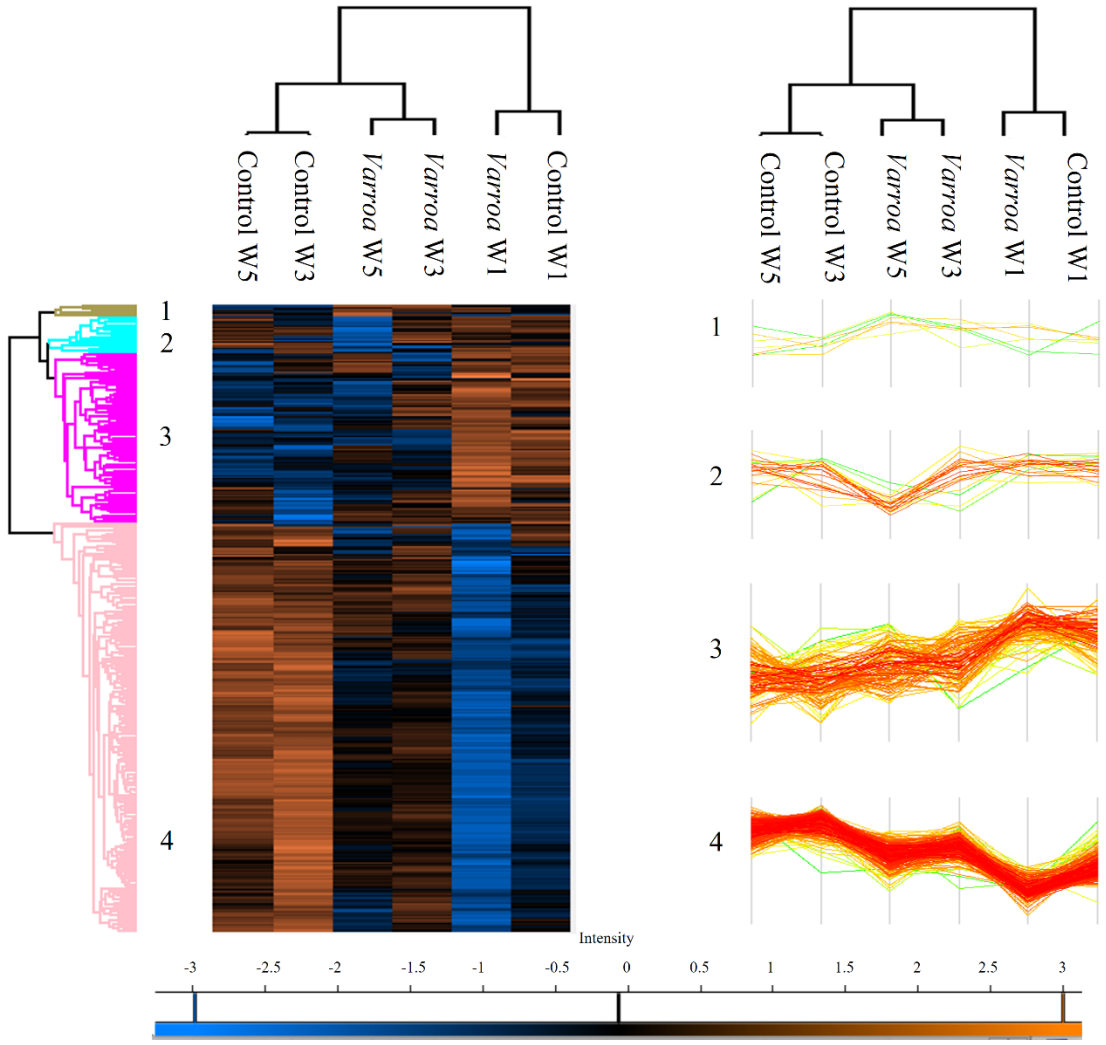


Figure 7.10: Hierarchical clustering of median intensity values for 222 SSDA proteins among the different samples from Control and *Varroa* groups collected from Weeks 1, 3 and 5. Cluster expression trends of identified proteins are represented by the coloured lines. Relative abundances (z-scored normalised) of individual proteins can be observed on the intensity spectrum.

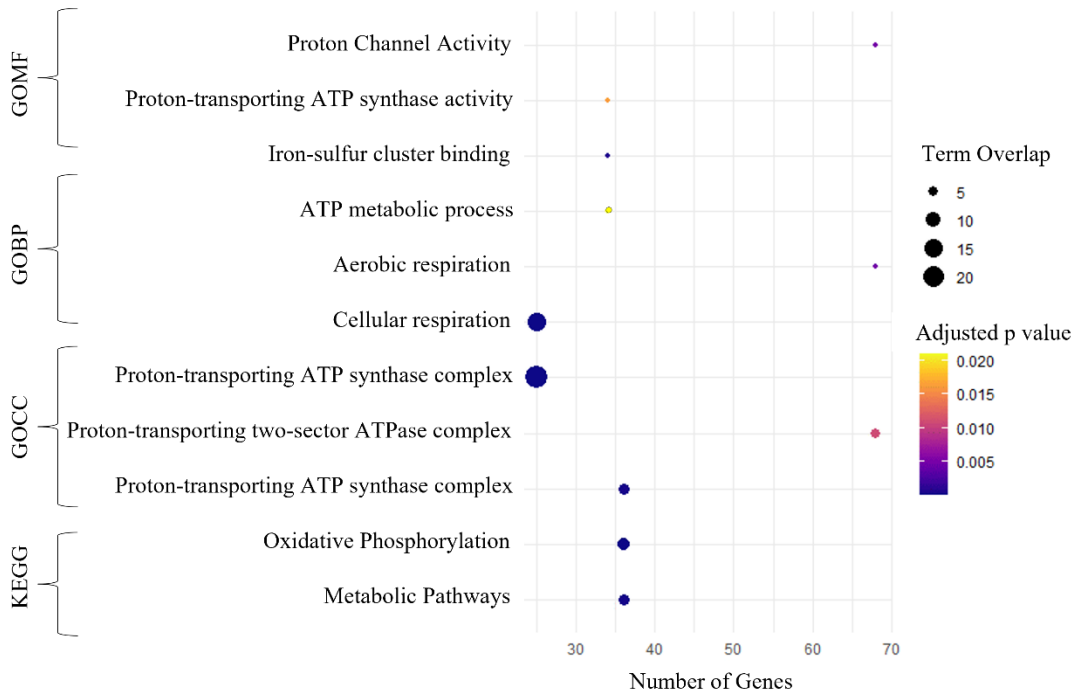


Figure 7.11: g:Profiler pathway analysis on proteins from Cluster 3 (Figure 7.10). Term overlap refers to the number of proteins identified within each gene ontology term (GOBP, GOCC, GOMF, KEGG). User threshold of significance was set as 0.05.

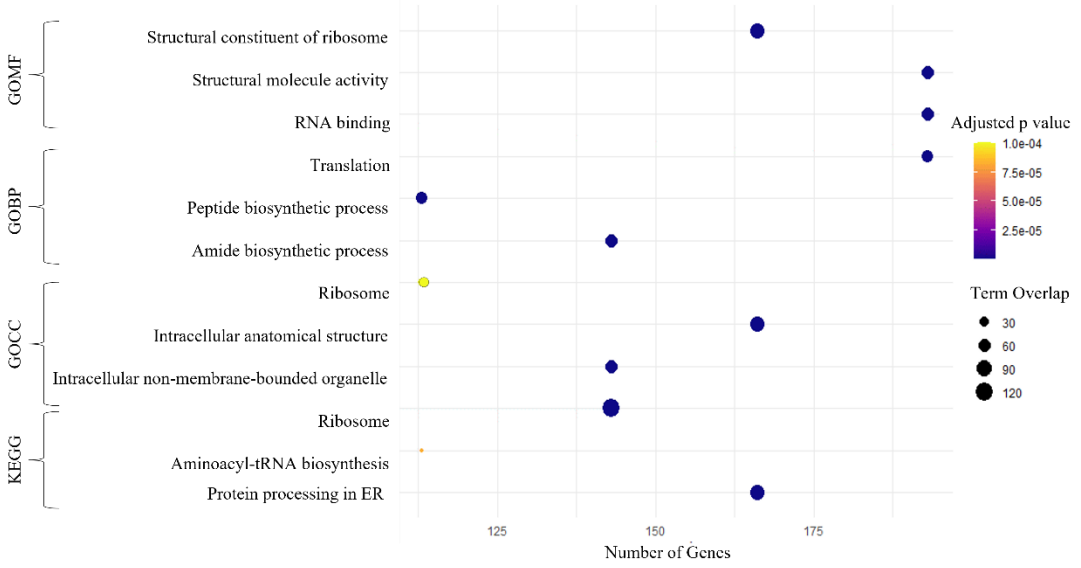


Figure 7.12: g:Profiler pathway analysis on proteins from Cluster 4 (Figure 7.10). Term overlap refers to the number of proteins identified within each gene ontology term (GOBP, GOCC, GOMF, KEGG). User threshold of significance was set as 0.05.

7.3.2 Proteomic analysis of Control samples compared with *Nosema* infected colonies

A total of 3,407 proteins were initially identified in Perseus analysis of the dataset investigating the proteomes of Control and *Nosema* samples from weeks 1, 3 and 5. This dataset was reduced significantly, through filtering steps to remove contaminant peptides, reverse hit peptides and peptides only identified by site. LFQ intensity values were Log_2 transformed and only proteins identified in at least three of the three replicates were included for further analysis. This resulted in a data matrix of 455 proteins. The matrix was further filtered through ANOVA significance testing (p value < 0.05) as only ANOVA significant proteins were used for analysis.

Principal component analysis was completed on ANOVA significant proteins and identified the clustering arrangement of the samples (**Figure 7.13**). Control Week 5 samples were clustered separately to the other samples. There was an overlap of *Nosema* samples collected in Week 1 and Week 5, yet Week 3 *Nosema* samples were clustered away from Weeks 1 and 5. Control Week 1 and 3 samples were clustered in close proximity with all *Nosema* samples. The cluster arrangement of samples indicates a similar proteome profile between the samples.

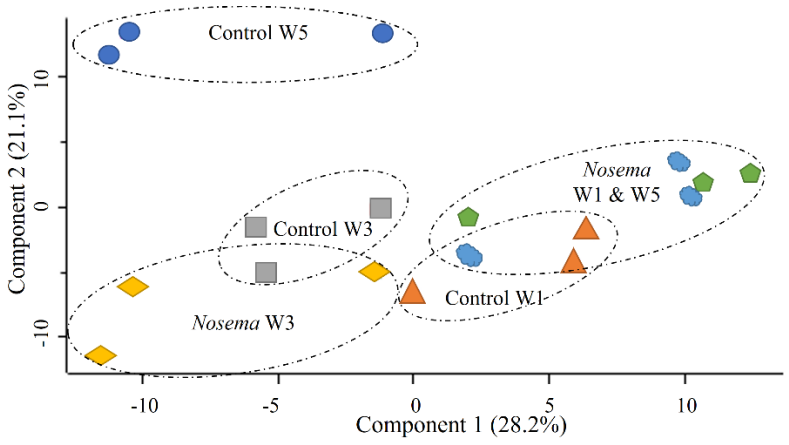


Figure 7.13: PCA analysis on *Nosema* and Control samples from Weeks 1, 3, and 5. Orange triangle – Control Week 1, Grey square – Control Week 3, Blue circles – Control Week 5, Green pentagon – *Nosema* Week 1, Yellow diamond – *Nosema* Week 3, Light blue hexagons – *Nosema* Week 5. The total variance of this PCA with the data matrix was 49.3%.

Student's t-tests were implemented to identify changes in the worker bee proteomes between Control and *Nosema* samples respective to each other as the disease trial progressed through the weeks. Proteins with a relative fold change > 1.5 and a -log p value > 1.3 were determined to be statistically significant differentially abundant (SSDA) proteins and were used for further analysis (**Supp. File 7.3**).

7.3.2.1 Control and *Nosema* infected Student's t-test comparisons

Volcano plot comparisons revealed very little difference between the Control and *Nosema* samples from Weeks 1 and 3 respectively. One protein was upregulated in *Nosema* sample Week 1 (mucin-19, rfc – 2.71) and only 10 proteins were upregulated in Control samples, with no pathway enrichment. Week 3 comparison of Control and *Nosema* infected samples resulted in 16 upregulated proteins in *Nosema* group and 10 in the Control group, no pathway enrichment was identified with either sample.

Week 5 *Nosema* samples had two cytochrome c oxidase subunits upregulated comparison to Control Week 5 samples (COX5b, rfc – 2.44 and COS6b, rfc - 2.94). One heat shock factor binding protein 1-like (rfc, 4.11), two cuticular proteins: cuticular protein 28 (rfc, 4.02), cuticular protein 1 (rfc, 3.90), one major royal jelly protein 1 (rfc, 3.80), and apidermin 3 precursor (rfc, 3.29), were all upregulated in Week 5 *Nosema* samples in comparison to Control Week 5 samples. Control Week 5 samples had 10 upregulated SSDAs. No pathway enrichment was detected for either set of SSDAs.

7.3.2.2 Control sample Student's t-test comparisons

Control sample comparisons between the three sample time points (Weeks 1, 3 and 5) were conducted. Week 1 and 3 Control sample comparison revealed the presence of ribosomal proteins in Week 3 samples (6 out of 20 proteins) in comparison to Week 1 samples, whereas only two ribosomal proteins were identified as SSDAs associated with Week 1 samples. Odorant binding protein 2 was the most abundant protein in Week 1 samples (rfc, 14.94) in comparison to Week 3 samples.

Week 1 and Week 5 Control sample comparisons identified 59 SSDAs upregulated in Week 1 samples of which were, two cytochrome c oxidase subunits

COX5b and COX6b (rfc, 2.21 and 3.90 respectively), three cuticular proteins - cuticle protein 28, cuticular protein 1, apidermin 3 precursor, cuticular protein analogous to peritrophins 3 (rfc, 3.28, 4.19, 4.66, and 5.08 respectively), heat shock factor binding protein 1-like (rfc, 4.41) and odorant binding protein 2 (13.03). Seven proteins were identified as SSDAs associated with Week 5 samples.

The final Control sample comparison was completed using Week 3 and 5 samples. Of the 42 upregulated SSDAs in Week 3 samples, Cytochrome c oxidase proteins - COX5b and COX6b (rfc, 1.91, 2.36 respectively) and heat shock factor binding protein 1-like (rfc, 3.08). Ten SSDAs were identified as upregulated in Week 5 samples.

7.3.2.3 *Nosema* Student's t-test comparisons

Nosema sample comparisons between the three sample points highlighted an increase in ribosomal and eukaryotic translation initiation factor proteins in Week 3 that was not observed in Weeks 1 or 5 (**Figure 7.14 & Figure 7.15**). The most abundant protein in Week 3 from both comparisons to Weeks 1 and 5 samples was hexamerin 70a (rfc, 75.24 and 103.74 respectively).

Student's t-test between Weeks 3 and 1 generated 33 upregulated and 19 downregulated proteins respectively. MAGUK p55 subfamily members 6IX2 and 7IX3 were increased in abundance in Week 1 samples compared to Week 3 samples (rfc, 1.52 and 1.53 respectively). *Nosema* sample comparison between Weeks 5 and 1 did not result in a large number of SSDAs (11 and 4 up and downregulated proteins respectively) indicating little variation in the proteome between the samples.

Comparison between Weeks 5 and 3 resulted in 26 upregulated and 54 downregulated proteins respectively. Ribosomal and eukaryotic translational initiation proteins were upregulated in Week 3 in comparison to Week 5. No pathway enrichment was detected in Week 5 SSDAs. MAGUK p55 subfamily member 6IX2 was also increased in abundance in Week 5 in comparison to Week 3 (rfc, 1.67).

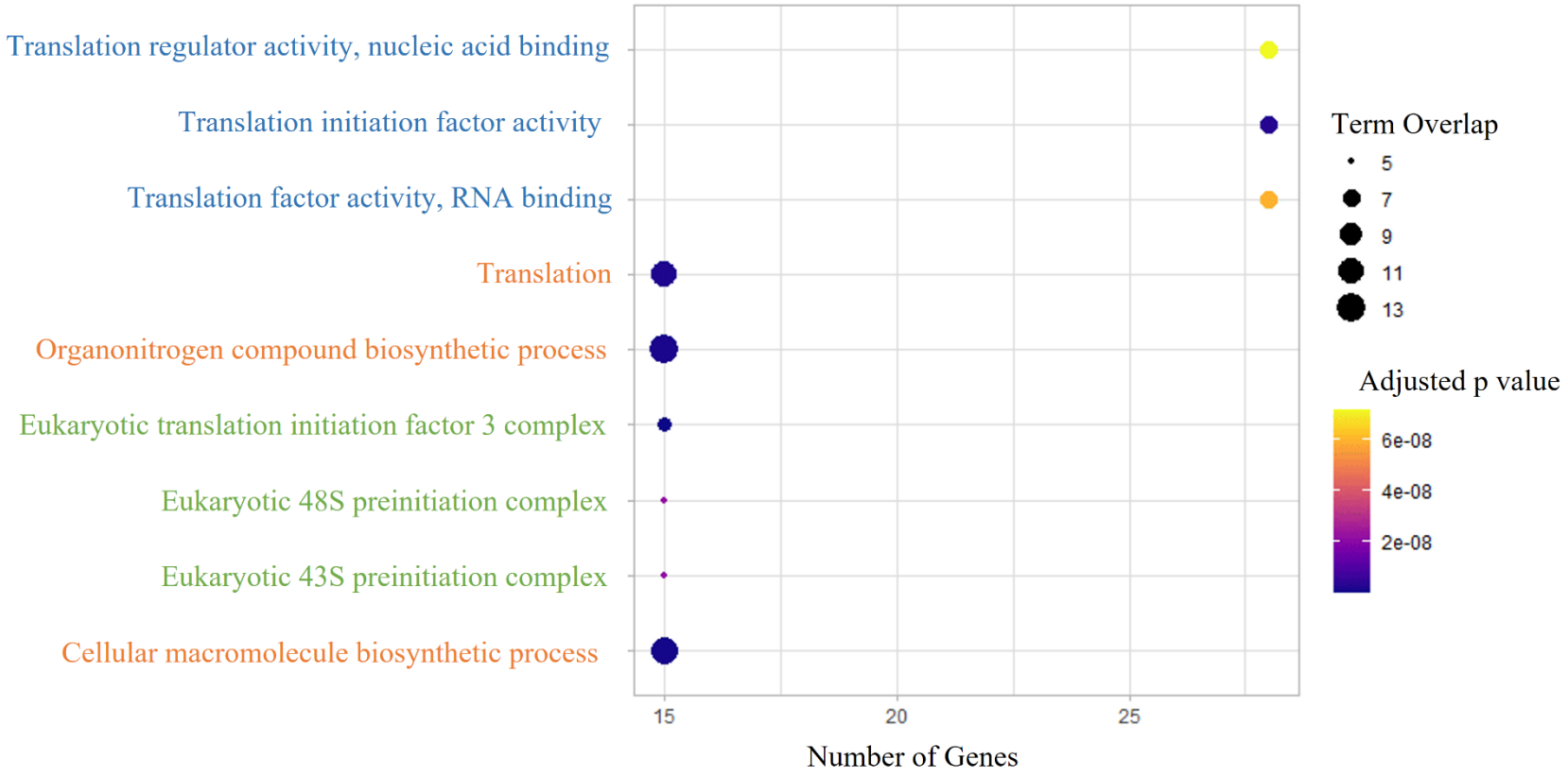


Figure 7.14: g:Profiler analysis on 33 SSDAs upregulated in Week 3 *Nosema* samples in comparison to Week 1 samples. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, and Green – GOCC.

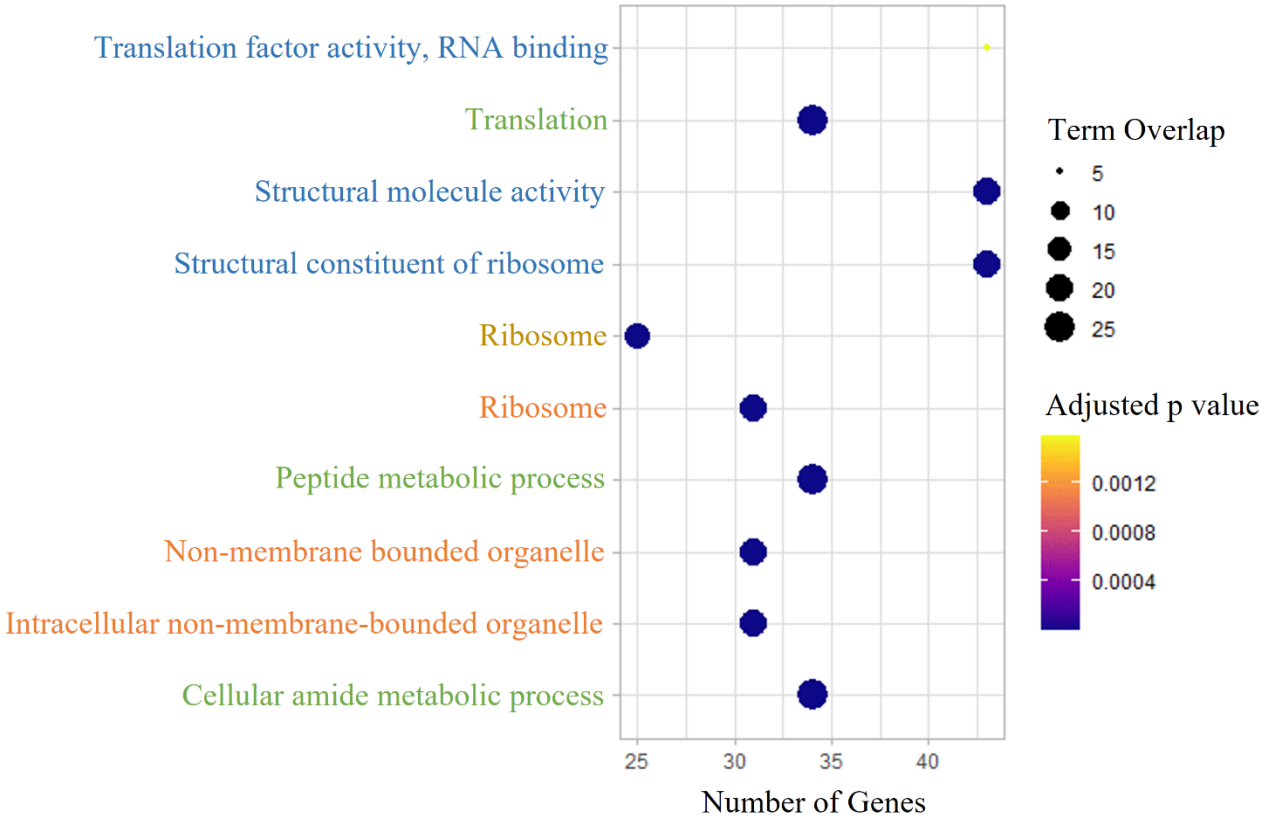


Figure 7.15: g:Profiler analysis on 54 SSDAs upregulated in Week 3 *Nosema* samples in comparison to Week 5 samples. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, Green – GOCC and Yellow - KEGG.

7.3.2.4 Hierarchical clustering analysis

Hierarchical clustering analysis was completed on all SSDA proteins was generated from all volcano plot comparisons. Euclidean distance analysis was performed on 230 z-scored normalised proteins (**Figure 7.16**). Samples were resolved into three groups: Week 5 Control samples, Weeks 3 Control and *Nosema* samples, and Week 1 Control and *Nosema*, with Week 5 *Nosema*. Proteins were clustered based on similar expression profiles which resulted in four clusters: Cluster 1 contained 92 proteins upregulated in Control Week 5 and 3 as well as Week 3 *Nosema* samples (**Supp. File 7.4**). No pathway enrichment was detected with the proteins within cluster 1. Pathway analysis identified an enrichment of proteins associated with the ribosome and structural molecular activity (**Figure 7.17**). Cluster 2 contained 96 proteins upregulated in *Nosema* Week 5 and 1 samples and Control Week 1 samples, no pathway enrichment was detected with the proteins within cluster 2. Cluster 3 consisted of 33 proteins downregulated in Control and *Nosema* Week 3 samples, no pathway enrichment was identified in cluster 3. Finally, Cluster 4 revealed 9 proteins downregulated in Control and *Nosema* Week 1 samples, no enrichment was detected.

The shift in proteome expression with *Nosema* Week 3 samples could be attributed to the increased presence of the microsporidian within the hives at the time the samples were collected. However, the increased presence of *Nosema* does not provide reason to why *Nosema* Week 3 samples were clustered with Control Week 3 samples.

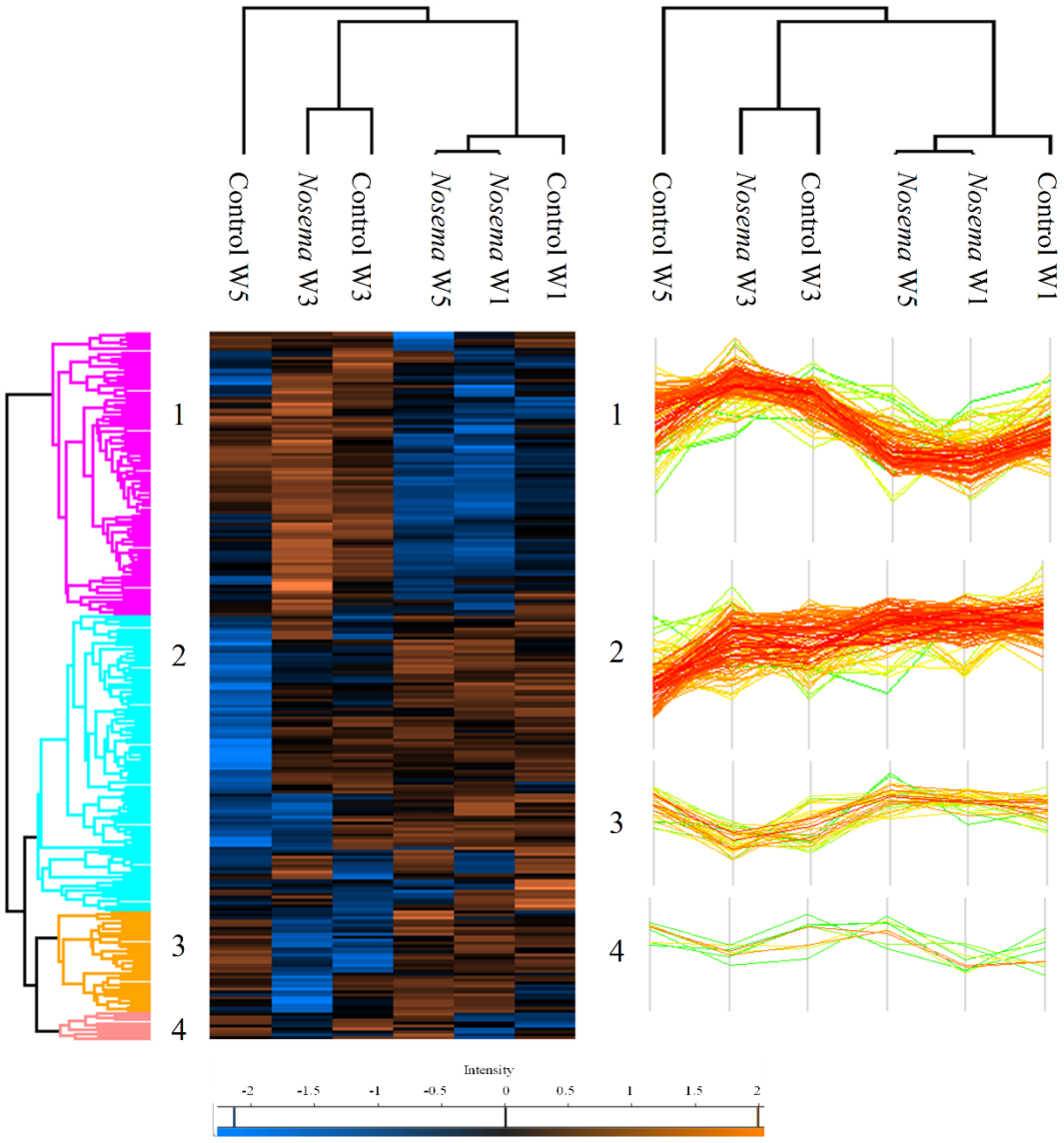


Figure 7.16: Hierarchical clustering of median intensity values for 230 SSDA proteins among the different samples from Control and *Nosema* groups collected from Weeks 1, 3 and 5. Cluster expression trends of identified proteins are represented by the coloured lines. Relative abundances (z-scored normalised) of individual proteins can be observed on the intensity spectrum.

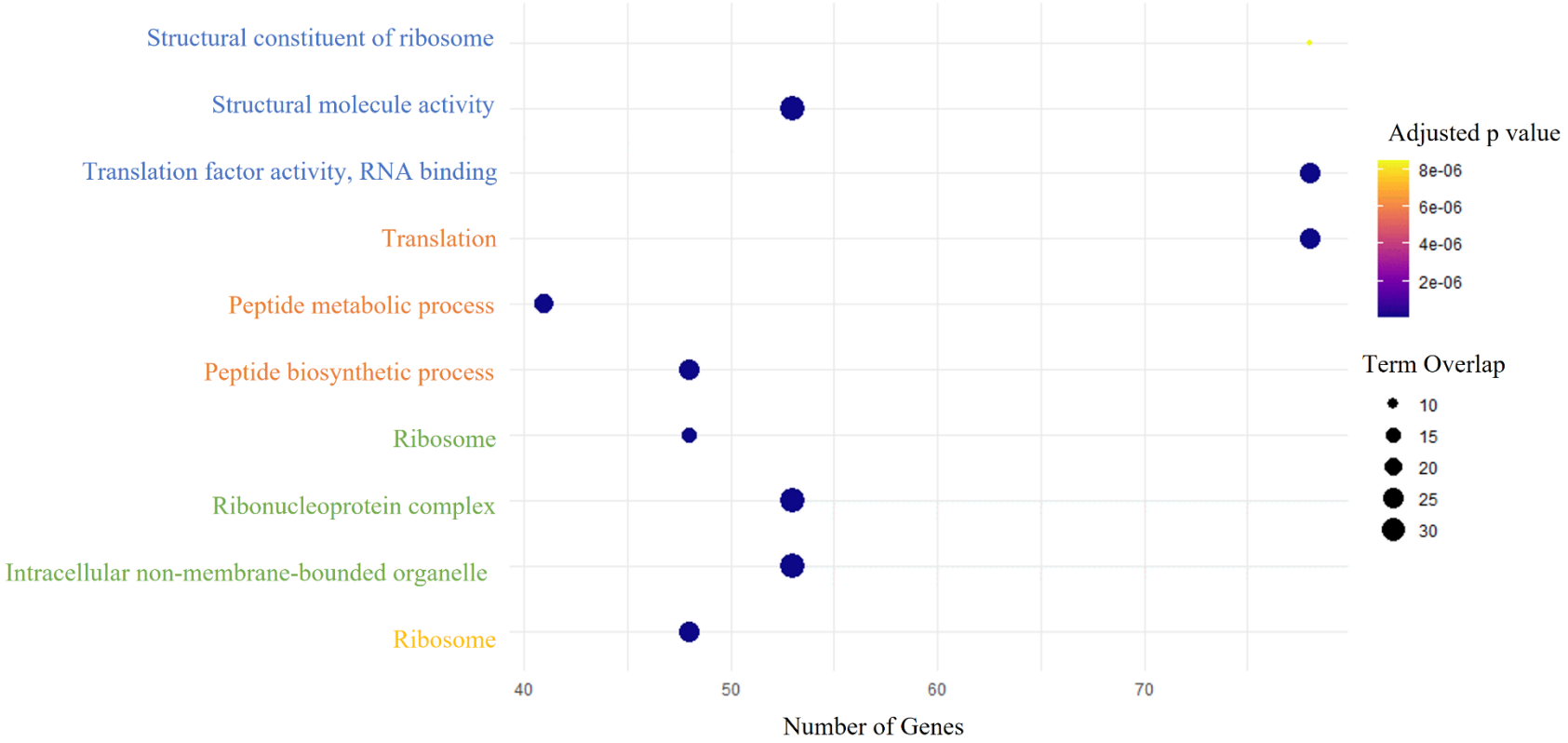


Figure 7.17: Gene ontology analysis on proteins from hierarchical analysis Cluster 1 (Figure 7.16). Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, Green – GOCC and Yellow - KEGG.

7.3.3 Proteomic analysis of Control samples compared with Double infected colonies

A total of 3,361 proteins were initially identified in Perseus analysis of the Control and Double infected samples collected from weeks 1, 3 and 5. This dataset was significantly reduced through filtering steps to remove contaminant peptides, reverse hit peptides and peptides only identified by site. LFQ intensity values were Log_2 transformed and only proteins identified in at least three of the three replicates were included for further analysis. The matrix was further filtered through ANOVA significance testing ($p < 0.1$) as only ANOVA significant proteins were used for analysis. This resulted in a data matrix of 500 proteins.

Principal component analysis was completed on 500 ANOVA significant proteins remaining in the data matrix. Analysis identified clustering arrangement of the samples based on the week they were collected (**Figure 7.18**). Three clusters were observed in the PCA graph, and unexpectedly Double and Control samples were clustered together but were separated based on the weeks that they were sampled.

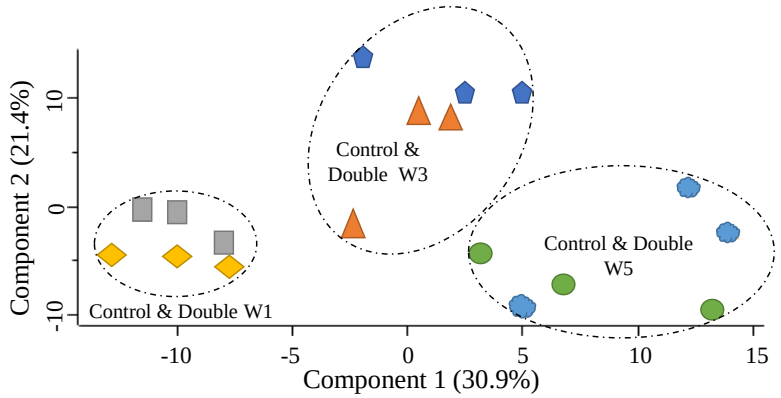


Figure 7.18: PCA graph on the proteomic analysis of double infected samples. Samples were clustered based on the week they were sampled. Yellow diamonds – Control W1, Grey squares – Double W1, Orange triangle – Control W3, Blue pentagon – Double W3, Green circle – Double W5, Light blue cloud – Double W5. Total variance of this PCA from the data matrix was 52.3%.

Student's t-tests were performed to identify changes in the worker bee proteomes of Control and Double infected samples relative to each other as the disease trial progressed through the weeks. Proteins with a relative fold change > 1.5 and a -

log p value > 1.3 were determined to be statistically significant differentially abundant (SSDA) proteins and were used for further analysis. Overall, a very small number of SSDAs generated between Control and Double comparisons from Weeks 1, 3 and 5 (**Supp. File 7.5**).

7.3.3.1 Control to Double Infected Student's t-test comparisons

Comparisons were completed between all Control samples. Week 1 samples had 24 upregulated proteins of which, cytochrome c oxidase - COX6b (rfc, 1.76), cuticular proteins - cuticular protein 4 (rfc, 7.61) and odorant binding protein 2 precursor (rfc, 9.70) increased in abundance in comparison to Week 3 samples that had 10 SSDAs. Week 1 and Week 5 comparison identified 59 and 3 SSDAs respectively. Apidermin 3 was the most abundant protein in Week 1 samples (rfc, 333.46) this was followed by several other cuticular proteins – cuticular protein 4, 1, and (rfc, 8.60, 6.45 and 5.36), odorant binding protein 2 (rfc, 11.53) and cytochrome c oxidase 5a (rfc, 1.70). Week 5 SSDAs were ubiquitin-conjugating enzyme E2-17 kDa (rfc, 16.61), alpha-glucosidase precursor (rfc, 4.80), and dehydrogenase/reductase SDR family member 4 isoform X1 (rfc, 1.51).

7.3.3.2 Double Infected Student's t-test comparisons

Comparisons between Double infected samples were competed from Weeks 1, 3, and 5. Analysis identified an increased abundance of cuticular proteins in Week 1 samples in comparison to Weeks 3 and 5 (**Table 7.3**). Three cuticular protein were identified as increased in abundance in Week 3 in comparison to Week 5 – cuticular protein 28 (rfc, 2.06), Cuticular protein analogous to peritrophins 3-C (rfc, 3.13) and cuticular protein 22 (rfc, 4.92). No cuticular proteins were identified as SSDAs associated with Double infected Week 5 samples.

Chemosensory proteins and odorant binding proteins were decreased in abundance in Double infected Week 5 samples in comparison to Week 1 and 3 (**Table 7.4**). Several cytochrome P450 proteins were identified as SSDAs associated with Week 5 samples in comparison to Week 1 - CYP6AS8 and CYP9Q2 (rfc, 5.51 and 1.53) and Week 3 – CYP6AS7 and CYP6AS8 (rfc, 3.60 and 2.86 respectively).

Table 7.3: Cuticular proteins increased in abundance (rfc) in Week 1 samples in comparison to Weeks 3 and 5

Protein name	Week 3	Week 5
Cuticular protein 1	5.18	6.24
Apidermin 3	105.14	2.52
Cuticular protein 14	-	3.11
Cuticular protein 2	-	3.12
Cuticular protein analogous to peritrophins 3-C	-	3.42
Cuticular protein 28	-	3.76
Apidermin 2	-	4.21
Protein obstructor-E	-	5.85
Cuticular protein 22	-	6.88
Cuticular protein 4	-	8.18

Table 7.4: Chemosensory odorant binding proteins increased in abundance (rfc) in Weeks 1 and 3 in comparison to Week 5.

Protein name	Week 1	Week 3
Odorant binding protein 2	9.87	-
Odorant binding protein 4	7.21	-
Chemosensory protein 1	4.00	3.43
Chemosensory protein 3	3.34	3.38

7.3.3.3 Hierarchical Clustering Analysis

Hierarchical clustering analysis was completed on 240 SSDAs from all volcano plot comparisons. Euclidean distance analysis was performed on z-scored normalised proteins and were grouped based on similarities of the protein profiles. Interestingly, Control and Double sample were clustered by the weeks they were collected (**Figure 7.19**) (**Supp. File 7.6**). Proteins were clustered into 3 groups: Cluster 1 contained 71 proteins and Perseus analysis identified an enrichment of proteins associated with the integral component of membrane in cluster 1 (p value = 1.153E-08). Cluster 2 contained 22 proteins but did not identify enrichment in pathway analysis. Cluster 3 consisted of 71 proteins, g:Profiler analysis identified a presence abundance of oxidative phosphorylation proteins and calcium ion binding proteins in pathway enrichment analysis (**Figure 7.20**).

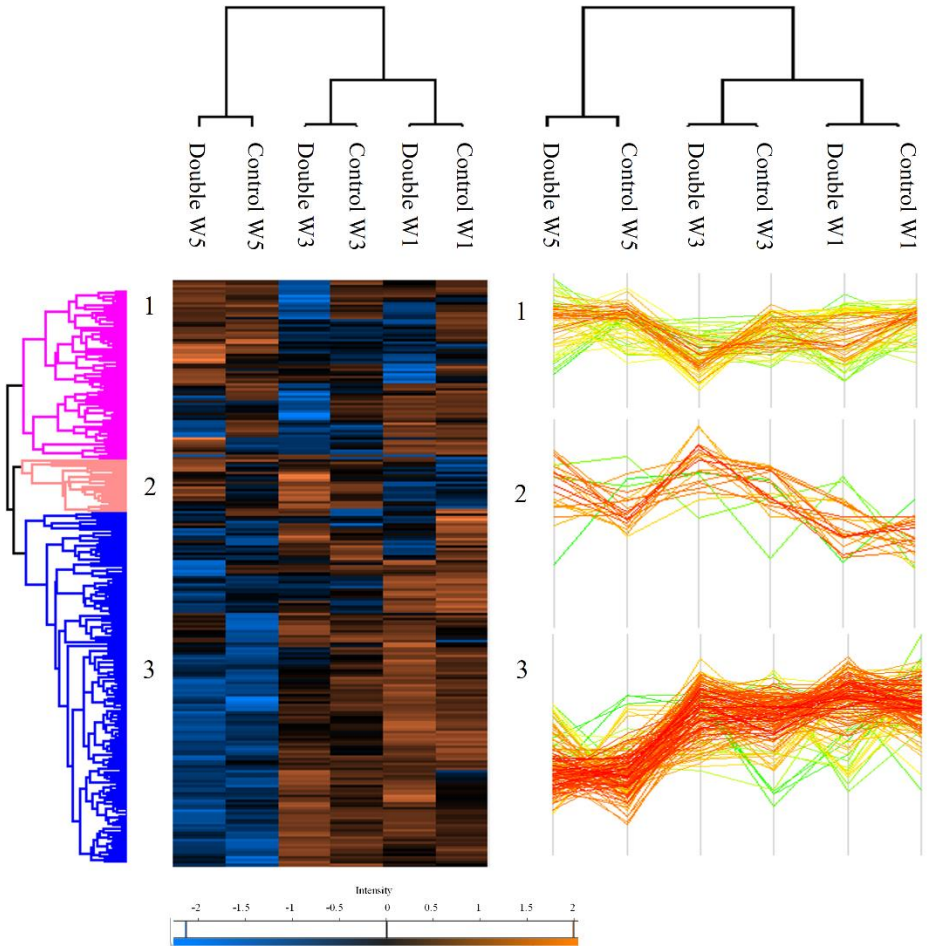


Figure 7.19: Heatmap clustering on the median intensity value of 240 SSDAs from Double and Control samples over three time points. Five clusters have been identified. Cluster expression trends of identified proteins are represented by the coloured lines. Relative abundances (z-scored normalised) of individual proteins can be observed on the intensity spectrum.

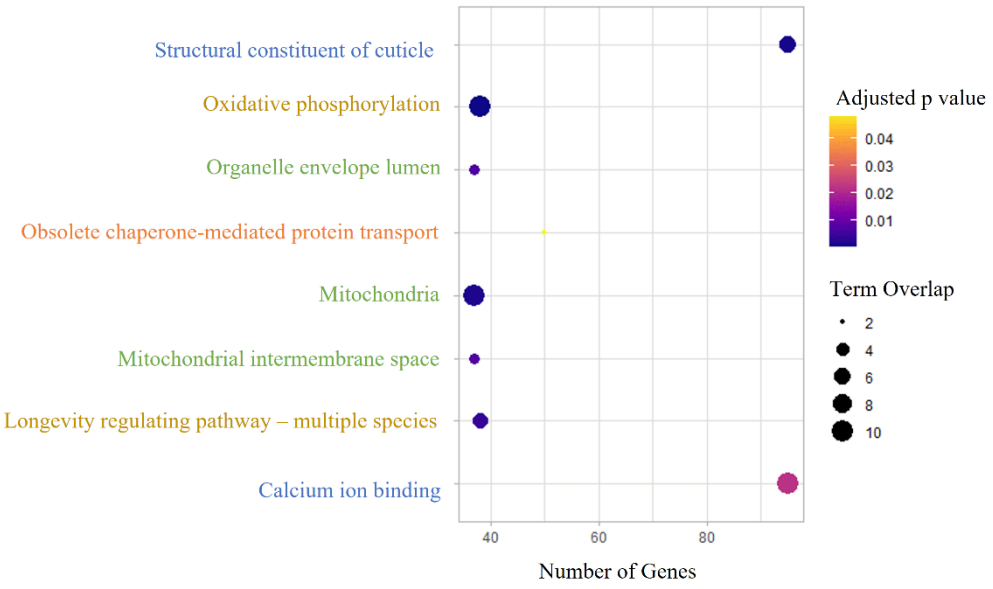


Figure 7.20: g:Profiler analysis on 71 SSDAs upregulated in Week 3 and 1 Double and Control samples. Term overlap refers to the number of proteins identified within each gene ontology term. User threshold of significance was set as 0.05. Colour code for Term analysis: Blue – GOMF, Orange – GOBP, Green – GOCC and KEGG – Yellow.

7.4 Discussion

Colony Collapse Disorder (CCD) is a major threat to beekeepers around the world and no single cause of CCD has been identified, instead numerous factors are thought to play contributing roles into the decline of honeybee colonies (Stankus, 2008; Rhodes, 2018a). The *Varroa destructor* is a consistent and widespread problem for western honeybee populations, since its host shift from the eastern honeybee populations (Rosenkranz *et al.*, 2010). The impact and interactions of the *Varroa* mite in honeybee colonies has been widely studied and characterised (Anderson and Trueman, 2000; Martin *et al.*, 2001; Rosenkranz *et al.*, 2010; Le Conte *et al.*, 2011; Traynor *et al.*, 2020). However, it was only recently that the feeding behaviour of *Varroa* mites was has been fully characterised, as it was thought the mites fed on the haemolymph of the worker bees, recent work however has demonstrated that *Varroa* mites feed on the fat body of worker bees (Ramsey *et al.*, 2019). Despite the volumes of work completed on the *Varroa* and its impact on honeybee colonies, there are aspects of the parasite-host relationship that are still being uncovered.

7.4.1 Discussion on analysis completed on Control Samples

The proteomes of the Control samples used in this study resembled the proteomes of the Apivar treatment groups used in the previous Chapter (**Section 5.3.2**) as Control samples consistently had increased abundance of ribosomal proteins in comparison to disease hives (*Varroa* and *Nosema*). This is further emphasised as proteomic comparisons between Control samples (Weeks 1, 3 and 5) showed an increase in cytochrome c oxidase subunits (COX5b and COX6b) and a number of cuticular proteins in Week 1 samples in comparison to Weeks 3 and 5 samples. Increase in cuticular and oxidative phosphorylation protein expression can be a mechanism of tolerance and/or resistance to exposure to insecticides in insects (Balabanidou *et al.*, 2018). Genes associated with the cuticle composition have been observed to be up-regulated in *Anopheles gambiae* insecticide resistant strains (Balabanidou *et al.*, 2016; Yahouédo *et al.*, 2017). Increase in cytochrome c oxidase proteins (COX5b and COX6b) could also be a proteomic response of the worker bees response to the presence of an insecticide, as cytochrome c oxidase subunit I was identified as more abundantly expressed in pyrethroid resistant *Blattella germanica* species (Pridgeon and Liu, 2003).

One protein that was consistently expressed as the most abundant protein in Week 1 Control samples in comparison to Weeks 3 and 5 Control samples was Apidermin 3. Apidermin proteins (1, 2, and 3) are predominantly found in proteins of hard cuticles, *apd-1* has the broadest expression mainly found in the epidermis underlying the cuticle designated for sclerotization (Kucharski *et al.*, 2007; Willis, 2010). Apidermin 2 and 3 (*apd-2* and *apd-3*) are associated with the tracheae and various parts of the digestive tract. *apd-3* is also expressed in external epidermis, including the eye where *apd-1* transcripts are not found (Willis, 2010). Apidermin 3 has been observed to be downregulated in deformed wing virus (DWV) bees only, but upregulated in *Varroa* and *Varroa* + DWV bees, however, it is still unclear why this occurs and what role *apd-3* plays during these infections (Erban *et al.*, 2019; Weaver *et al.*, 2021). Apidermin 3 has been observed to decrease in abundance in *Varroa* parasitised worker pupae, suggesting apidermin 3 expression may be influenced by age as in previous studies workers were adult bees (Surlis *et al.*, 2018). In the mono and dual infected hives apidermin 3 was identified as the most abundant protein in

Varroa Week 1 samples in comparison to Week 3 *Varroa* samples. Apidermin 2 was also identified as highly abundant in Week 1 dual infected samples in comparison to Weeks 3 and 5 dual infected samples.

In the *Nosema* mono infected colonies, apidermin 3 was identified as an SSDA in Week 5 *Nosema* samples in comparison to Week 5 Control samples. The abundance of apidermin 3 in Control Week 1 samples in comparison to Weeks 3 and 5 samples, Week 1 *Varroa* samples in comparison to Weeks 3 and 5 *Varroa* samples, and in Week 1 dual infected colonies in comparison to Weeks 3 and 5, indicate that apidermin 3 plays an important role in worker bees cuticular composition in response to *Varroa* mites and anti-*Varroa* treatment applications such as Apivar.

7.4.2 Discussion on proteomic analysis of mono-infected *Varroa* Samples

Work presented in this Chapter highlights variations of worker honeybee's proteome when exposed to *Varroa* parasitisation over time. Changes to the workers proteome were observed between *Varroa* infested colonies sampled at Weeks 1, 3, and 5. Week 3 samples had the highest number of *Varroa* mites identified through sticky inserts placed below the brood frames, Week 1 samples had the second highest mite fall count and Week 5 had the lowest. Despite the variation in mite fall count between Week 3 (1099) and Week 5 (196), the proteomes of these two sample points had a more similar protein profiles compared to the protein profile of Week 1 as observed in the hierarchical clustering analysis.

Several protein changes were observed between Week 1 to Weeks 3 and 5 samples such as the increase in major royal jelly proteins (MJPs) in Weeks 3 and 5 samples. The increased abundance of MRJ proteins in Weeks 3 and 5 in comparison to Week 1 could be linked to the increased exposure to *Varroa* in the colonies, an increase in MRJ proteins has previously been observed in *Varroa* infected hives (Erban *et al.*, 2019). MRJ protein 3 has a role in immune functionality and has been identified in the honeybee's brain (Okamoto *et al.*, 2003; Peixoto *et al.*, 2009; Ramanathan *et al.*, 2018; Erban *et al.*, 2019). Results from this Chapter identified MRJP3 as the most abundant protein in Weeks 3 and 5 in comparison to Week 1. The increase in MRJPs in the bee samples from the *Varroa* arm of the trial could be a mechanism to combat the parasitic effects of the *Varroa* mites over a period of weeks.

A small number of oxidative phosphorylation (OXPHOS) proteins were identified as upregulated in *Varroa* Week 1 samples in comparison to *Varroa* Weeks 3 and 5 samples. An increase in OXPHOS proteins in response to *Varroa* parasitisation has been observed in a previous work, however the increase was observed in parasitised drones and worker larvae (Lipiński and Żółtowska, 2005; Surlis *et al.*, 2018). The increased abundance of OXPHOS proteins in Week 1 samples could be an indicator of stress in the workers exposed to *Varroa* mites. More work would need to be completed on the proteomic response of worker honeybees to *Varroa* mite parasitisation to identify specific changes to the bees proteome. Future work would need to consider the application proteomic analysis on the abdomen samples.

In contrast there is an increase in metabolic and ribosomal proteins in Weeks 3 and 5 samples in comparison to Week 1. The level of metabolic increase in response to *Varroa* parasitisation in workers has been observed in previous work (Erban *et al.*, 2015; Aldea and Bozinovic, 2020). There is very little research published on the metabolic changes in honeybee workers in response to *Varroa* parasitisation using mass spectrometry techniques. An increase in ribosomal proteins has also been identified in previous studies of parasitised worker bees (Surlis *et al.*, 2015; Surlis *et al.*, 2018), and has also been associated with *Varroa* sensitive hygiene bees (Hu *et al.*, 2016). The increased abundance of metabolic and ribosomal proteins in *Varroa* samples Weeks 3 and 5 could be a mechanism of the worker bees to overcome parasitisation. However, it is not known if the workers sampled for this study were infected with *Varroa* mites as larvae. Therefore it is not possible to have definitive conclusions of the direct effect of parasitisation on adult workers in this Chapter. Instead, the results from this Chapter demonstrate a change in the proteomes of worker bees exposed to *Varroa* mites within their colonies, regardless of parasitisation.

7.4.3 Discussion on the proteomic analysis of mono-infected *Nosema* Samples

This work presented here investigated how the presence of *Nosema* infection in colonies causes changes in honeybee worker proteomes. The results presented above outline a deviation in the proteome of Week 3 *Nosema* samples in comparison to Week 1 and 5 *Nosema* samples. Week 3 samples had an increase in ribosomal and eukaryotic translation initiation factor proteins in comparison to Weeks 1 and 5 samples. An increase in cell ribosome levels has been detected in honeybee worker ventricular cells

in previous work, which is indicative of the pathology of *Nosema* (Higes *et al.*, 2007; Higes *et al.*, 2010). The increase in ribosomal and eukaryotic proteins in Week 3 correlates with a high presence of *Nosema* within the colonies. This might be an explanation to the high abundance of these proteins in Week 3 that was not observed in Weeks 1 or 5.

Hexamerin 70a was the most abundant protein identified in *Nosema* Week 3 samples in comparison to Weeks 1 and 5 samples. Hexamerin proteins have been characterised as storage proteins in winter worker bees and in honeybee larvae (Döke *et al.*, 2015; Lee and Kim, 2017). Hexamerins are well-studied proteins which are abundantly expressed in larval fat body used for amino acid storage, insect development and JH binding (Martins *et al.*, 2011; Lee and Kim, 2017). Presently there is very little research on the increased abundance of hexamerin in response to infection from *Nosema* spores.

Membrane-associated guanylate kinases (MAGUKs) p55 subfamily member 6 and 7 were increased in abundance in Weeks 1 and 5 samples in comparison to Week 3. MAGUKs are scaffold proteins and play a role in synapse formation and functionality (Olsen and Bredt, 2003; Oliva *et al.*, 2012). Mutation of the discs large (DLG) subfamily (which are part of the MAGUK family) disrupts the epithelial septate junctions and causes an overgrowth of the imaginal discs (Woods *et al.*, 1996; Oliva *et al.*, 2012). Like the increased abundance of hexamerin in response to *Nosema* infection, the decreased abundance of MAGUKs in Week 3 samples has not been widely investigated.

Studies have shown that *Nosema* infections causes changes to energy metabolism and immune response pathways (Kurze *et al.*, 2016). This was not observed in this analysis, however, that could be attributed to the experimental design. For future investigations of the impact of *Nosema* infections within colonies several factors should be considered: age of workers, tolerance to *Nosema* infections (Kurze *et al.*, 2016), time of year (Higes *et al.*, 2008) and anatomical sample used for proteomic analysis (Huang *et al.*, 2007).

Whole cell proteomic analysis on worker honeybees in response to *Nosema* infections can provide novel insights into how the host's respond to the presence of

Nosema in colonies and provide a better understanding into the overall impact *Nosema* has on honeybee colonies.

7.4.4 Discussion on the proteomic analysis conducted on Double infected Samples

The proteomic investigation of the dual infected colonies resulted in some unexpected results. As seen in the PCA, the dual disease samples were clustered close to the control samples. This indicates that worker bees in the disease colonies had a similar proteome to the worker bees in the Control hives. Volcano plot comparisons between Control and double diseased samples from Weeks 1, 3, and 5 resulted in a low number of SSDAs, further indicating little difference in the proteomes of samples.

Volcano plot comparisons between double diseased colonies revealed changes in the proteomes of worker bees sampled between Weeks 1, 3, and 5. Week 1 samples had an increase in apidermin 3 and 2 proteins in comparison to Weeks 3 and 5. Apidermin proteins have been observed to be upregulated in *Varroa* samples in previous proteomic investigations of diseased honeybee colonies (Erban *et al.*, 2019; Weaver *et al.*, 2021). However, the increase in apidermin proteins in Week 1 samples in comparison to Weeks 3 and 5, might suggest that the expression of apidermin in worker bees might be affected on the exposure and intensity of *Varroa* parasitisation within colonies. Erban *et al.* (2019) sampled the workers as they were emerging from the cells and therefore the increase in apidermin protein abundances may be affected by the age of the worker bees as age polyethism affects the protein expression within workers. As stated previously apidermin 3 precursor was identified as decreased in abundance in *Varroa* parasitised worker pupae (Surlis *et al.*, 2018). This provides an insight into how the age of the worker bee sample may affect the protein expression of the parasitised samples before even investigating how the prolonged exposure of the mites may further alter protein expression. Furthermore, changes to apidermin protein expression has not previously been observed in *Nosema* infected colonies, therefore it is not possible to identify why apidermin proteins are increased in abundance in Week 1 samples in comparison to Weeks 3 and 5 samples.

Interestingly, two chemosensory and odorant binding proteins were identified as increased in abundance in Week 5 samples in comparison to Weeks 1 and 3. Several cytochrome P450 proteins were also increased in abundance in Week 5 samples in

comparison to Weeks 1 and 3. An increase in OBP3, OBP14 and chemosensory protein 2 (CSP2) have been identified as increased in abundance in *Varroa* sensitive hygienic bees (Mondet *et al.*, 2015a). Colonies for this disease trial were not monitored for observing varroa sensitive hygienic behaviour and the age of workers were not known either. Both factors could influence the increased abundance of the chemosensory proteins in the Week 5 samples. Presently, there is little research on chemosensory and odorant binding proteins in response to *Nosema* infections. It is therefore difficult to understand what may have triggered the increased abundance of these proteins in Week 5 dual infected workers in comparison to Weeks 1 and 3 samples.

Overall, there was no significant protein changes between dual infected colonies and Control samples. This was not expected as there is a large record of publications that have investigated the negative impacts of *Varroa* (Kralj, 2004; Hu *et al.*, 2016; Gray *et al.*, 2019; Traynor *et al.*, 2020; Aldea and Bozinovic, 2020) and *Nosema* samples (Kurze *et al.*, 2016; Sinpoo *et al.*, 2018; Ponkit *et al.*, 2021) on bees. The proteomic data from the dual infected hives did not represent the overall health of the colony as two out of three colonies collapsed at the end of the experiment. The collapse of the colony was suggested to be a result of the dual infection levels of the *Varroa* and the *Nosema* within workers. There is a gap in the literature on dual infections for both *Varroa* and *Nosema* within honeybee colonies. Despite it being challenging to investigate the workers response to two pathogens further research is needed to gain a better understanding into how these infections affect honeybee colonies.

7.4.5 Factors in Experimental Design for Future Disease Trials

Future work investigating the proteomic response of *A. mellifera* to mono and dual infections of *Varroa* and *Nosema* should consider several factors. When determining how the Control colonies should managed, the question of whether an anti-*Varroa* treatments should be applied needs to be strongly considered. If treatments are to be applied to the Control samples, then it would be suggested that all colonies within the trial should be treated at the same time, and then introduce *Varroa* and *Nosema* infections into the disease trials. This would reduce the proteomic response to the treatments in Control samples and avoid what was observed in the results presened in this Chapter. It would set a better base line to the workers sampled in the trial and

avoid skewed proteomic responses in Control samples. When designing the disease trial, the position of the Control hives should also be taken into consideration. If placed in separate apiaries, the micro-climate surrounding the hives would be different and could therefore affect nutrient intake, foraging activity and overall activity within the hives, all of which may alter the proteome of the worker bees. However, if placed within the same apiaries, it is not possible to prevent drifting and subsequent spread of *Varroa* mites or *Nosema* infections from diseased hives to Control groups. With this, careful consideration needs to be applied when determining how to design the Control groups within the trials.

Another factor that should be strongly considered is the age of the bees (worker or drone) that will be sampled and used within the analysis. As stated above, studies investigating the effects of *Varroa* mites on worker bees identified two opposing results with regards to apidermin 3 protein, each study used different aged worker bee pupae (Surlis *et al.*, 2018) and a newly emerged worker bee (Erban *et al.*, 2019). Work has been completed on the changes in protein expression in honeybee workers as they age within the hive (Evans and Wheeler, 2001; Amdam *et al.*, 2009; Iovinella *et al.*, 2011; Ueno *et al.*, 2015; Dobritzsch *et al.*, 2019). It has been observed that *Varroa* mites preferably parasitise the nurse worker bees as opposed to forager worker bees (Xie *et al.*, 2016; Ramsey *et al.*, 2019). *Nosema* infections in worker bees have also been suggested to differ between the age of the worker bees, increasing hunger, disrupting foraging behaviours and infecting hive bees and forager bees at various levels of intensity (Higes *et al.*, 2010; Martín-Hernández *et al.*, 2012). Therefore, the age of the worker bee sampled in disease trials should be considered when considering the experimental design.

Several studies have found variations in infection rates of both *Varroa* and *Nosema* at different times of the year. *Varroa* population levels have been observed throughout the seasons and it has been observed that mite population increases from spring to autumn during which is the peak in brood production (Martin, 1998; Tentcheva *et al.*, 2004; Francis *et al.*, 2013). If measuring the change in proteomic response to the increased presence of *Varroa* mites within colonies, the time of the year should be considered as *Varroa* presence peaks in the summer months. However, if looking to investigate how *Varroa* parasitisation affects the emergence of winter

workers then the study should be designed to include the later summer months and the autumn months.

N. apis has was found to be infecting hives alone or in combination with *Nosema cerana* between March to June and from September to November in Spain, whereas *N. cerana* was identified in hives throughout the year (Martín-Hernández *et al.*, 2012). In general *Nosema* levels generally increase when the bees are exposed to prolonged confinement increasing the chances of in hive defecation. Specifically, in spring levels may spike as workers begin cleaning comb in preparation for the summer (Bailey, 1955; Office International des Epizooties, 2008). *N. cerana* has been observed to proliferate at slightly higher temperatures than *N. apis* which may be why it was observed all year round by Martín-Hernández *et al* (2012) (Fenoy *et al.*, 2009; OIE, 2018).

The duration of the trial and the time of year are other factors that should be observed in the experimental design. Length of the disease trial is an important factor to consider. If the trial is too long and the parasite load too high then there is a risk of losing the colonies to CCD. However, if too short then the full impact of the infections (*Varroa* or *Nosema*) may not be observed. The time of the year is also something to consider, if there is a high infection load when brood production is low, there is a further risk of collapse as there are little to no new bees that will replace the infected older workers.

7.4.5.1 Summary of experimental design points

- Whether to leave the colonies in hives with free access to foraging activities and interactions with other pollinators or hives. Or, to remove external factors and investigate the colony in laboratory based conditions, which can induce its own set of complications and stress related factors.

- To choose what cast to investigate, the proteomes of queens, workers, and drones all vary so determining the cast in which to conduct the experiment on should be a factor to consider.

- Determining the age of the worker or the worker caste, to complete the study with. Each worker caste will have a specific proteomic profile and should considered when determining sample regime.

- The season in which to complete and subsequently collect the samples. As seen in this thesis, worker bee's proteomes are influenced by the seasonality in which they are sampled. The time of year should be incorporated into the experimental design .

- When investigating anti-Varroa treatments on colonies, control samples need to be carefully considered. If control are collected just before treatment application as they were in this study, then disease effect on those control samples could be considered and analysed. The impact of the presence of the parasite or disease within colonies could alter the proteome of the control samples making it more difficult to determine a treatment effect in samples post-treatment application.

- If investigating the effect of diseases on the proteome of honeybees, several factors need to be considered. The time of year should be incorporated into the experimental design as levels of both Varroa and Nosema alter depending on the time of the year – both influenced by the size of colony and brood. Designing a control group several influences should be accounted for – application of treatment (if one is available), location of hives (if field trial, proximity to disease hives), age of queen, food supplementations and nutrient availability.

Chapter 8

General Discussion

8.1 General Discussion

The global importance of *Apis mellifera* in the world economy of food production and crop pollination is well known and documented (Klein *et al.*, 2006; Potts *et al.*, 2010; Rollin and Garibaldi, 2019). Insect pollination is one of the most important pollinator services to agricultural production yields with an estimated three quarters of major food crops being pollinated by animals (Rhodes, 2018b). Within the last 15 years there has been an increase in the attention to the health of honeybee colonies, specifically due to the devastating effects of Colony Collapse Disorder (CCD) (Ratnieks and Carreck, 2010; Russell *et al.*, 2013). CCD is characterised by the absence of workers in hives which contain enough food resources to discount starvation as a cause. No specific factor has been deemed to be responsible for CCD (vanEngelsdorp *et al.*, 2009) instead research has focused on a number of possible causes of CCD such as *Varroa* mites, environmental conditions and presence of insecticides in surrounding agricultural areas (Ratnieks and Carreck, 2010).

Hive losses are not always attributed to CCD which can occur through starvation, poor management, weather, presence of *Varroa* and viruses and potentially exposure to both commonly used agricultural treatments and insecticides. Winter losses are tracked each year through COLOSS surveys that occur worldwide, with overall winter loss rate in Ireland from 2018 to 2019 being 10.7% (Gray *et al.*, 2020). Colony failure may occur at any time of the year, however colonies are more likely to fail during winter due to lack of foraging, brood production and difficulties with combating low temperatures. It is therefore very important to better understand the changes worker bees undergo in preparation for winter, when exposed to commonly used treatments and wide-spread pathogens.

This work presented in this thesis sought to (1) identify changes in worker bees proteomes through a year in an age-polyethism study, (2) compare the proteome of winter workers in comparison to summer worker bees, (3) determine if any off-species effects can be detected using proteomics when worker bees are exposed to commonly used anti-*Varroa* treatments, and (4) assess the effects of the presence of *Varroa* and *Nosema* both singular and combined infections, on the proteome of worker bees sampled from infected colonies.

Previous work has been completed on investigating proteomic changes within hemolymph, hypopharyngeal glands, mandibular glands, fat body, antennas (Amdam *et al.*, 2005a; Feng *et al.*, 2009a; Huo *et al.*, 2016; Iovinella *et al.*, 2018). However, these studies have not utilised mass spectrometry analysis but instead used PCR, immuno-electrophoresis assay, 2D gel electrophoresis and Western blots techniques (Amdam *et al.*, 2004b; Feng *et al.*, 2009a; Iovinella *et al.*, 2011; Lee and Kim, 2017). Very few studies have involved the whole cell lysate of full anatomical samples of honeybees (Surlis *et al.*, 2018). The work presented in this thesis looked to utilise whole cell lysate proteomic analysis using label free mass spectrometry on head, abdominal and venom sac honeybee worker samples, to provide an overall insight into the proteome of worker bees as they age and exposed to different external environmental influences.

The first objective of this thesis was to analyse proteomic changes that occur in worker bees as they age within the colony during summer months. Age polyethism is the process whereby worker bees undergo physiological and biological adaptions (hypopharyngeal glands – MRJP production in nurse bees that switches to α-glucosidase in foragers (Kubota *et al.*, 2004)) to optimise their participation for roles within the colony that correlate to the age of the bee. Gaining an insight into the changes in worker bee proteomes profiles using whole cell lysate on head samples had not previously been conducted. Very few protein groups were significantly changed in abundance between samples of the different ages (newly emerged bees, early hive, late hive and forager bees). Instead it was observed that specific proteins were significantly changed in abundance between the samples of various ages.

Newly emerged worker bees had an increased abundance of short chain dehydrogenase reductase and hexamerin proteins, both of which have previously been identified as increased in abundance (Cunha *et al.*, 2005; Bitondi *et al.*, 2006; Martins *et al.*, 2010). Both proteins have been observed to be increased in worker pupae and have been suggested they play a role in cast differentiation (Evans and Wheeler, 2001; Guidugli *et al.*, 2004). Analysis identified that the profile of MRJPs followed the well documented activity of the hypopharyngeal glands with the highest point of protein abundance correlating with the largest size of the glands – nurse bees, and the lowest protein abundance with the smallest size of the glands – newly emerged worker bees

(Omholt and Amdam, 2004; Deseyn and Billen, 2005; Aurori *et al.*, 2014). Honey production proteins such as, α-glucosidase and α-amylase (Ohashi *et al.*, 1999) were detected in highest abundance in forager bees, which was expected as these workers are more involved in the production of honey (Kubota *et al.*, 2004). An interesting pattern in the abundance of Odorant Binding Proteins (OBPs) was observed throughout the samples with some OBPs being expressed in high abundance in newly emerged bees whilst others were in high abundance in forager samples. The shift in OBP abundance from workers of various ages has been demonstrated previously (Iovinella *et al.*, 2011). Interestingly, vitellogenin did not appear as one of the prominent proteins that changed between samples of various ages. This could be attributed to the anatomical sample that was used in this study, vitellogenin is the most abundant protein in the hemolymph (Amdam and Omholt, 2002) and therefore it's absence as a SSDA in this analysis could be attributed to the sample that was used for the proteomic analysis i.e. the head. Whole cell lysate using the head of honeybee workers provided an overall insight into the proteome of worker bees that has previously not been observed. This analysis allowed for individual protein profile patterns to be observed side by side, therefore giving a better insight into how the proteome of workers change as they age.

Studying the protein changes of worker bees at different ages has highlighted how the protein abundance is altered throughout the life of worker bees, which can be utilised to provide a more in-depth insight into how worker bees of different ages may react to disease, treatments, environmental and colony changes. One example of how age polyethism is a flexible and not strictly defined process was observed in analysis of June samples (**Section 3.4.1.1**). One of the colonies experienced a queen loss and it was observed that the foragers' proteomes from the queen-less colony resembled the proteomes of in-hive workers of queened colonies. The reversal or delay in age polyethism proteome progression due to the absence of a queen demonstrates how both external influences can affect the process of age polyethism within colonies.

Investigating protein profile changes in worker bees as they age within months allowed for a wider analysis on how age-polyethism may be affected by seasonal changes throughout the year. For the first half of the year (April to June) emerged bee proteome samples were clustered together in the PCA indicating a shared proteome of

newly emerged workers despite the difference in sample dates. However, the older workers– early and late hive bees and forager bees were clustered based on the months they were sampled (all May hive and forager bees together etc). This suggests that newly emerged bees are naïve to external stimuli or factors (weather, colony size), whereas in hive and forager samples are susceptible to external factors and therefore display a proteome similar to those with the same exposure to the external factors. Despite a divide in sample clustering of hive and forager samples between the months sampled there was a shared pattern in pathway expressions between newly emerged bees to hive and forager bees. Overall there was an increase in the abundance of proteins involved in: metabolic, carbohydrate, oxidative phosphorylation, and tricarboxylic acid cycle pathways in the hive and forager bees across the months. Whereas, newly emerged worker bees had proteins mostly involved in the ribosome and spliceosome pathways.

Analysis completed on the second half of year (July to October) revealed a further divide between samples. July and August emerged bee proteomes were clustered together but September and October emerged worker bee samples were clustered separately. This indicated a shift in the proteome of newly emerged bees as the colonies are sampled later in the year. Despite the divide in clustering between newly emerged bees, the proteomes of the hive and forager bees in September and October were clustered alongside workers of the same age sampled in July and August, indicating a shared protein profile between older bees. There was however, one group of proteins that was only increased in abundance in October hive and forager samples. This group of proteins was mostly associated with OXPHOS, mitochondria and ion transport pathways. It was this deviation in sample proteomes that triggered a further investigation to the emergence of winter workers from summer colonies.

The emergence of the winter worker from summer hives was investigated by completing a more in-depth analysis of sampled collected from July to October. It was suggested that winter workers emerged in hives in late Autumn, however, the exact time frame of their emergence is not fully characterised (Maurizio and Hodges, 1950; Döke et al., 2015; Kunc et al., 2019; Knoll et al., 2020). The work presented here suggests that workers in September and October emerge as winter workers, in contrast to previous studies that suggest that winter workers are derived from forager bees that

remain within the hive instead of transitioning from in hive bees to foragers (Amdam and Omholt, 2002; Aurori *et al.*, 2014; Döke *et al.*, 2015). This is an important factor to consider when preparing hives for winter in terms of applying anti-*Varroa* treatments and monitoring and feeding colonies, as it is essential for the survival for the colony for it to be as strong as possible when entering the winter months.

In addition to the age polyethism investigation, analysis was also conducted on characterising the proteome of winter worker bees. It began with a protein comparison between workers sampled within winter months (December 2019 – February 2020). However, this analysis did not provide any true insight into the proteome of winter workers, which can be attributed to the design of the analysis – comparing winter workers with other winter workers. A second approach was therefore taken to investigate the proteome of winter bees in comparison to summer workers.

Work was completed to investigate the proteomic differences between winter and summer honeybee samples (head, abdomen and venom sac) and highlighted fundamental differences between the workers' proteomes. Overall winter bees had a reduced proteome in terms of both number of proteins and the relative abundance of the proteins in comparison to summer workers. Proteomic analysis of head samples revealed an increase in several protein pathways in summer samples in comparison to winter samples: genetic information processing, energy metabolism and environmental information processing. An increased abundance of proteins associated with these pathways demonstrated the optimised proteome profile of workers in summer to aid in the roles they undertake and their navigation through the external environment (test flights, foraging, guarding the hive etc.). Three pathways associated with carbohydrate metabolism were increased in abundance in winter workers: galactose, ascorbate and aldarate metabolism and pentose and glucuronate interconversions. These pathways have links to energy metabolism, xenobiotic metabolism, a defensive role to protect cells against oxidative damage, and modulate humoral and cellular immune responses (Pristavko and Dovzhenok, 1974; Goggin *et al.*, 2010; Jiang *et al.*, 2019; Malka *et al.*, 2020).

Summer workers also had an increased abundance of major royal jelly proteins – two of which were exclusively expressed in summer workers. Hypopharyngeal glands in winter bees despite being hypertrophied are not observed to have a secretory

cycle (Brouwers, 1983; Deseyn and Billen, 2005), therefore it was not surprising that summer workers bees had a higher abundance of MRJPs. Storage protein hexamerin 110 was identified as exclusively expressed in winter worker bee head analysis. Hexamerin proteins have been identified in high abundance in both honeybee worker larvae (Cunha *et al.*, 2005; Bitondi *et al.*, 2006; Martins *et al.*, 2010) and in newly emerged worker bees, as observed in this thesis. Hexamerin however, is not fully characterised in tissue distribution and functionality so more research would need to be conducted to understand the role hexamerin plays in the longevity of winter worker bees. In both head and abdominal analysis, summer bees had an increased abundance of cuticular proteins in comparison to winter workers. This could be attributed to the lower rate of damage and activity the winter worker's exoskeleton is exposed to versus the high activity load and grooming that occurs in summer hives. The lower abundance of cuticular proteins in winter workers is something that should be investigated further – the cuticle is the initial physical barrier to both pathogens and foreign substances, if winter bees have a low abundance of cuticular proteins, but are then exposed to pathogens, treatments or other insecticides it could potentially weaken winter hives, leaving them more vulnerable to failure.

Winter bees had a reduced presence of cuticular proteins in comparison to summer workers in both the head and abdominal samples but there was an increased abundance of xenobiotic metabolism and a small number of antioxidant proteins. These proteins can provide a defence mechanism against reactive oxygen species. This combined with the presence of vitellogenin and apolipophorin-III within winter workers indicates an optimised defence mechanism honeybees that aids in reducing cellular stress whist adding in longevity (Amdam *et al.*, 2004b; Amdam *et al.*, 2005a; Seehuus *et al.*, 2006b).

The final section of the winter vs summer comparison was the proteomic analysis of the venom sac. This analysis revealed a significant difference between the venom sacs of the two sample groups. Summer workers had a much higher abundance of venom associated proteins in comparison to winter workers. The three most abundant proteins identified in the venom sac samples of summer workers bees were Api m6, Api m1 and Api m4, of which it is known that Api m6 and Api m4 are the most predominant proteins in bee venom (Hider, 1988; Peiren *et al.*, 2005). It was also

observed that numerous non-toxin proteins were increased in abundance in summer venom sac samples in contrast to winter samples. This indicates that proteins involved in these pathways are necessary to protect the secretory cells from the damage of bee venom (Danneels *et al.*, 2015; Hossen *et al.*, 2017; Scaccabarozzi *et al.*, 2021). The reduction in both bee toxins proteins and proteins involved in protecting secretory, could be a defence mechanism as means of limiting bee venom production, in order to reduce energy usage in their production and a protective mechanism from potential damage from the toxin itself.

The presence of *Varroa destructor* mites in colonies is widespread throughout apiaries across the world. *Varroa* have been a significant contributing factor to hive losses over the years from both direct parasitic impact and through the transmission of viruses (Rosenkranz *et al.*, 2010). Beekeepers across the globe are using various control methods in an attempt to reduce the negative impact *Varroa* mites have on their colonies. Anti-*Varroa* treatments are widely and commonly used, however, little research has been completed on the exact mode of action of these treatments and what potential side effects could occur from exposure.

There are three categories of anti-*Varroa* treatments – hard acaricides, soft acaricides and biotechnical/control methods. Only a handful of studies have been completed on how these treatments may have side effects on the colonies to which they are applied (Mondet *et al.*, 2011; Sabová *et al.*, 2019; Ye *et al.*, 2020). This work used proteomic techniques to investigate changes to the proteome of honeybees exposed to anti-*Varroa* treatments. The first treatment that was investigated was Mite Away Quick Strips (MAQs), which caused significant changes in mitochondrial (specifically cytochrome c oxidase), xenobiotic metabolism and cuticular proteins in worker bees. Formic acid (active ingredient in MAQs) is known to inhibit the activity of cytochrome c oxidase (Liesivuori and Savolainen, 1991). The presence of MAQs and therefore formic acid, triggered an off-species effect on the workers exposed to the treatment, as samples collected at the end of the first week (and therefore the end of the treatment) had an altered expression of cytochrome c oxidase levels in comparison to Control and Weeks 2 and 3 samples.

The second treatment that was investigated was Apivar (active ingredient is amitraz). This investigation found an increase in ribosomal proteins in bees exposed

to the treatment, which has previously been observed in response to amitraz exposure (Ye *et al.*, 2020). An increase in translational proteins associated with the transcriptome have been observed in insects when exposed to insecticides (Wei *et al.*, 2019; Ingham *et al.*, 2021), and when insects develop resistance to insecticides (Tan *et al.*, 2007; Sun *et al.*, 2011; Yu *et al.*, 2014). The increased presence of translational proteins in the proteomes of worker bees exposed to Apivar indicates an off species effect in the worker bees. The full scope on how Apivar effects the honeybee workers is still not fully understood and therefore need further investigations.

The final treatment that was investigated was Apiguard with thymol as the active ingredient. No large protein pathway changes were identified in worker bees following exposure to Apiguard. Thymol is known to be effective against *Varroa* populations within colonies, however it's mode of action and it's effect on *Varroa* mites is still unclear. An alteration in workers behaviour regarding the presence of Apiguard within colonies has been observed, with foragers that were not previously exposed to the treatment actively avoiding the gel (Mondet *et al.*, 2011). Similar to Apivar further studies need to be conducted on Apiguard exposure and mode of action need to be done to better understand how these treatments work.

More investigative research needs to be conducted to understand both the mode of action of these anti-*Varroa* treatments and how the colonies react to the presence of the treatments. For future work, proteomic investigation on a model insect such as *Galleria mellonella,* that has been used to determine the effects of antimicrobial agents, food additives such as the effects of caffeine (Maguire *et al.*, 2016; Maguire *et al.*, 2017; O'Shaughnessy *et al.*, 2022), is recommended. This would allow for a better insight into how the active ingredients of the anti-*Varroa* treatments may interact with the honeybees. As it has been previously noted that the behaviour of worker bees change when exposed to anti-*Varroa* treatments within colonies, and the insight that the proteome of worker bees changes as they age, these are two factors that should be considered and investigated in future research. Understanding how both the colony adapts and the true response of the worker bees to the exposure of a treatment will provide a better insight into how the combination of the *Varroa* mite and the presence of the treatments may influence the overall health of the colonies.

The final Chapter looked to characterised the effects of *V. destructor*, *Nosema* both as individual infections and as a dual infection in honeybee colonies. The impact of *V. destructor* on honeybee workers is still not fully understood, but recently it has been discovered that *Varroa* mites feed on the fat body of the worker larvae and not the haemolymph (Ramsey *et al.*, 2019). A small number of studies have been completed on proteomic changes that occur in workers parasitised by *Nosema* (Vidau *et al.*, 2014), however, more work needs to be conducted to better understand the mechanism of parasitisation. This Chapter looked to investigate the proteomic changes in worker bees sampled from single and dual parasitised colonies over the timeframe of five weeks.

Analysis on *Varroa* single infection samples identified an increase in MRJPs from samples that had the highest number of *Varroa* mites at the time of collection. An increased abundance of ribosomal proteins in bee samples was observed in colonies that had a longer exposure time to the presence of the mites in the hives. The work demonstrates that the presence and intensity of the infestation of *Varroa* mites within colonies affects worker bees. However, as stated above, the full impact *Varroa* mites have on worker bees and colonies they infect is still not clear.

The next section of the Chapter looked at the proteomic response of worker bees to the presence of single *Nosema* infections within colonies. It was discovered that the samples with the highest *Nosema* infection rate had the most divergent proteome in comparison to the other disease samples. An increase in ribosomal, eukaryotic proteins, and hexamerins were observed in the samples from the highest infection time points. In contrast these samples had a reduction in membrane-associated guanylate kinase proteins – scaffold proteins that play a role in synapse formation and functionality, if mutated can disrupt the epithelial septate junctions and causes an overgrowth of the imaginal discs (Woods *et al.*, 1996; Olsen and Bredt, 2003; Oliva *et al.*, 2012). This work demonstrates that the intensity of the infection can have a significant effect on the proteome of the worker bees. Further research would need to be conducted to be able to better understand the full impact *Nosema* has on worker bees at varying levels of infection.

The final section of the Chapter looked at the proteomic response of worker bees to dual infection of both *Varroa* mites and *Nosema*. Interestingly, the results of

the proteomic analysis revealed little variation between infected and Control samples. An increase in chemosensory proteins was observed in Week 5 samples in comparison to Weeks 1 and 3 dual infected colonies. This was not expected as infection levels in Week 5 were not significantly increased in comparison to Weeks 1 and 3. The increased abundance of chemosensory proteins in Week 5 may indicate a response of worker bees to a prolonged infection presence within colonies. A limitation to this investigation was the sample that was chosen for proteomic analysis. Only the head was chosen as a sample point, future work should consider analysis on both the abdomen and the hemolymph to investigate the impact single and dual infections of *Varroa* and *Nosema*.

8.2 Concluding remarks

As the dependency on insect pollination grows for global food production, so does the need to gain a better understanding to the biology of *A. mellifera*. It is well known that honeybees work collectively as a superorganism within hives to completed numerous tasks harmoniously. Work presented in this thesis highlights the complexity of investigating changes in honeybee worker proteomes in response to several factors: age, seasonality, parasitisation and treatments. This proteomic analysis has provided a novel insight into the adaptions that occur in worker bees in response to environmental changes. These can have numerous potential applications to future studies investigating the health and adaptations that worker bees have in *A. mellifera* colonies which may aid in reducing hive losses.

8.3 Future Work

1. The results presented here characterised the emergence of *A. mellifera* winter workers in autumn. Future work might examine the nutrient content of food given to larvae laid in autumn months to investigate if there is a content change in both volume and quality of bee milk.

2. Results in Chapter 3 identified proteomic changes that occur through age-polyethism. Future work may investigate the effect of altering the colony structure (removing the queen, brood etc.) on the proteome of worker bees.

3. This thesis investigated the effect of anti-*Varroa* treatments on the proteome of *A. mellifera* worker bees. Future work could investigate how treatment application to colonies may affect the colony health - egg laying, brood rearing, worker interaction with the treatment.

4. Future work may also consider conducting proteomic analysis on the affects of anti-*Varroa* treatments on simpler *in-vivo* models such as *Galleria mellonella.*

5. Work in this thesis was also conducted on samples exposed to *V. destructor* and *Nosema* infections both singular and dual infections. Future research might investigate the effects of these pathogens in a lab based setting

Chapter 9

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Chapter 10

Appendix

Supplementary datasets can be accessed with the link provided:

Chapter 2

Supp. File 2.1: 2-D gel images

Chapter 3

Supp. File 3.1: Volcano plot data from sample analysis between April through to June
Supp. File 3.2: Volcano plot data from sample analysis between July through to October
Supp. File 3.3: Hierarchical clustering analysis from all individual months from April to October

Chapter 4

Supp. File 4.1: Clusters generated from hierarchical analysis from April to June
Supp. File 4.2: Clusters generated from hierarchical analysis from July to October
Supp. File 4.3: Volcano plot analysis from samples between July to October

Chapter 5

Sup. File 5.1: Volcano plot analysis between winter and summer head, abdomen and venom sac samples

Chapter 6

Supp. File 6.1: Volcano plot analysis between Control to MAQ treatment samples
Supp. File 6.2: Hierarchical clustering analysis from SSDAs generated between Control and MAQ treatment samples
Supp. File 6.3: Volcano plot data generated from Control and Apivar treatment samples
Supp. File 6.4: Hierarchical clustering analysis from SSDAs generated between Control and Apivar treatment samples

Supp. File 6.5: Volcano plot data generated from Control and Apiguard treatment samples

Supp. File 6.6: Hierarchal clustering analysis from SSDAs generated between Control and Apiguard treatment samples

Chapter 7

Supp. File 7.1: Volcano plot data from *Varroa* and Control sample comparisons

Supp. File 7.2: Hierarchal clustering analysis from SSDAs from Control and *Varroa* samples

Supp. File 7.3: Volcano plot data from *Nosema* and Control sample comparisons

Supp. File 7.4: Hierarchal clustering analysis from SSDAs from Control and *Nosema* samples

Supp. File 7.5: Volcano plot data from Double infected and Control samples

Supp. File 7.6: Hierarchal clustering analysis from SSDAs from Double infected and Control samples