Investigating the effects of non-insecticide pesticides widely used in Irish agriculture on the bumblebee *Bombus terrestris* (L. 1758)

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by

Merissa George Cullen, B. Sc.

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Supervisor

Dr James Carolan,

Dept. of Biology,

Maynooth University,

Co. Kildare,

Ireland

Head of Department

Prof Paul Moynagh,

Dept. of Biology,

Maynooth University,

Co. Kildare,

Ireland

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Publications

Cullen, M.G., Bliss, L., Stanley, D.A., and Carolan, J.C. (2023). Investigating the effects of glyphosate on the bumblebee proteome and microbiota. *Science of The Total Environment, 864*, p. 161074.

Cullen, M.G., Thompson, L.J., Carolan, J.C., Stout, J.C., and Stanley, D.A. (2019). Fungicides, herbicides and bees: A systematic review of existing research and methods. *PLOS ONE, 14(12),* p. e0225743.

Oral Presentations

9th Maynooth Biology Research Day, 2022 - 'Is weedkiller affecting bees? An insight into the *Bombus terrestris* gut & pesticide risk assessment'

Irish Mass Spectrometry Society Meeting, 2021 - 'Profiling the effects of active ingredient and commercial formulated glyphosate on the brain and gut proteome of the bumblebee *Bombus terrestris* using mass spectrometry-based proteomics'

Irish Pollinator Research Network Meeting, 2021 - 'A comparative analysis of active ingredient and commercial formulated glyphosate on the brain and gut proteome of the bumblebee *Bombus terrestris*'

Environ, 2020 - 'A comparative analysis of active ingredient and commercial formulated glyphosate on the brain and gut proteome of the bumblebee *Bombus terrestris*'

SETAC Europe, 2020 - 'An analysis of glyphosate on the brain and gut proteome of the bumblebee *Bombus terrestris*'

Irish Pollinator Research Network Meeting, 2020 – 'A molecular analysis of the effects of active ingredient and commercial formulated-glyphosate on the bumblebee *Bombus terrestris*'

Maynooth University Three Minute Thesis Competition, 2019 – 'The effects of herbicides and fungicides on *Bombus terrestris* in Irish Agriculture'

8th Maynooth Biology Research Day, 2019 - 'A preliminary assessment of the effects of glyphosate on the bumblebee gut'

Maynooth Biology Post-Graduate Seminar Series, 2019 – 'Assessing hazards of pesticide use in Ireland for pollinators'

Irish Pollinator Research Network Meeting, 2019 - 'Preliminary results on the impact of glyphosate on *Bombus terrestris*'

Poster Presentations

EURBEE 9, 2022 – 'A comparison of glyphosate active ingredient and a glyphosate-based commercial formulation on the digestive tract proteome and microbiota of *Bombus terrestris*'

Outreach

RTÉ Brainstorm, 2022 – Guest article titled 'Memo for gardeners: pesticides are bad news for bees'

Maynooth University Biology Undergraduate Summer School, 2019 – Lecture titled **'**Pesticides 101: Advantages and disadvantages of pesticide use'

Maynooth University Women in STEM Society, 2019 – Guest lecture titled 'From B.Sc. to Ph.D.'

Foundations in Science Writing and Communication Elective, Maynooth University, 2019 – Guest lecture titled 'Pesticides and bees in Ireland'

Awards

Environmental Sciences Association of Ireland - Best Oral Presentation at Environ 2020

Peer-Review

Scientific Reports, Nature Publishing Group. ISSN: 2045-2322

Bulletin of Entomological Research, Cambridge Core. ISSN: 1475-2670

Conference Organisation

Maynooth University Biology Undergraduate Summer School, 2019 & 2022 – Co-founded, chaired, and organised in 2019, and acted as Post-Graduate Advisor for the organising committee in 2022.

Conference & Workshop Attendance

EURBEE 9, 2022 – Belgrade, Serbia

9th Biology Research Day, 2022 – Maynooth University, Maynooth, Ireland

Irish Mass Spectrometry Society Meeting, 2021 – Online

Startup Entrepreneur Experience, 2021 – Workshop by Spin Up Science, Online

Irish Pollinator Researchers Network Meeting, 2021 – Online

Environ, 2020 – Online

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Presentation Skills in an Online Environment, 2020 – Workshop by Michael Comyn, Maynooth University, Online

Python Programming for Biologists, 2020 – Royal Society of Biology, Glasgow University, Online

Irish Pollinator Research Network Meeting, 2020 – Maynooth University, Maynooth, Ireland

8th Biology Research Day, 2019 – Maynooth University, Maynooth, Ireland

Irish Pollinator Researchers Network Meeting, 2019 – DCU, Dublin, Ireland

Environ, 2019 – Carlow Institute of Technology, Carlow, Ireland

Boosting Your Bioinformatics Toolkit, 2019 – Workshop by Dr Andrew Lloyd, Carlow Institute of Technology, Carlow, Ireland

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Declaration

I have read and understood the Departmental policy on plagiarism.

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Signature:  ……

Merissa George Cullen

Date:……..09/06/2023……

Abbreviations

16S – 16S ribosomal RNA

2D – Two-dimensional

ABC - ATP-binding cassette

AKH - Adipokinetic hormone

AMP - Antimicrobial peptide

ANOSIM – Analysis of similarity

AOP - Adverse outcome pathway

Bp – Base pair

CASK - Calcium/calmodulin-dependent protein kinase

DAG - Diacylglycerol

DDA – Data dependent acquisition

DIA – Data independent acquisition

DMI - Demethylation inhibitor

EBI - Ergosterol biosynthesis inhibitor

EC - European Commission

EFSA - European Food Safety Authority

EPA - Environmental Protection Agency

EPPO - European Plant Protection Organisation

EU - European Union

FADH - Flavin adenine dinucleotide (FAD) + hydrogen (H)

FDR – False discovery rate

GABA - Gamma-aminobutyric acid

GAI – Glyphosate active ingredient

GCF – Glyphosate commercial formulation

GO – Gene ontology

GST – Glutathione S-transferase

HPLC - High pressure liquid chromatography

ITS – Internal transcribed spacer

Kb – Kilobase

kDa - Kilodalton

KEGG - Kyoto Encyclopedia of Genes and Genomes

LC - Liquid chromatography

LD50 - Lethal Dose 50

LFQ - Label-free quantitative

Lsd1 – Lipid storage droplets surface-binding protein 1

m/z – Mass to charge ratio

mRNA - Messenger RNA

MRPP - Multi-response permutation procedure

MS - Mass spectrometry

ms - milliseconds

MS/MS - Tandem mass spectrometry

mtDNA - Mitochondrial DNA

NADH - Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)

PAGE – Polyacrylamide gel electrophoresis

PAI – Prothioconazole active ingredient

PCA - Principal component analysis

PCF – Prothioconazole commercial formulation

PCoA - Principal coordinates analysis

PCR – Polymerase chain reaction

Ppm - parts per million

PPP – Plant protection product

RFLP - Restriction fragment length polymorphism

ROS - Reactive oxygen species

rRNA – Ribosomal RNA

SD – Standard deviation

SILAC – Stable isotopic labelling with amino acids in cell culture

SOD – Superoxide dismutase

SSDA - Statistically significantly differentially abundant

Ssp – Subspecies

STRING - Search Tool for the Retrieval of Interacting Genes/Proteins

TAE – Tris-acetate-EDTA

TMT – Tandem mass tagging

tRNA – Transfer RNA

UDP - Uridine diphosphate

Wt – Weight

Abstract

Although pesticides are a key driver of bee decline globally, the contribution of non-insecticidal pesticides, specifically herbicides and fungicides, is poorly understood. The herbicide glyphosate and the fungicide prothioconazole are amongst the most widely used pesticides in Ireland. Consequently, characterising their impact on Ireland's wild bee species is of urgent importance. Here, I investigated and characterised the impact of glyphosate and prothioconazole on the bumblebee *B. terrestris* at the molecular and organism level, in addition to two representative commercial formulations: Roundup Optima+® and Proline®, containing glyphosate and prothioconazole, respectively. Utilising mass spectrometry-based proteomics, DNA amplicon sequencing, and survival assays, I uncovered the impact of these pesticides and formulations on the digestive tract, brain, and fat body proteome, digestive tract microbiota, survival, behaviour, and food consumption in *B. terrestris*.

Neither pesticide altered survival or food consumption, but prothioconazole altered behaviour at field-realistic concentrations. Further, all treatments led to microbiota dysbiosis. Glyphosate and Roundup Optima+® consistently altered oxidative stress regulation and mitochondrial proteins in all organs and led to decreases in structural proteins in the digestive tract. Both glyphosate-based treatments altered synaptic transmission and signaling in the brain, and protein biosynthesis and energy homeostasis in the fat body. However, differential impacts were also observed. Further, prothioconazole and Proline® had differential impacts on all key organs, indicating the impact of co-formulants in formulations and solvents used for pesticide solubility on bees, leading to significant alterations to detoxification, neurotransmitter biosynthesis and cytoskeletal proteins, and oxidative stress in the digestive tract, brain, and fat body, respectively. Overall, this research uncovered the impacts of glyphosate and prothioconazole, as well as representative formulations, on *B. terrestris*, and raised important questions on the complexities of pesticide impacts on bees when used as part of a formulation.

Chapter 1
General Introduction

1.1 Insect Pollinators and Their Global Importance

Pollination is the process of pollen transfer from the male anther of a flower to the female stigma, where downstream ovules in the female ovary can be fertilized by nuclei from pollen grains, and the production of seeds (with or without fruit), can take place, resulting in the plant reproduction. For some plants, self-pollination is a viable option, but for many, self-pollination can lead to inbreeding depression in a population and result in less-than-ideal phenotypes such as reduced pollen load, fewer flowers and smaller seeds and fruit (Tuohimetsä *et al.*, 2014; Walker, 2020; Dung *et al.*, 2021). Cross-pollination between different plants of the same species allows for a new combination of genes and prevents inbreeding depression. One caveat for the plant is that a vector is required to transport pollen from one plant to the stigma of another. Over 10% of angiosperms rely on wind as a vector for pollination, and whilst wind pollination has evolved independently more than 65 times, it requires the production of much more pollen than animal-pollinated plants to produce the same number of seeds (Linder *et al.*, 1998; Ackerman, 2000; Walker, 2020). Approximately 87.5% of plants are pollinated by animals, the overwhelming majority of which are insects (>99%) (Ollerton *et al.*, 2011; Walker, 2020). Globally, an estimated $235-577 billion in revenue is generated from insect pollinated crops such as apples, strawberries and almonds (Gallai *et al.*, 2009; Potts *et al.*, 2017; Klein *et al.*, 2018). In addition, insect pollinators are vital for natural ecosystem functioning, contributing to the reproduction of many flowering plants (Ollerton, 2017; Klein *et al.*, 2018). More than 98% of flower-visiting insects belong to four groups: Lepidoptera, Coleoptera, Diptera and Hymenoptera, and 60% of Hymenoptera species visit flowers, including bees.

There are over 20,000 bee species which span every continent - except for Antarctica - and most bees have a unique mutualism with many animal-pollinated angiosperms,

as dissimilar to most insects, bees consume nectar and pollen throughout their entire lifecycle (Goulson, 2010; Walker, 2020).

Bees consume nectar through their proboscis, a mouthpart which can suck up nectar and forks into many parts at the tip which can be used to break up and separate food. Bees can indirectly collect pollen via pollen accumulation on the bees' body from contact with anthers and, as the bee visits different flowers, pollen can be transferred to different plants of the same species. Many bees will groom themselves to collect pollen for their own consumption or to nutritionally support their developing offspring (eusocial queens and solitary bees) or other members of the colony (eusocial bees). However, there are areas of the body which are difficult to reach and groom, which increases the likelihood that pollen grains in these areas will pollinate flowers visited in the future. Additionally, many bees collect pollen in pollen baskets or corbiculae, which are part of the hind leg tibia. Here, pollen grains are compacted for transport back to the colony or nest. In addition, bees are uniquely suited pollinators due to their ability to detect floral markings only visible in ultraviolet wavelengths which indicate where pollen and nectar are stored as well as the ability to learn and remember the shapes of flowers and detect various olfactory signals from floral resources with suitable pollen and nectar rewards (Gould, 1985; Hammer and Menzel, 1995; Gumbert, 2000; Deisig *et al.*, 2001; Orbán and Plowright, 2013). Whilst this is beneficial to the bee to avoid plants with low nutritional resources, it is also of benefit for plant reproduction to earn consistent visits from suitable pollinators (Goulson, 2010). Regardless of these benefits, bees may get more than they bargained for when foraging, ingesting pathogenic microorganisms and pesticide-contaminated pollen and nectar, which could have detrimental consequences (Krupke *et al.*, 2012; McArt *et al.*, 2014; Sanchez-Bayo and Goka, 2014; Zioga *et al.*, 2022).

1.2 Bee Decline

Bees are in decline in both abundance and diversity around the globe (Williams and Osborne, 2009; Potts *et al.*, 2010; Cameron *et al.*, 2011; Zattara and Aizen, 2021). In Europe, 9.2% of bees are threatened with extinction. However, there is a lack of baseline data for the abundance of many bee species, with too little information on over 50% of bee species in Europe to determine whether populations are stable, declining or increasing (Nieto *et al.*, 2014). Essentially, we are aware there is a problem, but we cannot grasp the magnitude of the problem.

In Ireland, the situation is similar. One third of Ireland's 100 bee species are threatened with extinction (Fitzpatrick *et al.*, 2006, 2007). The All-Ireland Bumblebee Monitoring Scheme reports a trend of a 4.1% decline in bumblebee species reported each year for the eight most common species, which includes *B. terrestris*, reported from 2012-2021. However, *B. terrestris* populations have remained stable (National Biodiversity Data Centre, 2021).

Whilst the situation regarding bee decline in Europe is far from well defined, trends in species with adequate data show that there is significant decline in wild bee species. In turn, major causes of decline have been identified through an abundance of research, which include but are not limited to habitat loss, commercial colony use, pathogens and parasites, and pesticides (Goulson *et al.*, 2008, 2015; Graystock *et al.*, 2013). It is important to note that there is no individual 'cause' of bee decline, rather, an amalgamation of various anthropogenic activities and an increasing human population has resulted in inhospitable conditions for bees. It seems an increased human population has resulted in more land and chemicals required for food and housing, which could have direct negative impacts on bees by contaminating and reducing the quantity and quality of food and habitat available for bees. With pollinators providing essential crop and wildflower pollination services (Gallai *et al.*, 2009; Garibaldi *et al.*, 2013; IPBES, 2019), it is our duty to determine landscape features and management practices which produce risks for bees, learn from our past mistakes, and use evidence-based mitigation strategies to reduce further declines.

1.3 Drivers of Bee Decline

There are various drivers of bee decline. This section, however, will focus on four prominent factors which, alone and in combination, contribute to an unfavourable environment for wild pollinators, leading to reduced survival and reproduction (Figure 1-1).

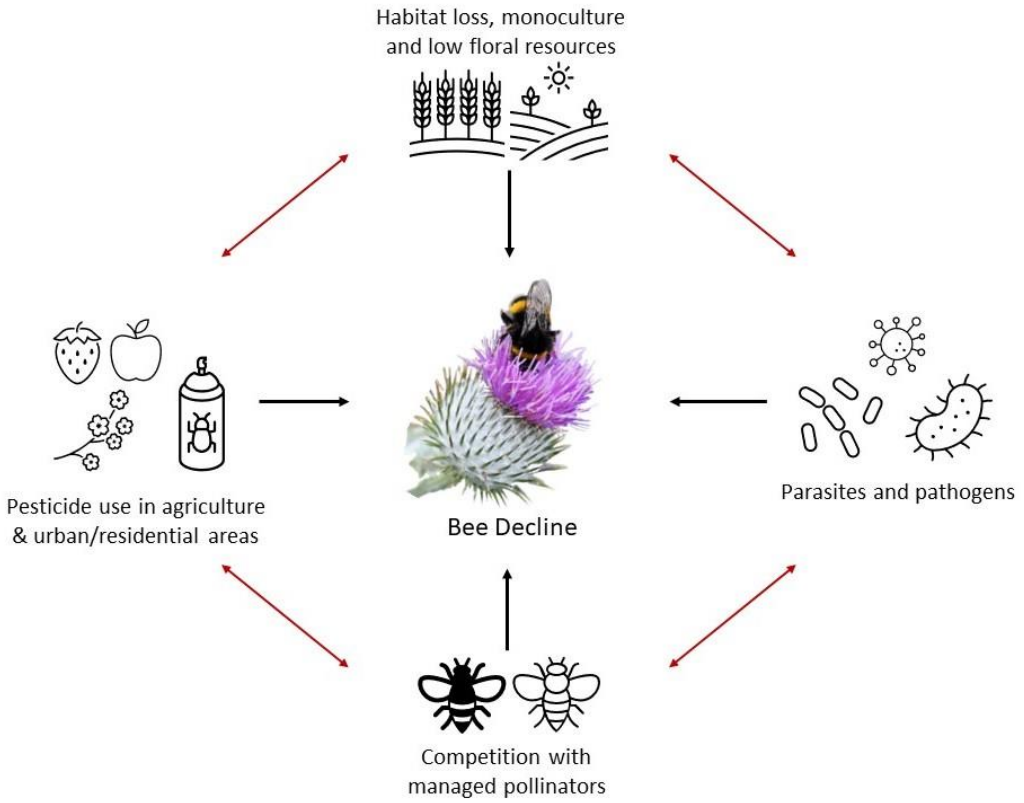


Figure 1-1 Drivers of Bee Decline. Habitat loss, pesticide use, commercial and managed colonies and parasites and pathogens are major drivers of bee decline individually. These stressors can interact in the environment, compounding negative impacts on bees and driving bee decline (Diagram redesigned after Goulson *et al.*, 2015)

1.3.1 Habitat Loss

In 2019, Ireland's Environmental Protection Agency (EPA) reported that 85% of assessed habitats had an unfavourable conservation status, with 46% of habitats in ongoing decline. No grassland, heathland or bog habitats were of favourable conservation status. As of 2019, 38% of orchid rich grasslands and 28% of hay meadows in Ireland have been lost (Biodiversity Working Group, 2020; EPA, 2020).

Agricultural intensification, forestry and urbanization play a significant role in habitat decline. Of Ireland's 7.04 million hectares, 67.6% of land area is used for agriculture and 9.5% is used for forestry. Agriculture negatively impacts over 70% of assessed habitats, forestry impacts over 30% of assessed habitats, and over 40% of impacted habitats have been disrupted by residential, commercial, industrial and recreational infrastructure and areas (NPWS, 2019). The main factors of agricultural practice in Ireland which contribute to the destruction of habitats and resulting pollinator declines are largely attributed to crop monocultures, a drive for increased productivity and the loss or neglect of hedgerows, farmland perimeters and scrub (EPA, 2020).

A range of suitable habitats which provide forage and nesting sites are required to support pollinators. Unfortunately, Ireland has become an increasingly inhospitable place for wild pollinators, as have our neighbouring countries. For example, in the UK, 97% of flower-rich grasslands were lost in the 20th century (Howard *et al.*, 2003) and in Germany, arthropod abundance declined by 78% between 2008 and 2017 in grasslands, with decline associated with agriculture (Seibold *et al.*, 2019). In 2017, Ireland outlined actions for biodiversity in the National Biodiversity Action Plan 2017-2021. However, the country's progress toward these biodiversity targets was deemed as too low (DCHG, 2019). As a European Union (EU)-member state, Ireland has further land-use and management changes to make in line with the EU Green Deal to reverse and mitigate biodiversity loss (The European Commission, 2019, 2020).

Changes to agricultural practice and an increase of suitable habitats may increase pollinator diversity and the pollination of crops. Whilst many insect-pollinated crops provide forage for bees, the 100 bee species present in Ireland need blooming flowers at various times of the year due to differences in lifecycles, habitats, and nutrition. As a result, bumblebee species with later queen emergence times are the most negatively impacted in Ireland (Fitzpatrick *et al.*, 2007). From an agricultural perspective, more visits from insect pollinators result in increased crop yield (Garibaldi *et al.*, 2013; Motzke *et al.*, 2015). However, pollinator richness and visitation rates to crops tend to decrease with increased distance from natural habitats (Ricketts *et al.*, 2008), making a case for the creation and maintenance of suitable habitats for bees in close proximity to farmland.

In addition, urbanisation has mixed impacts on pollinators. In some studies, urbanisation had a negative impact on the abundance and richness of bee species, with conflicting results on whether increased floral resources can mitigate bee decline in urban areas (Geslin *et al.*, 2016; Burdine and McCluney, 2019). However, some studies find that bumblebees can thrive in urban areas with a high density of private and community gardens providing nest sites and floral resources (Osborne *et al.*, 2008; Baldock *et al.*, 2019). Meadow plots in urban areas constructed from wildflower seed mixes also have positive impacts on bumblebee abundance (Blackmore and Goulson, 2014). However, increased garden management – for example, mowing and pesticide use - in private gardens can have negative impacts on bees (Smith *et al.*, 2006).

1.3.2 Managed and Commercial Bee Colonies

In 2019, more than 27,000 managed *A. mellifera* honeybee colonies were reported across Ireland, an increase of 26% since 2016 (DAFM, 2019). However, *A. mellifera* is just one of Ireland's 100 bee species, and the only one that is solely managed by beekeepers, with increased abundance possibly impacting wild bees. For example, high density or a high number of apiaries may lead to exploitative competition for floral resources with wild bee species. In a systematic review on the impacts of managed bees on wild bees by Mallinger *et al.* (2017), 53% of studies investigating competition found negative impacts of managed bees on wild bees and 70% of studies on pathogen transmission reported negative impacts of managed bees on wild bees.

Henry and Rodet (2018) found that wild bee abundance and nectar foraging success were negatively impacted proportional to the distance and density of nearby apiaries of managed honeybees in a protected site in France, with honeybee occurrence up to 15 times greater than wild bees. In addition, there was a decrease in larger bees with closer proximity to apiaries. In Scotland, Goulson and Sparrow, (2009) found significantly smaller bumblebees of various species in sites with honeybees compared to those without honeybees. In addition, Torné-Noguera *et al.* (2016) determined that honeybees accounted for most of the nectar and pollen consumption in rosemary and thyme-rich sites, with lower wild bee biomass closer to apiaries as a result of lower abundances of large bees in closer proximities to apiaries.

Size discrepancies in wild bumblebees due to competition with managed honeybees may alter reproduction and fitness. Elbgami *et al.* (2014) compared, over two years, the weight and reproductive success of *B. terrestris* colonies near and distant from honeybee apiaries. Bumblebee colonies near apiaries produced fewer and smaller queens and colonies weighed less than those further from apiaries. In addition, males were smaller, with offspring sex ratio biased towards males in colonies within closer proximity to apiaries. However, the competitive impact of managed bees on wild bees may be altered by landscape structure, with areas containing high proportions of semi-natural grassland possibly reducing the impacts of managed bees on bumblebee density near honeybee colonies (Herbertsson *et al.*, 2016).

In addition to managed honeybee colonies, the use of commercial bombus colonies can impact wild bee species negatively. Commercial bee colonies are utilized internationally and have created concern for wild bee species, with links to the parasites and pathogens transmission and the establishment of stable *B. terrestris* colonies in countries where they are non-native (Colla *et al.*, 2006; Kondo *et al.*, 2009; Graystock *et al.*, 2013; Schmid-Hempel *et al.*, 2014).

Commercial bumblebee colonies are of immense importance to ensure consistent crop yields in certain crop sectors across Ireland. Whilst this is important for the Irish economy and livelihood of Irish growers, commercial *B. terrestris audax* are reared artificially in continental Europe, leading to concerns around hybridization of commercial *B. terrestris audax* with native Irish populations as reproductives disperse from commercial colonies (Kraus *et al.*, 2011). There are no restrictions on the importation of commercial bumblebee colonies in Ireland as long as they are provided with a certificate of health from the provider (The European Commission, 2014). Presumably, this is because commercial *B. terrestris* colonies claim to be the same subspecies as those found in Ireland. Yet, using microsatellite and mtDNA sequencing, Moreira et al. (2015) discovered that Irish populations of *B. terrestris* were highly differentiated compared to British and continental European populations, despite *B. terrestris audax* recognised as the subspecies present in both Ireland and Britain. This is cause for concern for Irelands native *B. terrestris* colonies. In addition to competition with managed honeybees, commercial bombus colonies may add competitive stress for native bees. Ings et al. (2006) found that commercial bumblebee

colonies have a competitive advantage, with higher nectar foraging rates and the production of larger workers and more gynes compared to native wild colonies.

Taking steps to increase the number of native wild pollinators such as increasing the variety of floral resources available near crops and orchards, can increase crop yield (Blaauw and Isaacs, 2014). In addition, requiring a minimum distance between apiaries and farms utilising commercial bumblebee colonies could reduce the intensity of competitive stress for native bees (Henry and Rodet, 2018). Such actions towards utilising and attracting native bumblebee species rather than importing commercial colonies and increasing reliance on managed honeybee hives could maintain the economic benefits of bumblebees in Ireland and ensure the continued abundance, health, and genetic profile of native *B. terrestris* species.

1.3.3 Parasites and Pathogens

Whilst parasites and pathogens native to wild Irish bees may contribute to poor health along with stressors encountered in the environment, pathogen spill-over from commercial and managed colonies exacerbate the problem. Whilst some pathogens and parasites have a limited number of host species, for example, the *Varroa destructor* mite is associated with *Apis* species but has not been found in other bee species, others such as *Vairimorpha ceranae* and deformed wing virus, have a broader range and can pass between different bee species (Goulson *et al.*, 2015). In a horizon scan of future threats for pollinations, Brown et al. (2016) identified emerging RNA viruses as a high priority issue.

V. ceranae was recently reclassified using molecular phylogenetics and is often referred to as its former name, *Nosema ceranae* (Tokarev *et al.*, 2020). It is a microsporidian parasite which infects the midgut of bees after the ingestion of spores, which infect epithelial cells once germinated and are transmitted to other bees via a faecal-oral and oral-oral route (Gisder *et al.*, 2011; Smith, 2012). Implications of infection include increased mortality, immune system dysfunction and behavioural impacts (Paxton *et al.*, 2007; Paris *et al.*, 2018). *V. ceranae* was once thought of as a honeybee-specific parasite after transmission from *Apis ceranae*, the Asian honeybee, to other *Apis* species worldwide, most likely via global transportation of commercial and managed colonies (Higes *et al.*, 2006; Klee *et al.*, 2007). Soon after, *V. ceranae*

was found in *Bombus* species, along with *Apis*-associated deformed wing virus (DWV), demonstrating the momentum of pathogen spill-over from commercial and managed colonies to wild bees (Genersch *et al.*, 2006; Plischuk *et al.*, 2009).

DWV, in severe cases, can cause deformed wings (Highfield *et al.*, 2009) and reduced survival in bumblebees (Furst *et al.*, 2014). In England, wild bumblebees were found infected with DWV (Evison *et al.*, 2012), with high infections in honeybees possibly spilling over to wild bumblebees. Furst et al. (2014) determined DWV prevalence in wild bumblebees was higher in closer proximity to managed honeybees.

Multiple pathogens and parasites have been identified in commercial bombus colonies from various suppliers despite supplier claims that colonies were healthy (Graystock *et al.*, 2013; Murray *et al.*, 2013). Studies find that wild bumblebees near the sites of commercial colonies have a higher frequency of infection with multiple parasites and pathogens, including *V. ceranae,* deformed wing virus, *Crithidia* species and *Nosema bombi* (Murray *et al.*, 2013; Furst *et al.*, 2014; Graystock *et al.*, 2014). Further, *V. ceranae* displays higher virulence in bumblebees, leading to concerns around wild bee decline and the role that pathogen spill-over plays (Graystock *et al.*, 2013).

The dangers of disease to bee health may be compounded by other stressors such as habitat loss, competition with commercial and managed bees, and pesticide exposure (James and Xu, 2012; Goulson *et al.*, 2015; Paris *et al.*, 2017; Almasri, *et al.*, 2021). Further regulations are needed to prevent contact between commercial colonies and wild pollinators to mitigate interbreeding, disease transmission and prevent exacerbation of current environmental stressors encountered by native bees such as habitat loss and pesticides.

1.3.4 Pesticide Exposure

Pesticide exposure is a well-established risk to bees, but much of this research is limited to insecticides, and in recent decades, neonicotinoid insecticides in particular (Lundin *et al.*, 2015; Cullen *et al.*, 2019; IPBES, 2019).

Multiple studies have determined lethal and sublethal impacts of pesticides on bees (Di Prisco *et al.*, 2013; Bernauer *et al.*, 2015; Zhu *et al.*, 2017; Tomé *et al.*, 2020; Motta *et al.*, 2022). Pesticide exposure can interact with other stressors, such as

pathogens, parasites, and low forage resources, leading to negative impacts for bees including alterations to immunity, survival, and food consumption (Brown *et al.*, 2000; Tyler *et al.*, 2006; Di Prisco *et al.*, 2013; Castelli *et al.*, 2021). Whilst the majority of these studies occur in a laboratory setting (Lundin *et al.*, 2015; Cullen *et al.*, 2019), pesticide residue detection studies have confirmed pesticide contamination of nectar, pollen and bee colony materials, with many determining multiple pesticide residues in single samples, and some in bee-attractive non-target plants near agricultural sites (Krupke *et al.*, 2012; Lambert *et al.*, 2013; Tong *et al.*, 2018; Tosi *et al.*, 2018; Zioga *et al.*, 2020). Such residue studies confirm that pesticide exposure is likely for both native and managed colonies foraging in or near agricultural land. However, pesticide residue studies are limited by what pesticides researchers investigate, meaning pollen and nectar samples may contain a higher number of pesticides than the amount assessed for. In addition, the number of studies determining the floral residues and impacts of fungicides and herbicides for bees is far less than research investigating insecticides (Lundin *et al.*, 2015; Cullen *et al.*, 2019). As a result, our understanding of the impacts of fungicide and herbicide pesticides on bees alone and in combination with other pesticides, is weak. From studies conducted on interactions between pesticides, exposure to pesticide combinations can increase negative impacts such as increased mortality and alterations to reproduction, feeding and immunity in bees (Sgolastra *et al.*, 2017, 2018; Azpiazu *et al.*, 2019; Almasri *et al.*, 2021).

1.4 Pesticides

1.4.1 An Overview

Pesticides are chemical or biological substances which protect plants from harmful organisms or prevent the growth of unwanted plants. According to the European Commission (EC), a plant protection product (PPP) contains at least one pesticidal active ingredient and has at least one of the following functions: i) protects plants or plant products against pests or diseases before or after harvest, ii) influences the life processes of plants, iii) preserves plant products or iv) destroys or prevents growth of undesired plants or parts of plants (The European Commission, 2009).

The main pesticides used in agriculture are insecticides (target insect pests), fungicides (target fungal pests) and herbicides (target unwanted plants) (Figure 1-2). These pesticides are utilized to protect crops from damage and disease and increase crop yield in agriculture. Further benefits include increasing and ensuring crop quality, reducing tillage, reducing pest epidemics, controlling invasive species, increased farm revenue, and increasing the range of crops which can be grown in a particular area, ensuring food security (Cooper and Dobson, 2007). They also have beneficial amenity use in private and community gardens, parks, and golf courses (Kristoffersen *et al.*, 2008).

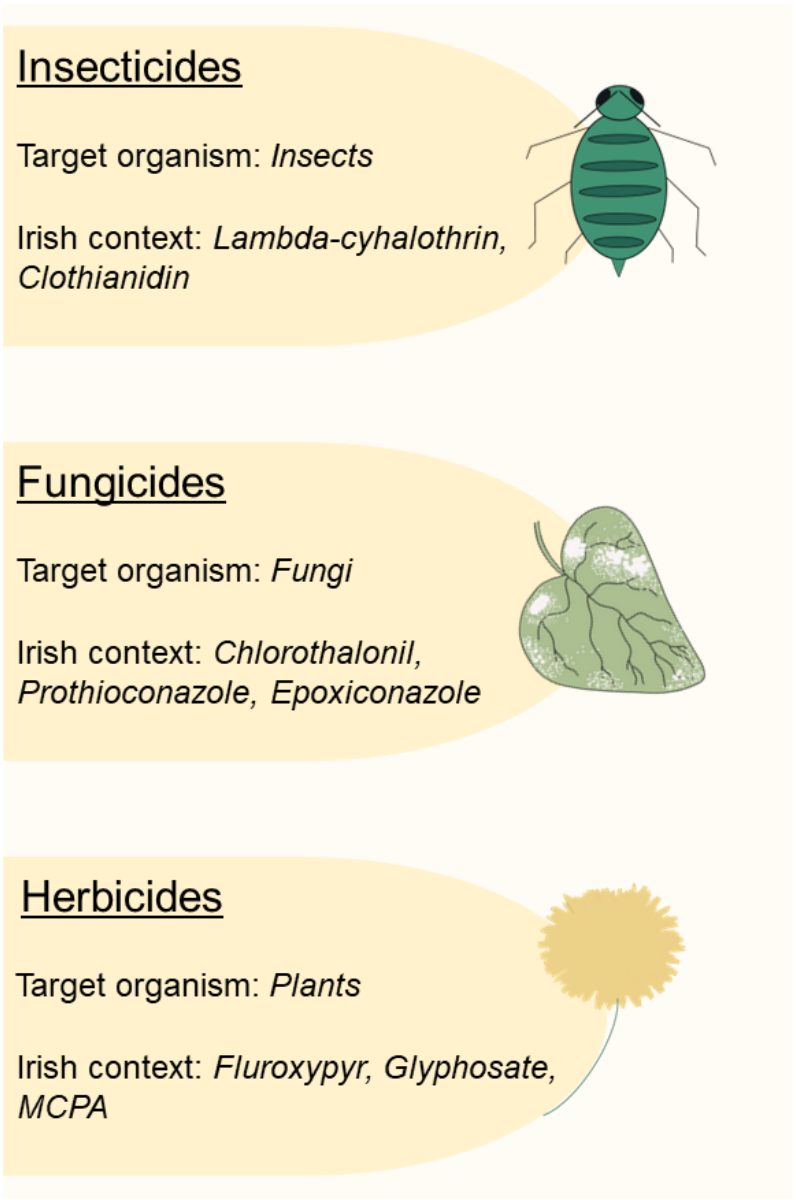


Figure 1-2 The Main Pesticide Classes used in Ireland. The top pesticides within these classes used in Ireland were informed by López-Ballesteros et al. (2022).

1.4.2 Pesticides in the European Union

Agricultural land covers 42% of the EU land area, with arable land accounting for 56% of all agricultural land (Perpiña Castillo *et al.*, 2018). As a result, there is a high demand for pesticides, with the EU accounting for 20.7% of the world's 2,661,124 tonnes of pesticide active ingredients used in agriculture in 2020 alone (FAO, 2020). To align with the high demand and usage of pesticides, the EU has multiple legislations for pesticide use including directive 2009/128/EC for sustainable pesticide use, regulation (EC) No. 1107/2009 for the approval of PPPs on the European market and regulation (EC) No. 396/2005 to determine maximum pesticide residue levels of

pesticides in food products for humans and animals. As of April 2022, 195 pesticides are explicitly banned or restricted in the EU (PAN International, 2022). As of November 2022, 943 pesticides are listed as 'not approved' for use in the EU, however, some may be approved in the future (The European Commission, 2022). A pesticide active ingredient may only be used in the EU after authorisation.

To receive authorisation for use of a pesticidal active ingredient under regulation (EC) No. 1107/2009, the substance must demonstrate "a clear benefit for plant production" and not be expected to "have any harmful effect on human or animal health or any unacceptable effects on the environment". To meet this criterion set out by the EC, the producer of the pesticide active ingredient must apply for approval to a rapporteur member state with a summary and complete dossier detailing intended use and the results of science-based assessments of the active ingredient. Open-access peer-reviewed literature investigating the active ingredient may also be included. Both the national authorities in the EU member states and European Food Safety Authority (EFSA) assess the dossier and if the pesticide active ingredient is approved, assessment and authorisation of pesticide products is conducted by the individual member state. New active ingredients can gain approval lasting 10 years, whilst renewals can be approved for up to 15 years (The European Commission, 2009).

Despite the EU possessing the strictest pesticide regulations in the world, and the major benefits of pesticides to the economy and food security, the widespread use and reliance of pesticides in agriculture has led to concerns for human and animal health, pest resistance, biodiversity and environmental damage and contamination (Bass *et al.*, 2014; Carvalho, 2017; Van Bruggen *et al.*, 2018; Meftaul *et al.*, 2020). In particular, major concerns have been raised regarding possible negative impacts of pesticides for declining insect pollinators, along with criticism of the current pesticide risk assessment required under regulation (EC) No. 1107/2009 for pesticidal impacts on bees (Sanchez-Bayo and Goka, 2014; Brühl and Zaller, 2019; Sgolastra *et al.*, 2019, 2020).

1.4.2.1 Pesticide Pollinator Risk Assessment

As part of the European Commission (EC) strategy for sustainable pesticide use, regulation (EC) No. 1107/2009 outlines the information and assessments required for

the approval of pesticides for use within the EU. This legislation states that pesticide products may only be approved for use within the EU if there is "negligible exposure of honeybees" or "no unacceptable acute or chronic effects on colony survival and development, taking into account effects on honeybee larvae and honeybee behaviour."

These parameters are investigated using a tiered risk-assessment on the pesticide's impact on the honeybee *A. mellifera L.* which is based on guidance developed in 2002 (The European Commission, 2002, 2009). These risk assessments mainly focussed on acute exposure to pesticides, with chronic exposures added to risk assessments in 2009. Tier one risk assessments are mandatory and involve a suite of laboratory tests on adult worker honeybees including acute and chronic oral and contact toxicity tests to determine the LD50 and hazard quotient. LD50 tests aim to determine the toxicity of the pesticide by calculating the concentration at which 50% of the test species perish. The hazard quotient is the ratio between the estimated environmental exposure of the bee to the pesticide and the acute oral or contact toxicity of the pesticide (European Court of Auditors, 2020). If the LD50 is high – generally, tests do not establish an LD50 at concentrations >100µg/bee - or the hazard quotient is <50, no further testing on bees is required. In addition, for insect growth regulators, bee brood feeding tests are required in tier one. If the results of tier one testing determines high mortality risk, an investigation into sublethal effects is carried out in tiers two and three under semi-field and field conditions following EPPO 170 (EPPO, 2010). Only formulated products are used in higher tiered testing which contain the pesticidal active ingredient along with co-formulants, a range of chemicals which increase the efficacy of the pesticide active ingredient. The formulation with the active ingredient to be evaluated is applied to crops at the highest application rate for intended use. Honeybee colonies are required to be exposed to the treated crops for seven days. If the assessor wishes to determine colony development, the bees are removed to a site with no bee-attractive crops until the test period reaches 28 days after the initial pesticide application. Mortality, foraging density, general behaviour, and colony-assessment data (i.e., proportion of adults and brood, signs of disease) are required pre-exposure and during the seven-day exposure period. Pollen collection, hive weight, detailed brood assessments and residues in collected matrices are optional.

There are no defined endpoints for tier two and three testing, which rely on expert judgement for analysis of results.

1.4.2.2 Limitations

Despite multiple EU action plans and legislations aimed at improving biodiversity and sustainable use of pesticides for mitigating bee decline (The European Commission, 2019, 2020), 9.2% of wild bee species are threatened with extinction across Europe, with over 50% of species too data-deficient to determine their conservation status (Nieto *et al.*, 2014). With agricultural intensification and pesticide use identified as major drivers of bee decline, the extrapolation of data from honeybee-centric pesticide risk assessments to nearly 2000 wild bee species across Europe with varying ecological and physiological traits is clearly inefficient and haphazard at best.

At the organism level, studies have determined that different bee species display varying sensitivity to pesticides (Arena and Sgolastra, 2014a; Sgolastra *et al.*, 2019). At a population level, considering whether the species is eusocial or solitary, ground or above-ground nesting, managed or unmanaged, a broad or narrow range forager in terms of foraging distance and preferred floral resources, different bee species will certainly have drastically different responses to an environment contaminated with pesticides (Arena and Sgolastra, 2014a; Sgolastra *et al.*, 2020; Schmolke *et al.*, 2021). For example, a pesticide with negative impacts on reproduction will impact *A. mellifera*, *B. terrestris* or a solitary bee species in drastically different ways (Rundlof *et al.*, 2015; Sgolastra *et al.*, 2019). A honeybee colony which hosts thousands of workers and has ongoing queen and colony production is far more likely to survive as a population through exposure to pesticide contamination than a bumblebee queen exposed directly after diapause, in a solitary phase of her lifecycle, requiring forage from thousands of flowers each day whilst attempting to initiate a colony – if she dies, the colony will never be.

Whilst EFSA advised an update on current risk assessments to include chronic and repeat exposures, a consideration of various routes of exposure e.g., contaminated pollen and guttation fluid, and for assessments to include *Bombus* and solitary bee species (European Food Safety Authority, 2013), the EC still does not require pesticide assessments on non-*Apis* species. The limitations of risk assessment to one bee species

with limited exposure routes and a lack of investigation into sublethal impacts were highlighted in the restriction of three neonicotinoid insecticides in the EU. From 2013, after decades of intensive agricultural use, neonicotinoids were banned for outdoor use due to overwhelming evidence of negative impacts on bees through academic research. These restrictions were made official through legislation in 2018, despite EU required risk assessments finding negligible impacts to honeybees in prior approval and renewal of these insecticides (European Commission, 2018c, 2018a, 2018b).

Before EU sanctioned restrictions, neonicotinoids were one of the most widely used insecticide groups in the world, with a pesticide market share of €1.5 billion in 2008 due to their efficiency in protecting crops against insect pests (Jeschke *et al.*, 2011). Neonicotinoids are insecticides which act on nicotinic acetylcholine receptors of insect nervous systems. They are also systemic, meaning the compound can be taken up by plants through the soil and travel throughout the plant to all tissues including pollen and nectar, making seed treatment a viable and easy option for farmers (Jeschke *et al.*, 2011; Simon-Delso *et al.*, 2015). Multiple studies determined neonicotinoid pesticides had lethal and sublethal impacts on bee immunity, foraging behaviour, wild bee nesting and colony growth, reproduction, and olfactory learning (Mommaerts *et al.*, 2010; Whitehorn *et al.*, 2012; Yang *et al.*, 2012; Di Prisco *et al.*, 2013; Lundin *et al.*, 2015; Rundlof *et al.*, 2015). In addition, neonicotinoids have high persistence in soil and plant materials. Along with the systemic nature of neonicotinoids, this led to unintentional contamination of plants and soils and a ubiquitous presence of neonicotinoids on and near land where it may have been applied years prior, both before and after 2018 restrictions (Botías *et al.*, 2015; David *et al.*, 2016; Zioga *et al.*, 2020). Today, neonicotinoids are still found in pollen, nectar, and food residues, possibly due to their high persistence, illegal use, or authorized emergency use (Lambert *et al.*, 2013; Zioga *et al.*, 2020; European Food Safety Authority *et al.*, 2022). For bee researchers and conservationists, the restriction of neonicotinoid use was welcomed, but it was not immediately enforced across member states. Between 2013 and 2019, member states granted 206 emergency authorisations for restricted neonicotinoid use (European Court of Auditors, 2020).

In addition, current pesticide risk assessments do not consider pesticide mixes which are likely to be found in bee-attractive crops, despite evidence of pesticide cocktails

in pollen and nectar of bees (David *et al.*, 2016; Zioga *et al.*, 2020) and in the residues of food pollinated by bees and apicultural products (European Food Safety Authority *et al.*, 2021, 2022). In EFSA's 2020 pesticide residue analysis of food from member states, up to 30 different pesticides were found in honey, with some exceeding maximum residue levels. In addition, in 2019, strawberries, which benefit from bee pollination, had one of the highest frequencies of multiple pesticide residues found in 63.6% of strawberry samples tested and 15.2% contained more than five residues (European Food Safety Authority *et al.*, 2021, 2022). Based on pesticide cocktails found in pollen, nectar and bee-pollinated crops, multi-pesticide exposure is likely and may have negative impacts on bee health through additive or synergistic pesticide activity (Biddinger *et al.*, 2013; Sgolastra *et al.*, 2017, 2018; Raimets *et al.*, 2018). A lack of multi-pesticide exposure assessments are a limitation for pesticide authorisation highlighted by the EFSA Scientific Committee, (2021).

Finally, there is ambiguity around the testing of pesticide formulations for impacts on bees. Pesticide formulations are what are applied to crops and sold for amenity use. They contain one or more pesticidal active ingredients which are designed to target specific pests. They also contain co-formulants, sometimes referred to as 'inert' ingredients due to their lack of pesticidal activity. Co-formulants are included in formulations to increase the efficacy of the pesticide active ingredient(s). They can include surfactants, which increase the surface area of the target plant and penetration of the active ingredient, solvents, and emulsifiers, which aid active ingredient solubilisation, and antifoaming agents and dyes. In addition, agricultural users may also add 'adjuvants' to tank mixes including formulations, which also aid in increasing pesticide active ingredient efficacy (Hazen, 2000). Whilst the pesticide active ingredient is tested according to regulation (EC) No. 1107/2009, individual co-formulants are not required to undergo the same risk assessment regimes for their impact on bees, and risk assessment of formulations is only guaranteed if the formulations contains more than one active ingredient or has toxicity ≥ the active ingredient (The European Commission, 2009, 2013). Whilst individual formulations need to be assessed for impacts to honeybees at the member state level, approval can be based on similarities to previously tested formulations including those with co-formulants which are 'equivalent.' What qualifies as an equivalent co-formulant is undefined (The European Commission, 2009). At the EU level, only one formulation

needs to be tested along with the active ingredient when determining terrestrial ecotoxicological impacts, and at that "it is not always necessary to generate experimental data with the formulation; instead the data on the active substance could be sufficient" (The European Commission, 2002).

In addition, formulations are not tested as stringently as active ingredients as they are tested comparatively against currently used formulations using mortality as an endpoint (The European Commission, 2009; Jess *et al.*, 2014). Further, whilst there is a list of banned co-formulants in the EU, many adjuvants added to tank mixes do not need to submit pesticide risk assessments on bees for approval. Co-formulants have generic use and can be formulated with several active ingredients, meaning their usage is likely to be higher than pesticide active ingredients themselves. In 2022, EFSA identified 182 co-formulants from only 82 pesticide formulations, highlighting their extensive use (European Food Safety Authority, 2022).

Whilst there is a lack of research on co-formulant impacts on bees, there is some evidence to suggest that co-formulants can have negative impacts for bee survival and learning (Straw *et al.*, 2022). More studies are using commercial formulations to determine impacts on non-target organisms (Mesnage *et al.*, 2015; Lopes *et al.*, 2018; Pochron *et al.*, 2020; Tóth *et al.*, 2020; Zhao *et al.*, 2020), although this can cause confusion as some authors refer to the impacts found in their studies as a direct result of the active ingredient, when any of the formulation ingredients, alone or in combination, could be leading to negative results found. One of the major blockages for academic researchers in determining the impacts of co-formulants used in formulations is that only co-formulants with hazardous impacts on humans are required to be listed as ingredients in the EU, with many formulation ingredients considered proprietary information (The European Commission, 2006, 2009). As a result of a lack of stringent regulatory assessments of pesticide formulations and a lack of information on which co-formulants are present in pesticide formulations marketed for agricultural and domestic use, the magnitude of pesticide co-formulant exposure and resulting impacts on bees is almost entirely unknown and difficult to investigate.

Across the board, the pesticide risk assessment process across the EU seems inadequate to protect bees from further decline. In addition to shortcomings associated with species tested, a lack of pesticide combination risk assessment and less stringent

testing of formulations and associated co-formulants, risk assessments are not publicly available for critique from the scientific community – an action which could increase public trust in the EU risk assessment process and allow experts to flag potential negative consequences of pesticide use. More stringent post-assessment monitoring of pesticide uses, residues and impacts on biodiversity could also aid in mitigating further biodiversity decline (Storck *et al.*, 2017).

"We need to dampen down the wild swings between using chemicals like neonicotinoids almost without constraint on the one hand and banning their use altogether on the other hand. Neither is sensible. This calls for a much more evidence-driven, risk-based way of regulating the use of pesticides" - (Boyd, 2018).

1.4.3 Pesticide Use in Ireland

In terms of agricultural land coverage, Ireland is amongst the top three EU countries with more than 60% of the island's 7.04 million hectares dedicated to agriculture (NPWS, 2019; Perpiña Castillo et al., 2018). As a result, nearly 3000 tonnes of pesticide active ingredient was used in the agricultural sector alone in 2020 (FAO, 2020). Due to Ireland's wet oceanic climate, the active ingredients most used in pesticide products on the market are herbicides and fungicides (Figure 1-3), with herbicides accounting for over 78% of active ingredients in pesticide products placed on the market in 2020 (DAFM, 2020). For example, in outdoor grown blackcurrants, which rely on insect pollinators, 50% of pesticides used are herbicides and 36% are fungicides (DAFM, 2018a). In top fruit crops, mainly apples, 85% of pesticides used were fungicides (DAFM, 2018b). According to DAFM pesticide usage reports from 2014-2017, chlorothalonil, prothioconazole and epoxiconazole are the most used fungicides in Ireland. However, chlorothalonil was banned for use in the EU in 2019. The most used herbicides are fluroxypyr, glyphosate and 4-chloro-2-methylphenocyacetic acid (López-Ballesteros *et al.*, 2022).

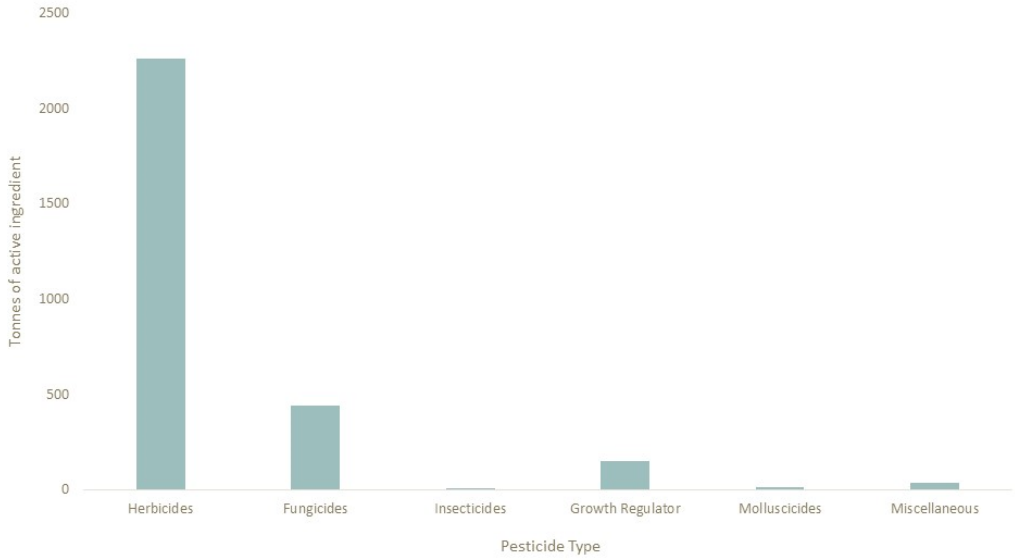


Figure 1-3 The Pesticidal Active Ingredients of Plant Protection Products Placed on the Irish Market in 2020. Herbicides accounted for 78% of pesticide active ingredients, followed by fungicides at 15% of the pesticide market and growth regulators at 5%. Insecticides were just 0.3% of the pesticide market (DAFM, 2020)

Considering one third of Irish bee species are threatened with decline and increased agricultural intensification and pesticide use are considered as major drivers of bee decline, it is important to understand how and if the pesticides which bees encounter will have negative impacts. As an EU member state, pesticide risk assessments follow those outlined in 1.4.2.1 in line with regulation (EC) No. 1107/2009. As such, only honeybees are a requirement for pesticide regulatory risk assessments, and risk relies on a high LD50 or hazard quotient of the pesticide to assess for sublethal impacts in the field. However, herbicides and fungicides generally have low LD50s, meaning sublethal tests are rarely conducted before approval of these pesticides. In an attempt to fill this knowledge gap, the sublethal impacts of some herbicides and fungicides on bees have been investigated in academic literature (Cullen *et al.*, 2019; Battisti *et al.*, 2021; Rondeau and Raine, 2022).

1.4.4 Herbicides and Fungicides

The assumption that low toxicity identified in EC-required pesticide risk assessments equates to negligible risks for non-target organisms is the same assumption that led to highly intensive neonicotinoid use for decades. Neonicotinoids, however, were designed to target insects. As bees are insects, it’s not surprising that intensive

neonicotinoid use led to hazardous conditions for bees (Goulson *et al.*, 2015; Lundin *et al.*, 2015). However, insecticides account for just 0.3% of the pesticide market in Ireland, with herbicides and fungicides dominating the market and agricultural sector, meaning bees are most at risk of exposure to these pesticides.

Residue studies have reported the presence of herbicides and fungicides in pollen and nectar collected by bees (Zioga *et al.*, 2020) and multiple sublethal effects have been demonstrated from exposure to herbicides and fungicides individually to the pesticidal active ingredient alone, within formulations, or in combination with other pesticides (Cullen *et al.*, 2019; Rondeau and Raine, 2022).

Fungicidal active ingredients make up more than 35% of the global pesticide market as fungal diseases threaten the yield and quality of cereals, oilseeds, fruits, and vegetables (Zubrod *et al.*, 2019). In Ireland, disease control programmes rely on azole fungicides with multiple applications per crop per growing season to control *Fusarium* and *Microdochium* species which are difficult to control using non-azole fungicides (Jess *et al.*, 2014). It is estimated that without fungicides, cereal crop yield would reduce by more than 40% due to the prevalence of Septoria tritici blotch, a disease caused by *Zymoseptoria tritici.* However, crop yields also rely on efficient pollination, with crops such as oilseed rape, strawberries, and apples - which benefit from insect pollination - relying on synthetic fungicides for disease control (DAFM, 2016, 2018a, 2018b). In bee and plant samples from formal incident monitoring of honeybee deaths in the EU between 1981 and 2007, 40% of analysed samples contained fungicides (Potts *et al.*, 2016). Despite low hazard quotients in regulatory risk assessments, fungicides can have sublethal impacts on bees, including negative impacts on learning, colony development, brood development, disease resistance and immunity (Mussen *et al.*, 2004; Decourtye *et al.*, 2005; Huntzinger *et al.*, 2008; Bernauer *et al.*, 2015; Cizelj *et al.*, 2016). In addition, some fungicides – particularly demethylation inhibitor (DMI) fungicides – can act synergistically with insecticides, increasing the magnitude of insecticide toxicity and negative impacts on bee health and survival (Biddinger *et al.*, 2013; Sgolastra *et al.*, 2017, 2018; Raimets *et al.*, 2018).

Herbicides are vital to increase crop yield by removing unwanted plants. They are also used in amenity settings to remove unwanted plants in gardens, parks, and golf courses in addition to being utilized by the transport sector to remove plant overgrowth which

could limit commuter vision and cause hazardous conditions (Cooper and Dobson, 2007). In addition, herbicides are used on hard surfaces in urban areas as weeds can grow in joints and cracks, causing damage to roads and paths, widening cracks, and inhibiting water run-off (Kristoffersen *et al.*, 2008). Herbicides are the most used pesticides across the EU, accounting for over 30% of pesticide sales across the EU in 2020 (EUROSTAT, 2020b). Recent research on herbicides, particularly glyphosate, has indicated risks for humans, animals, and the environment (Bridi *et al.*, 2017; Ford *et al.*, 2017; Tarazona *et al.*, 2017; A.G. Pereira *et al.*, 2018; Bali *et al.*, 2019; Tang *et al.*, 2020). In particular, glyphosate has gained attention for possible negative impacts on bees including impacts on bee survival, learning, brood development, immunity, and the digestive tract microbiota (Farina *et al.*, 2019; Motta and Moran, 2020; Odemer *et al.*, 2020; Motta *et al.*, 2022). The intensive use of herbicides may also indirectly impact bees by decimating important food sources for bees such as dandelions (*Taraxacum agg.*) which are a rich source of pollen and nectar (Hicks *et al.*, 2016). Worryingly, glyphosate residues are being found in non-treated and non-target plants which bees may forage on (Cebotari *et al.*, 2018; Botten *et al.*, 2021), with research showing that bees will forage on glyphosate-contaminated flowers indiscriminately (Thompson *et al.*, 2022).

Research into the impacts of herbicides and fungicides on bees is low compared to the abundance of investigations into the impacts of insecticides. Consequently there are extensive gaps in our knowledge of how these pesticides impact bees (Cullen *et al.*, 2019). It is important to understand if and how bees could be negatively impacted by our most utilized pesticides to avoid further declines which could have disastrous implications for native bee species, natural ecosystems, and the agricultural sector in Ireland.

1.5 The Current State of Knowledge on the Impacts of Herbicides and Fungicides on Bees

In 2019, Cullen et al. conducted a systematic review (Manuscript S1-1) of peer-reviewed literature to determine the gaps in our knowledge regarding the impacts of herbicides and fungicides on bees. We aimed to answer seven questions:

1) In what year and in what geographical location has existing research taken place?
2) Which bee species have been most studied?
3) How have bees been experimentally exposed to pesticides?
4) What methodological approaches have been used?
5) Which fungicides and herbicides have been most studied, and have studies investigated pesticide active ingredients or formulations?
6) Do studies claim to use field-realistic pesticide concentrations? And
7) What life stages, effect levels and types of effects have been investigated?

To answer these questions, we used the Web of Science Core Collection to gather literature in November 2018 using the search terms (fungicide*) AND (*bee OR *bees) and (herbicide*) AND (*bee OR *bees). This resulted in 437 publications. To meet the criteria for analysis in this systematic review, the paper must have investigated the direct impacts of at least one herbicide or fungicide on bees and must have included an investigation into mortality, sublethal behavioural impacts, susceptibility to stressors including other pesticides, impacts on the colony or population level, impacts on the molecular or physiological level, impacts on pollination services or modelling approaches focusing on the impacts of herbicides or fungicides on bees. After the removal of non-peer reviewed manuscripts and analysing the literature to determine the criteria were met, the final dataset included 89 papers. For each paper, full bibliographical reference, country, bee species, exposure method, methodological approach, pesticide formulation name (where relevant), active ingredient and pesticide substance group, whether the author claimed a field realistic concentration, and the effect level and type studied was collected.

We found that most studies were conducted in North America and Europe, with no studies conducted in Ireland. In addition, we found that most studies were conducted

on *Apis* species (67 papers) with the majority of these focusing on *A. mellifera.* In comparison, few studies were carried out on bumblebees and solitary bees. Thirteen studies investigated impacts on *Bombus* species, seven investigated *Osmia* species and *Megachile rotundata* was investigated in eight studies. Of the *Bombus* species investigated, eight studies included *B. terrestris*. We also determined that most studies used oral exposures (62 papers) and were carried out in the lab (74).

Seventy studies were carried out on fungicides, with 51 investigating ≥2 fungicides. The top fungicide groups investigated were triazoles (30 studies), strobilurins (15 studies) and imidazoles (14 studies). Propiconazole was the most investigated pesticide active ingredient, followed by boscalid, chlorothalonil, pyraclostrobin, iprodione, prochloraz and myclobutanil (Table 1-1). Only 29 studies determined the impacts of herbicides on bees, with 14 papers reporting on ≥2 herbicides. The top herbicide groups investigated were phosphonoglycine (15 studies) and triazines (8 studies), alkylchlorophenoxy (5) and bipyridylium (5). The phosphonoglycine pesticide glyphosate was studied the most (15) followed by atrazine, 2,4-D, paraquat and simazine (

Table 1-2). Most studies investigated pesticidal impacts at the organism level (68 papers) on adult bees (73 papers). Twenty-eight papers investigated impacts on the sub-individual (molecular or physiological) level, eight at a colony level and only one at a population level. Fifty-eight studies investigated multiple effect types, with mortality (63) and morphological/physiological (27) effects most studied. The remainder of effect types were investigated in ≤ 14 papers (Figure 1-4).

Table 1-1 The Most Common Fungicides Investigated. The fungicide active ingredient, chemical class, number of studies, whether the study investigated the fungicide active ingredient alone or as part of a formulation and whether the study investigated the fungicide alone or in combination with other pesticides was recorded.

Fungicide	Chemical class	No. studies (in formulation, active ingredient only)	Alone / in combination with other compounds
Propiconazole	Triazole	15 (8, 7)	11 / 8
Boscalid	Carboximide	13 (9, 4)	3 / 10
Chlorothalonil	Chloronitrile	12 (3, 9)	10 / 3
Pyraclostrobin	Strobilurin	12 (9, 3)	2 / 11
Iprodione	Dicarboximide	11 (9, 2)	10 / 3
Prochloraz	Imidazole	11 (2, 9)	10 / 7
Myclobutanil	Triazole	8 (3, 5)	6 / 2

Table 1-2 The Most Common Herbicides Investigated. The herbicide active ingredient, chemical class, number of studies, whether the study investigated the herbicide active ingredient alone or as part of a formulation and whether the study investigated the herbicide alone or in combination with other pesticides was recorded.

Herbicide	Chemical class	No. studies (formulation, active ingredient)	Individual / Combination
Glyphosate	Phosphonoglycine	15 (4, 11)	15 / 2
Atrazine	Triazine	6 (2, 4)	6 / 2
2,4-D	Aryloxyalkanoic acid	5 (1, 4)	5 / 1
Paraquat	Bipyridylium	5 (1, 4)	5 / 0
Simazine	Triazine	4 (0, 4)	3 / 1

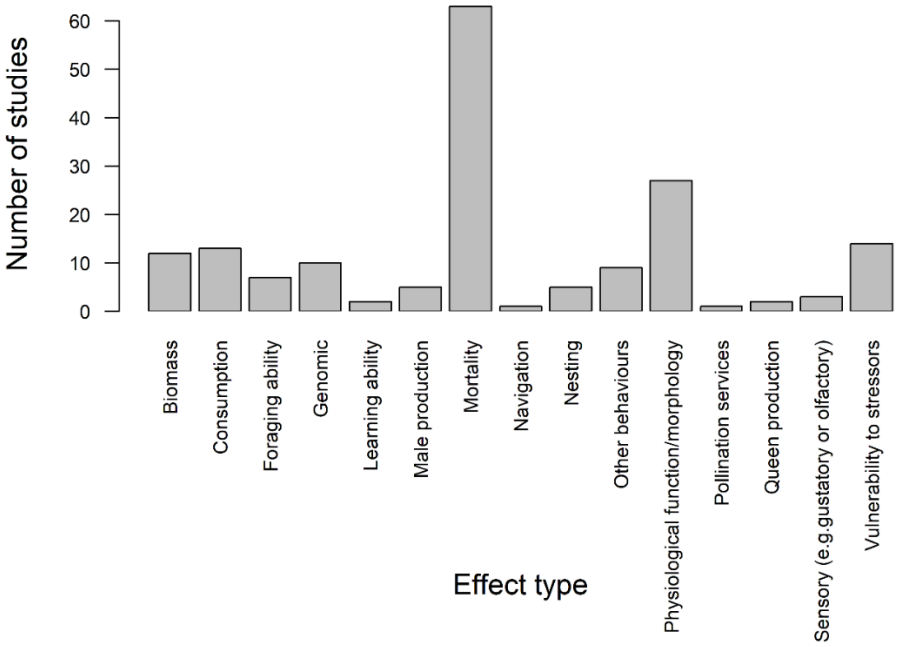


Figure 1-4 The Number of Studies Investigating Different Effect Types from Herbicide or Fungicide Exposure. Mortality and physiological function/mortality were the most investigated effects after pesticide exposure, followed by vulnerability to stressors, consumption, biomass, and genetic studies.

Overall, this review highlighted a dearth of research into herbicides and fungicides and their sublethal impacts on non-*Apis* species. A systematic review researching the impacts of neonicotinoids, a single chemical class of insecticides, on bees found 268 papers (Lundin *et al.*, 2015). In comparison, Cullen et al. (2019) found only 89 papers investigating the impacts of all chemical classes of herbicides or fungicides on bees. Different bee species display varying pesticide sensitivity (Arena and Sgolastra, 2014a), highlighting the need to determine the impacts of herbicides and fungicides on non-*Apis* species. In addition, only 29% of studies investigated sublethal impacts, an area which needs to be researched considering we are yet to determine if many herbicide or fungicide active ingredients have *any* effect on bees. If they do, their mode of action or targets within bees will need to be determined considering bees are non-target organisms. Most research investigating the impacts of herbicides investigated active ingredients whereas fungicide investigations displayed much more variation between investigations into active ingredients and formulations. It is important for future studies to be carried out on both the active ingredient and relevant commercial formulations to determine whether sublethal impacts may be caused by the pesticide itself, or co-formulants present in the formulation (Mullin *et al.*, 2015; Straw *et al.*, 2022).

Since Cullen et al. (2019), multiple peer-reviewed studies have been published investigating the impacts of herbicides and fungicides on bees at the lethal and sublethal level. In particular, there has been an increase in studies investigating the impact of the herbicide glyphosate on the bee digestive tract microbiota (Motta *et al.*, 2018, 2020; Blot *et al.*, 2019; Motta and Moran, 2020; Castelli *et al.*, 2021), although all studies were carried out on *Apis* bee species and mostly use the active ingredient above field-relevant concentrations. In addition, new research has highlighted negative impacts of herbicides on immunity, brood development, reproductive success, food consumption, detoxification, and metabolism (Odemer *et al.*, 2020; Zhao *et al.*, 2020; Araújo *et al.*, 2021; Graffigna *et al.*, 2021; Chen *et al.*, 2022; Motta *et al.*, 2022). Most were conducted on *A. mellifera*. For fungicides, negative impacts on bees have been detected, including synergism with insecticides, leading to alterations to reproductive success, metabolism, food consumption and learning (Iverson *et al.*, 2019; Alkassab *et al.*, 2020; Chen *et al.*, 2021; DesJardins *et al.*, 2021; Wang *et al.*, 2022) across different bee species.

Whilst research into herbicides and fungicides impacts on bees seems to be steadily increasing, there is still a lack of research on non-*Apis* species, particularly for research investigating herbicides. Further research gaps highlighted in Cullen et al. (2019) such as a lack of investigation into both active ingredients and formulations and molecular level studies still stand. Research into the molecular level impacts of herbicides and fungicides on different bee species could determine the tissues most impacted, guiding future research in a direction where target tissues, cells, receptors, or proteins can be more quickly and easily identified. In this way, likely outcomes could be more readily estimated, and further research into colony and population level impacts would have a steady foundation on which to build an overall understanding.

1.6 *Bombus terrestris*: the Buff-tailed Bumblebee

Bumblebees are of the genus *Bombus* and account for 250 species of bees worldwide. One of these species, *Bombus terrestris*, commonly known as the buff-tailed bumblebee, has become a popular model organism for bumblebee research. *B. terrestris* is of economic and ecological importance throughout Europe, present in many countries as both a wild and commercial pollinator (Estoup et al., 1996; Velthuis and Doorn, 2006; Moreira *et al.*, 2015; National Biodiversity Data Centre, 2021). There are nine known subspecies (ssp) of *B. terrestris*: ssp *africanus*, ssp *audax*, ssp *dalmatinus*, ssp *terrestris*, ssp *calabricus*, ssp *canaeriensis*, ssp *lusitanicus*, ssp *sassaricus*, and ssp *xanthopus* (Rasmont *et al.*, 2008). Subspecies may be distinguished by characteristic colouration and morphology via coat hair colour and patterns and leg or tibia colouration. The subspecies present in Ireland, and the focus of this research, *B. terrestris audax*, can be identified by a narrow yellow band directly below the head on the thorax, which can be mixed with black hairs, a single yellow band on the abdomen, and a buff-coloured tail (Figure 1-5) (Rasmont *et al.*, 2008). Visual identification of *B. terrestris* based on hair colouration and pattern alone may be difficult and can require mtDNA RFLP analysis to assist species identification as *B. terrestris* and *B. lucorum* have an identical appearance (Wolf *et al.*, 2010). To visually distinguish between the two species; *B. lucorum* banding are a lemon-yellow colour, compared to a darker yellow colour in *B. terrestris*. In addition, the tail of *B. lucorum* is white, compared to a buff-coloured tail in most *B. terrestris* queens and workers, but this distinction can be difficult to pinpoint with certainty.

Figure 1-5 The Characteristic Coat Colouration and Patterning of *B. terrestris audax*.

1.6.1 Life Cycle and Colony Roles

Bumblebee colonies have an annual lifecycle (Figure 1-6), with a thriving *B. terrestris* colony producing up to 350 worker bees. This is a mean feat for the single queen that starts the colony; it takes arduous work, persistence, and an element of luck.

In early Spring, the *B. terrestris* queen emerges from diapause, a prolonged state of decreased activity where queens survive on fat stores (Alford, 1969). The first port of call is to find a suitable nesting site which will be the home of her future colony. The nesting site will be underground and usually takes the form of an abandoned rodent burrow. Once the nesting site is considered suitable, the queen begins foraging for nectar and pollen to begin her colony, visiting approximately 6000 flowers a day. The queen can secrete wax from her ventral abdomen, which is used with pollen and nectar for the infrastructure of the nest. A wax pot is formed to store nectar and a pollen lump covered in wax is used to lay the first batch of 8-16 eggs which will become the queen's first adult workers. The egg contains a single diploid fertilized cell surrounded by a mesh-like material, the yolk, which will provide nutrients for the first days of development. A vitelline membrane surrounds the yolk and a hard chorion covers the membrane which functions to protect the egg (Stell, 2012). Bees are holometabolous and through various stages of metamorphosis and development, the adult worker will emerge approximately 4-5 weeks after the egg is laid. The queen incubates the eggs by sitting directly on top of the pollen lump and generating heat – this can be seen to

an extent with the naked eye as she pumps her abdomen. The queen can reach internal temperatures of 37-39°C which allow for an incubation temperature of 30-32°C (Goulson, 2010). During this period, the queen juggles foraging with brood incubation until the developing brood pupate. After approximately four days, eggs hatch into larvae which eat the pollen available inside their pollen lump, living together inside the pollen lump in what is known as a brood clump. As the larvae continue to develop, the brood clump disintegrates, and larvae generate their own cells from wax and silk. During this time they are fed a pollen and nectar mixture supplemented with proteins produced by the hypopharyngeal gland of the queen (Pereboom, 2000). After approximately two weeks, developing larvae spin a silk cocoon for pupation, which is a non-feeding metamorphosis period. During the pupation of the first batch of workers, queens tend to increase their foraging time to collect more pollen and nectar for the next batch of eggs to be laid. After another two weeks of development, the pupae will hatch, biting through their cocoon to emerge as adult workers which lack pigment – appearing white and black until their yellow bands develop, usually within 24 hours. In addition, their wings and exoskeletons will be soft and harden within 1-2 days (Alford, 2011). During this stage, newly hatched workers are referred to as callows. In a few days, some of these workers will assume the task of foraging for pollen and nectar, and the queen will stay inside the colony until her death. Others will aid the queen in caring for the next batches of developing brood in the same manner as the queen. In the following month, nest weight can increase up to 10-fold as more and more eggs are laid by the queen, with more and more workers available to care for an increased number of developing brood (Goulson *et al.*, 2002). During this period, pollen and nectar are stored in the empty cocoons from hatched workers and optimal temperature is maintained within the colony from heat produced by workers to incubate developing brood, or colony cooling conducted by wing fanning at the colony entrance. Between April and August, if there is enough food and workers to nurture developing queens, the colony will cease the production of workers and switch to producing reproductive bees: gynes (future queens) and males (Figure 1-7). Haploid (unfertilized) eggs are laid to produce males, while diploid (fertilized) eggs are laid to produce females. Whilst there is some uncertainty, it is widely accepted that larvae respond to a pheromone produced by the queen which directs their development towards becoming a worker. A lack of this pheromone is thought to alter physiological processes within the developing larvae to become a gyne (Cnaani *et al.*, 1997; Lopez-

Vaamonde *et al.*, 2007; Goulson, 2010). Males leave the colony to mate and do not return to the colony. Gynes will forage during the day to build up fat reserves and return to the colony at night. Eventually, after mating just once, gynes will find a suitable place to enter diapause (Estoup *et al.*, 1996; Schmid-Hempel and Schmid-Hempel, 2000; Baer *et al.*, 2001). Mated gynes will dig a few centimetres into disturbed soil to form a small chamber where they will remain over autumn and winter for the duration of diapause to hopefully begin their own colony in the spring. Since the colony of origin ceases to produce workers once reproductives are produced, it perishes – and the founder queen dies with them (Goulson, 2010).

Whilst bumblebees typically display this annual lifecycle, winter-active colonies are observed in countries with mild winter temperatures such as New Zealand. Increasing winter temperatures may be increasing the number of winter-active colonies in the UK and Ireland, too. Winter-active *B. terrestris* workers have been recorded in the UK as early as 1990 (Robertson, 1991; Stelzer *et al.*, 2010) and the National Biodiversity Data Centre in Ireland is currently undertaking a study to monitor the number of winter-active *B. terrestris* workers (National Biodiversity Data Centre, 2019).

Bumblebees do not display a rigid and obvious division of labour such as the age-based caste system found in *Apis mellifera* honeybee hives and questions remain unanswered about how labour is divided in bumblebee colonies. Younger bumblebees are more likely to perform tasks within the colony whilst older workers are more often found foraging (Goulson, 2010). However, this is not the rule, and some workers remain in the colony until death. Alternately, there is some evidence to suggest that task-specialization and labour division may be based on size as bumblebee worker size is highly variable, particularly in comparison to *A. mellifera* (Pouvreau, 1989; Kelemen *et al.*, 2022). This is known as alloethism. In general, larger workers are more likely to be foragers while small workers carry out tasks in the nest (Goulson *et al.*, 2002; Holland *et al.*, 2021).

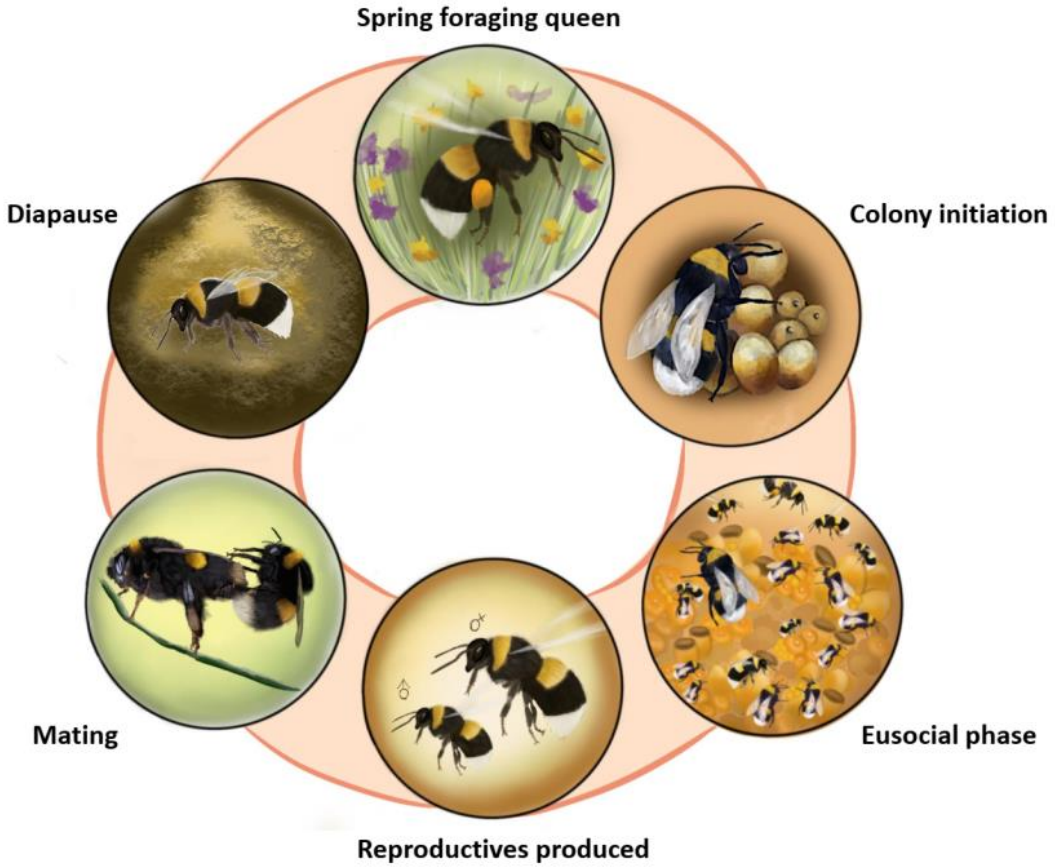


Figure 1-6 The Bumblebee Annual Lifecycle. In spring, the queen exits diapause to initiate a colony in a suitable underground environment, usually an abandoned rodent burrow. The queen lays her first batch of eggs and incubates these whilst foraging for pollen and nectar for their development. Once the brood develops into adult workers, the queen retires to lay eggs and care for developing brood full time whilst workers forage and assist her with developing brood care. After some months, reproductives are produced where males and gynes (new queens) leave the colony to mate. After mating just once, the mated gyne enters diapause and the origin colony dies. When she wakes up in spring, she will begin her own colony (illustration by S.J. Larragy).

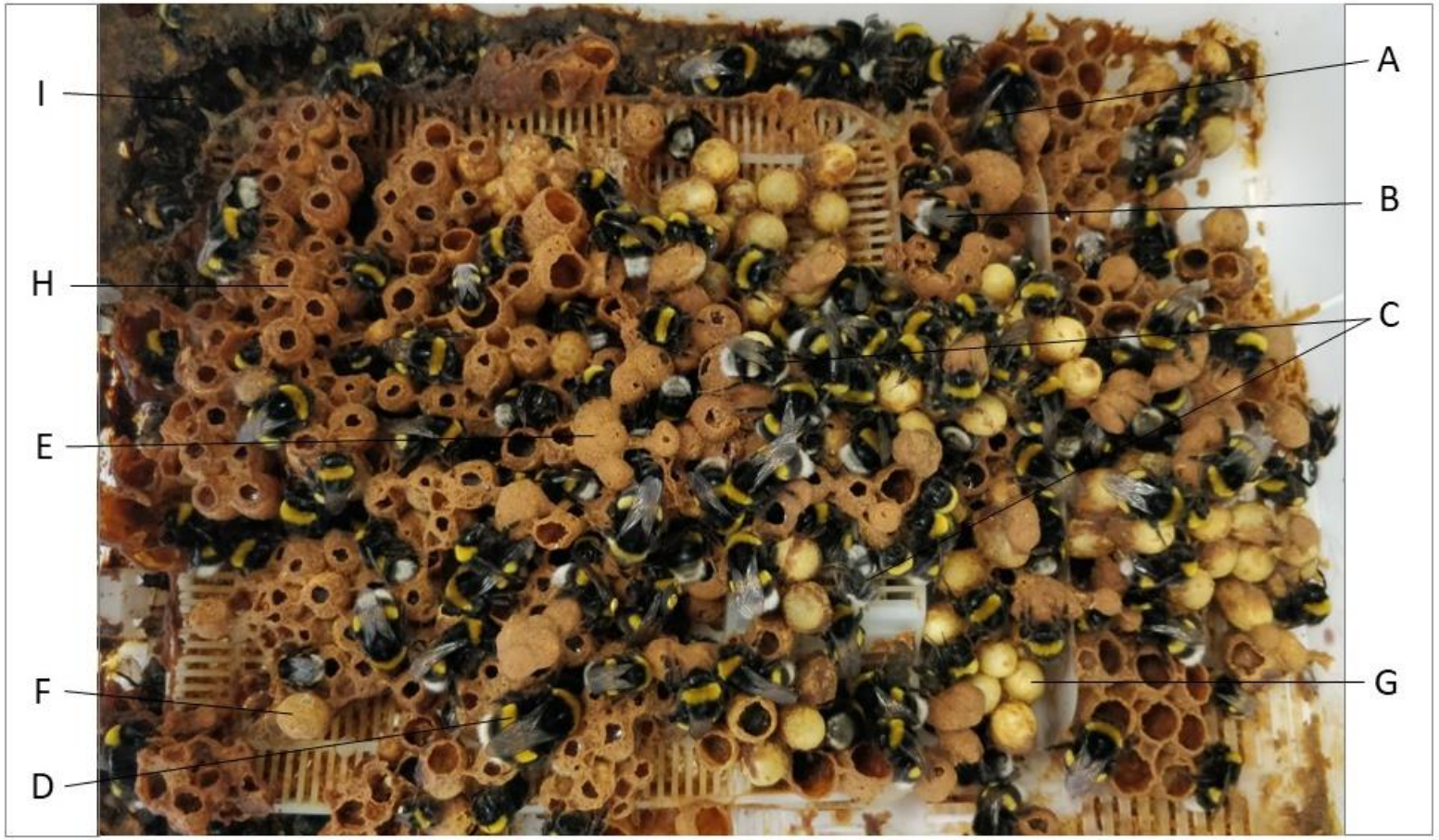


Figure 1-7. *B. terrestris* colony at the Stage of Producing Reproductives. (A) queen, (B) worker, (C) male callows, (D) gyne, (E) pollen lump with laid eggs, (F) larvae in individual cell, (G) pupae cocoons, (H) nectar pots, (I) corpse removal site.

Some theories as to why larger bees are more likely to be foragers relate to: larger eyes and ocelli enabling better vision compared to smaller bees (Kapustjanskij *et al.*, 2007), a smaller limb size in larger bees may reduce heat loss as heat is needed for flight (Peat *et al.*, 2005), larger bees can carry larger forage loads (Goulson *et al.*, 2002) and larger bees have a longer proboscis, giving them a unique advantage with deeper flowers (Pouvreau, 1989; Goulson *et al.*, 2002). However, small *B. terrestris* foragers can better trigger pollination in some plant species compared to larger workers (Stout, 2000) and bees with shorter tongues, a feature directly proportional to size, can forage faster on shallow flowers (Plowright and Plowright, 1997), leaving alloethistic task-division possibly determined by the floral resources available (Goulson, 2010). However, bumblebees display a high level of behavioural plasticity, with bees switching between nest and foraging tasks depending on the nutritional needs of the colony (Cartar, 1992; MacKenzie *et al.*, 2021). Less studied behaviours such as nest guarding and corpse removal show some elements of size-based task division, although behavioural plasticity is still observed (Munday and Brown, 2018; MacKenzie *et al.*, 2021).

1.6.2 Geographical Distribution and Habitat

Bees evolved from wasps from the superfamily Sphecoidea approximately 130 million years ago and both belong to the order Hymenoptera, with bees belonging to the family Apidae. Whilst the earliest known bee fossil – *Trigona prisca*, a stingless bee - can be dated back 74 to 94 million years ago, the earliest *Bombus* fossil is dated back 38-26 million years ago. Whilst unclear, bumblebee lineages are thought to have diverged 40-25 million years ago during a period of global cooling in Central Asia, dispersing through the west to Europe. A further spread to North America is dated back approximately 20 million years ago, and to South America 4 million years ago (Hines, 2008; Benjamin and McCallum, 2019).

B. terrestris can flourish in a wide range of habitats as it is a generalist forager. It is active in all countries around the Mediterranean Sea excluding Egypt (Rasmont *et al.*, 2008) and has recently spread north past Sweden and Norway and to the Altai Mountains (Rasmont *et al.*, 2015). Countries containing sea barriers greater than 10km seem to have restricted gene flow as subspecies have evolved where populations are physically separated e.g., subspecies of *B. terrestris* developed between Great Britain

and Europe. It is the only European bumblebee that has modified its lifecycle and phenology based on environmental temperatures, observed to produce 1-3 generations per year outside of traditionally active periods (Rasmont *et al.*, 2015). However, *B. terrestris* is also present as an invasive species due to commercial colony imports in Argentina, Chile, Japan, Tasmania, and New Zealand where it poses a threat to native bee species (Goulson, 2010; Rasmont *et al.*, 2015).

1.6.3 Economic and Ecological Importance

Most research on bees focuses on honeybees, but bumblebees are also important for the pollination of various food crops and wildflowers. As a natural consequence, greater species diversity is correlated with greater crop yield (Garibaldi *et al.*, 2013; Klein *et al.*, 2018; Katumo *et al.*, 2022). Differences in lifecycles, reproduction, habitat, and nutritional needs contribute to differences in pollination efficacy, but bumblebees also have a unique strategy for collecting pollen which is absent in honeybees known as buzz pollination. Blueberries, tomatoes, and strawberries are examples of food crops which benefit from buzz-pollination. To gain access to the pollen of these crops, which are only released when vibrated, the bumblebee can grip itself to the flower centre and rapidly contract their flight muscles to send vibrations throughout their body at approximately 400 Hz. This vibration is relayed to the anther via direct contact with the bees thorax to shake and release pollen from the anthers (Walker, 2020). In addition, honeybees are less likely to forage in cold temperatures or during periods of rain. Bumblebees will often forage regardless of environmental conditions due to their tolerance for colder conditions gained through various thermoregulatory adaptations including heat production via flight muscles and insulation from a dense coat of hair (Goulson, 2010). Some studies also demonstrate that bumblebees are more efficient pollinators: with bumblebees foraging earlier in the day, at a faster pace and with greater pollen deposition resulting in higher fruit set and weight compared to honeybees (Stanghellini *et al.*, 2002; Artz and Nault, 2011). Essentially, bumblebees are key pollinators of crops and wildflowers, particularly in countries with cold and unpredictable weather.

Ireland has 100 bee species: One honeybee, 21 bumblebee species and 78 solitary bee species (National Biodiversity Data Centre, 2021). Bumblebees provide value to natural and human populations via wildflower reproduction and food crop production,

although the value of the former is not well documented in Ireland. Animal pollination of crops including apples, strawberries and rapeseed contribute approximately €59 million per year to the Irish economy (Stout *et al.*, 2019). Apples and rapeseed are amongst the most valuable crops to the Irish economy based on the proportion consumed in Ireland, both of which are pollinated by bees (Stout *et al.*, 2019). Additionally, Burns and Stanley, (2022) determined that insect pollination dramatically increased the yield and monetary profit of apples in Ireland, with bumblebees important for the pollination of Jonagored apples along with honeybees and solitary bees, although honeybee visitation rates could vary. The value of animal pollination for strawberries, tomatoes and apples in Ireland are valued at €11.32 million, €0.69 million, and €4.41 million, respectively. As a result, Irish growers import commercial bumblebee colonies to ensure a high yield of these crops, with up to 2000 commercial *B. terrestris audax* colonies imported into Ireland each year (All-Ireland Pollinator Plan, 2020).

1.7 The Adverse Outcome Pathway

The adverse outcome pathway (AOP) is a framework developed by Ankley et al. (2010) to aid in the understanding and organisation of ecotoxicological effects of a chemical across different biological levels which may lead to adverse outcomes, from the molecular initiating event (e.g., receptor-ligand interaction, genotoxicity, protein oxidation or modifications) to population responses (Figure 1-8). The AOP is designed to permit the translation of molecular level findings to possible negative outcomes. Along with data from experiments carried out on different biological levels, a pathway can be created to effectively interpret and communicate possible impacts of pesticides on non-target organisms through the assembly and evaluation of causal relationships at various different endpoints (Ankley and Edwards, 2018).

Using a framework such as the AOP when investigating the risks of pesticides to bees can give a clearer understanding of how a chemical can negatively impact various bee species as well as identify research gaps and priorities going forward. The AOP has six primary areas of research suggested to uncover a chemical's negative impacts in a given species, including: the chemical properties of the toxicant, the molecular initiation event, cellular responses, organ responses, organism responses and population responses. In terms of the research available on how herbicides and fungicides impact bees, there is no research on molecular initiating events, and very few on most other areas of research suggested in the AOP, with most research focusing on organism level lethal events. As researchers, if we focus on filling the gaps of an AOP for a given pesticide, we may be able to reach a consensus on how particular pesticides may impact bees more quickly and effectively. However, caution should be taken when extrapolating from honeybee data to fill data gaps in an AOP for other non-*Apis* bee species, as differences between *Apis* and non-*Apis* bee species may alter the type and severity of impacts resulting from pesticide exposure (Gradish *et al.*, 2019; Sgolastra *et al.*, 2019). In the research presented in this thesis, I take the whole bumblebee system into account (Figure 1-9) – investigating the molecular level impacts of a herbicide and fungicide on key tissues – as well as at the organism level. This will give a holistic overview of the impacts of these pesticides on *B. terrestris* health and the ability to piece results together to determine possible knock-on colony and population level effects.

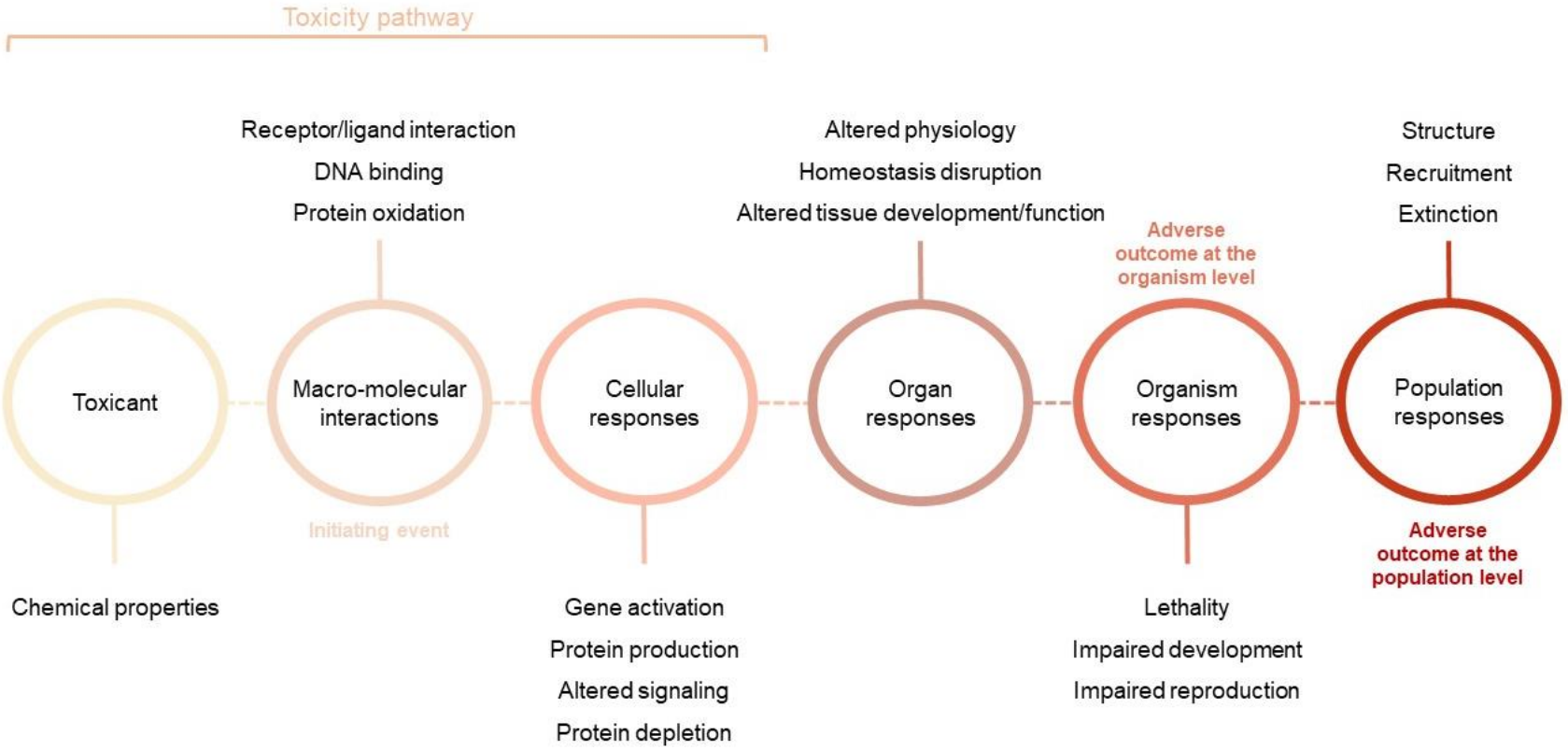


Figure 1-8 Key Elements of The Adverse Outcome Pathway. The toxicant, macro-molecular interactions and cellular responses allows the elucidation of possible negative impacts on biological pathways which could lead to higher order impacts at the organ, organism, and population level. In addition, determining an AOP pathway can aid in estimating and mitigating hazards and risks of exposure (Designed after Ankley et al. (2010).

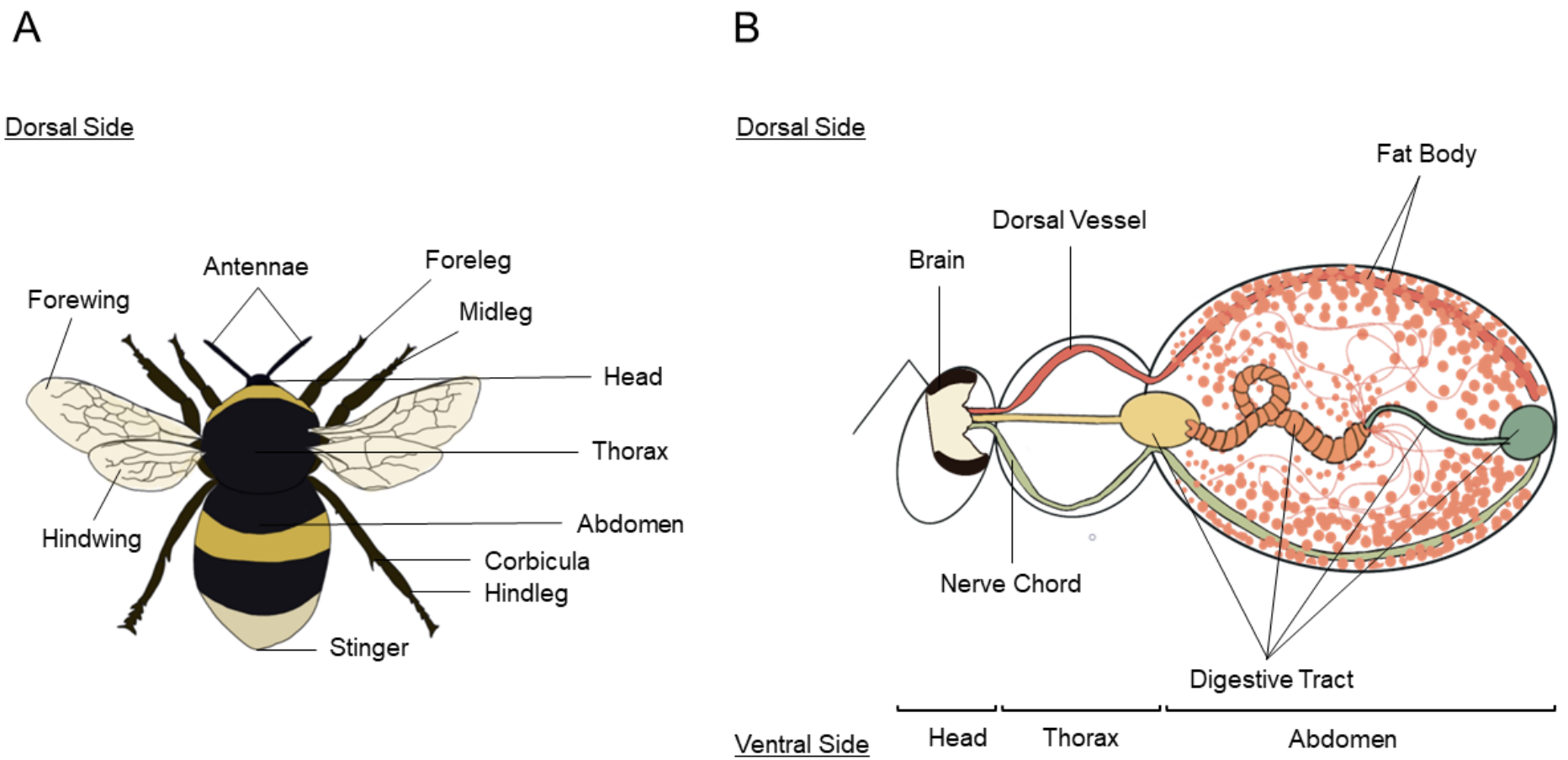


Figure 1-9 Anatomy of the Bumblebee. (A) Key external features of bumblebee anatomy, and **(B)** key internal features of bumblebee anatomy, including the brain, digestive tract, and fat body researched throughout this thesis.

1.8 Utilising Mass Spectrometry-based Proteomics

1.8.1 Mass Spectrometry: A Brief Background

Until 2015, the *A. mellifera* genome was the only bee genome sequenced (The Honeybee Genome Sequencing Consortium, 2006). The publication of the sequenced genome for *B, terrestris* and *B. impatiens* in 2015 greatly increased the scope of genomic, transcriptomic, and proteomic research which could be carried out on bumblebee species using these species as a model (Sadd *et al.*, 2015). Whilst genomics and transcriptomics have provided invaluable insights into the impacts of stressors on bees (Aufauvre *et al.*, 2014; Vázquez, Latorre-Estivalis, *et al.*, 2020; Zhao *et al.*, 2020; Castelli *et al.*, 2021; Motta *et al.*, 2022), proteins are the biochemical actors of the cell, with proteomics providing a phenotypic characterisation of cellular processes (Hora *et al.*, 2018; Colgan *et al.*, 2019; Zaluski *et al.*, 2020).

Before the 1990s, Edman degradation was relied on for peptide sequencing, a chemical process whereby amino acids were individually cleaved from a single protein for identification by high-performance liquid chromatography (HPLC) (Mann, 2016). However, throughout the 1990s, multiple discoveries would set the baseline for the high resolution, high throughput sequencing and analysis of complex peptide mixtures that can be achieved with mass spectrometry (MS)-based proteomics today (Mann *et al.*, 2013). These included the development of electrospray ionization (Fenn *et al.*, 1989), an online LC-MS infrastructure (Hunt *et al.*, 1992) and liquid chromatography tandem MS (LC-MS/MS) (Link *et al.*, 1999).

High resolution MS/MS coupled with HPLC separation leads to accurate protein quantitation and identification. HPLC pumps with tiny bead particles along a long column and gradient with a low flow ensure a steady elution of peptides from the HPLC column to the MS. In the MS, electrospray ionization is used for ionising peptide molecules and transferring them to the gas phase (Walther and Mann, 2010). Here, the mass-to-charge ratio (m/z) of peptide ions can be determined by behaviour in an electric field using several types of mass analysers which is later used for protein quantification and identification. For example, the quadrupole mass analyser consists

of four alternately charged cylindrical poles which detect m/z of peptide ions by their movement through the electric field created by the poles (van der Wal and Demmers, 2015). Quadrupoles allow for the selection of peptide ions with particular m/z charges or scanning of the range of m/z values to obtain a mass spectrum. From here, peptide ions can be captured in an ion trap. The orbitrap is a commonly used ion trap and consists of a spindle shaped electrode which ions oscillate around (Scigelova and Makarov, 2006). The oscillation frequency of peptide ions around the orbitrap allows accurate m/z measurements. Ion intensity is used in this research to determine relative abundance of peptides which uses the number of peptides detected in a specific retention time window to accurately determine abundance. This is more accurate than the alternative spectral counting method (van der Wal and Demmers, 2015). From the resulting MS/MS spectrum, the m/z ratios for peptide fragments detected are displayed. Mass differences of one amino acid between fragments can be used to determine the amino acid sequence and identity of proteins. To determine proteins present in the original biological sample, the peptide fragment spectrum and peptide masses are searched against a protein database and identifications can be verified using software such as MaxQuant (Cox and Mann, 2008).

1.8.2 Common Approaches to Mass Spectrometry-Based Proteomics

LC-MS/MS is commonly used for MS-based proteomics. Before the introduction of biological samples to LC-MS/MS, proteins are denatured, disulphide bonds are broken via reduction and alkylation, and proteases are used to produce a peptide mixture. Subsequently, the peptides can be purified from any surfactants and other impurities used for denaturation using desalting columns. For peptide quantification using LC-MS/MS, peptides can be labelled with tags which can be distinguished by their mass, or label-free quantitation (LFQ) can be used (van der Wal and Demmers, 2015). The most used quantitative proteomic profiling techniques are LFQ, ion-based isobaric labelling methods such as tandem mass tagging (TMT), and stable isotopic labelling with amino acids in cell culture (SILAC) (Johannsen *et al.,* 2021).

1.8.2.1 Label-Free Quantification

Label free approaches to quantitative proteome profiling are important for discovery-based quantitative proteomics. LFQ requires that each sample is analysed separately,

increasing the MS instrument run time. In addition, since each sample is run separately, sample preparation, handling, and sensitive high-resolution MS instruments are essential to ensure accurate data collection. However, LFQ MS approaches reduce the amount of sample processing and handling and is a more cost-effective alternative to labelling kits (Mann *et al.*, 2013). In addition, an unlimited number of samples can be analysed since this method does not rely on a finite number of labels. The main data acquisition techniques used for quantitative proteomics using LC-MS/MS, which differ in how identification (MS^2) data are acquired, are data dependent acquisition (DDA) and data independent acquisition (DIA).

1.8.2.1.1 LFQ Data Dependent Acquisition

In LFQ DDA, the ions for a selected m/z range are individually isolated and fragmented. These ions are selected from a full-scan mass spectra obtained in MS^1, where a specified number of the most intense peptide ions are selected, and the MS/MS spectra acquired for further isolation and fragmentation in MS^2. This results in thousands of MS/MS spectra for protein quantitation and identification. Quantitation involves a comparison of chromatographic peak intensities between sample sets and identification can be achieved by searching MS/MS spectra against a chosen protein sequence database (Bantscheff *et al.*, 2007). One drawback is that due to the nature of selecting parent ions from MS^1 with the highest intensity peaks, lower abundance peptides are often excluded (Dowling *et al.*, 2023).

1.8.2.1.2 LFQ Data Independent Acquisition

In LFQ DIA, a precursor mass range is selected and divided into narrow windows. MS2 data is acquired from all detected precursor ions in each m/z window until the entire mass range selected is covered. Peptides are quantified by comparing the chromatographic peak areas for precursor ions between samples. MS2 spectral libraries containing fragment ion maps are utilised for protein identification of peptides from the acquired datasets (Dowling *et al.*, 2023). With MS2 data acquired from all detected precursor ions, rather than a selected number of high intensity ions as in DDA protein quantification, DIA allows for increased coverage and reproducibility. However, a requirement for the generation of spectral libraries is a drawback in comparison to DDA protein quantification (Thermo Scientific, 2020).

1.8.2.2 Alternatives to Label-Free Quantification

In discovery-based proteomics, quantitative proteomic profiling can be carried out using labelling methods as an alternative to LFQ and can improve throughput of quantitative MS analyses and increase the number of peptides identified and quantified in a single analysis (Thermo Scientific, 2020). Two commonly used labelling methods are TMT and SILAC.

1.8.2.2.1 TMT quantification

TMT labelling is a multiplex technique, where samples from several experimental conditions can be analysed, allowing for greater throughput in quantitative proteomic profiling. This method involves the use of isobaric chemical tags with the same nominal mass and chemical structure with an MS/MS reporter group, a spacer arm, and an amine-reactive group, where amine-reactive groups bind to peptide N-termini or to lysine residues, where trypsin will cleave proteins during the digestion step of sample preparation. During MS analysis, the heavy carbon and nitrogen isotopes within tags allow each tag fragment to act as unique reporter ions during MS^2 and peptides are quantified based on reporter ion intensity (Dowling *et al.*, 2023). TMT labelling reduces the MS run time as samples can be combined and increases the number of peptides and proteins quantified and identified. However, experiments are limited as only 16 separate samples can be compared and quantified in a single LC-MS/MS run using TMT tagging.

Using TMT labelling, Sialana and colleagues investigated the proteomic changes linked to associative olfactory conditioning in the honeybee brain synaptosome (Sialana *et al.*, 2019). This study identified > 5000 proteins across experimental groups and characterised the proteomic changes associated with olfactory learning which included alterations to SNARE proteins and ABC transporter proteins, which are important for neurotransmitter signaling in the honeybee brain.

1.8.2.2.2 SILAC quantification

SILAC labelling relies on the metabolic incorporation of ‘heavy’ isotope-containing amino acids e.g. ^{13}C or ^{15}N-labeled amino acids into selected experimental groups, and the incorporation of amino acids with a natural isotope abundance for a different

experimental group, most often the control group. Up to five different conditions can be tested using this technique (Dowling et al., 2023). An equal protein concentration is taken from each cell culture experimental group and combined for digestion and LC-MS/MS. The mass difference between isotope labels allow for identification of the experimental group and relative peak intensities of isotopic labels are used to for protein quantification. SILAC increases the throughput of quantitative MS analyses and reduces the run-time required as samples are combined. However, there are currently no studies to my knowledge that use SILAC to investigate the bee proteome.

Langlands and colleagues used SILAC labelling to analyse the proteome of a *Drosophila* embryo mutant, depleted for a fibroblast growth factor receptor called heatless (Langlands *et al.*, 2019). This study used SILAC labelling in yeast and allowed fly larvae to feed on the resulting SILAC labelled yeast. This resulted in the incorporation of SILAC amino acid labels into the ovaries of mutant flies. This study characterised changes to the proteome of mutant embryos which included alterations to proteins associated with the cytoskeleton and nuclear transport, providing further insight into the molecular pathways involved downstream of heatless receptor signaling.

This technique is demonstrably more challenging for use in studying the impacts of herbicides and fungicides on the bumblebee proteome compared to LFQ techniques or TMT quantification due to the need for SILAC labels to be incorporated into the study organism metabolically. Whilst the technique used in Langlands and colleagues' study (Langlands *et al.*, 2019) could be considered, it would require a greater amount of physical labour, time, and protocol optimisation to be utilised in bee studies. However, SILAC labelling could be a viable option for cell culture studies, e.g., investigating the impact of pesticides on the microbiota of bumblebees by incorporating SILAC labels to the growth media of bacterial and fungal cultures commonly found in the bumblebee digestive tract after pesticide treatment. However, using SILAC for bumblebee cell studies would be much harder to accomplish, as there are no bumblebee commercial cell lines, which may reflect in the lack of peer-reviewed studies using SILAC labelling to investigate bees.

1.8.3 The Application of Mass Spectrometry to Investigate the Impact of Pesticides on Bumblebees

Advancements in MS-based proteomics allow us to take a temporal and spatial snapshot of the molecular state of cells or tissues in response to various stressors or conditions. In the case of investigating the impacts of herbicides and fungicides on bees, gaps in our knowledge include low toxicity profiles and a lack of research on sublethal impacts on non-*Apis* bees, leading to a vague-at-best characterisation of how certain herbicidal or fungicidal compounds impact bees. With a declining bee population (See section 1.2) and high agricultural dependence on both bees and pesticides, it is important to rapidly identify which pesticides have negative impacts on bees and exactly how the pesticide elicits negative impacts to understand the magnitude of exposure risks and quickly respond with mitigation measures. Consequently, using MS-based proteomics can lead to beneficial insights into the proteome of key bumblebee organs after pesticide exposure by uncovering alterations to the bumblebee proteome which could have negative consequences. These insights allow for evidence-based hypotheses and mitigation guidelines to be produced for the impact of herbicides and fungicides on bees.

1.9 Thesis Aims

The principal aim of this thesis was to determine the effects of the herbicide glyphosate and the fungicide prothioconazole, two commonly used pesticides in Irish agriculture, on the proteomes of the digestive tract, brain, and fat body in the important bumblebee species *B. terrestris*. In addition to investigating the impacts of technical grade glyphosate and prothioconazole, widely available commercial formulations containing either glyphosate or prothioconazole as the active ingredient were selected for investigation of impacts to the *B. terrestris* proteome. This was conducted to allow comparisons to be made between the tissues of active ingredient, formulation, and control-treated bees, determining if co-formulants present in either of the formulations may alter the proteomic response differentially to the active ingredient. MS-based proteomics was employed throughout this thesis as it permitted the identification and quantification of thousands of proteins from a single biological sample, thus providing deep insights into the molecular landscape of the organism and tissue under study. Through the assessment of physiological changes at the molecular phenotype level, insights enabled a characterisation of the bee proteome in key organs after exposure to glyphosate or prothioconazole.

To facilitate a deeper understanding of the impacts of glyphosate and prothioconazole on *B. terrestris*, bioassays were also utilised to determine if there were alterations to mortality, food consumption, and behaviour, along with DNA amplicon sequencing to determine if there were significant alterations to the digestive tract microbiota, after pesticide exposure. This research contributes to an AOP which can act as a basis to guide future investigations into the impacts of herbicides and fungicides on bumblebee species. In addition, by determining if the selected formulations have differential impacts on *B. terrestris* in comparison to the active ingredients alone – the depth of investigation required into the impacts of co-formulants on bees was further established. Overall, the results of this research provide an organismal and molecular level foundation for the impacts of glyphosate and prothioconazole active ingredients, and representative formulations, on *B. terrestris*, providing a deeper insight into the impacts of widely used pesticides in Irish agriculture on a native wild bee species.

Chapter 2

Materials and methods

2.1 Chemicals and Reagents

All oral solutions utilized in pesticide exposure assays contained Sucrose BioXtra > 99.5% (Sigma Aldrich). Pesticide active ingredients were analytical grade (PESTANAL®, Sigma-Aldrich International GmbH). Pesticide commercial formulations used were RoundUp Optima+® (Evergreen Garden Care UK Ltd, PCS No. 04662) and Proline® (Bayer Crop Science Ltd, PCS No. 03786). Sample processing for mass spectrometry utilized the following chemicals and reagents: lysis buffer (6M Urea (Sigma Aldrich), 2M thiourea (Sigma Aldrich), and one Complete™, Mini Protease Inhibitor Cocktail tablet (Roche Diagnostics), Qubit® Quant-IT™ protein assay kit (Invitrogen), 2-D clean up kit (GE HealthCare Life Sciences), Resuspension buffer (6M urea, 0.1M Tris HCl (Sigma Aldrich), pH 8.0), ProteaseMax™ Surfactant Trypsin Enhancer (Promega), Sequencing Grade Modified Trypsin, Porcine (Promega), Trypsin Resuspension Buffer (Promega), Pierce™ C18 spin columns (Thermo Scientific), Ammonium persulphate (Sigma Aldrich), Ultrapure Protogel® (National Diagnostics), Ultrapure 10x 0.25M tris/1.92M glycine/1% SDS (National Diagnostics), Protogel® stacking buffer (National Diagnostics), 4x ProtoFLOWGel resolving buffer (Flowgen Bioscience), and Tetramethyl Ethylenediamine (TEMED) (National Diagnostics), 4X SDS Sample Buffer (EMD Millipore), Blue Protein Standard broad range (BioLabs Inc New England), and InstantBlue™ (Expedeon). For DNA extractions, quality assessment and PCR sequencing, the following reagents were used: DNeasy Blood and Tissue Kit (Qiagen), 50x Tris-acetate-EDTA (TAE) buffer (Fisher Bioreagents), agarose (Sigma Lifescience), SYBR® Safe DNA gel stain 10,000x concentrate in DMSO (Invitrogen), HyperLadder™ 100bp (Bioline), HyperLadder™ 1kb (Bioline) and 5x DNA loading buffer blue (Bioline), PCR H_2O (distilled water twice filtered using a 50ml Filtropur S 0.2µm sterile syringe filter head (Sarstedt), 5x Green GoTaq® Flexi buffer (Promega), 25mM $MgCl_2$ (Promega), PCR nucleotide mix (Promega 10µm primers

515F (Sigma Genosys, forward primer sequence: 5'-GTGCCAGCMGCCGCGGTAA-3'), 806R (Sigma Genosys, reverse primer sequence: 5'-GGACTACHVHHHTWTCTAAT-3'), ITS1-1F-F (Sigma Genosys, forward primer sequence: 5'-CTTGGTCATTTAGAGGAAGTAA-3'), ITS1-1F-R (Sigma Genosys, reverse primer sequence: 5'- GCTGCGTTCTTCATCGATGC-3'). Unless otherwise stated, H_2O used for forming chemical mixtures was Milli-Q water 18MΩ obtained from Millipore Milli-Q ion exchange apparatus.

2.2 Bumblebee Maintenance

Bombus terrestris audax bumblebee colonies (Biobest, Agralan Ltd) were maintained at 23°C ± 5°C and 67% relative humidity ± 20%. Colonies were kept in darkness except during handling or observation in which case red light was used. Colonies were fed sugar solution (Biobest, Agralan Ltd) *ad libitum*, and 4 g of pollen (Biobest, Agralan Ltd) every two days. All colonies used were queenright, absent of reproductives, and acclimatized to their environment for at least 48 hours before engaging in experimental procedures.

2.3 Pesticide Preparation and Exposure

All pesticide solutions for oral exposure were prepared in 40% (w/v) sucrose solution, which was prepared by dissolving 400g of sucrose in distilled H_2O made up to 1000 ml. For pesticides with low water solubility, stock solutions were dissolved in acetone, and final concentrations for oral exposure were prepared in 40% (w/v) sucrose solution containing 0.3% acetone overall. All pesticide stock and treatment solutions were homogenized using a magnetic stirrer.

2.3.1 Glyphosate Preparation

Glyphosate active ingredient stock solution was prepared by dissolving technical grade glyphosate (PESTANAL®, analytical standard, Sigma-Aldrich International GmbH, <= 100% N-(phosphonomethyl) glycine)) in 40% (w/v) sucrose solution to give the final concentration required, as described in 3.3. Lower concentrations of glyphosate were prepared by diluting a calculated aliquot of glyphosate active ingredient stock solution in 40% (w/v) sucrose solution and mixed using a magnetic stirrer. Glyphosate commercial formulation stock solution was prepared by aliquoting a calculated amount of Roundup Optima+® into 40% (w/v) sucrose solution. The concentrations of RoundUp Optima+® were calculated based on glyphosate active ingredient content (containing 170 g/L glyphosate) in the formulation so that both the technical grade glyphosate and Roundup Optima+® treatments contained the same concentration of glyphosate. Roundup Optima® was purchased from a local retail outlet in Ireland. Required concentrations of glyphosate commercial formulation were prepared by dissolving a calculated aliquot of glyphosate commercial formulation stock solution in to 40% (w/v) sucrose solution and mixed using a magnetic stirrer. All glyphosate stock and exposure concentrations were prepared at a maximum time of 48 hours before first oral exposure experiments and kept at 4°C in labelled amber glass bottles. Solutions were mixed each day to ensure solutions were homogenous.

2.3.2 Prothioconazole Preparation

Prothioconazole active ingredient stock solutions were prepared by dissolving technical grade prothioconazole (PESTANAL®, analytical standard, Sigma-Aldrich International GmbH, 3H-1,2,4-Triazole-3-thione, 2-[2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2- hydroxypropyl]-1,2-dihydro)) in acetone to give the final

concentrations required, as described in section 2.3. Multiple prothioconazole active ingredient stock solutions were prepared so that lower concentrations of acetone could be achieved in the prothioconazole active ingredient treatment solutions for oral exposures, prepared by diluting a calculated aliquot of prothioconazole active ingredient stock solution in 40% (w/v) sucrose solution, leading to a final acetone concentration of 0.3%. All solutions were mixed using a magnetic stirrer. Prothioconazole commercial formulation stock solution was prepared by aliquoting a calculated amount of Proline® in 40% (w/v) sucrose solution. The concentrations of Proline® were calculated based on prothioconazole active ingredient content (containing 250 g/L prothioconazole) in the formulation so that both the technical grade prothioconazole and Proline® treatments contained the same concentration of prothioconazole. Required concentrations for pesticide oral exposure were prepared by aliquoting a calculated amount of prothioconazole commercial formulation stock solution into 40% (w/v) sucrose solution. All solutions were mixed using a magnetic stirrer. Prothioconazole active ingredient and commercial formulation solutions were prepared at a maximum time of 24 hours before first exposure and fresh prothioconazole stock and exposure solutions were prepared every two days to prevent any impacts of prothioconazole degradation on exposure experiments as prothioconazole aqueous photolysis is 2.1 days at pH 7. Prothioconazole solutions were kept at 4°C in labelled amber glass bottles covered in foil to prevent photolysis.

2.3.3 Pesticide Exposure Assays

Exposures were carried out following modified OECD 245 guidelines (OECD, 2017a). Bees were randomly chosen from each of four-five origin queenright colonies, disarmed using CO_2 and marked on the dorsal thorax with non-toxic paint in a colour assigned to their origin colony. Bees identified from each Biobest origin colony were then evenly allocated to technical replicate group isolation chambers so that each isolation chamber had an even mix of bees from each origin colony. In total, there were 12 (glyphosate) or 10 (prothioconazole) bees per isolation chamber unless otherwise stated. Group isolation chambers consisted of plastic chambers (11 cm x 7.5 cm x 17.5 cm) with a top-facing lid and a mesh lining for waste to fall through to a plastic bin or a plastic chamber (17cm x 14.7cm x 8.5cm) with a top facing lid lined with autoclaved sand where specified. Bees had *ad libitum* access to feeding tubes (15

ml falcon tubes with 2 mm feeding holes) filled with 40% w/v sucrose solution. Group isolation chambers were acclimatized and maintained at 23°C ± 3°C and 67% relative humidity ± 20%, unless otherwise stated, for the duration of the experiment and were continuously kept in the dark.

After overnight acclimatisation, each group had *ad libitum* access to their respective treatment or control solutions in feeding tubes as described above and were given a fresh suspension every day for the duration of the experiment (ten days for survival assays and five days for all other assays). Bee mortality and behaviour was recorded every 24 ± 2 hours from the first treatment exposure time for the duration of the exposure (See behaviour descriptions in Table 2-1). In bioassays carried out to determine survival, each treatment and control was carried out in triplicate with each group isolation box/treatment defined as one technical replicate.

Table 2-1. Conditions Recorded During Exposure Assays. Conditions were recorded for exposure assays for survival (ten days), proteomic (five days), and digestive tract microbiota (five days) analysis.

Condition Recorded	Description
Number of bees	Number of bees present in each group isolation chamber as the beginning of each 24 (± 2) hour period.
Environmental Observations	Relative humidity (%), temperature (°C)
Mortality	The number of bees dead.
Moribund	Bees unable to walk, showing very weak movement of leg or antennae and/or have a weak response to stimulation.
Affected	Bees are upright and attempting to walk but have a lack of coordination, hyperactivity, aggressiveness, rotations, or shivering.
Apathy	Bees showing low or delayed reactions to stimulation e.g. light or blowing, bees motionless
Comments	Response to stimulus (light blowing or light), number of bees surviving/expiring since last observation, behavioural or physical observations outside of those listed.

Statistical analyses were performed using Minitab® 20.3 using Anderson-Darling to confirm data followed the normal distribution. For survival assays, survival data was analysed using a Kaplan-Meier analysis using log-rank and Wilcoxon tests to determine if there were statistically significant differences in survival rates between treatments. Behavioural observations were analysed using a generalized linear model with treatment as the main factor and date, day, the number of bees present, temperature and humidity considered as covariates.

2.3.4 Sucrose Solution Consumption Recording and Analysis

Some pesticide exposure assays also aimed to determine sucrose solution consumption; three empty control chambers each contained the control solution (and three additional chambers contained an acetone control where exposure treatments contained acetone) in the same feeding tubes administered to bees to determine evaporation. All feeding tubes were weighed at each observation point and directly after fresh suspension of treatment every 24 ± 2.5 hours from the first treatment exposure time for the duration of the exposure. Consumption was calculated as the difference in feeding tube weight at each observation divided by the number of live bees that day. The average evaporation each day was deducted from the weight of feeding tubes accessed by bees. The amount of sucrose solution consumed was corrected for evaporation and was then divided by the number of bees present at the last observation to determine the amount of sucrose solution consumed per bee. The normality of consumption data was confirmed using an Anderson-Darling test. A two-sample t-test with 95% confidence interval was performed to determine any statistical significance in consumption between treatments. If data did not follow the normal distribution after transformations, a non-parametric Mann-Whitney test was carried out to determine differences in sucrose solution consumption. All consumption statistical analyses were carried out using Minitab® 20.3.

2.4 Mass Spectrometry Preparation and Analysis

At the end of the exposure period, bees were removed from their group isolation chambers one at a time and anaesthetised using CO_2 immediately before dissection and preparation of the relevant tissue for mass spectrometry. For all dissections carried out, dissections were alternated between bees from different exposure groups (e.g., exposure to treatment 1, exposure to treatment 2, control) to reduce any temporal bias in results.

2.4.1 Digestive Tract Dissection and Preparation for Proteomic Analysis

Bees were randomly selected from each group isolation chamber and the abdomen was removed. The abdomens were extended slightly while pinned to a wax dissection bed to view pleural membranes on the ventral abdomen; at this point, the bee was re-pinned to keep the abdomen in this position. Under a dissection microscope using a small sterile scissors, the cuticle was cut open to reveal the contents of the abdomen. If ovaries were present, these were first removed and discarded. Digestive tracts were cut directly below the crop to include the proventriculus to the end of the ileum. The digestive tract was then lifted from the abdomen with sterilized tweezers and placed into a 1.5ml Eppendorf containing 300 µl ice-cold lysis buffer, snap frozen in liquid nitrogen and kept at -20°C until all dissections from bees taking part in the exposure assay were completed. Between each bee, dissection tools were sterilized with ethanol. Once all tissue samples were dissected, samples were thawed on ice and homogenised for 30 seconds each. Samples were subsequently sonicated twice for 15 seconds and centrifuged at 9000rpm for five minutes at 4°C to pellet any remaining cellular debris. The supernatants were aliquoted in to clean 1.5 ml tubes and stored at -70°C until further mass spectrometry preparation of samples.

2.4.2 Fatbody Dissection and Preparation for Proteomic Analysis

Bees were randomly selected from each group isolation chamber and abdomens were pinned as outlined in 3.4.1. Using a small sterile scissors under a dissection microscope, the cuticle was cut around the perimeter of the bee abdomen and the ventral side of the abdomen was opened and pinned adjacent to the pinned dorsal abdomen. The dissection tract, any ovaries present, and any tracheal or muscle tissue blocking access to fat cells were removed and discarded. Fat cells were removed from

inside the dorsal and ventral abdomen using a sterile tweezers and placed into a 1.5 ml Eppendorf containing 300 µl ice cold lysis buffer, snap frozen in liquid nitrogen, and kept at -20°C until all dissections from bees taking part in the exposure assay were completed. Dissection tools were sterilized with alcohol between each bee. Once all tissue samples were dissected, samples were processed as outlined in 3.4.1.

2.4.3 Brain Dissection and Preparation for Proteomic Analysis

Bees were randomly selected from each group isolation chamber for dissection. For each brain dissection the abdomen was removed, and bees were held by the thorax. Antennae, proboscis, mandibles, and excess hair on the ventral head exoskeleton were remove with a small sterile scissors. A sterile blade was used to peel the exoskeleton from the head. Once the brain was visible, it was removed with a sterile tweezers and placed in a 1.5ml Eppendorf containing 300µl ice cold lysis buffer, snap frozen in liquid nitrogen, and kept at -20°C until all dissections from bees taking part in the exposure assay were completed. Dissection tools were sterilized with alcohol between each bee. Once all tissue samples were dissected, samples were processed as outlined in 3.4.1.

2.4.4 Protein Quantification and 2-D Clean Up

Protein quantification was carried out using Qubit® Quant-IT™ protein assay kit on a Qubit® fluorometer version 2.0 following manufacturer guidelines. 75-100 µg of protein was removed from each sample and processed with a 2-D clean up kit following manufacturer guidelines, to remove biological impurities. The resulting pellet was resuspended in 50 µl resuspension buffer (6M urea, 0.1M Tris HCl, pH 8.0), 10µl were allocated for post 2D clean up qubit quantification and gel electrophoresis, 20 µl was allocated for protein digestion and peptide purification, and 20µl was stored in a 1.5 ml Eppendorf tube at -20°C for the unlikely event of sample loss or further processing. 2D clean up qubit quantification was conducted using Qubit® Quant-IT™ protein assay kit on a Qubit® fluorometer version 2.0 following manufacturer guidelines.

2.4.5 One Dimensional Gel Electrophoresis and Protein Staining

One-dimensional, sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) was utilized to ensure sample quality before mass spectrometry. 10% ammonium persulphate, H_2O, 4X Protogel, resolving buffer, and TEMED solution were used to create SDS-PAGE resolving and stacking gels (

Table 2-2). Resolving gels were poured in the space between two 1mm plates set up following manufacturer guidelines. 120 µl butanol was aliquoted onto the surface of the resolving gel directly after pouring into the plates and given 40 minutes to ensure polymerisation. After this time, butanol was removed from the surface and the stacking gel was applied and left for 30 minutes. Gels were either used right away or stored at 4°C for up to two days. For each sample, 1-5µl sample was mixed with a calculated amount of 4X SDS sample buffer and 1 µl PCR water. Samples were run on 1D-SDS PAGE gel using a BioRad powerpack and gel rig at 120 V for 1.5-2 hours alongside a blue protein standard broad range ladder. Gels were removed from plates and the stacking portion discarded. Gels were then stained overnight with InstantBlue™ on a rocker and viewed using EpsonScan version 2.20 and ImageScanner III to determine sample quality before continuing with mass spectrometry analysis of samples.

Table 2-2. SDS PAGE Resolving and Stacking Gel Formulation.

Reagent	Resolving Gel (ml)	Stacking Gel (ml)
30% protogel	5	0.65
4X resolving buffer	3.1	-
Stacking buffer	-	1.25
H_2O	3.7	3
10% APS	0.125	0.025
TEMED	0.013	0.005

2.4.6 Protein Sample Digestion

A protein digestion protocol was followed to produce peptide products for mass spectrometry. 105 µl 50mM ammonium bicarbonate was added to 20 µl aliquots of each sample. For the reduction of protein disulfide bridges, 1µl of dithiothreitol was added to each sample and samples were incubated at 56°C for 20 minutes, vortexed directly before and ten minutes into the incubation period. Samples were removed from 56°C incubation, centrifuged for 3 seconds to collect all sample condensation at the bottom of the Eppendorf tube, and cooled at room temperature for five minutes. Following this, protein samples were alkylated by adding 2.7 µl iodoacetamide and incubated in darkness at room temperature for 15 minutes. Following reduction and alkylation, 1 µl of ProteaseMAX and 1µl of trypsin were added to all samples which were incubated at 37°C. After 15-17 hours, 1 µl trifluoroacetic acid was added to all samples to end digestion. Samples were incubated at room temperature for ten minutes and centrifuged for ten minutes at 10,800 rpm (13,000 x *g*).

2.4.7 Peptide Clean-Up and Purification

Digested samples were cleaned and purified using C18 spin columns following the manufacturer's guidelines. Purified peptides were eluted from the C18 columns into 1.5 ml Eppendorf tubes to collected 90 µl of purified peptides for each sample. Each sample was dried down using a SpeedyVac concentrator (Thermo Scientific Savant DNA 120) at medium heat for 1.5 – 2 hours. Once dried, samples were stored at 4°C until needed for mass spectrometry.

2.4.8 Mass Spectrometry

2.4.8.1 Calibration and Quality Control

Prior to each mass spectrometry run a series of instrumentation parameter checks were conducted. For the Q Exactive instrument the following parameters/parameter windows were required: Total Ion Chromatogram (TIC) <1000; TIC variation <10%; injection time <10 ms; background intensity > e^6; automatic gain control target = 100%; source turbomolecular pump speed = 100%; ultra-high vacuum turbomolecular pump = 100%, and lock mass variation ± 2. A 200ng loading of HeLa Protein Digest Standard (Pierce) was also run in quadruplicate prior to each run to determine if the Q Exactive was performing optimally. Raw files were searched using Proteome Discoverer version 1.4 (Thermo Fisher) against the UniProt protein set for *Homo sapiens* with the following parameters required before loading a sample: background intensity >1x e^9; number of spectra > 19000; number of protein groups > 2500, number of proteins with 2 or more peptides identified >1500, number of high confidence peptides > 9000, and retention times for key ions of 472.77 and 655.86 of 28.9 (± 0.2) and 36.7 (± 0.2) minutes, respectively. These reference values were determined for 200ng of the HeLa Protein Digest Standard run on the same methods, directly after a deep clean or service of the instrument.

After each run all raw files were inspected for spectral profile and background intensities to identify samples with dissimilar profiles to the majority. In addition, the sample that was loaded first was rerun at the end of the sample batch run to determine if retention time drift or a drop in sensitivity had occurred during the run. This was assessed through a principal component analysis (PCA) on the LFQ intensity values for all samples, where the pre- and post-run technical replicates were expected to resolve with one another. The PCA was also used to identify outliers in experimental groups. In cases where the pre- and post-run samples did not resolve with one another, or where the number of outliers resulted in too few replicates, the run was repeated. Only mass spectrometry runs and sample batches that passed all of the internal quality control checks described above are included in this thesis.

2.4.8.2 Peptide Sample Runs

On the day of a mass spectrometry run, dried samples were removed from the refrigerator and equilibrated at room temperature for ten minutes. Samples were resuspended in QExactive loading buffer (2% v/v acetonitrile and 0.05% v/v trifluoroacetic acid) to give a final concentration of 0.5µg/µl (unless otherwise stated). This was calculated using post 2D protein quantification values (see section 2.4.4). Samples were briefly vortexed and sonicated in a sonication bath for three minutes to improve peptide resuspension. Samples were centrifuged at 13,400 rpm (16,000 x g) for five minutes at room temperature and the supernatant was removed and placed into vials for mass spectrometry.

1µg of peptide mix for each sample was eluted onto the Q Exactive (Thermofisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. An increasing acetonitrile gradient was used to separate peptides on a Biobasic C18 Picofrit™ column (200 mm length, 75 mm ID), using a 120-minute reverse phase gradient at a flow rate of 250 nL/min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300-2000 Dalton) was carried out using the Orbitrap to select the 15 most intense ions prior to MS/MS. MaxQuant version 1.6.17.0 (www.maxquant.org) was used for protein identification and LFQ normalisation of all MS/MS data. The Andromeda search algorithm in MaxQuant was used to correlate all MS/MS data against protein reference sequences obtained from the National Centre for Biotechnology to correlate the data against the protein reference sequences derived from the *B. terrestris* genome (Sadd *et al.*, 2015) obtained from the National Centre for Biotechnology Information (NCBI) repository (17,508 entries, downloaded September 2018).

2.5 Proteomic Analysis

2.5.1 Annotation and Statistical Analysis

Perseus version 1.6.1.1 or 1.6.15.0 was used for data visualisation and statistical analysis. Normalized LFQ intensity values were used as a measure of protein abundance. The data was filtered for the removal of contaminants and peptides identified by site. LFQ intensity values were $\log_2$ transformed and samples were allocated to groups corresponding to treatment. Proteins absent from any samples in at least one group were not used for further analysis. A data imputation step was conducted to replace missing values with values that simulate signals of low abundant proteins chosen randomly from a distribution specified by a downshift of 2.1 times the mean standard deviation (SD) of all measured values and a width of 0.1 times this SD. Normalized intensity values were used for principal component analysis (PCA). A two-sample t-test was performed using a cut-off value of $p \leq 0.05$ to identify statistically significant differentially abundant (SSDA) proteins. Volcano plots were produced using VolcaNoseR (Goedhart and Luijsterburg, 2020) by plotting –Log p-values on the y-axis and Log_2 fold-change values on the x-axis to visualize differences in SSDA protein abundances between treatment groups. Hierarchical clustering of SSDA proteins was performed using z-score normalised intensity values to produce a heat map of protein abundance profiles for comparison between treatment groups.

2.5.2 Functional Annotation

The Search Tool for the Retrieval of Interacting Genes/proteins (STRING) version 11 (Szklarczyk *et al.*, 2019) (www.string-db.org) was used to map protein-protein interaction networks. Protein sequences were input into the STRING database and protein-protein interactions were analysed using the homologous *Apis mellifera* and *Drosophila melanogaster* match for each identified *B. terrestris* protein. To reduce redundant terms in functional enrichment analysis, where stated, STRING networks were sent to Cytoscape version 3.9.1. Here, functional enrichment terms from the genome were analysed and redundant terms were filtered with a redundancy cut-off value of 0.5 for all hits. Where stated, SSDA proteins were investigated for pathway enrichment and functional characterisation using Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (https://www.genome.jp/kegg/) using

BlastKOALA similarity tool (https://www.kegg.jp/blastkoala/). Resulting KEGG and protein IDs were used for gene ontology (GO) and BRITE enrichment to identify enriched biological pathways and protein functionality. Uniprot and QuickGO (www.uniprot.org; www.ebi.ac.uk/QuickGO) were used to further identify associated biological processes, molecular functions, and cellular compartments for SSDA proteins. Interpro (v.67.0) (www.ebi.ac.uk/interpro) was used to characterise protein superfamily and domain homologies of uncharacterised proteins to determine potential protein identity and function. Venn diagrams were produced to determine common SSDA proteins and relative fold changes from different treatment groups in comparison to their respective controls in glyphosate analysis experiments (http://bioinformatics.psb.ugent.be/webtools/Venn/). This was not carried out for prothioconazole experiments as the PAI treatment contained acetone.

2.6 Digestive Tract Microbiota DNA Extraction, Preparation, Sequencing, and Analysis

2.6.1 DNA Extraction and Quality Control

After pesticide exposure detailed in 3.3.3. Whole digestive tracts, excluding the crop due to its lack of an established microbiota (Martinson *et al.,* 2012), were dissected from bees similar to 2.4.1. DNA was extracted from individual digestive tracts using the DNeasy Blood and Tissue Kit following the manufacturer's instructions, with any changes detailed in this section. Dissected digestive tracts were homogenized in 200 µl buffer ATL. This was followed by the addition of 50 µl 10 mg/ml lysozyme. Samples were incubated for 30 minutes at 37°C and vortexed every five minutes at 800rpm. 300mg glass beads and 200µl buffer AL were added to each sample and samples were bead-beat for five minutes at 30Hz. Samples were incubated at 56°C and vortexed at 0 and 30 minutes at 800 rpm following the addition of 50 µl proteinase K. Extracted DNA was stored at -20°C before submission to NovoGene for 16S and ITS amplicon sequencing to determine the bacterial and fungal species present.

To determine DNA quality, extracted DNA was run on a 1% TAE, 1% agarose gel containing SyberSafe dye. DNA samples were mixed with 5x DNA loading buffer blue and run alongside a 100 bp and 1 kb DNA ladder. Gels were run at 120V for 40-60 minutes. For visualisation, gels were placed into an EtBr/UV transilluminator and observed using GeneSnap software version 7.05 (SynGene) for three seconds. To determine the presence of bacterial and fungal DNA, polymerase chain reactions (PCR) were carried out using primers to target the 16S and ITS regions of bacterial and fungal DNA, respectively. Before PCR preparation, all utensils and reagents needed were sterilised under UV light for 20 minutes. Master mix 1 and master mix 2 (Table 2-3) were made up and added to 1 µl of thawed DNA. Additionally, separate PCR cycles were used to determine bacterial and fungal (Table 2-4) DNA presence in samples using primers targeting 16S and ITS DNA regions. The resulting PCR products were run on a 1x TAE gel and visualised as outlined above. For final determination of DNA quality, DNA concentration and purity were measured for all DNA extraction samples using a NanoDrop™ spectrophotometer (ND-1000 3.7.1) using 1 µl buffer ATL as a blank sample. The concentration in ng/µl, absorbance at

260/280, and absorbance at 260/230nm were recorded. Once all quality checks were complete, DNA samples were sent to NovoGene Europe (UK) Ltd for further analysis.

Table 2-3. Master Mix 1 and Master Mix 2 Formulations for PCR of Bacterial 16S and Fungal ITS DNA fragments.

Reagent	µl/sample (Master Mix 1)	µl/sample (Master Mix 2)
PCR H_2O	14.75	12.05
5x Green GoTaq Buffer	5	5
$MgCl_2$ (25mM)	2.5	3
dNTPs (10mM)	0.5	0.5
515F (10mM)	0.5	-
806R (10mM)	0.5	-
ITS-1F	-	1
ITS-1R	-	1
GoTaq (5U/µl)	0.25	0.2
DNA template	1	1

Table 2-4. PCR Cycles for Attainment of PCR Products Containing Bacterial (PCR Cycle 1) or Fungal (PCR cycle 2) DNA From Primers Targeting the 16S and ITS Regions.

PCR Cycle 1		**PCR Cycle 2**	
Temperature (°C)	**Time (Minutes)**	**Temperature (°C)**	**Time (Minutes)**
94	5	95	3
35 cycles of		**40 cycles of**	
94	1	94	0.5
56	1	50	0.5
72	1	72	0.5
Final cycle			
72	10	72	30

2.6.2 PCR Amplification, Library Preparation, Sequencing, and Analysis

PCR amplification, library preparation, sequencing and statistical tests were carried out by NovoGene Europe (UK) Ltd. PCR amplification was carried out using primers specific for 16S (V4) and ITS (ITS1-5F) regions connecting with barcodes. 250 bp paired-end raw reads were generated from libraries sequenced on a paired-end Illumina platform NovaSeq 6000. Raw reads were filtered to obtain high-quality clean reads according to the Qiime (version 1.7.0) (Caporaso *et al.*, 2010). The SILVA138 database (http://www.arb-silva.de/) was used as a reference database for tag comparison using the UCHIME algorithm (Edgar *et al.*, 2011) to detect and remove chimera sequences. On the remaining tags, Uparse software (Uparse version 7.0.1090) was used to determine sequences with >97% similarity and these were clustered into operational taxonomic units (OTUs). A representative sequence for each OTU was screened using Qiime (version 1.7.0) in Mothur method against the SSUrRNA database from the SILVA138 database for species annotation. MUSCLE (version 3.8.31) was used to determine phylogenetic relationships of all OTU representative species. The data was normalized to obtain OTU abundance information and subsequent alpha and beta diversity analysis were carried out using this data. Wilcox and Tukey tests were performed to analyse the significance of differences between treatment groups. Taxonomy trees were created using independent NovoGene R&D software showing the top 10 genera in high relative abundance by default (DeSantis *et al.*, 2006). In addition to taxonomy trees, the relative abundances of the top 10 genera of each group and individual sample were displayed to demonstrate how they differ in relative abundance between treatment groups.

Alpha and beta diversity were calculated using QIIME (version 1.7.0) and displayed with R software (version 2.15.3). Several tests and parameters were used to determine alpha diversity i.e., species richness and evenness, and differences between treatment groups. Rarefaction curves were generated by comparing the number of OTU's identified (y-axis) and the sequence coverage (x-axis) to determine species richness and the sequencing depth used. Venn diagrams were generated to compare the number of unique and shared OTU's between treatment groups. A rank abundance curve was created to determine species richness and evenness of taxa across treatment groups. The rank abundance curve was generated by sorting OTU's in samples by their relative

abundance i.e., number of sequences identified, from large to small and assigning a sorting number. By plotting relative abundance on the y-axis and species rank on the x-axis, a general overview of species richness and evenness in each treatment group can be obtained. Further, Tukey and Wilcoxon tests were carried out on pairwise comparisons of observed species, Chao1, Shannon and Simpson indices to determine if there were significant differences in alpha diversity indices between treatment groups.

The determine beta diversity differences, i.e., community composition differences, weighted and unweighted unifrac distances were used. Unifrac distances involve calculating the distance between microbial communities in samples or treatment groups and using the square matrix of distance or dissimilarity between samples or groups to determine differences in beta diversity. Weighted unifrac data takes both the presence and relative abundance of taxa into account, whilst unweighted data takes only the presence/absence of taxa into consideration. Weighted unifrac can provide more information on overall community structure, whilst unweighted unifrac distances may give insight into the presence or absence of rare or low abundance taxa. PCA and Principal Coordinates Analyses (PCoA) were carried out to visualise distance between samples and treatment group community composition using dimensionality reduction. By summarising variables with orthogonal transformation, a PCA uses the minimum number of components to explain the most variance. PCoA was used to visualise the distance between samples using weighted and unweighted unifrac distances to account for the maximum distances between samples. In addition, boxplots were generated to show differences in beta diversity indices between groups where Tukey and Wilcox tests were carried out on beta diversity indices to determine significant differences in beta diversity between groups. Analysis of similarity (ANOSIM) and multi-response permutation procedure (MRPP) statistical methods were utilised to determine if there were statistically significant differences in bacterial and fungal community structure between treatment groups. ANOSIM is a non-parametric test which evaluates whether variation between groups is larger or smaller than variation within groups. In MRPP analysis, the differences in microbial community structure between groups are investigated for statistically significant differences using dimension reduction methods. Two-sample t-tests were used to determine significant differences in relative abundance of microbial genera and species between treatment groups ($p \leq 0.05$).

Chapter 3

Characterising the impacts of glyphosate and a glyphosate-based formulation on the digestive tract proteome and microbiota of *B. terrestris*

3.1 Introduction

3.1.1 The Digestive Tract

The digestive system is vital to meet the nutritional needs of the adult bumblebee. It plays a role in nutrition absorption, storage, food transportation, osmoregulation, and pathogen defence. The digestive tract is a tube of epithelial cells from the oral cavity to the anus and consists of three sections: the foregut, midgut, and hindgut (Figure 3-1). During development, the foregut and hindgut develop from invaginations that form on either end of the ectoderm of the developing embryo. The midgut arises from the endoderm. Midgut rudiments, tissues which form a bridge between the developing foregut, midgut, and hindgut, create the continuous tube of epithelial cells which will become the digestive tract (Stell, 2012; Klowden and Palli, 2022).

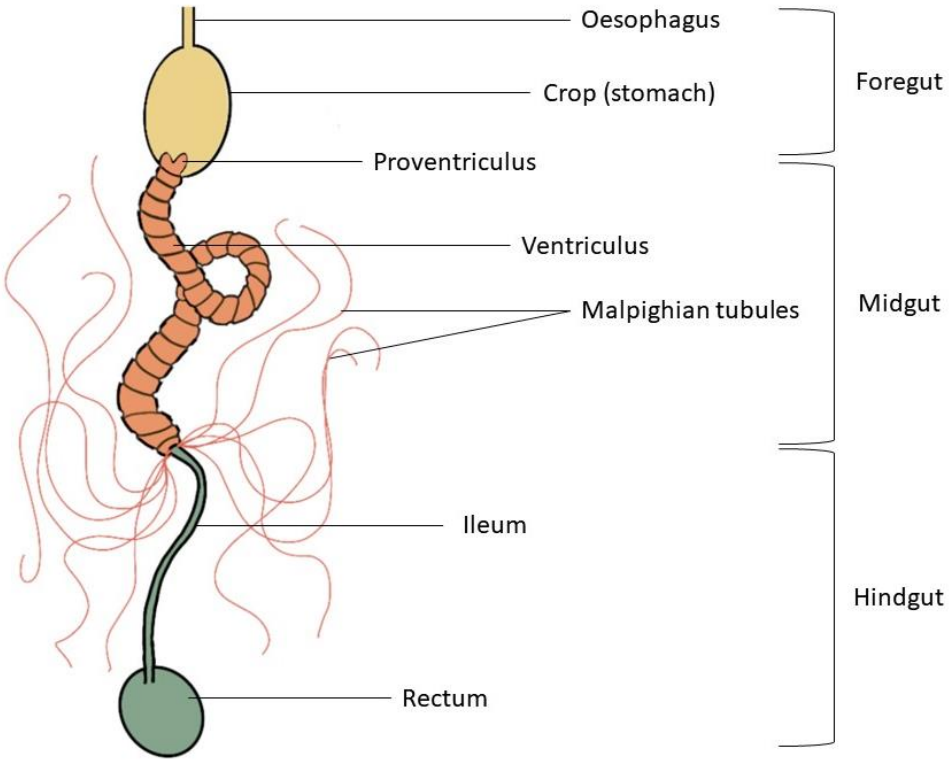


Figure 3-1 A labelled diagram of the bee digestive tract. The foregut includes the pharynx, salivary glands (not shown here), the oesophagus and the crop. The midgut begins at the proventriculus, and hindgut begins at Malpighian tubule protrusions.

3.1.1.1 The Foregut

The foregut consists of the oral cavity, salivary glands, pharynx, oesophagus, and crop. The bumblebee mouthparts can be considered as a pre-oral cavity. The main mouthparts for food ingestion to the oral cavity are the mandibles and proboscis. The mandibles are thick biting mouthparts with a sharp edge connected to the genae cuticle, powered by a pair of muscles in the head. The cuticle labrum plate rests between the mandibles and protects the soft epipharynx tissue which acts as a cushion for the proboscis to lean against when extended, creating an airtight seal. The proboscis requires this airtight seal as it used to aspirate nectar. The proboscis is a feeding tube with two overlapping galeae in the front and two labial palps in the back which aid in creating a vacuum when the proboscis is extended. Hairs which extend from the labial palps are also important in gathering sensory information. The glossa (tongue) exists in the centre of this structure and extends beyond the outer structures of the proboscis tube. The tongue has hair projections which increase the surface area of the proboscis. When the proboscis is not needed, it is folded behind the mandibles

(Stell, 2012). Whilst the structure of the proboscis creates a vacuum, the dilator muscles of the pharynx, situated at the front of the head, create suction for the aspiration of nectar through the proboscis and oral cavity through to the oesophagus. Head and thorax salivary glands secrete enzymes into the oral cavity where it travels with food to the pharynx passing the brain and down through the neck into the oesophagus. The oesophagus extends from the pharynx and distends once entering the petiole at the beginning of abdomen into the crop (stomach). The pharynx and oesophagus use peristalsis to move food through the digestive tract to the crop inner longitudinal and outer circular sheets of muscle. This is also useful in the regurgitation of nectar for feeding developing brood in the colony (Holtof *et al.*, 2019). Food that enters the crop will undergo some digestion from the salivary enzymes released in the oral cavity and some lipids can be absorbed here. The crop also has a storage function and can extend to carry up to 30% of the bees' weight in nectar (Stell, 2012). The foregut ends at the proventriculus, a sphincter muscle which protrudes into the crop and acts as a valve to regulate food entry to the midgut. Short spines in the proventriculus allow pollen to pass through to the midgut while nectar for the colony can remain in the crop (Klowden and Palli, 2022).

3.1.1.2 The Midgut

Through the proventriculus, food enters the midgut ventriculus where most digestion takes place. Due to its endodermal origin, the midgut is the only section of the digestive tract lacking a cuticle lining and consists of a single layer of epithelial cells. Many of these cells are enterocytes, although enteroendocrine cells are present along with immature intestinal stem cells (Klowden and Palli, 2022). All epithelial cells of the midgut rest on basal lamina and basement membrane networks surrounded by longitudinal and circular muscles to aid peristalsis. The majority of epithelial cells in the midgut are columnar enterocytes with microvilli at the apical membrane for increased surface area for the secretion of enzymes and absorption of nutrients (Huang *et al.*, 2015). Extensive networks of endoplasmic reticula are present in these cells for the efficient production of protein enzymes. Enteroendocrine cells play a role in the enteroendocrine system. Their presence in the midgut lumen allows for nutrient and xenobiotic sensing for production of necessary hormones and peptides and communication to other organs about the midgut environment via endocrine pathways

(Holtof *et al.*, 2019). Goblet cells are specialized enterocytes which may play a role in water flow for nutrient absorption in the midgut. Goblet cells form cavities inside the midgut epithelia and mediate ion transport across the midgut epithelia into the lumen via potassium antiporters and V-ATPase pumps. Midgut epithelial cells are continuously replaced by midgut progenitor cells in crypts from the opposite side of the midgut to the lumen which divide and specialize to become columnar enterocytes, enteroendocrine cells, or goblet cells.

As the food bolus enters and travels through the midgut lumen it is separated from direct contact with epithelial cells by the peritrophic matrix. The peritrophic matrix is a network of chitin microfibrils, peritrophins, glycoproteins and proteoglycans secreted by midgut epithelia (Holtof *et al.*, 2019). It was once believed that the sole purpose of the peritrophic matrix was to protect the midgut from damage from sharp-edged food and pathogens. Whilst this is of benefit, it seems the peritrophic matrix plays a much greater role in the efficient digestion of food in the midgut. By enveloping the food bolus, the peritrophic matrix creates compartmentalization in the ventriculus – an endoperitrophic space inside the peritrophic matrix and an ecoperitrophic space between the peritrophic matrix and the midgut epithelia within the ventriculus lumen (Figure 3-2). The peritrophic matrix network is permeable to allow the entry and exit of some enzymes and nutrients so digestion can occur in the endoperitrophic space. It is thought this may aid in countercurrent water flow, a process in which fluid is secreted at the posterior end of the midgut which allows the contents of the ectoperitrophic space to move back up towards to anterior midgut for further digestion and absorption (Jimenez and Gilliam, 1990). Due to this compartmentalisation, it is thought that digestion in the midgut occurs in stages. The first stage taking place in the endoperitrophic space, the second stage in the ectoperitrophic space and the final stage occurring along with absorption at the surface of midgut epithelial cells (Klowden and Palli, 2022). Once digestion and absorption take place, undigested food passes through to the hindgut.

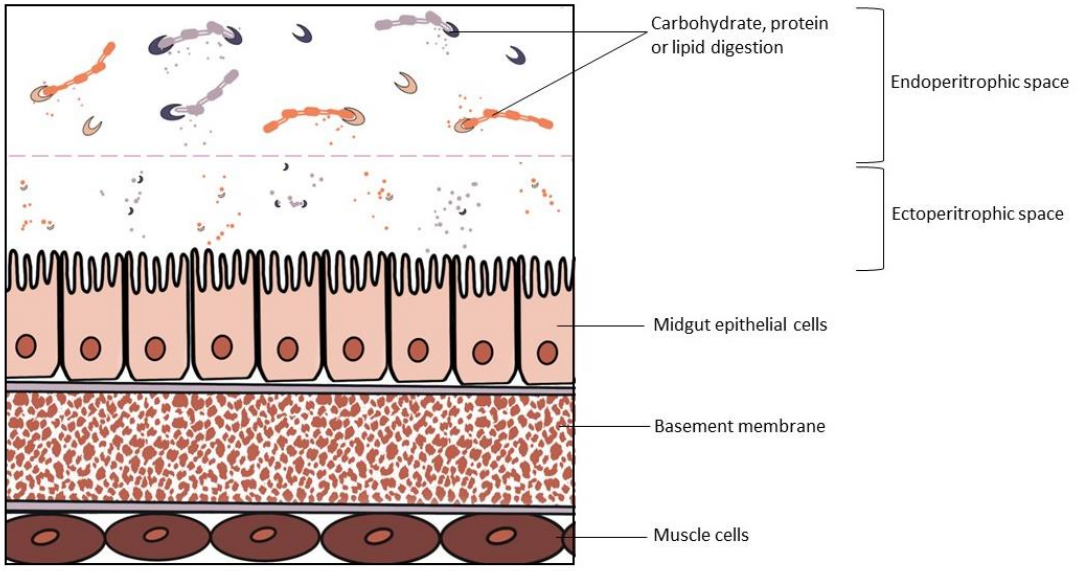


Figure 3-2 Midgut compartmentalization by the peritrophic matrix. The peritrophic matrix creates an endoperitrophic space where macronutrients are digested with pores which allow the entry and exit of enzymes and digested nutrients. Further digestion takes place in the midgut lumen outside of the peritrophic matrix - the ectoperitrophic space - before digestion by midgut epithelial cells (redesigned with modifications after Holtof et al. (2019)).

3.1.1.3 The Hindgut

The pylorus sphincter muscle marks the junction between the midgut and hindgut. The hindgut consists of Malpighian tubules, the ileum, and the rectum. Its main function is in osmoregulation and excretion. Approximately one hundred Malpighian tubules open into the junction between the midgut and hindgut. Malpighian tubules extend from this junction and lay freely in the abdominal haemocoel, where metabolic waste from the haemolymph diffuses into the lining of the tubule cells for transport to the ileum. The ileum is a narrow tube lined with cuticle which leads to the rectum, a distended sac with columnar epithelial cells clustering into rectal pads inside the rectum wall for fluid absorption and transport. From the contents delivered to the ileum by the ventriculus and Malpighian tubules, the ileum can selectively reabsorb amino acids, water, and ions as well as any useful nutrients available. Waste then passes through to the rectum where rectal pads can absorb water and remaining waste is stored as faecal matter until an appropriate time for excretion (Stell, 2012; Klowden and Palli, 2022;). Additionally, the hindgut is host to a

community of bacteria and yeast which form the specialized digestive tract microbiota (Martinson *et al.*, 2012).

3.1.1.4 The Digestive Tract Microbiota

The bumblebee digestive tract is host to approximately 30 million bacterial cells (Li *et al.*, 2015). The core taxa found in the bumblebee digestive tract are: *Snodgrasella, Gilliamella, Lactobacillus (Bombilactobacillus* and *Lactobacillus* near *melliventris*), *Schmidhempelia, Bifidobacteriaceae* (*Bifidobacterium* and *Bombiscardovia*) and *Enterobacteriaceae*. Non-core genera include *Apibacter, Acetobacteraceae, Fructobacillus and Pseudomonadaceae*. The majority of bee microbiota research to date has focused on honeybees (Hammer *et al.*, 2021; Hotchkiss *et al.*, 2022), which have differences in their core microbiota species diversity, lifecycle and nutritional needs compared to bumblebees (Leonhardt and Blüthgen, 2012; Kwong and Moran, 2016; Kwong *et al.*, 2017). In addition, whilst bumblebees contain many of the same core taxa as honeybees, there are differences, with *Schmidhempelia* and *Bombiscardovia* genera found only in bumblebees, and some core genera present are bumblebee-specific strains (Kwong *et al.*, 2017). These core taxa are generally present in bumblebees, regardless of an artificially-reared or wild life-history across Europe, Asia, and America (Hammer *et al.*, 2021). Such a distinct and well-established co-evolved microbiota is uncommon in insects, with the digestive tract microbiota of social bees closer to mammals than many insects (Kwong and Moran, 2016). Hammer et al. (2021) suggests that sociality present in both social bees and mammals gives an intergenerational transmission route to both groups and allows for host specialization unseen in non-social insects.

Symbiotic bacterial species are not present in developing brood and are obtained in the first days after emergence as an adult worker. Bumblebees acquire much of their core digestive tract microbiota through vertical transmission as newly emerged workers via the queen and other workers through a faecal-oral route with close contact required for transmission. In addition, bees can acquire non-core bacterial species through horizontal transmission from the environment through other colonies or species, likely when foraging or drifting into non-natal colonies, although drifting is of low occurrence in *B. terrestris* (Kwong *et al.*, 2014; Zanette *et al.*, 2014; Newbold *et al.*, 2015; Hammer *et al.*, 2021). In addition, yeasts present in nectar may rely on

this transmission route, relying on the host bumblebee for transmission between flowers and a safe place to reside over the winter within the queen's digestive tract (Pozo *et al.*, 2018; Praet *et al.*, 2018).

In recent years, a number of studies have highlighted the importance of the bee microbiota for nutrition, pathogen resistance, pesticide resistance, longevity and behaviour (Koch and Schmid-Hempel, 2011; Kwong *et al.*, 2017; Mockler *et al.*, 2018; Praet *et al.*, 2018; Li *et al.*, 2021; Steele *et al.*, 2021; Rutkowski *et al.*, 2022). However, the core species researched in association with the bumblebee digestive tract microbiota have mainly been elucidated via 16S rRNA gene sequencing which only provides information on the bacterial species present, with comparatively little attention given to the fungal species. Whilst some studies suggest fungal species are of little benefit or consequence to bees (Bosmans *et al.*, 2018; Pozo *et al.*, 2020), others highlight key benefits of fungal species in pathogen protection, nutrition and foraging behaviour (Herrera *et al.*, 2013; Stefanini, 2018; Parish *et al.*, 2020; Pozo *et al.*, 2021; Rutkowski *et al.*, 2022). Overall, the evidence of beneficial and mutualistic relationships between the bee host and hindgut microbiota is compelling, but the extent to which bees rely on their digestive tract microbiota community needs further research.

3.1.2 Glyphosate

Glyphosate ((N-phosphonomethyl) glycine) is one of the most widely used pesticides in agricultural and non-agricultural landscapes across the globe, and Ireland is no exception (Benbrook, 2016; Maggi *et al.*, 2019; López-Ballesteros *et al.*, 2022). Glyphosate is a non-selective, systemic herbicide and acts by inhibiting an enzyme - 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) – which is involved in the penultimate step of the shikimate pathway required for aromatic amino acid synthesis in plants and microorganisms (Steinrücken and Amrhein, 1980; Duke and Powles, 2008; Maeda and Dudareva, 2012) (Figure 3-3). The shikimate pathway is absent in animals, giving rise to the assumption that glyphosate exposure has little to no effect on non-target organisms. However, a growing body of evidence suggests that glyphosate can detrimentally affect animals. Numerous studies have now determined the impacts of glyphosate on learning and memory, reproduction, cell viability, the digestive tract microbiota and mitochondrial function in a wide range of animals

including mammals (George *et al.*, 2010; De Liz Oliveira Cavalli *et al.*, 2013; Ford *et al.*, 2017; Bali *et al.*, 2019; Tang *et al.*, 2020; Mesnage *et al.*, 2021), fish (Bridi *et al.*, 2017; A. G. Pereira *et al.*, 2018; Gaur and Bhargava, 2019; Panetto *et al.*, 2019), and invertebrates (Herbert *et al.*, 2014; Bailey *et al.*, 2018; J L Pereira *et al.*, 2018; Farina *et al.*, 2019; Motta and Moran, 2020; Gao *et al.*, 2021). As a result of possible negative impacts on mammals, there is concern for the safety of glyphosate for humans (Tarazona *et al.*, 2017). Due to glyphosate's intensive use in agricultural and non-agricultural settings, it is commonly found in food residues across the EU (European Food Safety Authority, 2021; 2022) and whilst authorization was originally due to expire on the 15th of December 2022, the EC extended glyphosate authorisation until December 2023 to allow for thorough risk assessment before reauthorisation or restriction.

3.1.2.1 Glyphosate's Mechanism of Action

Glyphosate inhibits growth and leads to the death of plants and microbes by disturbing the shikimate pathway. The shikimate pathway is found only in plants and microorganisms and produces chorismate as an end product. The shikimate pathway involves seven enzymatic reactions connecting carbon metabolism and aromatic amino acid synthesis, using intermediates of the glycolysis (phosphoenolpyruvate) and the pentose phosphate pathway (D-erythrose 4-phospate) to produce chorismate (Figure 3-3) (Maeda and Dudareva, 2012). This compound is essential to produce aromatic amino acids: L-tryptophan, L-tyrosine, and L-phenylalanine for protein synthesis and in synthesis of vitamins K1 and B9, plant defence and growth compounds and cell wall components (Herrmann and Weaver, 1999; Maeda and Dudareva, 2012). Whilst the disruption of aromatic amino acid synthesis is deemed as the major lethal impact of glyphosate exposure on organisms relying on the shikimate pathway, chorismate is also the precursor for secondary metabolites such as ubiquinone, lignans and tannins (Knaggs, 2001). Leading to multiple routes of lethality. When exposed to glyphosate, the inability of the organism to produce chorismate may lead to disrupted growth, development, defence, secondary metabolite synthesis and protein synthesis.

Glyphosate disrupts the shikimate pathway via the penultimate reaction involving 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS catalyses the

production of EPSP by transferring the enolpyruvyl of phosphoenolpyruvate to the 5-hydroxyl position of shikimate-3-phosphate produced earlier in the pathway. EPSP is used for the formation of chorismate, and the enolpyruvyl unit will eventually become the side chain of phenylalanine and tyrosine (Maeda and Dudareva, 2012). Glyphosate competitively inhibits the binding of phosphoenolpyruvate to EPSPS by occupying its binding site (Schönbrunn *et al.*, 2001). As a result, EPSP synthesis is reduced and with a lack of chorismate, aromatic amino acid synthesis and the synthesis of various compounds vital for physiological functioning cannot take place, leading to plant or microorganism death.

Whilst the shikimate pathway is present in plants, bacteria, fungi, and some protists, the enzymes involved display some differences across different organisms. For example, EPSPS's present in an organism can be classified based on glyphosate sensitivity with class I or class II EPSPS representing glyphosate-sensitive and glyphosate-resistant enzyme types, respectively. Whilst most bacteria and plants

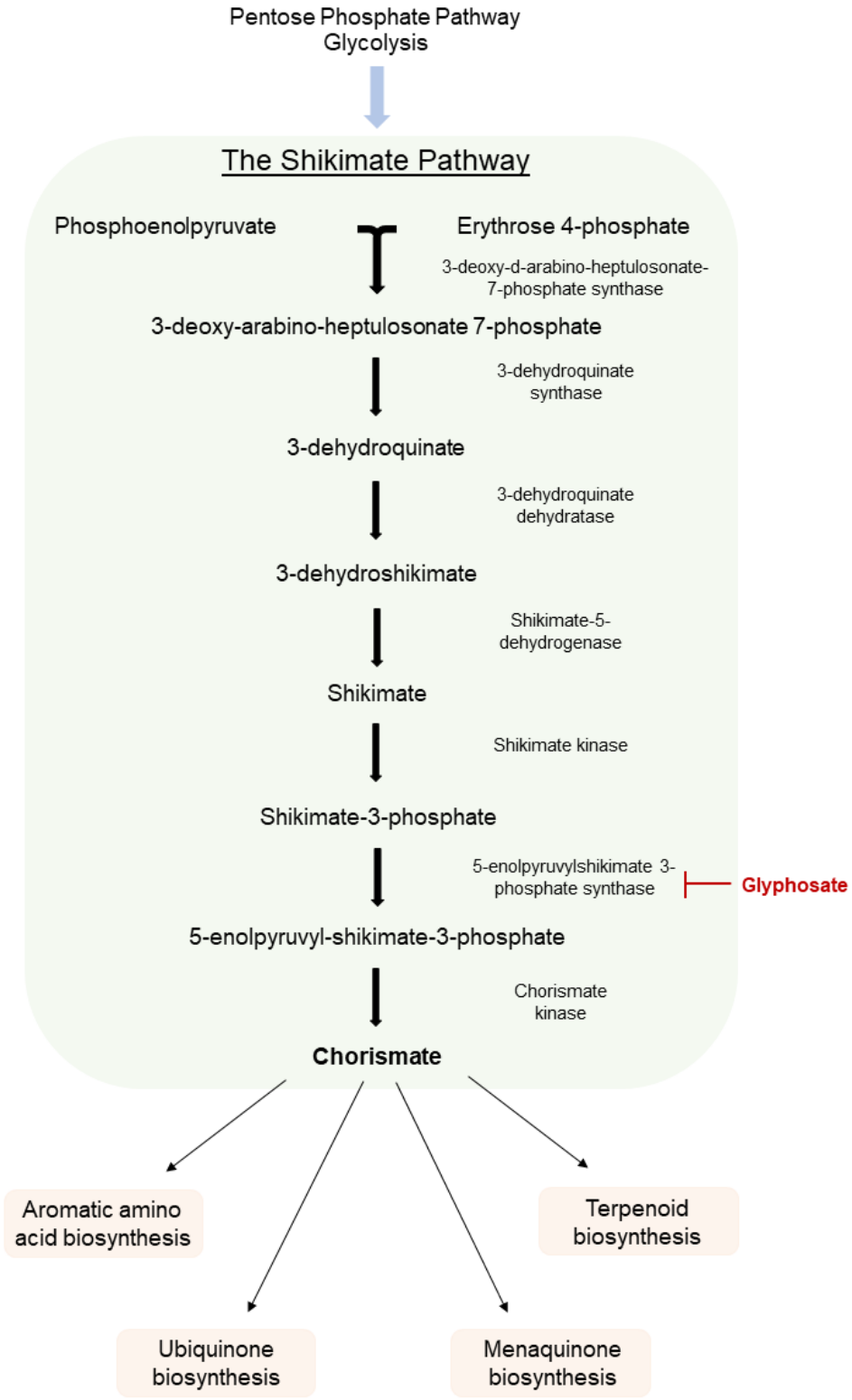


Figure 3-3 The shikimate pathway. The shikimate pathway produces chorismate, vital for aromatic amino acid biosynthesis and terpenoid biosynthesis in plants and microorganisms. Glyphosate disrupts the penultimate step in the shikimate pathway altering plant and microbe growth and development (after Mesnage et al. (2021)).

contain class I EPSPS, there are some exceptions, such as *Agrobacterium* spp. strain CP4 and *Bartonella apis* found in the *A. mellifera* digestive tract (Motta *et al.*, 2018; Padgette *et al.*, 1995). However, some *Snodgrasella alvi* strains from the honeybee digestive tract microbiota show resistance to glyphosate despite possessing a class I EPSPS, alluding to alternative glyphosate resistance mechanisms in some bacteria (Motta *et al.*, 2018).

Regardless of sensitive and non-sensitive EPSPS classes, at low doses, glyphosate may be beneficial for plant growth by inhibiting the growth of plant pathogens. In addition, a dose-response phenomenon known as hormesis has been observed in glyphosate-exposed plants, where a low dose of glyphosate leads to a somewhat beneficial response rather than the lethal response seen at higher doses (Brito *et al.*, 2018). Williams et al. (2015) discovered that glyphosate could increase the ear number and kernel mass of sweetcorn and Ather Nadeem et al. (2017) found growth and seed increases in various plant species exposed to low doses of glyphosate including the bee pollinated wildflower *Lathyrus aphaca*. It is unclear how widespread this phenomena is or how glyphosate exposure could lead to beneficial impacts on plants, however, it could lead to the overuse of glyphosate at high concentrations with the desire to kill target plants. This may lead to the development of glyphosate-resistant flowering weeds which could be attractive to insect pollinators whilst containing glyphosate residues (Brito *et al.*, 2018). Whilst bees may be exposed to glyphosate at higher concentrations via pollen and nectar before plant death (Thompson *et al.*, 2014, 2022), some studies have determined low glyphosate concentrations in pollen and nectar of non-target healthy plants (Cebotari *et al.*, 2018; Zioga *et al.*, 2022), resulting in chronic exposure of bees to glyphosate. However, the majority of studies investigating the impacts of glyphosate on bees or their microbiota focus on relatively high concentrations (Gregorc and Ellis, 2011; Motta *et al.*, 2018; Blot *et al.*, 2019; Chen *et al.*, 2022), resulting in a need for research of the impacts of glyphosate on bees at low doses which may be found in healthy, thriving, bee-attractive plants.

3.1.2.2 The Impact of Glyphosate on Bees

Pesticides have been implicated as one of the principal drivers of bee decline, but most research focuses on insecticides, with much less known about the impacts of non-insecticidal pesticides on bees (Cullen *et al.*, 2019). Given that glyphosate is one of

the most widely used pesticides worldwide (Benbrook, 2016; Maggi *et al.*, 2019), and bees could be exposed to glyphosate when foraging (Thompson *et al.*, 2022), determining whether it has impacts on pollinators is essential.

The impact of non-insecticidal pesticides such as glyphosate on important pollinator groups including bees is beginning to receive attention (Cullen *et al.*, 2019). Under certain conditions, glyphosate has been shown to impact bee survival (Dai *et al.*, 2018; Almasri *et al.*, 2020; Motta and Moran, 2020), learning and memory (Herbert *et al.*, 2014; Mengoni Goñalons and Farina, 2018; Farina *et al.*, 2019), brood development (Farina *et al.*, 2019; Odemer *et al.*, 2020), and immunity (Vazquez *et al.*, 2018; Zhao *et al.*, 2020; Almasri *et al.*, 2021; Castelli *et al.*, 2021; Motta and Moran, 2022). Additionally, glyphosate is known to affect the digestive tract microbiota of honeybees at some concentrations (Dai *et al.*, 2018; Motta *et al.*, 2018; Blot *et al.*, 2019; Motta and Moran, 2020) which may have consequences for development, nutrition, and pathogen protection (Kwong *et al.*, 2017; Zheng *et al.*, 2017; Stefanini, 2018; Miller *et al.*, 2021). Given that the shikimate pathway is present in microbes but not bees (Herrmann and Weaver, 1999) and the impacts of glyphosate on the digestive tract microbiota in honeybees, glyphosate may result in alterations to the digestive tract microbiota of bumblebees, too. However, it is unknown whether these effects are typical for all bees as little research exists that investigates the consequences of glyphosate exposure for bumblebees or solitary bees (Cullen *et al.*, 2019). In addition, pesticide sensitivity and differences in lifecycle, habitat, and nutrition can impact risk severity, resulting in a need for pesticide risk assessment on a wider range of bee species (Arena and Sgolastra, 2014a; Manjon *et al.*, 2018; Sgolastra *et al.*, 2020).

One of the difficulties associated with assessing the potential risks of pesticides to insects relates to the presence of co-formulants in commercial formulations used in both agricultural and non-agricultural settings. Most testing conducted by regulatory bodies for negative impacts of pesticides on bees, for example, focuses on the active ingredient (The European Commission, 2009; European Food Safety Authority, 2013). However, most commercially available products include the active ingredient and co-formulants, such as surfactants, antifoaming agents, solvents, and dyes that improve the efficacy of the pesticide. Under European Union regulation (EC) No. 1107/2009, individual co-formulants are not required to undergo testing of the same

stringency as those of active ingredients (The European Commission, 2009, 2013). In addition, formulations may not require testing for toxicity to honeybees if previously tested formulations contain co-formulants which are 'equivalent' (The European Commission, 2009) (See section 1.5.2).

Although the effects of glyphosate-based commercial formulations on various nontarget organisms is now being increasingly investigated in academic research (Mesnage *et al.*, 2013, 2015; J.L. Pereira *et al.*, 2018; Lopes *et al.*, 2018; Fantón *et al.*, 2020; Pochron *et al.*, 2020; Zhao *et al.*, 2020), whether these effects are attributable to the active ingredient or co-formulants is seldom determined. This represents a major knowledge gap in our attempts to mitigate the risks posed to pollinators by herbicides. Co-formulants are generally not subjected to the same testing rigour as the AI and where they have been tested, the focus has been on mortality, ignoring possible sublethal effects (Mesnage and Antoniou, 2018; Novotny, 2022; Straw *et al.*, 2022). In addition, many co-formulants are considered proprietary information, making it difficult and sometimes impossible to assess co-formulant impact on the organism being studied (Mesnage and Antoniou, 2018).

Overall, the widespread use of glyphosate and the lack of studies investigating impacts on bees, particularly in non-*Apis* species, has led to a weak understanding of how this pesticide may impact wild bee species such as bumblebees. To determine the possible impacts of glyphosate efficiently and rapidly on bumblebees, molecular analysis of the impact of glyphosate exposure is required. In this chapter, an assessment of the impacts of technical grade glyphosate and the glyphosate-based formulation, RoundUp Optima+®, at a field realistic concentration, on the bumblebee *Bombus terrestris* is described. The results of this research can be applied to the AOP framework developed by Ankley et al. (2010), which aims to understand and collate ecotoxicological effects of a chemical across different biological levels (See section 1.7). The digestive tract was investigated because i) it represents one of the most important organs in the bee for nutrition and pathogen resistance, ii) it is one of the first points of contact between ingested pesticides and host cells, and iii) it houses an important and diverse microbiota. Using a framework such as the AOP when investigating the impacts of pesticides can provide a clear understanding of how and where a chemical affects an organism and identifies knowledge gaps and research

priorities going forward. Molecular level effects of glyphosate will be determined in this chapter using LFQ mass spectrometry-based proteomics (Cox and Mann, 2007; Walther and Mann, 2010) on total protein extracts derived from the digestive tracts of bees after glyphosate AI or RoundUp Optima+® exposure. To assess the impact of glyphosate on *B. terrestris* at the organism level, survival, behaviour, and consumption are useful indicators to determine if there are impacts on basic functioning. Since glyphosate impacts the shikimate pathway, which is present in bacteria and fungi, analysis of DNA extracts from the digestive tract after glyphosate exposure can determine whether glyphosate impacts the digestive tract microbiota. Combined, this multi-level analysis will provide insight into the impacts of glyphosate exposure and the mechanisms behind them (if any) in *B. terrestris*, an ecologically important pollinator and organism which is missing the primary target pathway of this herbicide. Such an approach will also determine whether differences are observed in bees exposed to glyphosate alone or a glyphosate-based formulation, and therefore highlight the potential risks posed by co-formulants.

3.1.3 Chapter Aims

1. Determine the organism-level impacts of glyphosate AI and RoundUp Optima+® on *B. terrestris* by analysing mortality, behaviour, and sucrose solution consumption.
2. Characterise the impact of glyphosate AI and RoundUp Optima+® on the *B. terrestris* digestive tract proteome.
3. Determine if glyphosate AI or RoundUp Optima+® impact the digestive tract microbiota of *B. terrestris* using 16S and ITS DNA amplicon sequencing.
4. Compare and contrast the impacts of glyphosate AI and RoundUp Optima+® on *B. terrestris* digestive tract proteome and microbiota to ascertain if there are overlooked risks associated co-formulant mixtures present in a commonly used formulation.

- Populate an AOP model to determine the major impacts of glyphosate on the *B. terrestris* digestive tract based on this research, where knowledge gaps remain and highlight where further research is required to fully elucidate the mechanism(s) of action and downstream impacts of glyphosate exposure on bees.

3.2 Experimental outline

3.2.1 Survival Assay and Analysis

Glyphosate active ingredient (GAI) and RoundUp Optima+® commercial formulation (GCF) were prepared at 1, 10 and 100ppm in 40% (w/v) sucrose solution as described in section 2.3.1. The concentrations of RoundUp Optima+® were calculated based on glyphosate acid content in the formulation so that both the technical grade glyphosate and RoundUp Optima+® treatments contained the same concentration of glyphosate. Seventy-two bees were chosen randomly from each of four origin commercial colonies and evenly allocated to group isolation chambers (11cm x 7.5cm x 17.5cm) resulting in twelve bees per chamber. Bees were acclimatised overnight as described in section 2.3.3 at 23 ± 3°C and 58 ± 6% relative humidity. The following day, bees were exposed to either 1, 10 or 100ppm GAI or GCF or the control solution 40% (w/v) sucrose solution for ten days as described in section 2.3.3. Every 24 ± 2 hours, bees were observed for mortality and behavioural alterations (Table 2-1) and given fresh aliquots of treatment. This experiment was conducted in triplicate and data was analysed as described in section 2.3.3.

3.2.2 Glyphosate Exposure, Digestive Tract Sample Preparation for Mass Spectrometry and Proteomic Data Analysis

Exposures were conducted as described in section 2.3.3. Nine bees were randomly chosen from each of four origin commercial colonies with three bees per origin colony allocated to one of three group isolation chambers, in a comparable way to section 3.2.1. Each group isolation chamber comprised twelve bees in a plastic chamber (17cm x 14.7cm x 8.5cm). All bees were acclimatised overnight at 20 ± 2°C and 58 ± 5% relative humidity, as for the duration of the experiment and were continuously kept in the dark. The following day, bees were exposed *ad libitum* to either 1ppm GAI or 1ppm GCF in 40% (w/v) sucrose solution, or the control 40% (w/v) sucrose solution. Every 24 ± 2 hours, bees were observed for mortality and behavioural alterations (Table 2-1) and given fresh aliquots of treatment. A conservative and field realistic dose of 1ppm was selected for this experiment, based on the lowest concentration found in the crops of honeybees after consumption of treated forage, and concentrations found in untreated tree flowers, reported in Thompson *et al.,* (2014)

and Cebotari *et al.,* (2018), respectively. We deviated from the OECD exposure length of 10 days to an exposure length of 5 days as bees can realistically be exposed to glyphosate via pollen and nectar of treated plants for this period (Thompson *et al.,* 2022; Thompson *et al.,* 2014). Bees may also be exposed to glyphosate for longer periods from non-target plants containing glyphosate in pollen or nectar (Cebotari *et al.,* 2018).

Eight bees were randomly selected from each group isolation chamber after the five-day exposure and samples were processed, prepared, and run on the Q-Exactive mass spectrometer as described in section 2.4 with four digestive tracts (bees) per treatment analysed using LFQ mass spectrometry. All data analysis of results obtained from the LFQ mass spectrometer followed the protocol described in section 2.5 using Perseus v 1.6.1.1. Behaviour data collected from the pre-dissection exposure assay was analysed as described in section 2.3.3.

3.2.3 Glyphosate Exposure, Consumption Assay, and Digestive Tract Microbiota DNA Processing, Sequencing, and Analysis

Six bees were randomly selected from each of five origin colonies, with bees evenly spread across three group isolation chambers resulting in ten bees per isolation chamber (11cm x 7.5cm x 17.5cm). All bees were acclimatised to 25 ± 3°C and 77± 10% relative humidity for the duration of the exposure and were continuously kept in darkness. Bees were exposed to treatments *ad libitum* for five days as described in section 3.2.2. In addition, three empty isolation chambers were assembled containing the control solution only to determine evaporation each day to record consumption. Mortality, behaviour, and consumption were recorded every 24 ± 2.5 hours as described in sections 2.3.4 and 3.2.2. Consumption data was analysed as described in section 2.3.4 and mortality data was analysed as described in section 2.3.3. Behaviour could not be analysed as no behavioural alterations were observed. Digestive tracts were dissected from eight bees per treatment and DNA extraction, processing, sequencing, and analysis were conducted as described in section 2.6.

3.3 Results

3.3.1 Survival Assays

No significant differences were observed in survival between any of the pesticide treatments in comparison to the control treatments (Log-rank p=0.416, Wilcoxon p=0.436) (Figure 3-4; Table S3-1; Table S3-2).

3.3.2 Behavioural Alterations

From behavioural data recorded during ten-day survival assays, generalised linear models determined that date and day, i.e., the longer bees were contained in isolation chambers, to have a significant effect on the number of bees displaying moribund behaviour (p = 0.048 and 0.047, respectively) (Table S3-3). In addition, 10ppm GAI had a significant impact on the number of moribund bees (p = 0.044). There were no differences in the frequency of behavioural observations between bees exposed to any other treatment or during the five-day exposure conducted for the proteomics experiment. Behavioural analysis could not be conducted on data collected during exposures for the microbiota experiment as all values were zero.

3.3.3 Alterations to Sucrose Solution Consumption

A two-sample t-test did not determine any statistically significant differences in consumption of the GAI (P=0.124) or GCF (P=0.623) in comparison to the control group. In addition, there were no statistically significant differences in consumption between either glyphosate treatment (p=0.27) (Table S3-4; Table S3-5).

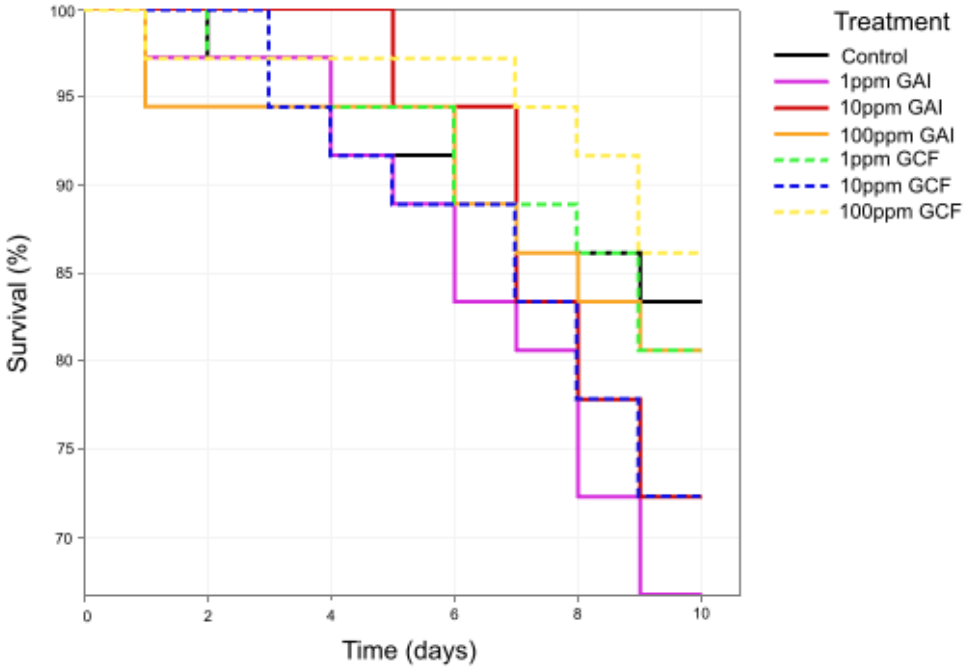


Figure 3-4 A Kaplan-Meier survival curve displaying survival over the duration of exposure to 1, 10 or 100ppm glyphosate AI or CF or control. There were no significant differences in survival between treatments.

3.3.4 LFQ Analysis of Glyphosate Exposure on the *B. terrestris* Digestive Tract

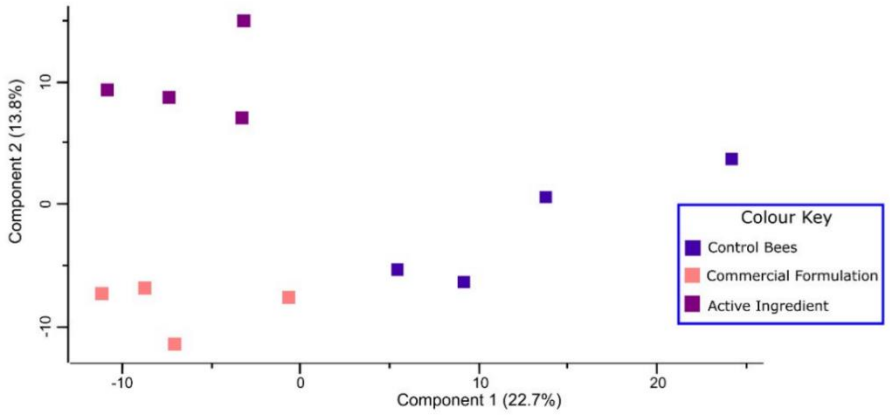
3.3.4.1 Identified and Quantified Proteins

In total, 2,613 proteins were originally identified from proteins extracted from the *B. terrestris* digestive tract, of which 1,365 proteins remained after filtering (Table S3-6). Principal component analysis (PCA) on all proteins resolved a clear difference between the three treatment groups (Figure 3-5A). Variance of both glyphosate-based GAI and GCF compared to the control samples indicate distinct differences. There is also a distinct variance between the proteomes of GAI and GCF bee digestive tracts, demonstrated by the separate clustering of GAI samples and GCF-exposed digestive tract samples in the principal component analysis.

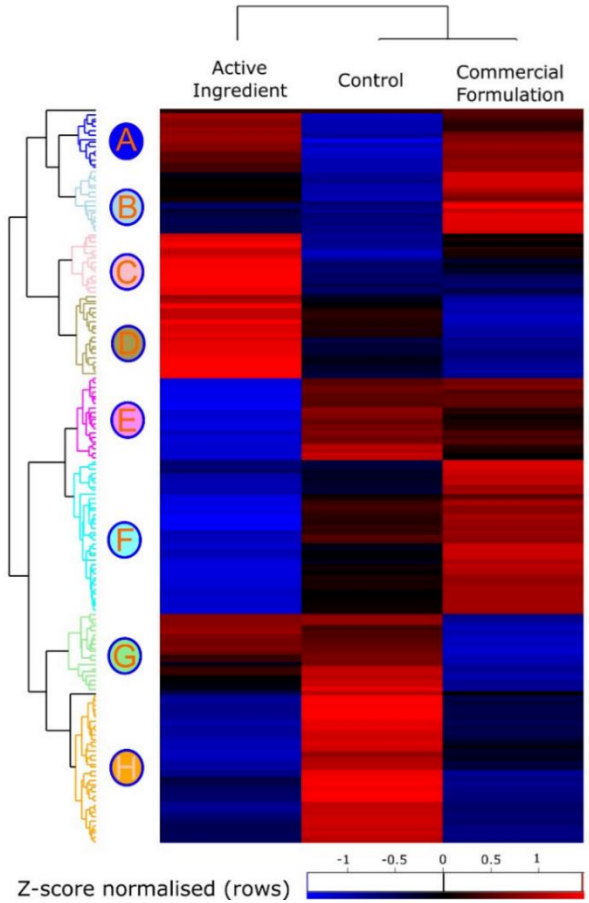
3.3.4.2 Two Sample T-tests

Two sample t-tests were performed amongst treatment groups to determine statistically significant differentially abundant (SSDA) proteins ($p \leq 0.05$, $S0 = 0.1$) and their relative fold differences (Table S3-7). STRING analysis was conducted on SSDA lists to identify pathways, processes, GO, KEGG terms and protein networks that were enriched in one protein set over another.

A



B



C

Cluster	Term description	Database	Term ID	FDR
A	Hydrolase	Uniprot	KW-0378	0.0232
B	Nucleotide metabolic process	Biological Process	GO:0009117	0.0495
	Hydrolase activity	Molecular Function	GO:0016787	1.91E-05
C	No significant enrichments			
D	Glycolysis / Gluconeogenesis	KEGG	dme00010	0.0042
E	Glutamine amino acid metabolism	Biological process	GO:0009064	0.00074
	Pyruvate metabolism	KEGG	dme00620	0.00021
	Aldehyde dehydrogenase family	PFAM	PF00171	0.0027
F	Mitochondrion	Cellular component	GO:0005739	1.66E-17
	Fatty acid beta-oxidation	Biological process	GO:0006635	2.34E-09
G	Lysosome	KEGG	dme04142	0.034
	Endocytosis	KEGG	dme04144	0.034
H	Endocytosis	Biological process	GO:0006897	0.0066
	Plasma membrane	Cellular component	GO:0005886	0.0171

Figure 3-5 There are Distinct Differences Between Glyphosate-Based Treatments. (A) A principal component analysis (PCA) demonstrates distinct treatment-dependant clustering of digestive tract proteomes. (B) Hierarchical clustering of z-score normalised values cluster the median protein expression values of SSDA proteins with a similar expression pattern in each treatment and (C) GO terms associated with each cluster were investigated to determine the processes represented by protein clusters.

3.3.4.2.1 Glyphosate Active Ingredient Versus Control

A total of 152 SSDA proteins (relative fold change (RFC) range: -222.68 to +18.07) were identified from the digestive tracts of GAI-exposed bees in comparison with control-treated bees, with 56 and 96 proteins having an increased and decreased abundance, respectively (Figure 3-6). The top 10 proteins with the highest increased abundance included the hydrolase glucosylceramidase (RFC: +18.07), an oxidase; laccase-1 (RFC: +9.56), a venom acid phosphatase; venom acid phosphatase Acph-1 (RFC: +2.78), the lysosomal protein; beta-hexosaminidase subunit (RFC: +2.02), the digestive serine protease; chymotrypsin (RFC: +1.50), a protein with carboxylic ester hydrolase activity; venom carboxylesterase-6 (RFC: +1.36), and two uncharacterized proteins: uncharacterized protein LOC100646617 (RFC: +2.82) and uncharacterized protein LOC100646009 (RFC: + 1.32) which has glutathione transferase and peroxidase activity. There were also proteins associated with 60S ribosomal subunit biogenesis (nucleolar protein 58, RFC: +5.28) and calcium homeostasis (regucalcin, RFC: +2.23). The top 10 proteins with decreased abundance included the transmembrane protein CD151 antigen (RFC: -10.43) and an uncharacterized protein; uncharacterized protein LOC105666313 (RFC: - 68.82). There were also proteins associated with microfibril formation (fibrillin-2, RFC: - 222.68), the basement membrane (collagen alpha-5(IV) chain, RFC: - 9.16 and collagen alpha-1(IV) chain, RFC: - 5.23), proteolysis and ubiquitin-mediated protein catabolism (uncharacterized protein LOC100644923, RFC: - 8.62, ubiquitin carboxyl-terminal hydrolase, RFC: - 10.42 and S-phase kinase-associated protein 1, RFC: - 5.67), cell adhesion (cartilage oligomeric matrix protein, RFC: 6.80) and transmembrane transport (sodium-coupled monocarboxylate transporter 2, RFC: - 8.37).

3.3.4.2.2 Glyphosate Commercial Formulation Versus Control

A total of 119 SSDA proteins (RFC range: -56.83 to +9.69) were identified from the digestive tracts of GCF-exposed bees in comparison with control-treated bees, with 47 and 72 proteins having increased or decreased abundance, respectively (Figure 3-7). The top 10 proteins with the highest abundance in comparison to the control included: a hydrolase involved in sphingolipid metabolism, glucosylceramidase (RFC:

+9.69), an oxidase; laccase-1 (RFC: +8.31), a serine-type peptidase; venom dipeptidyl peptidase 4 (RFC: +5.64) and an uncharacterized protein containing a chitin-binding type 4 domain; uncharacterized protein LOC100648508 (RFC: +7.30). There were also proteins associated with carboxylic ester hydrolase activity (venom carboxylesterase-6, RFC: +7.37), cellular protein modification (ubiquitin protein 5, RFC: +3.68), integral component of membrane (transmembrane protein 177, RFC: +3.27), mitochondrial respiratory chain complex I assembly (FAD-dependent oxidoreductase domain-containing protein 1, RFC: +3.15), trehalose metabolic process (trehalase, RFC: +2.33), and calcium homeostasis (regucalcin, RFC: +2.33). Of the top 10 proteins with the lowest abundance in comparison to the control there were the transmembrane protein CD151 antigen (RFC: -9.68) and an uncharacterized protein; uncharacterized protein LOC105666313 (RFC: -25.35). Other proteins were associated with microfibril formation (fibrillin-2, RFC: -56.83), lipid catabolic process (pancreatic lipase-related protein 2-like protein, RFC: -21.15), translation (eukaryotic translation initiation factor 2 subunit 1, RFC: -14.61), proteolysis (uncharacterized protein LOC100644923, RFC: -10.83), Arp2/3 complex-mediated actin nucleation (actin-related protein 2/3 complex subunit 3, RFC: -8.66), cell adhesion (cartilage oligomeric matrix protein, RFC: -6.79), signalling (protein slit-like isoform X1, RFC: -6.71) and, the basement membrane (collagen alpha-5(IV) chain protein, RFC: -5.52).

3.3.4.2.3 Glyphosate Commercial Formulation Versus Active Ingredient Alone

A total of 149 proteins (RFC range: -92.29 to +14.63) were identified from the digestive tracts of GCF-exposed bees in comparison with GAI-exposed bees, with 83 and 66 proteins having an increased and decreased abundance, respectively (Figure 3-8). Of the top 10 proteins with the highest abundance in the digestive tracts of GCF-exposed bees compared to the GAI-exposed bees, there were: a transmembrane helix protein (transmembrane protein 256, RFC: + 3.55), protein NipSnap (RFC: + 4.17), beta-lactamase (RFC: + 4.03) and two uncharacterized proteins; uncharacterized protein LOC100643115 (RFC: +14.63) and uncharacterized protein LOC100648508 (RFC: + 2.86), which has a chitin-binding type-4 domain. There were also proteins associated with translation (60S ribosomal protein L35a, RFC: +8.81), ubiquitin-dependant protein catabolic process (ubiquitin carboxyl-terminal hydrolase, RFC: + 5.65), protein localization to the membrane and negative regulation of peptidoglycan

recognition protein signaling pathway (transmembrane 9 superfamily member 2, RFC: + 4.60), carbohydrate metabolic process (myrosinase 1, RFC: + 4.17) and mitochondrial respiratory chain complex I assembly, with oxidoreductase activity (FAD-dependent oxidoreductase domain-containing protein 1, RFC: + 3.78). Of the top 10 most decreased proteins in GCF-exposed compared to GAI-exposed bees, there were: two uncharacterized proteins (uncharacterized protein LOC100651249, RFC: - 2.17 and uncharacterized protein LOC100646617 (RFC: - 4.46) and putative cysteine proteinase CG12163 (RFC: -1.44) which has cysteine-type peptidase and endopeptidase inhibitor activity. There were also proteins associated with sphingolipid metabolic process (glucosylceramidase, RFC: -1.86), ubiquitin-dependent protein catabolic process (E3 ubiquitin-protein ligase UBR4 isoform X1, RFC: - 2.01), carbohydrate metabolic process (L-lactate dehydrogenase-like isoform X2, RFC: - 2.04), lipid transporter activity (Niemann-Pick type protein homolog 1B, RFC: - 4.88) and three pancreatic lipases which are associated with lipid catabolic processes (RFC: - 2.05, - 2.15 and -92.29).

3.3.4.3 Gene Ontology and Pathway Analysis of SSDA Proteins

To determine the pathways and processes impacted in the brain and fatbodies of glyphosate-exposed bees, SSDA proteins from pairwise comparisons were analysed for enriched PPI networks, GO terms, KEGG pathways and Interpro domains using STRING v.11 using *D. melanogaster* as a reference genome (Table S3-8). BlastKOALA was used in the analysis of SSDA proteins against KEGG and BRITE databases (Table S3-9).

For SSDA proteins enriched in the digestive tract proteome of GAI compared to the control treatment group, there was an increase in proteins associated with carbohydrate metabolic process (11 proteins) and response to toxic substance (five proteins). BRITE analysis resolved an increase in proteins associated with membrane trafficking (five proteins) and the exosome (18 proteins). There was a decreased abundance in proteins associated with cell junction assembly (nine proteins), transport (28 proteins) and the TCA cycle (six proteins) (Figure S3-1). In addition, KEGG analysis resolved an enrichment in focal adhesion (five proteins) and amino acid biosynthesis (five proteins) pathways. BRITE analysis resolved enrichments in peptidases and inhibitors

(six proteins), mitochondrial biogenesis (four proteins), and transporters (eleven proteins).

SSDA proteins enriched in the digestive tract proteome of GCF compared to the control treatment group had an increased abundance associated with oxidative phosphorylation (four proteins), detoxification of reactive oxygen species (three proteins) and pyrimidine metabolism (three proteins). KEGG analysis resolved six proteins associated with oxidative phosphorylation. BRITE resolved enrichment in mitochondrial biogenesis (four proteins). Proteins with a decreased abundance were associated with the extracellular matrix (five proteins) and cell-cell junctions (five) (Figure S3-2). KEGG analysis resolved enrichment in the ribosome (three proteins), focal adhesion (three proteins), and protein processing in the endoplasmic reticulum (three proteins). BRITE analysis resolved enrichment in peptidases and inhibitors (four proteins), chaperones and folding catalysts (four proteins), membrane trafficking (nine proteins), transporters (five proteins), and cytoskeleton proteins (three proteins).

SSDA proteins in the digestive tract proteome were compared between GCF and GAI treatment groups directly. Proteins with an increased abundance in GCF in comparison to GAI treatment groups were associated with the tricarboxylic acid (TCA) cycle (eight proteins), oxidative phosphorylation (seven proteins) and fatty acid degradation (seven proteins). KEGG analysis resolved enrichments in amino acid biosynthesis (six proteins), the TCA cycle (eleven proteins), and oxidative phosphorylation (twelve proteins) pathways. BRITE resolved enrichment in peptidases and inhibitors (four proteins), mitochondrial biogenesis (nine proteins), and transporters (seven proteins). In comparison, SSDA proteins decreased in GCF compared to GAI treatment groups had roles in toxic substance response (five proteins), glycolysis and gluconeogenesis (six proteins), and amino acid biosynthesis (five proteins) (Figure S3-3). KEGG analysis resolved enrichment in co-factor biosynthesis (five proteins), and BRITE resolved enrichments in chaperones and folding catalysts (four proteins), membrane trafficking (six proteins), and cytoskeleton proteins (four proteins).

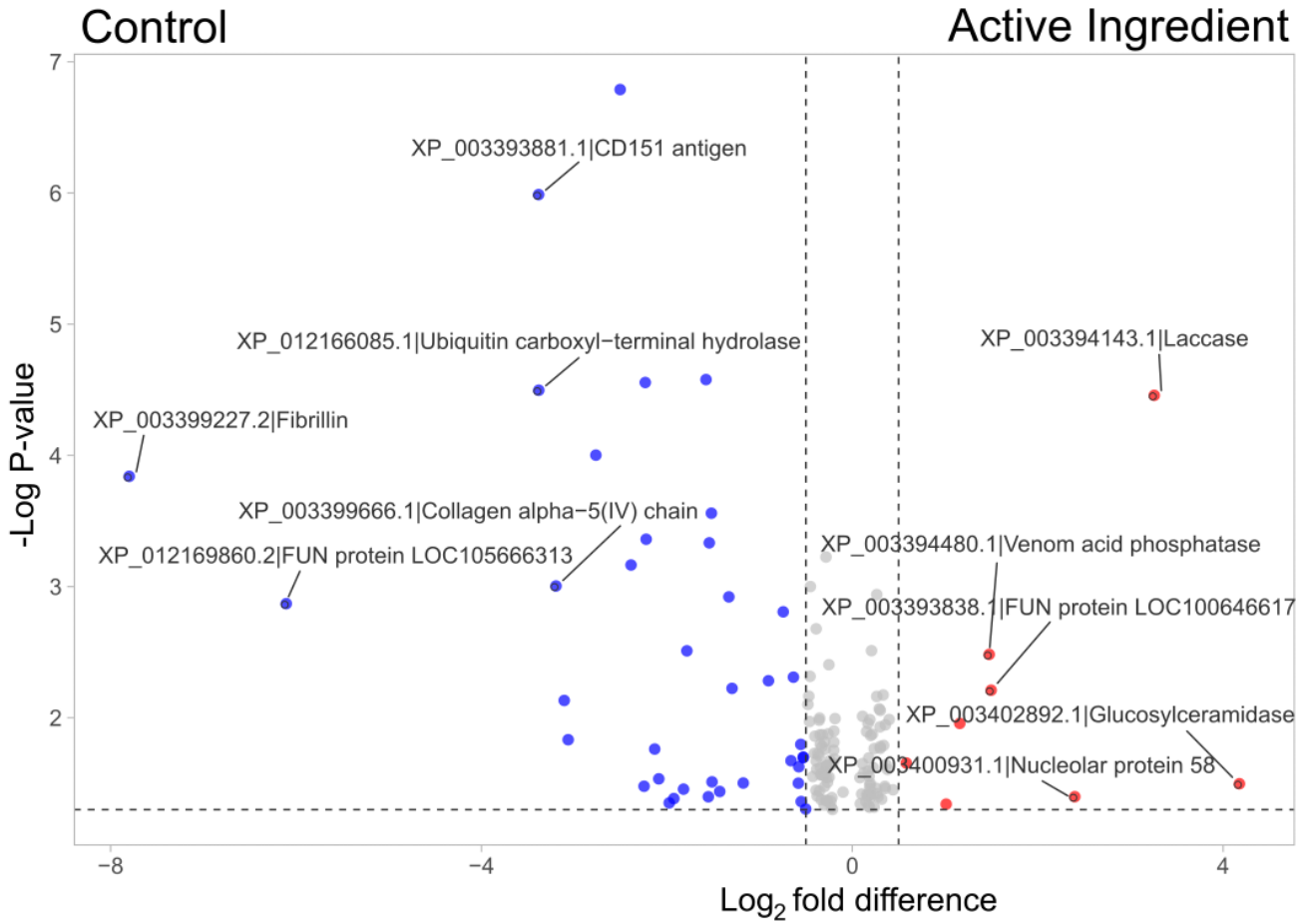


Figure 3-6 A two-sample t-test was performed to resolve and compare SSDA proteins in the digestive tract of *B. terrestris* after exposure to glyphosate active ingredient (GAI) or the control. SSDA proteins ($p \leq 0.05$) and the top five proteins with the most increased (right) and most decreased (left) abundance in comparison to the control are highlighted. Fibrillin was the most decreased SSDA protein and glucosylceramidase was the most increased SSDA protein after GAI exposure in comparison to the control.

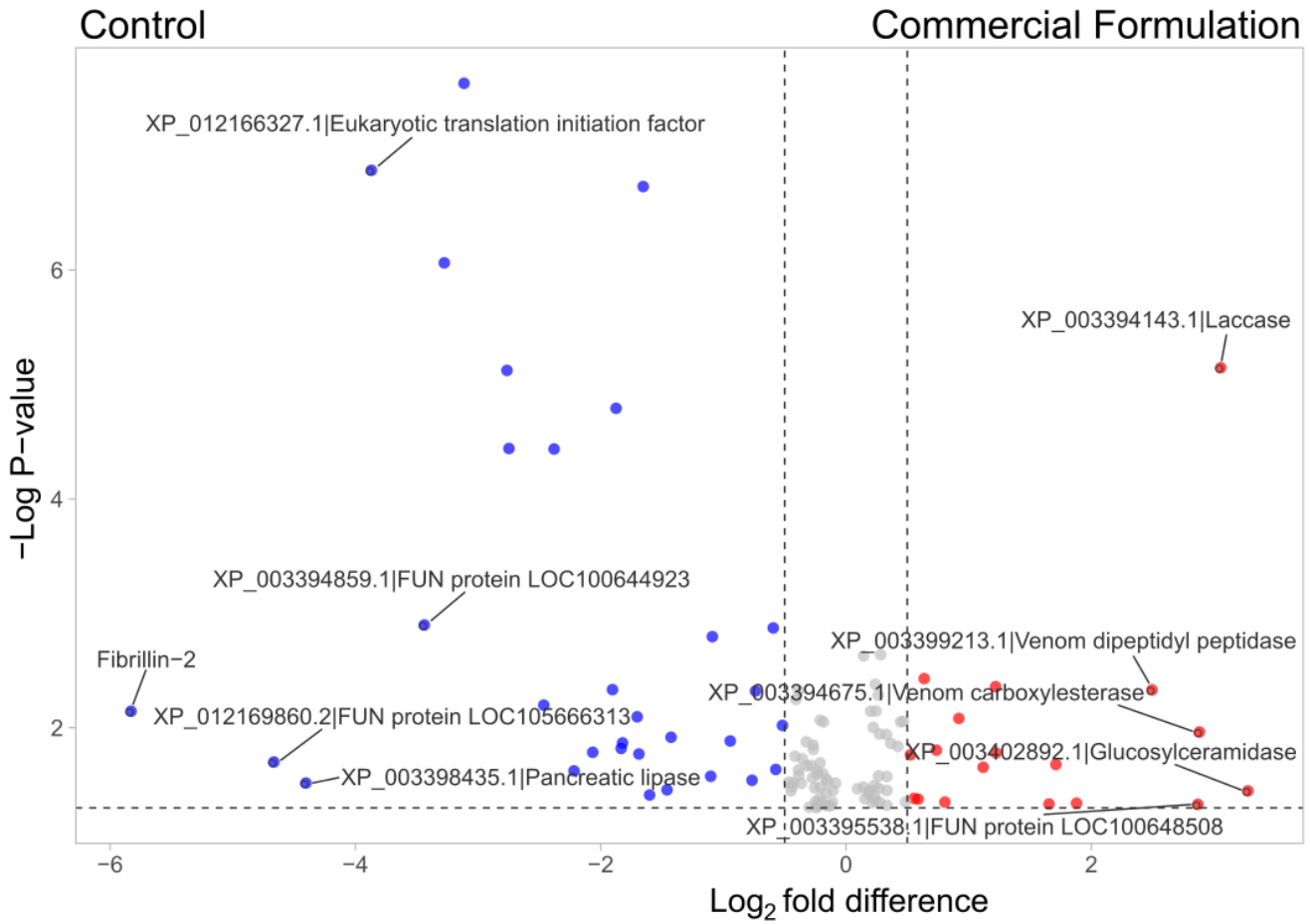


Figure 3-7 A two-sample t-test was performed to resolve and compare SSDA proteins in the digestive tract of *B. terrestris* after exposure to glyphosate commercial formulation (GCF) or the control. SSDA proteins ($p \leq 0.05$) and the top five proteins with the most increased (right) and most decreased (left) abundance in comparison to the control are highlighted. Fibrillin was the most decreased SSDA protein and glucosylceramidase was the most increased SSDA protein after GCF exposure in comparison to the control.

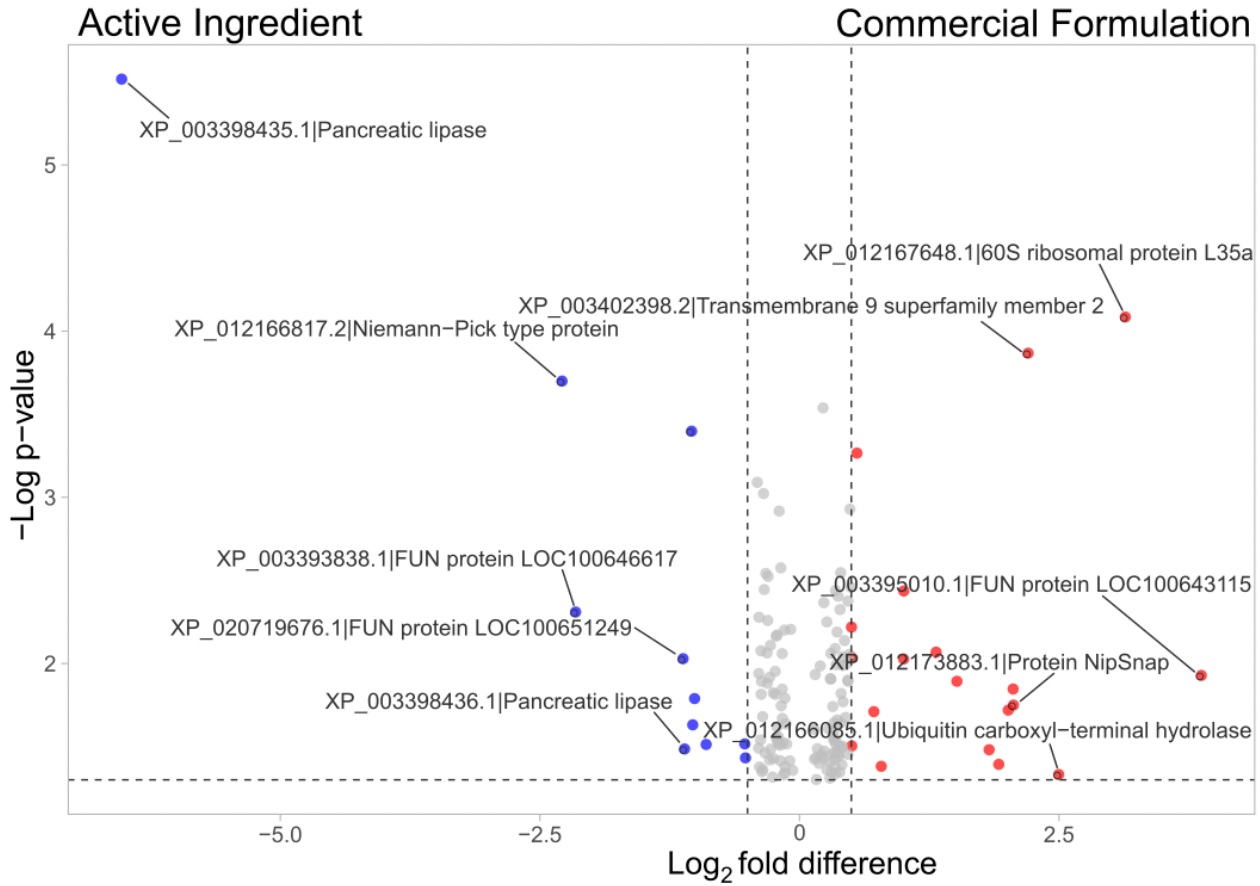


Figure 3-8 A two-sample t-test was performed to resolve and compare SSDA proteins in the digestive tract of *B. terrestris* exposed to glyphosate commercial formulation (GCF) or glyphosate active ingredient (GAI). SSDA proteins ($p \leq 0.05$) and the top five proteins with the most increased (right) and most decreased (left) abundance in GCF compared to GAI are highlighted. Lipase was the most decreased SSDA protein and functionally uncharacterized protein LOC100643115 was the most increased SSDA protein in GCF compared to GAI exposed bees, highlighting differences in response to co-formulants in GCF.

3.3.4.4 Hierarchical Clustering and Gene Ontology Enrichments

Hierarchical clustering was performed on mean z-scored, normalised LFQ values for 178 statistically significant proteins (ANOVA, Ben-Ho FDR <0.05), which resolved eight clusters (A-H). Each cluster represents a group of proteins with similar expression profiles in the GAI, GCF, or control-treated digestive tract (Figure 3-5B, Table S3-10). Proteins in each cluster were analysed using the STRING database using the equivalent *D. melanogaster* Uniprot identifiers to resolve enriched (FDR < 0.05) processes and pathways affected within or across treatments (Figure 3-5C; Table S3-11). Cluster A comprised 14 proteins with an increased abundance in both GAI and GCF-treated groups compared to the control group, with enrichment for hydrolases (five proteins). Cluster B included 15 proteins, four and seven of which were associated with nucleotide metabolic processes and hydrolase activity, respectively. Cluster D comprised 20 proteins with an increased abundance in the GAI-treated group, a decreased abundance in the control-treated group, and a further decreased abundance in the GCF-treated group. These proteins were associated with glycolysis/gluconeogenesis and co-factor metabolic processes (two proteins each). Cluster E contained 20 proteins with a decreased abundance in the GAI-treated group compared to GCF and control groups, nine of which were associated with the mitochondrion and several proteins were associated with glutamine metabolism. Cluster F contained 37 proteins with a low abundance in the GAI-treated group, a slightly higher abundance in the control-treated group, and an increased abundance in the GCF-treated group. Enriched processes in this group included fatty acid beta-oxidation (three proteins), oxidation-reduction processes (two proteins), mitochondrial transport (two proteins) and the TCA cycle/respiratory electron transport (three proteins). In total 19 proteins in cluster F were associated with the mitochondrion and when combined with cluster E (both clusters comprising proteins with reduced abundances in the AI-treated group), highlight the dramatic effect of the GAI on mitochondrial processes. Cluster G comprised 19 proteins with a low abundance in the GCF-treated group compared to GAI and control-treated groups, with significant enrichment in proteins associated with the fusome (two proteins). Cluster H consisted of 36 proteins with a low abundance in both glyphosate-based treatment groups in comparison to the control-treated group, with proteins associated with endocytosis (three proteins), regulation of intracellular pH (two proteins) and the

plasma membrane (six proteins). There were no significant enrichments in cluster C, which included 15 proteins with an increased abundance in the GAI-treated group compared to GCF and control-treated group.

3.3.4.5 Proteins With a Common Abundance Profile After Exposure to Either Glyphosate-Based Treatment

To investigate whether a consistent effect of glyphosate exposure could be identified, SSDA protein sets from both glyphosate treatments versus the controls were compared to find common proteins (Table 3-1). In total eight and 29 proteins were found in common in the increased and decreased glyphosate-treated digestive tracts, respectively. One of the major categories affected by both glyphosate-based treatments was cell structural integrity, comprising 11 proteins with lower abundance, some of which had the greatest fold change differences identified in the experiment. These included fibrillin, CD151 antigen, two collagen proteins and a cartilage matrix protein (combined fold change range of 3.7 to 222.7 across both treatments). 6 proteins associated with metabolism (4 amino acid/carbohydrate metabolism and 2 lipid metabolism) and 5 proteins associated with transport displayed similar fold change values and directions. Categories such as cell signalling, calcium transporting/signalling, gene regulation and protein modification/degradation all had multiple proteins with lower abundances in glyphosate-exposed bees whereas proteins associated with oxidative stress regulation had higher abundances. Individual proteins associated with immunity/detoxification, protein modification and cell migration guidance were identified as having a conserved response and one function-unknown protein (XP_012169860.2) had considerable relative fold change differences of 68.8 and 25.4 in the AI and CF-exposed digestive tracts, respectively, highlighting a potentially novel association with xenobiotic exposure

Table 3-1 Conserved Response to Both Glyphosate-Based Treatments. Functional categories, relative fold changes, MS measurements and characteristics for all SSDA proteins with similar expression profiles in both GAI and GCF treated bees. Relative fold changes and directions were determined against the procedural control.

Functional Annotation	Protein ID	Protein Name	RFC GAI	RFC GCF	FC Direction	No. of Peptides	Mol. wt [kDa]	MS/MS count
Cell structural integrity	XP_003399227.2	Fibrillin-2	222.7	56.8	Decreased	90	314.2	329
	XP_003393881.1	CD151 antigen	10.4	9.7	Decreased	4	26.5	16
	XP_003399666.1	Collagen alpha-5(IV) chain	9.2	5.5	Decreased	8	185.2	68
	XP_003399665.1	Collagen alpha-1(IV) chain	5.2	3.7	Decreased	15	193	197
	XP_003397666.1	Cartilage oligomeric matrix protein	6.8	6.8	Decreased	8	130.7	15
	XP_003392951.1	Protein BCCIP homolog	2.9	3.2	Decreased	4	34.4	22
	XP_012174910.1	Basement membrane-proteoglycan	2.5	2.1	Decreased	158	486	1519
	XP_012164850.1	Protein mesh	1.2	1.2	Decreased	50	153.9	495
	XP_003394511.1	23 kDa integral membrane protein	1.4	1.3	Decreased	3	27.2	27
	XP_012172164.1	DE-cadherin	1.3	1.2	Decreased	19	166.6	104
	XP_003397680.1	Innexin inx7	1.6	1.3	Decreased	9	45.9	55
Transport	XP_003402688.1	Facilitated trehalose transporter Tret1	4.7	5.2	Decreased	7	51	29
	XP_003399193.1	Na-independent sulfate anion transporter	4.4	3.6	Decreased	8	72.6	27
	XP_003396344.1	Organic cation transporter protein	3.4	2.7	Decreased	4	63	15
	XP_003398230.1	Anion transporter family member 2A1	2.9	2.1	Decreased	11	79.9	83
	XP_003398587.1	Choline transporter 1	1.5	1.5	Decreased	5	67.7	41
Metabolism	XP_003397815.1	Beta-hexosaminidase subunit beta	2	2.2	Increased	23	61.9	218
	XP_003398461.1	Succinate-semialdehyde dehydrogenase	1.4	1.2	Decreased	30	56.2	239
	XP_020718677.1	Amino acid transport protein rBAT	1.2	1.3	Decreased	10	69	64
	XP_003401829.1	ENT diphosphohydrolase 5	1.1	1.2	Increased	16	52.6	130

Lipid metabolism	XP_003402892.1	Glucosylceramidase	18.1	9.7	Increased	13	58.1	42
	XP_003402390.3	FGGY carbohydrate kinase	1.3	1.1	Increased	10	31.1	90
Protein modification/degradation	XP_003394859.1	Uncharacterized protein LOC100644923	8.6	10.8	Decreased	4	263.2	15
	XP_012174330.1	S-phase kinase-associated protein 1	5.7	4.7	Decreased	3	18.5	15
Gene regulation	XP_012164476.1	Regulator of chromosome condensation	4.7	3.3	Decreased	7	46.2	19
	XP_012175550.1	Histone H2B	1.1	1.1	Decreased	7	13.7	88
Cell signalling	XP_003401488.1	Uncharacterized protein LOC100644037	2.9	3.7	Decreased	7	75.7	19
	XP_003394892.1	Tubulointerstitial nephritis antigen	1.7	1.5	Decreased	26	51	266
Calcium transport/signalling	XP_003401730.1	Regucalcin*	2.2	2.3	Increased	19	36.9	227
	XP_020722918.1	Calcyphosin	1.6	1.7	Decreased	9	25.1	36
	XP_012175018.1	Plasma membrane Ca-transporting ATPase	1.5	1.4	Decreased	21	139.1	144
Oxidative stress regulation	XP_003399739.1	Venom carboxylesterase-6	1.4	1.4	Increased	14	65.6	116
	XP_003397315.1	Superoxide dismutase [Cu-Zn]	1.1	1.2	Increased	13	15.6	218
Function Unknown	XP_012169860.2	Uncharacterized protein LOC105666313	68.8	25.4	Decreased	7	30.7	53
Immunity/Detoxification	XP_003394143.1	Laccase-1	9.6	8.3	Increased	6	76	27
Protein modification	XP_003393428.1	Protein phosphatase methylesterase 1	3	3.1	Decreased	8	41.8	20
Guidance	XP_003403082.1	Protein slit	4.7	6.7	Decreased	10	109.1	26

*Also involved in lipid metabolism

3.3.5 The Effects of Glyphosate and Roundup Optima+® on the Digestive Tract Microbiota

2,128,021 effective tags representing the 16S rRNA V4 gene amplicon region were filtered out from 2,204,903 raw tags. For ITS (ITS1-5F) gene amplicon region, 1,169,831 effective tags were filtered from 2,320,223 raw tags (Table S3-12).

3.3.5.1 Bacterial and Fungal Community Composition

In general, the relative abundance of the top ten bacterial genera were not impacted by either glyphosate-based treatment (Figure 3-9) (Table S3-13). However, *Saccharibacter* species were nearly two times lower and four times lower in the GCF and GAI treatment groups in comparison to the control group. In addition, whilst the relative abundance of the genera *Vicingus* was low in all treatment groups, it was 14 times higher in the GAI treatment group and 15 times higher in the control treatment group compared to the GCF treatment group.

In general, Roundup Optima+® but not glyphosate altered the relative abundance of the top 10 fungal genera across treatment groups, with a reduction in the relative abundance of *Ascomycota* fungi, particularly *Candida* species (Figure 3-10). *Candida* were approximately four times higher in relative abundance in the control and GAI treatment group compared to the GCF treatment group. This was still the general trend when comparing the abundance of microbial genera across individual samples. GCF had a higher abundance of *Penicillium* and *Chaetomium* species. *Penicillium* was almost 15 times higher than the control treatment group and over 40 times higher than the GAI treatment group. *Chaetomium* was almost 30 times lower in the control treatment group and over 200 times lower in the GAI treatment group compared to the GCF group, with *Chaetomium* absent in many control and GAI treatment group samples. The genera *Mortierella* and *Pichia* were much lower in the control treatment group compared to both glyphosate-based treatment groups, with many control samples absent of these genera (Table S3-13). *Zygosaccharomyces* were four times lower in the GCF treatment group and 2.5 times lower in the control treatment group compared to the GAI treatment group.

All bacterial genus profiles were generally similar across samples except for gamb.07, gfmb.06 and gcmb.03, which had a relatively low abundance of *Snodgrasella* and

Gilliamella species – the core and most abundant genera found in other samples from all treatment groups. For the top 10 fungal genera, there were differences in the fungal abundance profile separate to the lower abundance of *Candida* found in the GCF-treated group. These included: gamb.01, gcmb.03, and gcmb.09 which had a greater abundance of *Zygosaccharomyces* than other samples and gfmb.01, gfmb.10, gcmb.05, and gcmb.07 which had minimal levels of *Candida* abundance. We could not conclude that these differences were due to origin colony differences (Table S3-14).

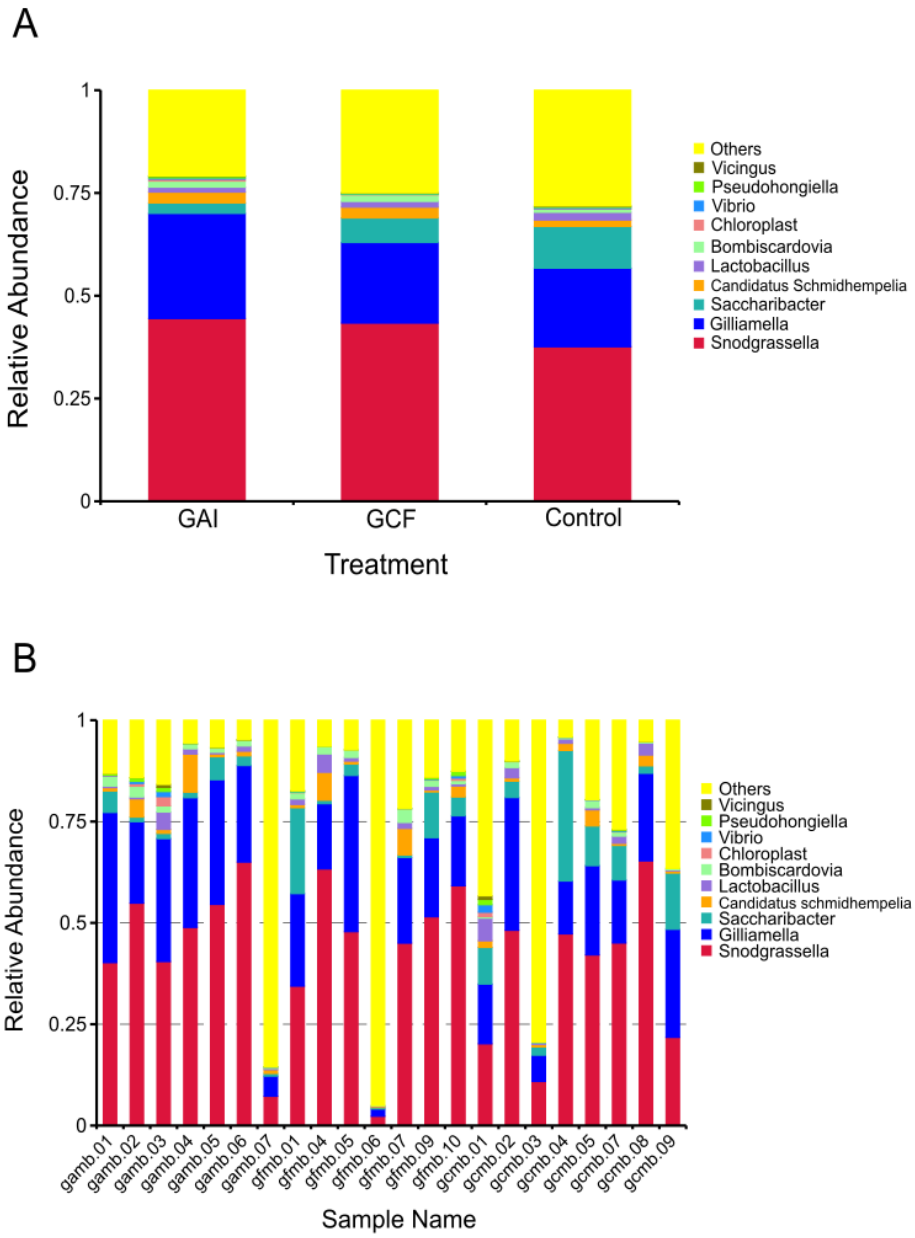


Figure 3-9 The Relative Abundance of the Top Ten Bacterial Genera. (A) The relative abundance of the top bacterial genera in each treatment group. **(B)** The relative abundance of the top ten bacterial genera in each sample. Digestive tract DNA samples were: Gamb (GAI treatment), gfmb (GCF treatment) and gcmb (control).

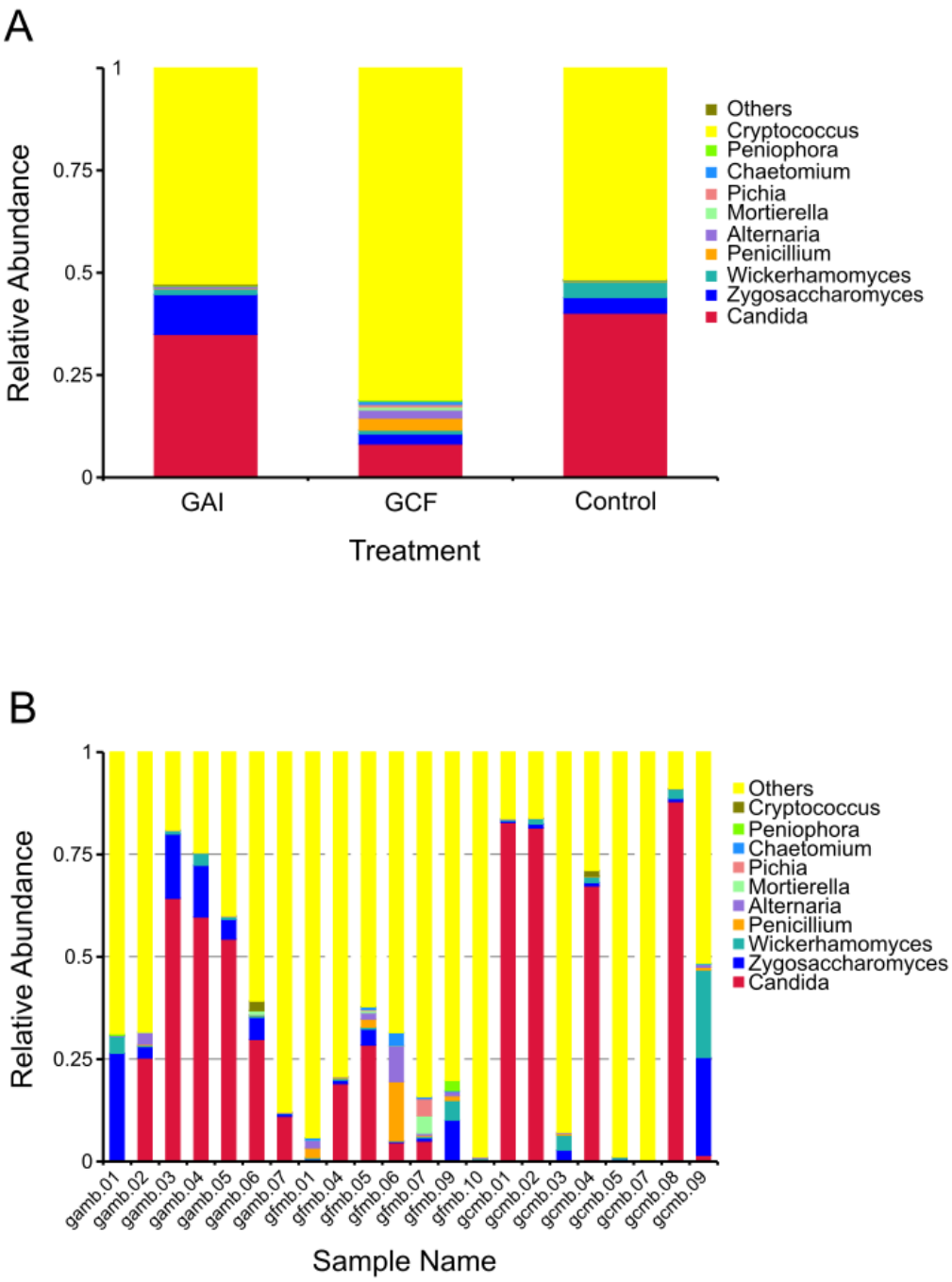


Figure 3-10 The Relative Abundance of the Top Ten Fungal Genera. (A) The relative abundance of the top fungal genera in each treatment group. **(B)** The relative abundance of the top ten fungal genera in each sample. Digestive tract DNA samples were: Gamb (GAI treatment), gfmb (GCF treatment) and gcmb (control).

Taxonomy trees were created based on the top 10 genera in high relative abundance by default for both bacterial and fungal genera. Bacteria represented 75.342% of the whole taxon. *Lactobacillus* bacteria were present in all samples representing 1.5% of the whole taxon and 1.99% of the selected taxon (Figure 3-11). *Lactobacillus apis* were present in similar abundance in each treatment group and represented 1.1% of bacteria, and 1.46% of the selected taxon. *Lactobacillus bombi* represented 0.155% of the whole taxon and 0.21% of the selected taxon, but the majority of *L. bombi* abundance was accounted for by the control group, with a much lower presence detected from glyphosate-based treatment groups. The bacterial genus *Bombiscardovia* represented 1.27% of the whole taxon and 1.69% of bacteria. It was present in similar abundance across each group, with a slightly higher abundance in glyphosate-based digestive tracts. *Saccharibacter* species accounted for 6.226% and 8.26% of the bacteria and proteobacteria, respectively, with a lower abundance found in the group exposed to GAI compared to GCF and control-exposed groups. Bacteria of the genera *Snodgrasella* accounted for 41.8% of bacteria and 55.5% of proteobacteria, however the species could not be identified, as the only identified species – *Snodgrasella alvi* – accounted for less than 0.02% of bacteria. This was also the case for *Gilliamella* species, accounting for 21.429% of bacteria and 28.44% of the proteobacteria, with only *Gilliamella apicola* identified with 0.04% of the selected taxon represented by this species. *Candidatus schmidhempelia* accounted for 2.283% of bacteria, with a lower abundance in the control compared to GAI and GCF-treated groups.

Fungi represented 38.091% of the whole taxon. *Candida* fungi were present in all samples representing 27.720% of the whole taxon and 72.77% of the selected taxon (Figure 3-12). *C. apicola* represented 55.85% of *Candida* and *C. bombi* represented 16.66% of the *Candida* genera. *C. magnoliae* accounted for 0.26% of *Candida* species. *Candida* species had a low abundance in the GCF-treatment group compared to the GAI and control-treatment groups. *Pichia* represented 0.212% of fungi and 0.56% of *Saccharomycetes*, with *P. membranifaciens* as the sole species identified, with most abundance originating from the GCF-treatment group. *Wickerhamomyces anomalus* accounted for 2.07% of fungi and 5.43% of *Saccharomycetes*, with a higher abundance in control-treated groups compared to both glyphosate-based treatment groups. *Cryptococcus neoformans*, accounting for 0.168% of fungi and 0.44% of *Basidiomycota*, had a much higher abundance in GAI-treated compared to other treatment groups. Many low abundance species were present, with most of the species' presence originating from the GCF-treatment group, including *Alternaria alternata, Penicillium bialowiezense, P. decumbens, Acremonium tubakii, Peniphora cinerea, Malassezia restricta* and *Mortierella chlamydospora.*

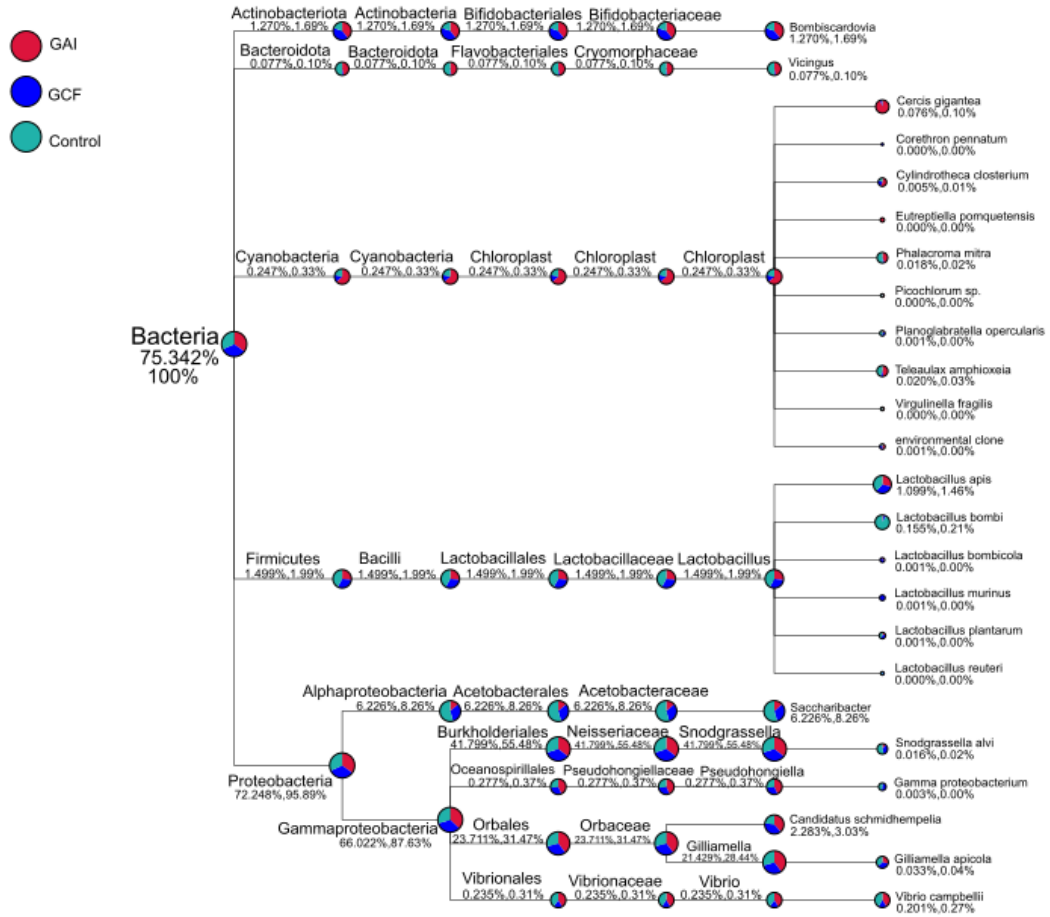


Figure 3-11 Group Taxonomy Tree for Bacterial Taxa Present in the *B. terrestris* Digestive Tract After Treatment with GAI, GCF or Control. The top 10 genera in high relative abundance were used by default to create the group taxonomy tree. The node size represents the relative abundance in each treatment group. The first number below taxonomic names demonstrate the percentage in the whole taxon and the second number represents the percentage in the selected taxon.

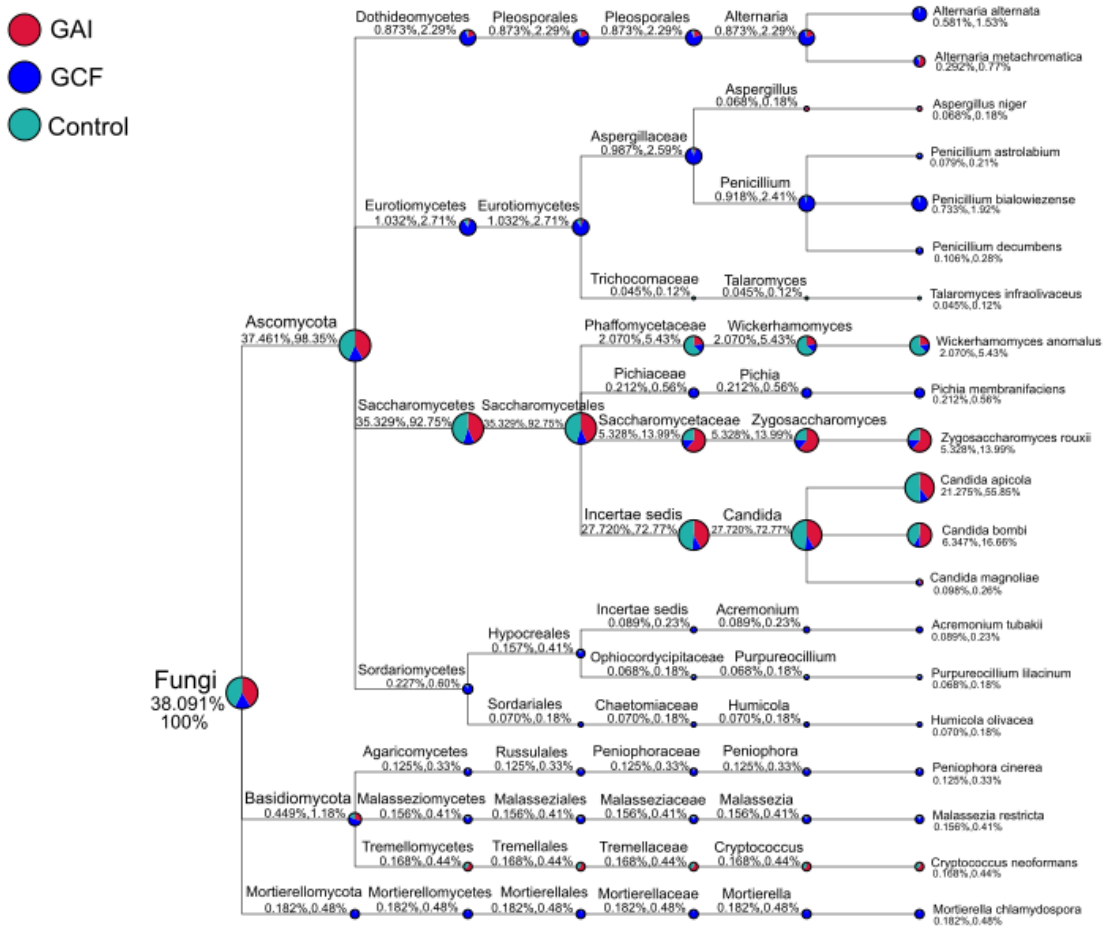


Figure 3-12 Group Taxonomy Tree for Fungal Taxa Present in the *B. terrestris* Digestive Tract After Treatment with GAI, GCF or Control. The top 10 genera in high relative abundance were used by default to create the group taxonomy tree. The node size represents the relative abundance in each treatment group. The first number below taxonomic names demonstrate the percentage in the whole taxon and the second number represents the percentage in the selected taxon.

3.3.5.2 Alpha Diversity Analysis of Bacterial and Fungal Taxa

Microbial community diversity within samples and treatment groups was analysed to determine species richness i.e., alpha diversity. OTU number was compared to sequence number to create rarefaction curves to determine species richness and diversity at the current sequencing depth within the digestive tracts of different treatment groups for. For OTU's identified from 16S amplicon sequencing, the curve generated from the GAI-exposed bees was less steep compared to GCF and control treatment groups, indicating a lower number of OTU assignments for the same amount of sequence coverage found in other treatment groups (Figure 3-13A). For OTU's identified from ITS amplicon sequencing, the rarefaction curve for the GCF treatment group was steeper than other treatment groups, indicating a higher fungal taxa richness than other groups, with a higher number of OTU assignments for the same sequence depth as other treatment groups (Figure 3-13B). Venn diagrams were used to analyse the number of normalised OTU's common and unique amongst samples from different treatment groups to determine species richness and diversity. From 16S amplicon sequencing, there were 440 OTU's common to all treatment groups (Figure 3-14A; Figure 3-14B). The control, GCF and GAI had 426, 385 and 145 unique OTU's respectively when compared to other groups. Amongst the glyphosate-based treatment groups, GAI and GCF, there were 518 common OTU's. GCF had 823 and GAI had 242 unique OTU's. From ITS amplicon sequencing, 259 OTU's were common between all treatment groups. The control had 256 unique OTU's compared to 278 and 683 in GAI and GCF treatment groups, respectively. Overall, GCF treatment groups had the largest number of unique identified OTU's, with 790 unique OTU's in a pairwise comparison with the GAI treatment group, which had 333 unique OTU's (Figure 3-14C; Figure 3-14D).

In addition, a rank abundance curve was created to determine bacterial and fungal richness and evenness of different taxa across treatment groups. The rank abundance curve was generated by sorting OTU's in samples by their relative abundance or the number of sequences included from large to small and assigning a sorting number. Based on the rank abundance curve, all treatment groups had a similar bacterial taxa richness and evenness (Figure S3-4). For fungal taxa, the GCF treatment group

displayed a higher relative abundance for ranked species before plateauing with other treatment groups at before reaching the species ranked 200 (Figure S3-5).

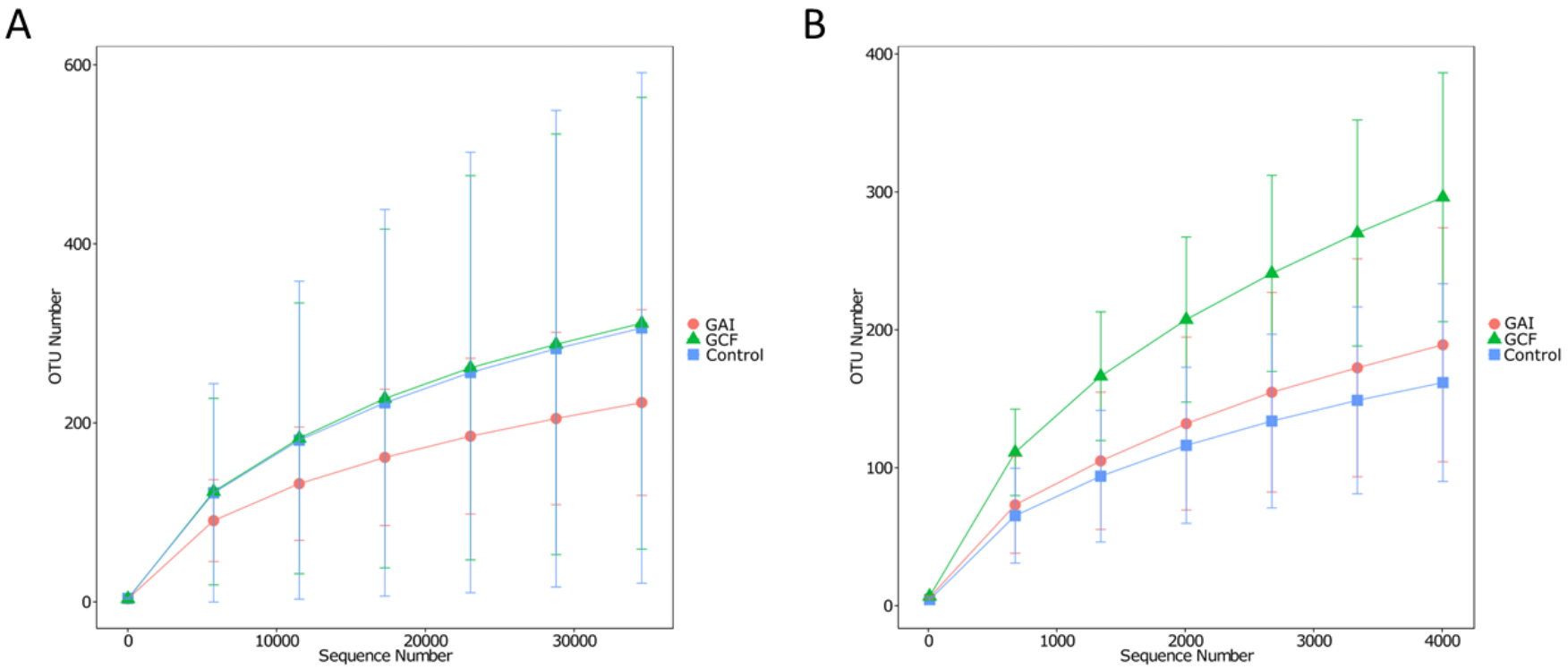


Figure 3-13 Rarefaction Curve for Taxa Identified in The Digestive Tract of *B. terrestris*. Comparing the OTU number to the number of sequences from sample groups gives an estimation of species richness in each treatment group. **(A)** For OTU identifications from 16S sequences, there were less OTU assignments compared to the number of sequences for GAI compared to other treatment groups. **(B)** For OUT identifications from ITS sequences, there was large variation amongst samples within groups, but GCF has greater species richness in comparison to GAI and control treatment groups determined by the number of OTU assignments compared to sequence number.

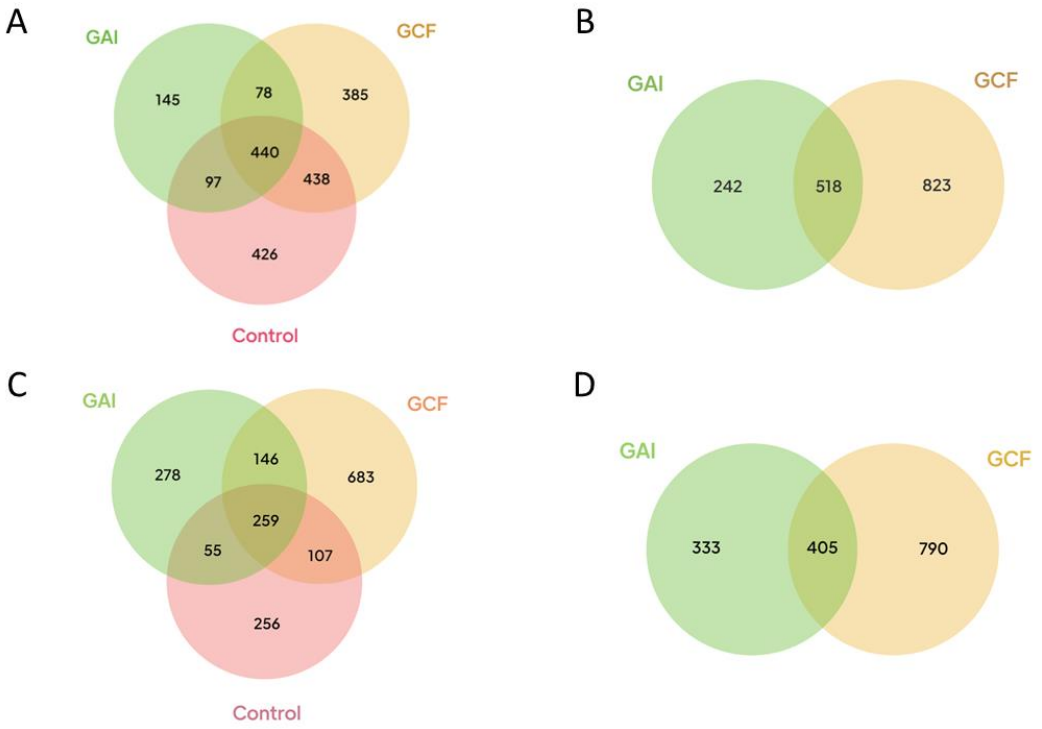


Figure 3-14 OTU's Common and Unique Amongst Treatment Groups. (A) There were 440 common 16S OTU's amongst all samples, and 518 between both glyphosate-based treatment groups. A relatively considerable number of unique OTU's were found in the control (426) and GCF (365) when comparing all groups, and in **(B)** GCF (823) when compared to GAI (242) alone**. (C)** There were 259 common ITS OTU's amongst all samples, 256 unique OTU's were found in the control which were not identified in the glyphosate-based treatment groups and **(D)** 405 between GAI and GCF treatment groups.

To determine alpha diversity, i.e. the species richness and evenness across samples and treatment groups, several statistical analyses were deployed. To take species evenness into account, i.e. the relative abundance of species, alpha diversity indices were determined with an OTU clustering threshold of 97% sequence identity similarity. There were no significant differences in bacterial alpha diversity amongst treatment groups using Wilcoxon and Tukey tests on Observed species, CHAO1, Shannon and Simpson indices (Figure 3-15; Figure 3-16; Figure S3-6; Figure S3-7; Table S3-15). Fungal alpha diversity was significant when comparing the GCF treatment group to the control treatment group (Wilcoxon Observed species $p = 0.012$; Wilcoxon Chao1 $p = 0.0123$, Wilcoxon Simpson $p = 0.0188$, Wilcoxon Shannon $p = 0.0187$) (Figure 3-17; Figure 3-18; Figure S3-8; Figure S3-9; Table S3-13).

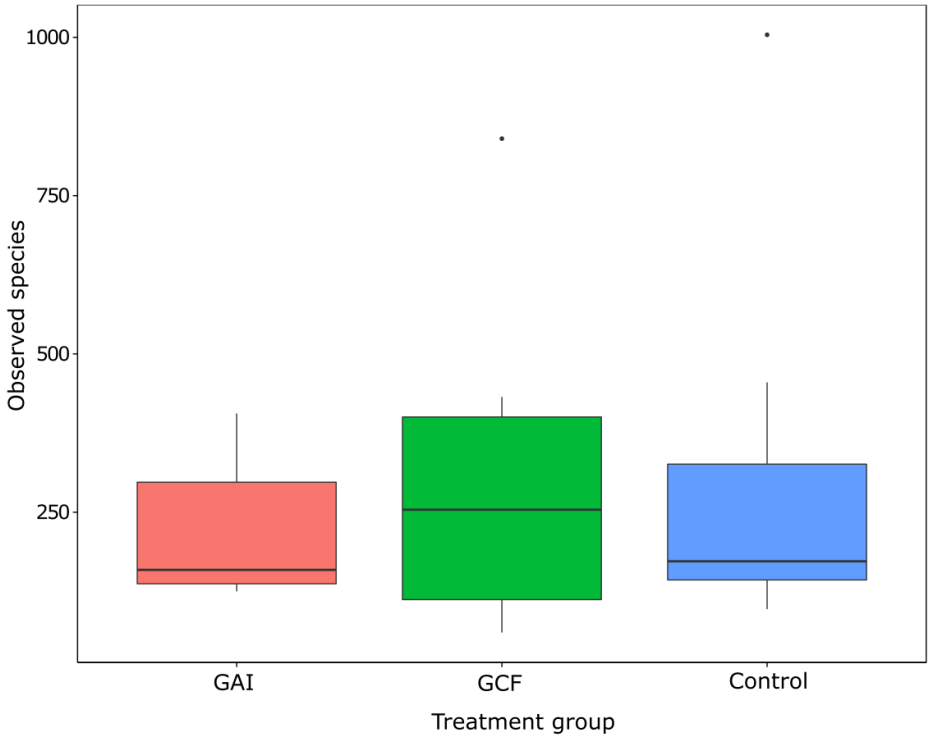


Figure 3-15 Boxplot of Observed Bacterial Species Per Treatment Group. The observed species count was compared amongst treatment groups to determine if there were any differences in bacterial species richness, with no significant differences in species count found.

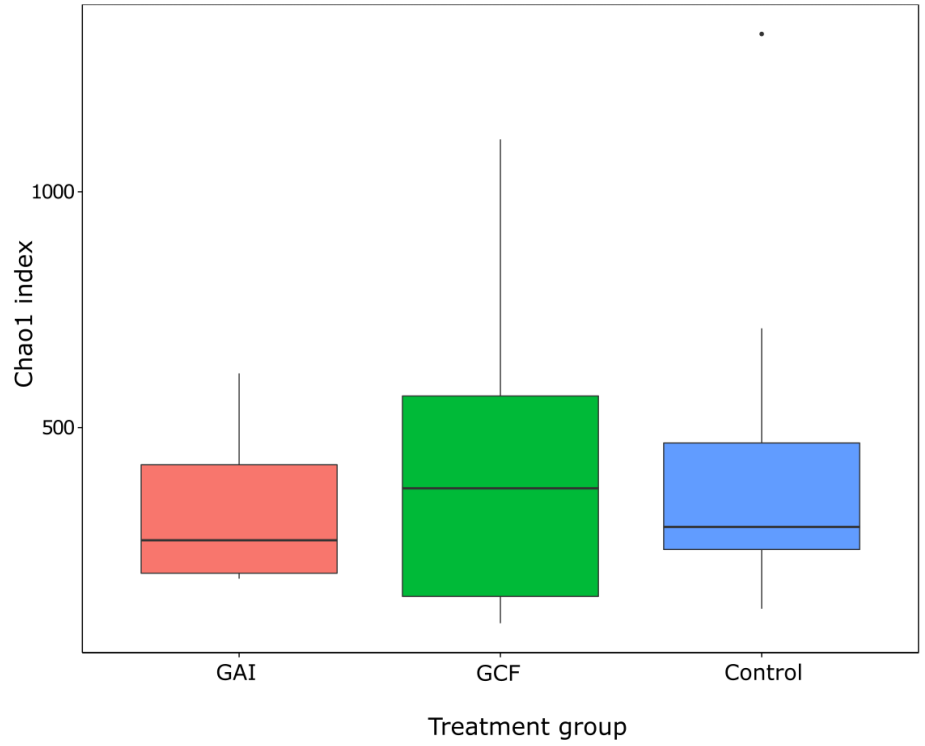


Figure 3-16 Boxplot of Chao1 Indices of Bacterial Species in Each Treatment Group. Chao1 estimation was determined to predict the alpha diversity of bacterial species in each sample, taking relative abundance of species into account. There were no significant differences in Chao1 alpha diversity indices between groups.

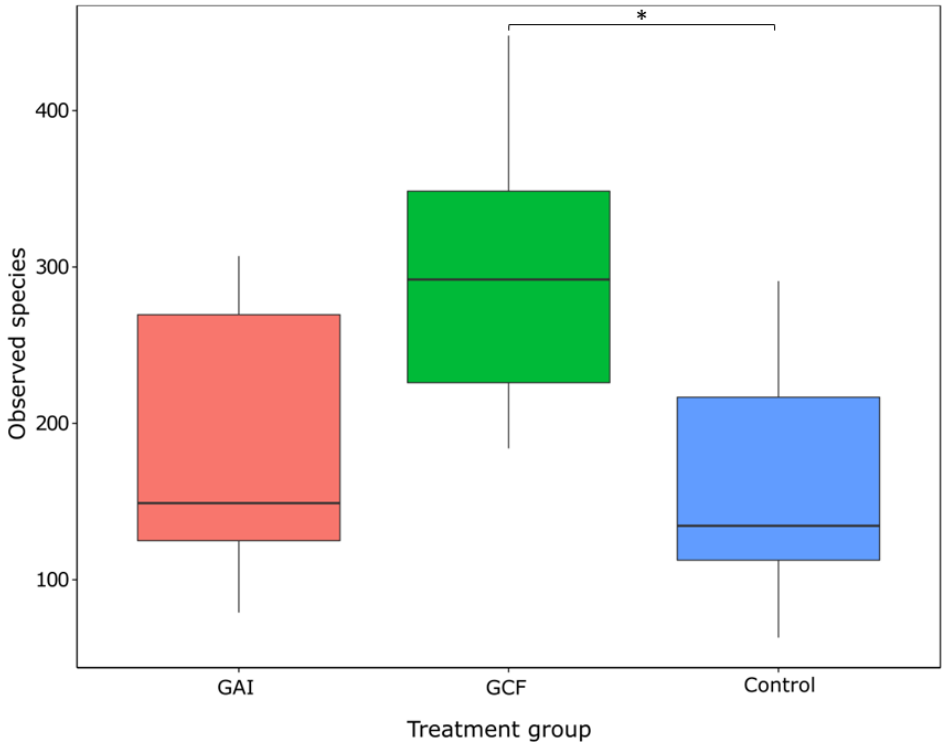


Figure 3-17 Boxplot of Observed Fungal Species Per Treatment Group. The observed fungal species count was compared amongst treatment groups to determine if there were any differences in species richness. There was a significant difference between GCF and control treatment groups (Tukey p = 0.022; Wilcoxon p = 0.012).

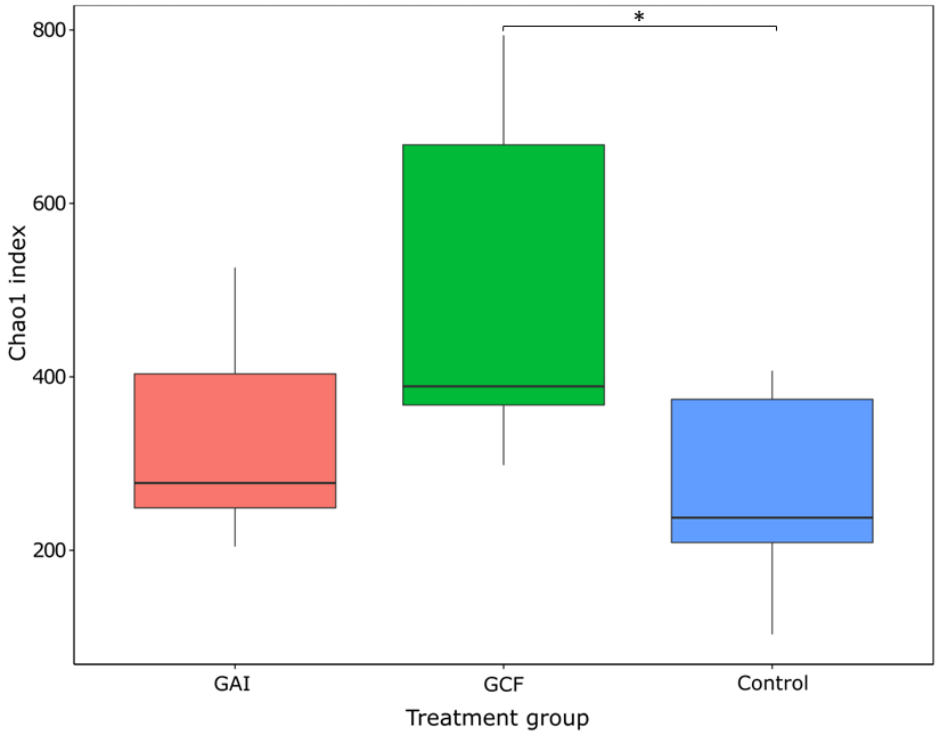


Figure 3-18 Boxplot of Chao1 Indices of Fungal Alpha Diversity in Each Treatment Group. Chao1 estimation was determined to predict the alpha diversity of fungal species in each sample, taking relative abundance of species into account. There was a significant difference in Chao1 indices between GCF and control treatment groups (Tukey p = 0.015; Wilcoxon p = 0.012).

3.3.5.3 Beta Diversity Analysis of Bacterial and Fungal Taxa

Whilst alpha diversity can estimate diversity within samples based on the number of species and their relative abundance, beta diversity is used to determine differences in the composition of microbial communities between samples and groups. To determine beta diversity, the difference between microbial communities was measured using the square matrix of distance or dissimilarity between group pairs using unweighted and weighted unifrac. Weighted unifrac data takes both the presence and relative abundance into account, whilst unweighted data takes only the presence/absence of bacterial taxa into consideration. Whilst weight unifrac can give more information on the overall community structure, unweighted unifrac can give an insight into the presence or absence of rare or low abundance taxa among samples.

A PCA and Principal Coordinate Analysis (PCoA) were used to visualise distance between samples and treatment group microbial community composition. By summarising variables with orthogonal transformation, a PCA uses the minimum number of components to explain variance. The results of the PCA demonstrate low bacterial and fungal variance in species composition between treatment groups, with most samples grouping together (Figure 3-19A; Figure 3-19B). The PCoA of bacterial weighted unifrac distances display a drift of control samples away from other treatment samples. However, there are no clear clustering between treatment groups, with some samples from each treatment group in close proximity to one another (Figure 3-19C). The PCoA of fungal weight unifrac distances demonstrate some distance between GCF and control-treatment samples, however there is no clear clustering of treatment groups (Figure 3-19D).

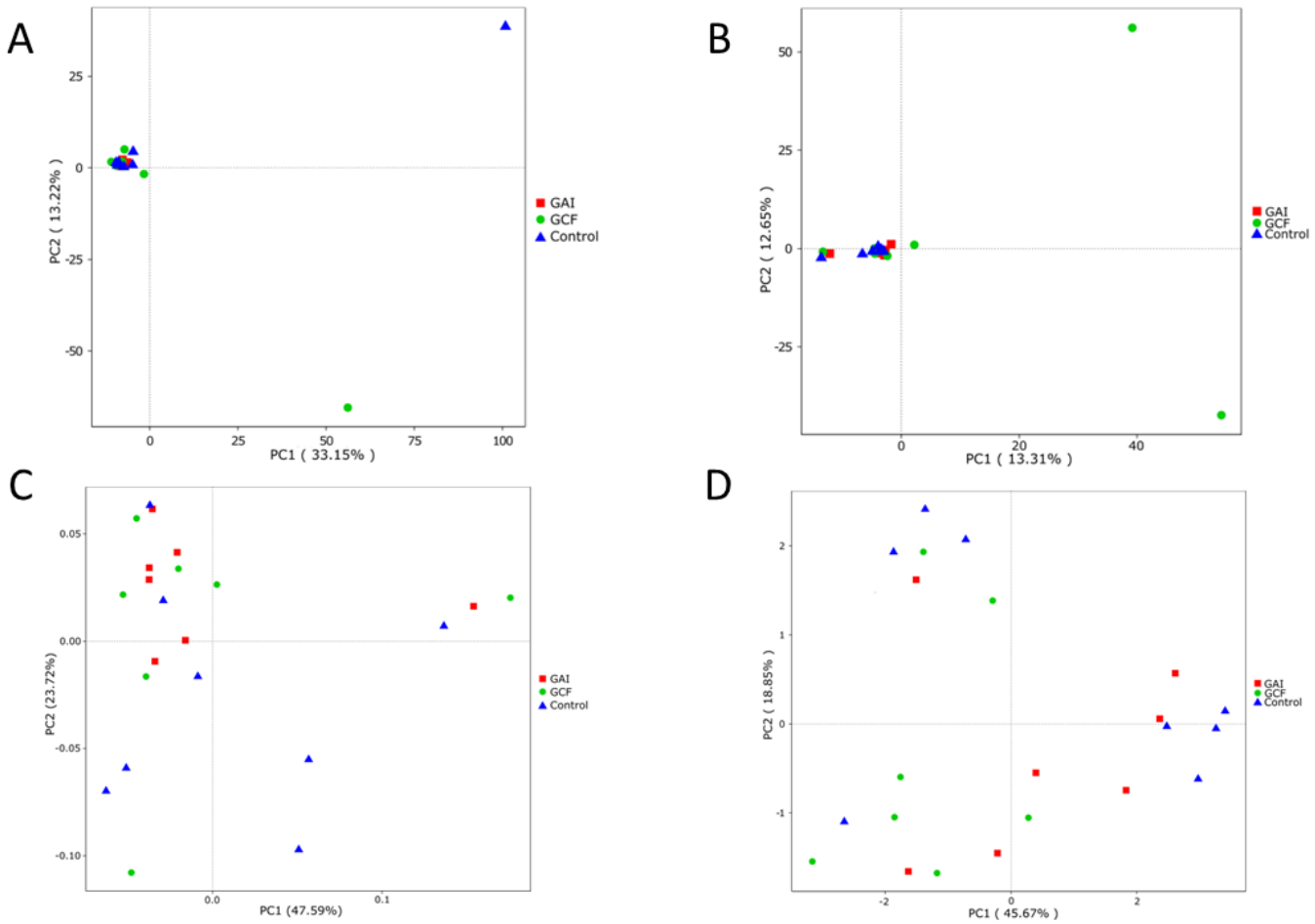


Figure 3-19 PCA and PCoA's were used to visualise the variance in microbial composition amongst treatment group samples. PCAs on **(A)** bacterial and **(B)** fungal taxa illustrate that most samples are clustered together, demonstrating low variance in bacterial and fungal composition. PCoA of **(C)** bacterial and **(D)** fungal weighted unifrac measurements, taking relative abundance and presence/absence of species into account, for both bacterial and fungal PCoA's, no clear clusters or distinctions were present between treatment groups.

Boxplots were used to demonstrate beta diversity indices and their differences between treatment groups with Wilcoxon and Tukey tests performed to determine if differences were significant (Table S3-16). Using weighted unifrac distance to consider both abundance and the presence or absence of bacterial taxa, overall, GAI had a lower beta diversity compared to other treatment groups, followed by GCF and the control treatment groups. Wilcoxon testing determined a statistically significant difference in beta diversity between GAI and control (Wilcoxon test, $p = 0.035$) (Figure 3-20A). Weighted unifrac distance of fungal samples was statistically significantly different between GAI and control treatment groups (Wilcoxon test, $p = <0.001$; Tukey test, $p = 0.031$) and between GCF and control treatment groups (Wilcoxon test, $p = 0.021$) (Figure 3-20B). The same statistical tests were used to determine statistically significant differences in unweighted unifrac distances between treatment groups (Table S3-16). There was a statistically significant difference in bacterial unweighted unifrac distance between GAI and GCF treatment groups (Wilcoxon test, $p = 0.003$; Tukey test, $p = 0.004$). There were no statistically significant differences in fungal unweighted unifrac distances between treatment groups.

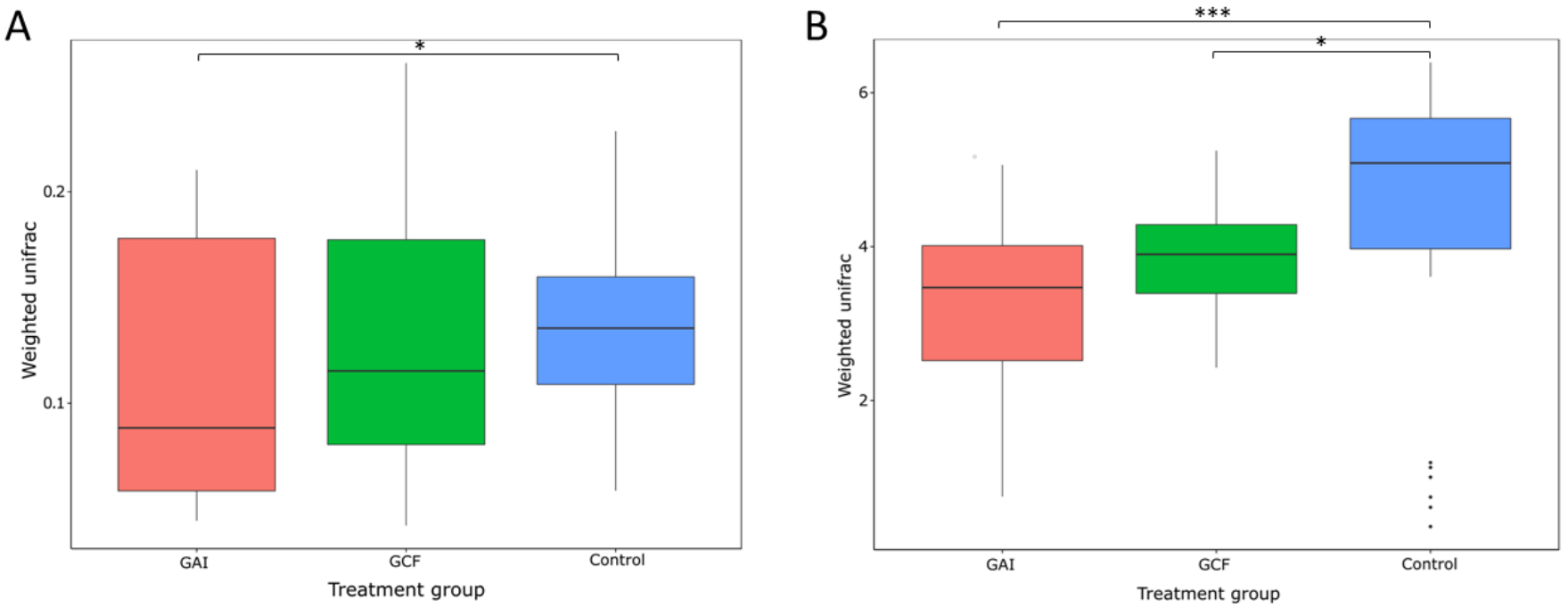


Figure 3-20 Boxplots and pairwise comparisons of weighted unifrac distances between treatment groups. (A) Tukey and Wilcoxon tests were conducted on bacterial weighted unifrac distances between different treatment groups. There was a statistically significant difference in beta diversity between GAI and control treatment groups (Wilcox p = 0.035). **(B)** Tukey and Wilcoxon tests were conducted on fungal weighted unifrac distances between treatment groups. There was a statistically significant difference in beta diversity between GAI and control treatment groups (Wilcoxon p = <0.001, Tukey p = 0.031), and GCF and control treatment groups (Wilcoxon p = 0.02).

Analysis of similarity (ANOSIM) and multi-response permutation procedure (MRPP) were conducted to determine if the differences in bacterial and fungal community structure between groups were significant (Table S3-17). ANOSIM revealed that inter-group variation is larger than intra-group variation of bacterial communities only when comparing the control group to the GAI-exposed group, indicated by a positive R-value (R = 0.035). However, the variation in community structure was larger within treatment groups than between treatment groups for all other pairwise comparisons. The opposite was true for fungal community variation, with the pairwise comparison of control and GAI treatment groups as the only pair displaying larger intra-group variation than inter-group variation (R = -0.034). ANOSIM did not determine any statistically significant differences between treatment groups for bacterial or fungal communities. MRPP analysis was also conducted to determine if there were statistically significant differences in microbial community structure between treatment groups. For bacterial communities, with a positive A-value when comparing GAI to control treated group determined larger inter-group variation than intra-group variation (A = 0.0076). All other bacterial pairwise comparisons of treatment groups had negative A-values, indicating a larger intra-group variation than inter-group variation. For fungal community structure, all groups displayed larger inter-group variation than intra-group variation. These results were not significant for both bacterial and fungal community structure differences between groups.

In addition, t-tests were performed to determine if there were significant differences in species abundance in pairwise comparisons between treatment groups. Overall, there were two bacterial genera and two bacterial species with significantly different relative abundances between GAI and GCF treatment groups. In a pairwise comparison of GAI and GCF treatment groups, the bacterial genera *Sva0485* and the species *L. murinus* and *Bifidobacterium bombi* were not present in GAI but were present in GCF (p = 0.03, p = 0.017 and p = 0.047, respectively). *Sporolactobacillus* genera were present in GCF, but not the GAI treatment group (p = 0.045). There was a statistically significantly lower relative abundance of the genera *Parabacteroides* (p = 0.022) and the species *Acinetobacter calcoaceticus (*p = 0.023) in GAI compared to control treatment groups (Table 3-2). *L. murinus* was significantly higher in GCF compared to its absence in the control treatment group (p = 0.017).

When comparing fungal genera and species relative abundance between groups, there were nine statistically significant differences found in GAI and GCF comparisons (Table S3-18). These included a higher relative abundance of *C. bombi* and *C. apicola* in GAI compared to GCF treatment groups ($p = 0.012$ and $p = 0.049$, respectively) and a statistically significant lower abundance of *Trichoderma asperellum* in GAI compared to the GCF treatment group ($p = 0.019$). *Rhodotorula mucilaginosa* and *Hannaella luteola* had a statistically significantly higher relative abundance in GAI compared to control treatment groups ($p = 0.032$ and $p = 0.039$, respectively). *T. asperellum* was significantly higher in GCF compared to the control group ($p = 0.019$) (Table 3-3).

Table 3-2 Statistically Significant Bacterial Genera and Species Relative Abundance Differences in Pairwise Comparisons of Treatment Groups. A t-test was conducted on the relative abundance of bacterial taxa in pairwise comparisons to determine statistically significant differences between treatment groups.

Comparison	Taxon	Average (GAI)	Average (GCF)	Average (Control)	Standard Deviation (GAI)	Standard Deviation (GCF)	Standard Deviation (Control)	P-value
GAI vs Control	Parabacteroides	4.14E-06	n/a	3.98E-05	1.09E-05	n/a	3.44E-05	0.02246
	Acinetobacter calcoaceticus	2.07E-05	n/a	3.62E-06	1.41E-05	n/a	1.02E-05	0.02307
GCF vs Control	*Lactobacillus murinus*	n/a	2.48E-05	0	n/a	2.00E-05	0	0.01669
GAI vs GCF	Sva0485	0	3.31E-05	n/a	0	3.09E-05	n/a	0.03002
	Sporolactobacillus	2.48E-05	0	n/a	2.60E-05	0	n/a	0.04527
	Lactobacillus murinus	0	2.48E-05	n/a	0	2.00E-05	n/a	0.01669
	Bifidobacterium bombi	0	2.07E-05	n/a	0	2.19E-05	n/a	0.04653

Table 3-3 Statistically Significant Fungal Genera and Species Relative Abundance Differences in Pairwise Comparisons of Treatment Groups. The top three lowest p-values were selected from each pairwise comparison.

Comparison	Taxon	Average (GAI)	Average (GCF)	Average (Control)	Standard Deviation (GAI)	Standard Deviation (GCF)	Standard Deviation (Control)	P-value
GAI vs Control	*Rhodotorula mucilaginosa*	5.7E-4	n/a	3.12E-05	5.13E-4	n/a	8.83E-05	0.03198
	Hannaella luteola	6.06E-4	n/a	0	6.09E-4	n/a	0	0.03887
	Rhodotorula	6.77E10-4	n/a	3.12E-05	6.23E-4	n/a	8.83E-05	0.03351
GCF vs Control	*Trichoderma asperellum*	n/a	2.35E-3	3.12E-4	n/a	1.71E-3	3.94E-4	0.01915
	Filobasidium	n/a	1.42E-4	0	n/a	1.33E-4	0	0.03002
	Tomentella	n/a	1.6E-3	0	n/a	1.55E-3	0	0.03440
GAI vs GCF	*Candida bombi*	9.4E-2	1.74E-2	n/a	5.79E-2	1.86E-2	n/a	0.01212
	Trichoderma asperellum	3.2E-4	2.35E-3	n/a	3.12E-4	1.71E-3	n/a	0.019606
	Candida	3.49E-1	8.16E-2	n/a	2.49E-1	1.11E-1	n/a	0.031252

3.4 Discussion

Glyphosate does not have a major direct effect on survival, behaviour, or sucrose consumption. It does affect the digestive tract proteome of *B. terrestris* and, depending on its consumption as the active ingredient alone or as part of a commercial formulation, differential effects were observed. Changes in the abundance of proteins associated with metabolism and the lysosome, were attributable to the co-formulants in the commercial preparation. In addition, the GAI and not GCF had a dramatic effect on the abundances of many mitochondrial proteins. However, a common glyphosate signature was also observed through effects on proteins associated with cellular structural integrity and adhesion, the extracellular matrix, oxidative stress regulation and metabolism. Additionally, through ITS and 16S amplicon sequencing, we investigated whether GAI or GCF had impacts on the digestive tract microbiota of *B. terrestris*. While neither the GAI nor GCF had significant impacts on bacterial species present, the GCF had a significant impact on *Candida* fungi in the *B. terrestris* digestive tract, once again highlighting potential effects caused by co-formulants.

3.4.1 Glyphosate Does Not Impact Survival, Behaviour or Sucrose Solution Consumption

Glyphosate, regardless of source, had no statistically significant impacts on survival or behavioural measures at 1, 10 or 100 ppm. Additionally, 1 ppm GAI or GCF had no impact on the consumption of sucrose solution, demonstrating that bees are neither attracted to nor repelled by glyphosate-contaminated sucrose solution any more than untreated sucrose solution. This result is further evidence that bees are at risk of glyphosate exposure, along with recent research by Thompson et al. (2022), which demonstrated that *B. terrestris* will indiscriminately forage on plants treated with glyphosate. Since glyphosate exposure is plausible in natural settings, it is important to investigate impacts on survival. Whilst there were no impacts on survival at 1, 10 or 100 ppm GAI or GCF after 10 days of exposure, some studies have found impacts at higher levels or over longer periods of exposure. Motta and Moran (2020) found that 16.91 and 169.1 ppm glyphosate exposure decreased honeybee survival, some which were actively obtaining their digestive tract microbiota while other groups had a pre-established microbiota, after 15-40 days of exposure. Interestingly, bees exposed to 1.691 ppm, a concentration closer to the lowest concentration used in this research,

led to higher survival rates in honeybees. Further, bees without an established digestive tract microbiota still displayed higher mortality than controls after exposure to 169.1 ppm glyphosate. This suggests that glyphosate exposure at higher concentrations can impact bee health independently of impacts on the digestive tract microbiota. However, from the evidence available, it is unlikely that bees would be orally exposed to such a high concentration for an extended period. The proteomic and DNA sequencing results of this research provide insight into the physiological processes altered by glyphosate in the digestive tract in addition to impacts on the microbiota. However, this research focuses on a single field-realistic dose of 1 ppm GAI or GCF for a shorter period of five days. Additionally, survival impacts observed in Motta and Moran (2020) in microbiota-free bees may be due to impacts on a different tissue than the one investigated in this chapter. Almasri et al. (2020) determined that glyphosate alters honeybee survival at much lower concentrations over a period of 20 days. In addition to concentration and exposure duration differences, glyphosate may alter honeybee and bumblebee species differently – but this is currently unknown.

3.4.2 Common Responses to Glyphosate Consumption, Regardless of Source

3.4.2.1 Cell Adhesion and Cell Structural Integrity

A major molecular effect of glyphosate exposure, whether ingested as an active ingredient or part of a commercial formulation, involved proteins associated with cell adhesion and cellular structural integrity. Of the 29 proteins with decreased abundance in common to both glyphosate-based treatments, 9 were associated with these processes. Several of these proteins represent some of the most differentially abundant proteins in terms of their relative fold changes (5 and 4 of the top 10 most decreased proteins in the glyphosate GAI and GCF treated digestive tracts, respectively). Alterations in these processes would have a marked effect on overall cell integrity, structure, and function. Constituents of the extracellular matrix including fibrillin-2, basement membrane-specific proteoglycan and collagens were significantly reduced in abundance and in fact fibrillin-2 had the highest decrease in fold change in comparison to the control group in both the glyphosate and GCF treatments by 222.68

and 56.83, respectively. Fibrillins are necessary for the formation of microfibrils; important components of the extracellular matrix and basement membranes of tissues which act as molecular scaffolds and impart structural integrity, strength, and growth factor regulation to tissues (Kumra and Reinhardt, 2018). A decrease in abundance was also observed for the tetraspanins, CD151 antigen and 23kDa integral membrane proteins. These plasma membrane-bound proteins have roles in cell adhesion, signalling and immunity via interactions with other proteins e.g. other tetraspanins, and integrins (Maecker *et al.*, 1997; Todres *et al.*, 2000; Zhuang *et al.*, 2007). Collagen IV proteins and heparan sulfate proteoglycans are components of the basement membrane which gives cells structural stability, regulate cell behaviour, and separate epithelial cells from the stroma of the tissue (Lunstrum *et al.*, 1988; Paulson, 1992; Tanzer, 2006; Altincicek *et al.*, 2009). Decreases in collagen proteins have previously been associated with wounding and haemocyte recruitment for cellular repair (Pastor-Pareja *et al.*, 2008; Saleh *et al.*, 2018) highlighting a potential mechanism for glyphosate toxicity (i.e. mechanical damage of digestive tract extracellular matrix or basement membrane). Further research into the histology of *B. terrestris* digestive tract after glyphosate exposure would shed light on this.

Several membrane-associated proteins with roles in cellular signalling were also affected by glyphosate-exposure. These include tubulointerstitial nephritis antigen and the basement membrane-specific heparan sulfate proteoglycan, both of which are involved in regulating major signalling pathways such as Wnt, Hedgehog and TGF beta (Lin and Perrimon, 2000; Theodosiou and Tabin, 2003; Logan and Nusse, 2004; Li *et al.*, 2007). Basement membrane proteoglycans are also involved in the regulation of the cytoskeleton itself.

Given that proteins involved in cell adhesion and the extracellular matrix are vital for cell signalling and tissue structural integrity (Gumbiner, 1996; Johansson, 1999) and the proper functioning of the basement membrane (Lunstrum *et al.*, 1988; Paulson, 1992; Tanzer, 2006), alterations in these processes in the insect digestive tract lining may represent a major detrimental effect of glyphosate exposure on bees.

3.4.2.2 Oxidative Stress Regulation, Pesticide Metabolism and Melanisation

Maintaining an equilibrium between free radicals and antioxidants is important to prevent oxidative stress and promote longevity and overall health. Several proteins with increased abundance in the digestive tract of bees exposed to both glyphosate-based treatments were associated with oxidative stress regulation, including superoxide dismutase and a venom carboxylesterase. Superoxide dismutase is involved in the destruction of free radicals and is expressed in response to oxidative stress in insects (Kim *et al.*, 2005; Choi *et al.*, 2006). Carboxylesterases are involved in lipid metabolism and pesticide detoxification via ester and thioester hydrolysis (Wheelock *et al.*, 2005) and are reported to be involved in insecticide metabolism and oxidative stress regulation in insects (Badiou-Benéteau *et al.*, 2012; Ma *et al.*, 2018; Mao *et al.*, 2021). The phenoloxidase enzyme, laccase, was also increased in both treatments, having remarkably similar increases in abundance. It participates in insect immunity and wound healing via melanin production through the catalysis of oxidation-reduction reactions. Laccase also has industrial use for the degradation of various pesticides (Jin *et al.*, 2016; Gangola *et al.*, 2018) which suggests a potential role of laccase in glyphosate metabolism. Multiple studies have identified the role of melanisation in insect immunity and wound healing, as well as the action of glyphosate in altering melanisation in insects (Galko and Krasnow, 2004; González-Santoyo and Córdoba-Aguilar, 2012; Parsons and Foley, 2016; Motta *et al.*, 2018, 2022). Melanisation is an important part of the insect immune response to mechanical injury or infection with pathogens, but if dysregulated, the insect immune response has the capacity to self-inflict major damage to the insect and must remain tightly regulated under normal physiological conditions (Theopold *et al.*, 2004; Dionne *et al.*, 2006; Jiravanichpaisal *et al.*, 2006; Eleftherianos and Revenis, 2011; Krautz *et al.*, 2014). Recent research suggests that glyphosate exposure inhibits phenoloxidase activity in the insects *Galleria melonella* and *Anopheles gambiae* in a dose-dependent manner (Smith *et al.*, 2021). The concentration used in this experiment was over 80 times less than the lowest glyphosate concentration used in the Smith et al. (2021) study, indicating that higher phenoloxidase abundance may be induced at lower field-realistic doses. Such increases in melanisation and oxidative stress regulation may also highlight a link to the alterations to the extracellular matrix and cellular integrity discussed above, as changes to some extracellular matrix proteins such as a decrease

in collagens are associated with mechanical damage and wound healing (Pastor-Pareja *et al.,* 2008; Saleh *et al.,* 2018).

The observed increase in proteins associated with oxidative stress regulation indicates that glyphosate, whether directly or indirectly, may create an environment in the digestive tract where there is oxidative stress. Reactive oxygen species (ROS) are important in melanisation, wound repair, and immunity (Nappi and Christensen, 2005; Zug and Hammerstein, 2015) and an estimated 90% of ROS are produced in the mitochondria, making mitochondrial ROS a likely source of oxidative stress (Balaban *et al.,* 2005). It is also known that exposure to glyphosate GCFs results in increased ROS levels and overall in reactive oxygen species and mitochondrial dysfunction in different species and cell lines (Chaufan *et al.,* 2014; Bailey *et al.,* 2018; A.G. Pereira *et al.*, 2018; Nerozzi *et al.,* 2020; Ravishankar *et al.,* 2020) highlighting an additional mechanism of potential glyphosate-induced impairment.

The increase in the abundance of antioxidant and detoxification proteins demonstrates potential mechanisms to counteract and prevent glyphosate-induced oxidative damage in bees. Considering the other findings, it does seem, however, that the response may be insufficient, particularly when coupled with the multiple stressors bumblebees face in the wild.

3.4.2.3 Lipid Metabolism and Calcium Homeostasis

Six proteins had an increased abundance across both treatments that are involved in metabolism, four of which are associated with lipid metabolism. These included FGGY carbohydrate, glucosylceramidase and venom carboxylesterase-6 (Acharya and Acharya, 2005; Zhang *et al.*, 2011; Singh *et al.*, 2017). The glycosyl hydrolase, glucosylceramidase, was the protein with the most increased abundance in both glyphosate treatments and had very similar RFCs indicating a clear and consistent response to glyphosate regardless of the source. Glycosylcerimidases engage in sphingolipid metabolism, which are important structural membrane proteins in insects. Carboxylesterases mainly catalyse the breakdown of water-soluble lipids and can be involved in the breakdown of longer, insoluble, fatty acid chains in the presence of surfactants but at a slower rate than more suitable lipases (Terra and Ferreira, 2012). Other lipid associated proteins: choline transporter protein and

phosphatidylethanolamine, had a lower abundance in glyphosate-treated digestive tracts. Choline transporters are found in cellular and mitochondrial membranes and are important for transporting the choline required for phospholipid biosynthesis into the cell. Phosphatidylethanolamine plays a role in ethanolamine transport and ultimately, phosphatidylethanolamine synthesis (Taylor *et al.*, 2021). Both proteins are also important for sphingomyelin production, which plays a structural role in membranes and changes in their abundance could interfere with lipid metabolism itself (Bridges, 1972; Michel *et al.*, 2006).

Another protein found to be of higher abundance in both glyphosate treatments was regucalcin (also known as smp-30), a calcium-dependent gluconolactonase also involved in regulating intracellular $Ca^{2+,}$ nucleic acid synthesis, proliferation, apoptosis, and intracellular signalling pathways (Yamaguchi and Murata, 2013; Marques *et al.*, 2014). Although regucalcin has also been linked to lipid metabolism in mice, little is known about its involvement in insects (Ishigami *et al.*, 2004; Yamaguchi *et al.*, 2004; Toprak *et al.*, 2020). Interestingly, regucalcin has been identified as an insecticide tolerance-related gene in the grain aphid *Sitobion avenae* after transcriptional increases of regucalcin in response to the neonicotinoid imidacloprid and the organophosphate chlorpyrifos (Wei *et al.*, 2019). While the increase in oxidative stress regulation and detoxification proteins could indicate defence against glyphosate toxicity, coupled with the increase in abundance of regucalcin in response to glyphosate, a conserved xenobiotic response may exist in insects.

Additional calcium transporting proteins were decreased in the digestive tracts of bees exposed to both glyphosate treatments, including plasma membrane calcium-transporting ATPase 2 and calcyophosin. Calcium is an important mineral for the regulation of a variety of biological functions via its presence as a co-factor or signalling molecule in insects (Taylor, 1987; Berridge *et al.*, 2000; Teets *et al.*, 2013; Collet *et al.*, 2021). Glyphosate is known to act as a chelating agent which can tightly bind to and sequester calcium from its surroundings (Fon and Uhing, 1964; Mertens *et al.*, 2018). Surprisingly, few studies have assessed the impact of glyphosate on calcium levels within animals, although Gaur and Bhargava (2019) reported calcium signalling alterations in zebrafish embryos after exposure to glyphosate. In insects,

calcium homeostasis needs to be maintained within epithelial cells to preserve calcium absorption in the midgut (Taylor, 1985; Taylor, 1987) whereas calcium influxes are involved in the repair of epithelial cells through ROS production, important for insect immunity and haemocyte signalling (Davis and Engström, 2012; Krautz *et al.,* 2014; Mikonranta *et al.,* 2014).

3.4.3 Technical Grade Glyphosate Cannot be Compared to Glyphosate-Based Formulations

Mitochondrial proteins were significantly affected by glyphosate exposure, but to different extents depending on the source of glyphosate. When compared to the respective control, 29 proteins associated with the mitochondrion were decreased in GAI, and 10 proteins associated with the mitochondrion were increased in the digestive tracts of GCF treated bees. GAI exposed digestive tracts had decreases in abundance in 48 mitochondria associated proteins when compared directly to GCF treatment groups, 22 of which are involved in the TCA cycle and oxidative phosphorylation. Despite this, some proteins associated with ROS production and energy metabolism were also differentially affected by GCF exposure. Glyphosate induced damage to and dysfunction of mitochondria has been widely reported in a number of animals (Peixoto, 2005; Lopes *et al.,* 2018; A. G. Pereira *et al.,* 2018; Neto da Silva *et al.,* 2020) and combined with findings here, highlight that the mitochondrion and its processes are particularly sensitive to glyphosate. Given the lack of a known target pathway for glyphosate in metazoans, but the universal presence of mitochondria (a remnant of a symbiosis with a prokaryote), investigation of the interaction between glyphosate and the mitochondrion at the molecular level is highly warranted.

The GAI and GCF also differentially affected endocytosis, protein degradation and modification, and plasma membrane proteins which could alter digestive system functioning. Proteins associated with endocytosis and the plasma membrane were decreased in the GCF treatment group, but some proteins associated with endocytosis were low after consumption of both treatments. When endocytosed molecules are fused with endosomes, they can then mature into lysosomes which rely on hydrolases – enzymes that were increased in both glyphosate treatments in the digestive tract - for digestion of these molecules. Previous studies have confirmed the effects of

glyphosate on lysosomal integrity (Lopes *et al.,* 2018); with one study by Mottier et al. (2020) demonstrating that a GCF and adjuvant alone can affect phagocytic activity and lysosomal membrane integrity in shellfish haemocytes with the GAI itself, glyphosate, resulting in little change. Proteins associated with protein degradation and modification, such as ubiquitin carboxyl-terminal hydrolase, ubiquitin-conjugating enzyme E2 J1, and NEDD8-activating enzyme E1 regulatory subunit, were increased in the GCF compared to GAI. Therefore, it seems that the co-formulants present in the GCF can cause alterations to important cellular processes themselves.

Proteins involved in glutamine amino acid and pyruvate metabolism were of low abundance in the GAI treatment group only, whereas proteins associated with energy production, namely glycolysis and gluconeogenesis had an opposite abundance profile. The effects of glyphosate on nucleic and amino acid metabolic processes have been previously reported for a wider range of animals including fish, frogs, and honeybee species (Rocha *et al.,* 2015; Wang *et al.,* 2019; Zhao *et al.,* 2020). Additionally, various proteins involved in lipid metabolism were decreased in the GCF but not the GAI. Lipases had notable RFC decreases in the GCF, with one having an RFC of -92.29 in comparison to the GAI, while proteins associated with carbohydrate metabolism such as myrosinase and trehalase were increased in the GCF treatment group, although some proteins associated with fatty acid degradation were increased in comparison to the GAI treatment group. These findings highlight a potential switch from lipid to carbohydrate metabolism attributable to the presence of one or more co-formulants. Metabolism is altered by GAI also, although proteins associated with energy production via TCA cycle and oxidative phosphorylation were decreased from exposure to glyphosate alone. Various proteins involved in carbohydrate metabolism were increased in abundance in the GCF but not GAI, indicating that the co-formulants may be resulting in alternative responses that may mask the glyphosate specific effects in this data. However, GAI had a higher abundance of SSDA proteins involved in glycolysis/gluconeogenesis and amino acid synthesis in comparison to GCF treatment groups. Shifts in metabolic processes have been previously reported at the transcriptomic and metabolic levels for two species of honeybees (Zhao *et al.,* 2020) and although the findings from this research are slightly different they are not unexpected given that different species display different sensitivities and responses to pesticides (Arena and Sgolastra, 2014b) and that this research focussed on a single

organ rather than the whole organism. Additionally, in this research, bees were exposed to a lower concentration of glyphosate which was estimated to be at or below residues found in glyphosate-treated areas (Thompson *et al.,* 2014; Cebotari *et al.,* 2018).

Results presented thus far demonstrate that the presence of co-formulants resulting in the resolution of a different proteomic profile in the bumblebee digestive tract after glyphosate exposure. Although these specific changes are discussed in terms of the effects of a formulation on protein function and abundance, it is necessary to consider that co-formulants may be acting as substrates for host enzymes and thus altering the direction of usual metabolic activities in the glyphosate-exposed bee. For instance, the main surfactant used in RoundUp Optima+® used in this research is alkylpolyglycoside which is made up of glucose and fatty alcohol, which could account for the shifts in metabolism reported here and elsewhere. What is clear, however, is that current policies on the listing and testing of all co-formulants in commercial formulations are inhibitory to achieving a full understanding of the effects and risks posed by GAI and co-formulants alike on bees.

3.4.4 Technical Grade Glyphosate and Roundup Optima+® Have Differential Impacts on the Digestive Tract Microbiota

The reported impacts of glyphosate on the bee microbiota (Dai *et al.*, 2018; Blot *et al.*, 2019; Motta *et al.*, 2020) led to an investigation on whether the glyphosate induced changes observed here at the proteomic level could be explained by an indirect consequence of alterations to the microbiome. In addition, most of these peer-reviewed studies focus on honeybees, bacteria only, do not compare GAI and a GCF and use relatively high concentrations of glyphosate.

Weighted unifrac distance was significantly different between GAI and control treatment groups, highlighting a significant difference in the community composition (beta diversity) of the digestive tract microbiota of bees exposed to GAI compared to the control. A t-test revealed a significantly decreased abundance of bacteria of the genus *Parabacteroides* and an increased abundance of the species *A. calcoaceticus* in the GAI compared to the control. In GCF treatment groups, *L. murinus* was present, with no presence in GAI or control treatment groups. Further, *B. bombi* was present

in GCF but not GAI treatment groups, indicating that co-formulants may alter how glyphosate impacts bacterial species. Castelli et al. (2021), studying honeybees, found that at GAI glyphosate concentrations 10 times that of the concentration used in this research (in addition to a *Nosema ceranae* infection), resulted in alterations to the relative abundance of *S. alvi* and *Lactobacillus* species. In addition, higher concentrations were also used by Motta et al. (2018) who not only reported changes in the honeybee digestive tract microbiota to *S. alvi*, but also demonstrated the possession of glyphosate-sensitive or insensitive EPSPS (the known target for glyphosate) in different bacterial species. This highlights another difference that may exist in some species between the *Apis* and *Bombus* microbiotas, which may explain why alterations to *S. alvi* were not observed. Mesnage et al. (2021) determined shikimate pathway perturbations in the digestive tract caecum of rats after GAI and GCF (RoundUp MON 52276) exposure, leading to an accumulation of metabolites found upstream of EPSPS. However, this was only significant at higher GAI and GCF concentrations, which may indicate that physiological changes observed at lower concentrations are consequences of damage to the host organism tissue and not a result of shikimate pathway alterations in the microbiota. In addition, metabolites were expressed in rat serum indicating oxidative stress, a pathway increased in both glyphosate-based treatments in this research, also. Whether this finding in *B. terrestris* is due to a downstream impacts of digestive tract microbial alterations or a result of direct impacts on the host organism is currently unknown. Research investigating the metabolome of the *B. terrestris* digestive tract could elucidate whether the shikimate pathway is impacted in the digestive microbiota after GAI or GCF exposure, further piecing together an evidence based AOP for glyphosate and co-formulant impacts on bee health.

The importance of fungi in the bee microbiota is understudied in comparison to bacterial communities, with fungi often seen as opportunistic or transient in bees, and therefore unimportant for survival (Batra *et al.*, 1973; Bosmans *et al.*, 2018; Hammer *et al.*, 2021). Despite this, some studies suggest fungi can impact foraging behaviour, nutrition, pathogen protection, longevity, and development in insects, including bees (Cheng and Hou, 2005; Herrera *et al.*, 2013; Stefanini, 2018; Parish *et al.*, 2020; Pozo *et al.*, 2020; Pozo *et al.*, 2021; Cullen *et al.*, 2021) making fungal presence, abundance, and community structure in bees worthy of investigation. Wilcoxon testing determined

that fungal alpha diversity (species richness) was significantly different in GCF compared to control treatment groups for all indices used. In addition, Wilcoxon testing determined a statistically significant difference in fungal beta diversity i.e. microbiota composition, between the GAI and control treatment groups and GCF and control treatment groups using weighted unifrac measurements, which takes both relative abundance and taxa presence/absence into account. When determining beta diversity based solely on unweighted unifrac measurements, i.e. on the presence of absence of taxa alone, Wilcoxon testing determined a significant difference between GAI and GCF treatment groups. In the GCF, fungal relative abundance and taxonomic composition were affected, with a statistically significant lower abundance in *Ascomycete*, particularly *Candida*, species observed. These included the yeasts *Candida apicola* and *Candida bombi*, which were statistically significantly decreased in GCF compared to GAI treated bees. A less common species, *Trichoderma asperellum* was observed in a statistically significant higher abundance in GCF treated bees compared to GAI and control treated bees. Along with the presence of bacterial species present in GCF which were not found in other treatment groups, these fungal species presence and significant increase in abundance may be a result of opportunistic colonisation due to lower *Candida* abundance, a core fungal genera found in the bee digestive tract microbiota. In the wild, this alteration to bee fungal communities in the digestive tract could lead to dysbiosis and perhaps infection with opportunistic pathogens (Näpflin and Schmid-Hempel, 2018; Tauber *et al.*, 2019; Pozo *et al.*, 2020). These findings further demonstrate the potential impact of co-formulants on biological systems as there were few changes in fungal community composition observed in GAI exposed bees, with GCF having the most exaggerated effect on the core bumblebee genera Candida, implicating one or more of the 'inert' ingredients, in this case, alkylpolyglycoside, nitroryl, or any of the unlisted components of Roundup Optima+®.

3.4.5 Glyphosate's Impact on *B. terrestris* Digestive Tract: An Adverse Outcome Pathway Model

Based on the AOP by Ankley et al. (2010) discussed in section 1.7, these results can be used to generate a putative model for glyphosate exposure to bumblebees. Through the successful application of high-resolution mass spectrometry, new insights into the

potential mechanisms and consequences of glyphosate exposure in an organism deficient in the primary target pathway for this herbicide have been elucidated in the digestive tract. The identification of proteins in the digestive tract affected by glyphosate, and the pathways, cellular processes, and structures they are involved in, along with the survival, behaviour, consumption, and microbiota research conducted, can populate the molecular, cellular, organ and organism level categories of an adverse outcome pathway to predict glyphosate's possible impacts on the digestive tract of bumblebees (Figure 3-21). This model involves a series of interrelated steps including i) attempts to metabolise/detoxify glyphosate; ii) major mitochondrial disruption either through the direct or indirect action of glyphosate, presenting a potential mode of action in nontarget organisms; iii) the production of reactive oxygen species and conditions of oxidative stress and mechanisms required for their contention; iv) major effects on cellular integrity through the disruption of extracellular matrix, basement membrane, and cell adhesion proteins; v) alterations to wound repair, melanisation, and signalling mechanisms and vi) alterations in carbohydrate and energy metabolism. However, due to differential impacts of GAI and GCF on the digestive tract proteome and microbiota, exposure to GCF's in the environment may alter this pathway depending on co-formulants present in formulations applied. In addition, these results may differ depending on exposure to higher or lower concentrations of glyphosate, or a more prolonged exposure to lower concentrations. Extensive links exist among all components listed above and although the direction and nature of these links under glyphosate exposure has yet to be fully elucidated, the discovery based quantitative proteomic approach adopted here has presented new hypotheses that can now be couched in molecular terms made available for future testing and bring us closer to a comprehensive understanding of how glyphosate alters bee health.

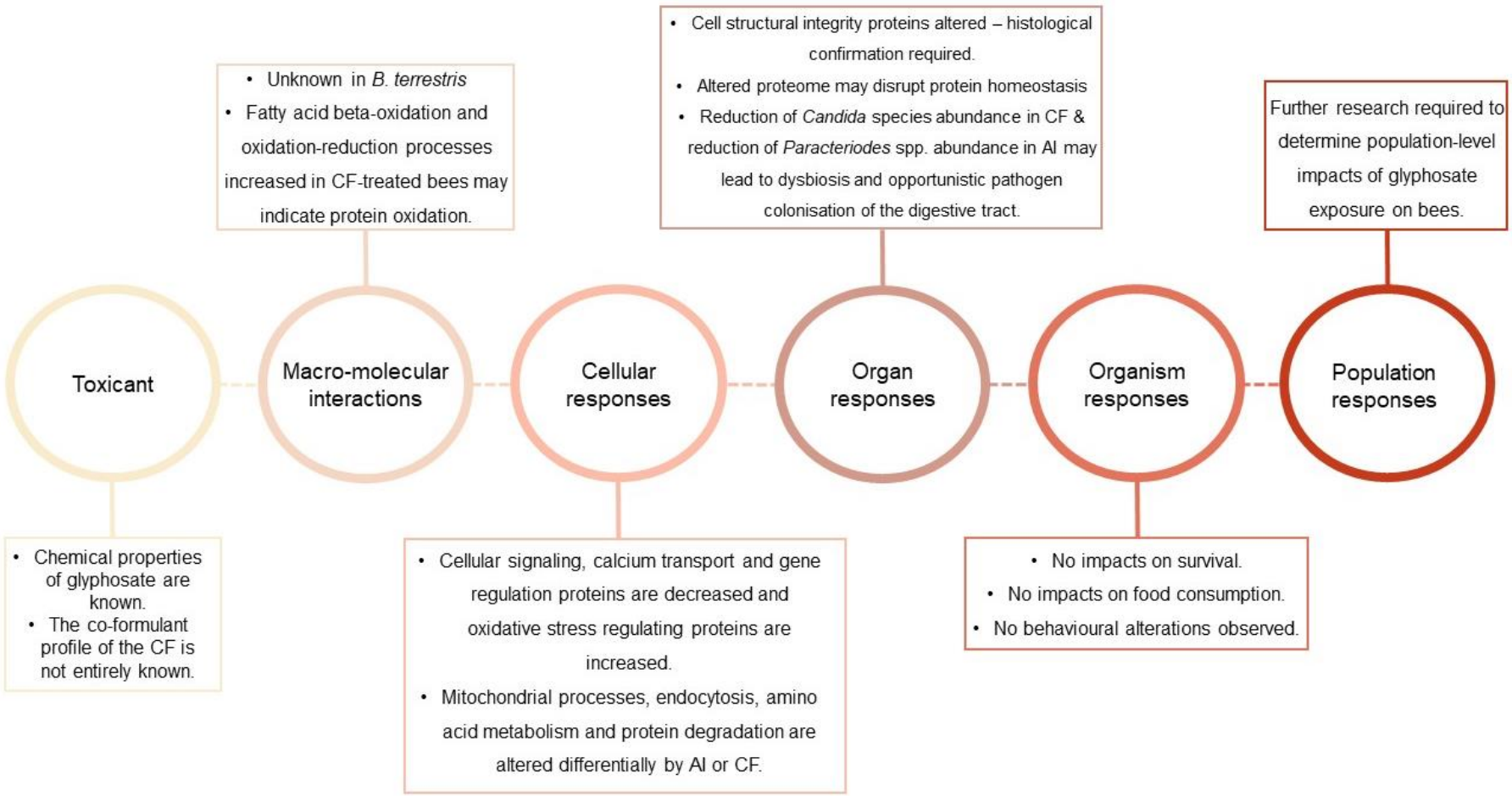


Figure 3-21 An Adverse Outcome Pathway on The Impacts of Glyphosate on *B. terrestris*. The results of this chapter were used to populate an adverse outcome pathway to demonstrate what is now known and what is still unknown in relation to glyphosate's (AI: glyphosate active ingredient; CF: RoundUp Optima+®) impact on the bumblebee digestive tract.

3.5 Conclusion

The results of this research demonstrate that glyphosate, even at low and conservative concentrations, can affect the non-target organism *B. terrestris*, in sub-lethal ways. Major cellular and physiological processes in the digestive tract proteome of *B. terrestris* were observed, regardless of whether glyphosate was ingested as an AI alone or part of the formulation Roundup Optima+®. Glyphosate altered the abundance of proteins associated with important processes including oxidative stress regulation, metabolism, cellular adhesion, and the extracellular matrix. Interestingly, endocytosis, oxidative phosphorylation, the TCA cycle, and carbohydrate, lipid, and amino acid metabolism were differentially altered depending on treatment with glyphosate AI or RoundUp Optima+®. Additionally, both technical grade glyphosate and RoundUp Optima+® significantly altered the digestive tract microbiota differentially. With RoundUp Optima+® significantly altering a core member of the fungal microbiota, highlighting the potential effects caused by the co-formulants in commercial formulations.

Chapter 4

Characterising the impacts of prothioconazole on the digestive tract proteome and microbiota of *B. terrestris*

4.1 Introduction

Fungicides are important for the protection of crops from fungal disease, particularly in wet climates where fungal growth is favoured. As a result, fungicides are the second most used pesticide class in Ireland, applied to over 300,000 hectares of agricultural land, with crops often treated multiple times per season (Jess *et al.*, 2014; López-Ballesteros *et al.*, 2022). In 2020, fungicides accounted for 15% of the pesticide active ingredients in agrochemical products on the Irish market (DAFM, 2020).

4.1.1 Ergosterol Biosynthesis in Fungal Pathogens

The cell membranes of Ascomycete and Basidiomycete fungal pathogens contain the sterol ergosterol. Ergosterol is crucial for maintaining plasma membrane fluidity, permeability, and the activity of plasma membrane-associated proteins such as ATPases which can impact nutrient acquisition (Hu *et al.*, 2017). In addition, ergosterol is important in stress responses, with ergosterol plasma membrane abundance important for cell maintenance under alcohol stress (Aguilera *et al.*, 2006). If ergosterol levels are low, fungi undergo altered endocytosis, cell fusion and cell shape, with cell membrane ergosterol instability leading to growth inhibition (Heese-Peck *et al.*, 2002; Aguilar *et al.*, 2010; Jordá and Puig, 2020). Ergosterol biosynthesis involves 30 enzymes and begins with acetyl CoA which is converted to squalene which subsequently undergoes multiple enzymatic conversions to produce ergosterol (Kuck and Vors, 2007). Ergosterol biosynthesis takes place in three modules, with the

final stage taking place in the endoplasmic reticulum membrane where ergosterol is transported to the fungal plasma membrane upon synthesis completion (Hu *et al.*, 2017). Ergosterol biosynthesis requires oxygen and iron. For many enzymatic steps, oxygen acts as an electron acceptor and heme, a co-factor whose biosynthesis requires oxygen and iron – is required for enzyme functionality for many enzymes in the ergosterol biosynthesis pathway (Jordá and Puig, 2020). Considering the importance of ergosterol for fungal growth and proliferation, ergosterol biosynthesis inhibitor (EBI) fungicides are utilized in agriculture to prevent and treat many crop diseases.

4.1.2 Triazole Fungicides

A chemical class of EBI fungicides called azoles are vital for controlling destructive fungal diseases in multiple food crops across the globe (Jess *et al.*, 2014; DAFM, 2018a, 2018b). Azoles are synthetic heterocyclic compounds with a pentagonal ring typically containing nitrogen, oxygen, and sulfur atoms. Azoles consist of the triazole and imidazole pesticide groups, with triazoles possessing an extra nitrogen atom in their heterocyclic rings (Martínez-Matías and Rodríguez-Medina, 2018). They are systemic compounds, travelling through plant vascular tissues to reach every part of the treated plant. Azoles competitively inhibit the enzyme lanosterol 14α-demethylase (CYP51), hence they are referred to as demethylation inhibitor (DMI) EBIs, CYP51 is a cytochrome P450 enzyme involved in catalysing the conversion of lanosterol to zymosterol after demethylation, reduction and desaturation reactions catalysed by CYP51, a C-14 reductase and a C-4 demethylation complex (Jordá and Puig, 2020). Zymosterol is an ergosterol precursor and subsequently is converted to fecosterol, which is converted to episterol, which is finally desaturated and reduced to ergosterol (Liu *et al.*, 2019). Azoles inhibit ergosterol biosynthesis by azole ring binding to the CYP51 iron atom within the heme group of the enzyme. Since the CYP51 binding site is occupied, it will not bind to lanosterol nor catalyse the conversion of lanosterol to ergosterol (Yoshida and Aoyama, 1987). As a result, ergosterol biosynthesis is drastically reduced, leading to growth inhibition in fungi. However, CYP51 enzymes are not exclusive to fungi and are found in most animals. As a result, azole fungicides can be hazardous for mammals, resulting in endocrine disruption, and display some antibacterial activity in addition to antifungal activity (Jackson *et al.*, 2000; Warrilow *et al.*, 2013).

4.1.3 Prothioconazole, An Important Fungicide for Irish Agriculture

DMI fungicides have approximately a 16% share in the global fungicide market, with the largest volume of azole fungicides sold in Europe (Jørgensen and Heick, 2021). Prothioconazole, a triazole fungicide, is one of the most used fungicides in Ireland (López-Ballesteros *et al.*, 2022). Prothioconazole was introduced to the market in 2004 as part of the pesticide product Proline®, produced by Bayer – a relatively new triazole fungicide, considering the first triazole was brought to market in 1976 (Kuck and Vors, 2007). Prothioconazole has a 1,2,4-triazole-3-thione toxophore moiety and a broad-spectrum range, covering a range of cereal, barley, and pulse crop fungal pathogens (Figure 4-1). In addition, it is effective against all economically destructive pathogens in oilseed rape (Kuck and Vors, 2007). As a result, prothioconazole is used in Ireland for the protection of crops from *Septoria tritici* blotch – a destructive wheat disease caused by *Zymoseptoria tritici*, *Microdochium* spp. and *Fusarium* spp. and is utilized in barley, wheat, oats, winter oilseed rape, vegetable and grassland and fodder crops, often applied multiple times over the season (Jess *et al.*, 2014; López-Ballesteros *et al.*, 2022). In 2015, prothioconazole was one of the most extensively used pesticides applied to carrots, cauliflower, and parsnips in Irish agriculture (DAFM, 2015). As a result of its intensive use in Ireland, bees may be exposed to prothioconazole when foraging for pollen and nectar.

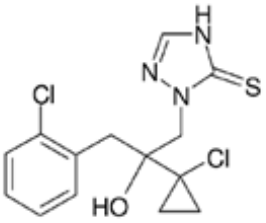
Structure/common name	Commercial data	Physicochemical data
 prothioconazole	Launched by Bayer CropScience in 2004 Patent no. DE19528046 (1994) Example trade name: Proline® Marketed by (for example) Bayer CropScience	139.1–144.5 °C Water solubility: 5 mg L^{-1} (pH 4, 20 °C); 300 mg L^{-1} (pH 8, 20 °C) Log P_{OW} = 3.82 (pH 7) Vapor pressure: $\ll 4 \times 10^{-4}$ mPa (20 °C)

Figure 4-1 Prothioconazole structure, commercial information, and physicochemical data. Prothioconazole is a broad-spectrum, systemic, triazole fungicide and is one of the most widely used fungicides in Ireland for protecting economically valuable crops against fungal pathogens (Figure from Kuck and Vors (2007)).

4.1.4 The Impact of Prothioconazole on Bees

Considering triazoles are designed as antifungal agents, they are not expected to have negative impacts on insect pollinators. EFSA suggests bees may be exposed to prothioconazole at foliar application when foraging on treated flowering plants with no exposure expected from seed application, despite the systemic nature of prothioconazole. The hazard quotients calculated from EU regulatory risk assessments for prothioconazole risk to bees was between 1.0 and 4.1, with a value of 50 or more indicating risk (EFSA, 2007). In a renewal assessment report in 2018, which includes the risk assessment of some prothioconazole formulations for impacts on bees, hazard quotients were higher for formulations in some assessments but still much below 50. All tests conducted indicated low toxicity to bees. However, regulatory risk assessments were only carried out to the full extent on *A. mellifera*, with acute contact toxicity to *B. terrestris* as the only other test conducted on a non-*Apis* bee species (EFSA, 2007; The European Commission, 2018). In addition, the prothioconazole formulation Proline® was not tested in regulatory assessments, despite it being the first prothioconazole product released to market (Kuck and Vors, 2007). Due to varying pesticide sensitivities across different bee species, and the possibility of sublethal impacts in wild bees not observed or tested for in *Apis* species (Arena and Sgolastra, 2014), it is important to gain an understanding of how prothioconazole may impact wild bees as well as honeybees.

Prothioconazole is widely used across multiple European countries. For example, it is applied to 20% of Germanys cultivated area which is equivalent to almost 10% of the total land area of country. It is also applied to 7% and 5% of the cultivated area of Estonia and Ireland, respectively, making it one of the top fungicides in these countries (López-Ballesteros *et al.*, 2022). Despite this, there is not much research on prothioconazole exposure risk or impacts on bees. Presumably due to its relatively recent arrival on the market compared to other fungicides widely used. From the few residue studies that investigate the presence of prothioconazole in pollen and nectar, it seems bees are at risk of exposure. A study investigating pesticide residues in honeybee pollen across Poland found prothioconazole present in 5% of samples, with prothioconazole displaying the highest concentration compared to other pesticides, detected at concentrations up to 356 µg/kg (Roszko *et al.*, 2016). However, pollen samples were collected from commercial beekeepers. Residue analysis from wild bees and bees foraging near farmland would be required to determine the exposure risk of prothioconazole from floral resources where it is applied most frequently. One study conducted on honeybee pollen from three agricultural sites in South Germany tested for the main prothioconazole metabolite, prothioconazole-desthio, which was found in 100 out of 281 analysed samples at a maximum concentration of 78.6µg/kg (Böhme *et al.*, 2018).

Along with a lack of prothioconazole residue data, there is a major lack of research investigating the impacts of prothioconazole on bees. In a systematic review conducted by Cullen et al. (2019) (Manuscript S1-1) which reported on peer-reviewed literature investigating the impacts of herbicides and fungicides on bees published as of November 2018, no research was conducted on the impacts of prothioconazole on bees. However, some research has investigated prothioconazole since, although research mainly focuses on prothioconazole synergistic action with other pesticides. Prothioconazole has been found to have relatively low synergistic effects on the insecticide flupyradifurone in acute contact toxicity assays and had only a weak inhibition of P450 enzyme activity in honeybees compared to other azole fungicides (Haas *et al.*, 2021). One study suggests prothioconazole may have an antagonistic impact with the pyrethroid insecticide alpha-cypermethrin after acute oral and contact exposure in honeybees (Taenzler *et al.*, 2022). Prothioconazole does not seem to enhance the toxicity of neonicotinoid insecticides in honeybees (Wood et al., 2019;

Haas and Nauen, 2021). In addition, prothioconazole is toxic to nectar yeasts, which may alter nectar chemistry, pollinator behaviour or the digestive tract microbiota of pollinators (Bartlewicz *et al.*, 2016). The impacts of other triazole fungicides on bees include reduced food consumption (Elston *et al.,* 2013), synergistic toxicity with neonicotinoid and pyrethroid insecticides (Pilling and Jepson, 1993; Sgolastra *et al.,* 2017; Robinson *et al.,* 2017; Han *et al.,* 2019), decreased weight and increased mortality (Han et al., 2019).

Considering the impact of pesticides on bee decline and the intensive use of prothioconazole, the scarcity of research on the impacts of prothioconazole exposure on bees is startling. As such, researchers and regulatory bodies are ill-equipped for reliably determining if prothioconazole exposure has negative effects on bees, particularly for non-*Apis* species. Mortality, behavioural and consumption studies can determine if there are short-term risks associated with prothioconazole exposure. To determine if prothioconazole alters digestive tract functioning, the application of LFQ-MS-based proteomics can characterise the proteome and associated biochemical changes within the digestive tract cells of bees – a guaranteed point of contact after pesticide ingestion. If there are alterations to the proteome in prothioconazole-exposed bees, characterising the impact at a cellular level will elucidate a toxicological profile for prothioconazole for the bee digestive tract, which is unknown. Considering the antimicrobial activity of prothioconazole, using amplicon DNA metagenomic sequencing can elucidate if prothioconazole impacts the digestive tract microbiota of bees. By investigating the survival, mortality, consumption, digestive tract proteome and microbiota profiles of both bees exposed to the prothioconazole active ingredient and a prothioconazole-based formulation, we can gain an awareness of the impacts of prothioconazole itself, and co-formulants present in a popular formulation. As a result, an overall understanding of the impacts of prothioconazole on *B. terrestris* at a whole organism and organ level for the digestive tract will be established, along with the knowledge of whether pesticide formulations require more in-depth analysis in future pesticide studies.

4.1.5 Chapter Aims

1. Determine the organism-level impacts of prothioconazole AI and a prothioconazole-based formulation, Proline®, on *B. terrestris* by investigating mortality, behavioural alteration, and sucrose consumption using bioassays.
2. Establish if prothioconazole AI and Proline® impacts the *B. terrestris* digestive tract proteome and if so, to characterise the digestive tract proteome of prothioconazole-exposed *B. terrestris*.
3. Characterise the digestive tract microbiota after prothioconazole active ingredient and Proline® exposure using 16S and ITS DNA amplicon sequencing.
4. Verify if there are differences in the digestive tract proteome and microbiota profiles between prothioconazole and Proline® in comparison to their respective controls.
5. Determine if the commonly used organic solvent in pesticide risk assessments, acetone, can impact the digestive tract proteome or microbiota at a low concentration.

4.2 Experimental outline

4.2.1 Prothioconazole Survival, Behaviour and Consumption Bioassays, and Analysis

Prothioconazole solutions were prepared as outlined in section 2.3.2 using technical grade prothioconazole and a prothioconazole commercial formulation Proline®. The following treatments were prepared for the survival, behavioural and consumption bioassays: control, acetone control, 0.3 ppm prothioconazole active ingredient (PAI), 0.3 ppm prothioconazole commercial formulation Proline® (PCF), 3 ppm PAI, 3ppm PCF, 30 ppm PAI or 30 ppm PCF. Acetone controls were the same as the procedural control of 40% (w/v) sucrose solution but contained 0.3% acetone. Acetone controls were included in exposure assays as the final treatment of PAI contained 0.3% acetone for PAI solubilisation. OECD no. 245 guidelines state that up to 5% acetone is acceptable for chronic oral pesticide exposure assays (OECD, 2017a). Concentrations of Proline® were calculated based on prothioconazole active ingredient content (containing 250g/L prothioconazole) present in the formulation so both the technical grade prothioconazole and Proline® treatments contained the same concentration of prothioconazole. Survival bioassays were conducted in triplicate with each isolation chamber and relevant treatment defined as one technical replicate as outlined in section 2.3.3.

Eight group isolation chamber boxes (11.5 cm x 8 cm x 17.5 cm) were reserved for bees, and six reserved for evaporation controls. There were n=10 bees per group isolation chamber, which consisted of an even number of bees from each of the five origin *B. terrestris audax* bumblebee colonies (Biobest, Agralan Ltd). This resulted in n=30 bees per treatment when results from all replicates were combined. Bees were acclimatised with *ad libitum* access to 40% (w/v) sucrose solution in darkness overnight at 23.3 ± 1°C and 71 ± 12% relative humidity which was maintained for the duration of the bioassay. The six evaporation control boxes were also filled with 40% (w/v) sucrose solution for overnight acclimatisation. The following day, feeders were weighed to determine sucrose solution consumption before replacement. Feeders were replaced with aliquots of one of the group isolation chambers assigned treatment. All feeders were weighed before introduction to their relevant group and three extra control and acetone control containing feeders were introduced to the six empty

evaporation control chambers. Each day, 24±2 hours from the initial exposure time, bees were observed for mortality, behavioural alterations, and consumption (see section 2.3.3 and 2.3.4). After ten days, all bees were anaesthetised and placed at 4°C. Statistical analyses were performed as outlined in section 2.3.3 and 2.3.4 using Minitab® 20.3. A non-parametric Mann Whitney test was used to determine statistically significant differences in consumption between different treatments as consumption data did not follow the normal distribution with $\log_{10}$, square root and inverse transformations unsuccessful in data normalisation.

4.2.2 Prothioconazole Exposure for Digestive Tract Proteome and Microbiota Characterisation

Prothioconazole exposure for digestive tract proteome and microbiota characterisation were conducted separately to survival bioassays as outlined in section 2.3.3. However, consumption was only calculated during the proteomic exposure assays and a two-sample t-test was utilized to determine statistically significant differences in consumption from proteomic exposure assays as data followed a normal distribution. There were n=10 bees per group isolation chamber, which consisted of an even number of bees from each of the five origin *B. terrestris audax* bumblebee colonies. For both exposure assays, bees were exposed for 5 days, and treatments consisted of 0.3 ppm PAI, 0.3 ppm PCF, acetone control or a non-acetone control. 0.3 ppm was chosen as the field-realistic treatment concentration for PAI and PCF for digestive tract proteome and microbiota characterisation as prothioconazole has been found in pollen samples up to 356 µg/kg (0.356 ppm) (Roszko *et al.*, 2016). No residues have been characterised in nectar to date (Rondeau and Raine, 2022). Chronic exposures were conducted as prothioconazole is applied to treatment crops multiple times throughout the growing season, meaning exposed bees are more likely to be chronically exposed to prothioconazole. In exposures before digestive tract proteome characterisation, bees were maintained in the dark at 27.1 ± 1.6°C and 64.5 ± 6% relative humidity for the duration of the exposure. For exposures before microbiota characterisation, bees were maintained in the dark at 24 ± 1°C and 72 ± 3% relative humidity. Every 24 ± 2 hours, bee were observed, and endpoints recorded similar to section 4.2.1. After five days of exposure, for each experiment, the digestive tracts of bees were removed for sample preparation.

4.2.3 Digestive Tract Sample Processing and Analysis

Digestive tracts (n=5) were dissected, homogenised, processed, and run on the Q-Exactive mass spectrometer as described in section 2.4 with four digestive tracts per treatment analysed using LFQ mass spectrometry. All data analysis of results obtained from the LFQ mass spectrometer followed the protocol described in section 2.5 using Perseus v. 1.6.15.0. Behaviour and consumption data collected from the pre-dissection exposure assay was analysed as described in section 2.3.3.

4.2.4 Digestive Tract Microbiota Sample Processing and Analysis

Digestive tracts were removed from n=6 bees per treatment. Behaviour and consumption were analysed as described in section 2.3.3 and 2.3.4. DNA extraction, processing, sequencing, and analysis were carried out as described in section 2.6.

4.3 Results

4.3.1 Survival Assays

Mortality was recorded for bees exposed to 0.3ppm, 3ppm and 30ppm PAI, PCF, acetone control or control treatments, respectively, for 10 days (Table S4-1). No significant differences in survival were found between any treatments (Log-Rank p-value = 0.177; Wilcoxon p-value = 0.309) over 10 days (Figure 4-2; Table S4-2).

4.3.2 Behavioural Alterations

For all exposure bioassays, a general linear model was conducted on the responses: moribund, affected, and apathy with treatment as the single factor and temperature, humidity, the number of bees and day and date as co-variates. In behavioural alterations recorded from ten-day survival assays, day and treatment had a significant impact on the rate of moribund bees ($p = 0.014$ and $p = 0.016$, respectively). The only treatment with a statistically significant impact on the rate of moribund bees was 0.3ppm PCF ($p < 0.001$) with a co-efficient value of + 0.1326. 0.3ppm PAI had a significant impact on the number of bees affected during survival assays ($p = 0.004$, coefficient = + 0.1025) (Table S4-3). Due to slightly higher number of moribund bees observed in the third replicate of survival exposure assays from 0.3 ppm PCF exposure, general linear models were carried out on the first two replicates only. This resolved still a significant impact of 0.3 ppm PCF on the number of moribund bees ($p < 0.001$) and 0.3 ppm PAI on the number of affected bees ($p = 0.013$) (Table S4-3). Whilst the number of bees present had a statistically significant impact on bees observed as affected ($p = 0.014$) during the five-day exposure period for the proteomic experiment, there were no statistically significant impacts of any treatment during the proteomic or microbiota five-day exposure (Table S4-3).

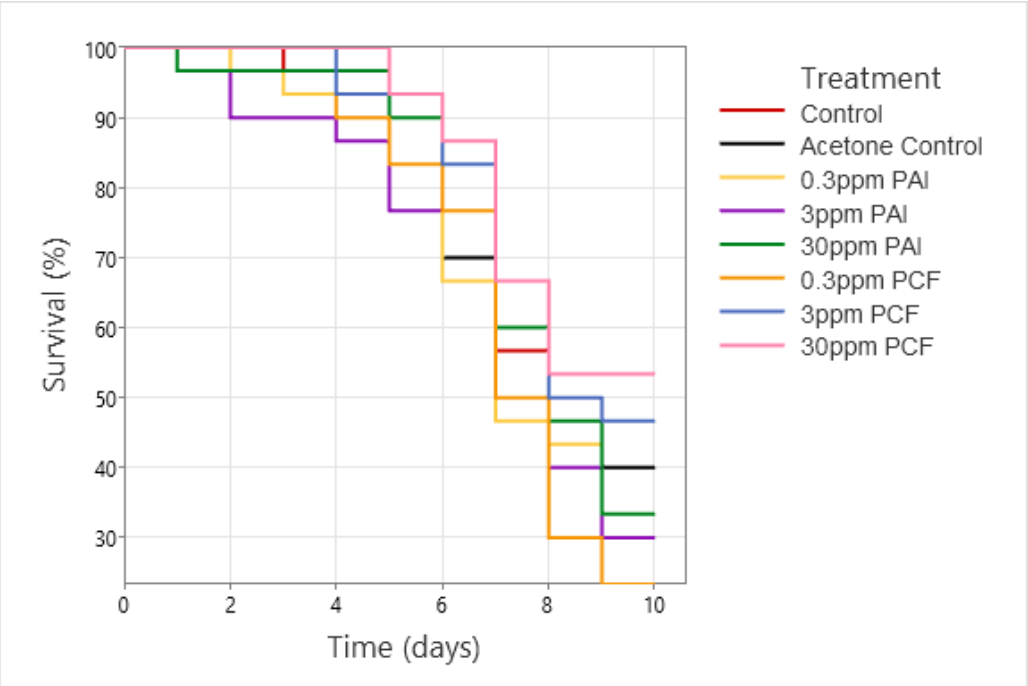


Figure 4-2 Kaplan-Meier Survival Curve. Log-Rank and Wilcoxon tests determined no statistically significant differences in survival between treatments and control bees after a 10-day exposure.

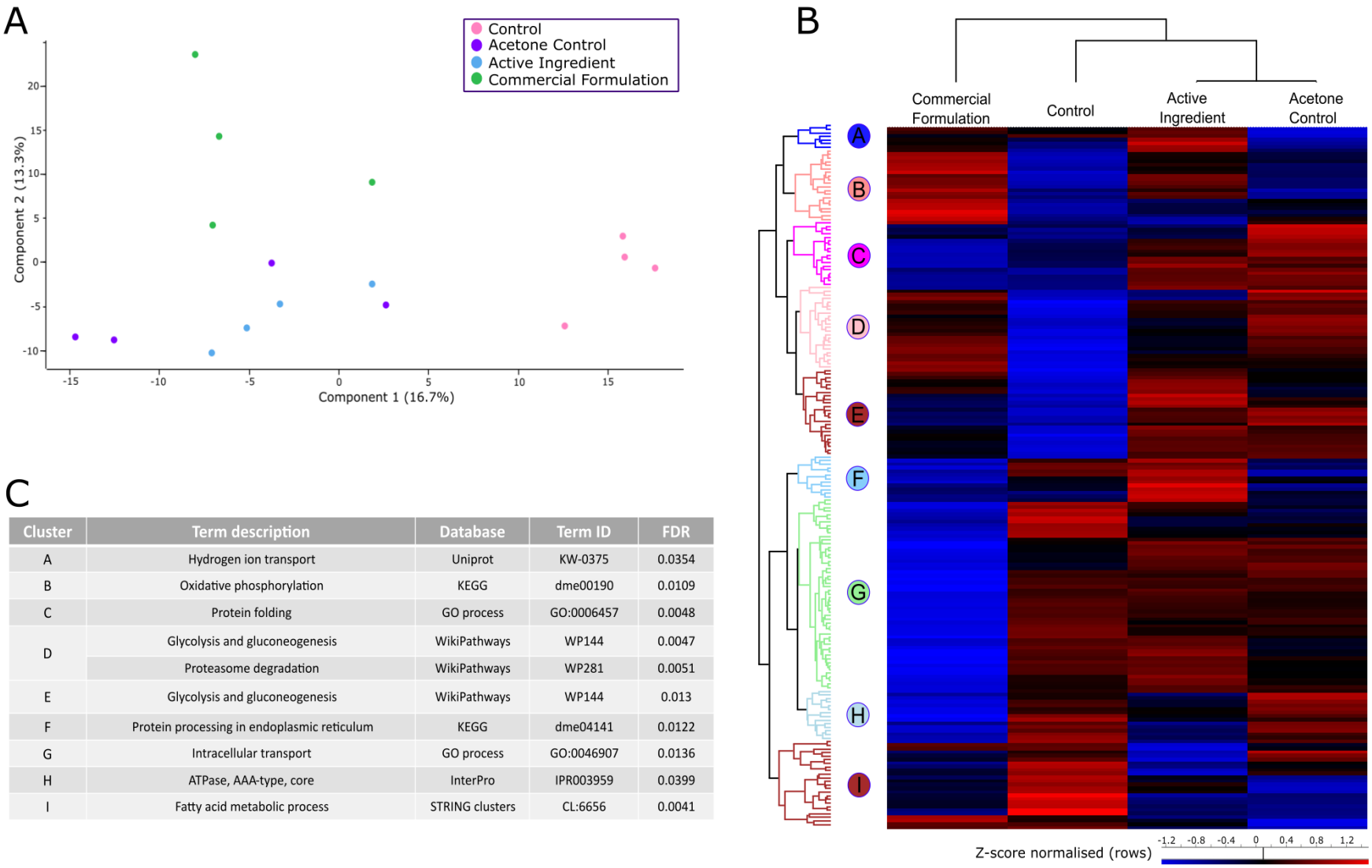
4.3.3 Prothioconazole Does Not Alter Sucrose Solution Consumption

From survival exposure assays, there were no statistically significant differences in the consumption of sucrose solution between any treatments. In the five-day proteomic exposure assay, consumption was statistically significantly different between the acetone and non-acetone controls ($p = 0.039$). Since this result was not observed in survival assays which were carried out in triplicate over ten days, statistical analysis was carried out to determine if there were significant differences in consumption between the control and acetone control treatment groups from these assays when taking only the first five days into account. However, there were no statistically significant differences in consumption over the first five days of exposure between the control and acetone control treatment groups ($p = 0.377$) (Table S4-4; Table S4-5).

4.3.4 LFQ analysis of prothioconazole exposure on the *B. terrestris* digestive tract

4.3.4.1 Identified and Quantified Proteins

2,599 proteins were identified from the *B. terrestris* digestive tract with 1,384 proteins remaining after post-imputation and $\log_2$ transformation (Table S4-6). PCA on all proteins determined variance of 16.7% in component one and 13.3% in component two (Figure 4-3A). Whilst the control treatment groups clustered together away from other treatment groups, and both the PAI and PCF-treatment groups cluster separately from each other, two samples from the acetone control-treatment group drift towards and mix with the two prothioconazole-treatment groups, determining variation between the control and the acetone control treated digestive tract proteome.



C

Cluster	Term description	Database	Term ID	FDR
A	Hydrogen ion transport	Uniprot	KW-0375	0.0354
B	Oxidative phosphorylation	KEGG	dme00190	0.0109
C	Protein folding	GO process	GO:0006457	0.0048
D	Glycolysis and gluconeogenesis	WikiPathways	WP144	0.0047
	Proteasome degradation	WikiPathways	WP281	0.0051
E	Glycolysis and gluconeogenesis	WikiPathways	WP144	0.013
F	Protein processing in endoplasmic reticulum	KEGG	dme04141	0.0122
G	Intracellular transport	GO process	GO:0046907	0.0136
H	ATPase, AAA-type, core	InterPro	IPR003959	0.0399
I	Fatty acid metabolic process	STRING clusters	CL:6656	0.0041

Figure 4-3 Differences can be observed in the digestive tract proteome between treatment groups. (A) A PCA demonstrates a distinct clustering of digestive tracts exposed to the control compared to other treatments. **(B)** Hierarchical clustering of z-score normalised values cluster the median protein expression values of SSDA proteins with a similar expression pattern in each treatment and **(C)** Enriched terms associated with each cluster were resolved using the STRING database to determine processes and pathways comparatively altered in each treatment group.

4.3.4.2 Two-Sample T-tests

Two sample t-tests were utilized for pairwise comparisons of treatment groups to determine SSDA proteins ($p \leq 0.05$, $S0 = 0.1$) and their relative fold differences in protein abundance (Table S4-7). STRING analysis was conducted on SSDA lists to identify pathways, processes, GO and KEGG terms and protein networks that were enriched in specific treatment groups. In addition, KEGG and BRITE analysis was carried out to determine SSDA protein functionalities and enriched processes and pathways.

4.3.4.2.1 Prothioconazole Active Ingredient vs Acetone Control

A total of 84 proteins were SSDA between the digestive tracts of bees exposed to PAI and its relevant acetone control (RFC range: + 8.24 to – 18.81), with 45 and 39 proteins having an increased and decreased abundance in PAI compared to the acetone control, respectively (Figure 4-4). The top 10 proteins with the highest increased abundance included: the extracellular matrix constituent; collagen alpha-5(IV) chain (RFC: + 8.24), uncharacterized protein LOC100643205 (RFC: + 6.88), dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit STT3A (RFC: + 5.25), translocon-associated protein (+ 5.17), the nitrobindin family member; THAP domain-containing protein 4 (RFC: + 3.87), the guanylate kinase; disks large 1 tumour suppressor protein (RFC: + 3.59), short/branched chain specific acyl-CoA dehydrogenase (RFC: + 3.17), the hydrolase; glucosylceramidase (+ 2.90), uncharacterized protein LOC100651605 (RFC: + 2.81) and putative ATP-dependent RNA helicase me31b (RFC: + 2.73). The top 10 proteins with decreased abundance included uncharacterized protein LOC100647671 (RFC: - 18.81), uncharacterized protein LOC100645163 (RFC: - 7.80), sugar transporter SWEET1 (RFC: - 6.80), the rab-associated protein; golgin subfamily A member 2 (RFC: - 5.36), C-Myc-binding protein (RFC: - 4.66), the aspartate carbamoyltransferase; CAD protein (RFC: - 3.19), uncharacterized protein DDB_G0272530 (RFC: - 3.13), and tyrosine-protein kinase Src42A (RFC: - 2.73) in addition to the membrane trafficking proteins; NECAP-like protein CG9132 (RFC: - 2.06), serine/threonine-protein kinase 25 (RFC: - 2.03).

4.3.4.2.2 Proline® vs Control

177 proteins were SSDA in the comparison of digestive tracts from bees exposed to PCF and its relevant control (RFC range: + 34.03 to – 40.60). 73 proteins had an increased abundance whilst 104 proteins had a decreased abundance in the digestive tracts of PCF compared to control-treated bees (Figure 4-5). The top 10 proteins with the most increased abundance included: uncharacterized protein LOC100647671 (RFC: + 34.03), the detoxification-associated protein: microsomal glutathione S-transferase 1 (RFC: + 9.79), multiple inositol polyphosphate phosphatase 1 (RFC: + 4.99), integrin beta-nu (RFC: + 4.80), the SNARE-associated protein; syntaxin-1A (RFC: + 3.79), an uncharacterized C97 family member peptidase: uncharacterized protein LOC100649546 (RFC: + 3.53), mitochondrial intermembrane space import and assembly protein 40-B (RFC: + 3.39), BAG domain-containing protein Samui (RFC: + 3.30), ethanolamine-phosphate cytidylyltransferase (RFC: + 2.93), and cAMP-dependent protein kinase type I regulatory subunit (RFC: + 2.77). The top 10 proteins with the most decreased abundance included renin receptor (RFC: - 18.82), 23 kDa integral membrane protein (RFC: - 15.79), aldehyde dehydrogenase, dimeric NADP-preferring protein (RFC: - 9.15), flexible cuticle protein 12-like protein (RFC: - 8.87), pancreatic lipase-related protein 2 (RFC: - 7.74), hemK methyltransferase family member 1 (RFC: - 7.11), putative adenosylhomocysteinase 2 (RFC: - 6.03), and uncharacterized protein LOC100644131 (RFC: - 5.56) in addition to the serine peptidases: transmembrane protease serine 9 (RFC: - 40.60), venom dipeptidyl peptidase 4 (RFC: - 7.33).

4.3.4.2.3 Acetone Control vs Control

There were 134 SSDA proteins identified when comparing the digestive tract proteomes of acetone control to control-treated bees (RFC range: + 55.33 to - 501.25). Of these, 91 proteins had an increased abundance, and 43 proteins had a decreased abundance in the digestive tracts of acetone control compared to the control-treated bees (Figure 4-6). The top 10 proteins with an increased abundance included: V-type proton ATPase 16 kDa proteolipid subunit (RFC: + 55.33), uncharacterized protein LOC100647671 (RFC: + 38.40), ethanolamine-phosphate cytidylyltransferase (RFC: + 4.38), eukaryotic translation initiation factor 1A (RFC: + 3.53), centrosomal protein of 290 kDa (RFC: + 3.40), mitochondrial intermembrane space import and assembly

protein 40-B (RFC: + 3.38), the membrane trafficking-associated protein: surfeit locus protein 4 (RFC: + 3.22), BAG domain-containing protein Samui (RFC: + 3.20) and the heat shock proteins: protein lethal(2)essential for life-like protein (RFC: + 5.53) and activator of 90 kDa heat shock protein ATPase (RFC: + 3.76). The top 10 proteins with a decreased abundance were: the immune protein: melittin (RFC: - 501.25), transmembrane protease serine 9 (RFC: - 115.06), phospholipase A2 (RFC: - 62.17), venom dipeptidyl peptidase 4 (RFC: - 11.07), short/branched chain specific acyl-CoA dehydrogenase, mitochondrial (RFC: - 8.05), protein FAM162B (RFC: - 7.86), uncharacterized protein LOC100647597 (RFC: - 7.20), uncharacterized protein LOC100644131 (RFC: - 5.93), the small ribosomal subunit protein: 28S ribosomal protein S29 (RFC: - 4.50), and uncharacterized protein LOC100643205 (RFC: - 4.21).

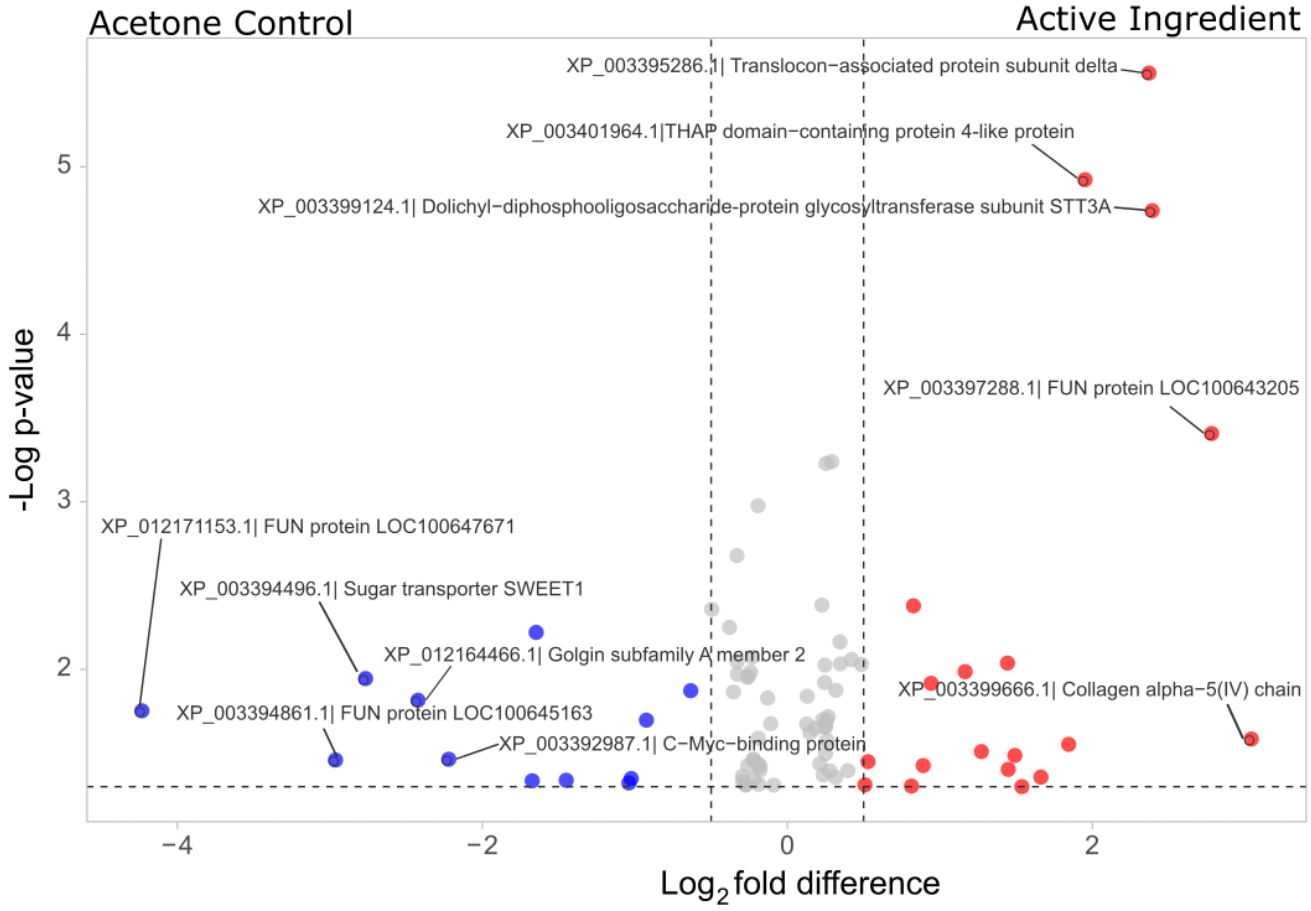


Figure 4-4 A two-sample t-test was performed to resolve and compare SSDA proteins in the digestive tract of *B. terrestris* after exposure to prothioconazole active ingredient (PAI) or its relative acetone control. SSDA proteins ($p \leq 0.05$) and the top five proteins with the most increased (right) and most decreased (left) abundance in PAI in comparison to the acetone control are highlighted. Functionally uncharacterized (FUN) protein LOC100647671 was the most decreased and collagen alpha-5(IV) chain was the most increased SSDA protein after PAI exposure in comparison to the acetone control.

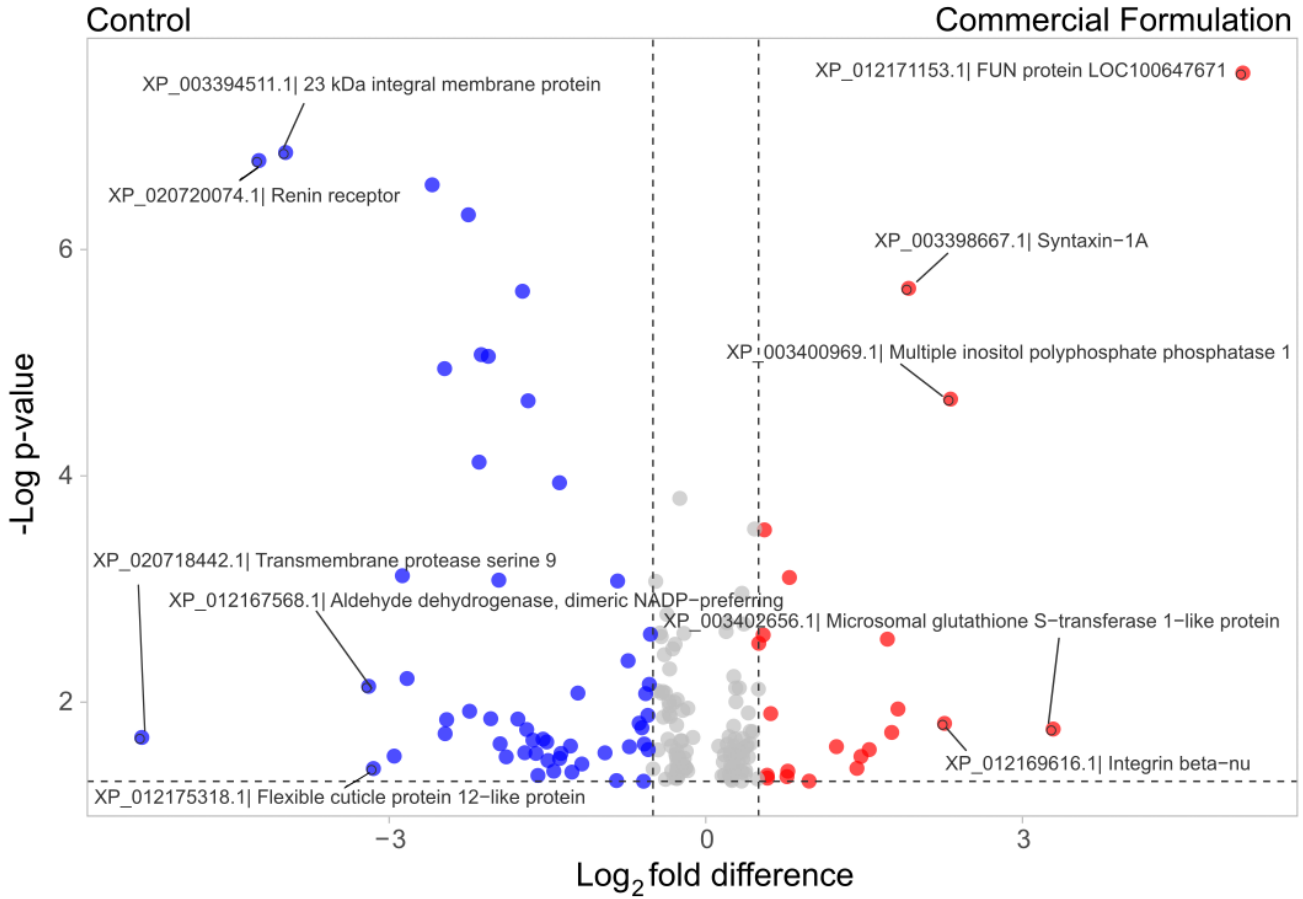


Figure 4-5 A two-sample t-test was performed to resolve and compare SSDA proteins in the digestive tract of *B. terrestris* after exposure to prothioconazole commercial formulation (PCF) or the control. SSDA proteins ($p \leq 0.05$) and the top five proteins with the most increased (right) and most decreased (left) abundance in PCF in comparison to the control treated group are highlighted. Transmembrane protease serine 9 was the most decreased and FUN protein LOC100647671 was the most increased SSDA protein after PCF exposure in comparison to the control.

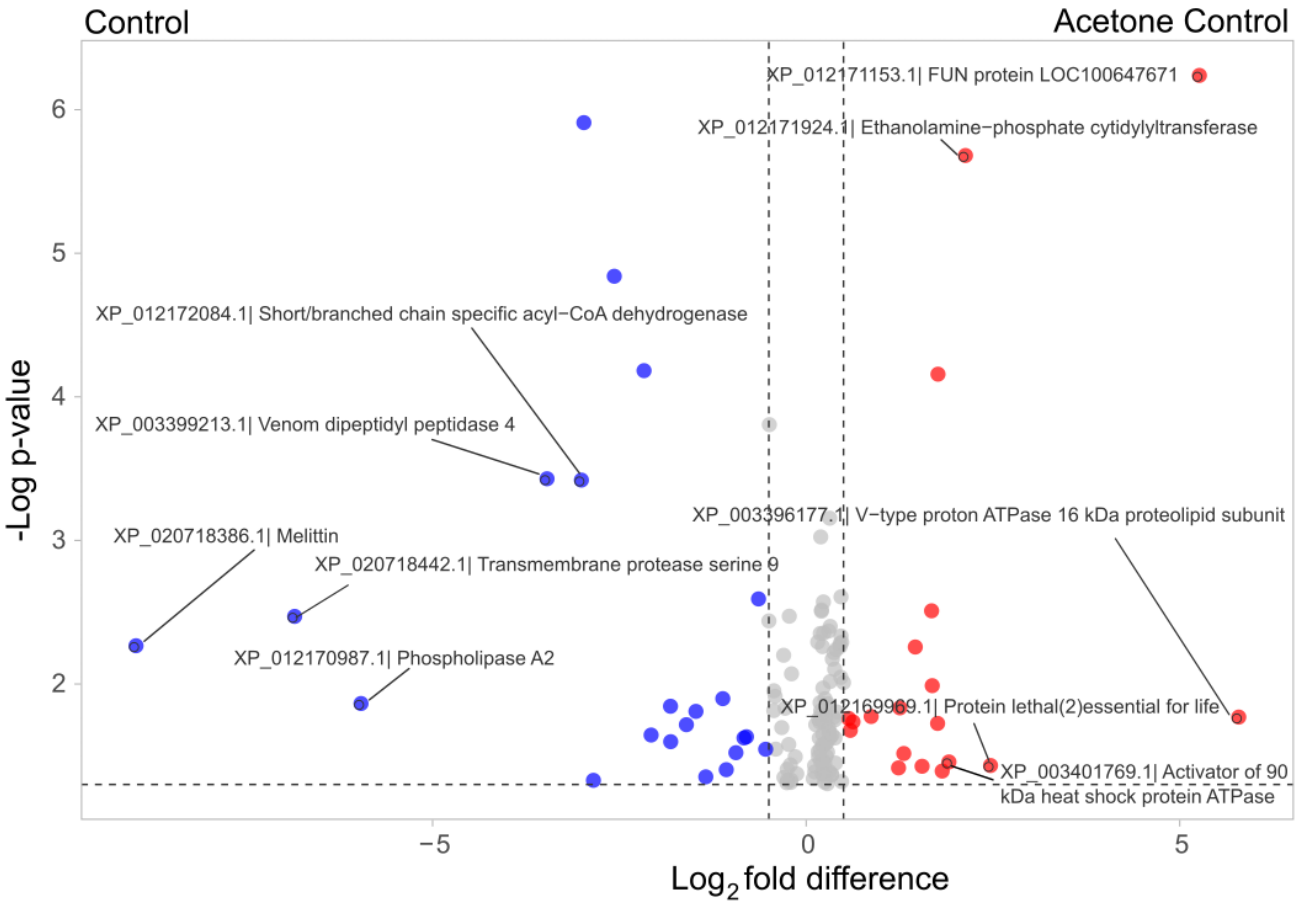


Figure 4-6 A two-sample t-test was performed to resolve and compare SSDA proteins in the digestive tract of *B. terrestris* after exposure to acetone control or the control. SSDA proteins ($p \leq 0.05$) and the top five proteins with the most increased (right) and most decreased (left) abundance in the acetone control in comparison to the control treated group are highlighted. Melittin was the most decreased and V-type proton ATPase 16kDa proteolipid subunit was the most increased SSDA protein after acetone control exposure in comparison to the control.

4.3.4.3 Gene Ontology and Pathway Analysis of SSDA Proteins

Enriched protein-protein interaction (PPI) networks and GO terms present in pairwise comparisons were determined using STRING v. 11 to analyse SSDA proteins. GO terms and protein functions were determined using *D. melanogaster* as a reference genome (Table S4-8). Enriched pathways, processes and protein functionalities were also determined using KEGG and BRITE (Table S4-9). Comparison of PAI to the acetone control resolved an increase in SSDA proteins associated with the basement membrane (three proteins), glycogen metabolism (three proteins), fatty acid metabolism (three proteins) and the electron transport chain (three proteins). In addition, seven proteins were enriched in signalling and cellular processes involving the exosome resolved using BRITE.

Proteins that were decreased in PAI compared to acetone control treatment groups were associated with actin cytoskeleton organization (six proteins) and vesicle coat (three proteins) (Figure S4-1). In BRITE enrichment, proteins were associated with membrane trafficking (nine proteins), the exosome (ten proteins) and the cytoskeleton (five proteins).

When comparing SSDA proteins in the digestive tract of PCF to control-treated bees, there was an increase in proteins associated with translation (eight proteins), cytoskeleton organization (eleven proteins), glutathione peroxidase activity (three proteins), and glycolysis and gluconeogenesis (four proteins). BRITE terms enriched included the ribosome (seven proteins), membrane trafficking (twelve proteins), the cytoskeleton (six proteins), and the exosome (sixteen proteins).

Proteins decreased in PCF in comparison to control treatment groups included proteins associated with translation (eleven proteins), endocytosis (seven proteins), the mitochondrial matrix (seven proteins), and proteasome degradation (four proteins) (Figure S4-2). BRITE enrichment included chaperones and folding catalysts (five proteins), membrane trafficking (fourteen proteins) and the exosome (eleven proteins).

When comparing the acetone control to control exposed treatment groups, increased SSDA proteins were associated with protein folding (twelve proteins), translation (eleven proteins), detoxification (five proteins), proteasome (six proteins), cellular response to stress (eighteen proteins) and the immune system (twenty-seven proteins). KEGG pathways enriched included amino acid biosynthesis (five proteins), endocytosis (seven proteins), and protein processing in the endoplasmic reticulum (six proteins). BRITE terms enriched included chaperones and folding catalysts (thirteen proteins), membrane trafficking (19 proteins), cytoskeleton (seven proteins) and the exosome (thirty-one proteins). SSDA proteins decreased in the acetone control compared to the control were associated with the mitochondrion (fifteen proteins), fatty acid metabolism (six proteins), the lysosome (three proteins), and the electron transport chain (three proteins) (Figure S4-3).

4.3.4.4 Hierarchical Clustering and Gene Ontology Enrichments

Hierarchical clustering was performed on mean z-scored normalised LFQ values for 195 statistically significant proteins (ANOVA, Ben-Ho FDR <0.05) which resolved nine clusters denoted A-I (Table S4-10). Each cluster represents a group of proteins with a specified expression profile, with an increased or decreased in abundance relative to other treatment groups (Figure 4-3B). Proteins in each cluster were analysed for enriched GO terms using the STRING v.11 database using the equivalent D. melanogaster reference genome and Uniprot identifiers to resolve enriched (FDR < 0.05) processes and pathways in each cluster (Figure 4-3C) (Table S4-11).

Cluster A contained seven proteins with enrichment for hydrogen ion transport (two proteins) with a decrease in the acetone control treatment group only. Cluster B had 20 proteins, with enrichments for oxidative phosphorylation (two proteins) which were increased in relative abundance in both prothioconazole-based treatment groups. Cluster C consisted of 18 proteins decreased in PCF and the control but increased in PCF and the acetone control. Enriched terms included protein folding (four proteins) and purine ribonucleotide binding (seven proteins). Cluster D resolved a cluster of 23 proteins which had a decreased abundance in the control treatment group only. These were associated with glycolysis and gluconeogenesis (two proteins) and proteasome degradation (two proteins). Cluster E had 24 proteins with an increased abundance in the PAI and acetone control treatment groups and a decreased abundance in the PCF

and control treatment groups. Enriched terms included glycolysis (three proteins) and increased mortality (17 proteins) observed in *Drosophila* species. In cluster F, there were no significant enrichments amongst the 12 proteins with a decreased abundance in PCF and acetone control treatment groups and a mixed abundance profile in the control treatment group, initially. However, using Markov cluster algorithm (MCL), enrichment was resolved for protein processing in the endoplasmic reticulum (two proteins). Cluster G comprised 53 proteins with a decreased abundance in PCF treatment groups only. These proteins were associated with cellular localisation (eleven proteins), intracellular transport (eight proteins), and the Golgi apparatus (six proteins). Cluster H had 14 proteins decreased in PCF and PAI treatment groups. After MCL clustering, two proteins were associated with ATPase, AAA-type, core. Cluster I contained 24 proteins with an increased abundance in the control treatment group only. After MCL clustering, three proteins were associated with fatty acid metabolic process and microbody.

4.3.4.5 Proteins With a Common Abundance Profile After Exposure to Either Prothioconazole-Based Treatment

Whilst SSDA proteins in PAI and PCF treatment groups could not be directly compared due to the presence of acetone in PAI, common proteins observed in PAI and PCF SSDA protein sets in comparison to their respective controls could be resolved. This was carried out to determine if there was a consistent impact of prothioconazole exposure on the digestive tract regardless of its ingestion as the active ingredient or within a commercial formulation. Two proteins were found in common in both the increased and decreased SSDA protein data sets of PAI and PCF in comparison to their relative controls (Table 4-1). The common proteins with an increased abundance in both treatments were cAMP-dependent protein kinase type I regulatory subunit (PAI RFC: + 2.42; PCF RFC: + 2.77), associated with protein phosphorylation regulation, and peptidyl-prolyl cis-trans isomerase-like protein (PAI RFC: + 1.19; PCF RFC: + 1.27), involved in protein folding. The two proteins with a decreased abundance in both PAI and PCF were transcription factor BTF3 homolog 4-like protein (PAI RFC: - 1.23; PCF RFC: - 1.35) and coatomer subunit epsilon (PAI RFC: - 1.23; PCF RFC: - 1.14). Transcription factor BTF3 homolog 4-like protein is associated with proteostasis and regulation of translocation to the mitochondria and

endoplasmic reticulum whilst coatomer subunit epsilon is associated with protein transport and Golgi homeostasis.

Table 4-1 A Conserved Response in *B. terrestris* Digestive Tract to Both Prothioconazole-based Treatments PAI and PCF. Functional categories, relative fold changes, MS measurements and characteristics for common SSDA proteins with similar expression profiles in both GAI and GCF treated bees. Relative fold changes and directions were determined against the relative control.

Functional Annotation	Protein ID	Annotation	RFC PAI	RFC PCF	FC direction	No. of peptides	Mol. wt [kDa]	MS/MS count
Protein phosphorylation regulation	XP_003397429.1	cAMP-dependent protein kinase type I regulatory subunit	2.42	2.77	Up	5	41.68	30
Protein folding	XP_003402266.1	Peptidyl-prolyl cis-trans isomerase-like protein	1.19	1.27	Up	9	22.777	131
Proteostasis	XP_003402732.1	Transcription factor BTF3 homolog 4-like protein	1.23	1.35	Down	9	20.024	158
Protein transport	XP_012169272.1	Coatomer subunit epsilon	1.23	1.14	Down	8	35.966	78

4.3.5 The Effects of Prothioconazole Active Ingredient and Proline® on the Digestive Tract Microbiota

2,292,178 effective tags representing the 16S rRNA V4 gene amplicon region were filtered from 2,377,649 raw tags. For the ITS1-5F gene amplicon region, 1,439,069 effective tags were filtered from 1,802,323 raw tags (Table S4-12).

4.3.5.1 Bacterial and Fungal Community Composition

The relative abundance of the top ten bacterial and fungal genera was analysed to determine the impacts of PAI, PCF or acetone used for solubilisation on the abundance of core microbial species (Table S4-13).

When analysing the relative abundance of the top 10 bacterial genera, the relative abundance of *Snodgrasella* was higher in the control compared to other treatments, and twice as high in the control compared to the acetone control (Figure 4-7). *Gilliamella* species were up to three times higher in relative abundance in the PAI treatment group compared to other treatments and was higher in PCF compared to both control treatment groups. *Lactobacillus* was 2-3 times lower in relative abundance in the PAI treatment group compared to other groups. In addition, species of the genera *Acinetobacter* were much higher in both acetone-containing treatments, the acetone control and PAI, compared to the control and PCF treatment groups. *Smithella* and *Lentimicrobium* species were higher in acetone-containing treatments compared to other treatment groups however this was due to one sample, pcmb.02, containing a higher relative abundance of *Smithella* and *Lentimicrobium* than other acetone control samples, with PAI treatment groups demonstrating a more consistently high relative abundance across samples.

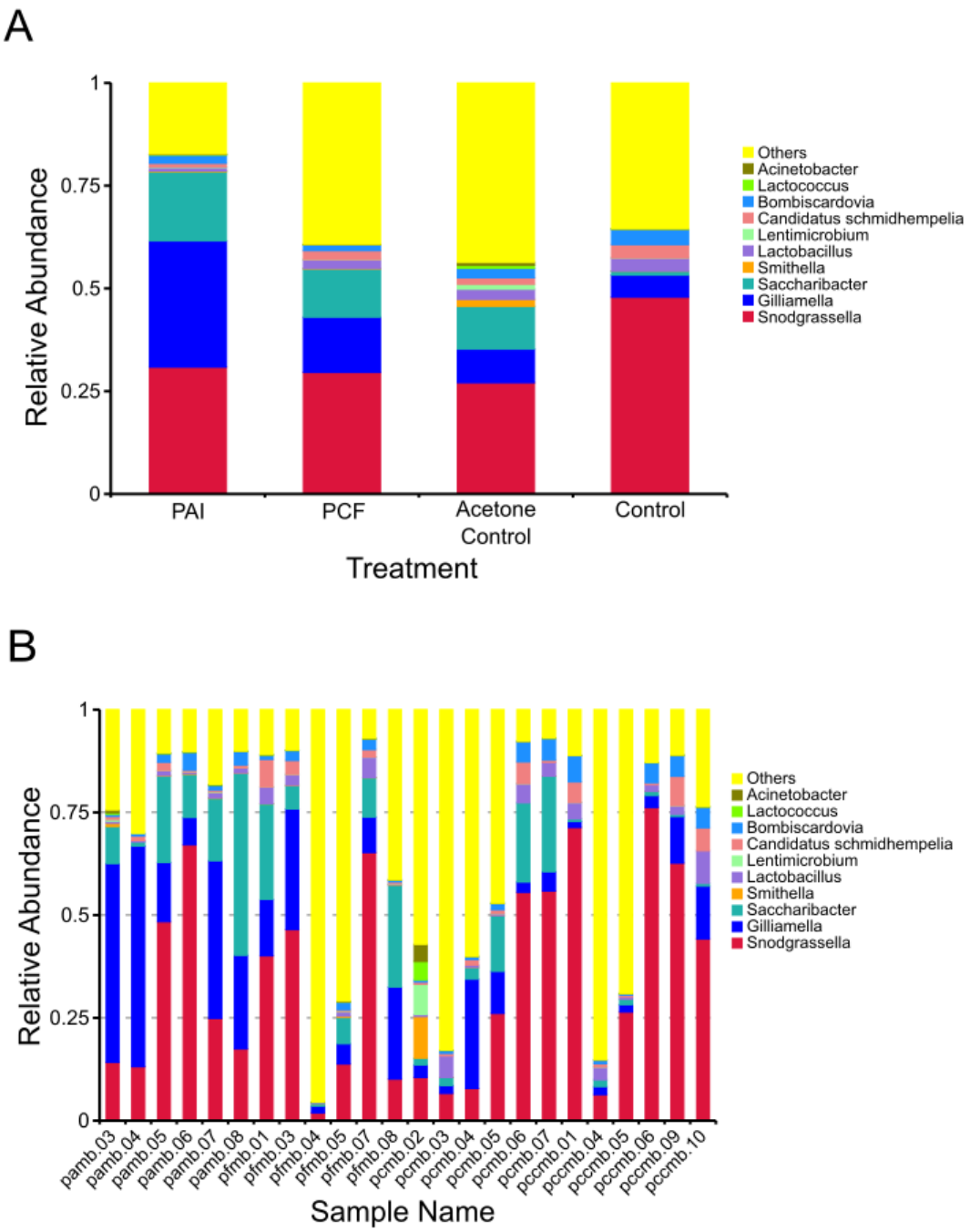


Figure 4-7 The Relative Abundance of the Top Ten Bacterial Genera. (A) the relative abundance of the top 10 bacterial genera in each treatment group and **(B)** in each sample. Digestive tract DNA samples were: pamb: prothioconazole active ingredient, pfmb: prothioconazole commercial formulation, pcmb: acetone control, and pccmb: control.

Analysis of the top 10 fungal genera resolved differences in the relative abundance of various genera across treatment groups and samples (Figure 4-8). *Candida* had the highest abundance in the control treatment group followed by the acetone control treatment group. In comparison, *Candida* species were of low abundance in both prothioconazole treatment groups with a relative *Candida* abundance over 20 times greater in the control compared to PCF and just over 10 times greater in the control compared to PAI (Table S4-13). This was the general trend observed across all samples from different treatment groups. In the acetone control and control treatment groups, *Wickerhamomyces* had a relatively increased abundance in comparison to prothioconazole treatment groups. The PAI treatment group a relatively higher abundance of *Thermoascus* in comparison to other treatment group samples, approximately 60 times higher than the control treatment group, 1.5 times higher than the acetone control treatment group and 30 times higher than the PCF treatment group. In addition, the acetone control had a higher abundance of *Thermoascus* compared to other treatment groups except PAI, approximately 40 times higher than the control and two times higher than the PCF treatment group. *Cryptococcus* was absent in all treatment groups except the control. In addition, *Chaetomium* was absent only in the control treatment group.

Whilst *Zygosaccharomyces* were higher in relative abundance in the PCF treatment group compared to other treatment groups, this may be due to Pfmb.01 acting as an outlier as it has a much higher abundance than other samples from the PCF treatment group which have a relative abundance of *Zygosaccharomyces* comparable to other treatment groups. This was also seen in the PAI treatment group, which displayed a much higher abundance of *Alternaria* and *Penicillium* than other treatment groups, however this was due to a much higher abundance in sample pamb.05, with other samples from the PAI treatment group possessing a much lower abundance of these genera. Alterations to bacterial and fungal genera abundance in individual samples could not be attributed to origin colony (Table S4-14).

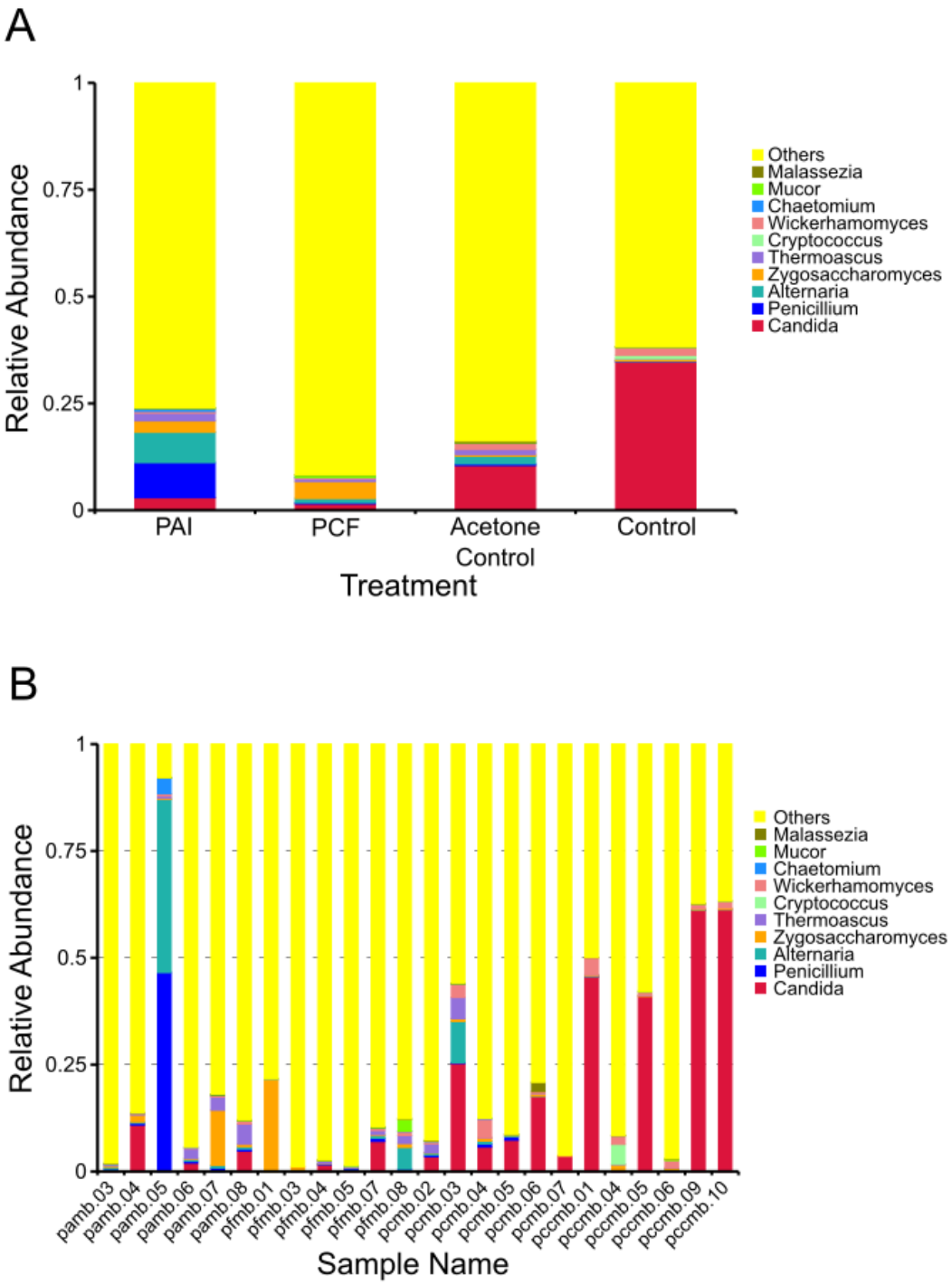


Figure 4-8 The Relative Abundance of the Top Ten Fungal Genera. (A) the relative abundance of the top 10 fungal genera in each treatment group and **(B)** in each sample. Digestive tract DNA samples were: pamb: prothioconazole active ingredient, pfmb: prothioconazole commercial formulation, pcmb: acetone control, and pccmb: control.

Taxonomy trees were created based on the top 10 genera in high relative abundance by default for both bacterial and fungal species to observe the relative abundance of microbial genera between different treatment groups (Figure 4-9; Figure 4-10).

Based on the group taxonomy tree created for bacterial genera, *Snodgrasella* accounted for 33.839% of bacteria, with a similar abundance in all treatment groups. However, individual species could not be identified. *Gilliamella* accounted for 21.83% of *Gammaproteobacteria* and 14.428% of bacteria, with over half of the comparative abundance contributed to the PAI treatment group, the remaining 50% of the abundance was similarly attributed to each of the other treatment groups. *Lentimicrobium saccarophilium* was mainly represented by the acetone control, with some presence in the PAI treatment group. *Lactobacillus* species were present in all samples, with the lowest relative abundance in the PAI treatment group and highest in the control treatment group. *Lactobacillus* species accounted for 2.164% of bacteria and 3.28% of *Lactobacillales*. Whilst some species were present in varying abundances in all treatment groups (*L. apis, L. bombi* and *Apis florea*), some were only present in the acetone control and control treatment groups. *L. delbrueckii* and *L. fermentum* were present in both control groups with most abundance represented by the acetone control treatment group. The majority of *L. reuteri, L. plantarum, L. mucosae,* and L. *salivarius* abundance was represented by the control treatment group. *L. apis* had the largest abundance in the *Lactobacillus* genera, representing 1.5% of bacteria and was much lower in abundance in PAI treatment groups compared to other treatment groups. *L. bombi* represented 0.14% of *Lactobacillus*, with a higher abundance in PCF treatment groups compared to other treatment groups.

Saccharibacter species accounted for 15.12% of *Proteobacteria* and 9.993% of all bacteria with a similar abundance across both prothioconazole and the acetone control treatment groups, and a low relative abundance in the control treatment group. In addition, *Bombiscardovia* was present in all treatment groups representing 2.385% of bacterial abundance. *Acinetobacter bohemicus* accounted for only 0.013% of bacteria and 0.02% of *Acinetobacter* bacteria, but most abundance could be attributed to the digestive tracts of bees from the acetone control group. *Lactococcus* species accounted for 0.223% of bacteria and 0.34% of *Lactobacillales*, with the majority of *Lactococcus*

abundance coming from the acetone control treatment group, followed by the PAI treatment group, which both contained acetone. The majority of *Lactococcus* representation came from *L. raffinolactis.* This relative abundance profile was also observed for *Smithella,* which accounted for 0.5% of bacterial abundance.

Based on the group taxonomy tree created for fungal genera, *Candida apicola* accounted for the highest relative abundance, representing 10.892% of all fungi selected and 50.34% of *Saccharomycete* abundance, with most abundance attributed to the control group, and minimal comparative abundance in prothioconazole treatment group. *Candida* bombi represented 1.5% of fungi and nearly 7% of *Saccharomycetes* with a similar abundance in all treatment groups except for the PCF treatment group, which had a much lower relative abundance. *Wickerhamomyces anomalus* accounted for 0.995% of fungi and 4.6% of *Saccharomycetes* with a similar abundance across all treatment groups except the PCF treatment group.

Thermoascus aurantiacus accounted for 0.919% of fungi and 4.25% of *Eurotiomycetes* abundance, with the highest abundance found in PAI, followed by the acetone control and PCF treatment groups. A relatively minimal abundance was present in control treatment groups. *Zygosaccharomyces rouxii* represented 1.886% of fungi and 8.72% of *Saccharomycetes*, with the highest abundance found in the prothioconazole treatment groups and a relatively low abundance in both control treatment groups. *Cryptococcus neoformans* accounted for 0.195% of bacteria and was only present in the control treatment group. *Alternaria alternata* represented 1.836% of fungi and 8.49% of *Dothideomycetes.* The *Alternaria* species present were *Alternaria alternata*, with most abundance represented by PAI treated groups, and *Alternaria metachromatica*, with most treatment represented by the acetone control, followed by PCF treated groups. A minimal abundance was accounted for from the control and PAI treatment groups in comparison. *Mucor circinelloides* accounted for 0.173% of fungi and 0.8% of *Mucoromycota*, with most abundance accounted for from the PCF treatment group, with some presence in the control treatment group also at a lower abundance. In addition, *Marasmius rotula* and *Entolom sericeum,* which represented 0.047% and 0.03% of all fungi abundance respectively, were only present in PCF treatment groups.

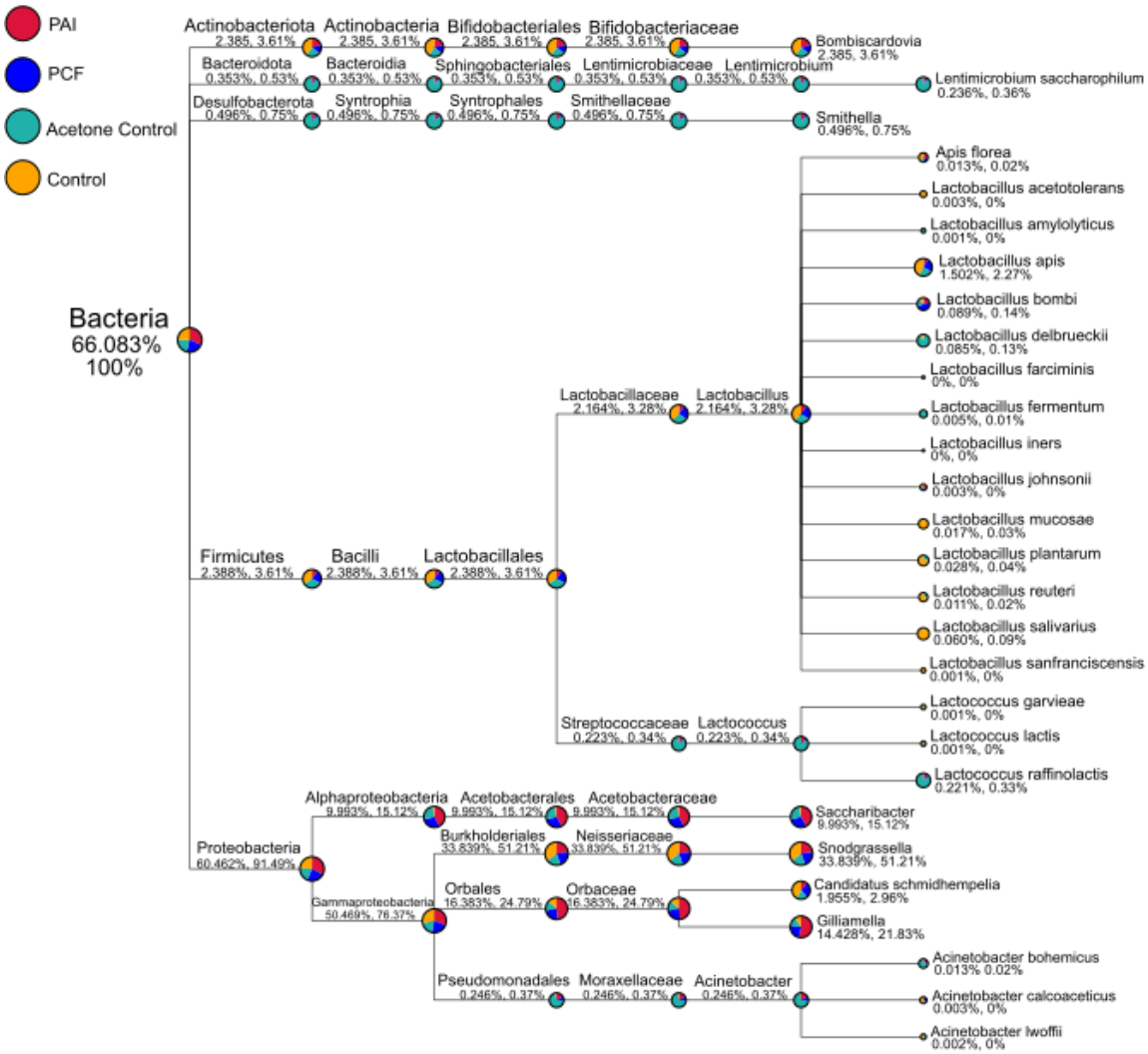


Figure 4-9 Group Taxonomy Tree for Bacterial Taxa Present in the *B. terrestris* Digestive Tract After Treatment with PAI, PCF, Acetone Control or Control. The top 10 genera in high relative abundance were used by default to create the group taxonomy tree. The node size represents the relative abundance in each treatment group. The first number below taxonomic names demonstrate the percentage in the whole taxon and the second number represents the percentage in the selected taxon.

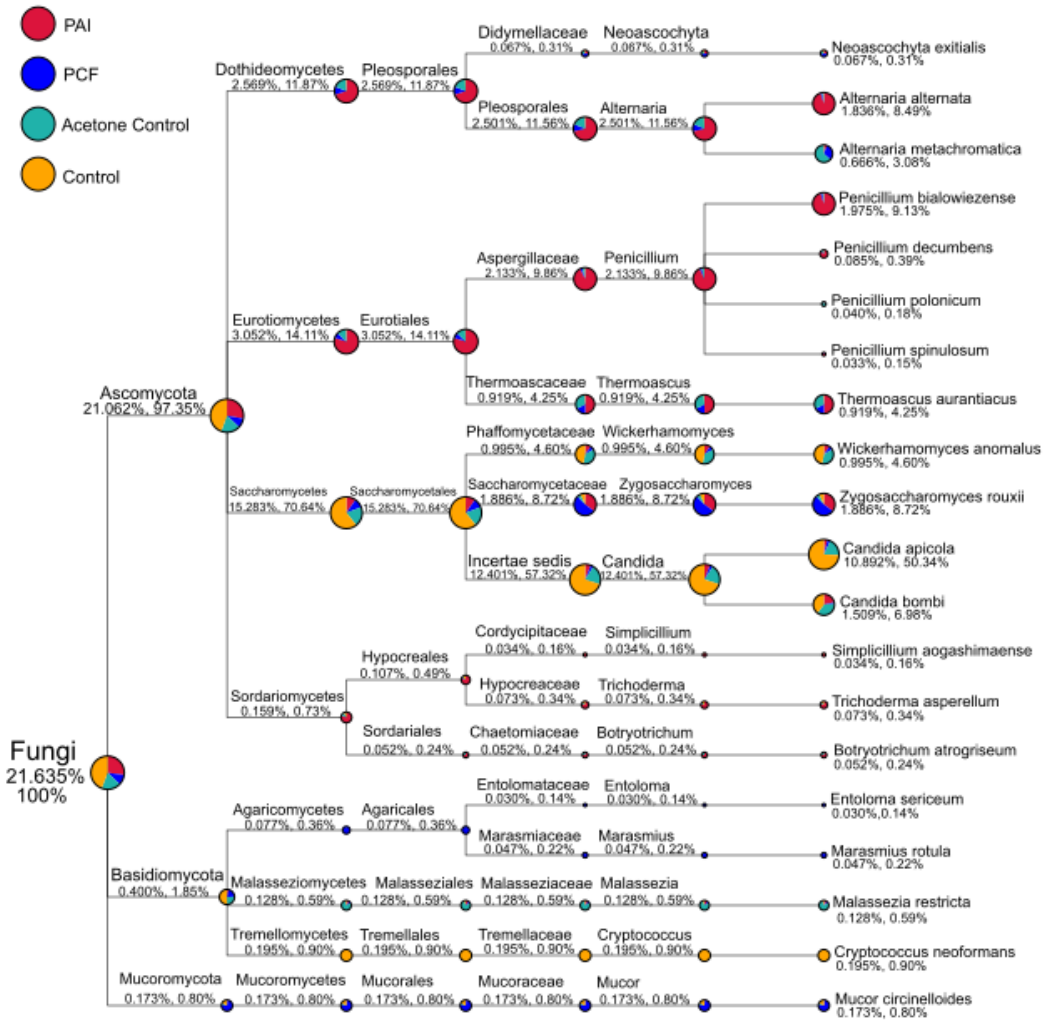


Figure 4-10 Group Taxonomy Tree for Fungal Taxa Present in the *B. terrestris* Digestive Tract After Treatment with PAI, PCF, Acetone Control or Control. The top 10 genera in high relative abundance were used by default to create the group taxonomy tree. The node size represents the relative abundance in each treatment group. The first number below taxonomic names demonstrate the percentage in the whole taxon and the second number represents the percentage in the selected taxon.

4.3.5.2 Alpha Diversity Analysis of Bacterial and Fungal Taxa

Comparisons of species richness were made between the microbiota of digestive tracts from different treatment groups. Initially, rarefaction curves were created to determine species richness and diversity at the given sequencing depth by comparing the number of OTU assignments from 16S and ITS sequencing and comparing this to the number of sequences identified. Based on rarefaction curves, there was a higher species richness in both prothioconazole treatment group samples compared to the acetone and control treatment group samples based on a higher number of OTU's identified at the same number of sequences (Figure 4-11A). This trend was similar in rarefaction curves based on ITS sequencing depth, however, the acetone control treatment group had a similar curve, and therefore species richness based on sequence numbers and OTU identifications, as the prothioconazole treatment group (Figure 4-11B).

Venn diagrams were used to analyse the number of normalised OTU's common and unique amongst different treatment groups to determine species richness. From 16S amplicon sequencing, there were 112 OTU's common between all four treatments (Figure 4-12A). The PAI, PCF, control and acetone control treatment group had 264, 637, 344, and 243 unique OTU's, respectively. 189 OTU's were common between PAI, PCF and the acetone control treatment group, whilst only 22 were common between PAI, PCF and the control treatment group. When comparing PAI directly to its relative acetone control treatment group, 126 OTU's were common between the groups, with 281 and 387 OTU's unique to PAI and the acetone control treatment group, respectively (Figure 4-12B). In a comparison of PCF directly with the control treatment group, only 57 OTU's were shared. The PCF treatment group had 685 unique OTU's and the control treatment group had 488 unique OTU's (Figure 4-12C). To determine the impact of acetone on 16S species richness, the number of shared OTU assignments were determined in the control and acetone treatment groups. There were 329 shared OTU's, with 406 and 567 unique OTU's in the control and acetone control treatment group, respectively (Figure 4-12D). From ITS amplicon sequencing, there were 156 OTU's common between all treatment groups (Figure 4-13A). PAI, PCF, control and the acetone control had 364, 227, 128, and 240 unique OTU assignments, respectively. 68 OTU's were common between PAI, PCF and the acetone control treatment groups, whilst 29 were common between PAI, PCF and the

control treatment groups. In a direct comparison of the number of OTU assignments in PAI and the relative acetone control treatment group, there were 124 shared OTUs (Figure 4-13B). The PAI and acetone control treatment group had 392 and 285 unique OTU's, respectively. There were 55 shared OTU assignments in a comparison of PCF and control treatment groups, with 260 and 173 unique OTU's in the PCF and control treatment group, respectively (Figure 4-13C). In addition, a direct comparison of the acetone control and control treatment group resolved 259 common OTU assignments, with 219 unique OTU's in the control treatment group and 428 unique OTU's in the acetone control (Figure 4-13D).

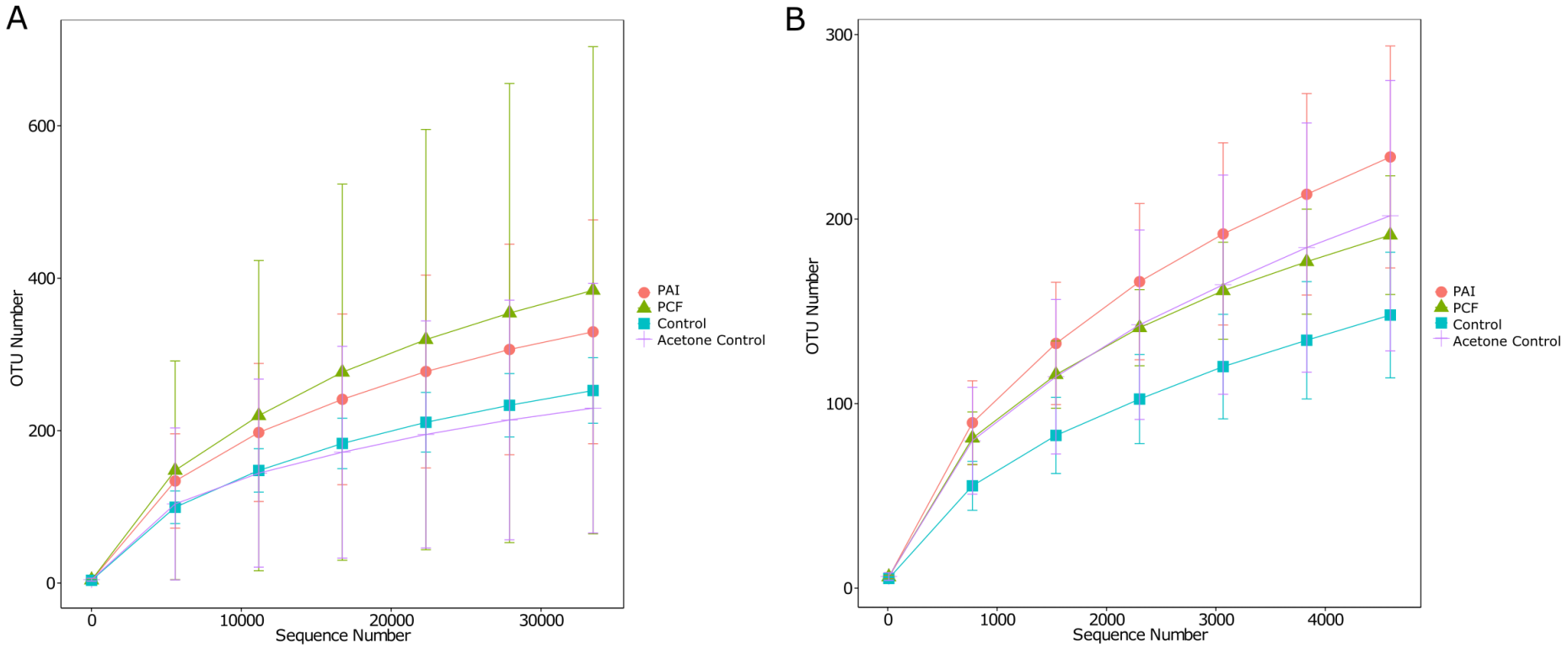


Figure 4-11 Rarefaction Curve for Taxa Identified in the Digestive Tract of *B. terrestris*. Comparing the OTU number to the number of sequences from sample groups gives an estimation of species richness in each treatment group. **(A)** For OTU identifications from 16S sequences, there were less OTU assignments compared to the number of sequences for the control groups compared to prothioconazole-based treatment groups. **(B)** For OTU identifications from ITS sequences, the control treated group had fewer OTU assignments per sequence number compared to other treatment groups.

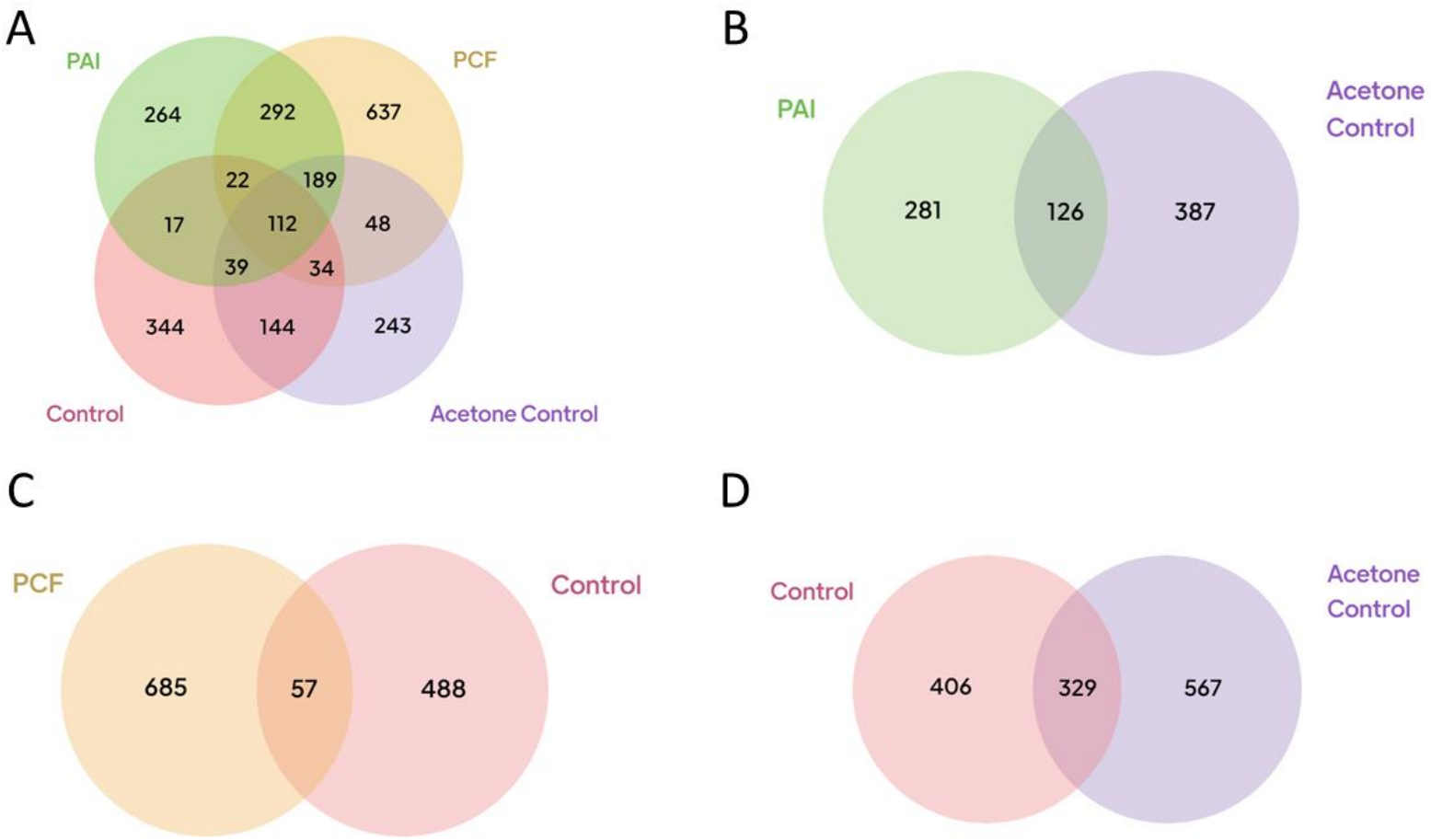


Figure 4-12 Common and Unique OTU's Identified from 16S Sequencing Amongst Different Treatment Groups. (A) There were 112 common OTU's between all treatment groups. (B) There were 126 common OTU's between the PAI and relevant acetone control group. (C) There were 57 common OTU's between PCF and the control treatment group and (D) there were 329 OTU's between the control and acetone control, with 567 and 406 unique OTU's in the acetone control and control treatment group, respectively.

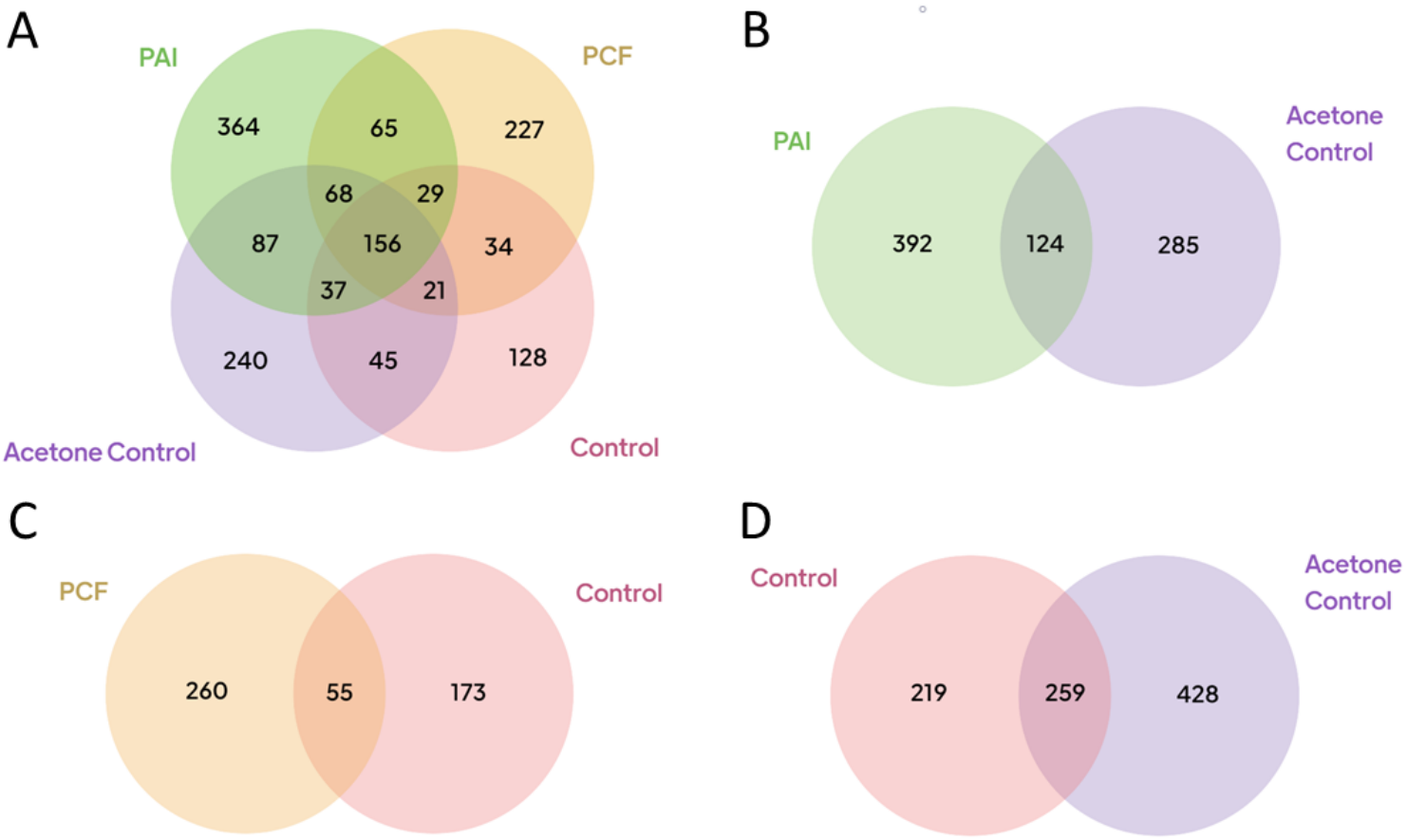


Figure 4-13 Common and unique OTU's identified from ITS sequencing amongst different treatment groups. (A) There were 156 common OTU's between all treatment groups. **(B)** PAI treatment group was compared to its relevant acetone control with 124 common unique OTU's between treatment groups. **(C)** The PCF treatment group was compared to the control with 55 common OTU's and **(D)** The acetone control had 428 unique OTU's compared to the control treatment group with 219 unique OTU's, there were 259 common OTU's between the control and acetone control.

Rank abundance curves were generated by sorting OTU's in samples by their relative abundance and assigning a rank number. This was done to analyse bacterial and fungal richness and evenness of different taxa across treatment groups. For 16S sequencing results, all treatments had a similar rank abundance curve except for the acetone control group, which had a rank abundance curve less steep than other treatment groups, indicating a generally higher relative abundance for ranked species compared to other treatment groups (Figure S4-4). For ITS sequencing, all treatment groups plateaued in the relative abundance of ranked species at approximately species rank 150. However, the ranked abundance curve illustrated a higher relative abundance for given species in the PCF treatment group, followed by PAI, the acetone control treatment group and the control treatment group, respectively (Figure S4-5).

Observed species, Chao1, Simpson and Shannon indices were generated for samples for both 16S and ITS sequencing results to determine alpha diversity. Tukey and Wilcoxon tests were utilised to determine pairwise treatment group variations in alpha diversity indices (Table S4-15). For bacteria present in the digestive tract, there were no significant differences in bacterial alpha diversity amongst treatment groups (Figure 4-14; Figure 4-15; Figure S4-6; Figure S4-7). When analysing fungal alpha diversity in the digestive tract, Wilcox testing of observed species, Shannon, and Simpson indices between the control and acetone control treatment group all found a significant difference in alpha diversity with a p value of 0.0326, 0.0225, and 0.0349, respectively. There were no significant differences in fungal alpha diversity between either prothioconazole-based treatment and their relative controls (Figure 4-16; Figure 4-17; Figure S4-8; Figure S4-9).

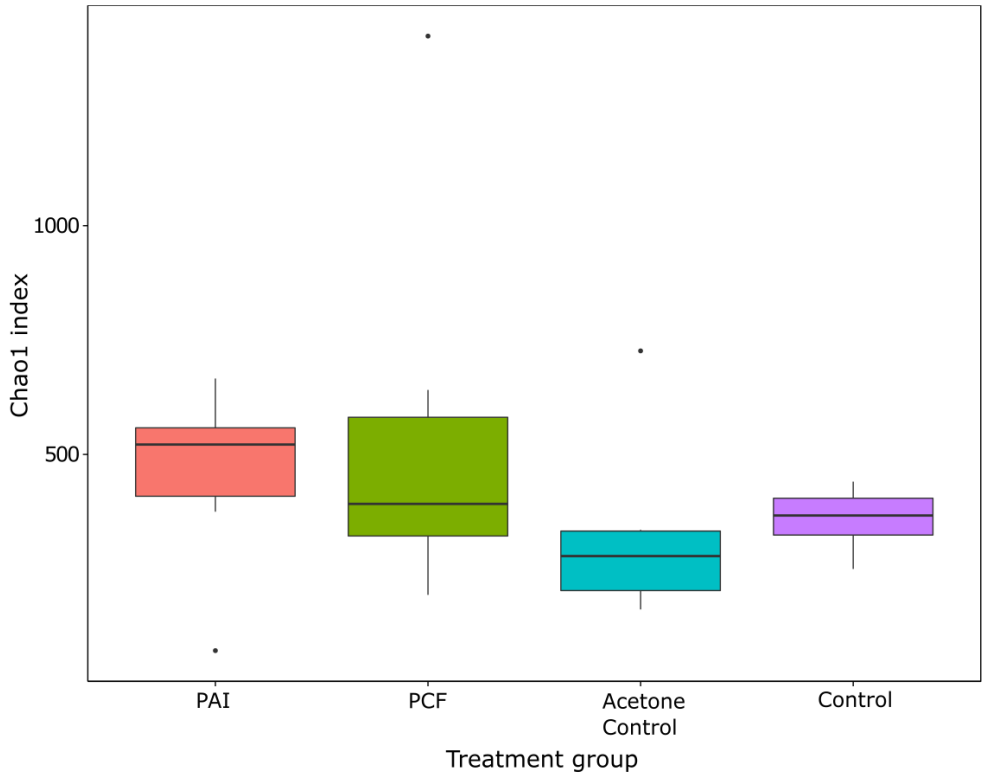


Figure 4-14 Boxplot of Observed Bacterial Species per Treatment Group. There were no significant differences in species count between treatment groups.

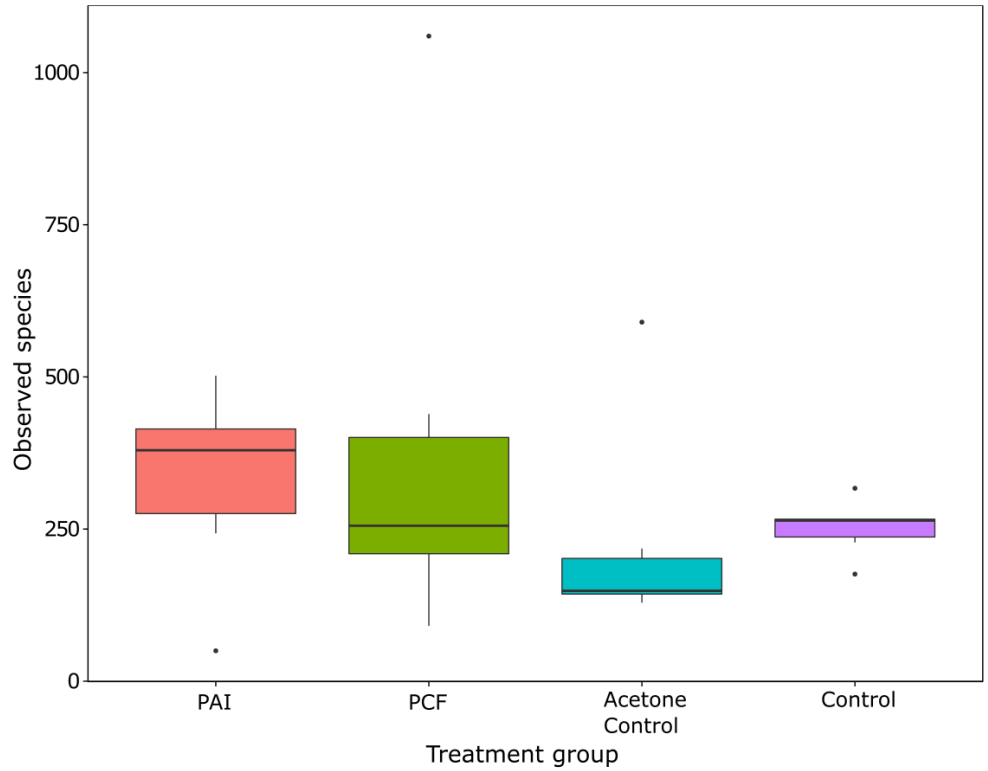


Figure 4-15 Boxplot of Chao1 Indices of Bacterial Species in Each Treatment Group. Chao1 estimation was determined for alpha diversity of bacterial species in each group taking relative abundance into account. There were no significant differences in alpha diversity between groups.

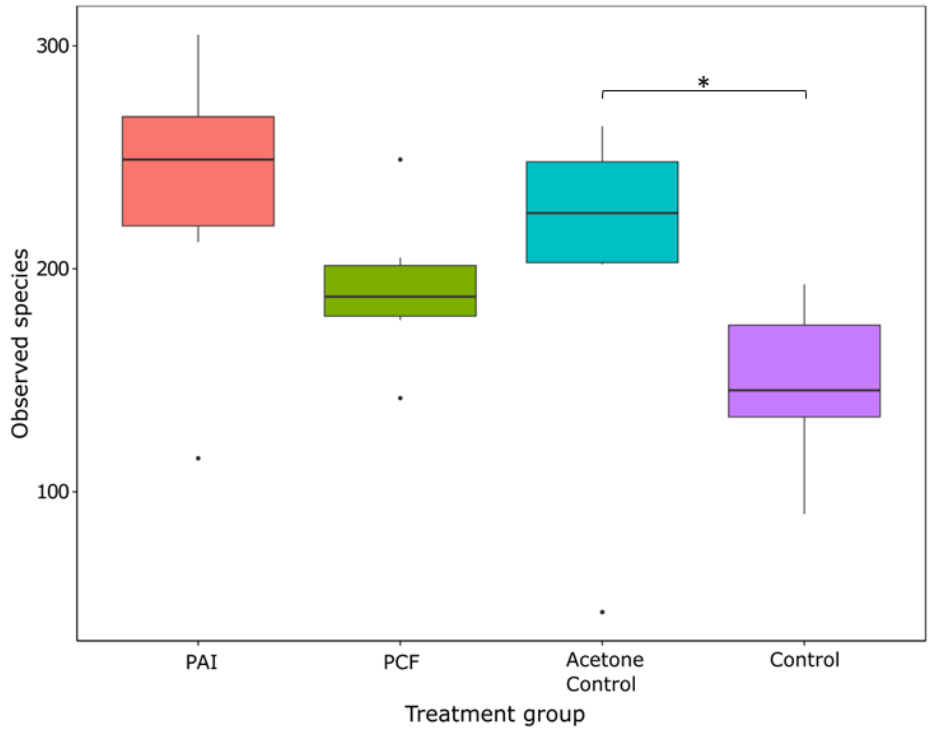


Figure 4-16 Boxplot of Observed Fungal Species per Treatment Group. Wilcoxon testing on observed species between groups determined a significant difference between the acetone control and control treatment groups (p = 0.033).

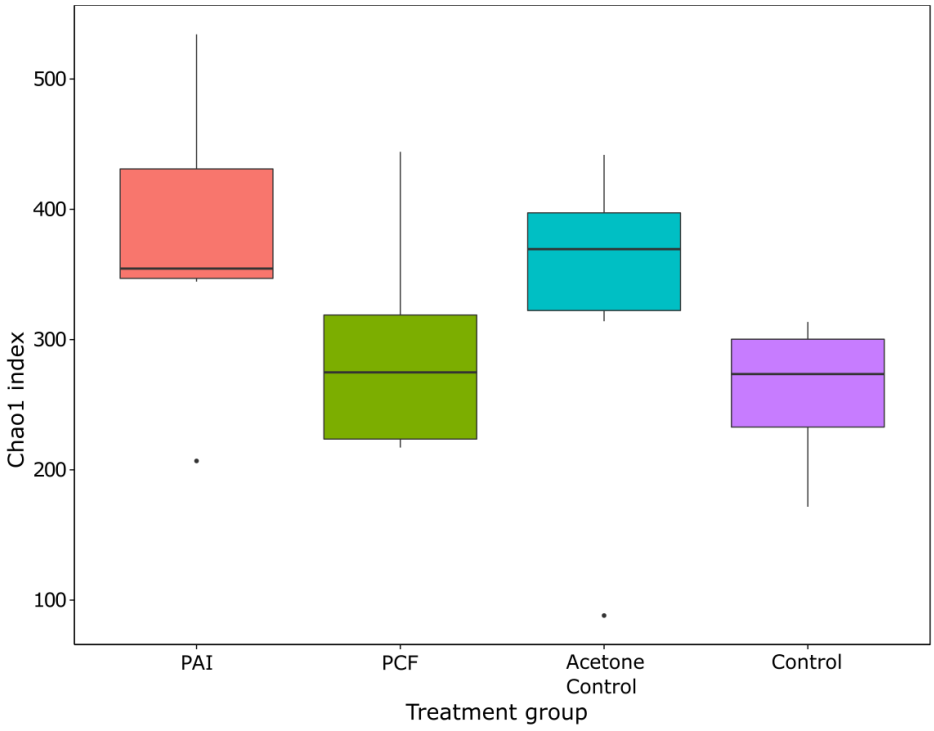


Figure 4-17 Boxplot of Chao1 Indices of Fungal Alpha Diversity in Each Treatment Group. There were no significant differences in alpha diversity based on Chao1 indices between treatment groups.

4.3.5.3 Beta Diversity Analysis of Bacterial and Fungal Taxa

To determine beta diversity – microbial community composition - the difference between microbial communities was measured using unweighted and weighted unifrac measurements and the square matrix of distance or dissimilarity between group pairs.

A PCA and PCoA were used to visualise variance and dissimilarity between samples and treatment group microbial community composition. Overall, PCA determined low variance between treatment groups for both bacterial and fungal microorganisms, with most samples clustering together (Figure 4-18A; Figure 4-18B). The PCoA of bacterial weighted unifrac distances display a drift of control samples away from other treatment samples. However, there are no clear clustering between treatment groups (Figure 4-18C). The PCoA of fungal weighted unifrac distance show clustering of PAI and PCF treatment groups together, with samples from the control treatment group clustering away from other treatments. Whilst samples for the acetone control treatment group cluster near the PAI and PCF treatment group samples, there is some drift of acetone control treatment samples toward the control treatment sample group cluster (Figure 4-18D).

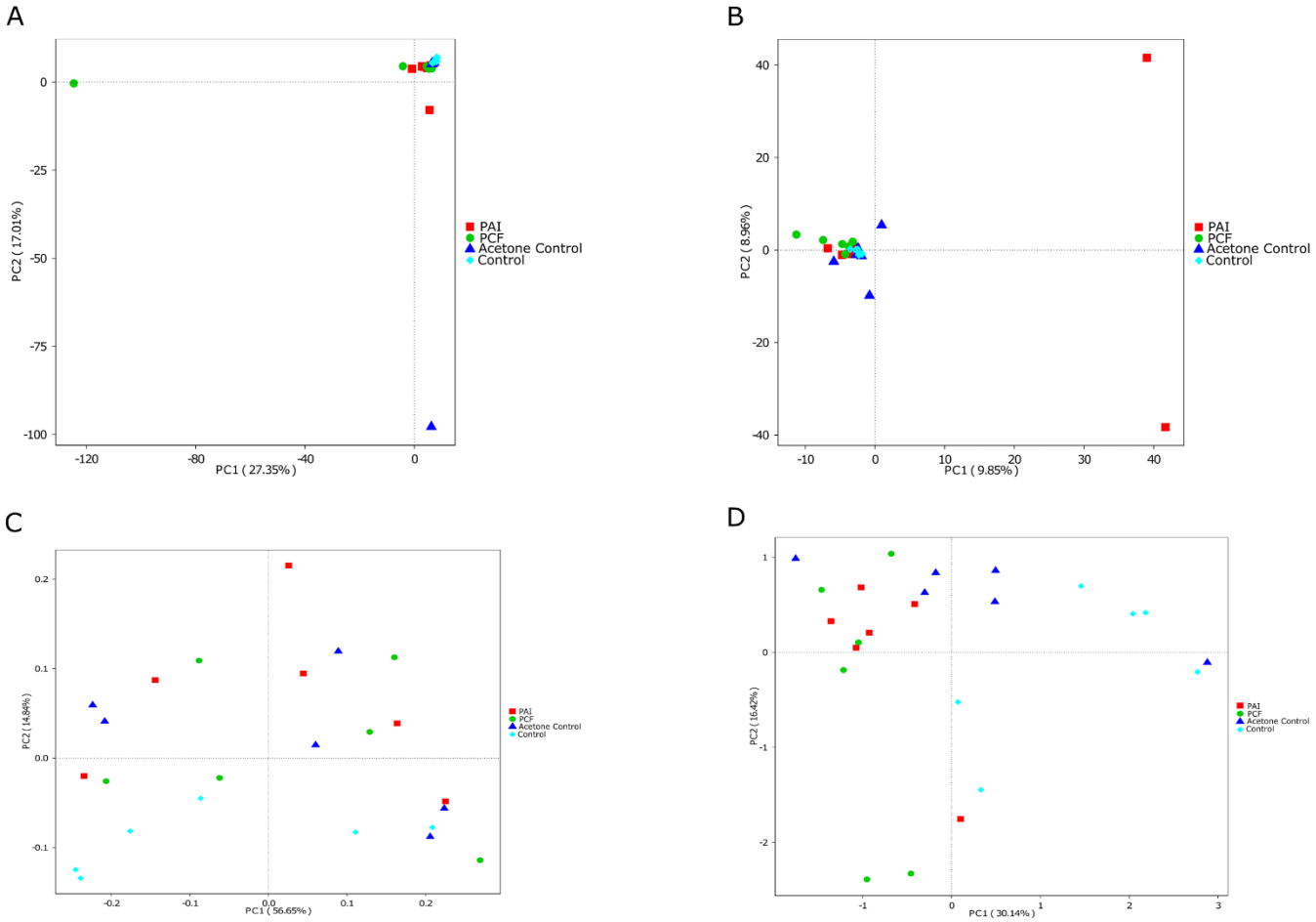


Figure 4-18 PCA and PCoA Were Used to Visualise the Variance in Microbial Composition Amongst Treatment Group Samples. PCA's on **(A)** bacterial and **(B)** fungal samples illustrate that there is little variance between treatment groups. PCoAs visualising **(C)** bacterial weighted unifracs illustrate control treatment samples drifting away from other treatment groups and **(D)** fungal weighted unifrac's illustrate clustering of all samples together except the control treatment group.

Wilcox and Tukey tests were performed on comparisons of weighted and unweighted unifrac distances between treatment groups to determine if beta diversity was significantly different between treatment groups (Table S4-16). Comparison of weighted unifrac distance between groups determined no statistically significant differences in bacterial beta diversity (Figure 4-19A). However, both Wilcoxon and Tukey testing found significant differences in fungal beta diversity between the PCF and control treatment groups (Wilcoxon $p = 0.005$; Tukey $p = 0.0328$) (Figure 4-19B). When considering unweighted unifrac distances, there was a significant difference in bacterial beta diversity between the PCF and control treatment group (Wilcoxon $p = 0.0063$). There were no significant differences found for fungal unweighted unifrac distances between groups.

ANOSIM and MRPP analyses were used to determine if differences in bacterial and fungal community structures were statistically significantly different between different treatment groups (Table S3-17). Both Anosim and MRPP analysis did not resolve any statistically significant differences in bacterial community structure between treatment groups. In Anosim analysis, all comparisons had an R value between 0.15 and 0.01, suggesting high overlap in bacterial community composition between groups. In MRPP analysis, all comparisons had an A value > 0, suggesting more similarity within groups than between groups. Both Anosim and MRPP analysis resolved statistically significant differences in fungal community composition between the PCF and control treatment groups (ANOSIM $p = 0.003$; MRPP $p = 0.007$) and the acetone control and control treatment groups (ANOSIM $p = 0.025$; MRPP $p = 0.017$).

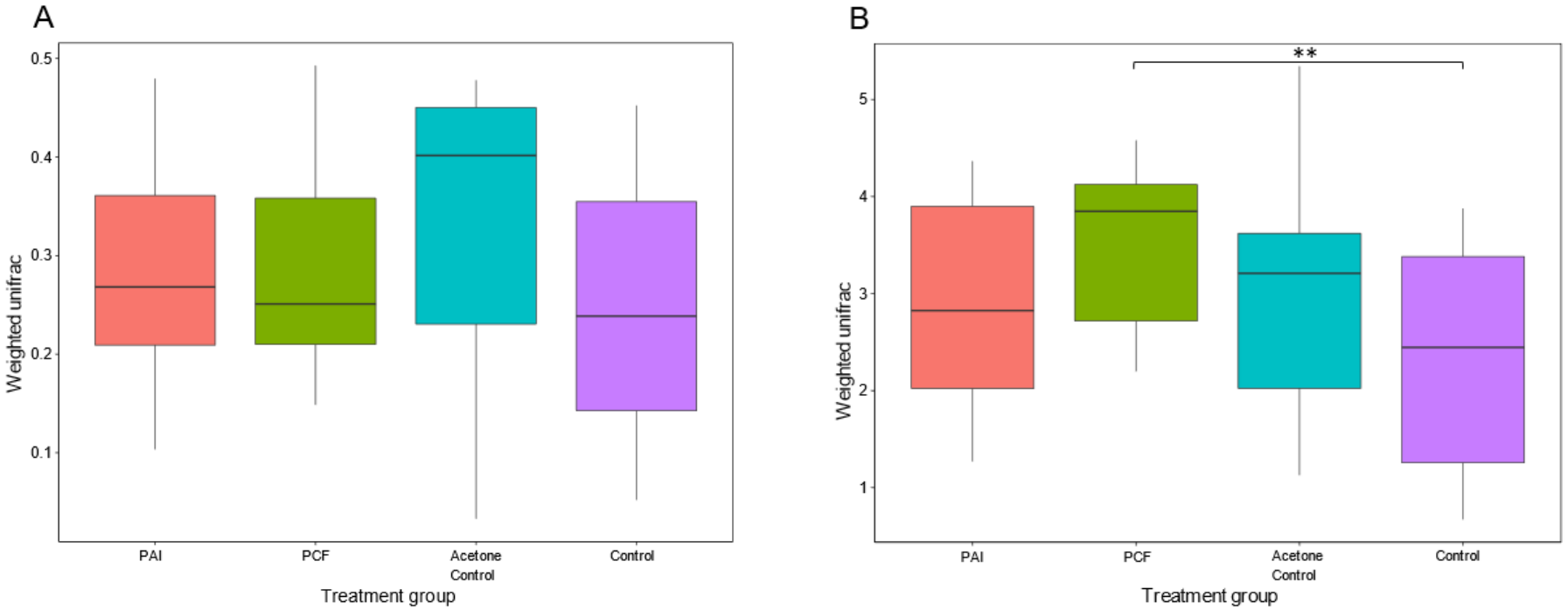


Figure 4-19 Boxplots of Weighted Unifrac Distances Between Each Treatment Group. Tukey and Wilcoxon tests were conducted to determine statistically significant differences in bacterial and fungal weighted unifrac distance comparisons between groups. **(A)** There were no statistically significant differences in beta diversity between treatment groups and **(B)** Both Wilcoxon and Tukey tests determined statistically significant differences in fungal beta diversity between PCF and control treatment groups (Wilcoxon $p < 0.01$, Tukey $p < 0.05$).

In Anosim analysis, the R value of the PCF-control group comparison was 0.47, showing differences with some overlap in community composition. For the acetone control-control group comparison, the R value was 0.255, suggesting a high overlap in community composition between these groups. MRPP analysis resolved A values > 0 for all comparisons, suggesting more similarity within groups than between groups.

T-tests were carried out on pairwise comparisons of bacterial and fungal genera and species relative abundance between treatment groups ($p \leq 0.05$) (Table S4-18). Overall, 24 bacterial genera and 10 bacterial species had statistically significant differences in relative abundance between the PAI and acetone control treatment groups. The bacterial genera *Geobacter* had a statistically significantly lower abundance in PAI compared to the acetone control ($p < 0.0001$) (Table 4-2). *L. salivarius* and *L. plantarum* had a significantly lower abundance in PAI compared to the acetone control ($p = 0.034$ and $p = 0.014$, respectively). In addition, the genera Mitochondria had a significantly lower abundance in PAI compared to the acetone control treatment group ($p = 0.009$). Whilst many differences found were a decreased abundance in PAI compared the acetone control group, the bacterial genera *Gilliamella, Pelagicoccus, Nitrosomonas* and *Thalassobaculum* were significantly higher in PAI compared to acetone control treatment group with p values of 0.034, 0.025, 0.029, and 0.03, respectively.

There were 41 bacterial genera and 15 bacterial species statistically significantly different in relative abundance when comparing the PCF treatment group to the control treatment group. *L. plantarum* and *L. acetotolerans* were not present in PCF but were present in the control treatment group ($p = 0.023$ and $p = 0.018$, respectively). *Lactococcus raffinolactis, Arcobacter butzleri, Tolumonas auensis,* and *Sphingobacterium multivorum* were present in the PCF treatment group but absent from the control treatment group ($p = 0.018$, 0.047, 0.007, and 0.025 respectively). The genera 'Mitochondria' was significantly lower in the PCF treatment group compared to the control treatment group ($p = 0.003$). The bacterial genus *Saccharibacter* had a significantly higher relative abundance in the PCF treatment group ($p = 0.046$) and the bacterial genus *Pseudomonas* had a lower relative abundance in the PCF treatment group compared to the control group ($p = 0.019$).

There were five genera and three bacterial species with a significant difference in relative abundance between the acetone control and control treatment groups. The genera *Hymenobacter* and *Helicobacter* were absent in the acetone control but present in the control treatment group ($p = 0.004$ and $p = 0.017$, respectively). All statistically significant differences found were as a result of a lower relative abundance in the acetone control compared to the control treatment group.

In a pairwise comparison of the relative abundance of fungal genera and species between PAI and acetone control treatment groups, there were two genera and two species with statistically significant differences (Table 4-3). *Rhodotorula* had a higher abundance in the PAI treatment group compared to the acetone control, which was accounted for by a single species, *Rhodotorula mucilaginosa* ($p = 0.025$). The genus *Aspergillus* had a higher abundance in PAI compared to the acetone control treatment group ($p = 0.005$), with *Aspergillus flavus* absent in the acetone control but present in the PAI treatment group ($p = 0.019$).

Three genera and three species had significantly different relative abundances between the PCF and control treatment group. Both *Candida* and *Wickerhamomyces* had a decreased abundance in PCF compared to the control treatment group ($p = 0.034$ and $p = 0.016$, respectively). *C. apicola* was the only *Candida* species identified as significantly reduced in PCF compared to the control group ($p = 0.036$). Statistically significant lower abundance of *Wickerhamomyces* was based on one species, *W. anomalus* ($p = 0.016$).

The fungal species *R. mucilaginosa* was present in the acetone control but not the control treatment group ($p = 0.013$). This was the only statistically significant difference when comparing fungal relative abundance in the control treatment groups.

Table 4-2 Statistically Significant Differences in the Relative Abundance of Bacterial Genera and Species in Pairwise Comparisons of Treatment Groups. A t-test was carried out on the relative abundances of genera and species to determine differences between treatment groups. The top three genera or species with the lowest p values are listed for each pairwise comparison.

Comparison	Taxon	Average (PAI)	Average (PCF)	Average (Acetone Control)	Average (Control)	Standard Deviation (PAI)	Standard Deviation (PCF)	Standard Deviation (Acetone Control)	Standard Deviation (Control)	P-value
PAI vs Acetone Control	*Geobacter*	1.99E-05	n/a	1.8E-4	n/a	3.62E-05	n/a	6.10E-05	n/a	0.0001
	Escherichia coli	1.2E-4	n/a	1.7E-3	n/a	1.9E-4	n/a	7.1E-4	n/a	0.0027
	Granulicatella	0	n/a	5.97E-05	n/a	0	n/a	3.27E-05	n/a	0.007
PCF vs Control	*Chloroplast*	n/a	4.2E-4	n/a	7.4E-3	n/a	5.7E-4	n/a	1.9E-3	0.0002
	Methyloversatilis	n/a	0	n/a	3.48E-05	n/a	0	n/a	1.22E-05	0.0009
	Tepidiphilus	n/a	0	n/a	5.48E-05	n/a	0	n/a	2.25E-05	0.0019
Acetone Control vs Control	*Hymenobacter*	n/a	n/a	0	4.98E-05	n/a	n/a	0	2.44E-05	0.004
	Helicobacter	n/a	n/a	0	7.97E-05	n/a	n/a	0	5.56E-05	0.017
	Lactobacillus acetotolerans	n/a	n/a	9.96E-06	9.46E-05	n/a	n/a	2.44E-05	6.66E-05	0.025

Table 4-3 Statistically Significant Differences in the Relative Abundance of Fungal Genera and Species in Pairwise Comparisons of Treatment Groups. A t-test was conducted on the relative abundances of genera and species to determine differences between treatment groups. The top three genera or species with the lowest p values are listed for each pairwise comparison.

Comparison	Taxon	Average (PAI)	Average (PCF)	Average (Acetone Control)	Average (Control)	Standard Deviation (PAI)	Standard Deviation (PCF)	Standard Deviation (Acetone Control)	Standard Deviation (Control)	P-value
PAI vs Acetone Control	*Aspergillus*	9.8E-4	n/a	2.5E-4	n/a	3.8E-4	n/a	3.2E-4	n/a	0.0055
	Aspergillus flavus	3.6E-4	n/a	0	n/a	2.6E-4	n/a	0	n/a	0.0199
	Rhodotorula mucilaginosa	1.08E-4	n/a	6.2E-4	n/a	1.2E-4	n/a	3.9E-4	n/a	0.0251
PCF vs Control	*Candida*	n/a	1.5E-2	n/a	3.5E-1	n/a	2.8E-2	n/a	2.8E-1	0.0338
	Wickerhamomyces	n/a	2.6E-3	n/a	1.9E-2	n/a	3.7E-3	n/a	1.1E-2	0.0165
	Others	n/a	9.1E-1	n/a	6.1E-1	n/a	8.4E-2	n/a	2.6E-1	0.0379
Acetone Control vs Control	*Rhodotorula*	n/a	n/a	6.2E-4	3.6E-05	n/a	n/a	3.9E-4	8.9E-05	0.0152
	Rhodotorula mucilaginosa	n/a	n/a	6.2E-4	0	n/a	n/a	3.9E-4	0	0.0129

4.4 Discussion

Overall, prothioconazole did not alter the survival or sucrose solution consumption of *B. terrestris*. However, low concentrations of prothioconazole alone and the commercial formulation significantly increased the observation of altered behaviour. In addition, prothioconazole ingestion led to differential physiological effects on the digestive tract, elucidated via proteomics and microbiota DNA sequencing. The active ingredient altered fatty acid metabolism, electron transport chain and cell structural integrity, while the commercial formulation altered glycolysis, translation, protein homeostasis and the cytoskeleton. Both prothioconazole-based treatments led to statistically significant alterations to the relative abundance of bacterial and fungal taxa, with the commercial formulation leading to statistically significant differences in the beta diversity of fungal taxa. In addition, through the inclusion of an acetone control to account for the acetone used to efficiently solubilise the active ingredient, it was determined that a low concentration of acetone can lead to significant impacts on the *B. terrestris* digestive tract including but not limited to impacts on metabolism, physiological stress and the species richness and community composition of the microbiota.

4.4.1 Survival, Behavioural and Consumption Alterations After Prothioconazole Exposure

There were no statistically significant impacts of any treatment group on the survival of *B. terrestris* over five days. In the ten-day exposure assay, 0.3 ppm PCF had a significant impact on bees with an increased occurrence of moribund bees, i.e., bees that were unable to walk with a weak response to stimulation ($p < 0.001$), whilst 0.3ppm PAI had significant effects on the number of bees affected, i.e., displaying reduced coordination and hyperactivity ($p = 0.004$). Both results had positive coefficient values, suggesting an increase in moribund and affected bees with an increased exposure to 0.3 ppm PCF and PAI, respectively. These results were not observed in the five-day exposure assays. However, the five-day exposure was not conducted in triplicate as was the case for the ten-day survival assays. As a result, the impacts of prothioconazole on the occurrence of moribund and affected bees could be

a result of a longer period of exposure or higher statistical robustness based on an increased number of samples analysed. Prothioconazole may lead to direct impacts between days five and ten of exposure, with the majority of moribund and affected bees observed after day five in ten-day assays. It should also be noted that the third replication of survival assays led to a higher level of moribund bees than observed in other replications, which could have altered the overall significance of PCF's significant association with moribund occurrence. To ensure this was not the case, a general linear model was carried out on the first two experiment replicates only, which resolved a significant impact of 0.3 ppm PCF on the number of moribund bees ($p < 0.001$), determining that this was not a false significant finding based on the higher number of moribund bees in this treatment in the third bioassay replicate. In addition, the number of affected bees was still significantly impacted by 0.3 ppm PAI exposure ($p = 0.013$).

There were no impacts of any prothioconazole treatment on sucrose solution consumption. However, compared to the non-acetone control, there was a significant decrease in sucrose solution consumption in the acetone control treated bees from the five-day exposure assay ($p = 0.039$). This was not observed in ten-day survival exposure assays, which were carried out in triplicate. Whilst it was thought that this could be due to an initial decrease in consumption at the beginning of exposure to the acetone control, resulting in a significant difference between acetone control and control consumption on day five but not day ten, this was unfounded when analysing data from the first five days of the ten-day survival assays ($p = 0.773$). This may be due to a lack of statistical power granted by the five-day exposure assays as technical replicates were not included. Alternatively, consumption data from ten-day exposure assays did not follow the normal distribution and could not be transformed, resulting in a reliance on non-parametric statistical analyses, which may not be as robust as the parametric two-sample t-test carried out for consumption analysis on results from five-day exposure assays.

The lack of a direct impact of prothioconazole on survival or consumption has been observed in the few studies that exist on prothioconazole impacts on bees. Wood et al. (2019) determined no impact of 0.36 ppm prothioconazole on honeybee worker survival or food consumption, a similar exposure concentration to the one used in this

chapter. However, prothioconazole exposure had synergistic effects in conjunction with exposure to the neonicotinoid insecticide thiamethoxam on pollen consumption, but not survival. In addition, Almasri et al. (2021) found no impact of the DMI triazole fungicide difenoconazole on honeybee mortality or food consumption after contact exposure. However, synergistic impacts were found for difenoconazole contact exposure after glyphosate oral exposure. In addition, difenoconazole and glyphosate exposure exacerbated the impact of *N. ceranae* infection. These results highlight that whilst prothioconazole and other triazole fungicides themselves may not have direct impacts on survival, in conjunction with other pesticides and pathogens that are likely encountered in the environment, dire consequences for survival may transpire, with multiple studies demonstrating increased mortality after dual insecticide-triazole fungicide exposure (Pilling and Jepson, 1993; Manning *et al.*, 2017; Han *et al.*, 2019).

One aspect of these findings which is less represented in current literature was the observation of significant impacts of lower concentrations of 0.3 ppm PAI and PCF on the number of affected and moribund bees, respectively. Considering there was no significant impact on mortality in 0.3ppm PCF, one may reach the conclusion that bees can recover from this moribund period. However, in the wild, a period of moribund activity could result in predation or starvation with nectar not as readily available as it would be in a laboratory setting. Another perplexing finding is that no significant increases in affected or moribund bees were observed after exposure to higher concentrations, 3 and 30 ppm of PAI or PCF. Following a basic dose-response relationship, higher doses would be expected to lead to a higher rate of negative impacts. However, this model does not consider the multiple molecular processes initiated to cope with xenobiotic stress and their complexities, such as those considered in a stress-response or adverse outcome pathway model (Ankley *et al.*, 2010; Guedes *et al.*, 2022). Still, despite the promotion of such pathways as informative models to consider in the design of future research, which can aid in understanding the impact of chemical stressors on organisms from the molecular initiating event up to the population through multiple biological organisation levels, research into sublethal impacts are less common and largely ignored compared to toxicity studies (Cullen *et al.*, 2019). In research by Wood et al. (2019), whilst non-significant, exposure to the insecticide thiamethoxam at 0.1 ppm had a higher mortality than exposure to 0.5 ppm over twenty days in *A. mellifera* brood. Exposure to 0.1 ppm thiamethoxam resulted

in a hazard ratio of 3.38, whilst exposure to 0.5 ppm led to a hazard ratio of 1.58. Additionally, Almasri et al. (2021) found that 0.01 ppm glyphosate oral exposure before difenoconazole contact exposure induced a significant toxic response, despite 0.1ppm glyphosate exposure also tested before fungicide exposure, which did not lead to a significant increase in mortality. Whilst this research determined sublethal physiological impacts by investigating enzyme marker activity, this was only carried out at a concentration of 0.01 ppm. Overall, these results suggest that exposure to low doses of prothioconazole, whilst not leading to direct mortality impacts, can lead to an increase in moribund and affected bees from PAI and PCF exposure, respectively. This highlights a major flaw which in pollinator-pesticide toxicity assessment: a lack of testing for sublethal impacts (Decourtye *et al.*, 2005; Cullen *et al.*, 2019). In addition, there is a lack of studies which investigate sublethal responses to varying doses of chemical stressors. Based on these findings, not only is further research needed on the sublethal impacts of fungicides on bees, but research investigating the impact of varying concentrations of pesticides, including triazole fungicides, on sublethal level endpoints are called for. Resolving the mechanistic processes behind a higher inhibitory response to a lower dose to pesticide would enhance understanding and mitigation efforts for the risk of pesticide to pollinators. Currently, lower doses are considered safer, however, this idea needs to be challenged by investigating the impacts of various concentrations of pesticides on a chosen sublethal endpoint.

4.4.2 The Impact of Prothioconazole on the Digestive Tract Proteome

4.4.2.1 Proline®-Associated Alterations to the *B. terrestris* Digestive Tract Proteome

Compared to the control, PCF exposure led to significant alterations to the digestive tract proteome including an increase in proteins associated with translation, cytoskeleton organisation, glutathione peroxidase activity and glycolysis/gluconeogenesis. A decrease in proteins associated with endocytosis, translation, the mitochondrial matrix, and proteasome degradation were also observed.

Proline®, the prothioconazole formulation used in this research (referred to as PCF), is used to control fungal diseases such as light leaf spot and *Phoma* leaf spot in bee-attractive crops such as oilseed rape. Whilst 25% of the formulation is represented by

the active ingredient prothioconazole, other co-formulants are present, resulting in an unknown impact of this formulation on bee health which cannot be elucidated through the investigation of prothioconazole alone. This is due to the unavailability of the full list of co-formulants in many formulations as they are considered proprietary information, making it difficult to fully investigate the impacts of pesticide formulations on non-target organisms in comparison to the main active ingredient. In Proline®, the only listed co-formulant is N, N, - Dimethyldecanamide in an indeterminate amount. This co-formulant is used as a solvent to create a prothioconazole emulsion as well as acting as a penetration enhancer for the transport of prothioconazole to its target organism (Irwin *et al.,* 1990).

Of the 73 SSDA proteins increased, eight were associated with translation and seven of these were components of ribosomal subunits. Of the 104 SSDA proteins decreased, eleven were associated with translation and eight of these were components of ribosomal subunits. Non-ribosome-associated proteins decreased included mitochondrial ribosome-recycling factor and two translation factors. Whilst it is unclear why some ribosomal proteins are increased whilst others are decreased, an overall disruption to protein translation and homeostasis, potentially associated with the mitochondria, is possible. Ribosomal proteins increased may be cytosolic or associated with other organelles whilst decreased ribosomal proteins may be associated with the mitochondria. Three out of twelve SSDA proteins with a decreased abundance annotated as mitochondrial were ribosomal proteins. In SSDA proteins with an increased abundance, none of the ribosomal proteins were annotated as mitochondrial. Further supporting this theory are seven decreased proteins associated with the mitochondrial matrix including mitochondrial ribosomal proteins L12 and S29, heat shock protein 75 kDa mitochondrial, and Lon protease, a protease involved in degradation of misfolded and oxidatively damaged polypeptides as well as regulatory proteins associated with mitochondrial gene expression regulation and mitochondrial integrity maintenance. Interestingly, alterations to mitochondrial-associated translation, including mitochondrial ribosomal proteins, were found in *A. mellifera* exposed to the triazole fungicide myclobutanil, which led to alterations to mitochondrial regeneration and ATP production (Mao *et al.,* 2017). The synthesis of new mitochondria rely on translocase complexes and other proteins involved in protein targeting, folding and assembly. These include chaperone and folding

catalysts, which were also significantly decreased in PCF compared to the control. This included heat shock protein 75kDa mitochondrial, hypoxia up-regulated 1 protein, ATP-Dependent Clp Protease (mitochondrial isoform), the TIM14 subunit mitochondrial import inner membrane translocase, and calreticulin. TIM23 translocase is important for protein transport into the mitochondria. Whilst TOM complexes transport proteins from the cytoplasm across the mitochondrial outer membrane, translocases TIM23 and TIM22 complexes are required for protein transport through the mitochondrial inner membrane (Jensen and Dunn, 2002). TIM14 is a component of TIM23 and interacts with other TIM proteins and mitochondrial heat shock proteins ATP-dependently to carry out mitochondrial protein transport. The TIM23 complex has membrane and import motor components, with TIM14 acting as an essential part of the mitochondrial import motor. As a result, TIM14 deficiency disrupts protein import into the mitochondria in yeast, which can have lethal consequences (Mokranjac *et al.*, 2003). Considering a statistically significant low abundance of a TIM14 subunit and chaperones including the heat shock protein 75kDa mitochondrial and calreticulin, in addition to identified mitochondrial-related translation proteins and the five proteins identified using BRITE as part of mitochondrial biogenesis, mitochondrial functioning or regeneration seem to be altered by exposure to Proline®. In addition, multiple proteins associated with the proteasome had a decrease in abundance, including proteasome subunit alpha type 7-1 and proteasome subunit beta type-3, indicating alterations to protein metabolism, processing, or homeostasis. In addition, SSDA proteins with the greatest decrease in abundance were transmembrane protease serine 9, (RFC - 40.6) and venom dipeptidyl peptidase 4 (RFC – 7.3), both of which participate in proteolysis. Triazoles are often used to increase the stability of pharmaceutical peptides as they permit high stability against proteases and isomerases (Staśkiewicz *et al.*, 2021). Interestingly, Xie et al. (2022) found that prothioconazole increased mitochondrial apoptosis in the liver of mice. Here we see in the *B. terrestris* digestive tract, that the formulation Proline® can alter translation and protein metabolism and homeostasis with a possible relationship to the mitochondria.

23% of identified proteins with glutathione peroxidase activity in the genome were SSDA increased after PCF exposure. These proteins included glutathione S-transferase, phospholipid hydroperoxide glutathione peroxidase, and one of the

proteins with greatest increase in abundance in the entire experiment, microsomal glutathione S-transferase 1, which had an abundance almost ten times higher than the control. Given the context of Proline® exposure, these proteins indicate defence against pesticide ingestion and destructive consequences as they are associated with xenobiotic detoxification and oxidative stress regulation. Papadopoulos et al. (2004) identified increased glutathione S-transferase (GST) production in honeybees in response to insecticides, low temperature, and starvation, indicating their importance in stress responses. In addition, honeybees exposed to acaracides to treat the parasitic mite *Varroa destructor* had increased GST activity (Wahida *et al.,* 2008). GSTs can neutralise xenobiotics via several mechanisms including conjugation of the reduced glutathione site (GS^-) with the electrophilic site of a toxic compound to form a thioester. This aids in neutralising the compound by occupying the electrophilic site to reduce damage to cellular components and by increasing the toxic compounds water solubility to aid in excretion (Enayati *et al.,* 2005). In addition, GSTs can (i) act as a cofactor for metabolic reactions, with GS^- removing a hydrogen atom from the substrate, (ii) reduce peroxide-containing compounds produced from oxidative stress caused by pesticides and reduce lipid hydroperoxides produced from an oxidative environment, and (iii) passively bind and sequester xenobiotic compounds (Pavlidi *et al.,* 2018). These mechanisms have been identified in the GST-associated resistance of insects to various insecticides (Clark and Shamaan, 1984; Vontas *et al.,* 2001; Enayati *et al.,* 2005; Riveron *et al.,* 2014; Pavlidi *et al.,* 2018), inferring a potential protective role of increased GSTs in the *B. terrestris* digestive tract after PCF ingestion.

Eleven SSDA proteins with an increased abundance engage in cytoskeleton organisation. These proteins included troponin C, actin, tubulin beta-chain, and cofilin/actin-depolymerising factor. The cytoskeleton and major components such as tubulin, an important microtubule component, and actin, an important intermediate filament component, are vital for cell structural integrity. Transmembrane protein complexes allow cell-cell adhesion by linking to the cytoskeleton. Alterations to cytoskeletal proteins may compensate for epithelial cell damage, with recent histopathological studies finding that prothioconazole exposure can lead to liver cell injury and colon structural damage in mice (Meng *et al.,* 2021; Tian *et al.,* 2022). Further, in an investigation of Proline® impacts on human dendritic cells, de Ávila et

al. (2022) found that Proline® induced cytoskeleton reorganisation and glucose metabolism alterations.

In addition to its structural role, the cytoskeleton plays a role in cell signaling and protein and RNA localisation. The transmembrane complexes which aid in cell-cell adhesion, which also act in localising and activating signalling molecules in association with the cytoskeleton, can trigger cytoskeletal reorganisation and alter cellular structure (Janmey, 1998). It is important to note that proteins involved in cell signaling pathways were also significantly increased after PCF exposure, including proteins with a high RFC such as integrin and multiple inositol polyphosphate phosphatase which had an abundance 4.8 and 4.9 times that of the control treatment, respectively. In addition, the protein with the highest abundance after PCF exposure is functionally uncharacterised, uncharacterised protein Loc100647671, and had an abundance 34 times higher than the control. Protein kinase C-like/Diacylglycerol (DAG)-binding and C2 domains were identified in this protein. DAG is an important 2nd messenger in cell signaling pathways and can activate serine/threonine protein kinases known as protein kinase C's, of which the N-terminal can bind DAG's. C2 domains are calcium dependent and found in endoplasmic reticulum membrane bound proteins. These proteins can bind lipids and transport phosphatidylinositol, a phosphatidylinostil-4,5-bisphosphate precursor– a 2nd messenger in the inositol triphosphate signaling pathway important for regulation of cell signaling and actin cytoskeleton organisation -, across the endoplasmic reticulum membrane (Berridge, 1993; Yang *et al.*, 2002). Therefore, this uncharacterised protein may play a key role in the digestive tract in protein transport, localisation and cell signaling.

As stated in Jager et al. (2013), all organisms obey mass and energy conservation laws. If there are increased resources allocated to detoxification, oxidative stress regulation, cytoskeleton organisation and translation, energy is needed. Further, it will come at the cost of other traits which could benefit from these energy resources, resulting in a trade-off. A need for increased energy resources may be reflected in this dataset by an increase in proteins involved in both glycolysis and gluconeogenesis including hexokinase type-2, malate dehydrogenase, phosphoglycerate mutase, and triosphosphate isomerase. From this research, we can assume that bees are not acquiring a higher than usual source of energy, as food consumption was not altered

after PCF exposure. As a result, we may assume that energy allocation to physiological processes have been altered in the digestive tract, which may have consequences for other physiological processes which may be reflected in statistically significant proteins with a decreased abundance after PCF exposure. Whether this could result in long term consequences needs further research, and an overall understanding of Proline® exposures impacts on general fitness would need an examination of multiple endpoints at various time points over various exposure periods. What these results do highlight, however, is that Proline® alters core physiological processes, including the induction of detoxification proteins in the bumblebee digestive tract. Whilst it is encouraging to see a defence mechanism mounted against Proline® in the digestive tract, other impacts of Proline® could lead to negative impacts either directly through alterations to the cytoskeleton, translation, energy allocation and protein metabolism, or indirectly, through a lack of energy resources allocated to other important physiological processes.

4.4.2.2 The Impact of Technical Grade Prothioconazole on the Digestive Tract Proteome

There were 45 SSDA proteins with an increased abundance and 39 SSDA proteins with a decreased abundance in PAI-treated bees compared to the acetone control. Proteins with an increased abundance were associated with basement membrane organization, fatty acid metabolism and the electron transport chain. Decreased proteins were involved in cytoskeleton organization and membrane trafficking.

Three proteins were increased with involvement in basement membrane organisation: Nidogen 2, laminin subunit beta 1, and the most increased SSDA protein, collagen alpha-5(IV) chain protein, which had an abundance 8.24 times higher than the acetone control. Both laminin and collagen IV are major constituents of the basement membrane, a type of extracellular matrix at the base of epithelial and endothelial tissues which separates epithelial and endothelial cells from the tissue stroma (Paulson, 1992). Changes to the basement membrane can regulate the cell environment and alterations may be associated with damage to epithelial cells (Altincicek *et al.,* 2009; Saleh *et al.,* 2018). The glycoprotein nidogen binds to both collagen IV and laminin, thought to function as a linker between the two basement membrane components, and is required for basement membrane maintenance in some

tissues. In fly embryos, laminin is vital for the assembly of all basement membrane proteins (Urbano *et al.*, 2009). In addition, nidogen may be important for lipid metabolism or storage, as a loss of nidogen in *Drosophila* led to reduced lipid content and smaller lipid droplets in the fatbody (Dai *et al.*, 2018). However, all components are important for proper basement membrane functioning, as laminin depletion results in low nidogen abundance in the gut, as demonstrated by Dai et al. (2018) in *Drosophila* embryos.

Both fatty acid metabolism and oxidative phosphorylation were altered after PAI exposure. The proteins 3-ketoacyl-CoA thiolase, acetyl-CoA carboxylase, and short/branched chain specific acyl-CoA dehydrogenase were all increased and associated with fatty acid metabolism. Subunits B and G of mitochondrial ATP synthase and subunits 4 of cytochrome c oxidase were increased, both involved in the electron transport chain which drives oxidative phosphorylation. In addition, glucose-6-phosphate 1-dehydrogenase was increased, which catalyses the rate-limiting step of oxidative phosphorylation. Not only is D-ribulose 5-phosphate produced from D-glucose 6-phosphate, which can be used in energy production, but cellular NADPH is produced in this process which is required for fatty acid biosynthesis (Park *et al.*, 2005). In previous studies, prothioconazole ingestion led to metabolic alterations in mice. Meng et al. (2021) observed lipid accumulation in mice liver cells. Additionally, prothioconazole altered the metabolite profile of the mouse digestive tract, with a significant decrease in α-glucose. In research by Tian et al. (2022), prothioconazole also altered the metabolic profile of mice liver cells. Gene transcripts and metabolites with known involvement in glycolysis and fatty acid synthesis were altered after prothioconazole exposure. Further, prothioconazole has been shown to alter lipid metabolism in zebrafish embryos and larvae (Tian *et al.*, 2019).

Interestingly, an attempt at detoxification can be observed in SSDA increased proteins after PAI exposure. The detoxification proteins peroxiredoxin 1 and cytochrome P450 6k1 were increased. Reactive oxygen species can be produced in reaction to xenobiotics, which can lead to oxidative damage of host DNA, proteins, and lipids. Peroxiredoxin 1 is an antioxidant thiol-specific peroxidase involved in reduction of reactive oxygen species such as hydrogen peroxide, to water and alcohols (Corona and Robinson, 2006). Cytochrome P450 on the other hand is a monooxygenase which can

oxidise steroids, fatty acids and xenobiotics using molecular oxygen to neutralise such compounds for excretion (Berenbaum and Johnson, 2015). Cytochrome P450s are involved in the detoxification of a number of pesticides in honeybees and some azole fungicides display synergistic toxicity with insecticides via cytochrome P450 inhibition, however, prothioconazole has not been found to inhibit cytochrome P450 activity (Haas and Nauen, 2021). An increase in basement membrane organisation proteins, lipid and carbohydrate metabolic proteins and detoxification proteins may represent an attempt to repair damaged digestive tract cells, provide the energy for such repair and detoxify prothioconazole itself.

Of the 39 SSDA proteins with a decreased abundance in PAI compared to the acetone control treatment group, six were associated with cytoskeleton organisation. Proteins associated with the cytoskeleton included the motor protein components myosin light chain and myosin vi-a, f-actin capping protein subunit alpha and actin-related protein 3, a component of the Arp2/3 complex which plays a role in actin polymerisation. Arp2/3 complex is important for actin filament nucleation, forming actin networks for cell motility and stability, in addition to playing functional roles in endocytosis and membrane trafficking (Goley and Welch, 2006). In addition, capping proteins, by binding to barbed ends of actin filaments, regulate actin cytoskeleton growth and motility and myosin, an actin-related motor protein, are involved in generating force on the cytoskeleton for multiple processes such as mitosis and axonal transport of cargo-containing vesicles (Carlier and Pantaloni, 1997; Vale, 2003; Kaksonen *et al.*, 2005; Goley and Welch, 2006). Interestingly, nine proteins were decreased after PAI exposure which were associated with membrane trafficking including proteins with the highest decreases in abundance in comparison to the acetone control such as golgin subfamily A member protein which was 5.4 times lower and NECAP-like protein cg9132 which was 2 times lower in abundance in PAI compared to acetone control treatment groups. According to Interpro, golgin subfamily A member proteins are Golgi auto-antigens, involved in maintaining Golgi structure and play an important role in restructuring of the Golgi apparatus during mitosis by interacting with small GTPase rab1b. In addition, golgin subfamily A proteins participate in vesicle tethering and fusion at Golgi apparatus cisternae, Golgi cisternae stacking, and regulating microtubule organisation. Overall, these proteins are important for protein trafficking in the Golgi apparatus (Weide *et al.*, 2001; Zhou *et al.*, 2014). NECAP-like protein

cg9132 is an accessory protein which binds to and aids major components of clathrin-coated vesicles, aiding in clathrin-coated vesicle formation (Ritter *et al.*, 2004). Overall, a decrease in proteins associated with cytoskeleton organisation with known roles in endocytosis and membrane trafficking, along with dramatic decreases in proteins involved in clathrin-coated vesicle formation and Golgi apparatus restructuring, suggests a role for prothioconazole on the impact of protein transport or synthesis, or perhaps, to the Golgi apparatus itself. In addition, the increase in proteins associated with basement membrane structure and metabolism highlight possible injury to digestive tract epithelial cells and a high energy requirement. Whether alterations to the Golgi apparatus are a direct consequence of injury or damage to epithelial cells, or a trade-off consequence of increased energy demand is yet to be elucidated.

4.4.2.3 Comparing the Impacts of Technical Grade Prothioconazole and Proline® on the Digestive Tract

Whilst results from the Proline® and prothioconazole treatment datasets cannot be directly compared due to the presence of acetone for technical grade prothioconazole solubilisation in PAI treated bees, the major processes altered can be qualitatively compared to one another to determine if both PCF and PAI have a similar impact or if the presence of co-formulants in the PCF alter the outcome of prothioconazole exposure in the *B. terrestris* digestive tract.

A direct comparison of PCF and PAI SSDA proteins (in respect to their relative controls) did not yield many common proteins across the two datasets. In both PCF and PAI, there were two shared proteins with similar RFCs in both SSDA proteins with an increased and decreased abundance. Proteins with an increased abundance were cAMP-dependent protein kinase type I regulatory subunit (RFC PAI: + 2.4; RFC PCF; + 2.8), involved in protein phosphorylation regulation, and peptidyl-prolyl cis-trans isomerase (RFC PAI: + 1.2; RFC PCF: + 1.3), involved in the acceleration of protein folding. Decreased proteins shared between the two datasets were transcription factor BTF3 homolog 4 (RFC PAI: - 1.2; RFC PCF: - 1.4), involved in the positive and negative regulation of protein translocation into the mitochondria and endoplasmic reticulum, respectively, and coatomer subunit epsilon (RFC PAI: - 1.2; RFC PCF; - 1.1), associated with Golgi homeostasis and essential for the retrograde

Golgi-to-ER transport (Letourneur *et al.,* 1994). Whilst this subset of proteins is far too small to elucidate a common impact of prothioconazole on the *B. terrestris* digestive tract, it seems that alterations to protein homeostasis and transport occur regardless of whether the prothioconazole was solely technical grade or part of a commercial formulation.

Exposure to both PAI and PCF led to an increase in detoxification proteins. For PCF, this involved GST proteins and a phospholipid hydroperoxide glutathione peroxidase, important in the neutralisation of xenobiotics and defence against oxidative stress. In PAI, detoxification proteins peroxiredoxin and cytochrome P450, a monooxygenase well documented in pesticide detoxification, were increased. Further, both pesticide treatments altered metabolism, although in different ways. PCF exposure altered glycolysis, while PAI exposure altered lipid metabolism and oxidative phosphorylation.

Interestingly, exposure to the triazole myclobutanil led to a higher amount of unmetabolized quercetin in the midgut and lower thoracic ATP levels compared to bees consuming quercetin alone (Mao *et al.,* 2017). In mice liver, prothioconazole exposure also increased lipid metabolism, but decreased glycolysis and glucose and fatty acid transport (Tian *et al.,* 2022). However, Proline contains N-N-Dimethyldecanamide along with other undisclosed co-formulants. N-N-Dimethyldecanamide is made up of methylated fatty amides which could alter energy resources available due to more energy resources allocated to catabolism of this compound, and/or the metabolic pathways required for its catabolism are different to those required for prothioconazole alone, leading to different metabolic alterations observed after PCF exposure. Jaffe et al. (2019) found that honeybees collected significantly less pollen from Proline®-treated cranberry trees compared to non-treated trees, however this cranberry pollen deficit was substituted with non-cranberry non-treated forage. Proline® altered foraging behaviour but not food consumption in this scenario. However, in areas of large monocultures, alternate forage may be sparse and the ability to forage for alternate food sources further afield will depend on the bee species. Whether the avoidance of honeybees to Proline®-treated crops is due to a deterrent effect has yet to be studied. As observed in this study, consumption differences were not statistically significant between PCF and PAI or control

treatments which indicates that, at least for *B. terrestris*, Proline®-treated food will be consumed if there are no other options. However, this could be different for pollen consumption, which was not investigated in this research.

Whilst PCF exposure led to an increase in proteins associated with cytoskeleton organisation, PAI exposure led to a decrease in cytoskeleton organisation proteins, determining in some cases, opposite impacts of PCF and PAI exposure on the *B. terrestris* digestive tract. Further supporting this, uncharacterised protein LOC 100647671 was the SSDA protein with the most decreased abundance after PAI exposure, nearly 19 times lower than the relative acetone control group. However, this same protein was the protein with highest abundance after PCF exposure, with an abundance 34 times higher than the control treatment group. Whilst this protein is uncharacterised, the presence of a protein kinase C/DAG-binding domain and C2 domain suggests this protein is a serine/threonine protein kinase C, which engage in cellular signaling events. C2 domains are membrane-bound endoplasmic reticulum proteins which bind lipids and transport precursors for phosphatidylinositol-4,5-bisphosphate, important for inositol triphosphate signaling which regulates cell signaling and actin cytoskeleton organisation (Berridge, 1993; Yang *et al.*, 2002). Whilst its exact function is unknown, this proteins link to cytoskeleton organisation, and the opposite profiles of cytoskeleton organisation in PAI and PCF, may give insight into the opposite abundance profiles of this protein. Regardless, the polarising impacts of PCF and PAI on the abundance of this protein are intriguing for future research on the impacts of triazole fungicides and co-formulants on non-target organisms.

4.4.2.4 The Acetone Impact: Differences Between the Acetone and Non-Acetone Control Treatments on the Digestive Tract

OECD guidelines on chronic and acute oral toxicity testing of chemicals on bees suggests the use of acetone as a solvent for low water solubility test chemicals. Guidelines suggest that treatment solutions with up to 5% acetone are permissible, but both a non-acetone and acetone control of the same acetone concentration must also be investigated (OECD, 2017a, 2017b). Accordingly, considering the low water solubility of prothioconazole, acetone was present in the PAI treatment group at 0.3%, resulting in the use of both a non-acetone control of 40% (w/v) sucrose solution and

an acetone control containing 0.3% acetone. This is a common practice amongst pesticide researchers investigating the impacts of low water-soluble pesticides on bees, presumably to avoid the complications that come with pesticide formulations and hidden co-formulants (Stanley and Raine, 2017; Sgolastra *et al.*, 2018; Strobl *et al.*, 2020). However, in this research, the digestive tract proteome of bees exposed to 0.3% acetone compared to the non-acetone control proteome gives cause for concern regarding the safety profile of acetone use in bee oral toxicity assays.

Acetone exposure led to an increase in detoxification proteins in the *B. terrestris* digestive tract. Glutathione-s-transferase, cytoglobin 2, glutathione synthetase, thioredoxin reductase 2, mitochondrial, and superoxide dismutase were SSDA proteins after acetone control exposure. Glutathione-S-transferase and glutathione synthetase are involved in the neutralisation of xenobiotics and reactive oxygen species produced from oxidative phosphorylation in the mitochondria or from oxidative damage as a result of pesticide exposure (Papadopoulos *et al.*, 2004; Wahida *et al.*, 2008; Marí *et al.*, 2009). From the same pathways, superoxide dismutase is responsible for clearing superoxide anions and thioredoxin reductase is involved in glutathione metabolism and the superoxide dismutase/catalase defence and thioredoxin system to reduce thioredoxin and allow it to act as an electron carrier, protecting proteins from oxidative stress via oxidative formation of disulfide bridges (Arnér and Holmgren, 2000). Cytoglobin is also involved in oxidative stress regulation, with superoxide dismutase activity to protect cells against superoxide radicals (Zweier *et al.*, 2021). Stadler et al. (2008) determined that acetone can induce lipid radical formation in mice livers after acute exposure. After five days of acetone exposure, evidence of protein oxidation and damage was observed. In the results presented in this chapter, the presence of proteins associated with reactive oxygen species neutralisation suggests an oxidative environment after acetone exposure. This can lead to the oxidation of proteins and lipids, leading to protein aggregation and dysfunction. In addition, eleven proteins associated with translation and nine proteins associated with the proteasome were increased, as well as thirteen chaperone and folding catalysts including five heat shock proteins, indicating possible oxidative damage to proteins, with an increased requirement for the removal of aggregated and damaged proteins as well as a need to replace these proteins with an increase in protein translation machinery such as ribosomal subunits and elongation factors. Two heat

shock related proteins were in the top ten most increased SSDA proteins after acetone control exposure. This included protein lethal(2) essential for life protein which, an alpha-crystallin chaperone-like protein with similarity to small heat shock proteins, with the ability to prevent denatured protein aggregation and increase cellular resistance to stress (Augusteyn, 2004). This protein had an abundance 5.5 times higher than the control. Further, activator of 90 kDa heat shock protein atpase had an abundance 3.8 times higher than the control. This protein binds to heat shock protein 90 to stimulate its ATPase activity and is a general up regulator of heat shock protein 90, increasing the efficacy of the cellular response to stress (Panaretou *et al.*, 2002).

Further, proteins associated with cytoskeleton organisation were increased, which are involved in cell structural integrity as well as membrane trafficking of proteins in the endoplasmic reticulum and Golgi apparatus, which could be required due to an increase in the translation of proteins (Egea *et al.*, 2015). Further supporting this theory are that 19 proteins associated with membrane trafficking and 31 proteins associated with exosomes were enriched in BRITE analysis of increased SSDA proteins following acetone control exposure.

Fifteen of the 43 SSDA proteins decreased after acetone exposure are associated with the mitochondrion. These included components of the electron transport chain and fatty acid metabolism, indicating disruption to energy metabolism via oxidative phosphorylation and fatty acid metabolism alterations. Two proteins were subunits of the cytochrome B-C1 complex, an enzyme important in the catalysis of ubihydroquinone oxidation and cytochrome c reduction in the electron transport chain. Further, subunit 4 of cytochrome c oxidase, the final enzyme in the electron transport chain, was significantly decreased in the acetone control treatment group compared to the control, indicating disruption to oxidative phosphorylation.

Phospholipase A2, a lipase involved in phospholipid metabolism and the promotion of cell lysis, was 62 times lower in the acetone control compared to the non-acetone control group. Melittin, a haemolytic and antimicrobial protein often associated with bee venom glands, was 501 times lower in the acetone control compared to the non-acetone control. This may be due to its link with phospholipase A2, with high melittin levels associated with high phospholipase A2 levels (Ferreira Junior *et al.*, 2010). In addition to phospholipase A2's lipase action on phospholipids, melittin is amphipathic

and binds to the negatively charged cell membrane surface, forming a pore as it inserts into the membrane lipid bilayer (Glättli et al., 2006). This action can cause intracellular molecules and ions to leak, increasing cell permeability and cell lysis. Interestingly, acetone ingestion has been associated with phospholipid membrane disruption and cell lysis in microorganisms. In a study of various organic solvents on several microorganisms, Dyrda et al. (2019) found that acetone was one of the most toxic solvents tested despite acetone considered as one of the lowest toxicity compounds of those tested. Authors determined this could be due to acetone's impact on the phospholipid membrane, as microorganisms with more phospholipid-rich membranes had lower viable cell counts after acetone exposure, although all microorganisms had lower cell counts compared to other solvents and the control. In addition, molecular dynamic simulations show that acetone favours partitioning into phospholipid bilayers, with higher acetone concentrations accumulating in the membrane, pushing phospholipid heads apart leading to alterations to phospholipid packing and increasing membrane fluidity (Posokhov and Kyrychenko, 2013).

Acetone is naturally produced in small amounts during fatty acid metabolism. Acetoacetate is a ketone body used for energy which can be decarboxylated to produce acetone. Stadler et al. (2008) found that increased levels of acetone, which can happen naturally during ketosis, increased the formation of free radicals in mice livers as a result of enhanced lipid peroxidation after acute exposure. After five-day exposures, free radical generation and markers of lipid and protein peroxidation were significantly increased. However, Buron et al. (2009) found that whilst acetone altered neuro-olfactory epithelium cells in mice, these cells were able to recover to their pre-acetone exposure state after 2-4 weeks, indicating that short-term exposure to lower concentrations may not have long-lasting impacts.

Overall, the presence of acetone seems to alter the digestive tract proteome by increasing oxidative stress, which could lead to protein and lipid oxidation, damaging proteins and leading to protein aggregation and dysfunction. In turn, chaperones would be required to assist in the removal of these proteins, as well proteases and the proteasome, of which there was an increase in our dataset. Further, a major decrease in melittin and phospholipase A2, proteins usually associated with cell membrane disruption, may have been decreased in an attempt to regulate cell lysis if acetone was

leading to increased cell lysis. An increase in cell membrane fluidity and damaged proteins may be the reason for an increase in cytoskeletal organisation and translation proteins to compensate for a loss of viable cells and functional proteins. In addition, a decrease in oxidative phosphorylation and fatty acid metabolism could be in response to increased protein and lipid peroxidation, as shown in mice livers, when there is an increase in acetone concentration. These processes naturally lead to a low level of reactive oxygen species and acetone, which could lead to further cellular stress.

The studies discussed in this section and the results presented in this chapter regarding the impact of acetone on the digestive tract proteome is a cause for concern given the reliance of acetone as a low toxicity solvent in toxicological and regulatory assessments. Despite accounting for acetone concentrations in pesticide treatments by using an acetone control, the impacts on the cellular oxidative environment and membrane fluidity could increase the negative impacts of pesticides tested. If pesticides more readily penetrate cells or are consumed in conjunction with increased cell lysis and oxidation conditions, the impacts of this stress on cellular functioning would be compounded. The regulatory restriction of the number of co-formulants available for use in formulations and a full suite of toxicity tests carried out on formulations used in the field would give a more realistic view of the impacts of pesticides as they would be encountered in the environment. Whilst acetone is sometimes necessary to increase the solubility of pesticidal compounds for testing, open access to the full co-formulation profile of formulations used in agriculture and gardens would provide a more realistic experimental set-up for researchers to determine pesticidal impacts on bees.

4.4.3 Prothioconazole Alters the Digestive Tract Microbiota

Whilst fungicides can impact the microbiome of mice and soil invertebrates (Jin *et al.*, 2018; Zhang *et al.*, 2019), there is a lack of research on the impacts of fungicides on the bee microbiota. Further, the majority of studies investigating the impacts of pesticides on the bee microbiota use 16S amplicon sequencing, but do not investigate gene regions which would give information on fungal genera (examples include Blot *et al.*, 2019; Motta and Moran, 2020; Motta *et al.*, 2020; Castelli *et al.*, 2021). Whilst fungicides can impact the microbiome of mice and soil invertebrates (Jin *et al.*, 2018; Zhang *et al.*, 2019), there is a lack of research on the impacts of fungicides on the bee

microbiota. Further, the majority of studies investigating the impacts of pesticides on the bee microbiota use 16S amplicon sequencing, but do not investigate gene regions which would give information on fungal genera (examples include Blot *et al.,* 2019; Motta and Moran, 2020; Motta *et al.,* 2020; Castelli *et al.,* 2021).

This research determined that prothioconazole altered the digestive tract microbiota of *B. terrestris.* Surprisingly, both prothioconazole-based treatments had a larger impact on the number of bacterial species with significantly altered relative abundances compared to fungal species. This was an unexpected finding given that prothioconazole is used as an antifungal agent. Both PAI and PCF exposure led to a decrease in *Lactobacillus* species and 16S OTUs assigned to the mitochondrion, which may explain alterations to mitochondrion-associated proteins in the proteome datasets. Several *Lactobacillus* species were absent from PCF treated bees, but not the control treated bees. When exposed to a different triazole fungicide, difenoconazole, mice exhibit alterations to their digestive tract bacterial microbiota, with alterations to the abundance of *Lactobacillus* species at low and high concentrations (Bao *et al.*, 2022), as seen for both prothioconazole-based treatments in this research.

After PCF exposure, fungal community composition was significantly altered, with the relative abundance of *Candida* species statistically significantly impacted in PCF exposed digestive tracts, but not PAI exposed bees, indicating the ability of co-formulants in Proline® to alter microbial species abundance differentially to prothioconazole. Bartlewicz et al. (2016) determined that prothioconazole was highly toxic to nectar yeasts, including *Candida* species, from concentrations as low as 0.06 to 0.5 ppm. This could be harmful for newly emerged worker bees who have yet to obtain their commensal microbiota. Whilst the majority of the digestive tract microbiota are obtained from other nest mates and/or the queen upon eclosure, it is thought that some core fungal species may be a result of ingestion when foraging for pollen and nectar (González *et al.*, 2005; Hammer *et al.*, 2021). In addition, the yeast profiles of pollen and nectar could be impacted by prothioconazole treatment, which could have downstream impacts for bee nutrition and crop pollination as studies have shown that the yeast profiles of nectar can alter foraging behaviour (Herrera *et al.*, 2013; Schaeffer and Irwin, 2014). Peng et al. (1984) found that honeybees can digest *Candida utilis*, which could provide nutrition for bees. Additionally, whilst no impacts

on consumption were observed in this investigation, some studies suggest that microbiota alterations can alter sucrose sensitivity and feeding motivation in honeybees, which could further alter the nutritional status of bees with altered digestive tract microbiota (Zheng *et al.*, 2017). Further, Hoang et al. (2015) found that yeast species in the *D. melanogaster* digestive tract had varying sensitivities to reactive oxygen species, which could explain alterations to fungal microorganisms in this investigation since the digestive tract proteome dataset of both PAI and PCF treated bees suggested oxidative environments.

A statistically significant increase in *Gilliamella* was observed in the PAI treatment group compared to its relative control. The digestion of nectar can be complimented by the host digestive tract microbiota. The most common fermentative bacteria in the bee digestive tract include *Gilliamella* and *Lactobacillus* species, which may have a role in the digestion of sugars (Kwong and Moran, 2016). Zheng et al. (2016) determined that certain *Gilliamella* strains can metabolise some of these sugars. Whilst this is strain dependent, amplicon sequencing cannot identify microorganisms at the sub-species level, so it cannot be determined if strains involved in the metabolism of these sugars were altered by prothioconazole.

Additionally, the fungal species *A. flavus* was present in the PAI treatment group, but not the acetone control treatment group. *Aspergillus* fungi are ubiquitous in the environment and opportunistically infect and cause disease in plants and animals, including insects (Kwon-Chung and Sugui, 2013; Becchimanzi and Nicoletti, 2022). Highlighting this, Foley et al. (2014) found that 30% of *Aspergillus* species identified in honeybee colonies were honeybee pathogens. One of these species was *A. flavus*, which led to significantly increased mortality in larvae and adult honeybees. In addition, *A. flavus* is noted as one of the main causes of stonebrood in honeybee larvae which, to my knowledge, has not yet been reported in bumblebees. Further, it is one of the most common *Aspergillus* species to infect bees (Becchimanzi and Nicoletti, 2022). Considering the high mortality and virulence reported from *A. flavus* infection in honeybees (Vojvodic *et al.*, 2011; Foley *et al.*, 2014), alterations to the digestive tract microbiota by prothioconazole could create the opportunity for *A. flavus* to colonise and proliferate to an abundance which could cause significant damage, disease, or mortality. Highlighting the impact of microbiota dysbiosis on the growth

of opportunistic pathogens, Miller et al. (2021) determined that the presence of *Bombella apis* could suppress *A. flavus* growth in honeybees. Perhaps if the experimental exposure period were to be extended, the impact of *A. flavus* colonisation in a perturbed digestive tract microbiota could have led to significant disease and mortality. However, since this is an opportunistic pathogen, it is impossible to tell if this would be the case, as mortality was not increased in ten-day survival assays conducted in triplicate. Whether this was due to the absence of *A. flavus* during these assays, or *A. flavus* promoting less toxicity in bumblebees compared to honeybees, is yet to be investigated. Additionally, *A. flavus* was not increased in PCF treated bees. Whether this is due to a difference in microbial species altered, e.g., a species increased in the PCF but not the PAI treatment group could have direct or indirect inhibition on *A. flavus* proliferation, or due to protective effects of one or more co-formulants, e.g., via an inhospitable environment for growth, is unknown.

In accordance with the impacts of acetone observed on the digestive tract proteome, when compared to the non-acetone control, acetone altered the digestive tract microbiota significantly. Both alpha and beta diversity were significantly different between the acetone control and control, indicating acetone mediated alterations to microbiota species richness and community composition. There were a number of significant differences in the relative abundance of bacteria in the acetone control group compared to the control group, including an absence of *Hymenobacter* and *Helicobacter* bacteria after acetone exposure. In addition, the fungal species *R. mucilaginosa*, a common human pathogen, was present in the acetone control but not the non-acetone control treatment group. Whilst there is little information on the impacts of *R. mucilaginosa* on bees, strain IM-2 has been identified as capable of detoxification of neonicotinoid insecticides (Dai *et al.*, 2010), indicating a possible role in detoxification of other xenobiotics such as acetone. Further, the impacts of acetone on the digestive tract microbiota are not fully unexpected. Dyrda et al. (2019) found that, despite acetone considered a low toxicity solvent, it displayed the highest toxicity to *E. coli, Bacillus subtilis* and *Saccharomyces cerevisiae* in cell viability assays, with the study suggesting toxicity as a result of alterations to the phospholipid membrane structure. Taken into consideration with the results found in this investigation, acetone could have serious impacts on both the digestive tract microbiota and epithelial cells. Further, these alterations could exacerbate any

pesticide impacts on host and microbiota cells. However, this requires further investigation.

4.4.4 Prothioconazole Impact on *B. terrestris* Digestive Tract: An Adverse Outcome Pathway Model

This research elucidated, for the first time, the impact of the commonly used triazole fungicide prothioconazole on the digestive tract of *B. terrestris* from the molecular to organism level. Based on prothioconazole active ingredient and the formulation, Proline®, impact on *B. terrestris*, we can begin to build up an AOP from the investigation thus far. The digestive tract is the first defence against pathogens, providing a physical and immune barrier to infection, in addition to being vital for nutrient digestion and absorption (Lemaitre and Miguel-Aliaga, 2013). In addition, it is the most likely point of contact for ingested xenobiotics such as pesticides, making the determination of pesticidal impacts on digestive tract health a worthy investigation. However, there are no studies on the impact of prothioconazole on the bee digestive tract, and very few on its toxicity to bees, with no research existing on prothioconazole direct impacts on bees prior to 2019 (Cullen *et al.*, 2019). Based on the intensive use of prothioconazole in Irish agriculture (López-Ballesteros *et al.*, 2022) and one third of Irish bees at threat of extinction, it is important to quickly and efficiently understand the possible dangers of prothioconazole use to employ mitigation strategies. To ensure this, using an AOP model can aid in clearly defining the known impacts of prothioconazole on pollinators whilst identifying major gaps for urgent research across multiple biological levels (Ankley *et al.*, 2010).

The research conducted in this chapter determined prothioconazole impacts on *B. terrestris* at the organism and molecular level on the digestive tract. Based on these findings, the identification of proteins, pathways, processes, and structures which may be impacted by prothioconazole can be elucidated to populate the molecular, cellular, organ and organism levels of an informative AOP based on possible negative impacts (Figure 4-20). Similar to the impact of glyphosate on the digestive tract, a number of interrelated pathways and processes were altered by prothioconazole including i) significant alterations in behaviour at low doses of prothioconazole active ingredient (behaviour affected) and Proline® (increased moribund incidence) but not high doses, ii) an attempt to detoxify prothioconazole, iii) an increased oxidative cellular

environment, iv) impacts on mitochondria and associated metabolism, altered differentially by the active ingredient and Proline®, v) alterations to cytoskeleton organisation, differentially impacted by both prothioconazole-based treatments, vi) alterations to protein translation, folding, and transport, and vi) differential alterations to the digestive microbiota, with Proline® leading to significant decreases in *Candida* relative abundance and significant alterations to microbiota community composition. Both prothioconazole-based treatments significantly impacted the relative abundance of *Lactobacillus*, with differential impacts on other bacterial species. Mortality and sucrose solution consumption were unimpacted.

Differential impacts of prothioconazole active ingredient and formulation highlight the ability of co-formulants, listed or unlisted, to alter pesticidal impacts on bee health. Further research is needed on the colony and population level, and additional molecular studies should be conducted that include various exposure periods, exposure routes, exposure to various co-formulants, recovery periods, and a deeper investigation on the impacts of opportunistic pathogens which may colonise the digestive tract after pesticide induced microbiota alterations. The exact nature of prothioconazole impacts on the digestive tract, similar to glyphosate, is yet to be fully elucidated. However, quantitative proteomics coupled with toxicity assays and amplicon sequencing of microbiota DNA, has enabled new hypotheses on how prothioconazole impacts the digestive tract, which can now be assessed further to elucidate a full AOP. With this insight we can then aid policy changes and mitigation strategies based on a comprehensive understanding of the impacts of triazole fungicides, including prothioconazole, on bees.

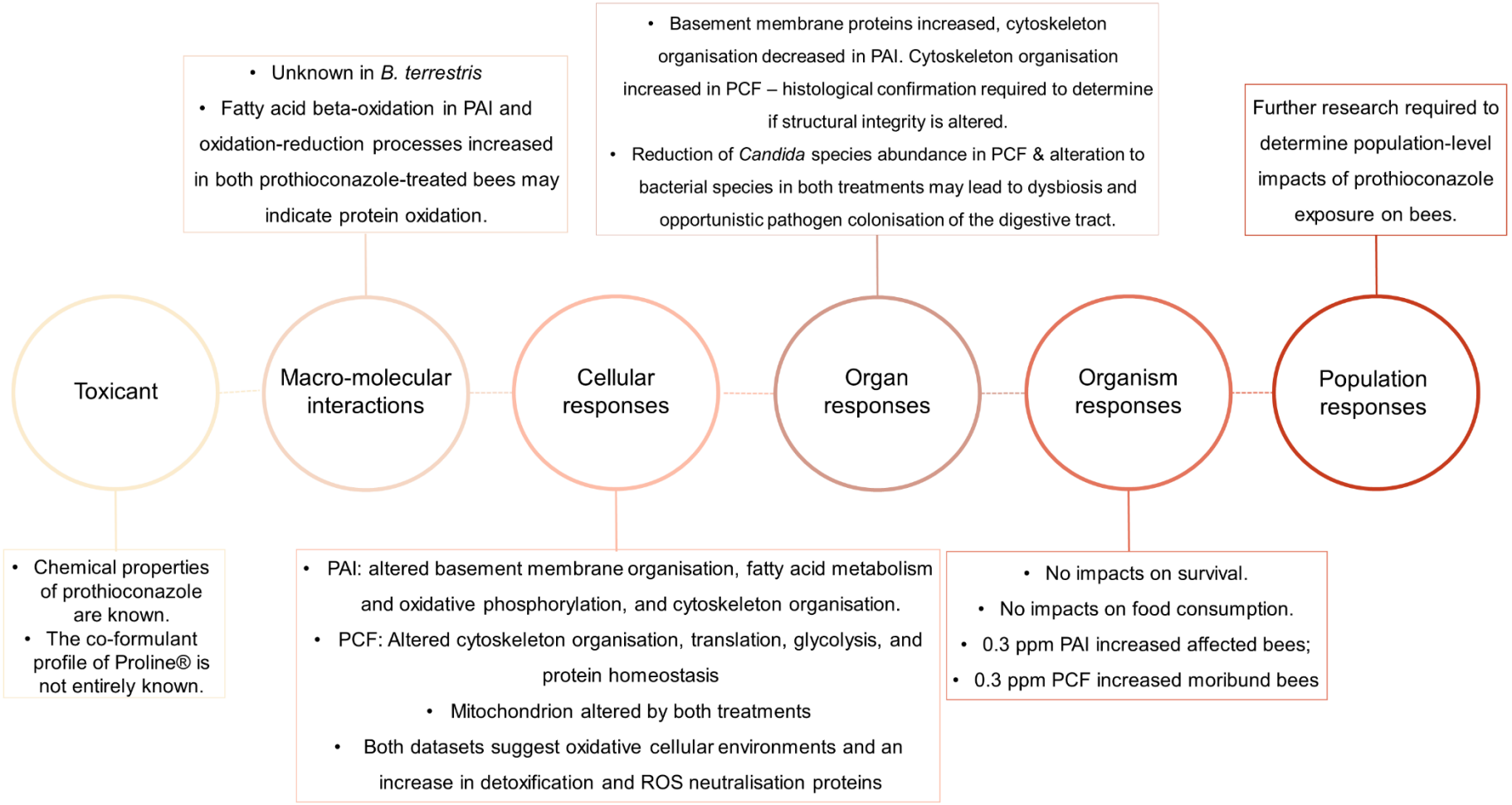


Figure 4-20 An Adverse Outcome Pathway for Prothioconazole Impacts on *B. terrestris*. The results of this chapter were used to populate an adverse outcome pathway to demonstrate prothioconazole active ingredient (PAI) and Proline® (PCF) impacts on the bumblebee digestive tract at the molecular and cellular level and on *B. terrestris* mortality, food consumption and behaviour at an organism level.

4.5 Conclusion

This chapter highlighted the impacts of the agriculturally relevant fungicide, prothioconazole, on *B. terrestris* mortality, food consumption, behaviour, digestive tract proteome and microbiota, resulting in an increased understanding of this fungicides potential negative effects on insect pollinators. In addition, by characterising impacts for both technical grade prothioconazole and the formulation Proline®, it was highlighted that the co-formulants present in commercial formulations can alter the impact of pesticides on bees. Prothioconazole did not impact mortality or food consumption, although some behavioural alterations were observed. Whilst prothioconazole increased proteins associated with the basement membrane, fatty acid metabolism and oxidative phosphorylation, Proline® exposure led to increases in cytoskeleton organisation, translation, and glycolysis. In addition, prothioconazole decreased proteins associated with cytoskeleton organisation and membrane trafficking, whilst Proline® decreased proteins also involved in translation, the proteasome and endocytosis. However, both prothioconazole treatments elicited an increase in proteins associated with detoxification and reactive oxygen species neutralisation, indicating an increased oxidative cellular environment after prothioconazole ingestion and both displayed impacts on the mitochondria. Further, both prothioconazole-based treatments significantly impacted the digestive tract microbiota, with more alterations to bacterial microbiota than fungal microbiota species. Whilst both treatments significantly altered the digestive tract microbiota, possibly leading to dysbiosis and opportunistic pathogen infection, some alterations differed. Proline®, but both technical grade prothioconazole, led to a significant difference in fungal community composition, with an observed decrease in the relative abundance of *Candida* species. Further, technical grade prothioconazole exposure led to increased relative abundance of *Gilliamella* species, which may play a role in nutrition, and increased *A. flavus* relative abundance, which is associated with disease in honeybees. In addition, the use and investigation of a low concentration acetone control, often considered as a low toxicity solvent, uncovered acetone-induced impacts on the digestive tract proteome and microbiota, including alterations to protein folding, translation, cellular stress responses and oxidative stress, and significant

impacts on the microbiota richness and community composition. This highlights a need for further research on the use of acetone as a solvent in pesticidal toxicity assays, as impacts from acetone exposure could exacerbate or alter the changes observed from pesticidal exposure.

Chapter 5
Investigating the effect of glyphosate and prothioconazole on the brain and fat body proteome of *B. terrestris*

5.1 Introduction

Whilst the type and magnitude of glyphosate and prothioconazole effects on the digestive tract proteome and microbiota discovered in chapters three and four were previously unknown, the presence of pesticide-induced alterations to the digestive tract were not unexpected. This is due to i) the digestive tract being a likely tissue to have direct contact with an orally ingested pesticide and ii) the recent increase in research on pesticidal perturbations to the digestive tract microbiota in various animals, including bees (Blot *et al.*, 2019; Motta and Moran, 2020; Bao *et al.*, 2022). However, there is truly little research on the impacts of non-insecticidal pesticides on other key bumblebee tissues (Cullen *et al.,* 2019). As a response to this gap in our understanding of non-insecticidal pesticides and bumblebees, this chapter aims to determine whether the impacts of glyphosate and prothioconazole can be determined beyond the digestive tract, specifically on two key tissues for bumblebee health: the brain and fat body.

5.1.1 The Bee Brain

The brain and ventral nerve cord make up the central nervous system of the bee. The brain consists of a large and complex network of neuronal groups above the oesophagus. A paired nerve trunk - the ventral nerve cord - connects the brain to groups of neurons along the ventral wall of the bee called ganglia which span the length of the bee body. This structure allows local control of movement, flight, and breathing at a segment level, and the firing of electrical impulses between ganglia and the brain at the systemic level. In this way, the overall control and co-ordination of the bee occurs in the brain. In addition, external and internal environmental information is processed in the brain, resulting in complex social behaviour, learning, memory, and sensory

integration in the bumblebee (Galizia *et al.,* 2012; Klowden and Palli, 2022; Stell, 2012).

Until 2021, bumblebee neurobiology researchers relied on the honeybee brain atlas constructed in 2005 (Brandt *et al.*, 2005; Rother *et al.,* 2021). Whilst both reconstructions confirm major similarities between the honeybee and bumblebee brain, there are differences in the volume of different areas and the number of defined sections (Rother *et al.,* 2021). The bumblebee brain consists of three main sections: the protocerebrum, the deutocerebrum and the tritocerebrum. Before discussing the main sections of the brain, we first need to consider the main cells which make up the brain: neurons and glial cells.

5.1.1.1 Neurons

Neurons are specialized nerve cells which facilitate electrical and chemical signalling. The main parts of a typical neuron are the soma (nucleus) and projections from the soma which include the axon and dendrites (Figure 5-1). The main organelles of the neuron are present in the soma, where a high demand for the production and packaging of proteins is facilitated via a high abundance of Golgi complexes and rough endoplasmic reticulum. The axon carries information away from the cell and dendrites, which are branched projections of the neuron, and can communicate chemically or electrically with other neurons. This is carried out via synapses which are the contact point between neurons. The synapse of a single neuron can communicate with hundreds of other neurons to integrate sensory inputs and outputs throughout the brain and central nervous system. An area of neuronal networks dense in synaptic connections and surrounded by glial cells is called a glomerulus (Klowden and Palli, 2022).

Neurons can be sensory, motor, interneurons, or neurosecretory neurons. Interneurons mediate the connections between sensory and motor neurons. Whilst sensory neurons transport information from sensory receptors, motor neurons are in close contact with muscles and regulate muscle contraction. Along the ventral nerve cord, ganglia contain groups of the somas of motor neurons and interneurons while sensory neuron somas are generally located near their respective sensory receptors. At the centre of a ganglion is the neuropil, which contains the axons, dendrites and synapses of the

ganglion neurons (Klowden and Palli, 2022). The bee brain, also known as the supraoesophageal ganglion, contains dense and diverse networks of neuropils, with 30 different neuropils identified in *B. terrestris* which comprise the three main sections of the brain (Rother *et al.*, 2021).

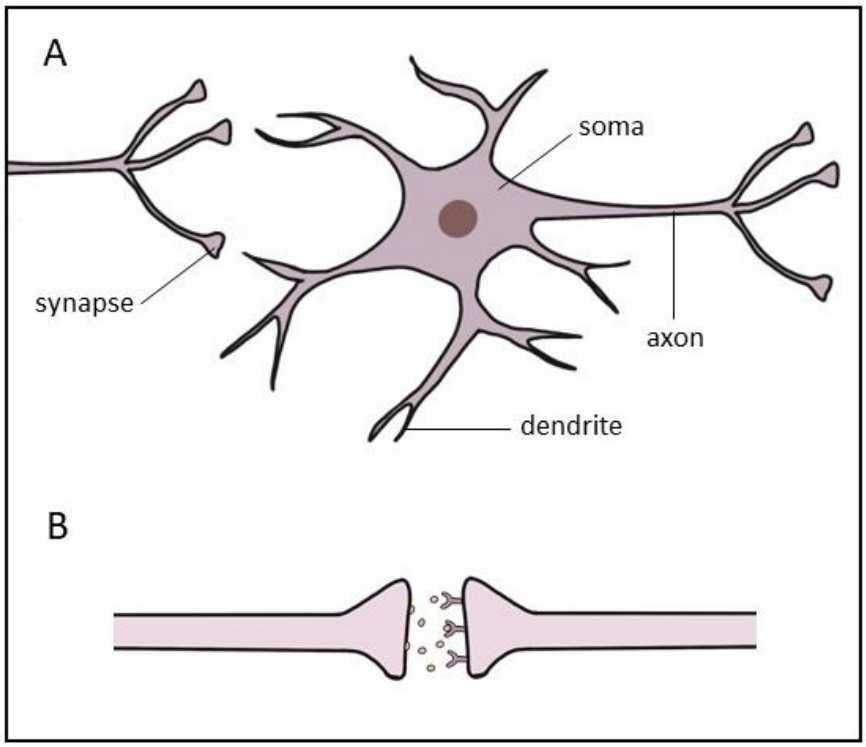


Figure 5-1 Neuron Structure and the Synaptic Cleft. (A) a typical interneuron schematic displaying the soma (cell body), dendrites, axon and synapses, and **(B)** the synaptic cleft, with the presynaptic neuron releasing neurotransmitter into the synaptic cleft, where neurotransmitters can bind receptors on the postsynaptic neuron membrane.

5.1.1.2 Glial Cells

Glial cells are specialized cells in the insect nervous system which aid in axon guidance and growth along with providing nutrients to neuronal cells. Additionally, glial cells are instrumental in protecting neurons via the blood-brain barrier and maintaining an environment conducive to the transport of electrical signals along neurons (Klowden and Palli, 2022).

The central nervous system is protected by two layers of glial cells. The outermost layer is made up of perineural glia, which secrete extracellular neural lamella. This population of perineural glia and lamella are referred to as the nerve sheath and play a major role in the blood-brain barrier. Perineural glia also provide neurons with nutrients via glycogen stores. The inner layer consist of subperineurial glia which are

large, flat, cells with an abundance of septate junctions to control the entry and exit of metabolites through the blood-brain barrier. Beneath this inner layer of subperineurial glia, cortex glial cells encase neuron somas and are responsible for outgrowth patterns from the soma. Neuropil glia surround the neuropil and can modulate transmission between neurons particularly in glomeruli. For example, astrocytes – a type of neuropil glial cell – can surround a synaptic junction and influence signal transmission via the regulation of ion concentrations and release of neurotransmitters or neuromodulators. Glial cells can also pair synapses with trachea (Klowden and Palli, 2022). This is essential, as the insect brain requires oxygen to maintain its high metabolic rate. Air sacs surround the brain which receive air from spiracles in the thorax. Air sacs in the brain lead to a network of branching tracheae, which are a network of interconnecting airways in insects, which further branch out until reaching target cells where gases can dissolve for metabolic use (Stell, 2012).

5.1.1.3 Neuronal Transmission

Ion transport across cellular membranes leads to a difference in electrical potential between the intercellular and extracellular space. In neurons, the electrical potential is approximately -70 mV in the absence of signalling from other neurons, this is known as the resting potential. The electrical potential changes substantially during stimulation. For example, when sensory receptors of sensory neurons detect light, touch or chemical signals, dendrites of the sensory neuron will depolarize proportional to the strength of the stimulation causing change in the electrical potential, this is known as receptor potential. The stimulation of receptor potential causes depolarization along the neuronal axon, known as an action potential, which travels to terminal synapses.

At terminal synapses, the electrical potential is converted to chemical energy. At the presynaptic synapse, the action potential delivered from the axon stimulates the opening of ion channels which allow the entry of calcium. An increase in calcium concentration stimulates synaptic vesicles containing neurotransmitters and neuromodulators, to fuse with the membrane and be released into the synaptic cleft, the space between the synapse of the depolarized neuron and other neurons in close proximity, at a rate which is dependent on the frequency and strength of depolarization reaching the synapse. At the synaptic cleft, neurotransmitters or neuromodulators can

bind to receptors on the postsynaptic membranes of other neurons, leading to ion channel alterations and depolarization of the receiving neuron which will travel along the axon of the postsynaptic neuron (Figure 5-1). Enzymes are present in the synaptic cleft which degrade neurotransmitters and neuromodulators after binding to prevent continual stimulation of neurons and to recycle metabolites needed for neurotransmitter synthesis back to the presynaptic neuron.

5.1.1.4 Neurotransmitters and Neuromodulators

The main excitatory neurotransmitters in insects are acetylcholine and glutamate at sensory receptors and neuromuscular junctions, respectively. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter. Multiple biogenic amines function as neuromodulators in the insect brain, including histamine, serotonin and octopamine. Octopamine is orthologous to norepinephrine in humans and in insects has an important role in a variety of physiological processes as both a neuromodulator in the nervous system and a hormone in the haemolymph and modifies sensory receptor sensitivity and regulates the response patterns of the insect (Klowden and Palli, 2022).

5.1.1.5 Major Neuropils of the Bee Brain

The neuropils identified in the *B. terrestris* brain can be divided into three segments. The first segment, the protocerebrum, receives information from sensory neurons in the compound eyes and ocelli. It contains various neuropils which act as distinct brain centres including the optic lobes, the central complex, the mushroom body, and the anterior optic tubercle (Galizia *et al.,* 2012; Klowden and Palli, 2022; Rother *et al.,* 2021). The deutocerebrum contains the antennal lobe and is responsible for sensory information processing (Figure 5-2). Lastly, the tritocerebrum functions in connecting the central nervous system to the visceral nervous system (Klowden and Palli, 2022).

5.1.1.5.1 The Central Complex

The central complex neuropil is in the centre of the bee brain and has four neuropil modules: the protocerebral bridge, the ellipsoid body, the fan-shaped body and the paired noduli (Rother *et al.,* 2021). The central complex is important in social insects as the navigational centre for the sensory integration of visual and olfactory signals and motor and muscle movements (Honkanen *et al.,* 2019).

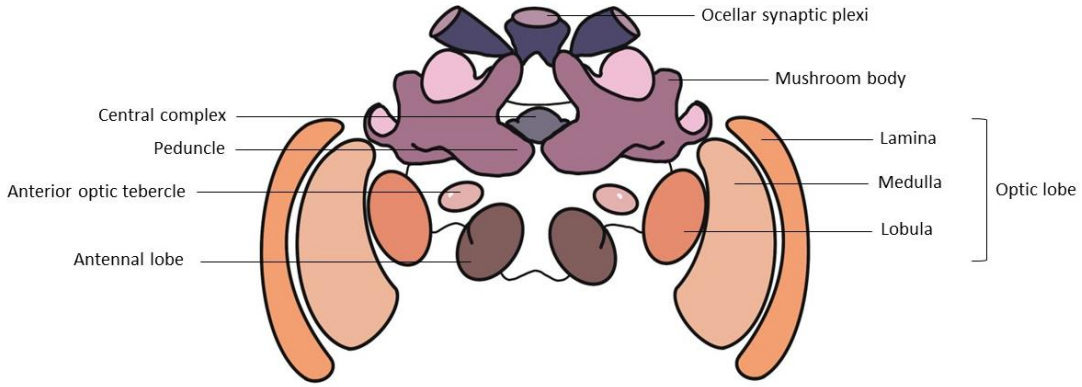


Figure 5-2 Frontal Diagram of Major *B. terrestris* Brain Neuropils. All major neuropils are colour coded for identification. The unshaded region represents unidentified neuropils.

5.1.1.5.2 The Mushroom Bodies

The mushroom bodies are dense glomeruli connected by a stalk like structure called the pedunculus. The calyces are the main sensory input region while the pedunculus and medial and vertical lobes are the main output regions (Galizia *et al.*, 2012; Klowden and Palli, 2022). There are approximately 368,000 specialized interneurons known as Kenyon cells in the mushroom bodies of bees – accounting for a large proportion of the 950,000 neurons throughout the bee brain (Galizia *et al.*, 2012). The mushroom body is larger in bees compared to other insects and is important in sensory integration, learning, memory and pattern recognition (Klowden and Palli, 2022). Presumably, Kenyon cells found in bees are of great importance due to their abundance in the mushroom bodies, however, their exact functions are unknown (Suenami *et al.*, 2018). The Kenyon cells of the mushroom body display plasticity, where new synapses can grow and undergo structural rearrangements depending on previous sensory inputs and experiences, making the mushroom body important for long-term memory (Szyszka *et al.*, 2008; Hourcade *et al.*, 2010).

5.1.1.5.3 The Optic Lobes

On each side of the mushroom bodies reside the optic lobes. The optic lobes are the largest part of the *B. terrestris* brain, taking up 33.5% of the entire brain volume (Rother *et al.*, 2021). The optic lobes have three distinct neuropils: the lamina, medulla and lobula complex. The optic lobes process sensory information from the compound eyes. There are approximately 4000 lenses in each compound eye of an adult worker bee where light hits the ommatidium – a structure underneath each lens with light

sensitive cells to detect and transmit light signals through nerves which exit the base of the ommatidium, bringing direct inputs from photoreceptors to the lamina of the optic lobe underneath (Stell, 2012). From the lamina, information is passed to the lobula complex via the medulla neuropil. From here, visual stimuli can be processed and integrated in the mushroom bodies (Klowden and Palli, 2022).

5.1.1.5.4 The Anterior Optic Tubercle

The anterior optic tubercle is located above the optic lobes and is one of the smallest neuropils of the bumblebee brain, taking up 0.4% of the entire brain volume (Rother *et al.,* 2021). This 200 μm wide neuropil is thought to receive visual information from interneurons of the optic lobes and is involved in polarization vision where it may carry light polarization signals to the protocerebrum (Pfeiffer and Kinoshita, 2012).

5.1.1.5.5 The Antennal Lobe

The antennal lobe is important for the detection of olfactory, temperature and humidity cues from receptors located on the antennae. The antennal lobe contains over 100 glomeruli where hundreds of thousands of receptor neurons synapse with hundreds of interneurons, which ultimately synapse with neurons that bring information to the mushroom bodies for processing (Klowden and Palli, 2022).

5.1.1.5.6 The Peripheral and Visceral Nervous System

The tritocerebrum is below the deutocerebrum and plays a vital role in connecting the central nervous system to the visceral nervous system. The peripheral nervous system includes neurons which stem from the central nervous system such as nerves in direct contact with muscles and sensory receptors. The visceral nervous system contains neurons which make connections with the digestive tract, heart and endocrine glands and forms connections with the peripheral nervous system ganglia which innervate the muscles of the digestive tract to regulate and modify various aspects of digestion and excretion. The tritocerebrum consists of circumoesophageal connections which connect the brain to the suboesophageal ganglia – ganglia which control the mouthparts - and ventral nerve cord of the central nervous system through the frontal ganglion. The frontal ganglion, below the main brain centres discussed above, plays a key role in the stomatogastric nervous system; part of the visceral nervous system which innervates the foregut and midgut. The recurrent nerve below the brain is

connected to the frontal ganglion which connects the hypocerebral ganglion to the corpora allata, corpora cardiaca and the foregut through oesophageal nerves that also innervate the ventriculus ganglia of the digestive tract. The frontal ganglion is also a source of neuropeptides involved in food-related learning and satiety in some insects (Hergarden *et al.,* 2012; Yamagata *et al.,* 2016). Overall, the brain and nervous system of bees is vital for memory, learning, and detection and response to the external and internal environment, altering and regulating physiological processes in various tissues including the digestive tract and endocrine glands.

5.1.2 The Fat Body

The fat body is often referred to as the insect equivalent of the mammalian liver. In truth, there is no homologous organ to the multifunctional insect fat body in non-insect species. The fat body can sense hormonal and nutritional signals and respond to the ever-changing metabolic, immunological, toxicological, and developmental needs of the insect via the mobilisation of nutrients from energy reserves, or with regulatory molecule synthesis and secretion, which result in alterations to physiological and metabolic processes within the insect (Li *et al.,* 2019).

5.1.2.1 Fat Body Cells

The fat body is an organ that consists of loose sheets of cells in the haemocoel of the abdomen, with some cellular aggregates physically contacting the digestive tract and reproductive organs. Trophocytes (adipocytes) are the main cells of the fat body and vital for the synthesis, storage, and mobilisation of energy. Specialized trophocytes are also present throughout the fat body which have alternative functions. These include urocytes which store and detoxify urates while bacteriocytes and mycetocytes are cells which contain microbial symbionts. In addition, oenocytes can be found within the fat body. These cells are specialized for the synthesis of cuticular lipids, proteins, and hydrocarbons. However, this section will mainly focus on trophocytes as they are the most abundant and well-characterised cells of the fat body.

5.1.2.2 Lipid Storage and Mobilisation

Trophocytes are specialized for lipid storage. Lipids are stored in these cells in the form of triglyceride, in organelles known as lipid droplets. Lipid droplets contain triglycerides inside a monolayer of phospholipid and cholesterol with proteins

embedded or associated with this monolayer. Lipid droplets mobilize stored triglycerides during non-feeding, starvation or periods of high-energy demand via lipolysis of triglycerides which hydrolyse to form diglycerides and fatty acids, which can be more easily transported to the haemolymph to meet the metabolic activity requirements of other tissues (Arrese and Soulages, 2010). The underlying mechanism of lipid mobilisation in the fat body involves adipokinetic hormone (AKH) (Gäde and Auerswald, 2003). AKH is synthesized by the corpus cardiacum in the brain and when released into the haemolymph, activates triglyceride lipase in the fat body for hydrolysis to diglyceride. High trehalose in the haemolymph inhibits the secretion of AKH, whilst elevated levels of octopamine, a neurohormone, stimulates AKH secretion. Diglycerides are transported through the haemolymph by lipophorins which are lipoproteins used to transport otherwise insoluble lipids to cells expressing lipophorin receptors (Klowden and Palli, 2022). Upon reaching the target cells diglycerides and fatty acids can be digested further by lipases into glycerol and fatty acids where glycerol is phosphorylated to glycerol-3-phosphate for entry into the glycolytic pathway and fatty acids can undergo β-oxidation in the mitochondria which removes acetyl CoA and hydrogen atoms from the fatty acyl CoA, generating FADH2 and NADH, where acetyl CoA can enter the citric acid cycle.

5.1.2.3 Carbohydrate Storage and Mobilisation

Trophocytes can also store carbohydrates as glycogen when carbohydrate intake is greater than required. Glycogen is a glucose polymer, stored in fat body trophocytes as an energy source where it can be hydrolysed into trehalose to meet energy demands when necessary. Trehalose is a glucose disaccharide and the main energy source circulating through the insect haemolymph, similar to glucose in vertebrates. Since trehalose is a disaccharide, it can diffuse more slowly into cells, allowing a higher concentration than glucose without consequence in the haemolymph. Additionally, glucose is readily absorbed in the insect midgut, so trehalose as the main carbohydrate energy source in the haemolymph avoids interference with this process as glucose is mainly taken into cells by passive diffusion. Upon absorption by the midgut, glucose is converted to trehalose using UDP-glucose from dietary carbohydrates or amino acids in the fat body. When the fat body trehalose reaches a threshold concentration UDP-glucose is used for glycogen synthesis. The release of hypertrehalosemic hormone from the corpus cardiacum is triggered by decreasing carbohydrate

concentration in the haemolymph, which activates a second messenger system resulting in the activation of glycogen phosphorylase, releasing glycogen-1-phosphate from glycogen which, along with UDP-glucose, is converted to glucose-6-phosphate, forming trehalose which is released for circulation in the haemolymph for delivery to cells which require energy. There, trehalose can be hydrolysed to glucose which can be oxidized to meet the metabolic requirements of the insect (Klowden and Palli, 2022). Alternatively, glucose-6-phosphate can enter glycolysis directly to meet the energy requirements of the fat body itself. Alternatively, AKH secretion can also activate glycogen phosphorylase to undergo the same energy mobilisation process from glycogen.

5.1.2.4 The Role of the Fat Body in Other Physiological Processes

The fat body is important for various physiological processes by responding to hormonal signals and nutrient sensing via amino acid transporters, resulting in the release of stored nutrients, synthesized proteins, and detoxification of harmful metabolic products (Klowden and Palli, 2022). The fat body is also the main site of vitellogenin synthesis and secretion. This lipoprotein is important for egg maturation, juvenile hormone regulation (Page *et al.,* 2012) and oxidative stress regulation (Seehuus *et al.,* 2006). Additionally, flight is energetically demanding, and bees are thought to rely on carbohydrate metabolism to fuel flight (Suarez *et al.,* 2005). Therefore, access to carbohydrates is important regardless of recent carbohydrate intake, with fat body storage and mobilisation of carbohydrates important to keep a steady supply of trehalose in the haemolymph. Finally, the fat body is a major site of immune and detoxification protein production. Detoxification takes place in three phases. In phase I, enzymes such as cytochrome P450 and esterase's transform and decrease the activity of toxic metabolic products and xenobiotics. In phase II, enzymes including glutathione-S-transferases (GSTs) metabolise products from phase I. In phase III, transporters such as ATP-binding cassette (ABC) transporters pump the transformed toxins out of the cell. Cytochrome P450, GSTs and esterase's are increased in the fat body of some insects after pesticide exposure, deemed important for pesticide detoxification (Birner-Gruenberger *et al.,* 2012; Mao *et al.,* 2019). In humoral immunity, antimicrobial peptides (AMPs) play a key role in mounting a defence against pathogens and wounding (Danihlík et al., 2016). AMPs are synthesized and secreted by the fat body, which is regulated by the recognition of

pathogen-related antigens via pattern recognition receptors and subsequent activation of immune pathways (Erler *et al.*, 2011).

5.1.3 The Impact of Glyphosate and Prothioconazole Exposure on the Bee Brain and Fat Body is Unknown

Many insecticidal pesticides negatively impact the bee brain and nervous system. However, many of these pesticides are designed to target the insect nervous system e.g., neonicotinoids target nicotinic acetylcholine receptors found in the brain, leading to adverse outcomes for functions that require proper brain and nervous system functioning, such as memory and learning (Blacquière *et al.,* 2012; Mengoni Goñalons and Farina, 2018; Muth and Leonard, 2019; Smith *et al.,* 2020). Some studies have determined impacts of glyphosate on the brain with impacts on learning, memory, sleep, and neurotransmitter activity in honeybees (Boily *et al.,* 2013; Mengoni Goñalons and Farina, 2018; Vázquez *et al.,* 2020; Hernández *et al.,* 2021). Whilst there remains few studies on the impacts of prothioconazole on bee health in any capacity, Almasri et al. (2021) found that glyphosate, alone and with the triazole fungicide difenoconazole, altered neurotransmitter levels in the honeybee head, suggesting that prothioconazole, as a triazole fungicide, could have impacts on the bee brain and nervous system.

The fat body is a vital tissue for detoxification, energy storage and metabolism (Li *et al.,* 2019). Some pesticides alter energy metabolism and detoxification enzyme activity; however few studies exist that have determined if the fat body tissue has been altered (Mao *et al.,* 2019; Zhao *et al.,* 2020; Almasri *et al.,* 2021). Glyphosate can suppress antimicrobial peptide gene expression in honeybees, which originate from the fat body (Motta *et al.,* 2022). In addition, glyphosate alters metabolism in bees (Zhao *et al.,* 2020). This suggests a possible impact of glyphosate on the fat body; however, no direct studies have been conducted on the fat body tissue itself. In addition, there are no studies published on prothioconazole exposure effects on the fat body.

Non-insecticidal pesticides, such as glyphosate and prothioconazole, are not designed to target insects, much less insect tissues in which glyphosate and prothioconazole are not known to have direct contact with upon ingestion. However, given the role of

pesticide exposure in ongoing bee declines (Goulson *et al.*, 2015) and a lack of research on non-insecticidal pesticide impacts on bees (Cullen *et al.,* 2019), particularly bumblebees, there is an urgent need to understand the roles of widely used herbicides and fungicides on overall bee health, regardless of anticipated impacts from mortality-based and non-tissue specific studies.

5.1.4 Chapter Aims

1. Determine if glyphosate exposure has statistically significant impacts on the brain and fat body proteome of *B. terrestris.*
2. Determine if prothioconazole exposure has statistically significant impacts the brain and fatbody proteome of *B. terrestris*.
3. Explore and characterise the effects that glyphosate and a glyphosate-based commercial formulation, Roundup Optima+®, has on the brain and fat body of *B. terrestris*.
4. Explore and characterise the effects that prothioconazole and a prothioconazole-based commercial formulation, Proline®, has on the brain and fat body of *B. terrestris.*
5. Establish if the commonly used solvent, acetone, investigated in chapter four for impacts on the digestive tract, also has implications for *B. terrestris* brain and fat body physiological functioning in comparison to a non-acetone control.

5.2 Experimental Outline

5.2.1 Glyphosate Exposure

Glyphosate active ingredient (GAI) and glyphosate commercial formulation RoundUp Optima+® (GCF) were prepared at 1 ppm in 40% (w/v) sucrose solution as described in section 2.3.1. Exposures were conducted as described in section 2.3.3. Before brain dissections, nine bees were randomly chosen from each of four origin commercial colonies with three bees per origin colony allocated to each of three group isolation chambers, resulting in twelve bees per isolation chamber (17 cm x 14.7 cm x 8.5 cm). All bees were acclimatised overnight with *ad libitum* access to 40% (w/v) sucrose solution at 20 ± 2°C and 58 ± 5% relative humidity, as for the duration of the experiment and were continuously kept in the dark. The following day, bees were exposed *ad libitum* to either 1 ppm GAI or 1ppm GCF in 40% (w/v) sucrose solution, or the control 40% (w/v) sucrose solution, with each group isolation chamber assigned to one of the three treatment options. Every 24 ± 2 hours, bees were observed for mortality and behavioural alterations (Table 2-1) and given fresh aliquots of treatment. On day five of exposure to glyphosate, seven bees from each treatment group were selected for brain dissections.

Before fatbody dissections, six bees were randomly selected from each of five origin colonies and allocated to each of the three isolation chambers, resulting in ten bees per group isolation chamber. All bees were acclimatised overnight and kept continuously in the dark with *ad libitum* access to 40% (w/v) sucrose solution at 23.1 ± 0.8°C and 75.5 ± 6.5 % relative humidity, as for the duration of the experiment. The following day, bees were exposed *ad libitum* to either 1ppm GAI or 1 ppm GCF in 40% (w/v) sucrose solution, or the control 40% (w/v) sucrose solution, with each group isolation chamber assigned to one of the three treatment options. Every 24 ± 2 hours, bees were observed for mortality and behavioural alterations (Table 2-1) and given fresh aliquots of treatment. On day five of prothioconazole exposure, eight bees were selected for fat body dissection from each treatment group.

5.2.2 Prothioconazole Exposure

Prothioconazole solutions were prepared and maintained as outlined in section 2.3.2 using technical grade prothioconazole (PAI) and a prothioconazole commercial formulation Proline® (PCF). 0.3 ppm PAI treatment was prepared in 0.3% acetone 40% (w/v) sucrose solution and 0.3 ppm PCF was prepared in 40% (w/v) sucrose solution alone. As a result, prothioconazole exposures required two controls, one consisting of 0.3% acetone 40% (w/v) sucrose solution and an identical non-acetone control. Exposures were conducted as described in section 2.3.3. Before both brain and fat body dissections, eight bees were randomly selected from each of five origin colonies and two were allocated to each of four group isolation chambers (11 x 7.5 x 17.5 cm) resulting in ten bees per group isolation chamber. Bees were acclimatised overnight with *ad libitum* access to 40% (w/v) sucrose solution and continuously kept in the dark for acclimatisation and the duration of the experiment. For exposures leading to brain dissections, bees were kept at 27.45 ± 1.25°C and 63.5 ± 8.5 % relative humidity. For exposures leading to fatbody dissections, bees were kept at 27.4 ± 1°C and 63 ± 5 % relative humidity. The day following overnight acclimatisation, bees were exposed ad libitum to one of four treatments: 0.3 ppm PAI, 0.3 ppm PCF, 0.3% acetone 40% (w/v) sucrose solution, or 40% (w/v) sucrose solution, with each group isolation chamber assigned to one of the four treatments. Every 24 ± 2 hours, bees were observed for mortality and behavioural alterations (Table 2-1) and given fresh aliquots of treatment. On day five of exposure, eight bees per treatment group were selected for both brain and fatbody dissections.

5.2.3 Brain and Fat Body Sample Processing and Analysis

All fat body and brain samples were dissected, homogenised, processed, and run on the Q-Exactive spectrometer as described in section 2.4 with four brain and fat body peptide samples ran per treatment. All data annotation, statistical analysis of results and functional annotation of each dataset obtained from the LFQ mass spectrometer followed the protocol described in section 2.5 using Perseus v. 1.6.15.0 for all datasets except for glyphosate-exposed brains, which were analysed using Perseus 1.6.1.1.

5.3 Results

5.3.1 LFQ analysis of glyphosate exposure on the brain and fat body proteome of *B. terrestris*

5.3.1.1 Identified and quantified proteins

There were 3,204 proteins identified from the *B. terrestris* brain, with 1,723 proteins remaining post-imputation and log_2 transformation (Table S5-1). 2,885 proteins were identified from the *B. terrestris* fat body, with 1,252 proteins remaining post-imputation and log_2 transformation (Table S5-2). PCA on brain samples had a variance of 27.1% in component one and 13.6% in component two (Figure 5-3A). Whilst there was some variation in the placement of control brain proteome samples, brain samples from GAI and GCF-exposed bees clustered together, away from the other treatment group, although some control brain samples clustered close to GCF treated bees. PCA on fat body samples had a variance of 25.8% in component one and 16.5% in component two (Figure 5-3B). Whilst fat body samples from GCF exposed bees clustered together, samples from control and GAI-exposed bees were more variable in their clustering pattern.

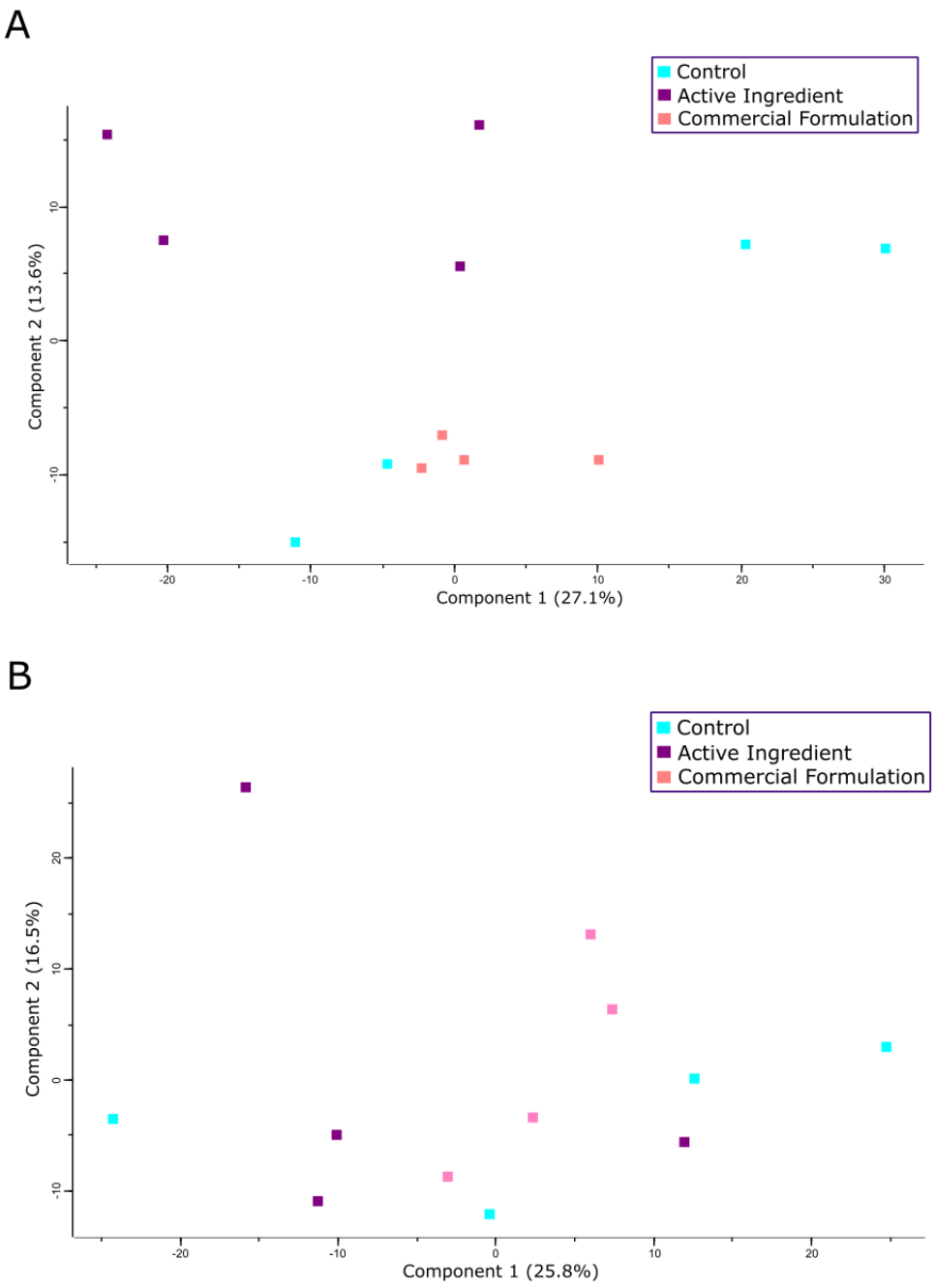


Figure 5-3 PCA Representing Variation Between Samples. In A) the PCA demonstrates variation in the brain proteome and in B) the PCA demonstrates variation in the fat body proteome, with each point representing the brain or fat body proteome of a single bee.

5.3.1.2 Two-Sample T-Tests

Two sample t-tests were utilized for pairwise comparisons of treatment groups to determine SSDA proteins ($p \leq 0.05$, S0 = 0.1) and their relative fold differences in protein abundance (Table S5-3; Table S5-4). STRING analysis was conducted on SSDA proteins found from pairwise comparisons of treatment groups from brain and fat body samples to identify pathways and processes impacted. In addition, KEGG and

BRITE analysis was carried out to determine enriched pathways and functionalities of SSDA proteins.

5.3.1.2.1 Glyphosate Active Ingredient vs Control

There were 105 SSDA proteins resolved from the brains of GAI exposed bees compared to control exposed bees (RFC range: + 35.4 to – 15.1). There were 67 proteins with an increased abundance and 38 proteins with a decreased abundance in GAI compared to control exposed bees (Figure 5-4A). The top ten SSDA proteins with the highest abundance in the brains of GAI-exposed bees were: putative mediator of RNA polymerase II transcription subunit 26 (RFC: + 35.42), alcohol dehydrogenase (RFC: + 14.23), adrenodoxin (RFC: + 11.56), neural/ectodermal development factor imp-l2 (RFC: + 9.25), 60s ribosomal protein l36 (RFC: + 7.18), hydroxymethylglutaryl-CoA synthase 1 (RFC: + 6.58), ecdysteroid-regulated 16 kDa protein (RFC: + 6.40), acyl-CoA-binding protein (RFC: + 5.37), glucose dehydrogenase [fad, quinone] (RFC: + 4.23), and geranylgeranyl pyrophosphate synthase (RFC: + 4.02). The top ten SSDA proteins with the lowest abundance in the brains of GAI-exposed bees compared to control-exposed bees were: uncharacterized protein LOC100649693 (RFC: - 15.12), syndecan (RFC: - 4.81), protein ndrg3 (RFC: - 4.67), upf0554 protein c2orf43 homolog (RFC: - 3.99), ras GTPase-activating protein 1 (RFC: - 2.90), arrestin (RFC: - 2.73), major facilitator superfamily domain-containing protein 6 (RFC: - 1.87), secretory carrier-associated membrane protein 5a (RFC: - 1.73), cytochrome c (RFC: - 1.67), and late histone H-1 (RFC: - 1.58).

Fifty-two proteins were SSDA in the fat bodies of bees exposed to GAI in comparison to control-exposed bees (RFC range: + 5.2 to -3.5). These included 15 proteins with an increased abundance and 37 proteins with a decreased abundance in GAI compared to control-exposed bees (Figure 5-5A). The top ten SSDA proteins with an increased abundance after GAI-exposure in the fat body were: coiled-coil domain-containing protein 58 (RFC: + 5.21), cuticle protein 38 (RFC: + 3.38), iron-sulfur cluster assembly enzyme iscu (RFC: + 2.59), tubulin-folding cofactor b (RFC: + 2.25), proteasome subunit alpha type-5 (RFC: + 1.85), serine proteinase stubble (RFC: + 1.76), nucleoporin p58/p45 (RFC: + 1.76), papilin (RFC: + 1.74), nuclease-sensitive element-binding protein 1 (RFC: + 1.58), and transcription elongation factor spt5 (RFC: + 1.45). The top ten SSDA proteins with a decreased abundance in the fat body

after GAI-exposure compared to control-exposure were: asparagine--tRNA ligase, cytoplasmic (RFC: - 3.47), caprin (RFC: - 3.05), lipid storage droplets surface-binding protein 1 (RFC: - 3.03), superkiller viralicidic activity 2 (RFC: - 2.29), spermidine synthase (RFC: - 2.28), ribonuclease UK114 (RFC: - 2.22), tryptophan 2,3-dioxygenase (RFC: - 2.03), fructose-1,6-bisphosphatase 1 (RFC: - 2.01), retinal dehydrogenase 1 (RFC: - 1.77), and serine--pyruvate aminotransferase, mitochondrial (RFC: - 1.74).

5.3.1.2.2 Glyphosate Commercial Formulation vs Control

There were 166 SSDA proteins resolved from the brain samples of GCF-exposed bees compared to control-exposed bees (RFC range: + 30 to – 7.6). This included 95 proteins with an increased abundance and 71 proteins with a decreased abundance in the brain after GCF exposure (Figure 5-4B). The top ten proteins with an increased abundance included: leucine-rich repeat-containing protein 70 (RFC: + 30.05), putative mediator of RNA polymerase II transcription subunit 26 (RFC: + 27.80), adrenodoxin (RFC: + 7.13), vacuolar protein sorting-associated protein 37b (RFC: + 4.58), eukaryotic translation initiation factor 3 subunit k (RFC: + 4.24), activated RNA polymerase II transcriptional coactivator p15 (RFC: + 3.64), CTP synthase (RFC: + 3.41), probable N-acetyltransferase san (RFC: + 2.95), F-bar domain only protein 2 (RFC: + 2.77), and tubulin gamma-2 chain protein (RFC: + 2.46). The top ten proteins with the most decreased abundance in the brains of GCF-exposed bees compared to the control-exposed bees were: 60S ribosomal protein L32 (RFC: - 7.59), endothelin-converting enzyme 1 (RFC: - 7.52), glutamate receptor 1 (RFC: - 6.86), spermine oxidase (RFC: - 5.79), vacuolar protein sorting-associated protein 13c (RFC: - 5.49), syndecan (RFC: - 4.75), cytochrome p450 6a2 (RFC: - 4.26), multidrug resistance protein homolog 49 (RFC: - 4.21), ER membrane protein complex subunit 1 (RFC: - 3.15), and inositol monophosphatase 2 (RFC: - 3.02).

In the fat bodies of GCF-exposed compared to control-exposed bees, there were 77 SSDA proteins (RFC range: + 7.3 to – 5.2). There were 39 proteins with an increased abundance and 38 proteins with a decreased abundance in the fat bodies of GCF-exposed bees compared to control-exposed bees (Figure 5-5B). The top ten proteins with an increased abundance included: uncharacterized protein loc100644966 (RFC: + 7.32), eukaryotic translation initiation factor 3 subunit g (RFC: + 6.35), coiled-coil

domain-containing protein 58 (RFC: + 5.29), methylglutaconyl-CoA hydratase, mitochondrial (RFC: + 3.74), ubiquitin-conjugating enzyme e2 g1 (RFC: + 3.37), short-chain specific acyl-CoA dehydrogenase, mitochondrial (RFC: + 3.35), iron-sulfur cluster assembly enzyme iscu, mitochondrial (RFC: + 3.03), ran-binding protein 3 (RFC: + 2.83), mitochondrial intermembrane space import and assembly protein 40-b (RFC: + 2.68), and tubulin-folding cofactor b (RFC: + 2.37). The top ten proteins with a decreased abundance in GCF-exposed fat bodies were: lipid storage droplets surface-binding protein 1 (RFC: - 5.15), receptor expression-enhancing protein 5 (RFC: - 4.52), collagen alpha-5(iv) chain (RFC: - 4.17), uncharacterized protein LOC100650927 (RFC: - 4.09), NADPH--cytochrome p450 reductase (RFC: - 3.14), UDP-glucuronosyltransferase 2b20 (RFC: - 3.06), cytochrome P450 4g15 (RFC: - 2.79), very-long-chain 3-oxoacyl-CoA reductase (RFC: - 2.52), cytochrome P450 9e2 (RFC: - 2.38), and E3 ubiquitin-protein ligase huwe1 (RFC: - 2.32).

5.3.1.2.3 Glyphosate Commercial Formulation vs Active Ingredient

A total of 130 SSDA proteins were identified from the brains of GCF-exposed bees compared to GAI-exposed bees (RFC range: + 43.3 to – 77.8). These included 88 proteins with an increased abundance and 42 proteins with a decreased abundance from the brains of GCF compared to GAI-exposed bees (Figure 5-4C). The top ten proteins with an increased abundance included: leucine-rich repeat-containing protein 70 (RFC: + 43.26), uncharacterized protein LOC100649693 (RFC: + 6.05), eukaryotic translation initiation factor 3 subunit k (RFC: + 5.89), RING-box protein 1a (RFC: + 4.58), endoribonuclease dicer (RFC: + 3.24), fasciculation and elongation protein zeta-2 (RFC: + 3.17), cullin-2 (RFC: + 2.94), uncharacterized protein LOC100642914 (RFC: + 2.76), tubulin alpha-1 chain (RFC: + 2.15), and arrestin (RFC: + 2.07). The top ten proteins with the most decreased abundance in the brain after GCF exposure compared to GAI exposure were: cytochrome b-c1 complex subunit 8 (RFC: - 77.78), 40s ribosomal protein s29 (RFC: - 8.09), spermine oxidase (RFC: - 7.89), ecdysteroid-regulated 16 kDa protein (RFC: - 6.76), sodium- and chloride-dependent GABA transporter 1-like protein (RFC: - 6.11), acyl-CoA-binding protein homolog (RFC: - 4.45), glutamate receptor 1 (RFC: - 4.05), isopentenyl-diphosphate delta-isomerase 1-like protein (RFC: - 3.89), dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 (RFC: - 3.85), and hydroxymethylglutaryl-CoA synthase 1 (RFC: - 3.19).

Further, 45 proteins were SSDA in the fat bodies of GCF-exposed bees compared to GAI-exposed bees (RFC range: + 6.3 to – 4.3), with 33 proteins with an increased abundance and 12 proteins with a decreased abundance (Figure 5-5C). Of the top ten proteins with an increased abundance in the fat bodies of GCF-treated bees compared to GAI-treated bees there were: eukaryotic translation initiation factor 3 subunit g (RFC: + 6.30), proteasome activator complex subunit 3 (RFC: + 5.26), protein cdv3 homolog A (RFC: + 4.67), nuclear autoantigenic sperm protein (RFC: + 3.55), bis(5-nucleosyl)-tetraphosphatase (RFC: + 3.10), protein prune homolog (RFC: + 3.00), ubiquitin-conjugating enzyme E2 g1 (RFC: + 2.98), ketohexokinase (RFC: + 2.79), molybdenum cofactor biosynthesis protein 1 (RFC: + 2.35), and superkiller viralicidic activity 2-like protein 2 (RFC: + 1.93). The top ten proteins with a decreased abundance were: vesicle-trafficking protein sec22b-b (RFC: - 4.30), guanine nucleotide-binding protein g(o) subunit alpha (RFC: - 4.00), cytochrome p450 6a2 (RFC: - 3.81), uncharacterized protein LOC100650927 (RFC: - 2.75), nuclease-sensitive element-binding protein 1 (RFC: - 1.66), ADP, ATP carrier protein 2 (RFC: - 1.59), glutamine synthetase 2 cytoplasmic (RFC: - 1.48), V-type proton ATPase subunit f (RFC: - 1.47), enolase (RFC: - 1.32), and guanine nucleotide-binding protein g(i)/g(s)/g(t) subunit beta-1 (RFC: - 1.27).

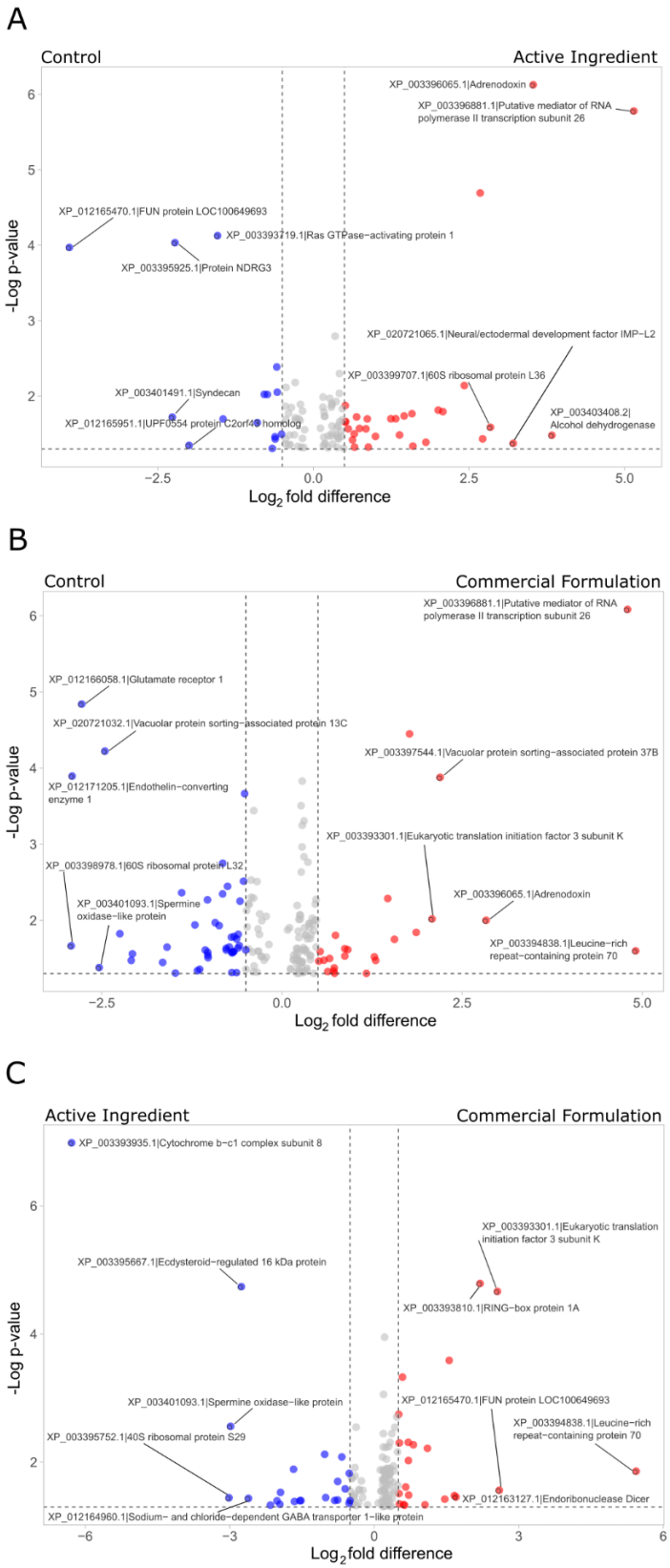


Figure 5-4 A Two Sample t-test was Performed to Resolve and Compare SSDA Proteins in the Brain of *B. terrestris* after Glyphosate Exposure. Volcano plots were generated to illustrate the number of SSDA proteins with an increased (red) and decreased (blue) abundance in the brain in pairwise comparisons of bees exposed to **(A)** GAI vs the control treatment, **(B)** GCF vs the control treatment, or **(C)** GCF vs GAI treatment. The five proteins with the highest and lowest RFCs were labelled.

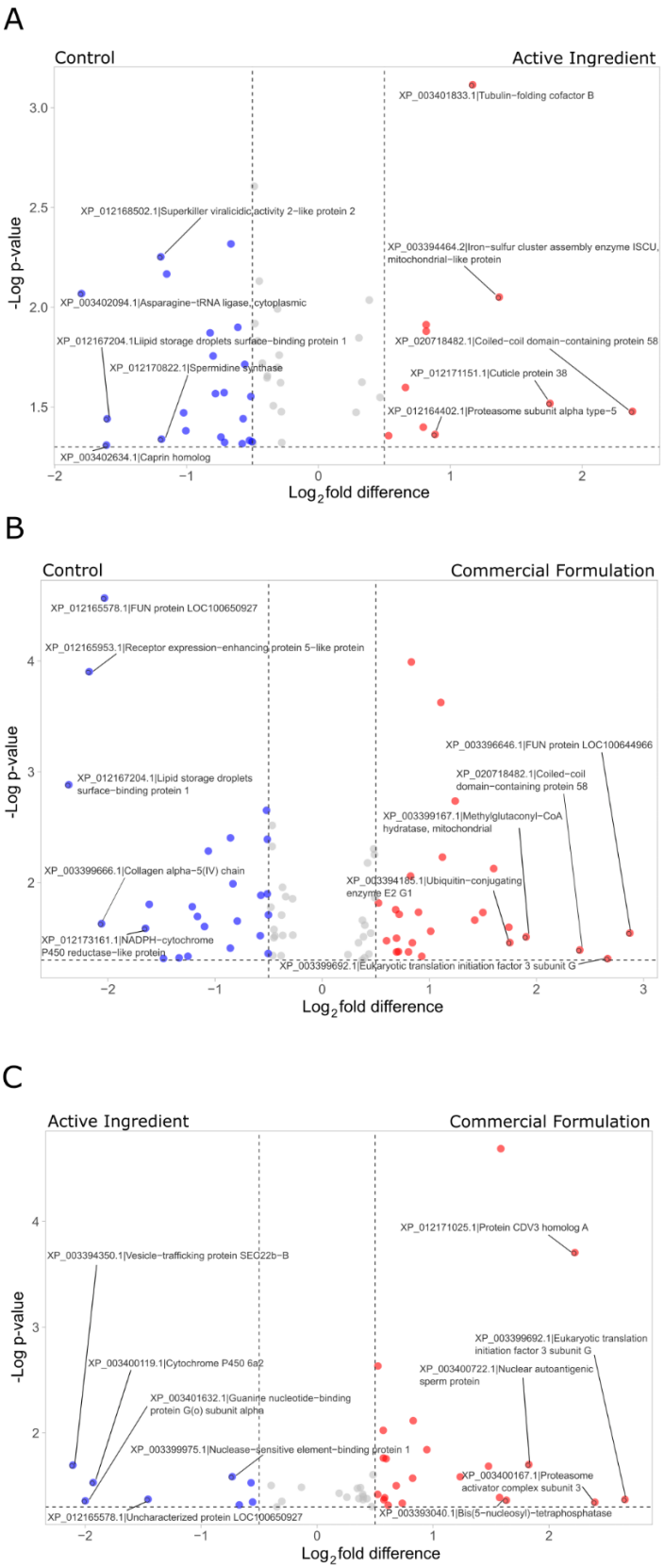


Figure 5-5 A Two Sample t-test was Performed to Resolve and Compare SSDA Proteins in the Fat Body of *B. terrestris* after Glyphosate Exposure. Volcano plots were generated to illustrate the number of SSDA proteins with an increased (red) and decreased (blue) abundance in the fat body in pairwise comparisons of bees exposed to **(A)** GAI vs the control treatment, **(B)** GCF vs the control treatment, or **(C)** GCF vs GAI treatment. The five proteins with the highest and lowest RFCs were labelled.

5.3.1.3 Gene Ontology and Pathway Analysis of SSDA Proteins

To determine the pathways and processes impacted in the brain and fat bodies of glyphosate-exposed bees, SSDA proteins from pairwise comparisons were analysed for enriched PPI networks, GO terms, KEGG pathways and Interpro domains using STRING v.11 using *D. melanogaster* as a reference genome (Table 5-1; Table S5-5; Table S5-6). BlastKOALA was used in the analysis of SSDA proteins against KEGG and BRITE databases (Table S5-7; Table S5-8).

5.3.1.3.1 Glyphosate Active Ingredient vs Control

Comparison of SSDA proteins from brains of GAI-exposed bees to control-exposed bees resolved an enrichment in increased abundance proteins associated with RNA transport (five proteins), lipid metabolism (five proteins), the proteasome (four proteins), and terpenoid backbone synthesis (four proteins) (Figure S5-1A). In addition, five proteins were enriched in messenger RNA biogenesis and nine proteins were enriched in membrane trafficking. SSDA proteins with a decreased abundance in the brain after exposure to GAI compared to the control treatment were associated with the TCA cycle (four proteins), RAVE complex (two proteins), the synapse (seven proteins), and the mitochondrion (nine proteins) (Figure S5-1B). Eight proteins were associated with membrane trafficking.

SSDA proteins with an increased abundance in the fat bodies of bees exposed to GAI compared to the control treatment group had enrichments for RNA metabolism (seven proteins), ABC-family protein mediated transport (three proteins) and proteasome degradation (two proteins) (Figure S5-2A). Three peptidases and one inhibitor were also enriched, along with proteins involved in messenger RNA biogenesis (three proteins), the spliceosome (two proteins), proteasome (three proteins), mitochondrial biogenesis (two proteins), and the exosome (two proteins). SSDA proteins with a decreased abundance in GAI-exposed fat bodies were associated with translation (eight proteins), glycolysis/gluconeogenesis (five proteins), and lipid droplets (two proteins) (Figure S5-2B). After BRITE analysis, six proteins were associated with the exosome.

5.3.1.3.2 Glyphosate Commercial Formulation vs Control

SSDA proteins with an increased abundance from the brains of GCF-treatment compared to control-treatment bees were involved in cellular responses to stress (eleven proteins) and unfolded protein binding (six proteins) (Figure S5-3A). BRITE analysis resolved nine chaperones and folding catalysts, nine membrane trafficking proteins, five chromosome associated proteins, and 12 exosome-associated proteins. Proteins with a decreased abundance were involved in neurotransmitter transport (six proteins) and oxidative phosphorylation (five proteins) and were associated with the ribosome – 15 ribosomal subunit proteins were decreased – and the endoplasmic reticulum, with seven proteins decreased (Figure S5-3B). KEGG analysis resolved an enrichment in oxidative phosphorylation (six proteins) and BRITE analysis resolved an enrichment in membrane trafficking (eleven proteins) and chromosome-associated proteins (six proteins).

SSDA proteins with an increased abundance in the fat bodies of GCF compared to control-treated bees played key roles in translational initiation (five proteins) and valine, leucine and isoleucine degradation (three proteins). In addition, proteins were involved in ubiquitin-like protein conjugating enzyme activity (three proteins), chaperone binding (three proteins), and superoxide dismutase complex (two proteins) (Figure S5-4A). BRITE analysis resolved five translation factor proteins, five proteins involved in mitochondrial biogenesis and seven exosome-associated proteins. SSDA proteins with a decreased abundance in the fat bodies of GCF compared to control-treatment bees were involved in translation (eight proteins), amino acid synthesis (four proteins), and collagen IV and G2F domain (two proteins) (Figure S5-4B). BRITE analysis resolved eight proteins associated with the exosome and five proteins involved in transfer RNA biogenesis.

5.3.1.3.3 Glyphosate Commercial Formulation vs Active Ingredient

GCF and GAI treated bees were subjected to a pairwise comparison and subsequent STRING, KEGG and BRITE analysis to resolve differences in the pathways and processes enriched from SSDA proteins to determine differences between Roundup Optima+® and technical grade glyphosate on the brain and fat body of *B. terrestris*. SSDA proteins with an increased abundance in the brain of GCF compared to GAI treated bees were involved in pyruvate metabolism and the TCA cycle (six proteins),

intracellular pH reduction (three proteins), rhodopsin-mediated signaling (three proteins) and regulation of synapse organisation (seven proteins) (Figure S5-5A). KEGG analysis resolved five proteins involved in purine metabolism and seven proteins involved in carbon metabolism, including PRPP biosynthesis. BRITE analysis of increased SSDA proteins resolved an enrichment in membrane trafficking (thirteen proteins), chromosome and associated proteins (six proteins), and exosome-associated proteins (eight proteins). SSDA proteins with a decreased abundance in the brain were involved in lipid metabolism (four proteins), proteasome degradation (three proteins), and the ribosome (six proteins) (Figure S5-5B). BRITE analysis further resolved five transporter proteins and six exosome proteins.

SSDA proteins with an increased abundance in the fat bodies of GCF compared to GAI treated bees were involved in pyruvate metabolism (three proteins), valine, leucine, and isoleucine degradation (four proteins), fructose and mannose metabolism (two proteins), RNA transport (three proteins) and fatty acid biosynthesis (two proteins) (Figure S5-6A). BRITE analysis resolved five proteins involved in mRNA biogenesis. SSDA proteins with a decreased abundance were involved in prostacyclin signalling through prostacyclin receptor (two proteins) and glycolysis and gluconeogenesis (two proteins) (Figure S5-6B). KEGG analysis resolved an enrichment in amino acid synthesis (two proteins), and the phagosome (two proteins). BRITE resolved proteins involved in mRNA biogenesis (two proteins), membrane trafficking (two proteins), and the exosome (six proteins).

Table 5-1 A Preview of STRING Enrichments from SSDA Proteins from Pairwise Comparisons of Glyphosate Exposure in the *B. terrestris* Brain and Fat Body. Relative fold change (RFC) direction, enriched terms, origin database and term ID, and false discovery rate (FDR) were recorded. Further enrichments can be found in the chapter 5 appendix (Table S5-5; Table S5-6).

Pairwise comparison	Tissue	RFC direction	Term description	Database	Term ID	FDR
GAI vs Control	Brain	Increased	Proteasome	KEGG	dme03050	0.0056
		Increased	Terpenoid backbone biosynthesis	KEGG	dme00900	0.0011
		Decreased	TCA cycle	STRING clusters	CL:6097	0.00011
		Decreased	Mitochondrion	GO component	GO:0005739	0.0139
	Fat body	Increased	RNA metabolism	Reactome	DME-8953854	0.00017
		Increased	Proteasome degradation	WikiPathways	WP281	0.0168
		Decreased	Translation	GO Process	GO:0006412	0.00055
		Decreased	Lipid droplet	UniProt Keywords	KW-0551	0.018
GCF vs Control	Brain	Increased	Cellular responses to stress	Reactome	DME-2262752	1.54E-06
		Increased	Unfolded protein binding	GO function	GO:0051082	0.00012
		Decreased	Ribosome	KEGG	dme03010	1.40E-14
		Decreased	Oxidative phosphorylation	KEGG	dme00190	0.0329
	Fat body	Increased	Valine, leucine, and isoleucine degradation	KEGG	dme00280	0.0055
		Increased	Superoxide dismutase complex	COMPARTMENTS	GOCC:1902693	0.0142
		Decreased	Translation	GO Process	GO:0006412	0.00079
		Decreased	Biosynthesis of amino acids	KEGG	dme01230	0.0019
GCF vs GAI	Brain	Increased	Pyruvate metabolism and TCA cycle	Reactome	DME-71406	0.00094
		Increased	Regulation of synapse organisation	GO process	GO:0050807	0.0266
		Decreased	Ribosome	KEGG	dme03010	0.00024
		Decreased	Proteasome degradation	WikiPathways	WP281	0.0041
	Fat body	Increased	Pyruvate metabolism	KEGG	dme00620	0.0034
		Increased	RNA transport	KEGG	dme03013	0.0447
		Decreased	Prostacyclin signalling via prostacyclin receptor	STRING clusters	CL:11704	0.045
		Decreased	Glycolysis and gluconeogenesis	WikiPathways	WP144	0.0047

5.3.1.4 Hierarchical Clustering

Hierarchical clustering of mean z-score normalised LFQ values of proteins found in the brain and fat body of *B. terrestris* was conducted (Table S5-9; Table S5-10). All proteins represented in the hierarchical clustering heatmap are statistically significant (ANOVA, Ben-Ho FDR <0.05) with each cluster revealing a group of proteins with a relative general expression pattern in the GAI, GCF or control treatment groups. Proteins in each cluster were analysed using STRING v.11 and *D. melanogaster* reference genome to reveal if specific processes, pathways, or protein functions were represented by any of the identified protein clusters.

Hierarchical clustering resolved eleven clusters in the brain (Clusters A-K) (Figure 5-6; Table S5-11). Cluster A included 5 proteins with a similar abundance across all treatment groups. There were no significant enrichments. In cluster B, there were 42 proteins with an increased abundance in both glyphosate-based treatment groups in comparison to the control treatment group. These proteins were associated with the regulation of SNARE complex assembly (vam6/Vps39-like protein and vacuolar protein sorting-associated protein 11 homolog) and ubiquitin-specific processing proteases (ubiquitin carboxyl-terminal hydrolase 14 and proteasome subunit beta type-3). Cluster C included 23 proteins that had low abundance in the control treatment group, an increased abundance in the GAI-treated group and a further increased abundance in the GCF-treated group. Three out of sixteen identified proteins were subunits delta, beta and theta of the T-complex protein associated with protein folding. Two proteins were associated with clathrin-coated pits (F-BAR domain only protein 2 and AP-1 complex subunit beta-1 isoform X1) and a further three proteins were associated with detoxification of reactive oxygen species and aldehyde metabolism (glutaredoxin-1, S-formylglutathione hydrolase and chloride intracellular channel exc-4). Cluster D comprised 18 proteins with a high abundance in the GAI-treated group compared to the GCF and control-treated groups. Five of the thirteen proteins identified were associated with lipid biosynthetic process (mevalonate kinase, ecdysteroid-regulated 16 kDa protein, geranylgeranyl pyrophosphate synthase, hydroxymethylglutaryl-CoA synthase 1, and fatty acid synthase). There were six proteins in cluster E that were of high abundance in the control-treatment compared to both glyphosate-treatment groups with no significant enrichments. Cluster F has eight proteins with a low abundance in GCF compared to GAI and control treatment groups

which also had no significant enrichments. Cluster G had 19 proteins, two of these proteins were ribosomal subunits (40S ribosomal protein S6 and 60S ribosomal protein L23a). Cluster H contained seven proteins of high abundance in the control treatment group compared to glyphosate-based treatment groups and had no significant enrichments. There were 20 proteins in cluster I, with low abundance in GAI, high abundance in control and a relative intermediate abundance in the GCF treatment group. Two proteins were associated with alanine, aspartate, and glutamine metabolism (glutamate dehydrogenase, mitochondrial, and putative glutamate synthase) and two were associated with the regulator of ATPase of vacuoles and endosomes (RAVE) complex (WD repeat-containing protein 7 and dmX-like protein 2). Cluster J had eleven proteins that were of a high abundance in GCF compared to control (intermediate abundance) and GAI (low abundance) treatment groups and included two proteins that were part of the Cul2-RING ubiquitin ligase complex (cullin-2 and RING-box protein 1A). Cluster K had six proteins with high abundance in GCF and low relative abundance in the GAI and control treatment groups; there were no significant enrichments.

Cluster	Term description	Database	Term ID	FDR
A	No significant enrichments			
B	Regulation of SNARE complex assembly	GO process	GO:0035542	0.0029
C	Chaperone	Uniprot	KW-0143	0.0199
D	Lipid biosynthetic process	GO process	GO:0008610	0.0035
E	No significant enrichments			
F	No significant enrichments			
G	Ribosome	KEGG	CL:437	0.0331
H	No significant enrichments			
I	Alanine, aspartate and glutamate metabolism	KEGG	dme00250	0.0123
J	Cul2-RING ubiquitin ligase complex	GO component	GO:0031462	0.0147
K	No significant enrichments			

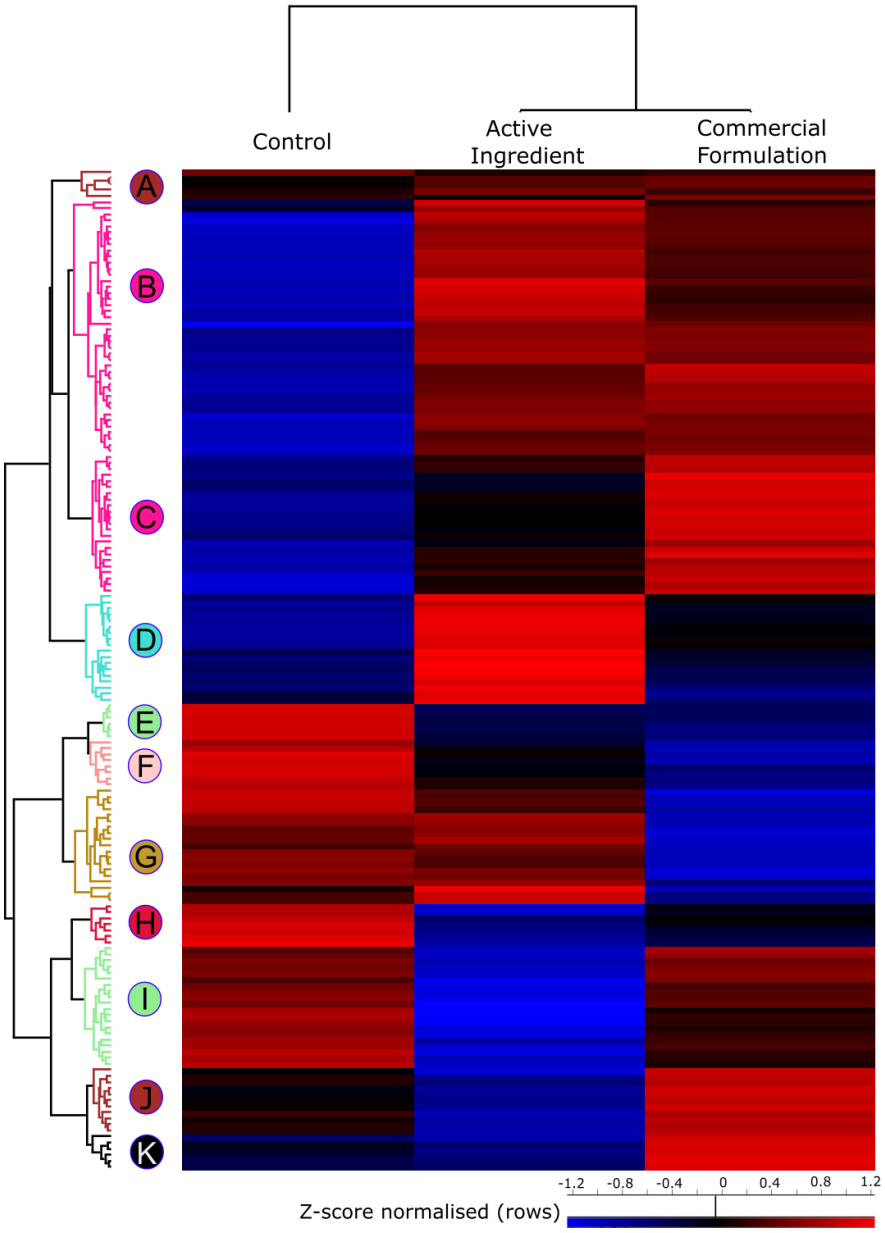


Figure 5-6 A Heatmap Produced from Hierarchical Clustering of Mean Z-score Normalised LFQ Values in the Brain Proteome of Glyphosate-exposed Bees. STRING analysis determined protein cluster characteristic descriptions. Each cluster is denoted alphabetically with blue representing decreased protein abundance and red representing protein abundance increases in each cluster.

Hierarchical clustering resolved seven clusters in the fat body (Clusters A-G) (Figure 5-7; Table S5-12). Cluster A had one protein with a high relative abundance in GAI compared to GCF and control treatment groups which was nuclease-sensitive element-binding protein 1. Cluster B had 18 proteins with increased abundance of proteins from both glyphosate treatment groups compared to the control treatment group. Two proteins in this cluster were associated with ubiquitin-conjugating enzyme E2 (ubiquitin-conjugating enzyme E2 variant 2 and SUMO-conjugating enzyme UBC9-B). Cluster C had three proteins increased in the control treatment group and cluster D had four proteins with a decreased in the GCF treatment group compared to other treatment groups, neither cluster resolved any significant enrichments. There were five proteins with a decreased abundance in GAI compared to GCF and control treatment groups in cluster E. Two of these proteins were associated with pyruvate metabolism (putative aldehyde dehydrogenase family 7 member and pyruvate carboxylase, mitochondrial). Cluster F had seven proteins with an increased relative abundance in the control group compared to GAI and GCF treatment groups. Two of these proteins were involved in amino acid biosynthesis (cystathionine beta-synthase and glyceraldehyde-3-phosphate dehydrogenase 2). Cluster G had eleven proteins with a relative increased abundance in the control, decreased abundance in GAI and an intermediate abundance in GCF treatment groups. Proteins were involved in glycolysis/gluconeogenesis (fructose-1,6-bisphosphatase 1 and retinal dehydrogenase 1) and the peroxisome (hydroxyacid oxidase 1 and serine--pyruvate aminotransferase, mitochondrial).

Cluster	Term description	Database	Term ID	FDR
A	No significant enrichments			
B	Ferrous iron binding	GO function	GO:0008198	0.0378
C	No significant enrichments			
D	No significant enrichments			
E	Pyruvate metabolism	KEGG	dme00620	0.0041
F	Biosynthesis of amino acids	KEGG	dme01230	0.0183
	Glycolysis/gluconeogenesis	KEGG	dme00010	0.0136
G	Arginine and proline metabolism	KEGG	dme00330	0.0136
	Peroxisome	KEGG	dme04146	0.0284

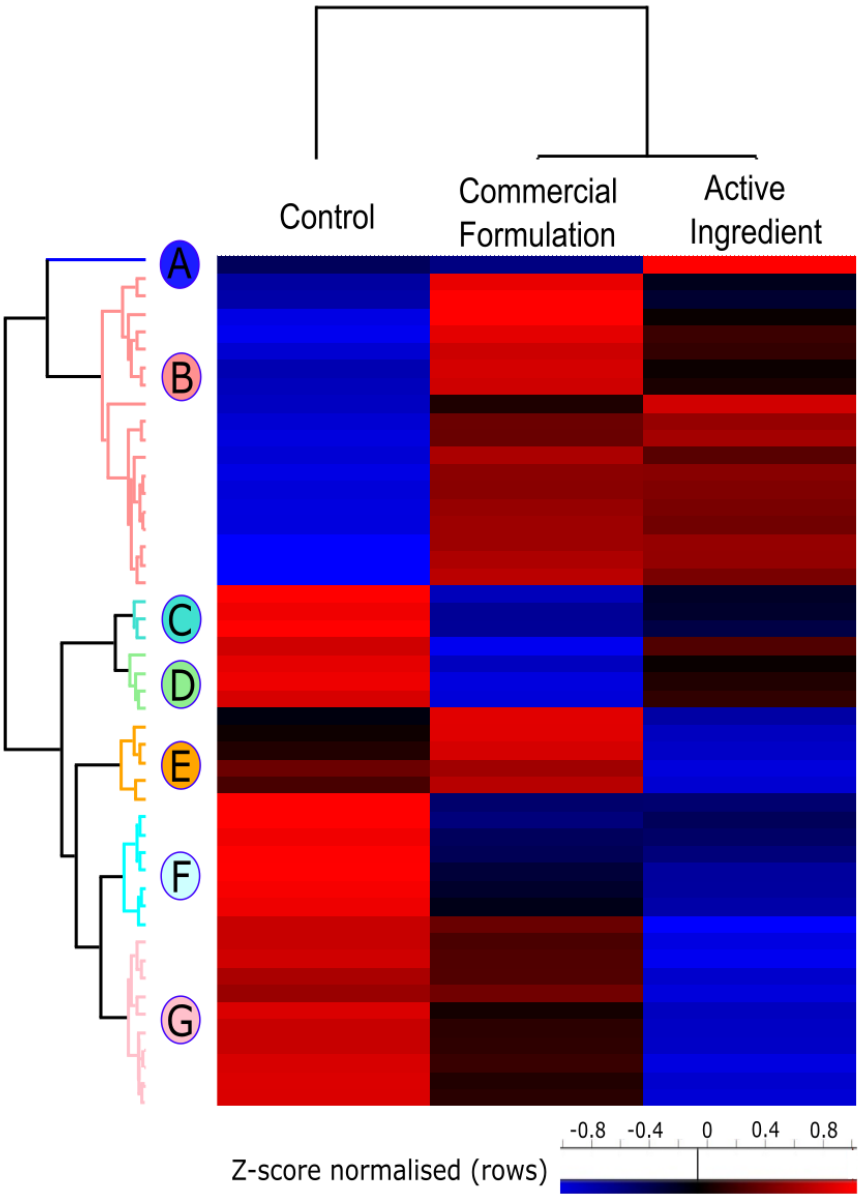


Figure 5-7 A Heatmap Produced from Hierarchical Clustering of Mean Z-score Normalised LFQ Values in the Fat Body Proteome of Glyphosate-exposed Bees. STRING analysis determined protein cluster characteristic descriptions. Each cluster is denoted alphabetically with blue representing decreased protein abundance and red representing protein abundance increases in each cluster.

5.3.1.5 Proteins With a Common Abundance Profile in the Brain and Fat Body After Exposure to Either Glyphosate-Based Treatment

To determine if there was a consistent glyphosate-effect on the brain and fat body of GAI and GCF-exposed bees, SSDA protein sets from both glyphosate treatments versus the control were compared to find common proteins with similar RFCs across treatment groups (Table 5-2; Table S5-13).

There were 24 increased and 10 decreased abundance proteins common in the brain proteomes of both GAI and GCF exposed groups. The protein common to both GAI and GCF treatment groups with the highest relative fold change was putative mediator of RNA polymerase II transcription subunit 26 which had an RFC of + 35.42 and + 27.80 in GAI and GCF treatment groups, respectively. Other proteins with an increased abundance in the brain after both glyphosate-based treatments were associated with the proteasome (proteasome subunit beta type-3, transitional endoplasmic reticulum ATPase TER94, and proteasome subunit alpha type-7-1), chaperonin-containing T-complex (T-complex protein 1 subunit eta and T-complex protein 1 subunit delta), and oxidative stress regulation (thioredoxin-2 and D-2-hydroxyglutarate dehydrogenase, mitochondrial). Two proteins with a decreased abundance in the brain were associated with long-term memory and the regulation of exocytosis (secretory carrier-associated membrane protein 5A and syntaxin-binding protein 5). The common protein with the lowest relative fold change in both treatment groups was syndecan with an RFC of – 4.81 and – 4.75 in GAI and GCF treatment groups, respectively.

There were 7 increased and 10 decreased abundance proteins common in the fat body proteomes of both GAI and GCF exposed groups. The protein common to both GAI and GCF treatment groups with the highest relative fold change compared to the control was coiled-coil domain-containing protein 58, also referred to as protein MIX23 with an RFC of + 5.21 and + 5.29 in GAI and GCF treatment groups, respectively. This protein is associated with mitochondrial protein importation. Further, two proteins common to both glyphosate-based treatment groups were involved in protein biosynthesis (transcription elongation factor SPT5 and eukaryotic translation initiation factor 2 subunit 1). Lipid storage droplets surface-binding protein 1 had the lowest relative fold change of all glyphosate-based treatment common

proteins with an RFC of – 3.03 and – 5.15 in the fat body proteome of GAI and GCF treatment groups, respectively. This protein is involved in lipid storage regulation and lipolysis activation. Four decreased abundance proteins common to both glyphosate-based treatments were associated with translation and protein biosynthesis (40S ribosomal protein S15, elongation factor 1-alpha, translation elongation factor 2, and leucine-tRNA ligase).

Table 5-2 Conserved Response to Both Glyphosate-based Treatments in the Brain and Fat Body. Functional annotations, RFC's ≥ 1.5 in both treatments, MS measurements and characteristics for all SSDA proteins with similar expression profiles in both GAI and GCF treated bees. Relative fold changes and directions were determined against the procedural control.

Tissue	Functional Annotation	Protein ID	Protein Name	RFC AI	RFC CF	FC direction	No of peptides	Mol wt [kDa]	MS/MS count
Brain	Carbohydrate metabolism	XP_003398652.1	Glucose-6-phosphate 1-epimerase	1.5	1.5	Increased	13	31.9	69
	Proteolysis	XP_012166535.1	CTP synthase	1.9	3.4	Increased	9	66.4	28
	Regulation of transcription	XP_003394768.1	Activated RNA polymerase II transcriptional coactivator p15	3.0	3.6	Increased	3	13.4	22
	Steroid biosynthesis	XP_003396065.1	Adrenodoxin	11.6	7.1	Increased	4	17.7	21
	RNA processing	XP_003396881.1	Putative mediator of RNA polymerase II transcription subunit 26	35.4	27.8	Increased	5	58.5	23
	mRNA splicing	XP_003393281.1	putative U5 small nuclear ribonucleoprotein 200 kDa helicase	1.8	1.8	Increased	31	242.6	83
	Signal transduction	XP_003393719.1	Ras GTPase-activating protein 1	2.9	2.3	Decreased	6	107.3	19
	Chromosome condensation	XP_012175524.1	Late histone H1-like	1.6	1.9	Decreased	13	23.3	89
	Transport	XP_012172275.1	Major facilitator superfamily domain-containing protein 6	1.9	1.6	Decreased	10	114.5	84
	Synaptic vesicle exocytosis	XP_012170680.1	Secretory carrier-associated membrane protein 5A	1.7	1.5	Decreased	9	39.2	72
	Serine proteinase inhibition	XP_003401213.2	Uncharacterized protein LOC100650789	1.5	2.0	Decreased	5	13.8	104
	Axon guidance	XP_003401491.1	Syndecan	4.8	4.8	Decreased	3	25.9	16
Fat body	mRNA transport	XP_012171232.1	Nucleoporin p58/p45	1.8	1.8	Increased	5	52.0	17
	Proteolysis	XP_020719744.1	Serine proteinase stubble	1.8	1.6	Increased	14	51.9	150
	Mitoprotein-induced stress response	XP_020718482.1	Coiled-coil domain-containing protein 58	5.2	5.3	Increased	7	19.1	35
	Iron-sulfur cluster assembly	XP_003394464.2	Iron-sulfur cluster assembly enzyme ISCU, mitochondrial	2.6	3.0	Increased	7	14.9	37
	Microtubule organisation	XP_003401833.1	Tubulin-folding cofactor B	2.2	2.4	Increased	6	28.0	31
	Regulation of lipid storage	XP_012167204.1	Lipid storage droplets surface-binding protein 1	3.0	5.2	Decreased	13	44.9	40

5.3.2 LFQ analysis of prothioconazole exposure on the brain and fat body proteome of *B. terrestris*

5.3.2.1 Identified and quantified proteins

There were 3,012 proteins identified from the *B. terrestris* brain, with 1,767 proteins remaining post-imputation and log_2 transformation (Table S5-14). 2,700 proteins were identified from the *B. terrestris* fat body, with 1,502 proteins remaining post-imputation and log_2 transformation (Table S5-15). PCA of brain samples had a variance of 22.9% in component one and 13.9% in component two (Figure 5-8A). In PCA of the brain proteome, PAI, PCF and control-treated samples mainly clustered together, whilst the acetone control-treated group showed some variation, drifting away from samples from other treatment groups. PCA on fat body samples had a variance of 15.8% in component one and 14.3% in component two (Figure 5-8B). Fat body samples from the control, PCF and acetone control treatment groups mainly clustered with other samples from their relative treatment group, with some overlap between treatment sample clustering. However, fat body samples from PAI-exposed bees showed more variance in clustering pattern compared to other treatment groups.

5.3.2.2 Two-Sample T-Tests

Two sample t-tests were utilized for pairwise comparisons of treatment groups to determine SSDA proteins ($p \leq 0.05$, S0 = 0.1) and their relative fold differences in protein abundance (Table S5-16; Table S5-17).

5.3.2.2.1 Prothioconazole Active Ingredient vs Control

There were 181 SSDA proteins resolved from the brains of PAI exposed bees compared to acetone control exposed bees (RFC range: + 10.6 to – 67.9). There were 108 proteins with an increased abundance and 73 proteins with a decreased abundance in PAI compared to control exposed bees (Figure 5-9A). The top ten SSDA proteins with the highest abundance in the brains of PAI-exposed bees were: splicing factor 1 (RFC: + 10.55), collagen alpha-1 (RFC: + 10.29), eukaryotic translation initiation factor (RFC: + 8.48), PRA1 family protein 3 (RFC: + 7.56), dolichol-phosphate mannosyltransferase (RFC: + 5.92), vesicular glutamate transporter 1 (RFC: + 5.30), hexaprenyldihydroxybenzoate methyltransferase (RFC: + 4.77), serine/threonine-protein phosphatase (RFC: + 4.29), uncharacterized protein LOC100646066 (RFC: + 4.26) and KH domain-containing protein(RFC: + 4.21).

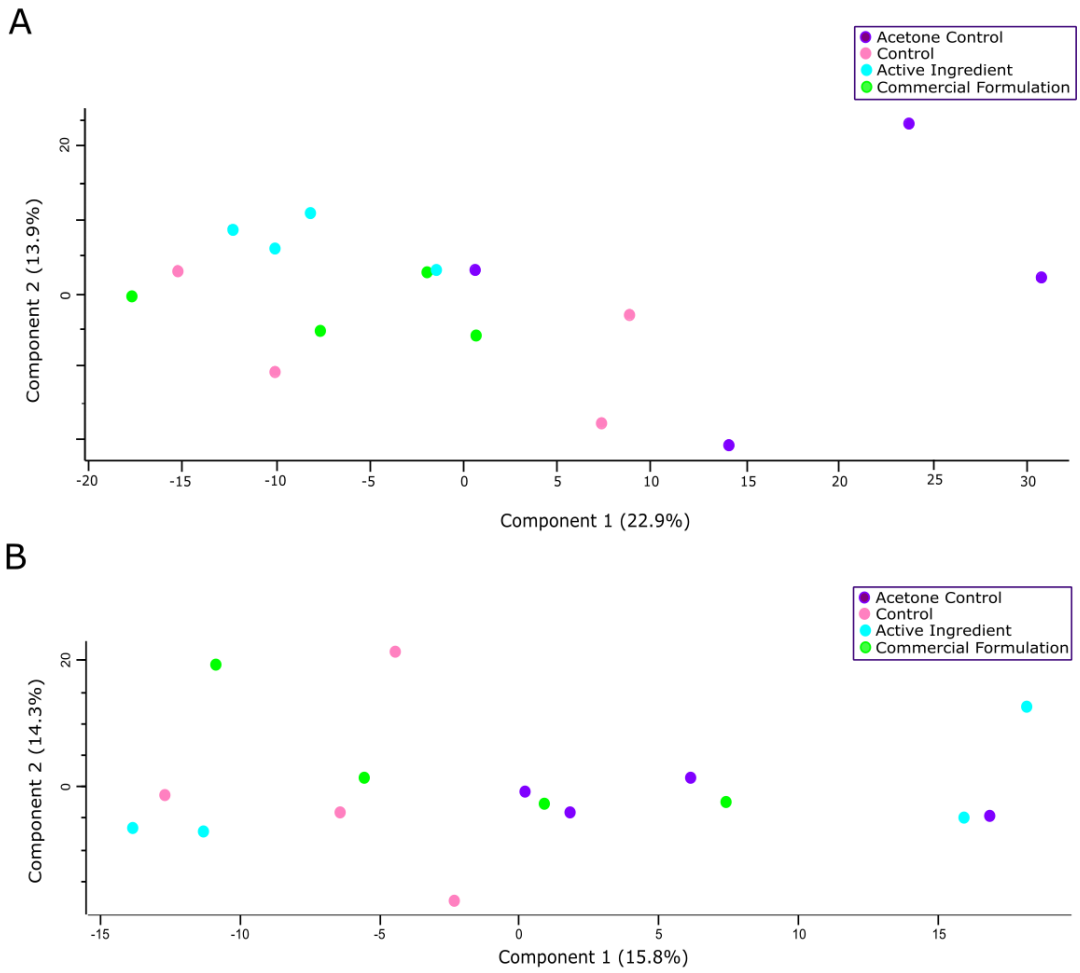


Figure 5-8 PCA Representing Variation Between Prothioconazole-Exposed Samples. In A) the PCA demonstrates variation in the brain proteome and in B) the PCA demonstrates variation in the fat body proteome, with each point representing the brain or fat body proteome of a single bee.

The top ten SSDA proteins with the lowest abundance in the brains of PAI-exposed bees compared to acetone control-exposed bees were: paramyosin (RFC: - 67.81), troponin T (RFC: - 46.93), troponin C (RFC: - 42.23), troponin I (RFC: - 36.26), myosin regulatory light chain 2 (RFC: -25.79), myosin heavy chain (RFC: - 25.42), uncharacterized protein LOC100647860 (RFC: - 17.98), myosin light chain (RFC: - 16.67), troponin C (RFC: -13.39) and tropomyosin-1 (RFC: - 13.19).

In the fat bodies of PAI-exposed compared to acetone control-exposed bees, there were 35 SSDA proteins (RFC range: + 6.5 to – 3.6). There were 22 proteins with an increased abundance and 13 proteins with a decreased abundance in the fat bodies of PAI-exposed bees compared to acetone control-exposed bees (Figure 5-10A). The top ten proteins with an increased abundance included: WW domain-binding protein (RFC: + 6.48), Golgi

phosphoprotein 3 (RFC: + 5.02), COP9 signalosome complex subunit (RFC: + 4.25), 85/88 kDa calcium-independent phospholipase (RFC: + 3.83), multifunctional methyltransferase subunit TRM112 (RFC: + 3.21), transportin-1 (RFC: + 1.45), nucleoporin Nup43 (RFC: + 1.4), RNA-binding protein squid (RFC: + 1.31), pyruvate carboxylase (RFC: + 1.24) and T-complex protein 1 (RFC: + 1.22). Although of relatively low fold differences, 6 of the 22 proteins with increased abundances were annotated as T-complex proteins. The top ten proteins with a decreased abundance in PAI-exposed fat bodies compared to the acetone control were: sterol O-acyltransferase (RFC: - 3.61), N(4)-(Beta-N-acetylglucosaminyl)-L-asparaginase (RFC: - 1.71), FAD-dependent oxidoreductase (RFC: -1.57), beta-ureidopropionase (RFC: - 1.43), acyl-CoA synthetase family member 2, mitochondrial (RFC: - 1.35), alpha-aminoadipic semialdehyde synthase (RFC: - 1.28), NHP2-like protein (RFC: - 1.27), glutaredoxin-C4 (RFC: - 1.26), eukaryotic translation initiation factor (RFC: - 1.25) and protein dj-1beta (RFC: - 1.20).

5.3.2.2.2 Prothioconazole Commercial Formulation vs Control

There were 49 SSDA proteins resolved from brain samples of PCF-exposed bees compared to control-exposed bees (RFC range: + 3.5 to – 12.0). This included 35 proteins with an increased abundance and 14 proteins with a decreased abundance in the brain after PCF exposure (Figure 5-9B). The top ten proteins with an increased abundance included: prostaglandin E synthase 2 (RFC: + 3.50), transmembrane emp24 protein (RFC: + 3.41), Wiskott-Aldrich syndrome protein (RFC: + 3.30), alpha/beta hydrolase (RFC: + 2.83), uncharacterized protein LOC100649490 (RFC: + 2.72), polypyrimidine tract-binding protein 2 (RFC: + 2.14), valine--tRNA ligase (RFC: + 1.80), 60S ribosomal protein L18 (RFC: + 1.32), centrosomal protein of 290 kDa (RFC: + 1.31) and 40S ribosomal protein S11 (RFC: + 1.30). The top ten proteins with the most decreased abundance in the brains of PCF-exposed bees compared to control-exposed bees were: putative mediator of RNA polymerase II transcription subunit 26 (RFC: - 12.04), RAC serine/threonine-protein kinase (RFC: - 5.85), ubiquitin-conjugating enzyme E2 (RFC: - 5.50), DNA fragmentation factor subunit beta (RFC: - 3.25), ATP-binding cassette sub-family F member 2 (RFC: - 3.17), uncharacterized protein LOC100648913 (RFC: - 2.22), methionine sulfoxide reductase (RFC: - 1.71), lissencephaly-1 (RFC: - 1.26), poly(ADP-ribose) glycohydrolase ARH3 (RFC: - 1.26) and S-adenosylmethionine synthase (RFC: - 1.19).

A total of 95 proteins were SSDA in the fat bodies of bees exposed to PCF in comparison to control-exposed bees (RFC range: + 8.3 to - 7.0). These included 33 proteins with an increased abundance and 62 proteins with a decreased abundance in PCF compared to control-exposed

bees (Figure 5-10B). The top ten SSDA proteins with an increased abundance after PCF exposure in the fat body were: translocon-associated protein (RFC: + 8.32), pre-mRNA-processing factor (RFC: + 6.53), ubiquitin-conjugating enzyme E2 (RFC: + 5.46), tumour suppressor candidate 3 (RFC: + 4.51), ras-like protein 3 (RFC: + 4.44), dentin sialophosphoprotein (RFC: + 3.75), uncharacterized protein LOC100649724 (RFC: + 2.86), nuclear ribonucleoprotein H (RFC: + 2.49), serine/threonine-protein kinase (RFC: + 2.08) and heat shock protein 83 (RFC: + 1.52). The top ten SSDA proteins with a decreased abundance in the fat body after PCF-exposure compared to controls were: histone H2A.V (RFC: - 6.99), vesicle-fusing ATPase (RFC: - 4.73), quinone oxidoreductase (RFC: - 3.96), cubilin (RFC: - 3.80), protein FAM151B (RFC: - 3.51), guanine nucleotide-binding protein (RFC: - 3.06), phosphatidylinositol-binding clathrin assembly protein (RFC: - 2.80), rab11 family-interacting protein (RFC: - 2.79), small nuclear ribonucleoprotein (RFC: - 2.76), and rabankyrin-5 (RFC: - 2.73).

5.3.2.2.3 Acetone vs Non-Acetone Control

Given the presence of acetone in only PAI treatments and the clear effect that acetone had on the digestive tract proteome in comparison to the non-acetone control (See Chapter 4), a comparison of PCF to PAI-exposed tissues could not be made. Instead, an analysis of the differentially abundant proteins that occurred between the acetone and non-acetone controls for both brain and fat body tissues was conducted to acquire additional evidence for the acetone effect observed first here in the digestive tracts of acetone control-exposed bees. In total, 76 SSDA proteins were identified from the brains of acetone control-exposed bees compared to control exposed bees (RFC range: + 33.4 to – 40.0). These included 45 proteins with an increased abundance and 31 proteins with a decreased abundance from the brains of acetone compared to non-acetone-exposed bees (Figure 5-9C). The top ten proteins with an increased abundance included: pancreatic lipase-related protein 2 (RFC: + 33.39), regucalcin (RFC: + 17.44), uncharacterized protein LOC100651683 (RFC: + 17.27), uncharacterized protein LOC100644055 (RFC: + 16.74), glucosylceramidase (RFC: + 9.59), fatty-acid-CoA ligase bubblegum (RFC: + 6.54), 27 kDa hemolymph protein (RFC: + 6.43), glucose-6-phosphate 1-dehydrogenase (RFC: + 5.77), glucose dehydrogenase (RFC: + 5.62), and uncharacterized protein LOC100649490 (RFC: + 4.72). The top ten proteins with the most decreased abundance in the brain after acetone exposure were: uncharacterized protein LOC100647721 (RFC: - 40.0), HIG1 domain family member (RFC: - 10.66), PRA1 family protein 3 (RFC: - 9.58), protein FAM162B (RFC: - 6.30), dolichol-phosphate mannosyltransferase (RFC: - 6.11),

vesicular glutamate transporter (RFC: - 5.83), hexaprenyldihydroxybenzoate methyltransferase (RFC: - 4.09), ATP-binding cassette sub-family F member 2 (RFC: - 3.98), constitutive coactivator of PPAR-gamma (RFC: - 3.90), and cytochrome P450 (RFC: - 3.38).

Further, 49 proteins were SSDA in the fat bodies of acetone control-exposed bees compared to non-acetone-control-exposed bees (RFC range: + 5.8 to – 3.8), with 21 proteins with an increased abundance and 28 proteins with a decreased abundance (Figure 5-10C). The top ten proteins with an increased abundance included: fatty acyl-CoA reductase 1 (RFC: + 5.80), elongation of very long chain fatty acids protein (RFC: + 4.90), fatty acyl-CoA reductase 1 (RFC: + 4.30), tumour suppressor candidate 3 (RFC: + 3.84), sterol O-acyltransferase 1 (RFC: + 3.17), ATP-binding cassette sub-family G (RFC: + 3.10), dentin sialophosphoprotein (RFC: + 2.95), RAC serine/threonine-protein kinase (RFC: + 2.94), very-long-chain 3-oxoacyl-CoA reductase (RFC: + 2.84), and protein phosphatase 2C T23F11.1 (RFC: + 2.57). The top ten proteins with the most decreased abundance in the fat bodies after acetone control exposure were: KH domain-containing protein (RFC: - 3.80), Golgi phosphoprotein 3 (RFC: - 3.74), epidermal growth factor receptor (RFC: - 3.63), rabankyrin-5 (RFC: - 3.47), pancreatic triacylglycerol lipase (RFC: - 1.87), SPARC (RFC: - 1.70), sorting nexin-6 (RFC: - 1.65), PTB domain-containing engulfment adapter protein 1 (RFC: - 1.63), sorting nexin-2 (RFC: - 1.61), and protein enhancer of sevenless 2B (RFC: - 1.59).

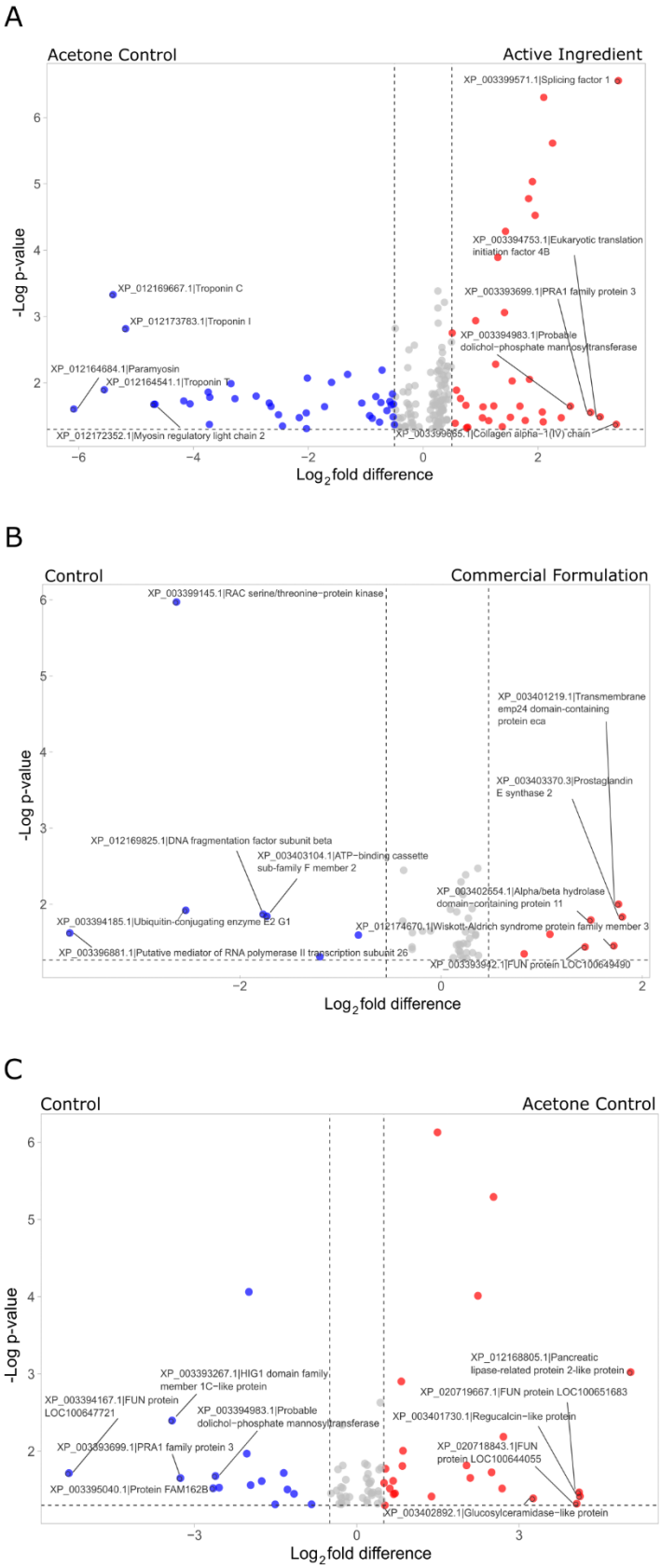


Figure 5-9 A Two Sample t-test was Performed to Resolve and Compare SSDA Proteins in the Brain of *B. terrestris* After Prothioconazole Exposure. Volcano plots were generated to illustrate the number of SSDA proteins with an increased (red) and decreased (blue) abundance in the brain in pairwise comparisons of bees exposed to **(A)** PAI vs acetone control, **(B)** PCF vs control, or **(C)** acetone control vs control. The five proteins with the highest and lowest RFCs were labelled.

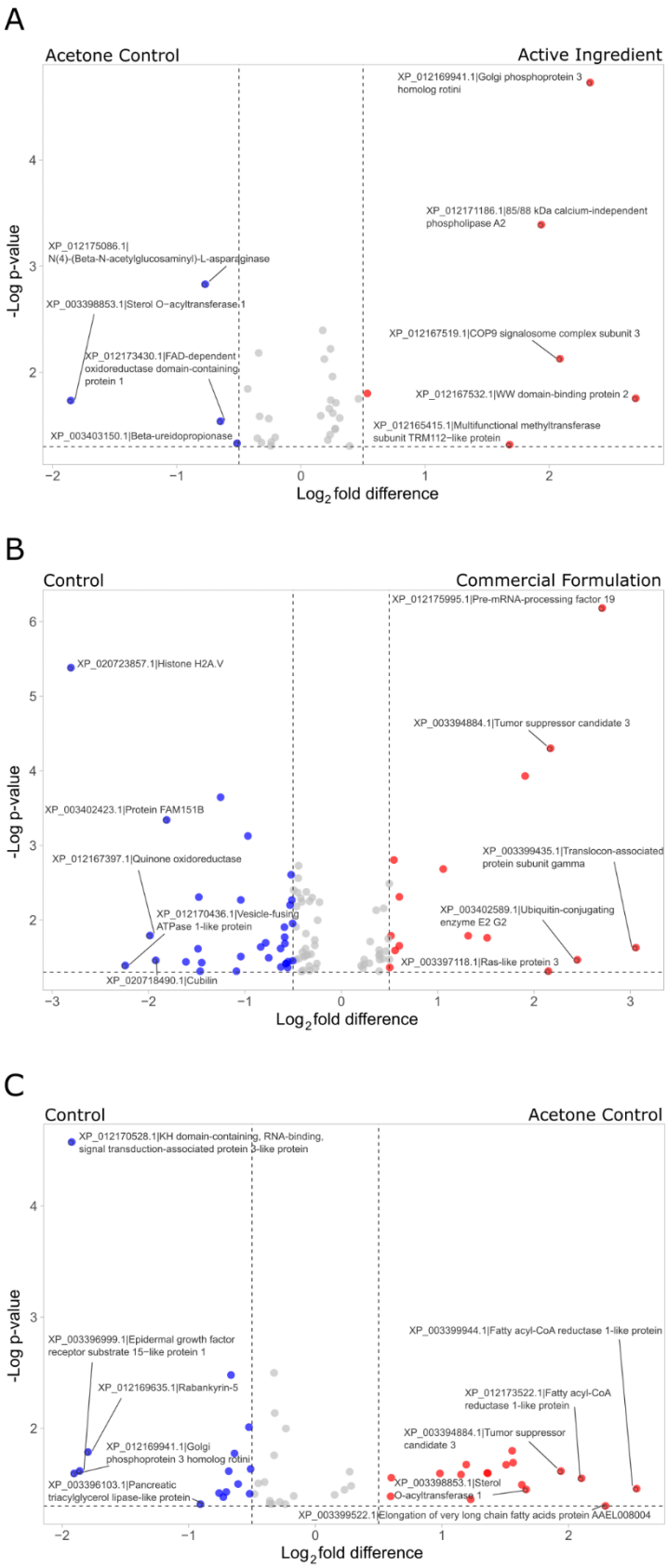


Figure 5-10 A Two Sample t-test was Performed to Resolve and Compare SSDA Proteins in the Fat Body of *B. terrestris* after Prothioconazole Exposure. Volcano plots were generated to illustrate the number of SSDA proteins with an increased (red) and decreased (blue) abundance in the fat body in pairwise comparisons of bees exposed to **(A)** PAI vs acetone control, **(B)** PCF vs control, or **(C)** acetone control vs control. The five proteins with the highest and lowest RFCs were labelled.

5.3.2.3 Gene Ontology and Pathway Analysis of SSDA Proteins

To determine the pathways and processes impacted in the brain and fat bodies of prothioconazole-exposed bees, SSDA proteins from pairwise comparisons were analysed for enriched PPI networks, GO terms, KEGG pathways and Interpro domains using STRING v.11 utilising the *D. melanogaster* reference genome (Table 5-3; Table S5-18; Table S5-19). BlastKOALA was also used in the analysis of SSDA proteins against KEGG and BRITE databases (Table S5-20; Table S5-21).

5.3.2.3.1 Prothioconazole Active Ingredient vs Control

SSDA protein comparisons of the brain proteome of PAI-exposed and acetone control-exposed bees resolved an enrichment in increased abundance proteins associated with cytoskeleton organisation (20 proteins), regulation of cell communication (25 proteins), the dopamine neurotransmitter release cycle and presynaptic cytoskeleton (five proteins), neurotransmitter biosynthesis, and excitatory synapse (three proteins) (Figure S5-7A). KEGG analysis resolved enrichments for the PI3K-Akt signaling pathway (nine proteins), endocytosis (five proteins), and the dopaminergic synapse (eight proteins). BRITE analysis resolved 21 proteins associated with membrane trafficking, five DNA repair and recombination proteins, and seven chromosome and associated proteins. Decreased abundance proteins in PAI compared to acetone-control exposed bees revealed an enrichment in cytoskeleton organisation (17 proteins), glycogen metabolism (four proteins), and the pentose phosphate pathway (three proteins) (Figure S5-7B). Further, 14 immune system proteins were enriched in the proteins with a decreased abundance dataset. KEGG analysis resolved enrichment in the pentose phosphate pathway (five proteins).

In the fat bodies of bees exposed to PAI in compared to the acetone control, increased abundance proteins were enriched for protein folding, with the six SSDA proteins identified all involved in the chaperonin-containing t-complex (Figure S5-8A). KEGG analysis resolved an enrichment in nucleocytoplasmic transport (two proteins) and BRITE resolved an enrichment in mRNA biogenesis (five proteins). SSDA proteins with a decreased abundance had no significant enrichments in STRING analysis with KEGG analysis resolving an enrichment in metabolic pathways (four proteins) and enzymes (nine proteins) (Figure S5-8B).

5.3.2.3.2 Prothioconazole Commercial Formulation vs Control

SSDA proteins with an increased abundance from the brains of PCF compared to control-exposed bees were enriched for translation (20 proteins) and mRNA processing (two proteins) (Figure S5-9A). SSDA proteins with a decreased abundance in brains of PCF compared to control-exposed bees had no significant enrichments in STRING analysis (Figure S5-9B). BRITE analysis resolved eight enzymes which included transferases, hydrolases, and an isomerase.

SSDA proteins with an increased abundance in the fat bodies of PCF-exposed bees compared to control-exposed bees were enriched for protein folding (six proteins) and cellular responses to stress (nine proteins) (Figure S5-10A). KEGG analyses resolved enrichments for pentose and glucuronate interconversions (three proteins) and protein processing in the endoplasmic reticulum (five proteins). BRITE analysis highlighted enrichments for chaperones and folding catalysts (eight proteins), membrane trafficking (four proteins), and exosome-associated proteins (nine proteins). SSDA proteins with a decreased abundance in PCF-exposed fat bodies compared to control-exposed fat bodies had enrichments for endocytosis (nine proteins), oxidative phosphorylation (eight proteins), and cytoskeleton organisation (eleven proteins) (Figure S5-10B). KEGG analysis revealed enrichment for PI3K-Akt signaling pathway (five proteins) and focal adhesion (five proteins) whilst BRITE highlighted enrichments in membrane trafficking (17 proteins).

5.3.2.3.3 Acetone Control vs Non-Acetone Control

SSDA proteins in the brains of bees exposed to the acetone control were compared to the brains of bees exposed to the non-acetone control. Proteins with an increased abundance in the acetone control compared to the non-acetone control had enrichments for the pentose phosphate pathway (four proteins), peroxisome (three proteins), and lipid metabolism (eleven proteins) (Figure S5-11A). BRITE analysis revealed enrichment in membrane trafficking (four proteins) and the exosome (ten proteins). Decreased abundance proteins were enriched in regulation of cell communication (nine proteins) and intracellular signal transduction (Figure S5-11B). KEGG analysis resolved enrichments in apelin signaling pathway (three proteins), phosphatidylinositol signaling system (three proteins), and axon guidance (three proteins). BRITE analysis resolved enrichments in protein kinases (four proteins), membrane trafficking (four proteins), and the exosome (three proteins).

From the fat bodies of acetone control compared to non-acetone control-exposed bees, increased abundance SSDA proteins were enriched for lipid metabolism (seven proteins) (Figure S5-12A). KEGG analysis resolved enrichment for fatty acid elongation (three proteins) and BRITE analysis resolved enrichments in the proteasome (two proteins), chromosome and associated proteins (two proteins), and transporters (two proteins). SSDA proteins with a decreased abundance in acetone control-exposed bees compared to control-exposed bees led to enrichments in vesicle-mediated transport (nine proteins) (Figure S5-12B). KEGG analysis resolved enrichment for oxidative phosphorylation (three proteins) and endocytosis (seven proteins) whilst BRITE analysis resolved enrichments for membrane trafficking (11 proteins), cytoskeleton proteins (five proteins) and the exosome (four proteins).

Table 5-3 A Preview of STRING Enrichments from SSDA Proteins from Pairwise Comparisons of Prothioconazole Exposure in the *B. terrestris* Brain and Fat Body. RFC direction, enriched terms, origin database, term ID, and FDR were recorded. Further enrichments can be found in the chapter 5 appendix.

Pairwise comparison	Tissue	RFC direction	Term description	Database	Term ID	FDR
PAI vs Acetone Control	Brain	Increased	Cytoskeleton organisation	GO Process	GO:0007010	5.03E-07
		Increased	Regulation of synapse assembly	GO Process	GO:0051963	6.64E-08
		Decreased	Cytoskeleton organisation	GO Process	GO:0007010	6.93E-07
		Decreased	Glycolysis, and Pentose phosphate pathway	STRING clusters	CL:5971	0.0001
	Fat body	Increased	Protein folding	GO Process	GO:0006457	0.00045
PCF vs Control	Brain	Increased	Translation	GO Process	GO:0006412	2.23E-21
		Increased	mRNA processing	WikiPathways	WP142	0.0289
	Fat body	Increased	Protein folding	GO Process	GO:0006457	0.0037
		Increased	Cellular response to stress	GO Process	GO:0033554	0.0078
		Decreased	Endocytosis	KEGG	dme04144	1.55E-06
		Decreased	Oxidative phosphorylation	KEGG	dme00190	3.24E-05
Acetone Control vs Control	Brain	Increased	Pentose phosphate pathway	KEGG	dme00030	6.40E-05
		Increased	Lipid metabolic process	GO Process	GO:0006629	0.00022
		Decreased	Regulation of cell communication	GO Process	GO:0010646	0.0276
		Decreased	Intracellular signal transduction	GO Process	GO:0035556	0.0362
	Fat body	Increased	Lipid metabolism	Reactome	DME-556833	0.0064
		Decreased	Vesicle-mediated transport	GO Process	GO:0016192	0.00067

5.3.2.4 Hierarchical Clustering

Hierarchical clustering of mean z-score normalized LFQ values of SSDA proteins (ANOVA, Ben-Ho FDR <0.05) found in the brain and fat body of *B. terrestris* was conducted (Figure 5-11; Figure 5-12; Table S5-22; Table S5-23). Each cluster represents a set of proteins with a general expression profile in PAI, PCF, acetone control and non-acetone control groups. Proteins in each cluster were analysed using STRING v.11 and *D. melanogaster* reference genome to reveal specific processes, pathways, or protein functions that were represented by the proteins of a given cluster.

In the brain dataset, 13 clusters were identified, of which eight including clusters A (4 proteins), D (17 proteins), E (17 proteins), F (3 proteins), G (3 proteins), H (8 proteins), I (1 protein), J (8 proteins) and K (6 proteins) had no significantly enriched ontology or pathway terms (Figure 5-11; Table S5-24). Cluster B comprised 18 proteins with a relatively low abundance in the acetone control only. This cluster had 2 guanylate kinase representatives which were disks large homolog 5 protein and peripheral plasma membrane protein calcium/calmodulin-dependent protein kinase type II alpha chain (CASK). Cluster C had 10 proteins with relatively higher abundances in both the PCF and PAI treatment groups in comparison to both control groups and included vesicle-associated membrane protein 2 and syntaxin 7, which are both SNARE associated proteins involved in vesicular transport. Of the 6 proteins in cluster L, which were relatively low in abundance in both the PCF and PAI treatment groups in comparison to both controls, included microtubule-actin cross-linking factor 1 and troponin C, proteins with EF-hand, calcium binding motifs that are associated with the cytoskeleton.

Cluster M was the largest cluster in the brain with 23 proteins with a high abundance in the acetone control treatment group compared to all other treatment groups. These proteins were associated with fatty acid metabolism (acetyl-CoA acetyltransferase, very long-chain-fatty-acid--CoA ligase bubblegum, and fatty acid synthase, pyruvate carboxylase, and isocitrate dehydrogenase), biosynthesis of amino acids (glyceraldehyde-3-phosphate dehydrogenase 2), the TCA cycle (pyruvate carboxylase and isocitrate dehydrogenase), and oxidative stress (catalase), further highlighting the impact of acetone on the bumblebee brain at the molecular level.

Hierarchical clustering of z-score normalised SSDA proteins from the fat bodies resolved nine clusters, seven of which had no significant gene ontology term or pathway enrichments

including clusters A (10 proteins), B (8 proteins), C (6 proteins), D (6 proteins), F (3 proteins), G (5 proteins), and H (11 proteins) (Figure 5-12; Table S5-25). Cluster E comprised 7 proteins with relatively high abundances in both the PCF and PAI treatment groups in comparison to both control groups. This cluster included proteins associated with protein folding, four of which were T-complex protein 1 subunits alpha, beta, epsilon, and theta. Cluster I comprised 14 proteins that had a low relative abundance in the PCF treatment group in comparison to PAI and control treatment groups. These included proteins integrin-linked kinase, talin-1, laminin subunit alpha, and parvin which are associated with cell adhesion.

Cluster	Term description	Database	Term ID	FDR
A	No significant enrichments			
B	Guanylate kinase homologues	SMART	SM00072	0.0177
C	SNARE interactions in vesicular transport	KEGG	dme04130	0.0067
D-K	No significant enrichments			
L	EF-hand, calcium binding motif	SMART	SM00054	0.0431
	Fatty acid metabolism	KEGG	dme01212	0.00097
M	Biosynthesis of amino acids	KEGG	dme01230	0.0013
	TCA cycle	KEGG	dme00020	0.0113

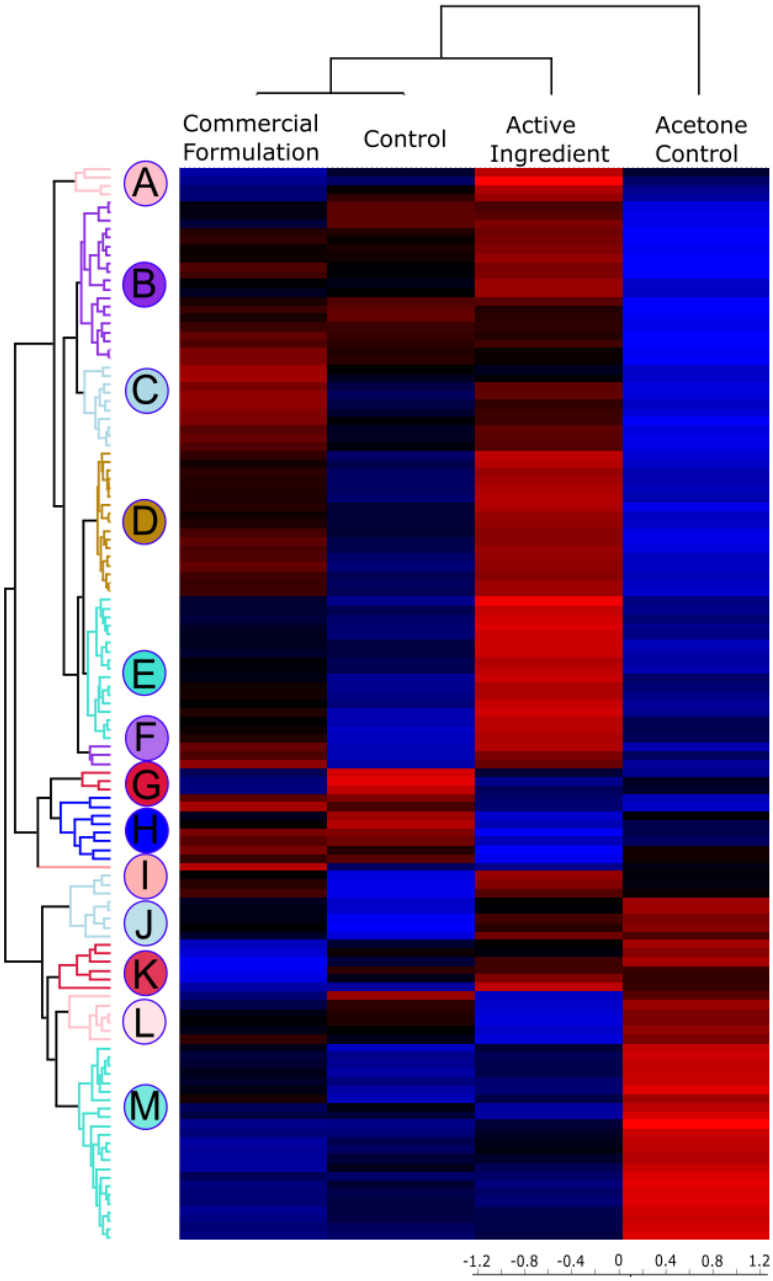


Figure 5-11 A Heatmap Produced from Hierarchical Clustering of Mean Z-score Normalised LFQ Values in The Brain Proteome of Prothioconazole-Exposed Bees. STRING analysis determined protein cluster characteristic descriptions. Each cluster is denoted alphabetically with blue representing decreased protein abundance and red representing protein abundance increases in each cluster.

Cluster	Term description	Database	Term ID	FDR
A-D	No significant enrichments			
E	Protein folding	GO process	GO:0006457	0.00055
F-H	No significant enrichments			
I	Localization of the PINCH-ILK-PARVIN complex to focal adhesions	Reactome	DME-446343	0.0268

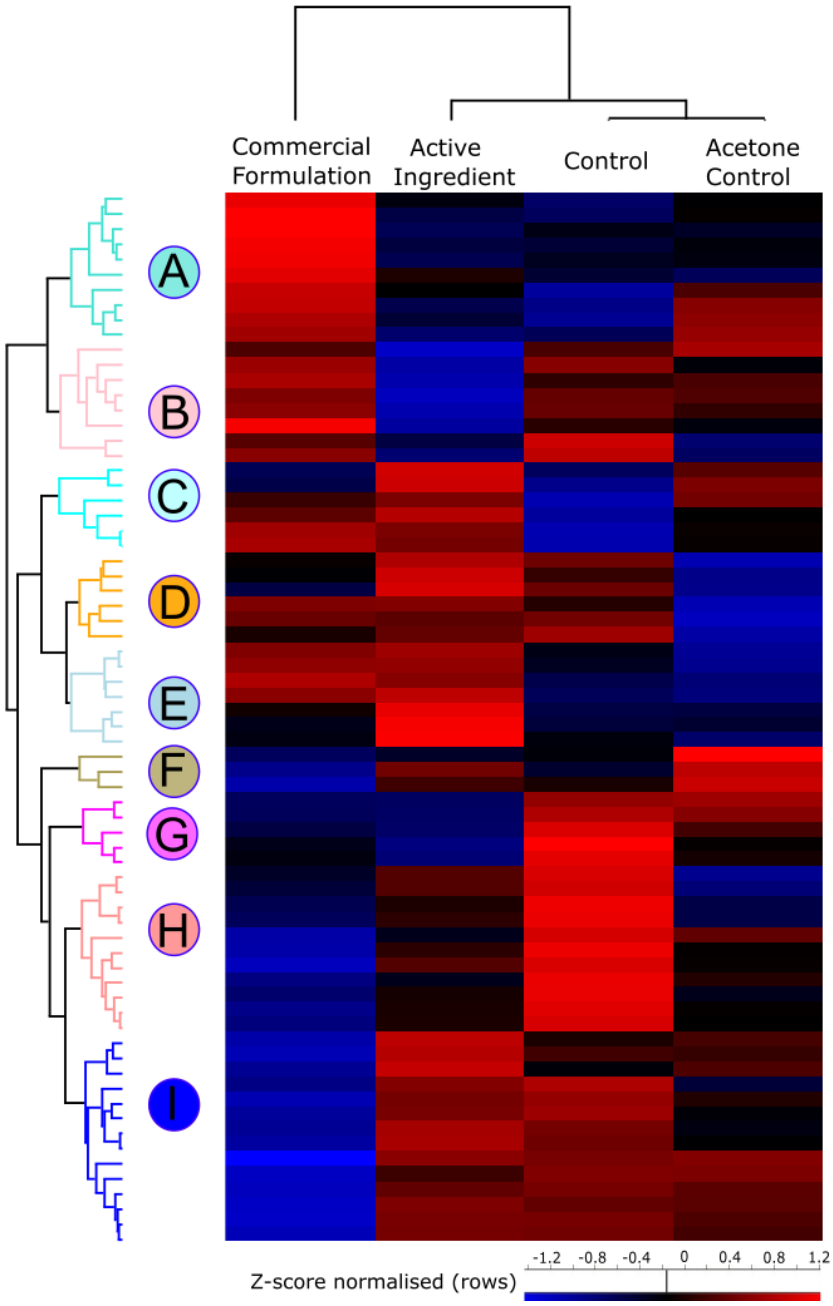


Figure 5-12 A Heatmap Produced from Hierarchical Clustering of Mean Z-score Normalised LFQ Values in The Fat Body Proteome of Prothioconazole-Exposed Bees. STRING analysis determined protein cluster characteristic descriptions. Each cluster is denoted alphabetically with blue representing decreased protein abundance and red representing protein abundance increases in each cluster.

5.3.2.5 Proteins With a Common Abundance Profile in the Brain and Fat body After Exposure to Prothioconazole Active Ingredient and Commercial Formulation

SSDA proteins from both PAI and PCF-exposed bees compared to their relative controls were analysed to find common proteins with a similar RFC direction across treatment groups (Table 5-4). This was carried out to determine if there was a consistent impact of prothioconazole on the brain or fat body proteome regardless of the presence of co-formulants in PCF or acetone in PAI.

In the brain, where were three common proteins between PAI and PCF treated bees with an increased abundance, and one common protein with a decreased abundance. Proteins with an increased abundance in both treatment groups were serine/threonine-protein kinase MARK2 (RFC PAI: + 1.36; RFC PCF: + 1.26), prostaglandin E synthase 2 (RFC PAI: + 2.87; RFC PCF: + 3.50), and alpha/beta hydrolase domain-containing protein 11 (RFC PAI: + 3.57; RFC PCF: + 2.83). Ubiquitin-conjugating enzyme E2 G1 was the only common protein with a decreased abundance with a RFC of – 5.73 and – 5.50 in the brain proteome of PAI and PCF exposed bees, respectively. There was one SSDA protein with an increased abundance and no common decreased abundance proteins in the fat body proteome of both the PAI and PCF treated bees. The one protein with an increased abundance was T-complex subunit alpha with an RFC of + 1.22 and + 1.15 in PAI and PCF treatments, respectively.

Table 5-4 Conserved Response to Both Prothioconazole-based Treatments in the Brain and Fat Body. Functional annotations, RFC's, MS measurements and characteristics for all SSDA proteins with similar expression profiles in both PAI and PCF treated bees. Relative fold changes and directions were determined against the relative procedural control.

Tissue	Functional Annotation	Protein ID	Protein Name	RFC AI	RFC CF	RFC direction	No of peptides	Mol wt [kDa]	MS/MS count
Brain	Protein phosphorylation	XP_012163188.1	Serine/threonine-protein kinase MARK2	1.4	1.3	Increased	13	124.9	51
	Prostaglandin biosynthesis	XP_003403370.3	Prostaglandin E synthase 2	2.9	3.5	Increased	9	46.4	33
	Cellular lipid metabolic process	XP_003402554.1	Alpha/beta hydrolase domain-containing protein 11	3.6	2.8	Increased	4	29.7	22
	Proteasome-mediated ubiquitin-dependent protein catabolic process	XP_003394185.1	Ubiquitin-conjugating enzyme E2 G1	5.7	5.5	Decreased	3	19.3	24
Fat body	Protein folding	XP_003395195.1	T-complex protein 1 subunit alpha	1.2	1.2	Increased	29	60.0	375

5.4 Discussion

Until recently, the impact of non-insecticidal pesticides on bees had largely been ignored in comparison to insecticides. However, there is still very little research on whether widely used herbicides and fungicides impact bees, with most studies focusing on mortality level effects using honeybees (Cullen *et al.,* 2019). Gaining an overall understanding of whether widely used pesticides have an impact on different tissues in bumblebees will aid to guide further research and inform science-based policy decisions for pesticide mitigation strategies in agricultural and horticultural spaces.

In this chapter, the brain and fat body proteome of *B. terrestris* was investigated after exposure to the herbicide glyphosate or the fungicide prothioconazole and a representative commercial formulation of each, to determine if these pesticides pose a risk to wild bees. Both glyphosate and prothioconazole exposure resulted in statistically significant differences in the proteomes of both tissues in *B. terrestris*. Whilst the glyphosate active ingredient and commercial formulation, Roundup Optima+®, showed some similarities in significant proteins altered, there were also distinctive differences between GAI and GCF in both tissues. However, a comparison of PAI and PCF exposure determined few proteins that had common profiles between treatments, perhaps due to the inclusion of acetone for active ingredient solubility, or the presence of co-formulants in the commercial formulation, Proline®. In addition, acetone-specific alterations were observed in both the brain and fat body in a comparison of acetone control to non-acetone control exposure. Overall, it was found that both glyphosate and prothioconazole have an impact on the brain and fat body of *B. terrestris* after oral exposure, both non-contact organs in a non-target organism. Different pathways and processes were significantly altered in the brain and fat body of both active ingredient and commercial formulation glyphosate and prothioconazole exposure groups, which may suggest individual co-formulant impacts or the ability of co-formulants to alter or compound active ingredient effects on the brain and fat body of *B. terrestris*.

5.4.1 The Impact of Glyphosate on the *B. terrestris* Brain Proteome

5.4.1.1 Glyphosate Active Ingredient Alters Lipid Metabolism, Protein Degradation, and Mitochondrial Proteins in the Brain Proteome

Exposure to GAI led to a statistically significant increase in the abundance of proteins involved in lipid metabolism and the proteasome, indicating alterations to metabolism and protein degradation or protein homeostasis. Alterations to metabolism and proteasome pathways have been identified on the proteomic and transcriptomic levels across multiple species including fish, mice, and honeybees, suggesting a common impact of glyphosate across multiple tissue types in different non-target organisms (Lopes *et al.,* 2018; Ganesan and Keating, 2020; Zhao *et al.,* 2020). In addition, glyphosate has been associated with oxidative stress conditions in the brain of both fish and honeybees (A.G. Pereira *et al.,* 2018; Zaluski *et al.,* 2020). This can lead to lipid peroxidation which could explain an increase in lipid metabolism to modulate lipids lost to oxidative damage. Further, an increase in proteasome-related proteins may serve to remove damaged proteins and lipoproteins from the brain. This conclusion is further supported by the increase in antioxidant SSDA proteins increased in the brain after GAI exposure, including thioredoxin and peroxidase. Thioredoxins are disulfide reductases that act as electron donors for enzymes such as thioredoxin peroxidases, whilst peroxidases reduce and neutralise reactive oxygen species which aids in defence against cellular oxidative stress as well as the redox regulation of protein functioning and signalling, which can regulate cellular processes such as apoptosis (Arnér and Holmgren, 2000; Corona and Robinson, 2006).

Proteins with a decreased abundance after GAI exposure were related to the TCA cycle and mitochondrion. Interestingly, multiple studies across a range of species have found that glyphosate alters mitochondrial functioning via disruption to mitochondrial metabolism, mitochondrial membrane polarisation and membrane permeabilization (Peixoto, 2005; Pereira *et al.,* 2018; Ravishankar *et al.,* 2020). Similar to the results presented here, Zhao et al. (2020) found alterations to synapse-related gene expression in whole bee transcriptome sequencing of two honeybee species after glyphosate commercial formulation exposure, revealing a possible role of glyphosate in altering synaptic transmission in the bee brain and nervous system.

5.4.1.2 Roundup Optima+® Alters Proteins Involved in Oxidative Stress Regulation, Oxidative Phosphorylation, and Protein Biosynthesis in the Brain Proteome

Interestingly, different brain processes were altered in response to GCF exposure in comparison to the control, with an increased in proteins associated with unfolded protein binding and cellular responses to stress. This included an increase in chaperones and folding catalysts such as heat shock proteins, which are important in preventing the aggregation of misfolded proteins and play a major role in protecting cells from oxidative stress by aiding the removal of damaged proteins. Oxidative stress regulators such as thioredoxin, glutaredoxin, and superoxide dismutase were also increased, indicating an increased oxidative environment in the brain after GCF exposure, similar to what was observed after GAI exposure.

In addition, GCF exposure led to a decrease in neurotransmitter transport, oxidative phosphorylation, and ribosomal subunit proteins, suggesting major alterations to neuronal ATP synthesis, neurotransmitter packaging and/or transport across the cell to synapses and the production of new proteins, which could include neurotransmitters. A decrease in neurotransmitter transport could reduce the efficiency, strength, or speed of neuronal transmission, particularly if reduced ribosomal subunits are associated with reduced neurotransmitter production. Interestingly, Bali et al., (2019) researched the mouse brain after exposure to a glyphosate formulation and found cognitive abnormalities along with significant alterations to acetylcholine esterase (AChE), superoxide dismutase and phenoloxidase activity, suggesting a glyphosate or co-formulant impact on neurotransmitter homeostasis and oxidative stress in the brain. Further, Glusczak et al. (2006) found that AChE activity decreased in the brain of fish after 96 hours of exposure to a range of concentrations from 3 – 20 ppm of the glyphosate-based herbicide Roundup®. Whilst this concentration is higher than what was used in the research presented in this thesis, Glusczak et al. (2007) repeated this research on the brain of a different fish species and found similar results at only 0.2 and 0.4 ppm of Roundup® exposure after 96 hours. By demonstrating impacts on AChE activity at a much lower concentration than the field-realistic concentration of 1 ppm used in this research, and up to 20 ppm used in previous research, these findings determine that glyphosate, at least when ingested as part of a formulation, can impact neurotransmitter homeostasis, and as a result, possibly neurotransmitter signaling in the brain of a non-target organism.

Further, decreased ribosomal subunit proteins seen here along with decreases in oxidative phosphorylation may be associated with alterations to the mitochondria. In chapter three,

glyphosate altered mitochondrial proteins and processes in the bumblebee digestive tract, and other studies have documented the impacts of glyphosate formulation on mitochondrial functioning and oxidative phosphorylation-associated proteins in the brain cells of fish and rodents (A G Pereira *et al.*, 2018; Neto da Silva *et al.*, 2020). Further, in the investigation of the nurse honeybee head proteome in response to pyraclostrobin formulation exposure, translation was significantly decreased in comparison to control bees, suggesting a reduction in translational proteins as a common xenobiotic response, or an impact of co-formulants such as surfactants common in pesticide formulations (Zaluski *et al.*, 2020). Didiot et al. (2012) identified glycoside molecules as translation inhibitors. Interestingly, an alkylpolyglycoside compound is used as a surfactant in Roundup Optima+®, which could explain why translation is altered after GCF exposure, but not after GAI exposure.

5.4.1.3 Roundup Optima+® and Glyphosate Differentially Impact Signal Transduction, Lipid Metabolism and Protein Homeostasis in the Brain Proteome

In a direct comparison of the impacts of GCF and GAI exposure on the brain, there were multiple differences in the pathways and processes altered. After GCF exposure, proteins associated with rhodopsin-mediated signaling and regulation of synapse organisation were increased in abundance in comparison to GAI exposure. Rhodopsins are G-protein coupled receptors which respond to photons coupled to light sensitive chromophores called retinal. Photon identification leads to isomerisation of retinal and subsequent conformational changes of rhodopsin to metarhodopsin which can activate a G-protein coupled signal cascade in photoreceptor cells, leading to further phototransduction and downstream sensory input and processing of visual information in the brain (Kiselev and Subramaniam, 1994; Stell, 2012). Along with an increase in regulation of synapse organisation, these results indicate differential alterations to phototransduction and possibly neurotransmitter signal transmission in the brain depending on glyphosate ingestion alone or as part of a formulation. Such alterations may provide the baseline mechanisms behind a recent study determining that the formulation Roundup Gold® alters fine-colour discrimination in *B. terrestris* (Helander *et al.*, 2023). Further research on the compound eye lens, underlying photoreceptors, optic lobe neuropils and bumblebee learning assays based on visual cues could reveal further information on whether glyphosate potentially alters bumblebee vision.

As discussed above, lipid metabolism, proteasome degradation and ribosomal subunit proteins were statistically significantly decreased in the brain after GCF exposure, however, these

processes were not significantly decreased after GAI exposure. This suggests that alterations to metabolism, proteolysis and translation observed in this research and other studies investigating the impact of glyphosate formulations may be attributed to the co-formulants. Whether this impact is due to individual co-formulant types, e.g. surfactants, specific surfactant classes e.g. glycosides, or the presence of co-formulants compounding the effects of glyphosate is yet to be determined.

5.4.1.4 Glyphosate Alters Protein Homeostasis, Oxidative Stress, and Synaptic Transmission-Associated Proteins in the Brain Regardless of Treatment Source

Of the 67 and 95 SSDA proteins with an increased abundance in the brain proteome of GAI and GCF-exposed bees in comparison to the control group, respectively, there were twenty-four proteins with an increased abundance in the *B. terrestris* brain after both GAI and GCF exposure.

Two common proteins were subunit proteins of the chaperonin-containing T-complex, essential for the folding of proteins involved in signaling and the cytoskeleton (Bigotti and Clarke, 2008; Kim *et al.*, 2013). Additionally, three common proteins were associated with the proteasome, indicating an increased need for protein degradation, possibly due to protein oxidative damage as antioxidant proteins were increased in both datasets, with D-2-hydroxyglutarate dehydrogenase mitochondrial, thioredoxin-2, and rab's geranylgeranyltransferase component A1 increased after exposure to both GAI and GCF. Further, the protein with the most increased abundance after GAI exposure and the second most increased abundance protein after GCF exposure was a putative mediator of RNA polymerase II transcription subunit 26, a key player in transcription regulation (Blazek et al., 2005; Yin and Wang, 2014). After exposure to a glyphosate commercial formulation, Zhao et al. (2020) also found an increase in mediator of RNA polymerase II subunit proteins in honeybees. Due to mediator of RNA polymerase II complex's wide range of regulatory roles in transcription, including both activation and suppression of transcription of type II genes, it is not possible to conclude the impact of its increased abundance after glyphosate exposure. However, to our knowledge, the involvement of this protein in pesticide resistance or susceptibility has not been documented in the literature thus far.

Of the 38 and 71 SSDA proteins with a decreased abundance in the brain proteome of GAI and GCF-exposed bees in comparison to the control group, respectively, there were ten proteins

common to both datasets. The protein with the largest decrease in abundance was syndecan, with a relative fold change of – 4.8 after exposure to both treatments. This protein is a cell surface proteoglycan and is important for axon guidance and cell to cell and extra-cellular matrix adhesions, processes required for the growth and maintenance of neural plasticity in the brain which could alter brain functioning (Wright and Harding, 2004). In addition, syntaxin-binding protein 5 and secretory carrier-associated membrane protein 5A were significantly decreased, both of which are associated with synaptic transmission and long-term memory. Whilst brain functioning after glyphosate exposure has not been investigated in bees, studies have demonstrated the negative impacts of glyphosate exposure on neurotransmitter levels and memory in mice and rats (Martínez *et al.*, 2018; Bali *et al.*, 2019).

Overall, these results demonstrate the ability of glyphosate, regardless of ingestion alone or with co-formulants, to alter protein folding, protein degradation, oxidative stress, translation, and synaptic transmission in the brain of *B. terrestris*.

5.4.2 Glyphosate Effects on the *B. terrestris* Fat Body Proteome

5.4.2.1 Glyphosate Active Ingredient Alters Translation, Protein Degradation and Energy Metabolism in the Fat Body Proteome

Proteins associated with RNA metabolism and protein degradation were increased in abundance after GAI exposure. Multiple proteins including nuclease-sensitive protein, heat shock protein70kDa, nucleoporin and a ribosomal subunit proteins were increased indicating alterations to RNA metabolism or transcription and translation processes. Proteasome subunits alpha 5 and 3 and serine protease stubble were increased, indicating an increase in protein degradation. In addition, mitochondrial ATP synthase subunit O was increased in abundance as well as the protein with the highest increase in abundance in comparison to the control, coiled-coil domain-containing protein 58, also known as MIX23, a mitochondrial intermembrane space protein. This protein aids in regulating mitochondrial protein import machinery and is commonly increased in abundance under stress conditions where mitochondrial protein import has been altered (Zöller *et al.*, 2020). This protein is crucial for protein transport into the mitochondrial matrix, particularly if TIM23 translocase function, activity or abundance has been decreased, however, there was no evidence of TIM23 decrease in abundance in this dataset.

Proteins with a decreased abundance after GAI exposure included subunit 2 of the COP9 signalosome, a proteolysis regulator, and subunit 2 of the SUMO-activating enzyme, involved in SUMOylation post-translational modification (Schwechheimer, 2004; Geiss-Friedlander and Melchior, 2007). In addition, eight proteins associated with translation including ribosomal subunit proteins, translation elongation factor 2, and tRNA ligases. Interestingly, two out of the six identified lipid droplet proteins in STRING were decreased after GAI exposure. These were the second and third most decreased proteins after GAI exposure: caprin and lipid storage droplets surface-binding protein 1 (Lsd1). Caprin is involved in the regulation of mRNA transport and translation, whilst Lsd1 is essential for lipolysis stimulation in the fat body, serving as an anchor for lipases. Further, this protein is involved in the regulation of lipid metabolism and storage in lipid droplets and acts with lipid storage droplet 2 to mediate energy homeostasis (Li *et al.,* 2019). In addition, five proteins associated with glycolysis were decreased, indicating glyphosate-induced alterations to energy metabolism in the fat body.

Overall, alterations to these proteins and processes suggest GAI-induced changes to protein homeostasis, protein stability and energy metabolism in the *B. terrestris* fat body. Based on an increased abundance of MIX23, alterations observed may be associated with mitochondria within the fat body, as observed in other tissues after glyphosate exposure throughout this thesis. In addition, major decreases in the abundance of lipid droplet proteins suggest alterations to energy homeostasis, which may have dire consequences for bees under starvation conditions.

5.4.2.2 Glyphosate Commercial Formulation Exposure Alters Translation, Oxidative Stress, Detoxification, and Basement Membrane Proteins in the Fat Body of *B. terrestris*

Translational initiation was increased in the fat body after exposure to GCF. Four of the increased proteins were associated with the translation initiation complex, including translation initiation factor 2 subunit 1, and subunits F, K, and M of eukaryotic translation initiation factor 3. Further, three proteins were increased which were associated with ubiquitin-like protein conjugating enzymes and nine proteins were increased which were associated with the mitochondrion. Indicating alterations to protein homeostasis, possibly associated with the mitochondria, as seen in the fat body after GAI exposure in section 5.4.2.1.

In addition, multiple proteins associated with oxidative stress regulation were increased in abundance. These included superoxide dismutase, phospholipid hydroperoxide glutathione

peroxidase, 10 kDa heat shock protein, and 60 kDa heat shock protein, mitochondrial. Phospholipid hydroperoxide glutathione peroxidase is involved in the reduction of phospholipid hydroperoxides and H_2O_2 and is up-regulated in response to oxidative stress conditions (Hu *et al.*, 2010). In addition, superoxide dismutase is increased under oxidative stress and neutralises reactive oxygen species such as superoxide conversions to O_2 and H_2O_2 (Choi *et al.*, 2006). In addition, heat shock proteins act as chaperones to prevent the aggregation of misfolded proteins and 60kDa heat shock protein promotes the refolding and assembly of unfolded proteins under stress conditions (Panaretou *et al.*, 2002; Dubey *et al.*, 2015).

Eight translational proteins including ribosomal subunits and elongation factors were decreased, indicating alterations to protein translation after GCF exposure in the fat body. In addition, cytochrome P450 9e2, cytochrome P450 4g15, and NADPH-cytochrome P450 reductase were decreased in the fat body of GCF exposed bees. Cytochrome P450s are monooxygenases involved in the breakdown of fatty acids and xenobiotics and are involved in pesticide detoxification in bees (Berenbaum and Johnson, 2015; Zhu *et al.*, 2017). Interestingly, Chen et al. (2022) found that exposure to Roundup® increased the gene expression of cytochrome P450 in the head of honeybees after acute exposure. These results are the opposite to what was found in this investigation, which may be due to tissue, species or temporal differences in exposure, or the lack of correlation between gene transcript and protein levels (Maier *et al.*, 2009). However, along with the data presented here, this research strengthens the hypothesis that glyphosate formulations affect genes and proteins involved in pesticide detoxification.

Further, collagen alpha-5(IV) and basement membrane-specific heparan sulfate proteoglycan were decreased after GCF exposure. Collagen alpha-5(IV), the third most decreased abundance protein, is a subunit of collagen alpha (IV). Collagen alpha (IV) is a crucial component of basement membranes and is secreted by the fat body, constituting approximately 50% of proteins in basement membranes (Kalluri *et al.*, 2003). In addition, basement membrane-specific heparan sulfate proteoglycans are required for basement membrane structural integrity and functionality (Pastor-Pareja and Xu, 2011). Whilst a decrease in basement-membrane proteins may alter the structure and functioning of fat body cells, alteration to translation proteins and collagen-IV may indicate a decrease in collagen IV production and secretion in the fat body, which could have consequences for other tissues in *B. terrestris* exposed to GCF.

Overall, GCF exposure led to altered protein homeostasis and increased oxidative stress, possibly associated with the mitochondria, in addition to alterations to detoxification and basement membrane proteins in the fat body.

5.4.2.3 Differential Impacts of Roundup Optima+® and Glyphosate on the Fat Body Proteome

A direct comparison of the fat body proteome between GCF and GAI-exposed bees was made to determine key differences in the pathways and processes impacted by the formulation and active ingredient alone. In comparison to GAI-exposed bees, there were 33 and 12 proteins increased and decreased, respectively, in the fat body of GCF-exposed bees. Whilst proteins increased in the fat body of GCF-exposed bees were associated with pyruvate metabolism, RNA transport and fatty acid synthesis, proteins increased in GAI-exposed bees were associated with prostacyclin signaling and glycolysis and gluconeogenesis.

Whilst individual analysis of the fat body proteome of bees exposed to each treatment in comparison to the relative control treatment revealed alterations to metabolism after exposure to both glyphosate-based treatments, it is interesting to note that metabolism is not altered in exactly the same way. For instance, pyruvate metabolism and fatty acid synthesis proteins have a higher abundance after GCF exposure whereas glycolysis proteins having an increased abundance in the fat body after GAI exposure. This may be due to the presence of co-formulants in GCF, which may require alternate pathways for catabolism and detoxification, or which may alter or compound the impact of glyphosate on the fat body. In addition, whilst translation-related proteins were impacted in both glyphosate-based treatments compared to the control, proteins associated with RNA transport, translation initiation factors, had a greater abundance in the fat body after GCF exposure compared to GAI exposure. Furthermore, whilst not much is known about prostacyclin signaling in insects, Ahmed et al. (2021) determined its involvement in the suppression of the immune response and anti-inflammatory signaling in moths, indicating possible differential impacts of GAI and GCF on the immune system.

5.4.2.4 Glyphosate Alters Protein Biosynthesis, Mitochondrial Proteins, and Lsd1 in the Fat Body Regardless of Treatment Source

In the fat bodies of glyphosate-exposed bees, there were seven and ten SSDA proteins with an increased and decreased abundance, respectively, common to both treatments in comparison to the control. Coiled-coil domain-containing protein 58, discussed in section 5.4.2.1 for its

importance in mitochondrial protein importation and its association with stress responses, was the most increased protein common to both treatments, with an RFC of 5.2 and 5.3 after GAI and GCF exposure, respectively. In addition, iron-sulfur cluster assembly enzyme ISCU, mitochondrial had an RFC of + 2.6 and + 3.0 after GAI and GCF treatment, respectively. This protein is important for cellular iron ion homeostasis and Fe-S cluster assembly. In the mitochondria, Fe-S clusters are important co-factors for electron transfer in the electron transport chain and redox reactions (Read *et al.*, 2021).

In addition, two proteins increased abundance in common were associated with protein biosynthesis. However, four out of the ten common proteins with a decreased abundance were also associated with protein biosynthesis, demonstrating glyphosate's ability to alter translation and protein homeostasis in the fat body regardless of its ingestion as GCF or GAI. Further, the protein with the highest decrease in abundance common after ingestion of both glyphosate-based treatments was lsd1, with an RFC of – 3.0 and – 5.2 after GAI and GCF exposure, respectively. As discussed in section 5.4.2.1, this protein is essential for lipolysis of triglycerides stored in fat body lipid droplets, which are required for energy metabolism and regulation of lipid storage in the fat body (Li *et al.*, 2019).

Overall, the SSDA fat body proteins in common to both GAI and GCF exposed bees in comparison to control-treated bees highlight the processes altered by glyphosate regardless of its ingestion alone or as part of a formulation. Based on these proteins, processes altered by glyphosate in the *B. terrestris* fat body include protein biosynthesis, mitochondrial functioning, and lipid droplet energy metabolism and storage.

5.4.3 The Impact of Prothioconazole on the *B. terrestris* Brain Proteome

5.4.3.1 Prothioconazole Active Ingredient Alters the Cytoskeleton, Neurotransmitter Biosynthesis and Energy Metabolism Proteins in the *B. terrestris* Brain

Exposure to PAI led to alterations in the abundance of SSDA proteins involved in cytoskeleton organisation. There were 20 proteins increased and 17 proteins decreased that were associated with cytoskeleton organisation in the brain of PAI-exposed bees in comparison to acetone control-exposed bees. Increased proteins included proteins involved in microtubule bundle formation such as microtubule-associated protein futsch, TPPP family protein CG45057 and dynamin. Both microtubule-associated protein futsch and TPPP family protein CG45057 are required for synaptic bouton growth by regulating synaptic microtubules, whilst dynamin is

involved in the production of microtubule bundles. The microtubule cytoskeleton is of great importance for neurons as microtubule bundles which run along axons are required for axonal transport, structural integrity, and signaling (Hahn *et al.*, 2019; Gutierrez *et al.*, 2021).

Interestingly, 14 out of the 17 decreased abundance proteins associated with cytoskeleton organisation were in the top 15 most decreased abundance proteins including paramyosin, troponin T, C, I, tropomyosin, myosin regulatory light chain 2, and myosin heavy chain. All members of the troponin complex, including multiple isoforms of the same subunits, were decreased. Whilst the troponin complex along with tropomyosin are typically associated with muscle contraction, they are important in cytoskeleton reorganisation and the development and maintenance of the nervous system. In addition, troponin proteins can play a role in transcriptional regulation and the immune response (Johnston *et al.*, 2017). In addition, myosin proteins are essential motor proteins associated with the actin cytoskeleton and play a role in the transport of proteins within neurons (Lewis *et al.*, 2009). Alterations to the cytoskeleton can have major impacts on neuronal cells as cytoskeletal structural and motor proteins are required for synaptic organisation, cell adhesion and transport of proteins, organelles, and neurotransmitters for maintenance of signal transmission and cellular processes within the brain (Prokop, 2020). Whilst it is unclear exactly how prothioconazole alters the cytoskeleton, alterations to troponin C gene expression has been observed in zebrafish embryos after exposure to the triazole fungicide difenoconazole (Zhu *et al.*, 2021). In addition, Guapo de Melo, (2020) found significant decreases in troponin, tropomyosin, paramyosin, and myosin protein abundances in the brain of *B. terrestris* after acute exposure to the neonicotinoid insecticide clothianidin, suggesting that cytoskeleton alterations may be associated with differential neuron physiology after pesticide exposure.

Proteins associated with neurotransmitter release cycle and the presynaptic cytoskeleton were increased after PAI exposure, including glutamate decarboxylase, an enzyme important in GABA production, and choline acetyltransferase, which engages in acetylcholine biosynthesis, an enzyme that is a marker for cholinergic neurons. Whilst GABA is a neuromodulator with inhibitory impacts at the post-synaptic membrane of neurons and alters the release of neurotransmitters at the presynaptic membrane, acetylcholine is an excitatory neurotransmitter in bees, activating nicotinic acetylcholine receptors for excitatory cholinergic neurotransmission in the brain (Barbara *et al.*, 2005; Klowden and Palli, 2022). The proteins CASK and vesicular glutamate transporter 1 were significantly increased also. CASK acts as a

scaffold for signaling and transmembrane molecules in neurons for synaptic development and plasticity in neurons whilst vesicular glutamate transporter 1, which had an RFC of + 5.3, is a neurotransmitter transporter associated with glutamate excitatory neurotransmission.

These findings indicate alterations to neurotransmission after prothioconazole exposure and may provide a potential explanation for an increase in bees with altered behaviour after PAI exposure seen in chapter 4. However, there was a significant decrease in proteins associated with glycolysis and the pentose phosphate pathway, as well as fatty acid biosynthesis, suggesting altered energy metabolism in the brain after PAI exposure. Since neurotransmitter synthesis is a high-energy cost process, alterations to metabolic processes could limit the energy required for neurotransmission or other vital cellular processes in the brain.

5.4.3.2 Proline® Alters Translation in the Brain Proteome

There were major alterations to translation proteins in the brain after PCF exposure a total of 20 out of the 35 SSDA proteins with an increased abundance associated with translation. Eighteen of these proteins were ribosomal subunit proteins. Interestingly, the protein with the highest increase in abundance was prostaglandin E2 synthase 2, a protein crucial for immunity in insects, regulating haemocyte spreading, nodule formation, phenoloxidase activity and antimicrobial peptide expression after immune challenges (Ahmed *et al.*, 2018). In addition, prostaglandins are involved in the regulation of antimicrobial peptide synthesis (García Gil de Muñoz *et al.*, 2008) and can induce gene expression in human immune cells (Baratelli *et al.*, 2005) . This may indicate an ability to increase the translation of target proteins and explain the major increase of ribosomal subunit proteins observed in this research.

Despite a major increase in translational proteins, putative mediator of RNA polymerase II transcription subunit 26 was the most decreased protein in the brain after PCF exposure with an RFC of -12.0 compared to control-exposed brain proteomes. Interestingly, this protein was the most increased in the brains of glyphosate-exposed bees, making alterations to this protein a potential biomarker of pesticide exposure. Further, an SSDA protein annotated as uncharacterised LOC100648913 with an RFC of -2.2 in comparison to the control exposed brain was identified as Amun using the *Drosophila* annotation, a protein associated with compound eye development. The overexpression of Amun can decrease transcription factors required for sensory organ functioning (Shalaby *et al.*, 2009) and decreased expression of Amun is associated with decreased Notch signalling which is important for cell renewal and

differentiation (Loza-Coll *et al.*, 2014), meaning a decrease of this protein may be required for the transcription and translational regulation of genes required for synaptic neuron differentiation, plasticity, or sensory functioning.

5.4.3.3 Common Prothioconazole and Proline® Associated Alterations in the *B. terrestris* Brain

The SSDA proteins present in the brain after exposure to PAI and PCF in comparison to their relative controls were analysed to determine common proteins in both datasets. There were three proteins with an increased abundance and one protein with a decreased abundance common to the brain proteome after exposure to both PAI and PCF treatments.

Prostaglandin E synthase 2 had an RFC of + 2.9 and + 3.5 in the brain after PAI and PCF exposure, respectively. As discussed in section 5.4.3.2, this protein is associated with prostaglandin synthesis, which has implications for immune signalling and gene expression. In addition, serine/threonine-protein kinase MARK2 and alpha/beta hydrolase domain-containing protein 11 had an increased abundance after exposure to both prothioconazole-based treatments in the brain. Whilst serine/threonine-protein kinase MARK2 is associated with the phosphorylation and alteration of several processes, including synaptic assembly and cytoskeleton organisation, alpha/beta hydrolase-domain containing protein may have a role in pesticide detoxification. Alpha and beta hydrolase enzymes have demonstrated an ability to degrade organophosphates via non-specific cleavage of ester bonds, creating an acid and alcohol as metabolites. However, this hydrolase domain may have a range of catalytic functions and could be associated with lipid metabolism (Montella *et al.*, 2012; Arya *et al.*, 2017). Ubiquitin-conjugating enzyme E2 G1 had an RFC of – 5.7 and – 5.5 in the brain after PAI and PCF exposure, respectively, indicating a decrease in proteasome mediated protein degradation.

A lack of common SSDA proteins in the brain of PAI and PCF exposed bees indicates differential impacts of prothioconazole on the *B. terrestris* brain depending on its ingestion as the active ingredient or as part of the formulation Proline®. However, there may be some similarities regarding immune signaling and decrease protein degradation. This may be due to the necessity to use acetone for PAI solubility, or the presence of co-formulants in Proline®, which could have additional impacts or compound the impact of prothioconazole on the *B. terrestris* brain proteome, resulting in differential impacts depending on the prothioconazole formulation make up.

5.4.4 The Impact of Prothioconazole on the Fat Body Proteome

5.4.4.1 Prothioconazole Active Ingredient Alters Proteins Associated with Protein Folding, Oxidative Stress, and the Mitochondrion in the Fat Body Proteome

PAI exposure led to alterations to protein folding in the fat body of *B. terrestris*. Of the 22 SSDA proteins with an increased abundance after PAI exposure, six were chaperonin-containing T-complex protein 1 subunits. The chaperonin-containing T-complex is made up of two rings of eight subunits and is required for the folding of actin and tubulin for microfilament and microtubule assembly (Grantham, 2020) and the prevention of protein aggregation (Tam *et al.*, 2009; Brehme *et al.*, 2014). In addition, nucleoporin and transportin-1 were increased, which participate in nucleocytoplasmic transport, important for the transport of mRNA and cargo proteins to and from the nucleus. Thirteen SSDA proteins had a decreased abundance in the fat body after PAI exposure. O-acyltransferase, a protein involved in cholesterol metabolism and homeostasis, had the most decreased abundance with an RFC of – 3.6. Four decreased abundance proteins were annotated as mitochondrial and involved in various processes such as mitochondrial respiratory chain complex I assembly and amino acid degradation. In addition, there was a decrease in glutaredoxin-C4 (glutaredoxin 1), an electron carrier protein involved in the glutaredoxin system, important for cell redox homeostasis without which the cell could be vulnerable to oxidative stress conditions. Glutaredoxin 1 is also associated with response to pesticide treatment in bees, making it's decrease in abundance in PAI-exposed fat bodies of some concern (Yao *et al.*, 2014).

5.4.4.2 Proline® Alters Protein Folding, Oxidative Stress, and Cytoskeleton Organisation Proteins in the Fat Body

Similar to the impact of PAI exposure, PCF exposure also led to alterations in protein folding proteins in the fat body of *B. terrestris*. With six proteins, including two chaperonin-containing T-complex proteins increased in abundance. However, nine proteins associated with cellular responses to stress were also increased, which was not observed in the fat body after PAI exposure alone. These proteins included three heat shock proteins, peroxiredoxin 1 and GST. This increase in chaperones and antioxidant proteins suggests oxidative stress in the fat body cells of PCF-exposed bees. Under normal circumstances, heat shock proteins act as molecular chaperones for protein folding, protein transport and the repair or degradation of proteins. However, under stress conditions, heat shock proteins have an increased expression to increase

cellular stress resistance. For example, under oxidative stress conditions, oxidised proteins can accumulate and aggregate, leading to cellular dysfunction, but an increase in heat shock proteins can prevent the accumulation of dysfunctional proteins by refolding damaged proteins or preventing further damage (Kalmar and Greensmith, 2009). Peroxiredoxins and GSTs are known to increase in abundance under oxidative stress conditions and are involved in the reduction of reactive oxygen species (Corona and Robinson, 2006) and GST has been previously implicated in pesticide detoxification in honeybees (Papadopoulos *et al.*, 2004; Wahida *et al.*, 2008).

PCF exposure also led to a decrease in SSDA proteins associated cellular pH alterations. Whilst STRING identified three proteins associated with this process, seven of the 62 SSDA proteins with a decreased abundance were subunits of V-type proton ATPase, a multi subunit protein involved in the acidification and pH maintenance of intracellular compartments such as the lysosome for activation of hydrolytic enzymes (Mindell, 2012). Interestingly, Zapata *et al.* (2022) demonstrated a link between V-type proton ATPase subunits with food intake in *C. elegans* and weight gain in mice, with V-ATPase disruption leading to decreased food intake and weight. These impacts were maintained upon re-introduction of V-ATPase, leading to the possibility of this protein as integral to metabolic memory. Additionally, proteins involved in oxidative phosphorylation were decreased, further suggesting alterations to metabolism in the fat body. Several proteins associated with the cytoskeleton and endocytosis were also decreased in abundance, including myosin-2 essential light chain, integrin-linked protein kinase, laminin subunit alpha and sorting nexins 2, 6 and 12.The profiles of these proteins may suggest alterations to endocytosis and cellular trafficking, which requires alterations to the cytoskeleton (Worby *et al.*, 2001; Schafer, 2002; Šamaj *et al.*, 2004).

Overall, these findings suggest increased oxidative stress in fat body cells, resulting in a need for molecular chaperones and antioxidants to prevent cellular damage and apoptosis in addition to alterations to endocytosis, pH maintenance, cytoskeleton organisation, and metabolism in the fat body after PCF exposure. Considering the high abundance of trophocytes within the fat body and its role in energy homeostasis, further research into a possible link between V-type proton ATPase alterations with food intake or weight gain could provide more insight on whether there are population level impacts of PCF on bumblebees. As discussed in chapter four, Jaffe et al. (2019) found decreased pollen foraging from prothioconazole treated cranberry trees in honeybees, which could indicate alterations to metabolism and food intake.

5.4.4.3 Common Alterations to the Fat Body Proteome after PAI and PCF Exposure

T-complex protein 1 subunit alpha, with an RFC of + 1.2 in both PAI and PCF was the only SSDA protein common to the fat body of both PAI and PCF exposed bees in comparison to their relative controls. This demonstrates that despite the exposure of bees to the same active ingredient, the formulation of the pesticide ingested can dramatically alter the processes and pathways impacted in the fat body.

5.4.5 Acetone Alters the Brain and Fat Body Proteome of *B. terrestris* at a Low Concentration

As discussed in chapter four, OECD guidelines on pesticide toxicity testing on bees determines that, if required for solubility, up to 5% acetone may be used in oral exposure regimes (OECD, 2017a). However, although acetone is considered to be a low toxicity solvent in pesticide regulatory assessments, this research determined acetone-specific impacts on the brain and fat body proteome of *B. terrestris* after oral exposure to 0.3% acetone 40% (w/v) sucrose solution.

In comparison to the non-acetone control, 45 and 31 SSDA proteins had an increased and decreased abundance, respectively, in the brain proteome of acetone control-exposed bees. Proteins with an increased abundance were involved in lipid metabolism and the pentose phosphate pathway and proteins with a decreased abundance were associated with intracellular signal transduction and cell communication regulation. Lipase had the highest abundance after acetone exposure, with an RFC of + 33.4, followed by regucalcin with an RFC of + 17.4 and a protein LOC100651683 annotated as Yellow-e2 with an RFC of + 17.2 in comparison to the control. Whilst lipase is a hydrolytic enzyme involved in the catabolism of lipids, regucalcin is involved in multiple processes such as the regulation intracellular Ca^{2+}, nucleic acid synthesis, and intracellular signaling, and may also play a role in lipid metabolism (Yamaguchi and Murata, 2013; Marques *et al.*, 2014). However, yellow-e2 has homology to major royal jelly proteins which constitute royal jelly secretion in *A. mellifera*. However, this protein is also related to protein yellow-f and f2, which are enzymes involved in the conversion of dopachrome to 5,6-dihydroxyindole for melanisation in *Drosophila* (Han *et al.*, 2002; Barek *et al.*, 2022).

In addition, proteins associated with oxidative stress regulation were increased in the brain after exposure to the acetone control, including catalase and peroxiredoxin-5, mitochondrial, indicating an oxidative cellular environment after acetone exposure, as observed in the

digestive tract in chapter four. Catalase protects cells from oxidative damage from H_2O_2 whilst peroxiredoxins are thiol-specific antioxidants that play a role in the reduction H_2O_2 as well as the detoxification of peroxides and sensing of H_2O_2 – mediated signaling events (Felton and Summers, 1995; Radyuk *et al.*, 2009). In addition, peroxiredoxin-5 can act as a negative regulator of immune signaling (Radyuk *et al.*, 2010), suggesting peroxiredoxin-5 could be increased in conjunction with yellow-e2 to control melanisation. Interestingly, acetone has been shown to increase lipid radical formation in mice (Stadler *et al.*, 2008). This may explain increased SSDA proteins involved in lipid degradation and synthesis to modulate the destruction and replacement of damaged lipids. In addition, acetone can accumulate in phospholipid membranes and increase membrane fluidity, which could alter cellular functioning and lead to leakage of important molecules (Posokhov and Kyrychenko, 2013).

The protein with the greatest decrease in abundance was uncharacterised protein LOC100647721 with an RFC of – 40.0 in the brain after exposure to the acetone control compared to the non-acetone control. This protein was identified as Pita, an architectural protein involved in the regulation of enhancer-promoter interactions, ultimately regulating transcription (Maksimenko *et al.*, 2015; Zolotarev *et al.*, 2016). In addition to alterations to signaling proteins, proteins associated specifically with synaptic transmission were decreased after acetone control exposure compared to the control, including CASK and vesicular glutamate transporter 1, which were significantly increased in abundance after PAI exposure in comparison to acetone. CASK is involved in synaptic development and plasticity in neurons whilst vesicular glutamate transporter 1 participates in glutamate excitatory neurotransmission. These results suggest acetone induced alterations to signaling and possibly neurotransmission in the brain of *B. terrestris*, which may be due to alterations to phospholipid membranes and/or increase oxidative stress.

In the fat body, there were 21 and 28 SSDA proteins with an increased and decreased abundance, respectively, after exposure to the acetone control in comparison to the non-acetone control. Similar to the brain, proteins with an increased abundance were associated with lipid metabolism, with four out of the top five most increased proteins associated with lipid metabolism. In addition, cytochrome p450 4g15 was increased, which is associated with the oxidation of steroids, fatty acids and xenobiotics for cell detoxification (Feyereisen, 1999). Proteins decreased in the fat body were mainly associated with transport, in particular vesicle-mediated transport associated with endocytosis and membrane trafficking, which included

sorting nexins, V-type proton ATPases and actin cytoskeleton-related proteins. Interestingly, these proteins were also decreased after exposure to PCF, which did not contain acetone, which may suggest alterations to membrane trafficking in response to non-pesticide xenobiotics that interact with fatty acids and lipids, such as acetone and chemical surfactants, which could alter, or compound pesticide impacts on the fat body.

These findings suggest that acetone could alter the impacts of prothioconazole on the brain and fat body proteome of bees and whilst PAI was directly compared to an acetone control, the impacts of acetone such as altered lipid metabolism, increased oxidative stress, and altered signaling in the brain, could compound or alter the impact of prothioconazole in the brain. Similarly, whilst co-formulants present in PCF could not be individually tested due to nondisclosure of formulation ingredients, co-formulants may also alter prothioconazole impacts on bees, meaning research investigating prothioconazole alone may not represent what pollinators are exposed to in the environment. Further, different co-formulants and solvents may have differential impacts, making it difficult to fully elucidate the impact of pesticides requiring organic solvents on bee health as demonstrated by this research. For example, an increase in moribund bees was observed in chapter four for PAI exposed bees, but not acetone control exposed bees. If acetone has alterations on membrane fluidity, neurotransmission, and oxidative stress, this may be modulated by oxidative stress regulators and alternative signaling mechanisms. However, if these alterations to the cellular environment of both the brain, fat body, and other tissues are compounded by prothioconazole-induced stress, this could be enough to induce irreversible physiological damage leading to intolerance of alterations to neurotransmission, lipid metabolism and signaling from acetone exposure. Since many co-formulants are designed to serve the same purpose as acetone, i.e. for active ingredient solubility, further testing of disclosed surfactants will be important to elucidate the impact of pesticides on bees as they may be ingested in the environment. Further, testing of acetone alterations and acetone alternatives for use in pesticide toxicity testing could provide a new suite of solvents with minimal impact on cellular lipid metabolism and phospholipid membranes, allowing for a more accurate investigation of pesticidal impacts on bees.

5.4.6 An Adverse Outcome Pathway Model for the Impacts of Glyphosate and Prothioconazole on the Brain and Fat Body Proteome of *B. terrestris*

Based on the AOP by Ankley et al., (2010), a framework which aims to accumulate and translate findings on the molecular level to the population level to determine how individual pesticides alter non-target organisms. The AOP framework allows the translation and communication of the potential negative impacts based on biological pathways altered after exposure. Here, we present a brief AOP for the impacts of glyphosate on the brain and fat body proteome (Figure 5-13) and the impacts of prothioconazole on the brain and fat body proteome (Figure 5-14) by translating the possible pathways and processes affected based on significant alterations to proteins within the brain and fat body proteome. As a result, these AOP models can guide future research and policies and aid in the understanding of impacts observed on learning, memory, detoxification, or energy homeostasis after exposure to either of these chemicals.

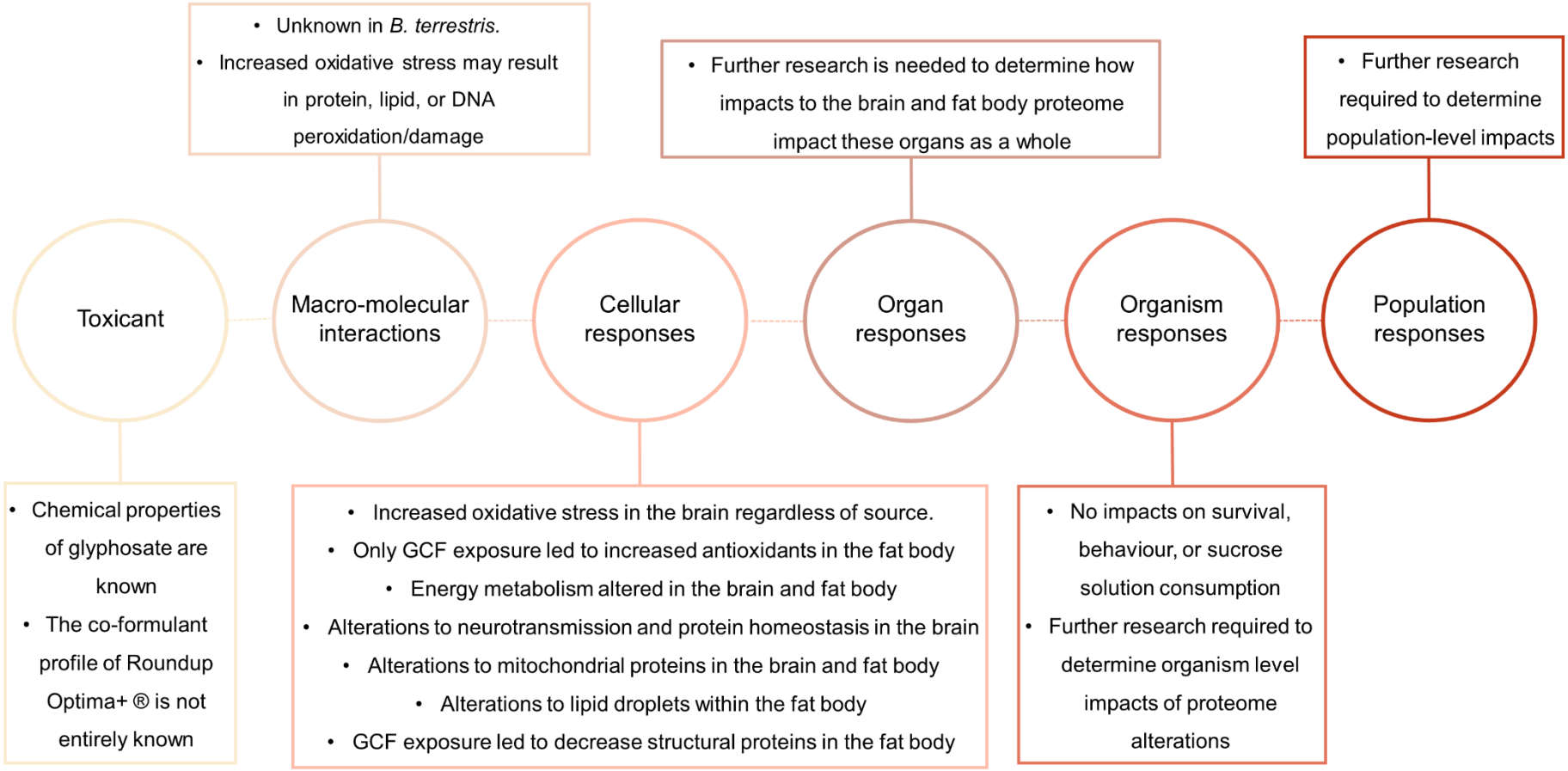


Figure 5-13 Adverse Outcome Pathway for Glyphosate Exposure in *B. terrestris* Brain and Fat Body. An AOP was filled to translate potential impacts on the brain and fat body of *B. terrestris* after glyphosate exposure based on the key findings from this chapter (After Ankley *et al.*, 2010).

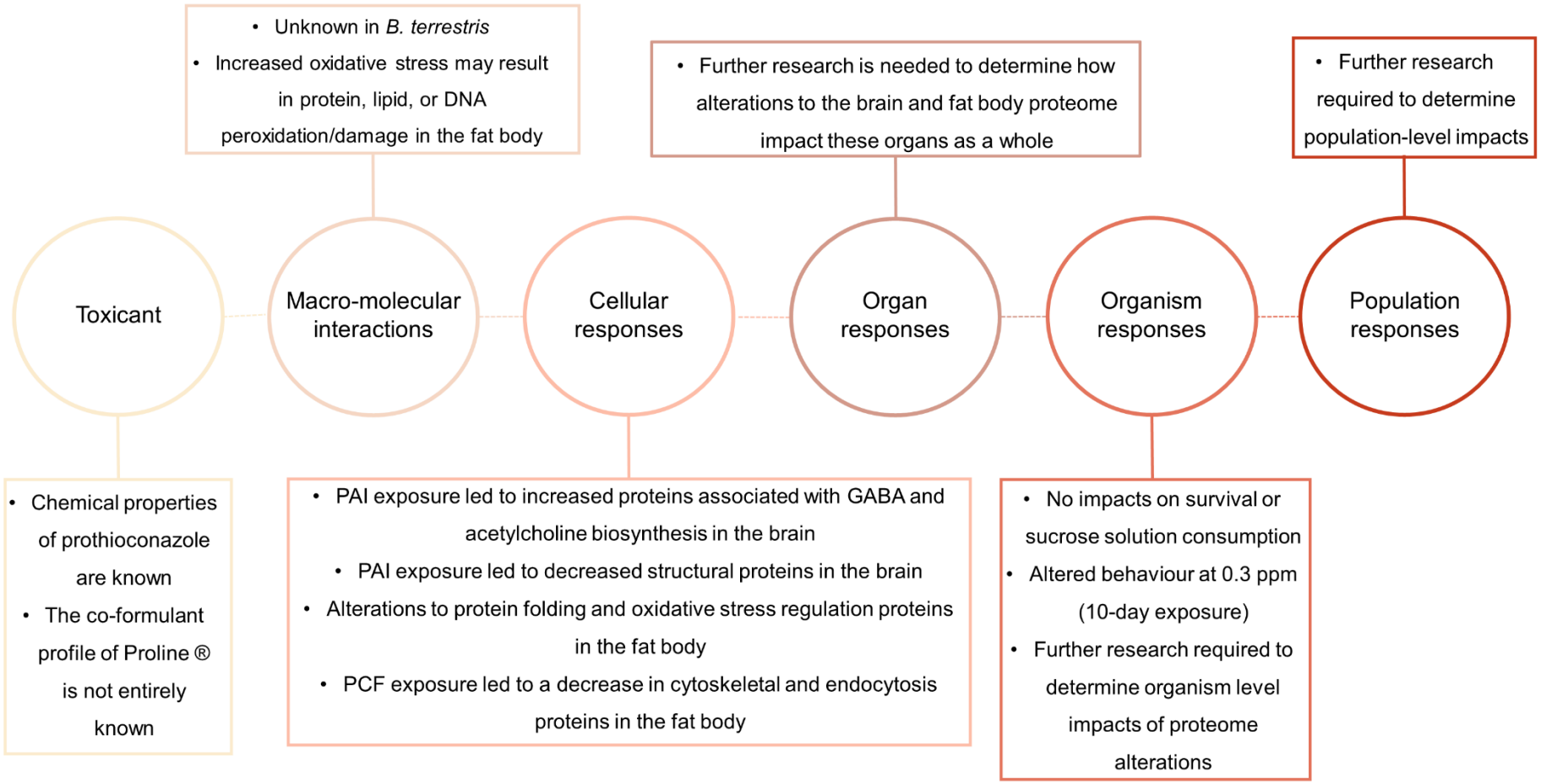


Figure 5-14 Adverse Outcome Pathway for Prothioconazole Exposure in *B. terrestris* Brain and Fat Body. An AOP was filled to translate potential impacts on the brain and fat body of *B. terrestris* after prothioconazole exposure based on the key findings from this chapter (After Ankley *et al.*, (2010)).

5.5 Conclusion

Whilst previous chapters highlighted the impacts of glyphosate and prothioconazole on behaviour, the digestive tract proteome, and the digestive tract microbiota, this chapter highlighted the impact of these agriculturally important pesticides on the brain and fat body proteome to determine risks to overall *B. terrestris* health. Whilst impacts on the digestive tract were somewhat expected based on the direct contact of orally ingested pesticides with this tissue, alterations to the brain and fat body proteome were unexpected as these are, as far as these pesticides are concerned, non-contact organs in a non-target organism. Whilst both the brain and fat body proteome were altered by glyphosate and prothioconazole treatments, in terms of the number of SSDA proteins observed, the brain seems to be more sensitive to both glyphosate and prothioconazole exposure.

It was demonstrated that the herbicide glyphosate altered protein homeostasis, oxidative stress and synaptic transmission in the brain proteome regardless of ingestion as the active ingredient alone or as part of the commercial formulation Roundup Optima+®. However, differential impacts were observed also, with effects on signalling important in visual information processing, synaptic organisation, translation, and lipid metabolism, which may indicate alterations to the *B. terrestris* brain based on the ingestion of co-formulants present in Roundup Optima+®. In the fat body, glyphosate altered protein synthesis, the mitochondria and lipid droplet proteins, which could have major consequences for energy homeostasis in *B. terrestris*. In addition, differential impacts were observed on proteins associated with energy metabolism, translation, and prostacyclin signaling, which could have implications for immune signaling.

Prothioconazole active ingredient also led to significant alterations to the brain proteome in proteins associated with the cytoskeleton, neurotransmitter biosynthesis, and energy metabolism. Proline® ingestion impacted the abundance of proteins associated with translation in the brain. In the fat body, prothioconazole active ingredient altered protein folding and oxidative stress, and whilst Proline® also altered these processes, cytoskeleton organisation proteins were also changed after Proline®

exposure. However, despite similar processes altered by prothioconazole-based treatments in the fat body, few alterations to proteins and processes common to either the brain or fat body tissues after prothioconazole active ingredient or Proline® exposure could be determined. As a result, the impact of acetone on both the brain and fat body were investigated. It was determined that acetone significantly altered proteins associated with lipid metabolism, signal transduction and oxidative stress in the brain, and proteins associated with endocytosis, membrane trafficking, and oxidative stress were altered in the fat body. Based on previous research on acetone impacts, these alterations may be due to acetone-induced oxidative stress and alterations to phospholipid membranes. These acetone-induced alterations, along with the co-formulants present in Proline®, highlighted the impact of solvents alone and in conjunction with pesticide exposure, challenging the idea that solvents are 'inert' ingredients with no impact on either target or non-target organisms.

Chapter 6
General Discussion

6.1 Thesis Background and Aims

In the new global economy that prioritises production and profit, pesticides are fundamental to worldwide agricultural systems, and Ireland is no exception. Over 60% of Irelands 7.04 million hectares are dedicated to agriculture, and nearly 3000 tonnes of pesticidal active ingredients were used in agriculture in 2020 (Perpiña Castillo *et al.*, 2018; FAO, 2020). In addition to their target organisms, pesticides are increasingly known to affect non-target and often beneficial organisms including insects (Blacquière *et al.*, 2012; Cullen *et al.*, 2019). Insect pollinators are vital for the pollination and reproduction of crops, with insect pollinated crops contributing an estimated revenue of $235-577 billion (Potts *et al.*, 2017). However, many insect populations and specifically bees are suffering from global decline in diversity and abundance (Potts *et al.*, 2010; Zattara and Aizen, 2021), with 9.2% of bee species in the EU and 33% of bee species in Ireland threatened with extinction (Fitzpatrick *et al.*, 2006; Nieto *et al.*, 2014). Whilst there are multiple factors associated with bee decline, such as habitat loss and disease, pesticide exposure plays a key role in bee decline with lethal and sublethal impacts identified for a range of pesticides (Di Prisco *et al.*, 2013; Zhu *et al.*, 2017; Motta and Moran, 2020). Non-insecticidal pesticides, such as herbicides and fungicides, are some of the most used pesticides globally, outweighing insecticide use in application, sales, and market value (US Environmental Protection Agency, 2017; Jess *et al.*, 2018; EUROSTAT, 2020a). In Ireland, herbicides accounted for over 78% of active ingredients found in pesticide products in 2020 (DAFM, 2020). However, most research investigating the impacts of pesticides on bees focuses on insecticides and honeybees, resulting in an urgent need to address the safety of non-insecticidal pesticide impacts on wild bees (Cullen *et al.*, 2019).

In 2018, neonicotinoid insecticides were restricted in the EU (European Commission, 2018a, 2018b, 2018c), a policy which was implemented after decades of intensive agricultural use and hundreds of peer-reviewed studies demonstrating their negative impacts on bees and probable contribution to bee decline (Blacquière *et al.*, 2012;

Goulson, 2013). This highlighted the short-comings of pesticide regulatory risk assessment procedures in the EU which these insecticides were approved under, including a narrow focus on just one species of bee, *A. mellifera*, and a focus on direct mortality effects, despite the knowledge of sublethal impacts negatively impacting bee health and longevity (Vandame *et al.*, 1995; Decourtye *et al.*, 2005; Balbuena *et al.*, 2015).

An additional issue relates to EC regulatory risk assessments, which mainly focus on pesticidal active ingredients despite pesticides used in agricultural and non-agricultural settings as pesticide formulations that contain both the pesticide active ingredient and co-formulants, also referred to as inert ingredients, which are used to increase pesticide solubility and efficacy. Less stringent assessment is required for the impacts of pesticide formulations on bees and no assessment requirements are set for individual co-formulants (The European Commission, 2009, 2013). Few studies have been carried out on the impact of co-formulants found in pesticide formulations on bees. Consequently, there is little scientific understanding of if and how pesticide formulations may impact bees (Straw *et al.*, 2022). Further, the co-formulants present in formulations – solvents, surfactants, dyes, and anti-foaming agents – are considered proprietary information, making it difficult to isolate and investigate individual co-formulants. In addition, a widespread belief that co-formulants are inert, i.e. that they do not have any impact on non-target organisms due to a lack of pesticidal activity, has led to a lack of investigation for impacts on bees. Straw and Brown, (2021) discovered that a co-formulant can damage the bumblebee digestive tract, and Mesnage *et al.* (2014) highlighted that pesticide formulations can be more hazardous than the pesticide active ingredient alone, however, these studies are few and far between, and a variety of co-formulants can be formulated with a range of active ingredients, making their usage likely higher than pesticidal active ingredients themselves, with EFSA identifying 182 co-formulants from 82 pesticide formulations (European Food Safety Authority, 2022).

Our published systematic review highlighted a major lack of research on the impacts of herbicides and fungicides on bees, particularly on non-*Apis* species (Manuscript S1-1). Further, we found very limited research on the impacts of the most used herbicides and fungicides on bees, indicating an urgent need to understand how the most used

pesticides in EU agriculture impact declining bee species if we are to employ strategic, useful, and science-based mitigation strategies to protect wild bees against further decline.

The herbicide glyphosate is the most used herbicide in the world, including Ireland, with systemic and broad-spectrum action targeting a range of plants and microbes via inhibition of the EPSPS enzyme, an important enzyme of the shikimate pathway for aromatic amino acid synthesis, which is not present in animals, including bees. Despite glyphosates widespread use and its detection in nectar and pollen of both treated and non-treated plants (Cebotari *et al.*, 2018; Zioga *et al.*, 2020, 2022), we are yet to understand if and how glyphosate impacts wild bees such as bumblebees. Whilst studies on glyphosate have increased in recent years, suggesting impacts on survival, immunity, learning and memory, and the digestive tract microbiota (Farina *et al.*, 2019; Motta and Moran, 2020; Motta *et al.*, 2022), the majority of studies focus on honeybees and use concentrations which are likely much higher than those ingested by bees in the environment (Thompson *et al.*, 2014; Cebotari *et al.*, 2018; Zioga *et al.*, 2022). Further, whilst an increase in digestive tract microbiota studies have found interesting results on the impact of glyphosate on bees, many focus solely on the bacterial microbiota, leaving the impact of glyphosate on the fungal microbiota unknown (Dai *et al.*, 2018; Blot *et al.*, 2019; Motta and Moran, 2020).

In Ireland, fungicides account for approximately 15% of pesticide active ingredients on the Irish market (DAFM, 2020), with fungicides applied, often multiple times per season, to over 300,000 hectares of agricultural land each year (López-Ballesteros *et al.*, 2022). Further, triazole fungicides account for the greatest weight of pesticides used globally (Zhang, 2018). Prothioconazole, a triazole EBI fungicide, is one of the most widely used fungicides in Irish agriculture (López-Ballesteros *et al.*, 2022), preventing fungal disease in crops by inhibiting ergosterol biosynthesis which is vital for cell membrane integrity and functioning in many fungal species (Jordá and Puig, 2020). Due to its widespread use, prothioconazole residues have been detected at concentrations up to 356 µg/kg in pollen (Roszko *et al.*, 2016; Böhme *et al.*, 2018; Rondeau and Raine, 2022). However, until 2019, there were no studies investigating the impact of prothioconazole on bees, and the few studies that exist have been restricted to investigating the potential of prothioconazole to synergise negative

impacts from insecticide exposure in honeybees (Wood *et al.*, 2019; Haas and Nauen, 2021; Taenzler *et al.*, 2022). Consequently, the impact of prothioconazole on bees is completely unknown.

The research conducted throughout this thesis was part of the Department of Agriculture, Food and the Marine funded PROTECTS (protecting terrestrial ecosystems through sustainable pesticide use). The principal goal of PROTECTS was to understand the impacts of the most used, but understudied, pesticides in Ireland on terrestrial ecosystems to provide baseline information in an Irish context to build towards effective, science-based mitigation strategies for reducing the impacts of pesticide use on terrestrial ecosystem services, particularly pollinators. Outputs from this project will support Irelands national action plan for sustainable pesticide use under the EU sustainable use of pesticides directive (Directive 2009/128/EC), reducing the risks and impacts of pesticides on pollinators and the environment.

Continued pollinator decline could be detrimental for food security and ecosystem health and with pesticide use linked to bee decline, it is urgently necessary to understand the impacts of the most widely used pesticides on bee health. We aimed to characterise the impact of these pesticides on the bumblebee species *B. terrestris*, using it as a model for wild bumblebees, which are important for pollination but typically less researched than their honeybee counterparts (Cullen et al., 2019). Utilising LFQ mass spectrometry-based proteomics, DNA amplicon sequencing and survival, consumption and behavioural assays, the presented research explores, for the first time, the impact of the herbicide glyphosate and the fungicide prothioconazole on *B. terrestris* key organs; the digestive tract, brain, and fat body in addition to impacts on survival, behaviour and feeding at field-realistic concentrations. Furthermore, in every experimental investigation, the impact of the pesticidal active ingredient were compared to a relevant pesticide commercial formulation to compare the impact of the active ingredient alone, and pesticide formulations which are used in agricultural and non-agricultural settings, on *B. terrestris*. This was conducted to allow comparisons to be made between the active ingredient, formulation, and control treated bees, determining if co-formulants may alter impacts to *B. terrestris* differentially to the active ingredient. As a result, we gained valuable information on similarities and differences of pesticide and co-formulant impacts on *B. terrestris* survival, behaviour,

feeding, digestive tract microbiota, and key organ physiological processes, highlighting that commercial formulations should be subjected to more stringent testing, and may not be comparable to the active ingredient within them.

This project was exploratory and discovery-based in nature, with LFQ mass spectrometry permitting the identification and quantification of thousands of proteins from a single biological sample, enabling the assessment of physiological changes at the molecular and cellular phenotype level for the characterisation of key organs indicating health status after exposure to glyphosate and prothioconazole. Along with DNA amplicon sequencing of the digestive tract microbiota, behavioural, survival, and consumption assays, this research provides an important opportunity to advance our understanding of glyphosate- and prothioconazole-based formulations and active ingredient impacts on bees. As a result of these investigations, there are several important areas where this study makes an original contribution to our understanding of the impacts of glyphosate and prothioconazole active ingredient and representative commercial formulations on *B. terrestris* at the molecular, cellular, and organism level. The organism and molecular level findings were incorporated into an adverse outcome pathway (AOP) framework as put forward by Ankley *et al.* (2010). The AOP serves to provide an outline of known and unknown impacts of chemical compounds on an organism from the molecular to the community level and collates research informed impacts and subsequent gaps in our understanding. The discoveries outlined in this thesis will guide further research to specific areas of concern, and aid in the production of science-based strategies for sustainable pesticide use, contributing to a holistic whole-view of the impacts of glyphosate and prothioconazole on bumblebees and mitigating the impact of pesticide use on pollinators.

For a comprehensive understanding of non-insecticide pesticide exposure on *B. terrestris,* it is important to gain insights on both the molecular and organism level to fully elucidate and understand possible lethal and sub-lethal impacts. In chapter three, we aimed to characterise the impact of glyphosate active ingredient and the glyphosate-based formulation RoundUp Optima+® on *B. terrestris*. For an initial understanding of this pesticides impact, we decided to investigate the impact of a five-day oral exposure at three difference concentrations ranging from field-realistic 1 ppm to higher-than-expected doses of 100 ppm on survival and behaviour. In addition, we

investigated the impact of 1 ppm glyphosate active ingredient and RoundUp Optima+® on sucrose solution consumption. To gain a molecular level insight on the impacts of glyphosate exposure, we utilised LFQ mass spectrometry-based proteomics and DNA amplicon sequencing to determine if there were statistically significant alterations to the digestive tract proteome and digestive tract microbiota after exposure to 1 ppm of glyphosate active ingredient or Roundup Optima+®. We focused on the digestive tract first as it is the main point of contact after ingestion and hypothesised that it is the tissue that is most likely to be impacted after ingestion of a xenobiotic with no known impacts on insects. LFQ mass spectrometry-based proteomics revealed insights into the impacts of the active ingredient and commercial formulation on the digestive tract proteome, whilst DNA amplicon sequencing allowed us to investigate the impact of field-realistic concentrations of the active ingredient and commercial formulation on both the bacterial and fungal microbiota.

In chapter four, we characterised the impacts of prothioconazole active ingredient and a prothioconazole-based formulation, Proline®, on *B. terrestris.* Similar to chapter three, we first investigated the impact of prothioconazole active ingredient and Proline® at the organism level, conducting survival, behavioural and consumption assays at three prothioconazole concentrations ranging from a field-realistic concentration of 0.3 ppm to higher-than-expected concentrations of 30 ppm for five days. Further, we investigated the impact of prothioconazole and Proline® on the digestive tract, with a similar hypothesis to that laid out for chapter three; since prothioconazole does not aim to target bees, the digestive tract will be the main point of contact and most probable point of pesticide-induced damage after oral ingestion. Using LFQ mass spectrometry-based proteomics and DNA amplicon sequencing, we explored the impact of prothioconazole and Proline® on the *B. terrestris* digestive tract proteome and both the bacterial and fungal microbiota. However, one caveat to investigating prothioconazole is its low water solubility, making the use of an organic solvent a necessity for the investigation of prothioconazole active ingredient, which was made up with 0.3% acetone for solubility. This was taken as an opportunity to also investigate the impacts of the solvent acetone, often used in pesticide toxicity assays, and considered safe for bees at concentrations up to 5% (OECD, 2017a). By utilising both a 0.3% acetone and non-acetone control in all experiments conducted in

this chapter, we were able to compare the impact of a low dose of acetone to the non-acetone control.

Finally, in chapter five, we aimed to characterise the impacts of glyphosate active ingredient, Roundup Optima+®, prothioconazole active ingredient, Proline®, and 0.3% acetone on non-contact organs in *B. terrestris*. After five days of exposure to field-realistic concentrations of these pesticides and a low concentration of the common solvent acetone, we utilised LFQ mass spectrometry-based proteomics to gain insights on impacts to the brain and fat body. The brain is vital for information processing, learning, and memory, whilst the fat body is a key tissue for energy homeostasis, xenobiotic detoxification, and the production of immune proteins, making these key tissues to the survival and longevity of bumblebees (Galizia *et al.*, 2012; Li *et al.*, 2019). By illuminating impacts of these commonly used pesticides and relevant formulations on the brain and fat body proteome, we can elevate our understanding of glyphosate and prothioconazole impacts, as well as co-formulant and solvent impacts, on key processes in tissues vital for bee health.

6.2 Key Findings

6.2.1 The Impact of Glyphosate and Prothioconazole on *B. terrestris* Survival, Behaviour and Food Consumption

We did not find any significant impacts of glyphosate, ingested as the active ingredient or commercial formulation, on *B. terrestris* survival, behaviour, or sucrose solution consumption. Furthermore, prothioconazole did not impact bee survival or sucrose solution consumption. However, we found that 0.3 ppm prothioconazole active ingredient and 0.3 ppm Proline® statistically significantly increased the incidence of affected and moribund behaviour in bees, respectively. Interestingly, these results may indicate a non-monotonic dose-response curve for prothioconazole exposure in bees, as only lower doses led to significant alterations to behaviour, an alternative to the classic dose-response relationship which associates higher doses with greater negative impacts, and lower doses with lower negative impacts (Beausoleil *et al.*, 2013; Vandenberg, 2013). However, some research on pesticide toxicity had similar findings, with *A. mellifera* brood displaying higher mortality after exposure to 0.1 ppm thiamethoxam than 0.5 ppm thiamethoxam (Wood *et al.*, 2019), and exposure to 0.01 ppm glyphosate leading to higher mortality rates when combined with difenoconazole exposure compared to 0.1 ppm glyphosate (Almasri *et al.*, 2021).

6.2.2 Glyphosate and Prothioconazole Alter *B. terrestris* Digestive Tract Microbiota

Whilst previous studies have identified that glyphosate alters the digestive tract microbiota in honeybees (Motta *et al.*, 2018; Dai *et al.*, 2018; Blot *et al.*, 2019), these studies focused on high concentrations of the active ingredient and investigated impacts on the bacterial microbiota alone, resulting in unknown impacts on the fungal microbiota. Research presented here, provided for the first time an insight into the impact of a field-realistic concentration of both glyphosate active ingredient and a commercial formulation on both bacterial and fungal species of the digestive tract microbiota in a bumblebee. We found that glyphosate differentially impacted the microbiota, depending on its ingestion as the active ingredient or Roundup Optima+®. After exposure to the active ingredient, bacterial and fungal beta diversity were significantly altered, with a relative decrease *Parabacteroides* bacteria and

Rhodotorula fungi in comparison to the control group. After Roundup Optima+® exposure, *L. murinus* were present, with no presence in active ingredient or control treatment groups. Further, *B. bombi* was present after Roundup Optima+® exposure, but not active ingredient exposure, indicating that co-formulants may alter how glyphosate impacts bacterial species, or alter bacterial species abundance regardless of glyphosate presence. This was highlighted in one of our major findings from chapter three, which was that Roundup Optima+® exposure led to significant alterations to fungal beta diversity, particularly, a significant decrease in the abundance of *C. apis* and *C. bombi* species present in the digestive tract, which was not found after exposure to the glyphosate active ingredient. Further, less common species such as *T. asperellum* were significantly increased in relative abundance in Roundup Optima+® treatment groups compared to active ingredient and control treatment groups. Along with the increased relative abundance of uncommon bacterial species after Roundup Optima+® exposure, which were not found in other treatment groups, these results may indicate opportunistic colonisation due to lower *Candida* abundance, a common fungal genera found in the *B. terrestris* digestive tract microbiota (Praet *et al.*, 2018). In the wild, this alteration to bee fungal communities in the digestive tract could lead to dysbiosis and perhaps infection with opportunistic pathogens (Näpflin and Schmid-Hempel, 2018; Tauber *et al.*, 2019; Pozo *et al.*, 2020). In addition, alterations to the microbiota overall may impact immunity and defence responses against pathogens (Kwong *et al.*, 2017; Praet *et al.*, 2018; Tauber *et al.*, 2019). These findings further demonstrate the impact of both glyphosate and co-formulants on the *B. terrestris* digestive tract, with co-formulants either alone or in combination with glyphosate leading to major alterations to core fungal species, implicating one or more of the 'inert' ingredients, in this case, alkylpolyglycoside, nitroryl, or any of the unlisted components of Roundup Optima+® as a promoter of major fungal microbiota disruption.

Another important finding was that prothioconazole altered the digestive tract microbiota. Unexpectedly, prothioconazole had a greater impact, in terms of the number of species with significant alterations to their relative abundance, on bacterial species than fungal species. Both prothioconazole treatments led to a significant decrease in the relative abundance of *Lactobacillus* bacteria, which has been observed with other triazole fungicides in mice (Bao *et al.*, 2022). However, only Proline®

exposure led to a significant difference in fungal beta diversity, with the relative abundance of *Candida* species decreased. These results are similar to those found after Roundup Optima+® exposure, although from the co-formulants listed, neither formulation shares a common co-formulant. Perhaps *Candida* is sensitive to the presence of certain types of co-formulants e.g. emulsifiers, or due to their intended use of increasing the efficacy of pesticides, perhaps *Candida* is more sensitive to a wide range of pesticides in the presence of surfactants and solvents, a hypothesis which needs to be further researched in order to gain insight into the impacts of various pesticides on the bee microbiota. Interestingly, Bartlewicz et al. (2016) found that prothioconazole is highly toxic to nectar yeasts, including *Candida* species, at concentrations as low as 0.06 ppm, and whilst *Candida* was decreased in relative abundance after prothioconazole active ingredient exposure, this was not significantly different in comparison to the acetone control, with *Candida* abundance decreased in the acetone control compared to the control. However, there was a significant difference in the relative abundance of *A. flavus* after prothioconazole active ingredient exposure compared to acetone control exposure. Interestingly, *A. flavus* can lead to stonebrood disease in honeybee larvae and increases mortality in both larvae and adult honeybees (Vojvodic *et al.*, 2011; Foley *et al.*, 2014). Based on these results, we hypothesize that prothioconazole exposure disrupts the microbiota, which can lead to opportunistic pathogen infection due to a decreased abundance of core microbial species in the microbiota.

6.2.3 Glyphosate Alters the Digestive Tract, Brain, and Fat Body Proteome of *B. terrestris.*

Investigating the impact of glyphosate on the proteome of three key tissues uncovered important insights into this pesticide, alone or as part of a commonly used formulation, on physiological processes important for *B. terrestris* health. Although there are differences between glyphosate active ingredient and the commercial formulation Roundup Optima+®, there were also common proteins and processes altered in the tissues suggesting a conserved ‘glyphosate’ impact, irrespective of its source.

One of our key findings was that glyphosate, regardless of its source, led to significant decreases in proteins associated with cellular adhesion, the extracellular matrix, and the basement membrane in the digestive tract, which could alter structural integrity of

digestive tract epithelial cells. Proteins with the largest decreases in abundance after exposure to both glyphosate active ingredient and Roundup Optima+® included fibrillin, collagen alpha (IV) chain proteins, cartilage oligomeric matrix protein and basement membrane proteoglycan. Of particular interest was the protein fibrillin, with had an RFC of -222.7 and 56.8 after exposure to the active ingredient or Roundup Optima+®, respectively. This protein is vital for the formation of microfibril components of the extracellular matrix and basement membranes, and act as molecular scaffolds for cellular structural integrity, strength, and growth factor regulation in tissues (Kumra and Reinhardt, 2018). In addition, decreases to collagen alpha IV proteins and heparan sulfate proteoglycans are vital for cell structural integrity as key components of basement membranes, with collagen alpha IV constituting approximately 50% of basement membranes (Paulson, 1992; Kalluri, 2003; Tanzer, 2006). Interestingly, decreases in collagen proteins have previously been associated with wounding and haemocyte recruitment for cellular repair (Pastor-Pareja *et al.*, 2008; Saleh *et al.,* 2018), which could indicate mechanical injury to digestive tract epithelial cells. Overall, these changes suggest major alterations to digestive tract epithelial cell structural integrity and provide a possible mechanism for glyphosate induced damage in the digestive tract after glyphosate ingestion.

Another major finding from this thesis was an increase in proteins associated with oxidative stress regulation in the proteome of all three tissues, indicating a potential defence response against pesticide-induced oxidative stress. This response was present regardless of glyphosate source. We hypothesised that increased ROS and resulting oxidative stress conditions in all three tissues may have led to oxidative damage to proteins, leading to significant alterations to proteins involved in protein homeostasis. However, in the digestive tract proteome, we also found a significantly increased abundance of the phenoloxidase enzyme laccase, which participates in insect immunity and wound healing through its role in melanin production. Melanisation is a key aspect of the insect immune response to pathogens or injury (González-Santoyo and Córdoba-Aguilar, 2012; Parsons and Foley, 2016). Further, ROS are important in melanisation and wound repair, which may further indicate mechanical injury to the digestive tract (Nappi and Christensen, 2005; Zug and Hammerstein, 2015). Additionally, some studies have found that glyphosate may alter melanisation in insects (Smith *et al.*, 2021; Motta *et al.*, 2022), however, these studies were conducted

in non-*Bombus* insects with exposure to higher glyphosate concentrations than used in this study.

Whilst alterations to antioxidants suggest increased oxidative stress in all three tissues, it is important to note that the majority of ROS originate from mitochondria (Balaban *et al.*, 2005). Interestingly, mitochondrial proteins were significantly altered after glyphosate exposure in all three tissues. Multiple studies across a range of species have found that glyphosate alters mitochondrial functioning via disruption to mitochondrial metabolism, mitochondrial membrane polarisation and membrane permeabilization (Peixoto, 2005; A.G. Pereira *et al.*, 2018; Ravishankar *et al.*, 2020). The results presented in this thesis indicate that mitochondria are also disrupted in multiple tissues of *B. terrestris* after glyphosate exposure, which may indicate a key mechanism of action of glyphosate against non-target organisms. Alterations to mitochondrial functioning would explain an increase in oxidative stress and may explain alterations to high energy cost processes such as neurotransmission in the brain and metabolism in the digestive tract and fat body, as many ATP-producing processes are carried out within the mitochondria, such as the TCA cycle and oxidative phosphorylation.

In addition to oxidative stress and mitochondrial alterations in the brain, glyphosate also led to significantly decreased abundances of proteins important for axon guidance, synaptic transmission, and memory, indicating for the first time that glyphosate at a field-realistic concentration can impact neurotransmission in the brain. Alterations to these processes may represent a mechanism behind the glyphosate induced alterations to behaviour and memory found in honeybees at higher concentrations (Mengoni Goñalons and Farina, 2018; Farina *et al.*, 2019; Hernández *et al.*, 2021; Luo *et al.*, 2021). In addition, we found that exposure to Roundup Optima+®, but not glyphosate active ingredient, led to a significant increase in the abundance of proteins involved in rhodopsin-mediated signaling and regulation of synapse organisation, which may indicate alterations to sensitivity to, or processing of, light sensory inputs and neurotransmission (Kiselev and Subramaniam, 1994; Stell, 2012). Alterations to multiple biological processes in the brain proteome after exposure indicate that that i) glyphosate may directly impact the brain of *B. terrestris*, passing through the blood brain barrier, and ii) Roundup Optima+®, whether due to a specific co-formulant or the presence of glyphosate with a specific type of co-

formulant e.g. a nonpolar solvent, alters metabolic processes in multiple types of cells in *B. terrestris*.

We also found that glyphosate alters protein biosynthesis and Lsd1 in the fat body regardless of treatment with the active ingredient or Roundup Optima+®. Lsd1 is a protein vital for lipolysis of triglycerides in the fat body for the mobilisation of substrates for energy metabolism and also plays a role in the regulation of lipid storage in the fat body (Li *et al.*, 2019). This protein had the greatest decrease in Roundup Optima+® exposed bees and was the third most decreased protein after exposure to the active ingredient. In addition to alterations to metabolism observed in the digestive tract and brain, this adds nuance to the impact of glyphosate on energy metabolism and high energy cost processes such as neurotransmission. Lipolysis and mobilisation of energy stores in the fat body could aid in meeting the energy requirements of *B. terrestris* under stressful conditions, however, a decrease in Lsd1 may alter the ability of the fat body respond to and meet the energy requirements for an appropriate stress response.

Glyphosate active ingredient and Roundup Optima+® differentially altered energy metabolism processes in the digestive tract, brain, and fat body proteome. These included significant alterations to proteins involved in lipid metabolism, oxidative phosphorylation, and glycolysis. Shifts in metabolic processes as a result of glyphosate commercial formulation exposure were previously found in honeybees (Zhao *et al.*, 2020). However, the research presented in this thesis reveals that glyphosate-induced metabolic shifts may be hard to predict as they could be dependent on the co-formulants present in formulations, as metabolic processes were differentially altered depending on exposure to glyphosate active ingredient or Roundup Optima+® in all three tissues investigated.

Interestingly, we also found that Roundup Optima+® exposed bees had a significantly decreased abundance of basement membrane proteins in addition to increased abundance of proteins associated with oxidative stress and detoxification in the fat body proteome. Some basement membrane proteins decreased in abundance were also decreased in the digestive tract of bees exposed to both glyphosate-based treatments, indicating that digestive tract cells may be more sensitive to xenobiotic damage than fat body cells. However, collagen alpha (IV) proteins - which were decreased in both

the digestive tract and fat body after glyphosate exposure - are secreted by the fat body, which may indicate glyphosate-induced alterations to collagen alpha IV synthesis and secretion in the fat body with knock-on impacts for other organs such as the digestive tract. However, this alteration to basement membrane proteins was only found in the fat body after Roundup Optima+® exposure but was found in the digestive tract proteome after exposure to both glyphosate-based treatments. Surprisingly, Larkin (2018) found an increase in antioxidant proteins and a decrease in structural proteins in response to neonicotinoid exposure and entomopathogenic fungal infection, which may suggest a more general xenobiotic response in the fat body to stressors. The author suggests that such stressors, which are known to lead to an increase in ROS, cause oxidative damage to structural proteins, which could lead to dysfunction and subsequent decreases in these proteins (Dalle-Donne *et al.*, 2001). Whilst this may be the origins of basement membrane and oxidative stress alterations in the digestive tract and fat body of glyphosate exposed bees, however it does not explain alterations to mitochondrial proteins, nor differential alterations to metabolism in all tissues, neurotransmission in the brain, or the microbiota within the digestive tract, suggesting that what we are observing in the fat body may be partly a general response to xenobiotics, and partly a result of the direct impact of glyphosate and/or Roundup Optima+® exposure.

These results suggest that, despite glyphosate's known MOA targeting a pathway in plants and microbes which is not present in bees, glyphosate has impacts on all key tissues investigated, which could lead to lethal consequences in conjunction with other stressors likely to be encountered in the environment. The main impact of glyphosate in all three tissues investigated, regardless of its source, seems to be an increase in oxidative stress and alterations to mitochondrial proteins. Further, glyphosate altered the metabolic profile of all three tissues differentially, depending on exposure to the active ingredient or Roundup Optima+®. In addition, glyphosate altered the bacterial and fungal microbiota of *B. terrestris*, with only Roundup Optima+® exposure leading to major alterations to core *Candida* species. Considering no statistically significant differences were found for glyphosate exposure up to 100 ppm in survival or behavioural assays, these impacts are more than likely sublethal, although exposure to nutritional stress, disease, and other pesticides in the environment could increase the risk of lethal consequences associated with glyphosate exposure.

6.2.4 Prothioconazole Alters the Digestive Tract, Brain, and Fat Body Proteome of *B. terrestris*

Due to the presence of acetone, which is required to solubilise neat prothioconazole, active ingredient treated bees could not be directly compared to Proline® treated bees. However, both the active ingredient and Proline® were compared to an acetone control and non-acetone control, respectively, and proteins in common could be directly compared between protein datasets in relation to their relative controls.

We found that both prothioconazole active ingredient and Proline® altered the digestive tract proteome. Bees exposed to the active ingredient had an increase in SSDA proteins associated with basement membrane organisation, fatty acid metabolism, the electron transport chain, and detoxification proteins. Interestingly, one of the most increased proteins was collagen alpha-5(IV), a basement membrane constituent decreased in the digestive tract after exposure to both prothioconazole-based treatments, and the fat body of glyphosate formulation exposed bees. Detoxification proteins were also increased, including peroxiredoxin 1 and cytochrome p450, which are involved in the neutralisation of ROS and detoxification of pesticides (Berenbaum and Johnson, 2015). Further, decreased proteins were associated with Golgi-membrane trafficking, which could have consequences for several physiological processes including protein transport, mitosis, and maintenance of Golgi structure (Weide *et al.*, 2001; Zhou *et al.*, 2014). Alternatively, Proline® led to an increase in proteins associated with glutathione peroxidases, with a major increase in GST, a protein involved in detoxification processes, indicating defence against pesticide exposure, which has been found in honeybees in response to multiple stressors including exposure to insecticides and acaracides (Papadopoulos *et al.*, 2004; Wahida *et al.*, 2008). Interestingly, proteins with an increased abundance were also involved in cytoskeleton organisation and glycolysis. A recent study found that Proline® alters cytoskeleton organisation and glucose metabolism in human dendritic cells (de Ávila *et al.*, 2022). This suggests a common impact of Proline® on different cell types from both a vertebrate and invertebrate non-target organism. Whilst the exact implications of these alterations are unknown, the cytoskeleton is important for cell structural integrity, cell signalling, and protein and RNA localisation, where alterations could have downstream negative impacts on multiple signalling processes within the digestive tract cells. Interestingly, translation was altered after Proline®

exposure, with decreased ribosomal proteins annotated as mitochondrial. In *A. mellifera*, the triazole fungicide myclobutanil altered mitochondrial regeneration and ATP production (Mao *et al.*, 2017), which could provide an explanation for mitochondrial ribosomal proteins altered, and an increase in glycolysis to increase ATP production. Other proteins vital for mitochondrial regeneration were also decreased, such as chaperone and folding catalysts and mitochondrial protein transporters, further suggesting alterations to either mitochondrial biosynthesis or protein translocation into and/or out of mitochondria.

These results highlight that prothioconazole, as part of a formulation or alone, elicits an increase in proteins associated with detoxification and oxidative stress regulation, and alterations to mitochondrial proteins, in the digestive tract. However, whether due to co-formulants in Proline® or the presence of acetone for active ingredient solubility, or both, these treatments had many differential impacts on the digestive tract proteome.

Both prothioconazole and Proline® exposure had significant impacts on the *B. terrestris* brain proteome. Prothioconazole active ingredient led to significant alterations to proteins associated with the cytoskeleton. Interestingly, the majority of the top 15 proteins with the greatest decreased relative fold change in comparison to the relative control were cytoskeletal proteins, including multiple troponin complex subunits, tropomyosin, and myosin. Similarly, these proteins were significantly decreased in abundance after exposure to clothianidin (Guapo de Melo, 2020), an insecticide with known impacts on neurotransmission in the brain, suggesting that prothioconazole may alter similar processes in the brain. Further supporting this, proteins associated with GABA and acetylcholine biosynthesis were increased, as well as proteins associated with synaptic development and neurotransmitter transport, indicating alterations to neurotransmission in the bee brain. These physiological changes may explain the significant behavioural alterations observed in chapter four, with exposure to 0.3 ppm active ingredient resulting in an increased incidence of bees with affected behaviour. In addition, prothioconazole active ingredient exposure led to a decrease in proteins involved in glycolysis, pentose phosphate pathway and fatty acid biosynthesis. Such alterations to energy metabolism may have negative impacts on energy demanding processes such as neurotransmission. Most proteins significantly increased in the brain after Proline® exposure were ribosomal subunits,

indicating an increase in protein biosynthesis. Whilst there were few SSDA proteins commonly shared between the two treatments, prostaglandin E2 synthase 2, a protein crucial for immunity in insects, had the highest relative fold increase after both treatments in the brain.

In the fat body, prothioconazole and Proline® exposure led to alterations to protein folding and oxidative stress. Whilst proteins associated with protein folding were significantly increased in abundance after exposure to both treatments, proteins associated with oxidative stress regulation and detoxification were differentially altered. Glutaredoxin-1, an electron carrier protein involved in the glutaredoxin system that also plays a role in the detoxification of xenobiotics, was decreased in the fat body of active ingredient-exposed bees (Yao *et al.*, 2014). Alternately, heat shock proteins, peroxiredoxin 1, and GST were increased in abundance after Proline® exposure. Whilst an increase in these proteins indicate increased oxidative stress, they also indicate increased resistance to cellular stress in the fat body of *B. terrestris* after Proline® exposure, a finding which was not observed after prothioconazole active ingredient exposure. Further, active ingredient exposure led to decrease in various mitochondrial proteins, a finding seen in multiple tissues exposed to glyphosate on a larger scale. Interestingly, proteins associated with the cytoskeleton and endocytosis had a decreased abundance in the fat body proteome of Proline® exposed bees in comparison to control exposed bees. Alterations to proteins associated with the cytoskeleton and specifically the basement membrane were also found in the digestive tract and fat body proteome of glyphosate exposed bees in addition to Larkin (2018) identifying significant decreases in structural protein abundance in the fat body proteome after exposure to pesticidal and pathogenic stressors. However, the decrease of endocytosis proteins such as sorting nexins 2, 6 and 12 along with cytoskeletal proteins in the fat body may suggest that these alterations are more in line with a decrease to endocytosis and cellular trafficking of proteins in the fat body, which rely on cytoskeletal alterations (Worby *et al.*, 2001; Šamaj *et al.*, 2004).

Overall, we found that prothioconazole and Proline® have significant impacts on the digestive tract, brain, and fat body proteome, which could have negative impacts on physiological functioning and health. However, prothioconazole active ingredient and Proline® had significant differential impacts on these tissues with few proteins in common between both treatments, perhaps due to the addition of co-formulants in

Proline® and the necessary addition of an organic solvent, in this case acetone, for active ingredient solubility, despite direct comparison of the active ingredient to the acetone control. Both prothioconazole-based treatments altered mitochondria in the digestive tract, but the active ingredient treatment altered basement membrane organisation, fatty acid metabolism, oxidative phosphorylation, and cytoskeleton organisation. Whilst Proline® exposure also altered digestive tract cytoskeletal proteins, Proline® led to an opposite abundance profile of cytoskeletal proteins compared to the active ingredient. In addition, Proline® altered translation, protein homeostasis, and glycolysis. In the brain, prothioconazole active ingredient led to alterations in the cytoskeleton, neurotransmitter biosynthesis and energy metabolism. In the fat body, the active ingredient altered protein folding and oxidative stress. However, Proline® led to major decreases in ribosomal subunits in the brain, and altered oxidative stress, protein folding, and cytoskeleton organisation in the fat body proteome. Due to our inability to directly compare tissues of *B. terrestris* exposed to prothioconazole active ingredient or Proline® due to acetone presence in the active ingredient-exposed bees, and the full formulation of Proline® considered proprietary information, we decided to investigate the impact of 0.3% acetone exposure in the digestive tract, brain, and fat body proteome, as well as the digestive tract microbiota, survival, behaviour, and sucrose solution consumption, in comparison to the non-acetone control.

6.2.5 The Acetone Impact: Acetone Alters the Proteome of Key Tissues and the Digestive Tract Microbiota of *B. terrestris* at a Low Concentration

Acetone is a commonly used solvent in pesticide toxicity assays as it is considered to have low toxicity. Further, it is recommended by OECD guidelines on pesticide toxicity testing in bees. However, we found that acetone led to multiple alterations in comparison to the non-acetone control at just 0.3% acetone concentration, despite OECD guidelines suggesting its use at up to 5% of any tested solution (OECD, 2017a). Despite comparing all experimental data from bees exposed to the prothioconazole active ingredient with the acetone control, *B. terrestris* exposed to prothioconazole active ingredient shared very few alterations with Proline® in all tissues examined.

We found that 0.3% acetone in a 40% (w/v) does not alter survival or behaviour, and whilst one five-day study suggested a significant decrease in sucrose solution

consumption when exposed to 0.3% acetone, this was not found in triplicate ten-day exposure assays. However, acetone had significant impacts on the digestive tract microbiota, and digestive tract, brain, and fat body proteome, which may alter or compound prothioconazole active ingredient impacts on these tissues. In the digestive tract, 0.3% acetone led to a significant increase in antioxidant and detoxification proteins, including GST and SOD, suggesting increased oxidative stress after acetone exposure. Further, we found that over one third of significantly decreased proteins were mitochondrial proteins, suggesting alterations to the digestive tract mitochondria, including proteins associated with energy metabolism such as components of the electron transport chain and fatty acid metabolism.

A major finding was a decrease in phospholipase A2 and melittin, proteins often associated with the venom gland, which had a relative fold change decrease of – 62 and – 501 in comparison to the control group. Phospholipase A2 and melittin are involved in phospholipid metabolism and cell lysis, and the abundance of these proteins are generally positively correlated (Ferreira Junior *et al.*, 2010). We hypothesise that acetone impacts membrane phospholipids and increase oxidative stress, leading to lipid and protein oxidation, which have been found in studies on micro-organisms and molecular simulations (Posokhov and Kyrychenko, 2013; Dyrda *et al.*, 2019). Further, in mice, acetone increases free radical formation and consequently, lipid peroxidation (Stadler *et al.*, 2008). These results may signify an attempt to cope with phospholipid membrane alterations and lipid peroxidation due to acetone induced increases in ROS.

In addition, acetone ingestion significantly altered alpha and beta diversity of the digestive tract microbiota. Acetone exposure led to the complete absence of *Hymenobacter* and *Helicobacter* bacteria in the digestive tract microbiota, which were present in control treated bees, as well as significant decreases in the relative abundance of *B. bombi*. Further, acetone exposure increased the relative abundance of the opportunistic fungal pathogen *R. mucilaginosa*, which was absent in the control treatment group, suggesting acetone-induced alterations to the digestive tract microbiota, which may have altered the impact of prothioconazole active ingredient on the digestive tract. These results align with the findings of Dyrda et al. (2019), who identified acetone-induced toxicity in multiple bacterial species and the yeast *S.*

cerevisiae. Acetone induced higher toxicity in microorganisms than all other solvents investigated, despite acetone's reputation as a low toxicity solvent.

In the brain proteome, acetone ingestion led to an increased abundance of proteins involved in lipid metabolism and antioxidants, indicating significant physiological alterations in the brain similar to those observed in the digestive tract. Whilst increased proteins in the fat body were also involved in lipid metabolism, cytochrome p450 was also increased, which is associated with the detoxification of xenobiotics (Feyereisen, 1999). However, proteins involved in endocytosis, membrane trafficking and the cytoskeleton were also decreased in the fat body proteome. Interestingly, similar proteins involved in endocytosis and membrane trafficking were significantly decreased in the fat body proteome after exposure to Proline®, which, to our knowledge, did not contain acetone. This suggests a common xenobiotic response in the fat body of *B. terrestris*, which may be in response to, or altered by, co-formulants with comparable properties to acetone such as nonpolar solvents or emulsifiers. Such alterations could compound or alter the impact of pesticides on the fat body. Consequently, research into the impacts of solvents and other co-formulants on pesticide-induced lethal and sub-lethal impacts are urgently required, considering pesticides are used in the environment as formulation mixes.

6.2.6 Co-Formulants Alter the Impact of Pesticidal Active Ingredients on *B. terrestris*

Both Proline® and Roundup Optima+® had significant impacts on *B. terrestris* which were not found after exposure to the pesticide active ingredients from these formulations, indicating either co-formulant-induced impacts, or the ability of some co-formulants to alter or compound pesticide-induced impacts on *B. terrestris*. To our knowledge, neither of these formulations had any shared co-formulants, although not all co-formulants were listed for either formulation.

In both cases, commercial formulations led to altered energy metabolism in multiple tissues in comparison to their respective active ingredients, indicating the ability of co-formulants to alter the metabolic profile of the digestive tract. Lipid metabolism proteins were significantly decreased in the digestive tract and brain of Roundup Optima+® exposed bees. Further, lipid metabolism associated proteins were

significantly decreased in the digestive tract of Proline® exposed bees, which were not found in groups exposed to the relevant active ingredient from these formulations.

In the brain, Roundup Optima+® altered rhodopsin signalling, which is important for light sensitivity and processing in the brain. This was not found for glyphosate active ingredient-exposed bees, suggesting that certain co-formulants alone or in combination with glyphosate, such as alkylpolyglycoside or nitroryl listed in this formulation, can alter vital signalling processes in the bumblebee brain. In the fat body proteome, Proline® led to significant decreases in proteins associated with cellular pH regulation, the actin cytoskeleton and endocytosis, indicating significant alterations the fat body physiological processes.

Another interesting finding was that, in the digestive tract microbiota, both formulations led to significant alterations to fungal community composition and significant decreases in the relevant abundance of *Candida* fungi in the digestive tract. This was not found for either of the active ingredients investigated throughout this thesis, suggesting the *Candida* may be sensitive to co-formulants present in both formulations. Whilst it is unknown if these formulations had the same or similar co-formulants due to incomplete listing of the full formulation profile, those listed suggest that these formulations contain different formulations. However, perhaps *Candida* are sensitive to chemicals with the same action as those present in these formulations e.g., to surfactants.

Overall, we found significant alterations in the digestive tract microbiota and all tissues investigated after formulation exposure which were not observed from groups exposed to the relevant active ingredient alone. Future research should determine whether impacts are observed based on the presence of commonly used co-formulants alone to guide further policy on stricter co-formulant regulatory assessments and, if necessary, restrictions. Further, given the abundance of studies testing the impacts of pesticide formulations on non-target organisms (Rocha *et al.*, 2015; A.G. Pereira *et al.*, 2018; Cullen *et al.*, 2019; Zhao *et al.*, 2020), researchers should be slow to attribute negative impacts found in non-target organisms exposed to formulations on pesticidal ingredients as co-formulants may alter or compound pesticide impacts, as demonstrated throughout this thesis. Finally, these findings, along with recent studies determining negative impacts of co-formulants (Mesnage and Antoniou, 2018; Straw

and Brown, 2021; Straw *et al.*, 2022), give further weight to a need for full disclosure of co-formulants present in commercial formulations, which would allow researchers to fully assess the impacts of pesticide formulations on organisms likely to be exposed in the environment.

6.2.7 Biomarkers of Pesticide Exposure

Throughout this thesis, I found that similar proteins or processes were significantly altered in one or more tissues exposed to either prothioconazole and glyphosate which can provide candidates for biomarkers of pesticide exposure and xenobiotic stress.

In the digestive tract, glucosylceramidase had an increased abundance after exposure to glyphosate active ingredient, Roundup Optima+®, and prothioconazole active ingredient, making it a potential biomarker for pesticide exposure. In addition, fibrillin had the greatest decrease in abundance whilst glucosylceramidase and laccase had the greatest increases in abundance, along with an increased abundance of SOD, after exposure to either glyphosate or Roundup Optima+® in the digestive tract. Further, glyphosate led to a decreased abundance of mitochondrial proteins in the digestive tract and fat body, regardless of exposure to the active ingredient or Roundup Optima+®. This suggests that mitochondrial proteins, fibrillin, glucosylceramidase, SOD, and laccase could make up a biomarker panel for identifying glyphosate exposure in bees.

In addition, a range of antioxidants could be used for detecting pesticide exposure. Whilst antioxidant proteins were increased in a number of tissues after exposure to either glyphosate or prothioconazole, the exact proteins were often different, or similar proteins had differential alterations. For example, superoxide dismutase had an increased abundance in the digestive tract after exposure to both glyphosate-based treatments but was only increased in the fat body after exposure to Roundup Optima+®. Further, peroxiredoxin and cytochrome P450 were increased in the fat body after exposure to prothioconazole active ingredient, but not proline®. Further collagen proteins were differentially altered based on pesticide exposure. Collagen-5 (IV) was increased in the digestive tract after prothioconazole active ingredient exposure, and collagen-5 (IV) and collagen-1 (IV) were decreased in the digestive tract after exposure to both glyphosate-based treatments. These results indicate that

testing for alterations to a range of antioxidants and collagen proteins may give insights into xenobiotic-induced stress and exposure but may not be useful for identifying which pesticides are present.

Despite a major increase in translational proteins in the brain after Proline® exposure, mediator of RNA polymerase II transcription subunit 26 had the greatest decrease in abundance in comparison to the control treatment group. Interestingly, this protein had the highest abundance in the brain proteome of glyphosate-exposed bees, making alterations to this protein a potential biomarker of pesticide exposure. Zhao et al. (2020) found an increase in subunit two of mediator of RNA polymerase II transcription in transcriptomics of whole honeybees exposed to a different glyphosate formulation for five days, further supporting the use of subunits of mediator of RNA polymerase II transcription as a biomarker of pesticide exposure.

Additionally, multiple structural proteins could be considered as biomarkers of pesticide induced alterations to brain functioning and neurotransmission. After prothioconazole exposure, we found major decreases in various structural proteins including troponin subunits, myosin, and tropomyosin. Interestingly, these proteins were also decreased in the *B. terrestris* brain proteome after acute clothianidin exposure, which has known negative impacts on neuronal transmission (Guapo de Melo, 2020), pushing these proteins forward as proteins of interest for further study as potential biomarkers of pesticide-induced alterations to brain physiology.

6.3 Towards an Adverse Outcome Pathway

The research presented in this thesis uncovered the effects of glyphosate and prothioconazole, and a representative formulation for each, on the digestive tract, fat body and brain proteome. In addition, these pesticides were investigated for impacts on the survival, behaviour, sucrose solution consumption, and the digestive tract microbiota. In each chapter, I used an AOP framework to produce an outline of known impacts of glyphosate and prothioconazole on *Bombus terrestris*. The AOP framework permits the translation of molecular level findings to predicted potential toxicity based on alterations to biological pathways and different organisational levels can be assembled to effectively interpret and communicate possible negative outcomes of pesticide exposure through the evaluation of causal relationships at various endpoints (Ankley and Edwards, 2018).

Here, for the first time I outline an AOP for glyphosate and prothioconazole on *B. terrestris*, from the molecular to the organism level, and outline knowledge gaps (Figure 6-1; Figure 6-2). Since glyphosate and prothioconazole are not designed to target bees, we are unsure of macro-molecular interactions which may trigger down-stream impacts on physiological pathways. However, an increase in antioxidant proteins in multiple tissues are exposure to both pesticides suggests that pesticide-induced oxidative stress and consequential damage to proteins, lipids, or DNA is a possibility. We also found that neither glyphosate nor prothioconazole had impacts on survival or sucrose solution consumption at field-realistic levels. However, whilst glyphosate did not alter behaviour, prothioconazole did at 0.3 ppm in the ten-day exposure assays only, despite up to 300 ppm exposures in the same assays, suggesting a non-monotonic dose-response relationship for prothioconazole which can guide future experiments and hypotheses. Further, we found that both pesticides altered the digestive tract, fat body and brain proteome, and digestive tract microbiota, which could have lethal consequences when combined with other environmental stressors (Sgolastra *et al.*, 2017; Al Naggar *et al.*, 2022). Further investigations on macro-molecular interactions, organism, and population responses are warranted to produce a complete AOP; however, this data serves as a guide for probable impacts and future experimental hypotheses.

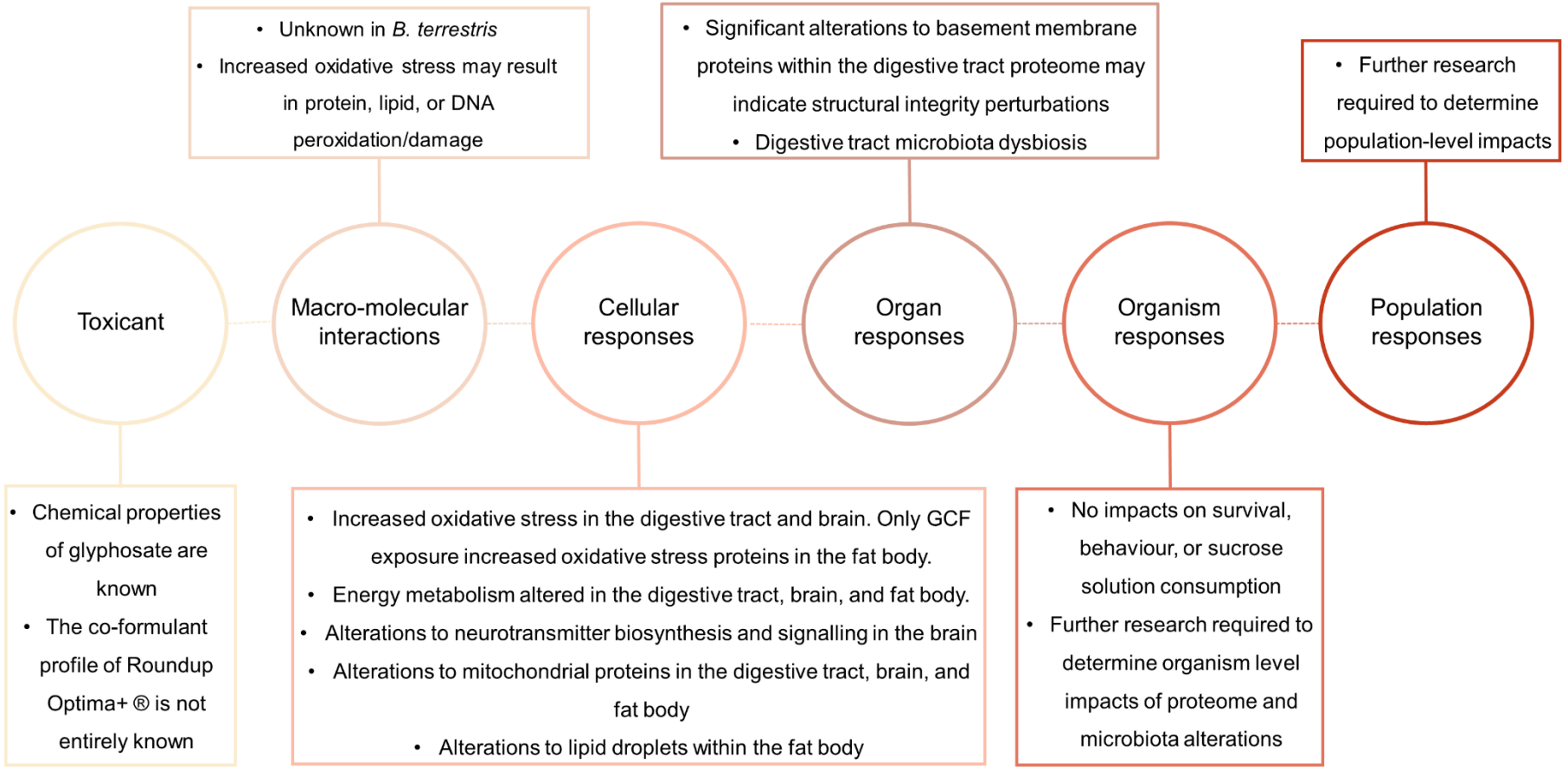


Figure 6-1 Adverse Outcome Pathway for Glyphosate Exposure in *B. terrestris*. An AOP was filled with some of the key findings from this thesis on the impact of chronic oral glyphosate exposure, at a low concentration, on *B. terrestris* (After Ankley *et al.*, 2010).

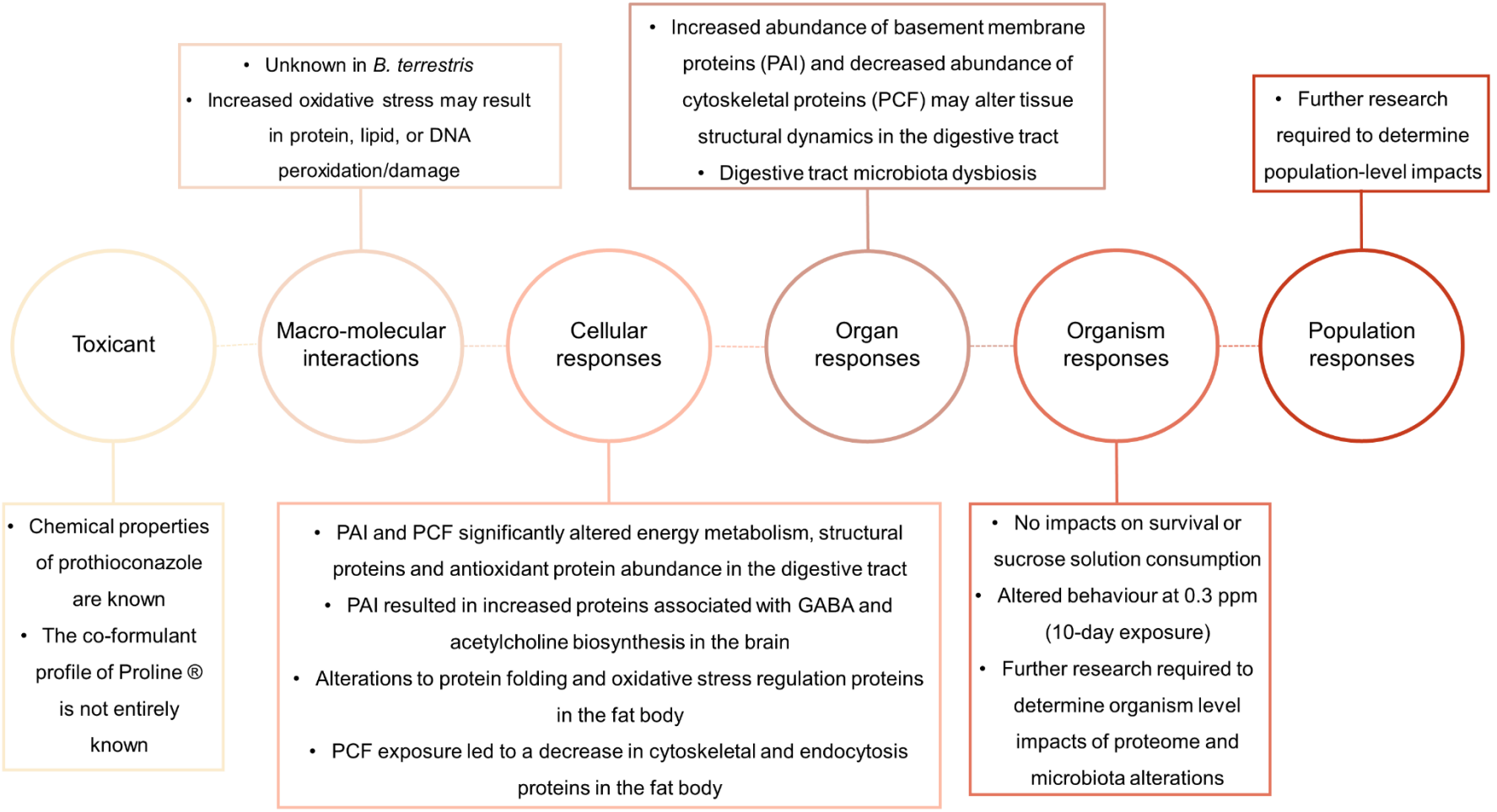


Figure 6-2 Adverse Outcome Pathway for Prothioconazole Exposure in *B. terrestris*. An AOP was filled with some of the key findings from this thesis on the impact of chronic oral prothioconazole exposure, at a low concentration, on *B. terrestris* (After Ankley et al. (2010)). PAI = prothioconazole active ingredient; PCF = prothioconazole commercial formulation.

6.4 Limitations

Whilst the results presented throughout this thesis provide much needed baseline information on the impacts of glyphosate and prothioconazole – including representative formulations for each – there were some limitations which should be acknowledged and addressed in the future.

One of the major limitations to this research is a lack of peer-reviewed research reporting on glyphosate and prothioconazole residues in pollen and nectar. We chose to expose bees to 1 ppm glyphosate for both glyphosate-based treatments based on a study by Thompson et al. (2014). This study used a worse-case scenario field-realistic spray regime and assessed concentrations in nectar and pollen collected by foraging bees. The mean glyphosate residue in nectar from honeybee hives was 0.99 mg/kg 7 days after application, whilst nectar samples taken from forager bees at various time points after application ranged from 2.78 to 31.3 mg/kg. Further, Cebotari et al. (2018) found glyphosate residues of 1 ppm in untreated flowers in a forestry setting. Therefore, we regard the exposure concentration of 1 ppm used in this study to be on par with or below probable field-realistic residues based on the current literature available. In addition, we chose a concentration of 0.3 ppm prothioconazole based on Raimets et al. (2020) who found up to 0.55 ppm, but a median concentration of 0.29 ppm, prothioconazole residues in pollen collected by honeybees in Estonia. To my knowledge, there have been no studies that quantify prothioconazole in nectar.

We also acknowledge that this study compared the glyphosate and prothioconazole active ingredient to a single glyphosate and prothioconazole-based commercial formulation. Whilst the commercial formulations chosen are widely used, there are many formulations available for use in agricultural and non-agricultural settings which contain a multitude of varying inert ingredients. However, one of our initial concerns was the use of glyphosate or prothioconazole active ingredient to determine harmful effects of exposure, when commercial formulations are applied in most settings. This study has demonstrated that glyphosate and prothioconazole can have differential effects depending on its use alone or as part of a commercial formulation. As a result, in future studies, the use of pesticides as the active ingredient alone or as part of a

commercial formulation in exposure experiments needs to be considered to acknowledge and determine effects of inert ingredients to avoid attributing all observed effects to the active ingredient alone. Further, a lack of disclosure of co-formulants presented on formulation packaging and SDS documents made it difficult to determine formulation make-up. For example, in Roundup Optima+® over 70% of co-formulants were disclosed as 'minor ingredients' and other than N-N-Dimethyldecanamide, no other co-formulants are listed for Proline®. However, based on the results of this thesis, where possible, investigation of individual co-formulants alone and combined with pesticidal active ingredients are required.

One of the biggest dissatisfying elements of this research was the requirement to use an organic solvent for prothioconazole solubility. Whilst the use of acetone was a necessity for prothioconazole active ingredient solubility, its presence limited the ability for direct comparison of prothioconazole active ingredient and Proline®. This made it difficult to find comparisons between prothioconazole-based treatments and, consequently, a lack of common proteins in the tissues investigated after exposure to either prothioconazole-based treatment could not be contributed to either the co-formulants in Proline® or acetone in prothioconazole active ingredient. We considered i) altering the protein formulation to contain 0.3% acetone, allowing all treatments to be compared to a single 0.3% acetone control. However, considering our lack of information around the co-formulants present in Proline®, we did not want to alter the formulation any further. Further, whilst we could consider prothioconazole active ingredient compared against 0.3% acetone control as taking account of acetone exposure, these compounds may not act in an additive way. Based on the impact of acetone found on all three tissues investigated, this may have compounded or altered the impact of prothioconazole. Acetone has been found to increase lipid peroxidation and disrupt phospholipid membranes increasing membrane fluidity (Stadler *et al.*, 2008; Posokhov and Kyrychenko, 2013). Prothioconazole-induced alterations to physiological pathways may then be altered based on the presence of acetone. However, the presence of co-formulants in prothioconazole formulations used in the field are used to solubilise prothioconazole also, making the identification of co-formulant alterations to important pathways of concern for research in the immediate future.

Finally, we are unaware how likely bees are to be exposed directly to co-formulants via pollen and nectar. A recent study by Balmer et al. (2021) found that specific co-formulants are detectable in fruit and vegetables, with varying half-lives depending on the co-formulant. However, we are currently unaware if co-formulants are present in pollen and nectar of plants, making it impossible to predict a realistic pesticide formulation exposure in a lab setting.

6.5 Recommendations for Mitigation Strategies and Future Research

This project was exploratory and discovery in nature as we set out to determine the potential impacts of non-insecticidal pesticides in bumblebees which were previously poorly characterised (Cullen *et al.*, 2019). The findings presented in this thesis provide new insights into the impacts of these pesticides on crucial biological processes in *B. terrestris* at field-realistic concentrations. However, there are still many unanswered questions about how these findings relate to the natural world or how they could interact with other stressors. Our discovery-based approach has resulted in clear directions for future researchers and further questions that need swift attention. Furthermore, limitations led to multiple concerns over EC regulatory assessments and policy-maker decisions which need urgent action. The following policy actions and research topics are therefore recommended:

1. EC regulatory assessments on the impact of pesticides are largely focused on pesticidal active ingredient impacts on honeybee mortality. It is recommended that the EC implement guidelines suggested by EFSA (European Food Safety Authority, 2013) to include a wider range of bee species and exposure routes. Further, assessment of individual co-formulants alone and in combination with the active ingredient it will be combined with in market formulations are needed to determine additive or synergistic impacts on bees. Additionally, further sub-lethal testing is required. As it stands, if no mortality is indicated, then sub-lethal impacts are not assessed.
2. Further, a major overhaul of guidelines and laws around the disclosure of co-formulants present in pesticide formulations is needed. As it stands, only ingredients with a hazard to human health are required to be listed, leading to non-disclosure of other co-formulants which are considered proprietary information. Since formulations are how pesticides are applied in real world scenarios, this makes determining the impact of pesticide exposure on bees difficult. Access to the full list of co-formulants used in marketed formulations would elevate researchers' abilities to accurately predict formulation mixes likely to have negative impacts, and those likely to have negligible impacts, on

bees, leading to evidence-based strategies for sustainable pesticide use and mitigation of bee decline.

3. Further research investigating the concentration of herbicides, fungicides, and co-formulants in pollen and nectar of treated and untreated bee-attractive plants is urgently required to design exposure experiments using well-informed and plausible concentrations and exposure routes.
4. Research on the impacts of pesticide formulations and active ingredients using field and semi-field studies are required to determine the impacts of glyphosate and prothioconazole on bees at the colony and population level. I have provided baseline information on the impact of glyphosate and prothioconazole on key tissues associated with immunity, nutrition, learning, and memory. As a result, key findings from field and semi-field studies on foraging, homing, and pathogen resistance can be compared to molecular level findings for a full understanding of these pesticide's impacts on bumblebees.
5. I found that the commonly used solvent, acetone, had negative impacts on *B. terrestris*, which may compound or alter pesticidal impacts observed. It is suggested that further research focuses on investigating the toxicity and sub-lethal impacts of a number of commonly used non-polar solvents and co-formulants to determine impacts on pollinators and microorganism. Consequently, solvents that are least likely to alter, or that have minimal impacts on, biological processes and survival can be determined. As a result, pesticide research can more confidently focus on elucidating the impact of pesticides alone.
6. Whilst prothioconazole is often applied multiple times per season, making chronic oral exposure the most likely route of exposure, glyphosate may have other routes of exposure which need to be explored. Based on 1 ppm glyphosate found in non-treated plants (Cebotari *et al.*, 2018), chronic oral exposure is likely, and Thompson and colleagues found that plants treated directly with glyphosate can take up to a week to fully perish, and that bees will indiscriminately forage on treated plants (Thompson *et al.*, 2022). However, further research on the biological pathways altered after acute exposure to higher doses of glyphosate and glyphosate-based formulations, based on exposure via recently-treated plants, is also required.

7. For glyphosate, my results indicate sub-lethal impacts via increased oxidative stress, alterations to digestive tract structural integrity, alterations to the mitochondria and energy metabolism, and disruption of key pathways in the brain and fat body. Further research is required on the molecular, cellular, organism, colony, and population level to determine the outcomes of these alterations in *B. terrestris*. Histopathological studies on digestive tract structural integrity, cellular and molecular assays investigating mitochondrial functioning and oxidative stress, organism level studies on pathogen resistance, learning and memory, and nutrition, and colony and population level studies on foraging, homing, and pathogen occurrence in bees are logical next steps for researchers to produce a full AOP to fully understand the impacts of glyphosate on *B. terrestris* and produce science-backed mitigation strategies for safe use.
8. For prothioconazole, I also found alterations to all three tissues investigated, as well as behavioural alterations at the lowest dose assessed. Surprisingly, significant alterations to vital processes in the brain were also observed. One likely impact of these alterations were the behavioural effects found in this research. Active ingredient exposed bees altered behaviour, and Proline® exposed bees had increased moribund incidence, which could be lethal in the wild, leaving bumblebees open to predation or adverse weather events. Research on memory, learning, foraging, light sensitivity and processing, and homing at multiple biological levels will give further insight into the impacts of prothioconazole on *B. terrestris*. Further investigation into structural proteins, immunity, and pathogen resistance after prothioconazole exposure is also warranted, as well as oxidative stress assays.
9. In addition, increased behavioural and moribund incidence after 0.3 ppm prothioconazole exposure, but not to higher doses, indicates a non-monotonic dose-relationship framework for prothioconazole exposure in bees. This needs further investigation and confirmation. Researchers should be cautious when concluding that prothioconazole has no impacts on bees based on experiments using high exposure concentrations.
10. Investigation into the utilisation of biomarkers suggested in section 6.2.7 for rapid detection of pesticide exposure or pesticide-induced stress would enable

effective and efficient pesticide exposure data during pollinator monitoring studies.

11. I found multiple proteins altered by pesticide or acetone exposure in the digestive tract and brain that are associated with the venom glands of bees such as venom dipeptidyl peptidase, melittin, and phospholipase A2. Since most research focuses on the role of these proteins in the venom gland, I could only hypothesise what their function may be in the brain and digestive tract. Further research on the role of proteins previously identified as venom gland proteins would aid in our understanding of how these proteins contribute to molecular processes in key bumblebee tissues.
12. Future research determining glyphosate and prothioconazole presence and concentration in different bumblebee tissues after exposure will aid in determining the likely source of stress from each tissue, e.g. are impacts found in the brain proteome due to direct translocation of pesticides throughout the haemolymph and across the blood brain barrier? or could alterations to the brain proteome be a knock-on effect from alterations to the digestive tract proteome and/or microbiota? This research could also determine the detoxification capacity of *B. terrestris* for these pesticides.
13. Proteome and digestive tract microbiota recovery was beyond the scope of this research. However, it would provide useful information for future mitigation strategies, particularly for pesticides like glyphosate where *B. terrestris* may have acute exposure to higher doses.
14. Finally, research shows that bees are likely to encounter multiple stressors such as pesticide cocktails, pathogens, and a lack of food (Goulson *et al.*, 2015; Herrera Lopez *et al.*, 2016; Al Naggar *et al.*, 2022). Further, different bee species may have different sensitivities to pesticides and differ in their ability to buffer negative impacts based on colony size, foraging distance, and life history traits (Arena and Sgolastra, 2014a; Schmolke *et al.*, 2021). Research on the impact of glyphosate and prothioconazole on different bee species at multiple biological organisation levels and in combination with other likely stressors are required.

6.6 Conclusion

This research investigated and consequently characterised the impact of glyphosate and prothioconazole on the proteome of key tissues, and the digestive tract microbiota, of *B. terrestris* at field realistic concentrations. I confirmed that these pesticides have little direct impact on survival but found significant alterations to key physiological processes which may lead to further decline in the environment when combined with multiple stressors, or if exposure if prolonged. Further, I identified that co-formulants and solvents can alter the impact of pesticides, either alone or via compounding or altering the impact of pesticides on key tissues. This study raised important questions on how we assess the impacts of pesticides on bees, and how complexities within the pesticide formulation market and a lack of research on non-insecticidal pesticide impacts on bees, particularly non-*Apis* species, make pesticide research an increasingly complex task. The findings of this research uncovered the impacts of a widely used herbicide and fungicide on *B. terrestris* and will surely be of interest to pesticide researchers and policy makers. Further research based on the guidance of these findings will lead to a greater understanding of the impact of glyphosate and prothioconazole on bees after exposure to both the active pesticidal ingredient and in combination with co-formulants. With further research expanding on the findings presented throughout this thesis, our understanding of the impacts of these widely used pesticides on bumblebees can be, in all their complexity, evolved.

Chapter 7

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Chapter 8

Appendix

Manuscript S1-1 and all supplemental tables listed in the List of Tables are available at: https://maynoothuniversity-my.sharepoint.com/:f:/g/personal/merissa_cullen_2015_mumail_ie/EjEo7wrRA-pBvbe5nON9d1QBd0_7sSHob1YWQG-Pp_33DA?e=AE66bd

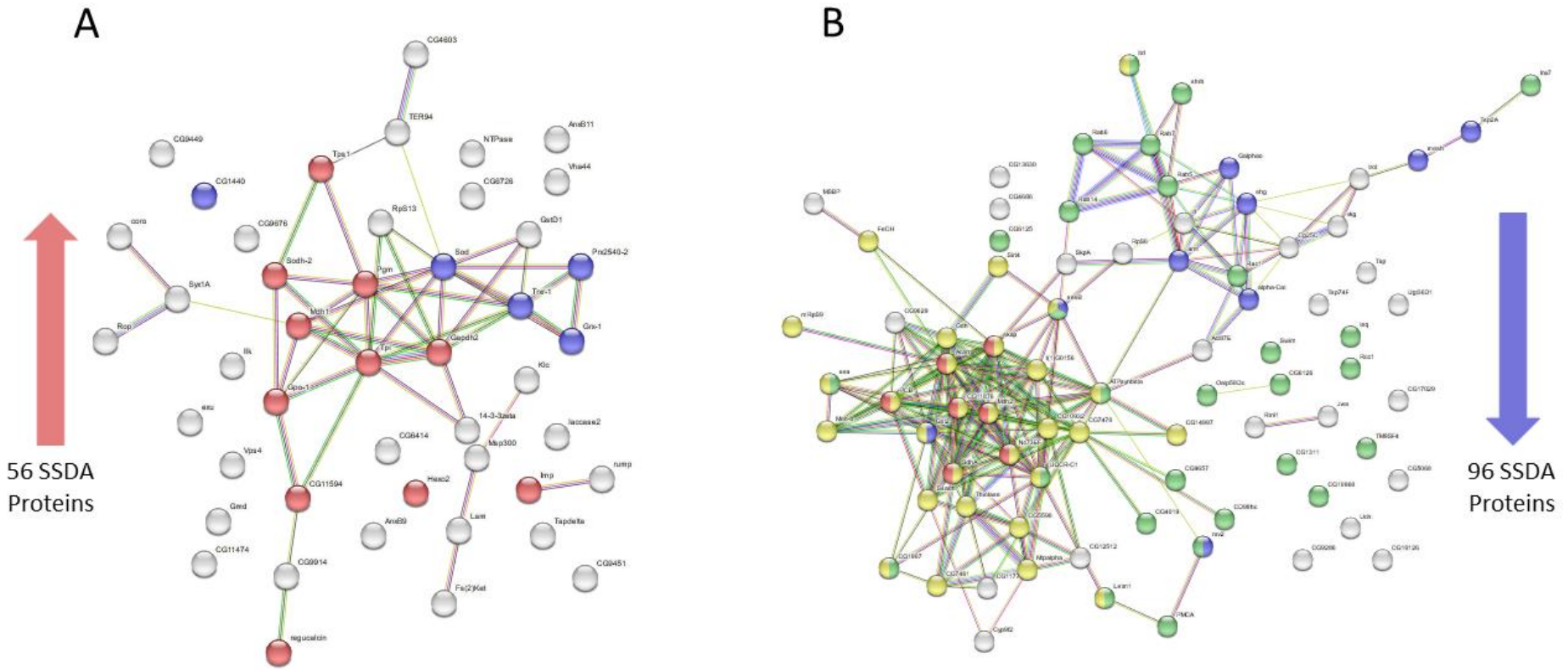

Figure S3-1 Protein-protein Interaction Network for SSDA Proteins Enriched in the Digestive Tract of GAI Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways from proteins with an increased abundance in the digestive tract of GAI compared to control exposed bees included carbohydrate metabolism (red) and response to toxic substance (blue). **(B)** Enriched pathways from proteins with a decreased abundance in GAI compared to the control exposed group included cell junction assembly (blue), transport (green), the cellular component 'mitochondrion' (yellow), and the TCA cycle (red).

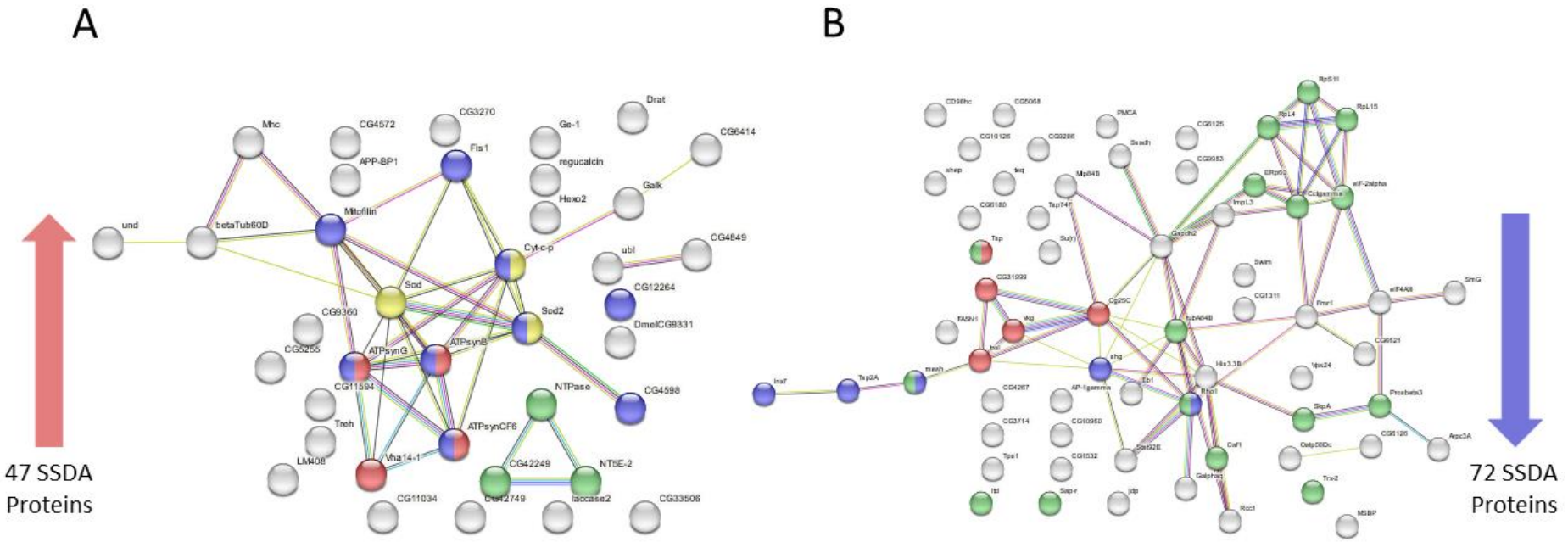

Figure S3-2 Protein-protein Interaction Network for SSDA Proteins Enriched in the Digestive Tract of GCF Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways from proteins with an increased abundance in the digestive tract of GCF compared to the control treatment group included oxidative phosphorylation (red), the cellular component 'mitochondrion' (blue), detoxification of reactive oxygen species (yellow) and pyrimidine metabolism (green). **(B)** Enriched pathways from proteins with a decreased abundance in the digestive tract of GCF compared to the control treatment group included collagen-containing extracellular matrix (red), cell-cell junction (blue) and protein metabolism (green).

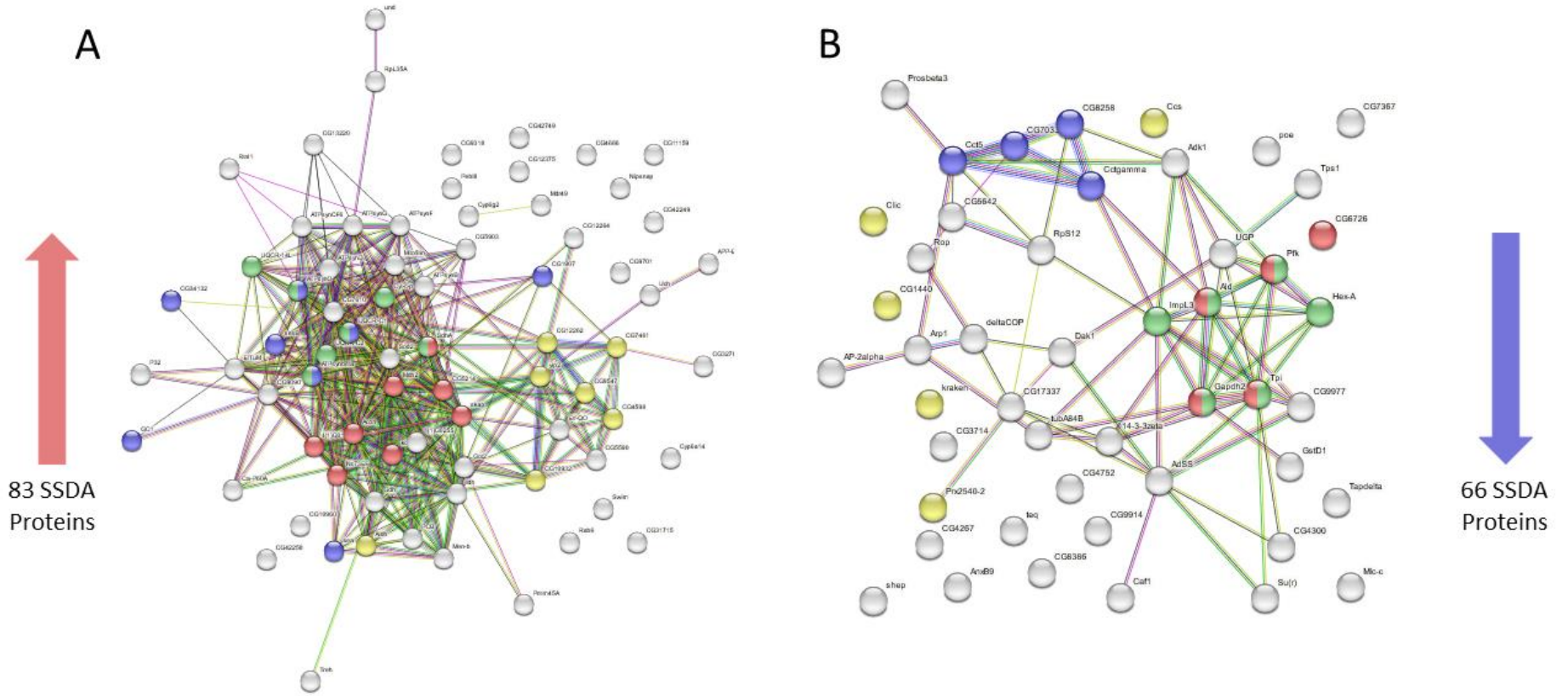

Figure S3-3 Protein-protein Interaction Network for SSDA Proteins Enriched in the Digestive Tract of GCF Compared to the GAI Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways from proteins with an increased abundance in the digestive tracts of GCF compared to GAI treatment group included TCA cycle (red), mitochondrial transport (blue), oxidative phosphorylation (green) and fatty acid degradation (yellow). **(B)** Enriched pathways from proteins with a decreased abundance in the digestive tracts of GCF compared to the GAI treatment group included response to toxic substance (yellow), chaperonin-containing t-complex (blue), glycolysis/gluconeogenesis (green) and amino acid biosynthesis (red).

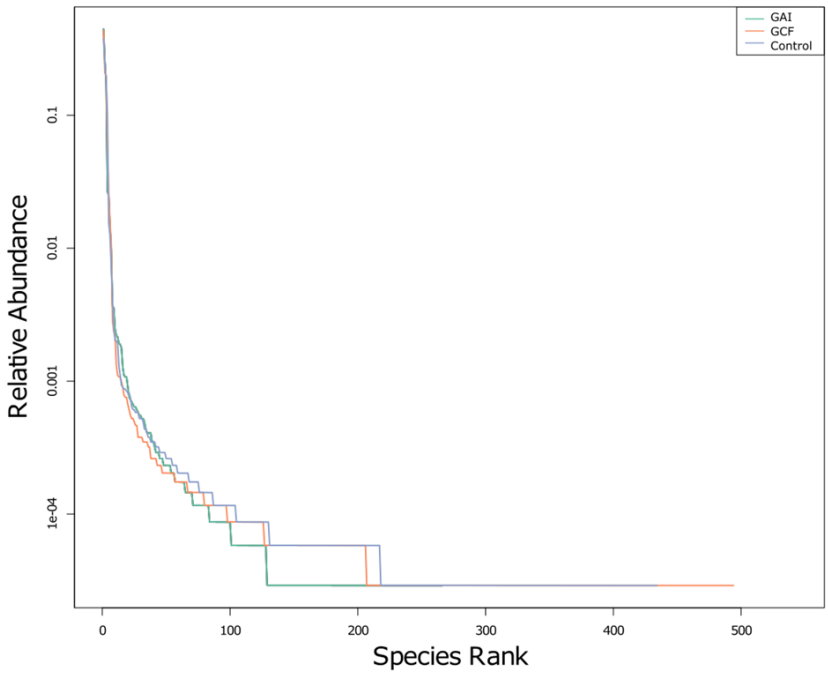

Figure S3-6 A Rank Abundance Curve for Bacterial Taxa in the Digestive Tract of *B. terrestris.* Based on the similar relative abundances (sequences) for the same number of identified OTU's, all treatments display similar bacterial species richness.

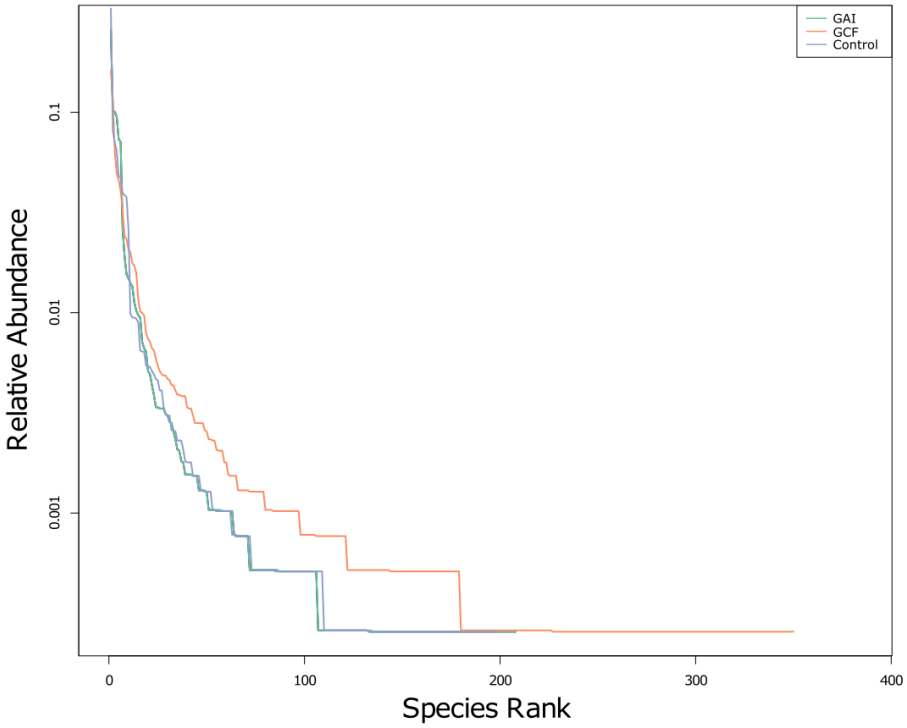

Figure S3-9 A Rank Abundance Curve for Fungal Taxa in the Digestive Tract of *B. terrestris.* The GCF treatment displays slightly higher relative abundance for species rank before plateauing along with other treatment groups before species ranked 200, indicating higher species richness in GCF compared to the other treatment groups.

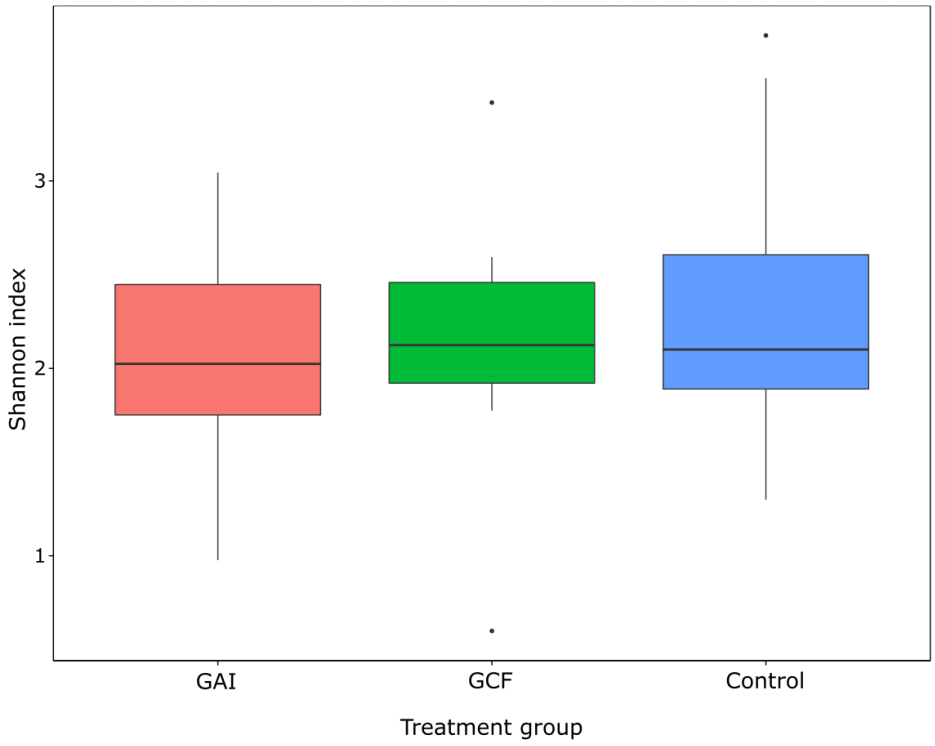

Figure S3-12 Boxplot of Shannon Indices of Bacterial Taxa in Each Treatment Group. Shannon indices were compared between groups. Similar to Chao1, Shannon diversity takes both species richness and evenness into account. There were no significant differences in alpha diversity between groups based on Shannon diversity indices.

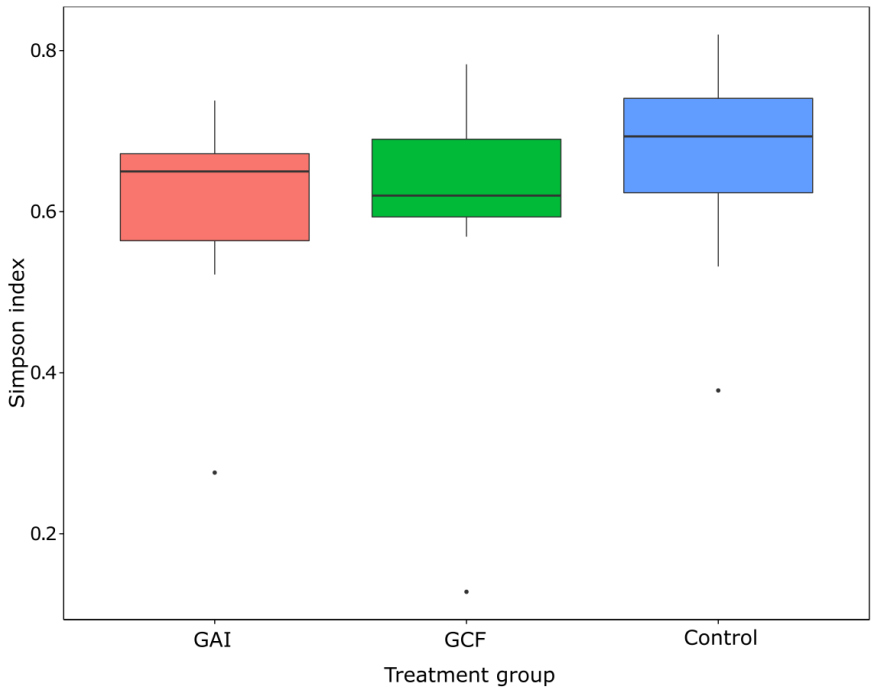

Figure S3-15 Boxplot of Simpson Indices of Bacterial Taxa in Each Treatment Group. Simpson indices were used to determine alpha diversity in addition to Shannon and Chao1 indices. There were no significant differences in alpha diversity based on Simpson indices calculated.

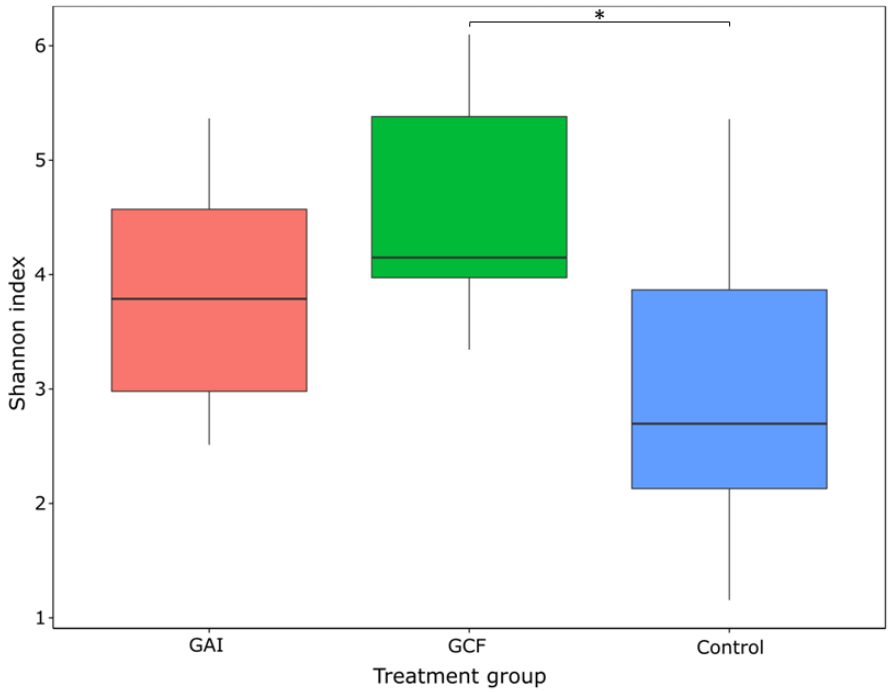

Figure S3-8 Boxplot of Shannon Indices of Fungal Taxa in Each Treatment Group. Shannon indices were compared between treatment groups. Shannon diversity was significantly different between GCF and Control treatment groups (Wilcoxon p = 0.019; Tukey p = 0.04).

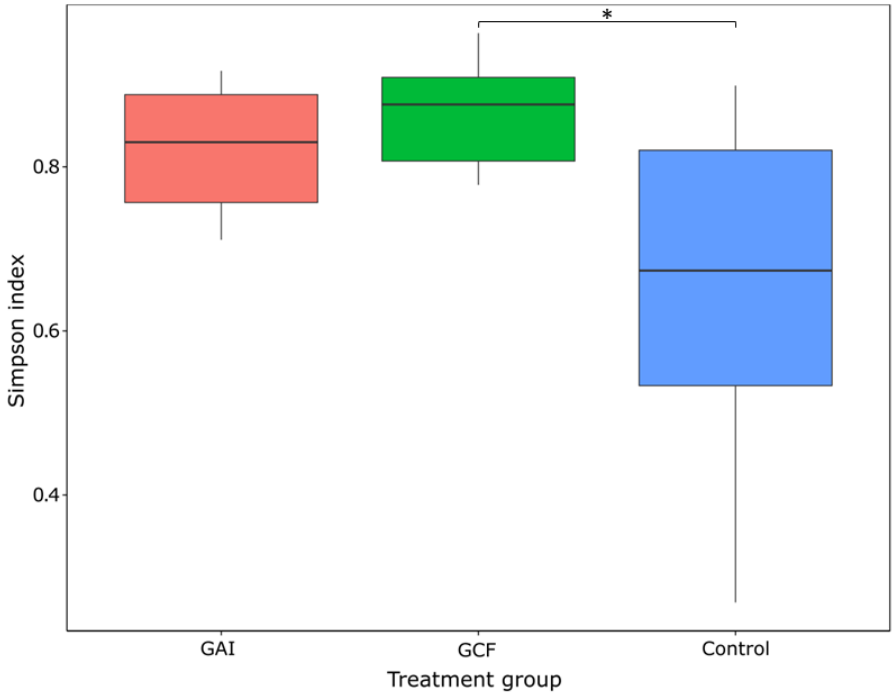

Figure S3-9 Boxplot of Simpson Indices of Fungal Taxa in Each Treatment Group. Simpson indices were used to determine alpha diversity. Simpson indices were significantly different when comparing GCF to control treatment groups (Wilcoxon p = 0.019; Tukey p = 0.022).

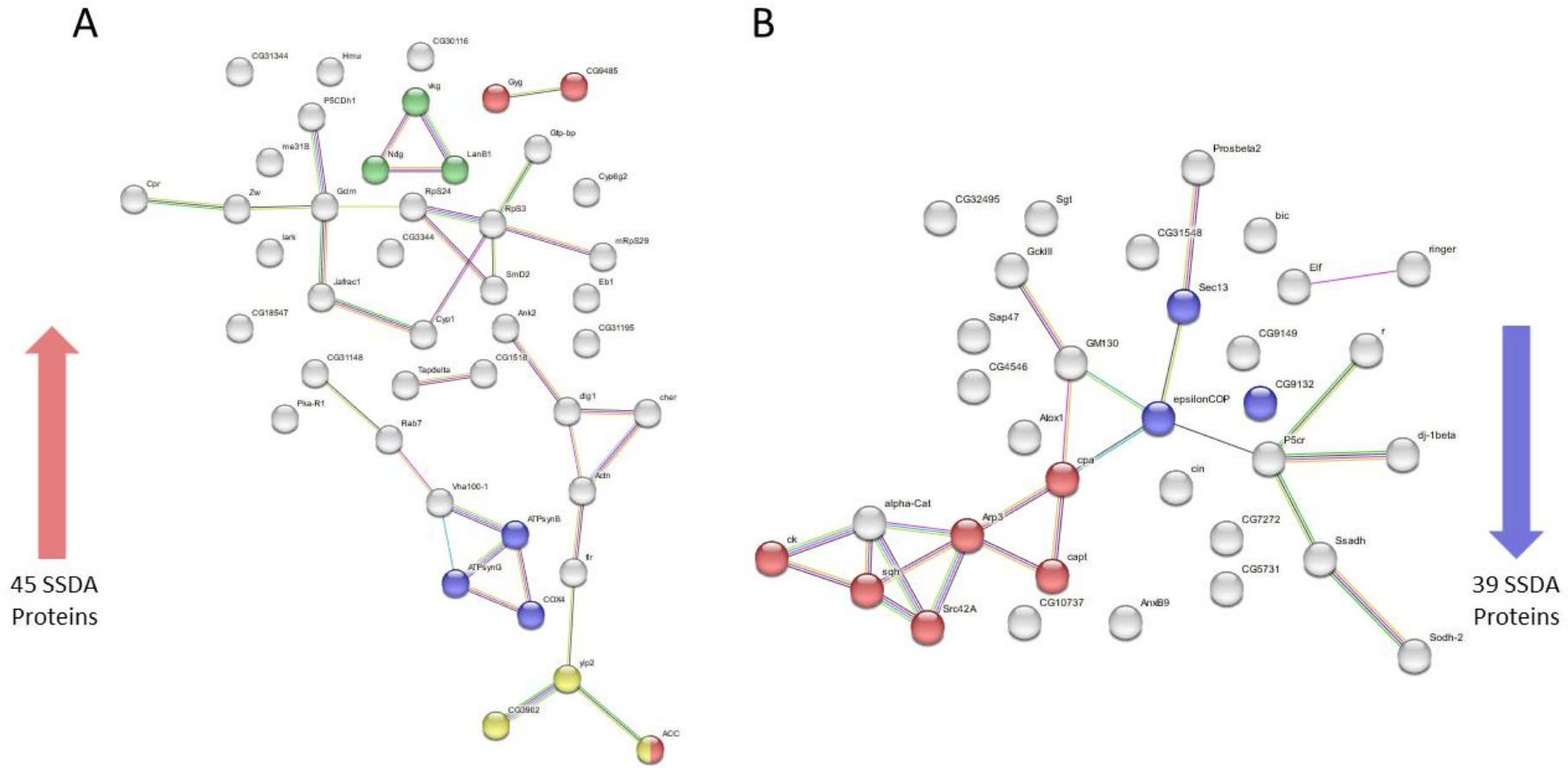

Figure S4-1 Protein-protein Interaction Network for SSDA Proteins Enriched in the Digestive Tract of PAI Compared to the Relative Acetone Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways from proteins with an increased abundance in PAI compared to the acetone control treatment group included basement membrane organization (green), glycogen metabolic process (red), fatty acid metabolism (yellow) and electron transport chain (blue). **(B)** Enriched pathways from proteins with a decreased abundance in PAI compared to the acetone control group included actin cytoskeleton organization (red) and vesicle coat (blue).

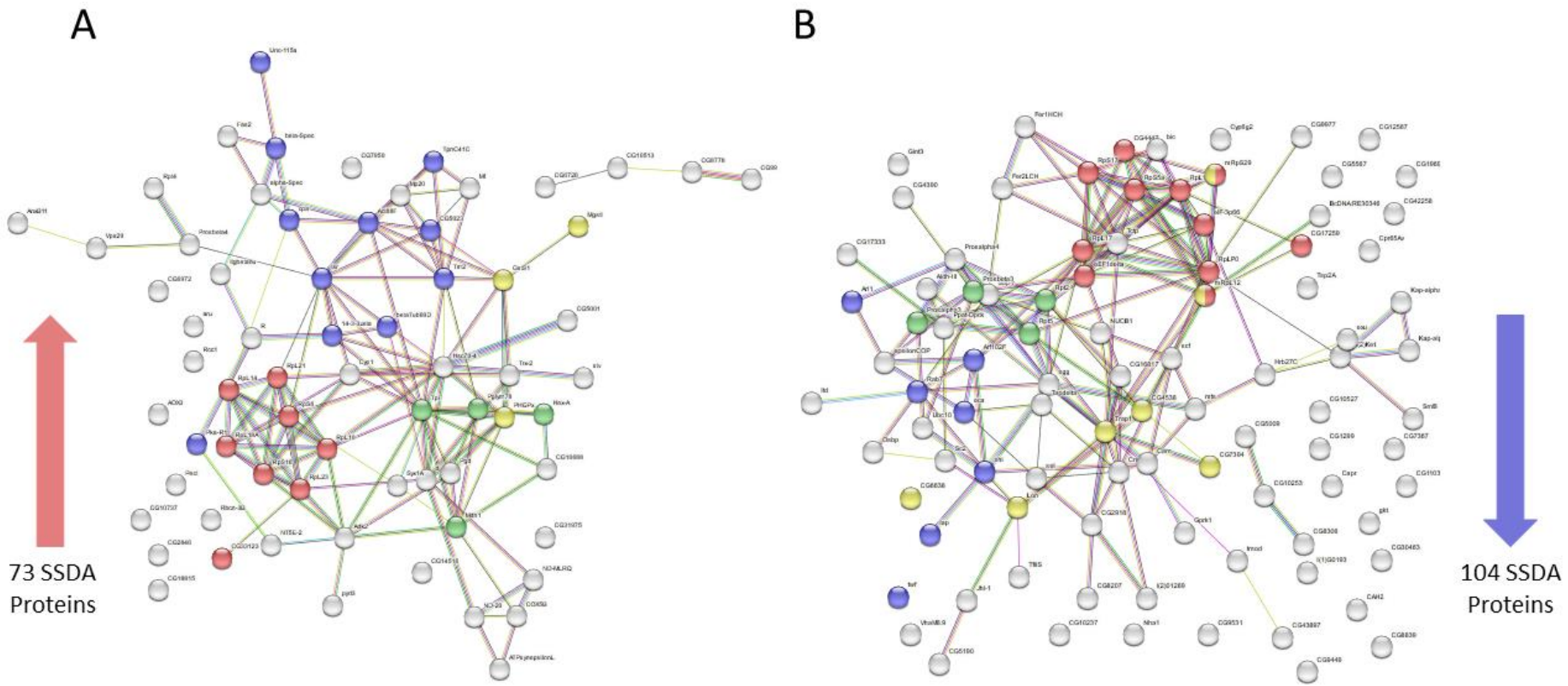

Figure S4-2 Protein- protein Interaction Network for SSDA Proteins Enriched in the Digestive Tract of PCF Compared to the Relative Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways from proteins with an increased abundance in PCF compared to the control treatment group included translation (red), cytoskeleton organization (blue), glutathione peroxidase activity (yellow), and glycolysis and gluconeogenesis (green). **(B)** Enriched pathways and terms from proteins with a decreased abundance in PCF compared to the control treatment group included endocytosis (blue), translation (red), mitochondrial matrix (yellow) and proteasome degradation (green).

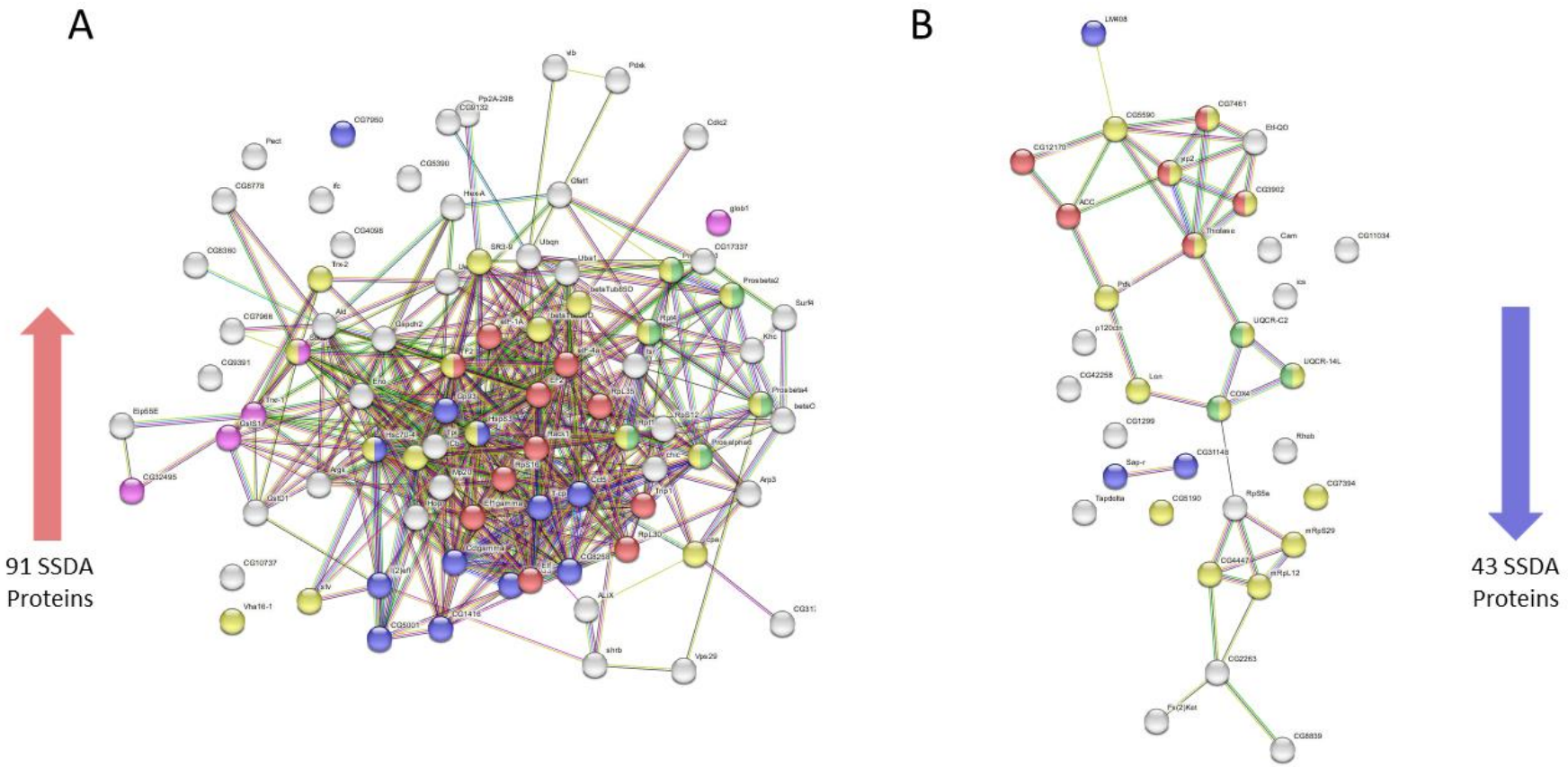

Figure S4-3 Protein-protein Interaction Network for SSDA Proteins Enriched in the Digestive Tract of Acetone Control Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and terms from proteins with an increased abundance in the acetone control compared to the control treatment group included protein folding (blue), translation (red), detoxification (purple), proteasome (green), and cellular response to stress (yellow). **(B)** Enriched pathways and terms from proteins with a decreased abundance in the acetone control compared to the control treatment group included mitochondrion (yellow), fatty acid metabolism (red), lysosome (blue), and electron transport chain (green).

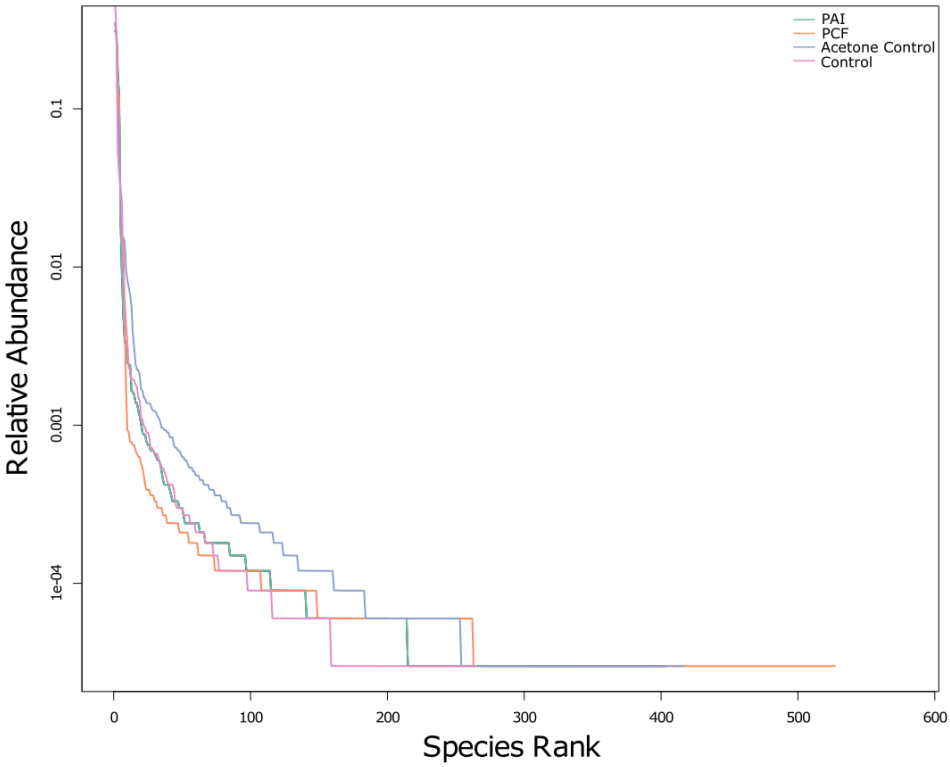

Figure S4-4 A Rank Abundance Curve for Bacterial Taxa in the Digestive Tract of *B. terrestris*. The acetone control had a higher relative abundance of bacterial species compared to other treatment groups.

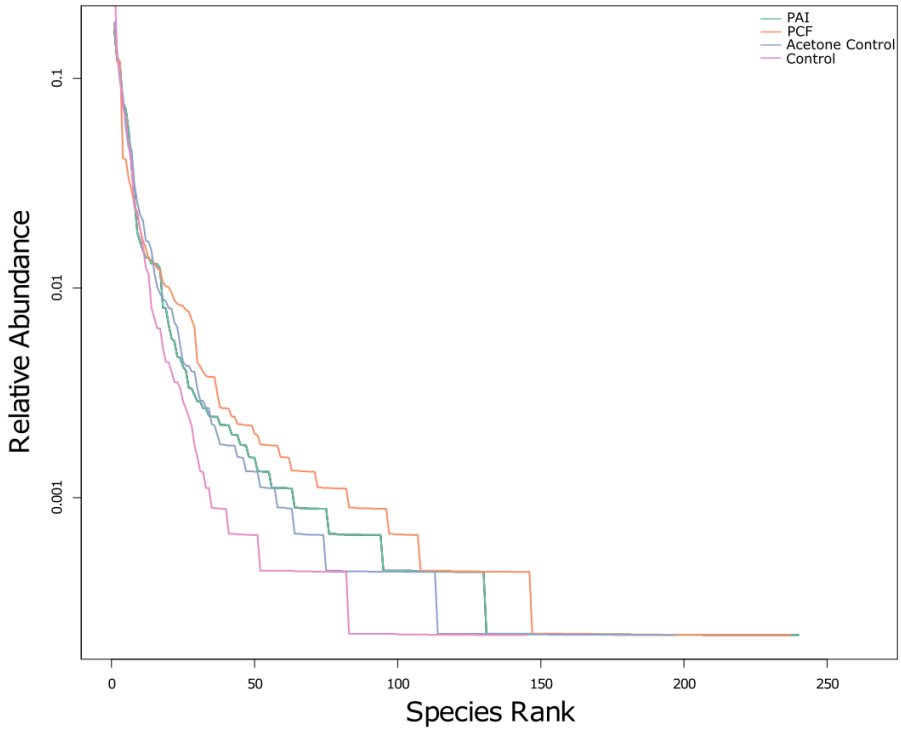

Figure S4-5 A Rank Abundance Curve for Fungal Taxa in the Digestive Tract of *B. terrestris*. The PCF and PAI treatment group had a higher relative abundance of fungal species compared to the control treatment groups, with the control treatment group displaying the lowest relative abundance.

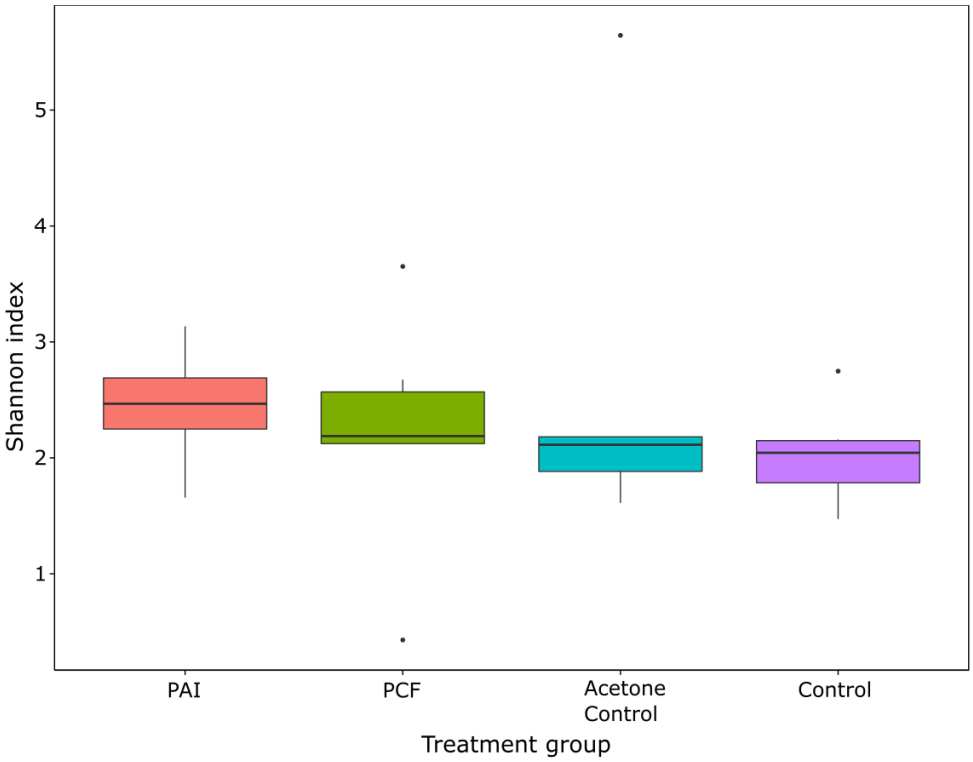

Figure S4-6 Boxplot of Shannon Indices of Bacterial Taxa in Each Treatment Group. Shannon indices were compared between groups to determine bacterial alpha diversity. Tukey and Wilcoxon tests determined no significant differences between treatment groups.

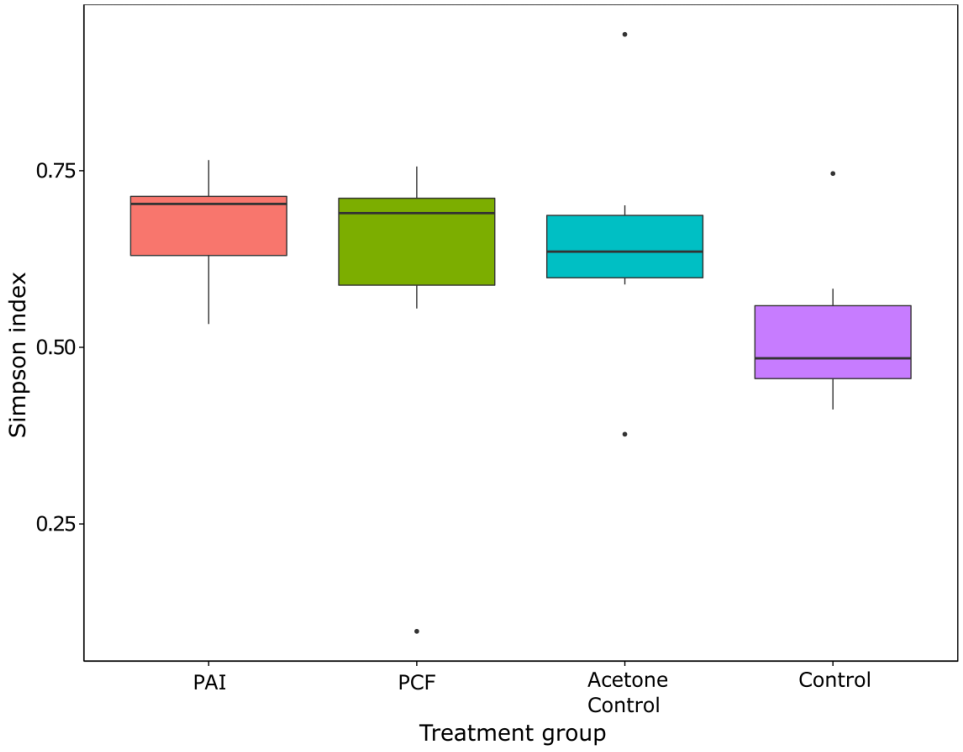

Figure S4-7 Boxplot of Simpson Indices of Bacterial Taxa in Each Treatment Group. Simpson indices were used to determine bacterial alpha diversity. Tukey and Wilcoxon tests determined no significant differences in Simpson alpha diversity between treatment groups.

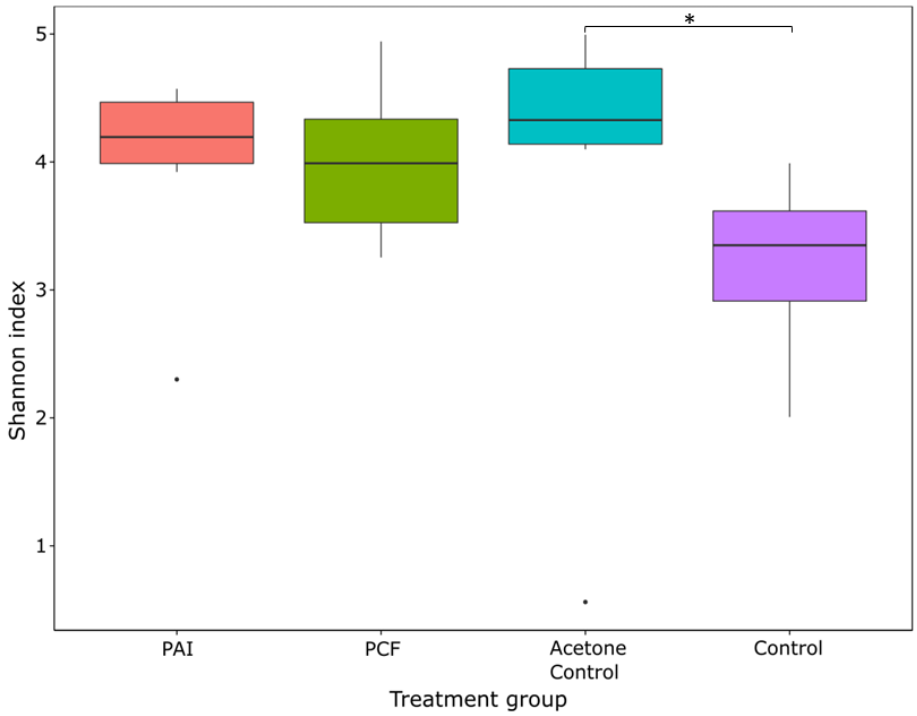

Figure S4-8 Boxplot of Shannon Indices of Fungal Taxa in Each Treatment Group. Shannon indices were used to determine fungal alpha diversity in different treatment groups. Wilcoxon testing determined a significant difference between the acetone control and control treatment groups (p = 0.023).

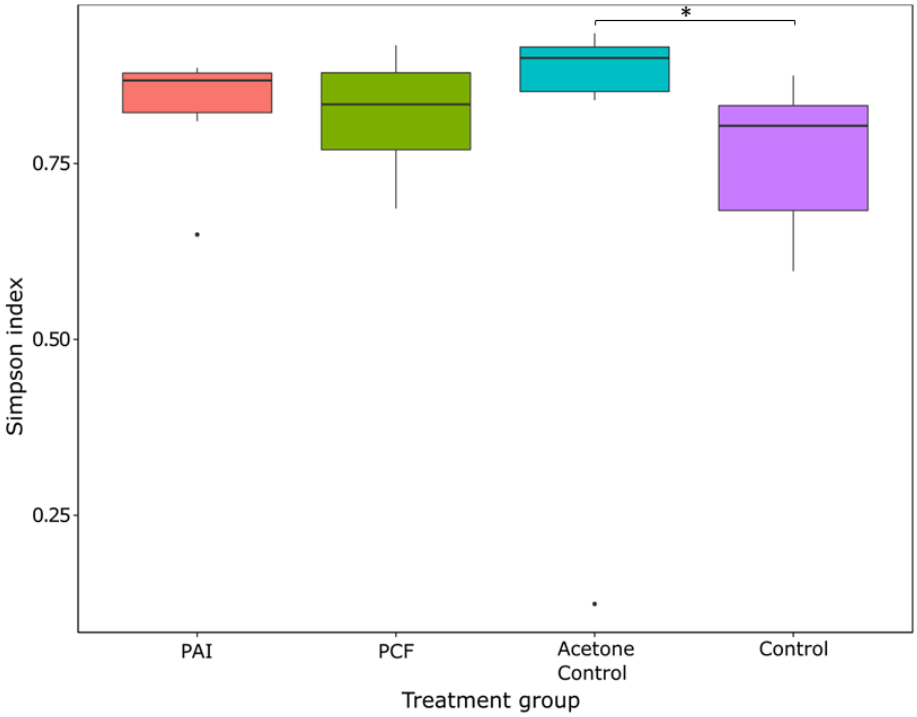

Figure S4-9 Boxplot of Simpson Indices of Fungal Taxa in Each Treatment Group. Simpson indices were used to determine fungal alpha diversity in different treatment groups. Wilcoxon testing determined a significant difference between the acetone control and control treatment groups (p = 0.035).

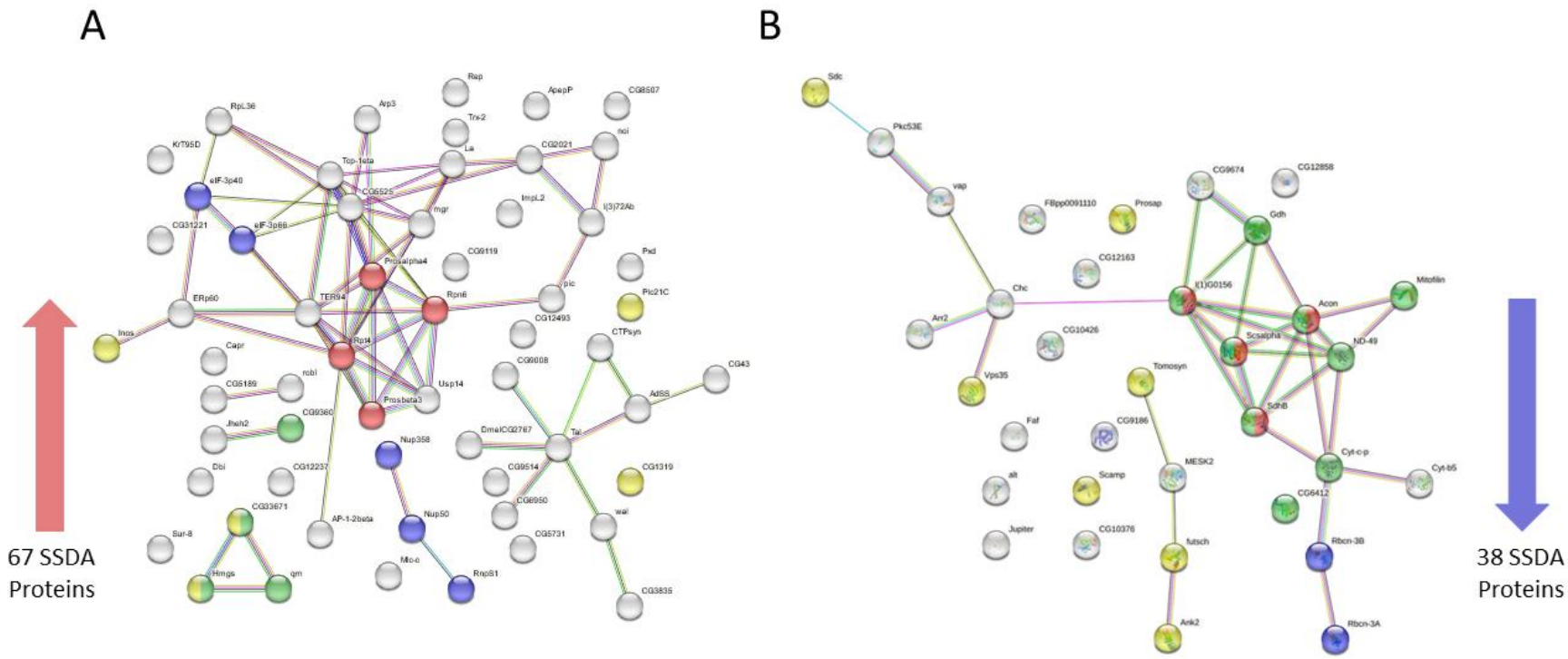

Figure S5-1 Protein-protein Interaction Network for SSDA Proteins Enriched in the Brain of GAI Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in GAI compared to the control treatment group included proteasome (red), RNA transport (blue), terpenoid backbone biosynthesis (blue), and lipid metabolism (yellow). **(B)** Enrichments from proteins with a decreased abundance in GAI compared to the control treatment group were associated with the TCA cycle (red), the synapse (yellow), the mitochondrion (green), and RAVE complex (blue).

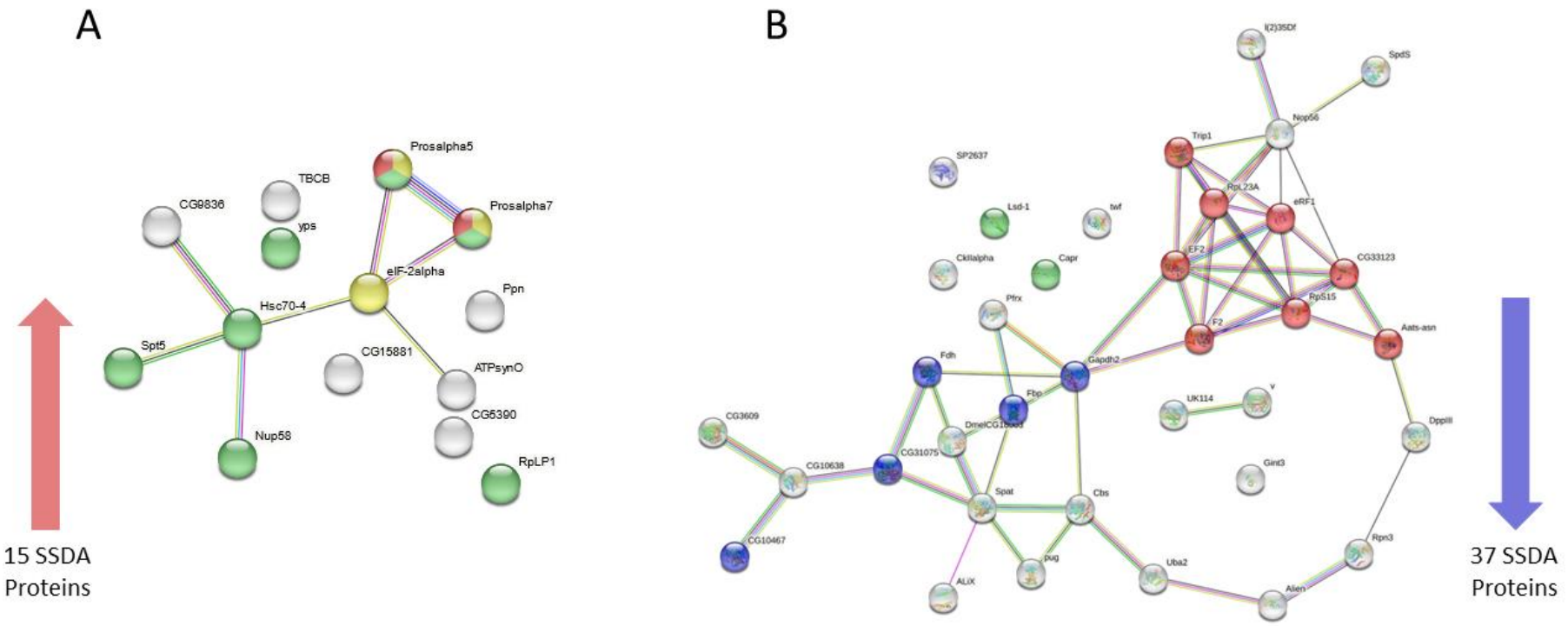

Figure S5-2 Protein-protein Interaction Network for SSDA Proteins Enriched in the Fat Body of GAI Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in GAI compared to the control treatment group included RNA metabolism (green), ABC-family mediated transport (yellow), and proteasome degradation (red). **(B)** Proteins with a decreased abundance in the fatbodies of GAI compared to the control treated group were involved in glycolysis/gluconeogenesis (blue), translation (red), and lipid droplet (green).

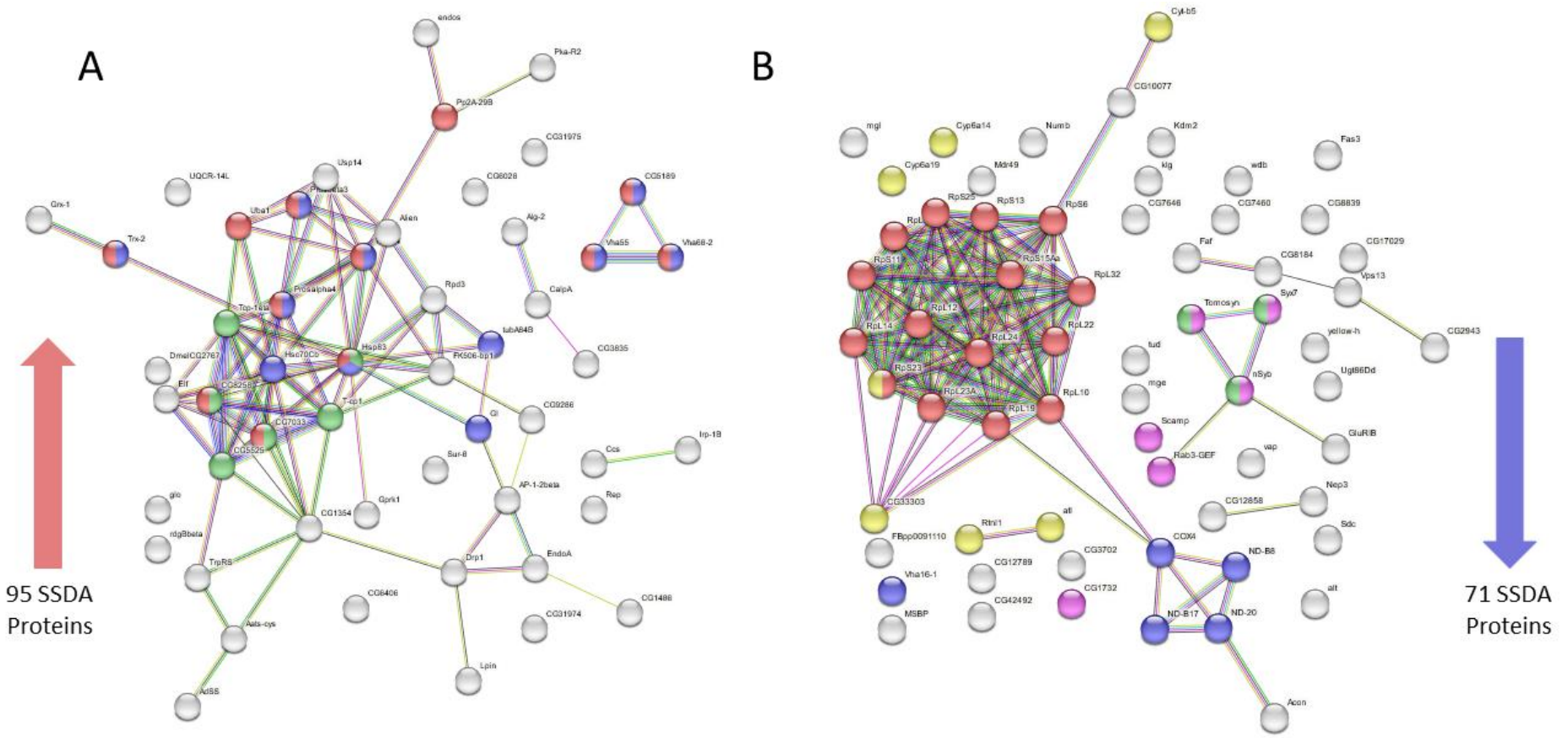

Figure S5-3 Protein-protein Interaction Network for SSDA Proteins Enriched in the Brain of GCF Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in GCF compared to the control treatment group included cellular responses to stress (blue), immune system (red) and unfolded protein binding/protein folding (green). **(B)** SSDA proteins with a decreased abundance in the brain proteome of GCF compared to the control treatment group were associated with the ribosome (red), oxidative phosphorylation (blue), neurotransmitter transport (purple), and endoplasmic reticulum (yellow).

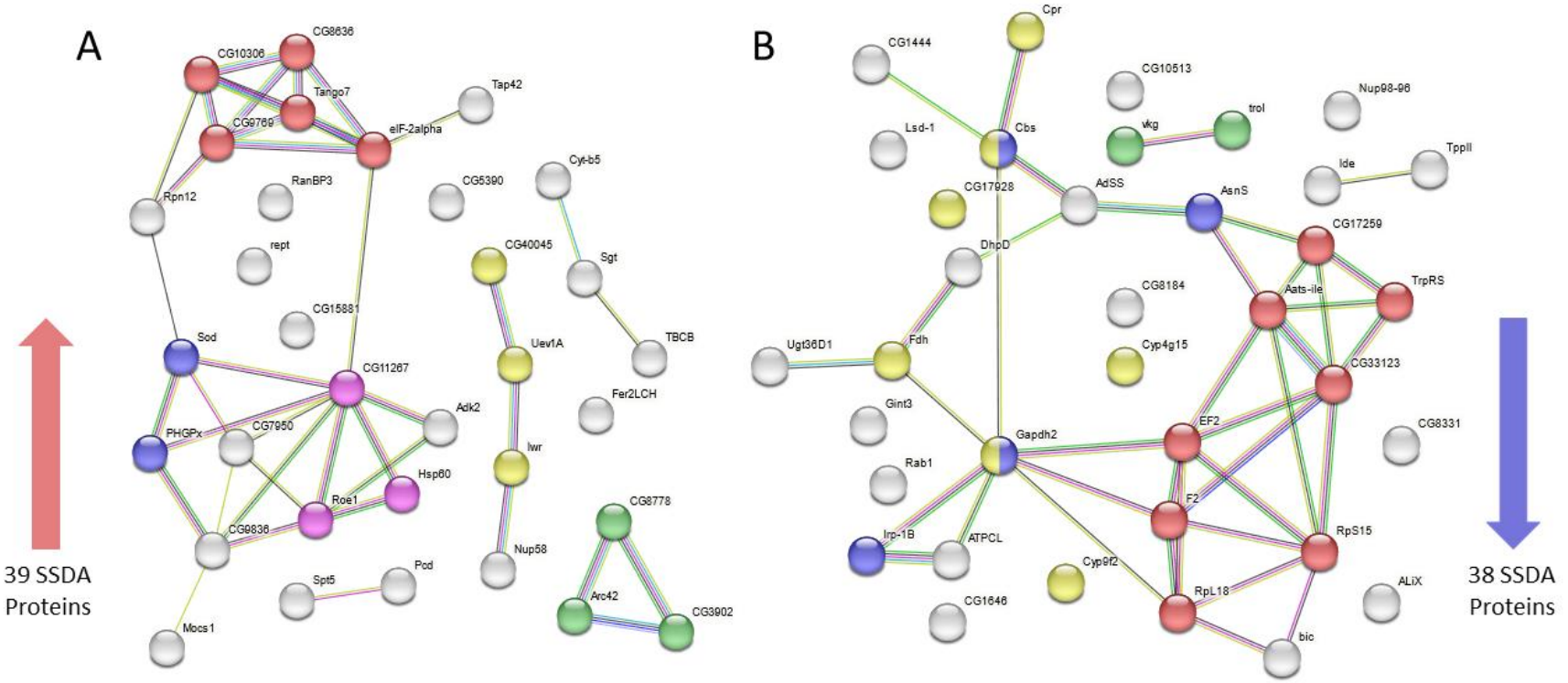

Figure S5-4 Protein-protein Interaction Network for SSDA Proteins Enriched in the Fat Body of GCF Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the fatbody of GCF compared to the control treatment group included translational initiation (red), ubiquitin-like protein conjugating enzyme (yellow), chaperone binding (purple), valine, leucine, and isoleucine degradation (green), and superoxide dismutase complex (blue). **(B)** Enriched pathways and processes from proteins with a decreased abundance in the fatbody of GCF compared to the control treatment group were associated with translation (red), collagen IV, non-collagenous, and G2F domain (green), amino acid biosynthesis (blue), and oxidoreductase (yellow).

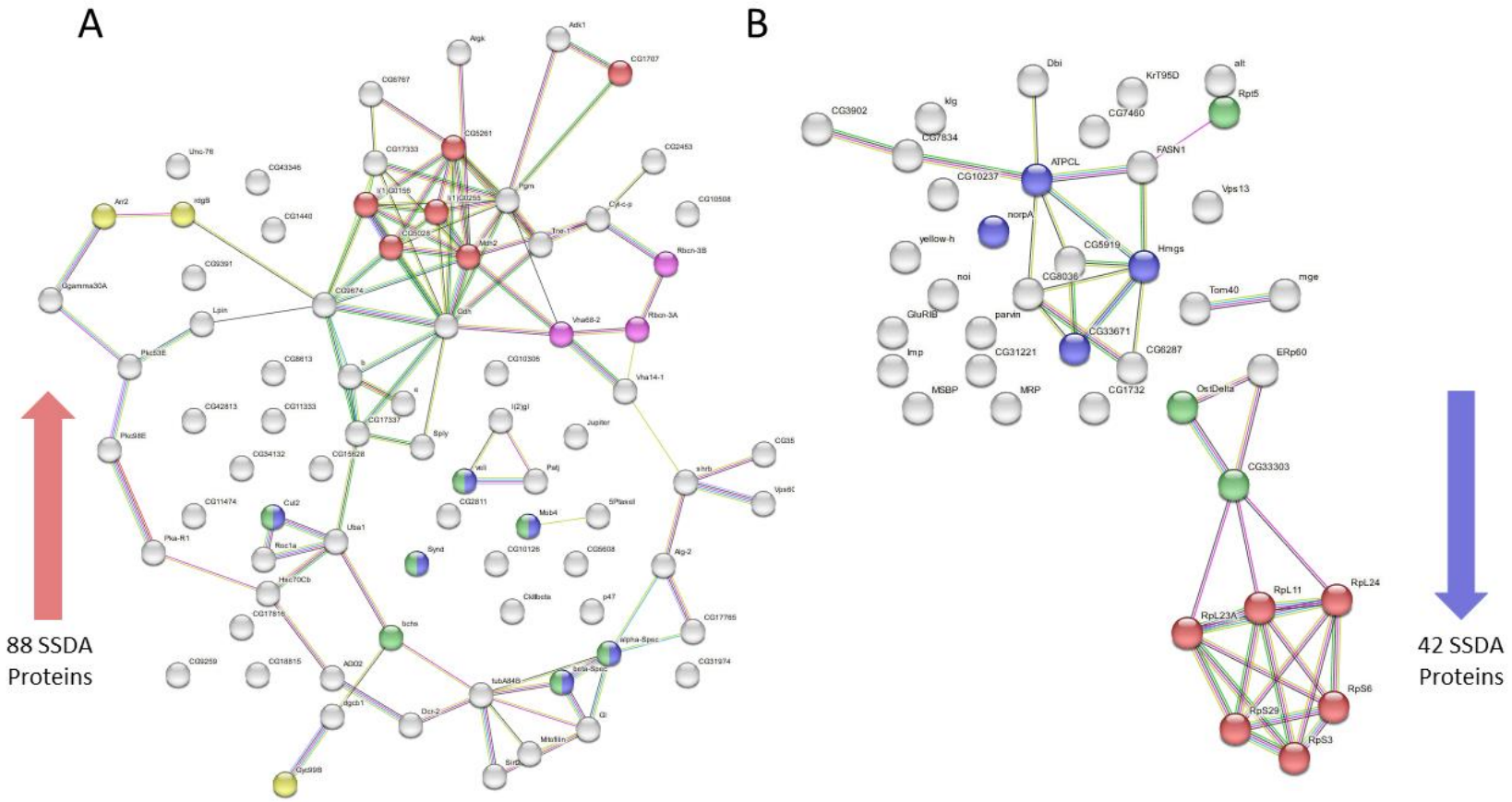

Figure S5-5 Protein-protein Interaction Network for SSDA Proteins Enriched in the Brain of GCF Compared to the GAI Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in GCF compared to the GAI treatment group included pyruvate metabolism and TCA cycle (red), intracellular pH reduction (purple), rhodopsin mediated signaling pathway (yellow), regulation of synapse organisation (green), and regulation of synaptic growth (blue). **(B)** Decreased proteins in GCF compared to GAI treatment groups were associated with the ribosome (red), lipid metabolism (blue), and proteasome degradation (green).

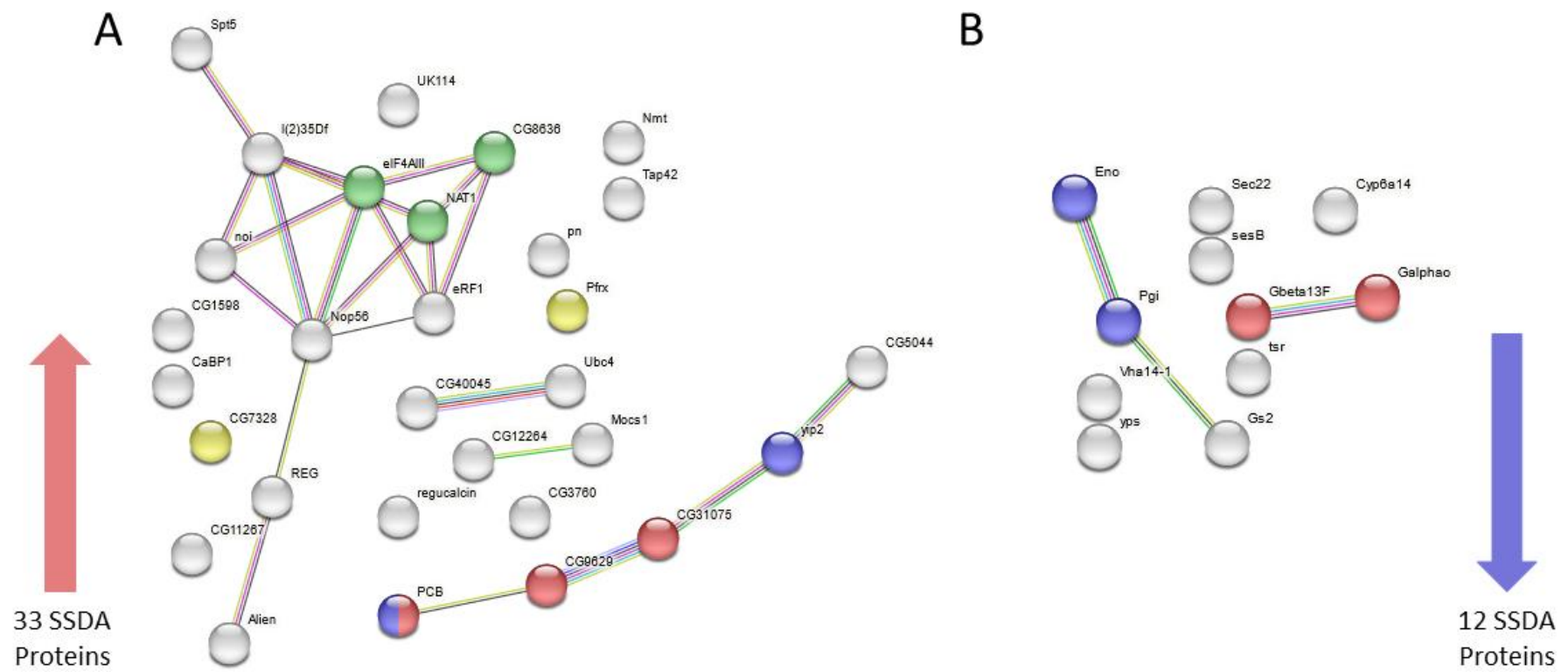

Figure S5-6 Protein-protein Interaction Network for SSDA Proteins Enriched in the Fat Body of GCF Compared to the GAI Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the fatbody of GCF compared to the GAI treatment group included RNA transport (green), pyruvate metabolism (red), fructose and mannose metabolism (yellow), and fatty acid biosynthesis (blue). **(B)** Enriched pathways and processes from proteins with a decreased abundance in the fatbody of GCF compared to the GAI treatment group included prostacyclin signalling through prostacyclin receptor (red) and glycolysis and gluconeogenesis (blue).

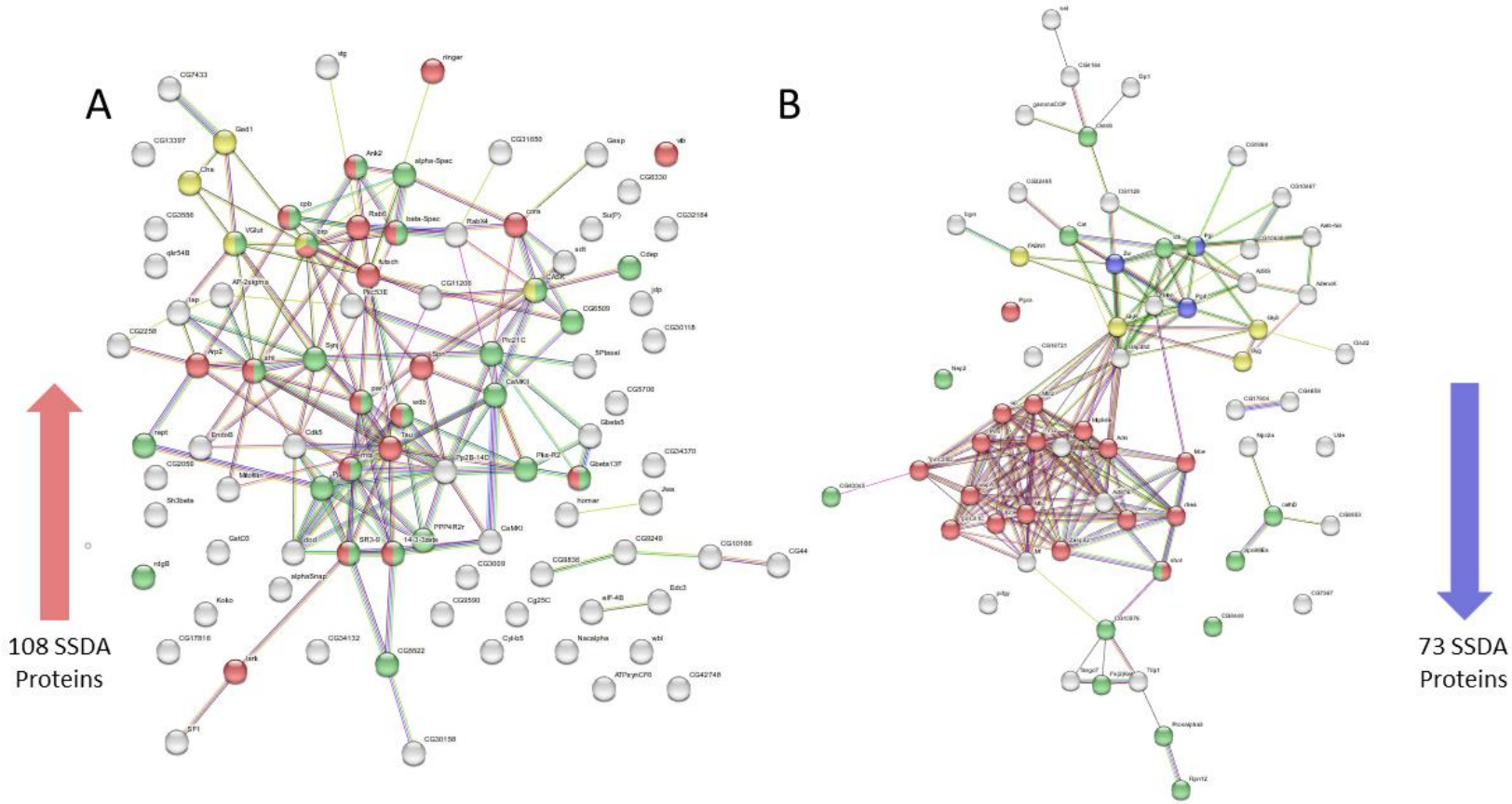

Figure S5-7 Protein-protein Interaction Network for SSDA Proteins Enriched in the Brain of PAI Compared to the Acetone Control Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the brain of PAI compared to the acetone control exposed bees included regulation of cell communication (green), cytoskeleton organisation (red), and dopamine Neurotransmitter Release Cycle, and presynaptic cytoskeleton (yellow). **(B)** Enriched pathways and processes with a decreased abundance in the brain of PAI exposed compared to acetone control exposed bees were associated with cytoskeleton organisation (red), glycogen metabolism (yellow), pentose phosphate pathway (blue), and the immune system (green).

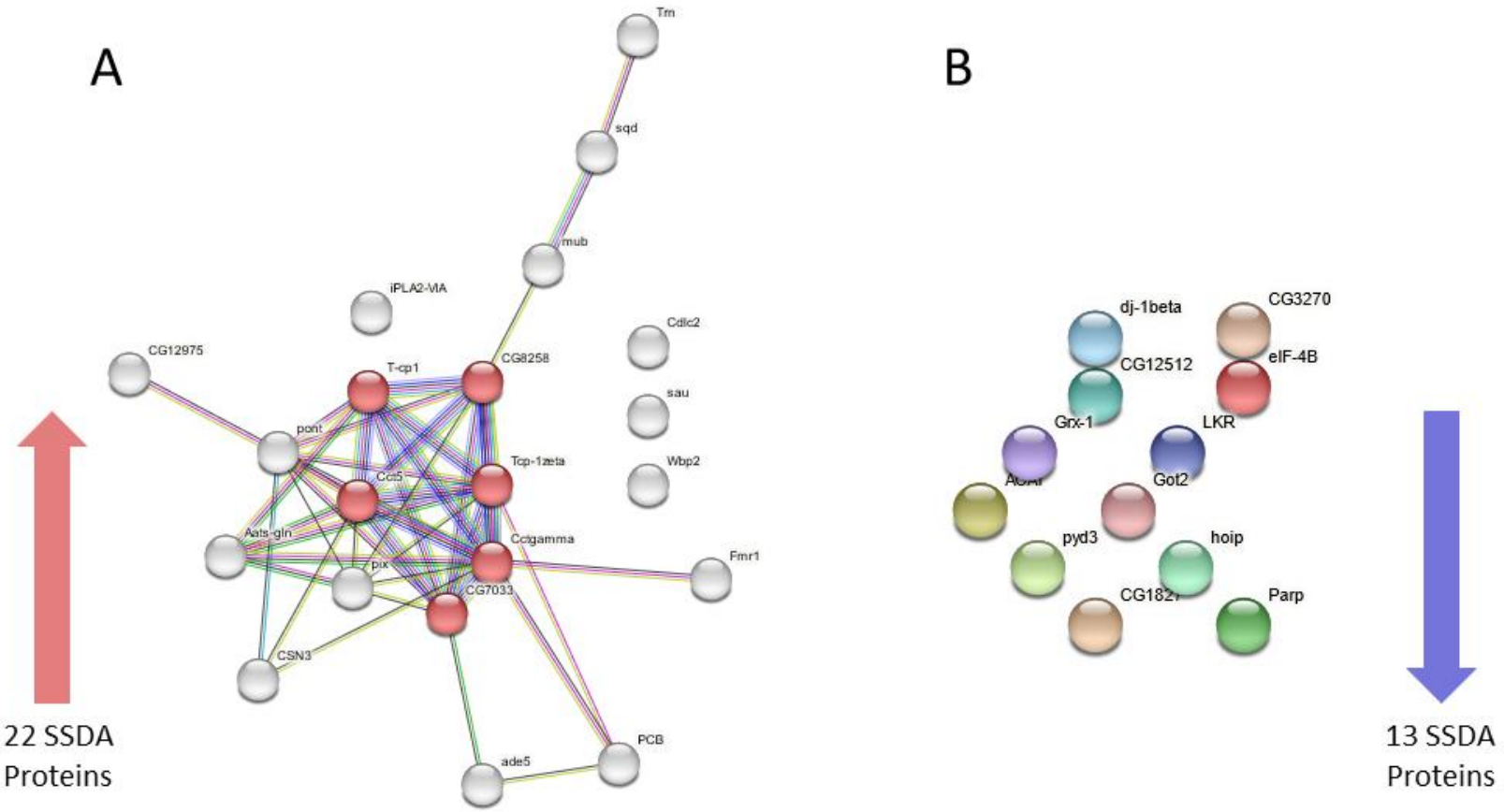

Figure S5-8 Protein-protein Interaction Network for SSDA Proteins Enriched in the Fat Body of PAI Compared to the Acetone Control Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the fatbody of PAI compared to the acetone control group were associated with protein folding (red) and **(B)** there were no enriched processes or pathways for proteins with a decreased abundance in PAI compared to the acetone control treatment group.

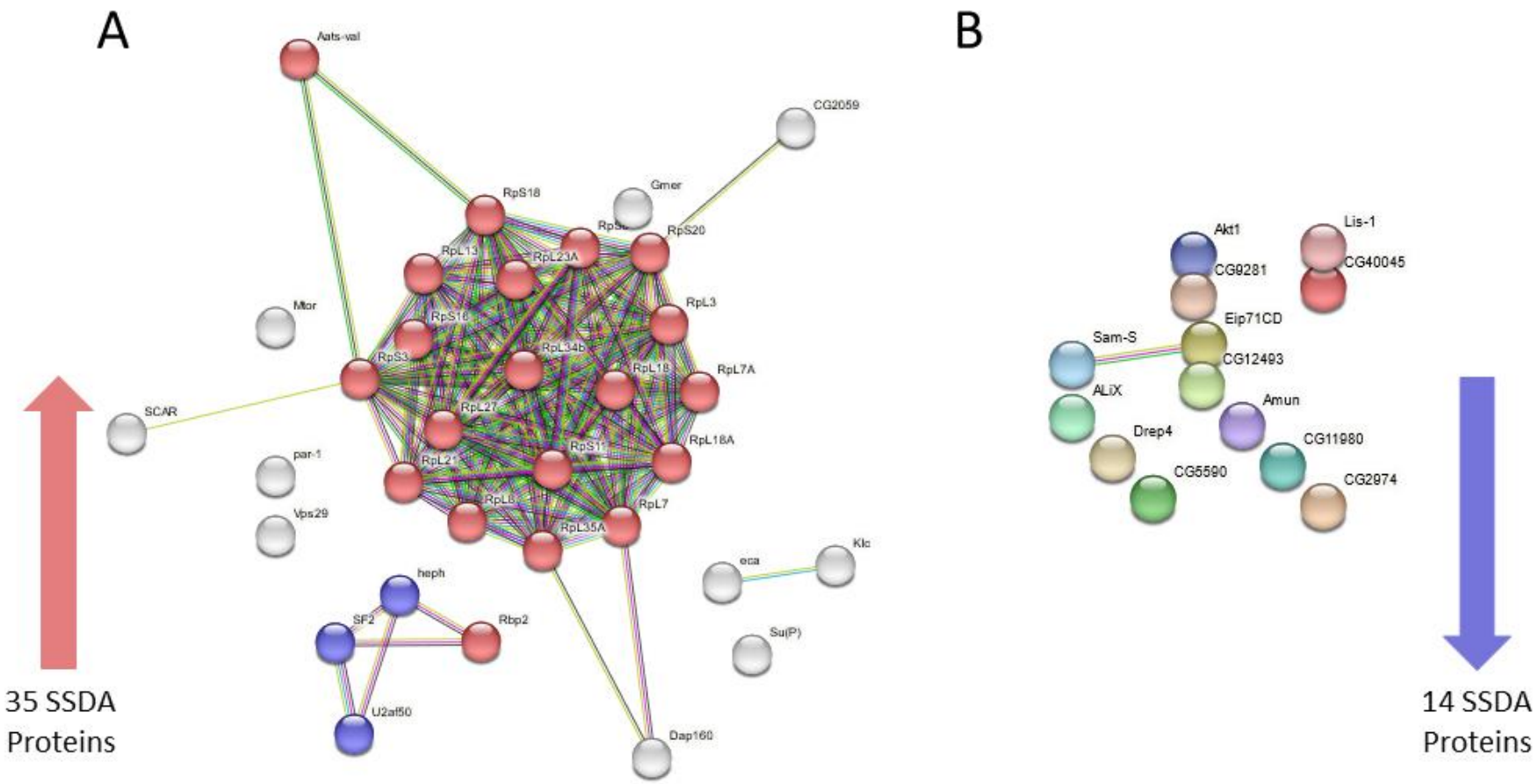

Figure S5-9 Protein-protein Interaction Network for SSDA Proteins Enriched in the Brain of PCF Compared to the Control Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the brain of PCF compared to the control group were associated with translation (red) and mRNA processing (blue). **(B)** there were no significantly enriched pathways or processes from decreased abundance proteins in the brain of PCF exposed bees compared to control exposed bees.

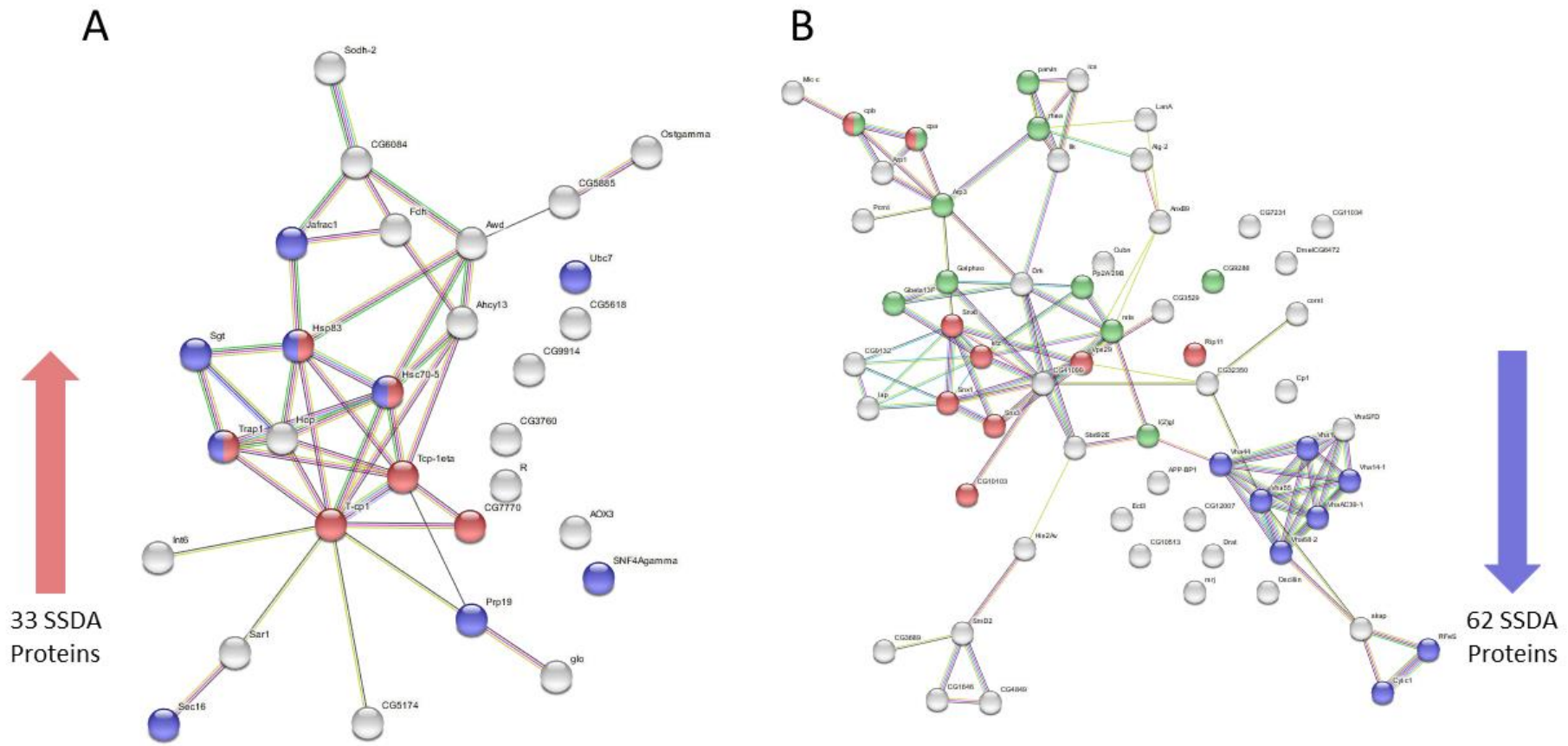

Figure S5-10 Protein-protein Interaction Network for SSDA Proteins Enriched in the Fat Body of PCF Compared to the Control Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the fatbody of PCF compared to the control group were associated with protein folding (red) and cellular response to stress (blue). **(B)** Proteins with a decreased abundance in the fatbody of PCF exposed bees were enriched for cytoskeleton organisation (green), endocytosis (red), and oxidative phosphorylation (blue).

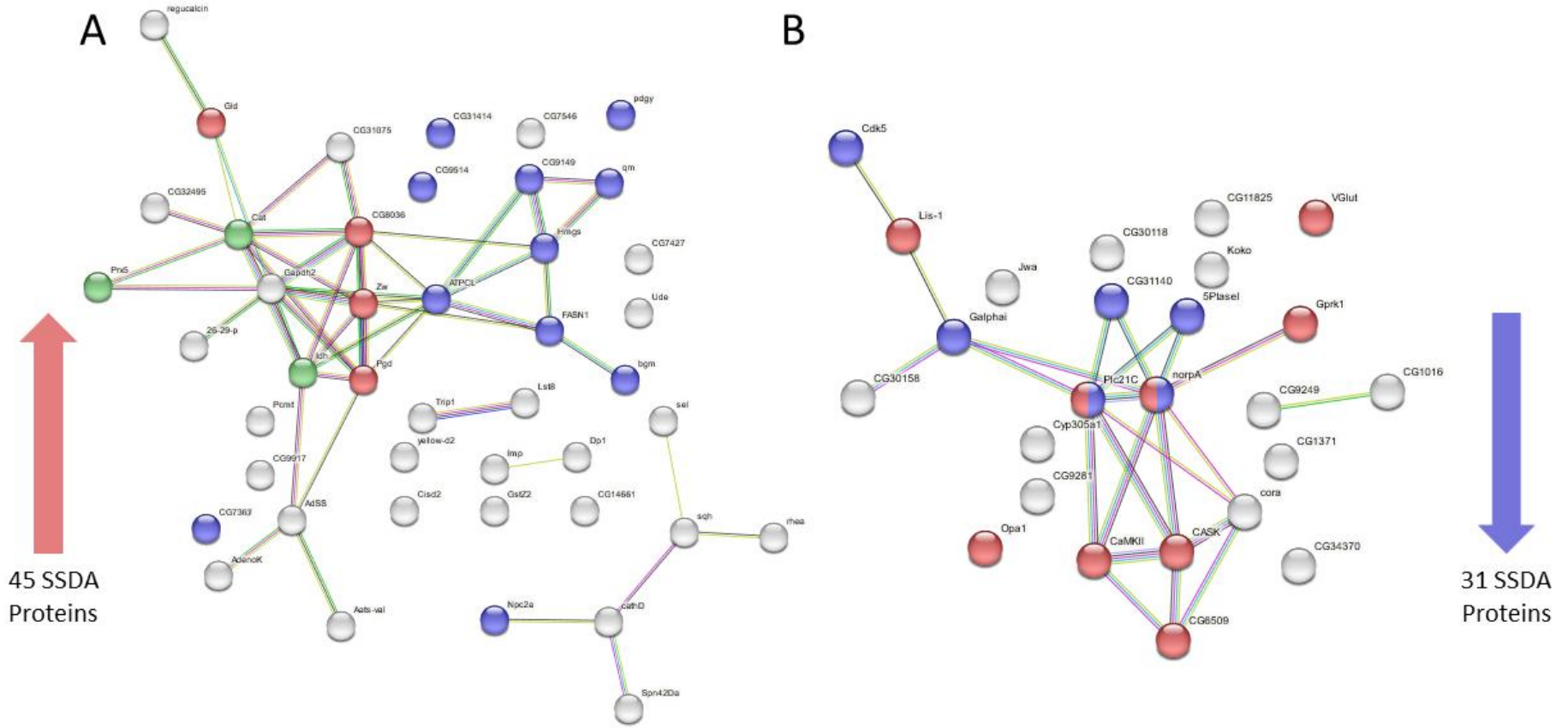

Figure S5-12 Protein-protein Interaction Network for SSDA Proteins Enriched in the Brain of the Acetone Control Compared to the Control Treatment Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the brain of acetone control compared to the control group were associated with the pentose phosphate pathway (red), the peroxisome (green), and lipid metabolism (blue). **(B)** Pathways and processes enriched for proteins with a decreased abundance in the acetone control compared to the control treatment group were involved in regulation of cell communication (red) and intracellular signal transduction (blue).

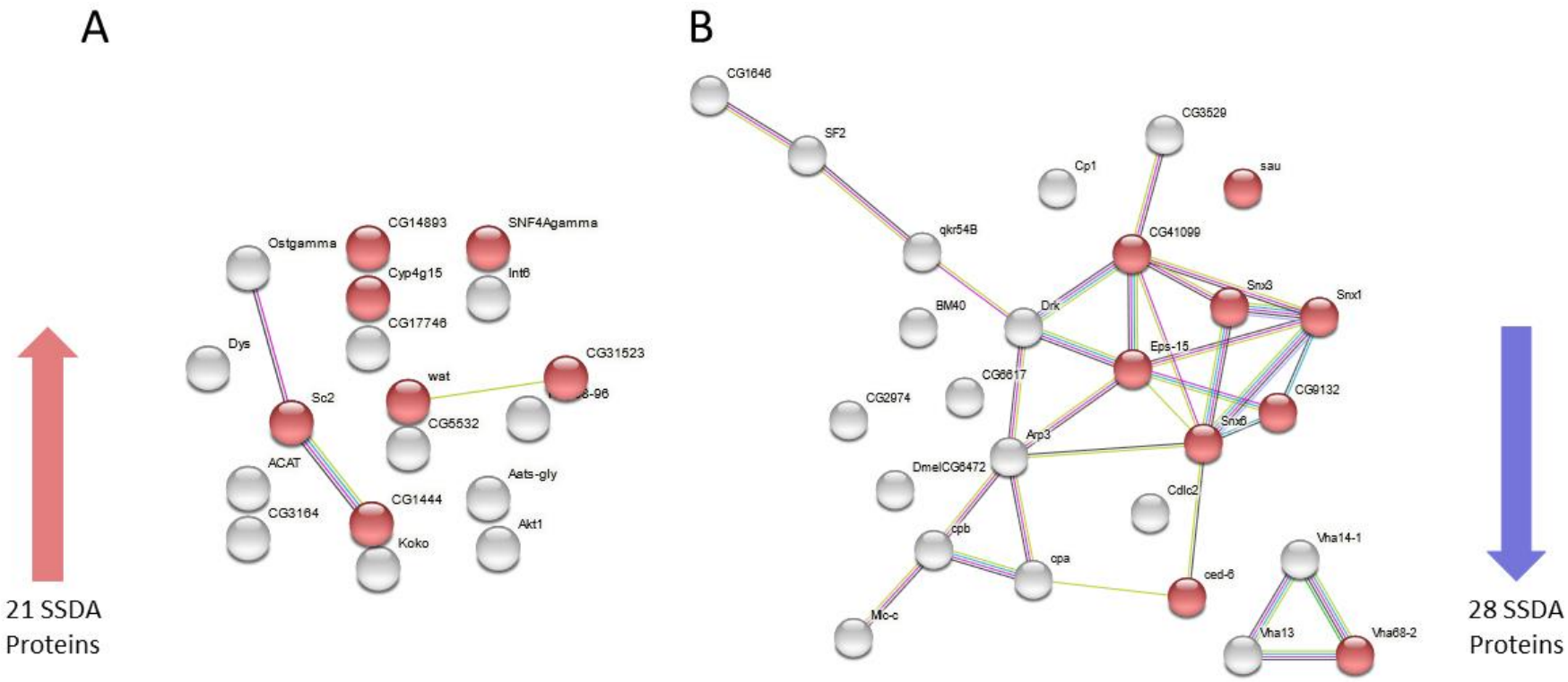

Figure S5-12 Protein-protein Interaction Network for SSDA Proteins Enriched in the Fat Body of Acetone Control Compared to the Control Group. Networks were produced using STRING (v.11). Nodes represent single SSDA proteins with lines between nodes representing known or predicted interactions. **(A)** Enriched pathways and processes from proteins with an increased abundance in the fatbody of acetone control compared to the control group were associated with lipid metabolism (red) and **(B)** decreased abundance proteins in the fatbody of acetone control compared to control exposed bees were associated with vesicle-mediated transport (red).