# A proteomic and cytological characterisation of the buff-tailed bumblebee (*Bombus terrestris*) fat body and haemolymph -An immune perspective


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by

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**Dissemination of research**

**Biology Department Seminar Series**

**2015** Proteomic characterisation of the immune response of the buff-tailed bumblebee *Bombus terrestris*. Larkin, D. E. and Carolan, J. C. (2015). Biology Department Seminar Presentation, Maynooth University. **Presentation.**

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**Annual Biology Department Research Day**

**2015** Characterisation of the immune response of the buff-tailed bumblebee *Bombus terrestris*. Larkin, D. E. and Carolan, J. C. (2015). Annual Biology Department Research Day, Maynooth University. **Presentation.**

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## Conference and Workshop Attendance

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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** _____

Dearbhlaith Ellen Larkin B.Sc.

**Date** _____

**Abbreviations**

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| 1D | One-dimensional |
| AA | Amino acid |
| AKH | Adipokinetic hormone |
| AMPs | Antimicrobial peptides |
| ApoD | Apolipoprotein D |
| ApoLp-III | Apolipoprotein-III |
| ATP | Adenosine triphosphate |
| BLASTp | Basic local alignment tool for proteins |
| BP | Biological process |
| CAT | Catalase |
| CC | Cellular component |
| CC | Corpus cardiaca |
| CLSM | Confocal laser scanning microscopy |
| cm | Centimetre |
| DAG | Diacylglycerol |
| DAPI | Diamidine-2-phenylindole |
| $dH_2O$ | Distilled water |
| DTT | Dithiothreitol |
| ER | Endoplasmic reticulum |
| FB | Fat body |
| FDR | False discovery rate |
| FSPBS | Filter sterilised phosphate buffered saline |
| *g* | g force |
| g | Grams |
| GI | Grey index |
| GI | Grey index |
| GO | Gene ontology |

| | |
|---|---|
| H&E | Haematoxylin and eosin |
| IAA | Iodoacetamide |
| ILPs | Insulin-like peptides |
| Imd | Immune deficiency |
| JAK/STAT | Janus kinase/ signal transducers and activators of transcription |
| JNK | Jun kinase |
| KEGG | Kyoto encyclopaedia of genes and genomes |
| LB | Luria broth |
| LC-MS/MS | Liquid chromatography mass spectrometry |
| LD | Lipid droplet |
| LFQ | Label-free quantitation |
| LPS | Lipopolysaccharide |
| M | Moles |
| m/z | Mass/charge ratio |
| MF | Molecular function |
| mg | Microgram |
| min | Minute (s) |
| ml | Millilitre |
| mm | Millimetre |
| mM | Millimoles |
| mRNA | Messenger RNA |
| MS | Mass spectrometry |
| nAChRs | Nicotinic acetylcholine receptors |
| nL | Nanolitre |
| OS | Oxidative stress |
| OXPHOS | Oxidative phosphorylation |
| PAGE | polyacrylamide gel electrophoresis |
| PCA | Principle component analysis |
| Pfam | Protein family |
| PGA | Potato glucose agar |

| | |
|---|---|
| PGRPLC | Peptidoglycan recognition protein LC |
| PGRPLE | Peptidoglycan recognition protein LE |
| PGRPSA | Peptidoglycan recognition protein SA |
| PMRS | Plasma membrane reticular system |
| PO | Phenoloxidase |
| ppb | Parts per billion |
| PPI | Protein-protein interaction |
| proPO | Prophenoloxidase |
| PRR | Pathogen recognition receptor |
| rcf | Relative centrifugal force |
| RH | Relative humidity |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RPs | Ribosomal proteins |
| rRNA | Ribosomal RNA |
| s | Second (s) |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| snRNA | small nuclear RNA |
| SOD | Superoxide dismutase |
| SSDA | Statistically significant differentially abundant |
| STRING | Search tool for the retrieval of interacting genes |
| TAG | Triacylglycerol |
| TBLASTN | Basic local alignment search tool for translated nucleotides |
| TCA | Tricarboxylic acid cycle |
| TFA | Trifluoroacetic acid |
| tRNA | Transfer RNA |
| UPR | Unfolded protein response |
| YEPD | Yeast extract peptone dextrose |

**Abstract**

Bees, including solitary and social, native and managed, are vital insect pollinators that provide essential ecosystem services. *Bombus terrestris* (Linnaeus) is a widespread and important bumblebee pollinator of wild and cultivated crops and although found commonly across Europe, is available commercially to supplement pollination requirements. Due to their activity, *B. terrestris* workers encounter a variety of diseases which in addition to habitat loss and agrichemical use, are key factors in global bumblebee declines. The profound economic and environmental consequences of this decline warrant a detailed investigation of the molecular and cellular aspects of bumblebee health. The principal components of the *B. terrestris* immune system, the fat body (FB) and haemolymph were characterised here using proteomic and cytological methodologies. The FB proteome is highly enriched in metabolic, detoxification and proteostasis processes whereas the haemolymph is enriched in cellular transport and immunity. At a cellular level the FB was shown to predominantly comprise adipocytes and oenocytes, while spherulocytes, oenocytoids and plasmatocytes were the most frequently found haemocytes in bumblebee haemolymph. The FB and haemolymph were also investigated under various stresses and contexts. In general, typical immune responses to microbial challenge were observed although immune signatures were lower than expected. Although specific responses to Gram-positive and Gram-negative bacteria and fungi were observed, a broad and conserved response to microbial challenge was found. The major responses in both the haemolymph and FB, however involved energy metabolism, protein processing and detoxification which provides insight into the mechanisms that support and regulate the immune response in bumblebees. Worryingly, pesticide exposure had a significant effect on the FB proteome and its ability to mount an immune response. Overall these results provide novel insights into molecular aspects of bee health and highlight the importance of nutrition and the risks posed by pesticides use on our important pollinator species.

# Chapter 1

## General introduction

## 1.1 Bumblebees

Bees are one of the most valuable and important insect groups, due primarily to their role as key ecological service providers through their pollination of wild and crop plant species. These essential insects are members of the order Hymenoptera (superfamily Apoidea), section Aculeata, which comprises hymenopterans whose females have stings including wasps, ants, and bees. Both bumblebees (*Bombus*) and honeybees (*Apis*) are members of this section. The colonial behaviour of bees can vary dramatically between species ranging from solitary (sub social) to colonies (eusocial) comprising hundreds to thousands of individuals. Both honeybee and bumblebee colonies are considered eusocial, as they have division of labour (egg-layer vs. foragers) among co-operating adult females of two generations, comprising mothers and daughters, living in a single nest. The two main groups of female castes within eusocial colonies are; workers, whose main responsibilities are foraging, brood care and defense and queens, who are almost solely responsible for egg laying. Colonies usually contain only one mated queen but can have one or numerous workers, usually unmated. In general, queens are much larger than her worker counterparts, but sometimes the difference is only in mean size (Michener 2007).

Bumblebees are large resilient insects with a body length between 7-27 millimetres; their bodies (abdomen and thorax) are covered in a thick layer of long alternatively coloured plumose hairs (Williams, 1998). Subtle colouration, banding and structural differences between bumblebee castes and species can be used to distinguish them morphologically (Figure 1.1). Queens can be easily distinguished visually as they are larger than both their worker and male counterparts. The two main types of bumblebee are; true and cuckoo bumblebees (obligate social parasites of true bumblebees). Worldwide there are approximately 250 bumblebee species, which can be further classified into 15 subgenera (Williams 1998; Williams et al. 2008). Although the majority of the bumblebee species in the world belong to the true bumblebee division (Kozmus et al. 2011), the remaining 45 species are cuckoo bumblebees, and belong to the subgenus *Psithyrus* (Pedersen 2002).

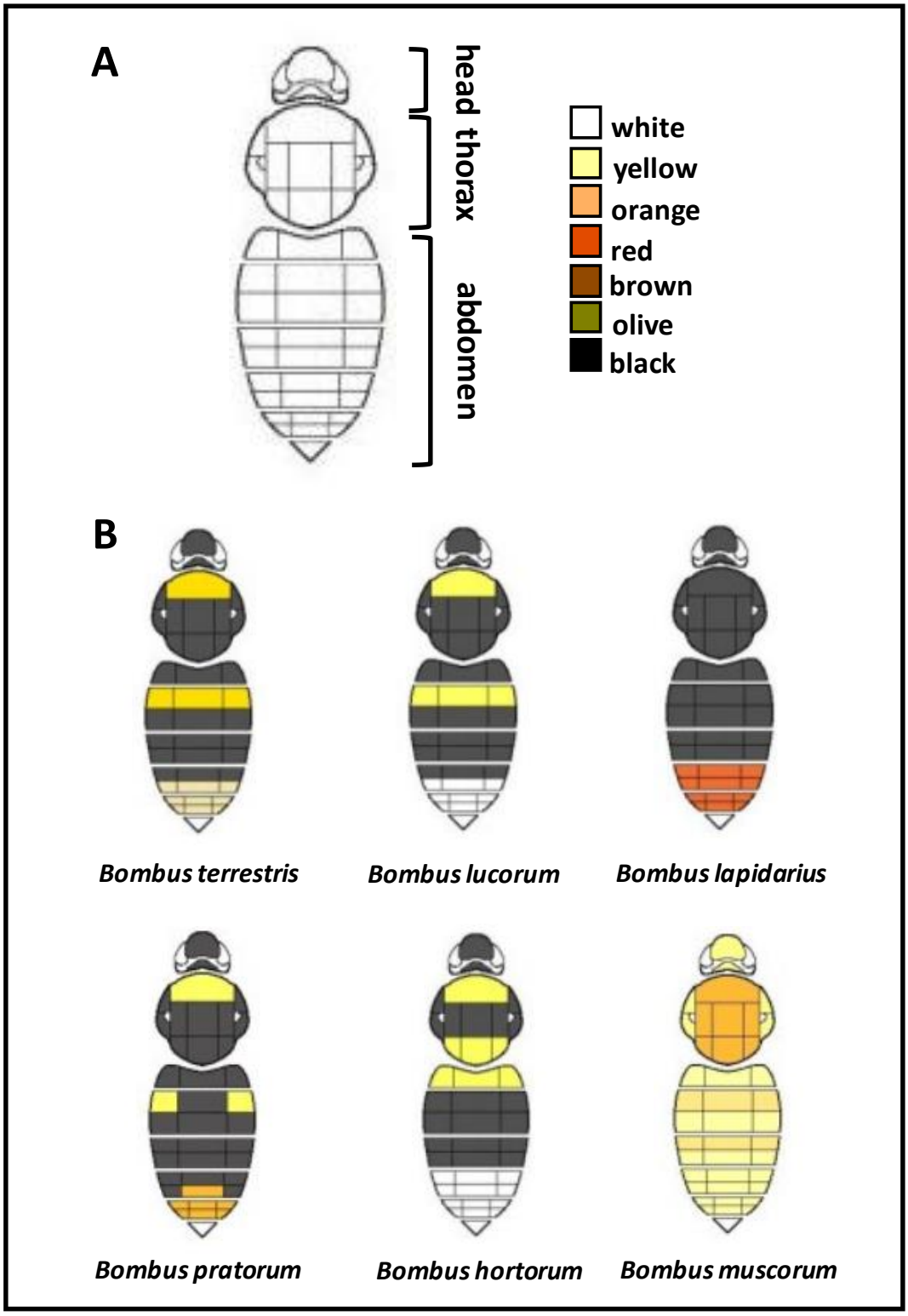


**Figure 1.1 Bumblebee banding patterns and colouration.** (A) Bumblebee species and castes can be distinguished through the colouration and banding pattern of the hairs on their head, thorax and abdomen. (B) The queens and workers of Irish bumblebee species such as *B. terrestris*, *B. lucorum*, *B. lapidarius*, *B. pratorum*, *B. hortorum* and *B. muscorum* are all identifiable based on these banding features. Adapted from Williams (2007).

### 1.1.1 Bumblebee anatomy

Like most insects the bumblebee body is composed of three distinct appendages, the head, thorax and abdomen. The head contains the mouthparts, antennae, eyes and brain, the thorax contains the salivary glands, oesophagus and flight associated muscles. The bumblebee worker abdomen contains all stinging apparatus along with digestive organs, fat cells, nerve chord and the dorsal vessel through which haemolymph is pumped into the haemocoel (Figure 1.2).

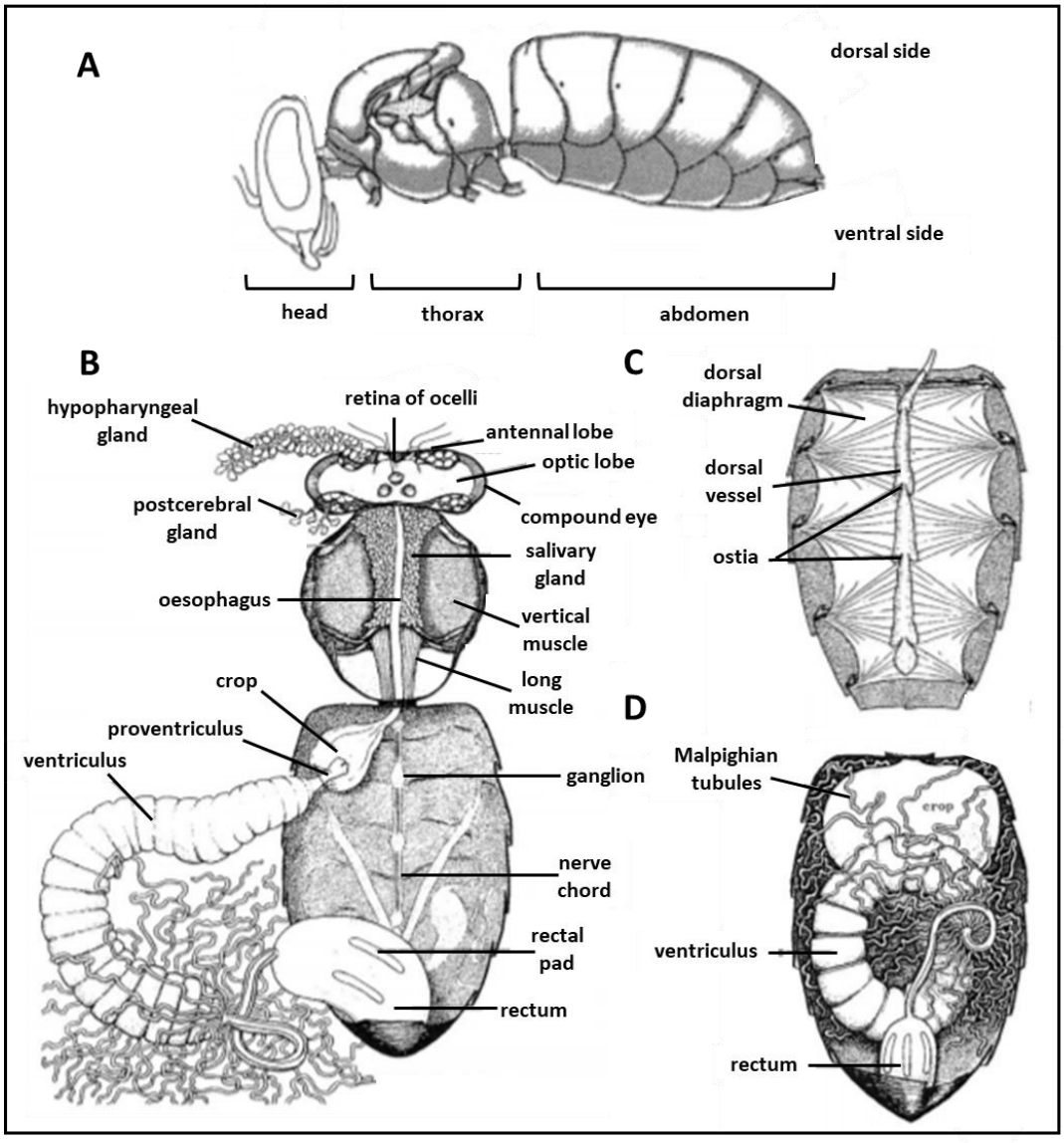


**Figure 1.2 Bumblebee anatomy.** (A) The bumblebee body is composed of three main parts, the head, thorax and abdomen. (B) Dorsal dissection of the bumblebee worker reveals, the underlying organs in all three sections. From this view the optic lobe, compound eye, retina and numerous glands can be observed in the head. Within the thorax, the oesophagus, salivary gland and long and vertical muscle are identifiable. The abdomen contains all major bee digestive system organs including the crop, the ventriculus or midgut, malpighian tubes and rectum. Removal of these organs reveals the nerve chord and associated ganglions. Adapted from Carreck et al. (2013).

## 1.2 *Bombus terrestris*

*Bombus terrestris* (Linnaeus 1758) the buff-tailed bumblebee, is one of Europe's most common bee species and key natural pollinators (Figure 1.3A). This social bee is a member of the family Apidae, genus and subgenus *Bombus* (Michener 2007). *B. terrestris* can be identified by a single yellow band at the top of the thorax (body), a single yellow band at the top of the abdomen and two white (buff) stripes at the bottom of the abdomen (tail). Typically *B. terrestris* colonies are located below ground and house annual colonies of around 100 - 300 workers which are produced and ruled by a single queen (Figure 1.3B) (Alford 1975; Erler et al. 2011). The *B. terrestris* life cycle begins when queens emerge in spring after several months of hibernation and establish a nest for the purpose of laying eggs (Figure 1.4). Eggs hatch into larvae after four days and need a further 10-14 days within which they are dependent on the queen and workers for feeding to pupate and metamorphose into an adult. Caste differentiation of females is apparent within the colony with a single queen solely undertaking the central reproductive duties whilst sterile female workers perform other tasks essential for colony success such as foraging and feeding larvae (Alford 1975). In *B. terrestris* colonies the switch from production of diploid workers to the reproduction of haploid males and gynes (virgin queens) is a notable event in the colony life cycle which represents a switch from growth to sexual maturation of the colony. This switch appears to be endogenously controlled by the queen but the exact mechanism by which this event occurs is unknown (Holland *et al.*, 2013). Once produced sexuals leave the colony to mate, after mating new queens go into hibernation whilst males and the remaining colony perish (Alford 1975).

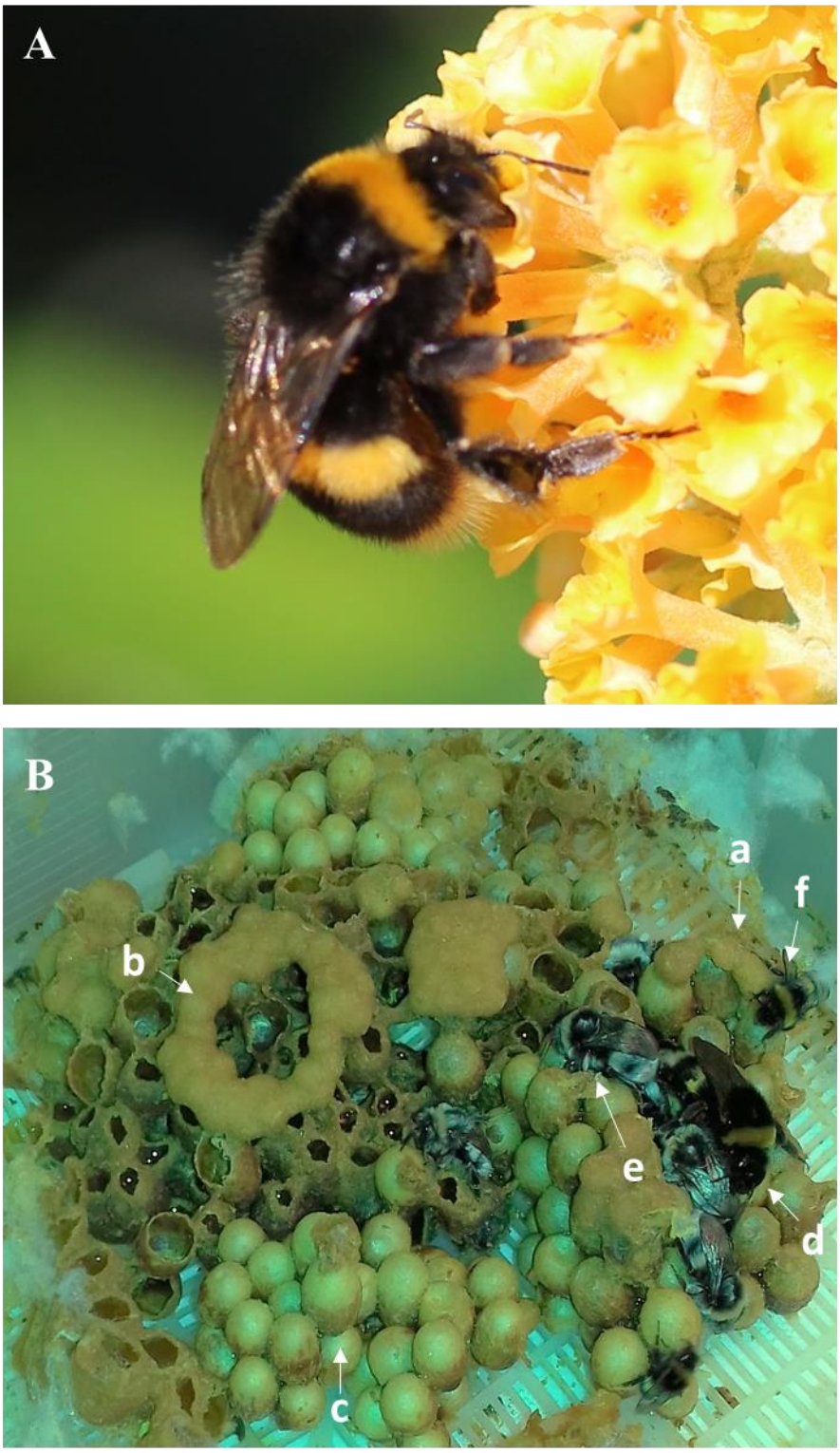


**Figure 1.3 *Bombus terrestris,* castes and colony.** (A) Subtle colouration, banding and structural differences between bumblebee castes and species can be used to morphologically distinguish them. *B. terrestris* can be identified by a single yellow band at the top of the thorax (body), a single yellow band at the top of the abdomen and two off white stripes at the bottom of the abdomen (tail). (B) Within the *B. terrestris* colony representatives of the main developmental stages are present; (a) newly laid eggs, (b) developing larvae, (c) cocooned pupae, (d) a single queen and both (e) newly emerged and (f) mature workers (Photo: D. E. Larkin).

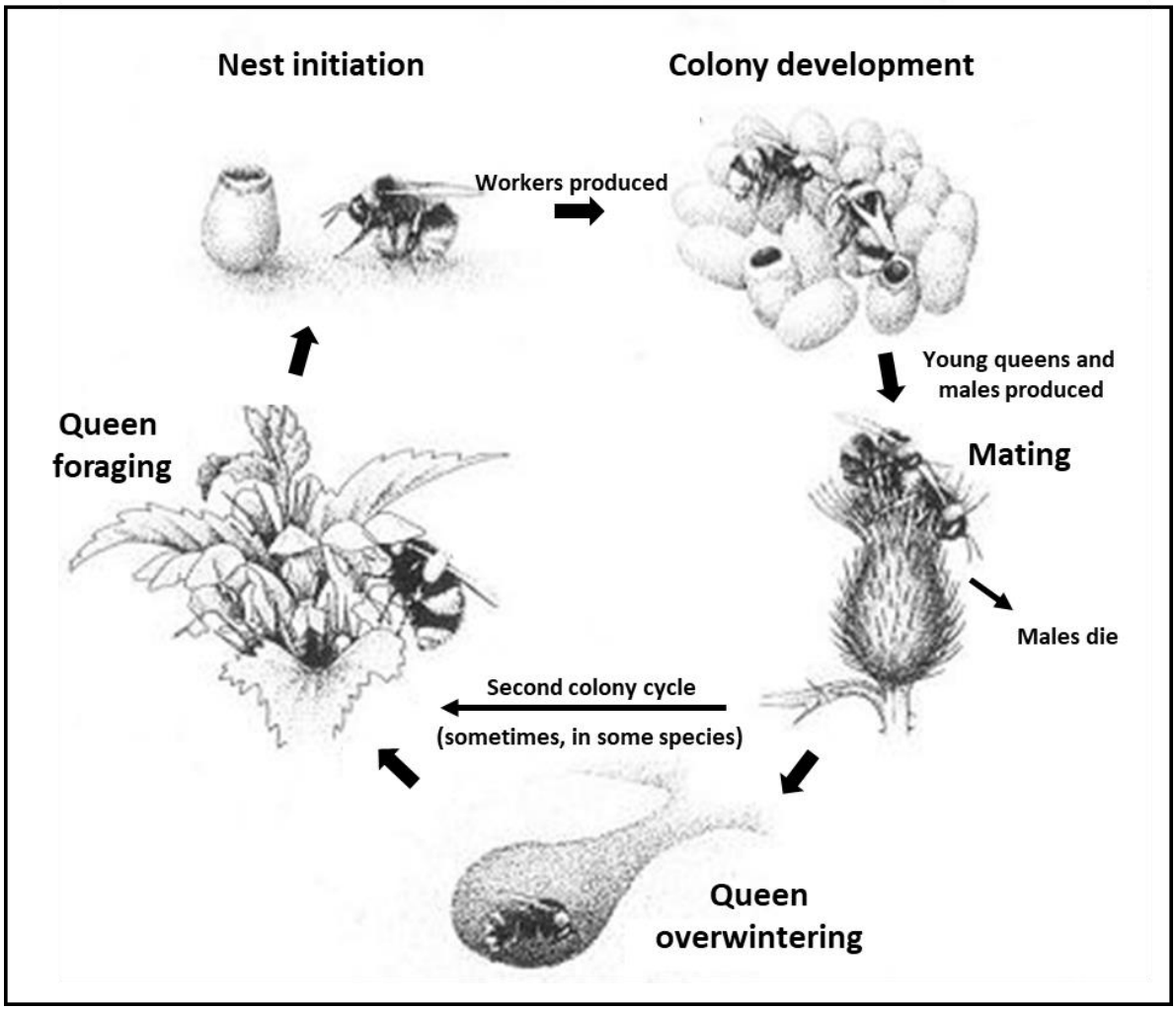


**Figure 1.4 Bumblebee colony life cycle.** An illustrative schematic of the bumblebee colony life cycle. Initiation of colonies commence when bumblebee queens emerge from hibernation, establish a nest and begin to forage intensely for provisions to support the developing colony. Eggs that are laid develop into female workers who then take over foraging duties for the colony while the queen's sole responsibility is reproducing. After substantial growth males and gynes (new queens) are produced within the colony. These new sexuals then leave the colony to mate, after mating new queens go into hibernation while males and the original colony die off. Adapted from Prys-Jones and Corbet (1987).

## 1.3 Global distribution and habitat

Bumblebees have a worldwide distribution, with the exception of sub-Saharan Africa and Oceania, and reach their highest diversity in temperate, alpine, and arctic zones (Goulson 2003a). In Africa, Asia and Europe bumblebees are particularly species rich in the Southern Tertiary Mountains which span from Tibet and the Himalayas westwards towards the Alps and Pyrenees; and in the north-temperate steppe zone running eastwards from the Baltic to inner Mongolia. In the Americas areas rich in bumblebee species include the Western Mountains which reaches from the Rockies southwest to the Andes (Williams 1998). *B. terrestris* is native to the Palaearctic and is common in many parts of Europe, North Africa, and parts of Western Asia (Sadd et al. 2015).

Bumblebees are most commonly associated with cool open unimproved, flower rich habitats (Williams 1988), from which they derive a continuous supply of forage plants throughout the colony life cycle (Carvell 2002). In addition, their habitats often experience one annual period of adverse weather (most often a cold winter) typical of subalpine meadows and north temperate grasslands (Williams 1998). The variety and quantity of bee species inhabiting a geographical area is heavily dependent upon the diversity and abundance of available plant species, as particular bee species have varied feeding and nesting requirements or are niche habitat specialists (Fitzpatrick et al. 2006).

## 1.4 Pollination

Pollination via bumblebee species, including *B. terrestris*, is essential for the maintenance of both native and economically valuable plant communities (Fontaine et al. 2005). The physical act of pollination happens through the transfer of pollen from the anther of one plant to the stigma on the same or different plants. Pollination is beneficial for both plant and bee as when a bumblebee visits a flower it drinks the nectar and collects pollen onto the pollen baskets on its back legs. On return to the nest the pollen is made into a pellet and fed to growing larvae whilst nectar is stored in honey pots for use at a later time (Fitzpatrick et al. 2006; Goulson et al. 2008). Although managed and wild bees are the most well-known for their contribution to pollination, many other insects and animals also participate including flies, beetles, moths, butterflies, wasps, ants, birds, and bats, among others (Figure 1.5) (Rader et al. 2016).

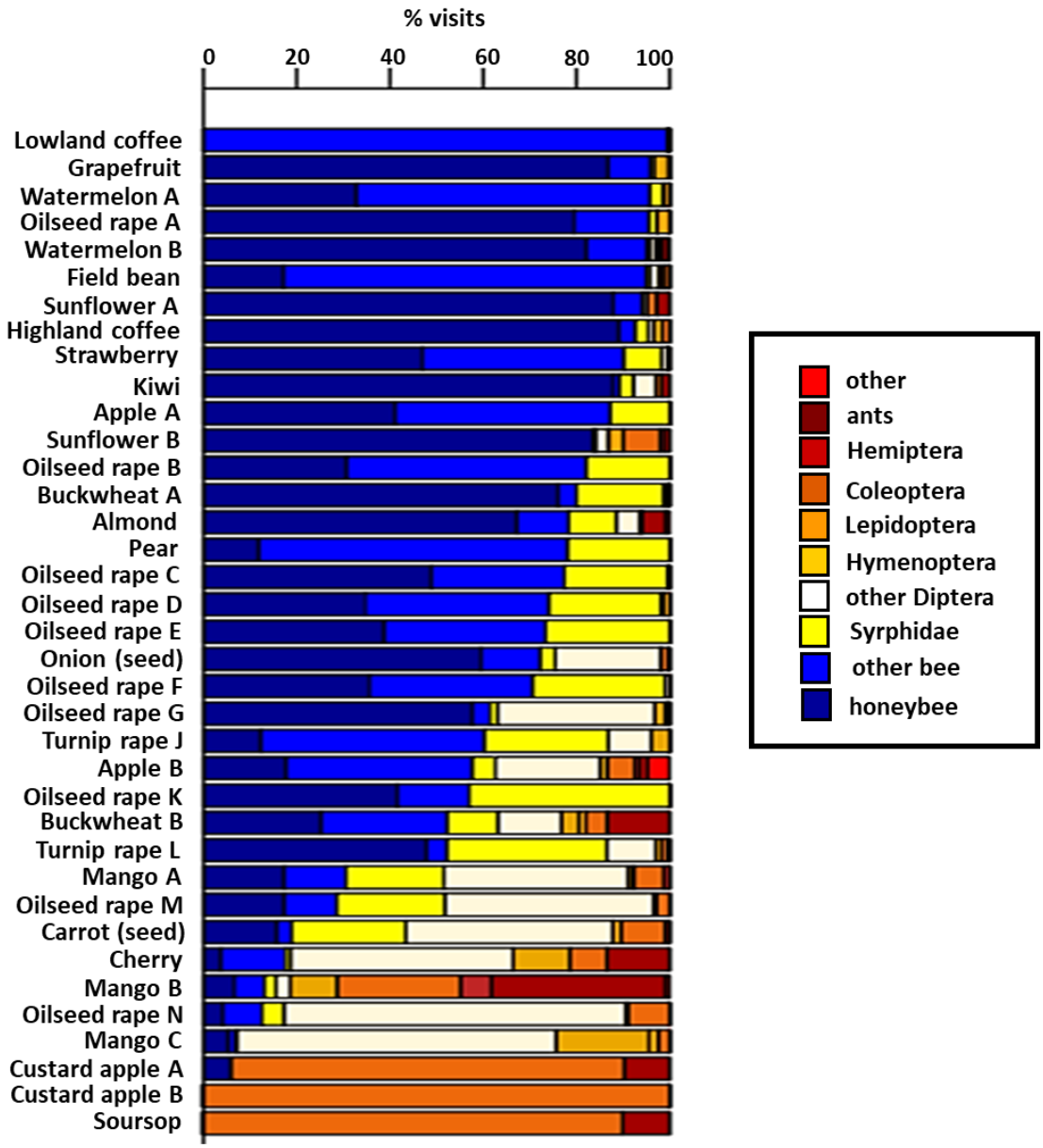


**Figure 1.5 Diversity of non-bee pollinators providing crop pollination services.** Rader et al. (2016) investigated the contribution of different insect groups to flower visitation across 37 crop studies for which visitation data were available. Crops are listed with the percent and type of insect visitor represented by coloured bars. Crops are ordered, top to bottom, from mostly bee-dominated to mostly non-bee–dominated. This study highlights the significant contribution that wild pollinators, other than bees, also make to global crop-pollination services.

Bees can be flower specialists or generalists, this preference is based upon the length of their proboscis (tongue) which determines whether it can reach the nectar in the flower, if a bee cannot reach the nectar it is unlikely to become a stable pollinator of that plant species (Michener, 2006). However, in regard to *B. terrestris* elements of physicality (robust size and long tongues) and ability to buzz-pollinate significantly increase the efficiency of pollen transfer in numerous economically important food crops such as

tomatoes and berries (Cameron et al. 2011). This efficacy has led to their domestication and further increased the reliance upon them to pollinate valuable economic crop species in order to meet growing demands for food production (Graystock et al. 2016). Over 70% of the food crops consumed by humans benefit from pollination (Klein et al. 2007), with the value of ecosystem services provided by pollinators to global agriculture estimated between €153 billion (Gallai et al. 2009) to €213–€522 billion per annum (Lautenbach et al. 2012; Potts et al. 2016).

### 1.5 Bumblebee declines, cause and effect

Recent recorded reductions in bumblebee populations in Europe and North America have led to environmental and economic concerns of global decline (Goulson et al. 2008; Williams and Osborne 2009; Szabo et al. 2012). For example of the 16 non-parasitic bumblebees native to the U.K., six have undergone considerable decline, one has been reintroduced after extinction (*Bombus subterraneus*), four are considered to be in decline and only six species are considered stable (Williams and Osborne 2009). Numerous stressors have been implicated as potentially contributing to losses of global bee populations including pesticides, parasites, pathogens, climate change and subsequently impaired nutrition, all of which can act both individually and in combination to negatively impact bee health (Figure 1.6) (Goulson et al. 2015; Smart et al. 2016). In order to effectively protect and conserve both wild and commercial bumblebee populations it is essential to understand the physiological mechanisms underpinning these interactions, both individually and in combination, in these important pollinators.

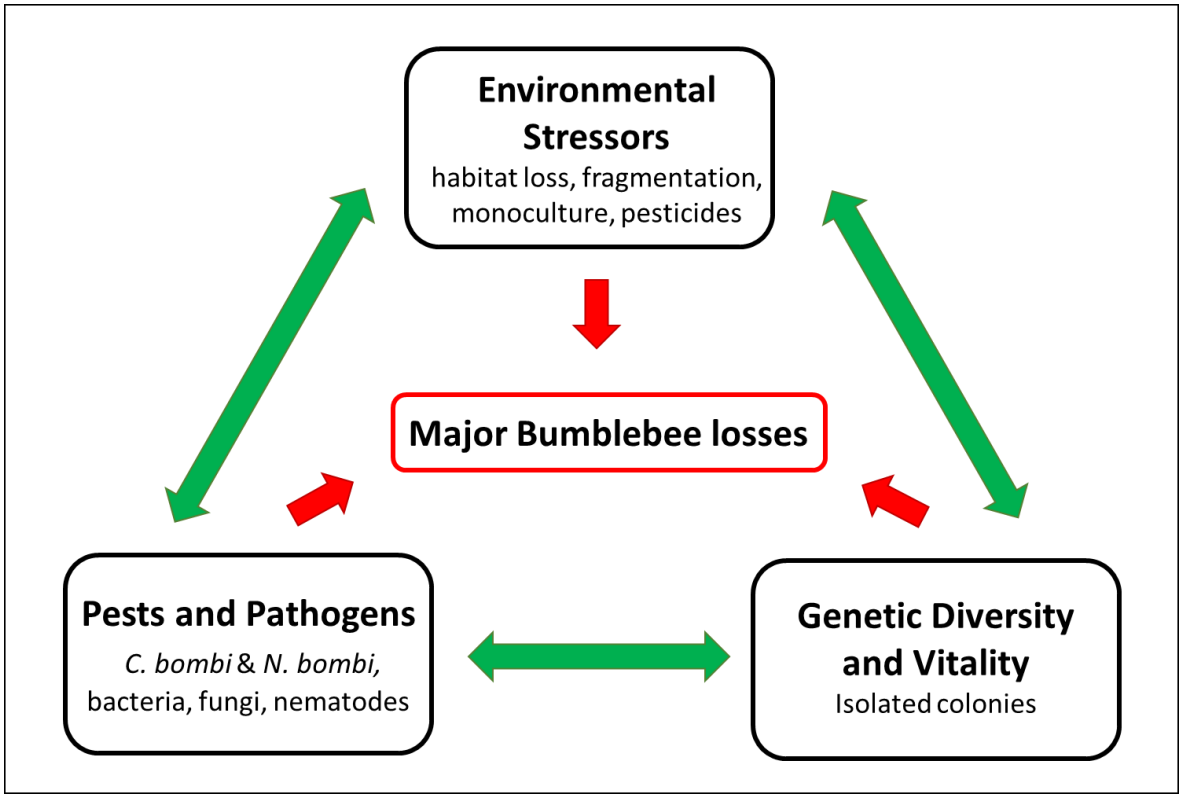


**Figure 1.6 Stressors associated with driving global bee declines.** Schematic outline of the stressors driving global bee declines and the potential interactions among multiple drivers. Black boxes represent the three main groups of stressors associated with bumblebee declines; red arrows represent direct pressures on bumblebees from stressors and green arrows represent interactions between stressors. Adapted from Potts et al. (2010).

### 1.5.1 Environmental stressors and reduced genetic diversity

Environmental stressors negatively impacting global bee populations include habitat fragmentation (Goulson et al. 2008), exposure to agrochemicals, climate change (Goulson et al. 2015) and decreased resource diversity (Biesmeijer et al. 2006). Changes in land use such as increased monoculture and urbanisation can reduce floral and habitat resources for bees (Rortais et al. 2005; Moritz et al. 2007) and also result in isolated colonies at risk of inbreeding and reduced genetic diversity (Goulson et al. 2008). Climate change can dramatically disrupt the normal phenological activity of bees at both colony and individual level (Stone and Willmer 1989; Potts et al. 2010) and change or limit the climatic distribution of bee species (Williams et al. 2007). Mismatches in temporal and spatial co-occurrence of floral resources and bees combined due to habitat fragmentation and seasonal changes can disrupt normal plant-bee interactions and as a consequence impact the ability of bees to obtain floral resources and pollinate (Memmott et al. 2007).

Reduced genetic diversity is a major driver of bumblebee declines, fragmentation and introduction of domesticated invasive pollinator species and subsequent interbreeding with wild populations can lead to decreased genetic diversity of native populations and could result in the extinction of local sub-species (Franck et al. 1998).

### 1.5.2 Pathogens and parasites

Most environments are abundant with infectious agents and invertebrates continually encounter frequent challenges to their immunocompetence (Loker et al. 2004). Bumblebees are no exception and in their role as pollinators they are frequently exposed to and infected by a broad range of parasites, parasitoids, and pathogens, including protozoans, fungi, bacteria, and viruses (Goulson et al. 2015). These pathogens often evolve a specialised virulence equilibrium with their hosts (Rabajante et al. 2015). However, this balance is delicate and any disturbance of the natural environment can unbalance this equilibrium, increasing the impact of pathogens as stressors on a population (Meeus et al. 2018). It is commonly accepted that parasites negatively impact bee health and drive bee declines both individually and in combination with other stressors (Goulson et al., 2015). Although numerous mechanisms are implicated in the spread of pathogens and parasites within bee populations, pathogen spillover from commercial colonies into wild populations has been recently and repeatedly demonstrated (Meeus et al. 2011; Whitehorn et al. 2013; Fürst et al. 2014). Commercial pollination is essential for numerous economically important crops worldwide, as demand for pollinator-dependent crops increases, so does the dependence on managed bees (Mallinger et al. 2017). The social nature and high population densities of most managed bee species (honeybees and bumblebees) facilitates the harbouring and spread of pathogens within colonies (Chen et al. 2006). In addition, the movement of colonies across large geographical regions for crop pollination services increases their potential to spread pathogens to wild bees including either novel or invasive pathogens (Goulson 2003a; Cameron et al. 2016).

Among the most prevalent pathogens infecting bumblebees are the protozoan trypanosome *Crithidia bombi* (Lipa & Triggiani) and the microsporidian *Nosema bombi* (Fantham & Porter) (Imhoof and Schmid-Hempel 1999). The obligatory gut parasite *C. bombi* is commonly observed in bumblebees with infection prevalence rates in workers of around 10–20% or higher (Shykoff and Schmid-Hempel 1991; Schmid-Hempel 1995).

Transfer between colonies occurs via horizontal transmission when healthy workers from uninfected colonies visit flowers previously contaminated by infected workers and ingest infective cells (Durrer and Schmid-Hempel 1994). Infected workers then return to the colony and cells are passed on to other colony members. The effects of *C. bombi* are characteristically varied and subtle, as expected for insect-pathogenic trypanosomes (Schaub 1994). In bumblebees, *C. bombi* has been shown to severely impact the survival of queens over wintering, colony founding success (Yourth et al. 2008), colony size, production of males (Brown et al. 2003) and foraging efficiency(Gegear et al. 2006). *N. bombi* infects the malpighian tubules, ventriculus, fat tissue and nerve tissues of bumblebees (Fries et al. 2001) and causes chronic rather than lethal effects (Macfarlane et al. 1995). Similarly to horizontal transmission in *C. bombi*, workers pick up *N. bombi* spores through exposure to contaminated shared food sources such as pollen or nectar (Durrer and Schmid-Hempel 1994; McIvor and Malone 1995; Fries et al. 2001).

Infection with these and other bumblebee parasites can substantially reduce the fitness and survival of individual bumblebees and the reproductive output of colonies (Brown et al. 2003; Otti and Schmid-Hempel 2008) resulting in (Genersch et al. 2006) lethal and sublethal effects at the population level. These include altered foraging behaviours (Otterstatter et al. 2005), reduction in their ability to monitor floral resources and make economic foraging decisions (Gegear et al. 2006). All of these changes represent the substantial cost of parasitism. The lack of understanding of the mode of transmission (Cameron et al. 2016) and bumblebee immune response to *C. bombi* and *N. bombi* is a major limiting factor in understanding whether these pathogens could cause the observed population declines.

### 1.5.3 Pesticides

The term pesticide includes a large variety of compounds such as insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators and others (Aktar et al. 2009). Classes of pesticides that are considered highly toxic to insect pests (i.e. direct contact toxicity days after application to crops) include carbamates, organophosphates, synthetic pyrethroids, chlorinated cylcodienes and chloronicotines (Claudianos et al. 2006). The introduction and use of synthetic insecticides coupled with the subsequent development and introduction of herbicides and fungicides has contributed dramatically to pest control and agricultural output (Aktar et al. 2009). These

benefits greatly contribute to pesticides dependence in modern agriculture (Ridgway et al. 1978) and although their use continues to be the primary strategy adopted globally to manage insect pest populations (Pimentel 2009) it is becoming ever more apparent that beneficial insects, including pollinators are being negatively impacted (McLaughlin and Mineau 1995; Di Prisco et al. 2013; Arena and Sgolastra 2014; Rundlöf et al. 2015; Czerwinski and Sadd 2017).

In recent years one of the more widely used pesticides are the neonicotinoids (Goulson 2013). These systemic insecticides upon application, spread throughout all plant tissues making them toxic to any insects that feed on the plant. This protects the plant from direct damage inflicted by herbivorous (mainly sap feeding) insects and indirectly by reducing insect vectored viruses (Simon-Delso et al. 2015). Exposure of bees to neonicotinoids occurs primarily through the ingestion of residues in the pollen and nectar of contaminated plants (Desneux et al. 2007; Sánchez-Bayo et al. 2016). Once ingested the neonicotinoid acts on the insect nicotinic acetylcholine receptor (Elbert et al. 2008) and has the ability to disrupt the neuronal cholinergic signal transduction (Fischer et al. 2014). They bind strongly to insect nicotinic acetylcholine receptors (nAChRs) in the central nervous system, causing nerve stimulation at low concentrations, but receptor blockage, paralysis and death at higher concentrations (Goulson 2013). Neonicotinoids bind preferentially to insect nAChRs in comparison to those of vertebrates, and are therefore selectively more toxic to insects (Tomizawa and Casida 2005). A major issue with the use of pesticides is the off-target effects on beneficial insects such as bees that become exposed during pollination activities (Botías et al. 2015). This exposure can have major negative impacts on the immunocompetence of individuals (Di Prisco et al. 2013), colony establishment (Woodcock et al. 2017), growth and development (Whitehorn et al. 2012). In some solitary bee species (*Osmia bicornis*) neonicotinoids have been shown to negatively impact reproduction (Woodcock et al. 2017), whilst in honeybees, exposure to neonicotinoids has been shown to increase the susceptibility of colonies to colony collapse disorder (Chensheng 2014).

In bumblebees, the disruption of neuronal cholinergic signal transduction can negatively impact homing, foraging (Stanley et al. 2016), mobility (Moffat et al. 2016), and in some cases can lead to the eventual death of target insects (Belzunces et al. 2012). In addition, the sublethal effects of pesticide exposure can detrimentally affect bumblebee fitness potentially increasing susceptibility to other stressors such as pathogens (Alaux, Ducloz,

et al. 2010; Fauser-Misslin et al. 2014). Furthermore, in social bees, pesticide-induced impairments on homing and foraging could have wide reaching impacts on overall colony function and may eventually lead to implications for the success of bumblebee colonies in agricultural landscapes and their ability to deliver crucial pollination services (Stanley et al. 2016). In addition, chronic and sub-lethal colony pesticide exposure which may not impact bumblebee fitness directly, but may enhance or act synergistically with other stressors such as pathogens and impair normal immune function (Goulson et al. 2008; Potts et al. 2010; Stanley et al. 2016; Baron et al. 2017). In order to effectively protect and conserve both wild and commercial bumblebee populations it is essential to understand the physiological mechanisms of the interactions both individually and in combination on these important pollinators.

### 1.6 Social immunity

Socially dwelling organisms such as *B. terrestris* are seemingly faced with a double-edged sword of high density and relatedness, a combination that facilitates elevated levels of parasite transmission toward which they direct both social and individual defences (Schmid-Hempel 1995; Barribeau and Schmid-Hempel 2013). In addition, to their individual immune response mounted via cellular and humoral defences, social insects also maintain a repertoire of behavioural adaptions to limit entry to the colony and infection of individuals by pathogens. These include; caste specialisation and co-operation (Barribeau and Schmid-Hempel 2013), highly specialised mechanisms of nest defence, recognition of nest mates, hygienic behaviour such as self and allopreening, and group effects known as social immunity (Cremer et al. 2007).

### 1.7 Innate immunity in insects

The first line of defence against pathogen entry in insects is a thick protective cuticle covered in antimicrobial proteins to protect their internal body cavity (Brey et al. 1993). If this barrier is broken by injury or infection pathogens are exposed to an inhospitable pH in the gut and a full spectrum immune response (Gerardo et al. 2010) mediated by the fat body, haemocytes and haemolymph (Krautz et al. 2014). Although invertebrates lack an adaptive antigen-based immune response they do possess an innate immune system (Cooper and Eleftherianos 2017). The innate immune response is composed of both

cellular and humoral elements which combine to contend with invasive micro-organisms that could potentially infect, kill or parasitize them (Figure 1.7) (Beutler 2004). This response is mediated through the insect barrier epithelia and the immune-related tissues such as the fat body and in the haemolymph (Gillespie et al. 1997). Cellular defences in insects involve immune cells called haemocytes that physically contend with invading microbes through processes including phagocytosis, encapsulation and nodulation (Lavine and Strand 2002). Humoral defences include responses mediated by soluble plasma proteins or fat bodies and includes melanin production, clotting and synthesis of antimicrobial peptides (AMPs) (Lemaitre and Hoffmann 2007). Recognition of parasites and pathogens induces processes such as melanisation, nodulation, cellular encapsulation and the prophenoloxidase (proPO) cascade (Barribeau and Schmid-Hempel 2013). These responses vary and are entirely dependent upon the type of pathogen encountered, with cellular reactions such as encapsulation more specific to large invaders such as nematodes or parasitoids and production of AMPs more likely in response to invasion by microbes (Gerardo et al. 2010).

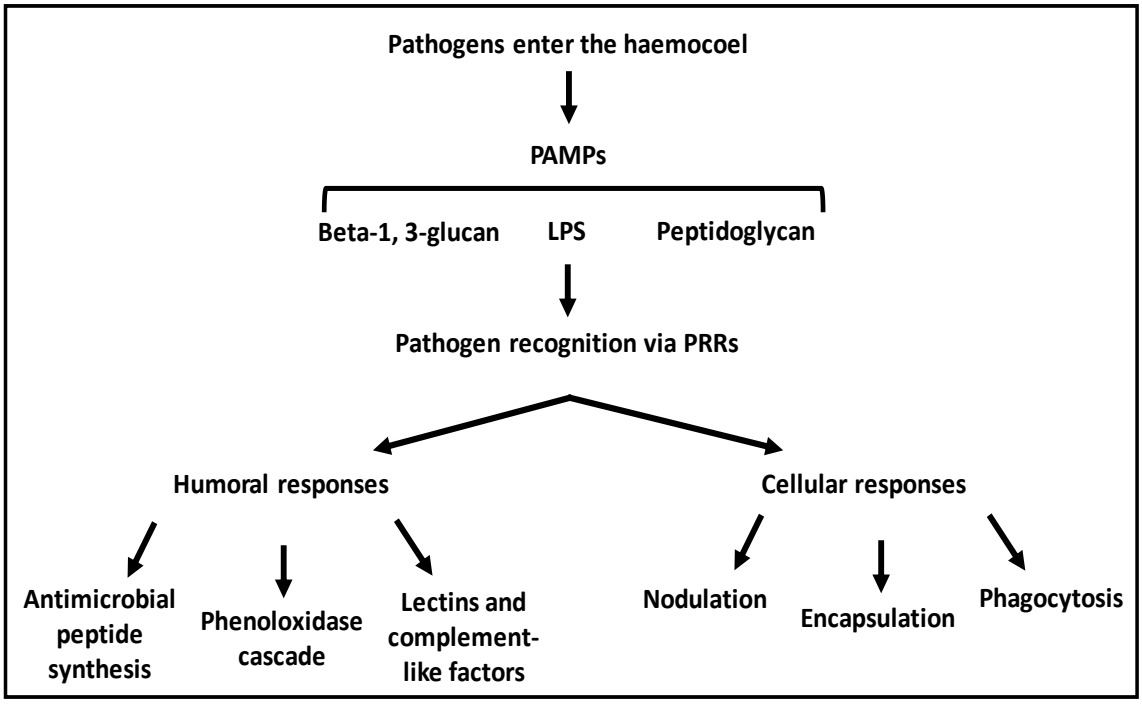


**Figure 1.7 General schematic of the cellular and humoral response elements of the insect immune response.** Pathogenic objects such as micro-organisms, which enter the haemocoel are recognised by a variety of pattern recognition molecules either free in the plasma or associated with various cell types. These pathogen recognition receptors (PRRs) recognise pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) on the surface of invading microbial cells. Recognition of pathogens within the haemocoel then leads to activation of cellular and humoral branches of insect immune response. Cellular defence includes activities such as phagocytosis, encapsulation and nodule formation, all of which work to trap micro-organisms within haemocytes and hence remove them from the haemocoel. Humoral responses include the antimicrobial peptides (AMPs), the phenoloxidase (PO) cascade and lectins and the complement system. Both PO and AMPs work to kill invading micro-organisms, while lectins and complement-like factors may act as recognition molecules and participate in the elimination of invading organisms. Adapted from Rowley and Powell (2007).

### 1.7.1 Pathogen recognition

When the preliminary defence behavioural, physical or mechanical strategies fail, pathogens enter the haemocoel or gut cavity of insects, an event that usually coincides with a wound or injury. The coagulation and melanisation cascades play central roles in insect immune responses to infection. As the elicitors of these cascades include cell wall components of micro-organisms, they represent major parts of the system by which infectious non-self molecules are recognised (Hoffmann 1995).

In insects, recognition of pathogens is achieved through pathogen recognition receptors (PRRs). These molecules are committed to recognising the presence of specific microbial or pathogen-associated molecular patterns (PAMPs) conserved across a broad spectrum

of microbial species (Takeuchi and Akira 2010). Recognition of PAMPs such as lipopolysaccharide (LPS) by the immune system initiates a number of distinct, interconnected signalling cascades such as Toll, Immune deficiency (Imd), Jun Kinase (JNK) and the Janus Kinase/ Signal transducers and activators of transcription (JAK/STAT) pathways (Strand 2008b; Mogensen 2009; Barribeau and Schmid-Hempel 2013) which cause the expression of immune related genes which express proteins that aid the immune response like cytokines or AMPs (Mogensen 2009). Unlike other pathogens, viruses are thought to be recognised via the recognition of damaged cells or apoptotic bodies (Zambon et al. 2005).

To date, there are few studies investigating the specific recognition system of pathogens in *B. terrestris*; and it is currently assumed that *B. terrestris* has some of the same general immune components as well characterised insects (Sadd et al. 2010). Although an immune study on the effect of the gut trypanosome *Crithidia bombi*, a common bumblebee parasite, on the expression of immune-related genes identified surface glycoprotein hemomucin as a potential recognition molecule involved in the detection of gut parasites (Schlüns et al. 2010) highlighting novel aspects of the bumblebee immune system. Binding of this molecule to a lectin was also found to be correlated with the expression of the antimicrobial peptide gene cecropin A1 in *Drosophila melanogaster* (Theopold et al. 1996). Currently it is only possible to identify known immune proteins that have been functionally characterised in model systems such as *D. melanogaster* however bumblebees may have species specific, novel or undiscovered immune proteins, defences and social behaviours that are involved in pathogen defence (Cremer et al. 2007; Barribeau et al. 2015). While elements of the *B. terrestris* immune response are similar to those of other insects, it is only through a comprehensive unbiased characterisation of the bumblebee immune response to a variety of microbial challenges that our reliance on homology-based comparisons will cease and our understanding of bumblebee immunity improved.

### 1.7.2 Haemocytes and cellular immunity

Cellular responses in insects are stimulated by wounding or parasite presence and mediated through the activity of haemocytes, that engage in a variety of central immune functions including phagocytosis, the formation of multicellular haemocyte aggregates, nodulation and encapsulation, and the production of reactive intermediates of oxygen and

nitrogen (Gillespie et al. 1997; Strand 2008b). In insect haemolymph, haemocytes and components of the PO system are always present or can be synthesised rapidly when required and therefore represent the first line of defence behind the cuticle (Schmid-Hempel and Ebert 2003).

Haemocytes are synthesized during embryogenesis and early developmental stages in hematopoietic organs and although they vary in size, structure and name, the primary functions of recognising foreign material and distinguishing between self and non self is conserved across all types (Park and Lee 2012). There are at least eight types of haemocytes in insects; prohaemocytes, plasmocytes, granular cells, coagulocytes, crystal cells, spherulocytes, oenocytoids and thrombocytoids. Although haemocyte density has been well characterised in bumblebees (Korner and Schmid-Hempel 2004; Moret and Schmid-Hempel 2009) little is known about the specific characteristics of the various subtypes and their corresponding function within the immune response. However, not all haemocyte-subtypes are found in every insect and at all times (Pandey and Tiwari 2012) as their presence, density and variety is subject to change over the course of an insect's life and in response to the presence of pathogens or wounding (Bergin et al. 2003).

Haemocytes are central to insect immune defence and play key roles in immune surveillance and defence against pathogens and parasites (Williams 2007). The insect cellular immune response encompasses all defence mechanisms and responses involving cells (Strand 2008a) and includes the initiation of wound repair to prevent pathogen entry into the haemocoel (Rowley and Ratcliffe 1978) through to the direct attack on invading microbes via encapsulation and aggregation (Williams 2007). Although circulating and sessile haemocytes are the principal cellular components, other cell types have immune function, including those in the fat body (Rowley and Powell 2007). The initiation of a response is dependent on the recognition of foreign material which induces effector responses and proteolytic cascades (Strand 2008a). Haemocytes detect foreign entities within the haemocoel either through direct interaction of surface receptors with molecules on the invading organism, or by indirectly sensing of humoral receptors which bind to and opsonise the surface of the invading organism. A number of inter- and intracellular signalling events must then coordinate effector responses (Lavine and Strand 2002). In *B. terrestris,* studies have shown that after infection with a microbial elicitor, such as lipopolysaccharide (LPS) or laminarin, there was an observed increase in haemocyte density illustrating their role in the defence and suggesting that sessile haemocytes are

being recruited to help contend with the infection. These recruited haemocytes also burst to release proPO in response to infection making these molecules available for melanisation (Korner and Schmid-Hempel 2004).

Once recruited to the site of damage or microbe location haemocytes can physically attach to their target in an attempt to restrict microbial proliferation and pathogenicity. The three main mechanisms involving haemocytes are encapsulation, nodulation and phagocytosis all of which are generally initiated by the presence of larger pathogens or parasites (e.g. nematodes, parasitoid eggs, protozoa etc.) in the haemocoel and haemolymph (Pech and Strand 1996).

#### 1.7.2.1 Encapsulation

Encapsulation occurs when larger microorganisms or damaged tissues are recognized and become surrounded by a multilayer of haemocytes (Rowley and Powell 2007). Haemocytes such as plasmocytes adhere and form multiple layers immobilising the invading body in a capsule. Both encapsulation and nodule formation ultimately lead to melanisation due to the activity of the enzyme phenoloxidase which is released from specialised haemocytes and activated via proteolytic cascades in the haemolymph (Cerenius et al. 2008). In bumblebees, decreased foraging activity is correlated with reduced encapsulation activity in males and foraging workers. In workers specifically, these decreases are thought to be due to the metabolic needs of foraging competing against important self-maintenance functions such as immune defence (König and Schmid-Hempel 1995), whilst there is still no known reason for this decrease in males (Baer and Schmid-Hempel 2006).

#### 1.7.2.2 Phagocytosis

Phagocytosis is a simple form of cellular defence initiated through the opsonisation activity of lectins that mark invading pathogens for destruction. Through this activity pathogens can then be recognised and engulfed by circulating haemocytes (Schmid-Hempel 2004). This then induces translocation of electrons from cytosolic NADPH to extracellular oxygen and produces superoxide which kills the pathogen but also results in inflammation and localised tissue damage at the site of infection (Baggiolini and Wymann 1990). Recently it has been shown that thioester-containing proteins that bind to pathogens in insects, may initiate cell lysis or phagocytosis (Blandin and Levashina

2004; Shokal and Eleftherianos 2017), although their link with phagocytosis in *B. terrestris* has yet to be confirmed.

#### 1.7.2.3 Nodulation

Through nodule formation or nodulation, micro-organisms become trapped in a central core of melanised haemocytes surrounded by a wall of flattened haemocytes, hence this cellular defence method isolates, neutralises and clears pathogenic particles from the internal environment of the host (Ratcliffe et al. 1985). The final stage in the contention of large pathogens/parasites is activation of PPO and deposition of melanin on the nodule (Pech and Strand 1996).

### 1.7.3 Humoral responses

In insects, humoral immune responses include all intracellular proteolytic cascades which regulate the initiation of melanisation and coagulation (Muta and Iwanaga 1996; Gillespie et al. 1997) and the synthesis of potent antimicrobial peptides (Meister et al. 1997). Central to the initiation and modulation of the insect humoral immune response are four canonical immune pathways; Toll, Imd (immune deficiency), JNK (Jun-kinase) and JAK/STAT (Janus kinase/ signal transducers and activators of transcription).

#### 1.7.3.1 Signalling pathways

The four principal pathways associated with insects humoral responses are characterised by the extracellular recognition of pathogens by membrane bound receptors that induce signalling cascades which ultimately result in the transcriptional activation of effector molecules in the nucleus (Erler et al. 2011). Although the components of each pathway vary slightly between insects, the majority of pathways and components are quite well-conserved across all taxa (Hultmark 2003). The Toll pathway is initiated by spätzle an extracellular cytokine in response to fungi and Gram-positive bacteria (Lemaitre and Hoffmann 2007). The Imd pathway is initiated in response to Gram-negative bacterial infection and is interlinked with the JNK pathway. Activation of the Imd pathway induces activation of the JNK pathway via TAK1 and can provide both positive and negative feedback on the expression of AMPs (Evans et al. 2006). The JAK/STAT pathway is thought to be activated by a haemocyte cytokine (Upd3) released in response to viruses or parasitoids binding to the domeless receptor during infection, this induces transcription of genes such as Vir-1 (Lemaitre and Hoffmann 2007). In insects this pathway is

necessary for initiating a number of complement-like proteins that are responsible for opsonising and inducing the phagocytosis of Gram-negative bacteria (Lagueux et al. 2000).

All four canonical immune pathways, in addition to antiviral processes, are present within *B. terrestris* (Colgan et al. 2011; Barribeau et al. 2015), yet some components are missing from each pathway when compared to model insects such as *Drosophila*. This is not surprising however, as *Drosophila* have an expanded immune repertoire to contend with their saprophytic life-style and bees may have many novel species specific immune responses which are yet undiscovered (Cremer et al. 2007; Barribeau et al. 2015).

In *B. terrestris* expression within these immune pathways varies across the developmental life cycle and sex (Colgan et al. 2011; Barribeau et al. 2015). All components of the Toll pathway are common to all life cycle stages except for Tube and Pelle, which are not expressed in the pupa and Tube being unique to the female adult stages (workers and gynes). In the Imd, JNK and JAK/STAT signalling pathways all components were expressed in all life stages, although the molecules TEPA, DREDD and TAK1 were observed only in workers (Colgan et al. 2011). With regards to sex, males in general appeared to have reduced expression of AMPs, PRRs and immune effectors compared to queens suggesting similarly reduced expression of the immune pathways they are associated with (Barribeau et al. 2015).

*B. terrestris* responses to Gram-negative bacteria and wounding involve increased expression of AMPs (abaecin, defensin 1 and hymenoptaecin) and a component of the Imd pathway transcription factor Relish, whilst wounding alone causes down regulation of the TEP A effector gene a member of the JAK/STAT pathway (Erler et al. 2011; Barribeau et al. 2015). Similarly, other studies have found links between the bumblebee immune response and elements of the mitogen-activated-protein kinase pathway, a large signal transduction pathway which can lead into other major signalling pathways such as the JAK/STAT pathway. Induction of AMPs through the Imd pathway has also been observed in bumblebees infected with the gut parasite *C. bombi* (Riddell et al. 2009). These findings illustrate the importance of these pathways in the bumblebee immune response to various pathogens and parasites.

### 1.7.3.2 Antimicrobial peptides

In terms of humoral mechanisms, invertebrate AMPs are key immune effectors in the neutralisation, elimination and destruction of pathogenic micro-organisms (Rowley and Powell 2007). These potent antimicrobial molecules have broad spectrum activity against both bacteria and fungi; and are key features of the innate immune response in insects such as Hymenoptera (Hoffmann 1995). AMPs are ubiquitous, lethal components of insect immunity synthesized *de novo* by the fat body and haemocytes in response to infection and rapidly released into the haemolymph (Bulet et al. 1999) at concentrations up to 100µM (Meister et al. 1997). They are systemically and regionally expressed within the insect and show a degree of specificity to the pathogen eliciting the response (Lehrer and Ganz 1999). These peptides are produced in large numbers at the site of infection and have a broad spectrum of antibacterial, viral, fungal, protozoan, and sepsis properties thought to be mediated through peptide-lipid or receptor interactions (Hancock and Diamond 2000).

In recent studies on the immune pathways of *B. terrestris* increased expression of AMPs (abaecin, defensin 1 and hymenoptaecin) has been observed in response to infection with Gram-negative bacteria and wounding (Erler et al. 2011; Barribeau et al. 2015; Sadd et al. 2015). Similarly, infection by *C. bombi* was found to induce differential upregulation of AMPs associated with the Imd pathway (Riddell et al. 2009) and reduction in AMP expression leads to an increase in *C. bombi* pathogenicity in infected bumblebees (Deshwal and Mallon 2014).

### 1.7.3.3 Melanisation, coagulation and the phenoloxidase cascade

The first line of defence and an initial physical barrier preventing pathogen entry and extreme haemolymph loss following wounding is the insect cuticle (Ashida and Brey 1995; Jiravanichpaisal et al. 2006). If this barrier is broken due to an inflicted wound, coagulation of the haemolymph assists in the efficient sealing of the wound in order to prevent pathogen entry and spread within the haemocoel (Bidla et al. 2005). Coagulation is a rapid and efficient activity which involves a combination of co-ordinated soluble and cell-derived factors (Söderhäll and Cerenius 1998; Jiravanichpaisal et al. 2006). Through this system pathogens which successfully breach the cuticle and gain entry to the haemocoel are exposed to a variety of cellular defensive mechanisms through which they

are trapped and immobilised, then humoral elements such as lectins and antimicrobial peptides participate in their neutralisation (Muta and Iwanaga 1996).

In response to pathogen infection, insects have the ability to mount numerous potent defensive reactions including the induction of proteolytic cascades which result in localised melanisation and coagulation (Eleftherianos and Revenis 2011). Proteolytic cascades play a major role in insect innate immune reactions as they can be induced more rapidly than immune responses which are dependent upon changes in gene expression (Cerenius et al. 2010). One of the most important proteolytic cascades in insects is the phenoloxidase (PO) signalling cascade, activation of this which is vital for initiation of responses such melanisation and coagulation.

In insects, wounding or the presence of harmful micro-organisms within the haemocoel results in melanin deposition around the damaged tissue or intruding organism (or object), known as melanisation. As a result, the melanin will physically immobilise the foreign entity and therefore limit or inhibit its capability to grow and spread. However, the most important outcome of melanin formation is the production of highly reactive and toxic quinone intermediates. It is well known that the activation of proPO plays a pivotal role in cuticular melanisation and sclerotisation in insects (Marmaras et al. 1996). Melanisation can be initiated by a variety of antigens but depends on the activation of the enzyme PO and the proPO cascade (Schmid-Hempel, 2005). Phenols are converted into quinones by PO which then polymerizes to melanin (Söderhall, 1998). This melanin is then deposited onto the surface of a pathogen where haemocytes aggregate to encapsulate it. In bumblebees increased proPO activity is linked with infection by the common gut pathogen *C. bombi*, which may indicate an important role for this response in defence and neutralisation of pathogenic entities within the haemocoel (Brown *et al.*, 2003).

Phenoloxidases are present in the insect haemolymph as zymogens which become activated upon detection of pathogens and produce indole groups which are then polymerised to melanin (Gonzalez-Santoyo and Cordoba-Aguilar, 2011). Upon the recognition of foreign objects proPO is activated by limited proteolysis, as a result cleavage of proPO to form PO will catalyse the early steps in the pathway to melanin formation (Figure 1.8) (Söderhäll and Cerenius 1998; Lemaitre and Hoffmann 2007). The next step in the cascade is performed by PO which catalyses the oxidation of phenols to quinones leading to spontaneous polymerization to form insoluble melanin. In addition

to the cleavage of proPO, triggering of this pathway results in the transcription of certain genes including those that encode serine protease inhibitors such as serpins which, through negative regulation, prevent premature and excessive activation (Eleftherianos and Revenis 2011). As quinone and melanin are both toxic to microorganisms, the presence of high PO activity in insects makes them more resilient to infection by microbes (Söderhäll and Cerenius 1998). In *B. terrestris* upon infection, prophenoloxidase (proPO) is activated and haemocytes are released from stocks, this activity represents the initial line of defence inside the cuticle (Korner and Schmid-Hempel 2004; Barribeau et al. 2015).

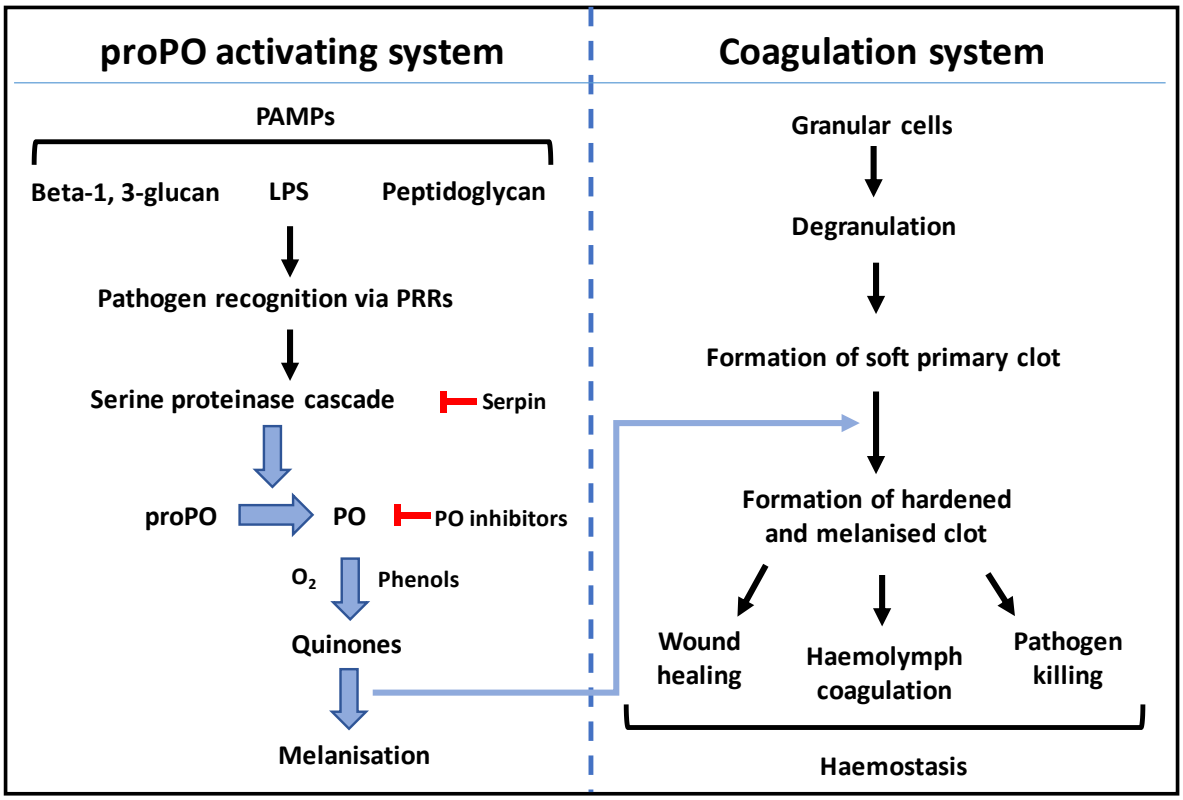


**Figure 1.8 Cross-talk between the phenoloxidase activating system, melanisation and haemostasis in insects.** General schematic illustrating cross-talk between prophenoloxidase (proPO)-activation, melanisation and the coagulation system in insects following pathogen infection. Phenoloxidase (PO) is known to be centrally involved in cross-linking and melanising the primary clot to produce the solid, melanised secondary clot, which is vital in sealing wounds and trapping pathogens. Lines and arrows represent the following signalling activities; activation (black), reaction (blue) and inhibition (red). Adapted from Cerenius and Söderhäll (2004); Eleftherianos and Revenis (2011).

## 1.8 Haemolymph

Insects have an open circulatory system through which haemolymph, their main extracellular fluid, is pumped by the dorsal vessel (Klowden 2007). As a result haemolymph is in constant and direct contact with all the internal organs and hence is the central medium responsible for transport and exchange of nutrients, hormones and waste materials between tissues and organs (Chan et al. 2006; Klowden 2007). Haemolymph is primarily composed of plasma and cellular haemocytes, with the main plasma components being; proteins, lipids, carbohydrates, amino acids, inorganic ions and hormones which are secreted into the haemolymph by the fat body (FB), haemocytes, epidermis and midgut epithelium (Gilbert and Chino 1974; Klowden 2007). Numerous factors such as developmental stage (Chan and Foster 2008), diet (Blatt and Roces 2001), temperature (MacMillan et al. 2015) and disease (Bergin et al. 2003) can influence the cellular and fluid composition of insect haemolymph.

In addition to its role in the transport of nutrients, waste products, hormones and water, haemolymph is also centrally involved in defensive and protective activities such as wound healing, coagulation and the immune response against invading micro-organisms (Lavine and Strand 2002). To effectively participate in these activities the haemolymph contains numerous vital immune components including haemocytes, antimicrobial peptides (AMPs) and elements of the signalling cascades such as serine proteases (Jiang and Kanost 2000) and the PPO signalling system (Chan et al. 2006). As the bumblebee haemolymph contains numerous elements of immunity and is regarded as the central medium responsible for transport and exchange of nutrients and hormones between tissues and organs, the analysis of its molecular composition essentially represents a snap shot physiological state of the bee. Hence, the haemolymph represents an excellent medium through which to investigate the molecular mechanisms associated with pathogen contention.

## 1.9 Fat body

Inside the cuticle is an aggregation of adipose tissue known as the fat body (FB), this essential organ is central to the maintenance of the insect physiological state and immuno-competence. The invertebrate FB is analogous to the liver and adipose tissue of vertebrates and is the main organ involved in energy metabolism and storage of proteins

and lipids (Azeez et al. 2014). However, their structures vary dramatically (Colombani et al. 2003) with the FB being unique to insects (Arrese and Soulages 2010). In insects the FB represents the primary site of intermediary metabolism, with protein synthesis and metabolic processes such as lipid, carbohydrate, amino acid and nitrogen metabolism occurring in the fat cells. In order to adapt to the insect's changing requirements the FB must perform multiple metabolic functions and integrate signals from other organs (Gäde 2004; Schooley et al. 2012). Proteins secreted by the FB into the haemolymph include; storage proteins used as amino acid reservoirs for morphogenesis, lipophorins responsible for lipid transport, vitellogenins for egg maturation and AMPs (Klowden 2007). The synthesis and secretion of most proteins into the haemolymph is a stage specific process (Arrese and Soulages 2010). The nutrient level stored in the FB controls many important aspects of the insect's life such as growth, morphogenesis and egg development (Mirth and Riddiford 2007). In addition to maintaining homeostasis and responding to the metabolic demands of the insect, the FB functions as an endocrine organ and plays a central role in mediating the humoral immune response. The structure and arrangement of the tissue allows for maximal haemolymph exposure which facilitates the fat body's rapid response to the insect's changing physiological needs through metabolic exchange (Arrese and Soulages 2010). This constant monitoring and mediation is essential during times of extreme energy demand like flight, when metabolic requirements increase from 50 – 100-fold (Beenakkers et al. 1985).

### 1.9.1 Fat body development and maturation

The invertebrate FB originates from cell clusters derived from embryonic mesodermal tissue. The organisation of cells in this cluster is determined by sub-patterning genes that control subdivision of the mesoderm and establish segment identity. This in turn activates transcription factors which specify the fat cell fate. Progenitor fat cells then proliferate forming the main three regions of the fat body; the dorsal fat cell projections, the lateral fat body and the ventral commissure (Hoshizaki 1998).

FB transformation during metamorphosis has been studied extensively in Diptera and Lepidoptera and is defined by a complete morphological change in body plan where some fat cells die through apoptosis whilst others are remodelled by autophagy in the latter stages of embryonic development (Arrese and Soulages 2010; Santos et al. 2015). In hemimetabolous insects the fat body remains into adulthood whereas in holometabolous

insects the FB cells disaggregate during metamorphosis and the organ can reform from these cells or the larval FB cells are completely removed and the adult FB is generated *de novo* (Arrese and Soulages 2010).

The functional role and cytological structure of the FB can change dramatically among insect developmental stages (Anand and Lorenz 2008). For example in the tobacco hornworm *Manduca sexta* the transition from larva to adult is accompanied by a switch from lipid storage to lipid metabolism (Tsuchida and Wells 1988). This switch to metabolism facilitates the degradation of larval FB triacylglycerol (TAG) to support their rapid growth, from typically several milligrams at hatching to 300 grams in the larval stage (Ziegler 1991). Similarly, lipid stores are mobilised during the pupal stage to support morphogenesis and are further relied upon as adult moths to meet the energy demands associated with both flight and reproduction (Ziegler and Van Antwerpen 2006).

### 1.9.2 Fat body structure

Fat bodies are complex lipid stores mainly located around the gut and adjacent to the integument of insects (Chapman and Chapman 1998). However, they may also be found as far as the head and thorax. In the haemocoel there is generally a peripheral or parietal layer located beneath the body wall and a perivisceral layer surrounding the alimentary canal (Figure 1.9) (Arrese and Soulages 2010).

They are pleomorphic and physically dynamic tissues that vary in structure between organisms, developmental stages, internal location and by differentiation between cells (Chapman and Chapman 1998). However, the general structure and arrangement of FBs are characteristically similar within species, with the same general layout observed within orders (Buys 1924). The FB is structurally heterogeneous and regional differentiation can be observed morphologically. This variation means that although the overall function of the tissue remains the same, there are some areas with multiple functions (Haunerland and Shirk 1995; Jensen and Børgesen 2000). A general characteristic conserved across all insects is the exposure of large areas to haemolymph, necessary for rapid metabolite exchange (Dean et al. 1985).

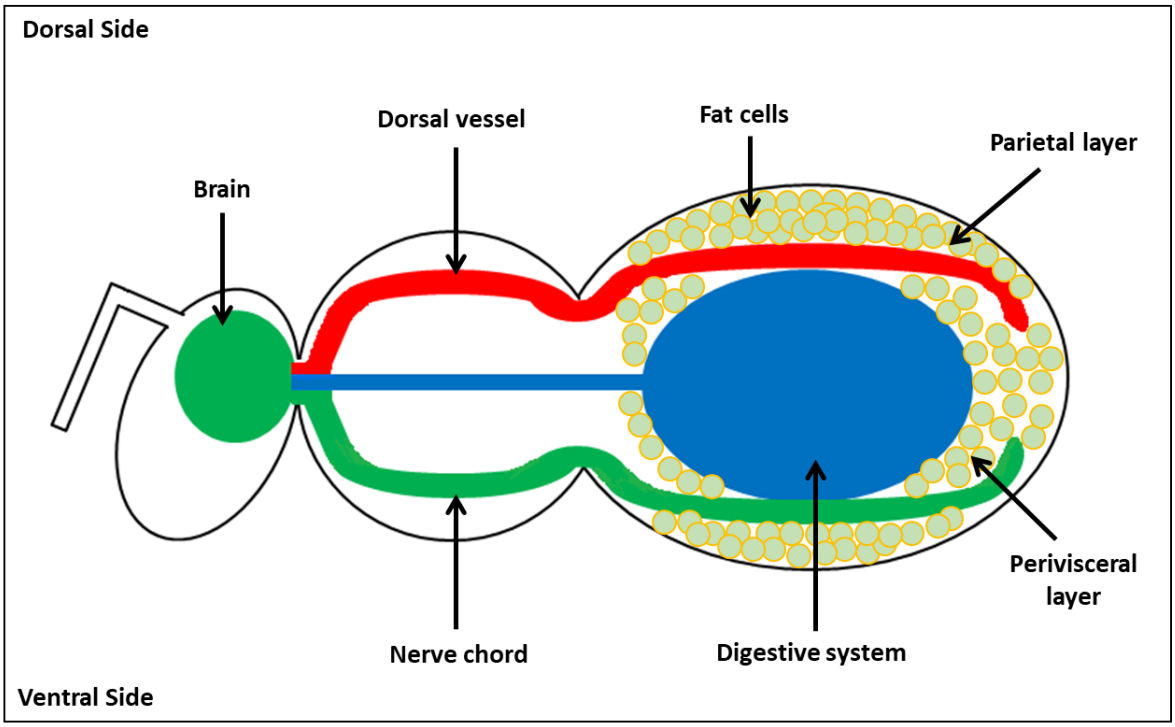


**Figure 1.9 General distribution of fat cells within the bee abdomen.** An overview of the insect body, highlighting the location of the fat body beneath the body wall cavity close to the dorsal vessel and alimentary canal can be observed. This location allows the fat body to interact easily and rapidly with both the circulatory and digestive systems. Adapted from Even et al. (2012).

They are pleomorphic and physically dynamic tissues that vary in structure between organisms, developmental stages, internal location and by differentiation between cells (Chapman and Chapman 1998). However, the general structure and arrangement of FBs are characteristically similar within species, with the same general layout observed within orders (Buys 1924). The FB is structurally heterogeneous and regional differentiation can be observed morphologically. This variation means that although the overall function of the tissue remains the same, there are some areas with multiple functions (Haunerland and Shirk 1995; Jensen and Børgesen 2000). A general characteristic conserved across all insects is the exposure of large areas to haemolymph, necessary for rapid metabolite exchange (Dean et al. 1985).

### 1.9.3 Cell types and structural composition

The FB has a highly variable cellular composition with only one cell type found in some orders and numerous cell types found in others. Six types of cells have been identified as part of the insect fat body including; adipocytes, urocytes, haemoglobin cells, mycetocytes, oenocytes and chromatocytes. The main cell type found in the majority of

fat bodies is the adipocyte or trophocyte. In orders where fat bodies are composed of a mix of cells the adipocyte is usually the most numerous cell variety. Adipocytes are composed of large lipid droplets containing triglycerides, glycogen and protein granules. Adipocytes are the target for several important hormones such as; neurohormones, juvenile hormones and ecdysteroids (Keeley 1978). Less common fat cells include haemoglobin cells, mycetocytes, oenocytes, urocytes and chromatocytes. Respiratory proteins such as haemoglobin were thought to be unnecessary in insects due to their efficient tracheal system. However, genes for haemoglobin have been present in every insect genome sequenced to date with haemoglobin cells identified in both fat bodies and tracheal systems of various insects (Hoshizaki 1998). Mycetocytes are fat cells present in cockroaches, aphids and some Hemipterans that contain symbiotic organisms inside their vacuoles (Dean et al. 1985). Oenocytes are ectodermal derived cells with links to synthesis of cuticular lipids, proteins and hydrocarbons and they can also be associated with either the fat or epidermal cells (Lockey 1988). Urocytes, are FB cells identified in cockroaches and locusts and are specialised to store urate (Arrese and Soulages 2010). Chromatocytes, the final type of fat cell have been observed in the aquatic larvae of two dipteran species (*Simuliidae* and *Thaumaleidae*). These cells are structurally distinctive with cells so flat the nucleus causes a bulge in their outline, and are thought to have been derived from adipocytes and therefore perform similar functions (Dean et al. 1985). Within bumblebees, all castes were revealed to contain both adipocytes and oenocytes (Alford 1969).

Structurally, fat bodies are commonly formed of thin sheets or ribbons of cells only one or two cells thick or of nodules of cells supported by connective tissue and trachea (Hoshizaki 1998). The structure of the FB as a tissue is determined by three main factors; cell size/shape, basal lamina and cellular junctions. The basal lamina is a porous sheet which functions to attach the FB to other tissues but also separate it from the haemolymph (Locke and Huie 1972; Ashurst 1982; Levinson and Bradley 1984). Although separated from the haemolymph, the FB still has the ability to both take up and secrete proteins through the basal lamina, suggesting that it may only serve as a barrier to contain some of the larger proteins and cells in the haemolymph (Dean et al. 1985). Between the fat cells are junctions which help form strong cellular adhesion within tissues. These junctions are thought to hold cells apart instead of together allowing for intracellular spaces used for the transport of proteins (Dean et al. 1985).

### 1.9.4 Storage and utilisation of energy and nutrients

Insects are constantly expending energy, so in the absence of food sources, like all animals, insects have the ability to store and utilise carbohydrates, proteins and fatty acids from their food as sources of energy (Arrese and Soulages 2010; Azeez et al. 2014). Fat reserves are the most important nutrient store in insects and are used to not only meet their daily life requirements for energy like flight (Beenakkers et al. 1985) but also the demands for major life events such as metamorphosis (Ziegler and Van Antwerpen 2006).

The two main energy reserves in animals are TAG and glycogen. Lipids are stored as TAG and carbohydrates are stored as glycogen. The mobilisation of these valuable energy reserves is controlled by two families of peptides; adipokinetic hormone (AKH) family and the insulin-like peptides (ILPs). AKH is produced and secreted by the corpus cardiaca, neurohemal tissue that is analogous to the vertebrate pituitary gland (Scarborough et al. 1984; Veelaert et al. 1998), whilst ILP is synthesized in the; median neurosecretory cells of the brain, corpus allatum, corpus cardiaca and some peripheral tissues including the FB (Hoshizaki 1998). Although fat reserve accumulation across insects varies; lipids are always the main component, representing more than 50% of the dry weight (Gilby 1965; Ziegler 1991). The ratio of glycogen to TAG can be altered across life cycles and in response to environmental stresses. TAGs are stored in a more anhydrous form than the hydrated, bulky glycogen and TAG be used directly for energy production through β-oxidation (Athenstaedt and Daum, 2006). TAG also has a higher caloric content per gram than glycogen (Arrese and Soulages 2010). Due to their high energy yield they account for more than 90% of the lipid stored in the FB (Bailey 1975; Canavoso et al. 2001).Through various metabolic pathways dietary fatty acids, carbohydrates or proteins (in the form of amino acids) can be converted to TAG and stored in the fat cells and it is this store that can be rapidly mobilised to support the metabolic and immune requirements of the insect when required (Arrese and Soulages 2010).

Glucose is essential in energy metabolism, heat generation and is also important in the chitinous exoskeleton. In the FB glucose is stored in its polymeric form glycogen which can be easily and quickly degraded to provide a source of glycolytic fuel (Steele 1982). Breakdown of carbohydrates to lipids by the insect metabolism is well studied and documented (Bailey 1975). The capacity of the FB for glucose lipogenesis is much

greater than that of glycogen synthesis which perhaps explains the high glucose to glycogen ratio content in the FB (Arrese and Soulages 2010).

Carbohydrates are stored in the FB as glycogen or trehalose (Hoshizaki 1998). Both are synthesized from uridine diphosphate (UDP)-glucose derived from dietary carbohydrates and amino acids. When trehalose, the major haemolymph sugar in most insects (Wyatt and Kalf 1957), reaches a certain concentration in the haemolymph the available UDP-glucose is converted into glycogen and stored (Arrese and Soulages 2010).

Lipophorins are the major lipoproteins found in haemolymph and act as re-useable, non-internalised transporters (Tsuchida and Wells 1988). During the passage to target organs or storage, most fatty acids are transported as DAG in lipophorins. One characteristic of lipid metabolism in insects is the tissue specificity of lipid delivery and hence the same lipophorins can selectively deliver energy to individual organs as required (Arrese et al. 2001). In many insect species lipids are also mobilised in the haemolymph in response to an immune challenge (Dean *et al*., 1985), although the nature of this mobilisation and the fate of the lipids are unknown. It is thought they could be involved in membrane biogenesis, haemocyte recruitment or as a source of energy (Arrese and Soulages, 2010).

### 1.9.5 Immune mediation

Although the FB is mainly associated with storage and metabolic functions, it is also involved in the regulation of haematopoiesis and innate immune homeostasis (Azeez et al. 2014). The *Drosophila* humoral immune response to microbial infection, including production of AMPs, pathogen recognition receptors (PRRs) and signalling cascades, is mainly undertaken by the haemocytes and fat cells (Keebaugh and Schlenke 2014). The fat cells of *Drosophila* have also been shown to synthesize the microRNA miR-8, which controls production and regulation of the AMPs drosomycin and diptericin (Choi and Hyun 2012). Many of the AMPs produced by the FB appear to be targeted at specific micro-organism e.g. drosomycin is active against fungi and defensins are active against gram-positive bacteria (Leclerc and Reichhart 2004).

The main stimulus for increased AMP expression is the recognition of microbial antigens such as peptidoglycan and lipopolysaccharides. In insects, recognition of pathogens is achieved through PRRs including; peptidoglycan recognition protein LE (PGRP-LE), peptidoglycan recognition protein LC (PGRP-LC) and peptidoglycan recognition protein

SA (PGRP-SA) stimulating the immune signalling cascades (Takehana et al. 2002). These molecules are involved in the recognition of specific microbial PAMPs conserved across a broad spectrum of taxa (Kimbrell and Beutler 2001). Following pathogen recognition, PRRs initiate the production and release of signalling molecules such as NFkB in the FB (Morishima et al. 1997) followed by the activation of immune pathways such as Toll, Imd and JAK/STAT that result in the expression of AMP genes and lysosomes (Figure 1.10) (Erler et al. 2011).

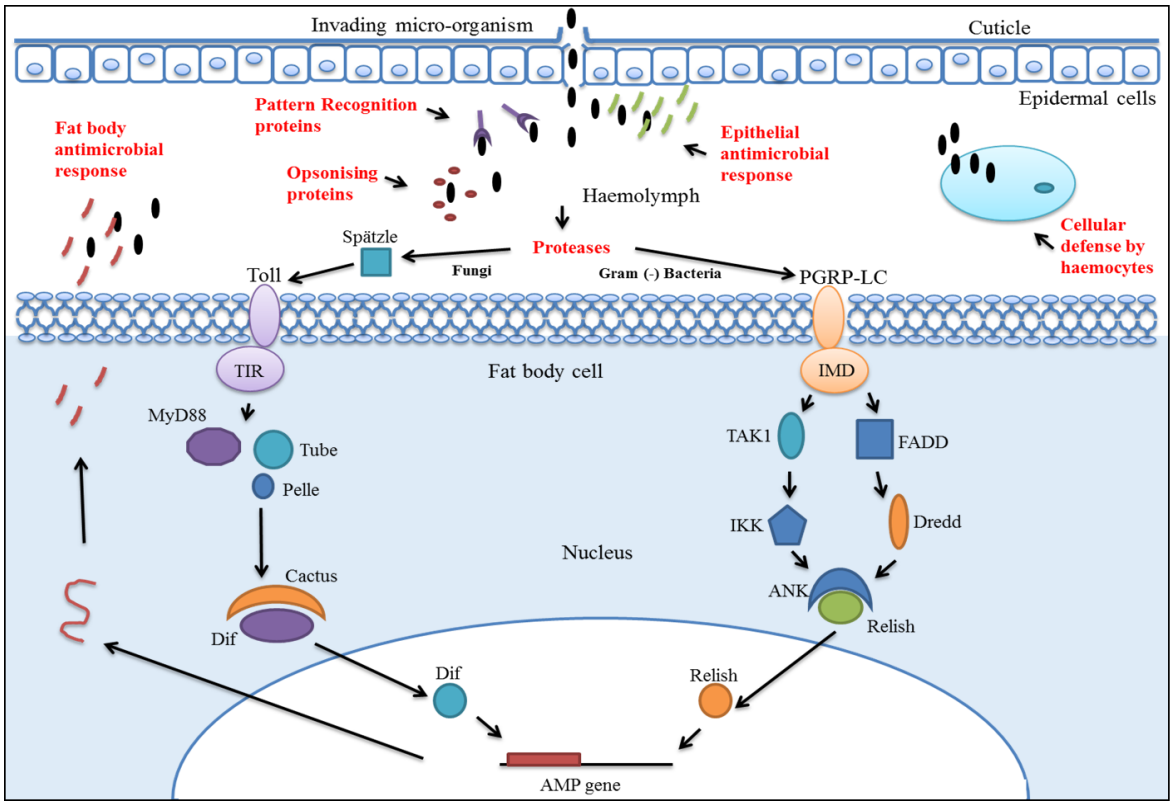


**Figure 1.10 Overview of insect immune response to various immunogenic elicitors.** Outline of all elements and sequential steps involved in the general innate immune response of insects. From the initial recognition of pathogen recognition receptors (PRRs) which recognise the presence of microbial antigens (lipopolysaccharide, Peptidoglycans and β-1, 3-glucans) to the pathways (Imd and Toll/Spätzle), proteolytic cascades (serpins, melanisation, coagulation), effector molecules (antimicrobial peptides and opsonisation factors) and cellular responses (phagocytosis, encapsulation) typically initiated in an insect in response. Adapted from Khush et al. (2002).

The location of the FB inside the cuticle and its large size, direct contact with the circulatory system and ability to rapidly synthesize and secrete antimicrobial peptides into the haemolymph so they can reach their effective concentrations contributes to this tissues efficiency as a lethal immune weapon against invading microbes (Tzou et al.

2002; Lemaitre and Hoffmann 2007). However, AMP production is not the only humoral immune response to take place in the FB. In *Drosophila* binding of different PGRPs can result in the production of other inducible factors such as cytokines, cytoskeletal remodelling components involved in phagocytosis and the prophenoloxidase (proPO) cascade (Azeez et al. 2014).

## 1.10 Application of molecular techniques to investigate the *B. terrestris* immune response

The importance of bumblebee health and survival has come to the fore due to their recent global declines with much research and study now focused on understanding the contributing factors to this decline. However, understanding the architecture of the bumblebee immune system is of equal importance to their continued survival, but is currently limited by a lack of experimental tools and resources including established cell lines, antibodies and functional information which for example would be readily available for model insect organisms such as *Drosophila*. This lack of resources in combination with the eusocial behaviour and a physiological state that is highly influenced by both internal and external colony environment means it is very difficult to ensure that sampled bees, even from a single colony, are similar from a genetic or physiological perspective. However, the recent completion of the *B. terrestris* genome has now provided the necessary foundation for post-genomic research such as proteomics on this key pollinator (Sadd et al. 2015) which now affords an unprecedented level of insight into the molecular basis of cellular, physiological and immunological processes and systems found within this organism.

The entire complement of proteins in a cell type or organism is referred to as the proteome (Oliver 2000). Proteomics is the term used to describe the global analysis of these proteins (Chambers et al. 2000) and is conducted through a combination of advanced techniques including mass spectrometry, amino acid sequencing, and downstream bio-informatic analysis to resolve, quantify, and characterise proteins (Blackstock and Weir 1999). Proteins represent the final functional element of gene expression and as genome sequence and protein function cannot be directly correlated due to host translational control and regulatory degradation activities (Gygi et al. 1999;

Lu et al. 2007), the expression levels of all proteins provide the most relevant and comprehensive characterisation of a biological system (Cox and Mann 2007).

High-throughput proteomics (HTP) aims to examine all dynamically changing proteins expressed by a whole organism, specific tissue or cellular compartment under defined conditions. The two main aims of this type of proteomic research are to identify all proteins derived from complex cell extracts and to quantify expression levels of all identified proteins (Kolker et al. 2006). Mass spectrometry (MS) –based proteomics has become one of the main tools increasingly used to accomplish these goals by identifying proteins through information derived from tandem mass spectrometry (MS/MS) and measuring protein expression by quantitative MS methods (Aebersold and Mann 2003; Walther and Mann 2010). As mass spectrometry is generally used in the analysis of samples containing thousands of proteins that can have large differences in concentration, sample preparation is of great importance to ensure the reproducibility and limit variability (Aebersold and Mann 2003).

Separation of cell lysates was originally carried out using gel-based methods. However, considerable bias exists in these methods particularly against proteins with isoelectric point extremes, high molecular weights and hydrophobic properties (Hart and Gaskell 2005; Garbis et al. 2005). This has led to increasing use of efficient in-solution protein digestion and preparation methods that result in the quantification and identification of thousands of proteins in a single sample (Gundry et al. 2009). Proteomic preparation for in-solution based analysis begins with denaturation of proteins via heat or surfactants to disrupt the protein tertiary structure and degradation of disulphide bonds via reduction and alkylation. This facilitates protein digestion by allowing proteases such as trypsin to maximally access all available cleavage sites. Peptides can then be subject to purification using desalting columns which remove the denaturants used in the digestion. Peptide mixtures can then be separated using online reverse phase liquid chromatography and analysed using mass spectrometry (LC–MS) with tandem mass spectrometry (LC–MS/MS) (Aebersold and Mann 2003; Gundry et al. 2009). Proteins may be isobarically or chemically labelled prior to analysis by mass spectrometry or analysed and quantified without a label (Label Free Quantification). The mass spectrometry of haemolymph and fat bodies samples conducted during this project followed this general proteomic preparation workflow.

## 1.11 Overview of thesis objectives

The primary goal of my research is to comprehensively elucidate the *Bombus terrestris* immune response and collect crucial information about the effects of biotic and abiotic stresses on our dwindling bumblebee populations. In insects the fat body, haemolymph and haemocytes of are central to mediating immune responses. Hence, using predominantly molecular based discovery tools such as mass spectrometry on the haemolymph and fat body, in tandem with cellular based bioassays, this project aims to piece together and investigate the primary components of the bumblebee immune system and other elements of the response to microbial challenge. As factors such as reduced genetic diversity, increased agrochemical insecticides and higher pathogen prevalence continue to have unknown individual and combinatorial effects on bees, studies on their contribution to bee declines are essential.

To provide in-depth functional analysis of the two key physiological elements of *B. terrestris* immunity, the haemolymph and FB, label-free quantitative (LFQ) mass spectrometry was applied to samples. It was anticipated that through mass spectrometry based proteomic approaches my research would, i) facilitate the identification and quantification of high numbers of proteins from a single sample type, ii) provide an objective and direct view of functional proteins, and iii) allow for quantitative analysis between treated and control bumblebee groups. Through this unbiased quantitative approach invaluable functional information on the proteins, processes and pathways active within these key immune samples will be obtained. Characterisation of the *B. terrestris* immune response will be facilitated through the stimulation of a response with a representative fungus (*Metarhizium anisopliae*), Gram-negative (*Escherichia coli*) and Gram-positive bacterium (*Staphylococcus aureus*). The microbial elicitors chosen are known to stimulate central insect immune pathways and hence induce a full spectrum immune response. This will allow for the identification of pathogen associated changes in both classical and novel immune proteins. In addition, gene ontology analysis will be conducted on all proteomic data to reveal the key associations between these stressors and the numerous biological processes active within the FB and haemolymph. Elucidating the general and specific immune signatures, will facilitate an understanding of the role of these proteins and pathways in a successful bumblebee immune response and provide a powerful platform from which future immune studies can be based. To

understand how pesticides, effect this response, FB and haemolymph from bees exposed to neonicotinoids and *M. anisopliae* both individually and in combination will be collected and analysed similarly. By comparing the normal immune profile to that of the pesticide exposed one, it is hoped that any potential effects on immunity will be revealed.

To compliment the proteomic based investigations, the morphology, density and activity of the cells central to *B. terrestris* immunity (haemocytes and fat cells) will also be characterised. As haemocyte density and activity are invaluable bioindicators of bee health these parameters will be quantified from bees challenged by both micro-organisms and pesticides, to understand and establishing profiles of cellular immune activity. In addition, the morphology of fat cells will be similarly studied to investigate the effect of these stressors on the size, quantity and composition of these central cells. It is anticipated that this research will provide novel insights into the bumblebee immune system and role of the fat body and provide an understanding of the individual and combinatorial effects of multiple stressors on bumblebee systems. Given the potential role of pathogens and pesticides in driving global bumblebee declines, understanding the architecture of the bumblebee immune system is of central importance to its continued survival.

### 1.12 Thesis hypotheses

Hypothesis-driven research has dominated scientific methodology over the last century and undoubtedly led to numerous important scientific advances (Weinberg 2010); however, developed frameworks of accumulated scientific knowledge are essential in order for meaningful hypotheses to be posed and tested (Tripathi et al. 2018). This information rich framework is not common in bumblebee studies but can be achieved through discovery-driven research projects. High throughput proteomics offers the opportunity to conduct a study in the absence of a clear hypothesis to test. This approach affords an unbiased, high throughput view of the changes that occur (in this case in the key immune isolates haemolymph and fat bodies) in bees under immune challenge. To date large scale investigations of immune response in bumblebees have characterised the mRNA levels of a panel of immune genes, or biochemical assays to assess enzyme activity in response to infection or wounding (Korner and Schmid-Hempel 2004; Riddell et al. 2011; Richter et al. 2012). Such targeted approaches are restricted and hence can suffer heavily from bias. What can be framed as a testable hypothesis suffers important

limitations based on what we can measure and what we already know. For example, using qPCR to measure mRNA levels is based upon the assumption that the target genes are involved in the immune response in the first place. More importantly such approaches assume that there is a strong correlation among transcript level and final protein abundance which is very rarely the case (Maier et al. 2009; de Sousa Abreu et al. 2009; Payne 2015). The discovery-based approach adopted here is free from such restrictions and biases and by examining the thousands of proteins routinely identified in a single sample between treatments, a broader systems level view of the response will be provided.

Although hypotheses can be generated prior to this project such as “that *E. coli* infection will alter the proteome of the bumblebee fat body”, I prefer to reserve the opportunity to generate hypotheses in light of my proteomic and cellular experiments, which will result in very focused and useful hypotheses that can be tested by future research. This approach aligns to one of the mantras of my current lab (Carolan Lab Maynooth University) that "the best hypotheses follow and do not precede proteomics" (Dr. J. Carolan personal communication).

# Chapter 2

## Materials and methods

## 2.0 General chemicals, reagents and sterilisation procedures

All chemicals were analytical, molecular or proteomic grade unless otherwise stated and were obtained from Sigma-Aldrich Co. Ltd. (Arklow, Ireland). For the analysis of proteins and preparation for mass spec preparatory, relevant products were sourced as following; Qubit™ fluorometer Protein quantification kits (Invitrogen, Thermo), 2D clean-up kits (GE Healthcare Life Sciences), C18 spin columns (Pierce), ZipTip® (Merck Millipore), sequence grade trypsin and Protease Max Surfactant trypsin enhancer were purchased from Promega. All SDS gel page reagents; Ultrapure Protogel, 4X Protogel, Resolving Buffer stock, acrylamide stock and tetramethyl ethylenediamine (TEMED) solution were sourced from National Diagnostics. Complete mini-series Protease inhibitor cocktail tablets for addition to proteomic collection buffers were sourced from Roche. All distilled $H_2O$; $dH_2O$ was purified with the Millipore Milli-Q apparatus to obtain milli-Q water 18MΩ.

## 2.1 Maintenance and preparation of bacterial and fungal strains

### 2.1.1 Bacterial culture mediums

Luria Broth (LB) nutrient agar plates were prepared by adding 40 grams of BD Difco™ powder per litre to $dH_2O$ and autoclaving at 121°C for 15 mins. Once hand warm the agar was poured into petri dishes (90 mm) and left to stand until fully set. Once set, plates were sealed and stored at 4°C until further use. Nutrient broth was prepared in a similar manner by adding 25 grams of LB broth powder (BD Difco™) to 1 litre of $dH_2O$ and sterilizing as above.

### 2.1.2 Maintenance of bacterial strains

*Escherichia coli* (DH52/pZB101 A1024230), a non-pathogenic lab strain and *Staphylococcus aureus* (ATCC-33591) were spread aseptically and maintained on LB nutrient agar (Table 2.1). Using a sterile loop from a single colony liquid cultures of LB nutrient broth were inoculated and grown overnight in a shaker at 200 rpm at 37°C to the early stationary phase. Insect immune response following treatment with peptidoglycan, lipopolysaccharide and β-1, 3-glucan is lower than that following treatment with live

pathogens (King and Hillyer 2012; Charles and Killian 2015). Hence, live micro-organisms were used for all infection-based experiments.

**Table 2.1 Microbial species and strains.** The species name, strain and source of all micro-organisms cultured for bumblebee infection experiments.

| Species | Strain | Source |
|---|---|---|
| ***Escherichia coli*** | DH52/pZB101 A1024230 | Antimicrobial resistance and microbiome laboratory, Maynooth University |
| ***Staphylococcus aureus*** | ATCC-33591 | Medical mycology unit, Maynooth University |
| ***Metarhizium anisopliae*** | F52 | Behavioural ecology laboratory, Maynooth University |

### 2.1.3 Bacterial cell culturing and enumeration

As described above liquid cultures of *E. coli* and *S. aureus* were grown overnight (37°C, 200 rpm) to reach early stationary phase or an $OD_{600}$ of 1.0. To prepare bacterial cells for infection work cultures were centrifuged (2,000 x *g* for 1 min), the supernatant was discarded, and the pellet resuspended in sterile Ringer's solution (Ringer hereafter) (NaCl, KCl, $CaCl_2$, $NaHCO_3$, Merck), this wash step was repeated twice more. The cell density of cultures was measured using a spectrophotometer (Eppendorf BioPhotometer) to get the $OD_{600}$. Using a standard light microscope (Olympus, CX22) and a Neubauer haemocytometer cell counts were then confirmed.

### 2.1.4 Fungal culture mediums

Potato glucose agar (PGA) was prepared by adding 50 grams (g) of extract per litre of $dH_2O$ and autoclaving at 115ºC for 15 min. Once the agar was hand hot it was poured into Petri-dish plates. Liquid cultures were prepared in yeast extract peptone dextrose broth (YEPD) (10 g yeast extract, 10 g glucose, 10 g bacteriological peptones in 1 litre of $dH_2O$).

### 2.1.5 Culture and maintenance of fungal strains

The Novozymes product Met 52 (*M. anisopliae*), was maintained as conidiospores on rice grains (Table 2.1). PGA plates were inoculated with conidiospores under aseptic conditions and grown at 25°C for 7-12 days. Once conidial growth was established the spores were used to inoculate blastospore cultures. To culture blastospores a loop of

virulent conidiospores from 15-day old culture was used to inoculate YEPD broth. Cultures were incubated in a shaker at 25°C at 200 rpm for three days to obtain blastospores.

### 2.1.6 Fungal cell preparation and enumeration

To isolate blastospores, cultures were filtered through two layers of miracloth and one layer of filter paper (Whatman™) to remove hyphae and cellular debris. The filtered culture was then centrifuged (2,000 x *g* for 1 min), the supernatant removed, and the spores resuspended in Ringer. This wash step was repeated twice to ensure the full removal of culture medium. Fungal cell concentrations were determined using a haemocytometer. Cells were diluted in sterile Ringer, pipetted up and down until suspension was homogenous and cell suspension (10 µl) was loaded under the cover slide of the haemocytometer before being counted under a light microscope at a magnification of 40X.

## 2.2 Insects, treatments and dissections

### 2.2.1 Bumblebee maintenance

Commercial *Bombus terrestris audax* colonies were maintained at 24±1°C (60% ± 5 relative humidity (RH)) in continuous darkness. Colonies were fed *ad libitum* on pollen (supplied by Agralan) and sucrose solution (0.5M). For the duration of experiments bees were housed in wooden holding boxes, with a perspex lid (depth 12 cm x width 16 cm x length 24 cm) at the same ambient environmental conditions as above. All necessary colony work was performed under red light conditions to minimise stress. Colonies throughout this thesis were sourced from two commercial suppliers Koppert Biological Systems and Biobest (via Agralan UK). Initial experimentation in this project was completed using Koppert colonies however once colonies developed exclusively for research purposes became available through Agralan, only research colonies were used from this point forward.

### 2.2.2 Bumblebee infection procedure

To prepare the bees for infection individuals were removed from the holding box one at a time and anesthetised using $CO_2$. The bee was then placed on a wax dissection platform and pinned down on its back by securing a strap across its thorax. Once the bee was in

position the abdomen was then gently stretched by using sterile tweezers to expose the site of injection, the interpleural membrane between the tergites of the abdomen. In order to infect the bees a small aliquot of each treatment was pipetted out onto parafilm and then drawn up into the tip of a syringe (Myjector® U-40 Insulin, Terumo®). The treatment was delivered via injection between the 3$^{rd}$ and 4$^{th}$ tergites on the left side of the abdomen. Wounding was limited by allowing only the very tip (2 mm) of the syringe to enter the bee. Post-infection each bee was transferred to new a holding box of the same dimensions and at the same ambient conditions. All bees subjected to the same treatment were infected and housed together.

### 2.2.3 Haemolymph collection

Bees were prepared for collection following the same procedure as above (section 2.2.2). While the bee was slightly stretched a small incision was made between the terga on the lower abdomen on the right-hand side of the body using sterile dissection scissors (Watkins and Doncaster). Haemolymph was drawn up using a micropipette capillary tube inserted into a pipette tip (p10) which was placed at the wound opening.

Haemolymph (approx. 20 µl) was deposited into a microtube on ice containing 50 µl of ice-cold filter sterilised phosphate buffered saline (fsPBS). All collections were kept on ice and had a further 50 µl of buffer added to them prior to centrifugation. All collections were spun down at 8,000 rpm for 5 minutes at 4˚C to remove cells and other debris. After centrifugation the supernatant was removed to a new microtube avoiding the pellet at the bottom of the tube. The samples were then stored at -70˚C for analysis at a later date.

### 2.2.4 Fat body dissection and collection

Bees were removed from their holding boxes, anaesthetised using $CO_2$ (as above) and euthanized. The connection between the thorax and the abdomen was then severed and the abdomen was pinned on a wax dissection bed. Abdomens were positioned slightly stretched to expose the pleural membranes of the ventral side of the abdomen. Once the abdomen was firmly pinned, sterile scissors were used to cut around the edge of the cuticle to allow the ventral side of the abdomen to be peeled back to reveal the contents of the haemocoel. The wax bed was then placed under a dissection microscope for removal of the organs and collection of the fat cells.

Fat cells were collected from both dorsal and ventral sides of the abdomen for every dissection. Sterile tweezers were used to gently pry the fat cells from muscle and trachea. Once the fat cells were dissected, they were removed to 300 µl of fsPBS. Samples were then homogenised using a motorised pestle (60 s per sample) and sonicated to release intracellular proteins (3 x 10 s). Homogenates were then centrifuged (5 mins at 9,000 *g*) to pellet cellular debris and the supernatant from each sample was then removed to a new tube and stored at -70°C for further analysis.

### 2.2.5 Pesticide preparation and exposure

A clothianidin stock solution (Fluka Analytical) was diluted to a concentration of 5 ppb in sucrose solution (0.5M) with acetone. Control bees for all pesticide work received sucrose solution containing the same concentration of solvent (acetone) as the neonicotinoid groups. In preparation for pesticide exposure all bees were starved for two hours by removing feeders and pollen from holding boxes. To expose bees, they were moved to a perspex holding box and provided aliquots (35 µl) of clothianidin solution. Bees were observed during this exposure period and were only considered exposed when they had consumed all droplets presented to them, bees that did not fully consume all provided pesticide solution were excluded from all further analysis.

## 2.3 Bioassays

### 2.3.1 Encapsulation/melanisation assay

The insertion of nylon filaments in the haemocoel provokes the encapsulation of the filament as well as the closure of the wound. The strength of this combined response can be assessed by measuring the degree of melanisation of the filaments.

#### 2.3.1.1 Filament preparation and inoculation

Nylon filaments (Efco creative emotions) were prepared under a stereoscopic microscope (Leica, EZ4) by measuring and cutting lengths (2 mm) of filament (diameter Ø 0.15 mm) in 100 % ethanol (Honeywell) using a sterile blade. To minimise any effect of the ethanol on the melanisation response all filaments were washed in sterile Ringer prior to insertion. To inoculate workers, they were anaesthetized with $CO_2$ and fixed on a wax bed as previously described (section 2.2.4). Abdomens were then gently outstretched

using tweezers to hold the last tergite on the abdomen and extend the abdomen down to reveal the interpleural membranes. Once outstretched a sterile needle was used to make a small entry wound between the 3rd and 4th sternites, through which the sterile monofilaments were inserted.

#### 2.3.1.2 Dissection and fixing

To remove the filaments abdomens were prepared as outlined in above (section 2.2.4). Once the abdomens were dissected most filaments were located near the site of infection. All recovered filaments were then subject to a two-step fixing procedure prior to mounting. Filaments were firstly incubated for one hour in a sterile watch glass containing ethanol and then transferred to a watch glass containing xylenes for a further hour. Control filaments (non-inserted filaments) were subject to the same fixing treatment prior to mounting.

Mounting slides were standard microscope slides modified to hold the filaments in line on the slide. To create an area on the slide to contain the filaments two coverslips were secured to the slide to create a holding gap (approx. 5 mm). Filaments were placed along this gap and Eukitt® was added (2 – 3 drops). Once the filaments positions were adjusted and air bubbles were removed a third coverslip was placed on top to seal the filaments in place. Slides were allowed to set over night at room temperature prior to imaging.

#### 2.3.1.3 Image analysis

The mounted filaments were photographed under constant magnification (4X), brightness (3.5) and contrast conditions using a Leica DM500 connected to LAS EZ imaging software (version 3.3). To analyse the degree of melanisation on filaments images were opened in ImageJ image analysis software version 1.51j. Images were scaled, converted to 8-bit grey-scale and made binary before measuring melanisation. The grey scale index (GI) measurement was used to obtain a representative value of filament melanisation. To measure GI the inner area of each filament was selected (using the tracing option) and measured. GI was measured on a scale from 0 (pure white) – 255 (pure black), hence higher amounts of melanisation correspond to higher GI measurements. To obtain the true GI measurement of each filament the mean grey values of control filaments were deducted from mean grey values of implanted filaments to account for the background level GI measurements of the nylon filaments.

### 2.3.2 Haemocyte counts

Haemolymph (10 µl) was collected (following set-up in section 2.2.3) into a 1.5 ml microcentrifuge tube containing 50 µl of ice-cold Ringer, the sample was then mixed by gently pipetting up and down (Moret and Schmid-Hempel, 2009). 10 µl of subsample was added to 10 µl of trypan blue (0.4% w/v), gently mixed and incubated for 3 mins at room temperature. 10 µl of this mix was loaded on a haemocytometer, left to stand for 10 minutes to allow the haemocytes to settle and finally, counted to measure the concentration of haemocytes.

### 2.3.3 Statistical analysis

Values from bioassays were square-root transformed to homogenise the variance and the relationship between haemocyte density or encapsulation response and treatment was investigated using univariate analyses of variance (ANOVA), differences were considered statistically significant at $P<0{\cdot}05$ and groups were compared using post hoc pairwise Tukey's comparisons. All statistical analysis was completed using GraphPad Prism v. 5.0 (www.graphpad.com).

## 2.4 Imaging and microscopy

### 2.4.1 Confocal fluorescent microscopy

To characterise ultra-structures of fat cells and haemocytes, fluorescent microscopy was conducted using the nuclear stain diamidino2-phenylindole (DAPI) (1 mg/ml) (1:500), the lipid stain Nile red (0.5 mg/ml) (1:1000) and the F-actin stain Rhodamine Phalloidin (1:1000) (Biotium Inc.). All fluorescently stained cells were imaged and analysed using the Olympus Fluoroview 1000 confocal laser scanning microscope (CLSM) under the supervision of Dr. Ica Dix, Biology Dept, Maynooth University.

#### 2.4.1.1 Fluorescent microscopy of fat cells

Fat cells were dissected (section 2.2.5) and collected into fsPBS. Collected fat cells were then added to 500 µl of fixative solution (4% paraformaldehyde) and incubated at 4°C for 60 min at to fix the cells. They were then washed twice and stored in fsPBS. Two fluorescent dyes were used to investigate the structure of fat cells; DAPI and Nile red. To

fluorescently stain fixed histological tissue and live cells DAPI (1 mg/ml) (15 µl) was added to the specimen and incubated for 15 mins, the slide was then washed with fsPBS and this step was repeated using Nile red (0.5 mg/ml) (15 µl). To stain live cells, the fat body was extracted and directly added to a slide and the dye was added as above. After the addition of the dyes a coverslip was placed on the slide and excess liquid was removed by placing Whatman paper at the edge of the slides to prevent cells from moving freely under the slide. The cover slides were then fixed *in situ* by applying a clear sealing solution around the perimeter of the slide which also prevented the sample drying out.

#### 2.4.1.3 Fluorescent microscopy of haemocytes

Cytological stains of haemolymph were prepared by smearing 10-15 µl aliquots across Polysine™ slides (VWR) and allowing 10 – 30 mins for haemocytes to adhere and haemolymph to dry. Prior to CLSM imaging cells were stained with diamidino2-phenylindole (DAPI) and rhodamine phalloidin (1:1000). To stain cells 20 µl of dye was added to the sample and incubated for 20 mins, the slide was then washed with fsPBS and this step was repeated using rhodamine phalloidin. A coverslip was then placed on the specimen and the excess liquid was drawn out by placing Whatman paper at the edge of the slides to stop cells from floating freely under the slide. The cover slides were then fixed in position by applying a clear sealing solution around the perimeter of the slide which also prevented the sample drying out.

### 2.4.2 Histology and cytology

#### 2.4.2.1 Haemocyte cytology

Cytological stains were prepared by smearing haemolymph (10-15 µl) across Polysine™ slides (VWR) and allowing 10 – 30 mins for haemocytes to adhere and haemolymph to dry. Preparations were then fixed in paraformaldehyde (4%) for 15 mins and rinsed three times with PBS. The slides were then stained as follows: incubated with haematoxylin (15 sec), thoroughly washed in $H_2O$, left to dry, stained with eosin (15 sec), washed in 70% ethanol to remove excess dye, washed in $H_2O$ and left to dry (adapted from Martin et al. 2007). Finally, mounting medium (Eukitt®) and a coverslip (22 x 50 mm) (VWR) was applied to all preparations. Haemocyte images captured at 100X using oil immersion on Leica DM1000.

### 2.4.2.2 Fat body histology

#### 2.4.2.2.1 Dissection and fixing

Bees were removed from treatment boxes, euthanized and then under a stereomicroscope their head and thorax were removed. The abdomen was snap frozen and cut either horizontally (transversally) or vertically (longitudinally) to create two even cuts to allow the diffusion of fixative. Samples were incubated in fixative solution at 4°C for 48 hours. Samples were then washed twice in fsPBS, placed in a shaker (25˚C at 300 rpm) and dehydrated in a graded series of increasing ethanol (70 – 100%). After dehydration samples were embedded in paraffin wax and cut into 20 - µm sections on the Shandon Finesse 325 microtome using a MX35 microtome blade (Ultra 34˚/80 mm, Thermo) (Table 2.2). Sections were mounted on glass slides, dried at 37°C overnight in preparation for fluorescent or haematoxylin and eosin (H&E) staining.

**Table 2.2 Bumblebee histology specimen processing.** Solutions and incubation times required for washing, dehydrating and waxing of histological specimens.

| Step | Duration (mins) | Solution |
|---|---|---|
| Wash | 10 | fsPBS |
| | 10 | fsPBS |
| Dehydrate | 30 | 50% ethanol |
| | 60 | 70% ethanol |
| | 60 | 100% ethanol |
| | 60 | 100% ethanol |
| | 60 | 50% ethanol, 50% xylenes |
| | 60 | 100% xylenes |
| Wax | 60 | Paraffin wax (59˚C) |
| | 60 | Paraffin wax (60˚C) |

#### 2.4.2.2.2 Histological staining

After drying the slides overnight at room temperature, the sections were placed in a glass staining tank and dewaxed by incubating in xylenes for 10 minutes. This step was repeated once. Samples were rehydrated by incubating in the following solutions for three minutes each; 100% ethanol, 70% ethanol, $H_2O$. Slides were then placed on tissue until fully dry. Once dry a drop of haematoxylin was added to each specimen using a Pasteur pipette. After five minutes the stain was removed, slides were washed with $H_2O$ (5 mins), dehydrated by washing with 70 % ethanol (five times) and set aside until fully dry. Eosin was then added to the specimens (as outlined above), slides were rinsed with 100% ethanol to remove excess dye and finally rinsed twice with xylenes (100%). All stained

tissues were then mounted in Eukitt™ mounting medium for further imaging and analysis.

#### 2.4.2.3 Imaging and statistical analysis

Bright field images of all samples were captured using a Leica DM1000 microscope. Confocal images were obtained using an Olympus Fluoroview 1000 with FV10-ASW 4.2 viewer. Measurements of both fluorescent and H&E stained images were carried out using the ImageJ image analysis software version 1.51j. Statistical analysis of fluorescently stained fat cells was performed using unpaired T-tests between treatment and control groups using GraphPad Prism v. 5.0 (www.graphpad.com). For all analysis a p-value of $< 0.05$ was deemed statistically significant.

### 2.5 Mass spectrometry preparation

#### 2.5.1 Haemolymph collection for proteomic analysis

Haemolymph was collected following a similar set-up as previously outlined (section 2.2.3). Collected haemolymph was deposited into a microtube on ice containing 50 µl of ice-cold collection buffer (fsPBS supplemented with a protease inhibitor cocktail tablet (Roche, Complete Mini)). All collections were kept on ice and had a further 50 µl of buffer added to them prior to centrifugation. All collections were spun down at 8,000 rpm for 5 min at 4˚C to remove cells and other debris. After centrifugation the supernatant was removed to a new microtube avoiding the pellet at the bottom of the tube. The samples were then stored at -20˚C for analysis at a later date.

#### 2.5.2 Fat body collection and preparation for proteomic analysis

For proteomic analysis fat cells were dissected as outlined above (section 2.2.4). Extracted fat cells were collected in 300 µl of ice-cold lysis buffer (6M urea, 2M thiourea supplemented with a protease inhibitor cocktail tablet, pH 8.0) and homogenised using a motorised pestle. Samples were sonicated (3 x 10 s) to rupture cell membranes and release intracellular proteins and centrifuged at 9,000 x *g* to pellet cellular debris. The supernatant was then removed to a new tube for further analysis and the pellet was discarded.

### 2.5.3 Protein quantification

Protein quantification of all sample types was carried out using the Qubit® Quant-IT™ protein assay kit on a Qubit® fluorometer version 2.0 following the manufacturer's operating guidelines. For both haemolymph and fat body samples 2 µl of sample was used for quantification. Sample was added to 198 µl of working buffer (199 µl of buffer B and 1 µl of dye A reagent), gently mixed and incubated (in the dark at room temperature) for 15 mins before measuring the protein concentration.

### 2.5.4 One dimensional gel electrophoresis and protein staining

One-dimensional, sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) separation of proteins was carried out using the following reagents; Ultrapure Protogel, 4X Protogel, Resolving Buffer stock, acrylamide stock and tetramethyl ethylenediamine (TEMED) solution (National Diagnostics). Separation of proteins was achieved by using polyacrylamide gel of varying percentages. The resolving and stacking gel layers were composed as outlined below (Table 2.3). Plates (1.0 mm) were set up following the manufacturers guidelines and filled with resolving gel. Butanol was applied to the surface and the gel was left to polymerise. Protein molecular weight ladder and laemmli type buffer were purchased from Bio-Rad laboratories (Hempel-Hempstead, Hertfordshire, UK).

**Table 2.3 SDS-PAGE gel preparation.**

| Reagent | Resolving gel | Stacking gel |
|---|---|---|
| 30% Protogel | 6 ml | 650 µl |
| 4X Resolving gel | 3.75 ml | - |
| Stacking buffer | - | 1.25 ml |
| $H_2O$ | 4.5 ml | 3 ml |
| 10% APS | 150 µl | 25 µl |
| TEMED | 15 µl | 5 µl |

### 2.5.5 2D Sample clean-up and purification

All samples were quantified using Qubit® protein assay. Samples were standardised based on quantification and a maximum of 100 µg of each sample was cleaned to remove biological impurities using the 2-D clean up kit following the guidelines provided by the manufacturer. The resulting pellet from this procedure was resuspended in 50 µl of

resuspension buffer (6M urea, 2M thiourea, 0.1M Tris-HCl, pH 8.0). This volume was then split into two 25 µl aliquots; one aliquot was frozen at -70˚C for further analysis and mass spec preparation procedure continued on the second.

### 2.5.6 Protein sample digestion

For protein digestion 25 µl of each sample was removed to a new microcentrifuge tube, 50mM ammonium bicarbonate (ambic) was added to each sample and proteins were reduced with 0.5M Dithiothreitol (DTT) at 56°C for 20 min. They were then alkylated by adding 0.55M iodoacetamide (IAA) and incubating at room temperature in darkness for 15 min. DTT acts as a reducing agent which cleaves disulphide bonds within proteins preventing the formation of inter/intracellular disulphide bonds between cysteine residues of proteins. Following this digestion was initiated by adding 1 µl of 1% w/v solution of Protease Max and 1 µl of sequence grade trypsin and incubating at 37°C for 18 hours. To terminate digestion 1 µl of 100% trifluoroacetic acid (TFA) was added, incubated room temperature for five min and centrifuged for 10 min at 13,000 x *g*.

### 2.5.7 Peptide clean-up and purification for LC-MS\MS

Digested peptide samples were cleaned and purified prior to mass spectrometric analysis using either C18 ZipTip® or C18 spin columns depending on the sample type and protein concentration. Both methods were performed following the manufacturer's guidelines. Eluted and purified peptides were dried in a Speedy Vac concentrator (Thermo Scientific Savant DNA 120) and resuspended in acetonitrile (2% v/v) and TFA (0.05% v/v). To aid resuspension samples were sonicated for 5 min at 16,000 x *g*. This supernatant was removed and used for mass spectrometry.

### 2.5.8 Mass spectrometric analysis

For mass spectrometry analysis 1 µg of digested sample was loaded on a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. The peptides were separated over a 2 – 40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120 min reverse-phase gradient at a flow rate of 250 nL $min^{-1}$ for fat body and 60 min gradient for haemolymph samples. All data from the QExactive was acquired in automatic data dependent switch mode. A full scan at 140,000

resolution with a range of 300 – 1700 m/z was followed by an MS/MS scan, resolution 17, 500 and a range 200 – 2000 m/z. From each scan the 15 most intense ions were selected for MS/MS analysis.

The analysis software MaxQuant v 1.5.0.8 (http://www.maxquant.com) was used for protein identification and label-free quantitation (LFQ) normalisation of all MS/MS data. The Andromeda search algorithm in MaxQuant was used to correlate all MS/MS data against the Bumblebee Genome (20,984 entries, downloaded 2015). Search parameters were set as follows: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm, fixed modification as cysteine carbamidomethylation, variable modifications as N-acetylation of protein and oxidation of methionine and a maximum of two missed cleavage sites allowed. The false discovery rate (FDR) was set to 1% for both peptides and proteins. LFQ intensities were obtained using the MaxLFQ algorithm from razor and unique peptides with a minimum ratio count of two peptides across samples. Only peptides with a minimum length of seven amino acids were considered for identification and proteins could only be considered identified when more than one unique peptide for each protein was observed.

## 2.6 Proteomic analysis

### 2.6.1 Protein annotation and statistical analysis

Data analysis, processing and generation of graphics for all identified proteins was completed using Perseus v 1.5.0.8. (www.maxquant.org/). Normalised LFQ intensity values were used as the quantitative measurement of protein abundance for subsequent analysis. The data matrix was first filtered for the removal of contaminants and peptides identified by site. LFQ intensity values were log2 transformed and each sample was assigned to its corresponding group. Proteins were filtered to identify and remove those not found repeatedly across treatments. A data-imputation step was conducted to replace missing values with values that simulate signals of low abundant proteins chosen randomly from a normal distribution.

Two sample t-tests were performed for all relevant comparisons using a cut-off of $p<0.05$ on the post imputated dataset to identify statistically significant differentially abundant (SSDA) proteins. Volcano plots were generated in Perseus by plotting negative log p-

values on the y-axis and $\log_2$ fold-change values on the x-axis for each pair-wise comparison to visualise changes in protein expression. The 'categories' function in Perseus was utilized to highlight and visualise the distribution of various pathways and processes on selected volcano plots. Normalised intensity values were used for a principal component analysis (PCA) which illustrates the degree of variation between all samples and demonstrates the quality of the replicates which show little variation by clustering together. Exclusively expressed proteins (those that were uniquely expressed or completely absent in one group) were identified from the pre-imputation dataset and included in subsequent analyses. 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.

Gene ontology (GO) mapping was also performed in Perseus using the *Apis* UniProt gene ID for all identified proteins to query the Perseus annotation file (downloaded January 2015) and extract terms for biological process, molecular function, Kyoto Encyclopaedia of Genes and Genomes (KEGG) name, KEGG pathway, protein family (pfam) and InterPro. GO and KEGG term enrichment analysis was performed on the major protein clusters identified by hierarchical clustering using a Fisher's exact test (a Benjamini-Hochberg corrected FDR of 2%) for enrichment in Uniprot Keywords, gene ontology biological process (GOBP), gene ontology cellular component (GOCC) and KEGG (FDR <2%).

### 2.6.2 Functional annotation

The Search Tool for the Retrieval of INteracting Genes/Proteins (STRING) v 10 (http://string-db.org/) was used to map known and predicted protein:protein interactions (Jensen et al. 2009). Uniprot gene lists (extracted from Perseus) were inputted and analysed in STRING using the medium to high confidence (0.5 – 0.7) setting to produce interactive protein networks for each group in all comparisons. GO term enrichment analyses for biological process, molecular function and cellular compartment were then conducted to identify potential pathways and processes that warranted further analysis. Such pathways were examined using the KEGG pathway analysis (http://www.kegg.jp/kegg/tool/map_pathway2.html), using the 'KEGG Mapper—Search & Colour Pathway' tool. To analyse the proteolytic repertoire of the fat body all identified proteins were blasted against the MEROPs database v 12.0

(https://www.ebi.ac.uk/merops/) to produce a list of all peptidases and inhibitors present (Rawlings et al. 2018).

To assist with the assignment of gene ontology (GO) terms and putative functional information to all proteins a basic local alignment search tool for proteins (BLASTp) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search of proteins identified was performed against the Uniprot sequence set for *Apis mellifera* and *Drosophila melanogaster* (WWW.Uniprot.org downloaded April 2017). The top Uniprot match for each identified protein was obtained and used to query the Perseus annotation file for *A. mellifera* and *D. melanogaster* (downloaded April 2017) and to extract terms for biological process, molecular function, Kyoto Encyclopaedia of Genes and Genomes (KEGG) name, KEGG pathway, protein family (pfam) and InterPro. The 'categories' function in Perseus was utilized to highlight and visualize the distribution of selected pathways and processes on volcano plots and heat map. Cellular component, molecular function, biological processes, KEGG pathways and immune function were further interrogated using the Cytoscape plugin ClueGO v 2.5.1 (http://www.cytoscape.org/). These annotations were visualized in histograms created by plotting the distribution of gene ontology (GO) results. STRING v 10.5 (https://string-db.org/) was used to resolve protein-protein interactions and to identify the functions and pathways that were most strongly associated with the protein list. To further investigate enriched pathways proteins were functionally characterized in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) PATHWAY database (http://www.genome.jp/kegg/pathway.html) by the BlastKOALA sequence similarity tool (http://www.kegg.jp/blastkoala/).

### 2.6.3 Immune annotation

A number of immune proteins were not identified through the gene ontology annotation steps described above. To ensure that all immune/defence associated proteins were annotated a master immune protein list for the entire predicted proteome was produced. The core immune protein list was obtained from the Bumblebee Genome Initiative (Sadd et al. 2015) using OrthoDB (https://www.orthodb.org/) to identify orthologues of previously characterised immune genes from other arthropods. BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searches were conducted against the NCBI Reference Sequence Database (RefSeq) (https://www.ncbi.nlm.nih.gov/refseq/) and a

reverse basic local alignment search tool for translated nucleotides (TBLASTN) was performed against available genome and short read archives.

All proteins identified in the bumblebee fat body and haemolymph were compared to this list to identify immune proteins. The GO terms obtained from the *Apis mellifera* and *Drosophila melanogaster* Uniprot homologues were also reviewed to confirm immune function. Additional proteins identified as having immune, defence or antioxidant functions were manually added to the master immune list using BLASTP searches when identified.

# Chapter 3

## Proteomic and morphological characterisation of the bumblebee (*Bombus terrestris* L.) fat body

## 3.1 Introduction

Anticipating and adapting to unfavourable environmental conditions by amassing energy reserves, such as lipids, carbohydrates and amino acids, is integral to survival as it reduces the negative effects of limited or unavailable food resources. The storage of energy is a ubiquitous and fundamental aspect of all biological systems and has important ecological consequences (Griffen 2017). Hence, systems capable of sensing and maintaining nutrient reserves are essential and must be able to integrate numerous signals from other organs. In insects the primary organ responsible for the monitoring, maintenance and management of energy stores is the fat body.

The insect fat body (FB) performs storage and endocrine functions similar to the vertebrate liver however their structures vary dramatically (Colombani et al. 2003). The FB is an organ of mesodermic origin comprised of cell clusters forming lobes that are supported by connective tissue and trachea (Hoshizaki 1998). The FB can be found throughout the insect body and is most abundantly distributed in the abdomen. Within the abdominal cavity the fat body is often present in two distinct layers, the parietal deposit, located just beneath the cuticle and the perivisceral lobes surrounding the organs within the haemocoel (Assis et al. 2014). The structure and density of the FB tissue arrangement allows for maximal haemolymph exposure which facilitates a rapid response to changing physiological needs through metabolic exchange (Arrese and Soulages 2010).

Although primarily involved in lipid accumulation and storage (Musselman and Kühnlein 2018), numerous other metabolic functions, including regulation of nutrient supply and storage, removal of toxic substances and participation in the immune response (Assis et al. 2014) are associated with the fat body. This physiological powerhouse also regulates the synthesis and utilization of numerous haemolymph proteins, circulating metabolites and energy reserves such as glycogen and fat (Arrese and Soulages 2010). Proteins secreted by the fat body into the haemolymph are mostly stage specific and include; storage proteins used as amino acid reservoirs in morphogenesis, lipophorins responsible for lipid transport, vitellogenins involved in egg maturation and antimicrobial peptides (AMPs) which are potent immune agents (Klowden 2007; Arrese and Soulages 2010). Haemolymph is vital for

insect immunity as it continuously stores and circulates immune components which are rapidly transported to the site of attack when required (Chan et al. 2006).

As in other insects, bumblebees require energy to maintain vital physiological energy demanding processes such as foraging, development and defence (Cartar and Dill 1990; Cartar and Dill 1991). These annual eusocial pollinators inhabit temperate regions with highly variable daily weather conditions including periods of wind, rain and cold temperatures, which often disrupt the regularity of foraging activity (Moret and Schmid-Hempel 2000). The ability to forage, store and maintain a reliable energy source is crucial as unlike their honeybee counterparts bumblebee colonies contain sufficient nectar stores to maintain colonies for a few days (Heinrich 1979; Dornhaus and Chittka 2005). After the initial colony establishment by the queen, the responsibility of colony survival lies with the workers whose main task, among others, is to supply the ever-growing colony with food. The continued survival of workers is dependent on their capacity to survive periods of variable food availability. Starved workers cannot typically survive for longer than 20-30 hours and therefore the failure to collect sufficient pollen and nectar can inevitably lead to the death of the colony itself (Cartar and Dill 1991; Moret and Schmid-Hempel 2000).

Bumblebee pollination is essential for numerous wild and economically important plant species. Globally insect pollination is estimated to contribute $215 billion to food production (Gallai et al. 2009), with 84% of all cultivated crop species in Europe directly depend on insect pollination (Williams 1994). Bumblebee pollination services are highly sought after by farmers due to their pollination efficiency which is responsible for significant increases in both the quality and quantity of a number of crops (Klatt et al. 2014).

However, over the last 50 years bumblebee numbers in Europe and the northern hemisphere have been decreasing rapidly (Goulson et al. 2005). Population declines are perpetuated by the simultaneous and chronic exposure of bees to drivers of decline such as habitat loss, climate change, fragmentation, pathogens and pesticides (Goulson et al. 2015). It is predicted that these losses will have profound economic and environmental consequences (Vanbergen and Initiative 2013). Continuing declines in wild and managed bee populations have a significant negative impact on crop production, with agricultural losses alone estimated at

over $200 billion per annum globally, and the losses from reduced pollination services in wild ecosystems is likely to surpass this (Gallai et al. 2009; Miller-Struttmann et al. 2017).

Bumblebees are models for the study of social aspects of disease pathology and immunity (Evans et al. 2006), however very little is known about the underlying molecular mechanisms of these and other parts of their physiology, including the fat body. Although numerous studies exist on the bumblebee fat body the majority of studies focus on glycogen and triacylglycerol content (Alford 1969; Röseler and Röseler 1986) and there is a gap in the knowledge of fat body function from the molecular perspective.

Proteins are central to most cellular enzymatic functions, regulatory activities, signal transduction and structural components (Garrels 2001). Hence, characterising all proteins expressed by the FB (the proteome), can reveal important aspects of reactivity, organisation and function in this central organ. Recent sequencing and annotation of the bumblebee genome now facilitates proteomics based mass spectrometry and an in-depth study of the molecular composition of the fat body (Sadd et al. 2015). To complement molecular based studies, cell morphology of the fat body cells will contribute important information regarding the density, composition and localisation of this important tissue throughout the abdomen of healthy workers.

With recent declines in bumblebee populations driven by stressors such as habitat loss, agrichemical exposure and disease the need for a comprehensive molecular characterisation of the bumblebee fat body and its role in immunity, metabolism and detoxification is greater than ever. In addition, the cell morphology and basic molecular mechanisms of this important organ will contribute significantly to the body of knowledge on bumblebee physiology and will benefit future physiological studies of this species. Formation of the FB proteome will also contribute to the identification of key immune proteins and proteases and inhibitors. Examining these proteins will provide further insight into the functionality of the FB. Understanding the bumblebee fat body proteome will facilitate the investigation and characterisation of key metabolic, immunological and regulatory processes that it is involved in and will provide a platform of knowledge from which future studies will be based.

### 3.1.1 Chapter three aims

- Conduct a morphological characterisation of the cells comprising the *B. terrestris* FB.
- Investigate and outline the molecular composition of the FB through the production of the first FB proteome for *B. terrestris*.
- Perform functional analysis of the FB proteome to reveal associations with biological processes.

### 3.1.2 Experimental outline

Morphological characterisation of the FB was completed through the preparation of histological sections of the abdomen and confocal microscopy of fluorescently stained fat cells. Samples for image analysis were obtained from workers sourced from commercial colonies (n = 4, Agralan). To prepare histological sections the abdomens of workers (n = 8), were collected, snap frozen, and fixed in paraformaldehyde. After fixing, samples were dehydrated in ascending ethanol dilutions (70 – 100 %) and finally embedded in paraffin wax. Histological sections of 20 µm were cut using a Shandon Finesse 325 microtome, stained with haematoxylin and eosin (H&E) and imaged using a Leica DM1000 photomicroscope and Olympus SZX16 camera. For this analysis both transversal and longitudinal sections were obtained.

Extracted fat cell lobes were further analysed using brightfield and confocal microscopy. For brightfield, cells were extracted, fixed, washed and mounted before imaging on a Leica DM1000 photomicroscope. To stain fat cells for confocal microscopy, cells were dissected (n = 4 bees), added to a slide and incubated in DAPI and Nile red. After incubation the excess dye was removed, a coverslip was placed on the specimen and a sealing solution was applied. Sections were similarly stained using this method. The slides were then imaged using an Olympus Fluoview 1000 confocal microscope.

To provide a comprehensive characterisation of the *B. terrestris* fat body proteome, fat body tissue was subjected to high resolution mass spectrometry. Fat cell masses were dissected from workers (section 2.2.4) and subject to mass spec preparation; protein quantification, 2D clean-up, digestion and peptide purification (C18). For mass spec analysis 1 µg of prepared tryptic peptides sample was loaded on a QExactive (ThermoFisher Scientific) high-

resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. The peptides were separated over a 2 – 40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120 min reverse-phase gradient at a flow rate of 250 nL $min^{-1}$ for fat body. Four independent biological replicates, sampled from two colonies (Koppert Biological Systems), were analysed in this study.

Initial protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.5.6.5 (http://www.maxquant.com). Data analysis and processing of the data was then performed using Perseus v 1.5.0.8. (www.maxquant.org/). To resolve protein-protein interactions and identify functions and pathways most strongly enriched within the fat body proteome STRING v 10 (http://string-db.org/) and the BlastKOALA sequence similarity tool (https://www.kegg.jp/blastkoala/) were used. To obtain a global overview of the proteome associated processes, gene ontology (GO) analysis was completed using the following GO assignment software; gene ontology enrichment analysis and visualization tool (GOrilla) (http://cbl-gorilla.cs.technion.ac.il/) and ClueGO v 2.5.1 (http://www.cytoscape.org/).

To analyse the proteolytic repertoire of the fat body all identified proteins were blasted against the MEROPs database v 12.0 (https://www.ebi.ac.uk/merops/) to produce a list of all peptidases and inhibitors present (Rawlings et al. 2018).

Prior to all sample collections, bees were removed from colonies in a randomised manner, acclimatised in wooden holding boxes (depth 12 cm x width 16 cm x length 24 cm), maintained under standard environmental conditions (24±1°C, 60% ± 5 relative humidity (RH), and supplied pollen and sucrose (0.5M).

## 3.2 Results

### 3.2.1 Histological analysis

A microanatomical structural analysis of the fat body and abdomen was conducted using histology (three sections per individual (n = 8)). The abdominal FB in *B. terrestris* is present only in a single layer, the parietal layer, located directly below the epidermal cell layer of the cuticle (Figure 3.1). The perivisceral layer which is commonly found in the abdominal cavity of many insects and surrounding the viscera is seemingly absent in *B. terrestris* workers.

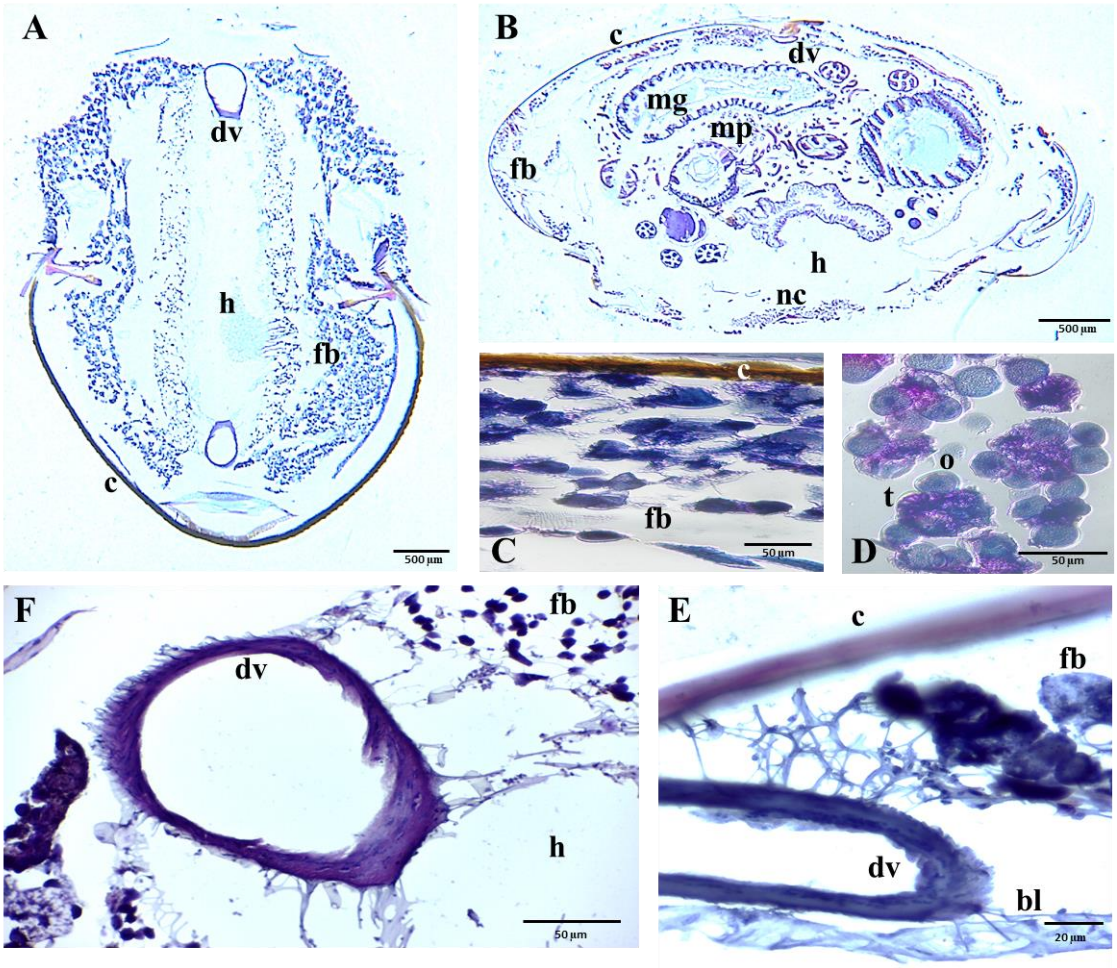


**Figure 3.1 Histological sections of the bumblebee worker FB stained with H&E.** (A)Longitudinal section showing the general distribution of the FB beneath the cuticle (c), (B) transversal section of *B. terrestris* abdomen outlining the location of the FB. (C) The parietal fat body is located between the cuticle (c) and basal lamina (bl) (F). (D) Trophocytes (t) in the FB can be distinguished by their large size and numerous lipid droplets. Oenocytes (o) can be observed in close association to trophocytes and are identifiable by their central circular nucleus. (E&F) The fat body is located close to the dorsal vessel and pericardial cells. Fb – fat body, h – haemocoel, dv – dorsal vessel, mg – midgut, mp – malpighian tubules, nc – nerve chord, bl – basal lamina.

In *B. terrestris* the parietal FB is in direct contact with the dorsal vessel but is separated from the haemocoel by a basal lamina, a thin membrane which supports the structure of the lobes and acts as a barrier between the organs in the abdomen (Martins et al. 2011) ( Figure 3.1). The parietal layer is composed of numerous cells that adhere to each other to form sheaths. Both histology and fluorescent microscopy indicate that these layers are composed of two distinct cell types, trophocytes and oenocytes. The two cell types are often in close association, although some areas were observed with a predominance of a single cell type. Often a higher proportion of oenocytes than adipocytes form the FB layers.

### 3.2.2 Fluorescent analysis

Confocal microscopy and fluorescent staining with the nuclear specific dye DAPI and the lipid staining dye Nile red facilitated a detailed examination of the individual cells forming the FB (Figure 3.2). An area comprising 20 adipocytes and oenocytes sampled randomly from four bees, was measured using ImageJ to evaluate the size of fat cells. The mean of each was obtained to get the average representative size of each cell type. Trophocytes are the larger of the two cell types (average area = 5516 $\mu m^2$, diameter range 45 – 200 µm). They contain a large irregular nucleus and numerous lipid droplets. Oenocytes are small circular cells (average area = 1840 $\mu m^2$, diameter range 20 – 110 µm), with a central uniform nucleus and a high lipid content in the cytoplasm.

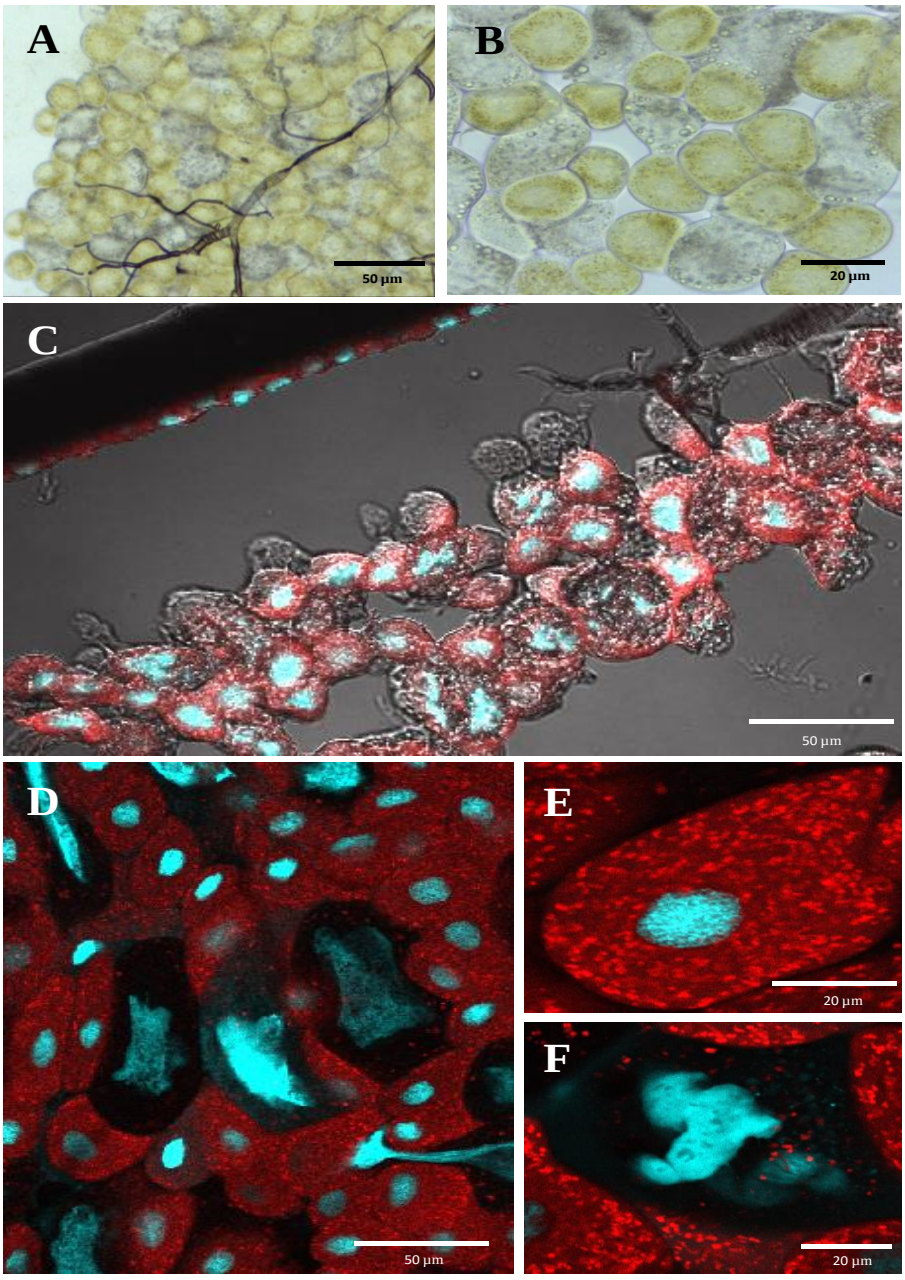


**Figure 3.2 Brightfield and confocal microscopy of fat cells.** Light micrograph highlighting the close association of the fat cells to each other and their characteristic yellow/brownish colour can be observed (A & B). Fluorescent based confocal microscopy images of the bumblebee fat body (C & D) resolving the two main cell types within; oenocytes (E) and adipocytes (F). The use of the nuclear membrane stain DAPI facilitated identification and characterisation of cells types; oenocytes have a large central uniform nucleus whilst the nucleus of adipocytes is large and multi-lobed. Oenocytes have a higher lipid concentration than that of adipocytes where lipids are contained in droplets dispersed throughout the cytoplasm. The distribution of the fat body within the bumblebee abdomen was investigated through fluorescent staining of histological sections, from this it was confirmed that the fat body is located directly beneath the cuticle and epidermis, in sheets of cells which are separated from the organs in the haemocoel by a basal membrane (C).

### 3.2.3 Protein identification and statistical analysis

LC-MS/MS was performed on four replicates of fat bodies and resulted in the initial identification of 2,466 proteins. The data matrix was then filtered for the removal of contaminants, reverse hit peptides and peptides identified by site. LFQ intensity values were $Log_2$ transformed and proteins not found in at least three of the four replicates were removed. These filtering steps resulted in the identification of 1,388 fat body proteins (Table A 3.1).

To identify the most and least abundant proteins within the proteome all protein hits were then ranked based on protein intensity. The top 50 proteins were further analysed for their associated biological function to provide an overview of the processes represented within the FB proteome (Table 3.1). Among those proteins most abundant within the proteome were some typical adult haemolymph proteins including vitellogenin, transferrin and apolipophorin, the latter being the most abundant protein in the haemolymph proteome. A high proportion of proteins associated with metabolism and energy were identified including those involved in oxidative phosphorylation (ATP synthase), carbohydrate (enolase, alpha-trehalose phosphate synthase, transketolase and pyruvate kinase), lipid (fatty acid synthase-like, ATP-citrate synthase and 3-ketoacyl-CoA thiolase) and amino acid metabolism (glutamate synthase and C-1-tetrahydrofolate synthase). Proteins involved in detoxification (catalase (CAT) and superoxide dismutase (SOD)), stress (heat shock proteins) and immune response (venom bombolitin, phenoloxidase and IRP30) were some of the most abundant proteins in the FB. In addition, structural (myosin, tubulin and paramyosin) and translation-associated proteins (elongation factor 1-alpha and translation elongation factor 2) were also identified within the top 50 FB proteins.

**Table 3.1 Top 50 most abundant proteins identified in the FB proteome.** The top 50 most abundant proteins within the FB proteome based on intensity listed in descending order include those involved in metabolism, immunity and protein processing.

| Intensity | Accession no. | Protein name |
|---|---|---|
| 8.64E+11 | XP_003397320.1 | Apolipophorins |
| 5.75E+11 | XP_012172140.1 | Transferrin |
| 5.15E+11 | XP_012164574.1 | Catalase isoform X1 |
| 4.9E+11 | XP_012176432.1 | Actin, clone 205-like |
| 4.25E+11 | XP_012163499.1 | Vitellogenin-like |
| 4E+11 | XP_012166988.1 | Myosin heavy chain, muscle isoform X9 |
| 3.49E+11 | XP_003398135.1 | Glyceraldehyde-3-phosphate dehydrogenase 2 |
| 3.42E+11 | AEN41592.1 | Venom bombolitin 1 |
| 3.4E+11 | XP_003400614.1 | ATP synthase subunit beta, mitochondrial |
| 3.37E+11 | XP_012166688.1 | Superoxide dismutase |
| 3.02E+11 | XP_003393705.1 | Transketolase-like protein 2 isoform X2 |
| 2.71E+11 | XP_012172464.1 | Peroxiredoxin 1-like |
| 2.7E+11 | XP_003392961.1 | Delta-1-pyrroline-5-carboxylate synthase |
| 2.14E+11 | XP_012174450.1 | Fructose-bisphosphate aldolase-like isoform X1 |
| 2.04E+11 | XP_003394310.1 | Heat shock 70 kDa protein cognate 3 |
| 1.99E+11 | XP_012174357.1 | Apolipophorin-3* |
| 1.96E+11 | XP_003395180.1 | Tubulin beta-1 chain |
| 1.91E+11 | XP_012175380.1 | Protein disulfide-isomerase |
| 1.73E+11 | XP_003397389.1 | Aldehyde dehydrogenase, mitochondrial |
| 1.71E+11 | XP_003396735.1 | ATP synthase subunit alpha, mitochondrial |
| 1.63E+11 | XP_003400137.1 | Pyruvate carboxylase, mitochondrial isoform X1 |
| 1.59E+11 | XP_012170810.1 | Enolase |
| 1.58E+11 | XP_012170987.1 | Phospholipase A2 |
| 1.57E+11 | XP_003399629.1 | 60 kDa heat shock protein, mitochondrial-like |
| 1.56E+11 | XP_012173498.1 | Elongation factor 1-alpha |
| 1.54E+11 | XP_012172367.1 | Aldose reductase-like |
| 1.43E+11 | NP_001267840.1 | Fatty acid synthase-like |
| 1.42E+11 | XP_003400548.1 | Phenoloxidase 2-like |
| 1.41E+11 | XP_003399519.1 | Lambda-crystallin homolog |
| 1.4E+11 | XP_003397462.1 | Heat shock 70 kDa protein cognate 4 |
| 1.39E+11 | XP_012171737.1 | ATP-citrate synthase |
| 1.31E+11 | XP_003396897.1 | Heat shock protein 83-like |
| 1.31E+11 | XP_012169580.1 | Transitional endoplasmic reticulum ATPase TER94 |
| 1.28E+11 | XP_003403028.2 | 3-ketoacyl-CoA thiolase, mitochondrial |
| 1.26E+11 | XP_003396466.1 | 3-hydroxyacyl-CoA dehydrogenase type-2 |

(Table 3.1 continued)

| Intensity | Accession no. | Protein name |
|---|---|---|
| 1.25E+11 | XP_003399611.1 | Cytochrome P450 4g15 |
| 1.23E+11 | XP_003395150.1 | Glutathione S-transferase-like |
| 1.19E+11 | XP_003403209.1 | Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase-like |
| 1.08E+11 | XP_003399865.1 | Probable D-xylulose reductase A |
| 1.03E+11 | XP_003395866.1 | Translation elongation factor 2 |
| 1.03E+11 | XP_012172756.1 | Arginine kinase isoform X2 |
| 1.03E+11 | XP_012175084.1 | Cytosolic 10-formyltetrahydrofolate dehydrogenase isoform X2 |
| 9.84E+10 | XP_012164956.1 | Alpha,alpha-trehalose-phosphate synthase |
| 9.7E+10 | XP_012164280.1 | C-1-tetrahydrofolate synthase, cytoplasmic isoform X2 |
| 9.56E+10 | XP_003400851.1 | Probable aconitate hydratase, mitochondrial |
| 9.55E+10 | XP_012175060.1 | Lysosomal aspartic protease |
| 9.46E+10 | XP_012164686.1 | Paramyosin, long form |
| 9.31E+10 | XP_012165021.1 | Transaldolase |
| 8.8E+10 | AEN62314.1 | IRP30 |
| 8.77E+10 | XP_012176722.1 | Pyruvate kinase-like isoform X3 |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

To investigate the processes associated with the most abundant proteins in the FB proteome functional analysis was conducted using GOrilla. All proteins within the dataset were compiled into a single ranked list based on protein intensity and from this the associated biological processes were examined (Figure 3.3). To focus on highly enriched GO terms the p-value threshold for this analysis was set at $10^{-6}$. This functional analysis revealed that, based on protein abundance, the most enriched processes are those associated with cellular (cytoplasmic translation) and metabolic roles (pyruvate metabolic processes).

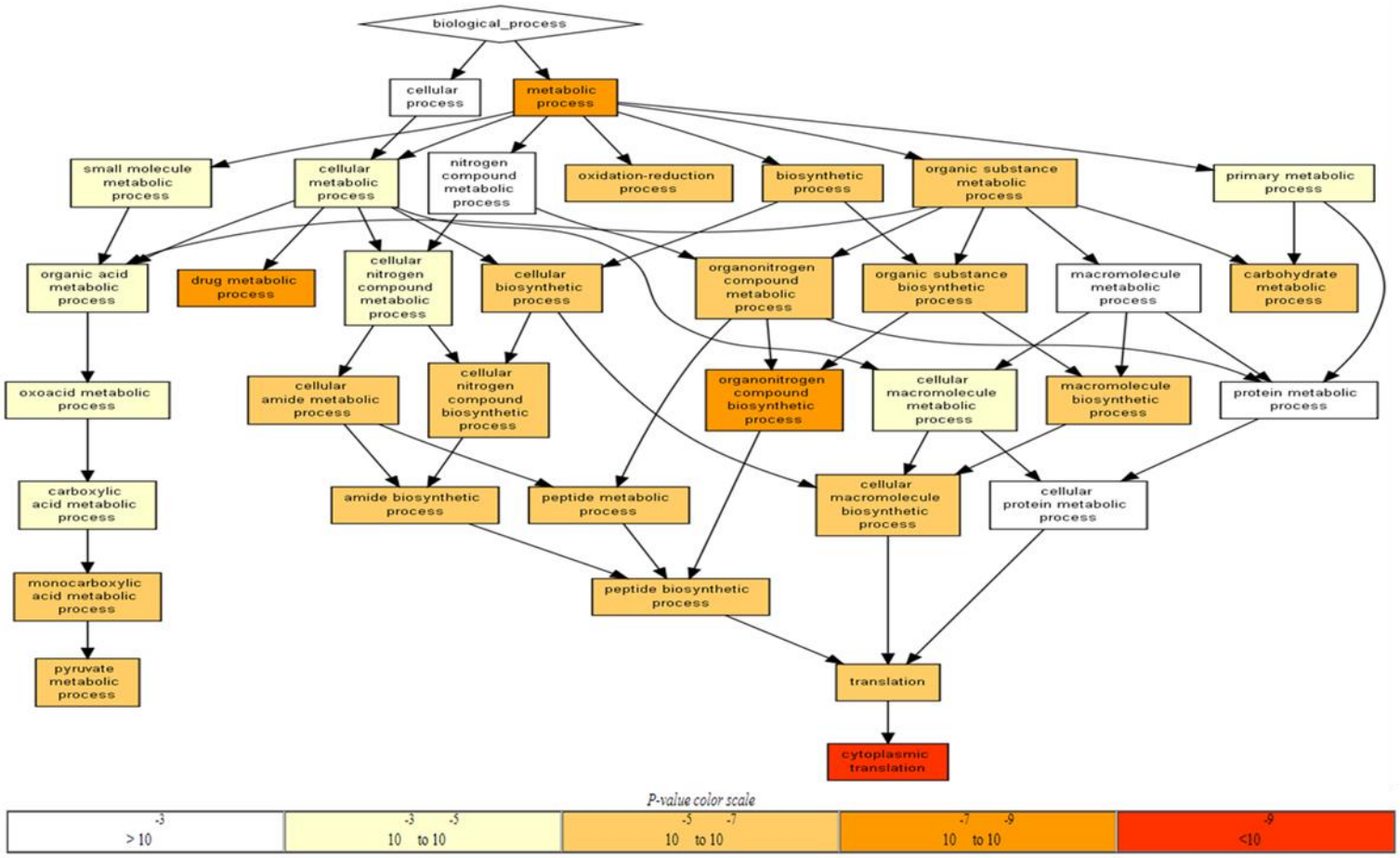


**Figure 3.3 Ranked hierarchy of enriched gene ontology biological processes within the *B. terrestris* FB.** Flow diagram of the overall organisation of biological processes based on protein intensity in the FB proteome (cut-off $p < 10^{-6}$). The colours in the p-value scale represent significance; white corresponds to lower significance ($p > 10^{-3}$) and red represents high significance ($p < 10^{-9}$).

### 3.2.4 Interaction network and pathway enrichment analysis

To identify the networks and pathways enriched in the FB proteome the search tool for the retrieval of interacting genes (STRING) and BlastKOALA (BK) were employed. Of all identified proteins, 1268 with functions based on homologous function in *Drosophila melanogaster* were selected for protein-protein interaction (PPI) network mapping using STRING. The resulting network indicated five main enrichments; carbon metabolism, oxidative phosphorylation, protein processing in the endoplasmic reticulum and ribosome and proteasome (Figure 3.4). Of the five main processes revealed to be enriched in this dataset, all were among the top 10 KEGG processes identified within this dataset (Table 3.2). Global analysis of all 60 KEGG processes (Table A 3.2) highlighted within this dataset revealed that processes such as the phagosome, RNA transport and biosynthesis and degradation of fatty and amino acids among the top 20 significantly enriched processes within this dataset (Table 3.2).

**Table 3.2 Top 20 KEGG processes enriched within the *B. terrestris* FB proteome.** The five processes identified STRING network analysis are among the top 10 KEGG processes identified for this tissue. Other process enriched include the TCA cycle, pyruvate metabolism and biosynthesis and degradation of fatty and amino acids. False discovery rate (FDR) is the representative enrichment score for each process and count in the gene set refers to the numbers of genes found within the dataset in relation to each process.

| Pathway description | Count in gene set | FDR |
|---|---|---|
| Metabolic pathways | 282 | 3.33E-85 |
| Ribosome | 76 | 3.45E-44 |
| Carbon metabolism | 62 | 1.71E-36 |
| Microbial metabolism in diverse environments | 76 | 3.33E-34 |
| Oxidative phosphorylation | 61 | 2.06E-26 |
| Protein processing in endoplasmic reticulum | 57 | 4.63E-26 |
| Biosynthesis of amino acids | 40 | 2.89E-25 |
| Valine, leucine and isoleucine degradation | 27 | 2.19E-23 |
| Proteasome | 33 | 3.50E-22 |
| Fatty acid degradation | 20 | 2.14E-13 |
| Phagosome | 30 | 7.77E-13 |
| RNA transport | 42 | 9.87E-13 |
| Glycolysis / Gluconeogenesis | 25 | 1.03E-12 |
| Citrate cycle (TCA cycle) | 22 | 1.60E-11 |
| Propanoate metabolism | 15 | 1.97E-11 |
| beta-Alanine metabolism | 14 | 4.17E-11 |
| Fatty acid metabolism | 21 | 4.17E-11 |
| Glyoxylate and dicarboxylate metabolism | 15 | 9.75E-10 |
| Tryptophan metabolism | 13 | 3.09E-09 |
| Pyruvate metabolism | 21 | 3.95E-09 |

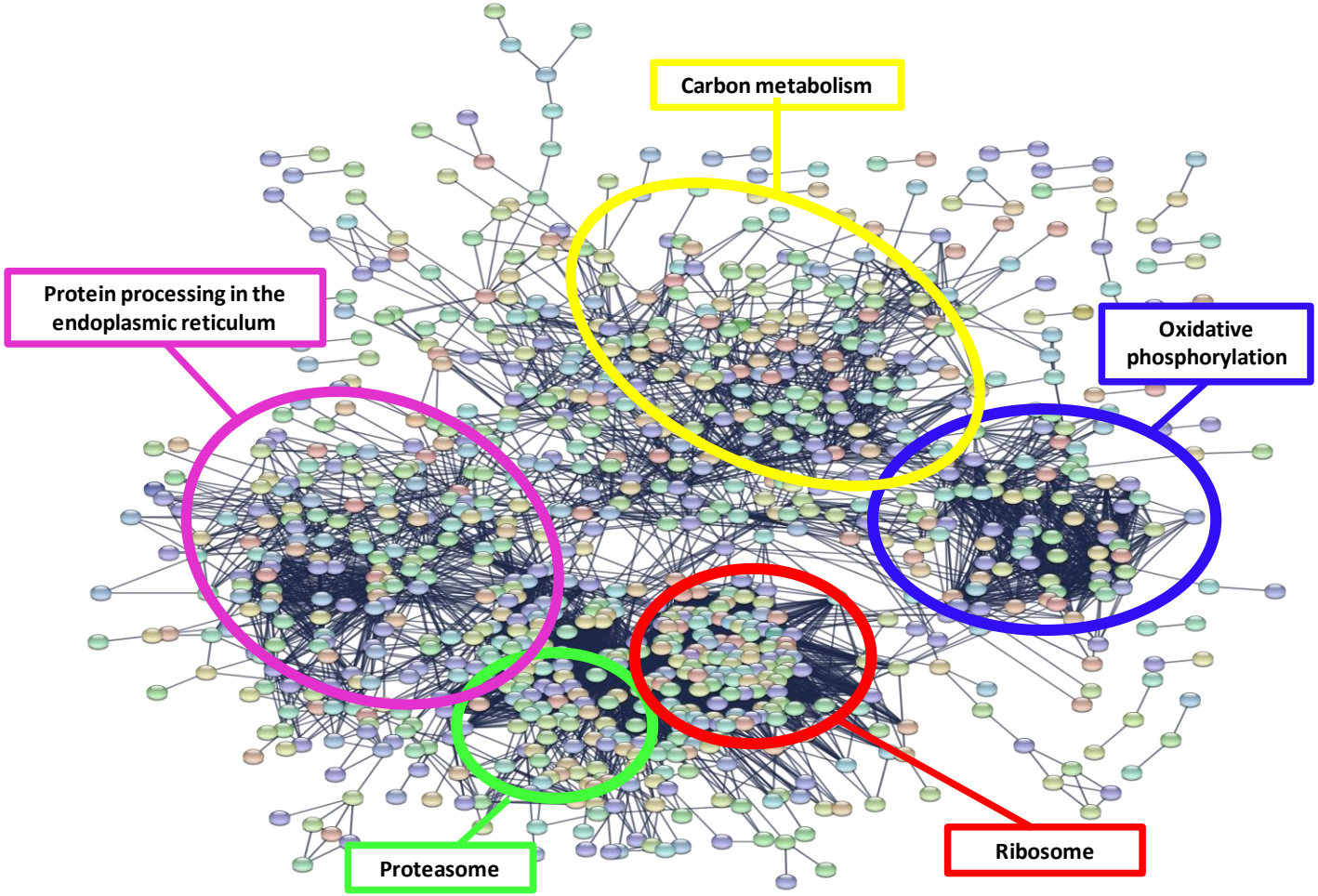


**Figure 3.4 Protein-protein interaction network of proteins identified in the *B. terrestris* fat body proteome.** Each node represents a protein and each linear connection represents an interaction, the extent of the confidence is represented by the width of each individual line. Within the network only interactions with the highest significance score (≥0.900) were included. Statistically enriched KEGG and biological process gene ontology descriptors were examined to identify clusters of proteins enriched within the FB proteome. The coloured circles highlight the most significant interacting groups within the network.

Overall the BK results indicated that the FB proteome contains proteins involved in biological processes such as genetic information processing, carbohydrate metabolism, energy metabolism, cellular processes, environmental information processing, lipid and amino acid metabolism (Figure 3.5) (Table 3.3). Further interrogation of the processes identified by BK analysis revealed a number of the specific pathways and processes found within the FB proteome.

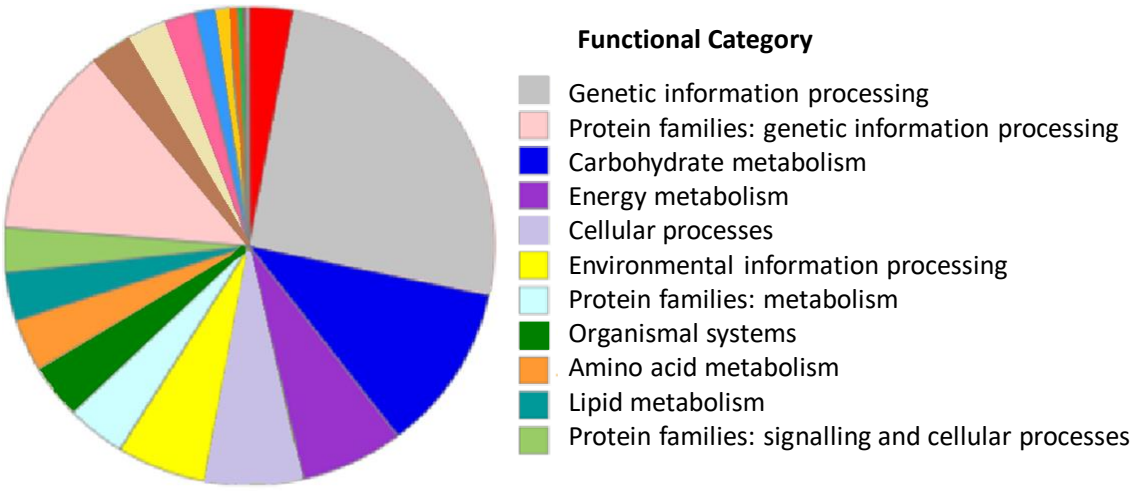


**Figure 3.5 BlastKOALA pathway analysis.** An overview of the diversity of biological processes in the fat body, the majority of which are normal house-keeping processes that could be observed in any typical cell. As expected, the main processes of energy storage and metabolism in bees; carbohydrate metabolism, lipid metabolism and energy metabolism are well represented in the fat body proteome.

**Table 3.3 Top five pathways identified across biological processes in BK analysis.** Within biological processes numerous pathways were identified. Protein number represents the number of FB proteins involved in each pathway. The five pathways containing the highest number of FB proteins were analysed.

| Biological process | Pathway | No. of proteins |
|---|---|---|
| Genetic information processing | Ribosome | 77 |
| | Protein Processing in ER | 58 |
| | RNA transport | 41 |
| | Proteasome | 35 |
| | Spliceosome | 18 |
| Carbohydrate metabolism | Glycolysis/ gluconeogenesis | 23 |
| | TCA cycle | 19 |
| | Pyruvate metabolism | 19 |
| | Propanoate metabolism | 15 |
| | Pentose phosphate pathway | 14 |
| Energy metabolism | Oxidative phosphorylation | 65 |
| | Carbon fixation in photosynthetic organisms | 13 |
| | Methane metabolism | 13 |
| | Carbon fixation in pathways in prokaryotes | 7 |
| | Nitrogen metabolism | 4 |
| Amino acid metabolism | Valine, leucine and isoleucine degradation | 24 |
| | Cysteine and methionine metabolism | 18 |
| | Glycine, serine and threonine metabolism | 15 |
| | Alanine, aspartate and glutamate metabolism | 14 |
| | Tryptophan metabolism | 12 |
| Lipid metabolism | Fatty acid degradation | 18 |
| | Fatty acid elongation | 8 |
| | Glycerophospholipid metabolism | 7 |
| | Glycerolipid metabolism | 6 |
| | Biosynthesis of unsaturated fatty acids | 5 |
| Cellular processes | Endocytosis | 33 |
| | Phagosome | 27 |
| | Lysosome | 25 |
| | Peroxisome | 17 |
| | Focal Adhesion | 17 |
| Environmental information processing | mTOR signalling pathway | 16 |
| | PI3K-Akt signalling pathway | 16 |
| | AMPK signalling pathway | 15 |
| | Rap1 signalling pathway | 12 |
| | MAPK signalling pathway | 10 |
| Organismal systems | Thermogenesis | 58 |
| | Retrogade endocannabinoid signalling | 30 |
| | Synaptic vesical cycle | 22 |
| | Cardiac muscle contraction | 16 |
| | Insulin signalling | 13 |

Within genetic information processing proteins involved in transcription (spliceosome), translation (ribosome and RNA transport) and protein folding, sorting and degrading (protein processing in the endoplasmic reticulum and the proteasome) were revealed (Figure 3.6). Metabolic pathways were represented by proteins involved in numerous pathways including those in carbohydrate (Glycolysis/gluconeogenesis, TCA cycle and pyruvate metabolism), energy (oxidative phosphorylation), lipid (fatty acid degradation) and amino acid metabolism (valine, leucine and isoleucine degradation) (Figure 3.7, 3.8 & 3.9). Cellular process-associated pathways included those mainly involved in endocytosis, the phagosome, lysosome and peroxisome (Figure 3.10). In addition, numerous FB proteins were present in insulin, glucagon and neuronal signalling pathways (Figure A 3.1).

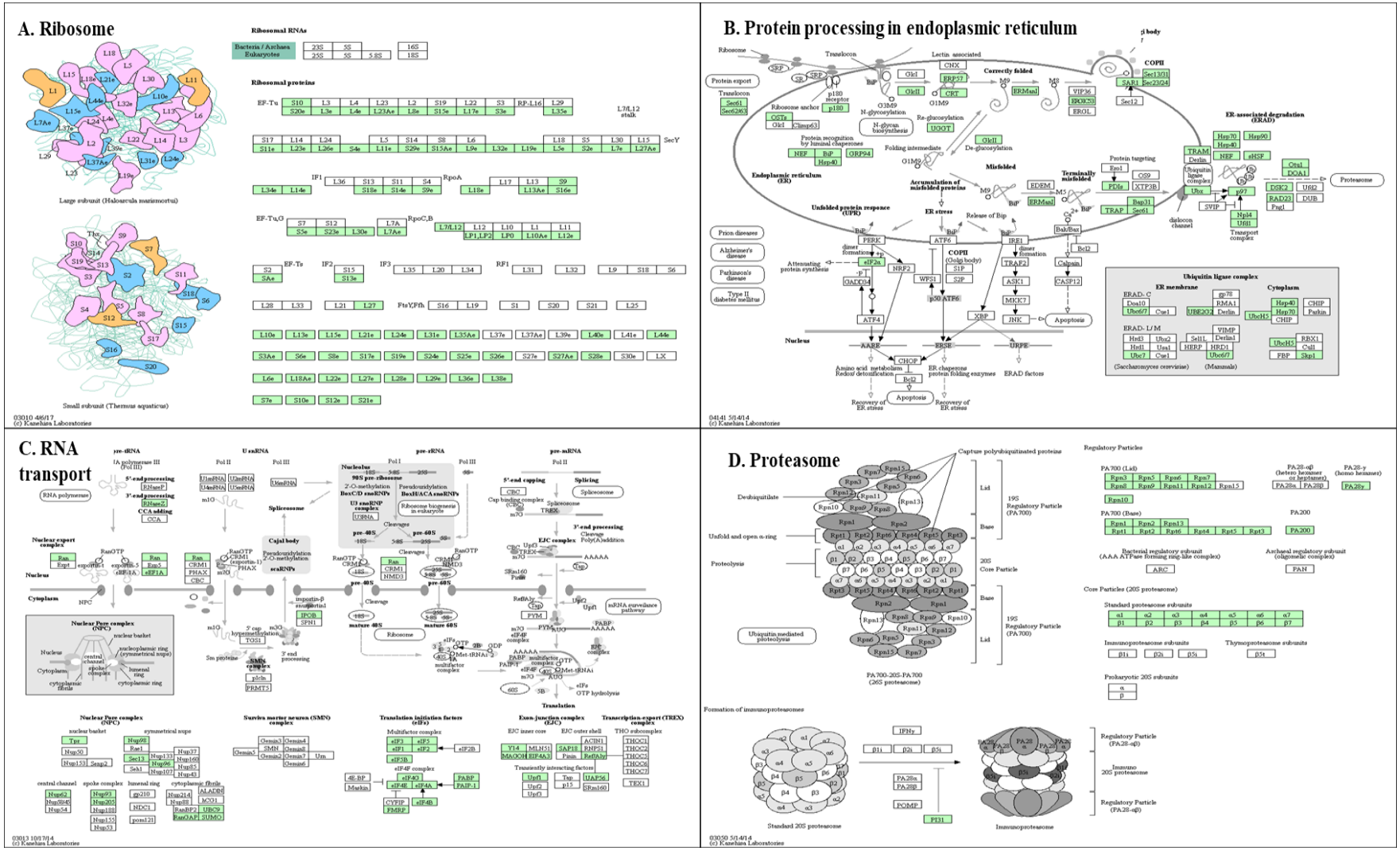


**Figure 3.6 Overview of the pathways within genetic information processes enriched in the FB.** Within each pathway diagram, green elements indicate members of the pathway identified as present in the FB proteome.

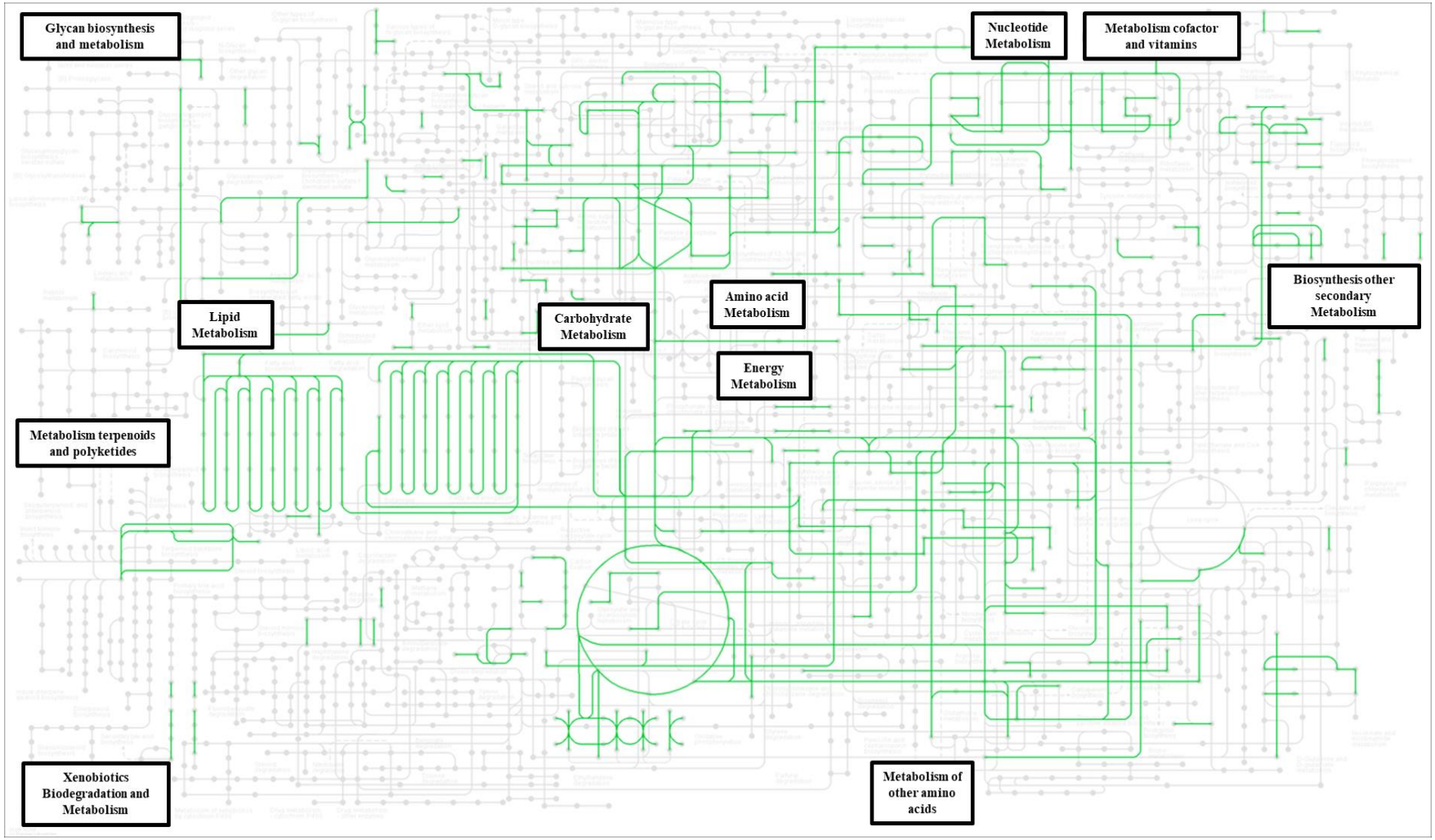


**Figure 3.7 Overview of all metabolic processes within the FB.** Global overview of the organisation of metabolic processes in the FB proteome. Green lines indicate the presence of those pathway elements in the *B. terrestris* FB proteome.

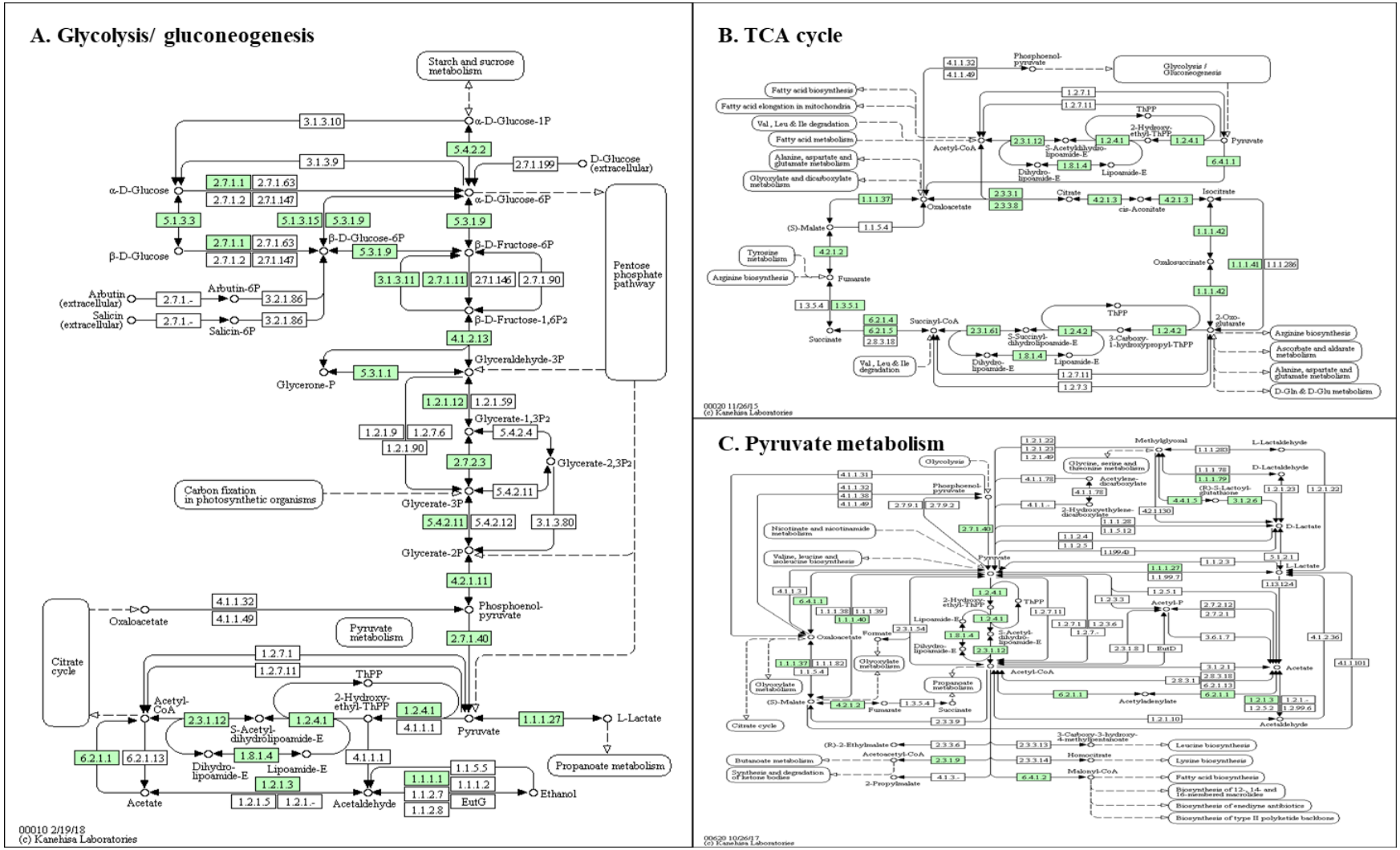


**Figure 3.8 Overview of the pathways within carbohydrate metabolic processes most enriched in the FB.** Within each pathway diagram, green elements indicate members of the pathway identified as present in the FB proteome.

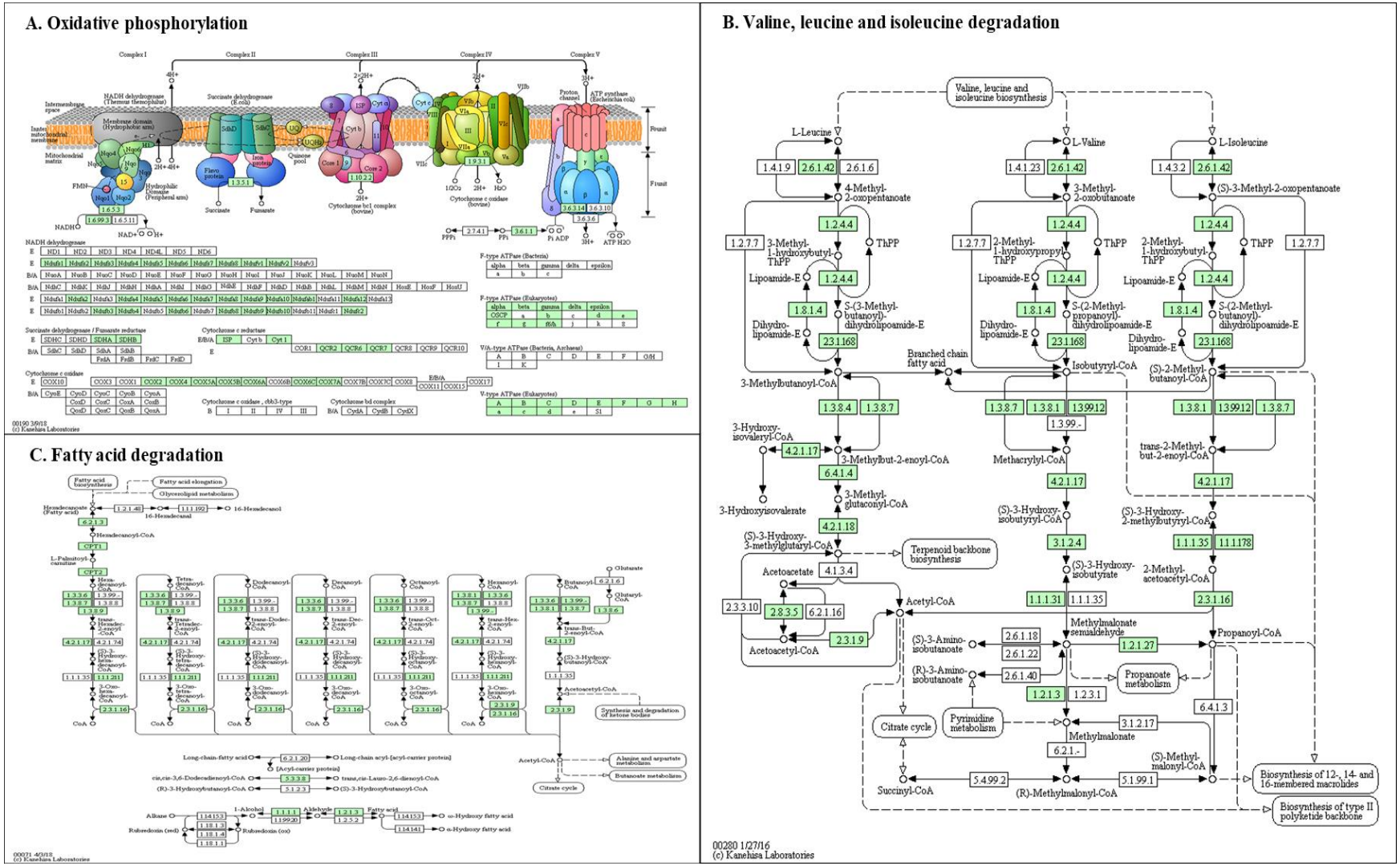


**Figure 3.9 Overview of the most enriched energy, amino acid and lipid metabolic processes within the FB.** Within each pathway diagram, green elements indicate members of the pathway identified as present in the FB proteome.

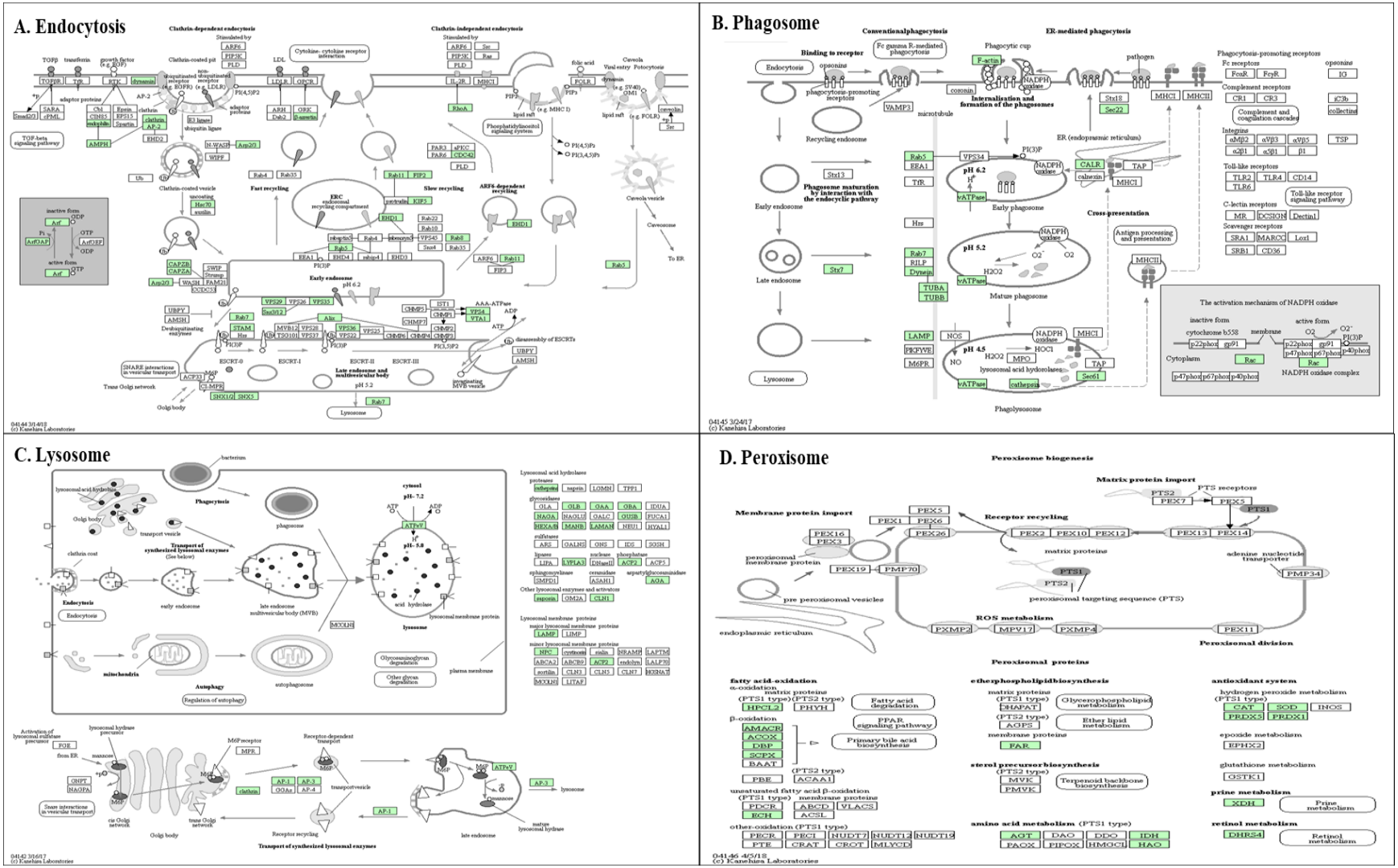


**Figure 3.10 Overview of the most enriched pathways within cellular processes in the *B. terrestris* FB.** Within each pathway diagram, green elements indicate members of the pathway identified as present in the FB proteome.

### 3.2.3 Functional analysis of the fat body proteome

To gain further insight into the biological processes associated with the FB proteome GO terms were obtained from the *D. melanogaster* genome for the proteomes of the FB (1388 proteins) and *B. terrestris* (20,984 proteins). To visualise these biological processes, the proteome was searched through ClueGO, using *Drosophila* as the reference genome. All significantly enriched biological processes ($p \leq 0.005$) are illustrated in Figure 3.11. Processes involved in protein homeostasis, energy metabolism and cellular transport were among the most represented in the protein set.

The enrichment in terms associated with biological processes was assessed using the gene ontology analysis software GOrilla. A list of enriched GOs and their corresponding p-values (threshold $10^{-8}$) were obtained by comparing the FB to a background *Bombus* proteome. From this it was revealed that processes such as cell redox homeostasis, carbohydrate metabolism, protein folding, and lipid oxidation are all significantly enriched with the FB proteome (Table 3.4).

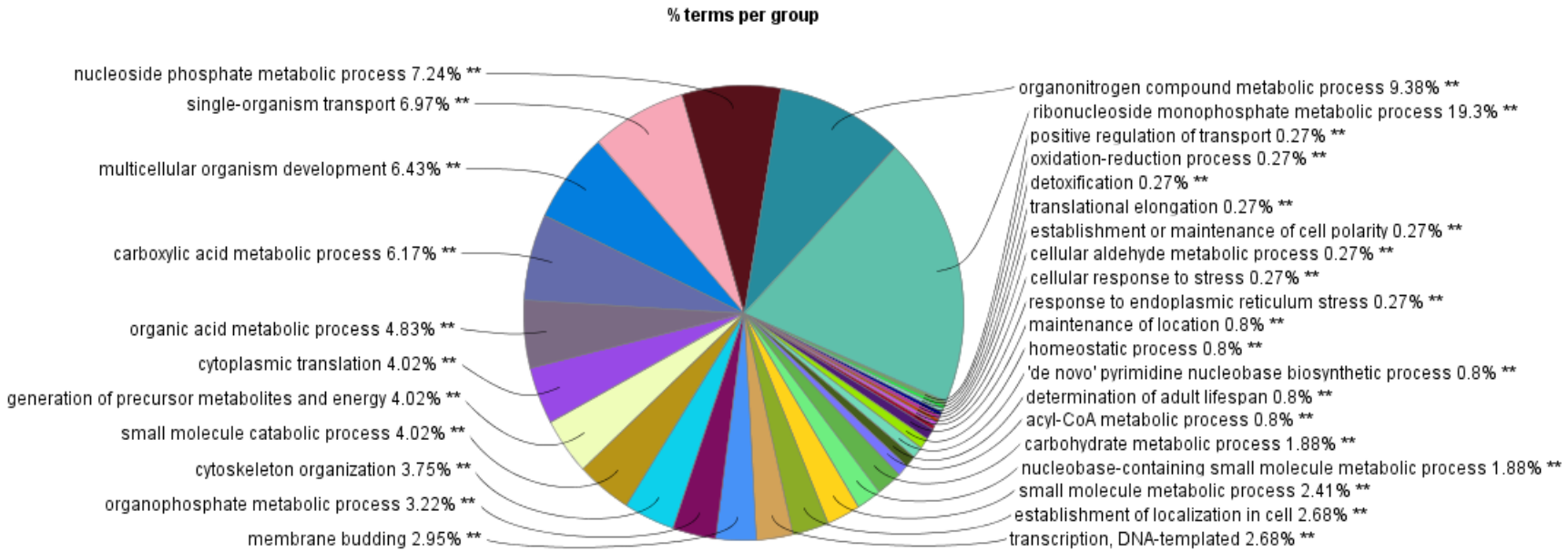


**Figure 3.11 Biological processes associated with the FB proteome.** ClueGO was used to investigate and visualise significantly enriched biological processes within the *B. terrestris* FB proteome. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$ statistical levels, respectively.

**Table 3.4 Top 30 biological processes enriched within the FB proteome.** The top 30 biological processes enriched within the FB in comparison to the entire *Bombus* proteome. To focus on highly enriched GO terms the p-value threshold for this analysis was set at $10^{-8}$. Hence, only GO terms with a p-value better than this threshold are reported. Number of genes refers to the total number of genes associated with a specific GO term.

| Description | GO Term | P-value | FDR q-value | Enrichment | no. of genes |
|---|---|---|---|---|---|
| Small molecule metabolic process | GO:0044281 | 5.11E-52 | 3.67E-48 | 2.5 | 545 |
| Carboxylic acid metabolic process | GO:0019752 | 8.61E-47 | 3.09E-43 | 3.07 | 284 |
| Oxoacid metabolic process | GO:0043436 | 3.07E-44 | 7.37E-41 | 2.95 | 297 |
| Oxidation-reduction process | GO:0055114 | 4.87E-44 | 8.76E-41 | 2.92 | 304 |
| Cytoplasmic translation | GO:0002181 | 5.69E-44 | 8.19E-41 | 4.94 | 82 |
| Organic acid metabolic process | GO:0006082 | 9.51E-44 | 1.14E-40 | 2.93 | 299 |
| Organonitrogen compound biosynthetic process | GO:1901566 | 9.97E-32 | 1.02E-28 | 2.32 | 422 |
| Metabolic process | GO:0008152 | 4.22E-31 | 3.79E-28 | 1.33 | 3065 |
| Generation of precursor metabolites and energy | GO:0006091 | 4.38E-28 | 3.50E-25 | 3.61 | 112 |
| Cellular amide metabolic process | GO:0043603 | 8.81E-26 | 6.33E-23 | 2.46 | 288 |
| Amide biosynthetic process | GO:0043604 | 9.25E-24 | 6.04E-21 | 2.68 | 208 |
| Nucleobase-containing small molecule metabolic process | GO:0055086 | 4.18E-23 | 2.50E-20 | 2.71 | 195 |
| Organonitrogen compound metabolic process | GO:1901564 | 1.08E-22 | 5.95E-20 | 1.45 | 1698 |
| Small molecule catabolic process | GO:0044282 | 2.03E-22 | 1.04E-19 | 3.42 | 102 |
| Catabolic process | GO:0009056 | 2.50E-22 | 1.20E-19 | 1.92 | 550 |
| Organic acid catabolic process | GO:0016054 | 2.75E-22 | 1.23E-19 | 3.87 | 74 |
| Carboxylic acid catabolic process | GO:0046395 | 2.75E-22 | 1.16E-19 | 3.87 | 74 |
| Organic substance catabolic process | GO:1901575 | 8.07E-22 | 3.22E-19 | 1.96 | 498 |
| Peptide biosynthetic process | GO:0043043 | 1.36E-21 | 5.15E-19 | 2.7 | 183 |
| Monocarboxylic acid metabolic process | GO:0032787 | 4.06E-21 | 1.46E-18 | 3.25 | 109 |
| Peptide metabolic process | GO:0006518 | 5.08E-21 | 1.74E-18 | 2.49 | 226 |
| Drug metabolic process | GO:0017144 | 5.46E-21 | 1.79E-18 | 2.66 | 186 |

(Table 3.4 continued)

| Description | GO Term | P-value | FDR q-value | Enrichment | no. of genes |
|---|---|---|---|---|---|
| Translation | GO:0006412 | 1.95E-20 | 6.11E-18 | 2.67 | 179 |
| Ribonucleoside monophosphate metabolic process | GO:0009161 | 2.40E-20 | 7.20E-18 | 4.14 | 57 |
| Electron transport chain | GO:0022900 | 5.04E-20 | 1.45E-17 | 4.03 | 60 |
| Purine nucleoside monophosphate metabolic process | GO:0009126 | 5.82E-20 | 1.61E-17 | 4.3 | 51 |
| Purine ribonucleoside monophosphate metabolic process | GO:0009167 | 5.82E-20 | 1.55E-17 | 4.3 | 51 |
| Cellular catabolic process | GO:0044248 | 8.94E-20 | 2.30E-17 | 1.91 | 497 |
| Ribonucleoside monophosphate biosynthetic process | GO:0009156 | 1.23E-19 | 3.06E-17 | 4.58 | 43 |
| Nucleotide metabolic process | GO:0009117 | 3.03E-19 | 7.26E-17 | 2.72 | 159 |

### 3.2.4.1 Immune protein identification

A list of all bumblebee immune proteins were compiled for the Bumblebee Genome using OrthoDB (https://www.orthodb.org/) to identify orthologues of previously characterised immune genes from other arthropods. The basic local alignment search tool for proteins (BLASTP) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search all identified genes (from the predicted genome) against the NCBI Reference Sequence Database (RefSeq) (https://www.ncbi.nlm.nih.gov/refseq/) and conducting a TBLASTN search of the searching genome assemblies and short reads archive (Sadd et al. 2015) to identify missing immune genes. All proteins identified in the bumblebee fat body were compared to this list to identify immune proteins. The GO terms obtained from the *Apis mellifera* and *Drosophila melanogaster* Uniprot homologues were also reviewed to confirm immune function. In total proteomic analysis identified 72 immune-related proteins, which post-filtering was reduced to 51 (Table 3.5).

**Table 3.5 Immune proteins identified in the *B. terrestris* FB.** The main immune-related proteins identified in the fat body include those involved in pathogen recognition reception (PRR), signal transduction, detoxification and antimicrobial activity. A number of uncharacterised proteins were also identified through immune annotation, these proteins have currently uncharacterized immune roles. References listed provide evidence for immune function for each protein.

| Immune function | Accession number | Protein name |
|---|---|---|
| **Antioxidant** | XP_012163601.1 | Glutathione S-transferase |
| | XP_012164108.1 | Probable phospholipid hydroperoxide glutathione peroxidase |
| | XP_012164574.1 | Catalase |
| | XP_012164907.1 | Thioredoxin-2 |
| | XP_012166348.1 | Peroxidase |
| | XP_012166540.1 | Superoxide dismutase |
| | XP_012166688.1 | Superoxide dismutase |
| | XP_012172050.1 | Glutathione S-transferase* |
| | XP_003397695.1 | Peroxiredoxin-6 |
| | XP_003401364.1 | Peroxiredoxin |
| | XP_003399714.1 | Peroxiredoxin-6 |
| | XP_003393349.1 | Heat shock 70 kDa protein cognate 5 |
| **Immune effector** | ADB29129.1 | Defensin 1 |
| | ADB29130.1 | Hymenoptaecin |
| | AEN41592.1 | Venom bombolitin 1 |
| | XP_003400548.1 | Phenoloxidase 2 |
| **Immune modulator** | NP_001267823.1 | Venom serine protease |
| | XP_003395081.1 | Venom protease |
| | XP_003395762.1 | Omega-conotoxin protein 1 |
| | AEN62314.1 | IRP30 |
| | XP_003393033.1 | Plasma protease C1 inhibitor |
| | XP_003395447.1 | Epididymal secretory protein E1 |
| | XP_003395667.1 | Ecdysteroid-regulated 16 kDa protein |
| | XP_003397328.1 | DEAD-box ATP-dependent RNA helicase 20 |
| | XP_003398065.1 | Putative ATP-dependent RNA helicase Pl10 |
| | XP_003398424.1 | Serpin B3 |
| | XP_003399186.1 | Antichymotrypsin-2 |
| | XP_003398065.1 | Putative ATP-dependent RNA helicase Pl10 |
| | XP_003401031.1 | Signal transducer and activator of transcription 5B |
| | XP_003401978.1 | Gametogenetin |
| | XP_003402576.1 | Serine protease inhibitor 3/4 |
| | XP_012163961.1 | Putative cysteine proteinase CG12163 |
| | XP_012163992.1 | Insulin growth factor-binding protein complex acid labile |
| | XP_012166473.1 | Plasminogen activator inhibitor 1 RNA-binding protein |
| | XP_012168271.1 | Protein argonaute-2 |

(Table 3.5 continued)

| Immune function | Accession number | Protein name |
|---|---|---|
| **Immune modulator** | XP_012172841.1 | Fibrillin-2 |
| | XP_012168652.1 | GTP-binding nuclear protein Ran |
| | XP_012169463.1 | Alaserpin |
| | XP_012172057.1 | Fragile X mental retardation syndrome-related protein 1 |
| | XP_012166473.1 | Plasminogen activator inhibitor 1 RNA-binding protein |
| | XP_003395653.2 | Serine protease easter* |
| | XP_012174357.1 | Apolipophorin* |
| | XP_003399683.1 | C-type lectin* |
| **PRR** | XP_003394802.1 | CD109 antigen |
| | XP_003397822.1 | Protein NPC2 homolog |
| | XP_003400160.1 | Peptidoglycan recognition protein |
| | XP_003400229.1 | MD-2-related lipid-recognition protein |
| | XP_012164498.1 | Chaoptin |
| | XP_012167632.1 | Beta-1,3-glucan-binding protein 1 |
| | XP_012173325.1 | Peptidoglycan-recognition protein 2 |
| | XP_012176660.1 | Leucine-rich repeat-containing protein 15 |

(*) indicates those proteins that have been reannotated as per section 2.6.3.

Within the 51 proteins comprising the FB immune repertoire is PRRs (8), antioxidants (12), immune effectors (4) and various other signalling modulators (27) (Table 3.5). To confirm the immune functionality of the FB immune repertoire, GO analysis was completed in Cytoscape using the *D. melanogaster* genome as the background reference (Figure 3.12). This functional analysis further confirmed the presence of immune proteins in the *B. terrestris* FB proteome which supports the role of FB in immunity.

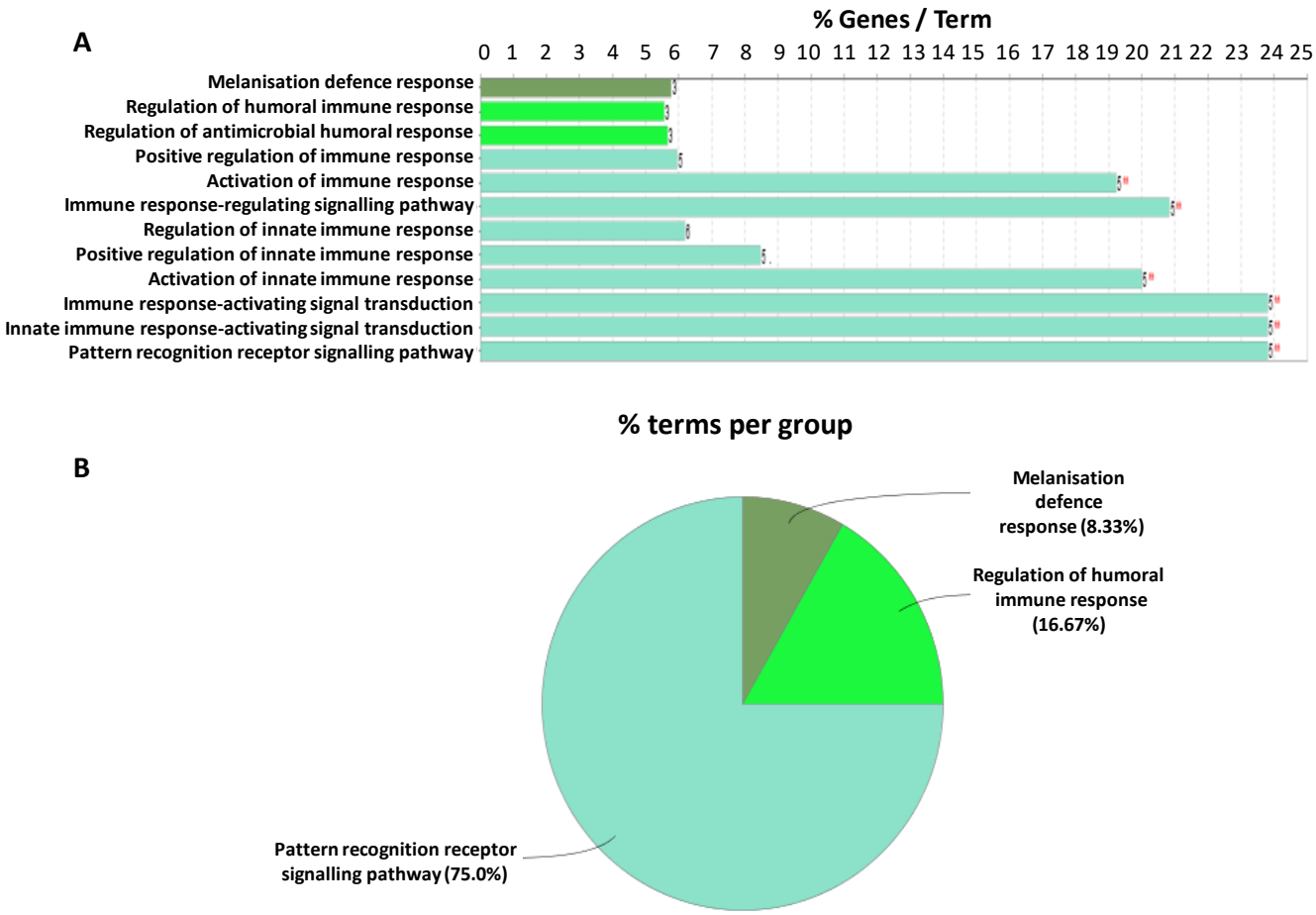


**Figure 3.12 Functional analysis of FB immune proteins.** Functional analysis of immune function of all identified immune-associated proteins in the FB proteome. (A) Bar chart representing all specific terms identified for immune proteins. The name of each functional class or pathway is assigned by the lowest p value of the terms of that class. Bars of the same colour indicate that those terms are categorised under the same functional group. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$statistical levels, respectively. (B) Pie chart contains functional labels of the most enriched terms only and provides an overview of the main functions indicated for the FB immune proteins.

### 3.2.4.2 Peptidase activity identification and classification

Proteolytic signalling cascades maintain numerous elements of insect physiology including immunity (Lu, Zhang, et al. 2014) and wound healing (Krautz et al. 2014). Hence, to efficiently and rapidly respond to changing physiological demands peptidases and inhibitors must be maintained at a basal level (Gubb et al. 2010). To investigate the abundance of peptidases and inhibitors in the FB proteome all proteins were blasted off the MEROPS database. Classification of proteins was completed in the database by assigning all identified peptidases or peptidase inhibitors to families based on statistically significant similarities in amino acid sequence, and further grouping families into clans when structural information supported homologous relationships (Alonso and Martinez 2017). The MEROPS database is periodically updated and in the most recent version 82 peptidase inhibitor families and 39 clans and 268 peptidases families and 62 clans are included (Rawlings et al. 2018).

In total 141 proteins were identified through the MEROPS search, of which 120 and 21 were proteases and their inhibitors respectively (Table 3.4). Both the peptidases and inhibitors represent proteolytic proteins from a diverse array of clans and families. The peptidases represented proteins belonging to 23 different clans and 43 families, and similarly the inhibitors are members of six different clans and families.

**Table 3.6 Peptidases and inhibitors of the FB proteome.** For Protease Class Aspartic (*A*), Cysteine (*C*), Threonine (*T*), Serine (*S*) proteases, Metalloprotease (*M*) and unknown (*U*). Number of proteins refers to numbers in each family. (I) indicates inhibitors and (P) indicates peptidases.

| Catalytic Type | Clan | Family | No. of proteins | Reference protein | Biological function | I/P |
|---|---|---|---|---|---|---|
| Inhibits serine endopeptidases | IM | I15 | 1 | Antistasin | Anticoagulant | I |
| Inhibits all endopeptidases | IL | I39 | 2 | Alpha2-macroglobulin | Antigen processing | I |
| Inhibits serine & cysteine endopeptidases | ID | I4 | 8 | Serpin | Immune modulator | I |
| Inhibits various kinases | JE | I51 | 1 | - | Kinase/chymotrypsin inhibitor | I |
| Inhibits metallopeptidase pappalysin | JB | I63 | 8 | Pappalysin-1 | Promotes cell growth | I |
| Contains a carboxypeptidase inhibitor | JK | I68 | 1 | - | Anticoagulation | I |
| *A* | AA | A1 | 1 | Nucellin | Proteolysis | P |
| *A* | AA | A9 | 1 | Spumapepsin | Proteolysis | P |
| *C* | CA | C1 | 2 | Cathepsin B | Lysosomal activity | P |
| *C* | CA | C111 | 8 | - | - | P |
| *C* | CD | C13 | 1 | Legumain | Lysosomal activity | P |
| *C* | CA | C2 | 1 | Calpain | Signal transduction | P |
| *C* | PC | C26 | 8 | Gamma - glutamyl hydrolase | Lysosomal activity | P |
| *C* | PB | C44 | 3 | - | autolytic cleavage | P |
| *C* | PC | C56 | 1 | - | Endopeptidase | P |
| *C* | CP | C97 | 1 | - | Proteostasis | P |
| *M* | MA | M1 | 1 | Aminopeptidase A | Immune modulator | P |
| *M* | MA | M12 | 2 | - | Signal transduction | P |
| *M* | MA | M13 | 6 | - | Proteolysis | P |
| *M* | MC | M14 | 2 | - | Antigen processing | P |
| *M* | ME | M16 | 5 | - | - | P |
| *M* | MF | M17 | 1 | Leucyl aminopeptidase | Antigen processing | P |
| *M* | MA | M2 | 1 | - | Antigen processing | P |
| *M* | MH | M20 | 2 | Angiotensin II | Vasopressor | P |
| *M* | MO | M23 | 10 | - | Proteolysis | P |
| *M* | MG | M24 | 3 | - | Proteostasis | P |
| *M* | MH | M28 | 2 | - | - | P |
| *M* | MJ | M38 | 1 | - | - | P |
| *M* | MA | M41 | 4 | - | Proteostasis | P |
| *M* | MA | M48 | 1 | - | Proteostasis | P |
| *M* | MA | M49 | 1 | - | Proteostasis | P |
| *M* | MM | M50 | 2 | - | Proteostasis | P |
| *M* | MA | M61 | 1 | - | Proteostasis | P |
| *M* | MP | M67 | 1 | - | Proteostasis | P |

(Table 3.6 continued)

| Catalytic Type | Clan | Family | No. of proteins | Reference protein | Biological function | I/P |
|---|---|---|---|---|---|---|
| *S* | PA | S1 | 9 | Chymotrypsin | Immune modulator | P |
| *S* | SC | S10 | 2 | - | Lysosomal activity | P |
| *S* | SK | S14 | 1 | - | Proteostasis | P |
| *S* | SJ | S16 | 1 | - | Proteostasis | P |
| *S* | SC | S28 | 2 | - | Lysosomal activity | P |
| *S* | PA | S29 | 1 | - | Proteostasis | P |
| *S* | SK | S49 | 1 | - | Signal transduction | P |
| *S* | SP | S59 | 1 | - | Proteostasis | P |
| *S* | unassigned | S72 | 1 | - | - | P |
| *S* | SB | S8 | 1 | - | Antigen processing | P |
| *S* | SC | S9 | 12 | - | Antigen processing | P |
| *T* | PB | T1 | 11 | - | Proteostasis | P |
| *T* | PB | T3 | 1 | Gamma - glutamyl hydrolase | Lysosomal activity | P |
| *U* | unassigned | U69 | 2 | - | - | P |
| ***S*** | **SR** | **S60** | **1** | **Lactoferrin** | **-** | **P** |

## 3.3 Discussion

The functional, pathway and protein level analysis of the FB proteome has revealed key involvement of this organ in immunity, proteostasis, metabolism and cellular processes, the majority of which are core cellular and molecular processes typical of most cell types. As predicted for the fat body, carbohydrate, lipid and energy metabolism are well represented in the functional analysis (Figure 3.4 &3.5) (Table 3.2 & 3.3). The global view of metabolism in the bumblebee FB is one of the constant monitoring, breakdown and recycling of nutrients (Figure 3.7). As expected, analysis also indicated the FB as being highly involved in protein processing, with numerous elements of translational mechanisms represented (Figure 3.3, 3.4 & 3.5). Analysis of the catalytic activity of proteins within the proteome revealed the majority were proteases, with limited numbers of inhibitors (Table 3.6). The presence of classical haemolymph proteins (apolipophorin, vitellogenin, transferrin etc.) (Table 3.1) and numerous immune proteins (AMPs, PRRs etc.) (Table 3.5) which are also commonly found in the haemolymph indicated the FB as a potential synthesis site for haemolymph proteins.

### 3.3.1 Key findings

- The *B. terrestris* fat body is composed of two cell types adipocytes and oenocytes, with the latter representing the most abundant cell type.
- Unlike queens of the same species, *B. terrestris* workers contain only one fat layer, the parietal layer.
- The *B. terrestris* FB is a highly metabolic and immune active tissue, which is involved in the monitoring, mediation and maintenance of internal homeostasis.

### 3.3.2 Fat body morphology

Two main cell types form the *B. terrestris* worker FB, adipocytes and oenocytes. The larger of the two are adipocytes, which are identifiable by their large multilobed nucleus and often large lipid droplets in the cytoplasm. Oenocytes can be distinguished from adipocytes by their small central uniform nucleus and lack of lipid droplets. However, although oenocytes lack lipid droplets, they are still lipid rich cells and are thought to be the source of cuticular lipids (Lockey 1985). Both cell types are typically intermixed throughout the FB, however within the worker FB oenocytes occur more abundantly. When found in lobes oenocytes appear to hold their circular shape more rigidly than that of adipocytes which mould their form to fit around each other and the oenocytes.

The observation of higher proportions of oenocytes than trophocytes in the worker FB is at odds with similar studies on Queens of the same species which had a higher proportion of adipocytes than oenocytes (Votavová et al., 2015). Analysis of sections also revealed that unlike other insects, *B. terrestris* workers appear to contain only one dominant layer of fat cells within the abdomen. This is referred to as the parietal layer and is located directly below the cuticle. Although adipocytes have been reported to be dispersed close to the gut of *B. terrestris* Queens, no fat cells outside the parietal layer were observed in their worker counterparts (Votavová et al., 2015). This perivisceral fat layer has also been found to be absent in adult mosquitos and the peripheral FB similarly forms a continuous layer of cells (Martins and Pimenta 2008; Martins et al. 2011). The biological significance for the absence of the perivisceral FB in adults mosquitos and *B. terrestris* workers remains unclear (Assis et al. 2014).

Fat cells were also observed surrounding the abdominal section of the dorsal vessel. Along this region an abundance of fat and pericardial cells is often observed near the ostioles, dorsal vessel valve openings, among which immune cells are dispersed. This close association between the fat body cells, dorsal vessel and pericardial cells represents a region where the fat body along with two or more cell types function as one biological system, in this case the hepato-nephrocitic system (Abdalla and Domingues 2015). This system is thought to exhibit biological homology to vertebrate hepatocytes and nephrocytes and therefore could function in detoxification (Abdalla et al. 2014). Due to this it has recently been investigated as a novel biomarker parameter for the study of ecological stresses, such as toxicants, on bumblebees (Abdalla and Domingues 2015).

### 3.3.3 Glycolysis, lipolysis, proteolysis and the maintenance of energy metabolism in the fat body

Insects are constantly expending energy, so in the absence of food sources, like all higher animals, insects have the ability to store and utilise carbohydrates, proteins and fatty acids from their food as sources of energy (Arrese and Soulages 2010; Azeez et al. 2014). Fat reserves are the most important nutrient store in insects and are used to not only meet their daily life requirements for energy like flight (Beenakkers et al. 1985) but also the demands for major life events such as metamorphosis (Ziegler and Van Antwerpen 2006). Through various metabolic pathways dietary fatty acids, carbohydrates or proteins can be converted to triacylglycerol (TAG) and stored in the fat cells, this store can be then mobilised to support metabolic needs (Arrese and Soulages 2010). The metabolic

processes that work in unison to manage storage and utilization of nutrients; as well as synthesis and maintenance of energy (adenosine triphosphate (ATP)); glycolysis, lipolysis, proteolysis and oxidative phosphorylation (OXPHOS) are all enriched within the FB proteome (Figure 3.4, 3.5 & 3.7). In accordance with the enrichment of these processes and the role of the fat body in energy metabolism, various enzymes involved in carbohydrate, lipid and protein metabolism were also identified in the proteome including; glyceraldehyde-3-phosphate dehydrogenase 2, aldehyde dehydrogenase, enolase and protein disulfide-isomerase (Table 3.1).

The two main energy reserves in animals are TAG and glycogen. Lipids are stored as TAG and carbohydrates are stored as glycogen. The mobilisation of these valuable energy reserves is controlled by two families of peptides; adipokinetic hormone (AKH) family and the insulin-like peptides (ILPs). AKH is produced and secreted by the corpus cardiac (CC) whilst ILPs are synthesized in the; median neurosecretory cells of the brain, corpus allatum, corpus cardiac and some peripheral tissues including the fat body (Hoshizaki, 2013).

Although fat reserve accumulation across insects varies; lipid is always the main component, representing more than 50% of the dry weight (Ziegler 1991). The ratio of glycogen to TAG can be altered across life cycles and in response to environmental stresses. TAGs are stored in a more anhydrous form than the hydrated, bulky glycogen. Storage as TAG allows it to be used for energy production through β-oxidation (Athenstaedt and Daum 2006). TAG also has a higher caloric content per gram than glycogen enabling them to produce twice as much metabolic water (Arrese and Soulages 2010). Due to their high energy yield they account for more than 90% of the lipid stored in the fat body (Canavoso et al. 2001).

Fat body accumulation is determined by the balance between fat synthesis (lipogenesis) and degradation (lipolysis/ fatty acid oxidation) (Kersten 2001). Lipogenesis takes place in the adipocytes and is the process in which fatty acids are synthesized and subsequent TAG production is also initiated. This process facilitates efficient storage of energy as fats by allowing fatty acids to be taken up and incorporated into the fat body.

In insects' fatty acids are necessary for high energy dependant skeletal muscular activities such as flying and the formation and secretion of both pheromones and eicosanoids (Arrese and Soulages 2010). Within the FB degradation of fatty acids was found to

enriched confirming the active breakdown of lipids within this tissue to support biological needs of the bee (Table 3.2) (Figure 3.9). Beta-oxidation is the metabolic breakdown of triglycerides into free fatty acids for energy. The result of this process is the production of acetyl-CoA, which can enter the tricarboxylic acid cycle (TCA) and be used by the electron transport chain to produce ATP.

In insects TAGs are transported through the haemolymph to appropriate tissues (muscle, fat cells etc.) by lipoproteins. The most abundant protein identified within the FB proteome was the lipoprotein, apolipophorin. Lipophorins are the major lipoproteins found in haemolymph and act as re-useable, non-internalised lipid transporters (Tsuchida and Wells 1988). During the passage to target organs or storage most fatty acids are transported as diacylglycerol (DAG) in lipophorins. One hallmark of lipid metabolism in insects is the tissue specificity of lipid delivery and hence the same lipophorins can selectively deliver energy to individual organs as required (Arrese et al. 2001).

In many insects' lipids are also mobilised in the haemolymph in response to an immune challenge (Cheon et al. 2006), the nature of this mobilisation and the fate of the lipids are unknown. It is thought they could be involved in membrane biogenesis, haemocyte recruitment or as a source of energy (Arrese and Soulages 2010).

Glucose is essential in energy metabolism, heat generation and is also important in the chitinous exoskeleton. In the fat body glucose is stored in its polymeric form glycogen which can be easily and quickly degraded to provide a source of glycolytic fuel (Steele 1982). Breakdown of carbohydrates to lipids by the insect metabolism is well studied and documented (Arrese and Soulages 2010; Kunieda et al. 2006). The capacity of the fat body for glucose lipogenesis is much greater than that of glycogen synthesis which perhaps explains the high glucose to glycogen ratio content in the fat body (Arrese and Soulages 2010).

The biochemical entry point for conversion of glucose to chemical energy is the glycolytic pathway. Through which glucose is metabolised by a series of ten reactions to form pyruvate, the end point of glycolysis. Among the numerous metabolic-associated proteins in the top 50 most abundant in the FB proteome, is pyruvate kinase, this enzyme catalyses the third irreversible step in glycolysis and controls the outflow from this pathway. This final step yields ATP and pyruvate, a central metabolic intermediate that can be oxidized further or used as a building block (Berg et al. 2002). Pyruvate is

oxidatively decarboxylated to form acetyl CoA in order to enter the TCA cycle. In this cycle, acetyl CoA is oxidised, and further ATP is produced. Within the FB proteome glycolysis/gluconeogenesis, pyruvate and TCA cycle pathways are present and statistically enriched confirming the breakdown of glucose for energy is actively undertaken by the FB.

Proteolysis refers to the process whereby proteins are broken down to their amino acid (AA) components. Within the FB a number of pathways and processes associated with the degradation of proteins were identified including the proteasome, AA degradation pathways and enzymes such as protein disulfide-isomerase. AAs can be used by insect cells for the biosynthesis of proteins and nucleotides but can also serve as an energy source through incorporation into the TCA cycle as intermediates (Pushparajan et al. 2017). Further to the identification of carbohydrate, lipid, protein and energy metabolism with the FB proteome, gene ontology mapping revealed that the FB comprised proteins associated with glucagon, insulin and neuronal signalling, confirming the FBs role in lipid and carbohydrate homeostasis.

Mitochondria are commonly referred to as the "Powerhouses of the cell" due to fact that the major part of ATP is synthesised by OXPHOS, which takes place in the inner mitochondrial membrane (Korla and Mitra 2014). The TCA cycle is the precursor for OXPHOS and is tightly associated with it both spatially and functionally and hence facilitates ATP synthesis in the mitochondria (Korla and Mitra 2014). Within the FB proteome OXPHOS was shown to be enriched through both protein interaction and pathway functional analysis. This indicates the FB is a mitochondrial-rich tissue and is centrally related to energy production in *B. terrestris* workers. Furthermore, carbohydrate, lipid and protein degradation all result in the production of TCA cycle precursors (acetyl CoA) which are also enriched in this proteome, this in indicative of all three processes contributing and actively participate in the production of energy within the *B. terrestris* FB.

### 3.3.4 Protein homeostasis in the fat body

Within the FB one of the major classes of biological processes revealed to be enriched was genetic information processing. This class includes the pathways responsible for protein production and degradation; the ribosome, protein processing in the endoplasmic reticulum, RNA transport, the proteasome and spliceosome. MEROPs analysis also

indicated numerous peptidases present in the FB as having roles in this proteolytic process including threonine peptidases which are the component peptidases of the proteasome. Further interrogation of the numbers of FB proteins involved in these pathways revealed that each is well populated with FB proteins. The biological enrichment and identification of numerous FB proteins in associated protein translation pathways confirms the role of the FB as a major site of protein synthesis activity. Other significant processes enriched within the proteome alongside protein synthesis machinery included cellular transport. These pathways are central to the movement of molecules in and out of the cell and include; endocytosis, phagosome, lysosome and peroxisome. They participate in the movement of particles and proteins in and out of the cell (AMPs, haemolymph proteins) and degradation of cytoplasmic constituents (house-keeping or immune-related).

Cells within all biological systems are continually subject a plethora of physiological and environmental stimuli. The response to which can range from acute to chronic and result in damage and dysfunction. Hence, cellular pathways that monitor protein synthesis, folding, and degradation are essential and highly conserved (van Oosten-Hawle and Morimoto 2014). The network responsible for maintenance of protein homeostasis (proteostasis) in an integrated and dynamic system of interconnected and processes which are stringently regulated by a variety of quality control mechanisms (Hetz and Glimcher 2011). Due to highly active protein synthesis machinery and secretory nature of the FB it is possible that pathways involved in both genetic information processing and cellular transport collaborate and coordinate to monitor proteostasis (protein homeostasis) within the FB.

Numerous elements of proteostasis are present in the FB from ribosomal subunits which initiate and translate mRNA, to protein synthesis checkpoints which remove mis-folded proteins to the proteasome for degradation. The processes within cellular transport may be exploited during proteostasis to remove misfolded or damaged proteins from cells.

### 3.3.5 The fat body immune association

Although the fat body is mainly associated with storage and metabolic functions, it is also involved in the regulation of haematopoiesis and innate immune homeostasis (Azeez et al. 2014). In *Drosophila* the humoral immune response to microbial infection, including production of AMPs, PRRs and signalling cascades, is mainly undertaken by the

haemocytes and fat cells (Keebaugh and Schlenke 2014). Similarly, in *B. terrestris* it was revealed that the fat body contains many proteins associated with central immune activities such as; pathogen recognition, cascade modulation, the prophenoloxidase cascade, antioxidant and antimicrobial activity. Among the most abundant proteins in this proteome were a variety of ubiquitous immune and defence proteins including venom bombolitin, numerous heat shock proteins, SOD, catalase, phenoloxidase and IRP30. Their abundance at similar and higher levels than metabolic and storage proteins which are traditionally linked to the function of the fat cells indicates the FB as a highly immune active tissue.

Functional analysis further confirmed the role of the FB in immune activities and indicated that PRR signalling pathway, melanisation and regulation of humoral immune response are particularly well represented in the FB. Numerous components of the PO cascade were also identified within the proteome including phenoloxidase, anti-chymotrypsin and serpins associated with initiation of this cascade . Melanisation can be initiated by a variety of antigens but depends on the activation of the enzyme phenoloxidase and the proPO cascade (Schmid-Hempel 2004). Phenols are converted into quinones by PO which then polymerizes to melanin (Söderhäll and Cerenius 1998). This melanin is then deposited onto the surface of a pathogen where haemocytes aggregate to encapsulate it. It has been shown in *B. terrestris* that upon infection prophenoloxidase (proPO) is activated and haemocytes are released representing the front line of defence inside the cuticle (Korner and Schmid-Hempel 2004).

The largest immune function represented within the FB was modulation of signalling. One of the FBs key immune functions is to release soluble factors into the haemolymph (Lemaitre and Hoffmann 2007). The immune factors it produces include antimicrobial peptides and signalling molecules, which are both numerous in the *B. terrestris* FB. Initiation of the transcription of these immune factors is completed via the Toll and Imd pathways which are located downstream of recognition molecules, also present in the FB proteome, that bind microbial elicitors such as peptidoglycan and beta-1,3 glucan (Lemaitre and Hoffmann 2007). Numerous peptidases and inhibitors within the FB proteome were also identified as having roles in immune signal transduction and regulation.

It is widely acknowledged that the FB is a powerful secretory organ of defence proteins (Krautz et al. 2014). Probably the most potent of all these proteins are AMPs. This investigation revealed the presence of six antimicrobial immune effectors in the FB, including two well-characterised AMPs (defensin and hymenoptaecin) and four venom proteins with novel antimicrobial activity (Moreau 2013). The main stimulus for increased AMP expression is the recognition of microbial antigens such as peptidoglycan and lipopolysaccharides. In insects, recognition of pathogens is achieved through PRRs stimulating the immune signalling cascades (Takehana et al. 2002). Once pathogens are recognised they initiate the production and release of eicosanoids such as NF-kB in the fat body (Morishima et al. 1997). They then initiate immune pathways such as Toll, Imd and JAK/STAT that result in the up-regulation of AMP genes and lysosomes and the subsequent production of antibacterial peptides (Erler et al. 2011; Barribeau et al. 2015; Sadd et al. 2015). The presence of these six AMPs in the FB proteome indicates that they are constitutively expressed in the FB, with one even among the most abundant proteins in the proteome (venom bombolitin). The presence of so-called venom proteins within the FB is not as a result of this tissue producing venom but merely due to the original identification and manual annotation of the individual protein.

Functional analysis further confirmed the role of the FB in immune activities and indicated the following as the most well-represented functions within the FB; PRR signalling pathway, melanisation and regulation of humoral immune response. Numerous components of the PO cascade were also identified within the proteome including phenoloxidase, anti-chymotrypsin and serpins associated with initiation of this cascade.

### 3.3.6 Reactive oxygen species and antioxidants

As in other organisms, insects must deal with a wide variety of potentially challenging environmental factors such as chemical, physical, and physiological stressors which can trigger an imbalance in homeostasis. Oxidative stress (OS) is one such imbalance and is characterised by increased production of reactive oxygen species (ROS) which can result in oxidative damage to proteins, lipids, and nucleic acids**.** As a consequence, the normal functions of cells, tissues, or even the whole organism may be seriously disrupted, which could detrimentally impact essential biological processes (Kodrík et al. 2015). To avoid or reduce this, organisms contain numerous antioxidant enzymes and substances, such as SOD, CAT, glutathione peroxidase, glutathione, ascorbate (vitamin C), tocopherol

(vitamin E), and thioredoxin (Bayir 2005), many of which were identified within the FB proteome.

Although ROS are implicated in numerous detrimental activities such as induction of oxidative damage they are also a normal by-product of metabolism and are involved in enzymatic reactions, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors, gene expression, and the antimicrobial action of immune cells (Bayir 2005). The presence of high amounts of antioxidants and enrichment of metabolic, detoxification and oxidation-reduction processes within the FB confirm it is highly metabolic tissue.

### 3.3.7 Peptidases and Inhibitors

Proteolytic signalling cascades are central to the activity of numerous biological processes as they facilitate rapid response to danger signals. Proteases modulate the activity and localisation of many proteins and protein-protein interactions (López-Otín and Bond 2008). Most protease components of extracellular signalling cascades are secreted as inactive zymogens, with each successive zymogen being activated by the previous protease in the cascade. Zymogens, particularly the first protease in a pathway, can often be autocatalytic, but amplification cascades can only respond to physiological challenges if they retain a low level basal activity (Gubb et al. 2010). Hence, their action is strictly controlled and imbalances in their activities are central in a number of pathologies (Turk et al. 2012). Inhibitors monitor accidental triggering of signalling and regulate the transduction and termination of signals once cascades are activated (Gulley et al. 2013a). Protease inhibitors function in limiting the activity of proteases by making a complex with them. They have the ability to inhibit both proteases with broad proteolytic actions (e.g. digestive enzymes) and those with specific target substrates (such as the component proteases of signalling cascades) (Gubb et al. 2010).

To further improve our knowledge of the proteolytic capacity of the fat body all identified proteins were blasted off the MEROPS database. Overall six families of inhibitors and 43 peptidases were identified. The six families of peptidase inhibitors identified had roles in biological processes ranging from anticoagulation and antigen processing to immune modulation and cell growth. The most well represented families of inhibitors were the I63 metallopeptidase pappalysin-1 inhibitor family and I4. I63 contains an inhibitor specific for the protein pappalysin-1, a function associated with this family of inhibitors

include inhibition of cell growth (Glerup et al. 2005). I4 proteins are inhibitors of both serine and cysteine endopeptidases. Among the most well-known proteins in this family are serine protease inhibitors (serpins).

Serpins are the largest and most important serine proteinase inhibitor family in higher eukaryotes (Gulley et al. 2013a). The majority of serpins inhibit chymotrypsin-type serine proteinases however there are serpins which can also act on cysteine proteinases (Schick et al. 1997; Kantyka and Potempa 2011). Although serpins mainly function as potent protease inhibitors, modulating a wide variety of proteolytic cascades hence controlling numerous physiological and pathological reactions (Bao et al. 2018), some perform other roles including hormone transport (Reichhart et al. 2011) and storage (Law et al. 2006). In *B. terrestris* the serpin 27A has been shown to be involved in suppression of the PO cascade activator serine protease 8 (Richter et al. 2012). Identification of high numbers of serpins within the proteome further confirms the enrichment of numerous immune signalling associated processes in the FB such as the melanisation defence response, regulation of the humoral immune response and PRR signalling pathways.

Among the 43 protease families identified in the FB, the three most well represented in descending order are; S9, T1 and M23. Family S9 contains a varied set of serine-dependent peptidases which are believed to be important for the degradation of biologically active peptides (Rawlings et al. 2018). The family T1 contains threonine peptidases which are the component peptidases of the proteasome (Tanaka 2009). The M23 peptidase family contains endopeptidases  some of which lyse bacterial cell wall peptidoglycans (Firczuk and Bochtler 2007). The abundance of peptidases associated with degradation of peptides and the proteasome corresponds to functional analysis of the FB proteome. Other processes associated with the peptidases identified in the FB proteome include; signal transduction, involvement in proteostasis and antigen processing.

### 3.4 Conclusion

The characterisation of all proteins expressed by the FB, has revealed important aspects of reactivity, organisation and function in this central organ. Proteomic based analysis of the FB revealed it is an essential regulatory organ with multiple functions ranging from nutrient storage and carbohydrate metabolism to immunity and defence response. As expected, the FB has key roles in energy metabolism and maintains production to meet

the demands of *B. terrestris* workers through integration of numerous metabolic pathways (lipid, carbohydrate and protein). The FB is also highly active site of protein synthesis, with all aspects of protein transcription, translation, folding and degradation enriched within the proteome. To chaperone proteins out of the FB and assist in mediation of proteostasis is a variety of cellular transport processes. Gene ontology analysis further confirmed the role of the FB in immune activities and identified the following as the most well-represented functions within the FB; PRR signalling pathway, melanisation and regulation of humoral immune response. In addition, numerous haemolymph proteins were identified within the FB including apolipophorin, vitellogenin and transferrin which may indicate the FB as a potential synthesis site for these and other haemolymph proteins. The comprehensive characterisation of the FB conducted here provides insight into these processes and will be invaluable to gaining insight into how the bumblebee contends with and is affected by environmental stresses.

As most stressors (pathogens and pesticides) of bumblebees are orally ingested and therefore interact within the abdomen, another important aspect for disease and toxicant histopathology is the digestive tract and HNS. Histopathology of segments of the digestive tract of *B. terrestris* has previously been exploited to study the structure and pathology of the important bumblebee specific parasite *Nosema bombi* (Larsson 2007).

# Chapter 4

## Characterisation of the total circulating haemolymph content of the Buff-tailed bumblebee (*Bombus terrestris*)

## 4.1 Introduction

Insects have an open circulatory system through which haemolymph, their main extracellular fluid, is pumped by the dorsal vessel via small arteries into the spaces surrounding tissues and organs called sinuses (Klowden 2007). Haemolymph is in direct contact with all the internal organs and as with blood in higher organisms, it is the central medium responsible for transport and exchange of nutrients, hormones and waste materials between tissues and organs (Chan et al. 2006; Klowden 2007).

This complex fluid is primarily composed of plasma and cellular haemocytes with the main plasma components being; proteins, lipids, carbohydrates, amino acids, inorganic ions and hormones which are secreted into the haemolymph by the fat body (FB), haemocytes, epidermis and midgut epithelium (Gilbert and Chino 1974; Klowden 2007). Numerous factors such as developmental stage (Chan and Foster 2008), diet (Blatt and Roces 2001), temperature (MacMillan et al. 2015) and disease (Bergin et al. 2003) can influence the cellular and fluid composition of insect haemolymph.

In addition to transporting nutrients, waste products, hormones and water, haemolymph is involved in many essential immune roles including protection and defence against; physical injury, invading pathogens or foreign bodies (Lavine and Strand 2002). Hence, this fluid contains numerous immune components including haemocytes, antimicrobial peptides (AMPs) and elements of the signalling cascades such as the prophenoloxidase signalling system (PPO) (Chan et al. 2006).

Within the insect haemolymph there are at least eight types of haemocytes including; prohemocytes, plasmocytes, granular cells, coagulocytes, crystal cells, spherulocytes, oenocytoids and thrombocytoids however not all are found in every insect (Pandey and Tiwari 2012). Haemocytes are synthesized during embryogenesis and the early developmental stages in hematopoietic organs and although they vary in size, structure and nomenclature, their main functions; to recognise foreign material and distinguish between self and non-self is conserved across all types (Strand 2008a). Recognition of pathogens stimulates haemocytes to attack and neutralise invading microbes via phagocytosis,

encapsulation, and nodulation. These responses are dependent on the recognition of objects as foreign to induce effector responses and proteolytic cascades (Strand 2008a).

Insect haemolymph is particularly protein rich and comprises proteins associated with defence, metabolism and nutrient storage. Many of the highly abundant haemolymph proteins seem to be conserved across phylogenetically diverse insects. The major groups of haemolymph proteins common to all insects include various storage proteins, lipoproteins, vitellogenins and inducible antibacterial proteins (Kanost et al. 1990). Representative of these groups are the most common adult haemolymph proteins which include; transferrin, vitellogenin and apolipophorin (Chan et al. 2006). Transferrin, a well-characterised iron transport protein participates in the movement of iron within the haemolymph to target tissues (Nichol et al. 2002). Insect transferrin has also been implicated as an infection-inducible gene (Yoshiga et al. 1997) and in innate immunity to fight microbial infections through iron sequestering strategies (Weinberg 1993). Vitellogenin is a common yolk precursor protein and hence a basic reproductive molecule (Guidugli et al. 2005). In honeybee (*Apis mellifera*) haemolymph, vitellogenin has also been revealed as a zinc carrier which is implicated in somatic maintenance (Amdam et al. 2012). Lipophorins are the major lipid transporting proteins of insect haemolymph. They can act as a reusable transporters shuttling and delivering lipids to target tissues without internalisation and degradation of the particle (Pennington and Wells 2002). Numerous novel roles for apolipophorins have emerged in pattern recognition and multicellular encapsulation reactions in the innate immune response (Whitten et al. 2004).

The bumblebee *Bombus terrestris* is a widespread and important European pollinator yet its physiology and in particular the specifics of its immune response are not yet fully understood. Although bumblebees are widely used models for the study of social aspects of disease pathology and immunity (Evans et al. 2006) and host-parasite interactions the underlying molecular processes involved in these events are still unknown (Mallon et al. 2003). With bee numbers across the world rapidly declining due to highly detrimental ecological and environmental changes, (Goulson et al. 2015) and with the spread of pathogens and parasites among bee species constantly rising, the ability to moderate drivers of bumblebee decline will require a thorough understanding of their defence and stress-associated mechanisms.

The aim of this chapter is to characterise the haemolymph proteome and profile the haemocyte composition and diversity in *B. terrestris.* Recent advances in molecular tools and the publication of the bumblebee genome (Sadd et al. 2015) have provided the basic molecular information to facilitate in-depth proteomic analysis on this important serum. This enables global analysis of protein composition provides the potential to gain insight into the numerous biological tasks associated with bumblebee haemolymph. To date there are few comprehensive published proteomes for bee haemolymph. A quantitative comparison of caste differences in honeybee haemolymph by (Chan et al. 2006) revealed notable changes in the molecular composition of haemolymph between the developmental stages and sexes. Gene ontology analysis of the *B. terrestris* queen haemolymph proteome by (Sadd et al. 2015) revealed the presence of proteins associated with metabolism, transport, olfaction, chemosensing and venom. In addition, numerous haemolymph associated proteins (HAPs) of unknown function were identified in the queen proteome. Comparisons between *B. terrestris* and *A. mellifera* queen haemolymph proteomes revealed similarities in the proteins involved in defence, immunity and antioxidant processes. The main differences in the proteomes were attributed to the *B. terrestris* HAPs, which indicated their potential roles in notable behavioural, physiological and social differences between these two species. The results of this cellular and molecular characterisation of the *B. terrestris* worker haemolymph proteome will provide a knowledge platform from which further research into mechanisms of defence and immunity can be based.

Bumblebee haemocytes are highly versatile immune cells and can vary dramatically in abundance in response to pathogens (Korner and Schmid-Hempel 2004) and across developmental stages (Moret and Schmid-Hempel 2009). Although they are a vital aspect of immunity and development currently there is little known about their population diversity in *B. terrestris* workers. This study aims to contribute to the knowledge of the types of circulating haemocytes in *B. terrestris* through examination of cytological preparations. Haemocytes have varying modes of action in wound and defence response, hence identifying the types of circulating haemocytes will complement defence-associated functional analysis of the haemolymph proteome.

Given that the fat body is in close proximity with the haemolymph and is recognised as a principal source of many haemolymph proteins, a comparison of haemolymph and fat body proteomes will also be conducted. This will result in the elucidation of important similarities and differences between these central tissues and will help determine the contribution of the fat body to the haemolymph.

### 4.1.1 Chapter four aims

- To establish the baseline level of circulating haemocytes within *B. terrestris* worker haemolymph.
- To comprehensively examine the molecular composition of worker haemolymph using proteomic analysis tools.
- To investigate the functionality of the haemolymph proteome through gene ontology analysis to reveal associations with biological processes.

### 4.1.2 Experimental outline

To investigate and characterise the composition of the total circulating haemolymph content, haemolymph was collected from commercial workers and subject to cytological and proteomic analysis. All bees were sampled at random from multiple commercial colonies. Selected bees were removed from the colony 48 hours prior to sample collection and housed in wooden holding boxes (depth 12 cm x width 16 cm x length 24 cm) with a perspex top. Bees were maintained under standard conditions (24±1°C, 60% ± 5 relative humidity (RH)) in continuous darkness and fed *ad libitum* on prescribed Agralan pollen and sucrose solution (0.5M).

The morphological analysis of the circulating haemocytes was performed using cytological smears prepared from haemolymph (10-15 µl) collected from workers (n = 4) and added directly to Polysine™ slides. Two collections and two subsequent sets of smears were prepared from each individual. All slides were incubated for 20 mins at room temperature to allow the haemolymph to dry and the haemocytes to settle and adhere to the slide. Once dry, preparations were fixed in 4% v/v paraformaldehyde. One of the two slides obtained per individual was subject to either haematoxylin and eosin (H&E) or fluorescent staining. H&E

stained slides were mounted and examined using light microscopy on a Leica DM1000 photomicroscope. Slides stained with fluorescent dyes (DAPI and Rhodamine phalloidin) were imaged using an Olympus Fluoview 1000 confocal microscope. Measurements for cell area and diameter were obtained using ImageJ analysis software and are presented as mean values ± standard error of the mean.

To establish the baseline level of circulating haemocytes within the haemolymph cell counts were conducted from bumblebee workers (n = 16) sampled from four commercially sourced colonies (Biobest, Agralan Ltd). Haemolymph (10 µl) was collected, diluted in 50 µl of ice-cold Ringer's solution and mixed by gently pipetting up and down following the methods of Moret and Schmid-Hempel, (2009). A 10 µl subsample was diluted (1:1) with trypan blue (0.4%) and a 10 µl aliquot of this mix was loaded on a haemocytometer. The slide was left to stand for 5 - 10 minutes to allow the haemocytes to settle prior to counting and calculation of final concentration.

To characterise the haemolymph proteome of *B. terrestris*, serum from three commercial workers (n = 2 colonies, Koppert Biological Systems) was collected (section 2.2.3) and prepared for LC-MS/MS analysis. From each bee approximately 20µl of haemolymph was collected using a microcapillary tip and deposited in ice cold FSPBS supplemented with protease inhibitor cocktail tablet (50 µl). All collections were centrifuged (8,000 rpm for 5 minutes at 4˚C) to remove debris and the supernatant was removed to a new microtube for further processing. This haemolymph isolation procedure was undertaken to minimise the time for potential modifications (proteolytic and chemical) of haemolymph proteins during collection and processing that could influence downstream proteomic analyses.

To prepare the samples for mass spectrometry the following steps were conducted; samples were quantified using the Qubit™ protein assay kit, contaminants were removed using a 2D clean-up kit, samples were subject to overnight tryptic digestion and finally, purified using C18 ZipTip® pipette tips. Tryptic peptides were then resuspended in 2% v/v acetonitrile and 0.05% v/v TFA and 1 µg was loaded onto a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer for analysis. Peptides were separated over a 2 – 40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 60 min reverse-phase gradient at a flow rate of 250 nL $min^{-1}$.

Initial protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.5.6.5 (http://www.maxquant.com). Data analysis and processing of the data was then performed using Perseus v 1.5.0.8. (www.maxquant.org/). To resolve protein-protein interactions and identify functions and pathways most strongly enriched within the proteome, STRING v. 10.5 (http://string-db.org/) and the BlastKOALA sequence similarity tool (https://www.kegg.jp/blastkoala/) were used. Using the gene ontology (GO) software; gene ontology enrichment analysis and visualization tool (GOrilla) and ClueGO v 2.5.1 (http://www.cytoscape.org/) a global overview of the biological processes within the proteome was revealed. To investigate and quantify the numbers of peptidases and inhibitors within the haemolymph identified proteins were blasted against the MEROPs database v 12.0 (https://www.ebi.ac.uk/merops/). Defence and immune constituents in the bumblebee haemolymph proteome were identified and characterised by incorporating immune information from the *Bombus* genome paper (Sadd et al. 2015) and self-annotation based on GO information sourced from *Apis mellifera* and *Drosophila melanogaster* genome as previously described (section 2.6.3).

Comparisons between the haemolymph and FB proteomes were made on a number of levels in order determine the contribution of the FB to the haemolymph and to identify key similarities and differences between these central tissues. Contrasts between the proteomes were made in respect to numbers of shared and unique proteins with specific emphasis on immune constituents, peptidases and inhibitors. Enrichment of biological processes identified within both proteomes were also compared using GOrilla to reveal potential shared and unique aspects of functionality within the proteomes.

## 4.2 Results

### 4.2.1 Haemocyte imaging and enumeration

To enumerate haemocytes circulating within haemolymph, samples from all workers were counted as described and the average of this count was taken as the baseline cell concentration. The average total haemocyte count of worker haemolymph was revealed as circa 2 x $10^6$ cells/ ml. After analysis of all prepared slides three distinct populations of haemocytes, population one (P1), two (P2) and three (P3), were identified across both H&E

(Figure 4.1) and fluorescently stained (Figure 4.2) samples. As cell membranes were not always easily definable in H&E stained haemocytes, measurements of cells and nuclei were taken from the fluorescently labelled samples (n = 6).

P1 haemocytes have central elongated nuclei and are identifiable by their oval cell shape which often appears with tapered ends (Figure 4.1A & 4.2B) (Table 4.1). P2 of circulating haemocytes had a low nucleus to cell membrane ratio could be observed with both smooth (Figure 4.2C) and pointed termini (Figure 4.1B) (Table 4.1). P3 haemocytes contained a large nucleus and hence a reduced amount of cytoplasm to the other identified cell types (Figure 4.1C & 4.2A) (Table 4.1). A number of cell particles with no identifiable nucleus were identified across H&E stained preparations (and incidentally absent from fluorescent images) most likely representing lysed, damaged or permeabilised cells (Figure 4.1D).

Bumblebee haemocyte subsets were compared to well-characterised haemocyte populations of other insect species including members of Hymenoptera (Amaral et al. 2010; Marringa et al. 2014; Richardson et al. 2018), Orthoptera (Yu et al. 2016), Lepidoptera and Diptera (Ribeiro and Brehélin 2006) to assist with classification and characterisation. These comparisons resulted in the annotation of P1 as spherulocytes, P2 as plasmatocytes and P3 as oenocytoids.

**Table 4.1 Morphometric analysis of circulating haemocyte populations identified in the haemolymph of *B. terrestris* workers.**

| | Haemocyte population | | |
|---|---|---|---|
| **Measurement** | **P1** | **P2** | **P3** |
| **C.D. (µM)** | 8.58 ± 1.25 | 9.62 ± 0.72 | 7.88 ± 0.65 |
| **N.D. (µM)** | 4.12 ± 0.43 | 3.83 ± 0.33 | 4.16 ± 4.06 |
| **C.A. ($\mu M^2$)** | 44.91 ± 8.66 | 71.47 ± 6.52 | 46.42 ± 4.06 |
| **N.A. ($\mu M^2$)** | 13.66 ± 1.99 | 11.39 ± 1.71 | 15.17 ± 0.24 |
| **CY.A. ($\mu M^2$)** | 31.47 ± 6.78 | 31.47 ± 6.12 | 31.25 ± 3.30 |
| **N/CY (%)** | 43 | 36 | 49 |

*Cellular diameter (C.D.); nuclear diameter (N.D.); cellular area (C.A.); nuclear area (N.A.); cytoplasmic area (C.A.); nuclear/cytoplasmic ratio (N/Cy). Measurements are average values with standard error of the mean.

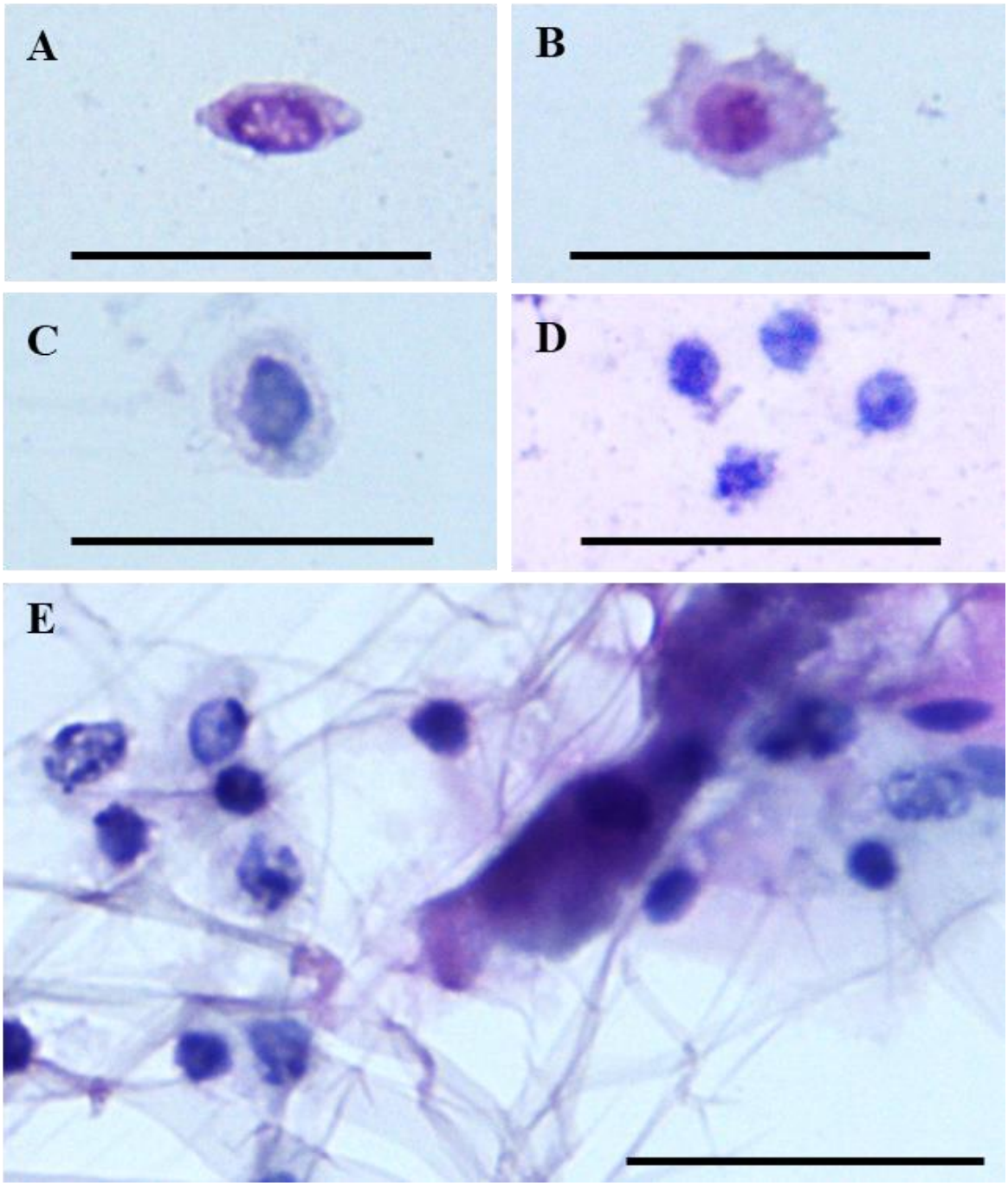


**Figure 4.1 Morphology and diversity of circulating haemocytes in *B. terrestris* haemolymph.** Cytological analysis of *B. terrestris* circulating haemocyte populations was conducted using light microscopy (40X) on fixed and H&E-stained cells. Three morphologically distinct cell types were identified; P1 (A), P2 (B) and P3 (C). (D) an additional cell particle that was routinely observed in H&E stained preparations and (E) the matrix that forms in the haemolymph and the agglomeration of haemocytes around and within it. Scale bars represent 10 µm.

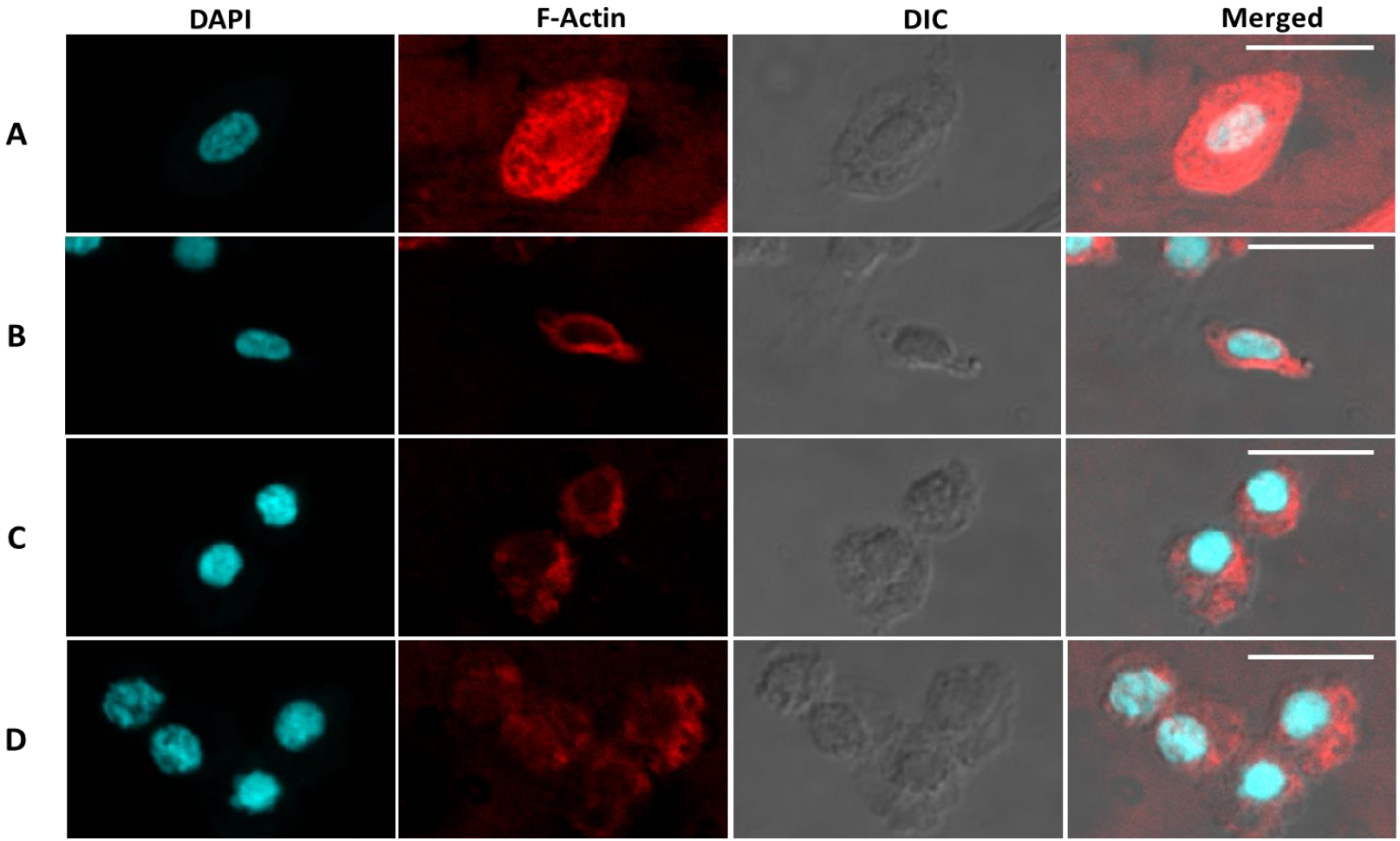


**Figure 4.2 Fluorescent micrographs of circulating haemocytes in *B. terrestris* haemolymph.** Fixed haemocyte images showing DAPI and F-actin staining, (A) oenocytoid, (B) spherulocyte, (C) plasmatocyte and (D) haemocyte aggregation. DAPI (blue) stains the nucleus and rhodamine phalloidin (red) labels polymerised actin (F-actin). Differential interference contrast microscopy (DIC) of the cell merged images on the right. Images were obtained by confocal microscopy. Scale bars represent 20 µm.

### 4.2.2 LC-MS/MS

LC-MS/MS was performed on three replicate samples and resulted in identification of a total of 453 proteins. This number was reduced after filtering the data matrix to remove contaminant peptides, reverse peptide hits and peptides only identified by site. LFQ intensity values were $Log_2$ transformed and only proteins found in all three replicates resulting in a final dataset of 207 proteins.

To assess relative protein abundance within the haemolymph proteome, proteins were ranked based on their mean intensity values (Table A 4.1). To obtain an estimate of relative protein abundance the peptide peak intensity approach was favoured over spectral counts as it is considered a more direct measurement of peptide abundance (Mallick and Kuster 2010; Matzke et al. 2013). The three most highly abundant proteins in the proteome were apolipophorin, transferrin and vitellogenin (Table 4.2). Other highly abundant proteins include proteins associated with the immune system including antioxidants (superoxide dismutase (SOD)), PRRs (leucine-rich repeat-containing protein 15-like isoform X2 and beta-1,3-glucan-binding protein 1), AMPs (hymenoptaecin, defensin and icarapin), signal modulators (IRP30, serpins and ecdysteroid-regulated 16 kDa protein), venom proteins (omega-conotoxin and venom dipeptidyl peptidase) and elements of the melanisation signalling cascade (phenoloxidase, anti-chymotrypsin and chymotrypsin inhibitor). Other notable proteins among the 50 most abundant in haemolymph proteome include hormone ecdysteroid-regulated 16 kDa protein, enzymes such as tryptase, spermine oxidase and dipeptidase and proteins associated with carbohydrate (enolase and alpha-glucosidase) and energy metabolism (arginine kinase).

**Table 4.2 The top 50 most abundant proteins within the haemolymph proteome.** The 50 most abundant proteins within the haemolymph proteome based on mean protein intensities listed in descending order. include those involved in metabolism and immunity.

| Intensity | Accession no. | Protein name |
|---|---|---|
| 3.13E+12 | XP_003397320.1 | Apolipophorins |
| 8.41E+11 | XP_012172140.1 | Transferrin |
| 7.03E+11 | XP_012163499.1 | Vitellogenin-like |
| 4.92E+11 | XP_003393266.1 | BMP-binding endothelial regulator protein* |
| 4.83E+11 | XP_012175191.1 | Chitinase-like protein Idgf4 isoform X2 |
| 4.68E+11 | XP_003400548.1 | Phenoloxidase 2-like |
| 4.59E+11 | XP_012174357.1 | Apolipophorin* |
| 3.21E+11 | XP_003402792.1 | General odorant-binding protein 56d-like |
| 1.56E+11 | XP_012166540.1 | Superoxide dismutase |
| 1.18E+11 | XP_012166633.1 | Tryptase-2-like |
| 1.17E+11 | XP_012176660.1 | Leucine-rich repeat-containing protein 15-like isoform X2 |
| 1.06E+11 | XP_012174479.1 | Dipeptidase 1-like isoform X2 |
| 8.98E+10 | XP_003393186.1 | BMP-binding endothelial regulator protein* |
| 8.93E+10 | XP_003401093.1 | Spermine oxidase-like |
| 8.28E+10 | AEN62314.1 | IRP30 |
| 8.03E+10 | XP_003403367.1 | Centromere-associated protein E isoform X2 |
| 7.85E+10 | XP_003399186.1 | Antichymotrypsin-2-like |
| 7.84E+10 | XP_012172841.1 | Fibrillin-2-like |
| 5.5E+10 | XP_012167632.1 | Beta-1,3-glucan-binding protein 1 |
| 4.92E+10 | XP_003395653.2 | Serine protease easter* |
| 4.5E+10 | XP_003398424.1 | Serpin B3-like |
| 4.27E+10 | XP_003395667.1 | Ecdysteroid-regulated 16 kDa protein |
| 4.06E+10 | XP_003396495.1 | Ejaculatory bulb-specific protein 3 |
| 4.02E+10 | ADB29130.1 | Hymenoptaecin, partial |
| 3.97E+10 | XP_012163782.1 | Papilin isoform X5 |
| 3.9E+10 | AFX62369.1 | Chymotrypsin inhibitor |
| 3.62E+10 | XP_003393256.1 | BMP-binding endothelial regulator protein-like |
| 3.36E+10 | D8KY58.1 | Kunitz-type serine protease inhibitor |
| 3.12E+10 | XP_003398082.1 | Poly(U)-specific endoribonuclease homolog isoform X1 |
| 3.02E+10 | XP_003394487.1 | Angiotensin-converting enzyme-like |
| 3.02E+10 | XP_003397300.1 | Esterase E4-like |
| 3E+10 | XP_012163961.1 | Putative cysteine proteinase CG12163 |
| 2.97E+10 | XP_012173053.1 | 27 kDa hemolymph protein-like |
| 2.74E+10 | XP_012170810.1 | Enolase |
| 2.66E+10 | XP_003395762.1 | Omega-conotoxin-like protein 1 |
| 2.52E+10 | XP_012172756.1 | Arginine kinase isoform X2 |
| 2.28E+10 | XP_003398135.1 | Glyceraldehyde-3-phosphate dehydrogenase 2 |
| 2.27E+10 | XP_003401878.1 | Hemocytin isoform X2 |
| 2.25E+10 | XP_012170795.1 | Peptidoglycan-recognition protein SC2-like |

(Table 4.2 continued)

| Intensity | Accession no. | Protein name |
|---|---|---|
| 2.15E+10 | XP_003401213.2 | Kazal-type proteinase inhibitor* |
| 2.08E+10 | XP_012164669.1 | Alpha-glucosidase-like |
| 2.03E+10 | XP_003399213.1 | Venom dipeptidyl peptidase 4 |
| 1.93E+10 | XP_012169463.1 | Alaserpin-like |
| 1.92E+10 | XP_003399187.1 | Antichymotrypsin-2-like |
| 1.91E+10 | XP_003400229.1 | MD-2-related lipid-recognition protein-like |
| 1.89E+10 | XP_012171993.1 | Trithorax group protein osa |
| 1.75E+10 | XP_003398804.1 | Gamma-glutamyl hydrolase A-like |
| 1.75E+10 | XP_012173325.1 | Peptidoglycan-recognition protein 2-like |
| 1.43E+10 | AGM53455.1 | Defensin-1, partial |
| 1.41E+10 | XP_003396228.1 | Icarapin-like |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

### 4.2.3 Protein interaction and pathway analysis

Identification of enriched networks and protein interactions within the haemolymph proteome was completed using the search tool for the retrieval of interacting genes (STRING). Functions for haemolymph proteins were obtained by using *Drosophila melanogaster* gene ontology resources as a reference. 162 proteins with functions based on protein homology to *D. melanogaster* were selected for protein-protein interaction (PPI) network mapping using STRING. The resulting PPI network constructed had an enrichment value of $p < 1.0e\text{-}16$. Statistically enriched KEGG and biological process gene ontology terms were examined to identify clusters of proteins enriched within the haemolymph PPI network. This revealed four significant KEGG processes within the network; the proteasome, glycolysis/ gluconeogenesis, lysosome and phagosome (Figure 4.3).

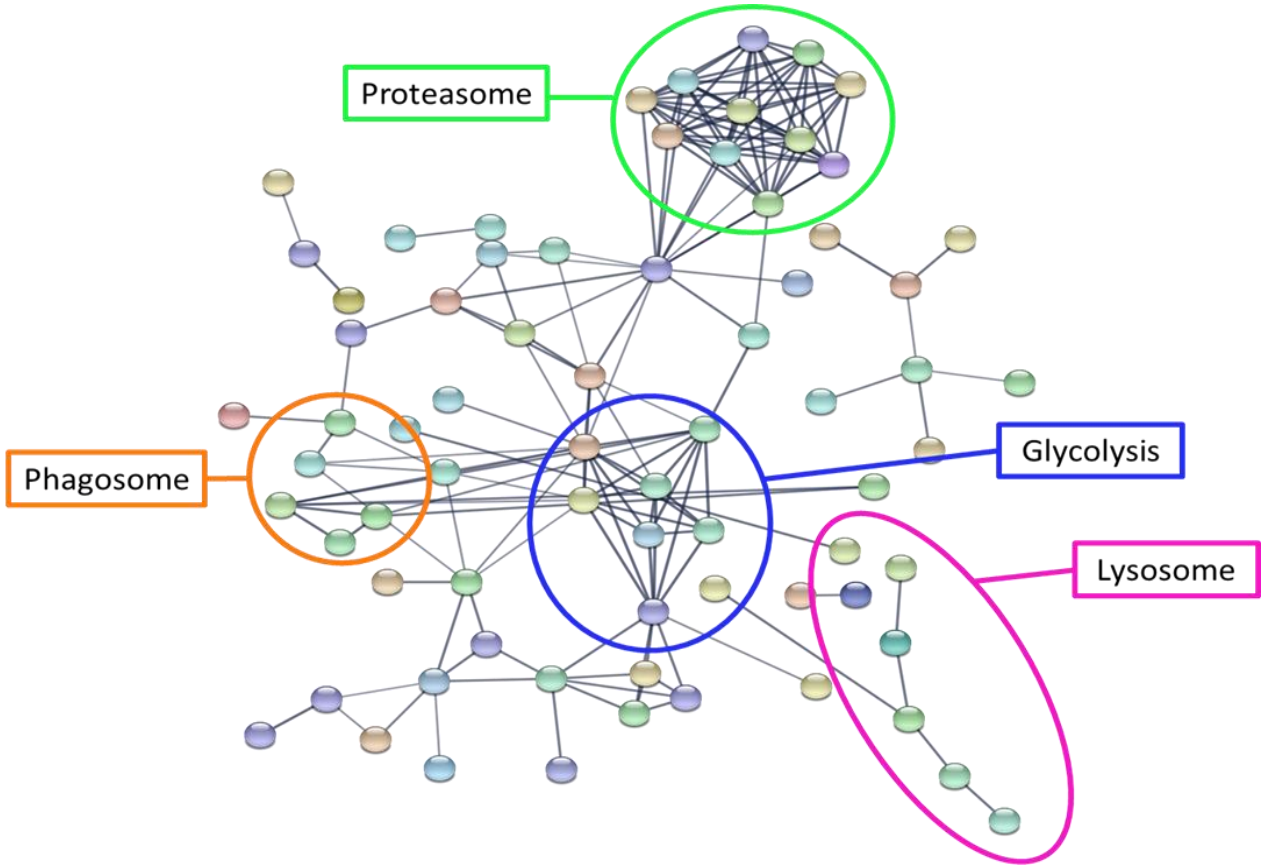


**Figure 4.3 Protein-protein interaction network of haemolymph proteins identified in *B. terrestris* haemolymph.** Interaction networks mapped using the STRING database. Each node represents a protein and each linear connection represents an interaction, the extent of the confidence is represented by the width of each individual line. Within the PPI network only interactions with a high confidence score (≥0.700) were included. Statistically enriched KEGG and biological process gene ontology descriptors were examined to identify clusters of proteins enriched within the haemolymph proteome. Coloured circles indicate the most significant interacting groups within the network.

Overall 14 KEGG pathways were associated with the haemolymph proteome (Table 4.3). The four processes indicated as most enriched within the STRING network analysis are all among the top 10 KEGG processes identified for this tissue. Other processes of interest enriched within the proteome include glycan degradation, biosynthesis amino acids and metabolism (carbon, galactose, starch and sucrose).

**Table 4.3 KEGG processes enriched within the haemolymph proteome.** False discovery rate (FDR) is the representative enrichment score for each process and count in the gene set refers to the numbers of genes found within the dataset in relation to each process.

| Pathway description | Count in gene set | FDR |
|---|---|---|
| Proteasome | 11 | 9.77E-10 |
| Glycolysis / Gluconeogenesis | 8 | 4.37E-06 |
| Lysosome | 10 | 4.37E-06 |
| Other glycan degradation | 4 | 0.00288 |
| Biosynthesis of amino acids | 6 | 0.00288 |
| Microbial metabolism in diverse environments | 9 | 0.00301 |
| Phagosome | 6 | 0.00301 |
| Carbon metabolism | 7 | 0.00343 |
| Galactose metabolism | 4 | 0.00918 |
| Metabolic pathways | 22 | 0.00918 |
| Glycosaminoglycan degradation | 3 | 0.00927 |
| Glycosphingolipid biosynthesis - ganglio series | 2 | 0.0215 |
| Glycosphingolipid biosynthesis - globo series | 2 | 0.0276 |
| Starch and sucrose metabolism | 4 | 0.0488 |

Analysis of the enrichment of biological processes and their respective associated pathways within the haemolymph proteome of *B. terrestris* was completed through BlastKOALA (BK). This analysis revealed genetic information processing, carbohydrate metabolism, organismal systems and cellular activities as well represented processes in the bumblebee haemolymph proteome (Figure 4.4).

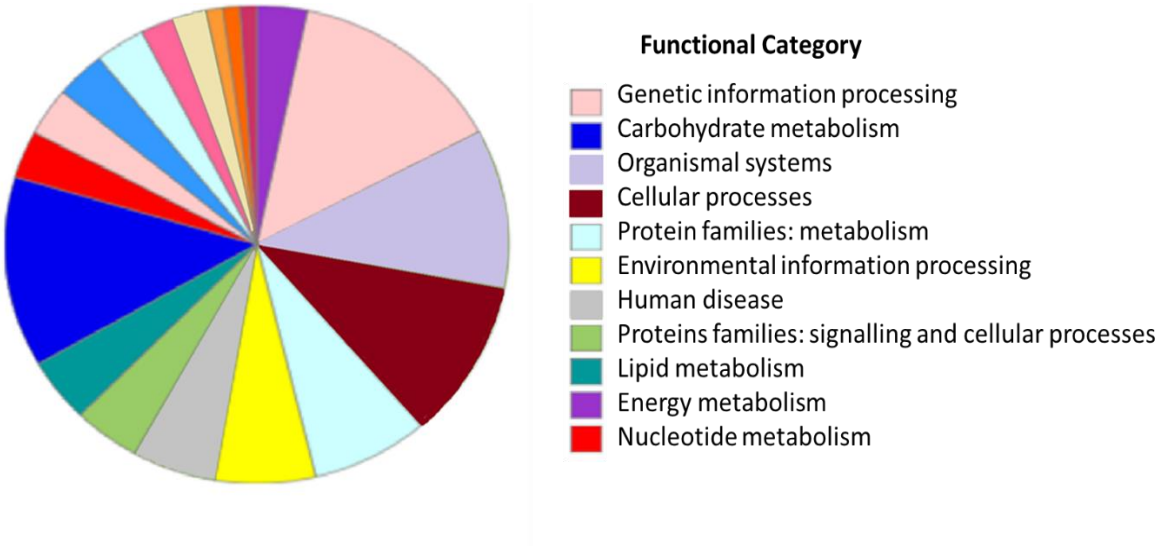


**Figure 4.4 BlastKOALA pathway analysis of the haemolymph proteome.** A global overview of biological processes associated with bumblebee haemolymph. Among the biological processes were those related to normal cell house-keeping including numerous metabolism-associated processes, genetic information processing, organismal systems and environmental information processing.

Further interrogation of the pathways enriched within each class of biological functions, as defined by BK, indicted that haemolymph proteins are present in pathways such as glycolysis, lysosome, phagosome and the proteasome (Table 4.4) (Figure A4.1). This further confirms the PPI results for the haemolymph proteome which also found significant enrichment for these pathways.

**Table 4.4 Top two pathways identified across biological processes in BK analysis of the haemolymph proteome.** Across each category of biological processes numerous pathways were identified. Protein number represents the number of haemolymph proteins involved in each pathway.

| Biological process | Pathway | No. of proteins |
|---|---|---|
| Carbohydrate metabolism | Glycolysis / Gluconeogenesis | 8 |
| | Pentose phosphate pathway | 3 |
| Energy metabolism | Oxidative phosphorylation | 3 |
| | Methane metabolism | 3 |
| Glycan biosynthesis and metabolism | Other glycan degradation | 4 |
| | Glycosaminoglycan degradation | 3 |
| Lipid metabolism | Sphingolipid metabolism | 3 |
| | Arachidonic acid metabolism | 2 |
| Nucleotide metabolism | Purine metabolism | 3 |
| | Pyrimidine metabolism | 2 |
| Cellular processes | Lysosome | 12 |
| | Phagosome | 6 |
| Environmental information processing | mTOR signalling pathway | 3 |
| | MAPK signalling pathway | 2 |
| Organismal systems | Protein digestion and absorption | 4 |
| | Renin-angiotensin system | 4 |
| Genetic information processing | Proteasome | 11 |
| | Protein Processing in ER | 2 |

### 4.2.3 Gene ontology and functional analysis

Functional analysis of the proteome was performed to gain further insight into the biological processes associated with *B. terrestris* haemolymph. Identification and visualisation of associated processes, was performed by searching the proteome through ClueGO, using *Drosophila* as the reference genome. All significantly enriched biological processes ($p \leq 0.005$) were illustrated on a pie chart (Figure 4.5). Carbohydrate metabolism, proteolysis and numerous immune-related activities (defence, melanin, wound response and detoxification) were among the processes revealed to be enriched within this proteome.

Using the gene ontology analysis software GOrilla haemolymph specific processes, were determined from those of normal house-keeping activities. GO terms were obtained for both proteomes from the *D. melanogaster* genome (haemolymph proteome 207, background proteome 20,984). A list of haemolymph-specific enriched GOs and their corresponding p-values (threshold $10^{-6}$) were then obtained by comparing the haemolymph to a background *Bombus* proteome. This examination revealed that processes such as carbohydrate metabolism, proteolysis, and immunity are all significantly enriched with the haemolymph proteome (Figure 4.6) (Table 4.5).

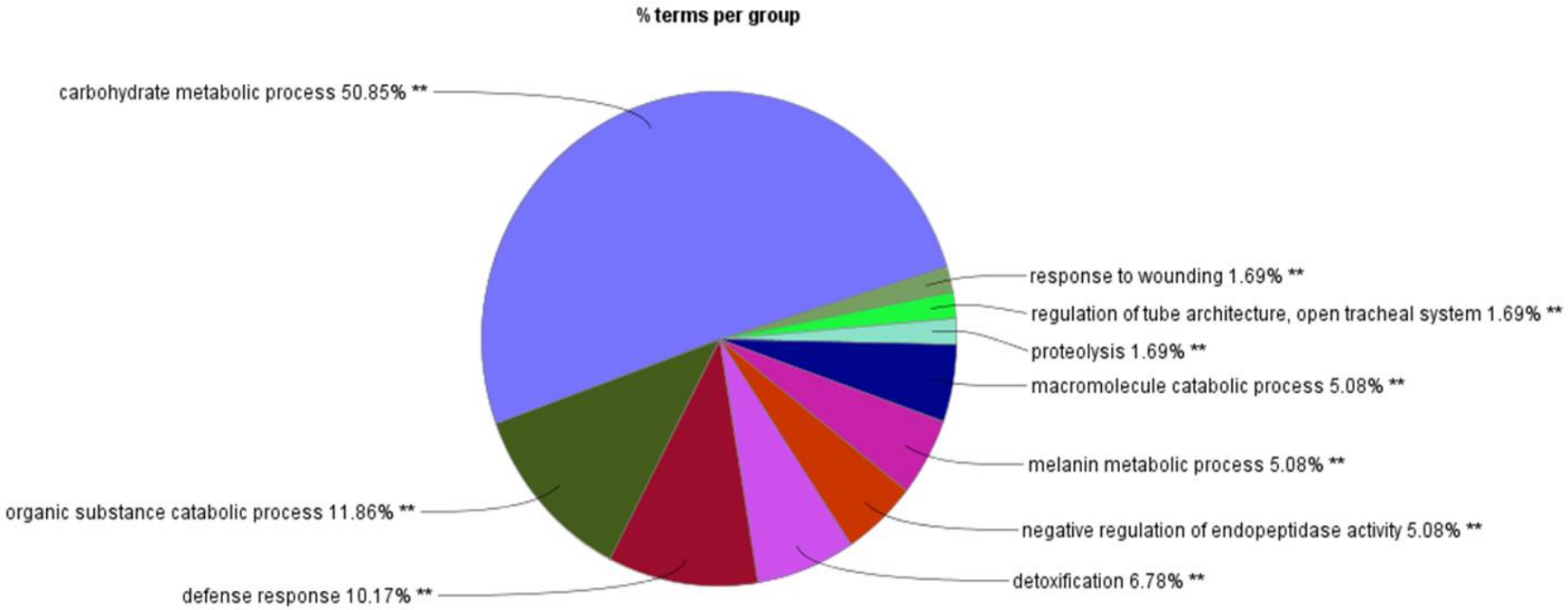


**Figure 4.5 Global overview of all biological processes associated with the haemolymph proteome.** ClueGO analysis revealed all significantly enriched biological processes within the *B. terrestris* haemolymph proteome. Only processes with $p \leq 0.005$ were included in the pie chart. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$statistical levels, respectively.

**Table 4.5 GOrilla analysis of the haemolymph proteome.** To highlight the most highly enriched GO terms within the haemolymph proteome the p-value threshold for this analysis was set at $10^{-6}$. Hence, only GO terms with a p-value better than this threshold are reported. Number of genes refers to the total number of genes associated with a specific GO term.

| Description | P-value | FDR q-value | Enrichment | No. of genes |
|---|---|---|---|---|
| Proteasomal ubiquitin-independent protein catabolic process | 1.24E-14 | 8.93E-11 | 33.69 | 14 |
| Defence response to Gram-positive bacterium | 3.01E-12 | 1.08E-08 | 28.3 | 15 |
| Response to other organism | 9.57E-12 | 2.30E-08 | 5.93 | 175 |
| Response to external biotic stimulus | 1.51E-11 | 2.73E-08 | 5.8 | 179 |
| Response to biotic stimulus | 1.51E-11 | 2.18E-08 | 5.8 | 179 |
| Multi-organism process | 3.35E-11 | 4.02E-08 | 5.29 | 205 |
| Proteolysis | 5.88E-11 | 6.05E-08 | 3.79 | 386 |
| Defence response | 1.26E-10 | 1.13E-07 | 5.21 | 199 |
| Carbohydrate metabolic process | 4.93E-10 | 3.94E-07 | 5.75 | 156 |
| Immune response | 1.42E-09 | 1.02E-06 | 6.17 | 130 |
| Catabolic process | 1.73E-09 | 1.13E-06 | 3 | 550 |
| Response to bacterium | 3.09E-09 | 1.85E-06 | 6.87 | 103 |
| Defence response to other organism | 4.07E-09 | 2.25E-06 | 5.77 | 139 |
| Organic substance catabolic process | 8.10E-09 | 4.16E-06 | 3.03 | 498 |
| Defence response to bacterium | 1.80E-08 | 8.65E-06 | 6.6 | 100 |
| Organonitrogen compound catabolic process | 3.09E-08 | 1.39E-05 | 4.08 | 243 |
| Carbohydrate catabolic process | 6.48E-08 | 2.74E-05 | 11.17 | 38 |
| Immune system process | 8.64E-08 | 3.46E-05 | 4.72 | 170 |
| Innate immune response | 9.04E-08 | 3.43E-05 | 7.08 | 80 |
| Humoral immune response | 1.32E-07 | 4.75E-05 | 10.35 | 41 |
| Response to external stimulus | 2.10E-07 | 7.19E-05 | 3.16 | 373 |
| Response to fungus | 2.29E-07 | 7.49E-05 | 11.79 | 32 |

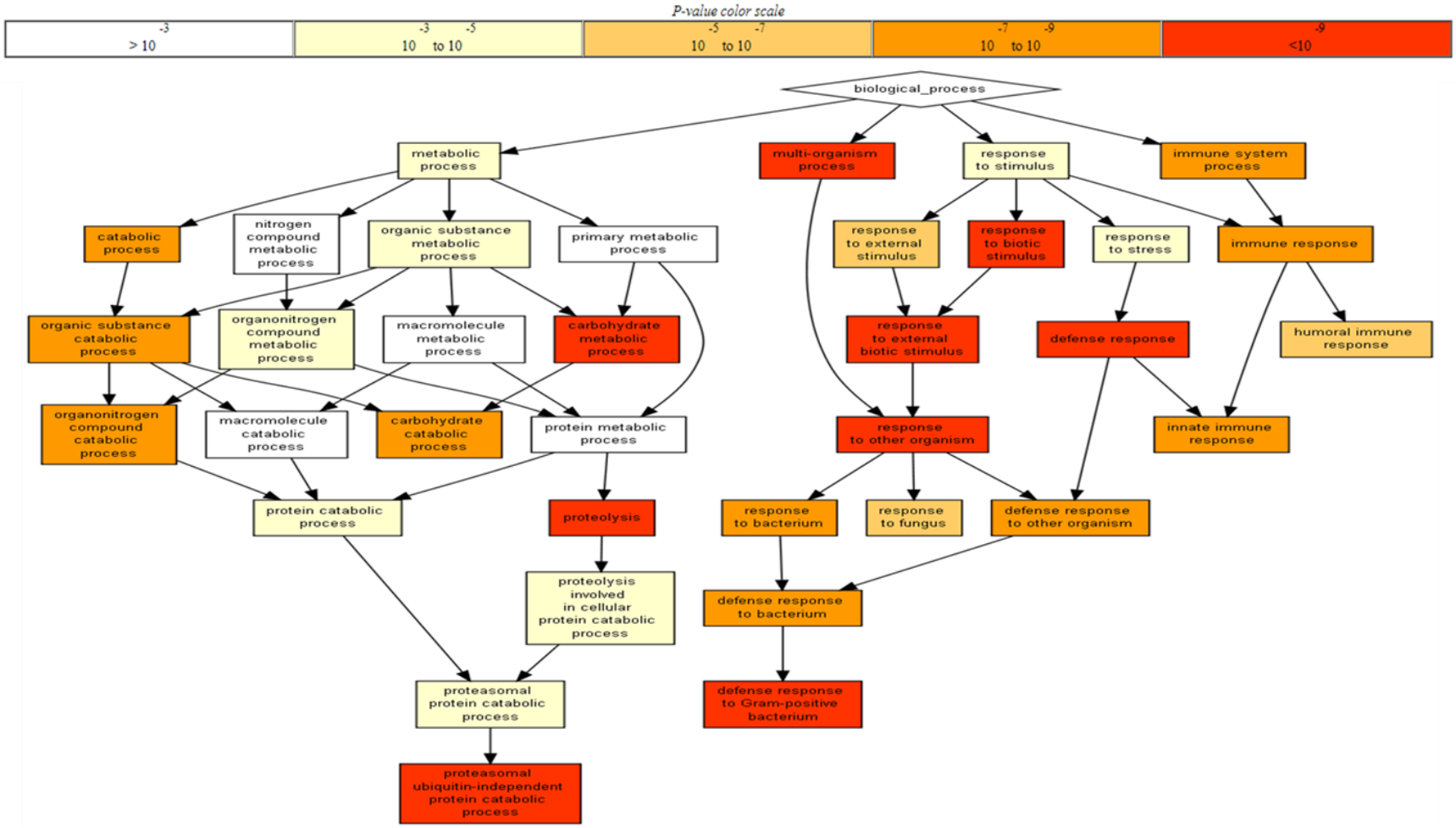


**Figure 4.6 Hierarchy of enriched gene ontology biological processes within the *B. terrestris* haemolymph proteome**. Flow diagram of the overall organisation of biological processes in the haemolymph proteome compared to a background whole *Bombus* proteome. The p-value scale corresponds significance to colour; white corresponds to lower significance ($p > 10^{-3}$) and red represents high significance ($p < 10^{-9}$).

As immune-related processes were consistently represented in the functional analysis of the haemolymph proteome. To further investigate the diversity of immune-related proteins in the haemolymph, defence and immune constituents were characterised by incorporating immune information from the *Bombus* genome (section 2.6.3). This investigation resulted in the identification of 45 immune-related proteins within the *B. terrestris* haemolymph proteome (Table 4.6). These proteins represented all the main functional classes of immunity including; immune effectors (7), PRRs (7), antioxidants (6) and immune signalling mediators (25).

To investigate the types of defence and immune-related roles haemolymph immune proteins participated in functional analysis was completed using the gene ontology mapping tool ClueGO using the *D. melanogaster* genome background (Figure 4.7). The most significant immune processes within the haemolymph proteome are pattern recognition receptor signalling pathway, melanisation defence response and regulation of innate immune response (Figure 4.7 B).

Metabolism and immunity are two of the main functional enrichments for this dataset. Numerous peptidases and inhibitors are involved in the stringent modulation of signalling for these important biological processes. Hence, investigation of the classes of peptidases and inhibitors in this proteome was completed to understand their role and further elucidate functionality of the haemolymph. Basic local alignment search tool for proteins (BLASTp) searches of all haemolymph proteins were performed against the MEROPS database identified 90 proteins with peptidase (55) or inhibitory (35) properties were identified (Table 4.7). Both the peptidases and inhibitors represent proteins from a diverse array of clans and families. Peptidases identified covered six classes of catalytic activity and inhibitors represented proteins belonging to nine clans and families.

**Table 4.6 Immune-related proteins identified in the *B. terrestris* haemolymph.** Numerous elements of insect immunity were identified in the haemolymph proteome of *B. terrestris* including antioxidants, immune signalling modulators, PRRs and AMPs.

| Immune function | Accession no. | Protein name |
|---|---|---|
| **Antioxidant** | XP_012166540.1 | Superoxide dismutase |
| | XP_012166688.1 | Superoxide dismutase |
| | XP_003395542.1 | Probable phospholipid hydroperoxide glutathione peroxidase |
| | XP_012166348.1 | Peroxidase |
| | XP_012164907.1 | Thioredoxin-2 |
| | XP_003397695.1 | Peroxiredoxin-6 |
| **Immune effector** | ADB29130.1 | Hymenoptaecin |
| | AGM53455.1 | Defensin-1 |
| | ACO90198.1 | Defensin |
| | ADB29128.1 | Abaecin |
| | XP_012170668.1 | Lysozyme |
| | XP_012175465.1 | Apidaecins type 73 |
| | XP_003400548.1 | Phenoloxidase 2 |
| **Immune modulator** | XP_003395081.1 | Venom protease |
| | XP_003395762.1 | Omega-conotoxin |
| | NP_001267823.1 | Venom serine protease precursor |
| | AEN62314.1 | IRP30 |
| | AFX62369.1 | Chymotrypsin inhibitor |
| | XP_003395653.2 | Serine protease easter* |
| | XP_003401213.2 | Kazal-type proteinase inhibitor* |
| | XP_012174357.1 | Apolipophorin* |
| | XP_012163150.1 | Neurotrypsin* |
| | XP_003398424.1 | Serpin B3 |
| | XP_003393295.1 | Serine protease snake |
| | XP_012174896.1 | Serine protease inhibitor 3 |
| | XP_003393033.1 | Plasma protease C1 inhibitor |
| | XP_012172791.1 | Multiple epidermal growth factor-like domains protein |
| | XP_012163961.1 | Putative cysteine proteinase CG12163 |
| | XP_012169463.1 | Alaserpin |
| | XP_012166703.1 | Leukocyte elastase inhibitor |
| | XP_012163992.1 | Insulin-like growth factor-binding protein complex acid labile subunit |
| | XP_003398909.1 | Furin-like protease 2 isoform X2 |
| | XP_003401209.1 | Four-domain proteases inhibitor |
| | XP_012172841.1 | Fibrillin-2-like |
| | XP_003395667.1 | Ecdysteroid-regulated 16 kDa protein |
| | XP_003399186.1 | Antichymotrypsin-2 |

(Table 4.6 continued)

| Immune function | Accession no. | Protein name |
|---|---|---|
| **Immune modulator** | XP_003399187.1 | Antichymotrypsin-2 |
| | XP_003396888.1 | Alpha-2-macroglobulin |
| **PRR** | XP_012173325.1 | Peptidoglycan-recognition protein 2 |
| | XP_003400229.1 | MD-2-related lipid-recognition protein |
| | XP_012176660.1 | Leucine-rich repeat-containing protein 15 |
| | XP_003396346.1 | Leucine-rich repeat neuronal protein 1 |
| | XP_012164498.1 | Chaoptin |
| | XP_003394761.1 | Beta-1,3-glucan-binding protein |
| | XP_012167632.1 | Beta-1,3-glucan-binding protein 1 |

(*) indicates immune proteins which have been reannotated as per section 2.6.3.

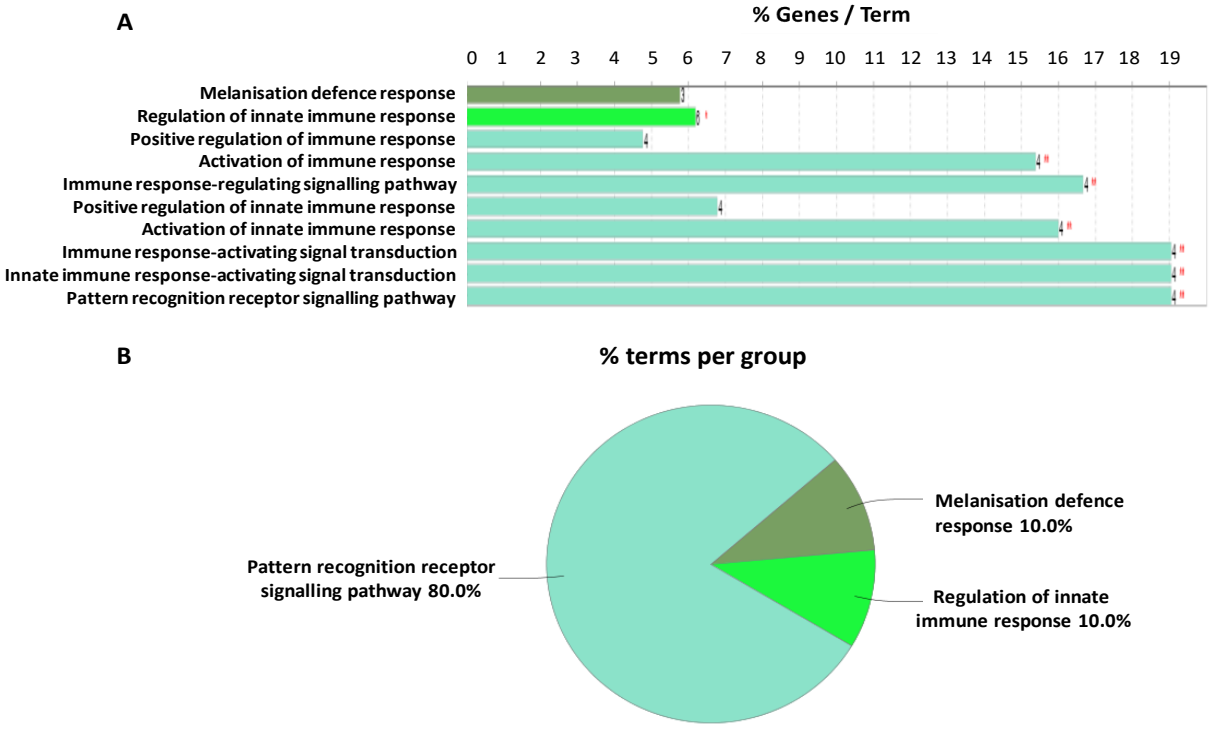


**Figure 4.7 Functional analysis of haemolymph immune proteins.** (A) Bar chart representing all specific terms identified for immune proteins. The name of each functional class or pathway is assigned by the lowest p value of the terms of that class. Bar colour indicates terms that are categorised under the same functional group. Single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$ statistical levels, respectively. (B) Pie chart contains functional labels of the most enriched terms only, providing a summary of the most enriched functions indicated for the haemolymph immune proteins. Only processes with $p \leq 0.005$ were included in the analysis.

**Table 4.7 Peptidases and inhibitors of the haemolymph proteome.** For Protease Class Aspartic (*A*), Cysteine (*C*), Threonine (*T*), Serine (*S*) proteases, Metalloprotease (*M*) and unknown (*U*). Number of proteins refers to numbers in each family. (I) indicates inhibitors and (P) indicates peptidases.

| Catalytic Type | Clan | Family | No. of proteins | Reference proteins | Biological function | I/P |
|---|---|---|---|---|---|---|
| **Inhibits serine endopeptidases** | IA | I1 | 4 | - | Inhibitors of serine endopeptidases | I |
| **Inhibits serine peptidases** | 1B | I2 | 3 | - | Inhibitors of serine peptidases | I |
| **Inhibitors all endopeptidases** | IL | I39 | 1 | Alpha2-macroglobulin | Antigen processing | I |
| **Inhibits serine & cysteine endopeptidases** | ID | I4 | 14 | Serpin | Immune modulator | I |
| **Inhibits various kinases** | JE | I51 | 1 | - | Kinase/chymotrypsin inhibitor | I |
| **Inhibits metallopeptidase pappalysin-11** | JB | I63 | 6 | Pappalysin-1 | Promotes cell growth | I |
| **Unassigned** | IZ | I59 | 1 | - | Anticoagulation | I |
| **Contains a carboxypeptidase inhibitor** | JK | I68 | 1 | - | Anticoagulation | I |
| **Inhibits serine & metallo endopeptidases** | IA | I8 | 4 | Chymotrypsin/elastase inhibitor | Immune modulator | I |
| ***A*** | AA | A1 | 1 | Nucellin | Proteolysis | P |
| ***C*** | CA | C1 | 3 | Cathepsin B | Lysosomal activity | P |
| ***C*** | PC | C26 | 1 | Gamma - glutamyl hydrolase | Lysosomal activity | P |
| **C** | PC | C56 | 1 | - | Endopeptidase | P |
| ***M*** | MA | M1 | 1 | Aminopeptidase A | Immune modulator | P |
| ***M*** | MA | M12 | 1 | - | Signal transduction | P |
| ***M*** | MA | M13 | 4 | - | Proteolysis | P |
| ***M*** | MC | M14 | 1 | - | Antigen processing | P |
| ***M*** | MJ | M19 | 1 | Membrane dipeptidase | Hydrolysis of dipeptides | P |
| ***M*** | MA | M2 | 1 | Peptidyl-dipeptidase A | Vasopressor | P |
| ***M*** | MH | M28 | 1 | Transferrin receptor | - | P |

(Table 4.7 continued)

| Catalytic Type | Clan | Family | No. of proteins | Reference proteins | Biological function | I/P |
|---|---|---|---|---|---|---|
| ***S*** | PA | S1 | 11 | Chymotrypsin | Immune modulator | P |
| ***S*** | SC | S10 | 3 | - | Lysosomal activity | P |
| ***S*** | SC | S28 | 1 | Pro-Xaa carboxypeptidase | Immune modulator | P |
| ***S*** | SK | S41 | 1 | - | Proteolysis | P |
| ***S*** | SR | S60 | 1 | Lactoferrin | - | P |
| ***S*** | SB | S8 | 1 | - | Antigen processing | P |
| ***S*** | unassigned | S81 | 1 | Destabilase | Lysosomal activity | P |
| ***S*** | SC | S9 | 3 | - | Antigen processing | P |
| ***T*** | PB | T1 | 13 | - | Proteostasis | P |
| ***U*** | unassigned | unassigned | 4 | - | - | P |

### 4.2.4 Comparative proteomic analysis between the haemolymph and FB

A comparison of the *B. terrestris* haemolymph and FB was carried out to compare protein repertoires and overall functionality of both proteomes. Initial comparisons of all proteins identified in both proteomes revealed 124 proteins in common to both sample types (Figure 4.8A). Functional analysis of common proteins using ClueGO indicated carbohydrate metabolism, detoxification, PRR signalling and the proteasome to be conserved processes across both FB proteomes (Figure 4.8B).

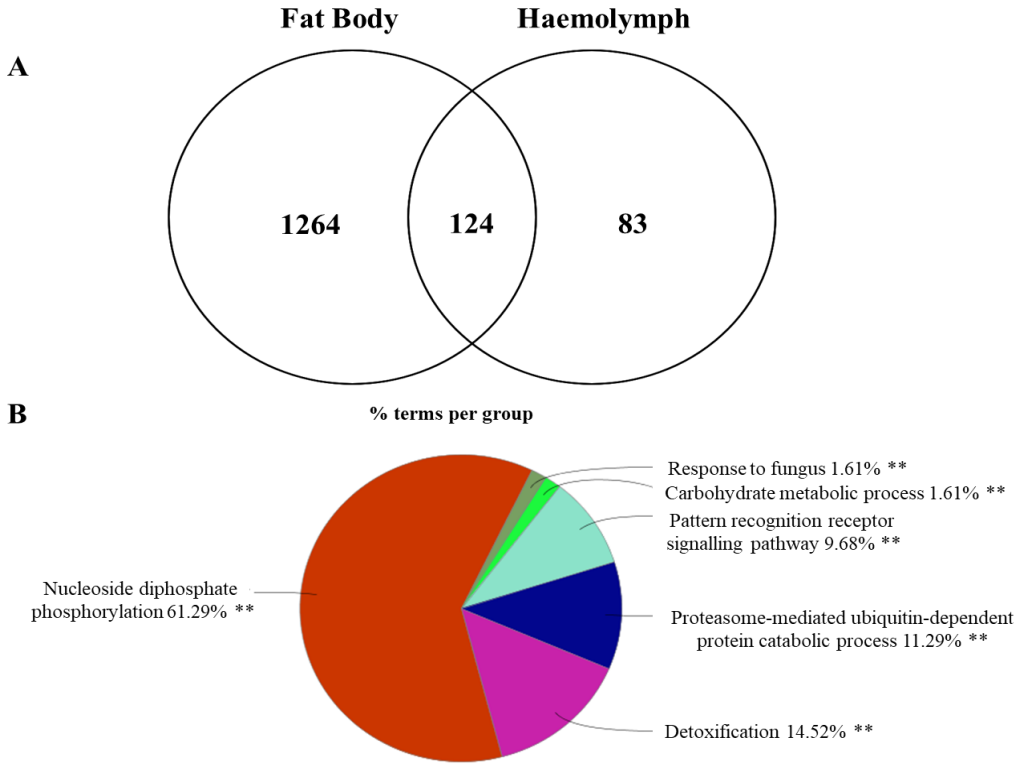


**Figure 4.8 Comparative analysis of the haemolymph and fat body proteomes.** (A) direct comparison of the FB (1388) and haemolymph (207) proteomes revealed 124 common proteins. (B) Functional analysis of common proteins. Only processes with $p \leq 0.005$ were included in the analysis. Single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$ statistical levels, respectively.

To investigate similarities in the immune repertoires of both proteomes, haemolymph and FB immune proteins were compared. The results of this comparison revealed 27 immune proteins common to both proteomes, with 24 and 18 unique immune proteins in the FB and haemolymph, respectively (Figure 4.9).

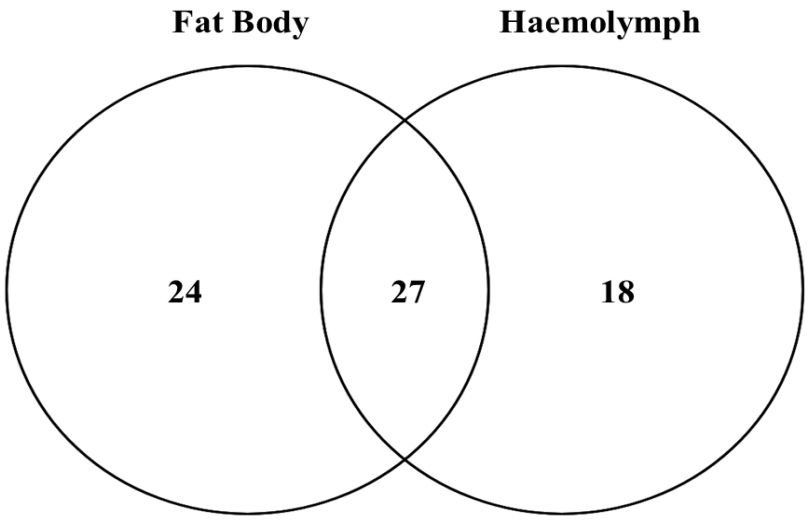


**Figure 4.9 Comparative analysis of the haemolymph and fat body immune proteins.** Comparison of FB (51) and haemolymph (45) immune proteins revealed 27 shared immune proteins.

The 27 common immune proteins present in both data sets included proteins from all immune function classes (Table 4.8). Overall 10 PRRs were identified of which five were common to both; MD-2-related lipid-recognition protein, chaoptin, beta-1,3-glucan-binding protein 1, peptidoglycan-recognition protein 2 and leucine-rich repeat-containing protein 15. In total 13 antioxidants were identified and of these five were found in the two proteomes; thioredoxin-2, peroxidase, peroxiredoxin-6 and two superoxide dismutase proteins. Globally nine immune effectors were identified from the proteomes of the haemolymph and FB, of these two were found in common in both (hymenoptaecin and phenoloxidase). These 15 included IRP30, apolipoprotein, ecdysteroid regulated 16 kDa protein and numerous serine proteases and elements of the melanisation cascade (antichymotrypsin-2). The largest class of immune proteins identified 37 proteins and contained those involved in immune modulation. Of the 37 identified, 15 were indicated as being present in both proteomes.

**Table 4.8 List of all common and unique immune proteins in the haemolymph and fat body proteomes.** All immune proteins identified from analysis of the fat body and haemolymph proteins were compiled and compared to identify those that were common and unique to a sample type.

| Immune function | Accession no. | Protein name | FB | Haemo |
|---|---|---|---|---|
| Antioxidant | XP_012163601.1 | Glutathione S-transferase | ✓ | - |
| | XP_012164108.1 | Probable phospholipid hydroperoxide glutathione peroxidase | ✓ | - |
| | XP_012164574.1 | Catalase | ✓ | - |
| | XP_012164907.1 | Thioredoxin-2 | ✓ | ✓ |
| | XP_012166348.1 | Peroxidase | ✓ | ✓ |
| | XP_012166540.1 | Superoxide dismutase | ✓ | ✓ |
| | XP_012166688.1 | Superoxide dismutase | ✓ | ✓ |
| | XP_003395542.1 | Probable phospholipid hydroperoxide glutathione peroxidase | - | ✓ |
| | XP_012172050.1 | Glutathione S-transferase* | ✓ | - |
| | XP_003399714.1 | Peroxiredoxin-6 | ✓ | - |
| | XP_003401364.1 | Peroxiredoxin | ✓ | - |
| | XP_003397695.1 | Peroxiredoxin-6 | ✓ | ✓ |
| | XP_012175811.1 | Heat shock 70 kDa protein 4 | ✓ | - |
| Immune effector | ADB29129.1 | Defensin 1 | ✓ | - |
| | ADB29130.1 | Hymenoptaecin | ✓ | ✓ |
| | AEN41592.1 | Venom bombolitin 1 | ✓ | - |
| | AGM53455.1 | Defensin-1, partial | - | ✓ |
| | ACO90198.1 | Defensin, partial | - | ✓ |
| | ADB29128.1 | Abaecin, partial | - | ✓ |
| | XP_012170668.1 | Lysozyme | - | ✓ |
| | XP_012175465.1 | Apidaecins type 73 | - | ✓ |
| | XP_003400548.1 | Phenoloxidase 2 | ✓ | ✓ |
| Immune modulator | NP_001267823.1 | Venom serine protease | ✓ | ✓ |
| | XP_003395081.1 | Venom protease | ✓ | ✓ |
| | XP_003395762.1 | Omega-conotoxin protein 1 | ✓ | ✓ |
| | AEN62314.1 | IRP30 | ✓ | ✓ |
| | XP_003393033.1 | Plasma protease C1 inhibitor | ✓ | ✓ |
| | XP_003393349.1 | Heat shock 70 kDa protein cognate 5 | ✓ | - |
| | XP_003395447.1 | Epididymal secretory protein E1 | ✓ | - |
| | XP_003395667.1 | Ecdysteroid-regulated 16 kDa protein | ✓ | ✓ |
| | XP_003397328.1 | DEAD-box ATP-dependent RNA helicase 20 | ✓ | - |
| | XP_003398065.1 | Putative ATP-dependent RNA helicase Pl10 | ✓ | - |
| | XP_003398424.1 | Serpin B3 | ✓ | ✓ |
| | XP_003393295.1 | Serine protease snake-like | - | ✓ |

(Table 4.8 continued)

| Immune function | Accession no. | Protein name | FB | Haemo |
|---|---|---|---|---|
| Immune modulator | XP_012174896.1 | Serine protease inhibitor 3-like isoform X2 | - | ✓ |
| | XP_012172791.1 | Multiple epidermal growth factor-like domains protein 10 | - | ✓ |
| | XP_012166703.1 | Leukocyte elastase inhibitor isoform X1 | - | ✓ |
| | XP_003398909.1 | Furin-like protease 2 isoform X2 | - | ✓ |
| | XP_003401209.1 | Four-domain proteases inhibitor-like | - | ✓ |
| | AFX62369.1 | Chymotrypsin inhibitor | - | ✓ |
| | XP_003401213.2 | Kazal-type proteinase inhibitor* | - | ✓ |
| | XP_012163150.1 | Neurotrypsin* | - | ✓ |
| | XP_003399186.1 | Antichymotrypsin-2 | ✓ | ✓ |
| | XP_003399187.1 | Antichymotrypsin-2 | ✓ | ✓ |
| | XP_003401031.1 | Signal transducer and activator of transcription 5B | ✓ | - |
| | XP_003401978.1 | Gametogenetin | ✓ | - |
| | XP_003402576.1 | Serine protease inhibitor 3/4 | ✓ | - |
| | XP_012163961.1 | Putative cysteine proteinase CG12163 | ✓ | ✓ |
| | XP_012163992.1 | Insulin growth factor-binding protein complex acid labile | ✓ | ✓ |
| | XP_012166473.1 | Plasminogen activator inhibitor 1 RNA-binding protein | ✓ | - |
| | XP_012168271.1 | Protein argonaute-2 | ✓ | - |
| | XP_012168652.1 | GTP-binding nuclear protein Ran | ✓ | - |
| | XP_012169463.1 | Alaserpin | ✓ | ✓ |
| | XP_012172057.1 | Fragile X mental retardation syndrome-related protein 1 | ✓ | - |
| | XP_012172841.1 | Fibrillin-2 | ✓ | ✓ |
| | XP_003395653.2 | Serine protease easter* | ✓ | ✓ |
| | XP_012174357.1 | Apolipophorin* | ✓ | ✓ |
| | XP_003396888.1 | Alpha-2-macroglobulin | - | ✓ |
| | XP_003399683.1 | C-type lectin* | ✓ | - |
| PRR | XP_003394802.1 | CD109 antigen | ✓ | - |
| | XP_003397822.1 | Protein NPC2 homolog | ✓ | - |
| | XP_003400160.1 | Peptidoglycan recognition protein | ✓ | - |
| | XP_003400229.1 | MD-2-related lipid-recognition protein | ✓ | ✓ |
| | XP_012164498.1 | Chaoptin | ✓ | ✓ |
| | XP_012167632.1 | Beta-1,3-glucan-binding protein 1 | ✓ | ✓ |
| | XP_012173325.1 | Peptidoglycan-recognition protein 2 | ✓ | ✓ |
| | XP_012176660.1 | Leucine-rich repeat-containing protein 15 | ✓ | ✓ |
| | XP_003394761.1 | Beta-1,3-glucan-binding protein-like | - | ✓ |
| | XP_003396346.1 | Leucine-rich repeat neuronal protein 1-like | - | ✓ |

(*) indicates immune proteins which have been reannotated as per section 2.6.3.

To compare the proteolytic and inhibitory capacity of the fat body and haemolymph MEROPS analysis results were compared. In total 199 proteins with either peptidase or inhibitory activity were identified. The FB proteome contained 109 unique proteins and similarly the haemolymph proteome contained 58 (Figure 4.10). Overall 32 common elements were identified of which 27 were proteases and five inhibitors (Table 4.8). Both the peptidases and inhibitors represented proteins from a diverse array of clans and families.

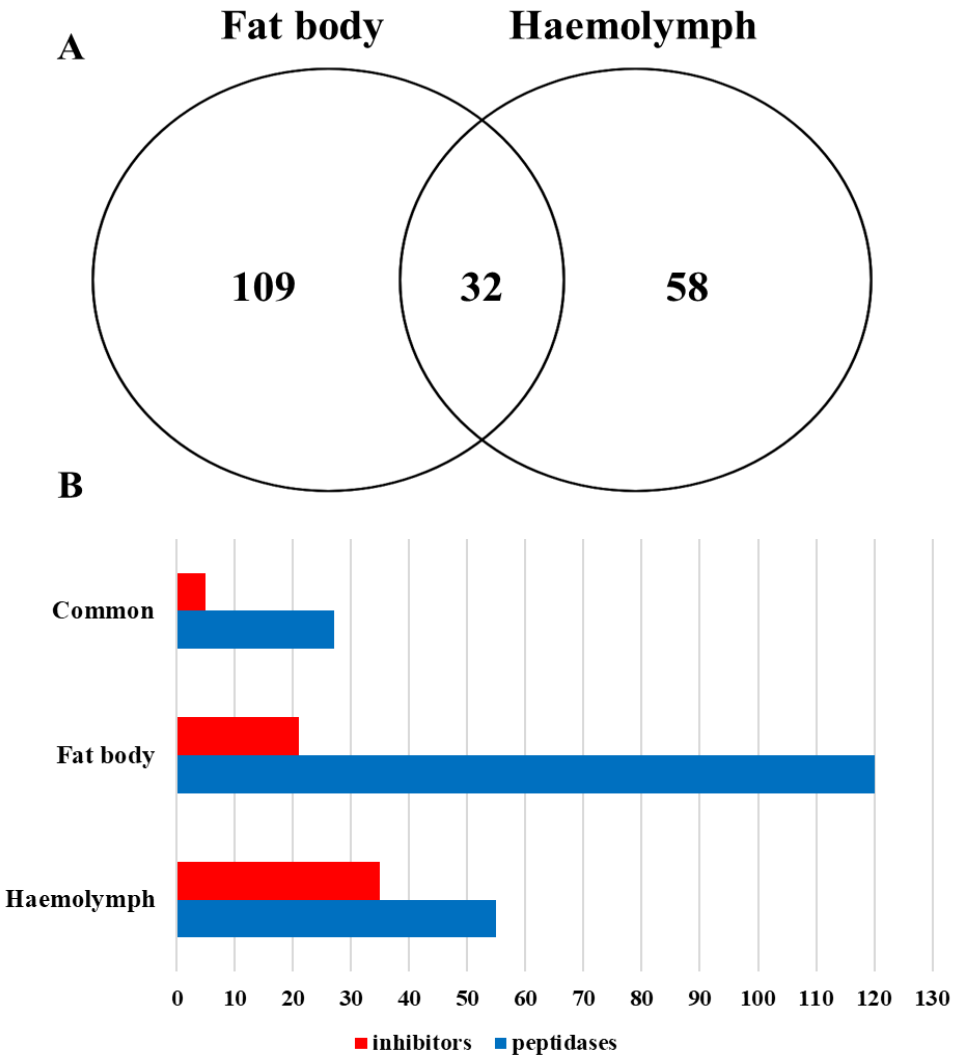


**Figure 4.10 Comparative analysis of haemolymph and fat body peptidase and inhibitor activity.** (A) Comparison of FB (141) and haemolymph (90) proteins revealed 32 shared and 109 and 58 unique peptidase/inhibitors, respectively. (B) Comparison of the numbers of peptidases and inhibitors in the FB, haemolymph and common to both.

**Table 4.9 The common peptidases and inhibitors in the haemolymph and fat body.** For Protease Class Aspartic (*A*), Cysteine (*C*), Threonine (*T*), Serine (*S*) proteases, Metalloprotease (*M*) and unknown (*U*). Number of proteins refers to numbers in each family. (I) indicates inhibitors and (P) indicates peptidases.

| Catalytic type | Clan | Family | No. of proteins | Reference proteins | Biological function | I/P |
|---|---|---|---|---|---|---|
| **Inhibitors of serine and cysteine endopeptidases** | ID | I4 | 1 | Serpin | Immune modulator | I |
| **inhibitor of the metallopeptidase pappalysin-11** | JB | I63 | 2 | Pappalysin-1 | Promotes cell growth | I |
| **Carboxypeptidase inhibitor** | JK | I68 | 1 | - | Anticoagulation | I |
| **I51 contains an inhibitor of serine carboxypeptidase Y** | JE | I51 | 1 | - | Kinase/chymotrypsin inhibitor | I |
| ***A*** | AA | A1 | 1 | Nucellin | Proteolysis | P |
| ***C*** | CA | C1 | 1 | Cathepsin B | Lysosomal activity | P |
| ***C*** | PC | C56 | 1 | - | Endopeptidase | P |
| ***M*** | MA | M2 | 1 | Transferrin receptor | - | P |
| ***M*** | MA | M13 | 2 | - | Proteolysis | P |
| ***M*** | MC | M14 | 1 | - | Antigen processing | P |
| ***M*** | MH | M28 | 1 | Transferrin receptor | - | P |

(Table 4.9 continued)

| Catalytic type | Clan | Family | No. of proteins | Reference proteins | Biological function | I/P |
|---|---|---|---|---|---|---|
| ***T*** | PB | T1 | 11 | - | Proteostasis | P |
| ***S*** | PA | S1 | 1 | Chymotrypsin | Immune modulator | P |
| ***S*** | SC | S28 | 1 | Pro-Xaa carboxypeptidase | Immune modulator | P |
| ***S*** | SC | S9 | 2 | - | Antigen processing | P |
| ***S*** | SC | S10 | 2 | - | Lysosomal activity | P |
| ***S*** | SR | S60 | 1 | Lactoferrin | - | P |
| - | unassigned | unassigned | 1 | - | - | P |

## 4.3 Discussion

Within insect haemolymph there is active chemical signalling between cells, tissues and organs by proteins, peptides and hormones (Grewal 2012). Many of these secreted proteins participate in maintenance and mediation of central biological processes such as immunity, development and metabolism. Hence, the application of LC-MS/MS to *B. terrestris* haemolymph and downstream analysis of this proteome provides an in-depth view of the molecular composition and underlying biological processes of this vital serum.

To complement the molecular analysis, the cellular portion of haemolymph, haemocytes, were also examined. Insect haemocytes are known to be involved in numerous physiological responses, including development and immunity. However, haemocyte populations of *Bombus* species have yet to be fully characterised. The completion of cell counts, and analysis of cytological preparations resulted in the identification and enumeration of circulating haemocyte populations of *B. terrestris*.

### 4.3.1 Key findings

- Within the *B. terrestris* haemolymph at least three cell types were identified; oenocytoids, plasmatocytes and spherulocytes.
- The *B. terrestris* haemolymph is a highly proteolytic and immune active serum which also participates which also participates in numerous aspects of normal cellular homeostasis, such as carbohydrate metabolism, protein degradation and cellular transport.
- Comparisons between the FB and haemolymph proteomes revealed numerous conserved biological functions such as carbohydrate metabolism, detoxification, the proteasome and PRR signalling.

### 4.3.2 *B. terrestris* haemocytes

The total haemocyte count in worker haemolymph is circa 2 x $10^6$ cells/ ml and three distinct morphotypes were identified (Figure 4.1). This count represents a baseline level of circulating immune cells and signifies a healthy worker maintained in favourable conditions. However, this number can vary dramatically in response to numerous factors such as age (colony and individual), stress, injury and infection. In bumblebees, worker

haemocyte numbers increase with the age of the colony (i.e. workers emerging early in the colony life cycle have lower concentration of haemocytes than those which emerge later) and in bees of increasing age the numbers of haemocytes have been shown to decrease (Moret and Schmid-Hempel 2009).

Haemocyte numbers in bumblebees have also been observed to both increase and decrease in response to immune challenge and wounding. Numbers increase for example, when pathogen recognition induces the release of sessile haemocytes into the haemolymph, these cells adhere to the surface of organs in the haemocoel until required (Korner and Schmid-Hempel 2004). Decreases in haemocyte numbers in response to infection are thought to be due to their participation in pathogen clearance and the formation of nodules (haemocyte aggregations) around invading pathogens (Doums et al. 2002).

The extracted haemocytes showed noticeable differences in morphology and after the examination of cells from all replicates, at least three distinct cellular morphotypes were identified (Figure 4.1 & 4.2). By making comparisons to haemocyte populations in well-characterised insects (Section 4.2.1) these three cell types were identified as spherulocytes, oenocytoids and plasmatocytes. The exact immune function of spherulocytes remains unclear (Hillyer 2016) however they have been implicated in coagulation of haemolymph (Soares et al. 2013). Oenocytoids contain high amounts of cytoplasmic phenoloxidase precursors and hence can contribute to melanisation (Jiang et al. 1997) and capsule formation in haemolymph (Lavine and Strand 2002). Plasmatocytes are considered key players in cellular immunity because of their ability to engulf and kill invading pathogens via phagocytosis (Kwon et al. 2014). Overall this indicates that cellular immune activities such as melanisation, capsule formation and phagocytosis are well-represented within the *B. terrestris* worker haemolymph.

A possible fourth haemocyte population was identified through H&E image analysis (Figure 4.1D). However, this population could not be verified through fluorescent and confocal image analysis (Figure 4.2). These cells may represent debris from permeabilised cells which have undergone cell lysis, death or degradation. Permeabilised cells have been identified in a number of other haemocyte studies (Marringa et al. 2014; Richardson et al. 2018). In addition, aggregation of haemocytes was observed across numerous slides. As suggested by Marringa et al., (2014) this indicates their potential

affinity for binding to other cells or surfaces and their likely role in wound repair and pathogen clearance.

Insect haemocytes are essential elements of cellular immunity and identification of the types of haemocytes is important, as it facilitates an understanding of the cellular capabilities of an insect (Soares et al. 2013). However, as previously reported comparisons can be difficult and yield few similarities (Lavine and Strand 2002; Ribeiro and Brehélin 2006; Marringa et al. 2014). However, more definitive characterisation would require additional molecular, ultrastructural, and functional studies of *B. terrestris* haemocytes to be conducted.

### 4.3.3 Haemolymph proteome

Proteomic analysis of *B. terrestris* worker haemolymph revealed that this complex serum is centrally involved in a number of important biological processes ranging from metabolism to immunity. Global gene ontology analysis of the proteome exposed the diverse biological processes haemolymph participates in, including; carbohydrate metabolism, defence response, detoxification, negative regulation of endopeptidase activity, melanin metabolism and response to wounding (Figure 4.5). Functional analysis of the pathways represented within the proteome also indicated carbohydrate metabolism, genetic information processing, organismal systems and cellular transport as the most enriched processes present in the haemolymph (Figure 4.4). Protein interaction analysis further confirmed this enrichment and revealed the most enhanced KEGG pathways within the proteome to be those associated with the proteasome, glycolysis/ gluconeogenesis and the lysosome (Figure 4.3) (Table 4.2). As expected for the haemolymph numerous immune proteins were identified within the proteome (Table 4.5). These proteins represented all major immune classes (AMPs, signal modulators and PRRs) and participated in PRR signalling pathways, melanisation defence and regulation of the innate response (Figure 4.7). In addition, the analysis of the catalytic activity of proteins within the haemolymph proteome revealed over 40% of the proteins identified as having catalytic activity which is expected for this highly metabolic and immune active serum (Table 4.6). Overall the haemolymph proteome can be described as a highly immune active serum which also participates in numerous aspects of normal cellular homeostasis such as carbohydrate metabolism, protein degradation and cellular transport.

#### 4.3.3.1 Haemolymph proteome immune repertoire

In insects, defence reactions are initiated by the recognition of pathogens which induce activation of proteolytic cascades in the haemolymph. These signalling cascades activate key defence responses which result in; melanisation of invading microbes, induction of cellular responses (phagocytosis or encapsulation) which neutralise foreign material, and the production of a potent humoral systemic response (Ferrandon et al. 2007). As expected, the haemolymph proteome of *B. terrestris* comprises numerous constituents of this immune response, ranging from pathogen recognition to effector production. Numerous proteins associated with processes such as melanisation, signal modulation, detoxification and antimicrobial response were all well-represented within the proteome. Confirmation of the immune significance of the haemolymph came from a review of the top 50 most abundant proteins in this proteome in which at least 20 typical immune proteins were identified. Highly abundant immune proteins identified within this proteome include those associated with the antioxidant system (SOD), PRRs (leucine-rich repeat-containing protein, beta-1,3-glucan-binding protein, MD-2-related lipid-recognition protein and peptidoglycan-recognition protein), AMPs (hymenoptaecin, defensin and icarapin), signal modulators (IRP30, serpins and ecdysteroid-regulated 16 kDa protein), venom proteins (omega-conotoxin and venom dipeptidyl peptidase) and elements of the melanisation signalling cascade (phenoloxidase, anti-chymotrypsin and chymotrypsin inhibitor).

Initiation of the innate immune response relies on recognition of evolutionarily conserved structures on pathogens, known as pathogen-associated molecular patterns (PAMPs), through a limited number of germ line-encoded PRRs (Mogensen 2009). Within the haemolymph proteome seven PRR proteins were identified. Of these, five were also among the top 50 most abundant proteins within the haemolymph. The variety and abundance of these essential molecules within the proteome is expected as pathogens recognition by PRRs stimulates immune responses through the activation of proteolytic cascades in the haemolymph and intracellular signalling pathways in immune-responsive tissues (Takehana et al. 2002). Recognition of PAMPs such as lipopolysaccharide (LPS) by the *B. terrestris* immune system induces the initiation of a number of distinct, interconnected signalling cascades such as Toll, Immune deficiency (Imd), Jun Kinase (JNK) and the Janus Kinase/ Signal transducers and activators of transcription (JAK/STAT) pathways (Barribeau and Schmid-Hempel 2013; Barribeau et al. 2015;

Sadd et al. 2015) which cause the expression of immune related genes which express proteins that aid the immune response like cytokines or AMPs (Mogensen 2009).

Within the haemolymph proteome of *B. terrestris* workers six proteins with classic antimicrobial properties (defensin, hymenoptaecin, abaecin, lysozyme and apidaecins) and two venom proteins (venom protease and omega-conotoxin) with novel immune functions were identified. These lethal components of insect immunity are synthesized de novo by the in response to infection and rapidly released into the haemolymph (Bulet et al. 1999). They are produced in large numbers at the site of infection and a have broad spectrum of properties (antibacterial, viral, fungal, protozoan, and sepsis) (Hancock and Diamond 2000) and show a degree of specificity to the pathogen eliciting the response (Lehrer and Ganz 1999). The presence of a variety of these proteins in the proteome of unperturbed workers indicates that proteins with antimicrobial properties may be constitutively expressed. Within the top 50 most abundant proteins in this proteome are four proteins with well-characterised antimicrobial properties (hymenoptaecin, omega-conotoxin, defensin and icarapin), their presence in this group may indicate that *B. terrestris* workers maintain high levels in the haemolymph in anticipation of infection by micro-organisms.

Another major element of innate defense in insects is the melanisation of pathogens and damaged tissues. During this process melanin surrounds invading pathogens limiting their spread and activity (Cerenius and Söderhäll 2004). In addition, during melanin formation, highly reactive and toxic intermediates which are important during defense against bacterial, fungal, and viral agents are produced such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates (González-Santoyo and Córdoba-Aguilar 2012). The active form of the enzyme phenoloxidase (PO) is responsible for this protective response where upon activation by tightly controlled proteolysis, PO will catalyse the initial steps in the pathway to melanin formation (Söderhäll and Cerenius 1998). Within the *B. terrestris* haemolymph proteome numerous members of the PO activating system were identified including phenoloxidase, chymotrypsin, anti-chymotrypsin along with numerous serpins. Gene ontology analysis of the proteome further confirmed the strong association between the haemolymph and the PO activating system with the identification of melanin metabolic processes, proteolysis and melanisation defence response as enriched terms within the proteome.

Functional analysis of enriched gene ontology biological processes within the haemolymph further confirmed the central role of this serum in immunity. Hierarchy analysis to investigate haemolymph specific biological processes revealed defence response and response to Gram-positive bacterium as two of the most enriched immune associated terms within the proteome. Functional analysis of all identified immune proteins within the haemolymph further confirmed this strong immune link and resulted in the identification of the most well-represented immune functions within the proteome; PRR signalling pathway, melanisation and regulation of innate immune response.

#### 4.3.3.2 Carbohydrate and energy metabolism in the haemolymph proteome

During periods of extreme physical exertion, when the energy demand cannot be readily satisfied by the available energy (ATP), energy stores are mobilised. In insects when energy reserves have to be mobilised, hormonal activation of the necessary catabolic enzymes, lipases and phosphorylases, causes the breakdown of nutrient stores (lipids and glycogen) and results in increased levels of lipids and carbohydrates in the haemolymph (Lorenz and Gäde 2009) which are then readily available to broken down to produce ATP (Kaufmann and Brown 2008). The hormones responsible for this activation in insects, are octopamine, the insect counterpart of adrenaline (Orchard et al. 1993) and the adipokinetic hormones (Lorenz and Gäde 2009).

One of the most consistently enriched biological processes identified in the haemolymph proteome was carbohydrate metabolism. Pathway analysis further revealed that the most significant associated pathways were that of glycolysis/ gluconeogenesis and pyruvate metabolism. Both pathways are involved in the breakdown of glucose which is essential in energy metabolism, heat generation, chitinous exoskeleton (Steele 1982) and serves as the main fuel for flight for bees (Suarez et al. 2005). When the demand for ATP is great, processes such as glycolysis, pyruvate metabolism and oxidative phosphorylation are necessary to drive production of ATP through the breakdown of nutrient stores. Within the 50 most abundant proteins in the haemolymph numerous proteins associated with glycolysis were; alpha-glucosidase which participates in carbohydrate degradation and enolase and glyceraldehyde-3-phosphate dehydrogenase which are responsible for catalysing the ninth and sixth step of glycolysis, respectively. In addition, energy associated proteins and processes such as arginine kinase and oxidative phosphorylation

were also abundant in haemolymph proteome further confirming its role in the supply and maintenance of energy. Metabolism-associated proteins within the haemolymph is advantageous for the insect in order to rapidly mobilise and participate in the breakdown and storage of energy to cells requiring it. The presence of numerous elements of carbohydrate and energy metabolism is expected within the haemolymph as it is the main transport medium responsible for transport and exchange of nutrients, hormones and waste materials between tissues and organs in insects (Chan et al. 2006; Klowden 2007).

#### 4.3.3.3 Protein production, digestion and absorption

Analysis of the functionality of the haemolymph proteome revealed genetic information processing among one of the most enriched biological processes. This biological group includes pathways involved in protein production and degradation including; the proteasome and protein processing in the endoplasmic reticulum. Enrichment analysis of the proteome further indicated the proteasome and proteolytic processes as having a significant role within the haemolymph. In addition, the presence of a high proportion of proteins belonging to the T1 protease family which contains threonine peptidases, the component peptidases of the proteasome (Tanaka 2009) further confirms the significance of the proteasome and hence its proteolytic activity within the *B. terrestris* worker haemolymph proteome.

In addition to proteasomal processes, cellular processes such as phagosome and lysosome were also identified as enriched in the proteome. The enrichment of processes such as the proteasome coupled with the presence of export processes (cellular processes) are indicative of high protein degradation. The haemolymph is constantly exchanging nutrients, hormones and waste products between tissues and organs hence it is expected that some proteins associated with cellular transport processes may also be present in the haemolymph as artefacts of the exchange of these materials (Mallick and Kuster 2010; Hou et al. 2016).

#### 4.3.3.4 Peptidases and inhibitors

In all living organisms proteases and their inhibitors control multiple biological processes (López-Otín and Bond 2008; Gubb et al. 2010). In insects many physiological responses are activated and mediated by signalling cascades including the phenoloxidase cascade (Lu, Zhang, et al. 2014), wound healing and coagulation processes (Krautz et al. 2014).

To investigate the proteolytic capacity of *B. terrestris* worker haemolymph all identified proteins were blasted off the MEROPS database. The blast results indicated the haemolymph as a highly catalytic serum, in which 90 of the 207 proteins forming the proteome were revealed as having either peptidase or inhibitory actions. Following further interrogation of these proteins it was found the haemolymph contains 55 proteins with peptidase activity and 35 with inhibitory activity. Overall nine families of inhibitors and 20 peptidases were identified.

Investigation of the properties of the nine families of peptidase inhibitors revealed their roles in numerous central biological processes ranging from anticoagulation and antigen processing to immune modulation and cell growth. The two most well-represented families of inhibitors in the haemolymph proteome were the I63 metallopeptidase pappalysin-1 inhibitor family and I4. The I4 family of proteins are inhibitors of both serine and cysteine endopeptidases. Among the most well-known proteins in this family are serine protease inhibitors (serpins). Serpins mainly function as potent protease inhibitors, responsible for the modulation of an array of proteolytic cascades hence controlling the resulting physiological and pathological reactions (Bao et al. 2018). In *Drosophila melanogaster*, serpins play a role in the modulation of the Toll signaling cascade in the haemolymph via the control of proteolytic cascades (Lemaitre and Hoffmann 2007). Similarly serpins have immune-association in *B. terrestris*, in which the serpin 27A has been shown to be involved in suppression of the PO cascade activator serine protease 8 (Richter et al. 2012). Investigation of the I63 family revealed it contains an inhibitor specific for the protein metallopeptidase pappalysin-1, functions associated with this family include negative regulation of cell growth (Glerup et al. 2005; Kantyka and Potempa 2011).

Proteases are central to the normal functionality of numerous vital biological processes such as DNA replication, protein transcription, inflammation, immunity and cell proliferation which they control through stringent modulation of the activity and localisation of many proteins and protein-protein interactions (López-Otín and Bond 2008). Proteases can be separated into five classes of proteolytic enzymes on the basis of their catalytic mechanism: aspartic, cysteine, metallo-, threonine and serine peptidases (Barrett et al. 2003). Within the haemolymph proteome all classes of peptidases were present and could be further categorised into 14 clans and 20 families. The two peptidase families with the highest number of members present in the haemolymph were T1 and

S1 with 13 and 11 members, respectively. The family T1 contains threonine peptidases which are the component peptidases of the proteasome (Tanaka 2009). The main function of the proteasome is the degradation of damaged or mis-folded proteins (Adams 2003). The S1 or chymotrypsin family are one of the most well-studied family of enzymes and in mammals perform a variety of roles in physiological processes such as digestion, coagulation, cellular and humoral immunity (Ross et al. 2003). In the honeybee (*Apis mellifera*) S1 family proteases have been revealed to have potential roles in immune response (Zou et al. 2006). Functions associated with other protease families in the haemolymph proteome range from immune modulation and signal transduction to proteolysis and lysosomal activity.

#### 4.3.3.5 Non-secreted cellular proteins in the haemolymph proteome

Within the proteome some cytosolic proteins were identified. Their presence in the proteome may indicate a role for the haemolymph in clearing apoptotic cells as observed for mammalian blood (Omenn et al. 2005) or it may be as a result of the collection procedure. In order to pellet and remove all haemocytes from the haemolymph a centrifugation step was included in the collection method. However, it is possible that during this process haemocytes were damaged, lysed or not fully pelleted and hence some remained in some form in the serum. In addition, the collection procedure included a small wound in the abdomen of the bee which as outlined by (Handke et al. 2013) may have resulted in tissue damage and consequential rupture of cells at the wound site may have induced release of non-secreted cellular proteins such as ribosomal proteins into the haemolymph. In conclusion, the non-secreted intracellular proteins detected in the haemolymph proteome may have originated from the lysis of any included cells such as haemocytes during collection or may be present as a result of haemolymph function. To definitively clarify the origin of all identified non-secreted cellular proteins additional experimentation will be required using a variety of methods with higher detection sensitivity than shotgun proteomics (Handke et al. 2013).

### 4.3.4 Comparative analysis of the haemolymph and fat body proteomes

Overall comparisons of the FB and haemolymph proteomes revealed 124 proteins common to both (Figure 4.8A). Functional analysis of these proteins revealed the conserved biological processes in the FB and haemolymph are those of normal cellular

house-keeping processes including carbohydrate metabolism, detoxification, the proteasome and PRR signalling (Figure 4.8B).

To investigate the immune repertoires of both proteomes comparative analysis of all identified immune proteins was completed. From this it was revealed that the FB and haemolymph have 27 common immune proteins from all major functional classes including five PRRs, five antioxidants, two immune effectors and 15 immune modulators. Further analysis of the differences between both proteomes revealed the FB has an abundance of antioxidants whereas the haemolymph is rich in immune effectors. Out of the total nine antioxidants identified across both proteomes eight were found in the FB and four were exclusive including glutathione S-transferase, glutathione peroxidase and catalase. The abundance of antioxidants in the FB may be present to combat redox molecules produced within this highly metabolic tissue. Of all 12 immune effectors identified across both proteomes, 10 were present in the haemolymph and five were unique. These included AMPs, venom proteins and phenoloxidase. The most numerous and diverse immune effectors were AMPs which included defensin, hymenoptaecin, abaecin and apidaecins. These lethal components of insect immunity are synthesized de novo by haemocytes and the FB in response to infection and rapidly released into the haemolymph (Bulet et al. 1999) hence it is expected that they would be present in higher numbers than in the FB. Proteolytic signalling cascades are vital for the activity of numerous biological processes hence it was expected that both proteomes would contain high numbers immune modulators.

Proteolytic enzymes have the ability to act as positive or negative effectors of a variety of biological processes either as non-specific catalysts of protein degradation or selective agents controlling physiological events (Neurath 1984). Comparisons of the proteolytic profiles of the FB and haemolymph proteomes revealed 32 shared and 109 and 58 unique peptidases and inhibitors, respectively. Overall analysis revealed higher amounts of peptidases than inhibitors in both proteomes. The common proteins included 27 proteases and 5 inhibitors.

The most abundant common family of inhibitors was I4. This family are inhibitors of both serine and cysteine endopeptidases. The most well-known proteins in this family are serine protease inhibitors (serpins) which modulate an array of proteolytic cascades and hence control a variety of physiological and pathological reactions including hormone

transport (Reichhart et al. 2011), storage (Law et al. 2006), the PO cascade (Richter et al. 2012) and the Toll signaling cascade (Lemaitre and Hoffmann 2007). Identification of high numbers of serpins within the FB and haemolymph further confirms the enrichment of numerous immune signalling associated processes in both such as the melanisation defence response, regulation of the humoral immune response and PRR signalling pathways.

Of the 27 proteases common to both proteomes, the most abundant family was T1. This family contains threonine peptidases the central component peptidases of the proteasome (Tanaka 2009). The main function of the proteasome is the degradation of damaged or mis-folded proteins which is a normal house-keeping function maintained in most cells (Adams 2003). Other common families of proteases included those that function in antigen processing, lysosomal activity and immune modulation.

### 4.4 Conclusion

This study provides a comprehensive overview of the molecular and cellular composition of the *B. terrestris* worker haemolymph. Through baseline cell counts and analysis of cytological preparations it was possible to determine the amount and variety of circulating haemocytes within an unperturbed worker. Three populations of haemocytes were identified within the haemolymph and were annotated as spherulocytes, plasmatocytes and oenocytoids. However, for definitive characterisation further molecular, ultrastructural, and functional studies of *B. terrestris* haemocytes are necessary.

Functional analysis of *B. terrestris* worker haemolymph revealed that this complex serum is centrally involved in a number of biological processes, ranging from metabolism to immunity. Global gene ontology analysis of the proteome exposed the diverse biological processes haemolymph participates in, including carbohydrate metabolism, defence response, detoxification, negative regulation of endopeptidase activity, melanin metabolism and response to wounding. As expected for the haemolymph, numerous immune proteins were identified including AMPs, signal modulators and PRRs. Overall the haemolymph proteome can be described as a highly proteolytic and immune active serum which also participates in numerous aspects of normal cellular homeostasis, such as carbohydrate metabolism, protein degradation and cellular transport.

Comparisons between FB and haemolymph proteomes revealed conserved biological functions such as carbohydrate metabolism, detoxification, the proteasome and PRR signalling. Analysis of the proteolytic profiles of the FB and haemolymph revealed numerous shared peptidases and inhibitors that participate in functions including immune signal modulation and proteolysis. Further analysis of the differences between both proteomes revealed the FB has an abundance of antioxidants whereas the haemolymph is rich in immune effectors.

# Chapter 5

## Label-free mass spectrometry-based analysis of the fat body, a central immune organ in the bumblebee *Bombus terrestris,* in response to wounding and bacterial challenge

## 5.1 Introduction

Bumblebees (*Bombus spp*.) are essential pollinators of native and economically valuable plant crops and communities (Fontaine et al. 2005). Elements of their morphology (robust size and long tongues) and ability to buzz-pollinate, significantly increase the efficiency of pollen transfer in numerous economically important food crops such as tomatoes and berries (Cameron et al. 2011). This efficacy lead to their domestication and further boosted our reliance on them to meet growing demands for food production (Graystock et al. 2016). Over 70% of the food crops consumed by humans benefit from pollination (Williams 1994; Klein et al. 2007), with the value of ecosystem services provided by pollinators to global agriculture estimated between €153 billion (Gallai et al. 2009) to €213–€522 billion per annum (Lautenbach et al. 2012; Potts et al. 2016).

Recent recorded decreases in bumblebee populations in Europe and North America have exacerbated environmental and economic concerns of global decline (Goulson et al. 2008; Williams and Osborne 2009). Numerous stressors have been implicated as potential contributors to global losses in bee populations, these include pesticides, parasites, pathogens, climate change and impaired nutrition, all of which can act synergistically to negatively impact bee health (Goulson et al. 2015; Smart et al. 2016). In order to effectively protect and conserve both wild and commercial bumblebee populations it is essential to understand the physiological and molecular mechanisms of infection, stress and defence in these essential pollinators.

Insects have an efficient and highly developed defence system to contend with and neutralise invading micro-organisms. This system is centred around three major responses; proteolytic cascades (coagulation and prophenoloxidase), cellular responses (encapsulation and phagocytosis) and synthesis and secretion of several potent effector molecules (antimicrobial peptides (AMPs)) (Lemaitre et al. 1996; Hoffmann and Reichhart 2002). The insect fat body (FB) is centrally involved in the initiation and modulation of these immune actions.

The FB is the homeostatic control centre for numerous vital physiological processes ranging from metabolism to immunity and detoxification. Although primarily responsible for lipid accumulation and storage (Musselman and Kühnlein 2018), numerous other functions are

associated with the FB, including monitoring, usage and storage of circulating metabolites, haemolymph proteins, energy reserves (glycogen and fat) (Arrese and Soulages 2010), removal of toxic substances and the immune response (Assis et al. 2014). The main role of the FB within the insect immune system is to release soluble factors which can be produced both constitutively and exclusively in response to immune stimulation, into the haemolymph, (Lemaitre and Hoffmann 2007). The FB achieves this through the initial recognition of pathogen recognition receptors (PRRs) which leads to the activation of serine protease and signalling cascades (Nappi et al. 2000). These signalling cascades (such as Toll) result in the transcription of numerous immune proteins which include AMPs, initiation factors essential for clot formation in response to wounding (Lemaitre and Hoffmann 2007) and prophenoloxidase cascade proteins (Zhu et al., 2003). Elements of these signalling pathways and genes encoding AMPs have both been identified within the FB of adult fruit flies (*Drosophila melanogaster*) (Lemaitre et al. 1996).

Given the contribution of disease to bee declines, the need for a comprehensive molecular characterisation of the bumblebee FB and its role in immunity, metabolism and detoxification is essential. Understanding the complex and intricate mechanisms by which the FB can both maintain homeostasis and mediate defence responses could provide novel insights into pathogenicity and immunity in bumblebees. As proteins are fundamental in the majority of cellular enzymatic functions, regulatory activities, signal transduction and structural components (Garrels 2001). Hence, the application of label-free quantitative mass spectrometry to characterise the FB proteome in response to bacterial challenge and wounding, enables an understanding of central aspects of the reactivity and immune function in this key organ. In addition, the analysis of immune stimulation at the cellular level could reveal potential pathological traits of bacterial stimulation in fat cells. This in combination with a proteomic immune response profile could lead to the discovery of key molecular and cellular biomarkers of infection and immunity in this key pollinator.

### 5.1.1 Chapter five aims

- To investigate the morphological properties of *E. coli* challenged fat cells; to identify pathogen-associated changes in their properties and composition.

- To utilise label-free mass spectrometry to investigate changes in the FB proteome in response to the presence of bacterial elicitors and wounding through functional and gene ontology analysis.

### 5.1.2 Experimental outline

Colonies of *Bombus terrestris* audax bumblebees (Koppert Biological Systems) were maintained in the lab under standard environmental conditions of 24±2˚C, continuous darkness or red light and fed *ad libitum* on sucrose solution (0.5M) and pollen (Agralan). Two colonies were chosen where the founding queen was present, only worker callows were emerging from hatching pupal cases, and sexual offspring, i.e., males and gynes, were absent. Colonies were screened for the presence of common bumblebee parasites via inspection of both the frass and digestive tracts of workers. Workers from both colonies were randomly selected and housed in wooden "holding" boxes (depth 12 cm x width 16 cm x length 24 cm) with a Perspex lid and maintained under the same conditions as above including access to a sucrose solution and pollen. Bees were acclimatised in these microcolonies for 24 hours prior to treatments and fat body collections.

To assess the effect of immune challenge on the fat body proteome, workers (n = 8 per treatment) were either injected with 2 µl of a suspension of either Gram-negative bacterium *Escherichia coli*, or with sterile Ringer's solution (Ringer hereafter) (Merck), or maintained as naïve controls. To prepare the bacteria for injection, a single colony was selected from an inoculated Luria Broth (LB) agar plate, added to LB broth and grown overnight at 37°C. Cultures were then centrifuged at 3,000 x *g* for 10 mins, the supernatant was removed, and the cells were resuspended in sterile Ringer solution, this step was repeated twice more to ensure the removal of all LB broth. Cell density was adjusted to obtain $10^7$ cells per ml and bees were injected between the third and fourth sternites on the left side of the abdomen with 2 µl of bacterial suspension. Once treated all bees were returned to holding boxes for a further 24 hours after which FB samples were collected for further processing. Inclusion of a Ringer

only treatment was completed to account for the FB response to wounding only and as a procedural control for the microbial treatment.

Fluorescent staining and confocal microscopy were utilised to investigate the morphological characteristics of *B. terrestris* fat cells after exposure with *E. coli*. Workers from four colonies were sampled (n = 8 per colony, Agralan). Aggregate masses of fat cells were dissected from the pinned abdomens of anaesthetised bees (as per section 2.2.4) and collected into filter sterilised phosphate buffered saline (fsPBS). All tissue samples for fluorescent staining (n = 3 per treatment) were incubated with the nuclear stain 4′,6-Diamidine-2-phenylindole (DAPI) (1 mg/ml) (1:500) (Sigma) and the lipid stain Nile Red (0.5 mg/ml) (1:1000) (Sigma) for 30 mins each, washing with fsPBS between the addition of the second dye. All images were captured using the Olympus Fluoview1000 confocal laser scanning microscopy (CLSM).

To assess changes in the FB proteome response to the presence of microbial elicitors, fat body tissue was collected (section 2.2.4), processed and subjected to high resolution mass spectrometry. After initial protein quantification, 100 µg of sample was removed and prepared for mass spec by the following steps; 2D clean-up, digestion and peptide purification (C18). For mass spec analysis 1 µg of prepared tryptic peptides sample was loaded on a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. The peptides were separated over a 2 – 40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120 min reverse-phase gradient at a flow rate of 250 nL $min^{-1}$. All data from the QExactive was acquired in automatic data dependent switch mode. A full scan at 70,000 resolution with a range of 400 – 1600 m/z was followed by an MS/MS scan, resolution 17, 500 and a range 200 – 2000 m/z. From each scan the 15 most intense ions were selected for MS/MS analysis. Four independent biological replicates, sampled from two colonies (Koppert Biological Systems), were analysed in this study.

The analysis software MaxQuant v 1.5.0.8 (http://www.maxquant.org) was used for protein identification and label-free quantification (LFQ) normalisation of all MS/MS data. Data analysis, processing and visualisation of the data was then performed using Perseus v 1.5.0.8.

(www.maxquant.org/). To identify statistically significant differentially abundant (SSDA) proteins two sample t-tests were performed for all relevant comparisons on the post imputated data using a cut-off of $p<0.05$. To visualize changes in protein expression for each pair wise comparison individual volcano plots were generated within Perseus by plotting negative log p-values on the y-axis and log2 protein fold-change values on the x-axis. Principal component analysis (PCA) of the data set was performed using normalized intensity values. Hierarchical clustering was performed on Z-score normalised intensity values for all significant proteins identified via a multi-sample ANOVA, by clustering both samples and proteins using Euclidean distance and complete linkage.

To resolve key protein-protein interactions and identify the most significantly enriched functions and pathways within the dataset STRING v 10 (http://string-db.org/) was used. A global overview of the biological processes enriched within the dataset was completed using the following gene ontology (GO) assignment software ClueGO v 2.5.1 (http://www.cytoscape.org/).

To assist with the assignment of gene ontology (GO) terms and putative functional information to all proteins a basic local alignment search tool for proteins (BLASTp) search of all identified proteins was performed against the Uniprot sequence set for *Apis mellifera* and *Drosophila melanogaster* (WWW.Uniprot.org downloaded April 2017). The top Uniprot match for each identified protein was used to query the Perseus annotation file for *A. mellifera* and *D. melanogaster* (downloaded April 2017) and to extract terms for biological process (BP), molecular function (MF), cellular component (CC), Kyoto Encyclopedia of Genes and Genomes (KEGG) name, KEGG pathway, protein family (pfam) and InterPro. To investigate defensive processes within the data-set, ClueGO was used to extract biological processes and immune functions using *D. melanogaster* terms.

## 5.2 Results

### 5.2.1 Fluorescent microscopy

The application of fluorescent staining with the nuclear specific dye DAPI and the lipid staining dye Nile red and their visualisation using CLSM facilitated a detailed examination of the individual cells forming the FB. Four images per FB dissection were captured from both treated (*E. coli*) and control (Ringer) samples. Images were analysed to calculate the number and ratio of the cells comprising the FB, measure the area of cell types and quantity the lipid droplets (LDs) in adipocytes. To measure the ratio of fat cells per treatment, all cells within each image were identified, counted and ratios (oenocyte count/adipocyte count) were calculated. An area comprising three adipocytes and oenocytes sampled randomly from each image (n = 12) was measured using ImageJ to evaluate the size of fat cells. In addition, the area of the three largest LDs present in adipocytes selected for measurement were also quantified using ImageJ. Statistical analysis of all comparisons was performed using unpaired T-tests between treatment and control groups using GraphPad Prism v. 5.0 (www.graphpad.com). For all analysis a p-value of $< 0.05$ was deemed statistically significant.(Figure 5.1).

Comparisons of the number of adipocytes and oenocytes comprising the FB revealed no significant change in response to microbial stimulation. The numbers of adipocytes were similar in both treatment and control groups (p-value 0.542) and an increase in oenocytes in response to *E. coli* was observed. However statistical analysis did not reveal any significance (p-value 0.0506) (Figure 5.1). Similarly, the investigation of fat cell area did not yield any significant differences (Figure 5.2). Cell counts were further utilised to calculate the ratio of cell types in both control and treatment groups (oenocyte/ adipocyte), this analysis revealed the ratio of both as 3:1 oenocytes to adipocytes. To investigate if stressors such as pathogens effect LD storage in *B. terrestris* adipocytes, LDs were quantified and compared in *E. coli* and control. This analysis indicated large increases in LD area in response to the presence of *E. coli* in the FB (Figure 5.3 & 5.4).

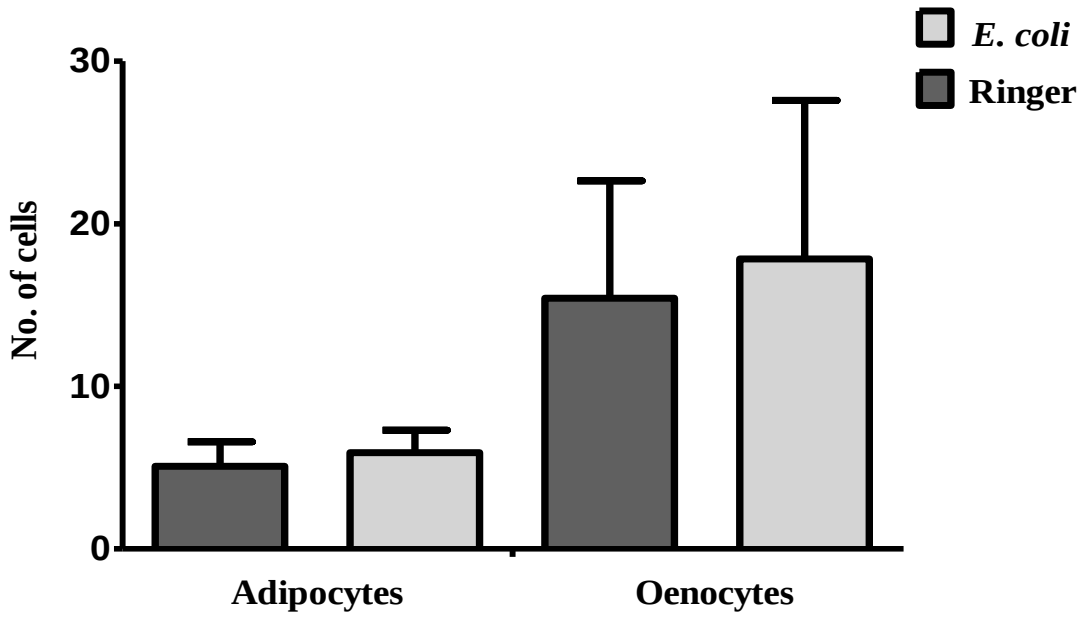


**Figure 5.1 Investigation of FB composition in response to treatment with a bacterial elicitor.** For all comparative analysis of the FB response to *E. coli*, Ringer was used as the procedural control. Adipocytes and oenocytes in both Ringer and *E. coli* treated FB samples were enumerated and compared (unpaired T-test) to investigate differences in the numbers of oenocytes and adipocytes composing the FB in response to bacteria.

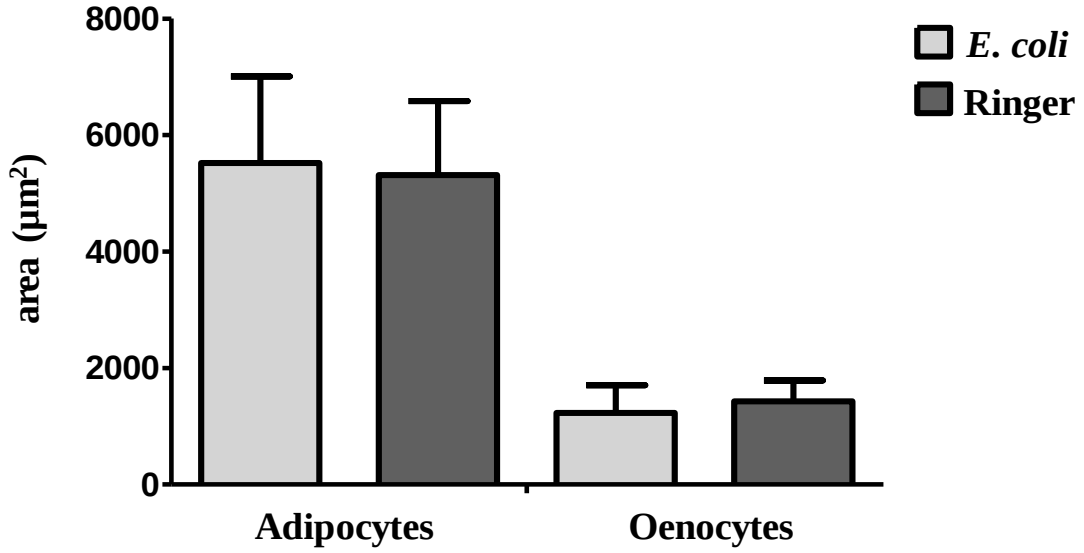


**Figure 5.2 Investigation of fat cell area in response to the presence of *E. coli*.** For all comparative analyses of the FB response to *E. coli*, Ringer was used as the procedural control. Adipocytes and oenocytes in both Ringer and *E. coli* treated FB samples were enumerated and compared (unpaired T-test) to investigate differences in the size (area) of oenocytes and adipocytes composing the FB in response to bacteria. The results revealed no significant differences in the area of adipocytes (p-value 0.17) or oenocytes (p-value 0.49) in response to the presence of *E. coli*.

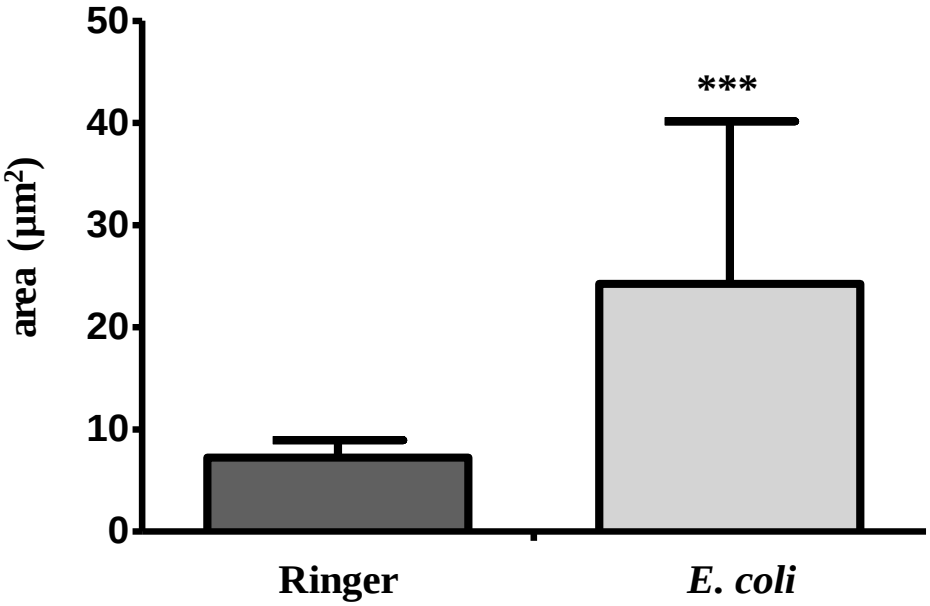


**Figure 5.3 Changes in LD area in adipocytes in response to Gram-negative bacteria.** For all comparative analysis Ringer was used as the procedural control. To investigate differences in the area of LDs in the FB in response to *E. coli* their area was calculated in control and treated samples. This analysis (unpaired T-test) revealed LD size is significantly changing in response to bacterial stimulation. (***) represents a p-value of $p<0.001$.

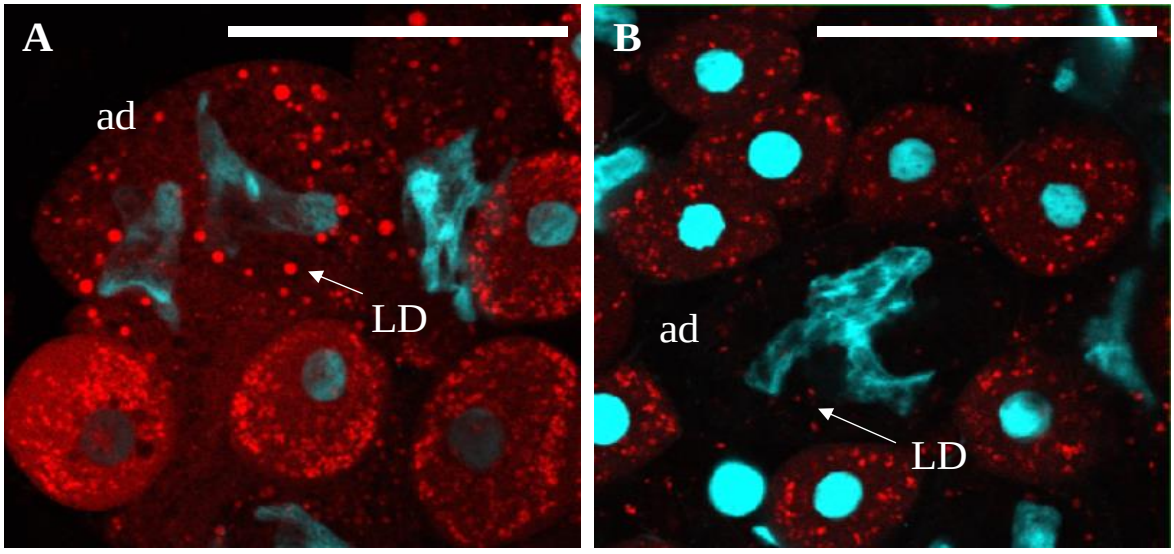


**Figure 5.4 Differences in adipocyte LD size in response to *E. coli*.** Fluorescent based confocal microscopy images reveal LD size in adipocytes (ad) in *E. coli* (A) and control (B) samples. The use of the lipid stain nile red facilitated the identification of LDs. Statistical analysis revealed LDs in control samples were smaller than those in *E. coli* treated FBs. Scale bar = 100 µm.

### 5.2.2 Identified and quantified proteins

All raw MS/MS files were imported into MaxQuant which was used to search the mass spectra against a protein database arising from the bumblebee genome (Sadd et al. 2015). This step identified the proteins present across all samples and calculated their relative abundance. Initially 2832 proteins were identified but were filtered to remove contaminant peptides, reverse peptide hits and peptides only identified by site. LFQ intensity values were then $Log_2$ transformed and only proteins found in at least three of four replicates were included resulting in 1660 proteins (Table A 5.1). In order to visualise similarities among replicates and samples, to visualise protein groups with similar expression profiles and to identify statistically significant protein differences a number of analysis procedures were carried out on the data, these were principal component analysis, hierarchical clustering, ANOVA analysis and Student's t-tests.

### 5.2.3 Principle component and hierarchical clustering analysis

To gain a global insight into the overall variation between samples, hierarchical clustering and PCA were performed to assess the variation of protein presence and abundance across all samples (Figure 5.5 & 5.6). PCA illustrates the degree of variation between all samples and demonstrates the quality of the replicates which show little variation by clustering together. However, both the PCA and clustering did highlight a high degree of variation between treatment groups.

To obtain a global view of protein presence/absence and abundance levels between different samples hierarchical clustering was performed (Figure 5.6). Hierarchical clustering, which groups samples with similar protein profiles, resolved eight clusters within all significant proteins (n = 471; Figure 5.6 A). Cluster A comprised 91 proteins displaying increases in abundance in response to both *E. coli* and wounding and included proteins associated with immunity (phospholipase A2 and omega conotoxin), detoxification (cytochrome P450, 10 kDa heat shock and thioredoxin) and carbohydrate (enolase and aldose reductase) and energy metabolism (NADH dehydrogenase and electron transfer flavoprotein). Functional enrichment of this cluster identified fatty acid metabolism as a central process. Clusters B and E included 33 and 52 proteins respectively, these clusters represented proteins decreasing

in response to both bacterial treatment and wounding. Within cluster B were proteins involved in physiological processes such as immune response (antichymotrypsin, venom dipeptidyl peptidase and alpha-2 macroglobulin) and carbohydrate metabolism (isocitrate dehydrogenase and beta-galactosidase). Cluster E contained numerous immune (defensin, icarapin, IRP30, phenoloxidase and chaoptin) and lysosome-associated proteins (Ras-related protein Rab-11A and -39B). Enrichment analysis revealed that proteins associated with the lysosome were well-represented, however no enrichment was obtained for cluster B. Proteins with decreased abundance in response to *E. coli* treatment comprised clusters C and D. Within the 44 proteins forming cluster C were a variety of cytoskeletal (troponin, myosin, laminin and alpha actinin), ribosomal (28S ribosomal) and immune (venom bombolitin) associated proteins. Cluster D comprised 50 proteins including those involved in detoxification (catalase, small heat shock protein and peroxiredoxin), protein synthesis (60S and 40S ribosomal proteins) and carbohydrate (glucose-1, 6-biphosphate synthase), fatty acid (fatty acyl-CoA reductase) and energy metabolism (acetyl-coenzyme A synthetase). GO enrichment analysis identified no enriched terms within cluster C and enrichment of the ribosome within cluster D. The clusters F, G and H were abundant in proteins associated with the response to Gram-negative bacteria. Cluster F comprised 45 proteins involved in numerous biological processes such as detoxification (thioredoxin), carbohydrate metabolism (maltase, trehalase and 6-phosphogluconate dehydrogenase) and immunity (peptidoglycan recognition protein, hymenoptaecin and chymotrypsin). Within the 103 proteins forming cluster G were proteins involved in the proteasome (26S proteasomal non-ATPase regulatory subunit, 7, 11, proteasome subunit alpha type-1 and -3), detoxification (superoxide dismutase) and immunity (chymotrypsin and leucine-rich repeat containing protein). Cluster H contained 53 proteins involved in the following physiological activities; detoxification (peroxiredoxin, cytochrome P450, superoxide dismutase and heat shock 70 kDa), immunity (protein toll, BMP-binding endothelial regulator protein and phospholipase A-2-activating protein) and carbohydrate (hexokinase, enolase and glucose-6-phosphate isomerase) and energy (ATP synthase and NADH-ubiquinone oxidoreductase) metabolism. Functional analysis of the clusters in this response revealed cluster G to be enriched with proteasomal proteins whilst no enrichments could be determined for clusters F and H.

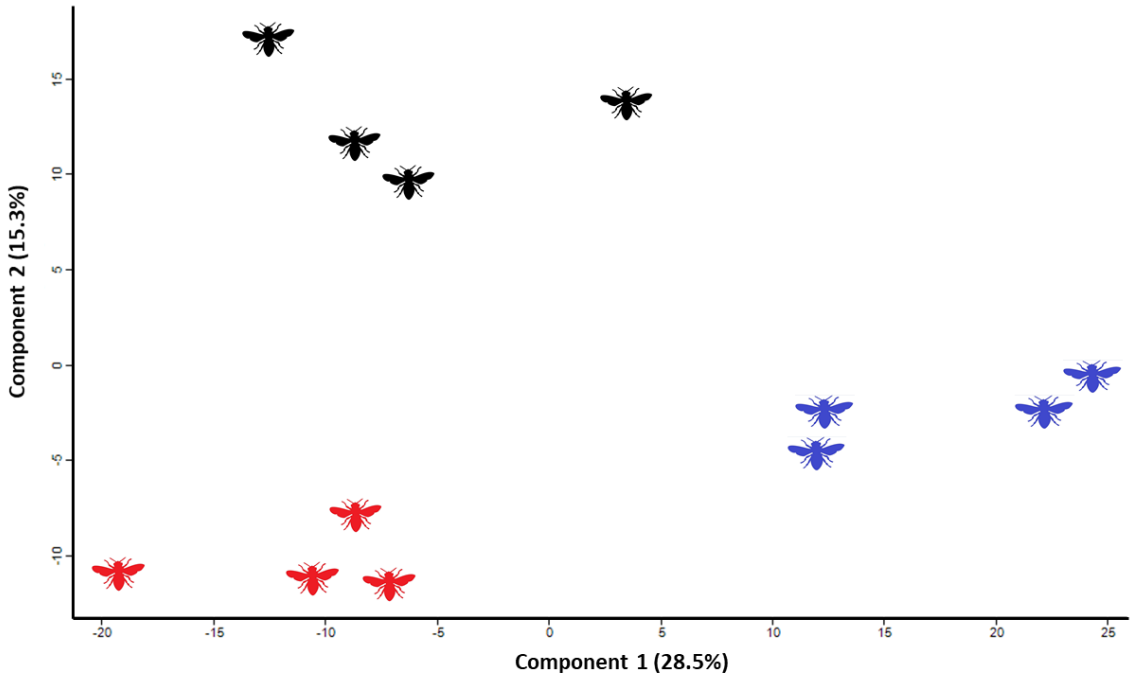


**Figure 5.5 Principal component analysis of all samples.** To gain a global view of the variation of proteins between different samples PCA was carried out. The coloured bees represent each individual sample of a treatment and are colour coded to identify the treatment; *E. coli* (red); ringer (blue) and naïve (black). Three clusters are apparent representing the three individual samples, with a clear similarity among the replicates of each sample. The x and y axis represent the principal directions in which the data varies, both of which account for 43.8% of the variance in the data.

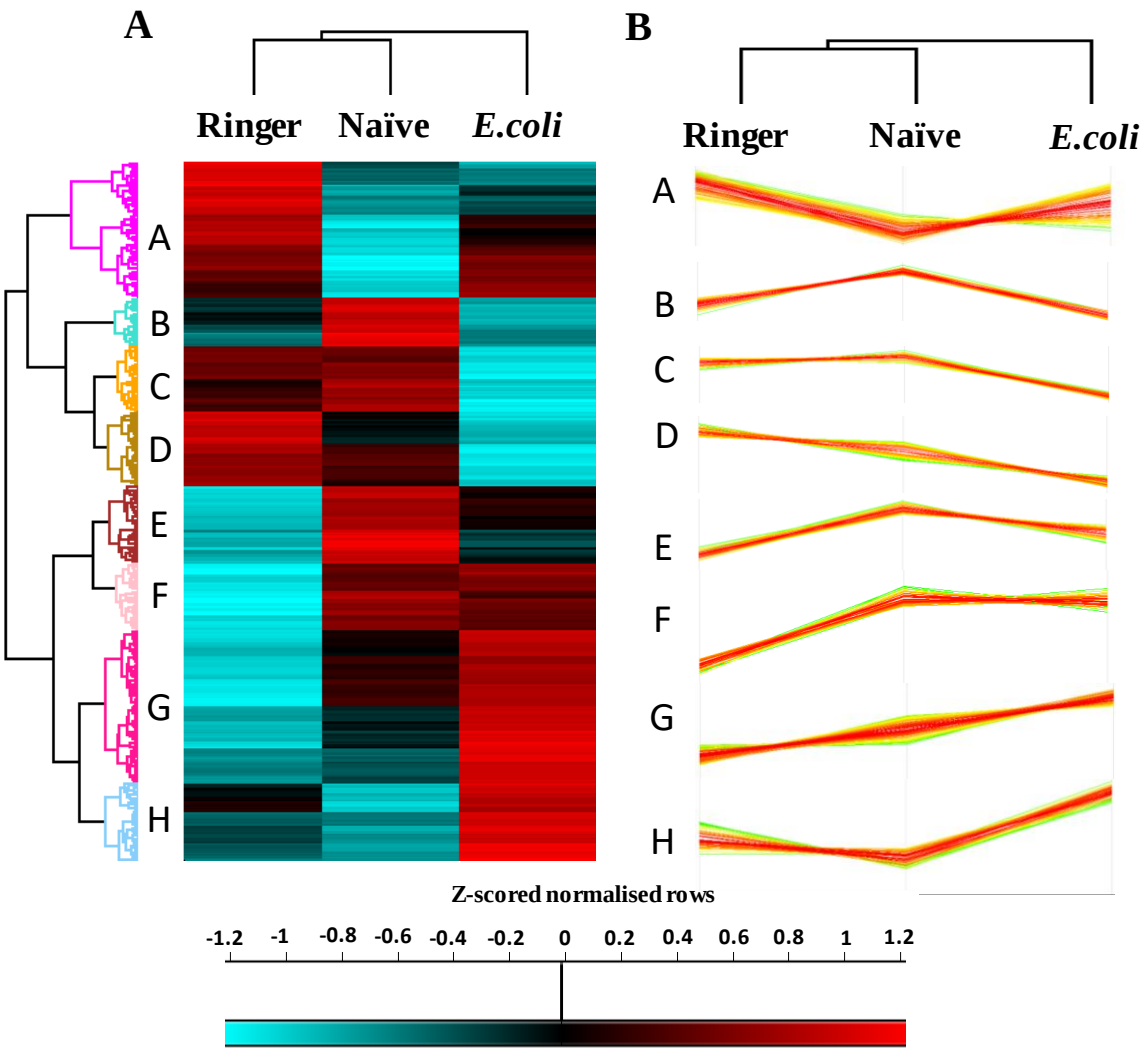


**Figure 5.6 Hierarchical clustering of all samples.** (A) Hierarchical clustering of the mean intensity values for 471 SSDA proteins among the different samples in response to *E.coli* and wounding. Heat map colours represent the relative expression of proteins in samples. (B) Cluster expression trends of identified proteins are represented by the coloured lines. These proteins have been divided into groups based on treatment specific expression profiles. Relative abundances (z-scored normalized) of individual proteins can be read from the intensity spectrum.

**Table 5.1 Significantly enriched gene ontology terms for biological processes.** A selection of significantly enriched KEGG terms (Fisher's exact test, FDR < 2%) identified in clusters A, D, E and G based on ClueGO analysis. No enrichment was identified for clusters B, C, F and H.

| Cluster ID | Cluster size | Term | P-value | Enrichment |
|---|---|---|---|---|
| A | 91 | Fatty acid metabolism | 0.00025 | 5.1758 |
| D | 50 | Ribosome | 3.22E-05 | 5.0027 |
| E | 52 | Lysosome | 3.09E-05 | 5.7395 |
| G | 103 | Proteasome | 3.86E-07 | 3.9412 |

### 5.2.4 FB Response to *E. coli*

Student's t-tests were performed between the different treatments to determine statistically significant differentially abundant (SSDA) proteins in response to the presence of bacteria and wounding (Figure 5.7 & 5.8) (Table 5.2 & 5.3). In total 414 SSDA proteins were identified in response to the presence of *E. coli* when compared to Ringer. Of this total number, 252 and 162 had increased and decreased abundances respectively (Figure 5.7). The ten most differentially increased abundance proteins included those associated with immunity (chymotrypsin and epididymal secretory protein), carbohydrate metabolism (chitinase, maltase and alpha-glucosidase), cell signalling (acetylcholine receptor) and proteases (trypsin and transmembrane protease serine) (Table 5.2). The ten proteins which the greatest decrease in abundance include those associated with; immunity (venom bombolitin), regulation of cellular processes (protein G12), carbohydrate (glucose 1, 6-biphosphate) and lipid metabolism (PI-PLCX) with the remaining proteins associated with proteostasis-related processes. Within the group of 252 proteins that had increased abundances in response to *E. coli* were numerous immune proteins such as; AMPs defensin and hymenoptaecin, PRRs peptidoglycan recognition protein and leucine-rich repeat containing protein, immune effectors phenoloxidase and venom carboxylesterase and antioxidants superoxide dismutase, peroxidase and thioredoxin (Table A 5.2). Within the decreased abundance dataset, immune proteins such as antioxidants (catalase and small heat shock protein) and immune modulators (plasminogen activator inhibitor) were also present. In addition, a variety of uncharacterised proteins were identified within the increased and decreased abundant proteins, indicating a potential novel role for these proteins in the immune response to bacteria in bumblebees.

**Table 5.2 Top 10 SSDA proteins in response to *E. coli* stimulation in the FB.** The top 10 most/least abundant proteins within the FB proteome in response to microbial stimulation based on intensity listed in descending order include those involved in metabolism, immunity and protein processing. Fold change represent the $\log_2$ mean LFQ intensity difference in *E. coli* compared to Ringer. Fold changes highlighted red and blue represent increased or decreased abundances, respectively, in response to *E. coli*.

| Fold change | Accession no. | Protein name |
|---|---|---|
| 6.78 | XP_003393535.1 | Chymotrypsin-1-like |
| 5.08 | XP_003400637.1 | Probable chitinase 2 |
| 4.98 | XP_003402225.2 | Maltase A1-like |
| 4.81 | XP_003401051.1 | Trypsin 3A1-like |
| 4.80 | XP_003395447.1 | Epididymal secretory protein E1-like |
| 4.59 | XP_003400107.2 | Transmembrane protease serine 9-like |
| 4.18 | XP_012165909.1 | Protein G12* |
| 3.89 | XP_003395914.1 | Maltase 1-like |
| 3.73 | XP_012164669.1 | Alpha-glucosidase-like |
| 3.60 | XP_003397879.1 | Acetylcholine receptor subunit beta-like |
| -2.95 | AEN41592.1 | Venom bombolitin 1 |
| -2.9 | XP_012174179.1 | 40S ribosomal protein S28-like |
| -2.86 | XP_012173446.1 | 40S ribosomal protein S30* |
| -2.51 | XP_012170854.1 | PI-PLC X domain-containing protein 3 isoform X2 |
| -2.44 | XP_012165957.1 | Cat eye syndrome critical region protein 5-like |
| -1.85 | XP_003399558.1 | J domain-containing protein isoform X1 |
| -1.64 | XP_012170683.1 | Secretory carrier-associated membrane protein 5A isoform X1 |
| -1.62 | XP_003393700.1 | Rab11 family-interacting protein 2 |
| -1.48 | XP_012166227.1 | ATP-binding cassette sub-family D member 1 |
| -1.44 | XP_012165551.1 | Glucose 1,6-bisphosphate synthase isoform X2 |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

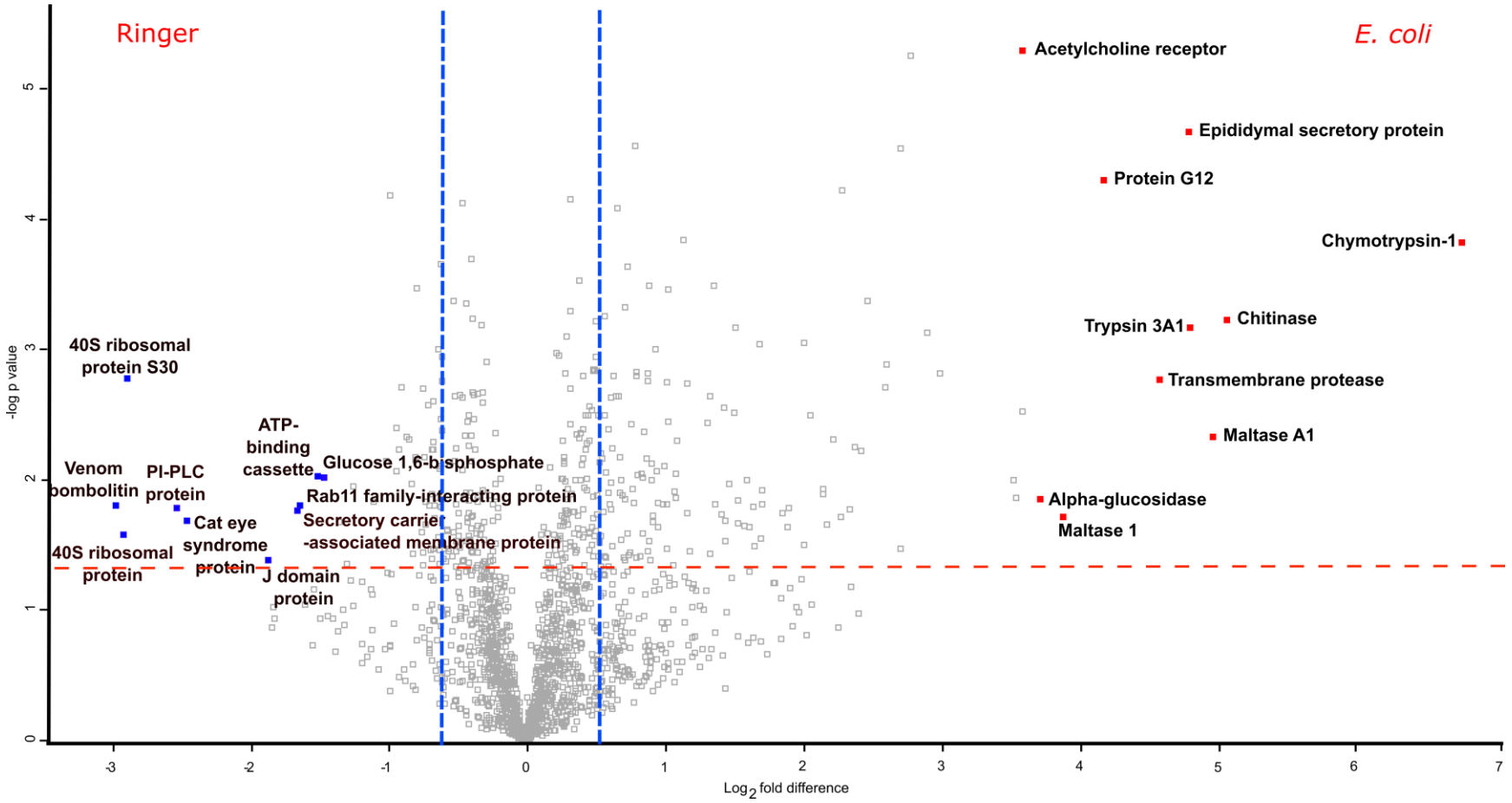


**Figure 5.7 Protein differential abundances of the *E.coli* response in the FB.** Volcano plots of the distribution of quantified proteins according to p-value ($-\log_{10}$p-value) and fold change ($\log_2$ mean LFQ intensity difference) in *E.coli* (red proteins) in comparison to Ringer samples (blue proteins). Proteins above the line are considered statistically significant (p-value <0.05) and those to the right and left of the vertical lines indicate relative fold changes ≥ 1.5. Annotated proteins represent the top ten increasing (red)/ decreasing (blue) proteins in response to wounding.

### 5.2.5 FB response to wounding

In response to wounding (Ringer in comparison the naïve equivalent), 295 SSDA proteins were identified in the *B. terrestris* worker FB (Figure 5.8). These included 124 and 171 proteins of increased and decreased abundance respectively (Table A 5.3). The most abundant of these proteins were those involved in fatty acid metabolism (long chain fatty acid and acyl-CoA-binding protein), lipid metabolism (phospholipase A2 and lipid storage droplets surface-binding protein), amino acid metabolism (ornithine aminotransferase) and protein transport (signal recognition particle 9 kDa protein and U6 snRNA-associated Sm-like protein LSm3) (Table 5.3). Proteins of decreased abundance in response to wounding included proteins associated with immunity (epididymal secretary protein E1-like and chymotrypsin), carbohydrate metabolism (maltase 1 and maltase A1), proteolytic activity (trypsin, natterin and transmembrane protease serine 9-like), regulation of cellular processes (protein G12), the ribosome (40S ribosomal protein S30) and cell communication (acetylcholine receptor subunit beta-like).

**Table 5.3 Top 10 SSDA wound response proteins in the FB.** The top 10 most abundant proteins increasing/ decreasing in the FB proteome in response to wounding based on intensity. Proteins are listed in descending order and include those involved in metabolism, immunity and protein processing. Fold change represent the $log_2$ mean LFQ intensity difference in Ringer when compared to Naïve treatments. Fold changes highlighted red and blue represent increased or decreased abundances, respectively, in response to wounding.

| Fold change | Accession no. | Protein name |
|---|---|---|
| 3.88 | XP_012170988.1 | Phospholipase A2-like |
| 3.76 | XP_012173446.1 | 40S ribosomal protein S30* |
| 2.46 | XP_012173524.1 | Enolase-phosphatase E1 |
| 2.39 | XP_003397572.1 | Long-chain-fatty-acid--CoA ligase 4 isoform X3 |
| 2.17 | XP_003394768.1 | Activated RNA polymerase II transcriptional coactivator p15 |
| 2.01 | XP_003398801.1 | Acyl-CoA-binding protein homolog |
| 1.98 | XP_012172166.1 | Ornithine aminotransferase, mitochondrial-like |
| 1.83 | XP_003402825.1 | U6 snRNA-associated Sm-like protein LSm3 |
| 1.81 | XP_012167207.1 | Lipid storage droplets surface-binding protein 1 isoform X2 |
| 1.72 | XP_012170030.1 | Signal recognition particle 9 kDa protein |
| -6.26 | XP_003395447.1 | Epididymal secretory protein E1-like |
| -5.47 | XP_003393535.1 | Chymotrypsin-1-like |
| -4.14 | XP_012165896.1 | Protein G12* |
| -4 | XP_003401051.1 | Trypsin 3A1-like |
| -3.92 | XP_003397879.1 | Acetylcholine receptor subunit beta-like |
| -3.81 | XP_003402992.1 | Protein G12-like |
| -3.75 | XP_003395914.1 | Maltase 1-like |
| -3.65 | XP_003398637.1 | Natterin-3* |
| -3.44 | XP_003402225.2 | Maltase A1-like |
| -3.36 | XP_003400107.2 | Transmembrane protease serine 9-like |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

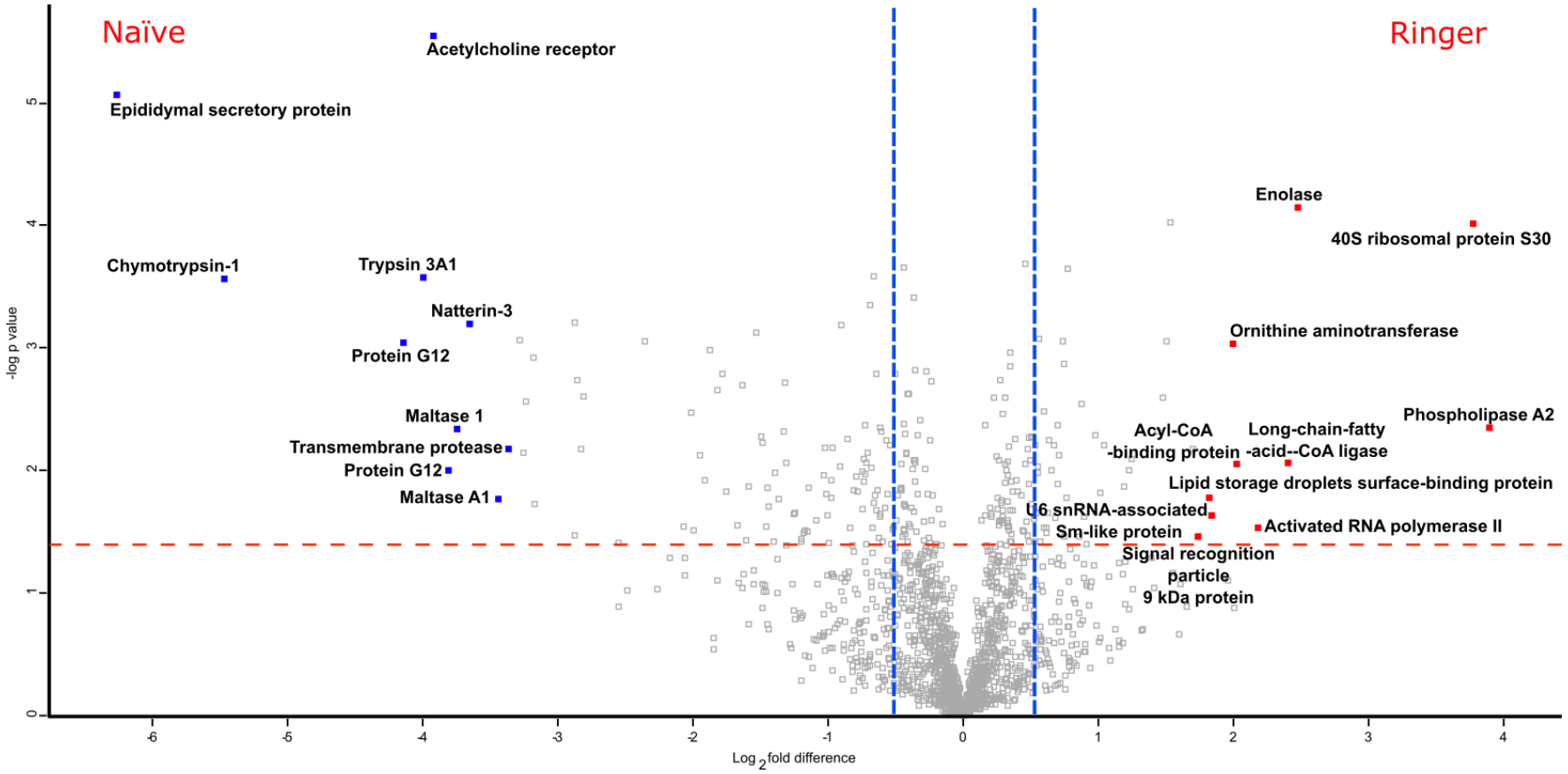


**Figure 5.8 Protein differential abundances of the wound response in the FB.** Volcano plot distribution of quantified proteins according to p-value ($-\log_{10}$ p-value) and fold change ($\log_2$ mean LFQ intensity difference) in Ringer-treated bees in comparison to their naïve counterparts. Proteins above the line are considered statistically significant (p-value <0.05) and those to the right and left of the vertical lines indicate relative fold changes ≥ 1.5. Annotated proteins represent the top ten increasing (red)/ decreasing (blue) proteins in response to wounding.

### 5.2.6 Protein Ontology

Using the search tool for the retrieval of interacting genes (STRING) it was possible to identify the networks and pathways enriched in the FB proteome in response to bacterial challenge and wounding (Figure 5.9). Protein-protein interaction (PPI) information was obtained from the STRING database using gene lists extracted for statistically significant differentially abundant (SSDA) proteins from pair wise t-tests ($p< 0.05$). Within the PPI network only interactions with the highest significance score ($\geq 0.900$) were included.

#### 5.2.6.1 FB response to microbial challenge

The SSDA proteins in response to Gram-negative bacterial challenge were categorised by their function, revealing their involvement in several biological pathways and activities including cellular transport, protein synthesis and degradation, and carbohydrate and energy metabolism (Figure 5.9A). Five processes were enriched within the proteins of increased abundance including carbon metabolism, oxidative phosphorylation, protein processing in the endoplasmic reticulum (ER), phagosome and proteasome whereas two processes, metabolic pathways, which encompasses elements of carbohydrate metabolism (Table 5.4) and the ribosome were identified within the proteins of decreased abundance.

KEGG pathway analysis was performed to obtain additional information on the enzymatic pathways and general processes responding to microbial challenge. Global analysis of the proteins with increased abundance revealed 21 KEGG processes. These included carbohydrate (glycolysis/ gluconeogenesis, pyruvate metabolism and the pentose phosphate pathway), lipid (glyoxylate and dicarboxylate metabolism) and amino acid metabolism (cysteine and methionine metabolism). The five pathways/processes identified from the STRING analysis above were present within the top 10 KEGG processes (Table 5.4) occurred. Six KEGG pathways were found to be enriched within the proteins of decreased abundance including the peroxisome, oxidative phosphorylation and tryptophan metabolism.

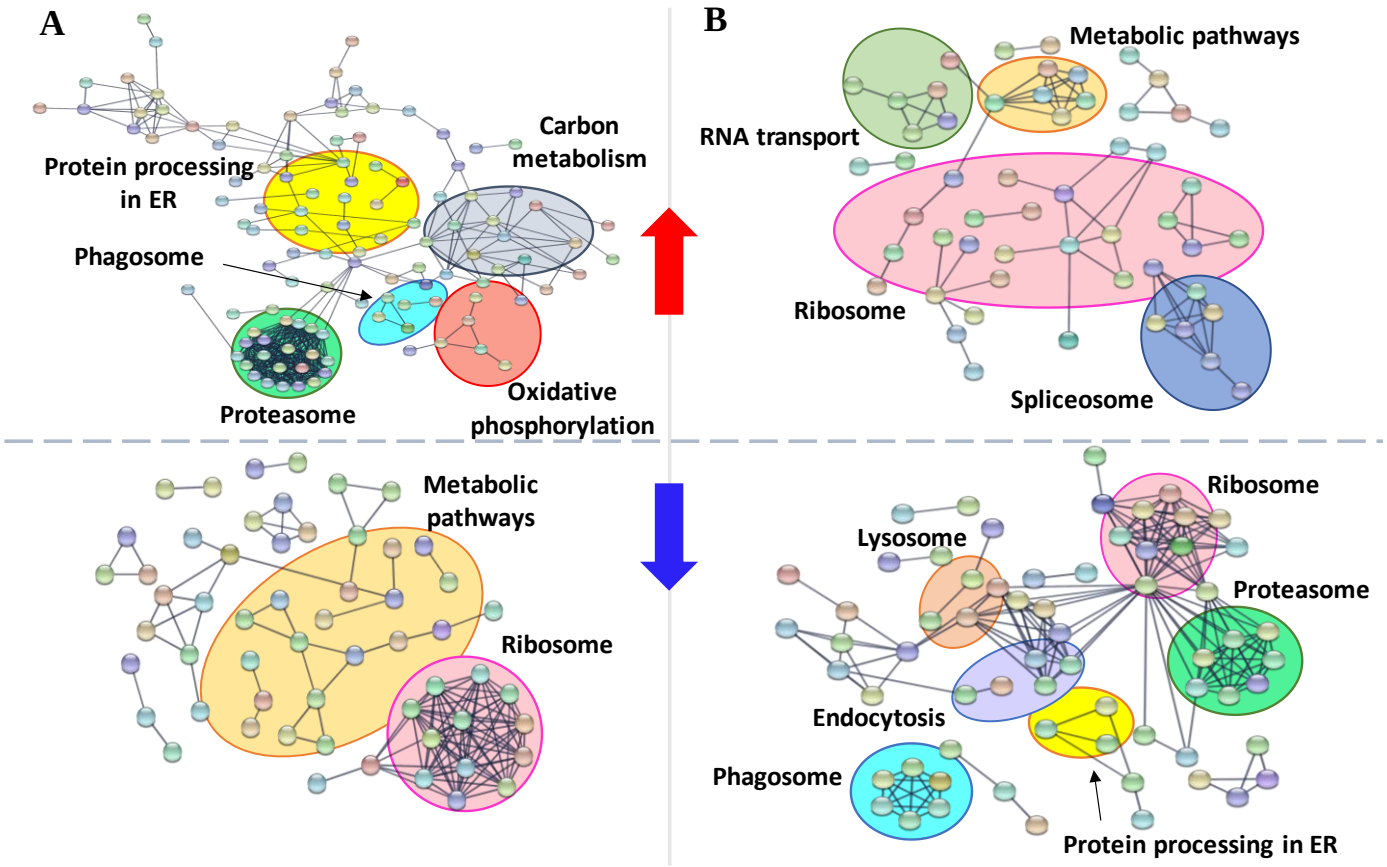


**Figure 5.9 Protein-protein interaction network of proteins identified in the *B. terrestris* fat body proteome in response to bacterial elicitors and wounding.** Each node represents a protein and each linear connection represents an interaction, the confidence is represented by the width of the individual line. Statistically enriched KEGG GO descriptors were examined to identify clusters of proteins enriched within the FB proteome in response to wounding and *E. coli*. Coloured circles highlight the most significant interacting groups in the network which are increasing (red) and decreasing (blue) in response to *E. coli* (A) and wounding (B). The continuous line divides *E. coli* and wounding T-test results and the broken line divides the increased/decreased protein sets.

**Table 5.4 Top 10 KEGG processes enriched within the *B. terrestris* FB proteome in response to bacterial presence.** Numerous metabolic process were revealed to be enriched in abundance in response to treatment with *E. coli*, whilst processes such as oxidative phosphorylation and the ribosome were enriched within the decreased abundance protein set. False discovery rate (FDR) is the representative enrichment score for each process and count in the gene set refers to the numbers of genes found within the dataset in relation to each process. Processes highlighted red and blue represent proteins of increased or decreased abundances, respectively, in response to *E. coli*, Processes are listed in descending order of enrichment.

| Pathway description | Count in gene set | FDR |
|---|---|---|
| Proteasome | 21 | 1.96E-22 |
| Carbon metabolism | 19 | 1.18E-12 |
| Metabolic pathways | 48 | 1.14E-10 |
| Microbial metabolism in diverse environments | 17 | 1.79E-07 |
| Protein processing in endoplasmic reticulum | 13 | 6.51E-06 |
| Pyruvate metabolism | 8 | 5.08E-05 |
| Glycolysis / Gluconeogenesis | 7 | 0.000471 |
| Pentose phosphate pathway | 5 | 0.000691 |
| Glyoxylate and dicarboxylate metabolism | 5 | 0.000764 |
| Cysteine and methionine metabolism | 5 | 0.00103 |
| Metabolic pathways | 31 | 2.18E-06 |
| Ribosome | 11 | 1.46E-05 |
| Tryptophan metabolism | 4 | 0.00232 |
| Oxidative phosphorylation | 8 | 0.00551 |
| One carbon pool by folate | 3 | 0.00756 |
| Peroxisome | 5 | 0.022 |

#### 5.2.6.2 FB wound response

PPI analysis of the SSDA wound responsive proteins revealed that processes associated with proteostasis, metabolism and cellular transport activities were generally enriched (Figure 5.9B). Processes increasing in response to wounding were categorised by their function, this resulted in the enrichment of four clusters on the PPI network; RNA transport, metabolic pathways, the ribosome and spliceosome. Similarly, processes decreasing in response to wounding were grouped resulting in the formation of six enrichment clusters; protein processing the ER, endocytosis, the lysosome, proteasome and ribosome.

The processes that were increasing and decreasing in response to wounding were further investigated by examining the enriched KEGG processes. In total 25 KEGG processes were identified as enriched within the increased protein dataset (Table A 5.3), of these the top ten were all associated with various types of metabolism including; carbohydrate (glycolysis/gluconeogenesis), amino acid (valine, leucine and isoleucine, tryptophan and beta-alanine) and fatty acid metabolism (fatty acid degradation). Overall ten KEGG processes decreasing in response to wounding were identified, this included the six processes revealed through PPI network enrichment and four additional processes (glycosaminoglycan degradation, oxidative phosphorylation, metabolic pathways and Glycosphingolipid biosynthesis).

**Table 5.5 Top 10 KEGG processes enriched within the *B. terrestris* FB proteome in response to wounding.** False discovery rate (FDR) is the representative enrichment score for each process and count in the gene set refers to the numbers of genes found within the dataset in relation to each process. Results presented in descending order of enrichment, represent processes enriched in both increase (red) and decrease (blue) protein sets.

| Pathway description | Count in gene set | FDR |
|---|---|---|
| Metabolic pathways | 36 | 2.46E-13 |
| Valine, leucine and isoleucine degradation | 8 | 1.05E-08 |
| Fatty acid degradation | 7 | 2.24E-07 |
| Glycolysis / Gluconeogenesis | 7 | 5.71E-06 |
| Microbial metabolism in diverse environments | 11 | 5.71E-06 |
| beta-Alanine metabolism | 5 | 7.50E-06 |
| Tryptophan metabolism | 5 | 8.67E-06 |
| Propanoate metabolism | 4 | 0.000376 |
| Histidine metabolism | 3 | 0.000446 |
| Carbon metabolism | 7 | 0.000446 |
| Lysosome | 12 | 3.77E-08 |
| Phagosome | 9 | 5.24E-06 |
| Proteasome | 7 | 4.90E-05 |
| Endocytosis | 7 | 0.00151 |
| Protein processing in endoplasmic reticulum | 7 | 0.0122 |
| Glycosaminoglycan degradation | 3 | 0.0131 |
| Ribosome | 7 | 0.0131 |
| Oxidative phosphorylation | 7 | 0.0149 |
| Metabolic pathways | 21 | 0.0149 |
| Glycosphingolipid biosynthesis - ganglio series | 2 | 0.0239 |

### 5.2.7 Functional analysis

In order to gain further insight into the biological processes associated with microbial challenge and wounding, GO terms were obtained for all identified FB proteins (1660 proteins) using *D. melanogaster* as the reference. To visualise wound and pathogen-associated biological processes, the proteome was searched through ClueGO. All the significantly enriched biological processes ($p \leq 0.05$) that were increased/ decreased in response to bacterial challenge and wounding were illustrated as pie charts (Figure 5.10 & 5.11). In addition, to examine the defence response activated within the FB to contend with bacterial elicitors and wounding all immune processes were examined following the same procedure as above.

Functional analysis revealed processes associated with stress response (cellular response to stress and reactive oxygen species), wound response and protein degradation (protein catabolism) are increased in response to *E. coli* presence and processes involved in cellular homeostasis, generation of energy and cellular transport (myofibril assembly) were among the most represented in the decreased abundance protein set (Figure 5.10).

Similarly, functional analysis of all increased/decreased biological processes in response to wounding in the FB was conducted (Figure 5.11), revealing an increase in processes including those associated with the production of new cells (mitotic cell checkpoint) and proteins (protein refolding and nuclear export) and electron transfer processes (oxidation reduction processes). The decreased processes represent similarly diverse biological processes including movement of molecules out of the cell (secretion by the cell and transmembrane transport), protein synthesis and degradation (proteolysis and cytoplasmic transport and translation) and inflammation.

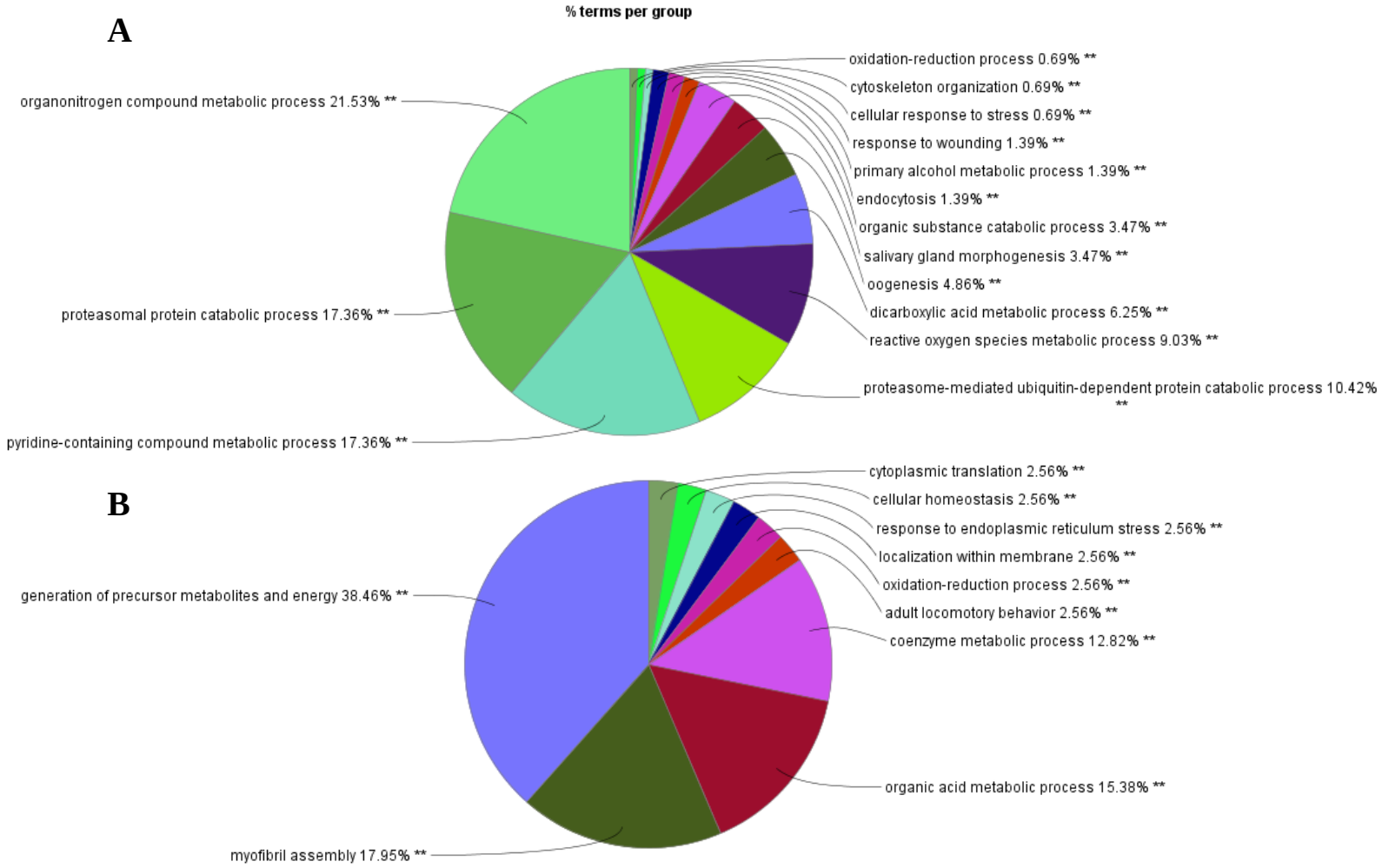


**Figure 5.10 Biological processes associated with the presence of Gram-negative bacteria in the FB.** Gene ontology terms for significantly enriched biological processes that were increased (A) or decreased (B) within the *B. terrestris* FB proteome in response to stimulation with *E. coli*. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$statistical levels, respectively.

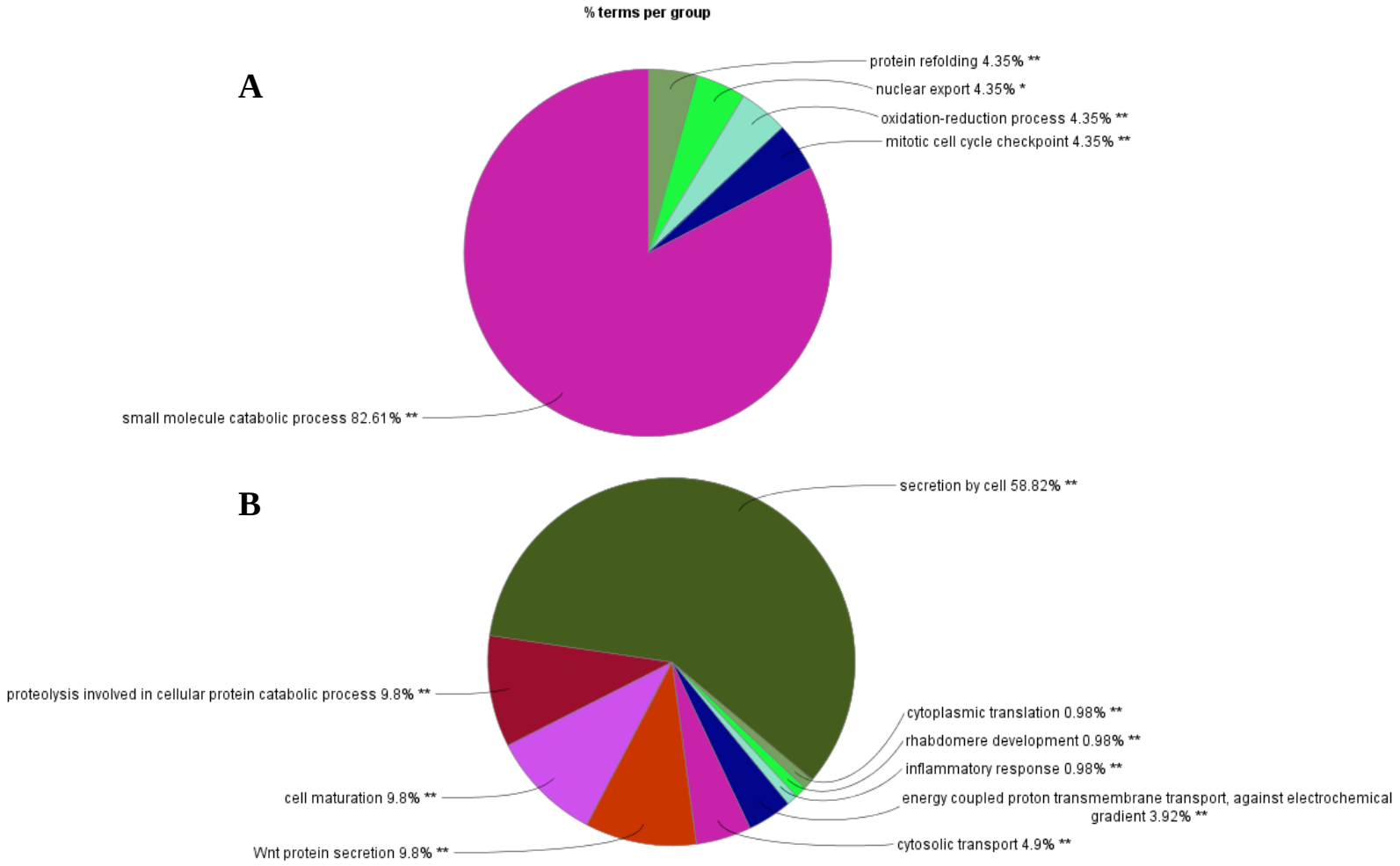


**Figure 5.11 Biological processes associated with wound response in the FB.** Gene ontology terms for significantly enriched biological processes that were increased (A) or decreased (B) within the *B. terrestris* FB proteome in response to wounding. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$ statistical levels, respectively.

### 5.2.7.1 Analysis of immune and defence-associated proteins in the FB response to *E. coli* and wounding

Given that, immune-related processes and proteins were consistently represented in both the statistical and functional analysis of the FB response to microbial challenge and wounding, further analysis of all immune-related proteins was conducted by incorporating immune information from the *Bombus terrestris* Genome Project (section 2.6.3). In total 29 immune associated proteins were identified in the FB response to *E. coli* and wounding (Table 5.6) representing all the major functional immune classes including antioxidants, immune effectors, immune modulators and PRRs.

The FB response to the presence of *E. coli* resulted in the differential abundance of 22 immune-associated proteins, 18 and four with increased and decreased abundances, respectively. Immune proteins of decreased abundance included immune effectors (venom bombolitin 1), antioxidants (catalase), and immune modulators (plasminogen activator inhibitor 1 RNA-binding protein-like and apolipophorin). Those proteins increasing in abundance to microbial stimulation included AMPs (defensin 1 and hymenoptaecin), immune effectors (PO), PRRs (peptidoglycan recognition protein and leucine rich repeat), immune modulators (epididymal secretory protein E1 and leukocyte elastase inhibitor) and antioxidants (heat shock kDa 70, SOD, peroxidase and thioredoxin). A number of immune proteins unique to the microbial response were also revealed and included five antioxidants, two immune effectors, six immune modulators and one PRR (Table 5.6).

Overall, in the FB response to wounding, 15 immune proteins were identified (three increased and 12 decreased) (Table 5.6). Proteins of increased abundance included two immune modulators (omega conotoxin and apolipophorin) and one antioxidant (heat shock 70 kDa). Those decreasing in response to wounding represented immune proteins from numerous functional classes such as antioxidants (thioredoxin-2 and glutathione S-transferase), immune effectors (defensin and phenoloxidase), immune modulators (epididymal secretory protein E1-like, c-type lectin and insulin-like growth factor-binding protein complex acid labile and IRP30) and PRRs (protein NPC2 homolog, peptidoglycan recognition protein-like, MD-2-related lipid-recognition protein-like and chaoptin-like). A

number of immune proteins unique to the wound response were also revealed including; one antioxidant, one immune effectors, two immune modulators and three PRRs (Table 5.6).

Analysis of the defence processes associated with *E. coli* and wounding, revealed immune processes increasing in abundance in response to the presence of microbial elicitors and proteins decreasing in response to wounding (Figure 5.12). Immune processes identified increasing in response to microbial stimulation included antimicrobial humoral response, positive regulation of immune response and melanisation defence response. Defence processes associated with proteins decreasing in abundance in response to wounding included activation of immune response, regulation of innate immune response and positive regulation of immune response. It was not possible to identify immune process associations for the proteins with increased abundance in response to wound or decrease abundance in response to treatment with *E. coli* using functional analysis.

**Table 5.6 Immune-related proteins identified in the *B. terrestris* FB in response to *E. coli* and wounding.** As expected, numerous elements of insect immunity were identified in the FB proteome of *B. terrestris* in response to treatment with *E. coli* (EvR) and wounding (RvN) including antioxidants, immune signalling modulators, PRRs and AMPs. The values for each protein represent the log2 fold change, positive values represent proteins with increased abundance and similarly negative values represent those proteins decreasing in abundance.

| Immune function | Accession no. | Protein name | Fold change | |
|---|---|---|---|---|
| | | | EvR | RvN |
| Antioxidant | XP_003393349.1 | Heat shock 70 kDa protein cognate 5 | - | 0.44 |
| | XP_012175811.1 | Heat shock 70 kDa protein 4 | 0.23 | - |
| | XP_012164574.1 | Catalase | -0.69 | - |
| | XP_012164907.1 | Thioredoxin-2 | 0.39 | -0.44 |
| | XP_012166348.1 | Peroxidase | 0.85 | - |
| | XP_012172050.1 | Glutathione S-transferase* | 1.15 | -0.46 |
| | XP_012166540.1 | Superoxide dismutase | 0.69 | - |
| | XP_012166688.1 | Superoxide dismutase | 0.5 | - |
| Immune effector | ADB29129.1 | Defensin 1 | 1.7 | -2.81 |
| | ADB29130.1 | Hymenoptaecin | 0.89 | - |
| | XP_003400548.1 | Phenoloxidase 2 | 0.78 | -1.48 |
| | AEN41592.1 | Venom bombolitin 1 | -2.95 | - |
| | AEN62314.1 | IRP30 | - | -2.01 |
| Immune modulator | XP_012172841.1 | Fibrillin-2 | 0.84 | - |
| | XP_003395762.1 | Omega-conotoxin-like protein 1 | - | 1.52 |
| | XP_012172057.1 | Fragile X mental retardation syndrome-related protein 1 | 1.19 | - |
| | XP_003395447.1 | Epididymal secretory protein E1 | 4.8 | -6.26 |
| | XP_003399683.1 | C-type lectin* | 0.42 | -0.73 |
| | XP_012163961.1 | Putative cysteine proteinase CG12163 | 0.31 | - |
| | XP_012163992.1 | Insulin-like growth factor-binding protein complex acid labile | - | -2.82 |
| | XP_012166473.1 | Plasminogen activator inhibitor 1 RNA-binding protein | -0.65 | - |
| | XP_012166703.1 | Leukocyte elastase inhibitor | 2.48 | - |
| | XP_012169166.1 | Interferon-inducible double-stranded RNA-dependent Protein kinase activator | 0.7 | - |
| | XP_012174357.1 | Apolipophorin* | -0.44 | 0.19 |
| PRR | XP_003397822.1 | Protein NPC2 | - | -1.95 |
| | XP_003400160.1 | Peptidoglycan recognition protein | 2.16 | -3.18 |
| | XP_003400229.1 | MD-2-related lipid-recognition protein | - | -0.8 |
| | XP_012176660.1 | Leucine-rich repeat-containing protein 15 | 0.79 | - |
| | XP_012164498.1 | Chaoptin | - | -2.35 |

(*) indicates those proteins that have been reannotated as per section 2.6.3.

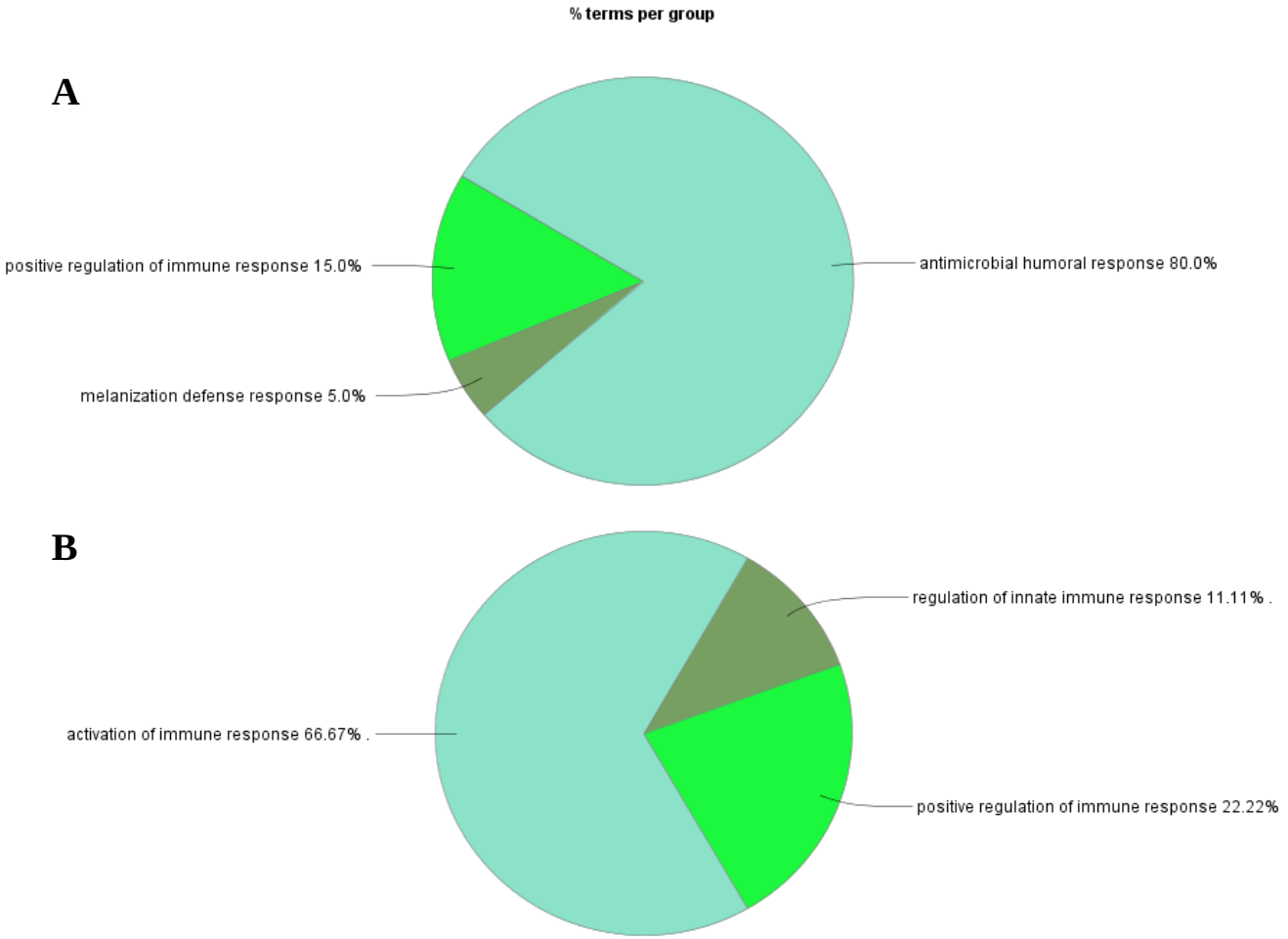


**Figure 5.12 Immune and defence processes associated with microbial treatment and wound response in the FB.** GO analysis software ClueGO was used to investigate and visualise significantly enriched biological processes (A) increased in response to microbial stimulation and (B) decreased within the *B. terrestris* FB proteome in response to wounding.

### 5.2.7.2 Analysis of proteins common to FB microbial response and wounding profiles

To investigate similarities and differences in the protein profiles of the microbial and wound response in the *B. terrestris* FB, all SSDA proteins identified in response to each treatment were compared, revealing 124 proteins in common, and 290 and 171 *E. coli*- and wound-specific proteins, respectively (Figure 5.13A). Within the proteins with similar profiles in response to both microbial and non-microbial challenge were typical haemolymph proteins (apolipophorin, vitellogenin and transferrin) along with numerous proteins associated with carbohydrate metabolism (trehalase, maltase and glucose dehydrogenase), the ribosome (40S and 60S ribosomal protein) and immune response. Common and unique immune associated proteins were revealed by incorporating immune information from the *Bombus* genome (as above). A number of common immune proteins were revealed such as antioxidants (thioredoxin 2), immune effectors (defensin 1, phenoloxidase 2 and apolipophorin), PRRs (peptidoglycan recognition protein) and immune modulators (c-type lectin and epidydimal secretory protein) (Table 5.6).

A number of proteins displayed divergent expression profiles in response to both *E. coli* challenge and wounding (Figure 5.14). 84 proteins were determined to have increased and decreased abundances to *E. coli* and wounding respectively and include biological processes associated with vacuolar transport, translation (mRNA localisation), terminal tracheal branching, response to fungi and protein degradation (proteasome) and secretion Figure 5.14A). The 40 proteins with increased and decreased abundances to wounding and *E. coli* respectively were associated with carboxylic acid and monocarboxylic acid metabolism (Figure 5.14B).

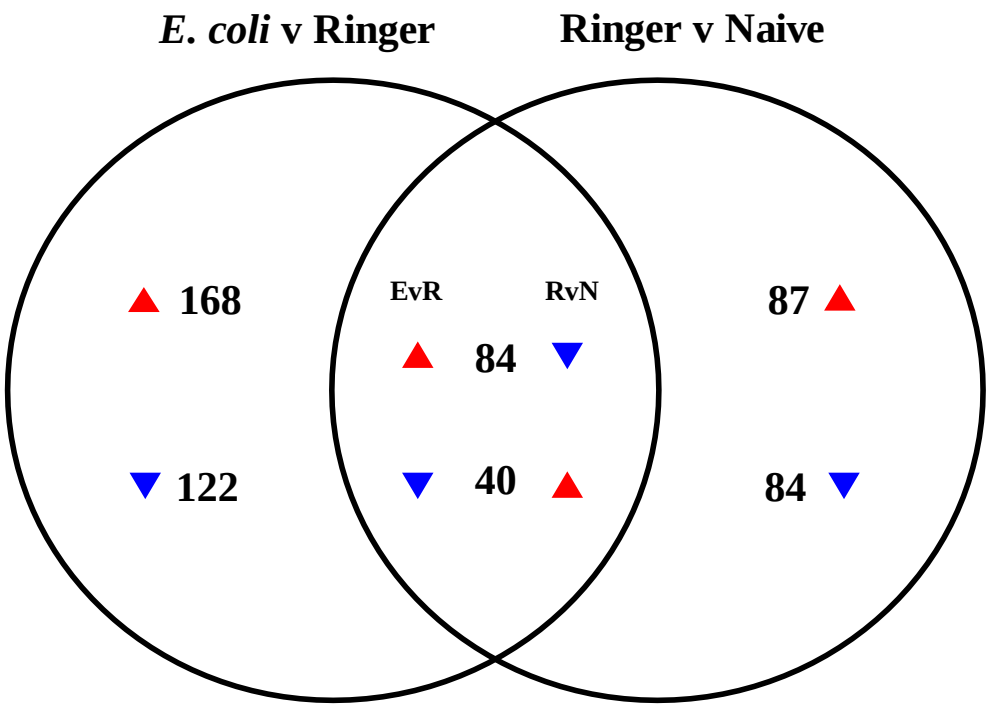


**Figure 5.13 Comparative analysis of the FB proteome in response to wounding and treatment with microbial elicitors.** Comparison of the SSDA proteins identified in response to *E. coli* and wounding resulted in the identification of 124 proteins conserved across both responses. Of this number 40 were common to both proteins increasing in abundance to *E. coli* and decreasing to Ringer. The remaining common 84 were common to those proteins increasing in abundance to wounding and decreasing in abundance in response to *E. coli*.

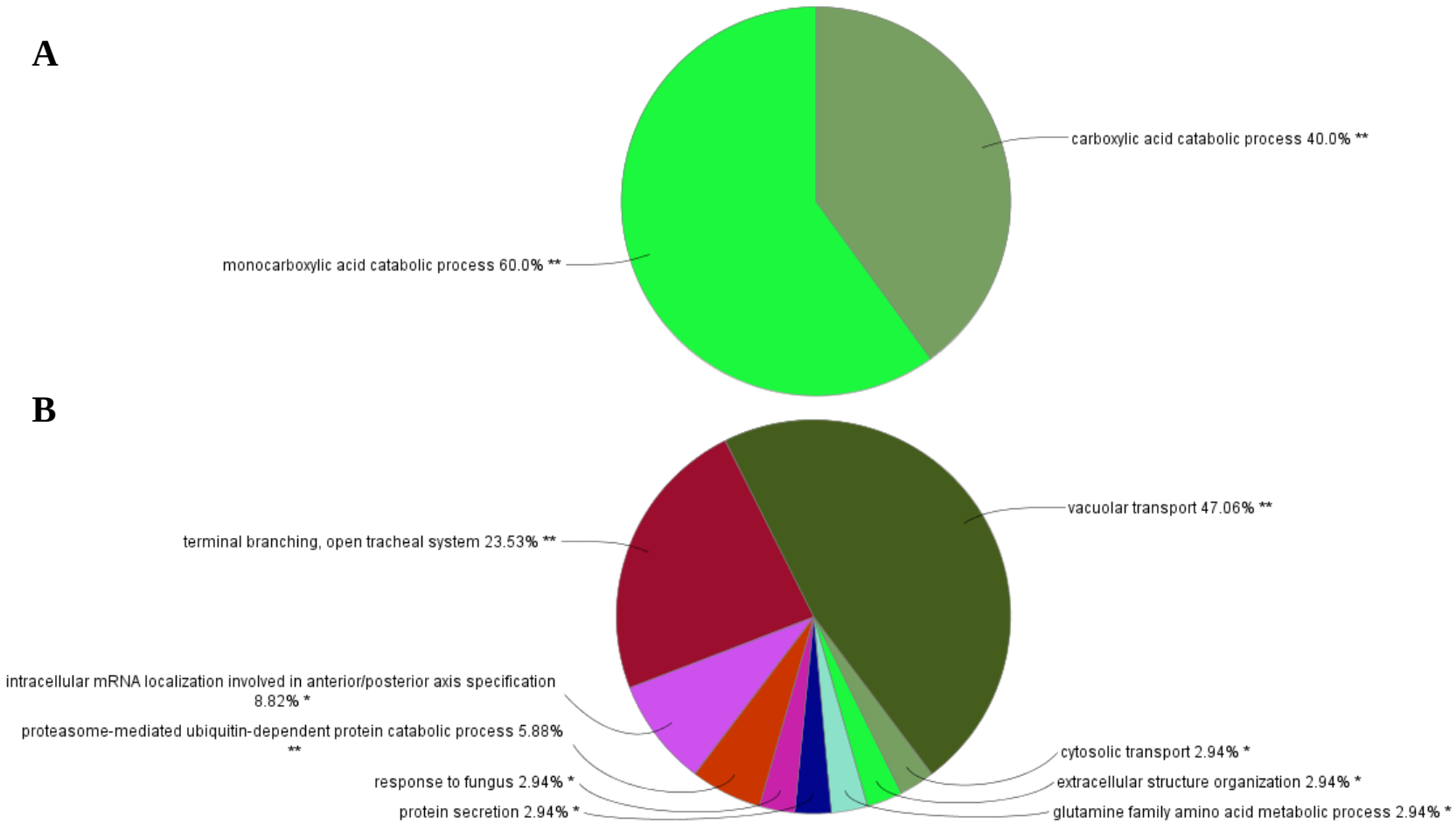


**Figure 5.14 Functional analysis of proteins common to the *E. coli* and wound response profiles.** Functional analysis of common proteins (A) increasing and (B) decreasing in response to *E. coli* and wounding revealed enrichment of BP such as the proteasome, translation and carboxylic acid metabolism. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$statistical levels, respectively.

## 5.3 Discussion

Through the application of LC-MS/MS, 1660 proteins were identified in the FB of *B. terrestris* workers, many of which displayed altered abundances in response to treatment with *E. coli* and wounding. Proteomic profiles of treatments and controls varied dramatically, highlighting strong signatures of protein abundance in response to each. Analysis of these signatures revealed the involvement of the following processes: the ribosome in response to wounding, phagocytosis, the proteasome and cellular detoxification in response to treatment with *E. coli* and fatty acid metabolism in response to both microbial stimulation and wounding. This increase in fatty acid metabolism coincided with an increase in lipid droplet size in the adipocytes forming the FB. An accumulation of lipid within these cells may indicate the rapid and dynamic role of FBs in mobilising energy stores to respond to microbial challenge and wounding. The proteomic response to *E. coli* involved immune, metabolism and proteostasis-related proteins whereas the response to wounding principally involved metabolism and cellular transport in addition to protein synthesis, processing and degradation. GO functional analysis of the SSDA proteins from each treatment using ClueGO revealed that an increase in proteasomal, stress and wounding responses and a decrease in cellular homeostasis, cytoplasmic translation and generation of energy are observed in response to microbial infection. Increases in protein folding, nuclear export and oxidation-reduction processes and decreases in cell secretion processes, inflammatory response, proteolysis and cell maturation were observed in response to wounding. Numerous defence-associated proteins were also revealed to be central to both responses with all classes of immune proteins present in both.

### 5.3.1 Key findings

- The bumblebee worker FB is composed of two cell types oenocytes and adipocytes. Although both are intermixed throughout this tissue, oenocytes are more abundant.
- Only a single layer of fat cells was present within the bumblebee abdomen, the parietal layer.
- The *B. terrestris* FB is a highly metabolic and immune active tissue, involved in the monitoring, mediation and maintenance of internal homeostasis.

### 5.3.2 Changes in FB composition in response to Gram-negative bacteria and wounding

In response to microbial challenge no significant changes in either fat cell size (area) or the ratio composing the FB was revealed. However, at the individual cell level, it was revealed that adipocytes exposed to pathogens have larger lipid droplets (LDs) than those of the controls. Similarly, oenocytes in immune stimulated FBs were observed to have a higher amount of lipids in comparison to non-infected controls, although this difference was not explored quantitatively in this study. Increased LDs are suggestive of an immune association between adipocyte LDs and the *B. terrestris* worker immune response. LDs are multifunctional structures present in most organisms, that are particularly abundant in the insect fat body, predominantly in the adipocytes, where they act as the central energy reservoir for the whole organism (Fujimoto et al. 2008; Barletta et al. 2016). The storage of lipids within the cytoplasmic LDs of adipocytes is a highly conserved feature, as its central role in energy homeostasis, membrane synthesis and cell signalling, provides an evolutionary advantage to organisms (Vallochi et al. 2018). LDs have been revealed to be present in in embryonic cell lines (Aag2 cells) of *Aedes aegypti*, where it has been shown that LDs are accumulated after exposure to bacterial and viral pathogens indicating their role in immune response (Barletta et al. 2016). Similarly, in mammalian immune cells, it has been shown that LDs participate in inflammation and infection associated processes, increasing in number in response to a variety of immune challenges and hence acting as reliable biomarkers of immune cell activity (Bozza et al. 2009).

### 5.3.3 Wounding and microbial challenge induces responses in energy, amino acid and protein metabolism

The insect immune response is an energetically and physiologically costly system (Schmid-Hempel 2004; Ardia et al. 2012). Hence, the generation of a successful immune response requires the efficient and targeted production and distribution of energy to the tissues mounting the response (Lee and Lee 2018). Given that the FB is the central metabolic tissue in bumblebees it was expected that elements of carbohydrate and energy metabolism would be abundant in this vital tissue. It is clear that the FB response to *E. coli* and wounding involves several key metabolic processes including carbohydrate (glycolysis/ gluconeogenesis, pyruvate metabolism and the pentose phosphate pathway), lipid (glyoxylate and dicarboxylate metabolism) and amino acid metabolism (cysteine

and methionine metabolism). These specific pathways suggest highly active catabolic processing within the FB, where numerous metabolic precursors (through degradation of carbohydrates, lipids or amino acids) are produced ensuring a continuous fuelling of cellular respiration pathways, which includes glycolysis, the TCA cycle and oxidative phosphorylation. TCA cycle precursors (acetyl CoA), were also more abundant in both wound and bacterial response profiles confirming that the principal response to wounding and microbial challenge in the FB is to increase available cellular energy (most likely in the form of ATP) in order to fuel the stress, repair and immune processes associated with defence against bacterial challenge. The centrality of these processes within immune response may perhaps indicate the FB as an interface for immunometabolism, the coordination between immunity and metabolism (Mathis and Shoelson 2011) in bumblebees.

Recent transcriptomic analysis of infection-induced responses in *Drosophila melanogaster* (Lee and Lee 2018) also revealed the expression of genes involved in metabolism with a suppression of anabolism-associated genes and increased expression of those involved in catabolism (Dionne et al. 2006; Chambers et al. 2012; Chakrabarti et al. 2014; Lee et al. 2018). Hence, it seems that microbial challenges induces a conserved response in both bees and flies where metabolic precursors from multiple sources are produced and fed into primary catabolic pathways to produce the energy required to mount the necessary immune response. Although the majority of metabolic processes were increased in response to wounding and bacterial presence, oxidative phosphorylation associated proteins actually decreased in response to wounding. As energy is central to supporting wound and stress response it is possible that this represents a requirement of a signal of microbial challenge to sustain oxidative phosphorylation that are absent in wounded bees.

Within the FB, proteins involved in tryptophan metabolism were decreased in abundance in response to *E. coli* challenge but were increased in response to wounding. Tryptophan is a glucogenic and ketogenic essential amino acid (AA) required by all forms of life and is essential for protein production and various other important metabolic functions (Moffett and Namboodiri 2003; Azeredo et al. 2017). As a glucogenic and ketogenic amino acid it can be shuttled into pathways that produce pyruvate and acetyl-coA directly (Owen et al. 2002; D'Mello 2003). Tryptophan is therefore a key intermediate metabolite and its regulation permits the control of numerous primary metabolic pathways either

towards anabolic processes or energy production. The divergence in tryptophan metabolism between wounded and *E. coli* challenged bumblebees also suggests a possible role in the immune system. In vertebrates tryptophan is essential in the induction of specific cellular pathways involved in immune response and signalling (Mellor and Munn 2003) and its depletion is a strategy employed by mammalian cells to restrict its uptake and use by invading pathogens (Richardson et al. 2015). The reduction in tryptophan metabolism in *E. coli* infected bees could represent a widely conserved defence mechanism against invading microbial organisms, although considerable research would be required to confirm that this is the case in bumblebees. However, what is clear is that through the control of tryptophan metabolism the bumblebee FB appears to have a mechanism to control potential energy production, a mechanism that can seemingly distinguish wounding from microbial challenge.

Within the FB response to *E. coli* and wounding numerous processes associated with the synthesis, processing, degradation and transport of proteins were identified including the proteasome, endoplasmic reticulum and ribosome. Within the cell there are a variety of RNA molecules; mRNA, small nuclear RNA (snRNA), ribosomal RNA (rRNA and transfer RNA (tRNA) each are synthesized in the nucleus and then transported to their sites of function throughout the eukaryotic cell by specific transport pathways (Nakielny et al. 1997). The spliceosome is also centrally involved in the protein translation process as it catalyses precursor mRNAs (pre-mRNAs) into functional mRNA (Will and Lührmann 2011) which can be used by ribosomes for protein synthesis (Zhou et al. 2015). Both processes are essential for normal cellular function and are enriched within the FB in response to wounding. The increased abundance of proteins associated with these pathways may be indicative of higher rates of protein synthesis in response to wounding. In response to *E. coli* an increase in translation would similarly be expected as immune proteins are synthesised to contend with the infection. However, decreased abundance of ribosomal proteins (RPs) was observed in the FB bacterial response profile. RPs have recently been implicated in a variety of ribosome-independent functions including tumorigenesis, immune signalling and development (Zhou et al. 2015). A recent study confirmed this RP immune function by revealing that translational inhibition facilitates a mechanism underlying damage-related signals (Vyleta et al. 2012). Hence, decreases in protein translation in response to bacterial elicitors may be a mechanism by which the FB initiates immune signalling. However, given the importance of energy production

(discussed above) it may be the case that in an attempt to channel available AAs into pyruvate production and the TCA cycle the FB restrict protein production itself.

The view outlined above that the FB regulates AA and protein metabolism to favour energy production is further supported by the increase in the abundance of proteasome proteins in response to infection. The proteasome is the main protein degradation system within the cell through which it participates in numerous essential cellular functions, such as protein quality control, transcription, cell signalling, and apoptosis (Rock et al. 1994; Finley 2009; Schmidt and Finley 2014). Increased numbers and activity of proteasomes would result in increased availability of recycled amino acids, which could foreseeably be channelled into energy producing pathways. In addition numerous immune functions have been associated with the proteasome including; the degradation of proteins for pathogen recognition mechanisms (Finley 2009), regulation of immune cell function through degradation of immune signal mediators and degradation of damaged proteins to counteract their cytotoxic potential (Kammerl and Meiners 2016). The increase in proteasomal proteins in response to bacterial elicitors as observed in the *B. terrestris* FB response may represent similar immune activities. In contrast, in response to wounding proteasomal proteins and cellular transport processes such as endocytosis and lysosome production, which may be exploited during proteostasis to remove misfolded or damaged proteins from cells, decreased in abundance. This decrease in the absence of immune elicitors may confirm the immune role of the proteasome in the FB pathogen response.

Similar to the proteasome the endoplasmic reticulum (ER) is primarily associated with protein processing. The ER functions as a protein-folding centre, responsible for the biosynthesis, folding, assembly and modification of numerous secreted and soluble proteins and membrane proteins (Kaufman 1999). The ER also acts as a calcium store, which receives and responds to growth factors, hormones, and stimuli that disrupt normal cellular energy levels, nutrient availability or redox status (Zhang and Kaufman 2008). Physiological states such as pathogen infection and alterations in redox status can increase or disrupt protein folding and create an imbalance between the protein-folding load and the capacity of the ER. This results in an accumulation of unfolded or misfolded proteins in the ER lumen, a state known as ER stress (Zhang and Kaufman 2008). To ensure correct protein folding and to prevent such an accumulation of unfolded or misfolded proteins, eukaryotic cells have evolved the unfolded protein response (UPR), which alters the transcriptional and translational activity of the cell to adapt to

homeostatic variations (Ron and Walter 2007; Zhang and Kaufman 2008). There is increasing evidence suggesting that protein folding and generation of ROS as a by-product of protein oxidation in the ER are closely linked events and may act as an adaptive mechanism to preserve cell function and survival (Malhotra and Kaufman 2007). Hence, the enrichment of these UPR associated processes within proteins increasing in abundance in the *B. terrestris* response to *E. coli* infection may suggest this mechanism is employed by the FB to adapt to damage and stress as a result of bacterial presence. However, as the ER also functions in protein folding and the initial production of proteins destined for secretion from the cell, the enrichment of this process may also be indicative of increased synthesis of central immune proteins such as AMPs in the FB. In contrast these processes are decreasing in abundance in response to wounding. This may be as a result of the stress of wounding disrupting protein folding and create an imbalance between the protein-folding load and the capacity of the ER resulting in ER stress (Zhang and Kaufman 2008). To prevent this imbalance the UPR alters the transcriptional and translational activity of the cell to adapt to homeostatic variations (Ron and Walter 2007; Zhang and Kaufman 2008). Hence, decreases in these normal house-keeping processes may be a result of all the UPR strategy trying to normalise protein folding and preserve cell function and survival (Malhotra and Kaufman 2007).

### 5.3.4 The FB immune response to wounding and microbial challenge

In insects the immune response is mediated by the recognition of pathogens by PRRs, which subsequently initiate immune signalling cascades resulting in the rapid synthesis and release of potent AMPs by the FB (Gottar et al. 2002). Within the FB response to wounding and *E. coli* numerous proteins from each class of immune protein were identified. In response to microbial elicitors it was expected that the FB would contain numerous PRRs. As expected, all identified PRRs within the FB *E. coli* response profile were increasing in abundance to the presence of bacterial elicitors. In contrast within the wound response profile although numerous PRRs were identified all showed decreased abundance. Reduction in the PRRs could be perhaps due to their degradation through the proteasome in redundancy from an absence of pathogens or their functioning in other novel wound or stress-related recognition roles. This highlights the importance and specificity of PRRs in the FB response to microbial elicitors. The recognition of pathogens via PRRs results in the initiation of signalling cascades responsible for modulation of immune response such as the production of AMPs. Hence, the observed

increased abundance of the majority of immune modulators in response to *E. coli* is expected. In contrast, immune modulators decreased in abundance in response to wounding including C-type lectin, which participates in the induction of innate immunity and inflammation following microbial infection and tissue damage (Kingeter and Lin 2012). However, two immune modulators, apolipophorin and plasminogen activator inhibitor, decreased in response to *E.coli*. Apolipophorin is a lipoprotein synthesised and secreted by the fat body which functions in lipid transport and immune response. Apolipophorin associated immune activities include detoxification of microbial cell wall components and stimulation of cellular activities such as haemocyte adhesion, phagocytosis and nodule formation (Zdybicka-Barabas and Cytryńska 2013). The decreased abundance of apolipoprotein in response to *E. coli* and its increased abundance under wounding may represent direct activity in the immune response to microbial pathogens. In addition, the serpin plasminogen activator inhibitor 1 RNA-binding protein-like was also decreased in response to *E. coli*. The majority of characterised arthropod serpins regulate innate immune responses by inhibiting the proteolytic cascades that take a central role in many immune reactions (Gulley et al. 2013b). Hence, similarly decreased abundance of this protein in the FB in result to bacterial treatment may be as a result of its involvement in immune signal regulation.

A variety of immune effectors including AMPs and PO were identified among both wounding and bacterial response profiles. Of those identified in response to *E. coli*, only one effector venom bombolitin, was not increasing in abundance. Immune effectors identified in response to *E. coli* included three AMPs and PO. In contrast all immune effectors identified in response to wounding were AMPs and all decreased in abundance. The increase in AMPs in response to bacterial treatment is expected as their fundamental biological role in insects is in the elimination of pathogenic micro-organisms (Diamond et al. 2009). The observed decreases in response to wounding may indicate that they are not produced in the absence of microbial elicitors or that levels are depleted after their secretion into the haemolymph to contend with a potential influx of microbes at the wound site.

Amongst the immune effectors increasing in response to Gram-negative bacteria was PO. Due to their open circulatory system insects must seal wounds quickly and efficiently to prevent bacteria entering the haemocoel, they do this using clotting (Theopold et al. 2004). Coagulation acts to close wounds, reduce loss of haemolymph and to trap

microbes, by forming a clot to prevent their entry into the haemocoel (Bidla et al. 2005). Prophenoloxidase is usually activated through a proteolytic cascade similar to the vertebrate clotting cascade (Theopold et al. 2004). The presence of PO in combination with numerous proteases from the S1 family is indicative of a melanisation response. The S1 or chymotrypsin family are one of the most well-characterised family of enzymes and in mammals perform a variety of roles in physiological processes such as digestion, coagulation, cellular and humoral immunity (Ross et al. 2003). In honeybees (*Apis mellifera*) this family of proteases has been implicated in immune response molecular activation mechanisms of the Toll and PO cascades (Zou et al. 2006; Jiang and Kanost 2000; Jiang et al. 2009). In addition, PO and numerous serpins are among the proteins decreasing in response to wounding. Hence, the presence of these proteases in combination with PO may indicate the activation of the PO cascade in response to the presence of bacterial cells or the depletion of this protein as it participates in coagulation and wound healing.

As expected in addition to typical immune proteins, numerous antioxidants were also identified as differentially abundant in response to wounding and bacterial treatment. Physiological stressors such as pathogen infection and wounding can trigger oxidative stress as a result of increased production of reactive oxygen species (ROS). This can result in oxidative damage to proteins, lipids, and nucleic acids. As a consequence, the normal functions of cells and tissues may be seriously disrupted, which could detrimentally impact normal biological processes (Kodrík et al. 2015). To contend with or reduce this, organisms produce numerous antioxidant enzymes and substances. In addition to stressors ROS are also a normal by-product of metabolism and are centrally involved in, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors, gene expression, and the antimicrobial action of immune cells (Bayir 2005). Hence, the observed increase in metabolic processes particularly those involving ATP production (described in section 5.3.2) would presumably explain the increases in antioxidant production to inhibit potential damage by increased ROS output. In contrast, wounding only, resulted in a reduced set of antioxidants which potentially correlates with the reduction in oxidative phosphorylation observed in wounded versus infected bumblebee FBs. Alternatively, the reduction in antioxidants in wounded individuals may represent their depletion through contention with the wound-associated oxidative stress.

In addition to immune and detoxification, proteins associated with the cellular transport activities such as the phagosome were also enriched. Phagosomes are intracellular vesicles formed by invagination of the plasma membrane around extracellular material (e.g. invading bacteria) formed through the process of phagocytosis (Shandala et al. 2013). These potent vesicles play essential roles both in the cellular immune response, through degradation of microbes, antigen presentation and immune signal transduction (Kagan and Iwasaki 2012; Shandala et al. 2013). Hence, the increase of phagosome-associated proteins in the FB response to *E. coli* may indicate the FB involvement in immune signalling or bacterial cell degradation via phagocytosis.

## 5.4 Conclusion

The protein expression profile of the *B. terrestris* FB was examined in response to microbial challenge and injury using proteomic analysis. This study demonstrates the complexity of the *B. terrestris* worker FB response to infection with Gram-negative bacteria and wounding, by revealing a large subset of SSDA proteins found in response to both stimuli. Cross-comparisons of both responses indicated the existence of tightly coregulated physiological processes that may be central to *B. terrestris* contending with a diversity of stressors.

An interesting and consistent observation was the high degree of similarity between the biological processes in response to both *E. coli* and wounding. Conserved wounding and infection associated processes included those associated with metabolism, proteostasis and cellular transport activities suggesting that manipulation of these normal biological processes is essential in the response to physiological stressors such as wounding and pathogens. In addition, a number of treatment specific signatures were also revealed. Both responses involved an enrichment in numerous energy associated processes including carbohydrate, lipid and AA catabolic pathways. As the FB is the main source of energy storage and metabolism, it was expected that these categories would be the most well represented. Immune and wound responses are energetically costly hence it was expected that metabolism-associated processes would be increased in response to both stimuli to maintain and mediate appropriate responses. However, although overall metabolism increased in response to bacteria and wounding, specific elements were decreasing

including oxidative phosphorylation in response to wounding and tryptophan metabolism in response to *E. coli*.

The wound response involved decreases in the abundance of proteins associated with oxidative stress, while all other elements of energy precursor production within this profile increased in abundance, indicating that oxidative stress may represent a microbe triggered process which initiates widespread production of energy from all the precursors accumulated. Similarly, in response to *E. coli* infection tryptophan metabolism represents an element of *B. terrestris* physiology which is limited by infection and hence abundant in wounding. The regulation of tryptophan controls the activity of numerous metabolic pathways towards either anabolic processes or energy production. The switch from anabolic to catabolic metabolism may be indicative of the FB fuelling increased energy production through degradation of these precursors to support the associated stress, immune and healing processes. In addition to proteomic results, analysis of the adipocyte lipid droplet content further indicated a link between infection and further highlight the importance of energy metabolism in the bacterial response.

Numerous elements of immunity were also represented within the FB response to *E. coli* and wounding including antioxidants, immune effectors, immune modulators and PRRs. These conserved proteins may represent the processes essential for a baseline defence response in the *B. terrestris* worker. Within the wound response an antioxidant (heat shock protein 70 kDa) and immune modulator (omega conotoxin) were the only two immune proteins increased in this response profile. The identification of immune proteins involved in the wound response is novel and has never been previously described in *B. terrestris*. As expected for the microbial immune response, all PRRs identified in response to *E. coli* had increased abundance, similarly the majority of immune effectors, which included AMPs (hymenoptaecin and defensin) and PO, immune modulators and antioxidants were increasing in response to the presence of bacteria within the haemocoel. Interestingly, within the FB wound response all PRRs decreased in the absence of pathogenic elicitors and all AMPs similarly decreased in abundance, indicating their activity is dependent on pathogen presence.

# Chapter 6

## Quantitative cellular and proteomic analysis of the systemic immune response in *Bombus terrestris* haemolymph to microbial challenge

## 6.1 Introduction

Bees naturally suffer from a broad range of parasites, parasitoids, and pathogens, including protozoans, fungi, bacteria, and viruses (Goulson et al. 2015). These pathogens often evolve a specialised virulence equilibrium with their hosts (Rabajante et al. 2015) and disturbances of the natural ecosystem can unbalance this equilibrium, increasing pathogen proliferation and their status as a stressor on a population (Meeus et al. 2018). Parasites, have long been known to negatively impact bee health and drive bee declines both individually and in combination with a variety of other stressors (pesticides, climate change etc.) (Goulson et al. 2015). Interactions between insects and pathogens are now considered to be highly multifactorial and context dependent (Brown et al. 2003; Prisco et al. 2013; Myers and Cory 2015). However, before these interactions and their impacts can be fully understood, it is crucial to obtain insights into the impact of a single driver, particularly one as intrinsic as disease.

Although numerous mechanisms are implicated in the spread of pathogens and parasites within bee populations, pathogen spillover from commercial colonies into wild populations has been recently and repeatedly demonstrated (Meeus et al. 2011; Whitehorn et al. 2013; Fürst et al. 2014). Commercial pollination is essential for numerous economically important crops worldwide, as demand for pollinator-dependent crops increases, so does the dependence on managed bees (Mallinger et al. 2017). The social nature and high population densities of most managed bee species (honeybees and bumblebees) facilitates the harbouring and spread of pathogens within colonies (Chen et al. 2006). In addition, the movement of colonies across large geographical regions for crop pollination services increases their potential to spread pathogens to wild bees including either novel or invasive pathogens (Goulson 2003b; Cameron et al. 2016). Transmission of infectious agents can occur when infected and uninfected individuals forage on the same flower (Durrer and Schmid-Hempel 1994) or come into contact with contaminated pollen (Singh et al. 2010) or faeces (Whitehorn et al. 2013).

Two of the most prevalent pathogens infecting bumblebees are the microsporidian *Nosema bombi* and the protozoan trypanosome *Crithidia bombi* (Imhoof and Schmid-Hempel 1999).

Infection with these intestinal parasites can substantially reduce the fitness of individual bumblebees and the reproductive output of colonies (Brown et al. 2003; Otti and Schmid-Hempel 2008). The accidental introduction of the gut parasite *N. bombi* to North America, via commercial bumblebee colonies coincided with a dramatic decline of four native bumblebee species since the 1990s, an event generally regarded as a major contributing factor in their decline (Cameron et al. 2011), although no conclusive evidence as of yet has been found to confirm these claims (Cameron et al. 2016). The lack of understanding of the mode of transmission (Cameron et al. 2016) and bumblebee immune response to *C. bombi* and *N. bombi* are major limiting factors in understanding whether these pathogens could cause the observed population declines.

Although bumblebees are widely used models for the study of social aspects of disease pathology, immunity (Evans et al. 2006) and host-parasite interactions the underlying molecular processes involved in these events are still unknown (Mallon et al. 2003). Although the innate immune response in bumblebees is similar to that of other social hymenopterans including the honeybee (*Apis mellifera*), there are also notable differences (Evans et al. 2006). Our understanding of the honeybee immune system including the pathways and mechanisms of their humoral and cellular processes exceeds that of the bumblebee. This discrepancy was due in part to the preferential focus on insects that are economically or medically relevant to humans. However with bumblebee numbers across the world rapidly declining due to highly detrimental ecological and environmental changes, (Goulson et al. 2015) and with the spread of pathogens and parasites among bee species constantly rising, it is essential that we gain insights into the drivers of this decline. This will only be achieved through comprehensive characterisation of their defence and stress-associated mechanisms at the molecular levels.

To combat infection with harmful and potentially pathogenic microbes and parasites, insects have a complex and dynamic immune response (Kurtz 2004) which can respond with high specificity to different types of parasites and infections (Barribeau and Schmid-Hempel 2013). This system is centred around three major responses; proteolytic cascades (coagulation and prophenoloxidase), cellular responses (encapsulation and phagocytosis) and synthesis and secretion of several potent effector molecules (e.g. antimicrobial peptides

(AMPs)) (Lemaitre et al. 1996; Hoffmann and Reichhart 2002). Insect haemolymph is centrally involved in numerous immune roles including protection and defence against; physical injury, invading pathogens or foreign bodies (Lavine and Strand 2002) and is an integral conduit of the insect immune response, containing numerous immune components including haemocytes, antimicrobial peptides (AMPs) and elements of the signalling cascades such as the prophenoloxidase signalling system (proPO) (Chan et al. 2006). Within the haemolymph there is also considerable crosstalk between humoral and cellular aspects of immune/defence systems. This crosstalk may involve haemocyte recognition of foreign objects either by direct interaction of surface receptors with molecules on the invading organism, or indirectly by recognition of humoral receptors that bind to and opsonize the surface of the invader. Inter- and intracellular signalling events then co-ordinate appropriate effector responses including phagocytosis or encapsulation (Lavine and Strand 2002). Hence, in order to gain a comprehensive view of the *B. terrestris* response to pathogen invasion it is necessary that both the cellular and humoral elements of the immune system are investigated.

In this study mass spectrometry-based proteomics was utilised to examine the humoral aspects of immunity in *B. terrestris* worker haemolymph in response to microbial challenge (fungal and bacterial). Proteins are central in the majority of cellular enzymatic functions, regulatory activities, signal transduction and structural components (Garrels 2001). Hence, the application of quantitative proteomics to provide a detailed characterisation and analysis of the immune response of *B. terrestris* provides valuable insight into the mechanisms of disease pathology and resistance in this highly social organism. To investigate immune response one representative fungus (*Metarhizium anisopliae*) and two bacteria, one Gram-negative (*Escherichia coli*) and one Gram-positive (*Staphylococcus aureus*) were chosen. The use of general non-bumblebee specific microbes was advantageous in this study due to i) the large body of literature and reference information existing for microbes commonly used in infection and immunology experiments and ii) the necessity to remove the signatures of manipulation by the natural pathogen (i.e. the pathogen has evolved specific strategies to immunosuppress or immunomodulate host systems). In addition, cellular bioassays were used in this study to characterise haemocyte associated responses to microbial challenge and map the induction and response steps of the cellular elements of the bumblebee immune

system. It is anticipated that taken together the molecular and cellular perspectives obtained in this study will resolve key and possibly novel aspects and components of the immune/defence systems in this important pollinator species.

### 6.1.1 Chapter six aims

- To investigate the effects of microbe presence within the *B. terrestris* worker haemocoel on the number of circulating haemocytes and strength of the encapsulation response.
- To comprehensively examine the molecular composition of worker haemolymph in response to microbial presence using mass spectrometry-based proteomics.
- To investigate the immune components of the haemolymph proteome after treatment with microbes using gene ontology analysis.

### 6.1.2 Experimental Outline

Colonies of *Bombus terrestris audax* bumblebees were obtained from Biobest (Belgium) via Agralan Ltd (Swindon, UK). The colonies were maintained in the lab under controlled environmental conditions, in continuous darkness or red light, at an ambient temperature of 24±2˚C and fed *ad libitum* on sucrose solution (0.5M) and prescribed pollen mix (Agralan). All colony work was completed under red light to minimise stress. Each worker bee was subjected to only one infection treatment and subsequently one haemolymph collection.

To investigate the *B. terrestris* worker immune response to microbial challenge, workers were infected with a representative Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacterium and an entomopathogenic fungus (EPF) (*Metarhizium anisopliae*). Both bacteria were cultured regularly in Luria broth (LB) and maintained on LB agar plates prior to use. To prepare the bacteria for infection, a single colony was selected from the agar plates and added to LB. Liquid cultures were grown overnight in shakers at 37°C. Cultures were then centrifuged at 3,000 x *g* for 10 mins, the supernatant removed, and the cells resuspended in Ringer's solution (hereafter Ringer throughout). This step was repeated twice more to ensure the complete removal of all culture medium. To prepare the cultures for infection the optical density (OD) was obtained, the cell density was then

adjusted using the appropriate buffer (Ringer) and the cell count was confirmed using a haemocytometer.

The Novozymes product Met 52 (*M. anisopliae*), was maintained as conidiospores on rice grains. PGA plates were inoculated with conidiospores under aseptic conditions and grown at 25°C for 7-12 days. Once conidial growth was established the spores were used to inoculate blastospore cultures. To culture blastospores a loop of virulent conidiospores from 15-day old culture was used to inoculate yeast extract peptone dextrose (YEPD) broth. Cultures were incubated in a shaker at 25°C at 200 rpm for three days to obtain blastospores. To isolate blastospores, cultures were filtered through two layers of miracloth and one layer of filter paper (Whatman™) to remove hyphae and cellular debris. The filtered culture was then centrifuged (2,000 x *g* for 1 min), the supernatant removed, and the spores resuspended in Ringer. This wash step was repeated twice to ensure the full removal of culture medium. Fungal cell concentrations were determined using a haemocytometer and adjusted accordingly with sterile Ringer solution.

The experimental design for this investigation involved five different experimental groups; three infections; *S. aureus*, *E. coli* and Met52 and two controls; Ringer and naïve bees. All micro-organisms were injected live to elicit a more rapid immune response (Charles and Killian 2015). Ringer was used as the buffer for the solutions containing the micro-organisms as it is similar in composition and pH to insect haemolymph and therefore should not elicit an immune response. This facilitates the examination of the bacterial immune response without interference. The response of naïve workers (un-infected or wounded) was measured as an additional control. Workers used for infection experiments (n = 10 per challenge) were kept in the colony until 24 hours prior to infection when they were selected at random from the colony and placed in six treatment specific holding boxes (wooden boxes with a Perspex top, depth 12cm x width 16cm x length 24cm) to acclimatize away from the colony environment.

To prepare the bees for infection individuals were removed from the holding box one at a time and anesthetised using $CO_2$. The bee was then placed on a wax platform and pinned down on its back by securing a strap across its abdomen. Once the bee was in position the body was then gently stretched using heat-flamed tweezers to expose the site of injection,

the gaps between the tergites of the abdomen. In order to infect the bees a small aliquot of each treatment (2µl of circa $3 \times 10^8$ cfu $ml^{-1}$) was pipetted out onto parafilm and then drawn up into the tip of a syringe. The bee was injected in the left side between the terga on the lower abdomen. Wounding was limited by allowing only the very tip of the syringe to enter the bee. Post-infection each bee was transferred to new a holding box of the same dimensions and at the same ambient conditions. All bees subjected to the same treatment were infected and housed together. Haemolymph collections were carried out 24 hours post infection on all infected bees to allow for a full spectrum immune response to mount within each individual. As Ringer was the solution for preparing micro-organisms, a group treated with Ringer solution only was also included in the experimental design. This group functioned as a procedural control for infection and wounding.

To investigate the effect of microbe presence on the level of circulating haemocytes and encapsulation response within the haemolymph, cell counts (n = 12) and encapsulation assays (n = 8) were conducted on bumblebee workers sampled from four colonies. Total haemocyte counts (THCs) were obtained for pathogen treated workers haemolymph (10 µl) was collected, diluted in 50 µl of ice cold Ringer and mixed following the methods of Moret and Schmid-Hempel, (2009). A 10 µl subsample was diluted (1:1) with trypan blue (0.4%) and a 10 µl aliquot of this mix was loaded on a haemocytometer. The slide was left to stand for 5 - 10 minutes to allow the haemocytes to settle prior to counting and calculation of final concentration.

To assess the strength of the encapsulation response, 2 mm nylon filaments were inserted into the bee abdomen 24 hrs after treatment with microbes following the infection treatment outlined above. After 24 hrs filaments were collected from bees via dissection. All recovered filaments were then subjected to a two-step fixing procedure prior to mounting. Filaments were incubated for one hour in a sterile watch glass containing ethanol and then transferred to a watch glass containing xylenes for a further hour. Control filaments (non-inserted filaments) were subject to the same fixing treatment prior to mounting. Mounting slides were standard microscope slides modified to hold the filaments in line on the slide. To create an area on the slide to contain the filaments two coverslips were secured to the slide to create a holding gap (approx. 5 mm). Filaments were placed along this gap and Eukitt® was added

(2 – 3 drops). Once filament positions were adjusted, and air bubbles removed a third coverslip was placed on top to seal the filaments in place. Slides were allowed to set over night at room temperature prior to imaging. Filaments were photographed using a Leica DM500 connected to LAS EZ imaging software (version 3.3) and the grey scale index (GI) measurement was obtained in ImageJ image analysis software version 1.51j.

To characterise the haemolymph proteome of *B. terrestris* in response to microbial presence, haemolymph from four commercial workers per treatment (n = 2 colonies, Koppert Biological Systems) was collected (section 2.2.3) and prepared for LC-MS/MS analysis. From each bee approximately 20µl of haemolymph was collected using a microcapillary and deposited in ice cold filter sterilised phosphate buffered saline (fsPBS) supplemented with protease inhibitor cocktail tablet (50 µl). All collections were centrifuged (8,000 rpm for 5 minutes at 4˚C) to remove debris and the supernatant was removed to a new microtube for further processing. This haemolymph isolation procedure was chosen to minimise the time for potential modifications (proteolytic and chemical) of haemolymph proteins during collection and processing that could influence downstream proteomic analyses.

To prepare the samples for mass spectrometry the following steps were conducted; samples were quantified using the Qubit™ protein assay kit, contaminants were removed using a 2D clean-up kit, samples were subject to overnight tryptic digestion and finally, purified using C18 ZipTip® pipette tips. Tryptic peptides were then resuspended in 2% v/v acetonitrile and 0.05% v/v TFA and 1 µg was loaded onto a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer for analysis. Peptides were separated over a 2 – 40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 60 min reverse-phase gradient at a flow rate of 250 nL $min^{-1}$.

Protein identification and label-free quantification (LFQ) normalisation of MS/MS data was conducted using MaxQuant v 1.5.6.5 (http://www.maxquant.com). Data analysis and processing of the data was then performed using Perseus v 1.5.0.8. (www.maxquant.org/). To identify statistically significant differentially abundant (SSDA) proteins Student's t-tests were performed for all relevant comparisons on the post imputated data using a cut-off of $p<0.05$. To visualize changes in protein expression for each pair wise comparison individual volcano plots were generated within Perseus by plotting negative log p-values on the y-axis

and log2 protein fold-change values on the x-axis. Hierarchical clustering was performed on Z-score normalised intensity values for all significant proteins identified via a multi-sample ANOVA, by clustering both samples and proteins using Euclidean distance and complete linkage.

The ClueGO application (v 2.5.1) within the functional analysis software Cytoscape (http://www.cytoscape.org/) was used for gene ontology (GO) enrichment analysis and visualization and provide a global overview of the biological processes changing within the haemolymph proteome. To supplement GO term assignment and provide putative functional information for identified proteins a basic local alignment search tool for proteins (BLASTp) search was performed against the Uniprot sequence set for *Apis mellifera* and *Drosophila melanogaster* (WWW.Uniprot.org downloaded April 2017). The top Uniprot match for each *Bombus* protein was identified and used to query the Perseus annotation file for *A. mellifera* and *D. melanogaster* (downloaded April 2017) and extract terms for biological process (BP), molecular function (MF), cellular component (CC), Kyoto Encyclopedia of Genes and Genomes (KEGG) name, KEGG pathway, protein family (pfam) and InterPro. Defence and immune components of the bumblebee haemolymph proteome were identified and characterised by incorporating immune information from the *Bombus* genome paper (Sadd et al., 2015) and additional manual annotations based on GO information sourced from *Apis mellifera* and *Drosophila melanogaster* genome as previously described (section 3.2.3). Immune function was investigated by selecting the GO immune function in ClueGO analysis software.

## 6.2 Results

### 6.2.1 Bioassays

Cellular defence in immune-challenged *B. terrestris* workers was examined using haemocyte counts and nylon filament-based encapsulation/melanisation assays. Information on the cellular aspect of *B. terrestris* immune response will reveal the activity of these two key cellular immune mechanisms in response to a range of pathogens and will contribute to the characterisation of the cellular response profiles to these elicitors. To enumerate haemocytes circulating within haemolymph, samples from all workers were counted as described (section

6.1.2) and the average of this count taken as the cell concentration. The grey scale index (GI) measurement was used to obtain a representative value of filament melanisation. GI was measured on a scale from 0 (pure white) – 255 (pure black), hence higher amounts of melanisation correspond to higher GI measurements. To quantify the grey index (GI) of each treatment, the average value for control filaments was subtracted from the average GI for all treatment groups to account for the filaments baseline GI.

Values from both bioassays were then square-root transformed to normalise the variance. The relationship between haemocyte density or encapsulation response and immune challenge was investigated using univariate analyses of variance (ANOVA), differences were considered statistically significant at $p<0{\cdot}05$ and groups were compared using post hoc pairwise Tukey's comparisons. All statistical analysis was completed using GraphPad Prism v. 5.0 (www.graphpad.com).

A significant difference in haemocyte number between control and immune challenged groups was observed (Figure 6.1). Increased numbers of haemocytes are observed in all immune challenged groups, with *S. aureus* resulting in the greatest increase. No significant difference in encapsulation was revealed between control and microbe treated groups (Figure 6.2). However, of the immune stimulated groups the highest encapsulation response was observed in *E. coli* and the lowest in *S. aureus*. The overall highest encapsulation response was observed in naïve bees and similar levels of encapsulation were revealed in control and *M. anisopliae* treatments.

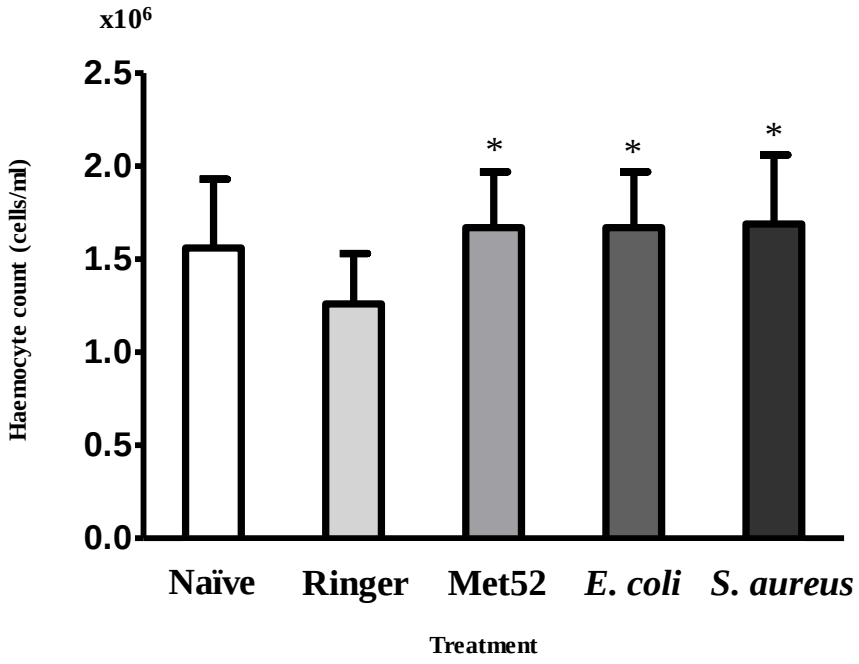


**Figure 6.1 Total haemocyte count of immune-challenged workers.** Circulating haemocytes in all treatments were enumerated and compared (ANOVA) to investigate differences in the numbers of immune cells circulating post immune stimulation. The results indicated that haemocyte numbers increase in response to microbial treatment, with significant increases observed in all immune treatment groups when compared to control groups. (*) represents a p-value of $p<0.05$ when compared to the control group (Ringer). Error bars represent standard deviation.

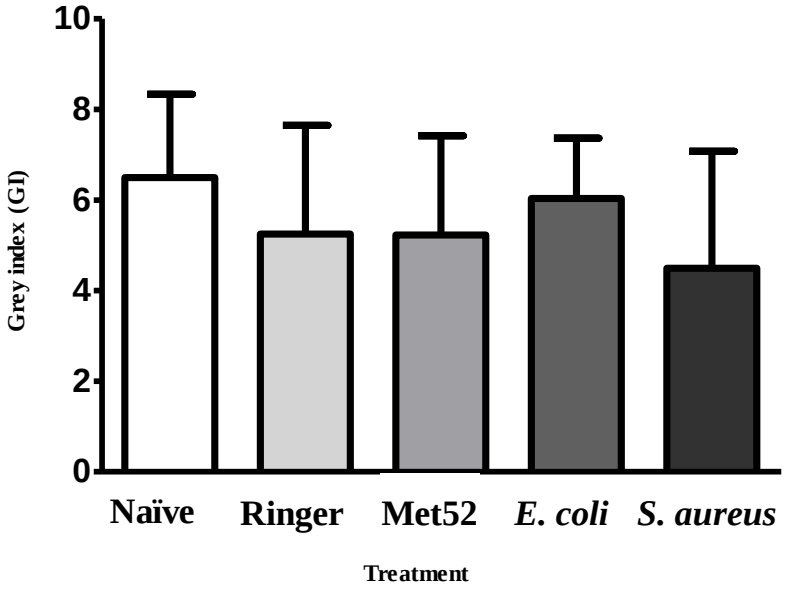


**Figure 6.2 Encapsulation response of immune-challenged workers.** The encapsulation response of all treatments was quantified and compared (ANOVA) to investigate differences in the response post immune stimulation. No significant differences were observed between the control and immune treatment groups. Error bars represent standard deviation.

### 6.2.2 Protein identification and quantification

All raw MS/MS files were imported into MaxQuant which was used to search the mass spectra against a protein database arising from the bumblebee genome (Sadd et al. 2015). Through this step proteins present across all samples were identified and their relative abundance calculated. This resulted in the initial identification of 791 proteins, however this number was significantly reduced through additional filtering to remove contaminant peptides, reverse peptide hits and peptides only identified by site. LFQ intensity values were then $Log_2$ transformed and only proteins found in at least three of four replicates were included resulting in 290 proteins (Table A 6.1). In order to visualise protein groups with similar expression profiles and to identify statistically significant protein differences within the protein set a number of analysis procedures were carried out including; hierarchical clustering, ANOVA analysis and Student's t-tests.

### 6.2.3 Hierarchical cluster analysis

Hierarchical clustering was performed on the 200 SSDA proteins to identify proteins with similar expression profiles across all groups, resulting in the identification of seven clusters (Figure 6.3). Cluster analysis revealed that group A contained 61 proteins increasing in abundance to the presence of bacteria and included proteins associated with detoxification (glutathione S-transferase and heat shock 10 and 70 kDa), cytoskeletal organisation and structure and carbohydrate metabolism. Functional enrichment of this response profile revealed and association with proteolytic and glycolytic processes (Table 6.1). The response profile of proteins increasing in response to treatment with *E. coli* was represented by the nine proteins forming cluster B. Proteins identified within this cluster included a central PO cascade protein (chymotrypsin inhibitor) and numerous cytoskeletal proteins (myosin, titin and tropomyosin). Functional analysis of this cluster resulted in enrichment of the myosin complex. Cluster C represented those proteins with increased abundance in response to the presence of microbial elicitors and comprised 18 proteins including immune (defensin, hymenoptaecin, venom serine protease and serpin B3), antioxidant (60 kDa heat shock and heat shock 70 kDa) and numerous proteasomal associated proteins. Enrichment analysis further confirmed the association with the proteasome and proteolytic activity. The response profile for proteins changing in abundance in response to the presence of *M. anisopliae* and

*E. coli* was represented by clusters D and E. Cluster D included 42 proteins increasing in abundance. This response profile included proteins involved in a variety of physiological processes including detoxification (glutathione S-transferase) and immunity (fibrillin, venom protease, omega conotoxin and chymotrypsin inhibitor). Cluster E represented 45 proteins decreasing in abundance and included a variety of immune proteins such as antioxidants (peroxidase), pathogen recognition receptors (PRRs) (beta-1, 3-glucan binding protein, chaoptin and MD-2-related lipid recognition protein), immune modulators (serine protease snake, antichymotrypsin and serine protease inhibitor 3) and immune effectors (lysozyme and icarapin). Functional enrichment of the *M. anisopliae* and *E. coli* response profiles revealed extracellular space associated proteins were well represented among cluster E, while no enrichment was obtained for cluster D. The response profile for proteins changing in abundance to treatment with *S. aureus* was represented by clusters F and G, with F representing proteins increasing in abundance and G including those decreasing in abundance. Cluster F included 10 proteins including a PPR (beta-1, 3-glucan binding protein, an immune modulator (leukocyte elastase inhibitor) and the antimicrobial peptide abaecin. Functional enrichments were not determined for clusters F and G.

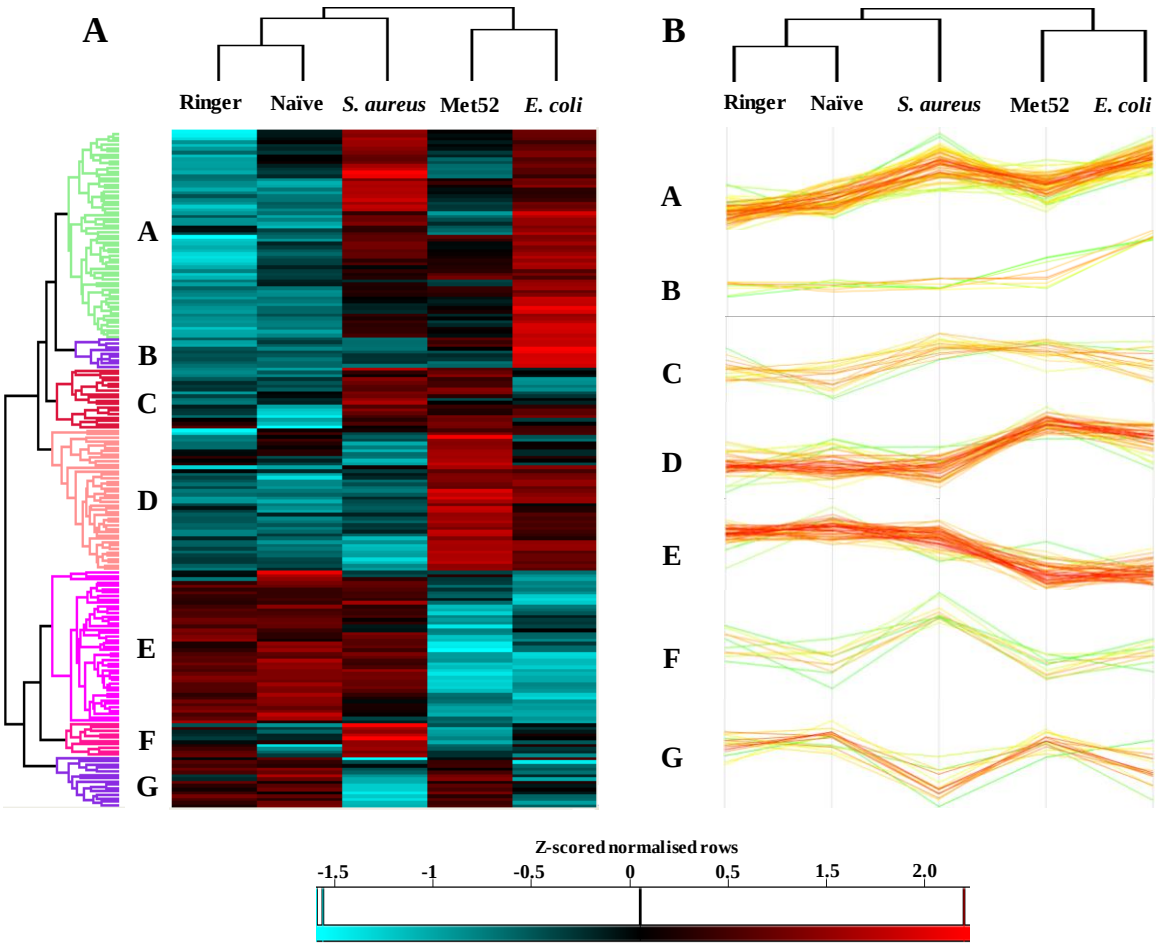


**Figure 6.3 Hierarchical clustering of haemolymph samples.** (A) Hierarchical clustering of the mean intensity values for 200 SSDA proteins among the different samples in response to immune challenge. Heat map colours represent the relative expression of proteins in samples. (B) Cluster expression trends of identified proteins are represented by the coloured lines. These proteins have been divided into groups based on treatment specific expression profiles. Abundance of individual proteins can be read from the intensity spectrum. Relative abundances (z-scored normalized) of individual proteins can be read from the intensity spectrum.

**Table 6.1 Significantly enriched gene ontology processes in *B. terrestris* worker haemolymph in response to treatment with microbes.** A selection of significantly enriched GOBP, CC, MF and KEGG terms (Fisher's exact test, FDR < 2%) in each of the identified protein clusters. No enrichment was identified for clusters D, F and G.

| Cluster ID | Cluster size | Type | Term | P-value | Enrichment |
|---|---|---|---|---|---|
| A | 61 | GOCC | Lipid particle | 5.89E-06 | 2.5884 |
| | | GOBP | Glycolysis | 1.48E-05 | 3.2787 |
| | | GOMF | Nucleotide binding | 1.62E-05 | 2.3845 |
| B | 9 | GOCC | Myosin complex | 6.40E-05 | 22.222 |
| C | 18 | GOMF | Threonine-type peptidase activity | 3.94E-10 | 10 |
| | | KEGG | Proteasome | 3.94E-10 | 10 |
| | | GOBP | Proteolysis involved in cellular protein catabolic process | 3.94E-10 | 10 |
| E | 45 | GOCC | Extracellular space | 0.0012035 | 2.328 |

### 6.2.4 Student's T-test analysis

Student's t-tests were performed between the different treatments to determine statistically significant differentially abundant (SSDA) proteins in response to microbial stimulation.

#### 6.2.4.1 Haemolymph response to treatment with *E. coli*

Student's t-test analysis identified 132 SSDA proteins were revealed in response to the presence of *E. coli* (Table A 6.2). Within this bacterial response profile 91 and 41 had increased and decreased abundances, respectively (Figure 6.4). The ten most differentially increased abundant proteins included proteins associated with glycolysis (probable phosphoglycerate kinase and phosphoglycerate mutase), oxidoreductase activity (trans-1,2-dihydrobenzene-1,2-diol dehydrogenase), lipid metabolism (inorganic pyrophosphatase), immunity (serine protease inhibitor 3/4), cytoskeletal formation (myosin heavy chain, muscle LIM and troponin) and cell adhesion (muscle-specific protein 20) (Table 6.2). Those proteins which had the greatest decrease in abundance include those associated with; carbohydrate degradation (alpha-glucosidase), hormonal signalling (protein takeout), proteolytic activity (zinc carboxypeptidase) and immunity (serine protease inhibitor ¾, BMP-binding endothelial regulator protein, chaoptin, insulin-like growth factor-binding protein, serine protease snake, four-domain proteases inhibitor and lysozyme). In addition, within the 132 proteins were 34 immune proteins, 15 increased and 19 decreased.

**Table 6.2 Top 10 SSDA proteins in the haemolymph in response to treatment with *E. coli*.** The top 10 most/least abundant proteins within the haemolymph proteome in response to *E. coli* infection based listed in descending order include those involved in cell adhesion, cytoskeletal formation and lipid, carbohydrate and energy metabolism. Fold change represents the $\log_2$ mean LFQ intensity difference in *E. coli* in comparison to Ringer. Fold changes highlighted red and blue represent increased or decreased abundances, respectively, in response to *E. coli*.

| Fold change | Accession no. | Protein name |
|---|---|---|
| 7.69 | XP_012166988.1 | Myosin heavy chain |
| 6.09 | XP_012170065.1 | Probable phosphoglycerate kinase |
| 5.74 | XP_012172950.1 | Muscle LIM protein Mlp84b |
| 5.68 | XP_012166074.1 | Titin |
| 5.57 | XP_012172948.1 | Muscle-specific protein 20 |
| 5.31 | XP_003395087.1 | Inorganic pyrophosphatase |
| 5.3 | XP_003401463.1 | Phosphoglycerate mutase 2 |
| 5.2 | XP_003394648.1 | Cytochrome c |
| 5.16 | XP_012164546.1 | Troponin T |
| 5 | XP_003403209.1 | Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase |
| -6.04 | XP_012164669.1 | Alpha-glucosidase |
| -5.19 | XP_012174011.1 | Zinc carboxypeptidase |
| -4.55 | XP_012170668.1 | Lysozyme |
| -3.99 | XP_003401209.1 | Four-domain proteases inhibitor |
| -3.31 | XP_003401334.1 | Serine protease snake |
| -2.53 | XP_012163992.1 | Insulin-like growth factor-binding protein |
| -2.35 | XP_012164498.1 | Chaoptin |
| -2.34 | XP_003393266.1 | Bmp-binding endothelial regulator protein* |
| -2.23 | XP_003397291.1 | Protein takeout |
| -2.22 | XP_003402576.1 | Serine protease inhibitor ¾ |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

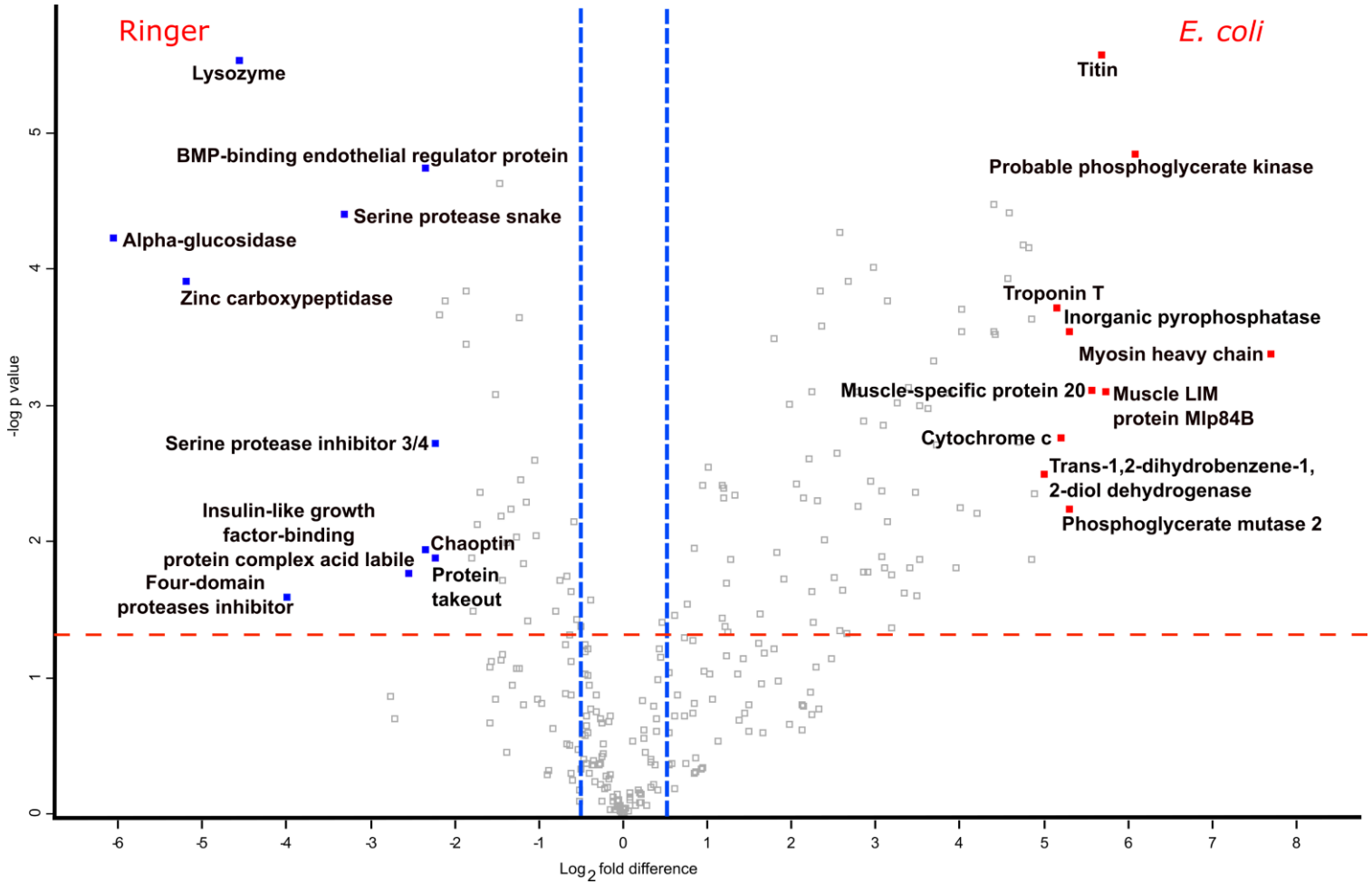


**Figure 6.4 Differential protein abundances of the haemolymph response to Gram-negative bacterial immune stimulation.** Volcano plot distribution of quantified proteins according to p-value (−log10p-value) and fold change (log2 mean LFQ intensity difference) in *E. coli* in comparison to Ringer. Proteins above the line are considered statistically significant (p-value <0.05) and those to the right and left of the vertical lines indicate relative fold changes ≥ 1.5. Annotated proteins represent the top ten increasing (red)/ decreasing (blue) proteins in response to wounding.

### 6.2.4.2 Haemolymph response to treatment with *S. aureus*

Within the haemolymph response to *S. aureus* 72 SSDA proteins were identified (Table A 6.3). This profile included 62 proteins increasing and 10 decreasing (Figure 6.5). Among the top 10 proteins increasing in response to Gram-positive bacteria includes those involved in the following processes; lipid metabolism (inorganic pyrophosphatase), chitin degradation (chitinase), cytoskeleton formation (transitional endoplasmic reticulum ATPase and cofilin/actin-depolymerizing factor) and immune proteins (peroxiredoxin, glutathione S-transferase, heat shock 83, 60 and 70 kDa) (Table 6.3). The ten proteins decreasing in response to treatment with *S. aureus* include seven immune proteins (apolipophorin, apolipophorin D, peroxidase, venom dipeptidyl peptidase, alpha-2-macroglobulin, venom protein and venom acid phosphatase), with the remaining three proteins involved in processes such as lipid signalling pathways (PI-PLC X domain-containing protein), oxidoreductase activity (prenylcysteine oxidase) and chitin degradation (chitinase). Globally within these SSDA proteins 21 immune proteins, seven decreasing and 14 increasing.

### 6.2.4.3 Haemolymph response to treatment with *M. anisopliae*

Student's T-test analysis of the global haemolymph response profile to treatment with the EPF (*M. anisopliae*) identified 85 SSDA proteins, 56 increasing and 29 decreasing (Figure 6.6) (Table A 6.4). The ten proteins increasing most in this response included proteins associated with a variety of biological processes including; immunity (peroxiredoxin, heat shock 10 and 60 kDa), cell signalling (neurotoxin), cytoskeletal formation (muscle LIM protein Mlp84B), proteolytic activity (neprilysin), lipid metabolism (inorganic pyrophosphatase), glycolysis (phosphoglycerate mutase and probable phosphoglycerate kinase) and carbohydrate metabolism (peritrophin) (Table 6.4). Of the top ten decreased abundance proteins four had roles in immunity (haemocyte protein-glutamine gamma-glutamyl transferase, serine protease snake, four-domain proteases inhibitor and lysozyme) and the remaining six had associations with the following biological activities; carbohydrate degradation (alpha-glucosidase), hormonal signalling (protein takeout), proteolytic activity (zinc carboxypeptidase and glutamate decarboxylase) and receptor binding (renin and vitellogenin receptor). Overall within this response profile 28 immune proteins were identified, 14 increasing and decreasing, respectively.

**Table 6.3 Top 10 SSDA proteins in the haemolymph in response to treatment with the Gram-positive bacteria *S. aureus*.** The top 10 most/least abundant proteins within the haemolymph proteome in response to microbial stimulation based on intensity listed in descending order include those involved in metabolism, immunity and chitin degradation. Fold change represents the $log_2$ mean LFQ intensity difference in *S. aureus* compared to Ringer. Fold changes highlighted red and blue represent increased or decreased abundances, respectively, in response to *S. aureus*.

| Fold change | Accession no. | Protein name |
|---|---|---|
| 6.2 | XP_003395087.1 | Inorganic pyrophosphatase |
| 6.13 | XP_003396897.1 | Heat shock protein 8 |
| 5.6 | XP_003399629.1 | 60 kDa heat shock protein |
| 5.54 | XP_012169580.1 | Transitional endoplasmic reticulum ATPase TER94 |
| 5.27 | XP_012174797.1 | Cofilin/actin-depolymerizing factor |
| 4.96 | XP_012172464.1 | Peroxiredoxin 1 |
| 4.8 | XP_012163601.1 | Glutathione s-transferase |
| 4.7 | XP_012170065.1 | Probable phosphoglycerate kinase |
| 4.4 | XP_003397462.1 | Heat shock 70 kDa protein cognate 4 |
| 4.23 | XP_012171737.1 | ATP-citrate synthase |
| -0.97 | XP_003399644.1 | Venom acid phosphatase Acph-1 |
| -0.92 | XP_012169952.1 | Prenylcysteine oxidase |
| -0.89 | XP_012167483.1 | PI-PLC X domain-containing protein 1 |
| -0.8 | XP_012171903.1 | Venom protein* |
| -0.73 | XP_003396888.1 | Alpha-2-macroglobulin |
| -0.68 | XP_003399213.1 | Venom dipeptidyl peptidase 4 |
| -0.63 | XP_003397064.1 | Peroxidase |
| -0.52 | XP_012166489.1 | Apolipoprotein D |
| -0.31 | XP_003397320.1 | Apolipophorins |
| -0.25 | XP_012175191.1 | Chitinase-like protein Idgf4 |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

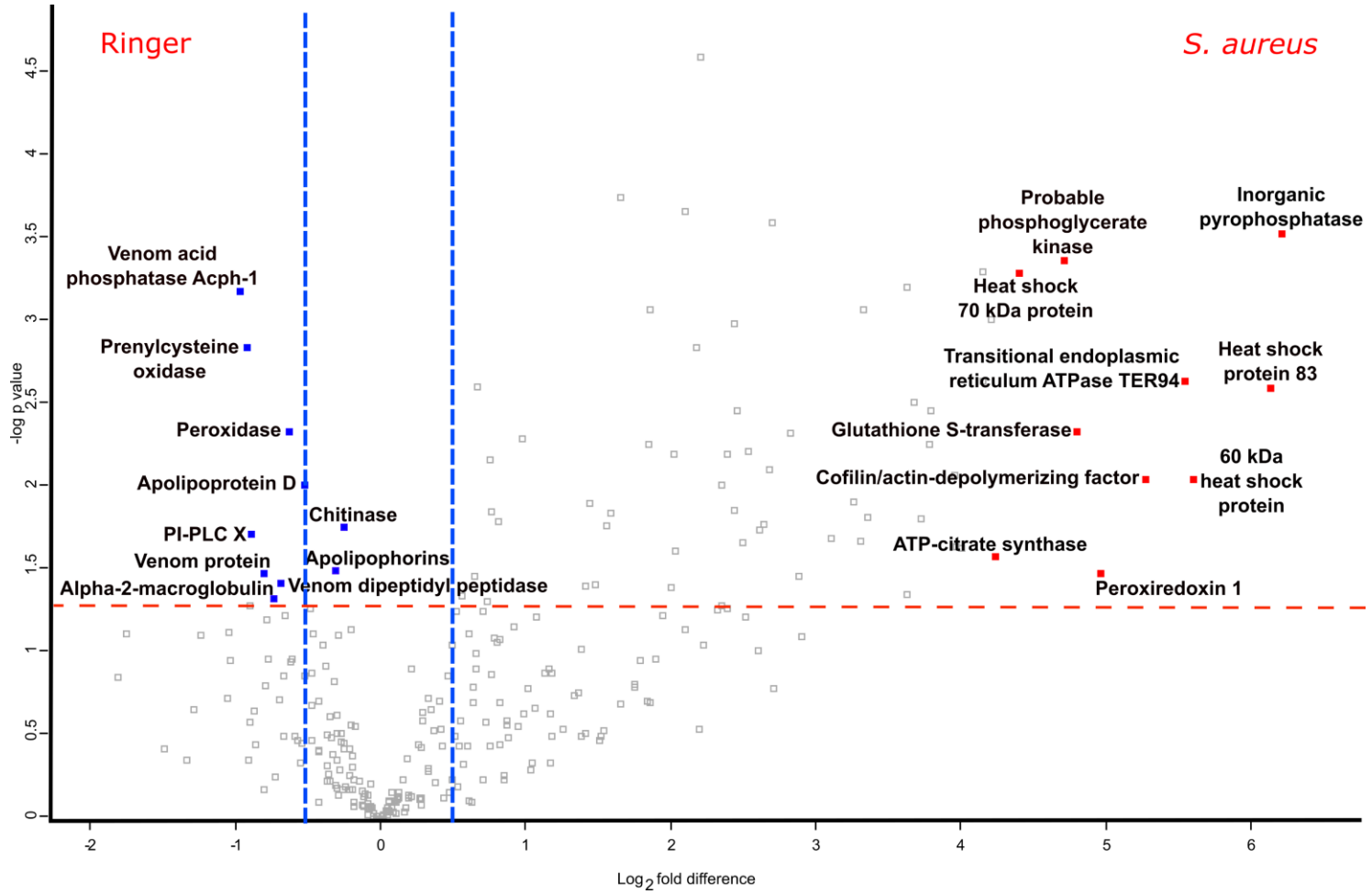


**Figure 6.5 Differential protein abundances of the haemolymph response to Gram-positive bacterial immune stimulation.** Volcano plot distribution of quantified proteins according to p-value (−log10p-value) and fold change (log2 mean LFQ intensity difference) in *S. aureus* in comparison to Ringer. Proteins above the line are considered statistically significant (p-value <0.05) and those to the right and left of the vertical lines indicate relative fold changes ≥ 1.5. Annotated proteins represent the top ten increasing (red)/ decreasing (blue) proteins in response to wounding.

**Table 6.4 Top 10 SSDA proteins in the haemolymph in response to treatment with entomopathogenic fungi (*M. anisopliae*).** The top 10 most/least abundant proteins within the haemolymph proteome in response to fungal infection based on intensity listed in descending order include those involved in immunity, proteolysis and lipid and carbohydrate metabolism. Fold change represents the $\log_2$ mean LFQ intensity difference in *M. anisopliae* compared to Ringer. Fold changes highlighted red and blue represent increased or decreased abundances, respectively, in response to *M. anisopliae*.

| Fold change | Accession no. | Protein name |
|---|---|---|
| 4.59 | XP_003399629.1 | 60 kDa heat shock protein |
| 4.11 | XP_003397695.1 | Peroxiredoxin-6 |
| 4.02 | XP_012172950.1 | Muscle LIM protein Mlp84B |
| 3.68 | XP_003398591.1 | Neurotoxin* |
| 3.63 | XP_003401463.1 | Phosphoglycerate mutase 2 |
| 3.56 | XP_012170065.1 | Probable phosphoglycerate kinase |
| 3.37 | XP_003403002.2 | Neprilysin |
| 3.26 | XP_003395087.1 | Inorganic pyrophosphatase |
| 3.16 | XP_012170152.1 | 10 kDa heat shock protein |
| 3.1 | XP_003393341.1 | peritrophin-1 |
| -6.24 | XP_012174011.1 | Zinc carboxypeptidase |
| -5.27 | XP_012164669.1 | Alpha-glucosidase |
| -5.21 | XP_012170668.1 | Lysozyme |
| -4.72 | XP_003401209.1 | Four-domain proteases inhibitor |
| -4.01 | XP_003401334.1 | Serine protease snake |
| -3.11 | XP_003402703.1 | Vitellogenin receptor |
| -3 | XP_012167292.1 | Renin receptor |
| -2.75 | XP_003401022.1 | Glutamate decarboxylase* |
| -2.71 | XP_003402321.1 | Haemocyte protein-glutamine gamma-glutamyl transferase |
| -2.7 | XP_003397291.1 | Protein takeout |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

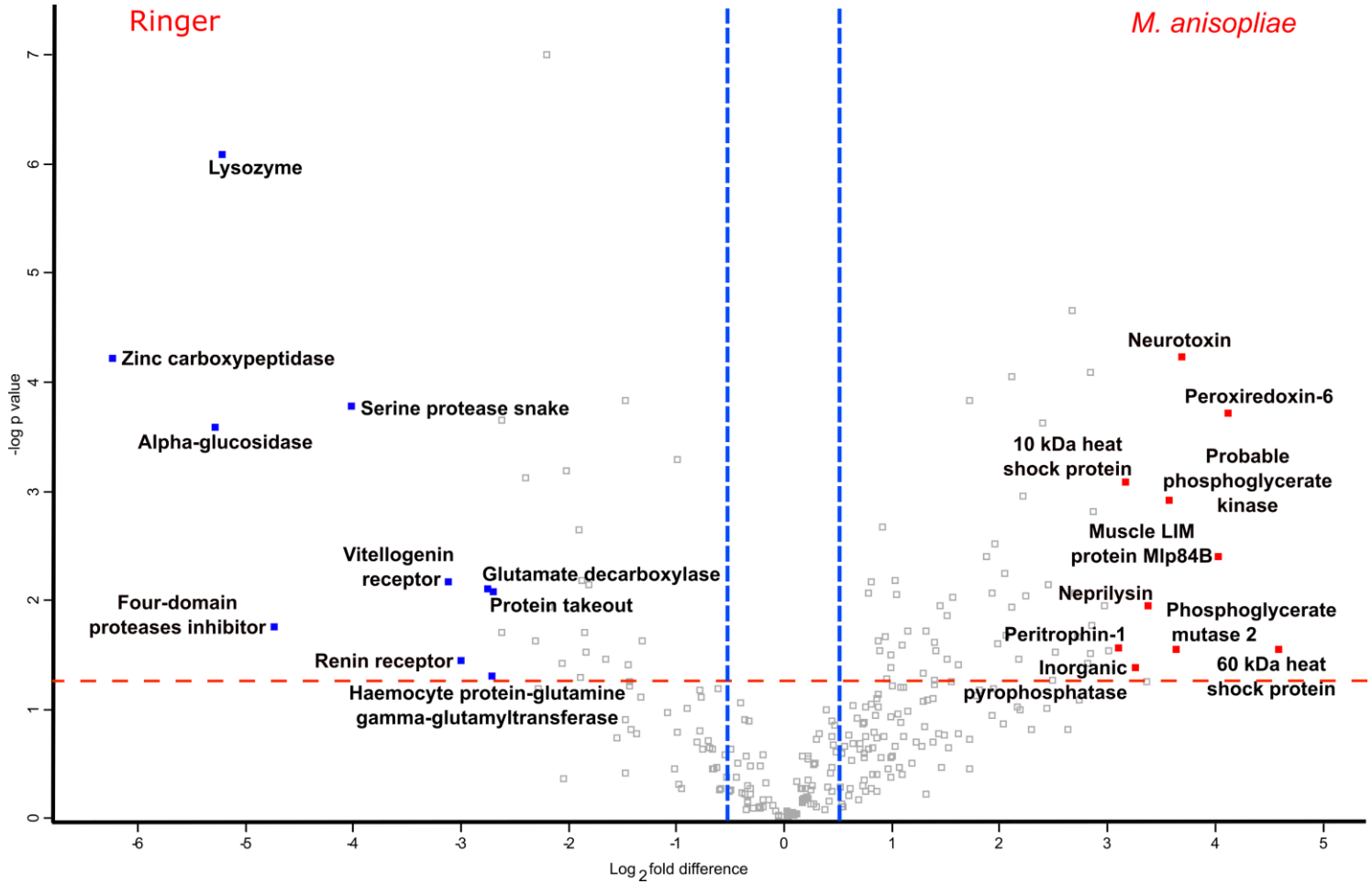


**Figure 6.6 Differential protein abundances of the haemolymph response to immune stimulation with the entomopathogenic fungi *M. anisopliae*.** Volcano plot distribution of quantified proteins according to p-value (−log10p-value) and fold change (log2 mean LFQ intensity difference) in *M. anisopliae* in comparison to Ringer. Proteins above the line are considered statistically significant (p-value <0.05) and those to the right and left of the vertical lines indicate relative fold changes ≥ 1.5. Annotated proteins represent the top ten increasing (red)/ decreasing (blue) proteins in response to wounding.

### 6.2.4.4 Comparative analysis of SSDA proteins in response to immune stimulation

Comparative analysis of the immune response profiles to all treatments revealed numerous proteins unique and common (Figure 6.7). Overall 17 proteins common to all treatment responses were revealed, one decreasing (prenylcysteine oxidase) and 16 increasing (Figure 6.7A). The most well-represented biological associations among those commonly increased were glycolysis (transketolase, aldose reductase, probable phosphoglycerate kinase and phosphoglycerate mutase) and antioxidant response (peroxiredoxin, glutathione S-transferase, superoxide dismutase and heat shock 70 kDa). Overall five immune and defence associated proteins were commonly identified increasing including; venom serine protease and all previously listed antioxidants (Figure 6.7B).

Within the 46 proteins unique to *E. coli*, 23 were increasing and decreasing, respectively. Within this total number, eight immune proteins were revealed (three increased and five decreased). Proteins increasing had the following functional associations; cytoskeletal formation (myosin heavy and light chain, titin, tropomyosin, tubulin, actin and troponin) and amino acid (glutamine synthase), carbohydrate (glycerol-3-phosphate dehydrogenase and L-xylose reductase) and energy metabolism (triosephosphate isomerase), while those decreasing were involved in iron storage (ferritin 3, ferritin subunit and transferrin), pathogen recognition (peptidoglycan-recognition protein 2) and immune modulation (insulin-like growth factor-binding protein complex acid labile subunit, serine protease inhibitor 3, chaoptin and serine protease easter) (Table 6.5).

Globally 22 proteins were revealed as unique to the haemolymph response to Gram-positive bacteria. This unique *S. aureus* profile contained 16 increasing and six decreasing proteins among which were numerous immune proteins (Figure 6.7). Those decreasing included venom proteins (venom protease and venom dipeptidyl peptidase), immune signal modulators (alpha-2-macroglobulin-like), proteins associated with chitin degradation (chitinase), lipid signalling (PI-PLC X domain-containing protein) and transport proteins (apolipoprotein). Those increasing represented proteins associated with the proteasome (proteasome subunit type alpha (1 and 5) and beta (1,4 and 6)), numerous proteins involved in the following energy metabolism associated pathways; glycolysis (fructose-biphosphate aldose), pentose phosphate pathway (phosphogluconate dehydrogenase) and citric acid cycle

(fumarate hydratase, isocitrate dehydrogenase and ATP citrate synthase). In addition, immune (IRP30) and detoxification (heat shock 70 kDa) proteins were also revealed in this set (Table 6.5).

The *M. anisopliae*-challenged haemolymph contained 19 unique proteins of which 14 increased in response to the EPF and five decreased (Figure 6.7). Immune proteins represented four of those increasing and one decreasing. The unique decreasing proteins included haemocyte associated proteins (haemocyte protein-glutamine gamma-glutamyl transferase), proteins involved in mediating biological regulation (vitellogenin and renin receptor) and an endonuclease (poly(U)-specific endoribonuclease). The increasing proteins contained numerous immune-associated proteins including omega conotoxin, kazal type proteinase inhibitor, fibrillin 2 and apolipophorin III (Table 6.5).

Twenty-nine proteins had common expression profiles to bacterial challenge. Within the 27 increasing are numerous structural proteins (muscle specific protein 20, troponin T, cofilin/actin, myophilin, filamin A and profilin), carbohydrate metabolism associated proteins (enolase, transaldose, arginine kinase, glucose-6-phosphate, malate dehydrogenase and fructose-biphosphate aldose), proteins involved in detoxification (peroxiredoxin 1, glutathione S-transferase and heat shock protein 83) and immune effectors (hymenoptaecin). The two proteins identified to be decreasing in response to bacteria have roles in immune modulation (venom acid phosphatase Acph-1 and apolipoprotein D). The *E. coli* and *M. anisopliae* response shared 45 common proteins. Within the 22 decreasing were numerous proteases (serine protease snake, serine carboxypeptidases, furin like proteases and putative cysteine proteases), inhibitors (serine protease inhibitors and anti-chymotrypsin) and numerous immune proteins including AMPs (icarapin) and PRRs (beta-1,3-glucan-binding protein 1). Proteins of increased abundance included cytoskeletal proteins (muscle LIM, laminin and alpha actinin), two inhibitors of the PO cascade (chymotrypsin inhibitor) and the antioxidant glutathione S-transferase. Shared responses to *S. aureus* and *M. anisopliae* included three immune proteins, two increased and one decreased. Increasing were heat shock proteins (60 kDa and 10 kDa) and decreasing in response to *S. aureus* and *M. anisopliae* was peroxidase.

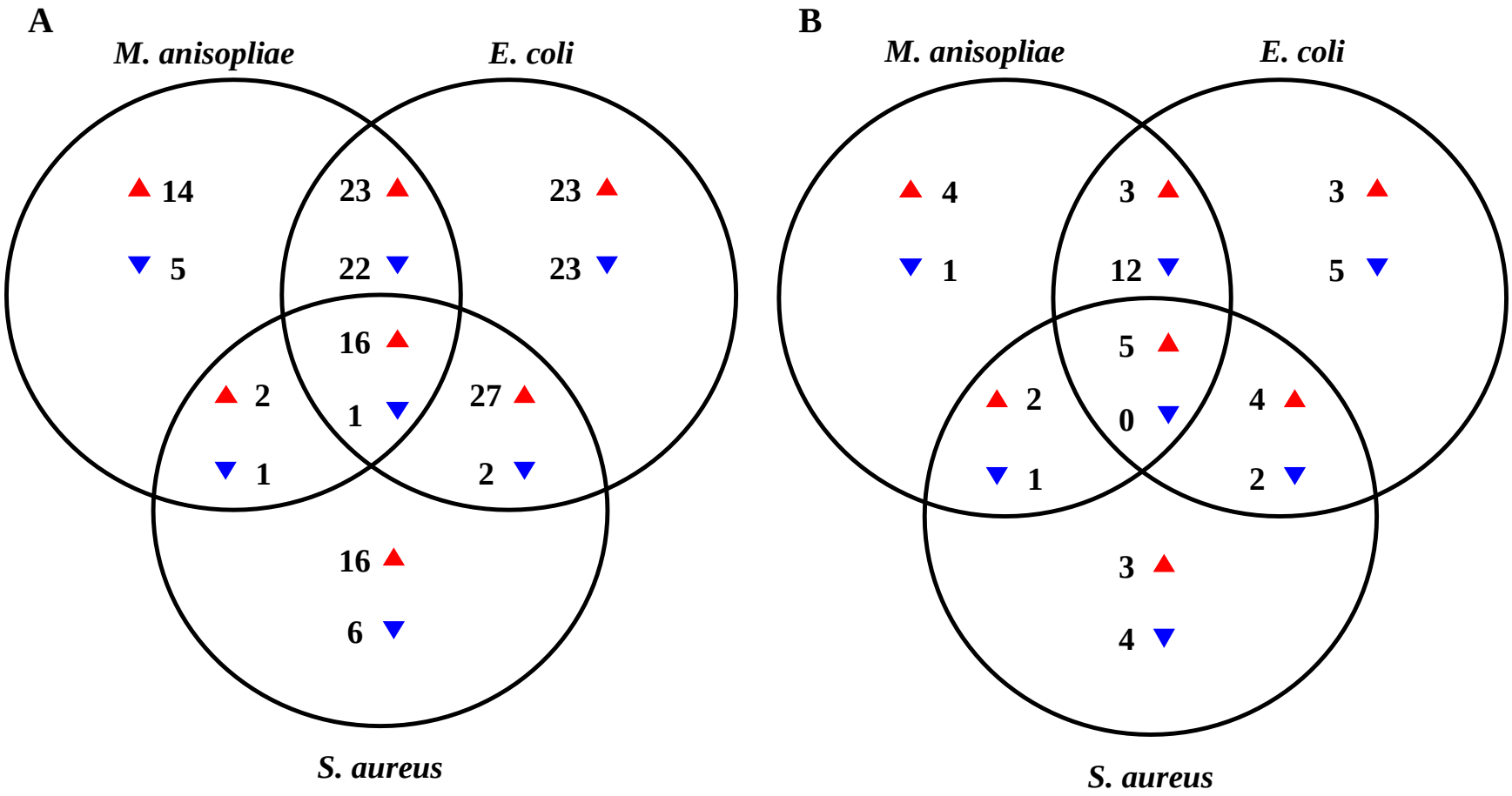


**Figure 6.7 Venn diagram analysis of common and uniquely abundant proteins in response to treatment with the three microbial elicitors.** (A) Numerous unique and common proteins were identified increasing (red arrows) and decreasing (blue arrows) in response to the three treatments. (B) Immune proteins specific and conserved across treatment groups were also identified.

**Table 6.5 Immune-associated proteins identified in the *B. terrestris* haemolymph in response to challenge with microbes.** As expected, numerous elements of the insect immune response were present including antioxidants (15), immune signalling modulators (27), PRRs (5) and immune effectors (4). The values for each protein represent the log2 fold change, positive values represent proteins with increased abundance and similarly negative values represent those proteins decreasing in abundance.

| Immune function | Accession no. | Protein name | Fold change | | |
|---|---|---|---|---|---|
| | | | *E. coli* | *S. aureus* | Met52 |
| Antioxidant | XP_003393349.1 | Heat shock 70 kDa protein cognate 5 | - | 2.35 | - |
| | XP_003394648.1 | Cytochrome c | 5.2 | - | - |
| | XP_003395150.1 | Glutathione S-transferase | 2.57 | 1.65 | 2.67 |
| | XP_003396897.1 | Heat shock protein 83 | 3.15 | 6.14 | - |
| | XP_003397064.1 | Peroxidase-like isoform X3 | - | -0.63 | -1.32 |
| | XP_003397462.1 | Heat shock 70 kDa protein cognate 4 | 3.62 | 4.41 | 2.17 |
| | XP_003397695.1 | Peroxiredoxin-6 | 4.58 | 3.8 | 4.12 |
| | XP_003399629.1 | 60 kDa heat shock protein, mitochondrial | - | 5.6 | 4.6 |
| | XP_003402976.1 | Heat shock protein beta-1 | 3.72 | - | - |
| | XP_012163601.1 | Glutathione S-transferase | 3.39 | 4.8 | - |
| | XP_012166688.1 | Superoxide dismutase | 1.33 | 0.75 | 0.78 |
| | XP_012170152.1 | 10 kDa heat shock protein, mitochondrial | - | 4.21 | 3.17 |
| | XP_012172050.1 | Glutathione S-transferase 1 | 3.11 | - | 2.52 |
| | XP_012172464.1 | Peroxiredoxin 1 | 3.41 | 4.97 | - |
| | XP_012175811.1 | Heat shock 70 kDa protein 4 | - | 3.26 | - |
| Immune effector | ADB29130.1 | Hymenoptaecin | 2.07 | 2.64 | - |
| | XP_003396228.1 | Icarapin | -2.17 | - | -2.02 |
| | XP_012170668.1 | Lysozyme | -4.56 | - | -5.22 |
| | XP_012171903.1 | Venom protein* | - | 0.8 | - |
| Immune modulator | XP_003395762.1 | Omega-conotoxin-like protein 1 | - | - | 3.01 |
| | AFX62369.1 | Chymotrypsin inhibitor | 2.4 | - | 1.95 |
| | XP_003393266.1 | BMP-binding endothelial regulator protein | -2.34 | - | -2.4 |
| | XP_003395653.2 | Serine protease easter * | -1.15 | - | - |
| | XP_003396888.1 | Alpha-2-macroglobulin | - | -0.73 | - |
| | XP_003397320.1 | Apolipophorins | - | -0.31 | - |
| | XP_003398909.1 | Furin-like protease 2 | -1.38 | - | -2.3 |
| | XP_003399186.1 | Antichymotrypsin-2 | -1.7 | - | -1.91 |
| | XP_003399187.1 | Antichymotrypsin-2 | -2.11 | - | -2.17 |
| | XP_003401209.1 | Four-domain proteases inhibitor | -3.99 | - | -4.72 |
| | XP_003401213.2 | Kazal-type proteinase inhibitor* | - | - | 2.11 |
| | XP_003401334.1 | Serine protease snake | -3.31 | - | -4.02 |

(Table 6.5 continued)

| | | | | | |
|---|---|---|---|---|---|
| **Immune modulator** | XP_003402321.1 | Hemocyte protein-glutamine gamma-glutamyltransferase | - | - | -2.71 |
| | XP_003402576.1 | Serine protease inhibitor 3/4-like, partial | -2.22 | - | -2.63 |
| | AEN62314.1 | IRP30 | - | 2.43 | - |
| | XP_003399213.1 | Venom dipeptidyl peptidase 4 | - | -0.69 | - |
| | XP_003399644.1 | Venom acid phosphatase Acph-1 | -0.74 | -0.97 | - |
| | NP_001267823.1 | Venom serine protease precursor | 2.35 | 2.02 | 1.93 |
| | XP_012163961.1 | Putative cysteine proteinase CG12163 | -1.5 | - | -1.48 |
| | XP_012163992.1 | Insulin-like growth factor-binding protein complex acid labile | -2.54 | - | - |
| | XP_012166489.1 | Apolipoprotein D-like | -1.46 | -0.52 | - |
| | XP_012172397.1 | Chymotrypsin inhibitor-like | - | - | 2.86 |
| | XP_012172841.1 | Fibrillin-2-like | - | - | 1.04 |
| | XP_012174357.1 | Apolipophorin-III-like protein precursor* | - | - | 0.92 |
| | XP_012174896.1 | Serine protease inhibitor 3-like isoform X2 | -1.44 | - | - |
| | XP_003401878.1 | Hemocytin isoform X2 | 0.47 | - | - |
| **PRR** | XP_012164498.1 | Chaoptin-like | -2.35 | - | - |
| | XP_012167632.1 | Beta-1,3-glucan-binding protein 1 | -1.23 | - | -0.99 |
| | XP_003400229.1 | MD-2-related lipid-recognition protein-like | -1.26 | - | -1.44 |
| | XP_012173325.1 | Peptidoglycan-recognition protein 2-like | -0.66 | - | - |

(*) indicates those proteins that have been reannotated as per section 2.6.3.

### 6.2.5 Protein ontology

To examine the biological processes associated with the haemolymph response profile of each microbial treatment, gene ontology analysis was completed in ClueGO using the *D. melanogaster* genome as the background reference (Figure 6.8). The results indicate that those proteins with increasing abundance in response to treatment with *E. coli* represent a diverse set of biological functions from muscle cell activity and cytoskeletal organisation to lipid and fatty acid metabolism. In addition, enrichment of immune and defence-related processes such as melanin processing, cellular detoxification and haemocyte proliferation were also revealed (Figure 6.8A).

Proteins of decreased abundance in this response comprised processes including protein processing and the following immune-related processes: defence response to Gram-positive bacteria, toll signalling pathway and response to fungi. The *B. terrestris* worker haemolymph response profile to *M. anisopliae* is composed of energy production (nucleoside diphosphate phosphorylation and amino acid metabolism), olfactory learning and cellular detoxification process increasing and processes-associated with defence against Gram-positive bacteria decreasing (Figure 6.8B). Functional analysis of the *S. aureus* response profile resulted in the identification of biological enrichments such as energy metabolism (pyridine nucleotide metabolism, glycolytic process, generation of precursor metabolites and ribose phosphate metabolism), however cellular oxidant detoxification and structure organisation and homeostasis were also identified (Figure 6.8C). No biological enrichments were attained for the proteins decreasing in abundance in the *S. aureus* response profile.

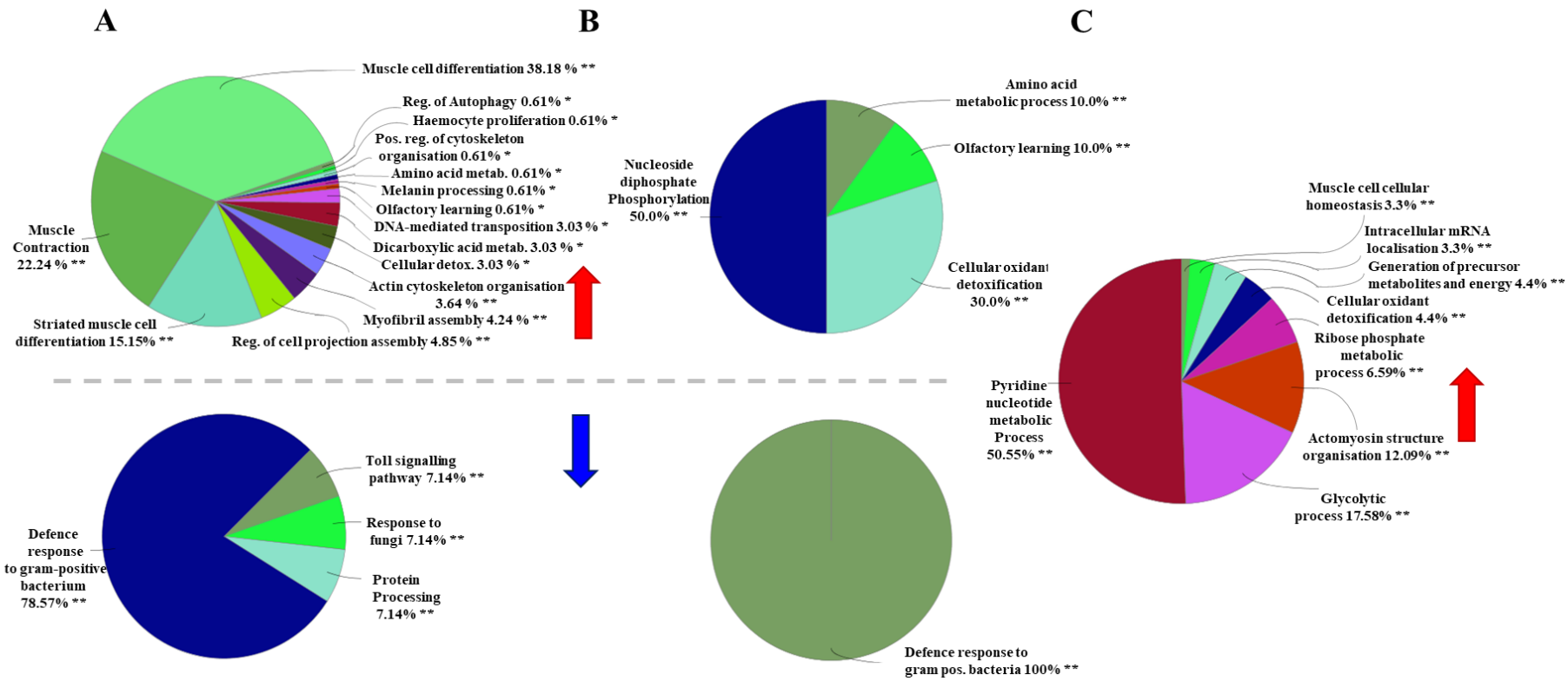


**Figure 6.8 Functional analysis of haemolymph pathogen response.** The most enriched terms for biological functions identified in the haemolymph proteome response to (A) *E. coli*, (B) *M. anisopliae* and (C) *S. aureus*. Single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$ statistical levels, respectively. The arrows indicate the direction the processes are changing in response to each treatment and broken line divides increase/decrease protein sets.

## 6.3 Discussion

With the implication of pathogens as major contributors to global bee declines, the need to understand the basic mechanisms of infection and immunity in important pollinators such as bumblebees has come to the fore. In this investigation *Bombus terrestris* workers were treated with a variety of microbial elicitors to elucidate the complex mechanisms of their immune response. A combination of both proteomic and cellular analyses of bumblebee haemolymph were employed in an attempt to assess both the cellular and humoral aspects of immunity. It was revealed that *B. terrestris* workers display qualitatively different and specific immune activities against both bacterial and fungal elicitors.

### 6.3.1 Key findings

- Observed increases in the THC of *B. terrestris* haemolymph in response to immune stimulation with micro-organisms (bacteria and fungus), coupled with no significant increase in encapsulation activity may indicate that haemocyte activity is preferentially involved in repair of the wound inflicted during infection.
- Proteomic analysis revealed quantitatively different signatures of protein abundance in response to the three microbial treatments. However, the overall haemolymph response to micro-organisms is a complex and multi-faceted process relying on the coordination of numerous biological activities.
- The response to bacterium resulted in significantly increased energy, detoxification and immune activities.
- Similarly, the response to fungus was rich in energy metabolism and immune proteins. Whilst metabolism associated proteins were increasing, the majority of immune proteins responsible for mediating aspects of the cellular and humoral defence to fungi were decreasing.

### 6.3.2 Variations in haemocyte density and encapsulation activity in response to microbes

The innate immune system of insects is composed of both humoral and cellular defence elements. Humoral defences include synthesis of AMPs, initiation and regulation of coagulation and melanisation cascades, and the production of reactive intermediates of oxygen and nitrogen. The cellular arm of defence is composed of circulating haemocytes

and includes haemocyte-mediated responses like phagocytosis and encapsulation which assist in neutralisation and removal of foreign particles including invading micro-organisms (Lavine and Strand 2002; Stofanko et al. 2008).

The THC of an individual bee can vary dramatically in response to numerous factors such as age (colony and individual) (Moret and Schmid-Hempel 2009), stress, injury and infection (Doums et al. 2002). Hence, enumeration of haemocytes can be used as a bioindicator of the physiological status of the bee. The THC in *B. terrestris* worker haemolymph increased significantly in response to immune stimulation with a variety of micro-organisms, a result similarly observed by (Korner and Schmid-Hempel 2004; Moret and Schmid-Hempel 2009). Increases in the THC may be a result of sessile haemocytes being recruited to help fight the infection (Korner and Schmid-Hempel 2004) and their role in wound repair (Evans et al. 2006). Recruited haemocytes also burst to release prophenoloxidase (proPO) in response to infection making these molecules available for melanisation (Korner and Schmid-Hempel 2004). The melanisation pathway which is central to encapsulation activity is initiated by the enzyme phenoloxidase whose precursor proPO is produced and released by haemocytes (Korner and Schmid-Hempel 2004; Evans et al. 2006). In response to immune stimulation with bacteria and fungi no significant change in encapsulation response was observed as previously expected. Numerous factors may be responsible for these results such as; i) the naturally high degree of variation in melanisation across bumblebee colonies which may have masked any significant changes; ii) the treatments used in this study may not have been sufficiently virulent to induce a response or iii) that *B. terrestris* workers have a low level non-specific encapsulation response (Allander and Schmid-Hempel, 2000; Mallon et al., 2003). In addition, the observed increases in THC in response to microbial treatment coupled with no significant change in encapsulation may also indicate that haemocyte activity is preferentially involved in the repair of the wound that was inflicted during treatment. In that scenario haemocyte recruitment to the wound site is essential in order to prevent additional or higher pathogen loads within the haemocoel.

### 6.3.3 Haemolymph response to treatment with fungi

In the haemolymph profiles of workers treated with *M. anisopliae*, differentially expressed proteins were over-represented by GO terms associated with energy metabolism, cellular detoxification and defence response against Gram-positive bacteria.

A variety of immune proteins involved in detoxification and immune modulation were present within those increasing in response to fungi. The only increased immune effector identified was venom serine protease precursor which has known antimicrobial activity (Choo et al. 2010). As expected, multiple antioxidants were increased in response to fungi and are most likely involved in mediating ROS in the haemolymph as a result of EPF infection. Immune modulators increasing in this response included elements of the PO cascade (chymotrypsin inhibitor), fibrillin which is a mediator and driver of cellular signalling (Zeyer and Reinhardt 2015) and apolipoprotein-III (apoLp-III) which along with fibrillin is unique to the response to fungi. It is known that apoLp-III stimulates antimicrobial peptide production in insect haemolymph, enhances phagocytosis, binds and detoxifies LPS, lipoteichoic acid, fungal conidia and β-1,3-glucan and therefore may act as a pattern recognition molecule for multiple microbial and parasitic invaders (Whitten et al. 2004). The *M. anisopliae* haemolymph response was rich in immune and metabolism-associated proteins. The immune response is an energy dependent process and these increased energy metabolism proteins and processes may represent an attempt to meet the demands of maintaining an immune response (Lee and Lee 2018). The high levels of detoxification proteins within this response are also expected as the bee attempts to contend with reactive oxygen species (ROS) produced as a result of increased metabolism (Bayir 2005) or the physiological stress induced by infection (Kodrík et al. 2015).

Proteins of decreased abundance in response to fungi included numerous defence-associated processes such as AMP icarapin, lysozyme and numerous immune modulators and key elements of the PO cascade (chymotrypsin and antichymotrypsin) and haemocyte associated proteins (hemocyte protein-glutamine gamma-glutamyltransferase) responsible for mediating aspects of the cellular and humoral insect immune response to fungi (Tanji et al. 2007). The decreased immune proteins may represent the effects of fungal infection, as EPFs manipulate and evade the host immune response by disrupting regulatory networks, including suppression of cytoskeleton formation and other features of the subcellular structure of host immune cells (Vilcinskas and Götz 1999) or may represented proteins depleted by their activity in immune signalling and responses.

### 6.3.4 Haemolymph response to bacterial challenge

#### 6.3.4.1 Haemolymph response to *E. coli*

Gene ontology analysis of the bumblebee haemolymph proteome in response to *E. coli* revealed a strong association of numerous central homeostatic and immune-related biological processes. These physiological activities included those associated with the generation of energy, protein processing, organisation of structural and cytoskeletal processes and immune processes. Within those proteins increasing in abundance in response to *E. coli* were a number of processes associated with the generation of energy. It is well reported that energy is essential to support normal homeostatic processes and immunity (Schmid-Hempel 2004; Ardia et al. 2012), hence increased energy production in the haemolymph response to the presence of micro-organism is expected as energy is rapidly directed to targeted tissues and cells mediating *B. terrestris*' immune response. Increases in energy precursor and production pathways leads to increased ROS production which induces enhanced expression of enzymatic and non-enzymatic antioxidants to contend with increased oxidative stress, hence an increase in energy production may also contribute to the detoxification activity of the haemolymph (Rand et al. 2015). Cellular detoxification is central to combating ROS produced from physiologically stressful events such as bacterial infection (Kodrík et al. 2015) and as expected numerous detoxification proteins (heat shock beta, cytochrome c and glutathione S-transferase) were identified increasing in abundance in the haemolymph response profile. Hence, within the *B. terrestris* haemolymph *E. coli* response energy production may perform the dual role of supporting energy requirements of immunity and inducing increased detoxification.

Biological responses reduced in response to *E. coli* included defence response to Gram-positive bacteria and fungi, protein processing and toll signalling. The decrease in central immune pathways (toll) and response may be indicative of these proteins being activated and used in the response to *E. coli* or may suggest an alternative or novel signalling and pathway response within *B. terrestris* to the presence of bacteria. In addition, decreases in protein processing may be as a result of general protein production being reduced to conserve energy and facilitate rapid production of antimicrobial proteins such as venom serine protease and hymenoptaecin. Interestingly, among the decreasing unique proteins were a variety of iron storage proteins and immune modulators including the cell protein

chaoptin, a leucine-rich repeat containing protein is involved in mediation of the adhesive interaction between cell surfaces most likely through a direct homophilic mechanism (Krantz and Zipursky 1990) and hence may be associated with the cellular immune response to *E. coli*. Iron sequestering has been revealed as an insect defence mechanism for limiting bacterial infection by reducing ferric ions that are essential for the growth and development of the invading pathogens (Yoshiga et al. 1997). The iron binding proteins identified within this response are associated with transport, storage and delivery (Tang and Zhou 2013) and hence decreases in these proteins may be indicative of their activity and involvement in iron sequestering within the *B. terrestris* haemolymph through binding and transport of iron molecules.

The most well represent group of processes within the protein set with increased abundance to *E. coli* were those associated with the cytoskeleton. Cytoskeletal components such as microtubules, actin and intermediate filaments form structural frameworks within cells which help determine their shape, cytoplasm organisation (Bearer 1992) and assist intracellular and whole cell movement (Cooper 2000). In insects reorganisation of the cytoskeleton is involved in haemocyte migration to wound sites (Zanet et al. 2009), nodule formation (Arai et al. 2013) and phagocytosis (Beck and Strand 2005). In addition, the immune processes represented in the proteins increasing in abundance to *E. coli* included haemocyte proliferation and melanin processing. The presence of numerous structural proteins associated with microfilament formation and cellular adhesion in combination with haemocyte proliferation may be indicative of active haemocyte division (Matozzo et al. 2008) or haemocyte migration (Moreira et al. 2013). Melanin synthesis occurs through activation of the PO cascade and results in the production of melanin, which can be used in encapsulation, coagulation and wound healing activities (Tang 2009). Hence, the enrichment of a combination of both cytoskeletal structural and haemocyte associated processes may indicate wound healing and clearance of *E. coli* cells within the *B. terrestris* haemolymph via cellular activities such as phagocytosis and encapsulation.

#### 6.3.4.2 Haemolymph response to *S. aureus*

Global analysis of the haemolymph response profile to Gram-positive bacteria revealed associations with structural, metabolic, detoxification and translation processes. Similar to the responses observed in fungi and *E. coli*, increases in energy metabolism are

recorded. This increase may be required to support the energetically costly immune response and the abundance of antioxidants may present an attempt to contend with ROS from metabolic or pathogen-associated stress (Bayir 2005; Kodrík et al. 2015). Similarly, to the response observed in *E. coli* numerous cytoskeletal and structural proteins may represent elements that assist in the maintenance of cell shape, participate in cellular division, and intracellular transport of molecules (Wulfkuhle et al. 1998). In addition, actin has been revealed to function as an extracellular immune factor which is externalized by insect immune cells upon immune challenge with bacteria and once externalized, can bind to the surface of bacteria. A functional role of this interaction with bacteria is to mediate their killing through either phagocytosis or direct antibacterial action (Sandiford et al. 2015). Enrichment of translation associated processes may be indicative of the production of immune effectors such as AMPs after downstream signalling. In addition, to translation proteins associated with the proteasome were abundant within the *S. aureus* response profile. The main function of the proteasome is in the degradation of damaged or mis-folded proteins (Adams 2003). Hence, it may be involved in the degradation of proteins damaged as a result of *S. aureus* infection or in amino acid recycling to fuel energy metabolism. GO assignment identified no specific processes or pathways that were enriched within decreased abundance proteins, however manual annotation revealed a variety of proteins as being associated with immune and detoxification function.

#### 6.3.4.3 Common responses to bacteria in *B. terrestris* worker haemolymph

The differentially expressed proteins with similar expression profiles to both Gram-positive and Gram-negative bacteria were those involved in energy production, detoxification, cytoskeletal and structural associated processes. Increased energy production is essential for fuelling the various elements of the response to bacteria including cellular repair, detoxification and immunity. Cellular detoxification and cytoskeletal associated processes are both highly involved in the immune response to pathogens. In insects structural and cytoskeletal regulation proteins, including actin, are produced in response to physiological stress (Courgeon et al. 1993) and have been implicated in the mediation of cellular immune activities such as phagocytosis (Rämet et al. 2002).

Insects contain a selection of antioxidant and detoxifying enzymes including superoxide dismutase (SOD), catalase, glutathione S-transferase (GT), peroxidase whose action is directed to the elimination of ROS and control of melanogenesis (Dubovskii et al. 2010). Antioxidants were among the most well-represented group of immune proteins in response to bacteria with seven identified and increasing in both. The high proportion of antioxidants within the haemolymph may be produced by the haemocytes and may represent an attempt to contend with an increase in oxidative stress and cell damage from bacterial infection and possibly increased energy production. Notably, peroxiredoxins were the most abundant class of detoxification proteins in response to both bacterial treatments. In *D. melanogaster* peroxiredoxins have similarly been shown to be central to the maintenance of redox homeostasis in response to bacterial infection (Ahn et al. 2012). However, recently, it has been proposed that peroxiredoxins may also play key roles in innate immunity and inflammation (Knoops et al. 2016). In addition to antioxidants, immune modulators and effectors were also commonly identified in the haemolymph bacterial response. Apolipoprotein D (ApoD) was the only immune modulating protein common to both responses. ApoD is a member of the Lipocalin family, an ancient group of small proteins engaged in a diverse array of physiological processes, however their general molecular function is binding and transporting hydrophobic ligands (Ganfornina et al. 2008). In insects, this protein has been shown to be involved in regulation of longevity and resistance to stress, locomotor behaviour, and degeneration in brain and other tissues upon aging (Sanchez et al. 2006) and hence may work in tandem with antioxidant enzymes to mediate the stress response to infection. Of the three immune effectors associated with this response, two were increasing in abundance and the other was decreasing. As expected for all identified immune effectors had antimicrobial-associated properties and included the AMP hymenoptaecin (Casteels et al. 1993), venom serine protease and venom acid phosphatase (Choo et al. 2010).

### 6.3.5 Conserved microbial response profiles in haemolymph

The unbiased analysis of total proteome changes in response to three different micro-organisms resulted in the identification of a number of both specific and conserved responses in *B. terrestris.* Comparisons of the gene ontology profiles of each treatment revealed processes associated with the generation of energy and precursor metabolites which are commonly increased in all pathogen profiles, further confirming the central role of energy metabolism in maintaining the energy demanding and physiologically

costly immune response (Schmid-Hempel 2004; Ardia et al. 2012). Five immune proteins, glutathione S-transferase-like, heat shock 70 kDa protein cognate 4, peroxiredoxin-6-like, superoxide dismutase and venom serine protease precursor were constitutively present in all three treatments and their abundance levels remained similarly high after all microbe-challenges. Detoxification proteins represented the majority of commonly maintained proteins suggesting they have a key role to play in contending with infection associated ROS. The presence of venom proteins increasing in response to all pathogens is expected due their antimicrobial properties (Moreau 2013).

In the response to both fungi and Gram-negative bacteria both the PRR beta-1, 3-glucan-binding protein 1 and MD-2-related lipid-recognition protein were identified. This is an unexpected observation as it is widely reported that responses to Gram-positive bacteria and fungi involve the same recognition and immune signalling pathways (i.e. Toll) (Lemaitre and Hoffmann 2007). Similarities between *E. coli* and *M. anisopliae* may be indicative of activation of both the Toll and Imd pathways which are shown to be initiated through recognition of microbial elicitors in particular peptidoglycan and fungal elicitors such as beta 1,3-glucans (Lemaitre and Hoffmann 2007; Wang et al. 2014). Similar results have been observed in *D. melanogaster* where classical Gram-negative binding proteins have been revealed to be involved in both recognition and initiation of the toll pathway in response to both Gram-negative bacteria and fungi (Levy et al. 2004).

Overall the haemolymph response to micro-organisms is a multi-faceted process reliant on the interaction of numerous biological processes. Production and maintenance of energy and management of ROS are both central to protection and defence from micro-organisms. In addition, a variety of immune components representing both cellular and humoral elements of immunity are necessary to appropriately attack and neutralise pathogens within the haemolymph.

### 6.4 Conclusion

This study provides a comprehensive overview of the molecular and cellular composition of the *B. terrestris* worker haemolymph in response to a variety of microbial treatments. Through haemocyte counts and quantification of the encapsulation response, characteristics of the cellular response to each treatment were assessed. It was revealed that there is a significant increase in the numbers of circulating haemocytes in response

to the presence of microbes confirming their importance in cellular immune activities. With regards to encapsulation activity, a slight increase was observed in response to bacterial treatments. However, this was not significant which indicates that it is a mechanism not principally involved in pathogen clearance.

Proteomic analysis revealed quantitatively different signatures of protein abundance in response to the three microbial treatments. It was revealed that decreasing within the fungal response profile, were numerous defence processes such as that of the toll pathway. These decreases may represent a dampening of the immune response by the invading fungi. The *E. coli* response contained numerous haemocyte-associated processes suggesting that there is a strong cellular element to the haemolymph response to Gram-negative bacteria which may help contribute to the maintenance of homeostatic processes, such as tissue repair and immune surveillance. The haemolymph response to *S. aureus* profile contained a high proportion of cytoskeletal and structural proteins which may represent elements involved in the maintenance of haemocyte shape, proliferation, and transport of molecules. Hence, these structural proteins participate in a variety of homeostatic and immune roles.

Comparative analysis of the response profiles of the various microbial treatments revealed that conserved biological functions such as energy metabolism, detoxification and a combination of immune elements are central to mounting a pathogen immune response. Analysis of the bacterial response profiles of *S. aureus* and *E. coli* revealed numerous shared processes most notably of which the presence of numerous cytoskeletal and structural proteins. These proteins may represent elements that assist in the maintenance of cell shape, participate in cellular division, and intracellular transport of molecule and have been implicated in the mediation of cellular immune activities such phagocytosis.

Functional analysis of *B. terrestris* worker haemolymph revealed this complex serum is centrally involved in a number of biological processes, ranging from metabolism to immunity. Global gene ontology analysis of the proteome exposed the diverse biological processes haemolymph participates in, including carbohydrate metabolism, defence response, detoxification, negative regulation of endopeptidase activity, melanin metabolism and response to wounding. As expected for the haemolymph, numerous immune proteins were identified including AMPs, signal modulators and PRRs. Overall

the haemolymph proteome can be described as a highly proteolytic and immune active serum, which also participates in numerous aspects of normal cellular homeostasis such as carbohydrate metabolism, protein degradation and cellular transport. This study will provide a platform from which other essential immune studies, such as that of the response to the bumblebee specific pathogens *N. bombi* and *C. bombi*, can now be based.

# Chapter 7

## A proteomic investigation of the combinatorial effects of pesticide and pathogen exposure on the immunocompetence of *Bombus terrestris*

## 7.1 Introduction

The global agricultural industry is under continuous pressure from a plethora of biological and anthropogenic sources. As the world population continues to rapidly grow, so too does the demand for food, fibre, and bioenergy leading to pressure for increased production from the same land area (Godfray et al. 2010). Such agricultural intensification results in activities and practices which remove limitations to plant productivity such as; irrigation, addition of fertilizers, crop breeding, mechanical loosening of the soil structure and replacement of biological pest and weed control with pesticides (Tilman et al. 2001). A positive impact of this intensification has been the ability to meet global food demands (Bommarco et al. 2013). However, numerous negative impacts also arise including loss of natural habitats through extensive land use, homogenisation of the agricultural landscape and the widespread use of agrochemicals (Hoekstra et al. 2005; Tscharntke et al. 2005).

Pollination, which is provided by a variety of insects, most notably bees is one of the principal ecosystem services most negatively affected by agricultural stressors. As a result there has been an increased dependence on commercial bumblebees and honeybees to fill this deficit in pollination services (Graystock et al. 2016). However, bees themselves are at risk from a variety of stressors implicated in their declines globally, including pathogens, pesticides and habitat loss (Goulson et al. 2015). Whilst biological factors such as infection by pathogens and parasites are undoubtedly involved in bee decline, it is also evident that agrochemicals such as pesticides are also a contributory factor (Sanchez-Bayo and Goka 2014).

There are four principal chemical classes of insecticides of major economic importance: organophosphates (introduced in the 1940s), carbamates (1950s), synthetic pyrethroids (1970s) and neonicotinoids (1990s). All four classes of compounds are designed to act on only three different target sites, with the biochemical mode of action of all resulting in the disruption of cholinergic nerve transmission in the insect central nervous system (Nauen 2006). Carbamates and organophosphates, both inhibit acetylcholinesterase, an essential enzyme in terminating nerve pulse depolarization (Nauen and Bretschneider 2002). Pyrethroids interfere with the sodium channels in the nerve membrane by prolonging the

open phase and hence cause neuronal hyperexcitation resulting in repetitive synaptic firing (Field et al. 2017). The neonicotinoids act agonistically and highly selectivity on insect nicotinic acetylcholine receptors (Nauen and Bretschneider 2002). The introduction and use of these pesticides coupled with the subsequent development and introduction of herbicides and fungicides has dramatically contributed to improved pest control and agricultural output (Aktar et al. 2009) and agrochemical use continues to be the primary strategy adopted globally to manage insect pest populations (Pimentel 2009). Yet paradoxically, beneficial insects including pollinators are negatively impacted (McLaughlin and Mineau 1995; Whitehorn et al. 2013; Rundlöf et al. 2015; Brandt et al. 2016; Stanley et al. 2016).

Neonicotinoids are the most widely used classes of insecticides in agriculture due to their efficacy in protecting crops against a wide range of insect pest species (Elbert et al. 2008; Goulson 2013). Neonicotinoid insecticides currently registered for use include acetamiprid, imidacloprid, thiacloprid, clothianidin, thiamethoxam, and dinotefuran (Sheets et al. 2016). All are water-soluble and when applied to seeds can dissolve in contact with water and are readily absorbed by the roots of the developing plants (Wood and Goulson 2017). These systemic insecticides can then spread throughout all plant tissues making them toxic to any insects that feed on the plant. This offers the plant protection from direct damage inflicted by herbivorous (mainly sap feeding) insects and indirectly from damage by plant viruses that are transmitted by insects (Simon-Delso et al. 2015).

There are three possible routes for exposure for insects: direct contact with sprays (on flowering crops or adjacent wild flowers), contact with contaminated foliage, and uptake of chemicals in nectar (Goulson et al. 2008). Neonicotinoid exposure in bees is primarily through ingestion of residues in the pollen and nectar of treated plants (Desneux et al. 2007; Sánchez-Bayo et al. 2016). It is thought that bumblebees are likely to be regularly exposed to low doses of pesticides (David et al. 2016). In the UK, chemical residues have been found both in crops such oilseed rape and in wild flowers co-occurring in these agricultural areas, both of which are common floral resources for several bumblebee species (Stanley et al. 2013; Botías et al. 2015; David et al. 2016). Neonicotinoid exposure has the ability to disrupt neuronal cholinergic signal transduction (Fischer et al. 2014) which can negatively impact homing, foraging (Stanley et al. 2016), mobility (Moffat et al. 2016) and in some cases can

lead to the eventual death of both target and non-target insects (Belzunces et al. 2012). In addition, the sublethal effects of pesticide exposure can detrimentally affect bee fitness potentially increasing susceptibility to other stressors such as pathogens (Alaux, Ducloz, et al. 2010; Fauser-Misslin et al. 2014). Furthermore, in social bees, pesticide-induced impairments on homing and foraging have wide reaching impacts on overall colony function and may eventually lead to implications for the success of bumblebee colonies in agricultural landscapes and their ability to deliver crucial pollination services (Stanley et al. 2016). To withstand future climatic and economic challenges, agriculture practices need to be more productive, stable, resilient and minimise their impacts on the environment (Foley et al. 2005). Hence, the development of alternative ecologically friendly strategies for pest control, with reduced effects on beneficial organisms is a priority (Toledo-Hernández et al. 2016).

Entomopathogens are naturally occurring micro-organisms (fungi, nematodes and bacteria) that are pathogenic to arthropods and are routinely exploited as biocontrol agents, an alternative to agrichemical based control methods. (Meyling and Eilenberg 2007). Entomopathogens such as the fungi *Metarhizium anisopliae* (Metchnikoff) and *Beauveria bassiana* (Bals.-Criv.) are widely used myco-biocontrol agents worldwide (Sandhu et al. 2012). Spray-based application of these agents yields poor biocontrol results and alternative delivery systems such as entomovectoring, using non-target insects to spread EPF spores, have been investigated (Mommaerts and Smagghe 2011). The flowers of agricultural crops play an important role in the life cycle of several important plant pathogens; hence flowers are more often the targets for EPF spores. Due to their capacity to transmit viruses and fungal and bacterial spores together with pollen (Ngugi et al. 2002) and their ability to deliver biocontrol agents directly onto the flower target, pollinators have been investigated as entomovectoring agents (Mommaerts and Smagghe 2011). EPFs are not insect species-specific and hence to avoid potential adverse effects on pollinators, their toxicity in both honeybees and bumblebees has been investigated. However, there are conflicting reports as to whether they pose a potential risk. Negative effects reported include the onset of mycosis in *B. terrestris* workers after wet treatment with both *M. anisopliae* and *B. bassiana* (Mommaerts et al. 2009). Contrastingly (Smagghe et al. 2013), when investigating the potential use of bumblebees as EPF biocontrol delivery systems through entomovectoring

found no behavioural or lethal effects of EPF exposure on bumblebees at concentrations as high as $10^7$ spores per gram.

In the context of recent bee declines, it is accepted that pathogens, including those that are generally benign, have the ability to exhibit context dependent virulence, where under specific conditions such as reduced host fitness or increased virulence expression they can have a significant effect on their host (Meeus et al. 2018). Context dependent virulence has become highly relevant in relation to recent global bee declines where it has also been established that the stressors driving bee declines (pesticides, pathogens etc.) can work both individually and in combination (Brown et al. 2003; Goulson et al. 2015; Kairo et al. 2017). Pesticides have been revealed to suppress the insect immune response therefore influencing the pathogenicity of micro-organisms such as *M. anisopliae* (Di Prisco et al. 2013). In order to limit global bee declines it is therefore vital to understand the interactions between stressors and their combinatorial effects on their hosts. The research conducted in this chapter aims to clarify if acute pesticide exposure at field relevant doses, increases pathogen virulence by reducing host fitness.

The insect fat body, although primarily involved in lipid accumulation and storage (Musselman and Kühnlein 2018), is implicated in numerous other functions including the removal of toxic substances and participation in the immune response (Assis et al. 2014). Fat body cells can participate in detoxification and homeostasis by recruiting, removing and storing toxic substances from the haemolymph (Martins et al. 2011). This physiological powerhouse also regulates the synthesis and utilization of numerous haemolymph proteins, circulating metabolites and energy reserves such as glycogen and fat (Arrese and Soulages 2010). The main role of the FB within the insect immune system is to release soluble factors into the haemolymph, which can be produced both constitutively and exclusively in response to immune stimulation (Lemaitre and Hoffmann 2007).

With recent declines in bumblebee populations driven by stressors such as agrochemical exposure and disease, the need for a comprehensive molecular characterisation of the bumblebee fat body and its role in immunity, metabolism and detoxification is greater than ever. In addition, the cell morphology and basic molecular mechanisms of this important organ will contribute significantly to the body of knowledge on bumblebee physiology and

will benefit future physiological studies of this species. The comprehensive characterisation of the FB proteome will also contribute to the identification of key immune proteins. Understanding the bumblebee fat body proteome will enable the investigation and characterisation of key metabolic, immunological and regulatory processes involved in the bumblebee response to exposure to pesticides and pathogens in combination and will provide a platform for future studies. In addition, cellular aspects of the bumblebee immune response will also be assessed through the completion of bioassays on haemocyte density and encapsulation activity. Investigating how cellular elements of the bumblebee immune response are influenced by exposure to pesticides and pathogens could provide important information on their functionality in this response.

### 7.1.1 Chapter seven aims

- To investigate the effects of neonicotinoid pesticide and pathogen exposure, both individually and in combination, on the numbers of circulating haemocytes and encapsulation activity within the haemolymph of *B. terrestris* workers.
- To utilise label-free mass spectrometry to investigate changes in the FB proteome in response to *M. anisopliae*, and neonicotinoid clothianidin exposure through functional and gene ontology analysis.

### 7.1.2 Experimental outline

To investigate the effect of pesticide exposure on the *B. terrestris* fat body proteome, label-free mass spectrometry was applied to both control and exposed groups of bees. In addition, the fat body response to the entomopathogenic fungi *M. anisopliae* and the combinatorial effect of pesticide exposure and *M. anisopliae* infection was also examined. Bioassays were completed for all treatments to assess the effect of pesticides, *M. anisopliae* and combination exposure on elements of the cellular immune response (total haemocyte count and encapsulation response) of *B. terrestris* workers.

Colonies of *Bombus terrestris audax* bumblebees were obtained from Biobest (Belgium) via Agralan Ltd (Swindon, UK) and maintained in the lab under controlled environmental conditions, in continuous darkness or red light, at an ambient temperature of 24±2˚C and fed *ad libitum* on a sucrose solution (0.5M) and prescribed pollen mix (Agralan). All colony

work was completed under red light to minimise stress to the animals. Each worker bee in this study was subjected to only one infection treatment and subsequently one haemolymph collection.

The experimental design for the investigation of fat body response to pesticides included a neonicotinoid exposed and control group. Examination of the combinatorial effect of pathogens and pesticides included two treatments, Met52 and a combination of clothianidin and EPF, and one control with Ringer's solution (hereafter Ringer). In this investigation all bees were fed sucrose containing acetone as control for the pesticide exposure. Workers used for infection experiments (n = 10 per challenge) were kept in the colony until 48 hours prior to infection when they were selected at random from the colony and placed in six treatment specific holding boxes (wooden boxes with a Perspex lid, depth 12cm x width 16cm x length 24cm) to acclimatize away from the main colony environment.

Clothianidin (99.9% analytical grade) was purchased from Fluka Analytical Ireland. A stock solution of 0.5mg/ml was prepared in 100% acetone and diluted to a final concentration of 5ng/ml or 5ppb with 0.5M sucrose. Control bees were fed sucrose/acetone solutions as a procedural control for the pesticide sample. The concentration of solvent for both the control and the exposed group was not greater than 1% v/v, as stipulated by guideline number 213 (OECD 1998) and the same concentration of solvent was present in the control and neonicotinoid groups (<0.1%). Prior to pesticide exposure all bees were starved for two hours by removing feeders and pollen from holding boxes and then moved to a perspex holding boxes where they were provided aliquots (35 µl) of clothianidin solution, accounting to a final exposure concentration of 0.175ng/bee. Bees were observed during the exposure period and were only considered exposed when they had consumed the full droplet, bees that did not consume the full aliquot of pesticide were excluded from the experiment.

The Novozymes product Met 52 (*M. anisopliae*) was maintained and prepared for infection as previously outlined (section 6.1.2). To prepare the bees for infection, individuals were removed from the holding box one at a time and anesthetised using $CO_2$. The bee was then placed on a wax platform and pinned down on its back by securing a strap across its abdomen. Once the bee was in position the body was then gently stretched using heat-flamed tweezers to expose the site of injection, the gaps between the tergites of the abdomen. In order to infect

the bees a small aliquot of each treatment (2µl) was pipetted out onto parafilm and then drawn up into the tip of a syringe. The bee was injected in the left side between the terga on the lower abdomen. Wounding was limited by allowing only the very tip of the syringe to enter the bee. Post-infection each bee was transferred to new a holding box of the same dimensions and at the same ambient conditions. All bees subjected to the same treatment were infected and housed together. For the combination exposure, workers were first exposed to pesticides (as above), and those bees considered exposed were then housed for a further 24 hours under standard conditions, after which they were treated with *M. anisopliae*.

To investigate the effect of pesticide and pathogen exposure on circulating haemocytes numbers and encapsulation activity within the haemolymph, cell counts (n = 12) and encapsulation assays (n = 9) were conducted in bumblebee workers sampled from four colonies. Total haemocyte counts (THCs) were obtained from haemolymph (10 µl), collected and diluted in 50 µl of ice cold Ringer and mixed following the methods of Moret and Schmid-Hempel, (2009). A 10 µl subsample was diluted (1:1) with trypan blue (0.4% w/v) and a 10 µl aliquot of this mix was loaded on a haemocytometer. The slide was left to stand for 5 - 10 minutes to allow the haemocytes to settle prior to counting and calculation of final concentration. The average of this count was taken as the cell concentration.

To assess the strength of the encapsulation response, nylon filaments (2 mm) were inserted into the abdomen of bees 24 hrs after treatment following the infection treatment outlined above. After 24 hrs filaments were collected from bees via dissection. All recovered filaments were then subject to a two-step fixing procedure prior to mounting. Filaments were firstly incubated for one hour in a sterile watch glass containing ethanol and then transferred to a watch glass containing xylenes for a further hour. Control filaments (non-inserted filaments) were subjected to the same fixing treatment prior to mounting. Mounting slides were modified to hold the filaments in line on the slide. To create an area on the slide to contain the filaments two coverslips were secured to the slide to create a holding gap (approx. 5 mm). Filaments were placed along this gap and Eukitt® was added (2 – 3 drops). Once filaments positions were adjusted, and air bubbles were removed a third coverslip was placed on top to seal the filaments in place. Slides were allowed to set over night at room temperature prior to imaging. Filaments were photographed using a Leica DM500 connected

to LAS EZ imaging software (version 3.3) and the grey scale index (GI) measurement was obtained in ImageJ image analysis software version 1.51j. The grey scale index (GI) measurement was used to obtain a representative value of filament melanisation. GI was measured on a scale from 0 (pure white) – 255 (pure black), hence higher amounts of melanisation correspond to higher GI measurements. To quantify the grey index (GI) of each treatment, the average value of control filaments was subtracted from the average GI of all treatment groups to account for the filaments baseline GI.

To provide a comprehensive characterisation of the *B. terrestris* fat body proteome in response to pesticides and pathogens, fat body tissue was subjected to high resolution mass spectrometry. Fat cell masses were dissected from workers (section 2.2.4) and subject to mass spec preparation, protein quantification, 2D clean-up, digestion and peptide purification (C18). For mass spec analysis 1 µg of prepared tryptic peptides sample was loaded on a QExactive (ThermoFisher Scientific) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. The peptides were separated over a 2 – 40% gradient of acetonitrile on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120 min reverse-phase gradient at a flow rate of 250 nL $min^{-1}$.for fat body. Four independent biological replicates, sampled from two colonies (Koppert Biological Systems), were analysed in this study.

Initial protein identification and label-free quantification (LFQ) normalisation of MS/MS data was completed using MaxQuant v 1.5.6.5 (http://www.maxquant.com). Data analysis and processing of the data was then performed using Perseus v 1.5.0.8. (www.maxquant.org/). To resolve protein-protein interactions and identify functions and pathways most strongly enriched within the fat body proteome STRING v 10 (http://string-db.org/) and the BlastKOALA sequence similarity tool (https://www.kegg.jp/blastkoala/) were used. To obtain a global overview of the proteome associated processes, gene ontology (GO) analysis was completed using the GO assignment application ClueGO v 2.5.1 within Cytoscape (http://www.cytoscape.org/).

## 7.2 Results

### 7.2.1 Bioassays

Investigating changes in the numbers of circulating haemocytes and strength of the encapsulation response can reveal either the immunologic efficiency, or vulnerability of these systems within the haemolymph of *B. terrestris* workers in response to exposure with pesticides and pathogens. This information will assist in the development of cellular response profiles to these stressors and will contribute to the understanding of how they detrimentally effect bees both individually and in combination.

In order to investigate the cellular mechanisms of defence in *B. terrestris* workers, post immune-challenge and exposed to toxicants, haemocytes were enumerated, and an encapsulation/ melanisation response evoked and measured using filaments. Values from both bioassays were then square-root transformed to normalise the variance and the relationship between haemocyte density or encapsulation response and immune challenge was investigated using univariate analyses of variance (ANOVA). Differences were considered statistically significant at $p<0{\cdot}05$ and groups were compared using post hoc pairwise Tukey's comparisons. All statistical analysis was completed using GraphPad Prism v. 5.0 (www.graphpad.com).

The results of the haemocyte analysis revealed decreases in circulating haemocytes in all treatments compared to naïve bees. The greatest decrease in haemocyte numbers was observed in bees treated with a combination of clothianidin and *M. anisopliae*. A slightly increased number of haemocytes was observed in *M. anisopliae* challenged groups, in comparison to those not containing an immune elicitor. No significant differences in total haemocyte count were revealed between Ringer and pathogen/pesticide samples (Figure 7.1). However, a significant decrease was observed between naïve bees and those treated with the combination of clothianidin and *M. anisopliae*.

Within the encapsulation response no significant differences were revealed between control and treatment groups (Figure 7.2). The encapsulation activity decreased slightly in both Ringer and pesticide groups and increased in *M. anisopliae* and combination treatments. The

greatest increase and decrease in this response was observed in *M. anisopliae* and pesticide groups, respectively.

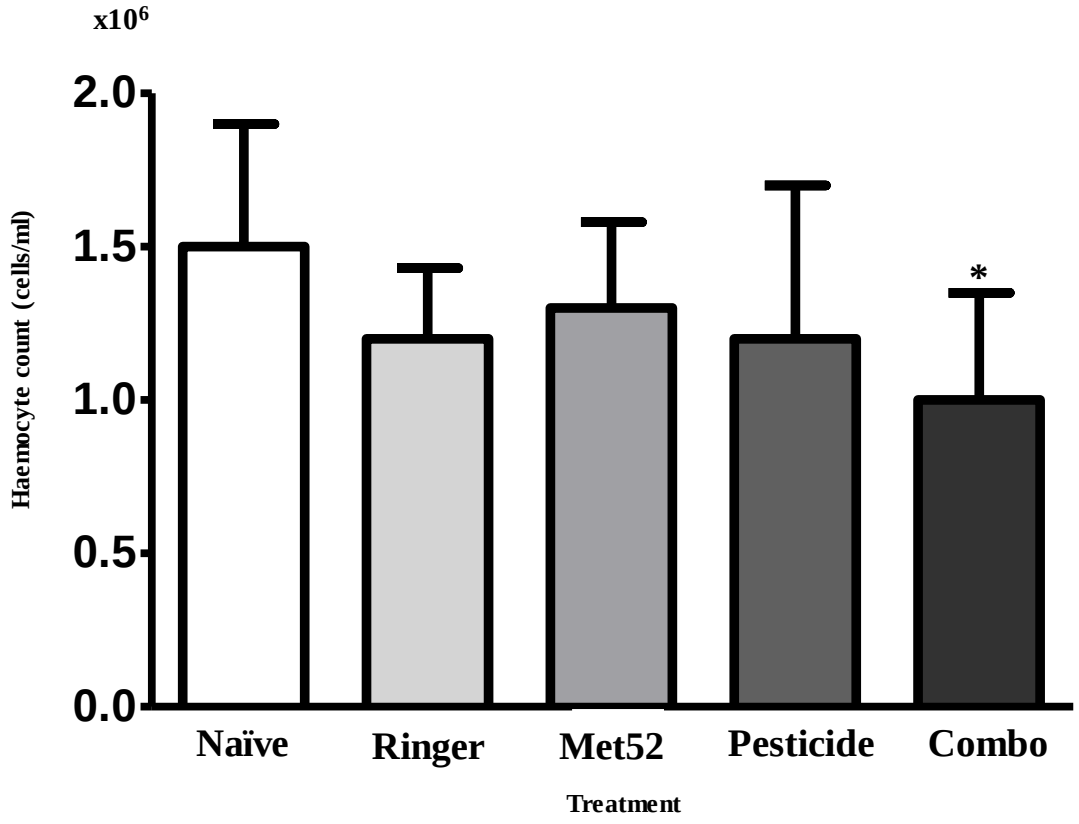


**Figure 7.1 Total haemocyte count of pesticide exposed and immune-challenged workers.** Circulating haemocytes in all treatments were enumerated and compared (ANOVA) to investigate differences in the numbers of immune cells circulating post treatment with pesticides and pathogens. Cell counts revealed decreases in all treated groups in comparison to naïve bees. The greatest decrease was observed in combination treated bees. In comparison to naïve bees this decrease was significant. (*) represents a p-value of $p<0.05$ when compared to the naïve group. Error bars represent standard deviation.

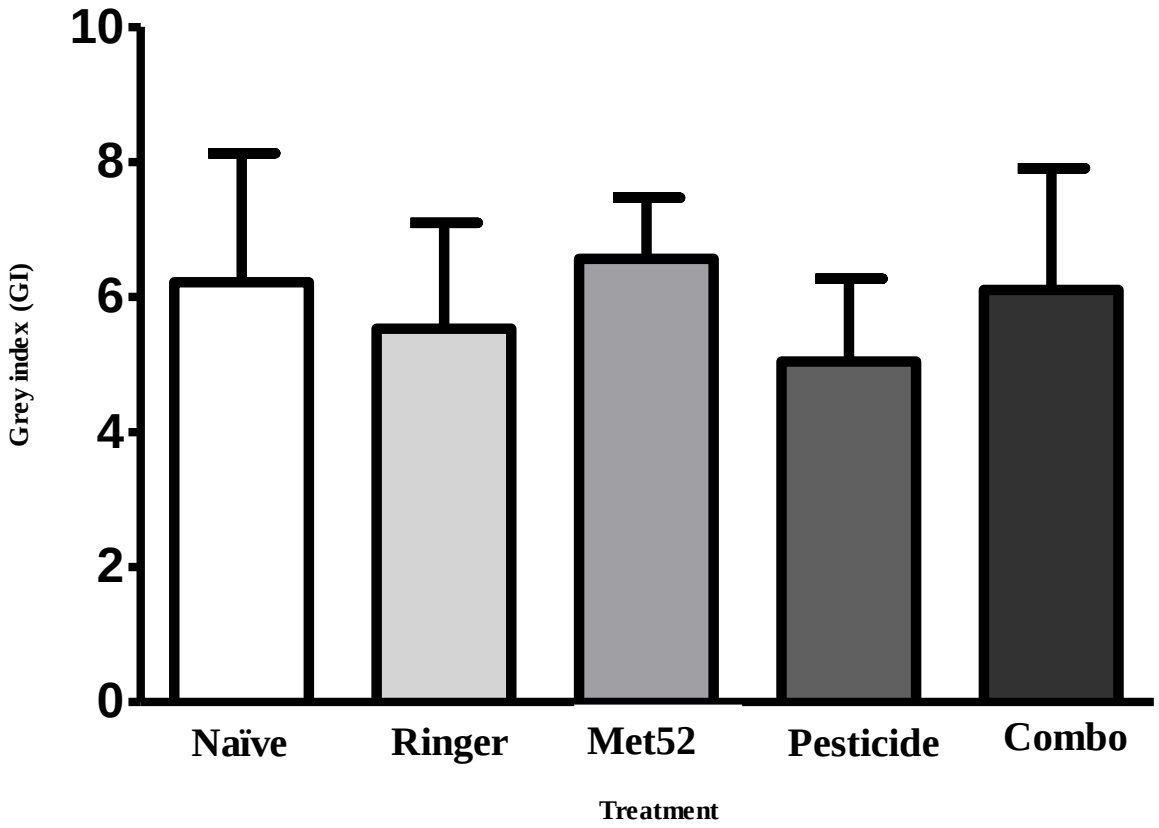


**Figure 7.2 Encapsulation response of pesticide exposed and immune-challenged workers.** The encapsulation response of all treatments was quantified and compared (ANOVA) to investigate differences in the response post immune stimulation. No significant differences were observed between the control and pesticide/ pathogen treatment groups. Notably Met52 and combination treated groups had higher levels of encapsulation activity than Ringer and pesticide groups. Error bars represent standard deviation.

### 7.2.2 Fat body response to pesticide exposure

#### 7.2.2.1 Protein identification and statistical analysis

LC-MS/MS was performed on four replicates of fat bodies from both pesticide and control treatments, this resulted in the initial identification of 2531 proteins. The data matrix was then filtered for the removal of contaminants, reverse hit peptides and peptides identified by site. LFQ intensity values were $Log_2$ transformed and proteins not found in at least three of the four replicates were removed. These filtering steps resulted in the identification of 1403 fat body proteins (Table A 7.1).

Student's t-tests were performed between the different treatments to determine statistically significant differentially abundant (SSDA) proteins in response to pesticides exposure (Figure 7.3) (Table 7.1). From this, 143 proteins were identified as SSDA in response to the presence of clothianidin exposure (Table A 7.2). Within this number, 59 were increasing and 84 were decreasing in abundance (Figure 7.3). The ten most differentially abundant proteins increasing in response to pesticide exposure included those involved in proteostasis, carbohydrate metabolism (ketohexokinase), cellular detoxification (probable phospholipid hydroperoxide glutathione peroxidase), lipid binding (apolipoprotein of lipid transfer particle-III) and cytoskeletal formation and structure (ankyrin repeat domain-containing protein 49) (Table 7.1). Among the proteins involved in proteostasis were those associated with degradation (ubiquitin-like modifier-activating enzyme and endothelin-converting enzyme 1), translation (probable splicing factor arginine serine rich 7) and transport (endoplasmic reticulum membrane protein complex).

Of the ten proteins with the greatest decreases in abundance, the majority were associated with cytoskeletal formation, structure (ankyrin, muscle M-line, titin and twitchin) and activity (calcium-transporting ATPase and sodium/potassium-transporting). The remaining proteins played roles in a diverse range of biological processes such as; carbohydrate metabolism (maltase), immunity (chymotrypsin), protein folding (putative protein disulfide-isomerase) and degradation (ubiquitin-conjugating enzyme E2 G2).

**Table 7.1 Top 10 SSDA proteins in the fat body in response to pesticide exposure.** The top 10 most/least abundant proteins within the fat body proteome after exposure to the pesticide clothianidin based on intensity listed in descending order include those involved in proteostasis, cellular detoxification, metabolism and cytoskeletal structure and activity. Fold changes highlighted red and blue represent increased or decreased abundances, respectively, in response to pesticide exposure.

| Fold change | Accession no. | Protein name |
|---|---|---|
| 3.65 | XP_003402417.1 | Ankyrin repeat domain-containing protein 49 |
| 2.76 | XP_003392943.1 | ER membrane protein complex subunit 8/9 |
| 2.29 | XP_003398753.1 | Ubiquitin carboxyl-terminal hydrolase isozyme L5 |
| 2.12 | XP_012172147.1 | Glutamate decarboxylase 2 |
| 2 | XP_012168607.1 | Ubiquitin-like modifier-activating enzyme 5 |
| 1.91 | XP_003399399.1 | Ketohexokinase |
| 1.83 | XP_012170197.1 | Endothelin-converting enzyme 1 |
| 1.8 | XP_003395542.1 | Probable phospholipid hydroperoxide glutathione peroxidase |
| 1.76 | XP_012164072.1 | Apolipoprotein of lipid transfer particle-III* |
| 1.68 | XP_012165763.1 | Probable splicing factor, arginine/serine-rich 7 |
| -5.27 | XP_003395914.1 | Maltase 1 |
| -3.88 | XP_012163879.1 | Sodium/potassium-transporting ATPase subunit alpha |
| -3.79 | XP_003393535.1 | Chymotrypsin-1 |
| -3.37 | XP_012163930.1 | Muscle M-line assembly protein unc-89 |
| -3.35 | XP_012166075.1 | Titin |
| -3.06 | XP_012172988.1 | Twitchin |
| -2.96 | XP_012163581.1 | Putative protein disulfide-isomerase* |
| -2.89 | XP_003399858.1 | calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum |
| -2.72 | XP_003402589.1 | Ubiquitin-conjugating enzyme E2 G2 |
| -2.62 | XP_012163412.1 | Ankyrin-2 |

(*) indicates those proteins that have been reannotated based on BLASTp ontology.

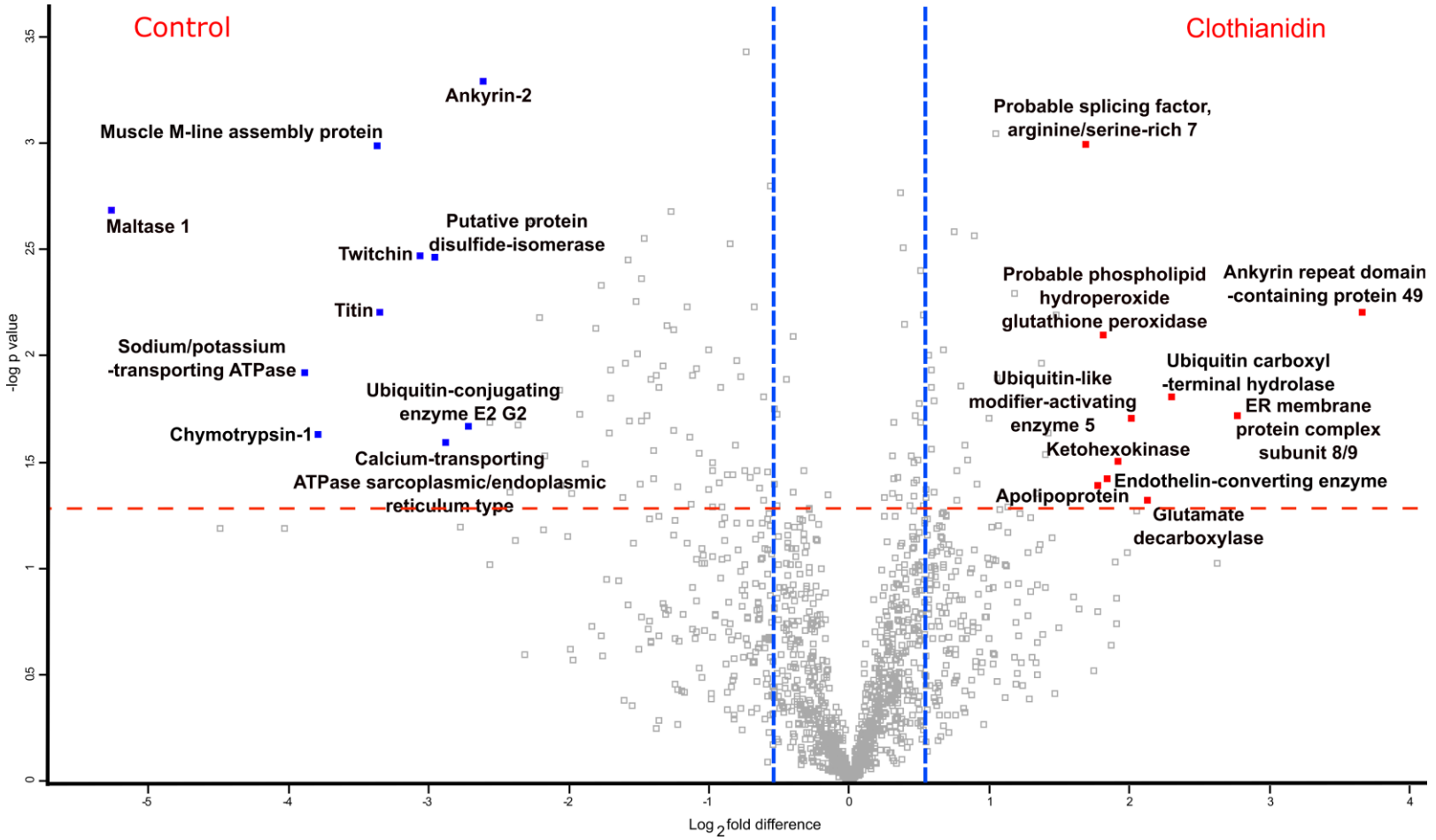


**Figure 7.3 Differential protein abundances of the fat body response to clothianidin exposure.** Volcano plot distribution of quantified proteins according to p-value (−log10p-value) and fold change (log2 mean LFQ intensity difference) in pesticide (clothianidin) in comparison to the control group. Proteins above the line are considered statistically significant (p-value <0.05) and those to the right and left of the vertical lines indicate relative fold changes ≥ 1.5. Annotated proteins represent the top ten increasing (red)/ decreasing (blue) proteins in response to wounding

### 7.2.2.2 Functional analysis

Investigation of the biological processes associated with the fat body response profile to pesticide exposure was completed using the gene ontology analysis package ClueGO, within the Cytoscape software. To complete this analysis the *D. melanogaster* genome was used as the background reference (Figure 7.4). Analysis of the proteins increasing and decreasing in abundance in response to pesticide treatment revealed the numerous biological processes activated within the FB to contend with ingestion of toxicants.

Among the proteins of increased abundance were those involved in processes and pathways such as protein degradation (protein catabolic process and protein modification by small protein removal) and carbohydrate (carbohydrate phosphorylation) and energy metabolism (pentose phosphate pathway) (Figure 7.4A). The processes represented in the decreased abundance protein set were cytoskeletal structure (myofibril assembly and muscle tissue & organ development), cell communication (cell-cell junction organisation), energy metabolism (proton transport, carboxylic acid and aerobic metabolism) and protein translation (pole plasma oskar mRNA) and transport (maintenance of protein location in the cell) (Figure 7.4B). In addition, less well-represented processes included melanin metabolism, cellular metal ion homeostasis, flight behaviour and mesoderm development.

Overall within this response 52 immune proteins were identified in the FB proteome (Table A 7.1). However, only four immune proteins were identified as SSDA in response to pesticide exposure. These included three with increased abundance (phospholipid hydroperoxide glutathione peroxidase, apolipophorin III and BMP-binding endothelial regulator) and one with decreased abundance (chymotrypsin 1). No immune function enrichment was revealed within this response profile.

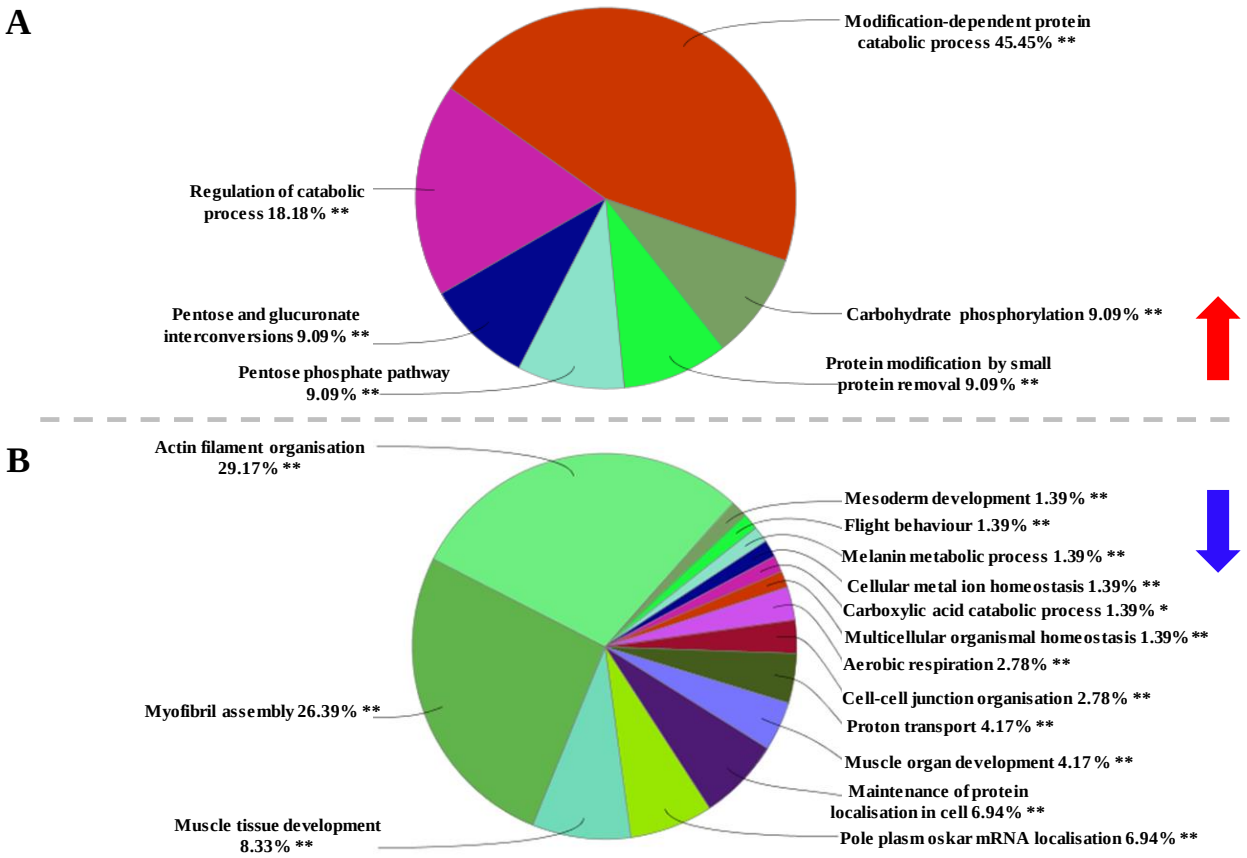


**Figure 7.4 Functional analysis of fat body response to pesticide exposure.** Functionally enriched terms provides an overview of the main biological and KEGG pathways for (A) increased and (B) decreased abundance proteins in the fat body proteome in response to pesticide response. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$ statistical levels, respectively. Values represent the percent of terms per group. Arrows indicate the direction processes are changing in response to treatments and broken line divides increase/decrease proteins.

### 7.2.3 Combinatorial response to pesticides and pathogens in the fat body

#### 7.2.3.1 Identified and quantified proteins

Following completion of the LC-MS/MS run, the raw MS/MS data files (n = 3) were imported to MaxQuant which searched the mass spectra against a protein database arising from the bumblebee genome (Sadd et al. 2015). This step identified the proteins present across all samples and calculated their relative abundance. Initially 3031 proteins were identified from the samples but with further filtering this number was reduced. Using the analysis software Perseus, the results were filtered to remove contaminant peptides, reverse peptide hits and peptides only identified by site. LFQ intensity values were then $Log_2$ transformed and only proteins found in all three of the treatment replicates were included. This resulted in the identification of a total of 1633 proteins (Table A 7.3).

#### 7.2.3.2 Hierarchical clustering

To gain a global view of the presence or absence and abundance levels of proteins between different samples hierarchical clustering was performed (Figure 7.5). Hierarchical clustering groups samples with similar protein profiles together and was completed by clustering z-score normalised average LFQ intensity values for all significant proteins (n = 121) for each sample group. From this general expression, profiles of all proteins across the treatments could be observed resulting in identification of eight clusters (Figure 7.5).

Cluster A contained 14 proteins with increased abundance in response to exposure to a combination of pesticides and pathogens. Proteins identified within this cluster participate in cytoskeletal formation (troponin, ankyrin), transmembrane active transport (plasma membrane calcium-transporting ATPase and calcium-transporting ATPase sarcoplasmic /endoplasmic reticulum), protein synthesis (RNA binding protein and elongation factor 1-alpha) and energy metabolism (ATP synthases, 2-oxoglutarate dehydrogenase and ADP, ATP carrier protein). Enrichment analysis of cluster A revealed involvement in purine nucleotide biosynthetic process and calcium ion homeostasis (Table 7.2). Cluster B comprised 31 proteins increasing in abundance in both bees treated with *M. anisopliae* and EPF and pesticides. Within this cluster proteins associated with muscle and cytoskeletal

structure (actin, myosin, myophilin, titin and sarcalumenin) were highly represented, while proteins involved in detoxification (small heat shock protein and thioredoxin) and cell differentiation and proliferation (ras-related proteins) were present to a lesser extent. Gene ontology analysis of this cluster revealed associations with striated muscle cell development and intracellular mRNA localisation involved in axis specification (Table 7.2). Proteins of decreased abundance in response to treatment with *M. anisopliae* were observed in clusters C, D and E. Cluster C contained 11 proteins associated with physiological processes such as detoxification (cytochrome P450) and energy metabolism (pyridoxal kinase, putative fatty acyl-CoA reductase and 6-phosphogluconate dehydrogenase). Within cluster D were nine proteins with involvement in the following processes; carbohydrate metabolism (transketolase), energy metabolism (ATP synthase, cytochrome b-c1 complex and 5-AMP-activated protein kinase catalytic subunit alpha-2) and DNA and RNA binding. The 16 proteins comprising cluster E included those involved in protein synthesis (eukaryotic translation initiation factor 5B) and degradation (26S protease regulatory subunit 4 and ATP-dependent Clp protease proteolytic subunit). Among the eight proteins in cluster F which represented a decrease in abundance in response to both *M. anisopliae* and combination treatment were numerous proteins with roles in oxidoreductase activity (cytochrome b-c1), and translation initiation (eukaryotic translation initiation factor 3 subunit C and M). Those proteins with decreased abundance in response to pathogen/pesticide exposure were represented by cluster G which contained 17 proteins involved in a diverse set of biological processes such as transcription (transcription elongation factor B polypeptide 1 and BTF3 homolog 4), translation (60S ribosomal protein L10), energy metabolism (phosphoglycerate kinase and NADH dehydrogenase)and immune response (neurotoxin). Cluster H comprised 15 proteins that increased in abundance in response to treatment with *M. anisopliae* including immune (IRP30 and venom serine carboxypeptidase), ribosomal (60S ribosomal protein L8) and transport (Golgi resident protein GCP60) associated proteins.

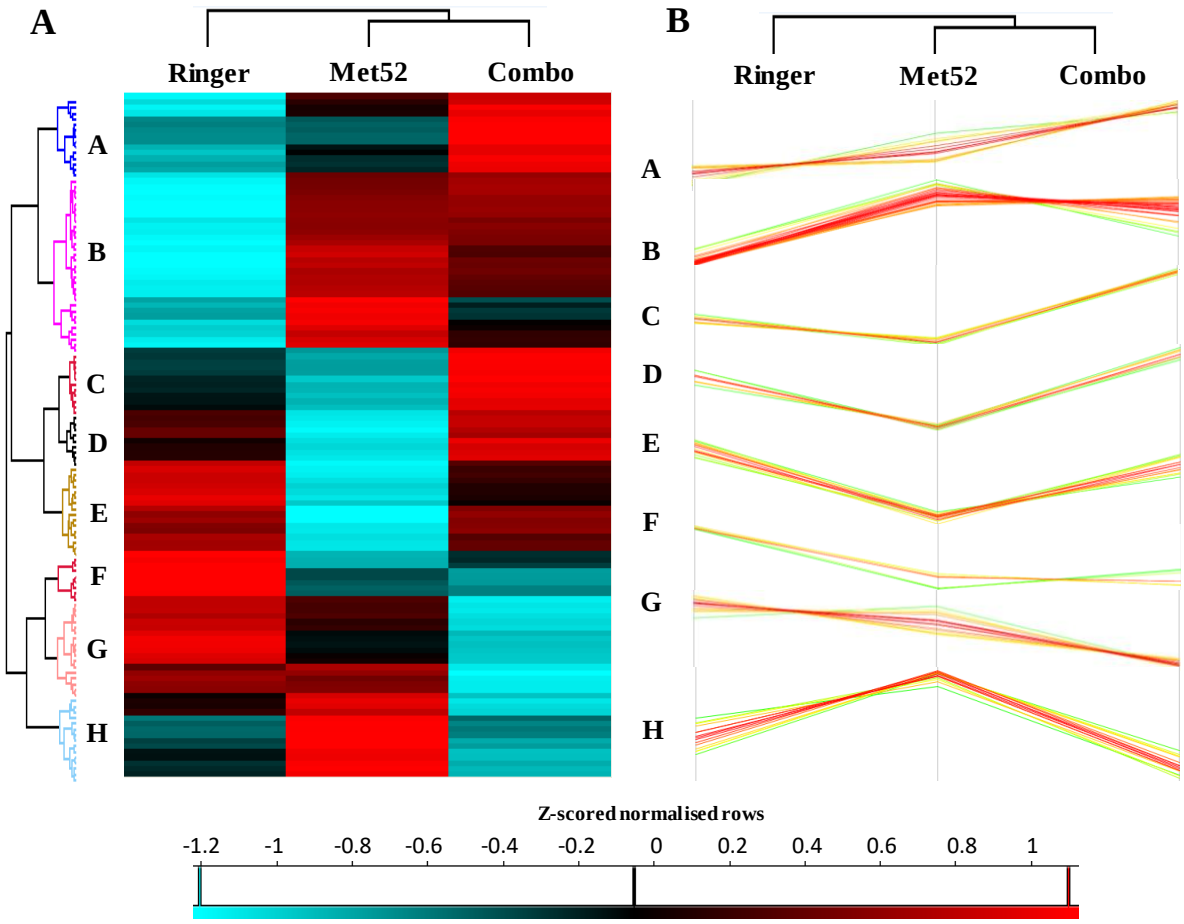


**Figure 7.5 Hierarchical clustering of FB samples.** (A) Hierarchical clustering of the mean intensity values for 121 SSDA proteins among the different samples in response to immune challenge. Heat map colours represent the relative expression of proteins in samples. (B) Cluster expression trends of identified proteins are represented by the coloured lines. These proteins have been divided into groups based on treatment specific expression profiles. Relative abundances (z-scored normalized) of individual proteins can be read from the intensity spectrum.

**Table 7.2 Significantly enriched gene ontology processes in *B. terrestris* worker FB in response to pesticide exposure.** Functional enrichment analysis of the proteins forming each response profile using ClueGO resulted in a selection of significantly enriched GOBP in each of the identified protein clusters. No enriched terms were identified for clusters C,D, E, F, G and H.

| Cluster ID | Cluster size | Type | Term | p-value |
|---|---|---|---|---|
| **A** | 14 | GOBP | Purine nucleotide biosynthetic process | 4.6 E-5 |
| | | GOBP | Calcium ion homeostasis | 1.4 E-5 |
| **B** | 31 | GOBP | Striated muscle cell development | 1.1 E-12 |
| | | GOBP | Intracellular mRNA localisation involved in axis specification | 5.6 E-4 |

#### 7.2.3.3 Statistical and protein annotation analysis

Pairwise Student's t-tests analysis identified 102 SSDA proteins ($p < 0.05$) in response to *M. anisopliae*, of which 49 and 53 had increased and decreased abundance, respectively (Table A 7.4). Within the FB response to the combined exposure with clothianidin and *M. anisopliae* 141 SSDA proteins were identified (Table A 7.5). Of this total number, 80 and 61 proteins had increases and decreases in abundance, respectively. To reveal the central biological processes involved in the fat body defence against these stressors, functional analysis of the proteins comprising each response profile was completed using the ClueGO gene ontology analysis tool (Figure 7.6).

Processes enriched within those proteins increasing in the FB in response to *M. anisopliae* included those involved in the adhesive contacts between neighbouring cells (adheren junction organisation), post-transcriptional regulation of gene expression (intracellular mRNA localisation) and development of striated muscle cells (Figure 7.6A). Within the proteins decreasing processes such as translation, cytoskeleton formation (cytoplasmic microtubule organisation) and pyridine metabolism were well-represented.

Functional analysis of proteins increasing in abundance in response to treatment with a combination of pesticides and pathogens revealed processes such as ion homeostasis, energy production (ATP biosynthetic process) and cytoskeleton formation (regulation of lamellipodium assembly and striated muscle development) are central to this response profile (Figure 7.6B). The combined treatment resulted in the decreased abundance of proteins associated with the regulation of translational initiation, centrosome localisation and long-term memory are centrally involved in this response profile.

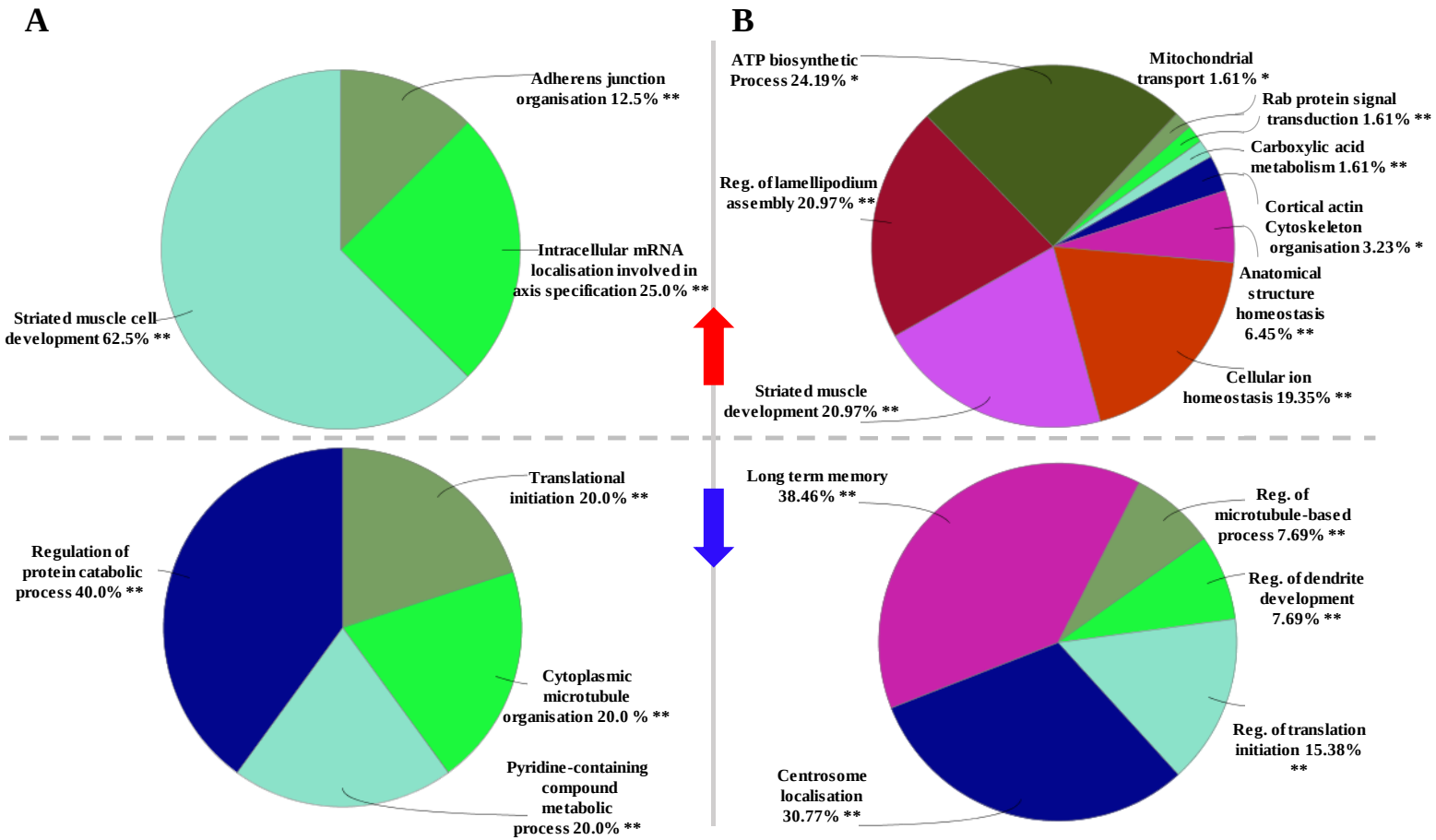


**Figure 7.6 Functional analysis of fat body response to pathogen and pesticide exposure.** Pie charts contains functional labels of the most enriched terms only and provides an overview of the main biological pathways indicated for the FB response to (A) *M. anisopliae* and (B) Combinational exposure to pesticides and pathogens. The single and double asterisks indicate significant enrichment at $p < 0.05$ and $p < 0.01$ statistical levels, respectively. Values represent the percent of terms per group. The arrows indicate the direction processes are changing in response to each treatment and broken line divides increase/decrease protein sets. Values represent the percent of terms per group.

52 immune proteins were identified in the FB proteome overall in this experiment. Of these only three and nine were revealed to be SSDA in *M. anisopliae* and combination treatment groups, respectively (Table 7.3). Among these were proteins from the following functional immune classes: antioxidant, immune modulator and immune effector. Notably, no pathogen recognition receptor-associated proteins were identified in response to either treatment. As a result of the small numbers of immune-associated proteins identified in response to *M. anisopliae* and combinatorial pesticide and pathogen treatment, it was not possible to obtain immune function enrichment values.

**Table 7.3 SSDA immune-associated proteins identified in the FB proteome response to pathogen and dual pathogen/pesticide exposure.** A variety of immune-associated proteins were identified across the response to pesticides and pathogens including antioxidants, immune effectors and immune modulators. The values for each protein represent the log2 fold change, positive values represent proteins with increased abundance and similarly negative values represent those proteins decreasing in abundance.

| Immune function | Accession no. | Protein name | Fold change | |
|---|---|---|---|---|
| | | | Combo | Met52 |
| Antioxidant | XP_003393865.1 | Cytochrome P450 9e2 | 2.44 | - |
| | XP_003400119.1 | Cytochrome P450 6a2 | 1.26 | - |
| | XP_003399611.1 | Cytochrome P450 4g15 | 1.18 | - |
| | XP_012170233.1 | Cytochrome P450 6k1 | 1.03 | - |
| | ABV48740.1 | Small heat shock protein | - | 2.01 |
| Immune effector | AEN62314.1 | IRP30 | - | 1.06 |
| Immune modulator | XP_003395653.2 | Serine protease easter* | -0.22 | - |
| | XP_012163150.1 | Neurotrypsin* | -0.25 | - |
| | XP_012173007.1 | Phospholipase A-2-activating protein | -0.38 | - |
| | XP_012172055.1 | Fragile X mental retardation protein | -0.39 | - |
| | XP_012166489.1 | Apolipoprotein D | -1.4 | - |
| | XP_003403029.1 | Toll-interacting protein | - | 0.10 |

(*) indicates those proteins that have been reannotated as per section 2.6.3.

## 7.3 Discussion

Numerous environmental and agricultural stressors have been implicated in the recent global declines in bee numbers both managed and wild. Among these stressors pathogens and pesticides are deemed to be of primary significance. The use of pesticides in crops that are also visited by pollinators is a controversial issue. On one hand, these agrichemicals are essential to protect and maintain the majority of economically valuable crops from insect pests. However, crops sprayed with pesticides include those pollinated by bumblebees and pesticide exposure has been implicated in negatively impacting bumblebee fitness by reducing the ability to mount an immune response (Alaux, Brunet, et al. 2010; Fauser-Misslin et al. 2014), forage and navigate (Stanley et al. 2016). Bumblebees are invaluable pollinators hence population losses have driven the development of pollinator friendly pest control strategies such as the development and use of EPF spores as biopesticides. The application of EPF spores to crops using sprays, yielded poor pest control results leading to the exploration of alternative application/delivery methods including entomovectoring, which reportedly result in improved pest control (Mommaerts and Smagghe 2011). The risks of this method to bumblebees have been investigated and the spore exposure concentrations which are non-toxic for these important pollinators are now known (Smagghe et al. 2013). However, most of these investigations have taken place under laboratory or greenhouse conditions and do not reflect realistic field conditions such as exposure to multiple stressors (Mommaerts and Smagghe 2011). Hence, it is important to understand how stressors which limit bumblebee fitness, such as pesticides could impact the potential pathogenicity of EPF spores.

### 7.3.1 Key findings

- It is evident that pesticides can work alone or in synergy with pathogens to alter normal cellular defence activities, such as encapsulation.
- The FB response to combinatorial exposure with both pesticides and pathogens varied considerably to the response to pathogens only, indicating pesticide exposure alters this response.
- In response to treatment with both clothianidin and *M. anisopliae*, proteins involved in immune modulation and long-term memory were among the most

abundant in the decreasing protein set. Indicating cognitive and immune functions are disrupted by neonicotinoid exposure.

### 7.3.2 Variations in haemocyte density and encapsulation activity in response to pathogen and pesticide exposure

Insect haemocytes are a central component of their cellular host defences and are essential in mechanisms which assist in neutralising pathogen threats such as phagocytosis, nodulation, encapsulation, and melanisation (Lavine and Strand 2002). The encapsulation and melanisation of intruding pathogens and wound closure are all haemocyte mediated processes (Evans et al. 2006). Haemocytes assist in wound repair and produce prophenoloxidase the precursor of phenoloxidase, the enzyme which catalyses the melanisation cascade (Korner and Schmid-Hempel 2004; Evans 2006). Enumeration of haemocytes can be used as a bioindicator of the physiological status of the bee as haemocyte number can vary dramatically in response to numerous factors such as age (colony and individual) (Moret and Schmid-Hempel 2009), stress, injury and infection (Doums et al. 2002). Similarly, encapsulation is a central cellular defence response hence quantifying the activity within the haemolymph may elude to the strength of the immune response and the degree to which encapsulation is involved. In addition, investigations in other key pollinator species such as *A. mellifera* are indicating that cellular immune competence is an important and informative parameter to study stressors and is central to developing new strategies to enhance bees' fitness (Negri et al. 2016).

Compared to naïve bees all treatments had decreased numbers of circulating haemocytes. In light of the infection method for Ringer and *M. anisopliae*, decreased cell numbers may be expected as haemocytes participate in clotting and wound healing from the inflicted injection wound (Babcock et al. 2008) and therefore would be removed from the circulating population. Decreased haemocyte numbers in fungal treated bees may be explained by the release of cytotoxic elements (destruxins) by *M. anisopliae* which help in its pathogenicity by damaging the cytoskeletal structure of haemocytes and hence altering haemocyte immune activities and inducing apoptosis (Vilcinskas and Götz 1999). However, the wound infliction hypothesis does not fit for our pesticide-exposed bees as they imbibed the treatment. Therefore, it seems that pesticides negatively impact the density of haemocytes circulating within the haemolymph, an effect previously seen in honeybees in response to field relevant doses to clothianidin ((Brandt et al. 2016)).

However, in comparison to Ringer treated bees both *M. anisopliae* and clothianidin showed slight increases in haemocytes. Overall the greatest decrease in haemocyte numbers was observed in bees treated with a combination of clothianidin and *M. anisopliae* suggesting a possible synergistic interaction between stressors negatively effects haemocyte numbers. Similar responses were observed by López et al. (2017) in honeybee larvae exposed to a sublethal dose of clothianidin in combination with *Paenibacillus larvae* spores. Interestingly the individual application resulted in an increase in haemocyte number whereas their combination resulted in significantly decreased THC. The authors hypothesised that observed increases in THC may indicate a role for haemocytes in both detoxification and bacterial clearance and combinatorial reductions may represent the cellular responses being overwhelmed as they contend with both stressors. In addition, to decreases in THC, López et al. (2017) also revealed larvae groups exposed to both stressors had a higher rate of mortality perhaps indicating a synergistic interaction between bacterial infection and pesticide exposure. It therefore seems that under combined exposure to pesticides and pathogens, bumblebee cellular defence systems are similarly affected.

No significant changes in encapsulation were identified here, which may be due to the naturally high degree of variation in melanisation response among bumblebees colonies (Doums et al. 2002; Mallon et al. 2003) or the insufficient stimulation of the immune response (Allander and Schmid-Hempel 2000). However, some general trends in encapsulation across treatment groups could be observed. In response to treatment with a fungal elicitor, encapsulation increased in comparison to the levels observed in both naïve and Ringer treated bees. Similar, increases in this response were observed in honeybees infected with the fungal pathogen *Nosema apis* (Dosselli et al. 2016), which indicates that the presence of fungal elicitors in general within the haemocoel of bees stimulates encapsulation activity. The greatest reduction in encapsulation activity was observed in bees exposed to clothianidin. Similar decreases were observed in honeybee workers exposed to clothianidin for 24 hours, indicating that pesticides have the ability to negatively impact encapsulation response even at the sub lethal field relevant doses (Brandt et al. 2016). The encapsulation response within bees exposed to a combination of *M. anisopliae* and clothianidin showed increased activity to those treated with pesticides only and slightly reduced in comparison to those treated with *M. anisopliae*. This may indicate a synergistic interaction between pathogens and pesticides on the

encapsulation activity of *B. terrestris* workers. The synergistic effects of pesticides and pathogens has similarly been shown to reduce the encapsulation activity in *D. melanogaster* larvae (Delpuech et al. 1996).

It is evident that altered haemocyte density together with a reduction in the encapsulation activity caused by clothianidin and a combination of both clothianidin and *M. anisopliae* exposure would likely impair the cellular immune defence capacity of *B. terrestris* workers and increase their susceptibility to stressors of decline, including pathogens. This work indicates that the cellular aspects of the *B. terrestris* immune response are negatively affected by sublethal, environmentally relevant concentrations of neonicotinoids as similarly observed in honeybees (Brandt et al. 2016).

### 7.3.3 *B. terrestris* FB response to treatment with the entomopathogenic fungi Met52

Proteomic analysis revealed that in response to treatment with the EPF *M. anisopliae* processes such as metabolism (carbohydrate and energy), proteostasis, cytoskeletal formation and intracellular junction activity were all centrally involved. In addition, and surprisingly, very few elements of the immune and antioxidant systems were identified within the FB response profile to EPF. In fact, the principal response involved metabolic processes and the majority of proteins with increase abundances were associated with the breakdown of carbohydrates and formation of TCA cycle precursors (acetyl CoA). This highlights the demanding energy requirement of pathogen contention, which requires a rapid and precise energy distribution to the appropriate cell or tissue for efficient self-protection (Lee and Lee 2018). However, elements of pyridine metabolism were represented among decreased abundance proteins. Pyridine nucleotides (NAD and NADP) play vital roles in metabolic conversions as signal transducers and in cellular defence systems. Both coenzymes participate as electron carriers in energy transduction and biosynthetic processes (Berger et al. 2004; Pollak et al. 2007). Hence, a decrease in these proteins may be a result of their activity in supporting energy metabolism, activity in cellular defence or as result of the immunosuppressive activity of EPFs.

Within the FB response to EPF treatment were numerous proteins involved in protein degradation, translation and transcription. Within proteins increasing in abundance, intracellular mRNA localisation was well represented. Localisation of mRNA serves several functions advantageous to protein expression in the setting of an immune response including the ability to; produce many copies of protein through repeated rounds

of translation making it a more efficient, to prevent the accumulation of protein in a location that may be harmful to the cell via translational repression, to allow a cell to rapidly respond to a stimulus without relying on transducing a signal to the nucleus by activating translation of a localised transcript and finally to serve as a scaffold for the assembly of protein complexes and function in a manner independent of simple protein translation (Blower 2013). Hence, enrichment of this process makes sense in the FB response to *M. anisopliae* as mRNA localisation can facilitate rapid production of immune active proteins such as IRP30 to participate in the defence response while maintaining intracellular homeostasis. In contrast, translational initiation was represented among proteins decreasing which may indicate a reduction in general translational activities within the cell to conserve energy or a switch to mRNA localisation as a more efficient means of protein translation. In addition to translation initiation, regulation of protein degradation was also decreased. As proteins play crucial roles in virtually all biological processes, the delicate equilibrium between their synthesis and degradation influences cellular homoeostasis (Dahlmann 2007). Protein degradation is controlled by the proteasome and through its involvement in degradation of mis-folded or damaged proteins the it participates in numerous essential cellular functions, such as protein quality control, transcription, immune responses, cell signalling, and apoptosis (Finley 2009; Schmidt and Finley 2014). Immune functions of the proteasome include degradation of products that can be used in antigen presentation to communicate the intracellular protein composition to the immune system (Finley 2009), regulation of immune cell function through degradation of inflammatory and immune mediators and degradation of damaged proteins to counteract the cytotoxic potential of damaged proteins (Kammerl and Meiners 2016). Hence, in response to EPF treatment proteolytic proteins may be reduced in order to maintain the homeostatic equilibrium of the cell or may be reduced during their activity in any of the various immune responses they act in.

Cytoskeletal components such as microtubules, actin and intermediate filaments are ubiquitous structures in eukaryotic cells (Bearer 1992). Within cells the cytoskeleton provides a structural framework serving as a scaffold that determines cell shape and general organisation of the cytoplasm. In addition to this role in structural integrity, the cytoskeleton is also responsible for cell movements. These include the movement of entire cells, but also the internal transport of organelles and other structures through the cytoplasm (Cooper 2000). In *D. melanogaster* actin-bundling proteins have been shown

to mediate several aspects of the cytoskeletal reorganization required for haemocyte migration to wound sites (Zanet et al. 2009). The actin cytoskeleton is also involved in mediating phagocytosis (May and Machesky 2001; Pearson et al. 2003). Similarly, fat body cells migrate to wound sites to participate in wound healing and to release antimicrobial peptides (Franz et al. 2018). In *M. anisopliae*-infected insects it is well documented that secondary metabolites secreted by the fungus alter and damage the cytoskeleton of haemocytes and hence inhibit their ability to perform cell movements (Götz et al. 1997) and phagocytose material (Vilcinskas et al. 1997) and can result in haemocyte apoptosis (Vilcinskas and Götz 1999). As insect fat cells perform similar cytoskeletal based immune activities, decreases in cytoskeletal processes in the fat body in response to EPF treatment may indicate cytoskeletal alterations as reported for haemocytes. Decreased abundance in these proteins in response to EPF may represent *M. anisopliae* induced damage to cytoskeleton proteins which could act to severely limit the fat cells ability to participate in cellular and humoral defensive responses. In addition, to cytoskeletal organisation, adherens junction organisation was also enriched within the EPF FB response. The adherens junction performs multiple functions including initiation and stabilisation of cell-cell adhesion, regulation of the actin cytoskeleton, intracellular signalling and transcriptional regulation (Hartsock and Nelson 2008). Hence, increased levels of adheren associated proteins may be present an attempt to rectify damage to the cytoskeleton or to assist in transcription.

Among the processes enriched within those proteins increasing in abundance in response to treatment with EPF was striated muscle cell development. Although muscle proteins are normally associated with movement and locomotion in animals, they are also been implicated in several atypical physiological processes including immune response (Chatterjee et al. 2016). Numerous insect studies have suggested that muscles might have a more direct role in immunity. In *Drosophila* muscle specific signalling pathways have been implicated in the regulation of oxidative stress (Tohyama and Yamaguchi 2010). Similarly, the expression of muscle structural genes in the initial stages of *Pseudomonas aeruginosa* pathogenesis in *Drosophila* are thought to function as a defence response (Apidianakis et al. 2005). Interestingly these muscle genes were not induced after sterile wounding, despite the fact that expression of tissue-reconstruction genes would be expected to aid in tissue repair (Apidianakis et al. 2005). Hence, the enrichment of muscle

associated processes here, may indicate their involvement in the FB defence against EPF infection.

The *B. terrestris* FB participates in various elements of immune response including pathogen recognition, detoxification, melanisation and regulation of humoral immune response. It was expected that elements of this response would be well-represented within the FB in response to *M. anisopliae* within the haemocoel. Unexpectedly, only five immune proteins were identified, all of which were increasing in abundance. However, of this number only three were significantly changing in response to this fungal elicitor. Identified immune proteins represented immune effectors (IRP30), modulators (venom serine carboxypeptidase and toll interacting protein) and antioxidants (cytochrome P450 and small heat shock). The presence of antioxidants is indicative of an insect defence strategy to protect against fungal infection through activation of detoxification systems. Through this reactive oxygen species (ROS) are produced and utilised as cytotoxic agents (Butt et al. 2016). The presence of the immune modulator venom serine carboxypeptidase may be indicative of phenoloxidase cascade induction, as similar venom serine proteins identified in the bumblebee *Bombus ignitus* have been revealed to act as prophenoloxidase activating-factors (Choo et al. 2010). Alternatively, they may act within the toll signalling pathway with the toll interacting protein. This pathway is one of the major regulators of the insect immune response (Tanji and Ip 2005), hence the presence of this toll-associated protein indicates it is involved in the *B. terrestris* antifungal response. IRP30 is a major immune responsive protein which has been revealed to be induced by bacterial infection in honeybees (Albert et al. 2011; Gätschenberger et al. 2013), similarly the identification of this protein within this response profile may indicate a role for IRP30 in the antifungal response in *B. terrestris*. The limited number of immune proteins identified here could also reflect the immunosuppressive activity of EPFs. It is known that fungi can manipulate and evade the host immune response by disrupting regulatory networks, including suppression of cytoskeleton formation and other features of the subcellular structure of host immune cells (Vilcinskas and Götz 1999). Alternatively, this reduced immune response may be a result of depletion due to their earlier use in the response or merely that the workers in this experiment were insufficiently stimulated to mount an immune response. It is difficult to determine which hypothesis is correct without further experiments looking at many timepoints and EPF doses.

### 7.3.4 *B. terrestris* FB proteome response to pesticide exposure

Exposure to field relevant doses of the neonicotinoid clothianidin resulted in changes to numerous physiological processes including proteostasis, melanin metabolism and metal ion homeostasis. However, the two processes most well-represented within this response were carbohydrate metabolism and cytoskeleton organization and assembly. Within the *B. terrestris* FB response to clothianidin exposure were numerous pathways associated with both carbohydrate and energy metabolism. This included an increase in carbohydrate phosphorylation and energy precursor activities such as the pentose phosphate pathway and pentose glucuronate interconversions. Like most animals, insects such as *B. terrestris* have the ability to store and utilise carbohydrates, proteins and fatty acids as sources of energy (Arrese and Soulages 2010; Azeez et al. 2014). This increased energy output may support the detoxification and immune responses or be a secondary method of inducing antioxidant production (Rand et al. 2015; Lee and Lee 2018). Increased ATP production may also be linked to maintenance of ion homeostasis in this energy dependent tissue wherein increases in energy precursor and production pathways leads to increased ROS production which induces enhanced expression of enzymatic and non-enzymatic antioxidants to contend with increased oxidative stress, hence contributing to the detoxification activity of the FB (Rand et al. 2015). Identification of antioxidants (phospholipid hydroperoxide glutathione and peroxidase) among the top ten proteins increasing in abundance in response to pesticides, may further indicate the role of detoxification activity in this response. Hence, the increases in energy production and detoxification in the FB may be an attempt to maintain protective, defensive and detoxification capacity in *B. terrestris* workers, an observation previously recorded in honeybees (Rand et al. 2015).

Proteins associated with cytoskeletal development and organization are among those with greatest decreases in abundance in response to pesticide exposure. These proteins are essential in numerous vital cellular processes. Within cells the cytoskeleton provides a structural framework serving as a scaffold that determines cell shape and general organisation of the cytoplasm (Bearer 1992). The observed decrease in these proteins may be as a result of increased levels of oxidative stress and subsequently ROS, causing damage to susceptible the actin cytoskeleton (Dalle-Donne et al. 2001). Damage to bumblebee fat cells and resulting apoptosis has previously been reported after exposure to low levels of environmental toxicant (cadmium 1 ppb) exposure (Abdalla and

Domingues 2015). This may indicate reduced cytoskeletal proteins represents fat cell damage which explains the reductions in numerous homeostatic processes observed in this study. In addition, these cytoskeletal proteins could be further investigated for their potential as markers and bioindicators of pesticide exposure in wild bee populations.

The FB contains numerous defence proteins such as antioxidant and immune proteins, and as such it was expected that high numbers of these central homeostatic and detoxification proteins would be identified in response to the toxicant. However, only three typical immune proteins were found, the immune modulators apolipophorin III, BMP-binding endothelial regulator and chymotrypsin. The presence of immune signalling modulators indicates a role for these proteins in toxicant response. The BMP signalling pathway is a conserved regulator of cellular and developmental processes in animals (Parker et al. 2004). In *Drosophila* this pathway is a key regulator of cellular cell growth, differentiation and morphogenesis and has been implicated in assisting *Drosophila* intestinal stem cells regenerate after damage via acute chemical or biological insults (Amcheslavsky et al. 2009; Buchon and Osman 2015). Hence, the presence of an element of this pathway may indicate similar regenerative roles in the FB to contend with oxidative stress damaged cells.

In insects it is known that apolipophorin-III participates in lipid transport and has numerous immune-associated activities (Zdybicka-Barabas et al. 2013). Lipoproteins, in addition to playing a role in lipid transport, may have protective functions. Among these are stimulation of antimicrobial peptide production, enhancement of phagocytosis and binding and detoxification of microbial elicitors (Whitten et al. 2004). Moreover, it has been suggested that cytokine-induced increases in serum lipid levels may play a role in insect defence responses by decreasing the toxicities of biological and chemical agents (Feingold et al. 1995). Identification of this protein within the FB pesticide exposure response profile may indicate a role for this protein in toxicant detoxification or associated signalling pathways. Within the proteins decreasing in abundance to clothianidin only one immune associated protein was identified, a major element of the phenoloxidase (PO) cascade, chymotrypsin. Within the PO cascade chymotrypsin is responsible for the cleavage of proPO to produce active PO which is essential for initiation of melanisation (Lu, Li, et al. 2014). Functional analysis also revealed melanin metabolism, a PO associated process enriched within proteins with reduced abundance in response to pesticides. Similar, reductions in constitutive PO activity have been

reported in the bumblebee *Bombus impatiens* after exposure to filed realistic doses (7 ppb) of the neonicotinoid imidacloprid (Czerwinski and Sadd 2017). PO activity is central to the rapid non-specific cellular responses mounted by insects in response to the presence of pathogens (Siva-Jothy et al. 2005). Hence, reductions in PO activity may represent a key mechanism by which pesticides disrupt immunocompetence in *B. terrestris* by limiting the ability of the bee to rapidly mount immune responses.

### 7.3.5 FB response to combinatorial treatment with pesticides and pathogens

Global analysis of the *B. terrestris* FB response to treatment with a combination of pesticide and EPF pathogen revealed that processes associated with energy production, translation and cytoskeleton organisation and activity are principally involved. Immune associated proteins identified within this response were limited and included only eight proteins. As previously observed in individual pesticide exposure results, combinatorial treatment resulted in similarly increased enrichment of carbohydrate and energy metabolism associated processes. In addition to metabolism, proteins associated with the formation of lamellipodium and striated muscle development were also identified. Although muscles are typically associated with movement and locomotion, recently their involvement in immunity (Chatterjee et al. 2016) and the regulation of oxidative stress has been revealed (Tohyama and Yamaguchi 2010). Hence, in the context of dual exposure an abundance of striated muscle associated proteins may be present to contend with the pathogen or to aid detoxification in response to the presence of a toxicant within the haemocoel. The enrichment of additional cytoskeletal and lamellipodia associated proteins may be indicative of fat cell movement towards the infection site and wound healing as their movement is facilitated by the formation of these actin-rich protrusions (Franz et al. 2018).

Among the protein set decreasing in response to dual exposure the following processes were enriched; long term memory, centrosome localisation and regulation of translation initiation. The reduction in abundance of proteins associated with memory and learning which supports recent studies that demonstrate neonicotinoid effects on higher cognitive function in bumblebees via disruption of neuronal homeostasis (Moffat et al. 2015). This results in impairment to learning (Williamson and Wright 2013), homing and foraging which can have larger implications on colony fitness and success (Stanley et al. 2016). Cross-talk between the fat body and brain to control foraging behaviour has previously

been reported in *A. mellifera* (Demas et al. 2011; Nilsen et al. 2011) and a similar interaction may take place in the FB of *B. terrestris* to control learning behaviour. The presence of these signals within the FB provides new insights into the wider molecular consequences of neonicotinoid exposure, in this case altering the FB potential to mediate and integrate hormones from other organs to maintain homeostatic and metabolic conditions in bumblebees (Arrese and Soulages 2010). Interestingly another higher-level process is negatively impacted by pesticide and pathogen exposure namely protein synthesis. This is observed through the down regulation of translation initiation. Clearly these basic cellular processes are distinctly altered in bumblebee fat bodies when in a stressed condition.

As with the individual treatments, the combined treatments results in the changes in abundance of relatively few immune proteins. The majority of those identified are associated with defence and antioxidant activity, with four of the nine falling into this category. Of the antioxidants revealed in this response all were cytochrome P450 variations. The identification of these proteins increasing in abundance in response to dual exposure is expected as these powerful antioxidants contend with infection (Vlachou et al. 2005) and toxicant originated oxidative stress (Terhzaz et al. 2015). All immune modulators identified within this response profile were decreasing in abundance to combinational treatment which may be as a result of their involvement in immune associated signalling or due to inhibition of their production by either pesticides (Di Prisco et al. 2013) or *M. anisopliae* (Vilcinskas and Götz 1999). In addition, it is possible that immunity is compromised due to direct or indirect toxic effects (Collison et al. 2016), or disruption of regulatory cross-talk between the nervous and immune system, which is targeted by the neonicotinoids (Demas, Adamo, & French, 2011; Di Prisco et al., 2013).

### 7.4 Conclusion

This investigation provides a comprehensive overview of the molecular composition of the *B. terrestris* fat body in response to exposure to clothianidin and *M. anisopliae* both individually and in combination. To compliment the molecular perspective and reveal characteristics of the cellular response, a number of assays were conducted to quantify haemocyte density and encapsulation activity in response to these stressors. Analysis of total haemocyte counts per treatment revealed decreases in all treatments compared to

unperturbed workers, with the greatest decrease observed in bees exposed to both stressors in combination. Encapsulation activity within exposed workers followed a similar trend with pesticide treated bees showing the lowest melanisation. Similarly, bees treated with both stressors in combination had reduced activity compared to those only treated with *M. anisopliae*. From these results it is evident that pesticides can work alone or in synergy with pathogens to alter normal cellular defence activities. This analysis indicates that cellular aspects of the *B. terrestris* immune response are negatively affected by pesticide exposure even at sublethal, environmentally relevant concentrations.

Proteomic analysis of the FB proteome revealed energy metabolism and cytoskeletal structure and organisation as the two most enriched processes altered under pesticide exposure. Energy metabolism is thought to be enriched in order to help maintain protective detoxification and stress responses, while decreases in the abundance of cytoskeletal proteins is thought to be a result of oxidative damage induced by both the pesticide and increased metabolism. Similarly, in the FB response to *M. anisopliae* energy metabolism was a central enriched activity. As defence is an energetically costly activity, energy production is increased to support and maintain an appropriate immune response. Protein synthesis associated proteins were also abundant within the fungal immune response in order to rapidly synthesise antimicrobial and immune proteins such as IRP30. Within proteins decreasing in abundance to *M. anisopliae* there were numerous cytoskeletal proteins. This decrease may represent *M. anisopliae* induced damage to cytoskeleton proteins, which may have a negative impact of the FB wound healing and immune response as fat cells utilise these structures to migrate towards wound sites where they release potent antimicrobial peptides. Hence, reduced abundance of these proteins could severely limit the fat cell's ability to participate in cellular and humoral defensive responses. The FB response to combination exposure with clothianidin and *M. anisopliae* varied considerably to the response to *M. anisopliae*, indicating pesticide exposure alters this response. Within this response as seen in both pesticide and *M. anisopliae* responses, energy metabolism was well represented among proteins with increased abundance. Similarly, to support the detoxification and immune responses. Within the proteins increasing in abundance were numerous cytochrome P450 proteins, which play an important role in both detoxification and pathogen response. Among the protein set decreasing in response to dual exposure was long term memory indicating one of the consequences of neonicotinoid exposure would be an effect on higher cognitive function

in bumblebees. In addition, all immune modulators identified within this response were decreasing in abundance which may illustrate that pesticides can inhibit activity of immune response.

Overall, despite low field relevant concentrations and acute exposure, the significant effect of insecticides on the *B. terrestris* FB proteome highlight the severe impact of these stressors both individually and in combination with pathogens on the fitness of bumblebee workers. Functional analysis of the FB response to clothianidin and *M. anisopliae* exposure revealed a broad range of biological functions are affected by these stressors. In particular, a reduction in immune activity was revealed in both bioassays and proteomic analysis. In the context of entomovectoring, these results suggest that if bees are exposed to stressors which negatively affect their immunocompetence, then the virulence equilibrium between bees and EPFs could be disrupted which could potentially result in safe spore loads becoming toxic. However, assessing the severity of this impairment will require further study. This investigation highlights the need for additional research on the molecular mechanisms by which bumblebees interact with pathogens and pesticides in order to better understand current global declines and to develop new interventions and treatments. Understanding how unperturbed healthy bumblebees process and contend with toxins under normal conditions will improve our understanding of how pesticides, on their own or in synergy with other stress factors including pathogens and parasites, lead to declines in bee fitness.

# Chapter 8

## General discussion

## 8.1 General discussion

As an important pollinator *Bombus terrestris* plays an essential role in the maintenance and productivity of both wild and commercial plant communities (Ashman et al. 2004; Aguilar et al. 2006). With global and Irish pollination services estimated at €153 billion and €53 million, respectively (Bullock et al. 2008; Gallai et al. 2009), and in an age of global food security concerns, the contribution of bees is of great importance (Potts et al. 2016). However, these valuable pollinators are currently facing unprecedented declines due to factors such as climate change, intensive agriculture, pesticide use, and the threat of pathogens and parasites (Potts et al. 2010; Goulson et al. 2015).

These observed declines, coupled with an ever-increasing reliance on bumblebee pollination services, have emphasised the need for a thorough understanding of *B. terrestris* health. In order to achieve this, we must first understand the systems that are found within the organism on the molecular and immunological levels. Hence, the primary goal of my research was to comprehensively elucidate the *B. terrestris* immune response and collect crucial information about the effects of biotic and abiotic stresses faced by our dwindling bumblebee populations.

With the majority of bumblebee studies focusing principally on environmental, behavioural and ecological aspects of bumblebee biology (Rutrecht and Brown 2008; Evans and Raine 2014; Kämper et al. 2016; Mobley and Gegear 2018) there is a significant gap in our knowledge regarding the molecular aspects of the defence mechanisms in this organism. Although numerous studies exist examining the interaction of bumblebees and pathogens (Imhoof and Schmid-Hempel 1999; Larsson 2007; Otti and Schmid-Hempel 2007; Rutrecht and Brown 2008; Baron et al. 2014; Blaker et al. 2014), little is known about the molecular mechanisms of those responses (Mallon et al. 2003). Due to the highly conserved nature of the innate immune system most studies use comparisons with model insects (*Apis mellifera and Drosophila melanogaster*) to propose and define aspects of the bumblebee immune response; however, comparisons are only useful for initial guidance and description, as these systems have major differences. Relying on comparative immunology alone results in a limited view of an organism's immune composition and the majority of *B. terrestris* immune studies are based on a selection of 20 – 30 immune proteins identified within these model organisms. However, investigating changes in this set of immune proteins alone as a measure of *B.*

*terrestris* immunocompetence may be highly biased and fail to account for species-specific immune complexities. Whilst some studies have made significant efforts to identify the immune component of bumblebees (Korner and Schmid-Hempel 2004; Erler et al. 2011; Barribeau and Schmid-Hempel 2013; Barribeau et al. 2015; Sadd et al. 2015), very little work exists that attempts to unravel the molecular mechanisms underlying bumblebee cellular and humoral defences.

It is widely acknowledged that disease is a major driver of global bee decline and a number of methodologies are available to assess immunocompetence and immune response in bees to a wide variety of microbial challenge. These methods include haemocyte counts, fat body (FB) quantification, encapsulation response, phenoloxidase activities, inhibition-zone assays and immune gene/RNA quantification using qPCR or RNASeq technologies (Allander and Schmid-Hempel 2000; Korner and Schmid-Hempel 2004; Moret and Schmid-Hempel 2009; Erler et al. 2011; Riddell et al. 2011; Brunner et al. 2013). Very little however is known about the immune response on the translational level, which is surprising given that the functional elements of the genome and the components that will ultimately respond to or contend directly with invading microbes are proteins. The research presented in this thesis therefore was based on extensive analysis of haemolymph and FB proteomes from infected and uninfected bees, which resulted not only in novel and significant insights into the diversity and complexity of the bumblebee immune response but also the mechanisms and pathways the control, fuel and support that response.

Bee haemolymph has been previously shown to be an excellent medium to study and examine elements of immunity, but also to ascertain the overall health status of the animal (Chan et al. 2006; Chan et al. 2009). However, the research presented here, represents (to the best of my knowledge) one of the first determinations of the processes, cells and proteins employed by *B. terrestris* in defence against a wide range of pathogens. The experiments based on the fat body resolved completely novel aspects of the bumblebee immune response and provide a valuable insight into mechanisms of disease pathology and resistance in this highly social organism. Elucidating the exact mechanisms of this response will provide a platform upon which future molecular and immune studies of this organism can be based. To complement the molecular perspective of the bumblebee immune system a cytological and histo-pathological perspectives were obtained through the examination of fat body and haemolymph cellular composition.

Infection studies with general pathogens were conducted to determine the typical immune response to individual classes of micro-organisms. Non-species-specific pathogens (*Escherichia coli*, *Staphylococcus aureus* and *Metarhizium anisopliae*) were chosen to provide a general view of pathogen response, in comparison to using naturally occurring bumblebee pathogens, such as *Crithidia bombi* and *Nosema bombi*, where understanding the basic immune response may be impeded by the adaptions and commensal interactions that are often observed in species-specific host-pathogen relationships. In addition, the microbial elicitors chosen have been shown to stimulate central insect immune pathways (Lemaitre et al. 1996; Lemaitre et al. 1997; Gregorio et al. 2001) and hence induce a full spectrum immune response. This facilitated the identification of pathogen associated global changes in both expected and novel immune proteins. The nature of these bee-pathogen interactions were further evaluated in bees exposed to sub-lethal doses of the neonicotinoid pesticide clothianidin to evaluate levels of context dependent virulence.

A prerequisite to investigating the immune response within the haemolymph and FB of bumblebees was a comprehensive and detailed characterisation of the physiological profiles of both central immune components. Proteomic analysis of the FB and haemolymph facilitated in-depth characterisation of the typical biological processes and functions within unperturbed bees. Within insects the FB is often described as the control centre of nutrient storage and utilisation (Arrese and Soulages 2010). Our analysis in *B. terrestris* confirmed this association and presented a global metabolic view of the bumblebee FB as one of constant monitoring, breakdown and recycling of nutrients. In addition to metabolism, numerous key regulatory processes ranging from immunity and defence to protein production and degradation were revealed to be enriched within the FB. The presence of numerous immune and haemolymph proteins was also revealed within the FB, which in combination with protein synthesis and transport implicates the FB as a principal site of synthesis of these key *B. terrestris* immune proteins. The immune function of the FB was also confirmed through gene ontology analysis which revealed its role in processes such as signalling, melanisation and the humoral immune response. In addition to the proteome, the morphological structure of the FB was characterised via microscopic analysis revealing that the *B. terrestris* worker FB is composed of adipocytes and a high proportion of oenocytes, which form a parietal layer of fat cells just below the

cuticle. These structural features are seemingly unique to workers and differ to those previously observed in queens of the same species (Votavová et al., 2015).

Profiling the *B. terrestris* haemolymph proteome revealed a highly proteolytic and immune active serum which participates in central physiological processes such as carbohydrate metabolism, protein degradation and cellular transport. The presence of numerous elements of carbohydrate metabolism and protein degradation in the haemolymph is expected, given its physiology function as the central medium for the transport and exchange of nutrients, hormones and waste materials between tissues and organs (Chan et al. 2006; Klowden 2007). Functional analysis revealed strong associations between the haemolymph and immune associated processes such as melanin metabolism, wound healing and detoxification. To investigate the cellular component of haemolymph, haemocytes, cytological analysis was conducted to determine the cell types present within *B. terrestris* serum. Although numerous studies have previously quantified the numbers of haemocytes (Korner and Schmid-Hempel 2004; Moret and Schmid-Hempel 2009), this investigation is the first to differentiate the populations of haemocytes found within *B. terrestris*. Cytological analysis revealed three distinct haemocyte populations in *B. terrestris* worker haemolymph; spherulocytes, oenocytoids and plasmatocytes all of which have roles in cellular processes such as melanisation, capsule formation and phagocytosis (Jiang et al. 1997; Lavine and Strand 2002; Soares et al. 2013; Kwon et al. 2014).

Comparisons between the FB and haemolymph proteomes revealed a number of common biological functions such as carbohydrate metabolism, detoxification, the proteasome and pathogen recognition receptor (PRR) signalling. A high proportion of immune proteins and numerous peptidases and inhibitors that participate in functions including immune signal modulation and proteolysis were also present in both the haemolymph and fat body cells. This strong correlation in immune associated functions may indicate the participation of the *B. terrestris* FB and haemolymph in immune cross-talk and mediation. Further analysis of the differences between both proteomes revealed that the FB has an abundance of antioxidants, whereas haemolymph is rich in immune effectors. The abundant levels of FB antioxidants suggests high production rates (or perhaps storage) reflecting their importance in the immune response and in the protection against the high levels of ROS produced within this highly metabolically active tissue. The

presence of large numbers of immune effectors within the haemolymph is expected as they can be rapidly transported to the site of infection or wounding when required.

The quantitative analysis of the *B. terrestris* FB proteome facilitated the global discovery and in-depth analysis of the immune signatures and biological processes associated with stress responses (bacterial challenge and wounding) in this important pollinator. Interestingly, a number of the most differentially abundant processes in response to wounding and infection are also those that are most enriched within the FB, including carbon metabolism, oxidative phosphorylation, protein processing in the endoplasmic reticulum, ribosomes and the proteasome. A common observation in both responses (bacterial challenge and wounding) was the enrichment of numerous energy associated processes including carbohydrate, lipid and amino acid catabolic pathways. This switch from anabolic to catabolic metabolism may be indicative of the FB fuelling increased energy production through degradation of these precursors to support the associated stress, immune and healing processes. This link between immune response and metabolic processes is known as immunometabolism (Mathis and Shoelson 2011) and similar infection-induced energy responses have been reported in *D. melanogaster* (Dionne et al. 2006; Chambers et al. 2012; Chakrabarti et al. 2014; Lee et al. 2018). The production of energy in microbe challenged bees was further evidenced by the increase in proteins associated with oxidative stress (that would protect cells and tissues from ROS by-products of energy production) and the metabolism of key glucogenic and ketogenic amino acids, particularly tryptophan. Tryptophan is a key intermediate metabolite for a wide range of anabolic or energy production processes, and its regulation could potentially represent a key mechanism for controlling the switch from routine to immune fuelling metabolism in bees and hence indicate the activity of immunometabolism coordination within the FB. Of significant interest was the observation that lipid droplets in the FB increased in number and size under microbial challenge highlighting a potential route for mobilising the energy required to mount the immune response through lipogenesis. Therefore, it seems that there is complex but highly dynamic molecular and cellular responses in bees, regulated by mechanisms that essentially result in the rapid production of fuel to contend with infection and wounding in this case. This demonstration of the importance of energy production and mobilisation in the response to microbial challenge or wounding induced stress, highlights one of the most important aspects of my research with respect to overall bumblebee health and declining

populations. If bees are not sufficiently resourced in terms of nutrition their ability to contend with even minor infections will be compromised. As we remove more and more natural habitats and reduce the availability of floral resources for our pollinators, we will have greater numbers of malnourished bees unable to contend with routinely encountered abiotic and biotic stresses.

In addition to metabolism, numerous immune elements were also represented within the FB response to *E. coli* and wounding including antioxidants, PRRs, immune effectors and immune modulators. The presence of bacteria within the haemocoel resulted in increased expression/abundances of all PRRs, the AMPs hymenoptaecin and defensin and phenoloxidase (PO) numerous immune modulators and antioxidants. Interestingly, without the microbe (wounding control) the identified PRRs and AMPs had decreased abundance, highlighting the specificity of the response and molecular recognition of the invading pathogen. Only two immune proteins increased in response to wounding; the immune modulator omega conotoxin and heat shock protein 70 kDa. It seems therefore that although a pathogen specific response can be observed, wounding itself triggers immune elements that may reflect the bee's system anticipating that wounding may be accompanied by infection at some point. The eventual recognition of the microbe then initiates an amplified and pathogen specific response. This association of immune elements with wounding alone, to my knowledge has not previously been demonstrated for *B. terrestris*.

To improve our understanding of immune response to a wider range of pathogens, bumblebee workers were challenged with blastospores of the commonly used entomopathogenic fungus (EPF) and biopesticide *M. anisopliae*. Similar, to *E. coli* and wounding, the principal response involved metabolic processes and the majority of proteins with increase abundances were those associated with the breakdown of carbohydrates and formation of TCA cycle precursors (acetyl CoA). Proteins decreasing in abundance in response to EPF included numerous cytoskeletal and structural components. These decreases may represent *M. anisopliae* induced damage to cytoskeleton proteins. Cytoskeletal proteins are central to the motility and immune activity of both haemocytes and fat cells (Zanet et al. 2009; Franz et al. 2018). In the FB they facilitate the migration of fat cells to wound sites to participate in wound healing where they release AMPs (Franz et al. 2018). Hence their decreased abundance in the FB

could act to severely limit the ability of fat cells to participate in cellular and humoral defensive responses.

Within the FB response to EPF a limited set of immune proteins were identified compared to those previously identified in response to both *E. coli* and wounding. The five identified immune proteins had increased abundance with only three of these proteins significantly changing in response to this fungal elicitor; IRP30, toll interaction protein and small heat shock. The toll pathway is one of the major regulators of the insect immune response (Tanji and Ip 2005), and the presence of this toll-associated protein indicates involvement of this pathway in the *B. terrestris* antifungal response. IRP30 is a major immune responsive protein which has been revealed to be induced by bacterial infection in honeybees (Albert et al. 2011; Gätschenberger et al. 2013). Similarly, the identification of this protein within this response profile may also indicate a role for IRP30 in the antifungal response in *B. terrestris*. The presence of antioxidants, such as small heat shock protein is indicative of an insect defence strategy to protect against fungal infection through activation of detoxification systems (Butt et al. 2016). The limited number of immune proteins identified here could reflect the immunosuppressive activity of EPFs as it is known that fungi can manipulate and evade the host immune response by disrupting regulatory networks, including suppression of cytoskeleton formation and other features of the subcellular structure of host immune cells (Vilcinskas and Götz 1999). These immunosuppressive qualities in bumblebees could present a challenge in using *M. anisopliae* as a biopesticide in pollinator dependent landscapes.

Given that the haemolymph is in direct contact with contents of the haemocoel (including invading microbes) and the demonstration that a considerable proportion of the haemolymph proteome originates from the FB, it was decided to focus on this fraction in terms of immune response. Haemolymph also represents a more accessible sample in comparison to FBs (that require dissections) and an additional pathogen (*S. aureus*) was included to provide an overview of response to fungi and both Gram-positive and Gram-negative bacteria. Proteomic analysis of microbial treated haemolymph revealed quantitatively different signatures of protein abundance in response to the three treatments. The response to *E. coli* comprised numerous central homeostatic and immune-related biological processes, such as generation of energy, protein processing, organisation of structural and cytoskeletal processes and immune processes. The majority of these processes were also enriched within the FB response, indicating a strong

connection among both (homeostatic and immune) physiological components. As outlined in the FB *E. coli* response the combination of increased detoxification proteins and energy metabolism may perform the dual role of supporting energy requirements of immunity and inducing increased detoxification to contend with pathogen induced oxidative stress. In addition to energy, haemocyte-associated processes such as cytoskeletal organisation, were also abundant suggesting that there is a strong cellular element to the haemolymph response to Gram-negative bacteria, which may help contribute to the maintenance of homeostatic processes, such as tissue repair and immune surveillance. Although numerous immune proteins were identified within this response, the majority had decreased abundances including those associated with the defence response to Gram-positive bacteria and fungi, protein processing and toll signalling. The decrease in central immune pathways (toll) and response may be indicative of these proteins being activated and used in the response to *E. coli* or may suggest an alternative or novel signalling and pathway response within *B. terrestris* to the presence of bacteria. One immune protein which had increased abundance in response to *E. coli* was the AMP hymenoptaecin which had a similar response profile in the *E. coli* FB and *S. aureus* haemolymph response profiles indicating a conserved function against a diversity of bacteria.

The haemolymph profile in response to *S. aureus* infection contained numerous cytoskeletal and structural proteins which, also outlined in the *E. coli* response, may represent elements involved in homeostatic and immune cellular activities, such as the maintenance of haemocyte shape, proliferation, and transport of molecules. Similarly, to *E. coli*, energy metabolism, detoxification and a combination of immune elements are central to mounting a pathogen immune response. In addition to energy processes, those associated with translation were also enriched which may be indicative of the production of immune effectors such as AMPs after downstream signalling. In addition, translation proteins associated with the proteasome were abundant within the *S. aureus* response profile. These processes are only increased in response to *S. aureus*, perhaps indicating their induction as Gram-positive specific response. The enrichment of translation associated processes may be indicative of the production of immune effectors such as AMPs after downstream signalling, whilst the proteasome may be involved in the degradation of proteins damaged as a result of *S. aureus* infection or in amino acid recycling to fuel energy metabolism.

In the haemolymph it was revealed that in response to fungi, numerous defence processes decreased in abundance which may represent immunosuppressive activities of EPF. In contrast within the FB-EPF response toll proteins increased in abundance, indicating haemolymph-based initiation via these proteins hence they are depleted. Their abundance in the FB may therefore be a result of this initiation and activity in the toll signalling pathway or may represent increased synthesis of toll proteins awaiting secretion into the haemolymph to replace the depleted levels. Similarly, to the FB response, energy metabolism was also enriched with the haemolymph EPF response profile.

To complement the proteomic analyses, the cellular composition of the *B. terrestris* worker haemolymph was also assessed. Haemocyte counts and quantification of the encapsulation response revealed characteristics of the cellular response to each treatment, including a significant increase in the numbers of circulating haemocytes in response to microbial presence, confirming their importance in cellular immune activities. With regards to encapsulation activity, a slight increase was observed in response to bacterial treatments. However, this was not significant indicating that it may not be a principal mechanism engaged in pathogen clearance.

Whilst biological factors such as infection by pathogens and parasites are undoubtedly involved in bee decline, it is also evident that agrochemicals such as pesticides are also a major contributory factor (Sanchez-Bayo and Goka 2014). Recent studies revealed that exposure to sublethal doses can detrimentally affect bee fitness potentially increasing susceptibility to other stressors such as pathogens (Alaux, Ducloz, et al. 2010; Fauser-Misslin et al. 2014). Currently, the majority of investigations on pesticide exposure in bumblebees focus on quantifying the behavioural effects (Morandin et al. 2005; Whitehorn et al. 2012; Fauser-Misslin et al. 2014; Stanley et al. 2016), meaning there is a lack of molecular studies and hence understanding of this response. To gain a global perspective of the FB response to a neonicotinoid pesticide, fat cells from clothianidin exposed bees were analysed. Numerous physiological processes were identified within this response, however, the two processes most well-represented were carbohydrate metabolism and cytoskeleton organization and assembly. Similar to the FB response to wounding, *M. anisopliae* and *E. coli*, an increase in carbohydrate metabolism and energy precursor activities was revealed. In addition to energy metabolism, numerous antioxidants (cytochrome P450) were identified among the top ten proteins increasing in

abundance in response to pesticides. Therefore, it was hypothesized that the increases in energy production and detoxification in the FB may be an attempt to maintain protective, defensive and detoxification capacity in *B. terrestris* workers, an observation previously recorded in honeybees (Rand et al. 2015). As similarly observed in the FB response to the EPF, cytoskeletal and structural proteins were decreasing in response to pesticide exposure. Toxicant induced damage to the cytoskeletal proteins of FB cells has previously been reported in bumblebees (Abdalla and Domingues 2015). Hence, the observed decrease in these proteins may be as a result of increased levels of oxidative stress as a result of toxicant exposure and subsequently ROS, causing damage to the susceptible actin cytoskeleton (Dalle-Donne et al. 2001). Pesticide exposure also had a significant effect on immune protein abundance. Within this response the only typical immune proteins identified were immune modulators and all were decreasing in response to pesticide exposure. Of these the most notable was chymotrypsin, a key protein in the phenoloxidase cascade. In addition to decreased abundance of this protein melanin synthesis was also revealed to be decreasing which taken together indicate that the potential for melanisation activity is limited. This could be a potential mechanism by which pesticides disrupt immunocompetence in *B. terrestris*.

Analysis of the combinatorial effect of pathogens and pesticides on the FB identified proteins associated with the formation of lamellipodium and striated muscle development. Although muscles are typically associated with movement and locomotion, recently their involvement in immunity (Chatterjee et al. 2016) and the regulation of oxidative stress has been revealed (Tohyama and Yamaguchi 2010). Hence, in the context of dual exposure, an abundance of these proteins may be present to contend with the pathogen or to aid detoxification in response to the presence of a toxicant within the haemocoel. Within the protein set decreasing in response to dual exposure the most notable enrichments were in protein synthesis and long-term memory-associated proteins. The reduction in abundance of proteins associated with memory and learning supports recent studies that demonstrate neonicotinoid effects on higher cognitive function in bumblebees via disruption of neuronal homeostasis (Moffat et al. 2015). This also potentially indicates for the first time in *B. terrestris* a novel cross-talk between the FB and brain to control learning behaviour. Learning impairments can disrupt homing and foraging which has larger implications on colony fitness and success (Williamson and Wright 2013; Stanley et al. 2016). The other central process negatively impacted by

both pesticide and pathogen exposure was protein synthesis. An increase in this process is observed in FBs of pesticides exposed bees hence, the decrease observed in response to combinatorial exposure may indicate a synergistic effect of these stressors on basic cellular processes such as protein production. Overall it is clear that neonicotinoid exposure, alters the FB's potential to mediate and integrate hormones from other organs to maintain homeostatic and metabolic conditions in bumblebees (Arrese and Soulages 2010).

As with the individual treatments, the combined treatment results in changes in abundance of relatively few immune proteins. The majority of those identified were associated with defence and antioxidant activity. The predominant type of antioxidants revealed in this response were cytochrome P450 variations. Their observed increases is expected as these powerful antioxidants contend with infection (Vlachou et al. 2005) and toxicant originated oxidative stress (Terhzaz et al. 2015). In addition to antioxidants, a variety of immune modulators were also revealed decreasing in abundance in response to combinatorial exposure, however these decreases could be a result of any of the following scenarios; (i) a result of their involvement in immune associated signalling, (ii) inhibition of their production by either pesticides (Di Prisco et al. 2013) or (iii) as a result of *M. anisopliae* immune suppression (Vilcinskas and Götz 1999). Assessment of cellular immune activities in response to pesticides and pathogens similarly revealed altered haemocyte density, together with a reduction in the encapsulation activity, which would likely impair the cellular immune defence capacity of *B. terrestris* workers and increase their susceptibility to stressors of decline, including pathogens. This pesticide induced immunocompromisation could have major implications for global bee health with pathogen prevalence on the rise.

Overall the research presented in this thesis revealed that pathogen and pesticide exposure has a significant effect on the *B. terrestris* FB and haemolymph proteomes, highlighting the severe impact these stressors, both individually and in combination, have on the fitness of bumblebee workers (Figure 8.1). Globally within the FB response to these stressors, energy metabolism and detoxification are central. Immune proteins are abundant within the FB, but not all are centrally involved in immune responses, instead it appears that the FB has the ability to produce stressor specific immune panels with AMPs, such as hymenoptaecin and defensin, involved in the bacterial response and IRP30 central to fungal responses. Notably, no immune effectors were identified in the

FB pesticide response profile which, in combination with a reduced diversity of immune proteins, indicates the effects of toxicant induced immunosuppression.

Functional analysis of *B. terrestris* worker haemolymph revealed this complex serum is centrally involved in a number of biological processes ranging from metabolism to immunity. Global gene ontology analysis of the proteome revealed the diverse biological processes haemolymph participates in, such as carbohydrate metabolism, defence response, detoxification, negative regulation of endopeptidase activity, melanin metabolism and response to wounding. As expected for the haemolymph, numerous immune proteins were identified including AMPs, signal modulators and PRRs, some of which were pathogen specific.

The enrichment of both energy and detoxification proteins throughout both haemolymph and fat body responses to stressors (chemical and biological) highlights the demanding energy requirement of pathogen contention, which requires a rapid and precise energy distribution to the appropriate cell or tissue for efficient self-protection (Lee and Lee 2018). The immune response is an energy dependent process and these increased energy metabolism proteins and processes may represent an attempt to meet the demands of maintaining an immune response (Lee and Lee 2018). The high levels of detoxification proteins within this response are also expected as the bee attempts to contend with reactive oxygen species (ROS) produced as a result of increased metabolism (Bayir 2005) or the physiological stress induced by infection (Kodrík et al. 2015).

In light of the global declines attributed to stressors such as pesticides and pathogens this thesis provides the first global insight into the pathways, processes and proteins centrally involved in the *B. terrestris* stress and immune responses. Analysis at functional level has provided novel insights into the bumblebee immune system and facilitated a basic understanding of the combinatorial effects of multiple stressors on bumblebee systems. Due to their novelty and level of insight, the results of this thesis have numerous potential applications such as development of disease biomarkers and improvements to bee health and may contribute the evidenced based research necessary to develop sustainable agrichemical legislation and wild and commercial bumblebee management strategies.

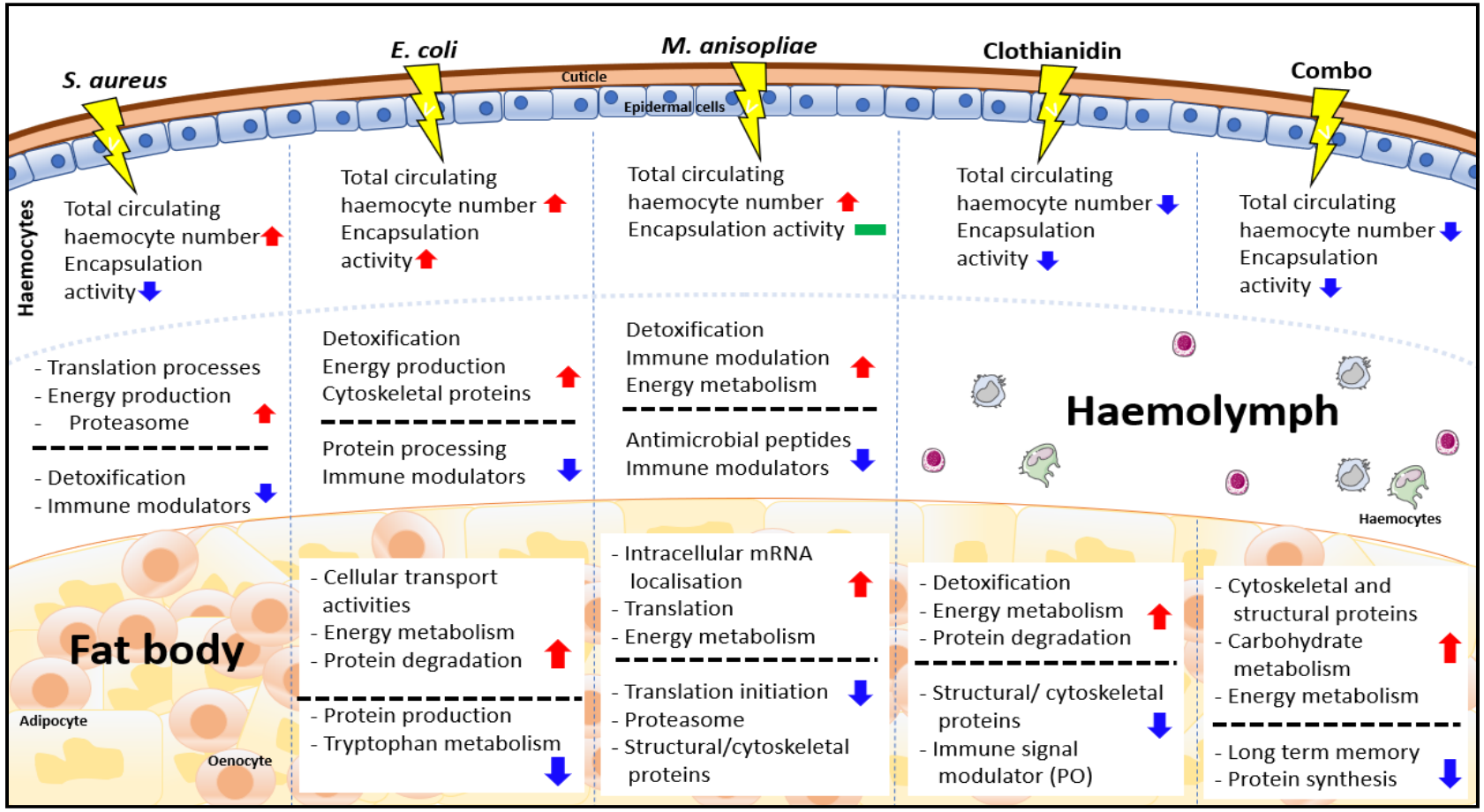


**Figure 8. 1 Overview of all proteomic and cellular results.** Overall this research reveals the variety of cellular and molecular changes which occur within the worker FB and haemolymph in response to treatment with pathogens and pesticides, both individually and in combination. Symbols represent the following results, red arrows represent increases, blue represent decreases, green vertical lines indicate no observed change and yellow lightning bolt indicates treatment.

## 8.2 Overall key findings

- The FB and haemolymph are both highly proteolytic and immune active fractions. Numerous conserved proteins and processes identified between them indicates elements of cross talk among these two central fractions.
- Energy metabolism and detoxification processes are central to both the haemolymph and FB stressor (chemical and biological) response. Increased energy metabolism highlights the demanding energy requirement of pathogen contention, which requires a rapid and precise energy distribution to the appropriate cell or tissue for efficient self-protection. Increased detoxification may be required to contend with increased oxidative stress from either elevated energy metabolism or pathogen infection.
- Tryptophan is a key intermediate metabolite for a wide range of anabolic or energy production processes, and its regulation could potentially represent a key mechanism for controlling the switch from routine to immune fuelling metabolism in bees and hence indicate the activity of immunometabolism coordination within the FB.
- Lipid droplets in the adipocytes of the *B. terrestris* FB increased in number and size under microbial challenge highlighting a potential route for mobilising the energy required to mount the immune response through lipogenesis.
- Pesticide exposure even at sublethal, environmentally relevant concentrations can work alone or in synergy with pathogens to alter normal cellular defence activities.

## 8.3 Future research hypotheses

The research conducted in this thesis is predominantly discovery-based, hence hypotheses on the components and signatures of the *B. terrestris* immune response were absent throughout, and I decided, instead to focus on clear experimental and analytical objectives. This decision was based in part on the biased nature of previous immune focused research upon which to build hypotheses and the fact that we know very little about the overall responses in bumblebees to microbial challenge. Through the completion of the non-biased discovery-based investigations presented throughout this thesis, we now have a knowledge platform upon which to build useful hypotheses on the

*B. terrestris* immune response. Listed below, therefore, are areas of future research that can build upon the framework of *B. terrestris* immunity built in this thesis, upon which we can now base specific hypotheses to test;

- Within the haemolymph immune response to microorganisms, a variety of conserved proteins were revealed. I recommend that this group is utilised to build a target panel of conserved proteins within the haemolymph in order to conduct a comprehensive temporal investigation of the *B. terrestris* immune response to microbial elicitors. Establishment of a complete proteomic temporal immune response could reveal key insights into the immune mechanisms employed within *B. terrestris*.
- Based on cytological characterisations, three distinct populations of circulating haemocytes were identified within the *B. terrestris* haemolymph. Further functional investigation of these cell types would provide a novel insight into the cellular immune capabilities of these cells and their potential contribution to immune response. In addition, once functionality is established, differential haemocyte counts in bees exposed to pathogens and pesticides could provide further insight into the cellular immune activities involved in these responses.
- A prevailing conservation across this research was the association of energy with immune response in both the FB and haemolymph. Future studies focusing on the link between these important systems could provide important insights into the centrality of immunometabolism in bumblebee immunocompetence. Specifically, the regulatory mechanisms that control tryptophan metabolism could be investigated as the central mechanism for switching between standard metabolism and immunometabolism.
- The lipid droplets in *B. terrestris* adipocytes increased in size in response to Gram-negative bacterial infection and I anticipate that similar increases would be observed in response to Gram-positive and fungal infection. Lipid droplet size should therefore be examined in response to numerous pathogens and parasites and evaluated as a potential cellular biomarker of disease.
- Detoxification proteins were highly abundant and numerous within the FB proteome of unperturbed bees and in response to infection and xenobiotic exposure. A key aim of future work would be to elucidate the centrality of detoxification proteins to *B. terrestris* immunocompetence.

- Although the acute and relatively low field relevant doses of clothianidin used to expose bees in this thesis resulted in low level suppression of key immune proteins, it is our hypothesis that after prolonged exposure (chronic) or increased concentrations (> 5 ppb) the immunosuppression would impact the cellular and humoral immunocompetence of workers more severely. This could result in the identification of clear biomarkers of xenobiotic stress.

# Chapter 9

## Bibliography

## 9.0 Bibliography


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# Chapter 10

## Appendix

## 10.0 Appendix

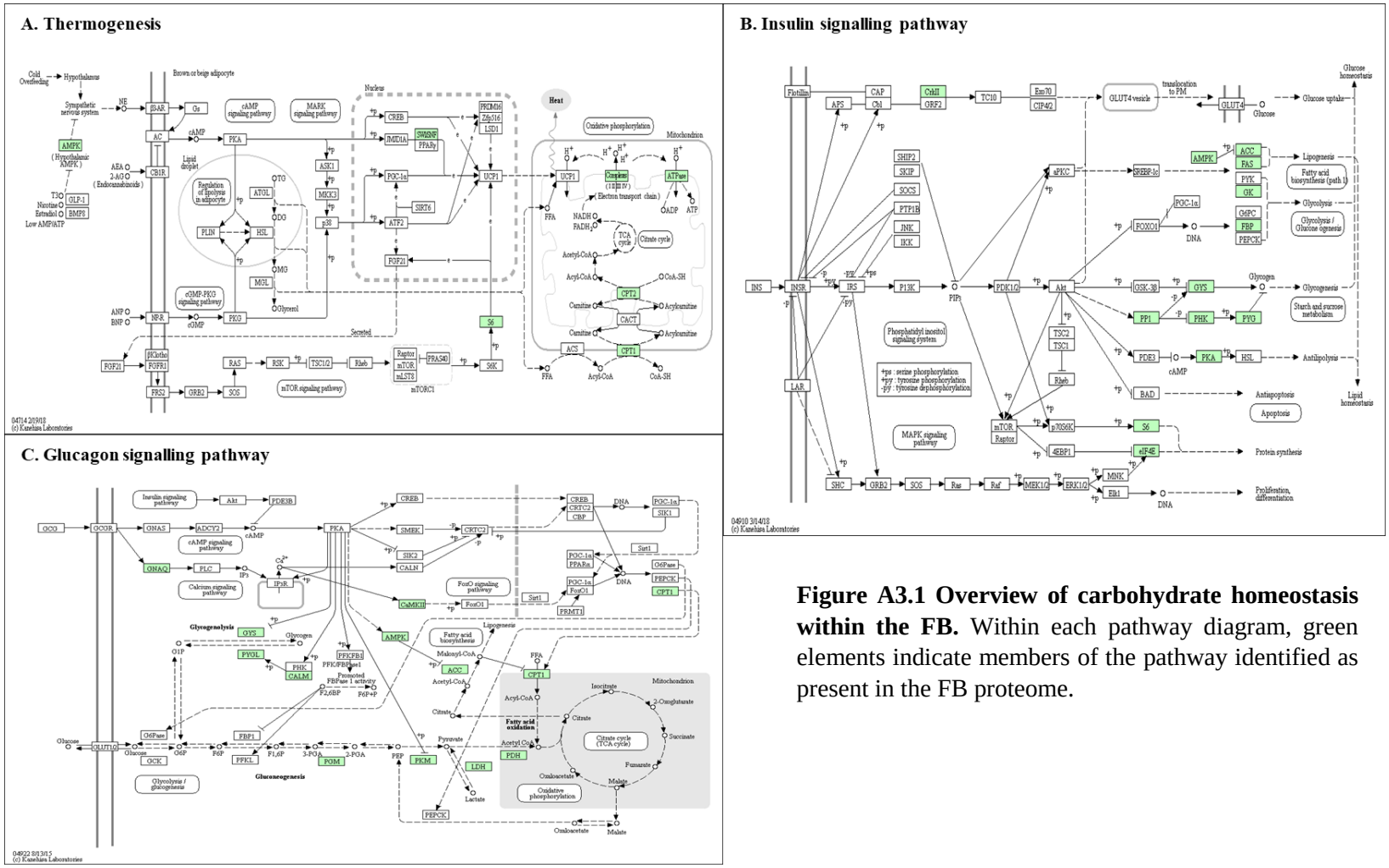
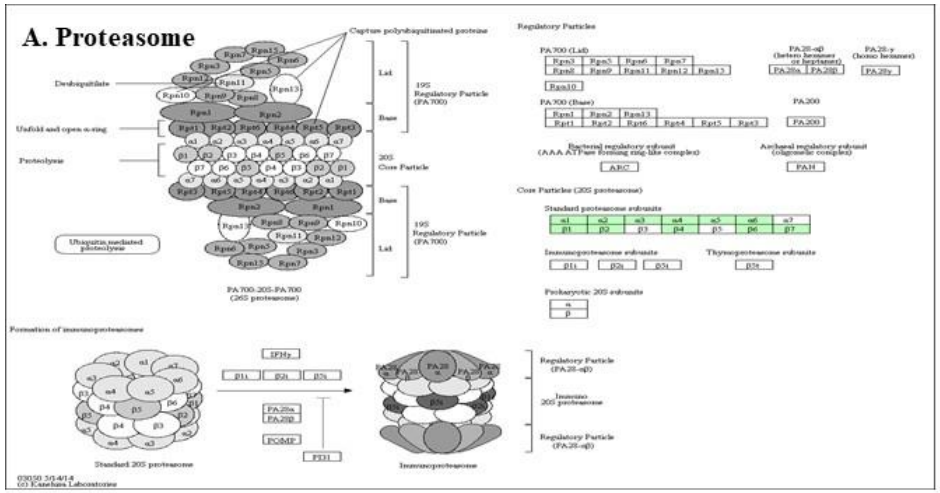
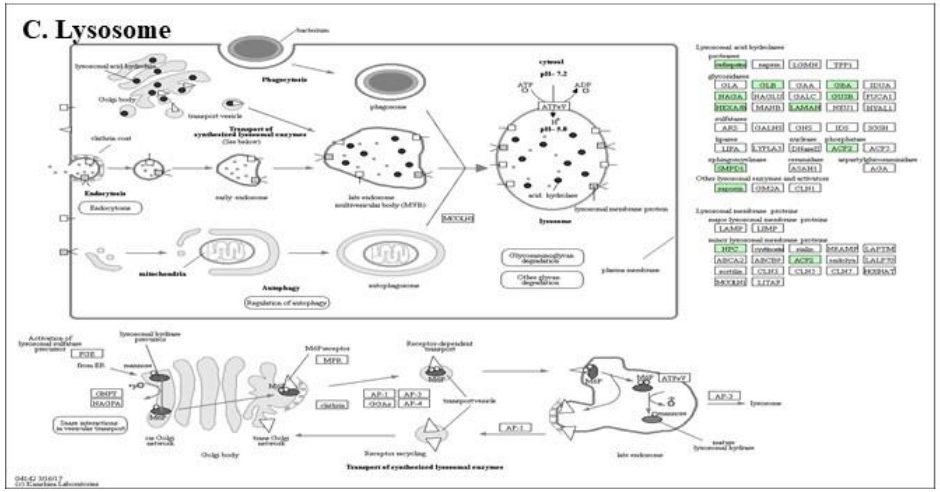



**Figure A3.1 Overview of carbohydrate homeostasis within the FB.** Within each pathway diagram, green elements indicate members of the pathway identified as present in the FB proteome.



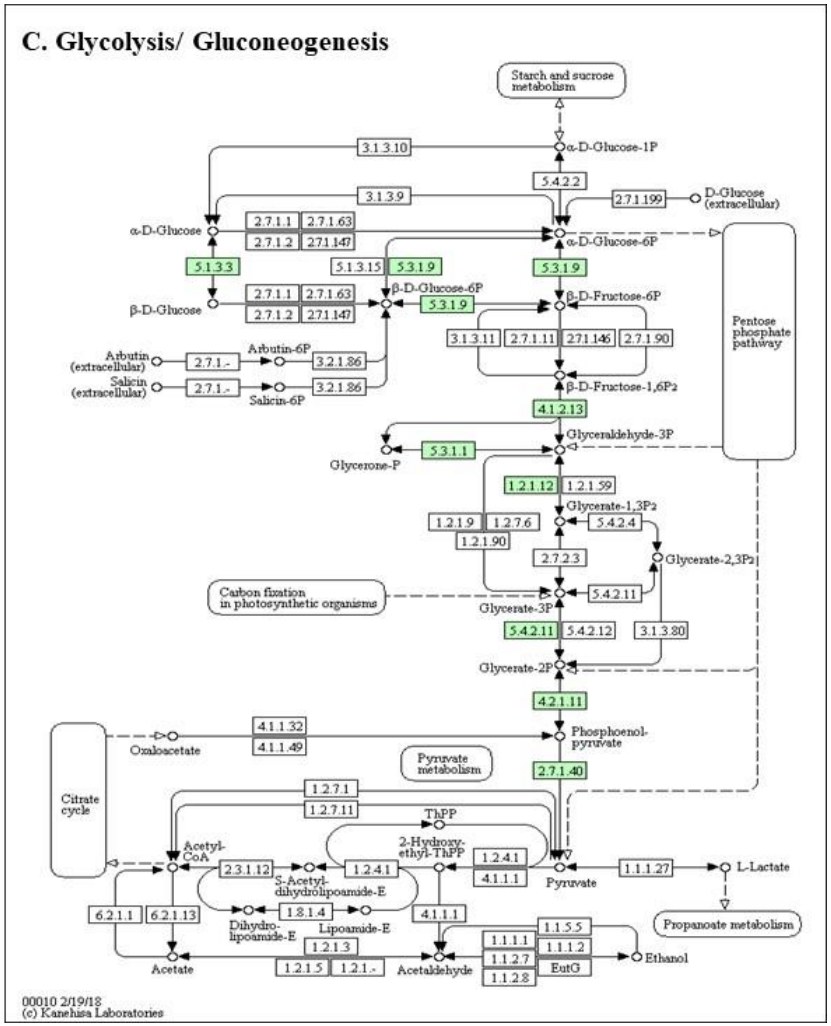




**Figure A 4.1 Top three biological pathways enriched within the haemolymph proteome.** Pathways are as follows; (A) proteasome, (B) lysosome and (C) glycolysis/gluconeogenesis. Elements of pathways highlighted green indicate proteins from that pathway present in the haemolymph proteome.

**The following appendix tables are located on the disc provided at the back of thesis or at the link below:**

- https://drive.google.com/drive/folders/1L59XJA33OO3PmBbTy5s30sAa9TAtsyJK?usp=sharing

**Chapter 3**

Table A3.1 All proteins identified in the FB proteome.

Table A3.2 All KEGG processes identified in the FB proteome.

**Chapter 4**

Table A 4.1 Proteins identified within the haemolymph proteome.

**Chapter 5**

Table A 5.1 List of all identified proteins in the FB proteome in response to *E. coli*.

Table A 5.2 List of all SSDA proteins in response to *E. coli* (*E. coli* versus Ringer).

Table A 5.3 List of all SSDA proteins in response to wounding (Ringer versus Naïve).

**Chapter 6**

Table A 6.1 All identified proteins in haemolymph proteome in response to microbial treatment.

Table A 6.2 All SSDA proteins identified in response to *E. coli.*

Table A 6.3 All SSDA proteins identified in response to *S. aureus.*

Table A 6.4 All SSDA proteins identified in response to *M. anisopliae.*

**Chapter 7**

Table A 7.1 All proteins identified within the FB proteome in response to pesticide exposure.

Table A 7.2 All SSDA protein identified in response to pesticide exposure.

Table A 7.3 All proteins identified within the FB proteome in response to pesticide exposure.

Table A 7.4 All SSDA protein identified in response to treatment with *M. anisopliae*.

Table A 7.5 All SSDA protein identified in response to a combination of pesticide and pathogen exposure.